Antibiotic Production by Dermatophyte Fungi

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Thirty-two clinical isolates of anthropophilic dermatophytes were examined for their capacity to produce antibiotics in liquid culture and on human stratum corneum *in vitro*. Antibiotics were detected and classified using agar diffusion bioassays and chromatographic analysis. Twenty-four isolates produced antibiotic substances in liquid culture filtrates; some strains produced more than one antibiotic. Only four isolates produced detectable levels of antibiotics when grown on stratum corneum unless an artificial sweat mixture was used as a nutrient supplement, when the number rose to 11. Representatives of all species studied produced benzyl penicillin-like substances. Some *Trichophyton* isolates also produced streptomycin-like antibiotics, a characteristic previously unrecorded for eukaryotic organisms. Other antibiotics, which apart from azalomycin F could not be properly classified, were produced by *Epidermophyton floccosum*. Antibiotic production occurred over the normal skin temperature range but sometimes the type of antibiotic produced and the frequency of detection appeared to be influenced by the incubation temperature.

INTRODUCTION

Although the production of antibiotics by dermatophyte fungi has long been known, there have been few systematic studies of either production or the ecological effects of production. The earliest reports of the production of antibacterial substances by dermatophytes appear to be those of Nakamura (1932) and Honda (1936). Peck & Hewitt (1945) believed the antibacterial product to resemble penicillin and Uri, Valu & Békési (1963) later demonstrated the production of benzyl penicillin by *Trichophyton mentagrophytes*. In a study of *Epidermophyton floccosum*, Nishio (1958) detected antibiotics in culture filtrates using column chromatography: penicillin-like and actinomycin-like antibiotics were indicated. Cole (1966) investigated the formation of 6-aminopenicillanic acid and penicillin by various fungi, and concluded that both substances were produced by *E. floccosum*. Geophilic dermatophytes were shown to produce antibiotics by Gip & Palsson (1970*a, b*), who found that *Microsporum cookei* inhibited *Staphylococcus aureus* strains both resistant and sensitive to benzyl penicillin. Wallerstrom (1967, 1968, 1969) concentrated on *E. floccosum* and related the 'epidermophyton factor' (EPF) to steroid antibiotics like fucidin. He also demonstrated that some *E. floccosum* strains produce penicillin.

If antibiotics are produced *in vivo* two effects might be noticed. One is the sensitization of the infected person towards the antibiotic produced. There is some epidemiological evidence that this occurs (Bolgar *et al.*, 1960; Schuppli, 1962; Blum & De Weck, 1966). The other effect might be a shift in the local bacterial flora towards antibiotic resistance. Wallerstrom (1968) was able to demonstrate that lesions infected with *E. floccosum* had a microflora

more resistant to EPF and penicillins than did the control samples (from persons with clinical lesions, but from whom no fungus was grown). In a study of experimental dermatophyte infection in human volunteers, Bibel & Le Brun (1975) found that the proportion of penicillin-resistant cocci at the site of infection increased during the course of infection, but decreased rapidly after the fungi became absent from the lesion.

In a preliminary study, Noble & Clayton (unpublished observations) investigated an unselected series of 98 patients with groin lesions resembling tinea cruris. Dermatophyte fungi were cultured as part of the routine clinical investigation; samples were obtained by scraping affected stratum corneum and were cultured on Sabouraud's agar. The bacterial flora was sampled using a cotton swab moistened in broth, and held in Stuart's Transport Medium until inoculated on to blood agar. Bacterial isolates were characterized as Gram-negative rods, *S. aureus*, other Micrococcaceae or diphtheroids. All Gram-positive species were tested for resistance to penicillin, tetracycline, neomycin, erythromycin, gentamicin and clindamycin using Oxoid Multodiscs. Gram-negative bacilli were tested for resistance to ampicillin, sulphonamide, sulphomycin sodium, colistin sulphomethate sodium, gentamicin, nitrofurantoin and kanamycin. Clear-cut results were not obtained; 57% of the 42 patients from whom fungus was cultured carried penicillin-resistant cocci compared with only 38% of the 56 patients from whom no fungus was cultured. However, this may not be surprising since natural variations among wild-type isolates of fungi producing penicillin have been reported for the *Penicillium notatum* group (Raper, Alexander & Coghill, 1944) and *Aspergillus nidulans* (Holt & Macdonald, 1968). The probability was that not all of the dermatophytes investigated in the initial study were antibiotic producers. To test this hypothesis it was decided to investigate a number of clinical isolates for their ability to produce antibiotics *in vitro*, both in a synthetic fermentation medium and on strips of human stratum corneum.

METHODS

Dermatophyte cultures. Thirty-two anthropophilic isolates cultured from tinea lesions at St John's Hospital for Diseases of the Skin were studied. Eight isolates were characterized as *Trichophyton mentagrophytes*, 11 as *Trichophyton rubrum* and 13 as *Epidermophyton floccosum*. Isolates were allocated consecutive domestic code numbers. All cultures were maintained on Sabouraud agar (Oxoid) slopes in 30 ml Universal bottles.

Bacteria used in bioassays. These were: *Bacillus subtilis* (NCTC 8236); *Escherichia coli* (NCIB 10224), resistant to streptomycin; *Escherichia coli* (NCIB 86), sensitive to streptomycin; *Staphylococcus aureus*, resistant to penicillin (a penicillinase producer which was resistant to 10 u. benzyl penicillin when tested by the disc method); and *Staphylococcus aureus*, sensitive to penicillin. The two *S. aureus* strains were of the same origin, the sensitive variant being derived from the resistant strain by loss of the penicillinase plasmid (Noble, 1977).

Antibiotic production medium. As penicillin was of particular interest in the study, a fermentation unit medium (FUM) specifically designed for benzyl penicillin production was used (Holt & Macdonald, 1968). A mycelial inoculum of the test fungus was added to 25 ml FUM in a 100 ml polypropylene conical flask. Three flasks were inoculated for each strain. The inoculum consisted of mycelium grown for 6 d at 30 °C in a 30 ml Universal bottle containing 3 ml Sabouraud broth (Oxoid). Flasks were incubated for 7 d on a rotary shaker (200 rev. min⁻¹ and 5 cm eccentric throw) at 26, 30 and 33 °C. Broth from the shake flasks was filtered through Whatman no. 1 filter paper, and the filtrates were used in bioassays and chromatography.

Antibiotic bioassay. Antibiotics were detected in culture filtrates using a standard agar-plate diffusion technique with or without penicillinase (BDH) incorporated at a concentration of 80 u. ml⁻¹ (Cole, Holt & Macdonald, 1976). Diagnostic Sensitivity Test Agar (DSTA, Oxoid) was seeded with *B. subtilis* spores. Assay plates were incubated at 37 °C for 16 h. Culture filtrates which produced inhibition zones were further analysed by paper chromatography.

Chromatography and bioautography. Crude concentrates were prepared by acetone extraction of filtrates and divided into two parts, one part being treated with penicillinase (80 u. ml⁻¹) to inactivate benzyl penicillin-like antibiotics. Antibiotics were characterized using the system devised by Betina (1964) in which the patterns of *R_F* values, obtained when different solvents are used in an ascending paper chromatographic system, are characteristic of particular antibiotics. A bioautographic procedure was used to visualize the distance run by the antibiotic. Dried chromatograms were applied to the surface of assay plates of DSTA seeded with *B. subtilis* spores. Plates were held at 4 °C for 3 h, followed by 16 h incubation at 37 °C.

Standard antibiotic preparations. Benzyl penicillin and streptomycin sulphate (Glaxo) were used for comparison in bioassays and chromatography.

In vitro growth of dermatophytes on human stratum corneum. The technique devised by Knight (1972) to study dermatophyte growth on stratum corneum was modified to permit detection of antibiotics excreted by the fungi. Layers of stratum corneum were removed from healthy skin on human forearms by applying a 4×2 cm strip of translucent adhesive tape ('Steridrape', Minnesota Mining and Manufacturing Co., St Paul, Minnesota, U.S.A.). Each strip was applied several times to the skin until it no longer adhered. Strips were fixed, stratum corneum uppermost, on to microscope slides resting on glass rods in Petri dishes and sterilized by ethylene oxide at least 3 d before inoculation.

For each isolate, the influence on antibiotic production of synthetic sweat mixture, a synthetic sebum preparation (Murphy, 1975) and a combination of both in equal proportions was investigated using the Steridrape technique. In each case, 5 µl of the appropriate sterile mixture or sterile distilled water were spread across the skin strips and allowed to dry. Sterile distilled water (8 ml) was placed in the bottom of each dish. Each inoculum consisted of 5 µl of spore suspension from a fungal isolate spread across the strip. Duplicate inoculated and control strips were incubated for 7 d at 30 °C. In addition, inoculated and control strips with sweat supplement or distilled water were incubated at 26 and 33 °C. Antibiotic activity was detected by the same procedure described for bioautography, the skin strip substituting for the chromatogram.

Where antibiotic production was positively demonstrated following fungal growth on stratum corneum, bioassays were performed to characterize the nature of the antibiotics more precisely. These bioassays involved the use of: (i) *B. subtilis* with and without penicillinase in the medium; (ii) penicillin-resistant and -sensitive isolates of *S. aureus*; (iii) streptomycin-resistant and -sensitive strains of *E. coli*.

RESULTS AND DISCUSSION

Using the classification of Betina (1964), at least three different antibiotics were recognized in culture filtrates of many of the 32 dermatophyte fungi examined. Figure 1 shows the summarized chromatograms for antibiotics produced by three representative isolates. For strains 3 and 9, the patterns of R_F values corresponded to those observed with known standards of benzyl penicillin and streptomycin sulphate respectively. The antibiotic activity of strain 3 was not detected after penicillinase treatment of the culture filtrates. Additionally, a penicillin-resistant strain of *S. aureus* was not inhibited by the antibiotic whereas a sensitive isolate was. After penicillinase treatment of the culture filtrates of strain 9, the antibiotic produced inhibited a strain of *Esch. coli* sensitive to streptomycin but not a resistant one. The R_F values of the antibiotic produced by strain 1 were similar to the published data for azalomycin F, but no authentic sample was available for comparison.

The antibiotics produced by the dermatophytes grown in liquid culture and on stratum corneum *in vitro* at different incubation temperatures are shown in Table 1. The capacity of anthropophilic dermatophytes to produce a range of antibiotics, some therapeutic, is obviously a common but probably not a universal characteristic. Some 75% of the isolates investigated produced antibiotics when grown in liquid culture, including all 8 *T. mentagrophytes* isolates, 7 of the 11 *T. rubrum* isolates and 9 of the 13 *E. floccosum* isolates. For Trichophyton isolates, the characterized antibiotics were benzyl penicillin-like or streptomycin-like. For *E. floccosum*, apart from benzyl penicillin-like and azalomycin F-like antibiotics, other antibacterial substances were produced by several isolates. Most of these fell within group 4 of Betina's classification, which includes azalomycin F and amphotericin B amongst others, but could not be characterized further by the patterns of R_F values. Although many isolates were able to produce more than one antibiotic, it is possible that the acetone extraction procedure chosen to concentrate the antibiotics from culture filtrates selected certain antibiotics. Others may have been present, but not concentrated. This is supported by the bioassay results for *T. mentagrophytes* isolates grown on stratum corneum where antibacterial substances other than those resembling benzyl penicillin and streptomycin were apparent. The greater frequency of detection of benzyl penicillin-like substances in liquid culture reflects the choice of the medium which is specially designed for benzyl penicillin synthesis. It may not be significant that detection of other antibiotics in culture filtrates was only demonstrated for penicillin-producing isolates. It could well be that a

Fig. 1. Summarized chromatograms of antibiotics extracted with acetone from culture filtrates of three representative dermatophyte isolates: ●, *T. mentagrophytes* strain 9 (streptomycin-like); ○, *T. rubrum* strain 3 (benzyl penicillin-like); □, *E. floccosum* strain 1 (azalomycin F-like). Solvents: 1, distilled water; 2, *n*-butanol saturated with water; 3, ethyl acetate saturated with water; 4, benzene saturated with water.

medium designed, for example, for streptomycin production might result in enhanced streptomycin detection. However it is noteworthy that FUM was able to support the production of a variety of antibiotics.

The detection of streptomycin-like substances in culture filtrates of over half of the Trichophyton isolates investigated, as well as on stratum corneum during growth of two *T. mentagrophytes* strains, was totally unexpected. If substantiated, this would appear to be the first record of streptomycin production, or similar aminoglycosides, by eukaryotic organisms.

The reduced frequency of antibiotic detection when dermatophytes were grown on stratum corneum *in vitro* was apparently not due to reduced diffusion rates. Standard preparations of benzyl penicillin and streptomycin sulphate diffused easily across both the stratum corneum and Steridrape, with and without synthetic sweat and sebum preparations, although the diffusion rate was greater for penicillin than streptomycin. The reduced frequency of detection may reflect either the poorer nutrient status of the stratum corneum compared to FUM, or complexing of antibiotics with non-diffusible substances in the stratum corneum.

The synthetic sebum and sweat preparations used as supplements represent fat-soluble and water-soluble components of skin secretions, respectively (Murphy, 1975). The sebum preparation appeared to be inhibitory to both antibiotic production and mycelial growth, the latter being very sparse. In contrast, the sweat supplement enhanced mycelial growth and antibiotic production on the stratum corneum and this probably reflects the increased nutrient status of the system. Such effects could be of ecological significance as areas of skin with high humidity and excessive sweating are often the foci of dermatophyte infections in adults. According to Noble & Somerville (1974) the skin temperature is maintained between 30 and 33 °C in a comfortable environment, though crural lesions must more closely approach 37 °C. The temperature range appears adequate for dermatophytes to produce antibiotics when growing on skin (cf. Table 1).

There is little evidence that dermatophytes make the skin totally unsuitable for multiplication of indigenous bacteria. On the contrary, dermatophytes may facilitate colonization by *S. aureus* which subsequently contributes to the appearance of symptoms (Marples &

Table 1. Antibiotics detected when dermatophytes were grown *in vitro* either in fermentation unit medium (FUM) or on stratum corneum (SC) with and without synthetic supplements

Domestic code number	FUM at:			SC+synthetic sweat at:			SC alone 30 °C	SC+sweat/ sebum mixture at 30 °C
	26 °C	30 °C	33 °C	26 °C	30 °C	33 °C		
<i>T. rubrum</i>								
2	P	PS	PS	—	—	—	—	—
3	P	P	P	—	P	P	P	P
4	P	PS	PS	—	P	P	—	—
5	P	P	P	—	P	—	—	—
18	—	PS	S	—	—	—	—	—
19	P	—	—	—	—	—	—	—
24	P	PS	S	—	—	—	—	—
<i>T. mentagrophytes</i>								
7	P	P	P	—	P	P	P	P
8	P	P	P	—	—	—	—	—
9	PS	PS	S	—	S	S	—	—
10	PS	PS	S	—	U	U	—	—
25	PS	PS	S	—	—	—	—	—
26	PS	PS	S	—	—	—	—	—
27	P	PS	PS	—	U	U	—	—
38	P	PS	PS	S	S	S	S	S
<i>E. floccosum</i>								
1	PA	PA	PA	—	—	—	—	—
14	PA	PA	PA	—	—	—	—	—
16	PU	PU	U	—	—	—	—	—
17	PU	PU	PU	—	—	—	—	—
32	PU	PU	U	—	U	U	U	U
34	PU	PU	PU	—	U	—	—	—
35	PU	PU	PU	—	U	U	—	—
36	PU	PU	PU	—	—	—	—	—
37	PU	PU	PU	—	—	—	—	—

P, Benzyl penicillin-like; acetone extracts of culture filtrates with R_F values characteristic of benzyl penicillin; antibiotic activity destroyed by penicillinase treatment; active against strains of *S. aureus* sensitive to benzyl penicillin, but not against resistant strains.

S, Streptomycin-like; acetone extracts of culture filtrates with R_F values characteristic of streptomycin; active against *Esch. coli* (NCIB 86) sensitive to streptomycin, but not against *Esch. coli* (NCIB 10224) resistant to streptomycin.

A, Azalomycin F-like; acetone extracts of culture filtrates with R_F values resembling published data for azalomycin F; active against bacteria used in bioassays except *Esch. coli* strains.

U, Uncharacterized; acetone extracts of culture filtrates with R_F values not resembling published data for antibiotics; antibiotic activity not destroyed by penicillinase treatment; active against all bacteria used in bioassays.

—, No antibiotics detected in amounts inhibitory to *B. subtilis* (NCTC 8236).

Bailey, 1957). Allen & Taplin (1974) suggested that the high rate of penicillin resistance amongst staphylococci in a human population in Panama, in the apparent absence of therapeutic penicillin, may be due to naturally occurring penicillin in the environment. Dermatophyte infections could provide an appropriate natural source. There are indications that cocci associated with tinea lesions do have a higher prevalence of antibiotic resistance (Wallerstrom, 1968; Bibel & Le Brun, 1975). Although it is not possible to extrapolate the results from experiments *in vitro* to conditions *in vivo*, this study suggests that genetic variability in the capacity of dermatophytes to produce antibiotics, together with fluctuations in temperature and skin secretions at the site of infection, might result in considerable variation in the antibiotic resistance of bacteria associated with tinea lesions. This would explain the poor correlation between the antibiotic resistance of bacteria and the fungal

flora of tinea cruris lesions noted by Noble & Clayton (unpublished observations, see Introduction).

It is necessary to assess the antibiotic production of the fungus while growing in skin in relation to the antibiotic resistance of the bacterial flora. Only Wallerstrom (1968) has attempted to do this. It would be interesting to know the antibiotic-producing potential of the strain used by Bibel & Le Brun (1975) in their experiments. Investigation of the antibiotic production by zoophilic dermatophytes could also prove rewarding, since Smith & Marples (1964) found that hedgehogs infected by dermatophytes carried a high proportion of penicillin-resistant *S. aureus* strains in their microflora.

Published studies on skin as a microbial habitat, and interactions between the constituents of the microflora *in vivo*, are still few. Selwyn (1975) has shown how staphylococci may produce antibiotics active against other cocci, and Noble (1977) has demonstrated that genetic variants may appear on skin in response to some unknown ecological pressure. Characterization of the different antibiotics synthesized by dermatophytes, conditions under which they may be produced in the living host, and possible effects on the resident microflora are now under investigation. It can be expected that such studies on the skin flora of man and other mammals will yield valuable results for microbial ecology in general.

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