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Interallelic complementation provides genetic evidence for the multimeric organization of the *Phycomyces blakesleeanus* phytoene dehydrogenase

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The *Phycomyces blakesleeanus* wild-type is yellow, because it accumulates β-carotene as the main carotenoid. A new carotenoid mutant of this fungus (A486) was isolated, after treatment with ethyl methane sulfonate (EMS), showing a whitish coloration. It accumulates large amounts of phytoene, small quantities of phytofluene, ζ-carotene and neurosporene, in decreasing amounts, and traces of β-carotene. This phenotype indicates that it carries a leaky mutation affecting the enzyme phytoene dehydrogenase (EC 1.3.-.-), which is specified by the gene *carB*. Biochemical analysis of heterokaryons showed that mutant A486 complements two previously characterized *carB* mutants, C5 (*carB10*) and S442 (*carB401*). Sequence analysis of the *carB* gene genomic copy from these three strains revealed that they are all altered in the gene *carB,* giving information about the nature of the mutation in each *carB* mutant allele. The interallelic complementation provides evidence for the multimeric organization of the *P. blakesleeanus* phytoene dehydrogenase.

Keywords: carotenoid; phytoene dehydrogenase; interallelic complementation; *Phycomyces blakesleeanus*.

Carotenoids represent one of the most abundant and widely distributed classes of pigment in nature. They are present in photosynthetic bacteria, cyanobacteria, algae and higher plants as well as in nonphotosynthetic bacteria and fungi [1]. Carotenoids are colour pigments in flowers and fruits and also in many crustaceans, insects, fishes and birds [2]. They play essential roles in photosynthesis [3], photooxidative protection [4], nutrition, vision and cellular differentiation [5]. Some carotenoids are used in the cosmetic and food industries and their potential use in disease prevention in humans and as antitumor agents is being considered [6,7]. Nowadays, there is considerable interest in the manipulation of carotenoid content and composition in plants to improve the agronomical and nutritional value for human and animal consumption [8].

Among fungi, β-carotene and neurosporaxanthin are the main carotenoids accumulated in the ascomycetes *Gibberella fujikuroi* and *Neurospora crassa*; astaxanthin predominates in the basidiomycete yeast *Xanthophyllomyces dendrorhous,* and β-carotene is the main carotenoid in the Mucorales *Blakeslea trispora*, *Mucor circinelloides* and *P. blakesleeanus* [9,10]. Mutants altered in the carotenoid pathway are detected by a change in colour due to the accumulation or lack of intermediate products or to overproduction of the end product. In Mucorales, many early studies on carotenoids biosynthesis were performed in *P. blakesleeanus* (reviewed in [11]) but recently carotenoid mutants of *M. circinelloides* have been isolated and investigated [12–15], because the lack of an efficient transformation system in *Phycomyces* impedes the isolation of genes by direct complementation and their functional analysis [16].

In fungi, the specific carotenoid pathway to β-carotene proceeds via three enzymatic steps carried out by the enzymes phytoene synthase, phytoene dehydrogenase and lycopene cyclase. The enzyme phytoene dehydrogenase is able to introduce four dehydrogenations in a substrate molecule to produce lycopene. Its coding gene is named *carB* in *Phycomyces* [17] and *Mucor* [18] and *al-1* in *Neurospora* [19]. A single bifunctional protein carries out phytoene synthase and lycopene cyclase activities in fungi. The existence of a bifunctional gene was proposed by Torres-Martínez *et al.* in 1980 for *Phycomyces* [20] and recently it has been shown to be a feature unique to fungal carotenogenesis. So far, the *crtYB* gene of *X. dendrorhous* [21], *carRP* of *M. circinelloides* [22] and *carRA* of *P. blakesleeanus* [23] have been the most extensively studied. The *al-2* gene of *N. crassa,* initially identified only as the phytoene synthase coding gene in this fungus [24], also shows this characteristic (quoted in [23]). The genes *carB and carRP* in *M. circinelloides* are 446 nucleotides apart and show a co-ordinated regulation of their expression by blue light, suggesting a bi-directional mode of transcriptional control [22]. In *P. blakesleeanus*, the genes *carB* and *carRA* also show a co-ordinated regulation by light (C. Sanz &

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M. I. Alvarez unpublished results) although the distance between the two genes is 1381 nucleotides [23].

In Mucorales, mutants altered in the gene *carB* are white and accumulate phytoene [15,25]. Another group of *carB* mutants (those which are leaky) are greenish, whitish or yellowish because they accumulate partially dehydrogenated products of phytoene [26–28]. Mutants altered in the P or A domains of the genes *carRP* or *carRA* of *Mucor* and *Phycomyces*, respectively, are white, accumulate no carotenoid or only traces of β-carotene, and are altered in the enzyme phytoene synthase [15,22,29]. In *Phycomyces*, white *carB* mutants and white *carA* mutants are easily distinguishable, because the latter are sensitive to vitamin A, which in this case restores function, i.e. carotene synthesis causing yellow colour [30]. Mutants disrupted in the R domain of both the *carRP* and the *carRA* genes are red, accumulate lycopene and are altered in the enzyme lycopene cyclase [15,22,25,31]. A third group of mutants altered in this bifunctional gene has been described for both Zygomycetes. In *Phycomyces* they complement neither the *carR* nor the *carA* mutants, and in *Mucor* they complement neither the *carP* nor the *carR* mutants. They are white, lack all carotenoids and have been considered mutants carrying mutations with deficiencies in both enzymatic activities [15,20,22,23,31].

In *Phycomyces* there are several types of mutants altered in the regulation of the carotenoid pathway. The *carC* mutants are whitish, because they produce only very small amounts of β-carotene [32]. Mutants disrupted in the genes *carS*, *carD* and *carF* are deep-yellow, because they overproduce β-carotene [33–35]. In *M. circinelloides* β-carotene overproducing strains have been found [29], but the regulation of the carotenoid pathway in *Mucor* seems to be different from that in *Phycomyces* [12,13].

The fungus *Phycomyces* remains multinucleate throughout the cell cycle. The mycelia are large coenocytes containing millions of nuclei and the asexual spores are multinucleate cells containing several nuclei, three to four being the most frequent number of nuclei per spore. These spores are formed by the division of a large mass of cytoplasm into multinucleate portions that develop strong cell walls in the sporangium. This packaging of nuclei into spores is random [36].

Quantitative complementation analysis has led to the hypothesis that the enzymes involved in the conversion of phytoene to β-carotene in *P. blakesleeanus* are organized as an enzyme aggregate [37,38]. This complex would consist of four copies of the enzyme phytoene dehydrogenase, which act sequentially on a molecule of phytoene, converting it successively in phytofluene, ζ-carotene, neurosporene and lycopene, and two copies of the enzyme lycopene cyclase, which covert first lycopene into γ-carotene and then γ-carotene into β-carotene. So far, no molecular evidence for such an enzymatic aggregate has been reported.

Among the *P. blakesleeanus carB* mutants previously isolated, two strains have been investigated in relation to the effects of the induced mutation on the activity of the enzyme: strain C5, which produces white mycelium and only accumulates high amounts of phytoene [25], and strain S442, producing a greenish mycelium and accumulating high amounts of phytoene and small amounts of phytofluene, ζ-carotene and neurosporene [28]. *In vitro* characterization of the phytoene desaturation reaction in these two strains revealed that the phenotypic block could be overcome by the addition of Tween 40 in strain C5, but not in strain S442. These observations indicated that while the catalytic activity of the phytoene dehydrogenase in strain S442 is directly affected by the mutation, strain C5 possesses a functional enzyme, likely altered in a region relevant for the correct spatial organization of the enzyme or of the enzyme complex [39].

In this paper, we report on the isolation and characterization of a new *P. blakesleeanus carB* mutant strain that shows interallelic complementation with two *Phycomyces* strains carrying different *carB* alleles. This provides genetic evidences for the multimeric organization of the enzyme phytoene dehydrogenase in *Phycomyces*. The nature of the mutations in the complementing *carB* alleles is presented.

EXPERIMENTAL PROCEDURES

Strains and growth conditions

The *P. blakesleeanus* strains used in this work are listed in Table 1. Growth media (SIV and SIVYC, minimal and rich medium, respectively) and growth conditions have been described previously [40–42]. For colonial growth, the pH of the media was lowered to 3.3. Minimal medium was supplemented as required with vitamin A (200 $\mu g \cdot mL^{-1}$). *Escherichia coli* strain DH5α was used for all cloning experiments and propagation of plasmids. It was grown under previously described conditions [43].

Table 1. *P. blakesleeanus* strains used in this work.

Strain[a]	Genotype[b]	Origin[c]	Phenotype[d]
NRRL1555	(–)	Standard wild-type	Yellow (β-carotene)
C2	*carA5* (–)	NRRL1555 by MNNG mutagenesis	White (traces of β-carotene)
C6	*carRA12* (–)	NRRL1555 by MNNG mutagenesis	White (traces of lycopene)
A98	*carC652* (–)	NRRL1555 by NQO mutagenesis	White (traces of β-carotene)
C5	*carB10*, *geo10* (–)	NRRL1555 by MNNG mutagenesis	White (phytoene)
S442	*carB401*, *mad107*, *carS42* (–)	C115 (*carS42*, *mad 107* (–))by MNNG mutagenesis	Greenish (phytoene)
A486	*carB679* (–)	NRRL1555 by EMS mutagenesis	Whitish (phytoene)

[a] Prefixes A, C, and S refer to strains isolated at the University of Salamanca, The California Institute of Technology and the University of Sevilla, respectively. [b] *car* indicates a mutant with abnormal carotene production; *mad* indicates a mutant with abnormal phototropism; *geo* indicates a mutant with abnormal geotropism; (+) and (–) indicate the two mating types. [c] MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; NQO, 4-nitroquinoline-1-oxide; EMS, ethyl methane sulfonate. [d] Colour of mycelium and main carotenoid accumulated.

Mutagenesis and isolation of mutants

Vegetative spores of *P. blakesleeanus* strain NRRL1555 were treated with ethyl methane sulfonate (EMS) (3%, v/v) in phosphate buffer (0.1 M, pH 7.0) at 22 °C during 4.5 h. The chemical was washed off with distilled water. Aliquots of the treated spores (5×10^3, viability about 3%) in distilled water were spread on SIVYC plates. Germinated spores were allowed to complete a full vegetative cycle and harvested as independent recycled spore pools. Aliquots from each pool were plated on acidified SIV medium plates. Strain A486 was identified by visual inspection of colonies derived from the mutagenic treatment and purified from a single spore.

Carotenoid analyses

Spores from the different *P. blakesleeanus* strains and from heterokaryons were plated on SIV plates and incubated during three days under continuous light at 22 °C. Mycelia were then scrapped off. A portion was used to determine the dry weight (1 h at 105 °C) and the rest was blended in a Sorvall Omni-Mixer with 20 mL methanol and 20 mL petroleum ether (boiling point 50–70 °C) for 3 min. The operation was repeated twice after changing the petroleum ether and the resulting fractions were combined. Spectrophotometric analysis of carotenoids in supernatant was performed in a Hitachi U-2000 spectrophotometer. For quantification of carotenoids, supernatant was concentrated in a rotoevaporator and chromatographed in alumina column [44].

For HPLC analysis, an aliquot of the supernatant was desiccated under nitrogen pressure and resuspended in petroleum ether/diethyl ether (9 : 1, v/v). Carotenoids were identified and quantified as previously described [22]. Phytoene, ζ-carotene and β-carotene were quantified after calibration making use of authentic standards.

Complementation analyses

Complementation analyses were performed by constructing heterokaryons following the procedures described previously [45], for surgical grafting of sporangiophores. In each case, heterokaryotic sporangia were identified by plating spores in acidified SIVYC medium. Only sporangia giving rise to different types of colonies were selected for further characterization.

Recombinant DNA procedures

Genomic DNA from *P. blakesleeanus* strains was isolated following the methods described by Möller *et al.* [46]. PCR amplifications of the genomic copies of the mutant *carB* alleles were carried out in a PerkinElmer 9700 Thermal Cycler. Two oligonucleotides were designed to amplify a genomic DNA fragment containing the entire coding region of the gene, flanked by 1302 bp and 352 bp at the 5′ and 3′ noncoding regions, respectively. Their sequences are: oligonucleotide A, 5′-AGTACAAA AGACAAGACT-3′ (nucleotide positions −1302 to −1285; and oligonucleotide B, 5′-GAGTCTGAGGTGCTGTAC-3′ (complementary to nucleotide positions +2287 to +2270) (numbering according to the sequence reported previously [17], accession no. X78434). PCR amplifications were performed in 50 μL final volume reactions containing 10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each oligonucleotide, 20 ng of genomic DNA and 2.5 U of Ampli*Taq* Polymerase (Applied Biosystems). Reaction mixtures were subjected to one cycle at 95 °C for 2 min; 40 cycles at 95 °C for 30 s, 60 °C for 60 s and 72 °C for 90 s; and a final additional extension period at 72 °C for 5 min. Amplified DNA fragments were purified from gels using the GeneClean Kit (Bio101) and cloned into pGEM-T-easy vector (Promega). Ligations, transformations of *E. coli* and plasmid amplifications were performed following standard procedures [43]. DNA sequencing was performed in an ABI 373 A automated DNA sequencer (Applied Biosystems).

Computer analysis

Nucleotide and amino-acid sequences were analysed using the Vector NTI Suite software package (InforMax, Inc.). Access to the PROSITE database of protein families and domains was carried out through the utilities offered by the ExPASy Molecular Biology server (http://www.expasy.ch).

RESULTS

Isolation and biochemical characterization of a new *car*- strain

Several colour mutant strains were obtained after EMS treatment of *P. blakesleeanus* strain NRRL1555 spores. One of these strains, A486, showed a characteristic whitish coloration. As mostly clean white mutants, altered either in the *carB* gene, in the *carRA* gene or in the *carC* gene, had been previously isolated, such a phenotype prompted us to characterize biochemically this mutant strain.

Figure 1 shows the HPLC elution profiles of the carotenoids accumulated by this strain when cultured in solid medium under continuous light conditions. For comparison, the elution profiles of the carotenoids accumulated by strain NRRL1555 have been included. Their analysis showed that β-carotene is the main carotenoid accumulated by strain NRRL1555. Small amounts of phytoene are also detected, while the three intermediates resulting from its sequential dehydrogenation, phytofluene, ζ-carotene and neurosporene, are hardly detectable. Strain A486 accumulated phytoene, phytofluene, ζ-carotene, neurosporene and β-carotene. Quantification of these products performed by spectrophotometric analysis and (when standards were available) by HPLC analysis (Table 2) showed that β-carotene represents about 90% of the total carotenoids accumulated by strain NRRL1555, and phytoene about 8%. Phytofluene, ζ-carotene and neurosporene, all together, represent less than 2%. Strain A486 accumulates mainly large quantities of phytoene, indicating that it is altered in the dehydrogenation of phytoene, probably in the *carB* gene, so far the only gene involved in this metabolic step reported in *P. blakesleeanus*. Small and decreasing amounts of phytofluene, ζ-carotene and neurosporene were also detected. No lycopene was found, while traces of β-carotene could be detected. Strain A486 was insensitive to vitamin A (data not shown), indicating that it is altered neither in the A domain of the *carRA* gene nor in the *carC* gene.

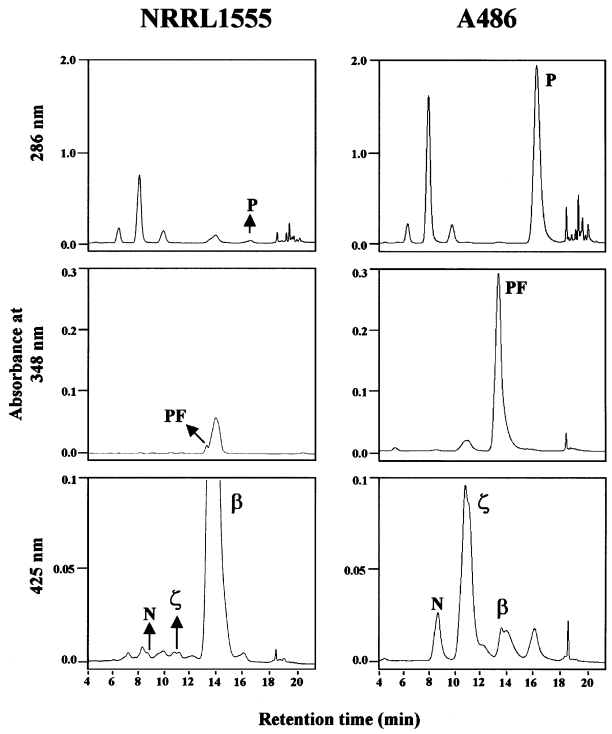

Fig. 1. HPLC elution profiles at 286, 348 and 425 nm of the carotenoids produced by the wild type strain NRRL1555 and by the mutant strain A486. (P, phytoene; PF, phytofluene; ζ, ζ-carotene; N, neurosporene; β, β-carotene).

Table 2. Quantification (μg per gram dry weight) by spectrophotometric analysis (SP) or by HPLC analysis (HPLC) of the amounts of the different carotenoids accumulated by strains NRRL1555 and A486. P, phytoene; PF, phytofluene; ζ, ζ-carotene; N, neurosporene; L, lycopene; β, β-carotene. ND, not determined.

	P	PF	ζ	N	L	β
NRRL1555						
SP	67	6	4	2	0	710
HPLC	58	ND	3	ND	0	687
A486						
SP	1854	301	83	22	0	9
HPLC	1686	ND	62	ND	0	8

Complementation analysis

To characterize genetically the mutant strain A486, a complementation analysis was performed by making heterokaryons between this mutant strain and representative strains altered in different carotenogenic genes whose mutations give rise to white or whitish mycelia. Table 1 summarizes the genotypes and phenotypes of the strains utilized in this analysis. When plating on acidified medium spores from heterokaryons A486*C2, A486*C6 and A486*A98, in all three cases yellow colonies were found in addition to colonies showing the colour of each of the two parental strains involved in the construction of each heterokaryon (data not shown). Therefore, the mutation in strain A486 complemented *carA, carRA* and *carC* mutations. These observations allowed us to discard any possible alteration in genes *carRA* and *carC* in strain A486, in agreement with the data derived from its biochemical characterization.

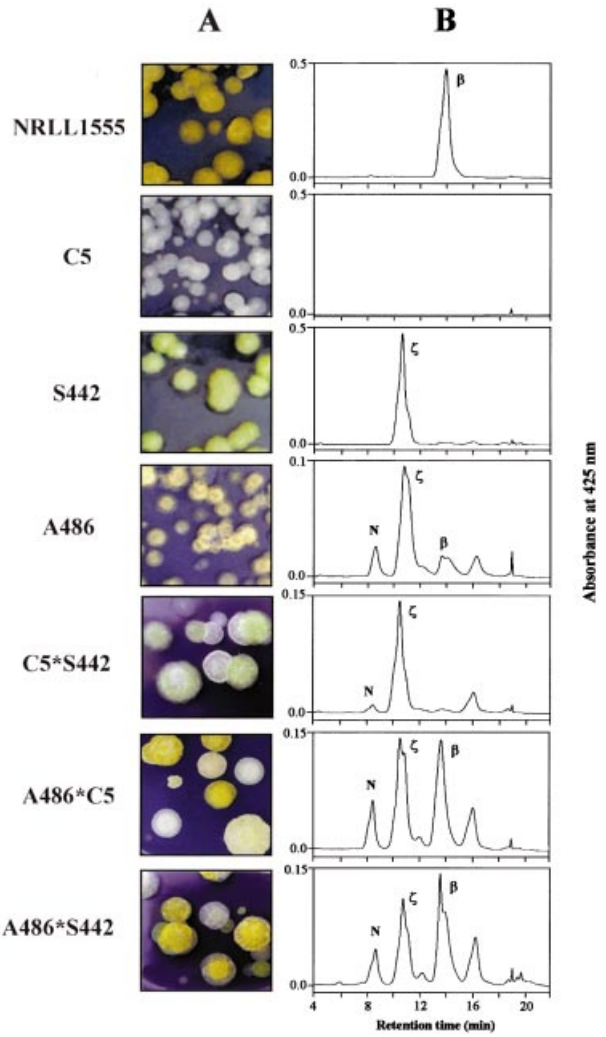

Fig. 2. Complementation analysis between strain A486 and the two carB mutant strains C5 and S442. (A) Colour of the colonies appearing in acidified SIV medium when plating spores from a single sporangium of the indicated origins. (B) HPLC elution profiles at 425 nm of the carotenoids accumulated in mycelia derived from the indicated strains or heterokaryons.

Interestingly, the mutation in strain A486 also complemented with mutations in strain C5 (*carB10*) and in strain S442 (*carB401*), two previously characterized *carB* mutants. As shown in Fig. 2A, spores from a sporangium from the heterokaryon A486*C5 produced three types of colonies of clearly distinguishable colour; whitish, white and yellow. Spores from a sporangium from the heterokaryon A486*S442 also produced three types of colonies: whitish, greenish and yellow. The heterokaryon C5*S442 was also constructed, but the derived spores only produced two types of colonies, white and greenish, indicating that mutations in these two strains do not complement.

To confirm that heterokaryons A486*C5 and A486*S442 accumulated β-carotene, HPLC analyses were performed.

Table 3. Quantification by HPLC analysis of the amount of β-carotene (μg per gram dry weight) accumulated by the *P. blakesleeanus* wild type strain NRRL1555, the carB strains C5, S442 and A486, and the heterokaryons formed between these mutant strains.

	NRLL1555	C5	S442	A486	C5*S442	A486*C5	A486*S442
β-Carotene	687	0	0	8	0	130	167

Carotenoids were extracted from mycelia of the wild-type, the mutant strains C5, S442 and A486 and the heterokaryons C5*S442, A486*C5 and A486*S442. In the case of the heterokaryons the mycelia were derived from a single sporangium. From the analysis of the HPLC profiles shown in Fig. 2B it can be seen that heterokaryons A486*C5 and A486*S442 accumulated 19% and 24%, respectively, of the amount of β-carotene produced by the wild-type (see Table 3).

These observations suggest that strain A486 is altered either in a new gene involved in carotenogenesis, or in the *carB* gene. In the latter case, these data would be indicative of interallelic complementation.

Cloning and sequence analysis of the *carB* mutant alleles

To check if strain A486 was altered in the *carB* gene, and in order to get further insights into the nature of the mutations of the *carB* gene in strains C5 and S442, the genomic copy of the *carB* gene from these three strains was amplified by PCR, cloned and sequenced. Two oligonucleotides were designed to amplify a 3589 base pairs DNA fragment comprising the entire *carB* gene coding region and 1302 base pairs of the 5′ noncoding region and 352 base pairs of the 3′ noncoding region (see Experimental procedures). In each case, two clones derived from two independent PCR reactions were sequenced to avoid errors introduced by the polymerase. In strain C5 (*carB10*) two close point mutations were found: the first one a C → T transition at position +1514, which produces a Ser444Phe substitution, and the second one, also a C → T transition, which causes a Leu446Phe substitution. In strain S442 (*carB401*) a G:A transition was found at position +1627 which determines a Gly482Ser substitution. In strain A486 a single point mutation, a G → A transition, was found at position +1459 which determines a Glu426Lys substitution. This observation confirmed that strain A486 was altered in the *carB* gene. The corresponding mutant allele was then named *carB679*.

DISCUSSION

Biochemical and genetic analysis demonstrate that strain A486 has acquired a leaky mutation in the *carB* gene, originating the mutant allele *carB679*. This strain was identified against the wild type yellow background of strain NRRL1555 because of its whitish coloration. Biochemically this mutant strain resembles very much a previously reported *carB* strain, S86 [26]. Both accumulate large amounts of phytoene and decreasing amounts of the successive intermediates resulting from the four sequential dehydrogenations of a substrate molecule, in very similar proportions in both strains. Traces of lycopene, the final product of the dehydrogenation reactions, are detected in strain S86, which harbours an additional mutation in the *carR* gene, while traces of β-carotene are found in strain A486, wild-type for this lycopene cyclase coding gene. As discussed in an earlier paper, this biochemical phenotype is only compatible with a single dehydrogenase enzyme entrusted with the four dehydrogenations of phytoene [26]. According to the model proposed on the basis of quantitative complementation studies, the four dehydrogenation reactions would be carried out in a specific sequence by four copies of the enzyme organized forming part of an enzyme complex [37,38].

The *carB* mutation in strain A486 complemented the *carB* mutations in strains C5 (*carB10*) and S442 (*carB401*). The finding that complementation between mutations occurs is indicative of mutations affecting different genes. However, complementation does not always imply that mutations reside in distinct and separate locations. In fungi there are well documented examples which demonstrate that in heterokaryons combining mutations from strains altered in the same gene, the wild type phenotype can be restored, at least partially [47–49]. Interallelic complementation is explained by the multimeric organization of the enzyme, which can cause the formation of hybrid oligomeric proteins in the heterokaryon (reviewed in [50]). The data derived from the complementation analysis performed in this work with three mutant strains altered in the *carB* gene, A486, C5 and S442, indicate that interallelic complementation between different *carB* mutations occurs, as β-carotene, the final product of the pathway, is produced in significant amounts in two heterokaryons, A486*C5 and A486*S442. It must be noted that strain S442 carries and additional mutation in a regulatory gene, *carS*, but no effect is expected for such a mutation in a heterokaryon, as it is recessive [33]. Hence, these observations provide genetic evidence for the multimeric organization of the *P. blakesleeanus* phytoene dehydrogenase enzyme.

A second mechanism that depends on the common organization of proteins into domains can not be excluded to explain interallelic complementation. It may be possible for two mutually fitting domains to pack together in a stable way even though they are contributed by two different mutant polipeptides. In *Phycomyces*, all the published data [37,38,51] are compatible with the first explanation (multimeric nature of the enzyme).

In many carotenogenic organisms similar enzymatic aggregates have been proposed which are associated with membranes [52,53]. This association implies the participation of membrane-bound enzymes. In *Phycomyces* the analysis of the deduced amino-acid sequence encoded by the *carB* gene reveals the presence of a transmembrane region near the C-terminus of the protein [17]. Deficiencies in phytoene dehydrogenase activity could therefore be caused by alterations in amino-acid residues essential for the catalytic activity itself, by mutations disturbing the organization of the enzyme complex, or by mutations in another protein of the enzyme complex. In order to improve our

understanding of the function and organization of the enzyme complex, the analysis of mutant alleles of the genes involved in the biosynthetic pathway can certainly provide valuable information.

In this work, mutations in the *carB* gene have been identified in the three mutant strains characterized. The mutation identified in strain S442 determines the amino-acid substitution Gly482Ser. This residue forms part of the 'bacterial-type phytoene dehydrogenase signature' (PROSITE accession no. PS00982, consensus pattern: ([NG]-x–[FYWV]–[LIVMF]–x–G–[AGC]–[GS]–[TA]–[HQT]–P–G–[STAV]–G–[LIVM]–x-(5)–[GS]) (where 'x' can be any residue), an amino-acid sequence located in the *P. blakesleeanus* deduced protein sequence near the C-terminus, between residues 471 and 491. The sequence VGA-THPG-G-P, located in the *P. blakesleeanus* phytoene dehydrogenase sequence between positions 475–486, has been postulated to be the carotenoid binding domain [54]. As the activity of this mutant enzyme could not be restored by the addition of Tween 40 [39], it can be concluded that the 482 Gly residue is important for the activity of the enzyme, likely being one of the residues mediating substrate binding.

In strain C5, Schmidt & Sandmann [39] found that the phytoene dehydrogenase activity was partially restored by treatment with Tween 40. Computer analysis of the *Phycomyces* phytoene dehydrogenase deduced protein sequence identifies several myristoylation sites (PROSITE accession no. PS00008, consensus pattern: (G–{EDRKHPFYW}–x-(2)–[STAGCN]–{P}). The addition of a hydrophobic myristate represents a potential mechanism by which an otherwise nonhydrophobic protein can become membrane bound. Interestingly, the two close mutations found in the *P. blakesleeanus carB10* allele determine two amino-acid substitutions that affect one of these myristoylation sites located between positions 443 and 448 (wild-type amino-acid sequence GSILGL). As almost any residue is allowed at position 4 of that consensus sequence, the substitution Leu446Phe probably does not alter the specificity of the sequence recognized by the enzyme responsible for this modification. However, charged residues, proline and large hydrophobic residues are not allowed at position 2 and therefore the substitution Ser444 → Phe likely alters that specificity and eliminates a possible myristoylation site. Although there is no direct evidence for the addition of a myristate group to this myristoylation site, it is interesting to note that a mutation leading to the loss of a functional myristoylation site could determine the alteration of the local molecular environment conditions required for the association of the different enzyme monomers or for their interaction with other membrane proteins. The observed *in vitro* activation of the enzyme could then be explained by a detergent-mediated spatial rearrangement of the enzyme complex, as suggested by Schmidt & Sandmann [39].

The mutation found in the *carB679* allele in strain A486 determines the substitution of an acidic amino acid (Glu) by a basic amino acid (Lys) at position 426. This causes a drastic reduction in enzyme activity, but it does not completely block it. Therefore, the characterization of this mutant allele allows the identification of an amino acid residue which is important, but no essential, for enzyme activity. Whether this residue plays a direct role in the catalytic activity or participates somehow in the establishment of a properly organized enzyme complex remains to be determined. But it is interesting to note that, although at a low rate, the enzyme aggregate in strain A486 is able to carry out the four successive dehydrogenations transforming phytoene to lycopene.

The data presented in this paper strongly support the model of an enzyme aggregate for the organization of the carotenogenic enzymes in *P. blakesleeanus* [37,38,51]. Molecular tools are already available which will make it feasible getting deeper insights into its organization and regulation.

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