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Efflux seems to be a general mechanism to resist tetracycline in the yeasts and possibly the moulds

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ABSTRACT

Objective: To isolate yeasts and moulds from selected natural systems and study the effect of tetracycline (Tet) on them so as to generate comprehensive data for further elucidation of transfer or evolutionary development of Tet resistance in general and in these lower eukaryotes in particular.
Methods: A total of 139 natural yeasts have been isolated from various ecosystems on potato dextrose agar medium. These along with model yeasts and selected natural and model moulds have been tested for their responses to Tet at various concentrations added to the growth media. The effluxed materials were obtained by vortexing and centrifugation of cells and tested against sensitive bacterium.
Results: It was found that Tet efflux was a general feature of natural yeasts and filamentous fungi (moulds) could resist Tet upto a concentration of 5 mg/mL. However, at a very high concentration (10 mg/mL) neither the yeasts nor the moulds could grow indicating that Tet is toxic for these eukaryotes at very high concentrations. The presence of Tet in the medium exaggerates filamentation in all the hyphal forming yeasts.
Conclusions: The results suggest efflux as the general mechanism of Tet-resistance in yeasts and moulds possibly acquired from bacteria via horizontal transfer.

1. Introduction

Recently, the application of tetracycline (Tet), a synthetic antibiotic for clinical and agricultural uses [1,2], has provoked widespread criticism because of the consequent evolution of resistant genes[3-5] and their prevalence in the environment[6-13]. The concern focuses mainly on the possibility of sharing of resistance genes between animals, soils and human bacteria via horizontal gene transfer and thus contributing to the worldwide problem of the increasing antibiotic resistance and multiresistance[14]. The fact that Tet does show activities against eukaryotes such as protozoan parasites and human cells is also important in this wake[15,16]. These necessitate the comprehensive evaluation of various natural taxa for the presence of Tet resistance genes or their ability to resist Tet[13,17].

Resistance to Tet in bacteria can arise through drug efflux, ribosomal protection proteins, 16S ribosomal RNA mutation, and drug inactivation through the action of a monooxygenase[18]. In eukaryotes, such as commonly used cell types as well as worms, flies, mice, and plants even at low concentrations, Tet induces mitochondrial proteotoxic stress leading to changes in nuclear gene expression and altering mitochondrial dynamics and function[19]. There is hardly any report on the resistance mechanism towards Tet in eukaryotic system.

Yeasts are unicellular fungi, though some of them are able to form pseudohyphae and even hyphae. Generally, they are saprophytes, and a few of them have been found to cause infections in immunocompromised people. Tet has been found to enhance growth and hyphal formation in *Candida albicans* (*C. albicans*)[20] and increase drug susceptibility to amphotericin B in *C. albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. This enhanced drug susceptibility is associated with the inhibition of mitochondrial function[21]. The chemically modified Tet has been found as effective control against *C. albicans* and many other keratogenic fungi[22].

In light of these, the aim of this study was to isolate yeasts and moulds from selected natural systems and study the effect of Tet on them so as to generate comprehensive data for further elucidation of transfer or evolutionary development of Tet resistance in general and in these lower eukaryotes in particular.

2. Materials and methods

2.1. Isolation of yeasts and moulds

Yeasts and moulds have been isolated on potato dextrose agar

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(PDA) medium[23,24]. Ponds and farm soils, pond water, flowers, fruits and leaves of various plants have been used as source materials for isolating yeasts. In the case of fruits, 1 g of partially rotten flesh was homogenized in 10 mL of sterile water, serially diluted to 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions and 0.1 mL of the fifth dilution was spread on to pre-poured PDA plates. In the case of flowers and leaves, aseptically cut materials were dipped in 10 mL of sterile water and shaken for 6 h, and the supernatant was then diluted suitably and inoculated as above. In the case of soils, 1 g of the sample was dissolved in 10 mL of sterile water and shaken for 1 h before inoculation. Inoculated plates were incubated at room temperature for 2–3 days and kept at lower temperatures (6–10 °C) to prevent fast development of moulds. Plates were examined after 7, 14 and 21 days of incubation and colonies of representative morphotypes were selected, purified and maintained on PDA slants at 4 °C. Moulds have been isolated from soils of Baramulla (Jammu and Kashmir).

2.2. Quality control organisms and determination of lethal concentration

Pichia stipitis (NCIM 3507), *Sachharomyces cerevisiae* (NCIM 3305) and *Aspergillus niger* (MTCC872) were used as controls. YGA (yeast extract, glucose and agar) medium supplemented with Tet in varied concentrations (200, 300, 400, 500 and 1000 mg per 100 mL medium) were inoculated with the yeast isolates along with the moulds. A YGA plate not supplemented with Tet was also inoculated with the same yeasts and moulds and incubated under parallel condition to serve as the control. The plates were inoculated at 25 °C for 3 days and the growth of the yeasts and the moulds was monitored visually. The experiment was repeated thrice. The concentration of Tet at which there was complete inhibition of growth was considered to be lethal.

2.3. Effect of Tet on yeasts and moulds

Tet was added to the cold molten (about 40 °C) YGA medium to the final concentration of 5 mg/mL of medium just before pouring into plates. The yeast and the mould isolates were patched on agar medium and then the plates were incubated at 25 °C for 10 days. A suitable control plate was kept to compare the growth rate. The cells from test and control plates were resuspended in sterile water separately, diluted to OD_{620} = 0.1 and 0.1 mL of this suspension was spread on to pre-poured PDA plates and incubated for 2–3 days at 25 °C.

2.4. Effect of Tet on long term culture of yeasts and moulds

The colonies grown in absence (control) and presence of Tet were kept at room temperature (25 °C) for 10 days followed by keeping them at 5 °C for 30 days. The cells from these colonies were picked up and inoculated onto fresh PDA media to test the viability.

2.5. Isolation of the effluxed material

The yeast and the mould colonies appeared on the Tet supplemented agar plates were carefully picked up and resuspended in 50 mmol/L sodium phosphate buffer (pH 7.2). The suspension was vortexed for 30 s and spun down at 3000 r/min for 5 min. The yellow supernatant thus obtained contained effluxed material that was stored at 4 °C.

2.6. Antibacterial test of the effluxed material

Freshly grown culture of *Escherichia coli* in nutrient broth was diluted in sterile water to the OD_{600} = 0.1. A 0.1 mL aliquot of the inoculums was spread onto nutrient agar medium. Filter paper discs, dipped in the yellow supernatant as obtained above were placed on a bacterial inoculated plate. The plate was incubated at 37 °C for 5 days to test for the inhibitory zone, if any.

2.7. Spectrophotometric assay of Tet

The concentration of Tet was assayed spectrophotometrically at 600 nm in μg/mL range against distilled water as blank.

3. Results

3.1. Isolation of yeasts and moulds

Altogether 139 yeasts were isolated from various sources (Table 1). Each of them represented a different morphotype. The yeasts, isolated from various natural and artificial ecosystems of Bhopal, were found to be different in cell morphologies (spherical, oval and rod shaped), colours (pink, brown, white, black and light yellow), growth rates (very slow to very fast), filamentation (non-filamented and filamented) and bud formation (non-budding and budding), which indicated that they are different species. The moulds with white or light-coloured colonies isolated from Baramulla soils were selected for the study. The identification of most of the yeast isolates was underway. The moulds were identified as *Truncatella angustata* BPF-5, *Pseudogymnoascus* spp. BPF-6. *Penicillium canescens* BPF-4 and *Penicillium* spp. BPF-8 and *Penicillium* spp. BPF-9.

3.2. Effect of Tet on the colour of yeast and mould colonies

Both the yeast and mould colonies on the Tet supplemented plate showed pale yellow coloration of their colonies after 24 h of incubation (Figure 1A). The intensity of coloration went on increasing in next few days and with the growth of the colonies. The colonies on the control plate were normal in colour (Figure 1B).

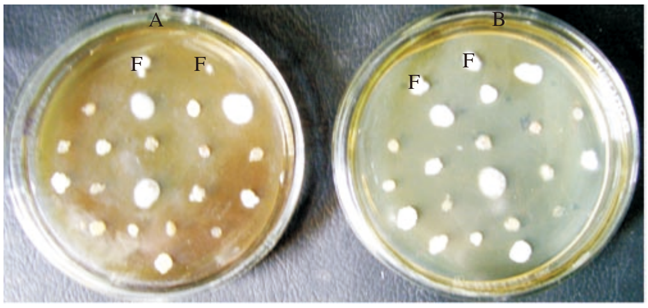

Figure 1. Colonies of representative yeast and mould (filamentous fungus) isolates (F) grown in Tet supplemented and control plates after 24 h of incubation at 25 °C. Only white mycelia bearing moulds are shown because of the ease of detection of color.

The yellow extract from the yeast and mould cells indicated the presence of Tet in them. When the effluxed material was soaked onto filter discs, it could inhibit bacterial growth as indicated by the zone of inhibition around the filter discs. This indicates that Tet was not metabolized by the yeasts or moulds, so that it had retained its antibacterial property. The yellow color of yeast cells was thus due to efflux of substance and that the material was accumulated on the cell surface.

Cells from both Tet supplemented and non-supplemented media kept for a month were found to be viable. This indicated that the efflux mechanism in these fungi was very efficient and upto a maximum concentration limit this could protect the cells and that the antibiotic effluxed from the cells remained immobilized on the surface of cells and does not seem to re-enter the cells despite its presence for 30 days. Furthermore, at much higher concentration of antibiotic, it seems that the rate of efflux becomes limiting leading to its accumulation of toxic level.

Table 1
Yeast isolates isolated from various sources.

Isolation number	Source	Sub-source
WFY1	Flour mill	Flour waste
SDY1	Timber	Saw dust
CUY1	Curd	
DY1, DY2, DY3	Gum	
PWY1, PWY2, PWY3	Pond	Water
LSY1	*Lagerstroemia speciosa*	Under canopy soil
BSY1	*Areca catechu*	Do
GSY1	*Gardenia* spp.	Do
PSY1, PSY2, PSY3, PSY4, PSY5, PSY6	Pond	Soil
NDY1, NDY2, NDY3, NDY4	Narmada river	Soil
SOY1, SOY2, SOY3	Soybean field	Soil
DSY1, DSY2, DSY3, DSY4, DSY5	*Dalbergia sisso*	Leaf
PFY1, PFY2, PFY3, PFY4, PFY5, PFY6	*Peltophorum ferruginum*	Leaf
CFY1, CFY2, CFY3, CFY4, CFY5	*Cassia fistula*	Leaf
BVY	*Bauganvillia* spp.	Leaf
MOY	*Moringa oliefera*	Leaf
OSY2, OSY4	*Ocimum sanctum*	Leaf
EJY2	*Eugenia jambolana*	Leaf
POY2	*Polyalthia longifolia*	Leaf
PGY1, PGY4	*Psidium guajava*	Leaf
TPY1, TPY2, TPY3	*Thevetia peruviana*	Leaf
ASY1, ASY2, ASY4	*Annona squamosa*	Leaf
VIY1, VIY2, VIY3	*Vernonia indica*	Leaf
LCY2	*Lantana camera*	Leaf
TIY1, TIY4, TIY5, TIY6, TIY7, TIY8	*Tamarindus indica*	Leaf
DRY1, DRY2, DRY7	*Delonix regia*	Leaf
CLY1, CLY2, CLY6	*Callistemon lanceolatus*	Leaf
DPY3	*Dillenia pentagyna*	Leaf
BIY3, BIY4	*Bombax insigne*	Leaf
PRY1, PRY2	*Plumaria rubra*	Leaf
SCY1, SCY2, SCY3, SCY4, SCY5	*Spathodia campanulata*	Leaf
FRY1, FRY 2, FRY4	*Ficus racemosa*	Leaf
CGY1, CGY2,CGY3,CGY4,CGAY	*Couroupita guianesia*	Leaf
GGY1,GGY2, GGY3	*Gardenia* spp.	Leaf
JLY1, JLY2	*Jatropha* spp.	Leaf
PJY1, PJY2, PJY3	*Putranjiva* spp.	Leaf
FFY1, FAY2, FAY5	*Accacia* spp.	Flower
RY3	*Rosa* spp.	Flower
KFY1, KFY2	*Nerium* spp.	Flower
MMY1, MMY2	*Quiscalis* spp.	Flower
BVY1, BVY2, BVY3, BVY4	*Bauhinia varigata*	Flower
PLY1, PLT2, PLY3, PLY4, PLY5	*Plumaria* spp.	Flower
BCY1, BCY2, BCY3, BCY4- BCY8	*Bombax ceiba*	
LY1	*Citrus limon*	Fruit
CHY1, CHY2, CHY3	*Achras sapota*	Fruit
PHY1, PHY2, PHY3, PHY4	*Phoenix sylvestris*	Fruit
MUY1, MUY2, MUY3, MUY4	*Musa paradisica*	Fruit
GY1, GY2, GY3 GY4, GY5	*Vitis vinifera*	Fruit

3.3. Effect of Tet on the morphology of yeasts

The yeast cells growing in presence of Tet showed dual effects. Non-filamentous yeasts showed normal morphology, while filament-forming yeasts (*Candida tropicalis*, *Candida, Issatchankia*, *etc.*) exhibited vigorous growth and filamentation in presence of Tet. Coincidently, the mould filaments also exhibited slightly enhanced growth in presence of Tet (Figure 2).

3.4. Inhibitory concentration of Tet

All the natural yeasts, selected moulds and control yeasts and moulds were found to grow at normal rate in presence of Tet at the concentration 1 mg/mL. They, however, showed varied rates of inhibition at higher concentrations. Their growth was completely inhibited at 10 mg/mL of Tet.

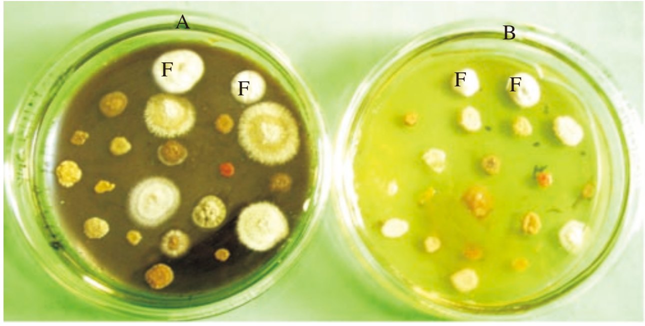

Figure 2. Colonies of representative yeast and mould isolates (F) grown in Tet supplemented and control plates after 15 days of incubation at 25 °C.

The normal growth at 5 mg/mL concentration of Tet indicated that yeasts and selected moulds are naturally resistant to this antibiotic (Figure 1A). This resistance is much higher (5 mg/mL) than that in bacteria (4 mg/L). The higher concentration, *i.e.* 10 mg/mL of Tet, however, found to be lethal for both the yeasts and the moulds.

4. Discussion

Both the yeasts and moulds showed efficient efflux of Tet, a mechanism earlier reported in prokaryotes. The bacteria are known to use four strategies to become resistant to Tet, limiting the access of Tet to the ribosomes, altering the ribosome to prevent effective binding of Tet, biosynthesizing ribosomal protection protein[25] and producing Tet-inactivating enzymes[18]. Out of these, efflux and ribosome protection are of major concern since same efflux and ribosome protection genes have been found in many different bacterial genera suggesting extensive horizontal transfer[17,26]. Conjugative plasmids and chromosomal elements (transposons) have been suggested to mediate this transfer[26].

All yeasts strains isolated and studied during this investigation shared one character, *i.e.* efflux of Tet. It remains to be known whether this efflux mode of Tet resistance has been acquired from bacteria via horizontal transfer as in bacteria[13,14] or evolved naturally. In the pathogenic yeast *C. albicans* and baker yeast *Sachharomyces cerevisiae* at least six genes *viz. CDR1, CAP1 and ERG11*[27] and *FLU1*[28] have been assumed to be involved in constituting multidrug transporter. The existence of other transport system involved in drug efflux is not yet known. Therefore, it is interesting to know that the same multidrug transporter is also used in the efflux of this primarily antibacterial drug.

Exaggeration in filamentation has also been found in both yeasts and moulds as a common response to Tet. The parallel behavior of the two taxa in presence of Tet is interesting that indicates a common molecular mechanism of filamentation or a common molecular target associated with filamentation. Since filamentation in pathogenic yeasts such as *C. albicans* has been reported to be stress-related[20], it seems therefore the presence of Tet in the cytoplasm, though for a transient period-causes stress in these yeasts and moulds. This is also to be noted that although yeasts and moulds are resistant to Tet upto a concentration of 5 mg/mL, their growth is completely inhibited at a higher concentration (10 mg/mL). This similarity in phenotypic response to Tet in yeasts and moulds did justify their inclusion in a common taxon, *i.e.* fungi.

Earlier, Tet was reported to inhibit some protozoas, such as *Giardia lamblia*, *Trichomonas vaginalis*, *Entamoeba histolytica*, *Plasmodium falciparum* and *Leishmania major* (lower eukaryotes) in addition to bacteria, but exact mechanism by which it exerts its effect on protozoans is not known[15]. Tet has also been reported to cause mitonuclear protein imbalance through their effects on mitochondrial translation in human cells[16,19] and has potential to be used in the treatment of bone metastasis[29].

Recently, Tet has been found to enhance the susceptibility towards amphoterecin B and decrease susceptibility towards terbinafine in *C. albicans, Cryptococcus neoformans* and *Aspergillus fumigatus,* which is indirectly due to the effect of Tet on mitochondrial function[21]. In another study on *Candida glabarata*, the increased efflux of drugs in mutant cells was found to be due to the overexpression of pump related genes, *CgDR1* and *CgDR2*[30]. These findings indicate the importance of Tet in the study of drug efflux in pathogenic yeasts and moulds.

The paper highlights a common drug efflux method of Tet resistance shown by all natural isolates of yeasts and moulds and the control microbes. Coincidently, this is one of the mechanisms which the prokaryotes use to resist the effect of Tet. The lower eukaryotes (yeasts and fungi) seem to inherit this mechanism from their prokaryotic progenitor as a method of choice. Alternatively, there has been very aggressive horizontal transfer of Tet gene, and if it is true than the finding reiterates the problems of the use of an antibiotic for long without monitoring its potential for the development of resistance gene. The finding may encourage further study into the mechanism applying a fungal system.

Conflict of interest statement

I declare that I have no conflict of interest.

Acknowledgments

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