NONSPORULATING, RESPIRATION-DEFICIENT VARIANTS OF *SACCHAROMYCES PASTORIANUS* INDUCED BY CYCLOHEXIMIDE

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ABSTRACT

LOMANDER, L. (Gothenburg University, Gothenburg, Sweden), AND K. GUNDERSEN. Nonsporulating, respiration-deficient variants of *Saccharomyces pastorianus* induced by cycloheximide. *J. Bacteriol.* **86**:956-965. 1963.—When grown on cycloheximide-containing solid medium, *Saccharomyces pastorianus* gave rise to rough colonies, consisting of two morphologically and physiologically different kinds of organisms, named primary colony organisms and secondary colony organisms. The primary colony organisms were slow-growing and cycloheximide-resistant as compared with the secondary ones which, like the parent organisms, were fast-growing in the absence of cycloheximide, but were inhibited when cycloheximide was incorporated in the medium. Cell aggregates, large swollen cells, and dwarf cells were found among the secondary colony organisms. The primary colony organisms were smaller than normal cells but were larger than the dwarf cells. The primary colony organisms also proved to have lost the faculty to sporulate, whereas the secondary colony organisms sporulated like the untreated parents. In contrast to the normal organisms and the secondary colony organisms, the primary colony organisms did not consume oxygen in manometric experiments with glucose as a substrate. On solid medium without cycloheximide, the latter organisms grew considerably better in a nitrogen atmosphere than in air.

In a recent investigation, Gundersen and Wadstein (1962) found that cycloheximide had a striking effect on the morphology of the cells of *Saccharomyces pastorianus*. When the organisms were grown in liquid cycloheximide-containing medium, cell aggregates and large swollen cells appeared. The authors also showed that large and small colonies appear on cycloheximide-free

agar medium, if the cells are previously exposed to 0.05 ppm of cycloheximide in liquid medium. Organisms resistant to 5 ppm of cycloheximide were obtained by transfer of *S. pastorianus* through a series of liquid cultures containing successively higher concentrations of cycloheximide.

The purpose of the present paper is to present the results of further investigations of the effect of cycloheximide on the cell and colony morphology of *S. pastorianus*. The influence of the antibiotic on the sporulation in this organism has also been investigated, and, finally, a study of the effect of cycloheximide on the respiration and anaerobic growth of *S. pastorianus* has been made.

MATERIALS AND METHODS

The organisms used were *S. pastorianus* Hansen, strain ATCC 2366, and a strain obtained from the central laboratory of the Pripp & Lyckholm group of breweries, Gothenburg, Sweden. The latter strain was designated the PL strain. Since the ATCC strain did not sporulate, all the sporulation experiments were carried out with the PL strain. Unless otherwise stated, the ATCC strain was used in all of the other experiments.

Stock cultures were maintained on a solid medium (pH 6.0), consisting of: glucose, 10 g; yeast extract (Difco), 2.5 g; KH_2PO_4 , 1 g; agar (Difco), 20 g; and distilled water to 1000 ml. Except for the sporulation experiments, this medium and a liquid medium of the same pH value and composition were used throughout. The media were sterilized by autoclaving at 115 C for 15 min, and the cultures were incubated at 28 C. Unless otherwise stated, all cultures were static.

Stock solutions of cycloheximide [β -(2-(3,5-dimethyl-2-oxo-cyclohexyl)-2-hydroxyethyl)-glutarimide] were prepared by dissolving 0.25 g of pure cycloheximide (obtained from The Up-

john Co., Kalamazoo, Mich.) in 5 ml of ethanol and diluting this to 100 ml with 0.067 M KH_2PO_4 . The 2500-ppm solution was sterilized by filtration and stored at 4 C. Dilutions were made in sterile 0.067 M KH_2PO_4 , and not more than 0.1 ml was added per 5 ml of medium to give the final concentrations.

The sporulation experiments were made according to a method described by Møller-Christensen (1955) with one modification: sterile distilled water was used instead of the sodium acetate solution.

Nuclear staining was done by the method of Ganesan and Swaminathan (1958), but instead of osmic vapors Carnoy's fluid was used as a fixative.

Measurements of cell diameters were made on microphotographs by means of an ocular from a microscope in conjunction with a scale which permitted an accuracy of 0.2 mm on the photograph.

Turbidimetric readings of cell suspensions were made in a Beckman model C colorimeter equipped with a red filter with maximal transmission at 660 μ . Determinations of cell numbers in suspensions were made by direct microscopic counts in a Helber blood-cell counting chamber.

Special techniques will be described in connection with the appropriate experiment.

RESULTS

Effect of cycloheximide on colony morphology. Approximately 0.05 ml of a suspension of *S. pastorianus* was spread on agar plates without cycloheximide and on plates containing cycloheximide in concentrations varying between 0.03 and 0.5 ppm. After 3 or more days of incubation of the cycloheximide plates, small smooth colonies appeared which, on further incubation, were more or less covered with a distinctly different layer of secondary growth. This secondary growth apparently started at the top of the primary growth, the final appearance of the colony being determined by the concentration of cycloheximide incorporated in the plates. The relation between colony morphology and cycloheximide concentration is illustrated diagrammatically in Fig. 1. With lower concentrations (0.03 to 0.05 ppm), the secondary growth in some instances completely covered the primary growth giving rise to a large smooth colony, but usually the secondary

FIG. 1. Diagrammatic representations of *Saccharomyces pastorianus* colonies formed on yeast extract agar containing different amounts of cycloheximide. (a) Normal colonies from plates without cycloheximide; (b and c) 0.03 to 0.05 ppm of cycloheximide; (d) 0.05 ppm; (e) 0.07 to 0.5 ppm; (f) >0.5 ppm. Dotted areas, normal organisms; white areas, secondary organisms; shaded areas, primary organisms.

growth appeared as sectors overlying the primary growth or as a starlike body resulting in a rough appearance of the colony as a whole (Fig. 1, c and d; Fig. 2). Eventually, a rough colony was rapidly transformed into a large smooth colony by complete overgrowth by the secondary organisms.

For identification, the organisms composing the secondary growth subsequently will be designated the secondary colony organisms, whereas the organisms in the small smooth colonies, upon which the secondary developed, will be designated the primary colony organisms. Organisms from plates not containing cycloheximide will be designated normal organisms.

When the concentration of the antibiotic was 0.07 ppm and higher, the secondary colony organisms never grew out on the agar surface, but grew on the primary colony organisms like a papilla (Fig. 1e, Fig. 3). When the concentration of cycloheximide was as high as 0.5 ppm, the secondary growth was considerably delayed (4 to 5 weeks) or was absent. In all experiments, the secondary growth had a faint yellowish color which disappeared on subsequent culturing in the absence of cycloheximide, whereas the primary growth was pure white. The colonies appearing on plates without cycloheximide were always smooth with an entire edge (Fig. 4).

It should be added that the PL strain of *S. pastorianus* also formed composite colonies on cycloheximide-containing agar. However, this strain was affected at slightly lower concentrations of cycloheximide than was the ATCC strain.

Growth and cycloheximide resistance in primary and secondary colony organisms. A thin suspen-

sion of *S. pastorianus* was spread on yeast extract agar containing 0.05 ppm of cycloheximide. After 5 days of incubation, it was possible, by means of a sterile glass needle, to pick up a small clump of cells of the primary and of the secondary colony organisms from a composite colony of a shape similar to Fig. 1d. The two kinds of organisms were streaked on cycloheximide-free agar and allowed to grow for 48 hr; then suspensions in sterile distilled water were prepared. A suspension of normal organisms was also made and used as a control. Approximately 0.05 ml of each of the three suspensions was spread on agar plates, containing 0, 0.05, and 0.1 ppm of cycloheximide, and incubated. The experiment showed that the primary colony organisms were much more resistant to cycloheximide than were the secondary colony organisms. On the other hand, the former were slow-growing compared with the latter. No differences as to resistance to cycloheximide and growth rate between the secondary colony organisms and the normal organisms were ever noted (Table 1).

These results indicate that the primary colony organisms are identical with the small colony organisms of *S. pastorianus*, and the secondary colony organisms with the large colony organisms

TABLE 1. Colony diameters of *Saccharomyces pastorianus* (normal, primary, and secondary organisms) on yeast extract agar with different concentrations of cycloheximide*

Organism	Cycloheximide (ppm)		
	0	0.05	0.1
Normal.....	5.7	3.0	1.3
Primary.....	3.3	3.4	3.3
Secondary.....	5.6	3.2	1.4

* Mean of 25 to 30 colonies of each organism and each cycloheximide concentration. Incubation: 3 days at 28 C. The results are expressed in terms of mean colony diameter (mm).

FIG. 8. Distribution curves of the cell diameters of normal *Saccharomyces pastorianus* and of organisms developed on agar plates in the presence of cycloheximide. (a) Normal organisms from cycloheximide-free agar medium, ○; primary colony organisms from 0.05 ppm of cycloheximide, ●; (b) secondary colony organisms from 0.05 ppm of cycloheximide, ●; secondary colony organisms after seven subsequent transfers on cycloheximide-free agar medium ●.

isolated from liquid cycloheximide-containing medium by Gundersen and Wadstein (1962).

Cell size and cell morphology of primary and secondary colony organisms. *S. pastorianus* was allowed to grow for 5 days on solid medium containing 0.05 ppm of cycloheximide. Rough colonies consisting of primary and secondary colony organisms appeared. Each kind of organism was

FIG. 2. Rough colonies on solid medium containing 0.05 ppm of cycloheximide. Primary (a), and secondary organisms (b); 8 days, 28 C.

FIG. 3. Composite colonies on solid medium containing 0.07 ppm of cycloheximide. Primary (a), and secondary organisms (b); 14 days, 28 C.

FIG. 4. Normal colonies on solid medium without cycloheximide; 7 days, 28 C.

FIG. 5. Phase-contrast micrograph of normal organisms; 72 hr, 28 C.

FIG. 6. Phase-contrast micrograph of primary colony organisms from solid medium containing 0.05 ppm of cycloheximide; 5 days, 28 C.

FIG. 7. Phase-contrast micrograph of secondary colony organisms from solid medium containing 0.05 ppm of cycloheximide. The arrow indicates an enlarged cell with five buds; 5 days, 28 C.

sampled and examined under a microscope. Normal organisms were also examined for purposes of comparison.

Primary and secondary colony organisms differed considerably in size and morphology from normal organisms. Normal organisms are shown in Fig. 5. Primary colony organisms were considerably smaller in size and generally had a more oval shape than the normal cells (Fig. 6). The secondary colony organisms consisted of those described by Gundersen and Wadstein (1962) as cell aggregates and large swollen cells which appeared when *S. pastorianus* was grown in liquid medium containing cycloheximide. In the present material, however, cell aggregates were sparse; instead, many large cells bearing from four to eight small single buds, similar to horned mines, were observed (Fig. 7). Dwarf cells, probably immature buds which had separated from a swollen mother cell, were also found.

To obtain a measure of the variation in cell size, the greatest diameter of 200 primary, secondary, and normal organisms was measured (Fig. 8). The primary colony organisms and the normal organisms showed normal limits of deviation, whereas the organisms of the secondary kind appeared to be of two significantly different sizes, both of which differed in size from the two former kinds of organisms. After repeated transfers on cycloheximide-free solid medium, the secondary colony organisms reverted to the size of the normal organisms (Fig. 8b), whereas the cell size of the primary colony organisms remained unchanged.

As it was of interest to ascertain whether there existed any morphological anomalies in the nuclear material of the primary and the secondary colony organisms, nuclear staining was carried out. For comparison, normal organisms were also stained.

The result showed that all different sizes of

cells, including the dwarf cells described above, contained a nucleus (Fig. 9 to 11).

Sporulation. As was stated above, it was not possible to use the ATCC strain for sporulation experiments. Instead, the PL strain, which sporulated amply in ordinary media, was used.

About 0.05 ml of a suspension of *S. pastorianus* was spread on solid medium containing 0.05 ppm of cycloheximide. After 8 days of incubation, the colonies appeared rough, as a consequence of the irregular secondary growth. Microscopy revealed that the secondary growth contained cell aggregates and abnormally large cells, which could not be seen in the primary growth where the cells were rather small. From one rough colony, the primary and the secondary colony organisms were isolated and streaked on cycloheximide-free agar, where they were allowed to grow for 48 hr. Cells from the 48-hr cultures were transferred to tubes containing 5 ml of liquid yeast extract medium and incubated for 24 hr; then sporulation experiments were started. After 48 hr of incubation, microscopy revealed that asci had developed in the sporulation cultures of the secondary colony organisms, whereas no asci were found in the cultures of the primary colony organisms. Repeated transfers of the latter to fresh liquid medium were made, and at each transfer a sporulation experiment was started. Not a single ascus was ever seen in any of the sporulation cultures of these organisms. Prolonged incubation (8 to 10 days) had no effect. It should be mentioned that the secondary colony organisms never sporulated as long as the cells were in contact with cycloheximide on agar medium.

It has already been stated that the secondary growth first becomes visible on the top of a small, smooth primary colony. It was thought that this secondary growth resulted from a sudden onset of multiplication of cells (or one single cell) lying close to the agar surface in the middle of the pri-

FIG. 9. Stained nuclei in normal organisms; 8 days, 28 C. (Micron marker on Fig. 12 applies to Fig. 9 to 13.)

FIG. 10. Stained nuclei in primary colony organisms grown on solid medium containing 0.05 ppm of cycloheximide; 12 days, 28 C.

FIG. 11. Stained nuclei in secondary colony organisms of the same origin as in Fig. 10. The arrow indicates an enlarged cell with seven buds.

FIG. 12. Asci formed in sporulation culture by organisms from a large colony on cycloheximide-free agar medium after previous growth in liquid medium containing 0.05 ppm of cycloheximide; phase-contrast micrograph.

FIG. 13. Cells from a sporulation culture of a small colony of the same origin as in Fig. 12. No asci are seen; phase-contrast micrograph.

mary colony. To investigate the probability of this assumption, young primary colonies were transferred from an agar plate containing 0.05 ppm of cycloheximide to tubes containing liquid cycloheximide-free medium. The transfers were made as soon as the primary colony had appeared, viz., after 3 to 4 days of incubation. To be sure that all the cells were transferred, even a piece of underlying agar was picked. Only one colony was transferred to each tube, and sporulation experiments were started after 5 days of incubation. After 48 hr of incubation of the sporulation cultures, asci could be seen in all of them, although not in the same abundance as in sporulation cultures of normal organisms, probably indicating that some of the cells were of primary origin, whereas those forming spores were secondary. The same experiment was repeated, with the exception that the transfer was made by lightly scraping off the colonies from the cycloheximide agar, without removing any of the agar. When the transfer was done in this way, no asci could ever be seen in the sporulation cultures. This finding was confirmed in several experiments where material from primary colonies was transferred into fresh liquid medium, followed by a sporulation experiment at each transfer.

It was demonstrated previously by Gundersen and Wadstein (1962) that large and small colonies appear on cycloheximide-free agar if *S. pastorianus* is first allowed to grow in liquid medium containing cycloheximide. As it was thought that organisms in the large colonies should sporulate in contrast to those in the small ones, the following experiment was carried out. *S. pastorianus* was grown in liquid medium containing 0.05 ppm of cycloheximide for 48 hr; then a number of cells were spread on solid medium without cycloheximide. After 8 days of incubation, large and small colonies had grown out. Cells from a large colony proved to sporulate (Fig. 12), whereas spores never occurred in sporulation cultures from the small colonies (Fig. 13).

Oxygen consumption and carbon dioxide production. Respiration experiments were carried out according to current methods in a Warburg respirometer (Braun, model V 85) with the normal ATCC and PL strains as well as with their offspring, the primary and the secondary colony organisms. The ATCC strain resistant to 5 ppm of cycloheximide (Gundersen and Wadstein, 1962) was also included. These experiments were carried out with exponentially growing cells har-

vested from yeast extract agar not containing cycloheximide. With the normal organisms and the secondary colony organisms, the age of the cell material at harvest was between 24 and 48 hr; with the slower growing primary colony organisms and the resistant organisms, the cells were harvested between the 48th and the 96th hours. To the Warburg flasks were added: 1 ml of yeast suspension corresponding to 1.4 mg (dry weight) of cells, 1 ml of 0.067 M phosphate buffer (pH 6.0) plus cycloheximide to give a final concentration of the antibiotic of 1 ppm, and 1 ml of 0.1 M D-glucose. To the center cup of the flasks was added 0.2 ml of 10% KOH. The temperature of the water bath was 28 C, and the shaking rate was 90 strokes per min. Carbon dioxide was determined by the direct method described by Umbreit, Burris, and Stauffer (1959).

The results of the respiration experiments are shown in Table 2. There seems to be no significant difference in gas exchange between the secondary colony organisms and the normal ones of either the ATCC or the PL strain of *S. pastorianus*. Oxygen consumption and carbon dioxide production and, consequently, also the respiratory quotient (RQ) agree closely in these organisms. The primary colony organisms, and also the cycloheximide-resistant organisms, however, proved not to consume oxygen under these conditions. Moreover, the carbon dioxide production of the latter organisms exceeded the carbon dioxide production of the normal organisms and the secondary colony organisms by 2.5 to 3 times. A comparable result was obtained by Whelton and Phaff (1947) with a nonrespiratory variant of *S. cerevisiae*.

Growth under anaerobic conditions. The results of the respiration experiments in the preceding section could be evaluated in such a way that the primary colony organisms of *S. pastorianus* differed from the normal organisms in being dependent on a purely fermentative, instead of a combination of a respiratory and a fermentative, type of metabolism. The failure to observe any oxygen consumption during these experiments of relatively short duration would not necessarily mean that oxygen is not consumed at all by these organisms. To investigate whether multiplication of the primary colony organisms would be possible in the absence of oxygen, plates of yeast extract agar inoculated with the different kinds of organisms, including the organisms re-

TABLE 2. *Oxygen consumption and carbon dioxide production by Saccharomyces pastorianus in the presence and in the absence of cycloheximide*

Strain	Cyclohex- imide	Q _{O₂} *	Inhibi- tion by cyclohex- imide	Q _{CO₂} [†]	Inhibi- tion by cyclohex- imide	RQ
	ppm		%		%	
ATCC normal organisms	0	49.6		84.8		1.7
	1	41.4	17	65.0	24	1.6
ATCC primary organisms	0	0		229.4		∞
	1	0	—	163.1	29	∞
ATCC secondary organisms	0	48.0		85.7		1.6
	1	38.6	20	65.0	24	1.7
ATCC organisms resistant to 5 ppm of cycloheximide	0	0		220.8		∞
	1	0	—	206.0	7	∞
PL normal organisms	0	25.0		55.5		2.2
	1	22.3	11	48.8	12	2.1
PL primary organisms	0	0		140.9		∞
	1	0	—	128.6	9	∞
PL secondary organisms	0	32.5		73.3		2.2
	1	26.7	18	56.5	22	2.1

* Expressed as microliters of O₂ per milligram (dry weight) per hour.† Expressed as microliters of CO₂ per milligram (dry weight) per hour.

sistant to 5 ppm of cycloheximide, were incubated for 4 to 7 days in Brewer jars in atmospheres of (i) air, (ii) nitrogen (O₂ content, 0.05% (v/v) or 0.4 mm of Hg, calculated), and (iii) hydrogen (O₂ content nil).

No growth took place in the hydrogen jars with either of the organisms (although growth was later resumed on subsequent aerobic incubation), whereas all of the organisms grew in air and in nitrogen. The primary colony organisms and the resistant ones, grew considerably better in a nitrogen atmosphere than in air, indicating that oxygen might be partly inhibitory to the growth of these organisms (Table 3). In these experiments, cycloheximide was not incorporated in the medium.

DISCUSSION

In considering the properties of the so-called primary colony organisms of this investigation, it soon becomes apparent that the organism corresponds very closely to the "petite colonie" yeast mutant investigated in detail by Ephrussi and his co-workers (Ephrussi, Hottinguer and

TABLE 3. *Growth of Saccharomyces pastorianus under aerobic and anaerobic conditions**

Strain	Aerobic		Nitrogen	
	No. of colonies	Diam (mm)	No. of colonies	Diam (mm)
ATCC normal organisms.....	59	4.0	62	4.1
ATCC primary organisms.....	64	2.2	60	3.5
ATCC organism resistant to 5 ppm of cycloheximide.....	65	2.0	68	3.4
PL normal organisms...	68	2.4	56	2.2
PL primary organisms..	71	1.2	58	2.2

* The values given indicate averages of colonies on three agar plates. Incubation: 5 days at 28 C. No organisms grew in hydrogen.

Chimenes, 1949; Slonimski, 1949; Tavlitzki, 1949). It also resembles a nonrespiratory variant of *S. cerevisiae* described by Whelton and Phaff (1947), the "secondary colony variant" described by Wild and Hinshelwood (1956), and several

similar variants formed by the action of many different agents, e.g., acriflavine, crystal violet, sodium azide, salts of heavy metals, etc. These organisms are all characterized by respiration deficiencies and slow growth. The organism investigated by Whelton and Phaff (obtained by treatment with ethylene oxide) also had other characteristics in common with the present primary colony organism, viz., a smaller cell size than normal cells, inability to form ascospores, and a much higher rate of fermentative carbon dioxide production.

In a recent review (Nagai, Yanagishima, and Nagai, 1961) organisms of this type were designated RD mutants (respiration-deficient mutants). Nuclear disturbances and changes in cytoplasmic properties, e.g., a reduction in the number of cytoplasmic granules, as well as several other anomalies in these organisms have been recorded.

As far as is known, respiration-deficient organisms formed by the action of cycloheximide have not previously been described. On the other hand, in view of the observation that cycloheximide has been found to interfere with the synthesis of protein and nucleoprotein (Kerridge, 1958) and to inhibit certain dehydrogenases in yeasts (Latuasan and Berends, 1958), this effect is not surprising.

Different explanations may be suggested to account for the events leading to the splitting of the normal organisms into two morphologically and metabolically different types when *S. pastorianus* is grown in media containing cycloheximide.

The most probable explanation of the origin of the secondary colony organisms seems to be that it is the inoculum cell which, after having overcome a period of growth depression, finally starts to grow normally. This theory is supported by the results obtained in the sporulation experiments.

The origin of the primary colony organisms is more difficult to understand. The possibility that a selection by cycloheximide of natural cycloheximide-resistant small colony mutants has taken place can be eliminated, since it was shown that every cell of a normal-organism suspension, which was spread on an agar plate containing cycloheximide, gave rise to colonies consisting of primary and secondary organisms.

A probable explanation might be that the primary colony organisms have arisen from one

of the younger buds of a cell aggregate formed by the action of cycloheximide. A typical cell aggregate is shown in Fig. 7. During repeated nuclear divisions, and the growth and reproduction of cytoplasmic structures in the expanding cell aggregate, increasing damage has probably occurred both in the nucleus and in the cytoplasm of every new-formed bud. However, it remains to be explained how these events lead to the formation of a colony consisting of primary respiration-deficient organisms, followed by a secondary growth of the same colony consisting of organisms with normal respiration and morphology.

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