FORMATION OF EXTRACELLULAR SPHINGOLIPIDES BY MICROORGANISMS

I. TETRAACETYLPHYTOSPHINGOSINE FROM *Hansenula ciferri*

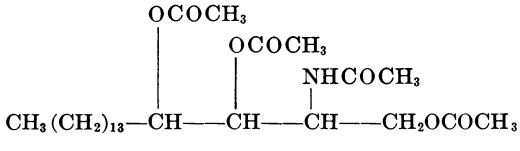
LYNFERD J. WICKERHAM AND FRANK H. STODOLA

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

Received for publication February 22, 1960

Lipides containing sphingosine and related hydroxyamino compounds are widely distributed in nature and are referred to as sphingolipides. Sphingosine and dihydrosphingosine, which have been obtained so far only from animal sphingolipides, are characterized by two hydroxyl groups; the analogous bases from plants, such as corn, soybeans, and various fungi, have an additional hydroxyl group and include the following: phytosphingosine (Carter et al., 1954); dehydrophytosphingosine (Mueller, 1953); and 2-amino-1,3,4-trihydroxy-*n*-eicosane (Reindel et al., 1940; Prošťenik and Stanacév, 1958).

Most sphingolipides are complicated molecules composed of at least three components; the best known examples are sphingomyelin, the cerebrosides, gangliosides, and phytoglycolipides. Some simpler sphingolipides, the ceramides, have also been described, in which only the amino group is in the combined form; *N*-lignocerylsphingosine is an example of this class. In this paper we record the isolation of still another type of sphingolipide in which all the functional groups of the base are esterified, not with the usual long-chain fatty acid, but with acetic acid. In chemical studies to be presented elsewhere, we have shown this new compound to be tetraacetylphytosphingosine (schema 1). It is produced extracellularly in the crystalline form by the yeast *Hansenula ciferri* strain NRRL Y-1031. This report appears to be the first on accumulation of a sphingosine-like compound outside the microbial cell, and describes the production of crude crystalline material and the relationship of sexual forms of the yeast to the formation of the compound.



Schema 1

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

MATERIALS AND METHODS

Cultures. H. ciferri NRRL Y-1031 was isolated by Ciferri and described by Lodder (1932). Filamentous forms of this strain were observed by Bedford (1942) and Wickerham (1951). Lodder and Kreger-van Rij (1952) reduced its rank to a variety of *H. anomala*, but Wickerham's opinion is that *H. ciferri* should be maintained as a separate species for reasons that are presented in the section on taxonomy.

The mating types used throughout this work for the production of crystalline material were derived from sporulated cultures by the heat treatment procedure devised by Wickerham and Burton (1954*a*). Vegetative cells were killed at a temperature that did not kill all the spores; 55 C, for *H. ciferri*. This simple procedure can be used without modification for obtaining ascosporic isolates of any genus of ascosporogenous yeasts (Wickerham and Burton, 1954*b*; Wickerham, 1958). Determination of the sex of ascosporic isolates, and the production and isolation of bisexuals from mating types of opposite sex, were according to procedures of Wickerham and Burton (1954*a*). Others used are mentioned at the pertinent points.

Medium. The culture medium used for the production of crystals was the yeast maintenance (YM) medium (Difco) devised by Wickerham for general cultivation and maintenance of yeasts. It contains 3 g each of yeast extract and malt extract, 5 g of peptone, and 10 g of glucose per liter of distilled water. In addition, the solid form contains 20 g of agar. The pH is not adjusted.

RESULTS

Production of crystals on solid media. In December 1956, cells were observed of mating type number 11 of *H. ciferri* NRRL Y-1031 that had been grown for 4 days at 25 C on a yeast maintenance agar slant. The cells were accompanied by numerous crystals, surprising because the parent heterothallic diploid culture had not been observed to produce them. The crystals ranged in size from about 15 μ in length to about 600 μ. These extremes were infrequent; a length of approximately 200 μ was common. Individual crystals were about 0.5 μ in diameter, and they were often compounded lengthwise to form flat, narrow ribbons and less regular lengthwise clusters from which single crystals separated and bent outward along the edges of the cluster. The interiors of the compounded crystals were hyaline and almost completely homogeneous, giving little or no suggestion they were composed of individual crystals. Most crystals were bent at sharp angles or in graceful curves. Rarely bundles of crystals have since been observed to be spirally wound together as compactly and as regularly as bundles of fibers in a rope.

A water mount of cells and crystals was warmed to a barely detectable degree until all crystals had melted and then was refrigerated for 23 min. The larger globules of tetraacetylphytosphingosine were still liquid, but those of about 6 to 15 μ diameter consisted of tiny needle-shaped crystals arranged in radial formation. The slide was replaced in the refrigerator and next morning all globules had converted to tiny crystals; in addition, long, very narrow crystals were scattered throughout the water. When the slide was slightly warmed, all crystals melted.

Serial transfers were made daily on yeast maintenance medium slants of the crystal-containing culture of *H. ciferri* mating type 11 (Y-1031-11) at 25, 28, and 32 C. Slant cultures at 25 C contained numerous crystals when 24 hr old, and globules were produced at 28 or 32 C. However, after 4 days of incubation at 32 C there were present tiny, narrow, bent, and generally ragged-appearing crystals, usually occurring singly, occasionally in small clusters, and occasionally radiating in large numbers from a single cell or clump of cells. From 40 to 60 such crystals have rarely been observed on a single cell. These tiny crystals did not melt even at 55 C. Apparently mating type 11 produces two types of crystallizable material. Under conditions of ordinary cultivation one type forms tiny crystals that melt at high temperature, and the other type forms long crystals that melt at low temperature. The low melter is in the liquid state when the culture is grown at 28 C or higher, and it is in the crystalline state when the culture is grown at 25 C, providing crystallization has been induced by crystals present in the inoculum or by previous exposure to low temperature.

Crystal induction. A petri dish containing yeast maintenance agar was streaked with cells of a crystal-containing (low melter) culture, then incubated for 5 days. Microscopic observation of the area of confluent growth, where the cells and crystals came off the loop in large number, showed abundant production of large crystals of the low melter, but individual colonies showed no crystals, only globules. These results were believed to be caused by the long crystals coming off quantitatively from the loop as it traversed the agar the first few times. Subsequently, the loop deposited cells but no crystals. Thus, crystals were not present in the isolated colonies to act as seeding centers for the conversion of globular material to crystals.

Ten isolated colonies were used to inoculate separate slants, which were incubated at 25 C for 48 hr. None had produced crystals at this time, though globules were present. Five of the cultures were then transferred at 24-hr intervals and the remainder at 48-hr intervals. Three was the minimal number of transfers at which crystals were observed, and some of the colony isolates were transferred many times with no crystals appearing. One of these isolates was placed in the refrigerator for 2 days; at the end of this time it contained low melting crystals, and typical crystals were produced on subsequent transfers on slants. All the cells of another slant, which contained globules, were transferred to 1 ml of sterile water; after the suspension was refrigerated for 24 hr, many very fine, long crystals were present. A loop of the suspension was streaked on a cold slant and incubated at 25 C over the weekend. The growth was then observed to have large crystals. A slant inoculated from it had crystals at 24 hr.

During induction by refrigeration the low melter crystallizes both as long narrow crystals in irregular clusters or singly, and as tiny crystals arranged regularly in spheres or other symmetri-

cal formations. It is desirable to transfer cells from a refrigerated culture to a fresh, cool slant and allow the culture to incubate at 25 C for a day or so. Then the long, large crystals are the low melting product and the short crystals are the high melting form. Cultures having lesser ability to synthesize crystals may be incubated longer before they are refrigerated, or the first slant after crystal induction may be incubated longer before it is observed for crystals, or both.

Isolation of crystalline material. To secure material for determining the molecular structure of the high and low melting crystals, mating type 11 was grown on agar in Erlenmeyer flasks and petri dishes. The flasks were of one-half and one liter capacity with approximately 50 ml of agar medium per small flask, and 100 ml per large flask. The flasks containing the media were cotton stoppered and autoclaved 20 min at 121 C (15 lb) the day before inoculation.

Inoculum was prepared by suspending the growth from ten 24-hr-old slants of mating type 11 in 100 ml of yeast maintenance broth. Two ml of inoculum were used per flask regardless of its size. The flasks or plates were tipped as necessary to spread the inoculum completely over the surface of the agar medium. As soon as the water in the inoculum was absorbed by the growing culture, the petri dishes were inverted. After 10 days of incubation at 25 C, the growth was washed off the agar with water after loosening with a soft-bristled brush. The aqueous suspension of cells and crystals was shaken with $\frac{1}{10}$ volume of petroleum ether (bp 40 to 60 C), the liquid centrifuged, and the filtered petroleum ether solution concentrated to an oil which crystallized on seeding or cooling to give a waxy solid melting

TABLE 1

Yields of crystalline material from cultures grown on solid yeast maintenance medium in flasks and petri dishes

Expt No.	No. of Flasks		No. of Petri Dishes	Cell Suspension	Yield of Crystals
	500-ml	1,000-ml			
				ml	*g*
1	63	21		5250	0.932
2	64	21		7450	0.772
3	51	15		3000	0.577
4	51	19	114	6050	0.935
5	54	22	133	4400	0.734

a little above room temperature. Yields on all runs are presented in table 1 to indicate the number of cultures harvested, the volume of cell suspension extracted, and the weight of crystals obtained.

The 0.577 g of crystals produced in experiment no. 3 corresponded to a weight yield of 1.4 per cent based on the glucose supplied, and 6.4 per cent of the total weight of cells and crystals was crystals. The weight of dried yeast cells in this experiment was 8.37 g.

Production of crystalline material in liquid media. Mating type 11 produced extremely few crystals in still liquid cultures, and yields were poor in shaken cultures. This mating type produces rather smooth, glistening growth on slants, a characteristic generally associated with hydrophilic cells. Another mating type of the same sex, designated as F-60-10, was selected for study because it produces hydrophobic cells. Its slant cultures are dry, white, and mat. The glistening types are relatively more fermentative, the mat types are relatively more oxidative in their metabolism (Wickerham, 1951). The growth of type F-60-10 contains small clusters of tightly agglutinated cells that are so cohesive they remain clustered when the more abundant loose cells of the culture are washed away with cold water. Mating type 11 also produces such localized clusters in its growth on slants, but its clusters are so weakly agglutinated that they disintegrate when attempts are made to grow them in shaken cultures.

Type F-60-10 was grown serially in flasks on a reciprocal shaker at 22 to 25 C. The loose cells were washed out of the culture every 3 or 4 days, then washed clusters received fresh medium. Such cultures yielded about 15 mg of crystals per 100 ml of culture after 4 days of incubation, but cultures inoculated directly from slants yielded only 3 to 5 mg. The washed clusters are shown in figure 1, and the crystals they contain appear in figure 2. The hydrophobic nature of the cells was further shown by their tendency to coat solid materials, such as sterile asbestos fibers, placed in the culture flasks at the time of incubation. Fine nylon fibers suspended into the medium from above caused most of the growth to accumulate on the fibers above the moving surface of the liquid. Acenaphthene crystals also served as centers on which the hydrophobic cells accumulated, thus escaping to a large extent the

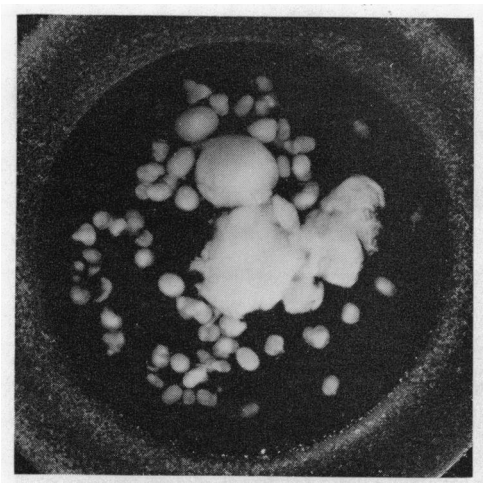


Figure 1. Washed clusters of cells from shaken cultures of mating type F-60-10 (×6; reduced slightly for reproduction here).

water of the culture medium. Incidentally, acenaphthene induced free cells to grow as large, loose, ramified chains of cells.

Taxonomy. The taxonomic standing of *H. ciferri* should be briefly mentioned. This species was described by Lodder in 1932 and reduced to a variety of *H. anomala* by Lodder and Kreger-van Rij in 1952. The variety is differentiated from the principal species by its lesser ability to produce pellicles on liquid media. Wickerham (1951) maintained *H. ciferri* as an autonomous species because of its ability to assimilate rhamnose and to produce true hyphae; neither of these capacities is possessed by *H. anomala*. Both positions are weak. It is true that *H. ciferri* produces weak pellicles on malt, wort, and yeast extract media used by zymologists. It is also true that most forms of *H. ciferri* produce strong pellicles in 24 hr on the chemically defined media commonly used for biochemical tests. Rhamnose is slowly assimilated, and the ability to produce true hyphae is heterozygous and therefore not possessed by all forms of *H. ciferri* that can be derived by appropriate selection and mating of ascosporic isolates.

Mating types of *H. ciferri* do not mate at all with mating types of *H. anomala*. This lack of sexual reaction would be an important means of separating the species if mating types of *H. ciferri* mated abundantly among themselves, but they do not. Mating types of yeasts most closely related to *H. ciferri* were carefully studied for their ability to produce crystalline products on yeast maintenance slants, using long periods of incubation before and after refrigeration to induce crystal formation. No crystals were observed in any of the cultures studied, which were: *H. anomala* var. *schneggii*, NRRL Y-993 and Y-840; *H. anomala*, Y-366-8, Y-407-15, Y-2067-6, Y-2067-7, Y-1653-10, Y-1653-15, Y-1656-18, Y-2153-4, Y-1737-9; *H. subpelliculosa*, Y-1683-11 and Y-1822-12. Some bisexuals were also studied. The mating types of *H. subpelliculosa* are uniformly smooth and glistening. Those of *H. anomala* vary from smooth glistening to mat and rugose, but generally are less mat than mating types of *H. ciferri*. The mating types of *H. anomala* var. *schneggii* are usually mat and rugose, and they are as aberrant in their sexual reactions as are the mating types of *H. ciferri*.

The following information is mentioned because it may be of use in studies of the biochemistry of *H. ciferri*. This species readily assimilates glucose, galactose, maltose, sucrose, trehalose, raffinose, melezitose, soluble starch, D-ribose, ethyl alcohol, glycerol, erythritol, adonitol, mannitol, sorbitol, α-methylglucoside, salicin, potassium gluconate, pyruvate, lactate, succinate, and citrate. An exogenous supply of vitamins is not required by the stock culture or by mating types 11 and F-60-10, but is required for growth by mating type 27.

Relation of crystal production to sexuality. Strain Y-1031 was subjected to a sexual and genetic study. Its ascosporic isolates mated sparingly and produced few ascospores, except for a single combination of mating types, numbers 11 and 27. This combination for some time yielded large masses of agglutinated ascospores. Therefore, 11 and 27 were the principal pair used in subsequent work, though others that possessed or lacked certain morphological and biochemical properties were also kept.

As already discussed, mating type 11 produced large, low-melting microcrystals when grown for 24 hr on yeast maintenance slants at 25 C. Under similar conditions neither the bisexual stock strain nor mating type 27 gave low-melting crystals; however, when slants of the bisexual were 48 hr old, a very few crystals were found during a long search; and when slants were aged for 2 weeks at 25 C, the low melter was present in fair numbers but disappeared from serial subcultures made at 24-hr intervals. Under the

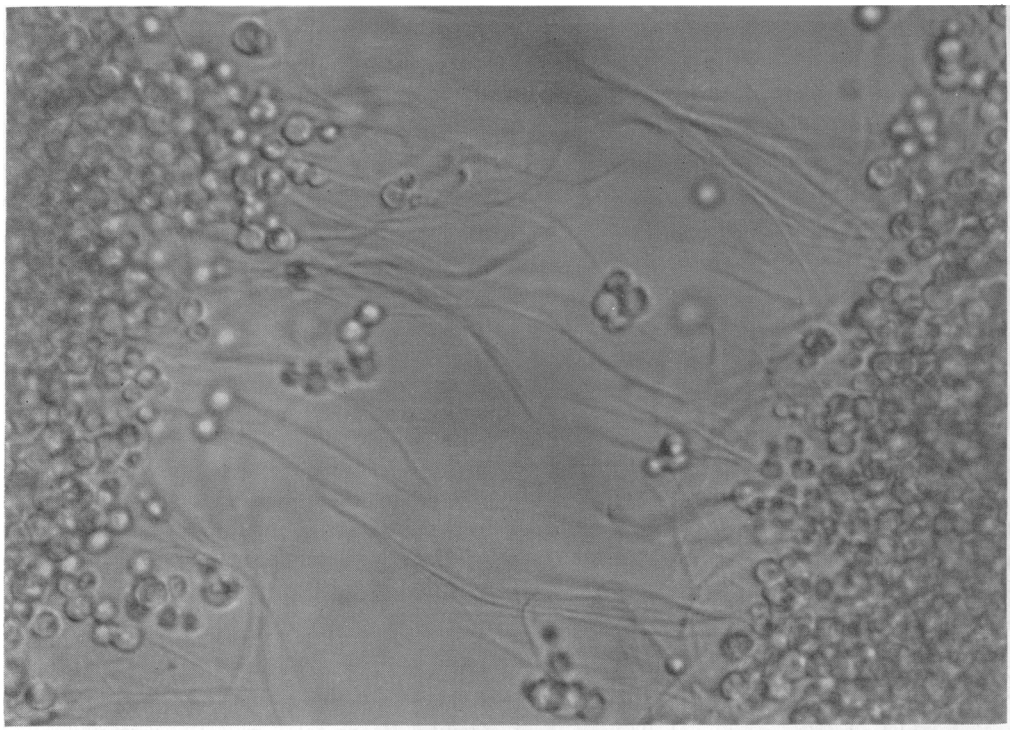


Figure 2. Washed clusters of cells magnified sufficiently to reveal crystals of tetraacetylphytospingosine (×880; reduced about 5 per cent for reproduction here).

same conditions mating type 27 produced neither low-melting nor high-melting crystals. Mating type 11 and the bisexual stock culture produced scant amounts of the smaller, high-melting crystals, which were more commonly seen in old slant cultures where they were observed to increase in number through 6 to 8 weeks of incubation.

Thus the questions arose: is the production of low-melting crystals characteristic of only one sex; do the bisexuals occupy an intermediary position? To obtain the answers, a series of bisexuals was selected to supply mating types by heat treatment of their sporulated cultures. The bisexuals were obtained primarily by mating opposite sexes of diverse properties, but a few were bisexuals that arose by spontaneous change to extreme mat type. The selected cultures formed a series ranging from presumed diploid through presumed polyploid, and from the least to the most oxidative. The latter type is characterized by the increasing ease with which pellicles are formed and by the increasing dryness of the colonies (figure 3, *A–C*).

Mating types obtained from the bisexuals by heat treatment were very diverse. Photographs of some individuals are shown in figure 3, *D–L*. Some ascosporic isolates were quite unstable, as shown in the photographs for mating type 27 (figure 3, *F–H*).

Ascosporic isolates of the same sex as 11 were arbitrarily designated as sex a, and those of the same sex as 27 were designated as sex b. Sex was determined by mixing each isolate with testers 11 and 27 separately, then observing which of the two mixtures produced zygotes and spores. Some isolates, presumably arising mainly as bisexual ascospores from polyploids, sporulated by themselves and generally produced few crystals. These were not included in the analysis of data.

The mating types and parent bisexuals were transferred daily three times; the third transfers were incubated 3 days at 25 C followed by 3 days in a refrigerator, then transfers were made to cool slants which were incubated at 25 C for 12 days. Microscopical observations for crystals started at the 8th day and continued through the 12th day. The bisexuals were observed at 12 days. The appearance of growth on the slants

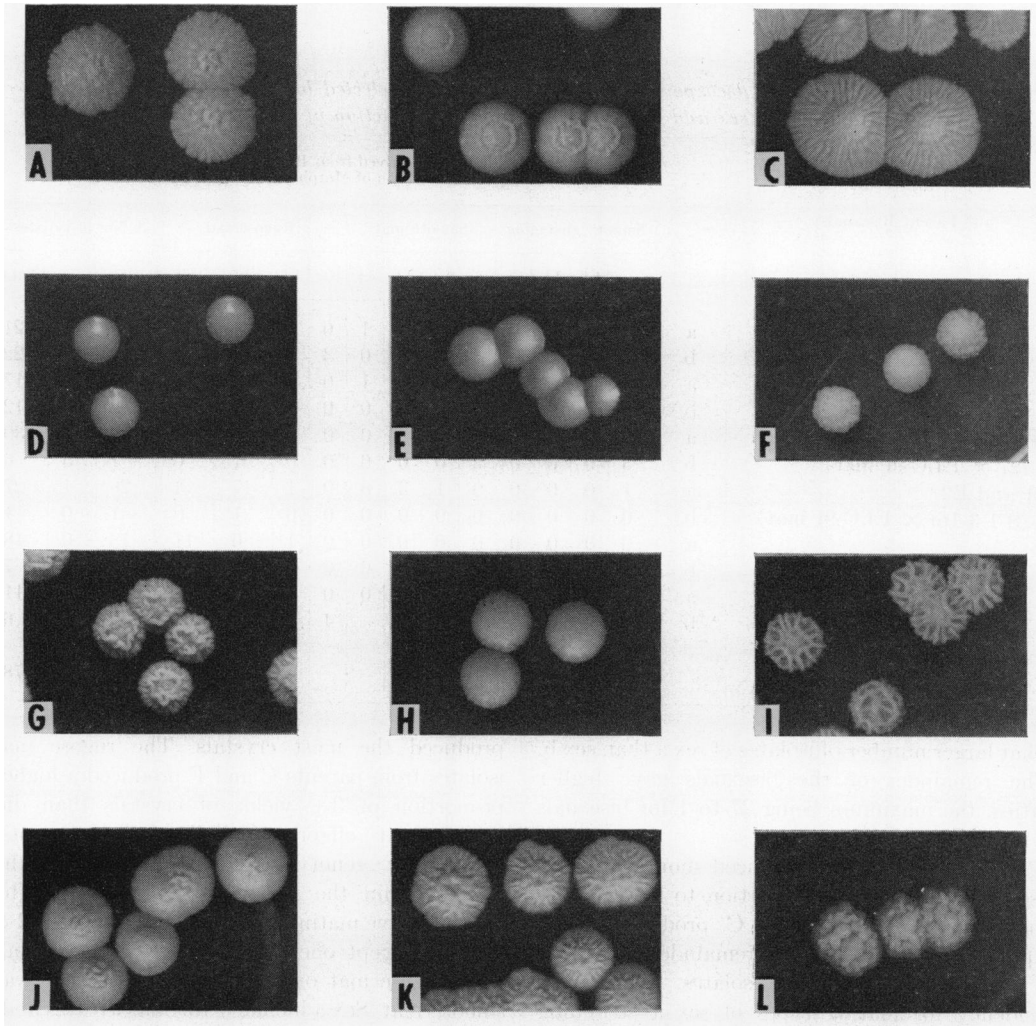


Figure 3. Six-day-old colonies on streak plates. The top row are some bisexuals from which mating types were obtained. Photograph *A* is of Y-1031 (11 × 27), representing cultures A and B of the text. Photograph *B* is the stock culture, the source of all cultures studied, and is bisexual C of the text. Photograph *C* is a strongly oxidative form that is representative of cultures in group G. *D* through *L* are mating types having various colony forms. Of particular note are Y-1031-11 (*E*), Y-1031-27 (*F*), S-1-3-1m (*J*), and ELC-9t mat (*K*), parents of some bisexuals which, in turn, yielded others of the mating types studied. Spontaneous variation within mating type Y-1031-27 is shown by photographs *F*, *G*, and H (×2; reduced about 5 per cent for reproduction here).

was recorded as smooth glistening, smooth mat, or rugose mat. Intermediates were placed in the group they most resembled. The relative number of crystals to cells was estimated microscopically as 1+ for scant, 2+ for moderate, and 3+ for abundant production, with 3+ being approximately equivalent to the yield given by mating type 11.

Parent bisexuals A and B yielded no low-melting crystals at 12 days; C, D, E, F, and G contained 1+ crystals at 12 days. These low yields are consistent with previous observations on the bisexual stock culture Y-1031.

The last column in table 2 shows that bisexuals A and B, possibly the only two that have one set of chromosomes of each sex, are the only two that produced approximately equal numbers of the two sexes. Stock culture C produced a some-

TABLE 2

Distribution of sexes among ascosporic isolates obtained from selected bisexuals of Hansenula ciferri, and the relation of sex and morphological type to production of low-melting crystals

Parent Bisexuals	Sex	Mating Types Derived from Parent Bisexuals Crystal Production of Morphological Types												No. of isolates		
		Smooth glistening				Smooth mat				Rugose mat						
		−	1+	2+	3+	−	1+	2+	3+	−	1+	2+	3+	+	−	Total
A and B	a	9	0	0	0	3	0	1	1	0	0	3	4	9	12	21
(11 × 27)	b	12	0	0	0	5	0	0	0	3	0	2	0	2	20	22
C	a	3	2	0	0	0	2	0	1	0	0	1	8	14	3	17
NRRL Y-1031	b	10	0	0	0	0	0	0	0	0	0	2	0	2	10	12
D1 and D2	a	0	0	0	0	1	0	0	0	0	2	13	14	29	1	30
(27 × ELC-9t mat)	b	3	0	0	0	2	0	0	0	0	0	1	0	1	5	6
E1 and E2	a	1	0	0	0	0	1	3	0	0	1	17	4	26	1	27
(S-1-3-1m × ELC-9t mat)	b	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1
F	a	0	0	0	0	0	0	0	0	0	1	6	11	18	0	18
(11 × S-1-3-1m ELC)	b	5	0	0	0	0	0	1	0	0	0	0	1	2	5	7
G	a	0	0	0	1	0	0	0	0	0	1	6	3	11	0	11
Radial types: PSH, 37, 68	b	2	0	0	0	0	0	0		4	0	0	0	0	6	6
														115	63	178

what larger number of isolates of sex a than sex b. The remainder of the bisexuals gave higher ratios, the maximum being 27 to 1 for bisexuals E1 and E2.

Bisexuals A and B produced more glistening ascosporic isolates in proportion to mat isolates than the other bisexuals, C produced about equal numbers, and the remainder produced a preponderance of mat isolates. There is a tendency for mating types of sex a stemming from A, B, and C to be mat, and for those of sex b to be glistening, but this tendency lessens among the mating types of the more oxidative bisexuals D through G. The latter tend to produce mat isolates in sex b also. Table 2 shows that most of the glistening mating types produced no crystals and that most mat mating types produced crystals. Thus, the more oxidative mating types usually produce low-melting crystals, whereas the less oxidative generally do not.

Most ascosporic isolates of sex a produced low-melting crystals, whereas those of sex b did not. Numerically, of 124 isolates of sex a, 106 (86 per cent) produced crystals; of 54 isolates of sex b, 8 (15 per cent) produced crystals. Of the three morphological types, so far as slant growth is concerned, the rugose mat isolates of sex a produced the most crystals. The rugose mat isolates from parents C and F produced a higher proportion of 3+ yields of crystals than did rugose mat offspring of the other parents. Apparently genotypic differences change the yields within the rugose mat phenotype. The eight positive mating types of sex b produced 2+ crystals except one that produced 3+, and all were rugose mat on slants except one that was smooth mat. Sex a included 109 mat isolates and, of these, 96 produced 2+ and 3+ crystals. Thus it appears that mat types of sex a are better producers of crystals than mat types of sex b.

All crystal-positive mating types of sex b were lyophilized to insure their availability indefinitely. All were rechecked for sex and, again, all conjugated with mating type 11 and none with mating type 27, thus confirming their original designation as sex b. Twelve bisexuals were isolated from the matings with type 11. At least one bisexual was derived from each of the eight crystal-positive mating types of sex b. All the bisexuals were capable of forming ascospores. Because both parents of these bisexuals produced crystals on slants, it was of interest to determine whether they would produce more crystals than bisexuals A through G.

The 12 bisexuals, all but one being mat and

rugose, were tested for crystal production. Parent bisexuals A, B, C, and D were included as controls. Slant cultures 48 hr old were refrigerated for 3 days; then transfers made to fresh slants were incubated for 2 days at 25 C, when they were observed for crystals. None of the controls yielded crystals during this short period of incubation following crystal induction. Three of the 12 bisexuals having both parents crystal-positive yielded more than 2+ and less than 3+ amounts of crystals; the others yielded 1+ or 2+. Apparently some bisexuals are moderately strong producers, if both parents are crystal producers.

DISCUSSION

The extracellular production of crystalline sphingolipides by yeasts must be rare. This occurrence is the only time low-melting crystals have been noticed by the senior author in over 27 years of observation. It seems possible that their appearance is the result of a deranged mechanism for lipide synthesis, or the lack of an adequate mechanism for combining the sphingolipide with other kinds of molecules. Until such time as fresh isolates of *H. ciferri* are obtained from nature, we shall not know for certain whether the production of the free lipides is a characteristic property of the species.

ACKNOWLEDGMENTS

The authors wish to express their appreciation for photographic work to Mr. Roland Haines and Mr. Robert Garrett of the Northern Regional Research Laboratory.

SUMMARY

Two new crystalline substances have been obtained from the yeast *Hansenula ciferri*. One is tetraacetylphytosphingosine, a compound not previously reported from natural sources. It is produced extracellularly when mating type 11 of *H. ciferri* Y-1031 is grown on a solid medium. This product was formed by a much higher percentage of mating types of one sex (a) than of the other sex (b). Oxidative mating types, which are characterized by mat growth on slants, produced more of the material than the fermentative, glistening types. The parent bisexual stock culture NRRL Y-1031 produced very little.

REFERENCES

BEDFORD, C. L. 1942 A taxonomic study of the genus *Hansenula*. Mycologia, **34,** 628–649.

CARTER, H. E., W. D. CELMER, W. E. M. LANDS, K. L. MUELLER AND H. H. TOMIZAWA 1954 Biochemistry of the sphingolipides. VIII. Occurrence of a long chain base in plant phosphatides. J. Biol. Chem., **206,** 613–623.

LODDER, J. 1932 Über einige durch das "Centraalbureau voor Schimmelcultures" neuerworbene sporogene Hefearten. Zentr. Bakteriol. Parasitenk. Abt. II, **86,** 227–253.

LODDER, J., AND N. J. W. KREGER-VAN RIJ 1952 *The yeasts, a taxonomic study.* North-Holland Publishing Co., Amsterdam.

MUELLER, K. L. 1953 The isolation of cerebrin base from plant phosphatides. Masters thesis, University of Illinois, Urbana, Illinois.

PROŠTENIK, M., AND N. Ž. STANACÉV 1958 Studien in der Reihe der Sphingolipoide. X. Über die Structur der Cerebrin-Base aus Hefe. Chem. Ber., **91,** 961–965.

REINDEL, F., A. WEICKMANN, S. PICARD, K. LUBER, AND P. TURULA 1940 Über Pilzcerebrin. II. Ann. Chem. Liebigs, **544,** 116–137.

WICKERHAM, L. J. 1951 Taxonomy of yeasts. 1. Techniques of classification. 2. A classification of the genus *Hansenula*. U. S. Dept. Agr. Tech. Bull. No. **1029,** 56 pp.

WICKERHAM, L. J. 1958 Sexual agglutination of heterothallic yeasts in diverse taxonomic areas. Science, **128,** 1504–1505.

WICKERHAM, L. J., AND K. A. BURTON 1954*a* A simple technique for obtaining mating types in heterothallic diploid yeasts, with special reference to their uses in the genus *Hansenula*. J. Bacteriol., **67,** 303–308.

WICKERHAM, L. J., AND K. A. BURTON 1954*b* A clarification of the relationship of *Candida guilliermondii* to other yeasts by a study of their mating types. J. Bacteriol., **68,** 594–597.