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 up to 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 monoxygenase[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
(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 $10^2$, $10^3$, and $10^4$ 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), Sachcharomyces 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$_{600}$ = 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.](Image)
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 Issatchenka, etc.) exhibited vigorous growth and filamentation in presence of Tet. Coincidently, the mould filaments also exhibited slightly enhanced growth and filamentation in presence of Tet.

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.

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 Saccharomyces cerevisiae at least six genes viz. CDR1, CAP1 and EGR1[27] and FLU[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 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.
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[20].

Recently, Tet has been found to enhance the susceptibility towards amphotericin 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 glabrata, 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.

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References

[1] Toomey KE, Barnes RC. Treatment of Chlamydia trachomatis genital infections. Rev Infect Dis 1990; 12(Suppl 6): S645-55.
[2] Roy Chowdhury P, McKinnon J, Wyrsh E, Hammond JM, Charles IG, Djordjevic SP. Genomic interplay in bacterial communities: implications for growth promoting practices in animal husbandry. Front Microbiol 2014; 5: 394.
[3] Loof T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, et al. In-feed antibiotic effects on the swine intestinal microbiome. Proc Natl Acad Sci U S A 2012; 109(5): 1691-6.
[4] Durso LM, Cook KL. Impacts of antibiotic use in agriculture: what are the benefits and risks?Curr Opin Microbiol 2014; 19: 37-44.
[5] Jechalke S, Heuer H, Siemens J, Amelung W, Smalla K. Fate and effects of veterinary antibiotics in soil. Trends Microbiol 2014; 22(9): 536-45.
[6] Barkovskii AL, Bridges C. Persistence and profiles of tetracycline resistance genes in swine farms and impact of operational practices on their occurrence in farms’ vicinities. Water Air Soil Poll 2012; 223(1): 49-62.
[7] Hong PY, Li X, Yang X, Shinkai T, Zhang Y, Wang X, et al. Monitoring airborne biotic contaminants in the indoor environment of pig and poultry confinement buildings. Environ Microbiol 2012; 14(6): 1420-31.
[8] Zhu YG, Johnson TA, Su QJ, Qiao M, Guo GX, Stedtfeld RD, et al. Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc Natl Acad Sci U S A 2013; 110(9): 3435-40.
[9] Hong PY, Yamarell AC, Dai Q, Ekizoglu M, Mackie RI. Monitoring the perturbation of soil and groundwater microbial communities due to pig production activities. Appl Environ Microbiol 2013; 79(8): 2620-9.
[10] Kyselková M, Jirout J, Chroňáková A, Vrchotová N, Bradley R, Schmitt H, et al. Cow excrements enhance the occurrence of tetracycline resistance genes in soil regardless of their oxytetracycline content. Chemosphere 2015; 93(10): 2413-8.
[11] Ling AL, Pace NR, Hernandez MT, LaPara TM. Tetracycline resistance and class 1 integron genes associated with indoor and outdoor aerosols. Environ Sci Technol 2013; 47(9): 4046-52.
[12] Wichmann F, Udikovic-Kolic N, Andrew S, Handelsman J. Diverse antibiotic resistance genes in dairy cow manure. MBio 2014; 5(2): e01017.
[13] Kyselková M, Kotrbová L, Bhumibhooman G, Chroňáková A, Jirout J, Vrchotová N, et al. Tetracycline resistance genes persist in soil-amended cattle feces independently from chlorotetracycline selection pressure. Soil Biol Biochem 2015; 81: 259-65.
[14] Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MO, Dantas G. The shared antibiotic resistome of soil bacteria and human pathogens. Science 2012; 337(6098): 1107-11.
[15] Ahler E, Sullivan WJ, Cass A, Braas D, York AG, Jensinger SJ, et al. Doxycycline alters metabolism and proliferation of human cell lines. PLoS One 2013; 8(5): e64561.
[16] Kobashi Y, Hasebe A, Nishio M, Uchiyama H. Diversity of tetracycline resistance genes in bacteria isolated from various agricultural environments. Microbes Environ 2007; 22(1): 44-51.
[17] Zhang Y, Snow DD, Parker D, Zhou Z, Li X. Intracellular and extracellular antimicrobial resistance genes in the sludge of livestock waste management structures. Environ Sci Technol 2013; 47(18): 10206-13.
[18] Zakeri B, Wright GD. Chemical biology of tetracycline antibiotics. Biochem Cell Biol 2008; 86(2): 124-36.
[19] Moullan N, Mouchiroud L, Wang X, Ryu D, Williams EG, Mottis A, et al. Tetracyclines disturb mitochondrial function across eukaryotic models: a call for caution in biomedical research. Cell Rep 2015; 10(10): 1681-91.
[20] McCool L, Mai H, Eissmann M, Larsen B. Tetracycline effect on Candida albicans virulence factors. Infect Dis Obstet Gynecol 2008; 2008: 493508.
[21] Oliver BG, Silver PM, Marie C, Hoot SJ, Leyde SE, White TC. Tetracycline alters drug susceptibility in Candida albicans and other pathogenic fungi. Microbiology 2008; 154(Pt 3): 960-70.
[22] Liu Y, Ryan ME, Lee HM, Simon S, Tortora G, Lauthon C, et al. A chemically modified tetracycline (CMT-3) is a new antifungal agent. Antimicrob Agents Chemother 2002; 46(5): 1447-54.
[23] Sahay S, Hamid B, Singh P, Ranjan K, Chauhan D, Rana RS, et al. Evaluation of pectinolytic activities for oenological uses from psychrotrophic yeasts. Lett Appl Microbiol 2013; 57(2): 115-21.
[24] Sahay S, Lone MA, Jain P, Singh P, Chouhan D, Shezad F. Cold-active moulds from Jammu and Kashmir, India as potential source of cold-active enzymes. Am J Curr Microbiol 2013; 1(1): 1-13.
[25] Li W, Atkinson GC, Thakor NS, Allas Ü, Lu C, Chan KY, et al. Mechanism of tetracycline resistance by ribosomal protection protein Tet(O). Nat Commun 2013; 4: 1477.
[26] Salyers AA, Speer BS, Shoemaker NB. New perspectives in tetracycline resistance. Mol Microbiol 1990; 4(1): 151-6.
[27] Sangalang D, Ischer F, Monod M, Bille J. Cloning of Candida albicans genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. Microbiology 1997; 143(Pt 2): 405-16.
[28] Calabrese D, Bille J, Sangalang D. A novel multidrug efflux transporter gene of the major facilitator superfamily from Candida albicans (Flu1) conferring resistance to fluconazole. Microbiology 2000; 146(Pt 11): 2743-54.
[29] Saikali Z, Singh G. Doxycycline and other tetracyclines in the treatment of bone metastasis. Anticancer Drugs 2003; 14(10): 773-8.
[30] Brunn S, Bergès T, Poupard P, Vauzelle-Moreau C, Renier G, Chabasse D, et al. Mechanisms of azole resistance in petite mutants of Candida glabrata. Antimicrob Agents Chemother 2004; 48(5): 1788-96.