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### LABORATORY IDENTIFICATION OF DERMATOPHYTES USING PROTOPLAST HYBRIDIZATION

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#### ABSTRACT

In this study techniques for laboratory identification of dermatophyte fungi through protoplast hybridization were established. Firstly, auxotrophic mutants of different species of microsporum and trichophyton were induced and identified. Secondly, protoplasts from these mutants were isolated by digestion of their mycelium with Novozyme 234 using  $\text{CaCl}_2$  (0.4M) as an osmotic stabilizer and glycine + HCl (pH 4.5) as the buffer system. Thirdly, isolated protoplasts from different species were fused using a solution containing 35% polyethylene glycol, 1 M KCl, 0.05 M  $\text{CaCl}_2$  and 0.05 M glycine with pH 6.1. Afterwards, the fusion products were plated onto minimal and complete media. It was found that protoplasts from different auxotrophic mutants from the same species hybridized and complemented each other and grew on both minimal and complete media, whereas mutants from different species did not have the ability to complement each other and therefore grew only on complete media. Information obtained in this study may prove useful for definite identification of suspected species of dermatophytes other than morphological criteria in laboratories.

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#### INTRODUCTION

Recently isolated protoplasts have served as useful experimental tools to study different aspects of medically important fungi. Protoplasts have been used to study the structure and biochemical composition of cell walls,<sup>1</sup> regeneration to vegetative mycelium,<sup>2,3</sup> fusion,<sup>4,5</sup> antibiotic action and metabolite production,<sup>6,7</sup> transfer,<sup>9</sup> and hybridization and transformation.<sup>10,12</sup> Protoplasts have also been used to obtain high yields of pure DNA.<sup>13</sup>

Despite the medical importance of dermatophytes, little is known about their molecular biology. Studies on the protoplasts of dermatophytes are rare when compared to those on the protoplasts of other medically important fungi.<sup>3,8,13</sup> Isolated protoplasts of dermatophytes could be used to provide a frame of references for future studies with these pathogenic fungi to elucidate better ways of controlling dermatophytoses, antibiotic sensitivity and resistance, the immunological responses (allergies) often associated with ringworms, details of cell wall synthesis, better understanding of pleomorphic phenomena, genetic mechanisms and

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taxonomical relationships of dermatophytes. Understanding of metabolic features of protoplasts will provide insight into the design of specific drugs to control the proliferation of these fungi in pathological situations. Also, revealing the enzymatic degradation of fungal cell walls will facilitate more detailed immunological and pathological investigation, and finally the definite identification of dermatophytes is made possible by protoplast hybridization.

In the clinical laboratory, identification of different species of dermatophytes is most often based on morphological study of the vegetative form, and sometimes the morphology of two species are so similar that their identification is difficult and indistinguishable. Therefore in this communication, we tried to establish protoplast hybridization as a useful technique to overcome these difficulties and identify the dermatophytes more easily through their genetic relatedness.

### MATERIALS AND METHODS

#### Organisms and growth conditions

*Microsporium gypseum*, *M. cookei*, *Trichophyton mentagrophytes* and *T. rubrum* were isolated from patients and cultured at 25°C on modified Sabouraud's agar slants containing 2% dextrose, 1% bactopectone, 1.5% agar, and 0.005% chloramphenicol. Clonal cultures of the fungi were obtained by the micropipette method.<sup>14</sup> Macroconidia in an aqueous suspension were picked up with a micropipette, transferred through several drops of water, and then inoculated into test tubes of nutrient media, one spore per test tube.

#### Induction of mutants

Mutagenesis in fungi was caused either by exposing the cultural suspension (resulting from clonal culture) to UV

radiation or by using N-methyl-N'-nitro-N-nitrosoguanidine (NTG).

Optimum conditions and details of methods of mutant induction have been established in our previous study.<sup>4</sup> A total of 250 putative mutants were screened by that method; among them four definite auxotrophs, including leucine, tyrosine, phenylalanine, and glutamic acid-requiring isolates of each species were identified. The mutants were cultivated on complete medium,<sup>3</sup> and maintained at room temperature.

#### Isolation and regeneration of protoplasts

Protoplasts were prepared and regenerated from each mutant using the procedures described previously.<sup>3</sup>

#### Hybridization of protoplasts

Protoplast hybridization between different mutants (Table I) was carried out according to a modification of the method of Anne and Peberdy.<sup>15</sup> One milliliter of washed protoplasts ( $10^8$  protoplasts) of each auxotroph was mixed and centrifuged at 1300 g for 5 minutes. Unless otherwise indicated the pelleted protoplasts were suspended in 1 ml of a prewarmed solution of 35% polyethylene glycol (PEG MW 6000-8000) in 1 M KCl and 0.05 M CaCl<sub>2</sub> and glycine, adjusted to pH 6.1. After incubation for 15 minutes at 32°C, the suspension was diluted with 6 ml minimal medium<sup>4</sup> containing 1 M KCl as osmotic stabilizer. The suspension was washed once with minimal medium adjusted to 1 M KCl, twice with 1 M KCl, and finally resuspended in 5 ml of 1 M KCl solution. Serial dilutions were made and plated onto minimal and complete medium<sup>4</sup> supplemented with 0.7 M KCl to select nutritionally-complementing hybridized protoplasts. The plates were incubated at 32°C for two weeks, the colonies showing prototrophic growth were counted and the frequency of protoplast hybridization between different markers was determined.<sup>4</sup>

Table I. Frequency of protoplast hybridization among mutants of dermatophytes.

Fusion Mixture	Hybridization Frequency
leucine auxotroph of <i>T. rubrum</i> × tyrosine auxotrophs of <i>T. rubrum</i>	47%
phenylalanine auxo. of <i>M. gypseum</i> × arginine auxo. of <i>M. gypseum</i>	53%
phenylalanine auxo. of <i>M. gypseum</i> × glutamic acid auxo. of <i>M. gypseum</i>	100%
phenylalanine auxo. of <i>M. gypseum</i> × phenylalanine auxo. of <i>M. gypseum</i>	0
phenylalanine auxo. of <i>M. gypseum</i> × arginine auxo. of <i>M. cookei</i>	0
phenylalanine auxo. of <i>M. gypseum</i> × arginine auxo. of <i>T. mentagrophytes</i>	0
leucine auxo. of <i>T. mentagrophytes</i> × tyrosine auxo. of <i>T. rubrum</i>	0



## RESULTS

Protoplast aggregation and fusion was observed immediately after PEG medium was added to the protoplast suspension. The membranes of two or more protoplasts were then observed to merge into one large structure containing the genome of both auxotrophs.

Hybridization between protoplasts of nutritionally-complementing auxotrophs of the same species was detected (Table I) by the formation of hybrid colonies on minimal medium. As Table I shows, hybridization occurred between protoplasts of different auxotrophs of *M. gypseum* as well as *T. rubrum*. Although fusion between the protoplast of the same marker or different species or genera may occur, their genomes do not complement each other. Therefore there will be no hybridization and no growth on minimal medium. Also, unfused protoplasts as well as mycelial fragments and conidia did not grow on minimal medium which was used as a control. The frequency of spontaneous reversion of protoplasts to prototrophy, as measured by plating the PEG-treated protoplasts of each auxotroph separately on minimal medium, was found to be in the range of  $10^{-6}$  to  $10^{-7}$ .

Crosses between protoplasts of different auxotrophs showed variable frequencies of hybrid formation (47-100%). The frequency of hybridization between phenylalanine and glutamic acid auxotrophs was higher than that for other markers (Table I).

The hybrid colonies (resulting from protoplast hybridization) were analysed with further cultivation on minimal medium to ascertain that their morphology are typic and the prototrophy was stable. Neither loss of prototrophy nor atypical morphology were detected for the hybrid colonies.

## DISCUSSION

A subject receiving much attention in developing countries is the distribution and pathogenicity of dermatophytes. Ringworm infections are still the most prevalent form of skin disease in these countries and cause a significant number of patients to be referred to medical clinics daily.<sup>16</sup> Although our knowledge of the distribution, pathogenicity and epidemiology of dermatophytes is fairly well established,<sup>16</sup> information concerning the molecular biology of these fungi is still at the preliminary stage. This research was undertaken to provide a frame of references for dermatophyte identification by protoplast hybridization. Perhaps the greatest interest in fungal protoplasts has been in the field of genetics following the development of new techniques in protoplast hybridization and transformation.<sup>4</sup>

Protoplast hybridization is a valuable tool for inducing genetic recombination and identification of fungi and is mostly used in the genera of penicillium, aspergillus and

candida.<sup>10,11,12</sup> In protoplast hybridization, the initial event is the aggregation and fusion of two or more protoplasts in the presence of PEG and osmotic stabilizers. The hybridization between protoplasts of nutritionally-complementing auxotrophs of dermatophytes was detected by formation of hybrid colonies on minimal medium (Table I). This means that hybridization occurs between the same species (with different markers) with the assurance that they represent a homogenous group of strains with respect to genetics relatedness. Complementation of genomes during protoplast hybridization may be due either to heterokaryosis or may occur as a consequence of karyogamy. Ferenczy et al.<sup>17</sup> obtained hybrid colonies between protoplasts of mutants of *A. nidulans* requiring lysine and methionine on minimal medium for identification of this species. Our results also showed that hybridization occurred between protoplasts of different auxotrophs of *M. gypseum* as well as *T. rubrum* resulting in identification of these species while the hybridization was unsuccessful using the same auxotrophs and different species or genera (Table I). It seems that in those fusion mixtures in which hybridization was not successful (Table I), this may have been due to their genomes not complementing each other. The frequency of hybrid formation during protoplast hybridization in dermatophytes was influenced by the particular auxotrophic markers used.

Crosses between phenylalanine and glutamic acid-requiring mutants yielded the highest frequency of detectable hybrids (Table I) while fusions between other markers yielded about 50% fewer. This difference could be related to the nature of mutation or different levels of complementation between different markers. The frequency of hybridization was reported to be less in other fungi such as aspergillus and trichoderma compared to dermatophytes.<sup>10,18</sup> One of the reasons for the high frequency of hybridization in dermatophytes could be the high viability and regeneration frequency of the protoplasts of these species.<sup>3</sup>

We believe that protoplast hybridization could help in the identification of dermatophytes when the phenotypical data cannot adequately separate them at the species level.

The taxonomy and identification of dermatophytes are based on macroscopic and microscopic morphological characteristics and sometimes two species from the same genus or different genera have such close morphology that they cannot be distinguished from each other and this consequently hampers our laboratory identification and management of the disease. This identificational problem most often occurs when fungi are at the pleomorphic stage.

Recently, DNA analysis has been reported to be a powerful tool for taxonomy and identification of dermatophytes.<sup>19,20</sup> DNA analysis of several non-pigmented strains of *T. rubrum* showed that they are genetically more closely related to *T. mentagrophytes* than *T. rubrum*. In the clinical laboratory it is occasionally difficult to distinguish

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these two species from each other. Also, *T. rubrum* and *T. tonsurans* are often difficult to differentiate.

These morphological problems reflect the close relatedness among these species and it seems that in cases in which morphological characteristics are not able to identify dermatophytes, more accurate methods for definitive laboratory identification are needed. Since DNA analysis is expensive and time consuming, the technique of protoplast hybridization was designed as a powerful tool to overcome these difficulties.

The data generated from protoplast hybridization studies in dermatophytes might be used as a framework for future studies to investigate taxonomical relationships on other than morphological criteria, and give a much clearer picture of genetic recombination and more precise identification of this group of medically important fungi.

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