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Short Communication

Lycopene cyclization in *Blakeslea trispora*

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Fungi produce and accumulate various carotenoids. Mycelia of the Zygomycete *Blakeslea trispora* contained β-carotene and its precursors γ-carotene and lycopene. When strains of opposite sex grew together, the β-carotene concentration increased fourfold, that of γ-carotene remained unchanged, and other intermediates practically disappeared. The inhibitors nicotine, 2-(4-chlorophenylthio)-triethylamine, α-picoline, and imidazole increased the concentrations of lycopene and γ-carotene and decreased those of β-carotene. From our quantitative results, we conclude that *Blakeslea* has two pathways for lycopene metabolism, of which other fungi have only one or the other. The main one, two cyclizations from lycopene to β-carotene, is carried out by an enzyme dimer, is stimulated by sexual interaction, and is sensitive to the inhibitors. The other pathway, a cyclization to γ-carotene, is not affected by mating or the inhibitors.

Key Words——*Blakeslea trispora*; carotene; cyclase inhibitors; lycopene cyclization; sexual activation.

Fungi produce many different carotenoids and some are attractive industrial sources for them (Vandamme, 1989; Bramley and Mackenzie, 1992). Industrial processes for β-carotene production (Ciegler, 1965; Ninet and Renaut, 1979) take advantage of its accumulation in submerged mycelia of *Blakeslea trispora* Thaxter, especially in "mated cultures", that is, cultures in which mycelia of the two sexes grow together (Lampila et al., 1985).

The last steps in the biosynthetic pathway consist in two cyclizations at the ends of the lycopene molecule, which is converted first to γ-carotene and then to β-carotene. Despite the enormous progress in the genetics and molecular biology of carotenoid production (Armstrong and Hearst, 1996), no fungal genes or enzymes for lycopene cyclization have been isolated yet. In another Zygomycete, *Phycomyces blakesleeanus* Burgeff, which accumulates essentially pure β-carotene, the cyclizations are carried out by an enzyme aggregate composed of two identical units, each responsible for one of the cyclizations. The existence and the mode of operation of this enzyme aggregate were deduced from the relative concentrations of lycopene, γ-carotene, and β-carotene accumulated when the pathway was partially blocked by genetic mutations (De la Guardia et al., 1971) or chemical inhibitors (Candau et al., 1991), and is not adequately modelled by metabolic control theories.

Lycopene cyclization in *B. trispora* can be blocked by mutations (Mehta and Cerdá-Olmedo, 1995), but these mutants cannot be used to investigate the operation of the pathway because of their lack of spores and the primitive status of the genetics of the organism. Lycopene cyclization is inhibited by imidazole, α-picoline, 6-methyl-2-aminopyridine (Ninet et al., 1969; Feofilova et al., 1995), and 2-(4-chlorophenylthio)triethylamine·HCl (CPTA) (Coggins et al., 1970) in *B. trispora* and by nicotine (Howes and Batra, 1970) in other organisms.

We observed that β-carotene was mixed with sizable amounts of its precursors in *B. trispora* mycelia of either sex grown separately, but was relatively free from them in mated cultures. The investigation of this disparity led us to recognize that lycopene metabolism can follow two alternative pathways in this fungus.

Materials and Methods

The wild-type strains NRRL2456, mating type (+), and NRRL2457, mating type (−), of *B. trispora* (Class Zygomycetes, Order Mucorales, Family Choanophoraceae) were cultured on minimal agar as described for *P. blakesleeanus* (Cerdá-Olmedo, 1987). Spores were collected from cultures grown on yeast nitrogen-base agar and kept in glycerol (250 g/l in water) at 4°C for up to one month prior to use. Plates were inoculated with 5000 spores and incubated in the dark at 30°C for 4 d. For mated cultures, 5000 spores of each strain were inoculated together.

Carotenes were extracted from lyophilized mycelia detached from agar media (Govind and Cerdá-Olmedo, 1986) and chromatographed on an aluminum oxide (Brockmann grade II–III) column (8 cm long, 1 cm wide) by elution with increasing concentration of ethyl ether in petroleum ether (Candau et al.,1991). The individual carotenes were quantified from their absorption coeficients (Davies, 1976). The dry mass was determined by weighing the lyophilized mycelia. The concentrations given are the means of at least two independent experimental results, with two determinations in each.

CPTA was a kind gift from Dr. Henry Yokoyama

(USDA Fruit and Vegetable Laboratory, Pasadena, CA). Nicotine was purchased from Sigma (St. Louis, MO), imidazole from Serva (Heidelberg, Germany), and α-picoline (2-methylpyridine) from Fluka (Buchs, Switzerland). Chemicals were added to the agar just before pouring the plates.

Results

In mated cultures, β-carotene constituted about 90% of the total carotenes; this allowed the development of *B. trispora* as an industrial source of β-carotene. In single cultures of the same wild types, β-carotene was less abundant and constituted less than 40% of the carotene mixture (Table 1). The absolute γ-carotene content was the same in mated and single cultures. These observations suggested a difference in cyclization between single and mated cultures.

Single cultures of the (−) strain were grown in the presence of CPTA, nicotine, and α-picoline. At the concentrations used in the experiments, these chemicals had little or no effect on growth and the total carotene content, but the proportions of different carotenes changed drastically (Figs. 1, 2). Under the effects of these chemicals, the β-carotene content was reduced and the lycopene content increased; at the higher concentrations, β-carotene practically disappeared. The effects of imidazole were similar to those of the other chemicals (Fig. 2), but the high concentrations (5 and 10 mM) needed to modify the composition of the carotene mixture in the mycelia were moderately toxic and reduced the mycelial dry weight by an average of 27%.

Discussion

We have observed that mycelia from mated and unmated cultures of *Blakeslea* differed considerably in their carotene contents, both qualitatively and quantitatively; CPTA, nicotine, α-picoline, and imidazole modified those contents: molecules with β rings were replaced by molecules with linear ψ ends. Our results can not be explained by the existence in *Blakeslea* of a single cyclase to carry out the two cyclizations needed to convert lycopene into β-carotene, although this simple explanation fits perfectly the results with a related fungus, *P. blakesleeanus* (De la Guardia et al., 1971; Candau et al., 1991). We propose that *Blakeslea* has two kinds of lycopene cyclase (Fig. 3). The first one, let us call it A, is a dimer aggregate that carries out the two cyclizations needed to convert lycopene to β-carotene. This activity is in-

Table 1. Carotene content of the (+) wild-type NRRL2456 and the (−) wild-type NRRL2457 grown separately or together ("mated"). Data are means and standard errors from 4 independent experiments (8 in the case of NRRL2457), with 2 determinations in each.

	NRRL2456	NRRL2457	Mated
Dry weight, mg per plate	154±10	119±6	139±10
Carotene, μg g^{-1} dry wt			
Phytoene	77±8	88±15	0
Phytofluene	7±2	9±3	0
Lycopene	21±2	16±2	6±2
γ-Carotene	48±5	43±4	44±3
β-Carotene	98±11	88±7	415±18
Total	251±23	244±23	464±18

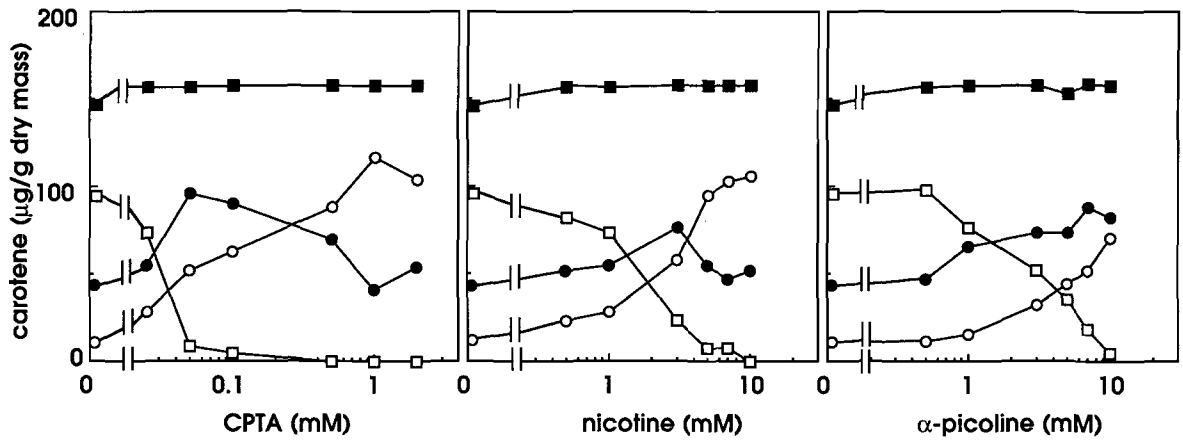

Fig. 1. Carotene content of wild-type NRRL2457 mycelia grown for four days in the presence of various concentrations of CPTA, nicotine, and α-picoline. Symbols: ○, lycopene; ●, γ-carotene; □, β-carotene; ■, total of the three carotenes.

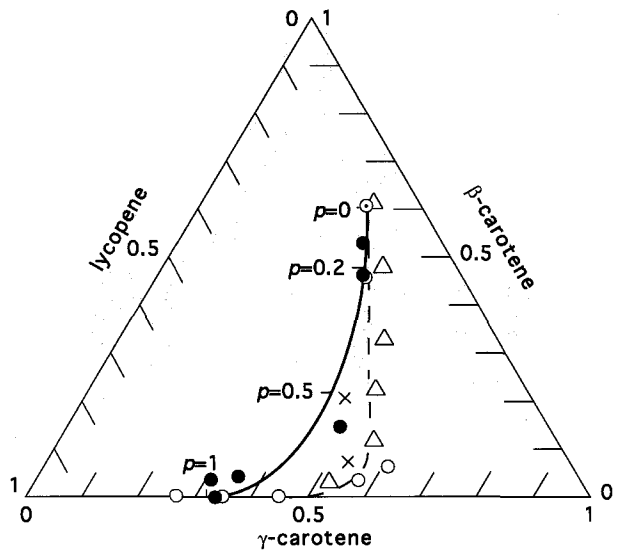

Fig. 2. Proportions of β-carotene, γ-carotene, and lycopene in wild-type NRRL2457 mycelia with respect to their sum. Calculated from the experimental values in Fig. 1 and additional results. The proportions are plotted as the distances of the symbols to the sides of the triangle (homogeneous coordinates). Symbols: ⊙, control; ○, CPTA; ●, nicotine; △, α-picoline; ×, imidazole (5 and 10 mM). The continuous line represents the predictions from the operation of two kinds of cyclase, one sensitive and one resistant to the inhibitors, as proposed in the text: lycopene $0.11+0.60p$; γ-carotene, $0.29+0.60p(1-p)$; β-carotene, $0.60(1-p)^2$. The broken line additionally assumes that resistant cyclases convert to γ-carotene one fourth of the lycopene molecules not metabolized by the inhibited ones: lycopene $0.11+0.45p$; γ-carotene, $0.29+0.75p-0.60p^2$; β-carotene, $0.60(1-p)^2$.

creased by sexual stimulation and inhibited by CPTA, nicotine, α-picoline, and imidazole. The second one, B, carries out only one cyclization, from lycopene to γ-carotene, and is not altered by either mating or the inhibitors.

This hypothesis readily explains the qualitative results. Sexual stimulation does not modify the γ-carotene content because this is due to B only. The activated metabolism in mated cultures hinders the accumulation of the precursor, phytoene. Inhibition of the first cyclase of the A aggregates leads to an increased accumulation of lycopene; a complete inhibition leads to the replacement of β-carotene by lycopene. Aggregates whose second cyclase is inhibited, but not the first, are responsible for increased accumulation of γ-carotene.

The same hypothesis allows a quantitative interpretation of the results. A proportion a of the lycopene formed in single, undisturbed cultures of the wild type is converted to β-carotene by the A aggregate; a proportion b is converted to γ-carotene by the B form; and the rest ($c=1-a-b$) accumulates as lycopene. From the values in Table 1 we calculate the parameters $a=0.60$, $b=0.29$, $c=0.11$.

Under a given exposure to a specific inhibitor, a lycopene molecule arriving at an A aggregate will have a probability p of finding the first cyclase blocked and being left unchanged, and a probability $1-p$ of finding the first cyclase active and being converted to γ-carotene. Let us assume that the second cyclase is identical to the first, being the product of the same gene, and is subject to the same probabilities p and $1-p$ of being found blocked or active by an incoming γ-carotene molecule. The proportion a of the lycopene that is processed by the A aggregates will have a probability p of remaining unchanged, a probability $p(1-p)$ of being converted to γ-carotene, and a probability $(1-p)^2$ of being converted to β-carotene. Inhibitors do not affect the fate of the lycopene molecules that are processed by the B cyclase or not metabolized. Under these assumptions, the carotene composition can be calculated as a function of p (continuous line in Fig. 2).

The experimental results were not very distant from this continuous line, but partially inhibited cultures contained more γ-carotene than expected. The deviations would be explained if the second cyclase was slightly more accessible to the inhibitors than the first; this is likely for the last unit of a membrane-bound enzyme aggregate. Another way of explaining the deviation would be that some of the lycopene molecules left untrans-

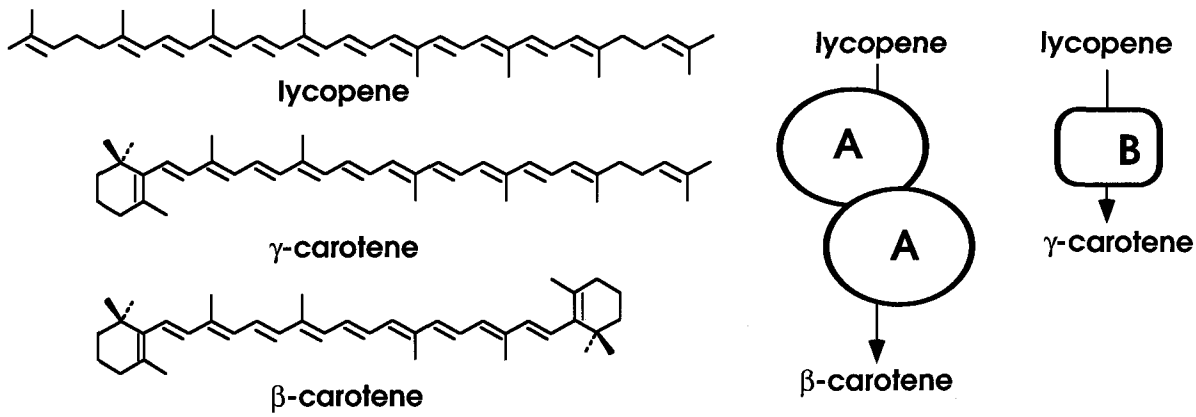

Fig. 3. Cyclization of lycopene to γ-carotene and β-carotene and proposed activities of two different lycopene cyclases, A and B, of *Blakeslea trispora*.

formed by inhibited A cyclases would not be stored away immediately, but taken up by the B cyclases and converted to γ-carotene; an excellent fit could be obtained in this way (broken line in Fig. 2).

The four chemicals tested, although very different in structure and properties, led to overlapping results in Fig. 2. This indicates that these results depend on the operation of the pathway, and not on chemical properties of the inhibitors other than their ability to block the A enzyme.

The two kinds of cyclases of *B. trispora* are found in other fungi but not together. Thus, the A aggregate of *B. trispora* is very similar to the cyclase aggregate of *P. blakesleeanus* (De la Guardia et al., 1971; Candau et al., 1991) in its mode of operation and sensitivity to inhibitors. The B enzyme of *Blakeslea* is probably the one present in the fungi that make γ-carotene but not β-carotene; these include different species of *Rhizophlyctis*, *Allomyces*, *Blastocladiella*, *Cheilymenia*, and *Scutellinia* (Emerson and Fox, 1940; Turian and Cantino, 1959; Davies, 1961; Goodwin, 1980; Schrantz and Lemoine, 1995). The metabolic diversity of the fungi is due not only to the presence of alternative pathways in different taxonomic groups, but sometimes to their coexistence in the same organism.

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