Enhancing the cultivability of bacteria with the combination of simple but unconventional methods

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INTRODUCTION

It is estimated that only 1-0.001% of bacterial species are cultivated yet, since not-yet-cultured prokaryotes are in specific physiological state or the applied cultivation methods do no fit with their growth requirements [1]. Even key microorganisms of the studied environment could be overlooked if they are "unculturable". Strain retrieval from a particular sample may depend on various factors: composition and nature of culture media (nutrients, pH, solid/liquid), incubation conditions (temperature, time, aerobic/anaerobic), pre-treatments (filtration, enrichment) and the investigated sample (biological interactions among species, sample matrix) [2, 3]. Recent studies have shown that only minor and inexpensive modifications may result in pure cultures of previously uncultivated bacteria.

OBJECTIVES

This study aimed to get pure laboratory cultures of not-yet-cultivated bacteria using simple but unconventional methods, which do not require expensive instrumental background.

STUDY SITES

Mohoş peat bog [M]
acidic, humic peat bog lake

lake in Ocna Mureş [U]
polluted, deep, saline lake

Cekend landfill site [C]
bioreactor treating leachate

GENERA DETECTED WITH CULTIVATION

M*		U			C		
Azospirillum-related genus	G	*Bacillus*		G	*Acinetobacter*	A	
Burkholderia	G	*Enterobacter*	A		*Advenella*	A	
Chitinophaga-related genus	G	*Labrenzia*	A		*Aequorivita*	A	G
Granulicella	G	*Marinobacter*		G	*Aquamicrobium*	A	G
Mucilaginibacter	G	Marivirga-related genus		G	*Bacillus*	A	G
Novosphingobium	G	*Pseudoalteromonas*	A	G	*Brevundimonas*	A	
Serratia	G	*Reinekea*		G	*Castellaniella*	A	
Telmatospirillum	G	*Roseovarius*		G	*Candidimonas*		G
Undibacterium	G	*Salinivibrio*	A	G	*Chelatococcus*	A	
Undibacterium-related genus	G	*Shewanella*	A	G	Derxia-related genus		G
		Tropicibacter		G	*Eoetvoesia*		G
		Vibrio	A	G	*Fictibacillus*		G
					Hydrogenophaga	A	
					Hyphomonas	A	
					Jonesia	A	
					Luteimonas		G
					Mesorhizobium	A	
					Noviherbaspirillum		G
					Paracoccus	A	G
					Parapusillimonas	A	G
					Prolinoborus	A	
					Pseudomonas	A	G
					Psychrobacter	A	
					Pusillimonas	A	G
					Rubellimicrobium		G
					Rufibacter	A	
					Sanguibacter	A	
					Simplicispira	A	G
					Sphingopyxis	A	
					Steroidobacter	A	

Aerobic plate counts (CFU/mL) on different media after various incubaction time

Medium *	*Incubation time*		
	7 days	*15 days*	*47 days*
MG	6.10×10^{4}	9.63×10^{4}	2.40×10^{5}
MA	7.36×10^{4}	9.60×10^{4}	1.14×10^{5}
UG	3.10×10^{4}	8.66×10^{4}	9.00×10^{4}
UA	6.00×10^{3}	1.23×10^{4}	1.36×10^{4}
CG	1.80×10^{7}	6.50×10^{7}	8.00×10^{7}
CA	2.90×10^{6}	1.02×10^{7}	2.00×10^{7}

* first letter stands for sampling site, second letter stands for gelling agent (G - gellan gum, A - agar)

Isolates were retrieved from media solidified with agar (A) or gellan gum (G); * due to unidentified technical difficulties, in the case of Mohos peat bog lake, we were not able to isolate strains from the medium solidified with agar.

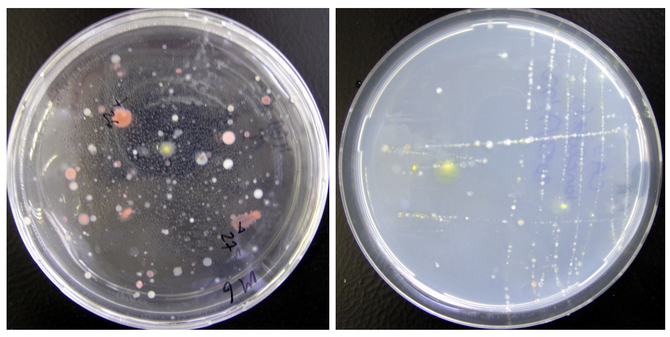
CULTIVATION STRATEGY

R2-based (~DSMZ medium 830) solid media were used adjusting their pH, organic carbon (at least threefold dilution of the original R2), salt and nitrogen content to the conditions presented in the sampling sites to mimic natural environment. For each medium type, two solidifying agents were applied, agar and gellan gum (in total, six different media were tested). Incubation time was also increased up to several weeks to enhance the retrieval of slow-growing bacteria. Furthermore, polyurethane foam-based *in situ* traps [4] were applied to enrich bacteria under natural conditions.

medium M	*medium U*	*medium C*
50 mL R2 medium	50 mL R2 medium	360 mL R2 medium
	50 g NaCl	1.81 g NH_4Cl
in 1 L distilled water	in 1 L distilled water	in 1 L distilled water
(pH 4.0)	(pH 8.0)	(pH 8.0)

media were solidified with 20 g/L agar (A) or 10 g/L gelrite (G)

polyurethane foam blocks used as *in situ* traps impregnated with different media

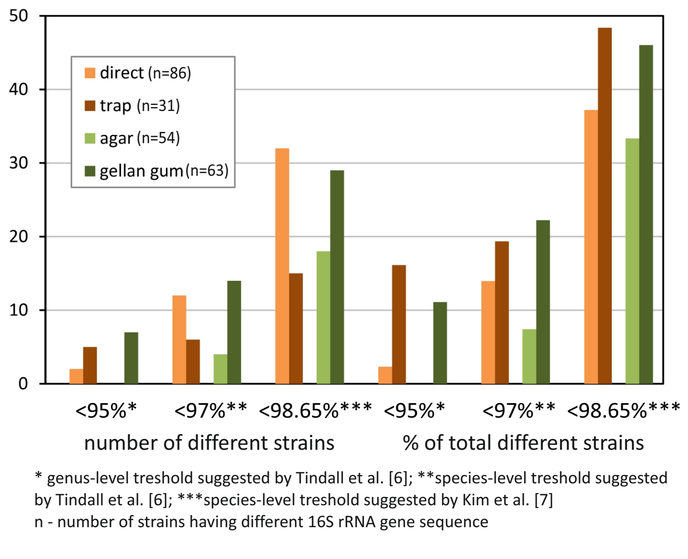


colonies grown on plates after spreading and streaking

RESULTS

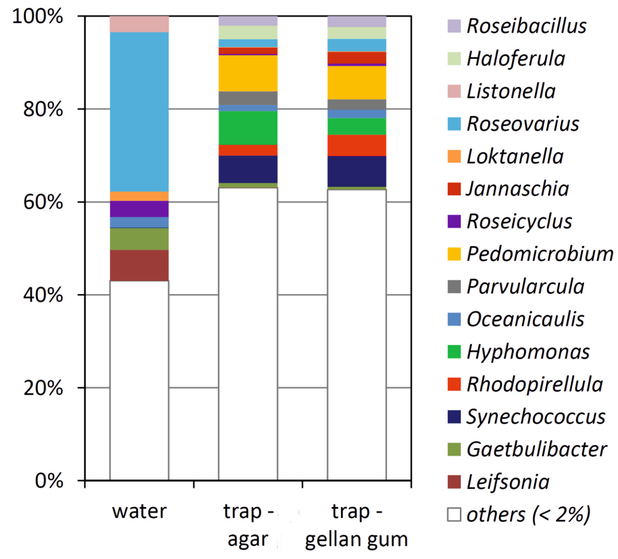
156 strains were isolated and sequenced. Strains shared 92-100% 16S rRNA gene similarities to the type strains of bacterial species with validly published names (identified using EzTaxon [5]) and were members of the phyla Proteobacteria, Firmicutes, Bacteriodetes, Actinobacteria and Acidobacteria.

Distribution of potential new genera and species within the different strain isolation methods based on 16S rRNA gene similarity values



* genus-level treshold suggested by Tindall et al. [6]; **species-level treshold suggested by Tindall et al. [6]; ***species-level treshold suggested by Kim et al. [7]
n - number of strains having different 16S rRNA gene sequence

Pyrosequencing analysis of Ocna Mureş lake [U] samples (lake water and *in situ* traps with different solidifying agents) based on the 16S rRNA gene



CONCLUSIONS

(1) Several potential new species has been isolated, which indicated the usefulness of the applied unconventional cultivation techniques. **(2)** Conventional laboratory media contain extremely high amounts of nutrients compared the conditions present in nature and this could be a key determining feature of selection during cultivation. **(3)** Gellan gum proved to be a superior solidifying agent compared to agar (higher plate counts, higher ratio of new species). **(4)** Comparison of the bacterial communities of water samples and those grown in the *in situ* traps showed that even cultivation conditions supposed to be close to the natural milieu have strong selective pressure on bacterial cultivation attempts.

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