APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1978, p. 639-642
0099-2240/78/0036-0639$02.00/0

Vol. 36, No. 5
Printed in U.S.A.

Carotene-Superproducing Strains of *Phycomyces*

F. J. MURILLO, I. L. CALDERÓN, I. LÓPEZ-DÍAZ, AND E. CERDÁ-OLMEDO
Departamento de Genética, Facultad de Ciencias, Universidad de Sevilla, Seville, Spain

Received for publication 20 August 1978

Production of β-carotene by wild-type *Phycomyces blakesleeanus* can be stimulated by light, chemicals, regulatory mutations, and sexual interaction between mycelia of opposite sex. Through genetic manipulations, we have isolated strains which have simultaneously and constitutively incorporated several of these stimulatory effects. In the dark and in a simple medium, some of the strains produce up to 25 mg of β-carotene per g (dry weight), or about 500 times the wild-type production under the same conditions. High lycopene-producing strains have also been isolated by using *carR* mutants, which are blocked in the conversion of lycopene to β-carotene. These strains should be useful in both industrial production of these pigments and basic research related to carotenogenesis.

The production of β-carotene by *Phycomyces blakesleeanus* depends on media and culture conditions but is generally low. In the dark, this fungus produces on the order of 50 μg of β-carotene per g (dry weight), making it inappropriate for industrial use (16).

β-Carotene production can be stimulated in several ways. Photoinduction results in an accumulation of about 500 μg/g under blue-light intensities of 2 W/m^2 (3), but light stimulation presents considerable practical difficulties in large-scale application.

Several chemicals stimulate carotenogenesis when added to the medium (22); up to 2,000 μg/g is accumulated in the presence of vitamin A (14), but the required concentrations of the vitamin are prohibitively large; up to 4,000 μg/g has been observed under the best conditions in the presence of β-ionone (16).

Mutations in the gene *carS* result in β-carotene contents of up to 4,000 μg/g in the dark. The *carS* mutants are still sensitive to vitamin A, but a double mutant, strain S106, has been obtained which reaches 6,000 μg/g. The new mutation, *car-102*, makes S106 insensitive to vitamin A. The stimulatory channel activated by vitamin A has thus become constitutive in this strain (18).

In the *Mucorales*, the interaction between mycelia of opposite sex leads to increased carotenogenesis through formation of trisporic acids (2, 6; A. Prieto, C. Spalla, M. Bianchi, and G. Briffi, Commun. Int. Ferment. Symp. London, p. 38, 1964). Mixed cultures of *Blakeslea trispora* strains of opposite sex in the presence of β-ionone have been considered promising for β-carotene production. However, it is difficult to maintain appropriate sex ratios in large cultures (10).

In *Phycomyces*, sexual stimulation occurs in single mycelia, called intersexual heterokaryons, which contain a mixture of nuclei of opposite sex (5). Such heterokaryons produce more than 400 μg of β-carotene per g (18), have a peculiar morphology, with formation of small aerial hyphae or pseudophores, and are unstable; they tend to segregate the components in homokaryotic form.

The genetics of carotene biosynthesis in *Phycomyces* has been reviewed (9; E. Cerdá-Olmedo and S. Torres-Martínez, Pure Appl. Chem., in press). Mutants in gene *carB* form phytoene, and mutants in gene *carR* produce lycopene (17, 21). All the intermediates from phytoene to β-carotene may be obtained in *Phycomyces*, either through the use of certain genetic combinations (1, 12) or through the addition of inhibitors (13, 19).

The objective of this work was to obtain *Phycomyces* strains that would yield high contents of β-carotene or other carotenes when grown on simple media in the dark.

MATERIALS AND METHODS

The strains of *P. blakesleeanus* used in this work are listed in Table 1. References to details about isolation, genotypes, and carotene production are given in the same table.

Heterokaryons were produced by using a previously described method (20). In the text, the two heterokaryon components are separated by an asterisk.

Cultures of homokaryons and heterokaryons were initiated as reported earlier (18). For all quantitative studies, cultures were grown on solid minimal medium

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

Strain	Genotype	Main carotenoid produced (μg/g, dry wt)	Reference
NRRL1554	Wild type (+)	β-Carotene (56)	18
M1	*carS43* (+)	β-Carotene (4,160)	18
S106	*carS42 car-102 mad-103* (−)	β-Carotene (5,595)	18
H7	*carR51* (+)	Lycopene (1,200)	This work
S136	*carR127* (+)	Lycopene (650)	This work
C9	*carR21* (−)	Lycopene (2,470)	21 and this work

(15), with glucose as carbon source, at 22°C for 4 days. In a few cases, cultures were grown on potato-dextrose agar (7), since its low cost would be an advantage in large-scale application. To observe distinct colonies, the minimal medium was supplemented with 1 mg of yeast extract per ml and acidified to pH 3.3.

The extraction of carotenes and their chromatographic separation and identification have been described previously (11, 12, 21).

Mutants were isolated after treatment with 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) per ml in pH 7.0 citrate-phosphate buffer, as previously described (8).

RESULTS AND DISCUSSION

Intersexual heterokaryons with *carS* mutations. Sexual interaction and regulatory mutations multiply each other's stimulatory effect on carotenogenesis when acting simultaneously in the intersexual heterokaryon M1 * S106. Some of the heterokaryotic mycelia contain 500 times more β-carotene than does the original wild type (Table 2).

These heterokaryotic mycelia are highly variable in β-carotene content, presumably reflecting variations in nuclear ratios. They are much less stable than the heterokaryons described by Heisenberg and Cerdá-Olmedo (15), since they tend to separate into mycelial patches of different colors and produce unexpectedly high proportions of homokaryotic spores. Sexual type is only one of the many differences in genetic background among the constituent nuclei, which are descended from very different wild types. The reasons for the instability are, thus, unclear. In any case, instability is a major drawback for the practical application of a strain.

Intersexual heterokaryons with balanced lethal mutations. The disadvantages of the intersexual heterokaryon M1 * S106 would presumably disappear in the diploid M1/S106. We have made considerable, but fruitless, efforts to isolate diploids from different *Phycomyces* heterokaryons.

An alternative would be to introduce recessive lethal mutations in both components of the heterokaryon. To this effect, spores from M1 * S106 mycelia were treated with NTG to a survival level of about 0.1%. Most nuclei are thus inactivated, and most survivors are homokaryotic. Among the heterokaryotic survivors, there are many that cannot segregate either or both components in homokaryotic form, owing to the introduction of recessive lethal mutations (8). A total of 117 survivors of the treatment, taken from among the most brightly colored, were tested for segregation by streaking their spores on acid medium. The heterokaryons S218 * S219 and S242 * S243 were obtained in this way. They produced considerable quantities of β-carotene (Table 2), and no homokaryons were found among their progeny, although they still showed a wide range of nuclear ratios, leading to variations in β-carotene content.

Variation in nuclear ratios could be limited if the lethal mutations were not totally recessive. If both heterokaryon components had mutations or sets of mutations that made mycelia with more than 70% of the corresponding nuclei inviable, nuclear proportions would be limited to the 30 to 70% range. Heterokaryotic S218 * S219 spores were treated with NTG, and a search for stable strains led to the isolation of S244 * S245 and S246 * S247. These heterokaryons are apparently very uniform and have high β-carotene contents (Table 2).

TABLE 2. *Production of β-carotene in intersexual heterokaryons M1 * S106 and their derivatives*

Strain	β-carotene (μg/g, dry wt)[a]
M1 * S106, mycelium A	14,340
M1 * S106, mycelium B	18,450
M1 * S106, mycelium C	20,325
M1 * S106, mycelium D	20,500
M1 * S106, mycelium E	25,342
S218 * S219	16,000
	(12,620)
S242 * S243	15,600
	(9,000)
S244 * S245	19,120
	(14,120)
S246 * S247	13,470
	(25,230)

[a] Numbers in parentheses correspond to potato-dextrose agar cultures. The others correspond to the usual minimal medium cultures.

High lycopene production. The lycopene content of strains C9 and H7 (Table 3) largely exceeds the β-carotene produced by the wild type. There is an apparent feedback regulation (18), so that the lack of the end product, β-carotene, stimulates the pathway.

The heterokaryon H7 * C9 produces no pseudophores and does not surpass the lycopene content of strain C9 alone (Table 3). This total lack of sexual stimulation supports the concepts that both pseudophore formation and carotenogenesis are activated by trisporic acids and that these acids derive from β-carotene (24). To exhibit sexual stimulation, a heterokaryon must thus be able to synthesize at least some β-carotene.

Strain S136 was isolated after treatment of spores of strain NRRL1554 with NTG. This strain contains lycopene (Table 3) as the main carotene, but also γ-carotene (110 μg/g), β-carotene (45 μg/g), and smaller amounts of phytofluene, ζ-carotene, and neurosporene. It presumably carries a mutation in gene *carR*, resulting in decreased cyclase activity, responsible for the production of the cyclated carotenes γ-carotene and β-carotene (12).

The heterokaryon S136 * C9 produces pseudophores, exhibits very intense red colors, and has high lycopene content. Spores of S136 *C9 were treated with NTG, and a search for stable heterokaryons resulted in the isolation of S183 * S184, S185 * S186, and S187 * S188 (Table 3). Although stability was achieved, optimum production was not. Further mutagenesis should bring this about.

Final comments. The strains obtained in this work reach very high carotene levels when grown on simple media in the absence of light or exogenous chemical stimulation. They should be particularly useful in the development of industrial processes for carotene production. There is room for further improvements and extensions. New stimulatory mutations, similar to mutation *car-102* in strain S106, could be introduced in *carS* (+), *carR* (+), and *carR* (−) genetic backgrounds and serve as the basis for a repetition of the process described in this paper. The intersexual heterokaryons could also carry mutant and wild-type alleles of genes *carB* and *carR* so that all the intermediates from phytoene to γ-carotene could be produced (1, 12). Media and culture conditions optimal for carotenogenesis in other organisms (10) should be tried out, since we have made no effort in this direction.

The strains obtained in this work should be particularly useful in the development of in vitro systems for carotene biosynthesis and for the isolation of mRNA's and proteins involved in the process (4, 23).

TABLE 3. *Formation of lycopene in homokaryons and intersexual heterokaryons containing carR nuclei*

Strain	Lycopene (μg/g, dry wt)
C9	2,470
H7	1,200
S136	650
H7 * C9 mycelium A	1,950
H7 * C9 mycelium B	2,575
H7 * C9 mycelium C	2,675
S136 * C9 mycelium A	9,780
S136 * C9 mycelium B	10,550
S136 * C9 mycelium C	12,540
S136 * C9 mycelium D	12,570
S136 * C9 mycelium E	14,600
S183 * S184	6,946
S185 * S186	5,830
S187 * S188	7,101

The successive increases in carotene content described in this paper support the hypothesis of the independence of the stimulatory effects of *carS* mutations, vitamin A, and sexual interaction (18).

ACKNOWLEDGMENTS

We thank M. I. Carretero for typing the manuscript and A. Fernández-Estefane for technical assistance.

Financial support for this study came from the Fundación Juan March.

LITERATURE CITED

1. **Aragón, C. M. G., F. J. Murillo, M. D. De la Guardia, and E. Cerdá-Olmedo.** 1976. An enzyme complex for the dehydrogenation of phytoene in *Phycomyces*. Eur. J. Biochem. **63**:71-75.
2. **Barnett, H. L., V. G. Lilly, and R. F. Krause.** 1956. Increased production of carotene by mixed + and − cultures of *Choanephora cucurbitarum*. Science **123**:141.
3. **Bergman, K., A. P. Eslava, and E. Cerdá-Olmedo.** 1973. Mutants of *Phycomyces* with abnormal phototropism. Mol. Gen. Genet. **123**:1-16.
4. **Bramley, P. M., and B. H. Davies.** 1975. Carotene biosynthesis by cell extracts of mutants of *Phycomyces blakesleeanus*. Phytochemistry **14**:463-469.
5. **Burgeff, H.** 1914. Untersuchungen über Variabilität, Sexualität und Erblichkeit bei *Phycomyces nittens* Kunze I. Flora **107**:259-316.
6. **Caglioti, L., G. Cainelli, B. Camerino, R. Mondelli, A. Prieto, A. Quilico, T. Salvatori, and A. Silva.** 1966. The structure of trisporic-C acid. Tetrahedron Suppl. **7**:175-187.
7. **Cerdá-Olmedo, E.** 1975. The genetics of *Phycomyces blakesleeanus*. Genet. Res. **25**:285-296.
8. **Cerdá-Olmedo, E., and P. Reau.** 1970. Genetic classification of the lethal effects of various agents on heterokaryotic spores of *Phycomyces*. Mutat. Res. **9**:369-384.
9. **Cerdá-Olmedo, E., and S. Torres-Martínez.** 1977. Biosíntesis de carotenos en *Phycomyces*, p. 277-287. *In* J. Cornudella, C. F. Heredia, J. Oró, and A. Sols (ed.), Avances de la bioquímica. Salvat Ed. S.A., Barcelona.
10. **Ciegler, A.** 1965. Microbial carotenogenesis. Adv. Appl. Microbiol. **7**:1-34.
11. **Davies, B. H.** 1965. Analysis of carotenoid pigments, p.

489-532. *In* T. W. Goodwin (ed.), Chemistry and biochemistry of plant pigments. Academic Press Inc., New York.

12. **De la Guardia, M. D., C. M. G. Aragón, F. J. Murillo, and E. Cerdá-Olmedo.** 1971. A carotenogenic enzyme aggregate in *Phycomyces*: evidence from quantitative complementation. Proc. Natl. Acad. Sci. U.S.A. **68**:2012-2015.
13. **Elahi, M., T. H. Lee, K. L. Simpson, and C. O. Chichester.** 1973. Effect of CPTA on the biosynthesis of carotenoids by *Phycomyces blakesleeanus* mutants. Phytochemistry **12**:1633-1639.
14. **Eslava, A. P., M. I. Alvarez, and E. Cerdá-Olmedo.** 1974. Regulation of carotene biosynthesis in *Phycomyces* by vitamin A and β-ionone. Eur. J. Biochem. **48**:617-623.
15. **Heisenberg, M., and E. Cerdá-Olmedo.** 1968. Segregation of heterokaryons in the asexual cycle of *Phycomyces*. Mol. Gen. Genet. **102**:187-195.
16. **Lilly, V. G., H. L. Barnett, and R. F. Krause.** 1960. The production of carotene by *Phycomyces blakesleeanus*. West Virginia University Agric. Exp. Stn. Bull. 441 T, Morgantown.
17. **Meissner, G., and M. Delbrück.** 1968. Carotenes and retinal in *Phycomyces* mutants. Plant Physiol. **43**:1279-1283.
18. **Murillo, F. J., and E. Cerdá-Olmedo.** 1976. Regulation of carotene synthesis in *Phycomyces*. Mol. Gen. Genet. **148**:19-24.
19. **Olson, J. A., and H. Kinzley, Jr.** 1962. The effect of diphenylamine on carotenoid, sterol and fatty acid synthesis in *Phycomyces blakesleeanus*. Arch. Biochem. Biophys. **97**:138-145.
20. **Ootaki, T.** 1973. A new method for heterokaryon formation in *Phycomyces*. Mol. Gen. Genet. **121**:49-56.
21. **Ootaki, T., A. C. Lighty, M. Delbrück, and W. J. Hsu.** 1973. Complementation between mutants of *Phycomyces* deficient with respect to carotenogenesis. Mol. Gen. Genet. **121**:57-70.
22. **Reyes, P., C. O. Chichester, and T. O. M. Nakayama.** 1964. The mechanism of β-ionone stimulation of carotenoid and ergosterol biosynthesis in *Phycomyces blakesleeanus*. Biochim. Biophys. Acta **90**:578-592.
23. **Schrott, E. L, and W. Rau.** 1975. Investigations to demonstrate the involvement of m-RNA in photoinduction of carotenoid synthesis. Ber. Dtsch. Bot. Ges. **88**:233-243.
24. **Sutter, R. P.** 1975. Mutations affecting sexual development in *Phycomyces blakesleeanus*. Proc. Natl. Acad. Sci. U.S.A. **72**:127-130.