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Inhibition of *Candida albicans* and Two Selected Gram-Negative Pathogens by Polar *Enterococcus faecalis* and *Carnobacterium* sp.

[1]R. Shekh, [1]K. Upadhyay, [2]S.M. Singh and [1]U. Roy
[1]Biological Sciences Group, BITS Pilani-Goa Campus, Goa 403726, India
[2]Polar Biology Laboratory, National Centre for Antarctic and Ocean Research,
Sada-Headland, Vasco-da-Gama, Goa, India

Abstract: The current study has the objectives to identify the polar microorganisms with the ability to produce antimicrobial substances with wide-spectrum potential to antagonize the multi-drug resistance *Candida albicans*, *Pseudomonas aeruginosa* and *putida*. As many as 218 bacterial strains were screened and isolated from 6 Antarctic Penguin rookery faecal samples at Larsemann Hills, East Antarctica and from arctic sea-water-glacier stream convergence samples for checking the production of antimycotic and antibacterial substances using the cut well agar assay. Seven selected bacterial isolates were grown at 15°C for 48 h and the cell free supernatant showed activity against either *Pseudomonas aeruginosa* and *putida* or four strains of *Candida albicans*. Three selected isolates produced antimicrobial substances (AMS) which inhibited 4 strains of multi-drug resistant *Candida* sp. and two other species of *Bacillus* inhibited one Candida strain. The isolates PR 210 and 211 were found to demonstrate a very strong fungicidal agent when concentrated. The present investigation led to the findings of the three AMS producers which were identified *Enterobacter hormaechii*, *Carnobaterium maltaromaticum*, *Enterococcus faecalis*, based on *16S rRNA* gene sequences and fatty acid compositions, respectively. The other two isolates were *Bacillus megaterium* and *B. mycoides* identified by 16 S rDNA phylogenetic analysis.

Key words: Antarctic, antimicrobial substances, antifungal substances, CFS, candidiasis

INTRODUCTION

Candidiasis encompasses infections that range from superficial, such as oral thrush and vaginitis, to systemic and potentially life-threatening diseases. *Candida albicans* is an opportunistic dimorphic fungus that causes mucosal and systemic infections in individuals undergoing immunosuppressive therapy for cancer or organ transplantation and in human immunodeficiency virus (HIV) infected patients. The majority of HIV infected patients (60 to 80%) develop one or more fungal infections during their illness, the most frequent being oropharyngeal candidiasis (Musial *et al*., 1988). Such an infection is so frequently associated with AIDS that it can be considered a criterion for the progression towards AIDS (Coleman *et al*., 1993). Fungal infections are widely treated with triazole antifungal agents, such as fluconazole. Unfortunately, long-term therapies have led to the emergence of fluconazole-resistant *C. albicans* strains that are cross resistant not only to other azoles but also to amphotericin B (Kelly *et al*., 1996). This obviously points to a pressing need for new antifungal agents, e.g., antimicrobial proteins or peptides (Hancock, 1999; Hancock and Chappel, 1999).

Antarctica and Arctic polar regions have been considered as a vast untapped reservoir of the microorganism with manifold antimicrobial potentials (O'Brien *et al*., 2004). The aim of the current study was to screen and isolate extensively the antimicrobial/mycotic principles (which might be

Corresponding Author: Utpal Roy, Biological Sciences Group, BITS-Pilani, Goa Campus, Goa 403 726, India

cold-active peptides) from the wild-type isolates from the soil and faecal samples of the Penguin rookery at Larsemann Hills, East Antarctica which might be promising to combat the multi-drug resistance human fungal pathogens because the rapid and continuous emergence of new drug-resistant microorganism have been a present day threat which the bacteriocins, bacteriocin-like inhibitory substances or antimicrobial peptides can solve. *Pseudomonas aeruginosa* is an important bacterial pathogen most frequently responsible for nosocomial infections; it is often resistant to many antibiotics used in causative therapy; moreover, *P. aeruginosa* is also able to form biofilms (Mittal *et al*., 2005). Today, the development of resistance to monotherapy is a problem and dual antimicrobial coverage is often needed.

In order to screen feces and feather samples from the Penguin rookery and arctic water we used the modified Trypticase broth as a medium for the production of antimycotic/antimicrobial substances. As many as 218 isolates were screened from the soil and faecal samples of Penguin rookery and Arctic glacier melt water and sea-convergence of arctic region at Ny Alesund, Norway.

All the samples were spread into MGYP agar (malt extract 0.3%, yeast extract 0.3%, peptone 0.5%, glucose 1% Hi-Media, India) with 1.9% NaCl (Gilbert *et al*., 2004) after appropriate dilutions and subsequently individual colony grown on the MGYP agar were transferred into the modified TSB broth (casein enzymatic hydrolyzate 1.7%, papaic digest of soyabean meal 0.3%, NaCl 0.5%, dipotassium phosphate 0.23%, dextrose 0.25%, 0.3% yeast extract, pH 7.4±0.20, E-Merck). All the incubations were carried out at 15°C for 48 h. The cell free supernatants were made from the 48 h old cultures by centrifuging at 4°C for 30 min at 12,000 rpm. The cell free supernatants were subjected to cut-well agar assay (Takahiro *et al*., 1991) using dried 30 mL MGYP agar plate on which 200 μL of freshly grown fungal pathogens were uniformly spread. All the wild-type isolates were screened for the production of antimicrobial substance against the different multi-drug resistant yeast strains like *Candida albicans* NCIM: 227, 857, 3129, 3471, 3557, MTCC: 183, 3958, 7315, *Candida kruzeii*, *Pseudomonas aeruginosa* and *Pseudomonas putida*. The yeast strains used as indicator organisms were checked for the antibiotic resistance in disc diffusion assay using various concentrations of paper discs of antifungal antibiotics like amphotercin B, fluconazole, nystatin, clotrimazole and itraconazole. In a freshly cut well 200 μL of cell free supernatant was added. The plates were incubated at 37°C for 2 days and observed for their antifungal activity by the appearance of zone of inhibition (Fig. 1) and the diameters of the inhibition zones were measured (Rammelsberg and Radler, 1990). In case of bacterial indicator organisms like *P. aeruginosa* and *Putida*, the brain heat infusion agar was used instead of MGYP agar. The indicator organisms were grown for 16 h before spreading on the BHI agar plates with the inoculums (1%). All the plates were incubated at 37°C for 24 h and were observed for the zone of inhibition.

Antibiotic Susceptibility Testing

The antimicrobial susceptibility of all the Candida strains was determined using antibiotic-impregnated with filter discs (Hi-Media). The filter discs were placed on the surface of YPD (Yeast peptone and dextrose, Hi-Media) and MGYP agar as well that previously had been seeded separately with the freshly grown organisms (24 h at 37°C). The plates were incubated at 37°C for 24 h and thereafter examined for zones of no growth around the discs. The following antibiotics were tested at various concentrations (mcg/disc): amphotericin B, fluconazole , itraconazole, clotrimazole and nystatin.

Fatty Acid Methyl Ester (FAME) Analysis

For extraction of fatty acid methyl esters, the fatty acids were prepared from 40 mg wet cell material harvested from a culture on TSB agar (30 g TSB, 15 g agar; Hi-Media, India) incubated for 5 days at 15°C. Whole-cell fatty acids were determined as described by Bozal *et al*. (2003). The extraction procedure and instruments were described previously by Pikuta and Hoover (2003).

Fig. 1: The cell free supernatant made after 48 h of incubation of the isolate AMG 108 (1 and 2 in duplicate), AMG 111 (3 and 4 in duplicate) showing the zone of inhibition against the *Candida albicans* strain NCIM 3471

Initially when the screening was performed against the strain *Candida albicans* NCIM 3471 and *Pseudomonas aeruginosa*, seven different microorganisms were found to inhibit the indicator strains mentioned above. These isolates were subjected to morphological testing, 16 S rDNA sequencing, fatty acid analysis and biochemical testing. The indicator organisms like NCIM 3471, MTCC 183, 223, 7315 used in the current experiments were turned out to be multidrug-resistant. In this study, four strains designated as AMG 108, AMG 111, PR-210 and PR-211 were found to exhibit considerably strong antimicrobial activity against the indicator organisms *C. albicans* NCIM: 3471, MTCC: 7315,183 and *P. aeruginosa*. The cell free supernatants obtained from the three isolates designated as AMG- 108, AMG-111, PR-210 and PR-211 showed the biological activity by demonstrating a strong zone of inhibition which was measured between 18 to 23 mm against the *Candida albicans* NCIM 3471.

Subsequently the cell free supernatant (CFS) from all the three isolates were tested further against the various strains of *C. albicans* MTCC: 183, 7315, 227 and *C. krusei* 3129. The zones of inhibitions were measured from 15 to 23 mm on the MGYP plates (Fig. 1). The 48 h old supernatants obtained from the growth of three promising bacterial strains AMG-108, AMG-111 and PR-210 at 15°C were concentrated using the Lyophilizer (Martin-Christ, Germany) in order to eliminate the highly volatile substances. The concentrated CFSs were subjected again to cut well agar assay against all the aforementioned strains of *Candida* sp. The concentrated CFS increased the zone of inhibition up to 23 to 25 mm against three strains NCIM: 3471, MTCC: 183 and 7315. In order to check the pH sensitivity the concentrated samples were adjusted to various pH's from 5 to 8 and the biological activity was checked by well diffusion techniques (Rammelsberg and Radler, 1990). The presence of *E. faecalis* corroborates the earlier findings regarding the presence of gram-positive bacteria *Streptococcus faecalis* from penguins (Soucek and Mushin, 1970). Members of the genus *Carnobacterium* have been isolated from a wide range of environments such as Salmonid fish (Hiu *et al*., 1984) and *C. maltaromaticum* were isolated from anoxic lake waters in Ace lake, Antarctica (Franzmann *et al*., 1991). Present finding of *Carnobacterium* sp. (AMG 111) from the Arctic glacier melt water at sea convergence area is consistent with earlier finding regarding the presence of *Carnobaterium* sp. from the Arctic and the Antarctic sea water as well as the deep sea (Groudieva *et al*., 2004; Newberry *et al*., 2004; Toffin *et al*., 2004; Lauro *et al*., 2007).

In this investigation, the specific identification of sea-ice bacteria was undertaken and the study indicates that the Arctic sea ice bacterial community is made up of mostly psychrotrophic halotolerant species and also the bacteria from the penguin rookery were psychrotrophic and some of them were producers of antimicrobial compounds. We have found out three wild-type isolates which were found to be strong producers of antimycotic substances with a broad-spectrum activity against the *Pseudomonas aeruginosa* and *P. putida*. We conclude by reporting for the first time a group of bacteria recovered from polar region which not only antagonize but also exhibit a strong fungicidal action against the selected multi-drug resistant *Candida* sp. and therefore the polar environments can be considered an unstinted source of microorganisms which might address the present-day multi-drug resistance problems.

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