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IN VITRO CAROTENOGENESIS BY WILD TYPE AND MUTANTS OF *PHYCOMYCES BLAKESLEEANUS*

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Key Word Index—*Phycomyces blakesleeanus*; Phycomycetes; mutants; carotenoids; biosynthesis.

Abstract—Cell extracts from shake cultures of the wild type and six mutant strains of *Phycomyces* converted [2-^{14}C]MVA into carotenes, squalene and prenyl phosphates. Oxygen was required for the desaturation of phytoene. When compared with the wild type, cells extracts of *carB* and *carR* mutants are much less effective in phytoene dehydrogenation and lycopene cyclization, respectively. This confirms previous conclusions about the biochemical functions of the *carB* and *carR* genes, which were based on genetic and *in vivo* studies. *CarA* strain mutants accumulate, *in vivo*, much less β-carotene than the wild type. This correlates with a 10-fold decrease in carotenogenesis *in vitro*. The addition of retinol to incubations of cell extracts of the wild type and C2 strains stimulated β-carotene formation. Both *carB* and *carR* mutants show enhanced total carotenogenic activities *in vitro* and the *carS* mutant shows a higher β-carotene-synthesizing activity than the wild type. It is suggested that the feed-back regulatory mechanism known to control this pathway operates at the level of enzyme synthesis.

INTRODUCTION

Carotenogenesis in *Phycomyces blakesleeanus* has been extensively studied from a genetic point of view. Both structural and regulatory mutants have been isolated and the corresponding mutations assigned, by complementation analysis, to several genes (see ref. [1] for a review). Linkage relationships of most of these genes have recently been reported [2].

Two structural genes, *carB* and *carR*, have been identified. Gene *carB* determines phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene)dehydrogenase; *carB* mutants are white and accumulate phytoene. Gene *carR* determines lycopene cyclase; *carR* mutants are red due to an accumulation of lycopene (ψ,ψ-carotene). From results of *in vivo* quantitative complementation analyses, it has been proposed that four copies of phytoene dehydrogenase and two copies of lycopene cyclase, integrated in an enzyme complex, carry out the four reactions leading from phytoene to lycopene and the two reactions required for the conversion of lycopene into β-carotene (β,β-carotene) [3, 4].

Gene *carA*, characterized by white mutants accumulating only traces of β-carotene, has been implicated in defective transfers of carotenes between enzyme aggregates [5]. When grown in the presence of retinol or other chemicals, *carA* mutants became coloured and accumulated appreciable amounts of carotenes [6, 7]. It is very likely that genes *carA* and *carR* correspond to different segments of a single, bifunctional gene [8].

Another regulatory gene, *carS*, is characterized by mutants accumulating much more β-carotene than the wild type. The *carS* gene product is apparently involved in a feed-back control mechanism operating in this pathway [9]. Other regulatory genes have been identified and related to the control of carotenogenesis by environmental conditions such as light or retinol, which stimulate carotene formation in *Phycomyces* [2, 10].

It should be noted that conclusions as to the functions of *car* genes have been based on analyses of *in vivo* accumulations of carotenes, and/or their precursors, by different strains. Studies on *in vitro* carotenogenesis by *Phycomyces* have also been reported. Cell extracts of wild type [11] and some mutants [12–14] have been investigated, but little is known about the activities and properties of the enzymes responsible for carotene formation in *Phycomyces* and the modifications caused by mutations at different genes.

Consequently, we have carried out a comparative study of *in vitro* carotenogenesis by a complete range of the best characterized *car* mutants of *Phycomyces*. This has allowed us to confirm the function of the structural genes and to gain biochemical information concerning control of the pathway by end product regulation.

RESULTS AND DISCUSSION

Carotenoid contents of shake cultures of Phycomyces

The amounts of carotenes in the C2, C5, C9 and C115 strains (Table 1) are similar to those found previously for dark grown cultures on a solid minimal medium [4, 9], but the wild type contained some 10-fold more β-carotene [4]. This stimulation is probably due to a combination of photoinduction and the presence of leucine in the liquid medium [15]. Mycelia of C5 and C9 contained, respectively, more phytoene and lycopene than the quantity of β-carotene in the wild type (Table 1), confirming the absence of end-product control in these mutants [9].

Table 1. Strains of *P. blakesleeanus* used in this work

Strain	Genotype	Phenotype	Carotene content: Major carotene (μg/g dry wt)	
NRRL 1555	(−)	Yellow, wild type	β-Carotene	500
C2	*carA*5(−)	White	β-Carotene	4
C5	*carB*10(−)	White	Phytoene	1950
C9	*carR*21(−)	Red	Lycopene	1940
C115	*carS*42(−)	Deep yellow	β-Carotene	2000
M1	*carS*43(+)	Deep yellow	β-Carotene	1936
M1 * S106	*carS*43(+) *carS* 42 *car*-102(−)	Deep yellow	β-Carotene	1980

Only genotypes relevant to carotenogenesis are indicated. 'Carotene content' refers to the major carotene in each case.

Table 2. The effect of oxygen on *in vitro* carotenogenesis in *Phycomyces*

Fraction	Strain C9 +O_2	C9 −O_2	M1 +O_2	M1 −O_2
Total lipid (TL)	25.9	22	107	92
Prenyl phosphates	21	20	21	27
Phytoene (P)	1.1	3.7	2.9	9.4
Lycopene (L)	5.5	1.1	1.8	0.06
β-Carotene (β-C)	0.43	0.07	5.2	0.09
Squalene (S)	1.3	1.1	3.1	3.0
Ratios ($\times 10^2$)				
β-C–TL	1.7	0.3	4.8	0.1
P–TL	4.3	16.6	2.7	10.2
L–TL	25.6	4.9	1.7	0.1
S–TL	4.8	4.9	28.6	32.9
(β − C + P + L)–TL	31.6	21.8	9.2	10.4

*All values are 10^{-3} dpm/mg protein from 1 μCi DL-[2-^{14}C]MVA and are the mean of duplicates. Protein concentrations: M1, 4.9 mg and C9, 1.6 mg per incubation.

In addition to lycopene, the C9 strain also contained phytoene (612 μg/g dry wt) and traces of phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene), ζ-carotene (7,8,7′,8′-tetrahydro-ψ,ψ-carotene), γ-carotene (β,ψ-carotene) and β-carotene. Similarly, the wild type strain contained phytoene (53 μg/g dry wt) and traces of phytofluene.

Incorporation of [2-^{14}C]*MVA into the terpenoids of cell extracts of* P. blakesleeanus

Cell extracts from all seven strains of *Phycomyces* were able to convert [2-^{14}C]MVA into carotenes, squalene and prenyl phosphates (Tables 2 and 3). Radioactivity in other carotenes could not be detected with a radiochromatogram spark chamber, suggesting that any incorporations were less than 100 dpm per compound.

The incorporation patterns in the presence or absence of oxygen (Table 2) indicate that the formation of both lycopene and β-carotene, *in vitro*, requires oxygen. Since phytoene desaturation needs NADP and FAD in *Phycomyces* [16], it is possible that the irreversible formation of NADPH and FADH, under anaerobic conditions, inhibits *in vitro* carotenogenesis. Squalene formation was not altered when oxygen was present (Table 2), although this is probably a reflection of the lack of sterol-synthesizing activities in the cell extracts, which itself may be due to insufficient amounts of NADPH in the incubation mixture [16].

After standardization of data from the different strains by reference in each case to mevalonate incorporation into total lipids, it can clearly be seen that cell extracts of strain C5 are much less effective than those of wild type in transforming phytoene into β-carotene (Table 3). This is in obvious agreement with the proposed absence of phytoene dehydrogenase activity in *carB* mutants [4].

The C9 and wild type cell extracts show similar *in vitro* capacities for β-carotene biosynthesis (Tables 2 and 3), although the *in vitro* accumulation of lycopene is almost 20-fold higher in C9 than in the wild type. Therefore, both the phenotypic and *in vitro* carotenogenic behaviour of C9 can be explained by a partial defect in lycopene cyclase [3], and also a higher level of carotenogenic enzymes in the mutant with respect to wild type. In addition, the higher total *in vitro* carotenogenic activities of C5 and C9 compared with the wild type, suggest that such feed-back regulation operates at the level of enzyme synthesis, and includes the enzymes responsible for both phytoene formation and its metabolism.

Feed-back control of carotenogenic enzyme syntheses is also supported by the high *in vitro* production of β-carotene by M1 cell extracts in comparison with wild type (Tables 2 and 3). M1 carries a mutation in gene *carS* which inactivates control by β-carotene in this mutant [9]. However, a similarly elevated *in vitro* production of β-carotene by C115 (Table 3), which also carries a *carS* mutation [9], was not found, although total carotenogenesis was some 1.3-fold greater than in the wild type (Table 3). We have no simple explanation for this anomaly.

Intersexual heterokaryosis is known to stimulate carotenogenesis in *Phycomyces* [9]. Heterokaryon M1 * S106, although showing an *in vitro* activity greater than wild type (Table 3), does not exhibit higher activity than M1 itself. Since under our growth conditions M1 * S106 does not accumulate more β-carotene than M1 (Table 1), no clear conclusion can be drawn about the effect of sexual

Table 3. Incorporation of [2-^{14}C]MVA into the terpenoids of *Phycomyces* cell extracts under aerobic conditions

Fraction	Strain				
	NRRL1555	C2	C5	C115	M1 * S106
Total lipid (TL)	137	129	112	114	117
Prenyl phosphates	193	168	83	176	153
Phytoene (P)	6.6	0.82	17	7.4	2.9
Lycopene (L)	1.8	0.39	1	2.0	3.2
β-Carotene (β – C)	1.6	0.08	0	1.2	3.4
Squalene (S)	75	67	49	54	60
Ratios ($\times 10^2$)					
β – C–TL	1.2	0.1	0.1	1.1	2.9
P–TL	4.8	0.6	15.5	6.5	2.4
L–TL	1.3	0.3	0.9	1.8	2.7
S–TL	54.5	52.2	43.6	47.8	50.8
(β – C + P + L)–TL	7.3	1.0	16.4	9.4	8.0

All values are 10^{-3} dpm/mg protein from 1 μCi DL-[2-^{14}C]MVA, and are the mean of duplicates. Protein concentrations: NRRL1555, 3.6 mg; C2, 1.4 mg; C5, 2.4 mg; C115, 2.8 mg; M1 * S106, 1.7 mg, per incubation.

interaction on the level of carotenogenic enzymes. The instability of this heterokaryon, which leads to a high proportion of homokaryotic spores [17], may partially explain its carotenogenic activities.

The biosynthesis of a small amount of β-carotene both *in vitro* and *in vivo* by the C2 strain (Tables 1 and 3) may be explained in a number of ways. There may be inherently low levels of the enzymes responsible for phytoene formation and metabolism, or else there is a rapid turnover of β-carotene once it has been synthesized. A recent investigation shows that the latter possibility is unlikely, and that an inability to transfer substrates between enzyme aggregates occurs in *carA* mutations [5]. Our results show that the formation of both squalene and prenylpyrophosphates *in vitro* are very similar in C2 and the wild type strain (Table 3). Further studies on this mutant are in progress.

The biosynthesis of carotenes *in vitro* that are not present in shake cultures is probably due to an alteration in the equilibria of carotenogenesis caused by the physical disruption of membrane-bound enzyme aggregates during the preparation of cell extracts, especially as this technique of cell disruption is known to break cell organelles [18]. It is noticeable, however, that no significant incorporations of [2-^{14}C]MVA into intermediates between either phytoene and lycopene or between lycopene and β-carotene were detected. This can be explained by the proposed existence of an enzyme complex of four dehydrogenases which catalyse the conversion of phytoene into lycopene and of two cyclases for the conversion of lycopene into β-carotene. Once the first substrate of each complex starts to be utilized it is apparently metabolized to the final product. Physical disruption may affect transfer between the two complexes and cause the accumulation of lycopene.

Cis *and* trans-*phytoene formation* in vitro

Following the incubation of C5, C2 and M1 * S106 cell extracts with [2-^{14}C]MVA, phytoene fractions were further resolved into the 15-*cis*- and all-*trans*-isomers. Radioactivity was detected in both isomers, but the ratios of cis–*trans*-phytoene were different for each mutant (Table 4). A control experiment, where ^{14}C-labelled *cis*-phytoene was incubated with a boiled cell extract and then re-isolated, showed that radioactivity in *trans*-phytoene was not significantly greater than the background value for the radioassay system.

The involvement of all-*trans*-phytoene in carotenoid formation in *Phycomyces* was implicated in earlier studies of the C115 [13] and C9 [19] strains. The strains capable of phytoene metabolism (C2 and M1 * S106) have high ratios of *cis–trans*-phytoene, in contrast to the *carB* mutant (C5) which produces more of the *trans*-isomer than *cis*-, *in vitro*. This suggests that *trans*-phytoene is part of the desaturation sequence, and that the low level *in vivo* [20] is a consequence of its rapid metabolism to phytofluene, once it has been formed by the isomerization of *cis*-phytoene.

Table 4. Incorporations of [2-^{14}C]MVA into *cis*- and *trans*-phytoene of *Phycomyces* cell extracts

	Incorporation (dpm)		
	C5	C2	M1 * S106
Cis-	1188	272	3012
Trans-	3162	93	220
Ratio *cis–trans*	0.38	2.92	13.7

Values quoted are dpm incorporated from 1 μCi DL-[2-^{14}C]MVA.

The effect of retinol on carotenogenesis by NRRL 1555 *and C2 cell extracts*

The increase of *in vitro* β-carotene formation by retinol (0.87 mM), in cell extracts of the C2 and wild type strains (Table 5), suggests that enzyme activities are stimulated by this compound. This is contrary to a previous report [6] which indicated that *in vivo* protein synthesis was elevated in both the wild type and mutants of *Phycomyces* in the

Table 5. The effect of retinol on carotenogenic activities of cell extracts from C2 and wild type strains of *Phycomyces*

	Strain			
	NRRL 1555		C2	
Fraction	Control	+Retinol	Control	+Retinol
Total lipid (TL)	52	50	252	250
Prenyl phosphates	157	198	270	285
Phytoene (P)	1.2	1.4	0.3	0.30
Lycopene (L)	0.77	0.29	0.09	0.09
β-Carotene (β − C)	1.8	5.8	0.13	0.24
Squalene (S)	14	9.4	59	70
Ratios ($\times 10^2$)				
β-G–TL	3.5	11.6	0.05	0.10
P–TL	2.4	2.8	0.12	0.12
L–TL	1.5	0.6	0.03	0.04
S–TL	28.0	18.8	23.0	28.0
(β − C + P + L)–TL	6.4	15.0	0.20	0.26

Values are 10^{-3} dpm/mg protein. Incubations contained 1 μCi DL-[2-^{14}C]MVA and retinol (87 μmol) solubilized in Tween 80 (5 mg); control incubations contained Tween 80 (5 mg). Protein concentrations: wild type, 3.6 mg; C2, 1.4 mg per 1 ml incubation.

presence of retinol. A more recent investigation, however, shows that for C2 at least, neither transcription nor translation are affected by retinol [5]. Two independent effects, therefore, may occur in the presence of this compound.

In conclusion, the *in vitro* activities of the *Phycomyces* cell extracts have confirmed the biochemical functions of *carB* and *carR* gene products and indicate the existence of a control of carotenogenic enzyme synthesis mediated by the end product of the pathway.

We feel that the present *in vitro* systems for carotenogenesis in *Phycomyces* can be used to investigate other aspects of this metabolic pathway for which physiological or genetic data are already available. Nevertheless, further improvements in efficiency of the system are certainly worthwhile, particularly purification of the carotenogenic enzyme complexes.

EXPERIMENTAL

Organisms and cultural conditions. The *Phycomyces* strains used in this study, together with their phenotypes and genotypes, are listed in Table 1. Heterokaryons are designated in the text by their two components separated by an asterisk. Cultures were maintained on agar slopes, while cell extracts and pigment analyses were made on mycelia grown in shake cultures for 54–60 hr in the light, at 24° [18].

Radiochemical. DL-[2-^{14}C]Mevalonic acid lactone (53 mCi/mmol) was obtained from Amersham International Ltd., Amersham, U.K. It was converted into the Na salt prior to use [12].

Preparation of cell extracts. These were prepared as described previously [12], except that 10 vol. 0.2 M Tris–HCl buffer, pH 8.0, were added to the sieved, lyophilized mycelia. Protein determinations were carried out using Folin reagent [21] as described elsewhere [22].

Incubation conditions. Incubations (1 ml) were carried out aerobically and anaerobically in Thunberg tubes, as described previously [12]. Retinal (0.87 μmol) was added to incubations as an emulsion in Tween 80 (5 mg) prior to addition of the cell extract. Tween 80 alone was added to control incubations in these cases.

Extraction of radioactive compounds. Prenyl phosphates were isolated from aliquots (5 μl) of the incubations as described previously [22]. EtOH (8 ml) and carrier terpenoids (1 ml, containing 100 μg each of phytoene, squalene, β-carotene, lycopene and ergosterol) were added to the remaining incubation mixture and the total lipids extracted as described elsewhere [22].

Extraction and quantification of carotenes from mycelia. These techniques have been described in detail in a previous publication [12]. Standard $E^{1\%}_{1cm}$ values were used to quantify the carotenes [23].

Purification of radioactive polyenes. Preliminary separations of carotenes, squalene and ergosterol from other lipids were achieved on columns of Al_2O_3 [12]. Purifications to constant specific radioactivities were carried out by TLC, as described previously [12]. 15-*Cis* and all-*trans*-phytoenes were separated on $AgNO_3$–Si gel (1 : 10) TLC, developed with petrol–EtOAc–di-*iso*-propylether (2 : 1 : 1) [23]. Radioactivity on thin layers was detected with a radiochromatogram spark chamber (Birchover Instruments Ltd., Letchworth, Herts, U.K.).

Radioassay. Terpenoids were eluted from thin layers with Et_2O and aliquots assayed by liquid scintillation counting [24] in a Beckman model L7500 counter equipped with automatic quench compensation. ^{14}C-labelled prenyl phosphates were assayed on DEAE-cellulose discs, as described previously [22].

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