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Separation of β-Factor Synthesis from Stimulated β-Carotene Synthesis in Mated Cultures of *Blakeslea trispora*

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Mated cultures of *Blakeslea trispora*, grown in a potato extract-glucose-thiamine medium, produced 10 to 15 times more β-carotene than either unmated culture. Mated, but not unmated, cultures produced a family of compounds (β factor) which stimulated carotenogenesis in unmated cultures. In fact, carotenogenesis was stimulated sixfold more in *minus* cultures than in *plus* cultures. By altering the relative amounts of *plus* and *minus* inocula used in fermentations of mated cultures, it was possible to separate the synthesis of β factor from the synthesis of extra β-carotene. The *plus* strain appeared to produce the β factor; the *minus* strain appeared to produce most of the extra β-carotene. Kinetic studies of β-factor formation suggested that physical contact between the two strains may be required to initiate β-factor synthesis.

Blakeslea trispora (7, 9), a phycomycete of the order *Mucorales*, is a heterothallic species (10) of *plus* and *minus* strains (sexes). Individual *plus* and *minus* strains can grow alone, produce asexual spores, and synthesize small amounts of β-carotene (6). Barnett et al. (2) observed that β-carotene production in the phycomycete *Choanephora cucurbitarum* was enhanced 15- to 20-fold by growing *plus* and *minus* cultures together. This observation was confirmed and extended to other members of the family *Choanephoraceae*, including *B. trispora* (1). In 1964, Prieto t al. (8) reported that mated, but not unmated cultures produced a family of acidic compounds which stimulated β-carotene synthesis in unmated cultures. These acidic compounds, termed β factor because they stimulated β-carotene synthesis, were found in the culture medium. O. K. Sebek and H. K. Jager (Bacteriol. Proc., p. 12, 1966) reported that acidic compounds from the medium containing mated cultures of *B. trispora* markedly increased β-carotene formation only in *minus* cultures. Our investigation of *B. trispora* confirmed these observations and revealed that β-factor synthesis and stimulated β-carotene synthesis can be separated in mated cultures. Our findings also suggested that physical contact between the two strains may be required to initiate β-factor synthesis.

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MATERIALS AND METHODS

Stock cultures of *plus* and *minus* strains of *B. trispora* (USDA, Northern Regional Research Laboratories, no. 9216 and 9159, respectively) were stored separately on PGA (0.4% potato extract, 2% glucose, 1.5% agar) slants containing 0.0002% thiamine-HCl. Cultures, kept at room temperature, were transferred every 2 weeks. All operations involving culture transfers were performed in a modified LabConco bacteriological hood (Laboratory Construction Co., Kansas City, Mo.) to prevent bacterial contamination.

Liquid cultures were grown in 500-ml DeLong flasks (with Morton closures) containing 100 ml of PGT (2% potato extract, 2% glucose, 0.0002% thiamine-HCl). Glucose (10 ml) and potato extract-thiamine (90 ml) solutions were combined (in the LabConCo hood) after autoclaving each separately for 25 min at 123 C. Lots 486270 and 510537 of Potato Extract (Difco) were used in these experiments; autoclaved PGT containing these potato extracts had absorbances at 325 mμ of 4.6 and 9.6, respectively.

Seed cultures for fermentations were prepared by inoculating separate flasks of PGT with chunks of agar containing either *plus* or *minus* mycelium and incubating these flasks for 2 days at 29 ± 2 C on a New Brunswick Gyrotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.) set at 250 rev/min.

Under these conditions, the mycelium formed one solid mass. Inoculum for the fermentation was prepared by aseptically transferring the drained, seed-culture mycelium to a 75-ml capacity Servall Omnimixer containing 40 ml of 0.9% saline, and grinding the mixture for 30 sec at 80% line-voltage. Equal volumes of inoculum were normally used in all flasks for a given set of fermentations. After a 6-day incubation period, at 29 ± 2 C on a Gyrotary shaker, the mycelium was collected on eight layers of gauze in a Büchner funnel on a suction flask. Excess medium was removed from the mycelium by holding a sheet of gum rubber over the funnel while applying suction. The mycelium was placed immediately in a vacuum oven at 30 C and was allowed to dry overnight. The filtered culture medium was stored at −14 C until analyzed.

Growth is expressed as milligrams (dry weight) of mycelium.

β-Carotene analyses were performed within 24 hr after collecting the mycelium. The weighed mycelium was ground in a Wiley Mill equipped with a 20 mesh screen. Preweighed portions (100 to 200 mg) of ground mycelium were placed in a filter paper holder within a butt tube. The β-carotene was extracted from this ground mycelium by gentle refluxing for 45 min with 60 to 70 ml of petroleum ether (Fischer Scientific Co., Pittsburgh, Pa.; boiling point range 37 to 60 C). Since all trans-β-carotene represents 95% of the colored carotenoids extracted from *B. trispora* (4), β-carotene concentration was calculated directly from the absorbance of the extract at 450 mμ, as compared with a standard curve (absorptivity = 277 per mg per ml). Concentration of β factor was routinely determined from the difference in absorbance (325 mμ) of medium from mated and unmated cultures. For measurements, the culture media were diluted 20-fold with 0.1 M tris (hydroxymethyl)aminomethane (Tris) sulfate buffer, *p*H 7.5. Chloroform extracts were prepared by adjusting 100 ml of culture medium to *p*H 2 with sulfuric acid, and shaking this mixture vigorously for 1 min with 100 ml of chloroform in a 300-ml separatory funnel. Tris buffer extracts were prepared by flash evaporating the chloroform extracts to dryness at 40 C and resuspending the residue in 10 ml of 0.1 M Tris-sulfuric acid, *p*H 7.5.

RESULTS

The extent of growth in *plus*, *minus*, or mated cultures of *B. trispora* during 6 days of fermentation in 100 ml of PGT was essentially the same, about 1,300 mg (dry weight) of mycelium from two flasks (Table 1). The amount of β-carotene synthesized by mated cultures was 12 times greater than the amount synthesized by either *plus* or *minus* cultures alone (Table 1). The yellow color of β-carotene appeared to be evenly distributed throughout the single, solid mycelial mass. The β-factor was found only in the medium of mated cultures, as determined by the ability of this medium to stimulate carotenogenesis in the *minus* strain (Table 1). This assay showed that medium from either unmated or mated cultures did not stimulate carotenogenesis in the *plus* strain. The Tris buffer extract of medium from mated cultures was serially diluted 512 times without loss of carotenogenic properties. Undiluted Tris buffer extracts of medium from unmated cultures exhibited no carotenogenic properties (Table 1).

TABLE 1. *Growth and β-carotene and β-factor production by mated and unmated cultures of blakeslea trispora*

Culture[a]	Growth[b]	β-Carotene/g of mycelium	β-Factor activity	
			CM[c]	TE[d]
	g	μg		
Plus	1.307	187	−	None
Minus	1.310	152	−	None
Plus and *Minus*	1.293	2,180	+	512

[a] Flasks containing 100 ml of PGT were inoculated with *plus* (5 ml), *minus* (5 ml), or *plus* (3 ml) and *minus* (2 ml) seed cultures. Flasks were incubated for 5.8 days.

[b] Growth: grams (dry weight) of mycelium from two flasks.

[c] The β-factor activity was determined qualitatively by spotting 0.1-ml portions of CM (culture medium) on filter-paper discs (13 mm in diameter). Discs were placed upon young *minus* mycelium, growing on 1.5% agar plates and containing Ciegler's medium (5), and then were incubated overnight. If mycelium surrounding disc was yellow, activity was present; if white, activity was absent.

[d] The β-factor activity was quantitated by assaying serially diluted portions of TE (Tris buffer extract) on *minus* mycelium. The greatest dilution to give a positive effect was recorded.

The absorbance spectra of culture media (A), chloroform extracts (B), and Tris buffer extracts (C) of *plus* (curve 1), *minus* (curve 2), and mated (curve 3) cultures are shown in Fig. 1. No new ultraviolet (UV)-absorbing material was present in any of the samples from unmated cultures (curves 1 and 2), when compared with the corresponding spectrum of uninoculated medium (curve 0). However, UV-absorbing material was present in fractions from mated cultures (curve 3), with the principal maximum at 325 mμ (328 mμ in the Tris buffer exrtact) and a second maximum, which was readily apparent in the Tris buffer extract, at 235 mμ. The ratio of absorbance of β factor at 328 mμ to absorbance at 235 mμ is 1.40. Under these experimental conditions, separate experiments revealed that 96 to 100% of β factor was extracted from the culture medium with chloroform. Since the total amount of net UV-absorbing material in the chloroform extract was essentially the same as the total amount of net UV-absorbing material present in the culture

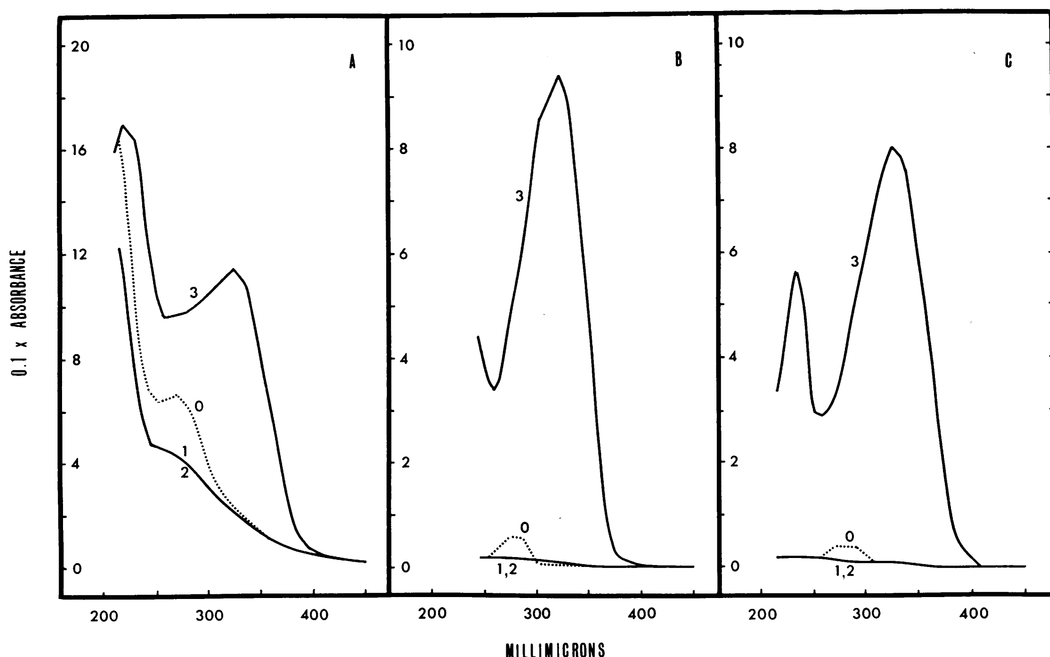

FIG. 1. *Absorbance spectra of culture media (A), chloroform extracts (B), and Tris buffer extracts (C) from plus (curve 1), minus (curve 2), mated (curve 3) cultures, and also uninoculated PGT (curve 0). For measurements, the culture media and chloroform extracts were diluted 20-fold with 0.1 M Tris-H_2SO_4 (pH 7.5) and chloroform, respectively. The Tris buffer extracts were diluted 200-fold with 0.1 M Tris-H_2SO_4 (pH 7.5) for measurements. The reference cell contained diluent employed for measurements.*

medium, the absorptivity of β factor in these two solvents must be very similar. Since the addition of PGT medium (0.05 volume) to the Tris buffer extract did not alter the absorbance of β factor more than 2% in this fraction, the recovery of only 85% of the UV-absorbing material from the chloroform extract in the Tris buffer extract represents either a loss of β factor or an alteration in its structure during preparation of the Tris buffer extract.

The β factor was completely extracted with chloroform from mated culture medium adjusted to *p*H 2. If the culture medium was adjusted to *p*H 7 and the β factor was extracted with chloroform, only half of the UV-absorbing material ($CHCl_3$ extract A) was removed from the culture medium. Further extraction of the culture medium (at *p*H 7) with chloroform removed very little additional UV-absorbing material. The remaining UV-absorbing material ($CHCl_3$ extract B) could be extracted with chloroform if the *p*H of the medium was lowered to 2. The UV absorbance spectra of Tris buffer extracts prepared from $CHCl_3$ extracts A and B revealed that both fractions have absorbance maxima at 235 mμ, that fractions A and B each have a second maximum at 307 and 337 mμ, respectively, and that the ratio of absorbance of the latter maxima to 235 mμ is 0.86 and 2.11 for fractions A and B, respectively. Equal amounts (325 mμ absorbance) of Tris buffer extracts A and B stimulated carotenogenesis in *minus* cultures (grown in PGT) at the rate of 137 and 207%, respectively, as compared with a rate of 210% for unfractionated β factor.

The response of unmated *plus* and *minus* cultures to a Tris buffer extract of β factor is shown in Fig. 2. Although β factor stimulated carotenogenesis in both sexes, carotenogenesis in *minus* cultures was stimulated sixfold more than in *plus* cultures. For example, in the presence of 21.8 units of β factor, there was a 422% increase in β-carotene production in *minus* cultures but only a 71% increase in *plus* cultures. The β factor apparently had no effect on growth of *plus* cultures but inhibited *minus* cultures.

Experiments in which the relative amounts of *plus* and *minus* inocula added to fermentation flask were varied are presented in Fig. 3. When 6 ml of *minus* inoculum and varying amounts of *plus* inoculum were used for fermentations (Experiment A), β factor synthesis was directly pro-

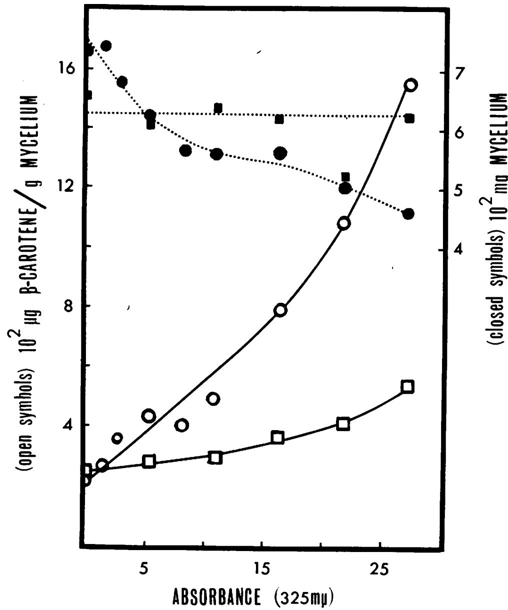

FIG. 2. *Stimulation by β-factor of carotenogenesis in unmated plus and minus cultures. Flasks containing 100 ml of autoclave-sterilized PGT and 10 ml of filter-sterilized 0.1* M *Tris-H_2SO_4 (pH 7.5), containing β factor as indicated, were inoculated with 4 ml of either plus (squares) or minus (circles) inoculum and were incubated for 6.3 days. The abscissa represents the net absorbance (325 mµ) of the culture medium owing to the presence of β factor at the time of inoculation.*

portional to the amount of *plus* inoculum used in the fermentation, and β-carotene synthesis was directly proportional to the amount of β factor produced, except at low concentrations of β factor. When 5 ml of *plus* inoculum and varying amounts of *minus* inoculum were used for fermentations (Experiment B), stimulated β-carotene synthesis was not proportional to β factor production. Synthesis of β factor reached its peak with only 1 ml of *minus* inoculum, whereas β-carotene production in the mycelium of this flask was only 30% of the β-carotene synthesis obtained with 3 ml of *minus* inoculum. On the basis of six experiments, we concluded that the highest yields of β-carotene were obtained with a 50 to 70% *plus* inoculum.

The effect of the time of combining *plus* and *minus* cultures on β factor and β-carotene production is shown in Fig. 4. Flasks containing 100 ml of PGT medium were inoculated with 4 ml of either *plus* or *minus* inoculum. The flasks were incubated at 29 ± 2 C on a Gyrotary shaker set at 250 rev/min. *Minus* cultures (and medium) were added to *plus* cultures at the indicated times and then incubated for an additional 5 days. Duplicate sets of unmated cultures were collected on preweighed membrane filters (Millipore Corp., Bedford, Mass.) and were dried and weighed to determine the amount of growth at the time of combining *plus* and *minus* cultures. These results indicated that there was no increase in mass

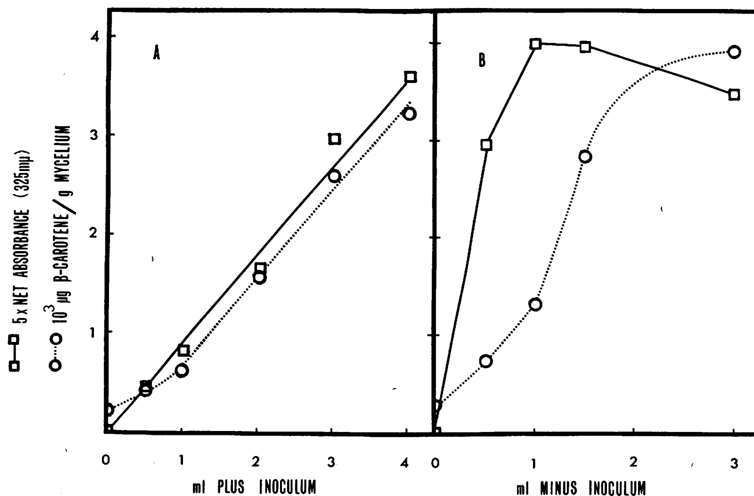

FIG. 3. *Effect of altering the ratio of plus and minus inocula in fermentations of mated cultures. In experiment A, each flask received 6 ml of minus inoculum and the indicated amounts of plus inoculum. In experiment B, each flask received 5 ml of plus inoculum and the indicated amounts of minus inoculum. In both experiments, flasks were incubated for 6 days.*

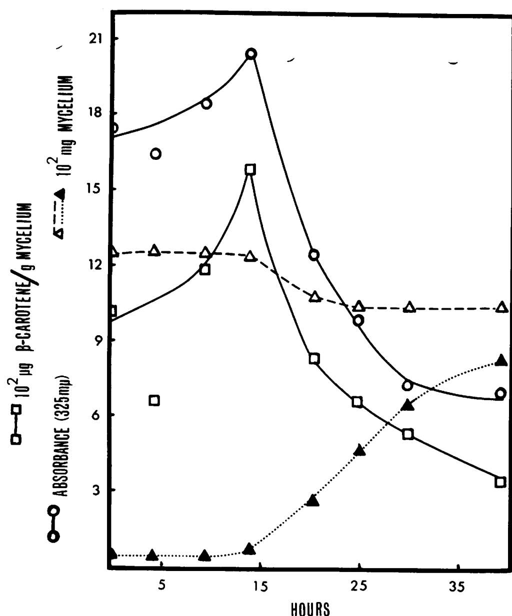

FIG. 4. *Effect of the time of combining plus and minus cultures on growth, and β-carotene and β-factor production. The abscissa represents the time after inoculation that cultures were combined. Cultures were then incubated for an additional 5 days before analysis. The dotted line represents the dry weight of mycelium of a duplicate set of cultures, collected at the time of combining plus and minus strains.*

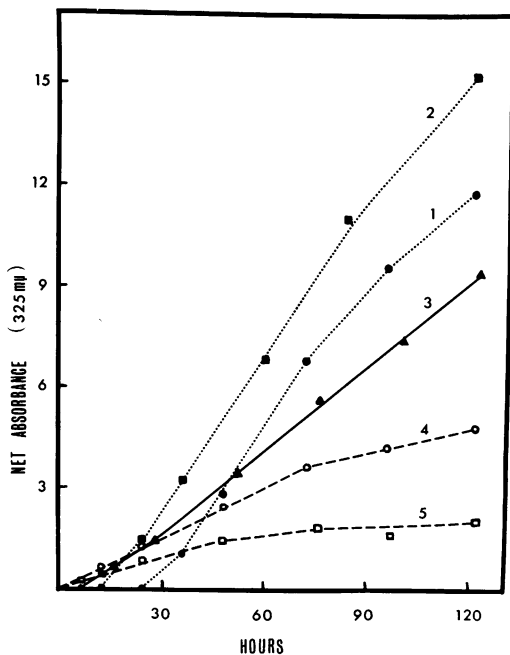

FIG. 5. *Kinetics of β-factor synthesis in cultures in which plus and minus strains were combined at the time of inoculation, 12, 20, 48, and 96 hours later (curves 1 to 5, respectively). Zero time on the abscissa represents the time plus and minus cultures were combined. At inoculation, flasks containing 100 ml of PGT received either 3 ml of minus or 6 ml of plus inoculum.*

during the first 14 hr of incubation. During this time the mycelium appeared as loose threads scattered throughout the medium. By the 20th hr, growth was underway and the mycelium of each unmated culture had already become one solid mass. Each flask containing cultures combined at 20 hr or later had two mycelial masses, one of which appeared a brighter yellow than the other after the 5-day period of incubation. (A three- to fourfold difference in β-carotene content of individual mycelial masses was detectable when *plus* and *minus* cultures were combined at 20 hr and were incubated for an additional 5 days.) The color in the individual masses appeared to be evenly distributed throughout the mycelium. Flasks containing cultures combined at 14 hr or earlier contained loose threads and little balls of mycelium in which the yellow color appeared to be localized in spots. When *plus* and *minus* cultures were combined at 20 hr or later, there was a progressive decrease in the total amount of β-carotene and β factor synthesized as compared with cultures combined at 14 hr or earlier.

Figure 5 depicts the kinetics of β-factor synthesis as a function of the time of combining *plus* and *minus* cultures. The initial rate of β factor formation, after a 26- to 30-hr lag from the time of inoculation, was the same in cultures combined either at zero time (curve 1) or at 12 hr (curve 2). However, the initial rate of β-factor formation in cultures combined at 20 (curve 3), 48 (curve 4), and 96 (curve 5) hr was 52, 33, and 18%, respectively, of the rate for cultures combined at zero-time. The appearance of the mycelium in cultures combined at various times was the same as that described for the cultures in the preceding experiment.

Figure 6 show the kinetics of β-factor formation after combining 32-hr-old unmated *plus* and *minus* cultures, which were left intact (curve 1), or dispersed for 10 sec (curve 2) or 30 sec (curve 3). The unmated cultures were dispersed in 40 ml of their respective culture medium in a 75-ml capacity Servall Omnimixer at 70% line-voltage. An additional 10 ml of 20% glucose was added to each flask of combined *plus* and *minus* cultures. The rate of β factor synthesis in the dispersed cultures was the same after different initial lag periods and was 2.2-fold greater than that of the intact cultures. In other words, the rate of β factor

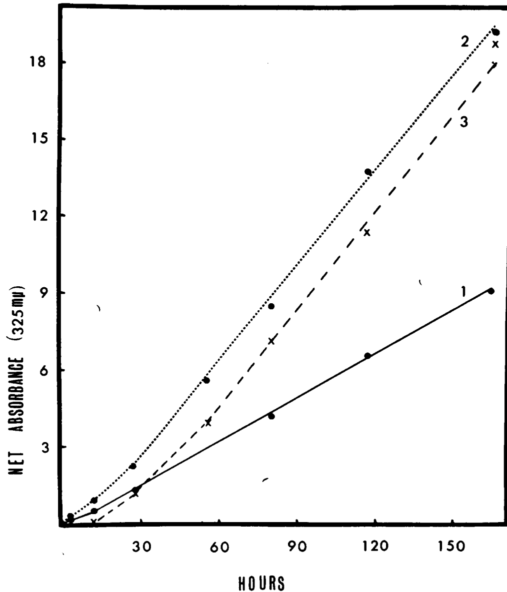

FIG. 6. *Kinetics of β-factor formation after combining 32 hr, unmated plus and minus cultures which were intact (curve 1), dispersed for 10 sec (curve 2) or 30 sec (curve 3). At inoculation, flasks containing 100 ml of PGT received either 3 ml of minus or 5 ml of plus inoculum.*

synthesis in intact cultures was 45% of the rate of β-factor synthesis in the dispersed cultures.

DISCUSSION

This investigation verified that mated, but not unmated, cultures of *B. trispora* produce a family of two or more compounds which accumulate in the surrounding medium. These compounds (β factor) stimulated carotenogenesis in unmated cultures, sixfold more in *minus* than in *plus* unmated cultures (Fig. 2). However, stimulation of carotenogenesis per unit of β factor in unmated *minus* cultures was only 20 to 30% of the carotenogenesis in mated cultures producing similar amounts of β-factor. These observations suggest that physical contact between the two strains may be required for effective transport of β-factor through the mycelial membrane. It is also possible that other factors may be involved in stimulating carotenogenesis, or that the structure of β-factor may have been altered during isolation, thereby lessening its effectiveness in stimulating carotenogenesis.

Caglioti et al. (3) reported esterifying β factor from the culture medium of *B. trispora* and resolving it into three compounds by silicic acid chromatography. The three components (A, B, C) represented 2, 10, and 80%, respectively, of the methyl ester of β factor added to the column. Physical and chemical analyses of fraction C indicated that it contained 19 carbon atoms, a hydroxyl group, a methyl-esterified carboxyl group, and a keto group conjugated with three ethylene bonds. Sebek and Jagar (Bacteriol. Proc., p. 12, 1966) recently reported resolving esterified β factor into four components.

By varying the relative amounts of *plus* and *minus* inocula used in fermentations of mated cultures it was possible to separate β-factor synthesis from enhanced β-carotene synthesis. Production of β factor was directly proportional to the amount of *plus* inoculum used in fermentations of mated cultures, in which excess amounts of *minus* inoculum were employed (Fig. 3A). Under these conditions β-carotene synthesis was directly proportional to the amount of β factor produced (except at low concentrations of β factor). However, when excess amounts of *plus* inoculum were used, β-factor production was maximal with 1 ml of *minus* inoculum, whereas β-carotene synthesis was only 30% of that obtained with 3 ml of *minus* inoculum (Fig. 3B). These observations suggest that the *plus* strain produces β factor on stimulation by the *minus* strain and that the *minus* strain produces most of the extra β-carotene formed in mated cultures. This latter conclusion is supported by our observations that (i) β factor-stimulated carotenogenesis in unmated *minus* cultures was sixfold greater than in unmated *plus* cultures (Fig. 2), and (ii) one mycelial mass contained three to four times more β-carotene than the other when *plus* and *minus* cultures were combined at 20 hr and incubated for an additional 5 days. Furthermore, microscopic examination of progamete formation on agar plates (containing 0.4% potato extract, 2% glucose, and 0.0002% thiamine-HCl) revealed that, just prior to zygospore formation, one progamete per pair appeared to be a brighter yellow than the other.

A study of the effect of the time of combining *plus* and *minus* cultures on β-factor and β-carotene syntheses revealed that cultures combined at 20 hr or later produced less of both compounds than cultures combined at 14 hr or earlier (Fig. 4). There was no growth during the first 14 hr of incubation. Cultures combined during this period subsequently grew as loose threads and balls. However, once growth began in unmated cultures, a single, solid mycelial mass formed, and this form was retained even when combined with the opposite strain. Thus, there was less physical contact between *plus* and *minus* strains when unmated cultures were combined after growth began rather than when they were combined before growth be-

gan. A study of the kinetics of β-factor production, as a function of time of combining *plus* and *minus* cultures, revealed that the rate of synthesis decreased inversely when compared to the extent of growth attained before unmated cultures were combined (Fig. 5). These observations suggested that either physical contact between *plus* and *minus* strains was essential for β-factor synthesis or that the age of the cultures themselves was critical for initiation of maximal β-factor synthesis.

In an attempt to resolve these possibilities, 32-hr-old unmated *plus* and *minus* cultures were either dispersed into loose threads before combining or were combined intact (Fig. 6). The intact cultures produced β factor at 45% of the rate at which dispersed cultures produced β factor. Unmated cultures, which were intact when combined at 20 and 48 hr, produced β factor at rates of 52 and 33%, respectively, of the cultures combined at zero time (Fig. 5). The 2.2-fold increase in the rate of β-factor production by the dispersed cultures over the intact cultures at 32 hr is essentially the same as the rate in cultures combined at zero time. These findings suggest that more surface area allows more physical contact and thus more β-factor production, and that physical contact between *plus* and *minus* strains may be necessary to initiate β-factor synthesis in mated cultures. The latter conclusion is supported indirectly by the fact that culture medium from either unmated culture did not stimulate β-factor synthesis in the opposite strain. Attempts to check this conclusion directly, by growing *plus* and *minus* strains on opposite sides of either a 1-μ (pore size) sintered-glass membrane or a 0.45-μ Millipore filter, were unsuccessful because the mold grew through the membrane or filter.

In summary, we conclude that physical contact between *plus* and *minus* strains was required to initiate β-factor synthesis in mated cultures. In addition, we found that the *plus* strain synthesized β factor when stimulated by the *minus* strain, and the *minus* strain synthesized most of the extra β-carotene produced by mated cultures.

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