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Mutations Affecting Sexual Development in *Phycomyces blakesleeanus*

(*car* mutants/defined medium)

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ABSTRACT **Although zygospore (mature zygote) formation in *P. blakesleeanus* occurs in liquid glucose–glutamate medium, morphological observations are made more easily when cultures are grown on 1-mm-thick agar medium. Zygophores (sexually differentiated hyphae) develop prior to physical contact in crosses of (+) and (−) wild types. Zygophores interlock upon contact and then undergo six successive morphological changes to become a zygospore. Mutants with abnormal carotene synthesis exhibit aberrant sexual behavior. Some zygospores do form in crosses of *car*A mutants and wild types. Only paired zygophores form in crosses of wild type(+) with *car*-42(−), a β-carotene-accumulating mutant. Zygophores form only on (−) in crosses of wild type(+) with *car*B(−), *car*R(−), *car*A*car*R(−), and *car*B*car*R(−) mutants, and only on (+) in crosses of *car*-43(+) with wild type(−), *car*-42(−), and *car*A(−) mutants. Zygophores do not form in crosses of *car*-43(+) with *car*B(−), *car*R(−), *car*A*car*R(−), and *car*B*car*R(−) mutants. These observations demonstrate that each mating type makes a chemical messenger that stimulates zygophore development in the opposite mating type.**

Blakeslee (1) discovered sexuality in the mucoraceous fungi in 1903. Copulating (+) and (−) mating types of the same species undergo successive morphological changes. Zygophores, sexually differentiated hyphae, develop from asexual hyphae. Progametangia develop from the stimulus of contact between (+) and (−) zygophores. Progametangia divide to form gametangia and suspensor cells. The gametangia fuse to form a zygote. The zygote increases in size and develops a thick, hardened, black wall to become a zygospore. Attempted copulations between opposite mating types of different species result only in zygophore and progametangia development. These observations suggest that the initial stages of sexual development are common to all species of mucoraceous fungi, which include *Phycomyces blakesleeanus*, *Mucor mucedo*, and *Blakeslea trispora*.

Burgeff (2) reported in 1924 that (+) and (−) cultures of *M. mucedo*, separated by a cellodin membrane, develop zygophores that grow toward their counterpart of the opposite sex. This observation suggests that diffusible and/or volatile substances pass between the sexes to stimulate the formation of zygophores and to direct their growth. From experiments performed 33 years later, Plempel (3) concluded that the (+) mating type makes a hormone that stimulates zygophore development in the (−) mating type, and the (−) mating type makes a hormone that stimulates zygophore development in the (+) mating type. Attempts by Plempel (4) to purify the two hormones from (+/−) cultures of *M. mucedo*, however, resulted in crystallization of a compound that stimulated zygophore development in both mating types. Van den Ende (5) then demonstrated that trisporic acid B and trisporic acid C, when applied separately in the *Mucor* bioassay, stimulate zygophore development in both (+) and (−) cultures.

Separate (+) and (−) cultures of *B. trispora* synthesize trisporic acid B and trisporic acid C when incubated with a neutral fraction isolated from the culture medium of the opposite sex (6). Tracer studies demonstrate that the neutral fractions contain mating type-specific precursors of the trisporic acids (7). These facts suggest that trisporic acids are intrahyphal chemical regulators of zygophore development and that the mating type-specific precursors are the interhyphal chemical messengers or hormones (6). Consistent with this suggestion are the existence of volatile mating type-specific agents that stimulate zygophore development in *M. mucedo* (8), and (−) mutants of *M. mucedo* that do not respond to trisporic acid but do form zygospores when mated with (+) wild type (T. Wurtz and H. Jockusch, personal communication).

Labeled β-carotene and labeled retinol are incorporated into trisporic acids in (+/−) cultures of *B. trispora* (9, 10). Numerous carotene mutants, which are also defective sexually, have been isolated from wild type *P. blakesleeanus* (11). Complementation analyses of the β-carotene-deficient mutants reveal three genes in carotene biosynthesis: *car*A, *car*B, and *car*R (12). Mutants in the gene *car*B are white and accumulate phytoene; mutants in the gene *car*R are red and accumulate lycopene. Mutants in the gene *car*A are white and produce minute amounts of β-carotene, but no other carotenes. In this report, I describe the morphological stages of sexual development in crosses of wild type, β-carotene-deficient, and β-carotene-accumulating mutants of *P. blakesleeanus*.

MATERIALS AND METHODS

Strains. The wild type strains of *P. blakesleeanus* Burgeff used in this work are NRRL1554(+) and NRRL1555(−). The β-carotene-deficient mutants used are listed in the legend to Table 1. The complete genotypes and origins of these mutants have been described (12, 13). The β-carotene-accumulating mutants used are M1 [*car*-43(+)] and C115 [*car*-42(−), formerly designated *Ph* 107 (14)]. M1 and C115 accumulate 28 and 17 times, respectively, more β-carotene than wild-type cultures when grown 4.5 days at 20° in darkness on glucose–asparagine–yeast extract agar medium (15). I isolated M1 from NRRL1554(+) after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, procedure 2 (16). The strains designated by C were isolated at the California Institute of Technology from the above wild types, those designated by S, at the Universidad de Seville from wild-type UBC24(−). All strains may be obtained from the Phycomyces Culture Col-

TABLE 1. *Sexual development in crosses of wild type and car mutants*

Cross*	Zygophores† (+)	Zygophores† (−)	Extent of sexual development‡
Wild type(+) × wild type(−)	0.5	0.5	S_8
*car*A(+) × wild type(−)	0.9	0.1	S_8
Wild type(+) × *car*A(−)	0.1	0.9	S_8
Wild type(+) × *car*B(−)	0	1	S_0–S_1
Wild type(+) × *car*R(−)	0	1	S_0–S_1
Wild type(+) × *car*A*car*R(−)	0	1	S_0–S_1
Wild type(+) × *car*B*car*R(−)	0	1	S_0–S_1
Wild type(+) × *car*-42(−)	0.5	0.5	S_2
car-43(+) × wild type(−)	1	0	S_1–S_0
car-43(+) × *car*-42(−)	1	0	S_1–S_0
car-43(+) × *car*A(−)	1	0	S_1–S_0
car-43(+) × *car*B(−)	0	0	S_0
car-43(+) × *car*R(−)	0	0	S_0
car-43(+) × *car*A*car*R(−)	0	0	S_0
car-43(+) × *car*B*car*R(−)	0	0	S_0

* The following mutants were tested—*car*A(+): C3, C152, C169, C170; *car*A(−): C2, C7, C8, S5, S14, S16, S23, S28, S40, S45, S48, S49; *car*B(−): C5, S20, S21, S22; *car*R(−): C9, C10, C11, C12; *car*A*car*R(−): C6, C143, C144, C171, C175, C177, C178, C179, C180; *car*B*car*R(−): C173, C174; *car*-42(−): C115; and *car*-43(+): M1.

† The relative number of zygophores produced by each mating type in a given cross.

‡ See *text* for the description of the stages.

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Medium. Sexual development was studied on a glucose–glutamate agar medium containing per ml: 20 mg of D-glucose, 1 mg of monosodium L-glutamate, 5 mg of KH_2PO_4, 500 μg of $MgSO_4 \cdot 7H_2O$, 28 μg of $CaCl_2$, 2 μg of thiamine·HCl, 2 μg of citric acid·H_2O, 1.5 μg of $Fe(NO_3)_3 \cdot 9H_2O$, 1 μg of $ZnSO_4 \cdot 7H_2O$, 300 ng of $MnSO_4 \cdot H_2O$, 50 ng of $CuSO_4 \cdot 5H_2O$, 50 ng of $NaMoO_4 \cdot 2H_2O$, and 15 mg of purified agar. The glucose was autoclaved separately. The final pH of the medium was 5.1. Monosodium L-glutamate and thiamine·HCl were obtained from Sigma Chemical Co., the purified agar from Difco Laboratories. All other chemicals were reagent grade.

Culture Methods and Analysis. Mycelia for inocula were obtained by plating sporangiospores at the edge of 10 cm in diameter petri dishes containing 25 ml of glucose–asparagine–yeast extract agar medium (14) and then incubating for 2–3 days at 22°. For observations, the (+) and (−) mycelia were inoculated 4 cm apart on a 10 cm in diameter petri dish containing 5 ml of glucose–glutamate agar medium and then incubated at 22° in darkness. Growth and sexual development were observed with the unaided eye and with stereo-dissecting and compound microscopes. Each experiment was performed at least three times. Photographs were taken on Kodak Panatomic X film with a Zeiss Photomicroscope.

RESULTS

Zygophores, sexually differentiated hyphae, develop prior to physical contact in crosses of (+) and (−) wild types (Fig. 1A). Zygophores of (+) and (−) mating types are indistinguishable. Zygophores are knobby structures: short, stubby, multibranched hyphae, which usually develop on the growing hyphal tips. Zygophores of opposite sexes grow towards each other. Sometimes zygophores change the direction of growth from that followed by the former asexual hyphae. As soon as the zygophores come into contact, the short multibranched hyphae interlock to form the first progametangial stage. Each short, stubby branch-hyphae of the paired zygophores then presumably branches more and interlocks with its counterpart of the opposite sex. Enlarged paired zygophores appear as knobby knots. The asexual hyphae, separate zygophores, and paired zygophores—designated S_0, S_1, and S_2, respectively—are in or at the surface of the agar. Subsequent stages of sexual development, designated S_3 through S_8, occur in the air above the agar surface. That is, the enlarged paired zygophores develop stout, sometimes yellow, paired pillars (S_3). The paired pillars separate, except at their base and apex, and enlarge to form a loop or the third progametangial stage (S_4). Septa form in both halves of the loop to delimit gametangia and suspensor cells (S_5). Thorn development is initiated on the suspensor cells (S_6). The gametangia fuse to form a translucent zygote that is surrounded by dark, sometimes black, thorns (S_7). The zygote then enlarges and develops a thick, hardened, black wall to become a zygospore (S_8). All stages of development can be seen on 1-mm-thick glucose–glutamate agar medium. Some zygophores of each mating type never pair with their counterpart of the opposite sex, and less than a third of the paired zygophores ever become zygospores. Eight to 10 times more zygospores form in 6 days when cultures are grown on 5- to 6-mm-thick glucose–glutamate agar medium. (The pH of the agar culture medium after 6 days is about 5.8.) However, zygophores do not form on the thicker medium until (+) and (−) hyphae have grown past each other. These zygophores are difficult to detect because the mycelial growth is so extensive. Zygospores also form in liquid glucose–glutamate medium when the cultures are *not* agitated.

Four types of aberrant sexual behavior are observed in crosses of *car* mutants with wild types and *car* mutants with *car* mutants (Table 1). (*i*) Zygospores are formed only in crosses of *car*A(+) mutants with wild type(−) and in crosses of wild type(+) with *car*A(−) mutants. Zygospore formation is at a reduced level in these crosses because 90% of the zygophores formed are on the *car*A mutant, whereas equal numbers of zygophores form on each mating type in crosses of (+) and (−) wild types. (*ii*) Sexual development does not proceed beyond the initial progametangial stage in crosses of wild type(+) and *car*-42(−), a β-carotene-accumulating mutant. (*iii*) Zygophores are formed only on the mutant in crosses of wild type(+) with *car*B(−), *car*R(−), *car*A*car*R(−), and *car*B*car*R(−) mutants and in crosses of *car*-43(+), a β-carotene-accumulating mutant, with wild type(−) (Fig. 1B). Furthermore, zygophores are formed only on *car*-43(+) in crosses with *car*-42(−) and *car*A(−) mutants. (*iv*) Zygophores are not formed in crosses of *car*-43(+) with *car*B(−), *car*R(−), *car*A*car*R(−), and *car*B*car*R(−) mutants. Consider a cross of wild type(+) with *car*-42(−): the zygophores are pale yellow and reddish orange, respectively, whereas the asexual hyphae are cream and pale orange. The knot that results from the pairing of zygophores has both pale yellow and reddish orange components. The two-colored appearance of paired zygophores is more striking in crosses of wild type(+) with C13 and C14. C13 and C14, which develop bright red zygo-

phores, are leaky *car*R mutants in which β-carotene represents 61% and 12%, respectively, of the total carotenes accumulated (12). Further sexual development is inhibited in these crosses. Zygophores develop only on the mutant in crosses of wild type(+) with (−) mutants accumulating negligible amounts of β-carotene. More zygophores develop in crosses with *car*R and *car*A*car*R mutants than with *car*B and *car*B-*car*R mutants. The zygophores of *car*R mutants are brighter red than the asexual hyphae. The zygophores of the *car*A-*car*R, *car*B, and *car*B*car*R mutants are as white as the asexual hyphae. Mutant zygophores usually develop under the surface of the agar. Some zygophores grow to the surface and enlarge. Some zygophores grow toward hyphae of wild type(+), surround it, and then enlarge. The latter enlarged knobby structures, which form in crosses of *car*R mutants, are one color—red, suggesting that the wild type(+) is not a participant in its formation. Similar observations were made in crosses of *car*-43(+) with wild type(−) and *car*A(−) mutants. The extent to which the zygophores of *car*-43(+) surround hyphae of their mate is related directly to the thickness of the agar medium. Finally, neither zygophore development nor sex-stimulated carotenogenesis is detectable in crosses of *car*-43(+) with (−) mutants accumulating negligible amounts of β-carotene.

DISCUSSION

The observations reported in this paper were made initially with cultures grown on a medium containing potato extract. Some observations were not reproducible with new lots of potato extract. For example, zygophores were not detectable with one new lot; zygospore formation decreased 20-fold with another lot. Thus, a defined liquid medium, in which zygospores form, was developed. Agar was added to the medium to facilitate morphological studies. Zygospore formation on the glucose–glutamate agar medium was as extensive as on the best lot of potato extract tested. These studies verify the report of Leonian and Lilly (17) that sexual reproduction in *P. blakesleeanus* occurs on a buffered medium containing only glucose, an amino acid, inorganic salts, and thiamine.

The stages of sexual reproduction described in this paper, S_1 through S_8, have been reported in part by one or more earlier workers (1, 2, 18, 19). All four workers have described S_3 through S_6 and S_8 in detail. Blakeslee (1) has described zygote formation, S_7. Orban (19) and Burgeff (2) have reported separate and paired zygophores, S_1 and S_2. I have shown that zygophores develop on both (+) and (−) mating types prior to hyphal contact, thereby demonstrating that sexual reproduction in *P. blakesleeanus* is initiated by interhyphal chemical messengers.

Auxotrophic, drug-resistant, and phototropic mutants of *P. blakesleeanus* are sexually normal. In contrast, *car* mutants are sexually defective (20). After Ootaki and coworkers (12) determined the genotypes of the β-carotene-deficient mutants, it was obvious from DeHaven's (21) work with 10 mutants that *car*A mutants formed zygospores at a reduced level when crossed with wild type and that all other *car* mutants were blocked at an early stage of sexual development. These conclusions were verified by Ootaki, Lockhart, and Anseau (personal communication), who tested all available *car* mutants. By using thin agar medium, I was able to detect zygophores and determine that they were: (*i*) formed predominantly by the mutant in crosses of wild types and *car*A mutants; (*ii*) formed only by (−) in crosses of wild type(+) with (−) mutants accumulating negligible amounts of β-carotene; (*iii*) formed only by (+) in crosses of *car*-43(+) with wild type(−), *car*-42(−), and *car*A(−) mutants; and (*iv*) not formed in crosses of *car*-43(+) with (−) mutants accumulating negligible amounts of β-carotene. These observations demonstrate that the (+) mating type makes a chemical messenger that stimulates zygophore development in (−), and that the (−) mating type makes a chemical messenger that stimulates zygophore development in (+). If trisporic acid is the only compound that stimulates zygophore development in *P. blakesleeanus*, these observations are consistent with the following four hypotheses. (*i*) β-Carotene is a precursor of trisporic acids (9). (*ii*) *Car*-43(+) is blocked in its metabolism

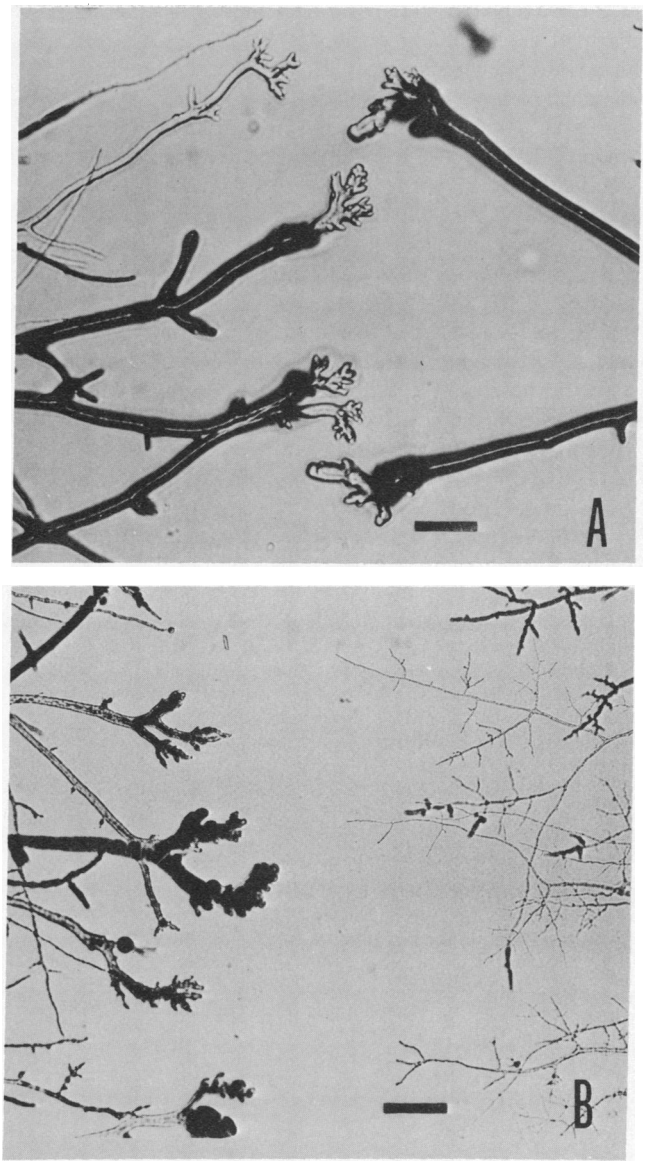

FIG. 1. Zygophores in crosses of (A) wild type(+) × wild type(−) and (B) *car*-43(+) × wild type(−). The (+) mating type is on the left in both photographs. The bars represent 21 μm in (A), 44 μm in (B). In (A), the asexual hyphae, which appear black, are at the surface of the agar, surrounded by water. In (B), the zygophores of *car*-43(+) appear black due to the high concentration of carotene.

of β-carotene. (*iii*) Trisporic acid biosynthesis proceeds via mating type-specific precursors (6, 7, 22). (*iv*) Trisporic acid is an intrahypal chemical regulator, rather than an interhyphal chemical messenger or hormone (6).

Trisporic acids, which are synthesized extensively only when (+) and (−) mating types are together, have been isolated from *B. trispora* (23), *M. mucedo* (5, 24), and *P. blakesleeanus* (21). Trisporic acids stimulate carotenogenesis (25, 26), syntheses of (+) and (−) mating type-specific precursors of trisporic acids (7, 22), and zygophore development (5, 6, 27, 28). However, 1,000 to 10,000 times more trisporic acids are needed to stimulate zygophore development in *P. blakesleeanus* than in *M. mucedo* (unpublished result). This result suggests that either the hyphal membrane in *P. blakesleeanus* is relatively impermeable to trisporic acids, or trisporic acid B and trisporic acid C are inactive metabolites of the zygophore-inducing compounds. The possibility of doing both genetic and biochemical experiments with *P. blakesleeanus* makes it an extremely useful organism for studying sexual development in mucoraceous fungi.

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