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Characterization of Yellow Mutants Isolated from the Red Yeast *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*)

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Yellow mutants of the astaxanthin producing red yeast *Phaffia rhodozyma* were obtained by nitrosoguanidine mutagenesis. The carotenoid composition of the yelow mutants, Yan-1 and Ny-1, was mainly β-carotene (> 95%) and torulene (< 5%). Therefore, the yellow mutants are carotene oxygenation deficient mutants (CODMs). CODMs produced decreased quantities of carotenoids compared to their red parents and this indicated that carotene may regulate its synthesis. CODMs, Yan-1 and Ny-4, on plates containing 50 μM antimycin, showed highly pigmented vertical papillae. Antimycin-induced mutants purified from the papillae showed increases in carotenoid content (up to 1 mg β-carotene/g yeast). CODMs, Yan-1 and Ay-1, were more sensitive to antimycin than red strains, Ant-1 and 67-385. This was probably due to lower antioxidant activity of β-carotene than that of astaxanthin. Light increased torulene and light+antimycin further increased the torulene. Yan-1 and Ny-4 could grow with succinate, though their red parents, Ant-1 and Anf-1p, could not. However, antimycin induced mutation of Yan-1 or Ny-4 destroyed the ability to grow with succinate.

The intact cell color of the wild-type yeast *Phaffia rhodozyma* is orange to orangish-red due to carotenoids, mainly astaxanthin (5). The yeast was isolated during the early 1970's from exudates of deciduous trees in mountainous regions of Japan and Alaska (25). It was originally designated "*Rhodozyma montanae*" (25), but its unusual characteristics and the lack of a Latin description allowed the change of the genus to *Phaffia* (22), in honor of Herman Jan Phaff. Since the basidiospore and the conjugation of *P. rhodozyma* were observed, the new name *Xanthophyllomyces dendrorhous* was recently proposed for *P. rhodozyma* (10).

Astaxanthin is an abundant carotenoid pigment in the marine environment (33). In nature, it probably originates in certain algae, fungi and small crustacea. These organisms are at the beginning of a food chain that leads to pigmentation of larger animals including salmonids (18, 19, 27, 28), crustacea (32) and birds such as the flamingo (8). Astaxanthin is an important constituent of aquaculture feeds because it provides necessary pigmentation, and it or precursor carotenoids may also contribute to the distinctive flavor of baked salmon (17). Pigments are an expensive component of salmon diets, and world-wide farm-rearing of salmon is expected to produce more than 400,000 metric tons in 1995 (2). Because of the increasingly strict regulations concerning the safety of chemicals as food additives, and the poor absorption of synthetic carotenoids compared to biological sources, natural carotenoid products may be given preference over synthetic pigments as color enhancers.

The yeast *P. rhodozyma* has been considered as a colorant for aquacultured animals (13, 16). The nutritional value of this yeast was good in the salmonid diet (16). The carotenoid absorption efficiency of processed yeast by salmon was superior to that of purified carotenoid. Also, this yeast can possibly be used for poultry feed to enhance the pigmentation of egg yolks (15). In some cases, an yellow colorant is preferred. For example, for the proper pigmentation of egg yolk, the yellow colorant can be added to poultry feed as a substitute for yellow pigments. The yellow colorant is also used in some countries for aquaculture. Purified β-carotene, for example, is used for some fresh-water fish culture in South Korea because of the preference for yellow coloration.

Yellow mutants are not only potentially useful for animal feed but also important for studies on the astaxanthin biosynthetic pathway and the regulation of *P. rhodozyma*. However, only few reports are available. Girard *et al.* (9) reported 3 kinds of color mutants that were defective in β-carotene oxygenation, phytoene dehy-

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drogenation, and phytoene synthesis. The authors proved part of the astaxanthin biosynthetic pathway proposed by Andrewes *et al.* (5). Schroeder and Johnson (29) reported that yellow mutants are more susceptible to oxygen radicals. In this study, I have isolated yellow mutants of *P. rhodozyma*, observed physiological changes in the yellow mutants and characterized them. Also, I report here on the presence of torulene in *P. rhodozyma* for the first time.

METHODS AND MATERIALS

Chemicals

Antimycin A (a mixture of antimycins A_1 and A_3; Sigma catalog #2006), β-carotene, N-methyl-N'-nitro-nitrosoguanidine, ethylmethane sulfonate, glucose, succinate, and hydrogen peroxide were purchased from Sigma Chemical Co., St. Louis, MO. All other inorganic chemicals were obtained in the highest grades available. Purity and sources of other chemicals used in this study have been described (3).

Yeast Strains and Growth

The sources of *P. rhodozyma* strain 67-385 (natural isolate) and other mutants were described in Table 1. Yeasts were maintained on 2% agar plates or slants in the refrigerator (4°C), or stored in a deep freezer (-70°C) as previously described (3).

P. rhodozyma was grown in 30 ml YM broth (yeast extract/malt extract/peptone/dextrose: Difco Co.) in 300 ml baffled flasks shaken at 150 rpm on an orbital shaker controlled at 20°C. Media were unbuffered and the initial and final pHs were 6.0 and 5.0, respectively, which was not inhibitory to yeast growth (in the presence of 0.2 μM antimycin the final pHs were lowered to 4.8). When antimycin was included in the media, it was first dissolved in a small quantity of ethanol (final concentration: less than 0.1%). Growth in liquid media was measured by determining the optical density at 660 nm as previously described (3).

Table 1. Description of *P. rhodozyma* strains used in this study.

Strain	Method of isolation	Parent	Main carotenoid
	Strains mentioned or used in this study		
67-385	Natural isolate	-	Astaxanthin
Ant-1	Antimycin agar[a]	67-385	Astaxanthin
Dant-1	Antimycin sensitivity	Ant-1	Astaxanthin
AAN-4	NTG[b] mutagenesis	Dant-1	Astaxanthin
2A2N	NTG mutagenesis	AAN-4	Astaxanthin
Anf-1p	NTG mutagenesis	2A2N	Astaxanthin
	Strains obtained and used in this study		
Yan-1	NTG mutagenesis	Ant-1	β-carotene
Ay-1, Ay-2, Ay-s1, Ay-s2, Ay-b1, Ay-b2	Antimycin agar	Yan-1	β-carotene
Ny-1, Ny-4	NTG mutagenesis	Anf-1p	β-carotene
ANy-1	Antimycin agar	Ny-4	β-carotene

[a]Antimycin agar, YM agar containing 50 μM antimycin (3). [b]NTG, N-methyl-N'-nitro-N-nitosoguanidine.

Antimycin-induced Mutation

Antimycin-induced mutation was performed by inoculation of the yeast on the YM agar plates containing 50 μM antimycin as described previously (3). These plates were incubated at room temperature for 1-2 months. After vertical papillae were obtained, they were purified on YM agar plates.

Nitrosoguanidine Mutagenesis

N-methyl-N'-nitro-nitrosoguanidine (NTG) mutagenesis was carried out by washing yeast cells in sodium citrate buffer (0.1 M, pH 5.5), resuspending them in the buffer, adding NTG (final conc.: 40 μg/ml), incubating for 20 min (more than 95% kill), washing with 0.1 M potassium phosphate (pH 7.0), and incubating the yeast in YM broth overnight (3). The yellow colonies on YM plates were isolated by visual examination.

Carotenoid Extraction and Analysis

For the quantitative analysis of carotenoid contents, one half to one ml of yeast culture was centrifuged and washed with distilled water. One ml of dimethylsulfoxide (DMSO) was added to dried yeast (31). Yellow mutants were pretreated with acetone before adding DMSO. One ml of acetone, petroleum ether and 20% sodium chloride solution were added serially. Upper petroleum ether layers were used for carotenoid quantification. The extinction coefficient ($E^{1\%}_{1\,cm}$) at 452 nm in petroleum ether of β-carotene used was 2592 (7). Experiments were repeated at least twice in independent trials to accurately assess carotenoid production

Individual carotenoids were analyzed by thin layer chromatography (TLC) and electronic absorption spectra (3). To prepare carotenoid extracts for chromatography, petroleum ether partitions were dried over anhydrous $CaCl_2$ and concentrated by evaporation in a stream of nitrogen. These were chromatographed on silica gel plates (Silica gel 60, 5×20 cm, 0.25 mm thickness, E. Merck, Darmstadt, West Germany) using 10% acetone/90% petroleum ether. After development, bands were scraped and eluted in acetone. Absorbance maxima (5, 7), R_f values (7), and cochromatography with standards (β-carotene and *trans*-astaxanthin) were used for identification of the pigments. Carotenoid concentrations were calculated using the extinction coefficients listed by Davies (7).

RESULT

Isolation of *P. rhodozyma* Yellow Mutants

Yellow mutants of *P. rhodozyma* were isolated by ni-

trosoguanidine mutagenesis. Absorbance spectra and TLC of carotenoid extract indicated that the yellow mutants, Yan-1 and Ny-1, contained β-carotene (more than 95%), torulene (1) and two unknowns (unknown #1 and #2) that were seemed like derivatives of torulene (Table 2). Trace quantities of torulene, unknown #1 and unknown #2 were also found in the astaxanthin producing red yeasts. The yellow mutants do not contain oxygenated carotenoid and thus they are carotene oxygenation deficient mutants (CODMs).

The carotenoid contents of CODMs severely decreased, compared to their parents. Three day old YM cultures of Yan-1 and Ny-4 produced 310 and 480 μg β-carotene/g yeast, whereas their red parents Ant-1 and Anf-1p produced about 900 and 1500 μg β-carotenoid/g yeast. The prolonged incubation of *P. rhodozyma* increased the carotenoid content (Table 3). However, the increase in the total carotenoid contents of Yan-1 or Ny-4 during the stationary phase was lower than that of Ant-1 (Table 3).

Antimycin-induced Mutation of CODMs

Strains, Yan-1 and Ny-4, produced highly pigmented vertical papillae (Fig 1a arrow I) on white star-shaped colonies (Fig 1a arrow II) when they were incubated on YM agar plates containing antimycin 50 μM for 1-2 months. Replating of type II colonies produced none or few colonies that showed the same morphology as the parents. Therefore, the white colonies were composed of dead cells and few parental-type live cells. Purification of the vertical papillae on YM agar plates produced smaller colonies than the parents (Fig 1b and 1c). Antimycin-induced mutants (Fig. 1 arrow III, IV, and V) of Yan-1 and Ny-4, produced higher quantities of carotenoids than the parents (Table 4). The antimycin-induced mutants varied in the contents of carotenoids and they produced more torulene (Table 5).

Physiological Changes in CODMs of *P. rhodozyma*

We reported that antimycin and light together induce the secondary respiratory chain which is resistant to antimycin (4). The induction of secondary respiratory chain improves carotenoid production up to 2-fold (4). Light alone increased the torulene content of strain Ny-4. Antimycin helped light but without light it decreased carotenoid production. Light+antimycin did not increase carotenoid production of strain Ny-4. This is probably due to the high sensitivity of CODMs to oxygen radicals (Fig. 2). Antimycin-induced mutants, Ant-1 and Ay-1, are more sensitive to antimycin than their parents, 67-385

Table 2. Properties of the carotenoids in yellow mutants of *P. rhodozyma*.

Carotenoid	Absorbance maxima (nm)[a]				Rf[b]
trans-Astaxanthin[c]			477		0.07
Phoenicoxanthin[c]			474		0.15
Unknown #1	382	461.5	487	518	0.19
Unknown #2	378	458.5	487	518	0.87
Torulene	391	461	487	518	0.90
β-Carotene		429	452	481	0.93

[a]Spectra recorded in acetone. [b]TLC solvent system was 10% acetone/90% petroleum ether on silica gel 60 with 0.25 mm thickness. [c]Not found from yellow mutants.

Table 3. Carotenoid production during the stationary phase in YM broth.

Strain	Incubation (day)	Growth (g/l)	β-Carotene (μg/g yeast)	β-Carotene (μg/l medium)
Yan-1	2	2.15	220	480
	5	4.17	390	1620
	14	3.57	490	1740
Ny-1	2	2.14	230	490
	5	4.24	440	1850
	14	3.43	590	2030
Ant-1[a]	3.5	2.46	930	2290
	8	2.13	1070	2280
	18	2.00	1650	3120

[a]Independent experiment from the experiment with Yan-1 and Ny-1.

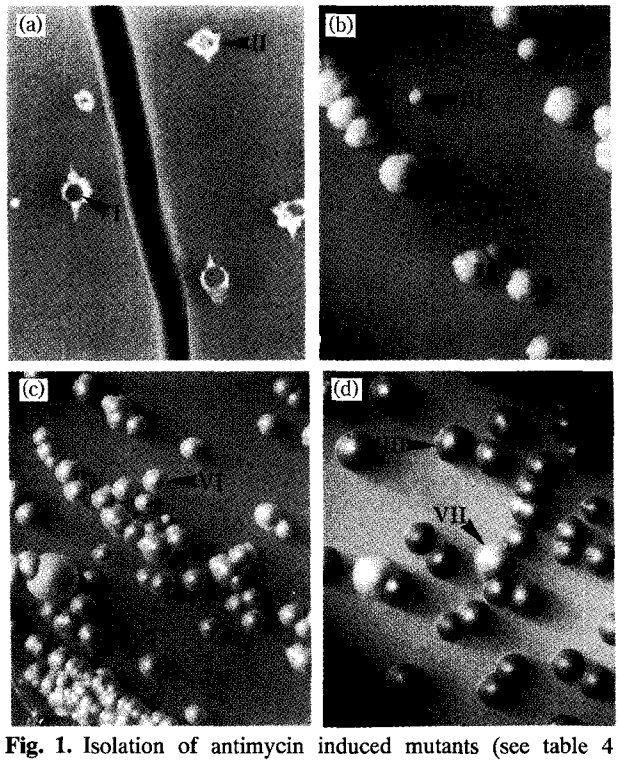

Fig. 1. Isolation of antimycin induced mutants (see table 4 for the description of arrows I-VIII).

a, Yellow mutant Ny-4 grown for 2 months on YM agar plate containing antimycin 50 μM; b, Replated vertical papilla of Yan-1 on YM agar plate; c, Replated vertical papilla of Ny-4 on YM agar plate; d, Ant-1 and 67-385 on YM agar plate.

and Yan-1, are (Fig 2). Interestingly, CODMs, Yan-1 and Ay-1, were much more susceptible to antimycin than red strains, 67-385 and Ant-1, were (Fig. 2). This was probably due to the excellent antioxidant activity of oxygenated carotenoids over β-carotene (21).

Antimycin-induced mutants grew lesser with succinate plus yeast extract 0.5% (Table 7). With succinate, CODMs produced more carotenoid than with glucose whereas red yeast did not.

Table 4. Carotenoid content of antimycin induced mutants.

Strain	Type in Fig. 1	Growth (g/l)	Carotenoid (μg/g yeast)	Carotenoid (μg/l medium)
Yan-1	V	3.18	400	1277
Ay-1	VI	2.84	960	2709
Ay-2	VI	3.27	740	2426
Ay-s1	III	3.80	580	2201
Ay-s2	III	2.74	800	2191
Ay-b1	IV	2.84	450	1291
Ay-b2	IV	3.16	440	1389
Ny-4	V	3.32	580	1915
ANy-1	VI	2.88	890	2564
67-385	VII	3.88	420	1638
Ant-1	VIII	3.66	1190	4365

Colonies were transferred to YM broth and grew for 3 days. The proper amount of yeasts were transferred to YM broth again and grown for 1 week. Finally 0.3 ml of fully grown yeast were inoculated into 30 ml of YM broth and grown for 4 days.

Table 5. Carotenoid composition of yellow mutants.

	% Carotenoid		
Strain	Yan-1	Ay-s1	Ay-1
Unknown #1	1.2	5.7	3.0
Unknown #2	1.7	2.5	2.1
Torulene	2.5	15.7	7.5
β-Carotene	94.5	72.2	81.2

DISCUSSION

Antimycin-induced mutants contain a higher level of heme containing catalase than their parents do (29). Antimycin-induced mutants appear to hydroxylate and desaturate carotenoid intermediates more readily as shown by analysis of the pigments (3), but appeared less prone to

Table 6. Effect of light and antimycin on growth and carotenogenesis of *P. rhodozyma*.

Condition	Dark	Dark+ Antimycin (0.2 μM)	Light	Light+ Antimycin (0.2 μM)
	% Carotenoid			
β-Carotene	94	95	90	87
Torulene	6	5	10	13
Unknown #2	0	0	tr	tr
Unknown #1	0	tr	tr	tr
	Growth (g yeast/l medium)			
	3.26	3.96	3.22	3.50
	Carotenoid (μg/g yeast)			
	650	640	500	540

Yeasts were grown under four 20 watt fluorescence bulbs, at a distance of 15 cm for 6 days at 20°C in YM broth.

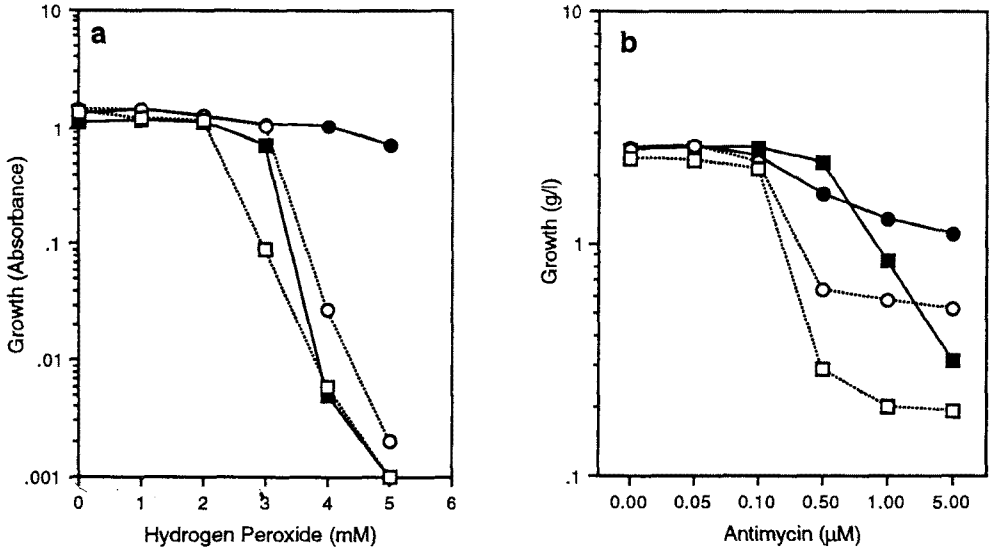

Fig. 2. Gorwth of *P. rhodozyma* strains with hydrogen peroxide and antimycin.
67-385, (-●-); Ant-1, (-■-); Yan-1, (-○-); Ay-1, (-□-). a, Hydrogen peroxide; b, Antimycin.

Table 7. Growth and carotenoid contents of antimycin induced mutants.

Strain	Carbon			
	Glucose 1%		Succinate 1%	
	Growth (g/l)	Carotenoid (μg/g yeast)	Growth (g/l)	Carotenoid (μg/g yeast)
Yan-1	4.25	370	1.48	480
Ay-1	3.80	700	0.54	710
Ay-s2	3.72	470	0.66	680
67-385	4.17	520	1.82	460
Ant-1	4.25	918	0.54	849

Yeasts grew in 0.5% yeast extract and 1% glucose or 1% succinate at 20°C for 5 days on orbital shaker.

carry out ring closure (Table 5). These reactions are known to be carried out by heme containing cytochrome-P-450 in sterol and steroid biosynthesis in yeasts and other organisms (11, 12, 20, 32). The P-450 system is involved in demethylation, desaturation and oxygenation of sterol intermediates (12, 32). Certain cytochrome P-450's have been postulated to have coevolved with sterol metabolism (23). The morphology of CODMs obtained from red antimycin induced mutants was very similar to the morphology of wild strains (Fig. 1). CODMs are deficient in oxygenation of carotenes that is probably performed by a heme containing enzyme. Antimycin is also related with heme, because it inhibits heme containing cytochrome b (12, 26). Therefore, carotenoid production is closely related to heme biosynthesis. Probably, certain heme mutations cause changes in morphology, in carotenoid contents, in carotenoid components, and in resistency to oxygen radicals.

Antimycin-induced mutation of antimycin-induced mutants was not possible, but that of strains, Yan-1 and Ny-4 (obtained from antimycin-induced mutatnts, Ant-1 and Anf-1P, respectively) was. Also, the morphology of CODMs from red strains showed the morphology of wild strains. CODMs showed increased carotenoid production and growth with succinate (Table 7), which indicated relations between mitochondria and carotenogenesis. An (1) also reported that mitochondrial structures of wild-type yeast 67-385 and mutant strain 2F-1 are different. The coincidence of mitochondrial mutation, color mutation, and morphological change indicates some relations between them.

Antimycin-induced mutation of CODMs produced new antimycin-induced mutants producing ~1 mg β-carotene/g yeast. However, carotenoid contents of yellow antimycin-induced mutants were still lower than those of red strains. β-carotene regulates its synthesis in *Phycomyces blakesleeanus* (6). *P. rhodozyma* might also have similar regulation to *Phy. blakesleeanus*, and thus the yellow mutants produce decreased quantities of carotenoids.

CODMs are more susceptible to DQ (29) and antimycin (Fig. 2) than red strains are. Also, white mutants were further more susceptible to DQ than yellow or red yeasts (29, 30). This is probably due to the protecting efficiency of oxygenated carotenoids over β-carotene or no carotenoid (21).

Oxygen radicals increased oxygenated carotenoids (3) and mono-cyclic carotenoids, 3,3'-didydroxy-3',4'-didehydro-β,ψ-carotene-4-one (HDCO) (3) and torulene (Table 6). Schroeder and Johnson (29) proposed that HDCO may come from the degradation of astaxanthin, since incubation of the red yeast decreased astaxanthin and increased HDCO. Johnson *et al.* (14) also proposed the pathway for the synthesis of β-zeacarotene that is produced by growth in a stressful condition. β-zeacarotene may also be a broken product of lycopene, neurosporene, or γ-carotene. Based on this idea, torulene also could be a broken product of β-carotene. However, the reactions are very specific, and thus this assumption is under question.

Many fungal species are generally regarded as safe (GRAS) by the regulatory authorities. No harmful effect of *P. rhodozyma* was reported in animal feeding tests (13, 15, 16). The β-carotene producing microorganism, *Blakeslea trispora*, produces 30 mg/g dry weight (24). The quantity of β-carotene produced by CODMs of *P. rhodozyma* is only 1/30 of that produced by *B. trispora*, at the present time. However, in the nutritional and safety aspects, after development of proper genetic manipulation, CODMs may be preferentially used for animal feed. Also, the yellow mutant is a potential genetic host for zeaxanthin, present in flavobacteria and corn.

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