Modification of Sexual Development and Carotene Production by Acetate and Other Small Carboxylic Acids in *Blakeslea trispora* and *Phycomyces blakesleeanus*

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In *Phycomyces blakesleeanus* and *Blakeslea trispora* (order Mucorales, class Zygomycetes), sexual interaction on solid substrates leads to zygospor production and to increased carotene production (sexual carotenogenesis). Addition of small quantities of acetate, propionate, lactate, or leucine to mated cultures on minimal medium stimulated zygospor production and inhibited sexual carotenogenesis in both *Phycomyces* and *Blakeslea*. In *Blakeslea*, the threshold acetate concentration was <1 mmol/liter for both effects, and the concentrations that had one-half of the maximal effect were <2 mmol/liter for carotenogenesis and >7 mmol/liter for zygosporogenesis. The effects on *Phycomyces* were similar, but the concentrations of acetate had to be multiplied by 3 to obtain the same results. Inhibition of sexual carotenogenesis by acetate occurred normally in *Phycomyces* mutants that cannot use acetate as a carbon source and in mutants whose dormant spores cannot be activated by acetate. Small carboxylic acids may be signals that, independent of their ability to trigger spore germination in *Phycomyces*, modify metabolism and development during the sexual cycle of *Phycomyces* and *Blakeslea*, uncoupling two processes that were thought to be linked and mediated by a common mechanism.

*Phycomyces blakesleeanus* and *Blakeslea trispora* are actual or potential sources of commercial carotenones used as provitamins, pigments, and antioxidants in the food and feed, pharmaceutical, and cosmetics industries (3, 10, 13, 26). Mutations in various genes (11, 20, 22) result in very high β-carotene contents or interrupt the carotene pathway, allowing production of lycopene and other carotenones. *Phycomyces* stops β-carotene biosynthesis through a feedback mechanism when a certain concentration has been reached and responds to four groups of activators with different modes of action typified by sexual activity, illumination, and addition of retinol or dimethyl phthalate to the medium (5).

Blakeslee (6) classified strains of Mucorales into two sexes, (+) and (−), by noting that when mycelia of opposite sexes meet on solid media, they become bright yellow and produce a succession of special structures, particularly zygospor. The enhanced coloration is due to increased accumulation of β-carotene, which is termed sexual carotenogenesis to distinguish it from the vegetative carotenogenesis that occurs in single-strain cultures. Sexual carotenogenesis and sexual morphogenesis are induced by trisporic acid C and related trisporoids, which are produced cooperatively by neighboring mycelia of different sexes (8, 34). Sexual carotenogenesis occurs in mated cultures that contain mycelia of the two sexes, in single-strain cultures of either sex exposed to natural or synthetic trisporides (17), in single-strain cultures of intersexual heterokaryons, whose mycelia contain nuclei of both sexes (22, 23), and in single-strain cultures of intersexual diploids (21). In all known mutants the carotene content is increased further by sexual stimulation.

*Phycomyces* and *Blakeslea* grow on acetate as a sole carbon source. Acetate must first be converted to acetyl coenzyme A (acetyl-CoA) by an acetyl-CoA synthetase (EC 6.2.1.1). Mutants of *Phycomyces* resistant to fluoracetate could not utilize acetate because of the loss of the acetyl-CoA synthetase encoded by the *facA* gene (15, 16). *Phycomyces* produces a different acetyl-CoA synthetase in response to carbon starvation (14).

Acetate also has regulatory effects on development. Very few vegetative spores of *Phycomyces* germinate after they are inoculated into a minimal medium in which growth and differentiation occur (28). Spore dormancy can be broken by various treatments, including exposure to heat, acetate, propionate, or other chemicals, some of which cannot be metabolized and others of which are toxic (29, 36, 38). The *ger* mutants of *Phycomyces*, isolated for decreased acetate or propionate spore activation, also are less responsive to activation with heat and do not exhibit the transient increase in the cyclic AMP level that immediately follows such treatments in wild-type spores. In these mutants a protein that detects heat shock, small carboxylic acids, and perhaps other compounds and that transduces the signal for germination may be altered (27, 37).

The objective of this study was to describe novel effects of acetate and other small carboxylic acids on the sexual processes of two zygomycete fungi. We examined whether the morphogenetic and metabolic changes induced by sexual interaction are tightly coupled as a single response. The results are significant for understanding sexuality in the Zygomycetes and for practical application of this sexuality in the carotene industry.

**MATERIALS AND METHODS**

Strains. NRRL1554 and NRRL1555 are natural (+) and (−) strains of *P. blakesleeanus*, respectively, and were obtained originally from the Northern Regional Research Laboratory (now the National Center for Agricultural Utilization Research, Peoria, IL). Strains F986 and F921 are wild-type (+) and (−) strains of *B. trispora*, respectively, and were obtained from VKM (All-Russian Collection of Microorganisms, Moscow, Russia). *Phycomyces* strains MU136,
MU138, and MU141 are fas4 mutants that are resistant to fluoroacetate and are defective for acetyl-CoA synthetase activity (16). Strains S437 and S440 are ger mutants whose spores show decreased activation by heat, acetate, and propionate (27). The mutants were isolated after treatment of NRRL1555 spores with N-methyl-N-nitro-N-nitosoguanidine.

**Culture conditions.** Cultures were grown for 4 days (unless indicated otherwise) at 22°C (Phycomyces) or at 30°C (Blakeslea) in the dark on cellophane disks (Sadipal, Gerona, Spain) placed on the top surface of 25 ml of agar medium in petri dishes (diameter, 85 mm). The minimal medium (9, 33) contained 20 g/liter glucose and 5 g/liter L-asparagine as carbon and nitrogen sources. Glutamate medium contained monosodium L-glutamate (1 g/liter) instead of asparagine. Potato dextrose medium prepared with fresh potatoes (9) gave more reproducible results than the dried commercial form of this medium. Phycomyces spores were collected from 4-day-old cultures by rinsing the cultures with sterile distilled water and then were activated by heat shock before plating (9). Blakeslea spores were collected from older cultures (1 to 2 weeks) with glycerol (1:3 [vol/vol] in water). Spore stocks were kept refrigerated for no more than 1 week. Each culture was started with 10⁵ spores, and half of each sex was used for mated cultures. Extracts obtained by freezing the media (−20°C for at least 2 h), thawing the media (22°C for 1 h), and centrifuging the liquid (1,000 × g, 10 min, 22°C) were used for glucose determination. Similar extracts obtained from media on which mated cultures of Blakeslea had grown for 2 days were used instead of water for preparation of minimal medium with trisporic acids.

**Quantification of zygospores.** Spores were plated directly on a medium without a cellophane disk. The zygospores of Blakeslea are distinct and small (diameter, ~50 μm) and were counted with a stereomicroscope. The zygospores of Phycomyces are much larger (diameter, ~500 μm), superimposed, and decorated with abundant thorns, which makes them difficult to count; these spores were scraped off the medium with no attempt at purification and weighed.

**Chemical analyses.** For carotene analyses, mycelia were scraped from the cellophane disks, lysophilized, weighed, and ground with a mortar and pestle in the presence of sand and petroleum ether (boiling point, 40 to 60°C). The extract was centrifuged (1,000 × g, 5 min, 22°C), vacuum dried, and dissolved in n-hexane. When possible, the procedures were carried out on ice under dim light, and the extracts were kept under a nitrogen atmosphere. A 10- to 20-μl aliquot of extract was loaded using a G1313A autosampler (Hewlett-Packard, Palo Alto, CA) into a C18 column (4.6 by 100 mm; 5-μm octyldecylsilane particles; Hypersil; Waters, Milford, MA) with a 10-mm refillable guard precolumn filled with the same material (Alltech, Deerfield, IL) in a series 1100 liquid chromatograph (Hewlett-Packard). The column was eluted at room temperature with methanol-chloroform-acetonitrile (47:47:6, vol/vol/vol) at a flow rate of 1 ml/min. The outflow was monitored with a diode array detector at 286, 450, 462, and 473 nm, where the absorption maxima of phytoene, β-carotene, γ-carotene, and lycopene, respectively. Concentrations were calculated following calibration with samples of the four carotenoids purified from Phycomyces cultures. The significance of differences in carotene concentrations was determined by standard tests (Student’s t test and nonparametric rank test).

Concentrations of trisporic acids were estimated (24) from A₃₂₅ values by using an extinction coefficient (1 cm, 1 g/liter) of 57.2. The glucose content was determined with an oxidase-peroxidase kit (Sigma Chemical Co., St. Louis, MO).

**RESULTS**

**Modification of sexual processes by acetate.** The presence of small amounts of acetate in the media modified the sexual interaction in mated cultures of both Phycomyces and Blakeslea (Fig. 1A). Zygospores, which are produced only when mating occurs, were much more abundant and appeared earlier in the presence of acetate than in the absence of acetate (Fig. 1B and similar observations with Phycomyces). Single-strain cultures were slightly colored (yellow for Phycomyces and orange for Blakeslea), and there was some variation among different wild-type strains. To properly identify the colors of mated cultures, zygospores must be scraped off the plates. Mated cultures in the absence of acetate and young mated cultures in the presence of acetate both had enhanced pigmentation due to sexual carotenogenesis. Older mated cultures in the presence of acetate appeared to contain approximately the same amount of pigment as unmated single-strain cultures contained.

**Zygospore formation.** Few zygospores were produced on minimal medium, but addition of acetate increased the zygospore density about 10-fold in Phycomyces cultures and about 25-fold in Blakeslea cultures (Table 1). The resulting values were greater than those obtained with glutamate medium, which is recommended (33) for the production of abundant zygospores on thin mycelia with well-separated hyphae. The threshold for a response to sodium acetate was ≥1 mmol/liter,
and the response was not saturated with 20 mmol/liter (Fig. 2); >7 mmol/liter was needed for the response to reach one-half the maximal value.

The effects of propionate, leucine, and lactate were qualitatively similar to the effects of acetate except for propionate at a concentration of 10 mmol/liter, in the presence of which there was not sufficient growth of Blakeslea for observation of any sexual response. In single-strain cultures these compounds failed to induce the formation not only of zygospores but also of zygothallus, the thickened hyphal tips that are the first morphological stage of the sexual response.

**Carotene production.** The increase in carotene content due to sexual interactions is particularly well known in Blakeslea, and mated Blakeslea cultures contained 4.2 ± 0.4 mg carotene/g (dry mass) or 13 times the average for the single-strain cultures (Table 2). The amount of carotenoids produced varied with the strain, and F921 contained more carotene than F986 contained. The sexual response of Phycomyces, 0.67 ± 0.04 mg carotene/g (dry mass), was only four times the average for single-strain cultures, which were similar in this respect. The increases due to sexual interaction were very significant (P << 0.001 for both Blakeslea and Phycomyces). Potato dextrose medium, which is commonly used in sexual cycle research, is not suitable for carotene production or sexual carotenogenesis, with single and mated wild-type strains of Phycomyces containing only 30 μg β-carotene per g (dry mass).

Mated cultures grown in the presence of acetate contained much less carotene than mated cultures grown in the absence of acetate (Table 2), and the effect was very significant (P << 0.001 with 10 mmol/liter in both Blakeslea and Phycomyces cultures). Acetate can be considered an inhibitor of sexual carotenogenesis. Mated cultures with acetate contained only a little more carotene than single-strain cultures without acetate contained; the increase (28% more on average with 10 mmol/liter acetate) was significant (P < 0.01) after all the results were pooled. The presence of acetate in single-strain cultures decreased the carotene content (with 10 mmol/liter acetate, to 70% of the content without acetate, on average); the difference was very significant (P < 0.001) only after all the results were pooled.

Leucine, DL-lactate, and propionate also were inhibitory, but DL-lactate was less effective with Blakeslea. In single-strain cultures, leucine slightly increased the carotene content, consistent with previous observations (12), and the other compounds appeared to be slightly inhibitory in some experiments.

The addition of acetate slightly increased the pH of the medium, but the results did not change when the experiments were repeated with media whose pH was adjusted to 5.4 ± 0.1 before inoculation.

With the exception of Blakeslea growing on media containing propionate, there was little or no variation in the overall growth of the cultures, as measured by the amount of dry mycelial mass per plate. For 4-day-old Phycomyces single-strain and mated cultures the average dry mycelial mass was 0.22 g per plate. Blakeslea strain F986 grew better than F921 (0.20 and 0.16 g/plate, respectively; significantly different at a P value of 0.01), and the average dry mycelial mass for mated cultures was 0.15 g/plate. In cultures of both fungi, glucose was depleted about 2 days after the cultures reached the maximal amount of dry mass. Thus, ~2.5 g/liter glucose remained in the medium of 4-day-old Phycomyces cultures.

For Blakeslea the threshold acetate concentration for inhibition of sexual carotenogenesis was <1 mmol/liter, and the response was saturated at ~5 mmol/liter. The threshold for Phycomyces was similar, but higher acetate concentrations were needed to saturate the response (Fig. 3).

The time courses of sexual carotenogenesis differed for Phycomyces and Blakeslea. Phycomyces, incubated at 22°C, was growing actively at 2 days, and most of the dry mass and most of the carotene in older cultures were produced on day 3 and the following days. In single-strain Phycomyces cultures, β-carotene is neither destroyed nor metabolized appreciably (4), but in mated cultures a considerable amount of β-carotene is used for production of trisporates (2). Sexual carotenogenesis increased with age, and the maximum amount of carotene (~0.8 mg total carotene per g [dry mass]) was present in older cultures (Fig. 4). Blakeslea incubated at 30°C grew most actively on the first day, when it produced one-half of the mature mass, and the remaining mycelial mass was produced by day 3. The maximum sexual carotenogenesis (~8 mg total carotene per g [dry mass]) occurred in

<table>
<thead>
<tr>
<th>TABLE 1. Zygospore production on different media*</th>
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<tbody>
<tr>
<td><strong>Medium</strong></td>
</tr>
<tr>
<td>Minimal (asparagine)</td>
</tr>
<tr>
<td>Minimal + acetate</td>
</tr>
<tr>
<td>Glutamate</td>
</tr>
<tr>
<td>Potato dextrose</td>
</tr>
<tr>
<td>Potato dextrose + acetate</td>
</tr>
<tr>
<td>Potato dextrose + asparagine</td>
</tr>
<tr>
<td>Potato dextrose + asparagine + acetate</td>
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* The values are means ± standard errors for 3 to 17 determinations in three or four independent experiments. The compounds added to the standard media were acetate at a concentration of 10 mmol/liter and L-asparagine·H₂O at a concentration of 2 g/liter.

FIG. 2. Dependence of zygospore production on acetate concentration. Mated cultures of Phycomyces wild-type strains (○) and Blakeslea wild-type strains (●) were grown on minimal medium with various concentrations of sodium acetate. The values are means for 3 to 17 determinations in one to four independent experiments relative to the values obtained for the controls in the absence of acetate. The average relative standard errors of the means were 12% for Phycomyces and 10% for Blakeslea. The acetate concentration is plotted on a logarithmic scale.
young cultures that were 1.5 to 2 days old. Production of β-carotene continued in older cultures, as observed for the phytoene content (Fig. 4) and for the very large amounts of trisporates found in mated cultures of this fungus (34). In the presence of acetate, sexual carotenogenesis occurred in young cultures (up to 1 mg carotene per g [dry mass]) but not in older cultures. Phytoene was abundant, but γ-carotene, lycopene, and other carotenes collectively comprised <10% of the total carotene in mated cultures without acetate and <0.1 mg/g (dry mass) in the other cultures.

**Acetate inhibition of sexual carotenogenesis in mutants.** To determine if acetate must be metabolized to be inhibitory, we tested three facA mutants which do not utilize acetate as a sole carbon source because they lack the required acetyl-CoA synthetase. To determine if acetate inhibits sexual carotenogenesis by the same mechanism that it uses to activate vegetative spores, we tested two ger mutants defective in spore activation by acetate, propionate, and heat (27). The results obtained with the mutants (Table 3) were indistinguishable from the results obtained with their parental strain (only 2 of the 30 pairwise comparisons between the fac mutants and the wild-type strains in Table 3 were significant at a P value of 0.05, and none was significant at a P value of 0.01). Mating increased the carotene content, and the increase was inhibited by acetate; both effects were significant for each of the five mutants and very significant (P < 0.001) for the pooled results for the three facA mutants and the pooled results for the two ger mutants.

These results were independently confirmed with single-strain cultures grown in the presence of trisporic acid extracts (~7 μmol/liter). These conditions resulted in a modest increase in the carotene content (43% on average) that was eliminated by acetate.

![FIG. 3. Dependence of β-carotene content on acetate concentration.](image)

![FIG. 4. Time courses for phytoene and β-carotene contents of single-strain and mated cultures of **Phycomyces** wild-type strains (○ and □) and **Blakeslea** wild-type strains (● and ▲, mated) grown on minimal medium with 10 mmol/liter sodium acetate (● and ▲, mated) and Blakeslea wild-type strains (● and ▲, mated) grown on minimal medium (○, □, and ▲) or minimal medium with 10 mmol/liter sodium acetate (●, ▲, and △). The values are means and standard errors for 2 to 15 determinations in one to four independent experiments.](image)

<table>
<thead>
<tr>
<th>Medium</th>
<th>Carotene content (mg/g [dry mass])</th>
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<tbody>
<tr>
<td></td>
<td>Blakeslea</td>
</tr>
<tr>
<td></td>
<td>F986</td>
</tr>
<tr>
<td>Minimal + sodium acetate (10 mmol/liter)</td>
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<tr>
<td>Minimal + potassium acetate (10 mmol/liter)</td>
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</tr>
<tr>
<td>Minimal + l-leucine (10 mmol/liter)</td>
<td>0.45</td>
</tr>
<tr>
<td>Minimal + L-lactate (20 mmol/liter)</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* Single and mated cultures were grown on minimal medium with the compounds indicated. The values are the mean total carotene contents (β-carotene and phytoene in the case of **Phycomyces**; β-carotene, γ-carotene, lycopene, and phytoene in the case of **Blakeslea** for at least two independent determinations. The average relative standard error of the means was 7%. 

![TABLE 2. Effects of acetate, propionate, lactate, and leucine on the carotene contents of **Phycomyces** and **Blakeslea**.](image)
tate; both effects were significant at least at a P value of 0.05 for the wild type, for the pooled results for the three facA mutants, and for the pooled results for the two ger mutants.

**DISCUSSION**

The increase in carotene biosynthesis and the induction of sexual morphogenesis that leads to zygospore production have been considered two indissoluble aspects of sexuality in the Mucorales, and both of these processes are induced by trisporic acids. Since trisporic acids are metabolites of β-carotene (2), sexual carotenogenesis was seen as part of an auto-catalytic cycle for sexual stimulation. Small amounts of trisporic acid precursors produced by the vegetative hyphae reach hyphae of the opposite sex, where the compounds are converted to trisporic acids that trigger sexual morphogenesis and sexual carotenogenesis (34). The additional carotene increases trisporic acid production, and the cycle is repeated. This hypothesis is supported by the observation that mutants unable to produce carotene also do not stimulate carotene production by their partners, and the mated pair of strains does not produce zygospores (33). The link between the two sexual responses has now been broken since we observed that acetate can also inhibit the metabolic response. Enhanced sexual morphogenesis occurred in the absence of the high carotene concentration that usually accompany it. Our results highlight our incomplete knowledge of sexuality in the Mucorales.

Sexual carotenogenesis occurs in both *Blakeslea* and *Phycomyces*, but mated cultures of *Blakeslea* contain more carotene than mated cultures of *Phycomyces* contain. The same sexual behavior need not occur in both organisms, and the organisms differ in morphology, in the regulation of carotenogenesis by light and chemicals (3), and in the production of trisporic acids (34).

Acetate is not a sexual hormone because in single-strain cultures it does not induce the appearance of zygophores, a conspicuous early stage of sexual development. Trisporic acids do induce zygophorogenesis in single-strain cultures of many Mucorales, and this is the basis for simple functional tests for the presence of these compounds in complex media (34).

The sexual and vegetative pathways for carotenogenesis are the same in both fungi and are blocked by the same car genes. Four genes that encode enzymes in the biosynthetic pathway, the early *hmgS* and *hmgR* genes (31) and the late *carRA* and *carB* genes (1, 30, 32, 35), occur only once in the *Phycomyces* genome. Acetate and the other chemicals evaluated in this study cannot block a special pathway, because no such pathway exists. Instead, they inhibit sexual activation of carotenogenesis and have little or no effect on the regulatory mechanisms that are known to operate during vegetative growth (e.g., feedback inhibition).

The threshold for the effect of acetate on sexual carotenogenesis was <1 mmol/liter, a concentration that provided less than 0.3% of the available carbon atoms and should not have caused major changes in metabolism. Under the conditions used in our experiments, acetate was not metabolized to a significant extent, because while glucose is present, acetyl-CoA synthetase is rare (16). Acetate also altered carotene production in mutants that cannot use acetate as a carbon source because they have mutations in the *facA* gene for acetyl-CoA synthetase (15, 16). Thus, acetate does not need be utilized as a carbon source to alter carotene metabolism, but instead it may act as a signal that prevents sexual carotenogenesis while increasing zygospore formation.

There are at least two signal transduction pathways for acetate in *Phycomyces*, based on observations of sexual carotenogenesis and the initiation of zygosporogenesis following exposure to acetate of ger mutants, which were isolated based on their inability to activate spores in response to acetate, propionate, and heat (27). Thus, the receptor thought to be mutated in the ger mutants is not used for the modification of sexual responses by acetate, although the two pathways could share later steps.

The two modifications of the sexual processes differ in their dependence on the acetate concentration, and the activation of zygosporogenesis is less sensitive to acetate than the inhibition of sexual carotenogenesis. Thus, the two effects are mediated by transduction pathways that are at least partially different.

All the compounds that we tested can be considered small carboxylic acids; l-leucine is converted to 2-keto-4-methylpentanoic acid when it enters the cells. The human genome contains a large family of G-protein-coupled receptors (18), two of which specifically bind carboxylic acids with one to six carbon atoms (7, 19, 25).

Glutamate minimal medium is better than the standard min-

<table>
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<tr>
<th>Medium</th>
<th>Carotene content (mg/g (dry mass))</th>
<th>Wild-type strains</th>
<th>facA mutants</th>
<th>ger mutants</th>
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<tr>
<td>Single strains</td>
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<tr>
<td>Minimal</td>
<td>0.17</td>
<td>0.19</td>
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<td>0.14</td>
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<tr>
<td>Minimal + trisporic acids</td>
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<td>0.22</td>
<td>0.32</td>
<td>0.26</td>
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<tr>
<td>Minimal + trisporic acids + sodium acetate</td>
<td>0.12</td>
<td>0.11</td>
<td>0.18</td>
<td>0.12</td>
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<tr>
<td>Mated with NRRL1554</td>
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<tr>
<td>Minimal</td>
<td>0.67</td>
<td>0.84</td>
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<td>0.47</td>
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<td>Minimal + sodium acetate</td>
<td>0.24</td>
<td>0.23</td>
<td>0.22</td>
<td>0.15</td>
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</table>

*The values are the mean total carotene contents (β-carotene and phytoene) for 2 to 15 determinations in two to four independent experiments. The average relative standard error of the means was 12%. The compounds added to the minimal medium were 10 mmol/liter sodium acetate and trisporic acids from mated *Blakeslea* cultures.
inal medium for zygospore production (33), which suggests that sexual development is favored by nitrogen starvation since the amount of nitrogen in glutamate minimal medium is <25% of the amount in the standard minimal medium. Potato dextrose medium also contains a low concentration of nitrogen and is a very good medium for zygospore production, but the low level of nitrogen is not the sole cause of this phenotype since addition of asparagine did not modify the production of zygospores (Table 1). We hypothesized that potatoes contain chemicals that could act like acetate in our experiments. This hypothesis was supported by the lack of synergy between acetate and potato-based media for zygospore production.

Our results have economic implications since fungal carotene production currently relies on sexual carotenogenesis. Media used in commercial carotenone production should contain little or no acetate, L-leucine, and lactate. Propionate also should be avoided due to its strong inhibition of Blakeslea growth. These chemicals might not be added intentionally to media but could result from the growth of bacterial contaminants in industrial fermentors.

The activation of dormant spores by small carboxylic acids can be easily explained for a saprophyte like Phycomyces, which grows too slowly to compete with bacteria but could feed on bacteria. Acetate and other small carboxylic acids are products of bacterial growth on carbohydrates and amino acids and could be suitable signals for breaking the dormancy of a predator of bacteria. Blakeslea is an opportunist on plant tissues, and its spores do not need to be activated to germinate. It is not obvious why either fungus has mechanisms to modify sexual processes in response to the same signals.

ACKNOWLEDGMENTS

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