Intersexual Partial Diploids of Phycomyces

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ABSTRACT

Sexual interaction between strains of opposite sex in many fungi of the order Mucorales modifies hyphal morphology and increases the carotene content. The progeny of crosses of *Phycomyces blakesleeanus* usually include a small proportion of anomalous segregants that show these signs of sexual stimulation without a partner. We have analyzed the genetic constitution of such segregants from crosses that involved a *carF* mutation for overaccumulation of β-carotene and other markers. The new strains were diploids or partial diploids heterozygous for the sex markers. Diploidy was unknown in this fungus and in the Zygomyctes. Random chromosome losses during the vegetative growth of the diploid led to heterokaryosis in the coenocytic mycelia and eventually to sectors of various tints and mating behavior. The changes in the nuclear composition of the mycelia could be followed by selecting for individual nuclei. The results impose a reinterpretation of the sexual cycle of Phycomyces. Some of the intersexual strains that carried the *carF* mutation contained 25 mg β-carotene per gram of dry mass and were sufficiently stable for practical use in carotene production.

*Phycomyces blakesleeanus* is a saprophytic fungus of the order Mucorales with vegetative and sexual life cycles (Figure 1). The mycelia belong to either of two mating types or sexes, called (+) and (−) (Blakeslee 1906). In the sexual cycle, thousands of haploid nuclei from mycelia of opposite sex enter a common cell, the zygospore, which eventually produces germospores; when adequately fed, these grow into normal vegetative mycelia of one sex or the other. Hans Burgeff (1928) conjectured that all the nuclei in the germospores from a zygospore descend from a single diploid nucleus. This conjecture was found to be true for most zygospores (Cerdá-Olmedo 1975). An additional conjecture of Burgeff was that the diploid nucleus undergoes meiosis and the meiotic products multiply by mitosis to produce the nuclei in the germospores. This conjecture was contradicted by results to be presented here.

A minority of the germospores produce anomalous mycelia that are thicker and yellower than normal mycelia, produce few sporangiophores, and are covered by many short aerial hyphae called pseudophores (Blakeslee 1906; Burgeff 1914; Orban 1919; Cerdá-Olmedo 1975; Eslava et al. 1975a,b). These were described by Blakeslee (1906) as “irregularly swollen, coiled and variously contorted outgrowths which, when well developed, form a dense felted covering to the mycelium” (p. 13). The anomalous mycelia do not carry out complete sexual reactions with tester strains of either sex, but some of their spore progeny do. Blakeslee called them homothallic, without proposing any specific genetic identity for them. Burgeff (1914) proposed that they carry different sexual markers in different nuclei and coined the concept and the term *heterokaryon* for them. In support of his interpretation he surgically grafted strains of the opposite sex to obtain heterokaryons with (+) and (−) nuclei, and these “intersexual heterokaryons” formed pseudophores and reproduced the general traits of the anomalous mycelia from the crosses.

The mycelia and other structures of Phycomyces and other Mucorales, such as *Blakeslea trispora*, are yellow because of the accumulation of β-carotene, a pigment, antioxidant, and provitamin of industrial interest (Vandamme 1989). Mixed cultures of opposite sex (“mated cultures”) contain more β-carotene than single cultures of the same strains; this stimulation is mediated by the production of trisporic acids (Cagliotti et al. 1966; Sutter 1987). Large-scale mated cultures of *B. trispora* are used in industry (Ninet and Renaut 1976), but present practical drawbacks, because they tend to become unbalanced during growth. Trisporic acids are too expensive and unstable to use as practical stimulants of β-carotene production. Intersexual heterokaryons of Phycomyces exhibit the sexual stimulation of carotenogenesis in single cultures (Murillo and Cerdá-Olmedo 1976), but are unstable during vegetative growth, because they tend to segregate mycelia with unbalanced nuclear composition and reduced stimulation. They can be stabilized by the introduction of complementing, recessive, lethal mutations in the constituent nuclei (Murillo et al. 1978) to form balanced-lethal intersexual heterokaryons, but they grow less well than the wild types.

The β-carotene content and therefore the color of the
and contain very little the mycelia. On the contrary, carA (Olmedo agents and subject to genetic changes (mycelia of Phycomyces is influenced by various external strain S236, marked with a 636 B. J. Mehta and E. Cerda Â-Olmedo 1987a). Mutations in genes carS (Olmedo et al. 1997) increase the carotene content of ancia contained viable germspores. Most (72%) of the mycelia produced by germspores and are "superyellow," that is, more deeply pigmented than the wild type, were obtained as siblings from a cross (Mehta et al. 1997); their respective mating types are (−), (−), and (+). They were crossed (Eslava and Alvarez 1987) with strain S236, genotype nicA101 (+), a nicotinic acid auxotroph with light-yellow mycelia (Salgado et al. 1989), and C2, genotype carA5 (−), an albino mutant (Ootaki et al. 1973).

To force segregation by shearing, pieces (10–20 mg wet mass) of 40-hr-old mycelium were suspended in 0.6 m sorbitol, exposed to ultrasound (twice, 30 sec, high power) in a Sonifier model 250, Branson Ultrasoundics Corporation (Danbury, CT), and plated on acid agar. A similar segregation was obtained by whipping the mycelial pieces in a Vortex mixer for 1 min. The shearing controls were heterokaryotic mycelia carrying nuclei of the albino mutant strain C5, genotype carB10 (−), and the red mutant strain C9, genotype carR21 (−) (Heinenberg and Cerda-Olmedo 1968; Ootaki et al. 1973; Aragón et al. 1976).

β-Carotene was measured spectrophotometrically after extraction of lyophilized mycelia (Govind and Cerda-Olmedo 1986). To test for sexual reactions, mycelia were subcultured on potato-dextrose agar between the standard (+) and (−) wild-type strains NRRL1554 and NRRL1555, respectively. Trisporic acids were measured spectrophotometrically after chemical extraction of culture filtrates (Sutter 1970). For this purpose, pieces of mycelia (about 5 mm across) were inoculated into petri dishes that contained 35 ml of a liquid minimal medium with monosodium L-glutamate (2.0 g/liter) and L-asparagine (0.2 g/liter) as nitrogen sources (Sutter and Whittaker 1981) or potato-dextrose medium and incubated for 8 days at 22° in the dark. Strains NRRL2456 and NRRL2457 of B. trispora were used as controls for the production of trisporic acids because of their effective sexual interaction (Anderson et al. 1958).

RESULTS

Crosses: Strains S561 and S562, carrying the carF181 allele for increased carotene content, were crossed with strain S236, marked with a nicA allele for nicotinic acid auxotrophy. The zygospores took 8–13 months to germinate. Out of 153 zygospores tested, only 34 germinated to produce a germ sporangium, and only 20 germ sporangia contained viable germspores.

Most (72%) of the mycelia produced by germspores from these crosses belonged to the eight kinds of segregants expected for three markers, carF, nicA, and sex,

melti of Phycomyces is influenced by various external agents and subject to genetic changes (Cerda-Olmedo 1987a). Mutations in genes carS (Murillo and Cerda-Olmedo 1976), carD (Salgado et al. 1989), and carF (Mehta et al. 1997) increase the carotene content of the mycelia. On the contrary, carA mutants are albino and contain very little β-carotene (Eslava et al. 1974).

We have investigated the genetic nature of the anomalous segregants from marked crosses. When combined with a carF mutation, such segregants contained large amounts of β-carotene.
each with two alleles (Table 1). They were either light yellow, like the wild type, or deep yellow, like the carF strains. The respective β-carotene contents were 0.065 ± 0.010 and 2.33 ± 0.08 mg/gdm (milligrams per gram dry mass, means and standard errors in 9 and 10 segregants, respectively). Many mycelia (27%) were unexpected, because of their intermediate color and carotene content (1.65 ± 0.99 mg/gdm, 9 segregants); the high variability was due in part to the frequent appearance of color and texture variations during vegetative growth (“sectoring”). A few germspores (~1%) produced very deep colored mycelia that contained more carotene than the most productive parent. In this article we will distinguish between “superyellow strains,” having about the same color and carotene content as the carF mutant homokaryons, and “hydroporous strains,” in which pigmentation and carotene content are significantly higher.

The cross of S563, another strain with the carF181 allele, and the carA albino strain C2 were even less fertile; 37 zygospores produced only three germangia, and only one of them contained viable germspores. The 80 colonies produced by these germspores ranged from white to hyperyellow. Many of them had an intermediate color between the wild type and the superyellow parent (0.21 ± 0.11 mg β-carotene/gdm, eight segregants).

**Hyuperyellow mycelia:** About 1% of the viable germspores produced hyperyellow colonies (29 in 2717 colonies grown from germspores from all three crosses). They contained 17.3 ± 1.5 mg β-carotene/gdm (range 24.5 to 10.6 in nine segregants tested). Those from the nicA crosses were either prototrophic or auxotrophic for nicotinic acid in about equal proportions (14 and 12, respectively). Hyperyellow mycelia were thick, had a velvety surface due to the presence of short and twisted aerial hyphae called pseudophores (Blakeslee 1906), and grew uniformly, except for the sporadic appearance of less-pigmented sectors, variable in extent and frequency. When confronted with wild-type mycelia of either sex, some hyperyellow mycelia showed no sexual reactions and others formed a few early structures of the sexual cycle, such as zygophores, but no gametangia or zygospores.

**Vegetative segregation of the hyperyellow strains:** In successive generations grown from vegetative spores, hyperyellow mycelia produced a variety of colonies that differed in pigmentation, carotene content, and sexual reaction (Figure 2). The color varied from hyperyellow to light yellow in the descendants of hyperyellow strains from nicA crosses and from hyperyellow to white in those of the carA cross. Repeated cycles of spore collection and plating failed to stabilize the hyperyellow phenotype. The more pigmented mycelia produced fewer sporangiophores and spores than the lighter ones, thus confirming the negative correlation between sporulation and carotene content that is observed with the carotene mutants. Mycelia with lighter colors became progressively more abundant in successive generations (Figure 3).

Some hyperyellow strains were relatively stable, while others produced many sectors in the course of vegetative growth. The tendency to produce sectors was increased by freezing and thawing, but many sectors maintained the hypervellow phenotype, allowing us to preserve such strains. When mycelial pieces that had been kept at −20° for up to nine months were inoculated on agar media, they produced mycelia with marked sectors of different colors. Cultures grown from the more pigmented sectors contained 11–20 mg β-carotene/gdm. These results confirmed that hyperyellow colonies were not genetically homogeneous, presumably because their nuclei were not identical.

The heterogeneity of the hypervellow mycelia was confirmed by shearing them either by exposure to ultrasonic waves or by mechanic stirring. Inoculation of the sheared mycelial suspensions on agar media led to the appearance of colonies with different colors. In two experiments with hypervellow mycelia from the cross S236 × S561, only 2–5% of the colonies observed after shearing were hypervellow and the others formed a continuum down to light yellow. Hypervellow mycelia could thus be purified from sectoring cultures. The alternative procedure, spore segregation, is impractical for hypervellow mycelia that sporulate very poorly or not at all.

As a control, the shearing treatments were applied to heterokaryotic mycelia carrying nuclei of two strains, C5 and C9, whose mycelia are white and red, respectively, because of structural mutations in the carotene pathway. These heterokaryons are yellow because of complementation and stable during vegetative growth (Heisenberg and Cerda-Olmedo 1968). The colonies produced by the fragments were yellow (68%), white (5%), or red (27%). The appearance of homokaryons indicated that many viable fragments contained one or few nuclei.

**Trisporic acid production by hypervellow mycelia:** Three hypervellow descendants of a hypervellow segregant of the carA cross produced trisporic acids when grown in pure culture in glutamate minimal medium. The respective analyses gave 0.32, 0.31, and 0.22 mg trisporic acids/gdm, while 0.44 mg/gdm was found in mated cultures of strains NRRL2456 and NRRL2457 of B. trispora, which were taken as positive controls. In potato-dextrose medium the hydroporous strains produced no detectable trisporic acids, while the mated Blakeslea controls reached 1.16 mg/gdm.

**Selection for uninucleate spores:** Resistance to 5-carbon-5-deazariboflavin offers the opportunity to select for single nuclei. The phenotype is due to darA mutations (Delbrück and Ootaki 1979; Roncero et al. 1984) that cause the inactivation of riboflavin permease. These mutations are very recessive, so that only uninucleate spores can give rise to spontaneous mutants, but so frequent that such mutants are found by plating a few million spores on minimal agar with the inhibitor. The nuclei in a mycelium can thus be sampled by select-


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*Germsporangia A from cross S236 × S561, germsporangia B from cross S236 × S562. Viability is the number of viable germspores in the germsporangium. Random mycelia grown from germspores from 12 germsporangia were subjected to analysis.

1 Proposed genotype. Cand N stand for the wild-type alleles of genes *carF* and *nicA*; c and n, for the respective mutant alleles; (ns), no sexual reaction. In brackets, alleles that may or not be present.
The germspores that gave rise to hyperyellow mycelia contained, at least in some cases, all the markers in the cross. Frozen mycelial pieces of a hyperyellow mycelium from cross S236 × S561 formed patches of different colors on nutrient agar; a total of 12 spontaneous dar mutants were isolated from a total of 6 × 10⁶ spores from different patches. To purify the mutants without contamination with spores on the agar, a total of 48 young aerial sporangiophores, without sporangia, were cut out and inoculated onto fresh agar. The resulting mycelia were tested for carotene content (from 0.9 to 7.1 mg β-carotene/gdm), auxotrophy, and sexual reactions. It is not surprising that none of the dar mutants contained as much carotene as the original hyperyellow strain (16 mg/gdm), because the hyperyellow mycelia sporulate very poorly; the spores came from sectors with lower carotene content.

The genotypes of the 12 dar mutants, listed with the conventions used in Table 1, were: four $cN(\sim)$; four $cN(+)(-)$; one $CN(+)(-)$; one $CeN(-)$; one $CeNn(-)$; and one $CeN(+)(-)$. These genotypes were deduced from the phenotypes and, in case of instability, from those of their progeny as well. Thus, the genotype $Ce$ was unstable for color, the genotype $(+)(\sim)$ was unstable for sex, and the genotype $Nn$ gave rise to nicotinic acid auxotrophs. The heterozygotes were phenotypically different from the homozygotes, except $Nn$, which may have been classified as $N$ if segregants were overlooked.

In short, the dar mutants contained all the markers in the cross, and therefore these must have been present in the founding nucleus of the original germspore, which must have been $CcNn(\sim)$, that is, the diploid $nicA101(+/)$ carF181(−).

**Genotypes of the anomalous segregants**: Because of the instability of the diploid nuclei, the mycelia became complex heterokaryons. The genotypes in Table 1 indicate predominant genotypes assigned by testing the segregants and their spore progeny for color, nicotinic acid requirement, and sexual reaction. A full diploid would have a carotene content intermediate between those of the haploid parents, but an early loss of the wild-type $carF$ allele would change the phenotype to hyperyellow.

The presence of both wild-type and mutant $carF$ alleles in one of the abnormal segregants of intermediate color and $(\sim)$ sex was confirmed by crossing it with the wild-type NRRL1554. The progeny germspores grew into $(\sim)$ and $(\sim)$ mycelia with wild-type to superyellow colors.

**DISCUSSION**

No rule is evident for the results on individual germ-sporangia (Table 1). The presence or absence of the different genotypes, and their frequencies, are erratic. More regular are the aggregate results obtained by adding the observations from different germ-sporangia. The frequencies of the eight normal genotypes approach
those expected for three independent genes. Such results are usual for Phycomyces crosses (Cerdá-Olmedo 1975; Eslava et al. 1975a,b).

The segregants that showed signs of sexual stimulation without a mating partner coincided exactly with detailed morphological descriptions in the literature (Blakeslee 1906; Orban 1919; Burgeff 1924). They may be called anomalous because they deviate from the wild-type phenotype and because they constitute a small minority of the progeny in Phycomyces crosses (2% in Table 1). The frequency varies considerably from one germ sporangium to another, and in rare cases they constitute the majority or the totality of the germ spores in a germ sporangium (Cerdá-Olmedo 1975). We have concentrated our analysis on those that were hyperyellow because of two practical reasons: easier analysis and possible applied interest.

The coexistence of determinants for both sexes in hyperyellow mycelia was confirmed by their sexual behavior and their vegetative progeny. The production of trisporic acids by hyperyellow mycelia grown in gluta mate medium provided an independent proof. These compounds are synthesized by a metabolic cooperation between both sexes of the Microales (Sutter 1987) and have been found in mated cultures of Phycomyces in glutamate minimal medium (Miller and Sutter 1984), but not in single cultures.

The sex markers are not the only alleles that can coexist in a germ spore. The abnormal segregants in Table 1 present examples of germ spores that carried both wild-type and mutant alleles of genes carF and nicA and a few of them carried the three pairs of alleles in the cross.

The germ spores that gave rise to hyperyellow mycelia could not be simple heterokaryons of two haploid genotypes, because they produced too many different kinds of progeny. The spores contain random samples of the nuclei in the sporulating mycelium (Heisenberg and Cerdá-Olmedo 1968); the spores from intersexual heterokaryons produce three kinds of mycelia: the same intersexual heterokaryons and normal (+) and (−) homokaryons. Our hyperyellow strains gradually produced a large variety of different phenotypes. In addition, germ spores, contrary to vegetative spores, derive from uninucleate primordia (unpublished observations by E. Cerdá-Olmedo) and must therefore be homokaryotic.

We propose that hyperyellow mycelia derived from germ spores that were diploid or aneuploid; at least they contained two copies of chromosome I, which carries the sex markers (Alvarez et al. 1980). Random chromosome losses during mycelial development led to heterokaryotic mycelia with complex mixtures of nuclei; “sectoring” was due to changes in the nuclear components or ratios thereof and increased when the mycelia were fragmented by freezing, stirring, or exposure to ultrasound. The mycelia showed the hyperyellow phenotype as long as they contained the markers for both sexes and at least a large majority of carF181 mutant alleles. The spores produced a diversity of mycelia because they contained random samples of the nuclei; but they tended to come from the less-pigmented sectors, because high carotene content and intersexuality reduce sporulation. Sampling single nuclei from the sporulating sectors of the hyperyellow mycelia supported our proposal: a mycelium from a single germ spore contained all the markers in the cross, and therefore the original nucleus in the germ spore primordium must have been diploid, at least for the marked chromosomes.

The single-meiosis conjecture of Burgeff requires that a germ sporangium cannot carry more than four different genotypes and that each allele cannot be present in more than two of these. Although seemingly upheld by previous results (Eslava et al. 1975a,b), this hypothesis was clearly contradicted by Table 1. Even if we limit ourselves to the normal segregants, only three germ sporangia in Table 1 were compatible with the hypothesis, and these were the least discriminating, because they were nearly sterile, having produced 16 germ spores or fewer.

No evidence for meiosis has ever been found in Phycomyces. Because meiosis is essentially a symmetrical and ordered process, the erratic genotype frequencies in single germ sporangia led to an alternative hypothesis: “that there is no meiosis at all, but that the diploid survivor(s) suffer repeated mitotic divisions in which frequent mitotic recombination and haploidization would occur, leading to haploid progeny nuclei” (Cerdá-Olmedo 1974, pp. 353–354). This is, in essence, the “parasexual cycle” of Aspergillus and other fungi (Pontecorvo 1956; Roper 1966). Mitotic recombination would have to be very active, because the recombination frequencies are high (Alvarez et al. 1993) and recombinants are found between mutations of the same gene (Eslava et al. 1975a). Chromosome nondisjunction during mitosis would lead quickly to haploidization if nuclei with fewer chromosomes multiply faster than those with more chromosomes. We have shown that at least some germ spores carry nuclei that have been formed through this process, and the rest of our observations are compatible with it. Diploidy and protracted haploidization represent novelties not only for Phycomyces, but for all Zygomycetes.

Our results do not exclude that some or most of the germ spores represent meiotic products if several ad hoc assumptions are made. The founder diploid nucleus in the zygospore (sometimes more than one) would multiply by mitosis; the resulting diploid nuclei would undergo separate meioses; the meiotic products would multiply by mitosis to produce the nuclei in the germ spores; and nuclear generation times would be very heterogeneous.

Partial diploidy could lead to modifications of duplicated genes, as reported for other fungi (Selker 1997).
This might be the cause of the high frequency of mutants in the germspores (Burgeff 1928), our finding of many segregants with incomplete sexual activity, and the differences between hyperyellow strains with respect to their stability during vegetative growth.

The hyperyellow mycelia constitute an innovation in the biotechnology of carotene production. Our hyperyellow mycelia grew well, were sensibly stable during vegetative growth, and contained as much β-carotene as balanced-lethal intersexual heterokaryons with carS mutations (Murillo et al. 1978), which grew worse. The high carotene content of the hyperyellow mycelia was due to a synergic stimulation of the pathway by the carF181 mutation and by sexual introduction. Introduction of additional synergic stimuli, such as carS mutations (Salgado et al. 1989), should result in further increases in carotene concentration.

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