A MODIFIED SCREEN FOR THE DETECTION OF CELL WALL-ACTING ANTIFUNGAL COMPOUNDS

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One would logically expect that antifungal agents which act *via* the inhibition of cell wall biosynthesis should show fungicidal activity and low toxicity as the cell wall is essential for fungal cell growth and is not synthesized by mammalian cells. A variety of cell wall-acting antifungal agents have been discovered and some of these have become economically important[1~3]. However, new nontoxic antifungal agents are needed, especially for medical applications[4]. While a variety of screens have been described for cell wall-acting antibacterial agents[5~8], less work has been published on the identification of agents which inhibit fungal cell wall synthesis.

A screen was recently described by SELITRENNIKOFF[9] in which cell wall-acting antibiotics are detected by their ability to block the regeneration of *Neurospora crassa* protoplasts. This assay is based upon the characteristics of a *N. crassa* strain which carries a temperature sensitive allele of *os*-1 (allele NM233(t)). Although strains carrying this mutation produce a normal cell wall, mutations at the *os*-1 locus permit *N. crassa* cells to divide and grow as protoplasts on osmotically supported media, providing that the cell wall is first removed either by enzymatic or antibiotic treatment[10]. Strains carrying a temperature sensitive *os*-1 allele are able to grow as protoplasts at the restrictive temperature (37°C) and will regenerate a cell wall if shifted to the permissive temperature (22°C). In the screen described by SELITRENNIKOFF, plates are inoculated with a preparation of protoplasts, shifted to the permissive temperature and antibiotic disks are placed on the media. Most antibiotics produce a clear zone of growth inhibition while cell wall-acting antibiotics produce a hazy zone containing protoplasts which are unable to regenerate but, lacking a wall, are resistant to the antibiotic. These zones are somewhat difficult to score because the protoplasts grow poorly at 22°C.

In addition, we were discouraged from employing this assay for mass screening because of the need to prepare and maintain large scale cultures of protoplasted cells. We argued, however, that it might be possible to employ this idea for screening without handling protoplasts by exploiting an inversion of the original principle. SELITRENNIKOFF's method was based on the preparation of protoplasts by antibiotic treatment (polyoxin to inhibit chitin synthesis and sorbose to inhibit glucan synthesis) and screening for antibiotics which maintained the protoplasted state. We reasoned that it should be possible to detect cell wall inhibitors by virtue of their ability to protoplast walled *os*-1 cells grown on osmotically supported media. It also seemed likely that the response might be improved for the following reason. In SELITRENNIKOFF's assay, good protoplast growth is necessary to distinguish general inhibitory antibiotics which kill protoplasts from cell wall inhibitory antibiotics which are inactive against protoplasts. However, this assay is performed at the permissive temperature (22°C), a temperature at which the *Neurospora* cells would be expected to grow poorly as protoplasts. In screening for antibiotics which induce protoplast formation, one would perform the assay at the restrictive temperature (37°C) which would encourage protoplast growth.

Materials and Methods

A medium containing Difco morphology agar

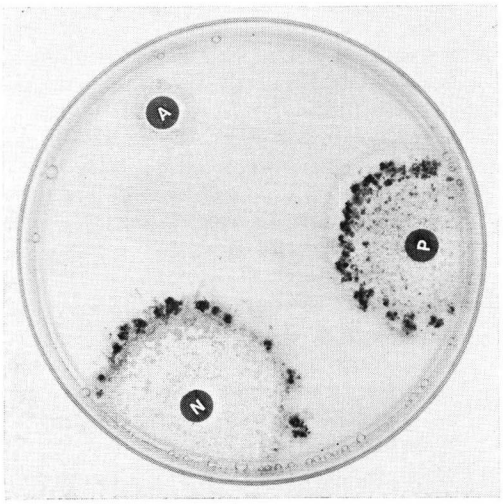
Table 1. Effects of various compounds tested in the cell wall-targeted screen.

Drug	Concentration (μg/disk)	Zone of inhibition	
		Diameter (mm)	Type
Econazole	20	31	Clear
Ketoconazole	20	22	Clear
Amphotericin B	20	12	Clear
Nystatin	20	18	Clear
Naftifin	20	—	
Tolnaftate	20	—	
Griseofulvin	20	—	
5-F-Cytosine	20	—	
Cycloheximide	20	36	Clear
Haloprogin	20	16	Clear
Aculeacin A	100	25	Turbid
Polyoxin ABG	40	25	Turbid
Nikkomycin XZ	20	35	Turbid

3.5%, yeast extract 0.1% and solidified with agar 1.5% was chosen because 1) it could be prepared, unlike VOGEL's media, from a small number of simple solid components and 2) it produced vigorous growth of the *N. crassa os*-1 strain. To this base medium, D-sorbitol was added to 7.5% as an osmotic stabilizer and L-sorbose was added to 2.5% as an inhibitor of glucan synthesis.

The above medium was inoculated with spores of strain FGSC1200 (*os*-1 allele NM233(t), mating type a, Fungal Genetics Stock Center, Arcata California, (U.S.A.) to a final concentration of $5\times10^4\sim10^5$ spores/ml and incubated with drug containing disks at the restrictive temperature, 37°C for at least 24 hours (36~48 hours optimum).

Fig. 1. Assay plate showing turbid zones produced by cell wall-acting compounds.
A; Aculeacin A, P; polyoxin ABG, N; nikkomycin XZ.

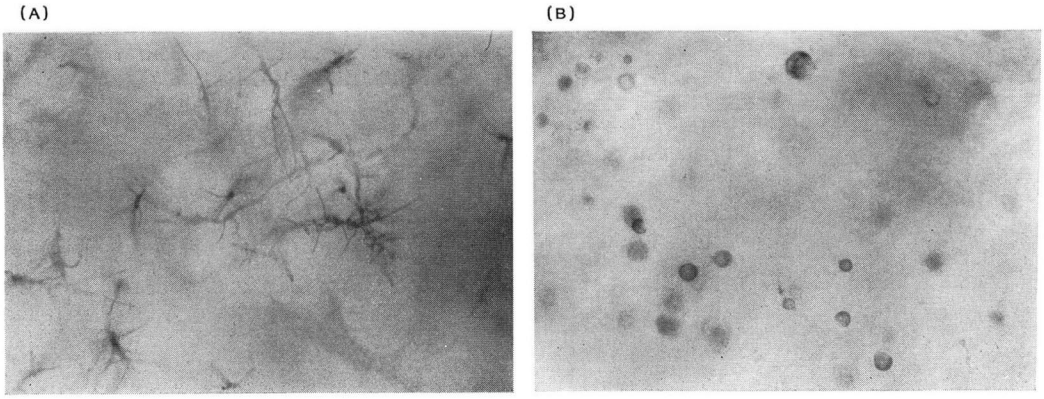


Results and Discussion

N. crassa os-1 protoplasts are formed by the combined action of a chitin synthesis and a glucan synthesis inhibitor. It therefore seemed logical that one of the two inhibitors would have to be incorporated into the medium in order to detect samples containing the other. We chose to incorporate a glucan synthesis inhibitor (sorbose) into the media for two reasons 1) the original regeneration assay only detected chitin synthesis inhibitors which raised the question of whether this type of assay would be capable of detecting glucan synthesis inhibitors and 2) the relative availabilities of sorbose and polyoxin made sorbose the practical choice for a large throughput screen.

Most antibiotics tested (see Table 1) produced either no response or produced a clear zone of inhibition. Three antibiotics [aculeacin A, polyoxin (ABG mixture) and nikkomycin (XZ mixture)] produced turbid zones. The polyoxin and nikkomycin zones were striking in that orange-colored material was discharged from the surface of the plate (see Fig. 1). An examination of the plates with an inverted phase contrast microscope indicated the presence of fairly normal looking hyphae in the turbid zones resulting from aculeacin treatment (Fig. 2). However, the polyoxin and nikkomycin zones

Fig. 2. Turbid areas surrounding drug disks were examined with an inverted phase contrast microscope at 100× magnification.
(A) Aculeacin A zone, (B) polyoxin ABG zone.



contained, in addition to normal hyphae, enlarged and ballooned hyphae which gave the appearance of being partially protoplasted and spherical cells which are likely to be cells that are largely if not entirely, protoplasted (Fig. 2). The orange colored material was amorphous by microscopic examination and appears to be secreted by the cells as a reaction to growth in the presence of a chitin synthesis inhibitor. The presence of this material, however, makes the response to chitin synthesis inhibitors very distinct and easy to score. Based upon the production of the orange-colored amorphous material, as little as 500 ng of polyoxin and 250 ng of nikkomycin could be detected.

Fermentations of known polyoxin and nikkomycin producing cultures (ATCC 19093 and ATCC 31160) produced clear positives in this assay. In a pilot screen of fermentations from randomly isolated actinomycetes, positives were obtained at a fairly high rate ($\sim 0.7\%$ of cultures tested). Preliminary characterization studies tentatively indicated that these fermentations contained polyoxins. This assay appears to be a sensitive and specific screen for polyoxins. We believe this screen to be simpler to perform and easier to read than the protoplast regeneration blockage screen. Potentially, additional screening, especially of unusual samples, could lead to the identification of novel cell wall inhibitors.

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