JOURNAL OF BACTERIOLOGY, July 1975, p. 118-122

Vol. 123, No. 1
Printed in U.S.A.

Mutant of the Yeast *Saccharomycopsis lipolytica* that Accumulates and Excretes Protoporphyrin IX

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Received for publication 11 April 1975

The red, water-insoluble pigment excreted by a mutant strain of the yeast *Saccharomycopsis lipolytica* is shown to be protoporphyrin IX. In genetic crosses the red phenotype has the properties characteristic of a defect in a single, recessive nuclear gene. The yield and ease of harvest of protoporphyrin IX from the yeast mutant indicate that this strain or its derivatives may be a valuable source of this substance.

Approximately 4 years ago an ascogenous strain of *Candida lipolytica* was isolated which opened up the possibility of a genetic study of this organism (15). Since this initial discovery of sexuality many genetic techniques have been developed which include methods for hybridization, mutant selection, sporulation, ascospore dissection, and genetic complementation (2, 3, 13). Following the proposal of Yarrow, the strains of *C. lipolytica* exhibiting sexuality will be referred to as *Saccharomycopsis lipolytica* (17).

A primary goal in developing the genetics of *S. lipolytica* was to study the genetic control of hydrocarbon metabolism, and for this purpose several mutant hunts for alkane-negative strains have been undertaken. In addition to the alkane-negative strains many other mutants with various phenotypes were identified during these experiments. These include auxotrophic, morphological, and pigmented strains (13). In contrast to unpigmented wild-type cells the color mutants include several strains that turn green on agar plates and a single one that forms red-pigmented colonies. This report describes some of the genetic and biochemical properties of the "red" mutant.

MATERIALS AND METHODS

Strains of S. lipolytica. The following strains were used: CX39-74B, genotype ± *trp1* ± *B* (haploid); B214, genotype *pop1 trp1* ± *B* (haploid); CX161-1B, genotype ± ± *ade1 A* (haploid); and CX270, genotype (B214 × CX161-1B) (diploid). Strain B214 was derived from CX39-74B by treatment with ultraviolet light (13). *pop1* refers to porphyrin accumulation (4). *A* or *B* denotes the bipolar mating type (3).

Media and genetic analyses. The compositions of the various media have been previously described (2). The methods employed for mutant induction and the genetic analyses have also been described (2, 3, 13).

Whole-cell preparations. Cells were grown to stationary phase either on YM agar plates (9 days, 23 C) or in aerated liquid YM medium (5 days, 23 C, New Brunswick rotatory shaker, 300 rpm). The plate cultures were scraped off the agar, suspended in distilled water, centrifuged (20,000 × *g* for 30 min), and the colorless supernatant fluid was decanted. Next the cells and overlaying red-brown precipitate were suspended in distilled water, divided into two aliquots, and recentrifuged (20,000 × *g* for 30 min). Cells from liquid medium were harvested, washed, and divided in the same manner.

Spectroscopic analyses and dry weight determinations. One aliquot of cells and colored sediment were suspended in pyridine, boiled for 10 min, and centrifuged to clarify. The spectrum of the colored supernatant fluid was analyzed on a Cary model 14 recording spectrophotometer. Yield of protoporphyrin IX was estimated by the molar extinction coefficient at 506 nm (7).

Dry weights were determined by vacuum drying the second aliquot of cells for 2 h at 90 C in a Thelco model 10 drying oven. The dried cells were cooled in a dessicator before being weighed in tared pans.

RESULTS

Isolation and phenotypic properties of the red mutant. The red mutant was the only one of its kind observed among approximately 2 × 10^5 colonies grown from cells mutagenized with ultraviolet light (13). The phenotype of the red strain is quite striking; on YM agar plates clones start to develop a red pigmentation within 24 h, and the color continues to deepen until at the end of 9 days of incubation (23 C) the colonies are a deep red-brown.

Different media and growth substrates were examined for their effect on the rate and intensity of pigment formation. With a given carbon

source color formation is enhanced in media enriched with peptone, yeast extract, and malt extract (YM medium) in comparison with synthetic basal medium. Variations in the carbon source resulted in differences in the rate of pigment formation. Acetate and *n*-hexadecane in place of glucose induce a more rapid coloring of the colonies even though growth is considerably slower. After 9 or 10 days of incubation, however, differences tend to disappear and colonies are deeply pigmented on all three substrates. The red phenotype can be recognized quite clearly after 48 h of incubation (23 C) on YM medium, and these conditions were adopted for scoring this marker.

The accumulation of the red pigment is apparently not seriously deleterious to growth. In liquid YM cultures the mutant gave a stationary-phase cell count of 6.8×10^7/ml compared to 8.0×10^7/ml for the parental strain, and on solid media mutant colonies were only slightly smaller than wild type.

In addition to hydrocarbons *S. lipolytica* can utilize certain fatty acids as carbon sources (3, 13). Surprisingly, colonies of the red mutant grown on plates with oleic acid as the carbon source remained white even after 10 or 12 days of incubation. The oleic acid was solubilized by use of a 2% concentration of the nonionic detergent Tergitol NP-40 (Sigma Chemical Co.), and this proved to be the reason for the lack of pigment formation. All the other media and carbon sources gave unpigmented colonies when Tergitol was added. Another detergent, Tween 40 (Nutritional Biochemicals), had a similar effect with the various media. Thus it seems that pigment formation is suppressed in the presence of these surfactants.

A deeply colored confluent growth of stationary-phase cells of the red mutant was scraped off the surface of YM plates, suspended in water, and centrifuged (10,000 × *g* for 10 min). A layer of flocculent, red-brown precipitate was found above the layer of cells which now appeared almost colorless. The supernatant fluid could be cleared of any remaining pigment by increasing the speed and time of centrifugation to 20,000 × *g* for 30 min. It is unlikely that the precipitate was the result of lysis and the release of intracellular contents since an examination of the pellet revealed little debris and no lysed cells in several microscopic fields. Thus the pigment appears to be excreted from the cells in a water-insoluble form. Under the microscope the colored precipitate consisted of irregularly shaped, red-brown particles up to 2 μM in size.

Spectroscopic analysis of the red pigment. Figure 1 presents the visible spectrum of the red pigment solubilized in pyridine. In addition to the four bands shown in Fig. 1, there was a strong absorption maximum in the Soret region (around 400 nm). This kind of spectrum is characteristic of an aetio-type free porphyrin and the positions of the maxima are identical to those reported for a naturally occurring porphyrin of this type, protoporphyrin IX (7). The relative heights of the absorption maxima are also in excellent agreement with the published values for protoporphyrin IX (Table 1). Furthermore the four-banded visible spectrum changed into a two-banded spectrum when a solution of the pigment in 0.1 M tris(hydroxymethyl)aminomethane (pH 7.1) plus 0.5% sodium dodecyl sulfate was acidified with HCl. The two new absorption bands were at 557 and 602 nm and gave a 557 nm/602 nm ratio of 2.1:1. This kind of spectral change is characteristic of the transition from a porphyrin-free base to the dication under acidic conditions and the values for the position and heights of the absorption maxima once again closely agree with those published for protoporphyrin IX (7). In accord-

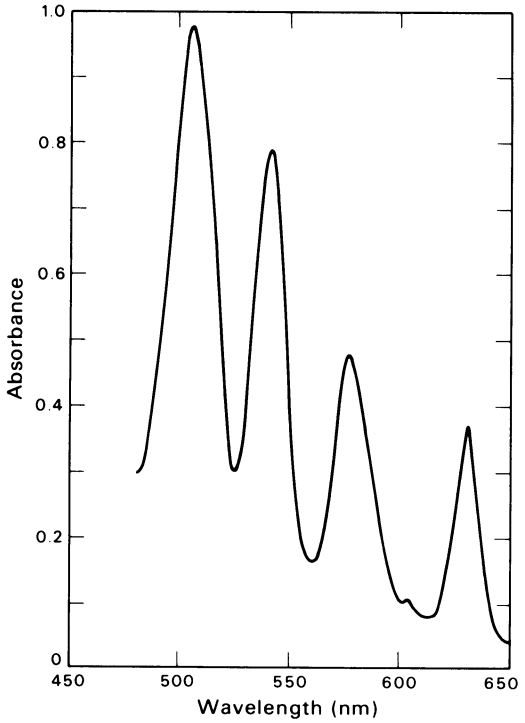

FIG. 1. *Four-banded visible absorption spectrum of the water-insoluble red pigment excreted by a mutant of S. lipolytica. The pigment was solubilized in pyridine as described in Materials and Methods.*

TABLE 1. *Spectroscopic properties of the red pigment accumulated by B214 popl trpl B compared to published values for protoporphyrin IX*

Absorption maxima (nm)	Ratio $\frac{A_x}{A_{631}}$ [a]	
	Published values of protoporphyrin IX[b]	B214 Red pigment
631	1.000	1.000
576	1.35	1.30
541	2.15	2.13
506	2.69	2.62
409	29.42	28.80

[a] Absorbance at x no. of nanometers divided by absorbance at 631 nm.

[b] See reference 7.

ance with the commonly accepted nomenclature for porphyrin-accumulating mutants, the gene responsible for the red phenotype is designated *popl* (4).

Genetic analysis. The *popl* mutant (B214 *popl trpl B*) was crossed to an unpigmented strain of opposite mating type. The heterozygous diploid remained unpigmented after 15 days of incubation indicating that *popl* is recessive to its wild-type allele. After sporulation the diploid was dissected, and *popl* was found to exhibit Mendelian segregation among the haploid spore clones (*popl*:± = 37:43). Furthermore an excess of parental over recombinant segregants (P/NP = 65/15) indicated linkage between *popl* and another marker in the cross (*adel*). This is the first case of intergenic linkage (18.8 map units) we have found in *S. lipolytica*.

Pigment formation among the *popl* segregants from the *popl* by wild-type cross (CX270) appeared to be strain dependent. The parental *popl* strain and some of the segregants produced very little pigment on synthetic medium plus glucose in contrast to certain other strains which became deeply colored on the same medium. The differences in pigment formation between strains were also apparent on YM medium although the contrast was not so pronounced. This result suggested that pigment production may be increased by coincidentally segregating modifying genes. The patterns of reversion among the *popl* segregants were likewise complex. The original mutant as well as segregants of a similar phenotype were never observed to revert to an unpigmented phenotype either spontaneously or among clones from approximately 2×10^7 cells which had been mutagenized with ultraviolet light. Another phenotypic class includes strains that were considerably darker on synthetic medium, grew almost as rapidly as the original mutant, and reverted spontaneously to a phenotype showing lighter pigmentation resembling that of the original mutant. Also observed were some slow-growing, deeply pigmented strains that reverted spontaneously to a faster-growing phenotype with lighter pigmentation or occasionally to a white revertant clone. Neither of the auxotrophic markers segregating in the cross (*adel*, *trpl*) co-revert with or correlate with the various *popl* phenotypes.

Yield of protoporphyrin IX from various popl strains. In Table 2 are presented quantitative determinations of the amount of protoporphyrin IX produced by strains representative of the various *popl* phenotypes. Visual impressions of the relative amounts of pigment produced by the strains on YM media are clearly reflected in the yields of protoporphyrin IX. Cells grown in aerated liquid medium produced less of the red-brown precipitate than those from agar plates and this, too, was reflected in the relative yields of protoporphyrin IX from liquid as compared to solid medium.

DISCUSSION

The close fit of the position and heights of the absorption maxima in Fig. 1 to the values published for protoporphyrin IX indicate that the red pigment is composed primarily of this material. Pigmented compounds that contain protoporphyrin IX or are chemically related to it include the metalloporphyrins, other porphyrin species and the hemoprotein enzymes such as the cytochromes or catalase. These

TABLE 2. *Yields of protoporphyrin IX from various popl strains of S. lipolytica*

Strain	Relative growth[a]	Color and relative intensity[a]	Yield[b] (mg of protoporphyrin IX/g (dry wt of cells)
CX39-74B ±	++++	White	<0.01 (plates)
B214 *popl*	+++	Red +	2.7 (plates)
CX270-40 *popl*	+++	Red ++	4.5 (plates)
CX270-40 *popl*			0.9 (liquid)
CX270-27 *popl*	+	Red ++	4.6 (plates)
CX270-27 *popl*			2.2 (liquid)

[a] Visual estimates of color intensity and growth were made on clones incubated for 9 days on YM plates (23 C).

[b] The red pigment was solubilized in pyridine and analyzed as described in Materials and Methods. A complete spectrum was recorded for all three *popl* strains to confirm the identity of the pigment as protoporphyrin IX. Yield was estimated by the molar extinction coefficient at 506 nm (7).

compounds also have strong and unique absorption maxima in the visible and Soret regions so that their presence in other than trace amounts could be detected in the spectrum of the pigment produced by the *pop1* mutant.

The accumulation of porphyrins has been observed in many microbial species including both photosynthetic and heterotrophic bacteria, yeast, and protozoa (10). The free porphyrins accumulated by these organisms are usually coproporphyrin III, coproporphyrin I, uroporphyrin I, or a mixture of different porphyrin species. Mutants of the yeast *Saccharomyces cerevisiae* have been isolated which accumulate coproporphyrin or complex mixtures of free porphyrins along with zinc metalloporphyrins (12, 14).

Mutants of *Escherichia coli* phenotypically very similar to the *pop1* strains of *S. lipolytica* have been studied by Cox and Charles (4). The *E. coli* mutants also were isolated on the basis of colony color, and the water-insoluble pigment they accumulate consists of a single porphyrin species, protoporphyrin IX. The major difference between the yeast and the bacterial *pop1* phenotypes appears to be in the relative yield of accumulated porphyrin. Under similar conditions (aerated liquid media) the yeast *pop1* strains yielded from 60 to 144 times more protoporphyrin IX per gram (dry weight) than did the *E. coli* mutant. If the yeast are grown on solid medium, yields up to 360 times that reported for the *E. coli pop1* mutant can be obtained.

The kinds of genetic lesions that might be responsible for the accumulation of protoporphyrin IX have been discussed by Cox and Charles (4). A block in heme synthesis could result in the accumulation and precipitation of water-insoluble protoporphyrin IX, a metabolite prior to heme in the synthesis of hemoproteins such as the cytochromes. Mutations that result in a defective ferrochelatase or a reduced cellular permeability to iron could have this effect. Alternatively *pop1* could represent a regulatory defect in porphyrin biosynthesis. This is perhaps more likely since *S. lipolytica* is an obligate aerobe and therefore dependent on the cytochrome respiratory enzymes for survival. There is evidence from *Rhodopseudomonas spheroides* that 5-aminolaevulic synthetase, the first enzyme in the biosynthetic pathway leading to protoporphyrin IX and hemoproteins, is regulated by both feedback inhibition and repression and that heme is the regulatory metabolite (11). Since the *pop1* phenotype of *S. lipolytica* is recessive, dominant regulatory (operator) mutations can apparently be ruled out.

Thousands of tons of hydrocarbon-grown yeast are currently being produced as animal fodder, and production is expected to increase dramatically in the future (6). The utilization of a *pop1* strain in some of these large-scale fermentations would yield substantial quantities of protoporphyrin IX as a by-product. The current method of preparing protoporphyrin IX for research purposes utilizes blood as the starting material and involves numerous purification steps to remove chelated metals, protein, and other porphyrin species (7). It seems reasonable to propose that the *pop1* yeast strains described in this report could be of value as a source of this material. Protoporphyrin IX, its metal adducts, and related derivatives have potential medical applications. They have an affinity for lymphatic tissue (9), and other studies involve their use in tumor diagnosis (1, 16), tumor therapy (5), and the suppression of heterologous rejection in tissue or organ transplants (8). The most important application promises to be the synthesis of protoporphyrin IX specifically labeled with ^{14}C, ^{13}C, and ^{15}N; the latter two derivatives are useful in spin resonance studies with reconstituted hemoproteins.

ACKNOWLEDGMENTS

This work was supported by grant GB-43736 from the National Science Foundation, by U.S. Atomic Energy Commission contract AT-(40-1)-4047, and by a Special Faculty Fellowship from the National Institute of General Medical Sciences (1-F14-6m-56001-01).

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