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# Structure and Function of the Genes Involved in the Biosynthesis of Carotenoids in the Mucorales

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**Abstract** Carotenoids are widely distributed natural pigments which are in an increasing demand by the market, due to their applications in the human food, animal feed, cosmetics, and pharmaceutical industries. Although more than 600 carotenoids have been identified in nature, only a few are industrially important (β-carotene, astaxanthin, lutein or lycopene). To date chemical processes manufacture most of the carotenoid production, but the interest for carotenoids of biological origin is growing since there is an increased public concern over the safety of artificial food colorants. Although much interest and effort has been devoted to the use of biological sources for industrially important carotenoids, only the production of biological β-carotene and astaxanthin has been reported. Among fungi, several Mucorales strains, particularly *Blakeslea trispora*, have been used to develop fermentation processes for the production of β-carotene on almost competitive cost-price levels. Similarly, the basidiomycetous yeast *Xanthophyllomyces dendrorhous* (the perfect state of *Phaffia rhodozyma*), has been proposed as a promising source of astaxanthin. This paper focuses on recent findings on the fungal pathways for carotenoid production, especially the structure and function of the genes involved in the biosynthesis of carotenoids in the Mucorales. An outlook of the possibilities of an increased industrial production of carotenoids, based on metabolic engineering of fungi for carotenoid content and composition, is also discussed.

*Keywords*: carotene, astaxanthin, vitamin A, food additives, *Mucor*, *Phycomyces*, *Blakeslea*

## INTRODUCTION

Carotenoids constitute one of the most widely distributed classes of pigments in nature [1]. These red, orange and yellow lipid-soluble compounds are found in plants and algae accompanying chlorophyll; in green plants they become evident in the autumn colour of leaves and contribute to the bright colours of many fruits and some flowers and roots. In the eubacterial community, anoxygenic photosynthetic bacteria, cyanobacteria and certain species of non-photosynthetic bacteria produce carotenoids. We can also find carotenoids in the animal kingdom [2]: in insects, crustacean shells, the plumage of birds, or fish flesh. Many fungi also produce carotenoids as secondary metabolites.

In addition to their obvious role as visually attractive natural pigments, carotenoids perform a variety of essential biological functions. A number of carotenoids are effective in preventing or controlling free radical generation. They provide photooxidative protection against potentially harmful singlet oxygen and reactive oxygen species in photosynthetic organisms [3] and are essential structural components of the photosynthetic antenna and reaction center complexes [4]. In plants, some of these compounds are precursors of the phytohormone abscisic acid, which modulates developmental and stress processes [5]. Certain carotenoids are precursors of vitamin A, retinal, and retinoic acid in mammals thereby playing essential roles in nutrition, vision, and cellular differentiation, respectively [6]. Carotenoids with provitamin A activity are essential components of the human diet, and there is increasing evidence that many carotenoids enhance the immune response [7] and inhibit proliferation of various types of cancer cells [8].

Carotenoids are used in animal feed (salmon, trout and poultry) to intensify the colour of the flesh or the egg yolk. Humans also consume large amounts of carotenoids added to foods, primarily to please the eye, but also because the carotenoid contents of foods change, due to harvesting, handling and processing methods [9], since carotenoids are degraded by light in the presence of oxygen. Most of these added carotenoids are synthesised by chemical methods, but there is increasing interest for carotenoids of biological origin. Natural mixed carotenoid supplements contain a variety of carotenoids, while the chemical compound added to foods is single species. Synthetic β-carotene is composed of all-trans isomers, while β-carotene from other sources of biological origin is composed of all-trans, 9-cis and other cis

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isomers, and is usually mixed with a small proportion of precursors [10].

Carotenoids were first isolated by 19th-century organic chemists: β-carotene in 1817, and the xantophylls in 1837 [11]. Many bacterial, fungal and plant carotenoid mutants have been isolated throughout the 20th-century, and the biochemical dissection of the pathway was first proposed by the 50's [12] in spite of the hydrophobic, membrane-bound nature of the enzymes involved. The advent of molecular genetics has allowed a detailed description of the genes and enzymes of the pathway, especially in bacteria, and this has been the start point for the cloning and analysis of carotenoid genes in fungi and plants. Since then, the progress has been fast, but much remains to be learned about the regulation of the pathway, as there exist many regulatory mutants which have not yet been characterised at the molecular level.

The abundant literature on carotenoids has been the subject of many books and reviews covering all aspects of the biochemistry and genetics of carotenoids in bacteria, fungi and plants [13-20]. In this review we summarise our knowledge of genes and enzymes for carotenoid production in the Mucorales, bearing in mind the current challenge for fungal carotenoid biotechnology.

## THE GENERAL CAROTENOID PATHWAY

The carotenoid pigments are only a small fraction of the vast group of chemical compounds known collectively as terpenoids. The common feature of all terpenoids is their synthesis from the building block isopentenyl pyrophosphate (IPP, $C_5$) (Fig. 1). The synthesis of IPP starts with the condensation of three molecules of acetyl-CoA to produce the $C_6$ compound 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and proceeds through mevalonate. A second route from pyruvate and glyceraldehyde-3-phosphate to IPP has been shown to operate in bacteria and chloroplasts [21-23], but probably not in fungi [24]. IPP itself is not reactive enough to initiate the condensation to higher terpenoids, and is converted into its isomer dimethylallyl pyrophosphate (DMAPP) by the IPP isomerase. Different prenyltransferases condense IPP units to generate geranyl pyrophosphate (GPP, $C_{10}$), farnesyl pyrophosphate (FPP, $C_{15}$) and geranylgeranyl pyrophosphate (GGPP, $C_{20}$). These intermediates may then be used as side chains for many molecules, undergo secondary transformations, or self-condense to the $C_{30}$ and $C_{40}$ precursors of sterols and carotenoids, respectively.

Most carotenoids contain a $C_{40}$ hydrocarbon skeleton that includes between 3 and 15 conjugated double bonds. The specific part of the carotenoid branch begins with the condensation, by phytoene synthase, of two molecules of GGPP to give rise to phytoene. Typically, a phytoene dehydrogenase introduces then several dehydrogenations into phytoene to produce neurosporene or lycopene. Two structurally and functionally distinct classes of phytoene dehydrogenase have been described.

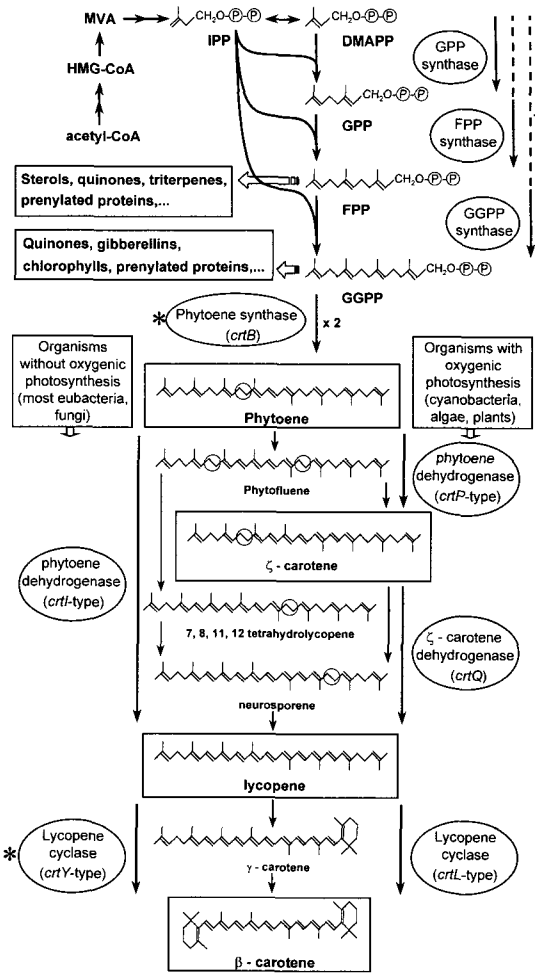


Fig. 1. β-carotene biosynthesis pathway from acetyl-CoA. The specific carotenoid branch is very similar in all organisms, but different enzymes are involved. The final product of each enzymatic step is boxed. An *asterisk* (*) indicates the particular situation present only in fungi: the same enzyme is involved in several steps (see text for more details).

In most eubacteria, fungi and anoxygenic photosynthetic bacteria, the *crtI*-type of phytoene dehydrogenase introduces three or four dehydrogenations to give rise to neurosporene or lycopene, respectively [16]. In contrast, in cyanobacteria, algae and higher plants the *crtP*-type of phytoene dehydrogenase converts phytoene into ζ-carotene, and then, a ζ-carotene dehydrogenase (*crtQ*) proceeds with the subsequent two dehydrogenations to lycopene [20]. The carotenoid biosynthesis pathways of different organisms diverge from neurosporene or lycopene to generate the diversity found in nature. While phytoene is a flexible and colourless molecule, the introduction of successive double bonds increases the rigidity, the hydrophobicity, and the ability to absorb visible light. The hydrophobic nature of carotenoids implies that their synthesis and intracellular location must be membrane-bound.

The next step in carotenoid biosynthesis is the cyclisation of lycopene, giving rise to α- or β-carotene through the intermediates δ- or γ-carotene. There are also several types of lycopene cyclase, the *crtY* type of eubacteria, and the *crtL* type from oxygenic photosynthetic organisms, which produce β-carotene by two successive β-cyclisations. A third type of lycopene β-cyclase is present in fungi and some bacteria as we will see below. In plants, the lycopene ε-cyclase introduces an ε-ring to the symmetrical lycopene or to the γ-carotene, giving rise to the monocyclic δ-carotene or the dicyclic α-carotene, respectively [20].

Xantophylls are oxygenated carotenoids. Zeaxanthin and lutein are synthesised by hydroxylation, in the 3 position, of each ring of β- and α-carotene, respectively. Another type of carotene modification widely distributed in nature is the introduction of keto groups in the 4 position of the rings. Among the carotenoids with this structure, we can find echinenone and cantaxanthin. In many bacteria, fungi, and some algae, the end product is the result of both of the above-mentioned modifications, as it happens with the diketo, dihydroxy compound astaxanthin [16,25,26].

## CAROTENOIDS IN FUNGI

Carotenoids are not as widely distributed in fungi as they are in plants. Most fungi produce no carotenoid at all, and those which do, show a carotenoid composition much simpler than photosynthetic organisms, often accumulating a single major carotenoid. Moreover, although many of the known carotenoids are not produced by fungi, most of the fungal carotenoids are the same that are found in plants and algae, which is of interest for fungal biotechnology. In the Mucorales *B. trispora*, *Mucor circinelloides*, and *Phycomyces blakesleeanus*, β-carotene is the main carotenoid; β-carotene and neurosporaxanthin predominate in the ascomycetes *Gibberella fujikuroi* and *Neurospora crassa*; and astaxanthin is the major carotenoid in the basidiomycetous yeast *X. dendrorhous*. Several fungi are the only producers of certain carotenoids. In *P. blakesleeanus*, most of the β-carotene is found in lipid globules (protein-coated oil drops), and some in a particulate vacuolar fraction [27]. β-carotene is synthesised in the membranes of these lipid globules [28].

The amounts and composition of carotenoids accumulated by fungi are highly variable, but natural concentrations are usually too low for commercial applications. The accumulation of carotenoids in fungi is affected by many environmental variables: it depends on the strain and culture conditions, it is influenced by light, or increases during the sexual interaction in the Mucorales [29]. Natural differences in carotenoid content and composition were found in several isolates of *P. blakesleeanus* [30-32] and *Ustilago violacea* [33]. The growth temperature and/or the composition of the medium also influence the accumulation of carotenoids. Peptone, acetate, leucine and other amino acids increase the β-carotene content of *P. blakesleeanus*, probably by direct conversion to HMG-CoA [34]. A common phenomenon for many fungi is that they accumulate higher amounts of carotenoids when grown under blue light than in the dark. This photocarotenogenic response [35-38], however, is not universal, since in other fungi, such as *B. trispora*, light has no effect [39]. Exposure of *X. dendrorhous* cells to high light intensities resulted in growth inhibition, decreased carotenoid synthesis, and changes in carotenoid composition [40]. In many species of the Mucorales, when mycelia of the opposite sex meet, the contact region becomes bright yellow due to the accumulation of β-carotene in sexual hyphae called zygophores. Similarly, mated cultures [41,42] or intersexual heterokaryons [43,44] show a sexual stimulation of carotenogenesis. The pheromones that initiate sexual development in the Mucorales are mating-type-specific derivatives of β-carotene and precursors of the trisporic acids [45,46]. The trisporic acids alone stimulate carotenogenesis in single cultures [47], but have no effect in fungi other than the Mucorales.

At least two different groups of chemicals [48-50] have been shown to exert a strong stimulation of carotenogenesis in the Mucorales: β-ring containing compounds (retinol or β-ionone) and several phenols (dimethyl phtalate or veratrol). The spot test devised by Cerdá-Olmedo and Hüttermann [51] provides a convenient method to screen new chemical activators in *P. blakesleeanus*, as well as to determine their potential synergistic relationships. It is important to note that the results for one fungus are often invalid for another: β-ionone is effective in stimulating carotenogenesis in *B. trispora* and *P. blakesleeanus*, but inhibits it in *G. fujikuroi* [52]; isoniazid is active for *B. trispora*, but not for *P. blakesleeanus* [15]. Unpublished observations suggest that in *M. circinelloides*, most of these chemicals do not enter the cells.

### Genetic Analysis of the Carotenoid Pathway

Many carotenoid-producing fungi are amenable to classical genetic analysis, but only a few have received appreciable attention [53,54]. Mutants altered in the carotenoid pathway are usually detected by a change in colour due to the accumulation or lack of intermediate products. *P. blakesleeanus* is probably the fungus in which the synthesis and regulation of carotenoids has been more extensively analysed [15,34]. The first *P. blakesleeanus* colour mutants were isolated in the 60's [31,55], and since then, many other mutants with altered carotenogenesis have been isolated. Genetic analysis of *P. blakesleeanus* carotenoid mutants revealed only two structural genes responsible for the conversion of phytoene to β-carotene: *carB* and *carR* [32]. Mutants altered in the *carB* gene are white and contain large amounts of phytoene. Other *carB* (leaky) mutants are greenish or yellowish and accumulate also phytofluene and ζ-carotene, partially dehydrogenated products of phytoene [56]. Mutants altered in the *carR* gene are red and accumulate lycopene and also other intermediates.

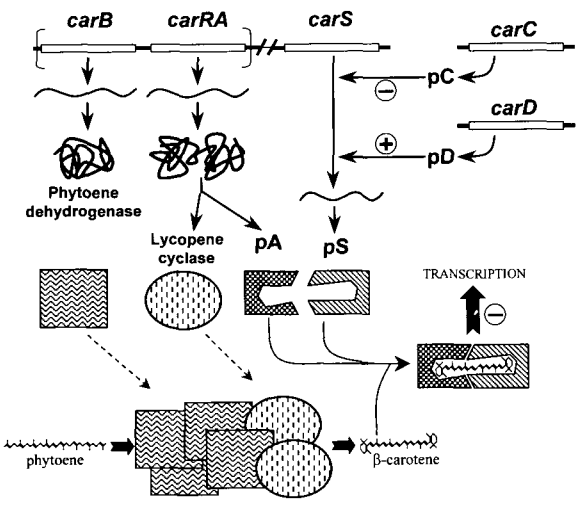


Fig. 2. Schematic representation of the β-carotene biosynthesis model of *P. blakesleeanus* [34]. Two closely linked structural genes (*carB* and *carRA*) give rise to three proteins (after cleavage of the *carRA*-encoded polypeptide). Two of these proteins (phytoene dehydrogenase and lycopene cyclase) arrange in an aggregate which transform phytoene into β-carotene, and the third protein is one of the regulatory proteins, that forms a complex with β-carotene and pS, the product of *carS*, acting negatively on the transcription of carotenogenic genes.

Some *carR* mutants are leaky and contain some β-carotene. Indeed, the *carR* gene is part of a bifunctional gene, *carRA*, responsible also for the A gene product, which was postulated to be involved in the regulation of carotenogenesis [57,58]. No mutants altered at the phytoene synthase have ever been described, and it was suspected that a single enzyme could catalyse the condensation of two molecules of either FPP or GGPP to produce squalene or phytoene, respectively [34]. Mutants impaired in this enzyme would then be lethal. This interpretation about the role of the A gene product has recently been revised [59,60].

Quantitative complementation analyses [61] led to the conclusion that conversion of phytoene to β-carotene is carried out by an enzyme aggregate: four copies of the phytoene dehydrogenase (the *carB* product) act sequentially on a substrate molecule and convert it successively into phytofluene, ζ-carotene, neurosporene, and lycopene. Then, two copies of the lycopene cyclase (the R part of *carRA* gene product) transform lycopene first into γ-carotene and then to β-carotene (Fig. 2).

Classical genetic techniques are well established in *P. blakesleeanus*. They have allowed the analysis of numerous carotenoid mutants, the setting up of a model for the production and regulation of carotenogenesis (Fig. 2) [15], and the construction of highly carotenogenic strains [62,63]. However, it is quite difficult to apply the molecular genetic techniques to this fungus, because of the lack of an efficient transformation system. Several years ago, we decided to use the related species *M. circinelloides* as a shuttle system to investigate the carotenogenesis pathway. This fungus has a very efficient transformation system and the plasmids used in transformation behave autonomously [64-66] or can be forced to integrate in the genome [67,68]. Moreover, exogenous genes, particularly those of *P. blakesleeanus*, are efficiently expressed in *M. circinelloides* [65,69,70].

The hunt for carotenoid mutants in *M. circinelloides* [59,71-73] resulted in a similar scenery to that in *Phycomyces*, with an important difference: mutants altered in the three enzymatic steps of the β-carotene synthesis from GGPP were found. Mutants affected in the *carB* and *carR* genes are very similar to those found in *Phycomyces*. However, *carP* mutants were affected at the phytoene synthase activity [59]. The *carP* mutants are similar to the *carA* mutants of *Phycomyces* in that they do not accumulate any carotenoid, and that they are altered in a bifunctional enzyme (the product of the *carRP* gene). In *M. circinelloides* it has been shown that this gene product has phytoene synthase and lycopene cyclase activities (Velayos *et al.*, submitted).

## Fungal Genes and Enzymes for Carotenoid Biosynthesis

Only a few fungal genes involved in the biosynthesis of carotenoids have been cloned to date. The genes and the corresponding enzymes are listed in Table 1. Representatives of all the structural genes involved in the biosynthesis of β-carotene have been isolated, but none of the astaxanthin-specific pathways are available yet.

The enzyme geranylgeranyl pyrophosphate synthase (GGPPS) can be considered to catalyse the previous step in the carotenoid pathway. Genes encoding GGPPS have been isolated from several fungi. A GGPPS gene has been cloned in *N. crassa*, *Nigrospora sphaerica* and *M. circinelloides*, while in *G. fujikuroi* two genes which are thought to be involved in different branches of the isoprenoid pathways, have been isolated [74]. In other fungi such as *P. blakesleeanus*, only the enzyme has been purified and characterised [75]. These enzymes belong to a family of prenyl synthases with different allylic substrate and chain length specificity [16]. In addition to their role in carotenoid biosynthesis, GGPP is a precursor of other important compounds such as quinones or gibberellins. Therefore, it is likely that the synthesis of GGPP is subject to complex regulation and possibly compartmentalisation. At least in *N. crassa* and *M. circinelloides*, it has been shown that light induces an increase in the transcription of these genes [36] (Velayos, unpublished).

In fungi, the specific carotenoid pathway to β-carotene proceeds via three enzymatic steps carried out by phytoene synthase, phytoene dehydrogenase, and lycopene cyclase. The deduced proteins of the fungal phytoene dehydrogenase encoding genes are of the *crtI*-type, able to introduce four dehydrogenations to produce lycopene. The *crtI* and *crtP* types of phytoene de-

Table 1. Cloned fungal genes for enzymes of the carotenoid biosynthesis pathway

| Organism | Gene | Encoded protein(s) | EMBL Acc. Num. | References |
|---|---|---|---|---|
| Ascomycota | | | | |
| *Cercospora nicotianae* | *PDH1* | phytoene dehydrogenase | U03903 | [113] |
| *Gibberella fujikuroi* | *ggs1* | GGPP synthase | X96943 | [114] |
| | *ggs2* | GGPP synthase | Y15280 | [74] |
| *Neurospora crassa* | *al-1* | phytoene dehydrogenase | M57465 | [88] |
| | *al-2* | phytoene synthase * | L27652 | [89] |
| | *al-3* | GGPP synthase | U20940 | [87] |
| | *wc-1* | transcriptional factor | X94300 | [92] |
| | *wc-2* | transcriptional factor | Y09119 | [93] |
| *Nigrospora sphaerica* | | GGPP synthase | AB037600 | |
| Basidiomycota | | | | |
| *Xanthophyllomyces dendrorhous* | *crtI* | phytoene dehydrogenase | Y15007 | [115] |
| | *crtYB* | phytoene synthase / lycopene cyclase | AJ133646 | [77] |
| Zygomycota | | | | |
| *Mucor circinelloides* | *carB* | phytoene dehydrogenase | AJ238028 | [60] |
| | *carRP* | phytoene synthase / lycopene cyclase | AJ250827 | |
| | *carG* | GGPP synthase | AJ276129 | |
| | *crgA* | putative transcriptional factor | AJ250998 | [94] |
| *Phycomyces blakesleeanus* | *carB* | phytoene dehydrogenase | X78434 | [90] |
| | *carRA* | phytoene synthase / lycopene cyclase | AJ276965 | |

*Reported as a phytoene synthase encoding gene, but homology data suggests that it could be a phytoene synthase / lycopene cyclase encoding gene.

hydrogenase are thought to have evolved as two unrelated types of proteins. However, a certain evolutive relationship cannot be discarded, as both types can be considered members of a FAD superfamily [60,76]. A single bifunctional protein carries out phytoene synthase and lycopene cyclase activities in fungi. The existence of a bifunctional gene (*carRP* of *M. circinelloides*, *carRA* of *P. blakesleeanus*, *crtYB* of *X. dendrorhous* and *al-2* of *N. crassa*) is a feature unique to fungal carotenogenesis. The first step specific to carotenoid biosynthesis, the synthesis of phytoene, is carried out by the C-terminus of this bifunctional polypeptide. The deduced amino acid sequences of these C-termini are very similar to phytoene synthases from all organisms, from bacteria to plants [16]. The only significant difference is that in fungi these sequences are fused to a polypeptide with lycopene cyclase activity. This may have occurred by fusion of the previously independent corresponding genes.

The N-terminus of the fungal protein is involved in the cyclisation of lycopene to give rise to β-carotene [77] (Velayos *et al.*, submitted). This part of the protein constitutes a novel kind of lycopene cyclase, completely unrelated to both the *crtL* and the *crtY* types. However, an interesting similarity has been found with some ORFs of unknown function in carotenogenic gene clusters of several bacteria (*Myxococcus xanthus* and *Mycobacterium marinum*) and with two ORF from *Brevibacterium linens* which very recently have been reported as lycopene cyclase [78]. This suggests that in some carotenogenic bacteria there are genes encoding polypeptides homologous to this fungal type of lycopene cyclase. These bacterial genes would encode polypeptides involved in lycopene cyclisation and, in the course of evolution, they probably became fused to the phytoene synthase encoding genes.

The lycopene cyclase part of the bifunctional polypeptide shows several transmembrane regions. This suggests that the phytoene synthase is anchored to the membrane through amino acids present in the N-terminus which are not involved in the phytoene synthase catalytic activity, but in the lycopene cyclase activity. When the N-terminus is removed, there is no phytoene synthase activity in *M. circinelloides*, but there is lycopene cyclase activity in the absence of the C-terminus (Velayos *et al.*, submitted), what suggests that anchorage to the membrane is essential for phytoene synthase activity. In other systems, the attachment of the soluble phytoene synthase to the membrane, which is necessary due to the insoluble nature of phytoene, is probably achieved by protein-protein interactions. These kinds of interactions are probably present between units of both the bifunctional protein and the phytoene dehydrogenase. A schematic model of a hypothetical carotenogenic aggregate in Mucorales that may probably be extended to the rest of fungi, is shown in Fig. 3. As it has been shown in *P. blakesleeanus*, four phytoene dehydrogenase and two lycopene cyclase molecules are involved in the synthesis of β-carotene. This implies the presence in our model of two mole-

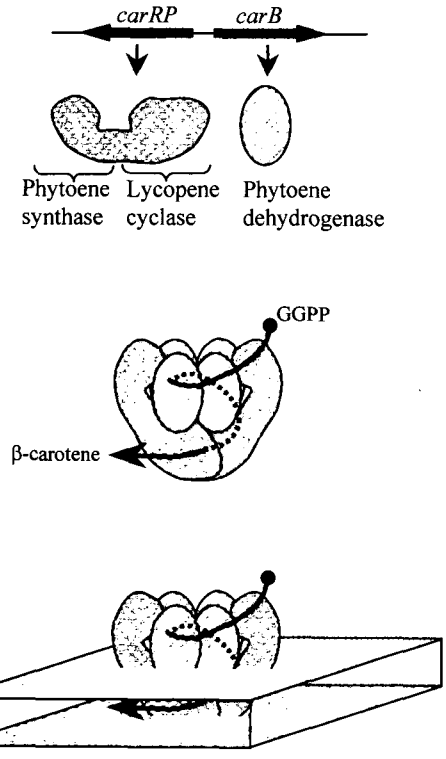


Fig. 3. Hypothetical model for a carotenogenic enzymatic aggregate in *Mucor circinelloides*. The same promoter controls the two structural genes *carB* and *carRP*; the encoded enzymes carry out the phytoene dehydrogenation (*carB*) and both phytoene synthesis and lycopene cyclisation (*carRP*). Two *carRP* and four *carB* products would interact forming an aggregate as previously proposed for *Phycomyces blakesleeanus* [34]: each phytoene dehydrogenase unit would perform a dehydrogenation and each lycopene cyclase a cyclisation. Two phytoene synthases are present but probably each one is able by itself to synthesise phytoene, which would pass through all the aggregate (only one of the two putative ways from GGPP to β-carotene is represented). Below, the probably actual situation of this aggregate anchored in a membrane (see text).

cules of phytoene synthase. There is no evidence that phytoene synthase works as a dimer. So, in our model, each unit of this enzyme synthesises phytoene, which probably pass trough the dehydrogenase and cyclase units of the aggregate up to β-carotene. It is likely that most of the phytoene molecules formed in an aggregate are transformed into β-carotene, but we cannot discard the possibility that any of the intermediate products are released to the membrane and even incorporated to a different aggregate.

## The Regulation of the Pathway

The particular features of the pathway in each organism affect the final carotenoid accumulation. From a theoretical point of view, this accumulation relies on the availability of substrates, the different mechanisms of regulation, and the effect of external factors. The existence of hyperproducing carotenoid mutants suggests that there must be regulatory mechanisms. This kind of mutants has been found in several fungi: *P. blakesleeanus* [62], *B. trispora* [79], *M. circinelloides* (Velayos, unpublished), and *X. dendrorhous* [80,81]. In *P. blakesleeanus*, a large number of mutants has been analysed, and different regulatory genes have been detected. Alteration of these genes can result in either deep yellow (*carS, carD, carF*) or light yellow (*carC*) mycelia. Mutations in the *carB* or in the *carR* part of the *carRA* gene result in a general activation of the pathway leading to a fifty-fold increase in carotenoid content, that is phytoene or lycopene, the intermediates accumulated. The same occurs in wild type when specific inhibitors of phytoene dehydrogenase or lycopene cyclase are used [43]. For a long time, it has been supposed that the A part of the *carRA* gene encoded a regulatory polypeptide involved in the transference of substrates in and through the enzymatic aggregates [58]. According to the model put forward several years ago, the product of the genes *carA* and *carS*, together with β-carotene, mediate this end-product inhibition of the pathway [49], as it is summarised in Fig. 2. This kind of end-product regulation seems to be absent in other fungi such as *M. circinelloides* [71], *B. trispora*, or *G. fujikuroi* [82]. When β-carotene and the *carS* gene product (pS) are present, they join pA and form a complex, which acts negatively on gene transcription; pA is thus sequestered and carotenogenesis would stop after enough β-carotene has been produced. Therefore, this regulation would be different from "feedback" and "repression" since no early enzymatic reaction would be blocked [34]. Very recent results make necessary to look over this model: since pA is in fact the phytoene synthase, the action of β-carotene and pS over pA could now be interpreted as feedback or repression mechanisms over the phytoene synthase, the first enzyme of the pathway, as it usually happens in many biosynthetic pathways. An important fact to be taken into account is the restoration of function in the *carA* mutants when vitamin A is added [48]. Obviously, this would be possible only if the mutations in the *carA* portion of the *carRA* gene give rise to a phytoene synthase with potential activity which is not brought about under normal *in vivo* conditions: vitamin A could somehow stabilise the defective enzyme and restore its function. Different *carRA* alleles are now being analysed to test the correlation between the mutations they carry and the vitamin A effect (Sanz, unpublished).

Several external factors have been shown to exert a positive or negative effect on carotenoid production of wild type and/or mutants. Most of them are chemical compounds, whose mode of action is not known. As we saw earlier, the effect of a chemical is not the same in different fungi [82].

Since carotenoids are derived from essential compounds (FPP, GGPP), such substrates are always available, although their destination to the specific branches of the isoprenoid pathway may be different. In several

fungi, such as *P. blakesleeanus* and *G. fujikuroi*, compartmentalisation of sterols and carotenoids has been described [83,84]: the same pathway is differentially controlled regarding the final product. While substrates are always available for sterol synthesis, they can be restricted for non-essential pathways, as it happens with carotenoids in fungi. A higher availability of these substrates can result in an increase in carotenoid production; this has been shown when multiple copies of the gene encoding IPP isomerase are introduced in bacterial cells [85,86].

Probably, the key mechanism of regulation operates at the transcriptional level. This kind of regulation has been found in most fungi in which different genes involved in carotenogenesis have been isolated. The most general agent that regulates the expression of these genes is blue light. A light-induced increase in transcript accumulation of several carotenogenic genes has been shown in *N. crassa* [87-89], *M. circinelloides* [60], and *P. blakesleeanus* [90]. A negative effect has been reported in the carotenogenesis of *B. trispora* and *X. dendrorhous* [39,40]. The action of blue light on carotenoid production is exerted through gene transcription, although other mechanisms could be involved. A sequence named APE (*al-3* proximal element) was defined in the promoter region of the gene *al-3* of *N. crassa*, and shown to be involved in blue light regulation [91]. Similar sequences have been reported for other blue-light induced genes in *N. crassa* [91], *P. blakesleeanus* [90], and *M. circinelloides* [60]. It is also possible that other sequences of the promoter regions are necessary for this and/or other kind of regulation.

A particularly interesting cluster-like organisation of the structural genes *carB* and *carRP* is found in *M. circinelloides*. Both genes are only 446 bp apart, and oriented in opposite directions. Their expression is regulated by light, as it happens with the *carG* gene (see Table 1), and seems to be co-ordinated: northern analyses showed that blue light induces the transcription of both *carB* and *carRP* genes in a very similar way (Fig. 4). The presence of several APE-like motifs in this short promoter suggests that the effect of these sequences can be bi-directional (Velayos *et al.*, submitted). In other fungi, these two genes are not so closely linked, so a different regulation could exist.

Little is known about the molecular nature of the regulatory genes involved in the carotenogenesis in fungi. Only three of these genes have been isolated to date: the *wc-1* and *wc-2* genes of *N. crassa* [92,93] and the *crgA* gene of *M. circinelloides* [94]. The *N. crassa* white-collar mutants (*wc-1* and *wc-2*) are pleiotropically defective in all blue light induced phenomena. Both genes encode proteins with a zinc finger DNA binding domain and a putative dimerisation domain (PAS). These proteins are blue-light-activated transcription factors and elements of the blue-light signal transduction pathway in *N. crassa* [95]. The *crgA* gene of *M. circinelloides* has a dominant-positive effect on light-regulated carotenogenesis in this fungus. This gene was originally identified in transformation experiments as a genomic DNA fragment causing carotenoid overaccumulation in the dark [94]. The *crgA* product displays several structural features such as a RING-finger zinc binding domain and glutamine-rich regions. Several observations suggest that this gene acts as a negative regulator in the dark.

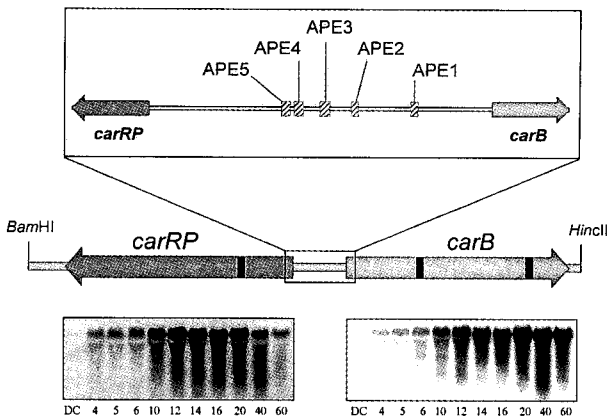


Fig. 4. Schematic representation of the organisation of the *Mucor circinelloides* structural genes specifically involved in β-carotene biosynthesis. Above, distribution of the APE (*al-3 proximal element*)-like sequences (see text) probably involved in light regulation of transcription. Below, an example of northern hybridisation with either *carRP* (left) or *carB* (right) probes of total RNA from *M. circinelloides* 3-day-old mycelia (grown in darkness) after a pulse of blue light (4 W/m$^2$ for 4 minutes or 960 J/m$^2$). It shows the positive and very similar effect of blue light on transcript accumulation of both genes; numbers indicate the time after the start of irradiation when the mycelia were harvested (only the first 4 minutes of this time were of irradiation); DC, darkness control.

## INDUSTRIAL PRODUCTION AND BIOTECHNOLOGY

From the technological point of view, the industrial production of carotenoids by fungi is feasible. Several carotenoid-producing fungi are the source of well-established industrial products (like the rennin or lipases from *Mucor* sp.). However, there is only one example of industrial production of fungal β-carotene. The process was first developed in the USA [96], and later improved in France [97,98], to get up to 30 mg of β-carotene per g dry weight or about 3 g/L. The fermentation process is currently used in Russia and Ukraine, and employs submerged cultures of *B. trispora* with aeration and agitation. Significant disadvantages of this process are the need for joint fermentation of two strains of opposite sex, and the use of chemical additives. Several factors affecting this production have been reviewed [99].

The research towards a possible industrial production has concentrated on fungi producing β-carotene or

astaxanthin and it has been undertaken only at the laboratory level. As we commented earlier, the carotenoid content of fungi is usually too low for industrial purposes. Strain improvement, optimisation of the medium and culture conditions, and the search for chemical activators are some but different strategies that have been followed to increase the carotenoid content of these fungi. In *P. blakesleeanus*, mutations in the *carS* gene increase the production of β-carotene up to 3-5 mg/g dry weight, and the use of intersexual heterokaryons carrying *carS* and balanced lethal mutations [62,100] has allowed overproductions of up to 25 mg β-carotene/g dry weight. Random mutagenesis has also been used in other fungi to isolate carotenoid hyperproducing mutants [79-81] (Velayos, unpublished). Strain improvement needs not be restricted to the accumulation of carotenoids, but it can be directed to develop strains that better fit the industrial production. In *P. blakesleeanus*, surface cultures are the most productive [101], since there is a decrease in carotenoid production in shaken cultures. The growth temperature for *B. trispora* and *P. blakesleeanus* must never be above 25°C, and this requires more energy to lower the culture temperature. The cell walls of *X. dendrorhous* are so thick that they cannot be used directly for animal feed, and must be partially lysed with *Bacillus circulans* [102]. In this context, *Mucor* offers a great advantage: it is a dimorphic fungus, that is, it can grow either aerobically as a mycelium, or anaerobically as budding yeasts. Monomorphic mutants of *Mucor* that grow exclusively as yeasts under aerobic conditions, and which are not altered in their carotenogenic activity, have been isolated [103] (Iturriaga, unpublished). This type of morphology is preferred by the fermentation industry because it allows the growth in submerged conditions, the biomass production is usually higher, and the cells are more easily separated from the culture media. Moreover, these yeast cells grow perfectly well at about 30°C. Experiments are currently in progress in our laboratory on the above-mentioned aspects.

Media composition is another important aspect in the fermentation industry. The best media employed in the production of β-carotene with *B. trispora* are composed of grain and legume seeds extracts, hydrocarbons (kerosene) and oils (soya or cotton-seed oil). The addition of microbial biomass (spent *B. trispora* mycelia, after the extraction of β-carotene, and cells of various bacteria, yeast and fungi) to mated *B. trispora* cultures also stimulates carotenogenesis [104]. It has also been observed that *Phycomyces* can grow on various simple media, composed of cheap sub-products of biological industries, increasing the biomass and maintaining its β-carotene concentration [15].

The industrial purification of β-carotene is complicated and expensive, and may not be necessary for many practical applications. For use in animal feeds, the carotene-rich mycelium may be dried, blended with different additives, and powdered. The increase in carotenoid production may be combined with other practical applications of the Mucorales. It has been shown that overproduction of β-carotene is accompanied by an increase in the total fatty acid content in *Phycomyces* which can reach up to the 50 % dry weight [105]. Polyunsaturated fatty acids and γ-linolenic acid (which are essential to mammals) predominate in the composition of the total oils of both *Phycomyces* and *M. circinelloides* [106]. Oil dissolves and stabilises β-carotene, so a carotene-rich oil saves in the extraction, purification and conservation of β-carotene. Moreover, the carotene-rich oil-rich mycelia might be used directly for animal feed. *Mucor* mycelia are also used in microbial biotransformations of steroids [107,108], and it has been shown that mycelial dead biomasses are effective in heavy metal biosorption [109,110].

The advent of molecular genetics has allowed an in-deep characterisation of the carotenoid pathway in fungi. One of the most exciting prospects on carotenoid biosynthesis is the development of novel strategies for the production of specific carotenoids irrespective of the organism employed. Recently, the phytoene synthase gene of daffodil has been introduced in rice. This transgenic rice accumulates phytoene, a compound with provitamin A activity, in the endosperm [111]. Bacterial genes for carotenoid biosynthesis have also been introduced and expressed in otherwise colourless organisms such as *Saccharomyces cerevisiae* or *Candida utilis* [112], two organisms used in well-established industries. Among the most recent findings is the use of multiple copies of IPP isomerase to increase the availability of substrates and the production of carotenoids [85,86].

## CONCLUSIONS AND OUTLOOK

Knowledge of the biology of carotenoids in fungi is required, not only to understand their basic processes, but also to develop biotechnological strategies for the industrial production of carotenoids. The isolation of several carotenogenic genes, including regulatory ones, has opened up the way to study the regulation of the pathway. In addition to understanding the control of gene expression, it is equally important to determine the relationships of the gene products in the carotenoid complexes. The use of several Mucorales, especially *P. blakesleeanus*, has allowed one of the most detailed models about carotenogenesis in any organism. The detection of a novel kind of carotenogenic gene has occurred at almost the same time in several fungi, but the versatility of *M. circinelloides* about its mutants allows a promising progress in the study of the molecular basis of carotenoid production and regulation. The structural gene organisation for carotenoid biosynthesis in this fungus is very attractive since it is the closest example among fungi to a co-ordinated regulation. This is important not only by itself, but from an applied point of view, as the improvement of gene transcription would be easier for biotechnological purposes. Further studies will take place at all levels of research during the next years. They will surely increase our basic knowledge, and contribute to develop a successful and commer-

cially viable technology for the production of carotenoids by fungi.

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