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BACTERIAL DIVERSITY IN THE URANIUM MILL-TAILINGS GITTERSEE AS ESTIMATED VIA A 16S rDNA APPROACH

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Abstract

Bacterial diversity in a soil sample collected from uranium mill-tailings called Gittersee and situated near the city of Dresden, Germany, was analysed by using a culture-independent 16S rDNA approach exploiting PCR amplification primers 7F and 1513R. The results were compared with those obtained earlier analysing the same sample by using another primer pair, namely 43F-1404R [1]. The two 16S rDNA approaches demonstrated that *Proteobacteria* were the most predominant group in the sample, followed by *Cytophaga/Flavobacterium/Bacteroides* and by Gram positive bacteria with low and with high G+C content, too. A large number of 16S rDNA sequences from two libraries were identical or almost identical. However, the ratio between the bacterial groups represented in them significantly differed.

7F-1513R primer set retrieved in addition to the above mentioned sequences also 16S rRNA genes, those of green non-sulphur bacteria and representatives of AD1 and OP11 divisions. The latter indicates that 7F-1513R primer set seems to be more reliable in analyses of bacterial diversity.

Key words: bacterial diversity, 16S rDNA retrieval, uranium mining wastes

Introduction. Microorganisms living in extreme terrestrial environments play an integral and often unique role in the ecosystem functions [2]. Soil microbial communities are among the most complex, diverse and important assemblages in the biosphere [3–7]. For better understanding of biogeochemical processes occurring in metal-polluted environments, it is important to investigate the diversity of bacteria and their natural metabolic activities. Microbial communities in extreme environments consist of not yet cultured bacteria [8, 1]. For this reason, microbial diversity in such habitats is usually investigated by molecular methods. Uranium mining wastes represent a serious environmental problem because their soils, sediments and waters contain large amounts of U and other toxic metals. It was demonstrated that they are occupied by a large number of very diverse bacterial populations [9, 1]. These bacteria are a subject of spe-

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cial investigation because they possess capabilities to survive in metal-contaminated habitats and some of them even to biotransform heavy metals and radionuclides. The latter makes them prospective for bioremediation [10].

The goal of this work was to investigate bacterial community structure in a soil sample collected from uranium mill-tailings Gittersee in Germany by using the 16S rDNA primer set 7F-1513R and to compare the results with those previously obtained by using another primer pair, 43F-1404R, in order to estimate the reliability of the mentioned two approaches.

Materials and methods. Site history. The Gittersee mill-tailings represents remainders from a uranium manufacturer. It is situated near the city of Dresden and was in operation from 1950 to 1962. During that time about 7000 tones of uranium were produced. Until 1992 this uranium containing waste pile was used as an industrial and municipal depository [11].

Description of the sample. The soil sample Gittersee-2 (Gitt-2) was collected in 1998 from a depth of 2 to 3 m by drilling. The sample was frozen and kept at –80 °C for further analyses. It contains the following metals (in ppm) as measured by ICP-MS: Al– 14300, Ca– 2680, Cr– 60, Fe–22800, Mn– 487, Co– 13, Ni– 34, Cu– 38, Zn– 98, As– 62, Sr– 46, Ag– 0,25, Cd– 2.8, Sn– 3.3, Hg– 0.5, Pb– 16, Th– 1.4 and U– 14.

Isolation of soil DNA. Total DNA was recovered by direct lysis according to the protocol of Selenska-Pobell et al. [9] and stored at −20 °C.

Amplification of 16S rDNA fragments. 16S rDNA fragments were amplified by PCR from the purified total soil DNA. The primers used for this were the bacterial-specific 7F (5'-AGAGTTTGATCCTGGCTCAG-3') and the universal 1513R (5'-GGTTACCTTGTTACGACTT-3'). PCR amplifications were performed as described previously in [12].

Construction of 16S rDNA clone library. The resulting amplicons were cloned by using TOPO^{TM}-TA plasmid vector in *E. coli* as specified by the manufacturer (Invitrogen, The Netherlands). One hundred sixty six (166) single white colonies were randomly chosen for further analysis.

Restriction fragment length polymorphism (RFLP) analysis. All 166 amplicons were digested in parallel by using the frequently cutting endonucleases *Msp*I (5'-C ↓ CGG-3') and *Hae*III (5'-GG ↓ CC-3') (Gibco BRL, Gaithesburg, USA). The digested amplicons were separated electrophoretically in 3.5% low melt agarose gels (Biozyme, Oldenburg, Germany) for 5 h at 100 V and grouped on the basis of their fingerprints similarity.

Sequencing of 16S rDNA fragments. All 16S rDNA fingerprints found more than once in the clone library were united in groups. One representative of each group as well as several individual clones were sequenced by using the primers 7F, 342F and 1513R as published earlier [9, 12].

Phylogenetic analysis. Phylogenetic analysis of the obtained 16S rDNA sequences could be found in an earlier published paper [12].

Results and discussion. RFLP analysis of the constructed 16S rDNA$_{7F-1513R}$ library showed that 60% of the clones possessed individual profiles. This indicates a very high diversity in the studied sample. The latter is in agreement with the results obtained earlier by analysing the same sample applying 16S rDNA$_{43F-1404R}$ retrieval [1].

The remaining 40% of the fragments were divided into RFLP groups which were further analysed as described in Material and methods. The results of this analysis are presented in Fig. 1. Most of the sequenced 16S rDNA fragments showed a high identity of 95% and more to other database members. Two 16S rDNA sequences Gitt2-GS-71 and Gitt2-GS-73 were evaluated as possible chimeras.

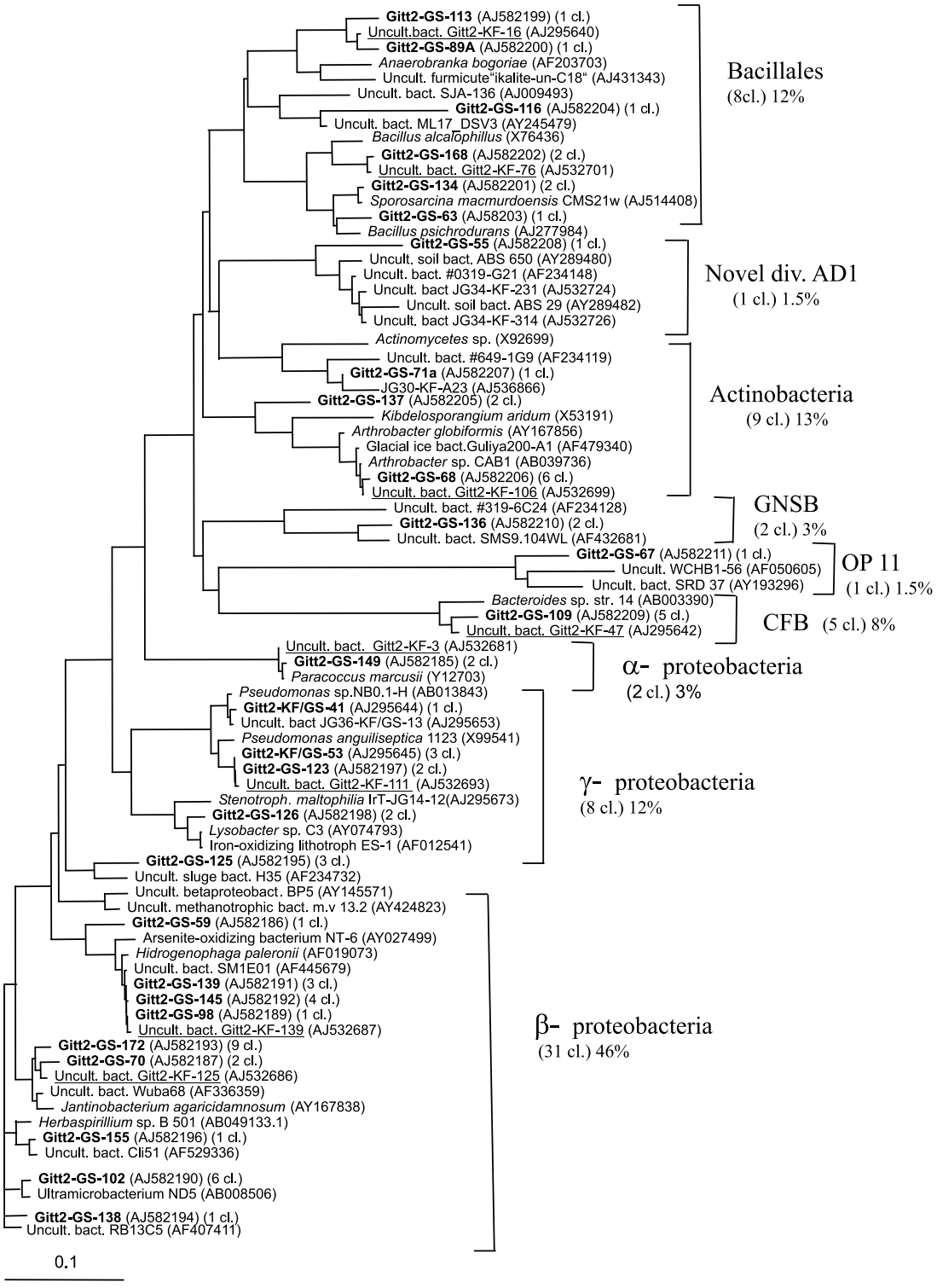
The most abundant group of sequences was affiliated to proteobacterial 16S rRNA genes. 46% of them represented beta-proteobacteria. The most frequently encountered sequence Gitt2-GS-172, representing nine clones, was affiliated with 97% identity with

16S rRNA gene of *Jantinobacterium agaricidamnosum* and with the environmental sequence Wuba 68. It shares relatively high percentage of identity also with the sequence Gitt2-KF-125. The latter was formerly retrieved in the same sample by using the mentioned primer set 43F-1404R [1]. To this cluster (see Fig. 1) also belongs the sequence Gitt-GS-70 which represents 2 clones. The sequences Gitt2-GS-145 representing 4 clones, Gitt2-GS-98 representing 1 clone, and the sequence Gitt2-GS-139 representing 3 clones, were grouped into a second cluster and they were affiliated with a very high identity of 99% to the 16S rDNA of an uncultured bacterium SM1E01 and to 16S rRNA gene of the hydrogen oxidizing bacterium *Hydrogenophaga palleronii* [13, 14]. Interestingly, the sequence Gitt-KF-139, retrieved in the same sample by the primer pair 43F-1414R, shares also 99% identity with the sequence Gitt2-GS-139. Relatively high is the identity of these two sequences with 16S rRNA gene of an isolate from a gold mine called NT-6 (see Fig. 1). This isolate represents a heterotrophic arsenite-oxidizing bacterium [6]. The sequence Gitt2-GS-102 representing six clones showed 98% similarity with the 16S rDNA of the ultramicrobacterium ND5 recovered from an urban soil [15]. Three clones represented by the sequence Gitt2-GS-125 were related to sequence H35 found in a nitrifying-denitrifying activated sludge [16].

Three sequences Gitt2-GS-138, Gitt2-GS-155, and Gitt2-GS-59 representing individual clones were also affiliated to beta-proteobacteria. The first of them was related to sequence RB13C5 retrieved from monochlorobenzene contaminated ground water. The second one was affiliated with sequence Cli51 identified in a perchloroethylene contaminated site and also with the 16S rRNA gene of the nitrogen fixing strain *Herbaspirillum* sp. B501 described in [17]. The third sequence belongs to the above described cluster including Gitt2-GS 98, 139, 145, and also Gitt2-KF-139 (see Fig. 1). Analysis of the beta-proteobacterial sequences retrieved from the sample Gitt2 demonstrated that the two primer sets, 7F-1513R and 43F-1513R, retrieved often the same sequences of this subdivision.

However, as evident from the comparative analysis of the sequenced clones of the two libraries, 16S $rDNA_{7F-1513R}$ and 16S $rDNA_{43F-1404R}$ (see Fig. 2), beta-proteobacterial sequences strongly predominated the first of them, whereas the second one was predominated by gamma-proteobacterial sequences.

12% of the analysed clones of 16S $rDNA_{7F-1513R}$ library were affiliated with gamma proteobacterial 16S rRNA genes. The sequences Gitt2-KF/GS-53 and Gitt2-GS-123 share 99% identity with the sequence Gitt2-KF-111 retrieved formerly from the same sample (see Fig. 1). The closest 16S rRNA gene of a cultured bacterium to the mentioned three sequences belongs to *Pseudomonas anguilliseptica* 1123/5 [18]. The second cluster of gamma-proteobacteria in 16S rDNA $_{7F-1513R}$ library consisting two clones was represented by sequence Gitt2-GS-126. The latter was closely related to the 16S rRNA gene of *Lysobacter* sp. C3 and to 16S rDNA of the uncultured iron-oxidizing lithotroph ES-1 recovered from ground water [19]. The third reference 16S rDNA of Gitt2-GS-126 sequence belongs to the isolate *Stenotrophomonas maltophilia* IrTz-JG14-12 recovered from a soil sample of a uranium mining waste pile located near the town of Johanngeorgenstadt in Germany. The sequence Gitt2-KF/GS-41 was almost identical with sequence KF/GS-JG36-13, retrieved also from the mentioned uranium mining waste pile [9]. Both sequences demonstrated 99% identity to *Pseudomonas* sp. clone NB0.1-H found at 6292 m depth in Japan Trench sediment. As it is seen in Fig. 1 according to gamma-proteobacteria, the use of two primer sets reveals an overlapping of representatives of this subclass and demonstrates that these bacteria are really predominant in both clone libraries. In contrast to the case of the beta-proteobacterial sequences, the amount of gamma-proteobacterial sequences was much higher in 16S $rDNA_{43F-1404R}$ library than in 16S $rDNA_{7F-1513R}$. The reciprocal relationship between beta- and gamma-proteobacterial sequences in the two clone li-


Gitt2-GS-113 (AJ582199) (1 cl.)
Uncult.bact. Gitt2-KF-16 (AJ295640)
Gitt2-GS-89A (AJ582200) (1 cl.)
Anaerobranka bogoriae (AF203703)
Uncult. furmicute"ikalite-un-C18" (AJ431343)
Uncult. bact. SJA-136 (AJ009493)
Gitt2-GS-116 (AJ582204) (1 cl.)
Uncult. bact. ML17_DSV3 (AY245479)
Bacillus alcalophillus (X76436)
Gitt2-GS-168 (AJ582202) (2 cl.)
Uncult. bact. Gitt2-KF-76 (AJ532701)
Gitt2-GS-134 (AJ582201) (2 cl.)
Sporosarcina macmurdoensis CMS21w (AJ514408)
Gitt2-GS-63 (AJ58203) (1 cl.)
Bacillus psichrodurans (AJ277984)
Bacillales
(8cl.) 12%
Gitt2-GS-55 (AJ582208) (1 cl.)
Uncult. soil bact. ABS 650 (AY289480)
Uncult. bact. #0319-G21 (AF234148)
Uncult. bact JG34-KF-231 (AJ532724)
Uncult. soil bact. ABS 29 (AY289482)
Uncult. bact JG34-KF-314 (AJ532726)
Novel div. AD1
(1 cl.) 1.5%
Actinomycetes sp. (X92699)
Uncult. bact. #649-1G9 (AF234119)
Gitt2-GS-71a (AJ582207) (1 cl.)
JG30-KF-A23 (AJ536866)
Gitt2-GS-137 (AJ582205) (2 cl.)
Kibdelosporangium aridum (X53191)
Arthrobacter globiformis (AY167856)
Glacial ice bact.Guliya200-A1 (AF479340)
Arthrobacter sp. CAB1 (AB039736)
Gitt2-GS-68 (AJ582206) (6 cl.)
Uncult. bact. Gitt2-KF-106 (AJ532699)
Actinobacteria
(9 cl.) 13%
Uncult. bact. #319-6C24 (AF234128)
Gitt2-GS-136 (AJ582210) (2 cl.)
Uncult. bact. SMS9.104WL (AF432681)
GNSB
(2 cl.) 3%
Gitt2-GS-67 (AJ582211) (1 cl.)
Uncult. WCHB1-56 (AF050605)
Uncult. bact. SRD 37 (AY193296)
OP 11
(1 cl.) 1.5%
Bacteroides sp. str. 14 (AB003390)
Gitt2-GS-109 (AJ582209) (5 cl.)
Uncult. bact. Gitt2-KF-47 (AJ295642)
CFB (5 cl.) 8%
Uncult. bact. Gitt2-KF-3 (AJ532681)
Gitt2-GS-149 (AJ582185) (2 cl.)
Paracoccus marcusii (Y12703)
α- proteobacteria
(2 cl.) 3%
Pseudomonas sp.NB0.1-H (AB013843)
Gitt2-KF/GS-41 (AJ295644) (1 cl.)
Uncult. bact JG36-KF/GS-13 (AJ295653)
Pseudomonas anguiliseptica 1123 (X99541)
Gitt2-KF/GS-53 (AJ295645) (3 cl.)
Gitt2-GS-123 (AJ582197) (2 cl.)
Uncult. bact. Gitt2-KF-111 (AJ532693)
Stenotroph. maltophilia IrT-JG14-12(AJ295673)
Gitt2-GS-126 (AJ582198) (2 cl.)
Lysobacter sp. C3 (AY074793)
Iron-oxidizing lithotroph ES-1 (AF012541)
γ- proteobacteria
(8 cl.) 12%
Gitt2-GS-125 (AJ582195) (3 cl.)
Uncult. sluge bact. H35 (AF234732)
Uncult. betaproteobact. BP5 (AY145571)
Uncult. methanotrophic bact. m.v 13.2 (AY424823)
Gitt2-GS-59 (AJ582186) (1 cl.)
Arsenite-oxidizing bacterium NT-6 (AY027499)
Hidrogenophaga paleronii (AF019073)
Uncult. bact. SM1E01 (AF445679)
Gitt2-GS-139 (AJ582191) (3 cl.)
Gitt2-GS-145 (AJ582192) (4 cl.)
Gitt2-GS-98 (AJ582189) (1 cl.)
Uncult. bact. Gitt2-KF-139 (AJ532687)
Gitt2-GS-172 (AJ582193) (9 cl.)
Gitt2-GS-70 (AJ582187) (2 cl.)
Uncult. bact. Gitt2-KF-125 (AJ532686)
Uncult. bact. Wuba68 (AF336359)
Jantinobacterium agaricidamnosum (AY167838)
Herbaspirillium sp. B 501 (AB049133.1)
Gitt2-GS-155 (AJ582196) (1 cl.)
Uncult. bact. Cli51 (AF529336)
Gitt2-GS-102 (AJ582190) (6 cl.)
Ultramicrobacterium ND5 (AB008506)
Gitt2-GS-138 (AJ582194) (1 cl.)
Uncult. bact. RB13C5 (AF407411)
β- proteobacteria
(31 cl.) 46%
0.1

braries (see Fig. 2) can be explained by possible preferential PCR amplification due to the primers used.

Only one sequence, Gitt2-GS-149, representing two clones in 16S rDNA$_{7F-1513R}$ library was affiliated to alpha-proteobacteria. This sequence was closely related to the 16S rRNA gene of *Paracoccus marcusii* [20] and was almost identical to sequence Gitt2-KF-3, retrieved formerly from the 16S rDNA$_{43F-1404R}$ library. Analogically to the case of gamma-proteobacteria, the primer set 43F-1404R retrieved alpha-proteobacteria in higher percentages than the set 7F-1513R (see Fig. 2).

Due to previously described PCR biases caused by the primer's nature [21] our results demonstrate that the two primer sets allow reliable identification of alpha-, beta-, and gamma-proteobacteria but in considerably different ratios. Interestingly, in the above mentioned uranium mining waste pile Johanngeorgenstadt alpha-proteobacteria were the most predominant group identified both by 16S rDNA$_{7F-1513R}$ and by 16S rDNA$_{43F-1404R}$ approaches [1, 12]. The latter indicates that the bacterial communities structure in uranium wastes is site-specific.

The second and the third predominant groups of bacteria in the Gitt2 soil sample were affiliated to bacilli and actinobacteria (see Fig. 1). The cluster of bacilli related sequences includes sequence Gitt2-GS-134 which was affiliated with 99% identity to the 16S rRNA gene of *Sporosarcina macmurdoensis CMS 21w*. Two clones represented by sequence Gitt2-GS-168 were affiliated with sequence Gitt2-KF-76, identified in the same sample by the alternative primer pair, 43F-1404R. Both sequences were related to the 16S rRNA genes of *Bacillus alcalophilus*. Sequence Gitt2-GS-63 was affiliated with the 16S rRNA gene of *Bacillus psyhrodurans* [5]. The individual sequence Gitt2-GS-116 was related to sequences ML17_DSV3 and SJA-136 retrieved from environmental samples [22]. Two sequences, Gitt2-GS-89A and Gitt2-GS-113, were affiliated with similarity of 97% and 95%, respectively, to the sequence KF-Gitt2-16, retrieved formerly from the sample by using the primers 43F and 1404R. The closest 16S rRNA gene to these sequences belongs to *Anaerobranca* sp., but the identity shared was very low (about 87% and 88%). The latter indicates that the sequences Gitt2-GS-89A, Gitt2-GS-113 as well as KF-Gitt2-16 represent a novel lineage of bacilli. Representatives of bacilli were identified in sample Gitt2 by using the primer pair 43F-1404R as well. Their amount was, however, significantly larger (see Fig. 2).

Nine of the clones of the 16S rDNA$_{7F-1513R}$ library were affiliated with actinobacterial 16S rRNA genes (see Fig. 1). Sequence Gitt2-GS-68 representing six clones was affiliated with 98% identity to the 16S rRNA gene of *Arthrobacter* sp. CAB1 and to sequence Gitt-KF-106. The latter retrieved by using the alternative primer pair. Sequence Gitt2-GS-137 representing two clones was related with an identity of 95% to the glacial ice bacteria Guliya 200-A1 and shared the same percentage of identity with the above mentioned sequence Gitt-KF-106. Both sequences Gitt2-GS-68 and Gitt2-GS-137 as well as the sequence Gitt-KF-106 fell into subclass *Actinobacteridae* of Actinobacteria. The individual sequence Gitt2-GS-71a (1081 bases) represents a part of sequence Gitt2-GS-71 which was evaluated as a possible chimera. First part of this chimerical sequence consisting of 260 bases was affiliated to gamma-proteobacteria whereas the main part of 1081 bases was affiliated to the subclass *Rubrobacteridae* of Actinobacteria. Interestingly, sequence Gitt2-GS-71a was identical to sequence JG34-KF-A23, retrieved from the uranium mining waste pile near Johanngeorgenstadt by using the 16S rDNA$_{43F-1404R}$ approach. Both sequences shared a relatively high similarity with the clone #649-1G9 described as yet-to-be-cultured Rubrobacterium (4). As it is shown

Fig. 1. Phylogenetic tree of Gitt2 16S rDNA sequences retrieved by using 7F-1513R PCR primer pair (given in bold). Matched sequences retrieved in the same sample with the alternative PCR primer pair 43F-1404R are underlined

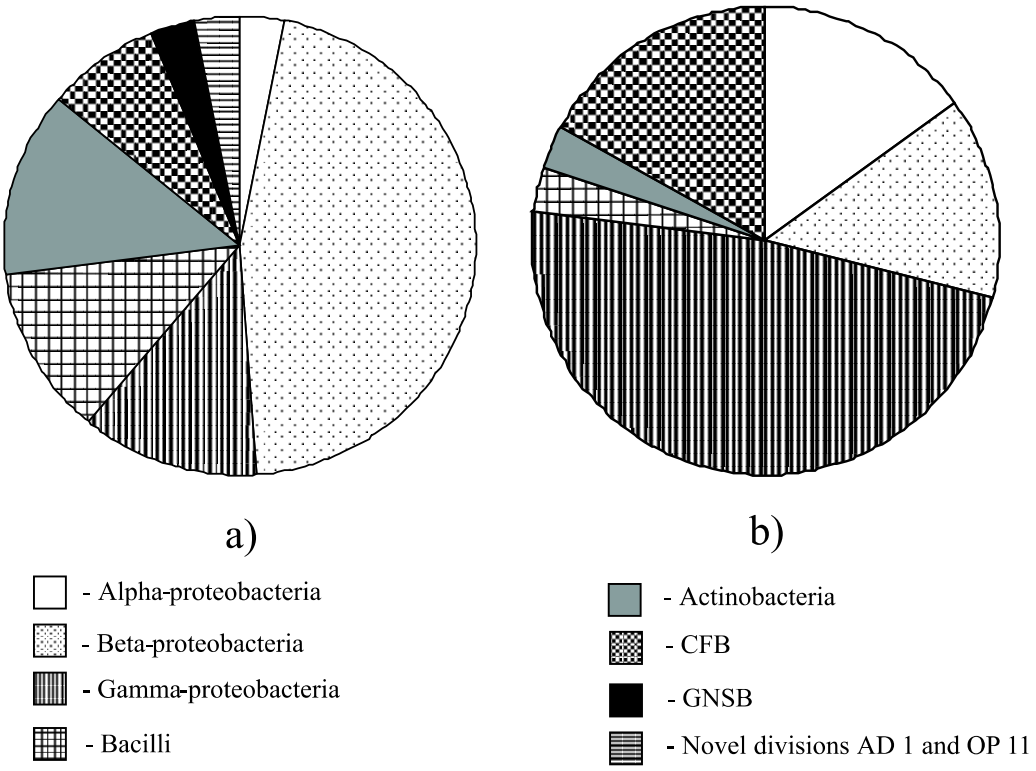


Fig. 2. Size of the bacterial groups (given in percentages) identified in sample Gitt2 by using: a) 16S rDNA$_{7F-1513R}$, and b) 16S rDNA$_{43F-1404R}$ retrievals

in Fig. 2, the 16S rDNA$_{7F-1513R}$ approach was more successful in identification of *Actinobacteria* than the 16S rDNA$_{43F-1404R}$ one.

Five of the clones of 16S rDNA$_{7F-1513R}$ library represented by the sequence Gitt2-GS-109 were affiliated to *Cytophaga/Flavobacterium/Bacteroides* group of bacteria. This sequence was closely related to the previously described sequence KF-Gitt2-47 retrieved by using the primer pair 43F-1404R. Both sequences were related to a strain of *Bacteroides* sp. (see Fig. 1) The number of CFB clones in the library 16S rDNA$_{43F-1404R}$ was higher than those retrieved from 16SrDNA$_{7F-1513R}$ (see Fig. 2).

The main distinction between the two clone libraries, however, was that 16S rDNA$_{7F-1513R}$, in contrast to 16S rDNA$_{43F-1404R}$ one, contained green non-sulphur bacterial (GNSB) sequences and also sequences representing novel bacterial divisions (see Figs 1 and 2). One of the sequences of 16S rDNA$_{7F-1513R}$ library, Gitt2-GS-136, was affiliated with relatively high identity with sequences #0319-6C24 and SMS9.104WL representing not yet cultured GNSB. Another one, Gitt2-GS-55 shared an identity of 96% with the newly described sequence ABS-650 of the so-called division AD1 [7]. In 16S rDNA$_{7F-1513R}$ library constructed for Gittersee 2 sample AD1 group was represented only by one sequence. The latter indicates that the population of AD1 cluster was not predominant in the sample Gitt2 and possibly, therefore, not detected by 16S rDNA $_{43F-1404R}$ retrieval. Sequence Gitt2-GS-67 shares 94 per cents of identity with sequence WCHB1-56 retrieved from hydrocarbon contaminated aquifer [3] and 93% identity with the sequence SRD37 retrieved from a sulphur river filaments [23] (see

Fig. 1). Both reference sequences were affiliated to the candidate division OP11 [8] consisting of yet to be cultured bacteria.

Conclusions. The comparative analysis of the two 16S rDNA clone libraries constructed by using primer pairs 7F-1513R and 43F-1404R demonstrates that bacterial communities of the uranium mill-tailings Gittersee are very complex and diverse. Proteobacteria are the most predominant division in the studied sample Gittersee-2. However, the proteobacterial alpha-, beta- and gamma-subdivisions were represented in different ratios inside the two clone libraries. The remaining clones of the two libraries were affiliated with 16S rRNA genes of CFB, bacilli and actinobacteria, also retrieved in significantly different ratios. The primer pair 7F-1513R showed advantages in amplification of 16S rDNA sequences from green non-sulphur bacteria and also from the novel divisions AD1 and OP 11. The latter were not identified in the 16S rDNA$_{43F-1404R}$ library constructed for the Gittesee-2 sample. These results demonstrate the necessity of using more than one PCR pair for direct molecular analyses of environmental samples, in order to obtain more profound and reliable information about the structure of the natural bacterial communities.

28 16S rDNA sequences obtained in this work were deposited to the EMBL under accession numbers AJ 295644, AJ 295645 and AJ 582185 – AJ 582211.

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