chro- and halotolerant (grew in a range 1-30°C and 10-15% NaCl). Pigmented strains of yeast (black and red) were highly resistant to UV ($LD_{99,99}$ compounded 600-1200 J/m^2), while for white yeast $LD_{99,99}$ – 250 J/m^2. ***Conclusions***. Strategy of a survival of microorganisms in Antarctic is directed on natural selection and sampling of microorganisms which initially were psychrotolerant and UV-resistant. If to take into consideration aerosol, ornithogenic and anthropogenic transfer of a microflora to Antarctic, the presented data allows to assume that the low temperature, high level UV, and also geographical isolation of islands are the primary cause for formation and evolution of microbic communities in Antarctic.

Key words: strategy of survival, antarctic bacteria/yeast, resistance to UV, psychrotolerance, halotolerance, evolution.

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PHYLOGENETIC FINGERPRINTING OF MICROBIAL COMMUNITIES IN METAL POLLUTED SOILS USING RIBOSOMAL INTERGENIC SPACER ANALYSIS (RISA)

Introduction

Soils constantly act as a sink for many hazardous contaminants and maintaining soil fertility and health is a big challenge. Hence, protecting the diversity of microbial communities is an important part of soil conservation. Microbial communities are among the most important soil components playing a key role in, e.g. nutrient cycling, plant symbioses, decomposition, metal and mineral transformations, and other critical ecosystem processes. In particular, fungi are known to play fundamental biogeochemical roles in most terrestrial ecosystems and many can survive and grow under extreme conditions, and are capable of utilizing a large range of simple and complex organic substrates [1]. However, fungi have frequently been ignored or neglected in biodiversity conservation.

In this study, it was attempted to demonstrate the benefits of culture-independent PCR-based fingerprinting technique called ribosomal intergenic spacer analysis (RISA) in the examination of the effects of a polycyclic aromatic hydrocarbon (pyrene) either alone or in combination with increasing concentrations of copper on fungal communities in soil microcosms.

Ribosomal intergenic spacer analysis (RISA) is a rapid and powerful tool for characterizing complex microbial communities and for detecting community composition changes in response to environmental disturbance [3]. Shifted RISA bands can be excised, cloned and sequenced to identify the populations involved in any community adaptations. This technique is based on gel separation of gene fragments by length differentiation of the internal transcribed spacer (ITS) region of the ribosomal DNA in fungi.

Materials and Methods

The microcosms were designed to resemble the PAH and metal contaminated ecosystem by using sandy soil [pH 6.7 & water content 19% (w/w)]. The soil has no previous history of PAH and metal contaminations. Microcosms were prepared by weighing 50 g (dry weight) soil into glass jars (59 mm diameter, 98.5 mm height, Sigma, Germany). A 10 mm hole was made in polypropylene lid and this hole was plugged with a polyurethane foam stopper to allow air flow. Soil guideline of the Ministry of the Environment of the Province of Ontario (MOE) was used to spike soil samples (Pyrene: 250 mg/kg, copper: 300 mg/kg, *see* Table 1). 250 mg of pyrene (Sigma, Germany) was dissolved in analytical grade acetone (BDH, UK) and mixed with 50g of dried sandy soil thoroughly by shaking vigorously. The soil samples were left for 2 hrs to evaporate acetone. Then cupric phosphate [Cu3(PO4)2] was added to the soil. Water content was maintained at 19% by addition of sterile Milli-Q water as necessary. Microcosms were incubated in dark at 20°C. On Day 21 soil samples were taken for molecular characterisation.

For RISA, 0.5 g aliquots of each soil sample were taken from each microcosm vessel in triplicate on day 21 and DNA was extracted by using an Ultra-CleanTM Soil DNA isolation kit (Mo Bio, Carlsbad, CA, USA) according to the manufacturer's protocol. A purification procedure was performed by using a High PureTM PCR product

clean-up kit (Roche, Mannheim, Germany) according to the manufacturer's instructions except for the application of elution buffer. The products were eluted in sterile Milli-Q water as the elution buffer supplied may interfere with the PCR and sequencing processes. The fungal intergenic region containing the two ITS and the 5.8S rRNA gene (ITS1-5.8S-ITS2) was amplified using primer set ITS1f (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). Negative controls were included in each reaction to ensure no contamination in experimental materials.

Table 1. Concentrations of pyrene and copper in the microcosm experiment (mg kg^{-1})

Pollutant	Treatment				
	C	P[a]	Pyrene + Cu[b]		
	Control soil	Pyrene	(1)	(2)	(3)
Pyrene	-	250	250	250	250
Cu	-	-	300	600	900

[a] Soil spiked with pyrene only (250 mg kg^{-1});
[b] Soils spiked with pyrene and cupric phosphate: (1) 300 mg kg^{-1}, (2) 600 mg kg^{-1} and (3) 900 mg kg^{-1}

To facilitate treatment comparisons within single gels, all PCR products from each treatment were pooled prior to analysis. Electrophoresis was performed with a Bio-Rad Protean II Xi Cell (Bio-Rad, Hercules, CA, USA) and 5% nondenaturing polyacrylamide gels were casted for RISA. Twenty microliters of (approximately 400 ng) of each PCR product was mixed with 2x gel loading dye (Bio-Rad) and electrophoresed at 60 V for 12 h at room temperature with 1·tris acetate EDTA (TAE) buffer along with standard marker Step Ladder, 50 bp (Sigma, St Louis, MO, USA). Gels were stained with SYBR Green I (Sigma-Aldrich) for 30 min, and gel images were taken by using a ProX-PRESSTM 2D Proteomic Imaging System (PerkinElmer, Munich, Germany). Individual bands of interest were excised from polyacrylamide gels with a sterile surgical blade under UV. DNA was extracted by using the crush and soak method [2] with slight modification. A small hole was made using a syringe needle in the bottom of a 0.2-ml PCR tube, and the excised gel slices were placed in this tube. The PCR tube was placed into a 1.5-ml tube, and the assembly was centrifuged at 16 110 g for 10 min until the entire gel slices were collected in the lower tube. Then 1 ml of crush and soak solution containing 500 mmol l-1 of NH4OAc (BDH), 0.1% SDS and 0.1 mmol l-1 EDTA (Sigma-Aldrich) was added to the crushed gel slices and incubated overnight at 37°C with shaking at 150 rpm. After centrifugation for 10 min, the supernatant was recovered avoiding transfer of fragments of acrylamide. One volume of ice-cold molecular grade ethanol (Sigma-Aldrich) and 0.1 volume of 3 mol l-1 NaOAc (pH 5.2) (BDH) were added to re-precipitate DNA. The resulting pellet was rinsed once with 70% ethanol, dried under vacuum and re-suspended in 20 µl of 1x·tris EDTA (TE) buffer (pH 8.0). The recovered DNA was then stored at -20°C until use.

Each band was re-amplified using the original primer set as described previously and the PCR products were further purified using a High PureTM PCR product clean-up kit. The re-amplified PCR products were cloned into the pDrive Cloning Vector (Qiagen, Hilden, Germany) according to the manufacturer's protocol and sequenced. The sequences obtained from RISA bands were compared to ITS gene sequences in the NCBI GenBank database using the BlastN search option and their closely related sequences were downloaded. The recovered sequences as well as the closest identified relatives were manually imported and aligned in Molecular Evolutionary Genetics Analysis (mega) Software ver. 4.0 using ClustalW. Evolutionary distances were calculated using the method of maximum composite likelihood and phylogenetic trees were generated from the distance matrixes using a neighbour-joining tree-building algorithm. The robustness of inferred tree topologies was evaluated by using the bootstrap test (1000 bootstrap replication) available in MEGA 4.0, and the branching nodes only with bootstrap values of >50% are indicated. The main focus of this study was on RISA band CoPa1 disappearing in the presence of copper contamination. DNA sequences obtained from the clones of this band were submitted to the NCBI database with accession numbers FJ235855 and FJ235856.

Results and Discussion

This study describes a microcosm-based approach performed to examine the effect of pyrene alone and pyrene supplied in combination with insoluble copper phosphate on soil fungal populations. The microcosm was established in the context of PAH-bioremediation because pyrene, a fused tetracyclic aromatic hydrocarbon, is often employed as an indicator for monitoring PAH-contaminated sites. The microcosms were designed to resemble a PAH and metal co-contaminated soil ecosystem because many PAH polluted sites are usually co-contaminated with various metal species. The original soil was artificially contaminated with pyrene (250 mg kg-1) and Cu (≥ 300 mg kg-1) equivalent to the MOE soil guideline limit. It is well known that Cu contamination negatively affects soil microbial biomass and their enzymatic activities and many microorganisms are generally assumed to be sensitive to Cu stress unless they can express some mechanism of resistance. Cu and Cu-containing compounds have been widely applied as bactericides and fungicides in agriculture. It can be assumed that Cu contamination may reduce the metabolic potential of any indigenous PAH-utilizing microbial populations. This could partially explain the reason why biotechnologies for in situ organic pollutant treatment may be seriously limited by the presence of toxic metals. In this study, we have focussed on fungal populations. Fungi offer certain advantages over bacteria for high molecular weight PAH bioremediation in soil habitats because initial attack on high molecular weight PAHs by fungal exoenzymes appears to be more likely than attack by bacterial intracellular enzymes alone. It was hoped that excision and identification of shifted RISA gel bands quantification would provide information about the dominance of particularly competitive fungal species or the elimination of sensitive ones.

The band(s) CoPa1 shifting in the presence of copper in microcosm became the main focus of this study. It was excised from day 21 RISA gels (Fig. 1) for microbial identification. Two clone insets from CoPa1 RISA band were randomly selected for DNA sequencing analysis because the excised DNA may consist of several phylogenetically different sequences even though it shows a single band. In our case, both replicates of each F-RISA band were identical (Table 2), but comigration of phylogenetically heterogeneous sequences can also occur. The gel band and its putative taxonomic affinities were assigned to at least genus level and the band was identified as Cryptococcus pseudolongus which was able to endure pyrene contamination, but was susceptible to copper toxicity.

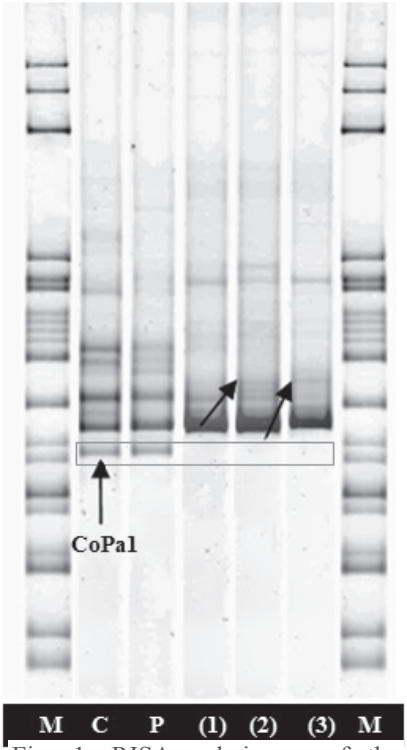

Fig. 1. RISA gel image of the fungal community composition in microcosm soil samples (day 21). Lanes **M**: marker; lane **C**: control soil; lane **P**: pyrene-spiked soil; lanes **(1), (2), (3)**: pyrene and Cu-spiked soils. Two arrows on lanes (2) and (3) also show potentially interesting bands that manifest only at high Cu contamination.

Table 2. Description of RISA band CoPa1 clones and their putative taxonomic affinities.

Accession number	Length (bp)	Presence in RISA profile			Closest match	Sequence Similarity/ Overlap (%)	Putative taxonomic affinity
		C	P	P+Cu			
FJ235855	492	+	+	_	*Cryptococcus pseudolongus* (AB105353.1)	100/97	*Cryptococcus pseudolongus*
FJ235856	492	+	+	_	*Cryptococcus pseudolongus* (AB105353.1)	100/97	*Cryptococcus pseudolongus*

Conclusions

RISA is a rapid and simple culture-independent approach for phylogenetic fingerprinting of dynamic microbial, fungal communities in polluted soils.

The members of communities responding certain way to organic and inorganic contaminants in soil can be visualised by RISA and phylogenetically identified.

It was shown by RISA followed by band identification that copper-sensitive fungal community member belongs to species Cryptococcus pseudolongus.

For practical use of microorganisms identified as perspective by phylogenetic insights provided by RISA, culture-independent approaches should be combined with conventional culture-dependant approaches involving isolation of alive members of microbial communities.

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PHYLOGENETIC FINGERPRINTING OF MICROBIAL COMMUNITIES IN METAL POLLUTED SOILS USING RIBOSOMAL INTERGENIC SPACER ANALYSIS (RISA)

Aims. The aim of this study was to demonstrate the benefits of culture-independent PCR-based fingerprinting technique called ribosomal intergenic spacer analysis (RISA) in the examination of the effects of a polycyclic aromatic hydrocarbon (pyrene) and selected toxic metal (copper) on fungal communities in soil microcosms. ***Methods.*** Shifts in soil fungal communities in response to pyrene and copper contamination were studied by ribosomal intergenic spacer analysis (RISA). ***Results.*** It was found that CoPa1 band was present at the same position in control and pyrene lanes, but it showed very weak intensity or disappeared in all Cu-added soils. This band of microbial community member susceptible to copper toxicity was identified as *Cryptococcus pseudolongus*. ***Conclusions***. RISA is a useful culture-independent approach for phylogenetic fingerprinting of dynamic microbial, fungal communities in polluted soils. The members of communities responding certain way to organic and inorganic contaminants in soil can be visualised by RISA and phylogenetically identified with the perspective of their use as bioindicators of specific contaminants.

Key words: RISA, soil, copper, pyrene, fungi, communities.