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ζ-CAROTENE AND OTHER CAROTENES IN A *PHYCOMYCES* MUTANT

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Key Word Index—*Phycomyces*; carotene biosynthesis; phytoene; phytofluene; ζ-carotene; colour mutants.

Abstract—Strain S442, a new mutant of the fungus *Phycomyces blakesleeanus*, has a greenish colour and a distinct green fluorescence under long-UV light. Carotene analyses reveal the presence of phytoene, ζ-carotene, phytofluene, an unidentified compound, and neurosporene (in descending order of abundance). Genetic analysis shows that the new mutation occurs at gene *carB*, whose protein product catalyses the four dehydrogenations of phytoene to lycopene via phytofluene, ζ-carotene, and neurosporene. S442 offers no indication of a specific ζ-carotene dehydrogenase. The residual dehydrogenase activity in S442 is inhibited by diphenylamine. The high ζ-carotene content makes S442 a good source of this compound.

INTRODUCTION

The fungus *Phycomyces blakesleeanus* owes its yellow colour to all *trans* β-carotene; under our usual growth conditions other carotenes are present only as traces. Mutants of various colours differ from the wild type either in the concentration of β-carotene or in the presence of considerable amounts of pathway intermediates. Thus, *carS* mutants overproduce β-carotene and are deep yellow, *carR* mutants overproduce lycopene and are red, *carB* mutants overproduce phytoene and are white [1].

The conversion of phytoene to lycopene is mediated by a dehydrogenase, the product of gene *carB*; four copies of the dehydrogenase, assembled in an enzyme aggregate, perform the four dehydrogenations that convert phytoene successively into phytofluene, ζ-carotene, neurosporene, and lycopene [2]. Leaky mutations in gene *carB*, resulting in partially defective dehydrogenases, give rise to yellowish mutants, which contain in decreasing proportions phytoene and its desaturation products [3]. There is no hint of specific gene functions for the different dehydrogenations (there being no mutants specifically accumulating ζ-carotene or any other desaturation intermediate). The order of the four dehydrogenations is presumably determined by the three-dimensional structure of the enzyme aggregate.

Chemical inhibitors of the dehydrogenase have the same effects as leaky mutations in gene *carB*: mycelia grown in the presence of diphenylamine [4–7] and Δ^2-2-methylhepten-6-one [8] contain phytoene, phytofluene, ζ-carotene, and neurosporene.

The activity of the pathway is stimulated by a variety of external agents, such as illumination [9–11], retinol [12], and dimethyl phthalate [13]. These agents increase the β-carotene content and enhance mycelial colour.

This paper reports on a *Phycomyces* mutant with an unusual greenish colour and a peculiar carotene composition.

RESULTS

The deep-yellow strain C115 was used in a search for new colour mutants. The spores were treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, the survivors were plated out, and about 5×10^5 of the resulting colonies were screened visually for colour variations. Many mutants exhibited known phenotypes. A colony with a striking greenish colour was isolated, subcultured several times from single spores, and designated strain S442. The novel strain grows normally and its sporangiophores sprout out in haphazard directions, like those of the parent strain. Hyphae, sporangiophores, and sporangia of S442 shine distinctly green under a long-UV lamp (Sylvania Blacklite Blue P20T/2).

Strain S442 contains phytoene, phytofluene, ζ-carotene, an unidentified compound, and traces of neurosporene (less than 3 μg per g dry weight), but no β-carotene, γ-carotene, or lycopene. Mycelial colour and fluorescence remain largely stable for many days after the cessation of growth. The carotene content of dark-grown mycelia does not vary between days 3–12 of incubation (Fig. 1, left). The carotenes are gradually lost under the light; phytofluene is the most affected and phytoene the most stable (Fig. 1, right).

ζ-Carotene and the unidentified compound have the same absorption spectrum, but are easily resolved on aluminum oxide columns. If we assume that both compounds have the same extinction coefficient, the unidentified one is present at about 10 μg per g dry wt in all dark-grown mycelia and at about 30 μg per g dry wt in all light-grown mycelia.

The addition of yeast extract (1 g/l) to the minimal medium substantially accelerates growth, but does not affect the carotene content of 4-day-old mycelia (3545 μg phytoene, 127 μg phytofluene, 634 ζ-carotene, and 3 μg neurosporene per g dry wt).

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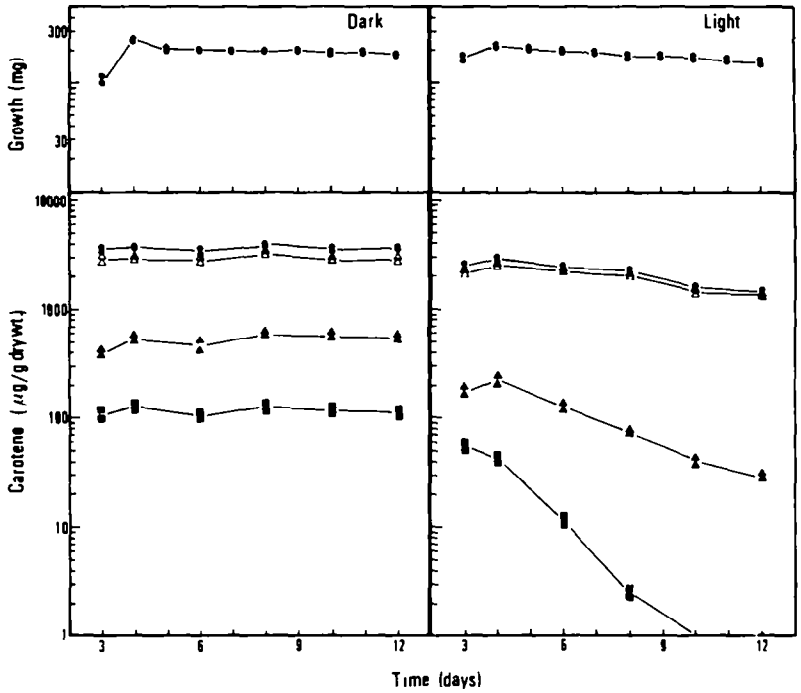

Fig. 1. Growth and carotene accumulation in strain S442 grown on minimal agar in the dark (left) or in the light (right). *Above*: mycelial dry wt per 8 cm Petri dish. *Below*: phytoene (△), phytofluene (■), ζ-carotene (▲), and sum of carotenes (●) in the dried mycelia.

The carotene analyses might lead to the suspicion that strain S442 has a specific defect in the dehydrogenation of ζ-carotene, perhaps a mutation affecting a hypothetical 'ζ-carotene dehydrogenase'. Complementation analyses (Fig. 2), on the contrary, reveal that S442 contains a *carB* mutation, and therefore a modified version of the enzyme responsible for all four dehydrogenations in the pathway. The critical observation is that heterokaryons C5 * S442 are in all respects intermediate between the C5 and S442 homokaryons. If strain S442 had a mutation in a gene other than the *carB* gene mutated in strain C5, the heterokaryons would contain the normal products of both genes and would produce β-carotene, like the wild type.

Retinol has practically no effect on the carotene profile of strain S442; dimethyl phthalate increases the total carotene content, owing to an extra accumulation of phytoene; diphenylamine does not affect the total, but increases the relative proportion of phytoene at the expense of the less saturated intermediates (Fig. 3).

DISCUSSION

The greenish colour of S442 stands out prominently against the deep-yellow background of strain C115, but would not be very noticeable against the wild type. This, and the extreme rarity of the mutation, explains why such mutants have not been found before. The best way to tell S442 apart from the wild type is to observe the strong green fluorescence of phytofluene under 'black' (ultraviolet B) light. The low frequency of the genetic change represented by strain S442 suggests a very specific modification of a gene product.

The high ζ-carotene content in strain S442 is not due to the loss of a gene product specifically needed for ζ-carotene dehydrogenation. The new mutation disrupts all four dehydrogenations and is genetically assigned to the gene *carB*, responsible for the common dehydrogenase.

The four dehydrogenations are not equally affected by the new mutation; if they were equally affected, one would expect decreasing concentrations of phytoene, phytofluene, ζ-carotene and neurosporene, as in the leaky *car B* mutants reported above [3]. About 84% of the carotene molecules in strain S442 remain as phytoene (failure of the first dehydrogenation); of those dehydrogenated to phytofluene, about 17% remain as such (failure of the second dehydrogenation); of those dehydrogenated to ζ-carotene, about 99% remain as such (failure of the third dehydrogenation); and no molecules are found to go beyond neurosporene.

The different failure rates of the four dehydrogenations are not contradictory with the structural modification of a single dehydrogenase coded for by gene *carB*. Four identical copies of an enzyme may tackle different substrates with varying effectiveness or may interact in different ways with their neighbours in the membrane.

The stimulation of the carotene pathway by dimethyl phthalate increases the accumulation of phytoene, but not of its derivatives. This indicates that the dehydrogenase in strain S442 works at top capacity in the absence of the phthalate.

The residual dehydrogenase activity in strain S442 is sensitive to diphenylamine; in the presence of 75 μM of this compound the failure rates are about 92% for the first dehydrogenation and 64% for the second; the other dehydrogenations are undetectable.

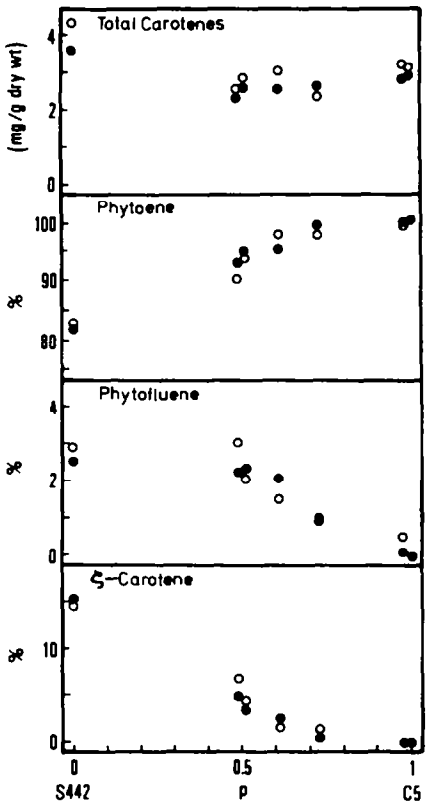

Fig. 2. Carotene analyses of five heterokaryons C5 ∗ S442 containing different proportions, *p*, of C5 nuclei; of the C5 homokaryon ($p = 1$), and of the S442 homokaryon ($p = 0$). (○), the sum of the carotenes and the percentages of phytoene, phytofluene, and ζ-carotene in cultures grown on minimal agar; (●), cultures grown on minimal agar supplemented with yeast extract (1 g/l). Neurosporene and an unidentified compound, not shown in this figure, were estimated to represent together less than 1 % of the total; β-carotene and other carotenes were absent.

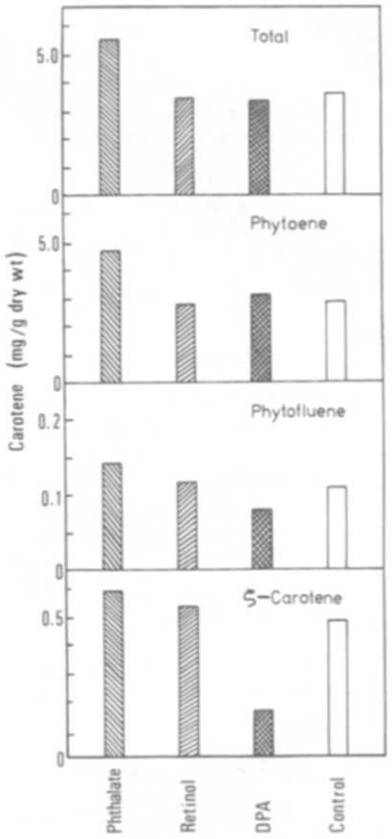

Fig. 3. Effect of dimethyl phthalate (2 mM), retinol (1 mM), and diphenylamine (75 μM) on the carotene content of S442 mycelia cultured on minimal agar in the dark. The bars indicate the average of 2–9 determinations.

The unidentified compound in strain S442 may well be an oxidation product of ζ-carotene, but it is unlikely to be an analytical artifact, since it is systematically more abundant in the cultures grown in the light than in those grown in the dark.

The observations on the response of strain S442 to light and chemicals do not require mutation *carB*401 of strain S442 to play a role in the regulation of the pathway. Light and retinol fail to stimulate carotenogenesis in strain S442, but this is a general feature of the strains with *carS* mutations or lacking β-carotene [14].

ζ-Carotene is widely distributed in the carotene-synthesizing organisms. Appreciable amounts have been found in some cases, for example in mutant maize [15], mutant tomato [16], and in etiolated barley treated with certain herbicides [17]. The high ζ-carotene content and the easy culture and handling make *Phycomyces* strain S442 a convenient source of ζ-carotene for biochemical and other work.

EXPERIMENTAL

Strains. Phycomyces blakesleeanus strain C115, genotype *carS*42 *mad*-107 (−), is a deep-yellow mutant; under normal conditions its mycelia contain about 3 mg β-carotene per g dry wt; its sporangiophores sprout out in all directions and are deficient in phototropism. Strain C5, genotype *carB*10 *geo*-10 (−), is a white mutant with a fast gravitropic response; under normal conditions its mycelium contains about 2 mg phytoene per mg dry wt.

Media and culture conditions. Minimal agar [18] contained 2 g/l L-asparagine · H_2O as nitrogen source and 15 g/l agar. Cultures were started with heat-shocked (48°, 15 min) spores or with mycelial pieces (about 1 mm^2) and incubated at 23°, either in the dark or under white light (0.75 W/m^2) from a battery of five fluorescent tubes (Sylvania F40T121D, 120 cm long).

Mutagenesis. Spores were treated for 30 min with 100 μg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml [19], with a survival rate of 3 %, and plated on minimal agar supplemented with yeast extract (1 g/l) at about 1000 survivors per plate.

Carotene analyses. Petrol (bp 40–60°) extracts of the mycelia [20] were layered on Al_2O_3 columns (Brockmann grade II, 3 cm long, 1 cm wide) and eluted with increasing concentrations of Et_2O in petrol. The different carotenes were quantified from their absorption coefficients [21].

Genetic analyses. The heterokaryon C5 ∗ S442 was made by sporangiophore grafting [22]. The nuclear proportions in the heterokaryon were calculated from the segregation frequencies in vegetative spores [23].

Chemical modification of carotenogenesis. Retinol acetate was dissolved in EtOH and polyoxyethylenesorbitan monooleate (Tween 80) and added to the melted medium at a final concn of 2 ml/l EtOH and 4 ml/l Tween 80. Dimethyl phthalate and diphenylamine were dissolved in EtOH and added to the melted medium at a final concn of 8 ml/l EtOH.

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