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# New Mutants of *Phycomyces blakesleeanus* for β-Carotene Production

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**The accumulation of β-carotene by the zygomycete *Phycomyces blakesleeanus* is increased by mutations in the *carS* gene. The treatment of spores of *carS* mutants with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine led to the isolation, at very low frequencies, of mutants that produced higher levels of β-carotene. Strain S556 produced about 9 mg of β-carotene per g of dry mass when it was grown on minimal agar. Crosses involving strain S556 separated the original *carS* mutation from a new, unlinked mutation, *carF*. The *carF* segregants produced approximately as much carotene as did *carS* mutants, but they were unique in their ability to produce zygospores on mating and in their response to agents that increase carotenogenesis in the wild type. The carotene contents of *carF* segregants and *carF carS* double mutants were increased by sexual interaction and by dimethyl phthalate but were not increased by light or retinol. Mixed opposite-sex cultures of *carF carS* mutants contained up to 33 mg of β-carotene per g of dry mass. Another strain, S444, produced more β-carotene than did S556 but was marred by slow growth, defective morphology, and bizarre genetic behavior. In all the strains tested, the carotene concentration was minimal during the early growth phase and became higher and constant for several days in older mycelia.**

All-*trans* β-carotene is responsible for the light-yellow color of *Phycomyces blakesleeanus*, a heterothallic fungus of the class Zygomycetes and the order Mucorales (6). Many external factors influence the color of the mycelium because they activate or inhibit carotene biosynthesis. Among these factors, sexual stimulation (12), blue illumination (2), and the addition of retinol (10) and dimethyl phthalate (8) to the medium represent four separate mechanisms of activation (3).

Superyellow mutants, which contain high carotene concentrations, occur commonly after mutagenesis. These mutants stand out against the light-yellow background of the wild type (28). Superyellow phenotypes are produced by mutations in either of two genes. Recessive *carS* mutations abolish the end product regulation of the pathway, and strains that carry them contain 2 to 5 mg of β-carotene per g of dry mycelium, that is, up to 100 times the wild-type level (18, 19). Recessive *carD* mutations increase carotene content to 1 mg per g of dry mass, but they are not useful for industrial carotene production because *carS carD* double mutants contain about as much carotene as do *carS* single mutants (26). Other regulatory mutations (*carA*) are semidominant and result in white mycelia with very low β-carotene contents (10, 15, 17, 22).

*carS* mutants do not represent a natural ceiling for carotene accumulation in *P. blakesleeanus*, since their carotene contents can be increased by sexual interaction (18), light (2), and dimethyl phthalate (3). We tested the assumption that additional mutations could increase the β-carotene contents of *carS* strains.

## MATERIALS AND METHODS

*P. blakesleeanus* Bgff. strains (Table 1) were grown from spores (sporangiospores) on minimal agar at 22°C (5), unless otherwise stated. Heterokaryons were prepared by sporangiophore grafting (21), with their spores giving rise to mycelia with different proportions of the two kinds of nuclei (14). Crosses were set up and analyzed by the method of Eslava and Álvarez (11).

Spores from *carS* superyellow strains C115 and S276 were exposed to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (0.1 mg/ml) to survival levels of 0.34 and 14% as previously described (25). About one-third of the survivors of this treatment are functionally uninucleate and can express recessive mutations. Putative mutants were detected as deeply pigmented (hyperyellow) spots and were purified by isolating the more pigmented colonies in repeated cycles of vegetative growth from spores.

The carotene contents were measured spectrophotometrically (12). Prior to extraction, spores were lyophilized and homogenized with glass beads (0.5 mm in diameter) in a Braun (Melsungen, Germany) homogenizer. Mycelia were extracted after 4 days of growth, unless otherwise stated. Exposures to chemical activators and light were carried out as previously described (3, 26). Here, retinol is used as an abbreviation for retinol acetate; Tween 80 is polyoxyethylenesorbitan monooleate.

## RESULTS

**Mutant isolation.** Putative hyperyellow mutants were tested in repeated subcultures from spores. Strain S556, isolated from *carS* mutant S276, contained 9.2 ± 1.6 mg of β-carotene per g of dry mycelium (the mean and standard error of four determinations). Four other mutants isolated in the same set of experiments contained more carotene than their parent did but not as much as S556 did and were not analyzed further. Strain S444, isolated from *carS* mutant C115, contained 12 ± 1.9 mg of β-carotene per g of dry mycelium (the mean and standard error of four independent determinations). Mutants such as S444 and S556 are rare; over two million colonies were screened for their isolation. Superyellow mutants are produced from the wild type at least 100 times more frequently.

S556 produced normal mycelia but fewer sporangiophores and therefore fewer spores than the wild type or the original *carS* mutant did. S444 grew slowly; its hyphae were thicker than were those of the wild type, and it produced few and stubby sporangiophores. S444 spores gave rise to mycelia of different

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

| Strain[a] | Genotype[b] | Sex[c] | Origin | Reference |
|---|---|---|---|---|
| B36 | *nicA101* | (+) | C269 × S102 | 23 |
| C2 | *carA5* | (−) | NRRL1555 | 16 |
| C115 | *carS42 mad-107* | (−) | NRRL1555 | 16 |
| C131 | *imb-2* | (−) | NRRL1555 | 13 |
| NRRL1555 | Wild type | (−) | | |
| S102 | *nicA101* | (−) | NRRL1555 | 23 |
| S226 | *carD172 nicA101* | (−) | NRRL1555 | 24 |
| S276 | *carS42* | (+) | C242 × (C115 * S102) | 23 |
| S444 | Unknown | (−) | C115 | |
| S556 | *carS42 carF181* | (+) | S276 | |
| S561 | *carF181* | (−) | (S556×B36) * S102 | |
| S562 | *carF181* | (−) | (S556×B36) * S102 | |
| S563 | *carF181* | (+) | (S556×B36) * S102 | |
| S564 | *carF181* | (+) | (S556×B36) * S102 | |
| S565 | *carS42 carF181* | (+) | (S556×B36) * S102 | |
| S566 | *carS42 carF181* | (+) | (S556×B36) * S102 | |
| S568 | *carS42 carF181* | (−) | (S556×B36) * S102 | |

[a] NRRL1555, originally obtained from the Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill., is the standard wild type. Strain B36 was from the Max Planck Institute, Berlin, Germany; strains with C designations were from the late M. Delbrück, California Institute of Technology, Pasadena; strains with S designations were from our laboratory.

[b] *car*, mutation that affects carotenogenesis; *mad*, mutation that affects phototropism; *nic*, auxotrophy for nicotinic acid; *imb*, mutation that affects the production of sporangiophores.

[c] (+) and (−) are the standard designations of the two sexes in the Mucorales.

TABLE 2. Recombination of *carS* and *carF* mutations[a]

| Type of strain and phenotype[b] | Inferred genotype | Zygospores[c] | Frequency (*n*) |
|---|---|---|---|
| Parental | | | |
| Light yellow | (+) | Normal | 101 |
| | (−) | Normal | 103 |
| Hyperyellow | *carS carF* (+) | None | 61 |
| | *carS carF* (−) | None | 13 |
| Recombinant, superyellow | *carS* (+) | None | 59 |
| | *carS* (−) | None | 62 |
| | *carF* (+) | Some | 22 |
| | *carF* (−) | Some | 17 |
| Asexual | | | |
| Light yellow | | None | 5 |
| Superyellow | | None | 9 |
| Hyperyellow | | None | 10 |
| Total | | | 462 |

[a] Analysis of prototrophic mycelia grown from germspores of the cross (S556 * B36) × S102 {genotypes, [*carS42 carF181* (+) * *nicA101* (+)] × *nicA101* (−)}.

[b] Phenotypes and amounts of β-carotene: light yellow, about 0.1 mg per g of dry mass; superyellow, about 3 mg per g of dry mass; and hyperyellow, about 9 mg per g of dry mass.

[c] Relative numbers of zygospores when strains were confronted on agar medium with a wild-type strain of the opposite sex.

colors, from hyperyellow to orange and reddish. None of the sectors could be purified and stabilized through repeated subcultures from spores. Spores of strain S444 were often morphologically abnormal and had lower rates of germination (56 to 76% in sectors of different colors) than those of C115 (81% under the same conditions) and the wild type (about 100%).

**Genetic analysis of mutants.** Strain S556 presumably carried the *carS42* mutation of its parent and an additional mutation. The two mutations could not be separated by recombination after a normal cross because, like its parent strain (S276), strain S556 was infertile when it was confronted with strains of the opposite sex; their interaction stopped before the production of zygospores (cells that contain nuclei of both mating strains). This difficulty was circumvented by using a helper strain (24). The cross of the heterokaryon B36 * S556 and strain S102 produced zygospores that, after a long dormancy, yielded germsporangia with haploid germspores. These recombination products are functionally equivalent to vegetative spores and start the vegetative life cycle. Both kinds of nuclei in the heterokaryon were fertile, but all the descendants of B36 and S102 were auxotrophs because of the *nicA101* allele in both strains. Prototrophic germspores should represent half of the progeny of S556 and S102.

Twenty-nine germsporangia from the heterokaryon cross (of 130 tested) contained prototrophic germspores. Their distribution in germsporangia was very irregular (range, 1 to 363; mean, 40; standard deviation, 70). The phenotypes of 462 prototrophic colonies produced by germspores (mean, 16 per germsporangium; standard deviation, 16) are shown in Table 2. About 5% of them were asexual and were not considered further. The following four classes were seen in the progeny: wild-type and hyperyellow parental classes and two superyellow recombinant classes that differed in fertility. The superyellow fertile class presumably carried the new mutation, henceforth called *carF*, responsible for the hyperyellow color in the *carS* background. The *carF*, *carS*, and sex markers are certainly not closely linked and are probably independent, given the usual irregularities of *Phycomyces* sexual segregations (4) and the reduced viability of the more highly pigmented spores.

Four *carF* segregants, S561, S562, S563, and S564, were isolated for further work. These segregants contained more carotene than did *carD* mutants, about as much carotene as did *carS* mutants, and less carotene than did *carS carF* double mutants.

S444 was derived from C115, a (−) strain that carries out the early stages of the sexual cycle, but there was no sign of sexual activity when S444 mycelia were confronted with test mycelia of either sex. As we did with S556, we used helper strain S102 to make three heterokaryons (S102 * S444), which presumably differed in the proportions of the two kinds of nuclei. Few zygospores were produced, but most germsporangia were fertile (Table 3). All prototrophic germspores found in 98 germsporangia gave rise to light-yellow colonies with normal, phototropic sporangiophores, and none was deeply pigmented; 31 of these colonies from 8 germsporangia were (+), and only 1 was (−). Many prototrophic colonies exhibited a wild-type growth habit. However, others grew slowly into thin mycelia unable to extend themselves to fill the whole plate; their growth improved in complex medium (10 g of yeast extract per

TABLE 3. Genetic analysis of strain S444[a]

| Cross no. | No. of germsporangia examined | No. of germsporangia with viable germspores | No. of germsporangia with prototrophic germspores | | |
|---|---|---|---|---|---|
| | | | Normal progeny only | Normal and abnormal progeny | Total |
| 1 | 108 | 95 | 19 | 49 | 68 |
| 2 | 15 | 12 | 3 | 7 | 10 |
| 3 | 24 | 21 | 3 | 17 | 20 |

[a] Strain B36 [genotype, *nicA101* (+)] was crossed with three mycelia of the heterokaryon S102 * S444 [genotype, *nicA101* (−) * *carS42 mad-107* UNK (−), where UNK is an unknown genetic change].

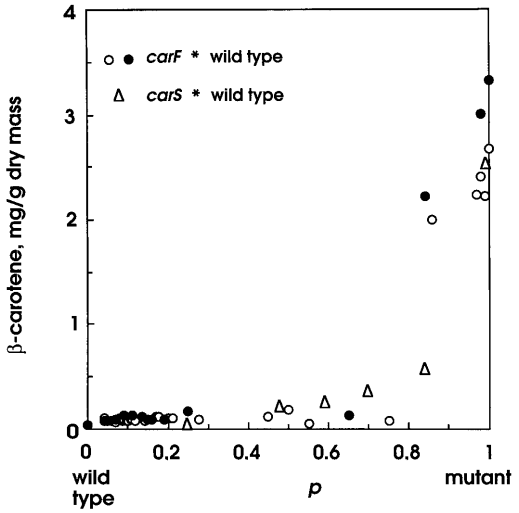


FIG. 1. Recessivity of the *carF* mutation. Shown are β-carotene concentrations in 4-day-old heterokaryotic mycelia (C131 * S561 [○], C131 * S562 [●], and C115 * NRRL1555 [△]) as a function of the proportion (*p*) of mutant nuclei in the heterokaryon.

liter, 5 g of peptone per liter, 20 g of D-glucose per liter). In short, the unknown genetic change that converted strain C115 into strain S444 did not behave as a Mendelian marker but resulted in aberrant segregants, in which some of the markers found in their parents appeared to be missing.

*Phycomyces* heterokaryons are very stable. The relative proportion of nuclei that constitute them can take practically any value, remains constant during vegetative growth, and can be calculated from the phenotype frequencies among spores collected from the heterokaryon (14). These peculiarities allow quantitative investigations of the recessivity and dominance of mutations. The *carF* mutation is very recessive. Heterokaryons that carried *carF* and wild-type nuclei contained much less carotene than did the *carF* strain, except when *carF* nuclei represented over 80% of the nuclei in the heterokaryon (Fig. 1). On the contrary, the observation that S102 * S444 heterokaryons were hyperyellow indicated that the unknown genetic change in strain S444 is dominant.

**Time course and activation of carotene synthesis in mutants.** In the wild type and in *carF* and *carF carS* mutant strains, the mycelial mass and β-carotene content remained approximately constant after the growth phase for many days; standard analyses are usually done with 4-day-old mycelia. The carotene content dropped during spore germination and reached a minimum in young mycelia. In the wild type, spores had more carotene than did adult mycelia (Fig. 2).

Strain S444 reached its maximal production of β-carotene toward the end of the growth phase. Unlike other carotene superproducers from our experience, strain S444 was at least as productive in liquid medium as it was in solid medium (Fig. 3).

The carotene contents of *carF* and *carS carF* mutants were increased by the addition of dimethyl phthalate to the medium but were not increased by the addition of retinol or by blue illumination (Table 4). The carotene contents of *carF* and *carS carF* strains increased when they were grown in mixed cultures with similar strains of the opposite sex (mated cultures) (Table 5). The effect was particularly marked in *carF carS* double mutants, whose mated cultures produced up to 33 mg of β-carotene per g of dry mycelium. The carotene content of strain

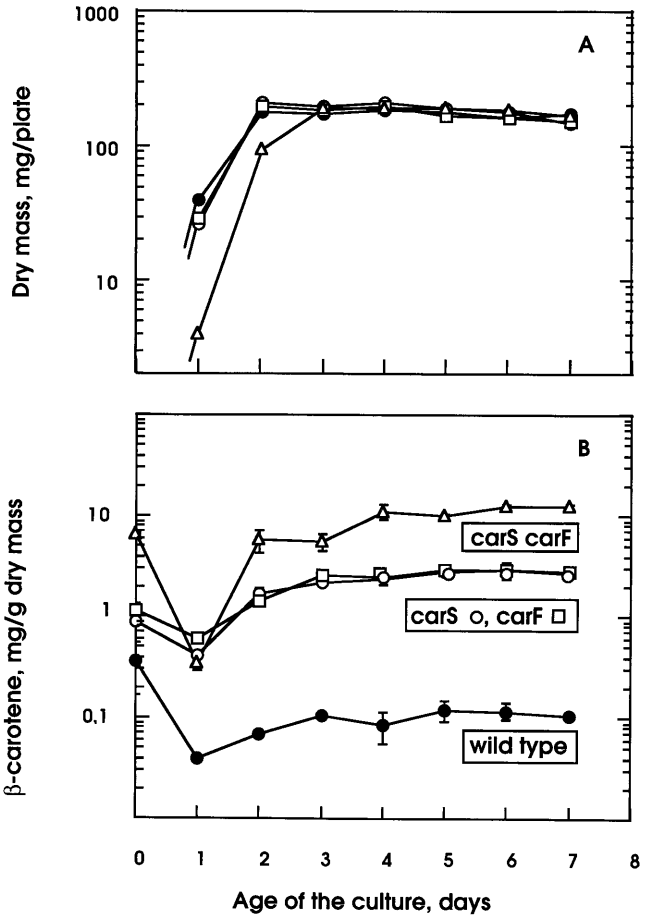


FIG. 2. Growth rates (A) and β-carotene concentrations (B) of various strains (S556 [△], S561 [□], C115 [○], and NRRL1555 [●]) as a function of age.

S444 was not further increased by light, retinol, dimethyl phthalate, or the proximity of (+) strains.

**Crosses with other *car* mutants.** Crosses of *carF* and other *car* strains were not fertile enough for quantitative analyses of

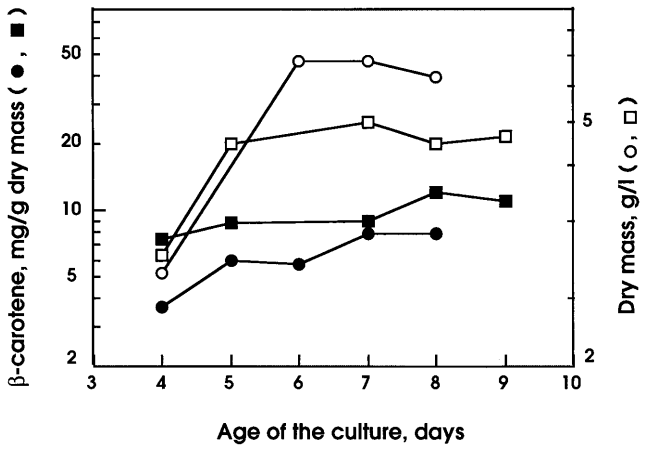


FIG. 3. Growth rates and β-carotene concentrations as a function of age in cultures of strain S444 grown in liquid medium (80 ml in 250-ml flasks) (squares) and solid medium (25 ml of agar medium on 10-cm-diameter petri plates) (circles). l, liter.

TABLE 4. Physical and chemical activation of carotene production in various strains

| Mutation(s) and strain | β-Carotene (mg/g of dry mass)[a] | | | | | |
|---|---|---|---|---|---|---|
| | White light[b] | | Retinol[c] | | Dimethyl phthalate[d] | |
| | − | + | − | + | − | + |
| Wild type, NRRL1555 | 0.10 ± 0.01 | 0.8 ± 0.1 | 0.11 ± 0.004 | 2.1 ± 0.1 | 0.11 ± 0.004 | 1.0 ± 0.03 |
| *carF* | | | | | | |
| S561 | 2.3 ± 0.01 | 2.7 ± 0.04 | 3.7 ± 0.3 | 2.6 ± 0.2 | 2.8 ± 0.1 | 4.4 ± 0.3 |
| S562 | 2.7 ± 0.001 | 2.8 ± 0.3 | 4.8 ± 0.01 | 4.1 ± 0.3 | 3.1 ± 0.1 | 3.9 ± 0.9 |
| *carS carF* | | | | | | |
| S565 | 6.5 ± 0.3 | 7.1 ± 0.1 | 7.2 ± 0.3 | 6.6 ± 0.8 | 7.5 ± 0.3 | 9.7 ± 0.6 |
| S566 | 7.9 ± 0.3 | 7.3 ± 1.0 | 10.4 ± 0.8 | 8.5 ± 1.4 | 7.8 ± 0.4 | 8.9 ± 0.4 |
| S568 | 6.8 ± 0.5 | 6.3 ± 0.1 | 6.7 ± 1.2 | 6.9 ± 0.8 | 6.2 ± 0.9 | 7.7 ± 0.4 |

[a] Data are the means ± standard errors of two to four independent determinations of 4-day-old mycelia with (+) and without (−) the indicated activator.
[b] From a battery of fluorescent lamps. Fluence rate, 4 W $m^{-2}$.
[c] Retinol acetate was predissolved in ethanol and Tween 80. The final concentrations were as follows: retinol, 0.75 mM; ethanol, 10 ml/liter; and Tween 80, 40 ml/liter. The solvents were applied to the corresponding control.
[d] Dimethyl phthalate was predissolved in ethanol. The final concentrations were as follows: dimethyl phthalate, 2 mM, and ethanol, 10 ml/liter. The solvent was applied to the control.

recombination, but they did support the distinction of the *carF* mutation from *carD* and *carA*.

Light-yellow, wild-type-like recombinants were produced by the cross of *carF* strain S564 and *carD* strain S226, but our analysis was limited to the single viable germsporangium produced by 1 of the 14 zygospores obtained.

Similarly, yellow recombinant colonies were produced by the cross of *carF* strain S563 with *carA* strain C2, but our analysis was restricted again to the single viable germsporangium produced by 1 of the 37 zygospores obtained. With 0.1 to 0.6 mg of β-carotene per g of dry mycelium, the yellow recombinants were intermediate between the *carA* and *carF* parents. Other germspores in the germsporangium gave rise to white (*carA*) colonies and unexpected hyperyellow colonies that are not analyzed here.

## DISCUSSION

The new mutants have the highest carotene concentrations found in homokaryotic *Phycomyces* strains. Their low frequencies, even after exposure to a powerful mutagen, suggest that the search for *Phycomyces* genes whose mutations result in superproduction of β-carotene is largely complete.

The *carF* mutation led to increased carotenogenesis, like the *carD* and *carS* mutations, but from its unique phenotype and the isolation of recombinants, we concluded that the *carF* mutation occurred in a previously unknown gene. A comparison of the phenotypes of representative carotene-superproducing mutants is shown in Table 6.

The original *carF carS* double mutant grew somewhat more slowly and produced fewer sporangiophores and spores than the wild type did. Strains with modifications in their β-carotene contents usually deviate from normal sporangiophore development (9, 13). The failure of *carS* mutants to produce zygospores has previously been attributed to their high carotene contents. However, this is no longer possible because the *carF* mutant, which produced about as much carotene, formed some zygospores with viable germspores.

The superyellow *carF* mutant probably contains a modified gene product that does not result in a loss of function. The recessive nature of the *carF* mutation can be explained if the normal gene product, but not the mutant, inhibits carotenogenesis; however, this hypothesis requires the loss-of-function mutations to be lethal because otherwise these mutants would be expected to be superyellow. Another possibility is that the mutant gene product activates carotenogenesis. The active *carF* gene product would be a protein homotetramer with freely mixing mutant and wild-type subunits; the superyellow phenotype would require all subunits to be mutated. This hypothesis is supported by the observation (Fig. 1) that the β-carotene contents of heterokaryons with *carF* and wild-type nuclei are approximately proportional to $p^4$, where $p$ is the proportion of *carF* mutant nuclei in the heterokaryon. According to this hypothesis, strains with loss-of-function mutations in *carF* could be white or wild-type yellow. No *carF* mutants have been found among the superyellow and white mutants previously characterized (6, 26, 27).

The synergy of *carF* and *carS* mutations to increase carotenogenesis suggests different mechanisms of action; the lack of activation of carotene biosynthesis by retinol in both mutants suggests that the normal products of both genes mediate the action of retinol. We have found no simple hypothesis to explain this apparent contradiction. It has been proposed (3, 6) that the carotene pathway is regulated by the interaction of the

TABLE 5. Sexual activation of carotenogenesis in *carF* and *carF carS* strains

| Strain(s) in culture | | β-Carotene content (mg/g of dry mass) |
|---|---|---|
| (+) | (−) | |
| *carF* strains | *carF* strains | |
| S563 | None | 2.7 ± 0.5 |
| None | S561 | 2.0 ± 0.4 |
| None | S562 | 2.0 ± 0.4 |
| S563 | S561 | 8.8 ± 1.9 |
| S563 | S562 | 8.9 ± 2.4 |
| *carF carS* strains | *carF carS* strains | |
| S565 | None | 6.9 ± 0.1 |
| S566 | None | 10.5 ± 0.8 |
| None | S568 | 8.0 ± 0.3 |
| S565 | S568 | 24.0 ± 1.1 |
| S566 | S568 | 32.8 ± 1.6 |

[a] Data are the means ± standard errors of two independent determinations of 4-day-old single and mixed cultures.

TABLE 6. Comparison of representative carotene-overproducing strains[a]

| Strain (genotype) | β-Carotene content (mg/g of dry mycelium)[b] | Retinol[c] | Dimethyl phthalate[c] | Blue light[c] | Sexual interaction[c] | Zygospore formation[d] |
|---|---|---|---|---|---|---|
| NRRL155 (wild type) | 0.1 | + | + | + | + | Yes |
| S225 (*carD*) | 1 | + | + | + | + | Yes |
| C115 (*carS*) | 3 | − | + | + | + | No |
| S561 (*carF*) | 3 | − | + | − | + | Yes |
| S566 (*carS carF*) | 9 | − | + | − | + | No |
| S444 | 12 | − | − | − | − | No |

[a] Based on the results of this work and the results in references 2, 3, 12, 19, and 24.
[b] Approximate results for mycelia under the culture conditions used in this work.
[c] +, increase in β-carotene content; −, no increase or a slight decrease.
[d] When strain was confronted on agar medium with a wild-type strain of the opposite sex.

*carS* and *carA* gene products with β-carotene (or retinol). It is intriguing that our *carA carF* recombinants are similar to *carA carS* recombinants in color and in carotene contents (15, 27).

Strain S444 is not a simple Mendelian mutant with a defined modification in a specific carotenogenic function. The nature of its genetic changes is hard to establish by the techniques of genetics and molecular biology available for *P. blakesleeanus*, and its slow growth discourages exploitation of its high β-carotene content.

The lowest β-carotene contents were found in rapidly growing young mycelia, where glucose metabolism kept the intracellular oxygen concentrations low. The time course of carotene contents meets the expectations of biochemical and morphological modifications brought into play against rising intracellular oxygen concentrations as metabolism dwindles. Our carotene-overproducing mutants are not fully constitutive; their carotene contents still mirror their overall metabolic activities.

The mutants reported here make *P. blakesleeanus* more attractive for biotechnological production of all-*trans* β-carotene (1, 7). The purified β-carotene in the market comes essentially from chemical synthesis. An industrial process with another zygomycete, *Blakeslea trispora* (20), has found only marginal application. The current trend toward natural food colors and the discovery of new beneficial effects of carotene, beyond its role as provitamin A, should stimulate demand and encourage the development of a biological production process.

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