Initiation of Dermatophyte Pleomorphic Strain Sporulation by Increased Aeration

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A normally asporogenous pleomorphic strain of Microsporum gypseum was induced to sporulate by controlled aeration and dehydration. Aeration of the pleomorphic strain under optimal cultivation conditions caused the initiation of a sporulation cycle with equivalent growth parameters and percentage intracellular water loss as the wild-type strain. Initiation of sporulation was not due to alteration of the medium's nutrient concentration or consistency, concentration of fungal growth by-products, or removal of volatile "staling factors." Macroconidia formed by the pleomorphic colonies were of characteristic wild-type morphology, but germinated to form typical pleomorphic colonies, indicating that the induced sporulation was strictly phenotypic and reversible. Other asporogenous pleomorphic strains from different dermatophyte genera also were induced to form macroconidia by aeration, suggesting a similarity in sporulation induction in Microsporum sp., Epidermophyton floccosum, and Trichophyton violaceum. Initiation of sporulation by aeration further suggested that the pleomorphic mutation was one which affected the sensitivity of the pleomorphic aerial hyphae to natural sporulation inducers (i.e., decreased humidity) and did not represent a loss in the ability to form fertile macroconidia.

Pleomorphism in dermatophytes presents a problem in both culture collection maintenance and the identification of these organisms. This spontaneous mutation occurs at a high frequency in many dermatophyte species when clinical specimens are grown on artificial culture media (2), and the concomitant loss of wild-type pigmentation and spore-forming ability makes generic and species identification impossible.

Stimulation of sporulation of microconidiate pleomorphic strains by use of increased carbon dioxide tensions has been reported previously (1, 5), but induction of sporulation in asporogenous pleomorphic strains has not been successful (6). Described here is a new method for sporulation induction in pleomorphic strains, utilizing controlled aeration and dehydration. A possible explanation for the observed early induction of sporulation by aeration is proposed and the results of this investigation suggest that further elucidation of this effect should prove to be important in the study of fungal differentiation and in the identification of these organisms in clinical laboratories.

MATERIALS AND METHODS

Organisms. A pleomorphic strain of Microsporum gypseum (R87P1), derived as a spontaneous mutant of the wild-type strain (R87) previously described (13), was used in the majority of studies of growth and sporulation induction. Other pleomorphic strains used in this study also were isolated as spontaneously occurring pleomorphic patches within wild-type colonies which had originally been isolated from clinical specimens. All pleomorphic strains were free of microconidia and macroconidia during the duration of their respective wild-type strain sporulation cycles.

Culture medium and aeration conditions. All cultures, unless otherwise specified, were cultivated on a medium containing: neopeptone (Difco), 1% (w/v); glucose, 1% (w/v); agar, 1.8% (w/v); and distilled water (pH 6.5). The medium was routinely sterilized by autoclaving at 121°C for 15 min except for the more concentrated types of media which were sterilized by Millipore filtration. Culture fluid by-products were collected from 7-day-old liquid-grown cultures by filtration, concentrated by flash evaporation (27°C), and sterilized by Millipore filtration before addition to the culture medium as 0.5, 1.0, 5.0, and 7.5% supplements.

The mycelial inoculum used was pregrown in
liquid medium for 5 days at 27 C using a Burrell wrist action shaker (Burrell Corp., Pittsburgh, Penn.). The mycelia then was collected aseptically by filtration and washed with 250 ml of sterile physiological saline (pH 6.6). A mycelial inoculum which could be dispensed by pipette was prepared by shearing the washed hyphae in a VirTis homogenizer (Model 23, VirTis Co. Inc., Gardiner, N.Y.) for 30 sec at approximately 16,000 rev/min. All cultures were inoculated on agar media by using one drop of sheared mycelial suspension dispensed centrally onto the surface of the medium.

Cultures for nutritional shift-down were grown in liquid medium as previously described (13). After growth for specific periods of time, the submerged mycelia were harvested aseptically by filtration and washed with 250 ml of sterile physiological saline, and the resultant mat was removed from the filter and placed in a petri plate containing 1.8% (w/v) agar (Difco) without additional nutrients.

Aerated cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml of agar medium. Glass tubing was arranged as shown in Fig. 1A with the air inlet tube 1.5 cm above the growing colony. Cultures for dry weight and percentage water determinations were grown on the surface of 8.7-cm cellophane discs placed on deep petri plates containing 70 ml of medium. Aeration of the petri plate cultures was conducted in the apparatus shown in Fig. 1B, employing the glass tubing and stopper assembly depicted in Fig. 1A, and enclosing the petri plate culture in a 16.51 by 20.96 cm plastic bag ("Baggie" brand, Colgate-Palmolive Ltd., Toronto, Canada). This method proved to be superior to the flask method, both in ease of sampling and for direct microscope observations. Aeration was supplied by a small aquarium pump (Tomofuji Co., Japan), and the air was sterilized by passage through a 17 by 2 cm tube containing sterile cotton wool. Dry or humid air was generated by directing the airstream through a 17 by 2 cm tube containing sterile calcium chloride, or a 250-ml Erlenmeyer flask half-filled with sterile distilled water. Air flow was monitored by screw clamps and monitored with an RGI air-flow meter (Roger Gilmont Instruments, Inc., Great Neck, N.Y.). Incubation of fungal cultures under increased carbon dioxide tensions has been described previously (6).

Growth measurement and calculations. Growth was measured as dry weight, after desiccation in vacuo over CaCl₂ at 27 C, or as amount of hyphal extension (radius [mm] per day). Dry weight of mycelia from solid culture media was estimated by using colonies grown on tared cellophane discs. The weight of intracellular water was estimated after the method of Ito and Fuji (8), as the difference in fungus weight after drying for 3 hr at 37 C and then after drying for 20 hr at 110 C. The percent intracellular water was calculated as weight of intracellular water divided by dry weight (37 C, 3 hr) times 100%.

Estimation of spore induction and maturation. Spore formation studies followed the stages of development previously described for the wild-type strain by El-Ani (6). Spore induction was designated as the time at which thin-walled, bulbous, aseptate macroconidia first appeared; spore maturation was reported when the macroconidia became thick-walled, septate, and free from the vegetative hyphae (Fig. 2). Isolation of mature macroconidia and germination procedures have been described previously (10). Photographs of lactophenol cotton blue-stained specimens were taken with a Zeiss microscope (green filter).

RESULTS

Optimum physical conditions for pleomorphic strain sporulation. The pleomorphic strain (R87P1) of M. gypseum was characterized by absence of reverse pigmentation, fluffy white mycelia, and reduced ability to sporulate, with only very few macroconidia initiated after 12 days of growth. The wild-type strain (R87) was tan in color, granular, and produced abundant macroconidia after 4 days of growth (Fig. 3). Comparison of growth curves shows that strain R87 initiated sporulation prior to rapid growth, whereas strain R87P1 initiated very sparse sporulation in the stationary phase of growth. Despite the wide differences in sporulation times, there was an identical percentage of intracellular water loss at the time of mature spore appearance in both strains (Fig. 3).

To determine the effect of aeration and drying on sporulation, the apparatus in Fig. 1 was devised. Colonies of the pleomorphic strain were pregrown for 1 to 7 days (in 250-ml flasks) and aerated at 50 cm³/min to determine if the colony could be stimulated to sporulate earlier than normal. As shown in Fig. 4,
strain R87P1 was induced to form spores by 8 days total growth, with optimal sporulation induction occurring when the colony was pregrown for 4 days. Aeration prior to this time or after it increased the duration of the sporulation period. When colonies pregrown for 4 days were exposed to different airflow rates, optimum sporulation was obtained with air flow rates of 25 to 40 cm³/min (Fig. 5). Air flow rates of 75 and 100 cm³/min gave slower spore induction but more rapid spore maturation. Aeration alone did not seem to be the cause of early sporulation induction, as humid aeration at 40 cm³/min did not stimulate sporulation as well as aeration at 40 cm³/min without a water trap. Similarly, rapid dehydration (dry air at 40 cm³/min) did not stimulate induction of spores as well as 40 cm³/min of air flow rate under normal conditions, but it did increase rate of spore maturation. These results suggested that both controlled aeration and controlled dehydration were important for early spore induction and maturation.

To determine if early spore induction was dependent on continuous aeration, colonies of strain R87P1, pregrown for 4 days, were separately exposed for periods of 1 to 4 days to aeration at 40 cm³/min. Although there were more spores initiated when the aeration period was long, the pleomorphic strain completed sporulation by 8 days total growth, in all cases, indicating that continuous aeration was not necessary for the completion of the sporulation cycle.

To investigate the effect of aeration on strain R87P1, dry weight, and percentage of bound water loss, it was necessary to treat celophane-grown colonies in the apparatus reported in Fig. 1B. To prevent too rapid dehydration of the colony and the medium, air flow was reduced to 10 cm³/min, resulting in delayed sporulation initiation (8 days) and spore

Fig. 2. Microscopic appearance and diagrammatic representation of developing strain R87P1 macroconidia. Spore development proceeds clockwise from the uninduced pleomorphic hyphae. Spore initiation first was marked by hyphal tip swelling and formation of a basal septum. Further septation, dividing the swollen hyphal tip into three compartments, and cell wall thickening marked the formation of immature spores. Mature spores were divided into at least four compartments and separated from the vegetative hyphae.
maturation (10 days) (Fig. 5 and 6). The aeration-induced lag phase of 4 days prior to spore initiation, rapid growth, spore maturation, and 25% bound water loss (Fig. 6) paralleled the spore initiation cycle of strain R87 (Fig. 3). This similarity suggested that spore production in both strains was equivalent, but the initiation step was lacking in strain R87P1.

**Optimum cultural conditions for sporulation.** As one of the most obvious visible effects of additional aeration was the dehydration of the culture medium, it was important to ascertain the optimum cultural conditions for sporulation of both strains, and to determine whether the concentration of the culture medium was responsible for the observed pleomorphic strain sporulation induction. As shown in Fig. 7, concentration of the medium nutrients permitted mycelial growth and repressed sporulation in both strains. Wild-type *M. gypseum* sporulated best at low concentrations of glucose-neopeptone. The pleomorphic

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**Fig. 3.** Comparison of strains R87 and R87P1 dry weight (○), at 37 C, 3 hr, and percentage intracellular water (O), on cellophane-grown cultures, 27 C. Spore types diagrammed correspond to stages of spore development in Fig. 2. Percentage intracellular water loss was calculated as maximum percentage of intracellular water minus percentage of intracellular water.

**Fig. 4.** Early induction of strain R87P1 sporulation by aeration of pregrown colonies. The dark bar within the vertical shaded bar represents immature spores; the top of the shaded bar represents mature macroconidia. Total growth time was calculated as the sum of days pregrown plus sporulation time. Air flow rate: 50 cm³/min, 27 C.

**Fig. 5.** Variable sporulation response of 4-day pregrown strain R87P1 to different air-flow rates. The dark bar within the vertical shaded bar represents spore initiation; the top of the shaded bar represents mature macroconidia. Humid or dry air aeration was treated as described in Materials and Methods. The control flask did not receive additional aeration. Total growth time was calculated as 4 days pregrown plus sporulation time.
strain displayed a narrower optimal nutrient concentration range for sporulation than strain R87, exhibiting a definite requirement for exogenous nutrients and a period of vegetative growth prior to sporulation. Both strains displayed optimum sporulation at 1% glucose-neopeptone concentration in the medium.

As shown in Fig. 8, when liquid-grown strain R87 was shifted down from complete medium to agar medium devoid of nutrients, it was capable of sporulation throughout its life cycle, exhibiting maximal sporulation ability prior to the rapid growth phase. The mycelia removed from the submerged culture were completely free of conidia prior to shift-down, indicating that spore initiation and maturation could be completed in the absence of exogenous nutrients. Strain R87 macroconidia, when placed on the shift-down medium, germinated, formed limited submerged hyphae, formed several aerial hyphae which initiated secondary macroconidial formation after 1 day, and completed sporulation after 3 days. The secondary conidia thus formed also were capable of germination and sporulated after 6 days on the agar surface, but the tertiary macroconidia formed in this cycle were only capable of germination and limited vegetative growth on minimal medium. These results suggested that the mature macroconidium contained sufficient endogenous reserves to complete germination, outgrowth and sporulation, and that extensive vegetative growth prior to sporulation was not a prerequisite in the wild strain. The pleomorphic strain, however, was only capable of limited sporulation over a narrow range in the stationary phase of growth, and after a definite period of vegetative growth during which exogenous nutrients were required (Fig. 8). These results paralleled the sporulation cycles for strains R87 and R87P1 reported in Fig. 2, and further suggested that the early sporulation of aerated strain R87P1 prior to the rapid growth phase (Fig. 6) was a result of true initiation of processes which were not normally expressed in that growth phase.

Growth of either strain on 1% glucose-neopeptone medium solidified with 1.8 to 10% agar (w/v) did not enhance growth or sporulation initiation, and restriction of nutrient diffusion or fungal exoenzyme diffusion by growing the colony on cellophane or dialysis membranes delayed sporulation by 2 days in

Fig. 6. Comparison of the dry weight and appearance of macroconidia in aerated (○) and nonaerated (●) strain R87P1 colonies. The arrow indicates the beginning of aeration. Spore types diagrammed correspond to the stages of spore development in Fig. 2. Cultures were grown on cellophane discs and aerated at 10 cm/min, 27° C.

Fig. 7. Optimum nutrient (glucose plus neopeptone) concentration for strain R87 and strain R87P1 sporulation. Spore development was determined at 3 days (●), 4 days (○), 10 days (■), 12 days (△), 14 days (▲), and 16 days (▽). The dotted line represents percentage of sporulation of strain R87 after 10 days and strain R87P1 after 16 days, where percentage of sporulation was calculated as colony surface area sporulating divided by total colony surface area times 100%. The spore types diagrammed correspond to the stages of spore development in Fig. 2.
both strains. Thus, physical conditions (i.e., aeration) which would result in an increased gel concentration in the culture medium and caused decreased nutrient or exoenzyme diffusion would appear to delay sporulation.

When either strain was grown on media containing various amounts of concentrated culture fluid by-products, neither growth nor sporulation was stimulated. Strain R87P1 was not stimulated or inhibited in sporulation abilities by either its own culture fluid by-products or the by-products of strain R87. Another approach to this consideration was to grow strain R87P1 on cellophane discs (which were perforated to allow hyphal penetration of the medium). Aeration of the colony was carried out to induce sporulation and then the sporulating aerial hyphae were stripped off, so that only the submerged hyphae remained. When the new emergent aerial hyphae were examined for spores, no spores were seen after 10 days despite the fact that the previous aerial hyphae had been sporulating and the culture was now a total of 20 days old. These results suggested that an inducer of sporulation was not present in the wild-type strain medium or concentrated in the pleomorphic strain medium by aeration.

In addition to medium dehydration, another effect of aeration could be the removal of volatile substances, CO₂, and other gaseous "staling factors" possibly causing the early initiation of sporulation. When a flask (from Fig. 1A) of strain R87P1 was connected to a flask of strain R87 so that the volatile materials from strain R87P1 would pass over the strain R87 colony, no delay in wild strain sporulation was observed. Also, the accumulation of possible sporulation inhibitory volatile materials produced by pleomorphic cultures is disproved by the observed sporulation of strain R87P1 cultures at 12 to 14 days (Fig. 3), as this would be the time when any volatile substances would be most concentrated.

When strain R87 was grown in the presence of added concentrations of 5, 10, and 15% CO₂, growth and sporulation were completely inhibited. The inhibition was reversed by removing the cultures from the CO₂ atmosphere. Growth of the pleomorphic strain was not affected by the increased CO₂ tensions, but sporulation did not occur in the presence of 5 to 15% CO₂ additions.

Nature of the pleomorphic strain reversion. The pleomorphic spores formed by aerated colonies were of characteristic wild-type morphology (Fig. 2). Unlike strain R87 conidia, strain R87P1 conidia would not germinate in physiological saline (37 C), but did form discrete germ tubes in nutrient media (37 C). The conidia were fertile and formed typical pleomorphic strain colonies which sporulated very sparsely in 12 to 14 days without additional aeration as did the parent pleomorphic colony. No revertants to strain R87 morphology, pigmentaton, or sporulation characteristics were observed.

To determine if the initiation of strain R87P1 sporulation by aeration was unique to this particular pleomorphic strain, or was a characteristic shared by pleomorphic strains of other genera and species, numerous independently isolated pleomorphic strains were aerated as described for strain R87P1. The results of this screening suggest that the M. gypseum pleomorphic strain used here was not unique in its response to aeration, and that sporulation in different dermatophyte genera and species may be initiated in a similar manner (Table 1). The morphology and manner in which the induced pleomorphic conidia arose from the vegetative hyphae were characteristic of the respective wild-type strain. The induced macroconidia were fertile and germinated to form pleomorphic colonies which were indistinguishable, one from the other.
Pleomorphic species response to aeration

| Pleomorphic strain                  | No. of strains tested | Appearance of mature macroconidia |  
|-------------------------------------|-----------------------|-----------------------------------|
|                                     |                       | Aerated culture                   |
|                                     |                       | Days aeration | Total days growth | Control culture | Total days growth |
| Microsporum gypseum (R87P1)         | 1                     | 4 | 8 | 14 |
| Microsporum gypseum (R87P12)       | 1                     | 4 | 8 | 14 |
| Microsporum gypseum (R87P8)        | 1                     | 5 | 9 | No spores* |
| Microsporum gypseum (R87P14)       | 1                     | 5 | 9 | No spores* |
| Microsporum cookei                 | 1                     | 5 | 10 | 16 |
| Microsporum canis                 | 1                     | 7 | 13 | 19 |
| Microsporum fulvum                 | 1                     | 4 | 10 | 20d |
| Epidermophyton floccosum           | 3                     | 7 | 13 | 16 |
| Trichophyton violaceum            | 1                     | 2* | 15 | 20d |

* No aeration.
* Air flow rate at 40 cm³/min, 27 C.
* After 20 days growth.
* Microconidia induced, no macroconidia.

**DISCUSSION**

Pleomorphic species have been previously described as a degeneration, due to the apparent loss of wild-type functions and characteristics (15). Induction of sporulation in pleomorphic strains by aeration, however, suggests that pleomorphism is not a complete sporulation degeneration, as only the ability to initiate wild-type functions within the normal time sequence has been lost, not the real ability to perform these sporulation functions.

Alteration in nutrient agar or concentration of growth by-products do not appear to be responsible for early spore induction in the pleomorphic strain, nor does the removal of volatile "staling factors" appear to stimulate sporulation. Although carbon dioxide has been found to stimulate sporulation in microconidiate Trichophyton mentagrophytes, Trichophyton rubrum, and Trichophyton meginiti cultures (1, 5), this gas was found to inhibit sporulation in both strain R87P1 and R87 of *M. gypseum*. Carbon dioxide, similarly, has been found to inhibit the growth and sporulation of an asporogenous *T. mentagrophytes* pleomorphic strain (5) and to inhibit fruit-body formation in basidiomycetes (12, 14).

The most striking similarity concomitant with the appearance of mature spores in both strain R87 and R87P1 is the percent of intracellular water loss. Water loss has also been reported to be associated with spore induction and maturation in slime molds, agarics, and in polypores (13, 14). The primary effect of aeration, however, may be an alteration in cell surface properties, with intracellular water loss being a secondary result.

Spore formation in *M. gypseum* occurs only on aerial hyphae, and it has been suggested by Morton (11) that the emergence of submerged hyphae into aerial conditions triggers a cell surface change, due to the creation of an air to water interface, which in turn acts as a sporulation stimulus. It is possible that the pleomorphic hyphae are unable to undergo normal wild-type strain cell surface change on emergence into aerial conditions, and hence do not initiate sporulation. This lack of response to aerial conditions is perhaps reflected by the intracellular water loss results reported, which show that the aerial hyphae of strain R87P1 required exposure to aerial conditions for twice as long as the wild-type strain to lose the same percentage of intracellular water. Additional aeration of the pleomorphic strain resulted in more rapid water loss, probably through alteration of the air to water equilibrium at the cell surface. When sporulating strain R87P1 hyphae were removed from the agar surface or when pleomorphic spores were germinated, the new emergent aerial hyphae were typically asporogenous and pleomorphic, indicating that the effects of aeration are not transmitted to the submerged vegetative hyphae and that the aeration effect was strictly phenotypic and reversible.

Recently it has been shown that hyphal branching and elongation are characteristic of a given environment, and that growth activity is expressed at the hyphal tip (7, 9). Cessation of apical tip elongation and branching, without restricting nuclear division, cell enlargement, and septation, could describe the processes giving rise to macroconidia in *M. gypseum* and
other dermatophytes. Tatum et al. have shown that pleiotropic effects leading to altered gross morphology are genetically determined in Neurospora, and may represent alteration of single enzymes regulating cell wall and membrane (lipid) synthesis (4, 16). Thus, the pleomorphic strain could arise from a similar pleiotropic mutation, resulting in the inability to balance vegetative hypha elongation and spore development, possibly due to alteration of enzyme pathways involved in cell wall or membrane synthesis. The primary locus of this mutation could also be a defect in a single regulatory enzyme, as genetic studies of M. gypseum have suggested that the pleomorphic mutation is the result of a single gene alteration (17).

The results of this study suggest that the pleomorphic mutant may be a valuable tool in the elucidation of the intracellular events controlling and coordinating hyphal elongation and sporulation. The results also suggest that the mutation giving rise to pleomorphism may be similar in different dermatophyte genera and suggests a relationship between the initiation of Microsporum sp. and Epidermophyton floccosum macroconidia formation and that of Trichophyton violaceum microconidiation. Aeration of asporogenous pleomorphic clinical dermatophyte specimens to assist in mycologic diagnosis may also prove to be a valuable technique.

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