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METAGENOMIC ANALYSIS OF SOIL MICROBIAL COMMUNITIES

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Abstract - Ramonda serbica and *Ramonda nathaliae,* rare resurrection plants growing in the Balkan Peninsula, produce a high amount of phenolic compounds as a response to stress. The composition and size of bacterial communities in two rhizosphere soil samples of these plants were analyzed using a metagenomic approach. Fluorescent *in situ* hybridization (FISH) experiments together with DAPI staining showed that the metabolically active bacteria represent only a small fraction, approximately 5%, of total soil bacteria. Using universal bacteria – specific primers 16S rDNA genes were amplified directly from metagenomic DNAs and two libraries were constructed. The Restriction Fragment Length Polymorphism (RLFP) method was used in library screening. Amongst 192 clones, 35 unique operational taxonomic units (OTUs) were determined from the rhizosphere of *R. nathaliae*, and 13 OTUs out of 80 clones in total from the library of *R. serbica*. Representative clones from each OTU were sequenced. The majority of sequences from metagenomes showed very little similarity to any cultured bacteria. In conclusion, the bacterial communities in the studied soil samples showed quite poor diversity.

Keywords: Metagenome, RFLP, OTU, FISH

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INTRODUCTION

Communities of natural microorganisms often encompass a bewildering range of physiological, metabolic, and genomic diversity. To explore and potentially exploit the microbial diversity present in the soils, direct cultivation or indirect molecular approaches are used. The traditional methods of cultivation and isolation of microorganisms involves samples of as little as 0.1% to 1% of soil bacteria (Torsvik et al., 2002).

In order to circumvent some of the limitations of cultivation approaches, indirect molecular methods have been developed. Their use in phylogenetic analysis of complex ecosystems such as soil have demonstrated that the multitude of discrete prokaryotic species represented in a single sample goes far beyond the numbers and phenotypes of known cultured microorganisms (Hugenholtz, 2002; Handelsman, 2004; Daniel, 2005).

Molecular approaches in assessing bacterial diversity are based on the analysis of total DNA isolated from environmental samples (metagenomic DNA). Analysis of PCR-amplified 16S rDNA from metagenomic DNA using universal bacterial primers cloned into a vector, followed by Restriction Fragment Length Polymorphism (RFLP) and sequencing, is a basis for phylogenetic studies of bacterial communities (Guazzaroni et al., 2010).

The development of fluorescent *in situ* hybridization (FISH) over the last decade has had a great impact on microbial diversity studies, allowing the fast screening of bacteria present in the sample (Kobabe et al., 2004). In addition, the PCR-based approach which samples the total DNA present in a sample cannot distinguish living from dormant or dead bacteria. FISH targets 16S rRNA, thus assessing only metabolically active bacteria which have high cellular rRNA content. Moreover, specific probes can be used to monitor microorganisms at different levels of taxonomic specificity (López-Archilla et al., 2004; Grenni et al., 2009). Since the PCR and FISH methods are not necessarily mutually exclusive, both can be used in

parallel to catalogue and compare microbial diversity in different soil samples, and to follow dynamic changes in bacterial community structure due to altered environmental factors.

The aim of this study was to analyze bacterial diversity from two rhizosphere soil samples of *Ramonda serbica* and *Ramonda nathaliae,* rare resurrection plants growing in the Balkan Peninsula. These species are capable of surviving long dry periods, passing quickly from anabiosis to full biological activity. Stress induces the mass production of reactive oxygen species (ROS). To defend themselves against ROS, higher plants produce phenolics as radical scavengers and release them into the soil (Rice-Evans et al. 1996). Studies on *R. serbica* have shown that this plant synthesizes a high amount of phenolic compounds as a response to stress caused by dehydration (Sgherri et al., 2004). Since phenol is a well-known biocide, the rhizospheres of these two plants were especially interesting for the analyses of autochthonous bacterial communities.

In order to assess the microbial diversity in the two rhizosphere samples, a metagenomic approach based on the construction of 16S rDNA libraries has been used. The FISH technique, together with other staining procedures, has been used to estimate the number of both total and actively growing bacteria in soil.

MATERIALS AND METHODS

Sample collection

Rhizosphere soil samples of two resurrection plants, *Ramonda serbica* and *Ramonda nathaliae*, were used. Soil samples were collected in the spring of 2004 from Jelašnička gorge, Serbia, when these plants were not fully hydrated.

DAPI staining and FISH

The rhizosphere soil samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (10 mM sodium phosphate buffer, 130mM sodium chloride; pH7.2) for 3 h at 4°C. Subsequently, the samples were washed three times in PBS to remove excess fixative. The pellets were first resuspended in 4`,6-diamidino-2-phenylindole (DAPI) solution (50μg of DAPI per ml of PBS) and incubated for 5 min. Excess DAPI was removed by washing in PBS. The experiment was carried out in duplicates. Subsequently one sample per rhizosphere was hybridized with rhodamine-labeled 16S rRNA probe, EUB 338 (Lee et al., 1999) and the other with rhodamine-labeled nonsense probe (NONEUB 338). Bacteria were collected by membrane filtration of the samples through a 0.2μm pore size Millipore polycarbonate filter. The cells were transferred from the filters by placing them face down onto microscope slides containing a drop of sterile water. After being air dried for about 30 min, the filters were removed from the slides, and the slides were treated with methanol:formalin solution (90:10 vol/vol) for 15 min, rinsed with distilled water, and air dried. Bacteria were counted under a fluorescent microscope (Olympus BX51).

Extraction of DNA

Metagenomic DNAs were extracted by a slightly modified Saano and Lyndstrom method (1995) using electroelution in DNA purification.

PCR amplification and libraries construction

The bacterial 16S rRNA genes were amplified using universal bacterial primers 27F (Lane et al., 1991) and 1392R (Marchesi et al., 1998). The PCR reaction was performed following touchdown conditions: 1) 3 min at 95° C; 2) 15 cycles of 40 s at 95° C, 1 min of annealing, decreasing temperature by 1° C in each subsequent cycle, starting from 60° C, extension for 2 min at 72° C; 3) 10 cycles of 40 s at 95° C, 1 min at 45° C, 2 min 72° C; 4) 10 min extension at 72° C.

The amplified products were purified with a QIA quick PCR purification kit (Qiagen, Hilden, Germany). The purified products were cloned into the pUC19 vector.

Table 1. Actively growing bacteria in two rhizosphere samples.

Rhizophere soil sample	CFU per gram of soil	Actively growing bacteria per gram of soil (FISH)
Ramonda serbica	$9x10^5$	$2x10^9$
Ramonda nathaliae	$1.86x10^6$	$9x10^8$

Screening of libraries

The isolated vectors from the recombinant clones were subjected to Restriction Fragment Length Polymorphism (RFLP) analysis using *Rsa*I endonuclease following the manufacturer's instructions (Fermentas, Vilnius, Lithuania). All sequences with same RFLP pattern were classified as one operational taxonomic unit (OTU).

DNA sequencing and in silico analysis

Representative clones from each OTU were selected for sequencing using M13/pUC universal sequencing primers. Sequencing was performed on an Applied Biosystems ABI Prism 3100 DNA Sequencer (Servizio di Sequenciamente di DNA del C.R.I.B. – Universita di Padova). The resulting sequences were compared with those available at both GenBank and Ribosomal Database Project (RDP) to determine their approximate phylogenetic affiliation (RDP; Cole et al., 2009). Chimeric sequences identified by independently comparing the alignments at the beginning and at the end of each sequence, and the alignments of the entire sequences, were excluded from further analyses.

RESULTS AND DISCUSSION

Soil samples from the rhizosphere of two endemorelict plants, *Ramonda serbica* and *Ramonda nathaliae*, collected at the beginning of spring, were subjected to the DAPI staining and FISH analyses to monitor the size and metabolic activity of the microbial community. DAPI nonspecifically binds to DNA, thereby detecting living, dormant and dead bacteria. The metabolically active bacteria in the soil samples were estimated using a rhodamine-labeled universal probe for the domain *Bacteria* (EUB 338). In order to compensate for the non-specific staining, a nonsense probe (NONEUB 338) was used. As nonspecific staining may result from the binding of either the oligonucleotide or the fluorochrome component of probe, correction was made by subtracting counts obtained by the non-specific nonsense probe from counts obtained by specific staining. Control experiments were performed with excess unlabeled probe. FISH and DAPI staining results have shown that only 5% of bacteria were actively growing while the others were dormant in the form of spores, or dead.

The FISH analysis results were compared with the traditional counting of colony forming unit (CFU) method. In the case of *R. serbica,* there was approximately a ten thousand fold greater number of growing bacteria determined by the FISH compared to the CFU counts (Table 1). For the *R. nathaliae* soil sample there was approximately a thousand fold greater number of viable cells obtained by FISH. As expected, the two methods showed that only a small fraction of bacteria can be assessed with traditional plating methods.

In parallel, the metagenomic DNAs isolated from the two rhizosphere soil samples were used as templates in PCR amplification with bacteria specific primers to construct 16S rRNA gene libraries. In total, 192 clones from the *R. nathaliae* rhizosphere (designated as RN1-192) and 80 clones from the *R. serbica* rhizosphere (designated as RS1-80) were obtained. The libraries were screened by the RFLP method. In the *R. nathaliae* 16S rDNA library, 35 OTUs were identified, while in *R. serbica* library, 13 OTUs were identified. Representative clones from each OTU were sequenced, and the analysis showed the presence of only 4 and 10 different 16S rDNA sequences in the metagenome of *R. serbica* and *R. nathaliae*, respectively (sequences accession numbers DQ398028 - DQ398040). Exclusion of chimeric inserts and sequences that could not be placed within currently recognized bacterial divisions is one of the reasons for the

A)

OTUs	SOURCE TAXON	SEQUENCE MATCH	SEQUENCE ACCESSION NUMBERs (NCBI)
	Uncultured bacterium	0.519	AY212633
RS1	Acidovorax sp.	0.528	AY258065
	Uncultured alpha proteobacterium	0.779	AY922144
RS2	Alpha proteobacterium CRIB-04	0.770	DQ123621
	Uncultured bacterium	0.764	AB177159
RS19	Pedomicrobium australicum	0.779	X97693
	Uncultured beta proteobacterium	0.519	AB076866
RS21	Diaphorobacter nitroreducens	0.520	AB076855
	Uncultured rumen bacterium	0.181	AB185606
RN1	Prevotella sp. oral clone FW035	0.174	AY349394

RN9	Planctomyces sp.	0.823	X81953
	Uncultured Acidobacteria bacterium	0.246	AY281358
RN11	Bacteria	0.246	Z95722
	Uncultured Chloroflexaceae	0.189	AF421750
RN29	bacterium		
	Streptomyces sp	0.196	AM039887
	Uncultured bacterium	0.561	AJ863173
RN30	---		
	Uncultured bacterium	0.519	DQ067003
RN38	---		
	Uncultured bacterium	0.772	DQ086464
RN44	Pseudomonas fulgida	0.778	AJ492830
	Uncultured actinomycete	0.968	AB015562
RN46	Microbacterium sp. oral clone AV005b	0.956	AF385527
	Uncultured alpha proteobacterium	0.814	AF509580
RN50	Sphingomonas rhizogenes	0.817	AY962684
	Uncultured bacterium	0.684	AY218692
RN65	----		

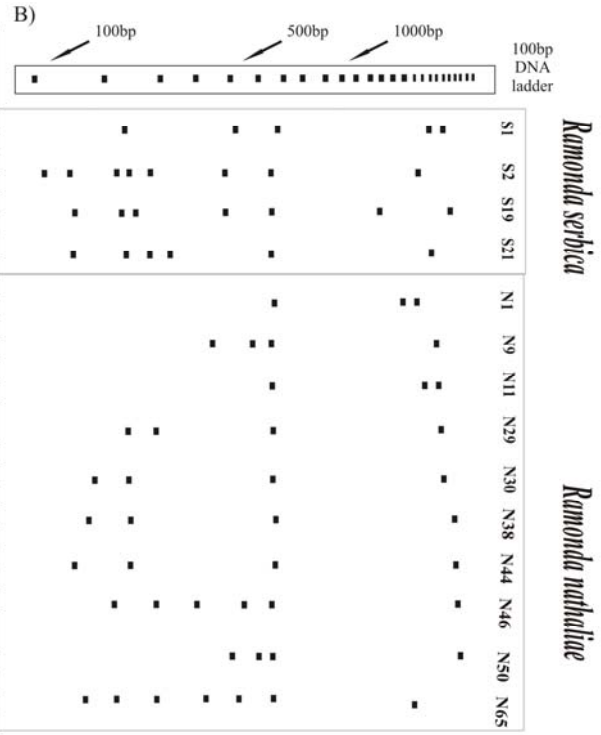

Fig. 1. Analyses of 16S rDNA libraries derived from metagenomic DNAs isolated from the rhizospheres of *R. serbica* and *R. nathaliae*. A) Sequence match algorithm scores of OTU representatives from both libraries. Dashes indicate that there was no sequence similarity with either uncultured or cultured bacteria in the RDP database. B) Schematic presentation of *Rsa*I RFLP patterns of sequenced OTU representatives. 100 bp molecular weight ladder is presented above the graph.

decrease in distinguished OTU numbers. In addition, the RFLP pattern depends on insert orientation and thus influences OTU discrepancy before and after sequencing.

In silico analyses showed that the most abundant bacteria in the two libraries belong to the class of yet uncultured bacteria (Figure 1 A and B). In the soil of *R. serbica,* 40% of all sequenced 16S rDNAs showed a similarity to the group of uncultured α-Proteobacteria and more than 30% to the group of uncultured β-Proteobacteria. Most sequences that showed similarities with the 16S rDNA of previously cultivated bacteria belonged to the Proteobacteria (15%). In the *R. nathaliae* soil, more than half of the sequences showed similarities with uncultured bacteria, about 25% were similar with actinomycetes, more than 10% with acidobacteria, and approximately 30% with other uncultivated bacteria. About 15% of the sequences showed a similarity to the 16S rDNA of previously cultivated streptomycetes and 10% to cultivated pseudomonades.

R. serbica synthesizes a high amount of phenolic compounds as a response to stress and the concentration of phenolic acids in the first phase of rehydration is four fold higher than in dehydrated plant (Peterson 2001, Sgherri et al. 2003). The soil samples analyzed in the present study were collected when the plants were not fully hydrated. As phenol has a toxic effect on the majority of bacteria, the low diversity in the studied soil samples could be the consequence of elevated phenol concentration.

In general, there is a considerable variability in the abundance of different phyla and class members in different environmental samples, judging by the

abundance of 16S rRNA genes. the wealth of species may be extremely high, as has been shown by Torsvik and colleagues, who identified more than 4000 different species in 1 g of forest soil (Torsvik et al., 1990). On the other hand, 80 to 100% of the California coastal bacterioplankton community is represented by a single bacteria group (Rehnstam et al., 1993).

It is important to consider that the libraries of PCR-amplified 16S rRNA genes do not always represent a complete or accurate picture of the bacterial community. Moreover, all of the currently published sequences of 16S rRNA genes represent just a small fraction of the overall bacterial diversity (Schloss et al., 2004). In addition, there may be biases in the contributions of various bacterial groups in libraries. As different soil micro-organisms have different susceptibilities to cell lysis methods, the number of 16S rDNA sequences present in the libraries depends on the extraction method (Gabor et al., 2003). So far metagenomic approaches have only scratched the surface of the genomic, metabolic and phylogenetic diversity stored in the soil metagenome, but their application is very important since metagenomics shows superiority over traditional cultivating methods. Although considerable progress in the characterization of microbial communities by metagenomic approach has been made, a further improvement of sequencing technologies and bioinformatics tools is required.

Analyses of the bacterial communities' structure in the soil samples taken when *R. serbica* and *R. nathaliae* were under stress showed that only a few bacterial phyla were present. It would be interesting to investigate if there is a change in bacterial community composition and size due to plants' specific life cycles, i.e. change in phenol concentration.

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REFERENCES

Cole, J. R., Wang, Q, Cardenas, E., Fish, J, Chai B., Farris, R. J., Kulam-Syed-Mohideen, A. S., McGarrell D. M., Marsh T., Garrity G. M., and *J. M. Tiedje* (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* **37** (Database issue), 141-145.

Daniel, R. (2005). The metagenomics of soil. *Nature Rev.* **3**, 470-477.

Gabor, E. M., de Vries, E. J. and *D. B. Janssen* (2003). Efficient recovery of environmental DNA for expression cloning by indirect methods. *FEMS Microbiol Ecol.* **44**, 153-163.

Grenni, P., Gibello, A., Caracciolo, A.-B., Fajardo, C., Nande, M., Vargas, R., Saccà, M.-L., Martinez-Inigo, M.-J., Ciccoli, R., and *M. Martin* (2009). A new fluorescent oligonucleotide probe for in situ detection of s-triazine-degrading Rhodococcus wratislaviensis in contaminated groundwater and soil samples. *Water. Res.* **43**, 2999-3008.

Guazzaroni, M.-E., Golyshin, P. N., and *M. Ferrer* (2010). Analysis of complex microbial community through metagenomic survey, In: Metagenomics: Theory, Methods and Application, (Ed. D. Marco), 55-79. Caister Academic Press, Norfolk, UK.

Handelsman, J. (2004). Metagenomics: Application of genomics to uncultured microorganisms. *Microbiol. Mol. Biol. Rev.* **68**, 669-685.

Hugenholtz, P. (2002). Exploring prokaryotic diversity in the genomic era**.** *Genome Biol.* **3:** reviews 0003.1-0003.8.

Kobabe, S., Wagner, D., and *E.M. Pfeiffer* (2004). Characterisation of microbial community composition of a Siberian tundra soil by fluorescence in situ hybridization. *FEMS Microbiol. Ecol.* **50**, 13-23.

Lane, D. J. (1991). 16S/23S rRNA sequencing. in: nucleic acid techniques in bacterial systematics. United Kingdom: Chichester E, Stackebrandt, MM Goodfellow (ed), pp. 115-175.

Lee, N., Halkjaer, P., Andreasen, P. H., Juretschko, S., Nielsen, J. P., Schleifer, K.H., and *M. Wagner* (1999). Combination of fluorescent in situ hybridization and microautoradiography-a new tool for structure-function analyses in microbial ecology. *Appl. Environ.Microbiol.* **65,** 1289-1297.

López-Archilla, A. I., Gérard, E., Moreira, D., and *P. López-García* (2004). Macrofilamentous microbial communities in the metal-rich and acidic River Tinto, Spain. *FEMS Microbiol. Lett.* **235**, 221-228.

Marchesi, J. R, Sat,o T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., and *W. G. Wade* (1998). Design and evaluation of useful bacterium-specific PCR primers

that amplify genes coding for bacterial 16S rRNA. Appl *Environ Microbiol* **64**, 795-799.

Peterson, D. (2001). Oat antioxidants . *J. Cereal. Sci.* **33**, 115-129.

Rehnstam A., Bäckman S. S., Smith D. C., Azam F., and *A. Hagström* (1993). Blooms of sequence-specific culturable bacteria in the sea. *Microbiol. Ecol.* **102**, 161-166.

Rice-Evans, C. A., Miller, N. J., and *G. Paganga* (1996). Structure-antioxydant activity relationship on flavonoids and phenolic acid. *Free Rad. Biol. Med.* **20**, 933-956.

Saano, A., and *K. Lindstrom* (1995). Small scale exstraction of DNA from soil with spin column cleanup, In: Molecular microbial ecology manual, (Eds. A. D. L. Akkermans, J. D Van Elsas, F. J. De Bruijn), 1-6. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Schloss P. D., and *J. Handelsman* (2004). Status of the microbial census. *Microbiol. Mol. Biol. Rev.* **68**, 686-691.

Sgherri C., Cosi E., and *F. Navaro-Izzo* (2003). Phenols and antioxydative status of Raphanus sativus grown in copper excess. *Physiol. Plant.* **118**, 21-28.

Sgherri, C., Stevanović, B., and *F. Navari-Izzo* (2004). Role of phenolics in the antioxydative status of the resurrection plant Ramonda serbica during dehydration and rehydration. *Physiol. Plant.* **122**, 478-485.

Torsvik V., Gokskøyr J., and *F. L. Daae* (1990). High diversity in DNA of soil bacteria. *Appl. Environm. Microbiol.* **56**, 782-787.

Torsvik, V., Daae, F. L., Sandaa, R.A., and *L. Øvreås* (2002). Microbial diversity and function in soil: from genes to ecosystems. *Curr. Opin. Microbiol.* **5**, 240-245.

МЕТАГЕНОМСКА АНАЛИЗА ЗЕМЉИШНИХ МИКРОБИОЛОШКИХ ЗАЈЕДНИЦА

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Ramonda serbica и *Ramonda nathaliae,* ретке биљке „васкрснице“ које расту на Балканском полуострву, у одговору на стрес продукују велике количине фенола. Бактеријске заједнице пореклом из ризосфере ових биљака анализиране су метагеномским приступом. Флуоресцентна „in situ“ хибридизација (FISH) и DAPI бојење показали су да у анализираним земљиштима има свега 5 % метаболички активних бактерија. Употребом прајмера специфичних за бактеријску ДНК умножени су гени за 16S рДНК и конструисане су две генске библиотеке. Библиотеке су претраживане уз помоћ RFLP методе. Од укупно 192 клона добијена из узорка ризосфере *R. nathaliae* идентификовано је 35 оперативних таксономских јединица (OTJ), док је из узорка ризосфере *R. serbica* добијено 13 OTJ од укупно 80 клонова. Представници сваке OTJ су секвенцирани. Анализиране заједнице одликује веома мали диверзитет и већина добијених секвенци је показала малу сличност са ДНК секвенцама до сада култивисаних бактерија.