

**Microbial diversity in ground water at the deep-well monitoring site S15  
of the radioactive waste depository Tomsk-7, Siberia, Russia**

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*If the Lord Almighty had consulted me before  
embarking on Creation, I should have  
recomedned something simpler.*

(Alfonso X, Spanish King of Castile and Leon)



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## Introduction

The concept of underground storage of radioactive wastes is applied since decades with the primary objective to permanently isolate the wastes from the biosphere (Bachofen et al., 1998; Christofi and Philip, 1997; Stroes-Gascoyne and West, 1996). As a result of these anthropogenic activities the contamination of the subterranean sediments and ground waters with uranium and other hazardous metals is a serious environmental problem worldwide. Many countries are involved in research programs that examine the underground radioactive wastes injection option of repository for the future, which requires an extremely good knowledge of the subsurface environment to be used as a host (Pedersen, 1996). It was demonstrated, that these extreme habitats are occupied with viable and metabolically active microorganisms, which exploit a wide range of redox reactions and thus influence the geochemical conditions there (Ehrlich, 1998; Lovley and Chapelle, 1995; Pedersen, 1997).

Number of studies have shown that microorganisms may effectively interact with the introduced toxic metals and radionuclides via direct and indirect mechanisms, which cause mobilization or immobilization of the metals (McLean and Beveridge, 2001; Merroun and Selenska-Pobell, 2001; Selenska-Pobell, 2002; Benyehuda et al., 2003). These activities encompass biotransformations such as oxidation (Beller, 2005; Lack et al., 2002) and reduction (Lovley, 2002; Shelobolina et al., 2004); biosorption by cell surface polymers (Hafez et al., 2002; Selenska-Pobell et al., 1999; Vieira and Volesky, 2000); uptake of metals inside the cells (Francis et al., 2004; McLean and Beveridge, 2001; Merroun et al., 2003; Suzuki and Banfield, 2004); induction of metal precipitation and generation of minerals (Douglas and Beveridge, 1998, Macaskie et al., 1992; Merroun et al., 2005; Renninger et al., 2001, 2004); or alteration of metal speciation caused by microbially induced redox changes in the environment (Bosecker, 1997).

The above described microbe-metal interactions may strongly influence the fate of the hazardous metals in and outside the habitat where they have been deposited (Banaszak et al., 1999; Francis, 1998; Lloyd and Lovley, 2001; Pedersen, 1996; Selenska-Pobell, 2002). A detailed understanding of the underlying mechanisms is therefore needed to predict the future migration of the radionuclides and to develop bioremediation strategies (Bachofen et al., 1998; Pedersen, 1996). For this reason, information is required about the structure, composition, distribution and activities of the microbial communities in these extreme habitats. The physiological and genetic diversity of the microbial community is a function of the habitat characteristics and their changes may be used as indicators for environmental

perturbation and for natural degradation and attenuation processes (Macalady and Banfield, 2003).

During the last years increasing number of studies were focused on investigation of microbial communities associated with different pristine and contaminated subsurface environments (Chandler et al., 1998; Fields et al., 2005; Frederikson et al., 2004; Nazina et al., 2004; North et al., 2004; Tiago et al., 2004). For revealing the complexity of the microbial communities there a variety of culture-dependent and -independent approaches were used. One of them, the direct molecular 16S rDNA retrieval, expanded greatly our knowledge about the composition and diversity of the microbial communities. Based of such analyses, entirely new lineages were discovered and it was demonstrated that some of their members can be major constituents of a given habitat (Dojka et al., 2000; Hugenholtz et al., 1998; Pace, 1997; Webster et al., 2004).

Applying the 16S rDNA molecular approach, bacterial diversity was studied in a deep-subsurface clay environment (Boivin-Jahns et al., 1996). Similar environments are a possible choice as depository sites for long life nuclear wastes. Members from the groups of Proteobacteria and Gram positive bacteria were identified. The authors raised the question about the origin of these microorganisms and hypothesized that they became trapped during the sedimentary processes, millions of years ago or migrated from the surface sediments. However, according to them the latter variant is less possible, because of the low permeability of the clay formation. Lawrence and colleagues showed the presence of bacteria in similar geological deposits applying variety of culture-dependent and -independent approaches (Lawrence et al., 2000). They demonstrated that the bacteria were non-homogeneously distributed within the studied sediments and supported the contention that they may survive for geologic time periods. The occurrence of nanobacteria in clay-minerals and other geological deposits implies about their possible role in formation of the latter (Folk and Lynch, 1997). These bacteria are characterized with very small-sized cells. Some of them are starvation forms, while others remain small even under nutrient-rich conditions (Torrella and Morita, 1981). Ultramicrobacteria were shown to be abundant in freshwater (Hahn et al., 2003) and marine environments (Haller et al., 1999), copiotrophic urban soil (Iizuka et al., 1998). However, almost no information exists about the composition and diversity of the small-sized microbial fraction within the subsurface environments so far. Recently applying the 16S rDNA retrieval, Miyoshi and colleagues reported the presence of microorganisms in ground waters that pass through 0.2  $\mu\text{m}$  pore-sized filters (Miyoshi et al., 2005). Interestingly, most of them represented novel bacterial lineages like Candidate divisions OD1 and OP11.

The Äspö deep hard rock laboratory has been constructed as a part of the Swedish program, which examines the option for a geological disposal of radioactive wastes (Pedersen, 1996). The extensive culture-dependent and independent studies demonstrated the presence of viable microorganisms, comprising iron-, sulphate-reducing and homocetogenic bacteria, and methanogenic Archaea (Pedersen et al., 1996; Pedersen, 1997; Kotelnikova and Pedersen, 1997). The distribution of these diverse metabolic groups of organisms was shown also in ground waters at depths of up to 1300 m at other sites of the Fennoscandian Shield (Haveman and Pedersen, 2002). The stable total cell numbers of the cultured organisms with increasing depth at this area indicated that there must be sufficient energy and carbon sources to support the microbial populations. Therefore, a model has been proposed of a hydrogen-driven biosphere in deep igneous rock aquifers in the Fennoscandian Shield (Pedersen, 1997). The presence of lithotrophic based ecosystems was reported also for other subsurface environments (Anderson et al., 1998; Chappelle et al., 2002; Bach and Edwards, 2003; Stevens and McKinley, 1995).

The lithoautotrophic microorganisms are capable to derive their energy via oxidation and reduction of different inorganic substrates and can use CO<sub>2</sub> as a sole carbon source. There are six known CO<sub>2</sub> fixation pathways found within the three domains of life - Bacteria, Archaea and Eucarya (Macalady and Banfield, 2004). In addition, some bacterial and archaeal groups, like *Thermotogae*, *Nitrospirae* and *Thermoplasmata*, include autotrophs that use unknown CO<sub>2</sub> fixation pathways. Considering the lack of organic electron donors and acceptors in most of the subsurface environments, it is surprising that almost no information exists about the composition and diversity of the subsurface lithoautotrophic communities so far (Alfreider et al., 2003; Lawrence et al., 2000). For determining the organisms involved in the CO<sub>2</sub> fixation, as well as in other crucial processes within the subsurface like sulphate reduction, ammonia-oxidation, nitrate reduction and others it is advantageous to focus directly on the functional genes of interest. Recently, Alfreider and colleagues have studied the diversity of the form I (*cbbL*) and form II (*cbbM*) of the large subunit of the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), involved in the CO<sub>2</sub> fixation, in subsurface environment (Alfreider et al., 2003). The analyses demonstrated the presence of various obligate and facultative lithoautotrophic Proteobacteria which possess the form I and II of the enzyme. Bacteria, harbouring the form II of RubisCO were reported also for subsurface low permeability geological deposits (Lawrence et al., 2000).

As a part of the Canadian program of subsurface disposal of radioactive wastes, extensive microbiological studies were performed in deep granite formations of the Canadian Shield (Stroes-Gascoyne and West, 1996). Significant number of bacteria was found in the nutrient-

poor ground waters from this site (Jain et al., 1997). They encompass denitrifying, N<sub>2</sub>-fixing, iron- and sulphate-reducing bacteria. No iron-oxidizing or methanogenic organisms were detected. The majority of the isolates were Gram negative bacteria.

The presence of active microbial populations was demonstrated also for a variety of pristine subsurface habitats. For example, psychrotolerant and microaerophilic bacteria were cultured from boreal ground water (Männistö and Puhakka, 2002). Representatives mainly of Actinobacteria, but also of the  $\alpha$ -subclass of Proteobacteria and *Bacteroidetes* (formerly *Cytophaga/Flavobacterium/Bacteroides*) were isolated from alkaline ground waters (Tiago et al., 2004). Interestingly, only few of the isolated strains were able to grow in laboratory conditions at pH values, characteristic for their indigenous habitat. Studying the attached and unattached bacterial communities in an acidic crystalline rock aquifer, it was demonstrated that the majority of organisms were suspended in the ground water and were not attached to the rock surface (Lehman et al., 2001). The analysis of the phylogenetic diversity of microorganisms in a deep subsurface paleosol has shown that the most frequently occurring bacteria there were members of Proteobacteria, Gram positive bacteria and *Bacteroidetes* (Chandler et al., 1998). In addition novel bacterial and archaeal clusters were identified as well. Recently, applying the 16S rDNA molecular approach, members of Euryarchaeota and Crenarchaeota were found for the first time in fully oxic, temperate groundwater environment (O'Connell et al., 2003). Their functional significance in such habitat not typical for the known representatives of the domain Archaea remains to be determined.

Extensive microbiological studies were performed also on subsurface environments, naturally containing uranium deposits or anthropogenically contaminated with radioactive and other wastes (Elias et al., 2003; Nazina et al., 2004; Selenska-Pobell, 2002; Suzuki et al., 2003). These extreme habitats are reservoir of unusual microorganisms, which may possess properties that can be used for development of bioremediation approaches. The fission reactors in the Oklo region are the best known natural analogue for geological disposal of radioactive wastes (Pedersen et al., 1996). Applying the 16S rDNA retrieval, Pedersen and colleagues demonstrated the presence of diverse Proteobacteria and Gram positive bacteria there. Interestingly each of the studied boreholes was dominated by bacterial species that did not dominate in any of the other wells.

The Hanford and the Savannah River Sites in USA represent areas, where large amounts of radioactive wastes were stored in tanks below the surface since the middle of the last century (Domingo et al., 1998; Fredrickson et al., 2004). The occurrence of viable microorganisms was shown by Fredrickson and colleagues in the high-level nuclear waste-contaminated vadose sediments at the Hanford Site. The majority of the isolated organisms were

*Arthrobacter* spp. In addition, Gram positive bacteria with low G+C and different subclasses of Proteobacteria were isolated as well. Some of the cultured strains were able to survive acute doses of ionizing radiation. Microbiological studies, performed at the Savannah River Site demonstrated the ability of the indigenous microorganisms to colonize in high densities the introduced metal coupons in this oligotrophic and radioactive environment (Domingo et al., 1998). Some of them were sulfate-reducing and acid-producing bacteria, which is important in the context of the microbial-influenced corrosion of metal components in the radioactive storage basins.

Injection of mixed radioactive and other industrial wastes into deep geological horizons has been practiced in several sites in Russia, since the 1960s (Nazina et al., 2004; Wickham et al., 2003). Recently, Nazina and colleagues have shown the presence of variety of microorganisms, like aerobic heterotrophs, anaerobic denitrifiers, fermenters, sulfate reducers and methanogens in formation waters from the deep repository site Severnyi, Siberia. Interestingly, the penetration of the radioactive wastes, containing also organic compounds, led to stimulation of the microbial activities and formation of methane and hydrogen sulfide. The authors suggest that the production of biogenic gas may result in local increase of the pressure in the depository and consequent discharge of wastes onto the surface.

High numbers of nitrate- and sulfate-reducing bacteria were found in the aquifer contaminated by the Shiprock uranium mill tailings (Elias et al., 2003). Dominance of ammonia-oxidizing bacteria of the  $\beta$ -subclass of Proteobacteria was demonstrated by both 16S rDNA and *amoA* retrievals in that site (Ivanova et al., 2000). These bacteria seem to be the likely source of high nitrate levels there. The authors suggest that before the stimulation of the microbial uranium reduction, the removal of nitrogenous compounds should be undertaken at this site. Multiple influence of nitrate on uranium solubility during bioremediation of uranium-contaminated subsurface sediments was presented also by others (Finneran et al., 2002). Recently Beller reported on nitrate dependent U(IV) oxidation by a chemolithoautotrophic bacterium *Thiobacillus denitrificans* (Beller, 2005). The oxidation of the insoluble form of U(IV) to U(VI) is of particular concern because the latter is highly toxic due to its solubility in ground waters. In addition, the soluble U(VI) compounds can be easily mobilized and transported outside the depository.

The presence of diverse bacterial communities was reported for soil, water and sediments of different uranium mining waste piles (Selenska-Pobell, 2002). The predominant bacterial groups there, identified via different molecular approaches, represented organisms which are known to biotransform metals.

Recently, Fields and colleagues studied the microbial diversity in ground waters contaminated with nitric acid and uranium wastes using the 16S rDNA retrieval (Fields et al., 2005). They observed that the diversity there was lower in comparison with that at a pristine site. The majority of the clones encountered in the impacted ground waters were related to *Azoarcus* spp. from the  $\beta$ -subclass of Proteobacteria. Populations of *Pseudomonas* spp. were present at the both contaminated and uncontaminated sites, but at different relative abundance. *Arthrobacter* and *Novosphingobium* spp. were identified only at the not contaminated site.

Microbial communities formed on the surface of exogenously introduced substrata in uranium-contaminated and pristine subsurface environments were investigated by Reardon et al. (2004) applying the 16S rDNA approach. The hematite-associated communities at both areas were represented mainly by members of the  $\beta$ -subclass of Proteobacteria. However, the community formed at the contaminated site was not so diverse, compared to that in the pristine area. Recently, it was reported, that  $\beta$ -proteobacteria, like *Thiobacillus denitrificans* and *Nitrosolobus multiformis*, dominate in oligotrophic and microaerophilic uranium contaminated sediments (Suzuki et al., 2003). After the incubation of these sediments under anaerobic conditions with organic substrates, shifting of the microbial community to representatives of *Firmicutes* like *Clostridium* and *Desulfosporosinus* spp. occurred. The authors suggest, that these organisms could be the major contributors to U(VI) reduction at this site.

From the described studies it is obvious that the knowledge about the physiological and genetic diversity of the microbial communities is crucial for monitoring the fate of the wastes and for applying the correct remediation strategies.

Recently, *in situ* bioremediation strategies were undertaken in order to stimulate the activities of bacteria, capable to reduce uranium, in uranium-contaminated subsurface habitats (Anderson et al., 2003; North et al., 2004). The addition of acetate stimulates the activity of *Geobacter* species and decreases the concentrations of the soluble form of uranium in the ground water. However, the authors have shown that when the conditions no longer favored the growth of *Geobacter* spp., uranium removal was less effective.

The process of uranium reduction is generally limited to anaerobic conditions. Furthermore it is questionable if the addition of electron donors like acetate is appropriate for long-term immobilization of uranium. Acetate is the preferential substrate for variety of microorganisms and their activation may lead to undesired effects, like for example production of biogenic gases (see above Severnyi site, Nazina et al., 2004). It should be noted, that in contrast to the different organic contaminants, the radionuclides and other heavy metals cannot be destroyed but only concentrated or converted to a solid form for recycling or final disposal (Macaskie et

al., 2000). Therefore, before applying of these *in situ* bioremediation strategies, the fate of the reduced U(IV) must be understood (Suzuki et al., 2005).

An alternative bacteria-based method for removal of toxic metals and radionuclides from contaminated sites is the development of bio-ceramic filters based on indigenous microorganisms, which are adapted to the extreme conditions of the site. For instance, a strain of *Bacillus sphaericus*, isolated from a uranium mining waste pile was used for the preparation of biological ceramics (biocers) (Raff et al., 2003). A high binding capacity of uranium and copper was demonstrated for the sol-gel immobilized cells, spores, and purified surface-layer protein of this strain. Moreover, it was shown that this biocers are suitable for reversible usage.

Anderson and Pedersen reported on *in situ* development of bacteriogenic iron oxides by *Gallionella ferruginea* biofilms which concentrate lanthanides and actinides from the ground water at concentrations higher than inorganic material (Anderson and Pedersen, 2003). The formation of the biofilms of this bacterium, which is frequently occurring at this site, was enhanced in reactors, installed 300 m below the surface at the Äspö area.

The aims of this work were to study the overall microbial diversity, applying the 16S rDNA retrieval, in ground waters at one deep-well monitoring site of the radioactive waste depository Tomsk-7 in Siberia; to analyze the oligotrophic and autotrophic bacterial populations, the latter based on the RubisCO approach; to culture some indigenous bacteria and to study their interactions with uranium and other heavy metals.

Tomsk-7, renamed recently to Seversk, is an industrial-scale complex for enriched uranium production (formely also for plutonium) located near the town of Tomsk in Siberia, Russia (Wickham et al., 2003). Disposal activities at this site constitute one of the world's largest discharges of radioactive material to the environment. The wastes were injected directly into porous and permeable sandstone horizons separated by clay horizons. The studied monitoring well S15 is located about 2 km apart from one of the radioactive waste injection areas in Tomsk-7. The well uncovers aquifer at 290 to 324 m below the land surface, which corresponds to one of the radioactive disposal horizons (EC Project: FIKW-CT-2000-00105; <http://www.galson-sciences.co.uk/BORIS>). Extensive hydrogeochemical studies were performed at different sites of the Tomsk-7 injection area in order to understand the behavior of a wide range of radionuclides, their transport and retardation in ground waters. However, almost no information exists about the structure of the natural microbial communities in this area and their ability to interact with the radioactive wastes. Previous microbiological studies,

performed on the same site, were based only on culture-dependent methods, which could not estimate the real microbial diversity (Wickham et al. 2003).

This work contains four chapters. In the first two of them the diversity of the bacterial and archaeal populations at the S15 monitoring well was studied via the 16S rDNA molecular approach in order to obtain detailed information about the composition of the microbial communities in this extreme habitat. Analyses were performed on the whole biomass of one sample, and on different biomass fractions, from a parallel sample. The fractioning gave an opportunity to identify representatives of small-sized and planktonic bacteria, which were masked when the analyses are performed on the whole biomass. In the third chapter the lithoautotrophic bacteria at the studied site were retrieved applying the RubisCO approach. These bacteria seem to be abundant in the studied oligotrophic subsurface habitat.

Of special interest was also to isolate and characterize bacterial strains from the S15 monitoring well in order to determine their heavy metal resistance and to study their interactions with uranium. These studies were performed by using techniques like X-ray absorption spectroscopy (XAS), transmission electron microscopy (TEM), energy dispersive X-ray (EDX) analysis and flow cytometry and are presented in chapter IV. Our results can serve for risk assessments of the studied Siberian subsurface environment, where deposition of radioactive wastes is still performed.

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## Chapter I

### **Molecular Bacterial Diversity in Water at the Deep-Well Monitoring Site at Tomsk-7**

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## Abstract

Bacterial diversity was studied in a water sample collected from a depth of 290–324 m below the land surface from the S15 monitoring site near the city of Tomsk, Siberia, Russia. In order to overcome the inherent problems of bacteria culturing, we applied a direct molecular approach based on PCR amplification and cloning of the 16S rDNA of the members of the natural bacterial communities. Sixty-five percent of the cloned sequences were almost identical to the 16S rRNA genes of *Dechlorosoma* sp. belonging to the Rhodocyclus group of the  $\beta$ -subclass of Proteobacteria. There was a relatively high number of sequences that shared a high identity with the 16S rRNA genes of some cultured  $\gamma$ -Proteobacteria (6.9%),  $\alpha$ -Proteobacteria (4.6%), and with the sequences of the mostly uncultured members of *Cytophaga/Flavobacterium/Bacteroides* (5.4%), Actinobacteria (4.6%), and *Planctomycetales* (2.3%). The rest of the retrieved sequences were clustered in small groups, each containing about 1% of the total number of clones. These matched with the 16S rDNA sequences of *Bacillales*, *Holophaga/Acidobacteria*, Nitrospira, and  $\delta$ -Proteobacteria, respectively.

## Introduction

Investigation of bacterial communities in extreme terrestrial environments such as uranium mining wastes or other heavy metal and radionuclide contaminated sites is important for understanding the role of these communities in the biogeochemical processes of those environments. It has been reported that many bacteria can interact with toxic metals and radionuclides via oxidation (Di Spirito and Tuovinen, 1982, Lack et al., 2002), reduction (Lovley, 1993 and 2002), bioaccumulation (Macaskie et al., 1992, McLean and Beveridge, 2001, Selenska-Pobell et al., 1999), and biomineralization (Douglas and Beveridge, 1998). These biotransformations lead to changes in the metals' mobility and can strongly influence their fate in the environment (Francis, 1999, Merroun and Selenska-Pobell, 2001, Merroun et al., 2003, Selenska-Pobell, 2002). However, because of our limited knowledge of the nutrient requirements and other life necessities of most bacterial species in nature, presently only a few percent can be cultured under laboratory conditions. Fortunately, problems with culturing natural bacterial communities have been largely overcome during the last decade by the application of direct molecular approaches, such as 16S rDNA retrieval. The use of such culture-independent techniques in microbial ecology has substantially expanded the current knowledge surrounding microbial diversity and activity (Dojka et al., 2000; Hugenholtz et al., 1998a, b; Ka et al., 2001; Pace, 1997; Pedersen et al., 1996a). Moreover, the cultivation of novel bacterial isolates with previously undescribed metabolic characteristics from a large variety of environments is becoming routine and is no longer particularly surprising, although



it is still very exciting (Bruns et al., 2003; Cho and Giovanoni, 2004; Jansen et al, 2002; Kato et al., 1998; Liu et al., 1997; Michaud et al., 2004; Straub et al., 2001; Straus et al., 1999; Takai et al., 2001). Here, bacterial diversity was studied in water collected from the S15 monitoring well, located near to the Borehole Radioactive Waste Injection Site Tomsk-7 in Siberia, Russia.

## **Materials and Methods**

### **Water Sampling**

Monitoring well S15 corresponds to Aquifer II, which is located 290–324 m below the land surface (EC Project: FIKW-CT-2000-00105; <http://www.galson-sciences.co.uk/BORIS>). First, underground water from the well was pumped out by an electric pump. Then, three water samples, each with a volume of 1 L were poured into sterile glass vessels filled with nitrogen gas to keep the samples isolated from contact with the atmosphere. The biomass from the water samples was concentrated via subsequent filtration on three types of filters: one glass fiber filter with a pore size of 1.2  $\mu\text{m}$ , and two nitrocellulose filters with pore sizes of 0.45 and 0.22  $\mu\text{m}$ . The filters were kept frozen at  $-20^{\circ}\text{C}$  for further analyses.

### **DNA Extraction**

Total DNA was extracted from the water samples using the method described by Selenska-Pobell et al., (2001). This procedure includes direct bacterial lysis followed by purification and concentration using AXG-100 Nucleobond-type anion exchange cartridges (Machery-Nagel, Düren, Germany). The DNA investigated in this study, called S15A, was recovered from the total biomass of one of the water samples, which was concentrated on the three different types of filters, mentioned above. The filters were shaken together for 1 hr at  $37^{\circ}\text{C}$  in 20 mL of buffer G3 with a pH of 8 (50 mM EDTA, 50 mM Tris-HCL, 0.5% Tween 20, 0.5% Triton X100, lysozyme 4 mg/mL) and then centrifuged. The supernatant was purified following the protocol of Machery-Nagel for extraction of bacterial genomic DNA. The obtained DNA pellet was washed with 70% ethanol, dried at room temperature, and then dissolved in 50  $\mu\text{L}$  of TE buffer (10 mM Tris, 1 mM EDTA, pH 8). Using one of the other water samples from well S15, DNA was recovered from each of the two biomasses collected on 0.45  $\mu\text{m}$  (S15B) and on 0.22  $\mu\text{m}$  (S15D) filters in separate.

### **Polymerase Chain Reaction (PCR) Amplification**

The PCR amplifications of the 16S rDNA fragments from the DNA samples were carried out in a Biometra thermal cycler (Göttingen, Germany). Reaction mixtures had a final volume of 20  $\mu\text{L}$  and contained 200  $\mu\text{M}$  deoxynucleotide triphosphates, 2.5 mM  $\text{MgCl}_2$ , 10 pmol of each primer, 1–5 ng of template DNA, and 1 U *AmpliTaq* gold polymerase with the corresponding

10x buffer (Perkin Elmer). The PCR primers used to amplify the 16S rRNA gene sequences were as follows: bacterial 16S<sub>7-21:F</sub> (5'-AAGAGTTTGATY-MTGGCTCAG-3') and universal 16S<sub>1492-1513:R</sub> (5'-TACGGYTACCT TGTTACGACTT-3'), *E. coli* numbering. Prior to the amplification, the DNA was denatured at 95°C for 3 min. This step was followed by 25 polymerization cycles consisting of three steps: 90 sec at 94°C, 40 sec at 55°C, and 90 sec at 72°C. At the end of the reaction an extension for 20 min at 72°C was performed.

### **Construction of the 16S rDNA Libraries**

The amplified 16S rDNA fragments were directly cloned in *E. coli* using a TOPO<sup>TM</sup>-TA cloning vector (Invitrogen, The Netherlands) following the instructions of the manufacturer. A total of 135 white colonies were randomly picked and cultured overnight at 37°C in 2 mL Luria Broth (LB). The size of the 16S rDNA inserts was checked by *in situ* PCR using the forward M13 (-40) (5'-GTTTTCCAGTCACGA-3') and the reverse M13 (5'-CAGGAAACAGCTA-TGAC-3') primers, followed by agarose gel electrophoresis with subsequent ethidium bromide staining. One hundred thirty clones possessing correct 16S rDNA inserts were stored as glycerol cultures at -80°C for further analysis.

### **Restriction Fragment Length Polymorphism (RFLP) Typing**

For screening the 16S rDNA diversity of the clone library, the PCR products were digested in parallel with three frequently cutting endonucleases: *MspI*, *HaeIII*, and *RsaI* (Gibco BRL; Life Technologies, Inc.; Gaithersburg, MD, USA). The digests were analyzed electrophoretically in 3.5% Small DNA Low Melt agarose gels (Biozyme, Hessisch Oldenburg, Germany). The RFLP patterns obtained were compared and grouped in 16S rDNA RFLP types.

### **Sequence Analysis**

One representative of each RFLP group as well as all the individual clones were sequenced. Direct sequencing of the PCR products was performed using standard bacterial 16S rDNA primers on an ABI PRISM 310 Genetic Analyser (Perkin Elmer, Foster City, California, USA). The following program was applied: denaturation at 96°C for 2 min followed by 25 cycles of 45 s at 96°C, 15 s at 55°C, and 4 min at 60°C. The 16S rDNA sequences were compared with those available in the GenBanks by BLAST analysis. The CLUSTAL W alignment program was used for sequence alignment. Phylogenetic trees were generated based on the results of the neighbor-joining algorithm with distance analysis with Jukes-Cantor corrections according to the PHYLIP v.3.5 package (Felsenstein, 1993). The sequences were checked for the presence of chimeras by using the RDP CHECK\_CHIMERA program.

## 16S rDNA Sequence Accession Numbers

The 16S rDNA sequences retrieved in the S15A sample were deposited in the European Molecular Biology Library (EMBL) database under accession numbers AJ534658–AJ534692.

## Results and Discussion

### Estimation of Diversity of the Cloned 16S rDNA Fragments by RFLP Typing

The full-length 16S rDNA inserts of 130 clones were categorized by RFLP analysis using *MspI* and *HaeIII* enzymes. As a result eleven groups were found that possessed identical or almost identical 16S rDNA RFLP patterns. Nineteen clones possessed inserts with individual patterns not closely related to any other in the library. One of the RFLP groups was extremely large and consisted of 85 clones. They were additionally screened with the *RsaI* enzyme, which also revealed identical RFLP profiles, confirming that all the clones possess the same sequence. The other RFLP groups consisted of two to five clones (see Table 1).

### Sequence Analysis of the Cloned Environmental 16S rDNA Fragments

Figure 1 and Table 1 represent analysis of the sequences representing the above-mentioned RFLP groups and the individual RFLP types from the 16S rDNA library of the S15A sample.

### Proteobacteria

As evident from the results shown in Figure 1, the two representative sequences (S15A-MN2 and S15A-MN6) from the most abundant RFLP group shared more than 99% similarity with the 16S rRNA genes of the *Dechlorosoma* sp. PCC. *Dechlorosoma* genus belongs to the Rhodocyclus group of the  $\beta$ -subclass of Proteobacteria and was described as consisting of (per)chlorate-reducing bacteria (CIRB) (Coates et al., 1999b). These bacteria seem to be very significant to remediation of (per)chlorate-contaminated environments because the end product of their dissimilatory (per)chlorate reduction is the harmless chloride (O'Connor and Coates, 2002). It was also reported that at the intermediate step of this process, (per)chlorate-reducing bacteria produce extracellular O<sub>2</sub>, which can be used by hydrocarbon-oxidizing bacteria in anaerobic environments (Coates et al., 1998). The true environmental role of *Dechlorosoma*, however, has yet to be determined because they are ubiquitous in a broad range of environments, including pristine ones. This suggests that their distribution is not restricted by (per)chlorate availability. Recently, it was demonstrated that several (per)chlorate-reducing organisms (including *Dechlorosoma* species) are capable of anaerobic Fe(II) oxidation. The end product of this metabolism is amorphous Fe(III) oxide, which can immobilize heavy metals and radionuclides (Lack et al., 2002).

In addition to the large group of *Dechlorosoma* sp. 16S rDNA specific sequences, several additional clones of the S15A library contained inserts that shared a high degree of identity

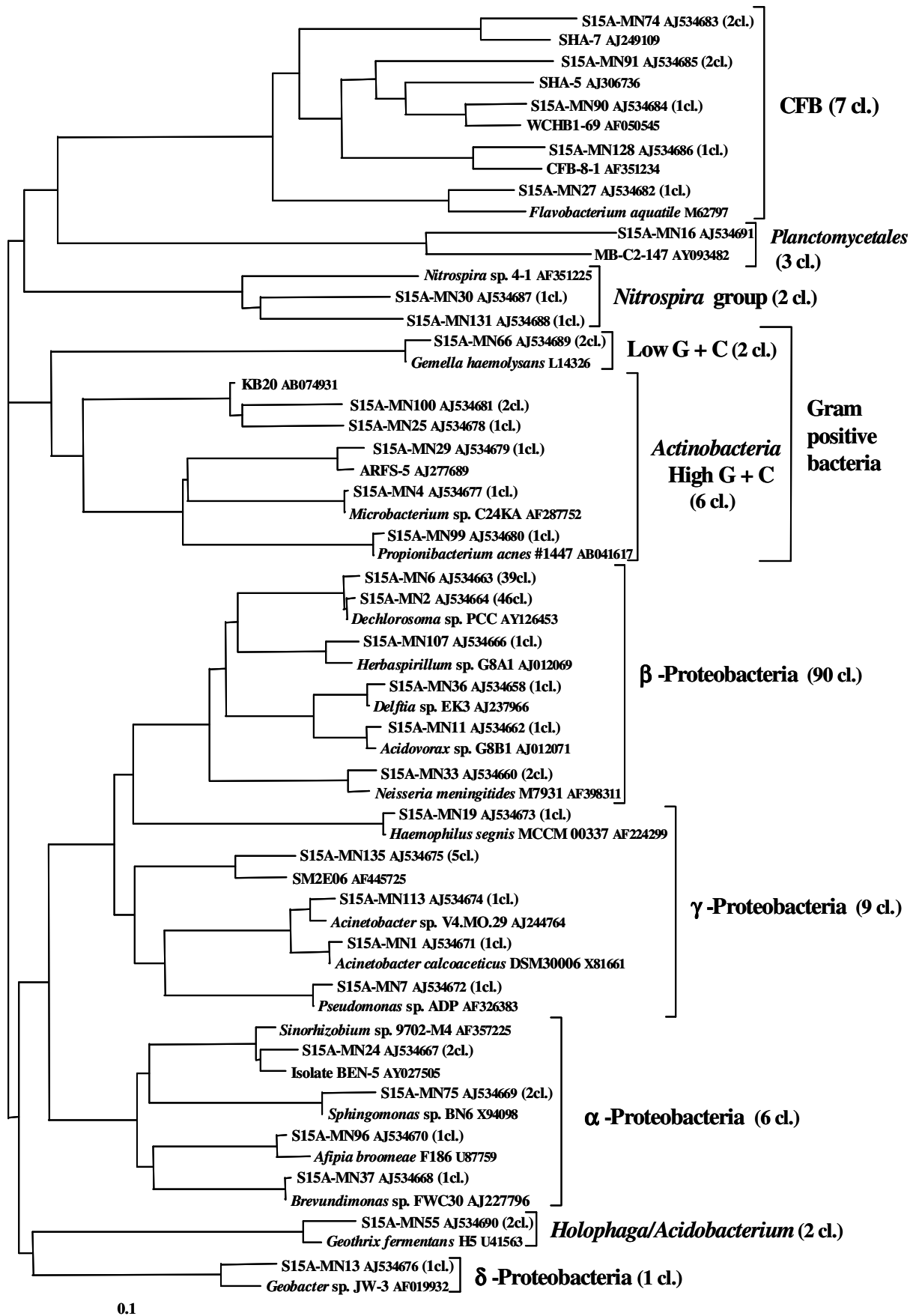


Fig. 1. Phylogenetic tree of the 16S rDNA sequences retrieved from the S15A sample. The number of clones is shown in brackets.

with other  $\beta$ -proteobacterial 16S rRNA genes. One of the sequences, S15A-MN36, showed a high degree of similarity with 16S rDNA of *Delftia* sp. strain EK3. The latter was isolated from a biofilm reactor capable of degrading 1,3-dichloropropene (Katsileva et al., 1999). The sequences S15A-MN107 and S15A-MN11 were related to the 16S rRNA genes of two other  $\beta$ -proteobacterial strains, namely, *Herbaspirillum* sp. G8A1 and *Acidovorax* sp. G8B1. These strains were isolated from freshwater ditches and are capable of anaerobic mineralization of quaternary carbon atoms (Kniemeyer et al., 1999). Interestingly, the predominance of  $\beta$ -proteobacterial sequences also was demonstrated in the samples collected from the boreholes of natural fission reactors in the Oklo region of Gabon, Africa, by Pedersen et al. (1996b). Snaider et al. (1997) reported the predominance of representatives of the Rhodocyclus group in a bacterial community found within an activated sludge of a municipal wastewater treatment plant. According to Snaider and his colleagues, members of this group are often underestimated when cultivation techniques are applied. The sequence S15A-MN33 shares an identity correlation of 97% with the 16S rRNA gene of the human pathogen *Neisseria meningitides* M7931 and represents a RFLP group consisting of two members. Interestingly, in recent times it was published that large nonpathogenic bacterial populations occupying various soil and water environments are phylogenetically related to different animal and human pathogens. The isolated representatives of such populations possess a high level of ecological and metabolic diversity, and their role in the natural environments has yet to be clarified (Coenye et al., 2003; Drancourt, 2000; Hauben et al., 1999;).

The second numerically predominant cluster of the library consisted of a group of five 16S rDNA cloned fragments represented by the sequence S15A-MN135 and four individual sequences, all related to  $\gamma$ -proteobacterial 16S rRNA genes. The closest matching sequence (SM2E06) in the Gene Bank to the S15A-MN135 was retrieved from a travertine depositional area at Mammoth Hot Springs, Yellowstone Natural Park. The individual sequence S15A-MN7 shares a 98% identity with the 16S rRNA gene of the *Pseudomonas* sp. strain ADP. The latter uses atrazine as a sole source of nitrogen for growth and, furthermore, can metabolize this compound under nongrowth conditions, which is a significant ability in the context of environmental bioremediation (Mandelbaum et al., 1995). Another individual sequence, S15A-MN19, was related to the 16S rRNA gene of *Haemophilus segnis* MCCM 00337 (Olsen et al., 2001).

The sequences S15A-MN113 and S15A-MN1 shared an identity on the species level (i.e., more than 98%) with the *Acinetobacter* spp. 16S rRNA genes. Sequences closely related to the latter also were found in other subterranean environments, including different boreholes,

Table 1. Affiliation of the 16S rDNA sequences of the S15A sample (1.2, 0.45 and 0.22  $\mu\text{m}$ )

Clone name (Accession number)	Number of clones	Closest phylogenetic relative (EMBL No.)	BLAST % of similarity
		<b><math>\alpha</math>-Proteobacteria</b>	
S15A-MN24 (AJ534667)	2	<i>Sinorhizobium</i> sp. 9702-M4 (AF357225)	97
		Arsenit-oxidizing bact. BEN-5 (AY027505)	97
S15A-MN96 (AJ534670)	1	<i>Afipia broomeae</i> F186 (U87759)	98
S15A-MN37 (AJ534668)	1	<i>Brevundimonas</i> sp. FWC30 (AJ227796)	99
S15A-MN75 (AJ534669)	2	<i>Sphingomonas</i> sp. BN6 (X94098)	97
		<b><math>\beta</math>-Proteobacteria</b>	
S15A-MN2 (AJ534664)	46	<i>Dechlorosoma</i> sp. Iso1 (AF170350)	99.2
S15A-MN6 (AJ534663)	39	<i>Dechlorosoma</i> sp. PCC (AY126453)	99.8
S15A-MN107 (AJ534666)	1	<i>Herbaspirillum</i> sp. G8A1 (AJ012069)	97
S15A-MN36 (AJ534658)	1	<i>Delftia</i> sp. EK3 (AJ237966)	99
S15A-MN11 (AJ534662)	1	<i>Acidovorax</i> sp. G8B1 (AJ012071)	99
S15A-MN33 (AJ534660)	2	<i>Neisseria meningitidis</i> M7931 (AF398311)	97
		<b><math>\gamma</math>-Proteobacteria</b>	
S15A-MN7 (AJ534672)	1	<i>Pseudomonas</i> sp. ADP (AF326383)	98
S15A-MN19 (AJ534673)	1	<i>Haemophilus segnis</i> MCCM 00337 (AF224299)	98
S15A-MN135 (AJ534675)	5	Uncultured bacterium SM2E06 (AF445725)	95
S15A-MN113 (AJ534674)	1	<i>Acinetobacter</i> sp. V4.MO.29 (AJ244764)	98
S15A-MN1 (AJ534671)	1	<i>Acinetobacter calcoaceticus</i> DSM30006 (X81661)	99
		<b><math>\delta</math>-Proteobacteria</b>	
S15A-MN13 (AJ534676)	1	<i>Geobacter</i> sp. JW-3 (AF019932)	97
		<b><i>Cytophaga/Flavobacterium/Bacteroides</i></b>	
S15A-MN74 (AJ534683)	2	Uncultured bacterium SHA-7 (AJ249109)	92
S15A-MN91 (AJ534685)	2	Uncultured bacterium SHA-5 (AJ306736)	93
S15A-MN90 (AJ534684)	1	Uncultured eubacterium WCHB1-69 (AF050545)	94
S15A-MN128 (AJ534686)	1	Uncultured CFB group bacterium 8-1 (AF351234)	94
S15A-MN27 (AJ534682)	1	<i>Flavobacterium aquatile</i> (M62797)	94
		<b>Gram+, High G+C; Actinobacteria</b>	
S15A-MN100 (AJ534681)	2	Uncultured bacterium KB20 (AB074931)	95
S15A-MN25 (AJ534678)	1	Uncultured bacterium KB20 (AB074931)	93
S15A-MN29 (AJ534679)	1	Uncultured bacterium ARFS-5 (AJ277689)	97
S15A-MN4 (AJ534677)	1	<i>Microbacterium</i> sp. C24KA (AF287752)	99.7
		<i>Microbacterium kitamiense</i> A1 (AB013920)	98
S15A-MN99 (AJ534680)	1	<i>Propionibacterium acnes</i> #1447 (AB041617)	99
		<b>Gram+, Low G+C; Firmicutes</b>	
S15A-MN66 (AJ534689)	2	<i>Gemella haemolysans</i> (L14326)	98
		<b><i>Holophaga/Acidobacterium/Geothrix</i></b>	
S15A-MN55 (AJ534690)	2	<i>Geothrix fermentans</i> H5 (U41563)	96
		<b><i>Nitrospira</i> Group</b>	
S15A-MN30 (AJ534687)	1	Uncultured <i>Nitrospira</i> sp. clone 4-1 (AF351225)	91
S15A-MN131 (AJ534688)	1	Uncultured bacterium FW118 (AF524003)	92.7
		Uncultured <i>Nitrospira</i> sp. clone 4-1 (AF351225)	92.6
		<b><i>Planctomycetales</i></b>	
S15A-MN16 (AJ534691)	3	Uncultured bacterium MB-C2-147 (AY093482)	90
		<b>Novel or chimeras</b>	
S15A-MN46	1	Uncultured bacterium ZZ14AC20 (AY214201)	89
S15A-MN56	1	<i>Natronoanaerobium</i> G-M16NWC-4 (AJ271452)	89

indicating that *Acinetobacter*-related bacteria might be common in the groundwater (Pedersen et al., 1996 a, b). The closest matching sequence to S15A-MN1 was the 16S rRNA gene of *A. calcoaceticus* DSM30006, a strain capable of degrading poly-nuclear aromatic hydrocarbons (PAHs) (Mueller et al., 1997).

Several sequences retrieved in sample S15 were closely related to  $\alpha$ -proteobacterial 16S rRNA genes of strains that are also involved in interactions with PAHs (see Table 1 - the sequences S15A-MN75 and S15A-MN24 and their matches in the Gene Bank). The strain *Sphingomonas* sp. BN6 was described by Nohynek et al. (1996) as PAH-degrading, whereas the strain *Sinorhizobium* sp. 9702-M4 was characterized as transporting PAHs and toxic metals in the environment via extracellular polymers (Janecka et al., 2002). Interestingly, the next related sequence to S15A-MN24, BEN-5 represents the chemolithoautotrophic arsenite-oxidizing bacterium, recovered from a gold mine (Santini et al., 2000). The individual sequence S15A-MN37 reveals a very high identity of 99% with the 16S rRNA gene of *Brevundimonas* sp. FWC30, which was isolated from untreated influent sewage at the Gold Bar facility in Canada (Abraham et al., 1999). The sequence S15A-MN96 shares a 98% similarity with the 16S rDNA of another member of  $\alpha$ -Proteobacteria, *Afipia broomeae* F186. Only one individual sequence was found in the S15A library that matched with 97% the 16S rRNA gene of *Geobacter* sp. JW-3. Members of the  $\delta$ -proteobacterial family Geobacteriaceae are very effective metal reducers and were found in a large variety of environments where dissimilatory metal reduction is an important process (Anderson et al., 2003; Lovley, 2002; Nevin et al., 2003).

Looking at the results presented in Figure 1, it is noticeable that the main part of the sequences of the S15A 16S rDNA clone library is closely related to the 16S rRNA genes of Proteobacteria, especially those of the  $\beta$ -proteobacterial genus *Dechlorosoma*. Here an important question arises: In the retrieved populations of sample S15A, were *Dechlorosoma*-species-specific 16S rDNA sequences really predominant or was their predominance in the constructed library a result of biases (described by Derakshani et al., 2001; Hansen et al., 1998) of the approach used that were a result of preferential amplification or cloning of these particular rDNA fragments? In order to answer the important question of bias surrounding the eventual “masking effect” of the “*Dechlorosoma*”-specific  $\beta$ -proteobacterial sequences, which might have concealed the rest of the sequences of the natural bacterial populations in sample S15A, we recently analyzed a second water sample collected in parallel with the sample S15A from the same site. In contrast to sample S15A, which is the subject of this work and from which DNA was recovered from the total biomass collected on all three filters (with pore-sizes of 1.2, 0.45, and 0.22  $\mu\text{m}$ ) (see Materials and Methods), in the case of the

second sample, three DNA fractions were recovered from each of the filters separately. Two 16S rDNA libraries for the DNA recovered from the filters with 0.45 and 0.22  $\mu\text{m}$  pore-sizes were constructed using the same primers as those used for the S15A sample. The library constructed for the DNA recovered from the biomass concentrated on the 0.45  $\mu\text{m}$  filter, called S15B, was dominated by the same *Dechlorosoma* sp. related sequences as those found in the S15A library but to a lesser extent (see Table 2).

Table 2. Size of the 16S rDNA groups of sequences (given in %) found in the libraries S15A, S15B and S15D

Bacterial groups	S15 clone libraries		
	S15A: 1.2, 0.45, 0.22 $\mu\text{m}$	S15B: 0.45 $\mu\text{m}$	S15D: 0.22 $\mu\text{m}$
Proteobacteria			
$\alpha$	4.6	5.1	7.1
$\beta$ / <i>Dechlorosoma</i> sp.	69/65	39/28	8.1
$\gamma$	6.9	5.8	13.6
$\delta$	0.77	2.2	–
CFB	5.4	4.4	55
Low G+C	1.5	9.5	–
High G+C	4.6	5.1	1.1
<i>Holophaga/Acidobacteria</i>	1.5	1.4	–
<i>Nitrospira</i>	1.5	–	–
<i>Planctomycetales</i>	2.3	–	–
Cyanobacteria	–	19.7	13.6
<i>Deinococcus/Thermus</i>	–	0.7	–
Termite group I	–	0.7	0.5
OP8	–	1.4	–
OP11	–	–	0.5
TM7	–	0.7	–
Novel or chimeras	1.5	3.6	0.5

Of special interest in regard to the above question is the library constructed for the DNA recovered from the biomass collected on the 0.22  $\mu\text{m}$  filter. In the 16S rDNA library of this sample, called S15D, the  $\beta$ -proteobacterial sequences were not any more predominant, although they were present in the sample. Moreover, the 16S rDNA inserts of one of the RFLP groups found in the S15D library were almost identical to the 16S rRNA gene of *E. coli* K12. This group represented, however, only 9.8% of the total number of cloned sequences (Nedelkova and Selenska-Pobell, 2004, and to be published). About 55% of the clones of the S15D library possessed sequences that shared more than 98% identity with 16S rDNA genes of members of the *Cytophaga/Flavobacterium/Bacteroides* (CFB) group (see Table 2). Almost 14% of the S15D clones contained sequences that were very distantly related to 16S rRNA genes of an uncultured cyanobacterial group. The latter sequences were found in about



20% of the clones of the S15B library as well (detailed analyses of the S15B and S15D libraries will be soon published). From the results presented in Table 2, one can conclude that, on one hand, the *Dechlorosoma* sp.-related sequences had really masked the CFB and many other 16S rDNA sequences that were not amplified or were not successfully cloned from the DNA recovered from the total biomass collected on all three filters from the S15A sample. On the other hand, it seems that the problem is not just a preferential amplification of the Proteobacteria, because in the S15D library even  $\gamma$ -proteobacterial sequences were not amplified with a strong preference. We feel that the results presented in Table 2 indicate that the *Dechlorosoma* sp.-related sequences in the S15A library most probably represent a dense  $\beta$ -proteobacterial population. The cells of this population are either larger than 0.45  $\mu\text{m}$  or they are associated with colloid particles which are larger than 0.45  $\mu\text{m}$ . The main part of these bacterial cells seems, however, to be associated with colloid particles bigger than 1.2  $\mu\text{m}$ . The exact size of this *Dechlorosoma* sp.-related population should be estimated more precisely by using-real-time PCR (work in preparation in our laboratory). In addition, efforts to culture and study representatives of this species are in progress in our laboratory.

#### ***Cytophaga/Flavobacterium/Bacterioides* (CFB) Group**

Seven of the clones of the S15A library possessed inserts that shared a relatively high identity (over 92%) with 16S rRNA genes of CFB representatives (see Figure 1). The S15A-MN91 and S15A-MN74 sequences represent two RFLP groups, each consisting of two clones. They were found to be related to the rDNA sequences SHA-5 and SHA-7 retrieved from an anaerobic 1,2-dichloropropane-dechlorinating bioreactor (Schlötterburg et al., 2000). The individual sequence S15A-MN90 of the library reveals an identity of 94% with the 16S rDNA WCHB1-69, which was found in the methanogenic zone of a hydrocarbon- and chlorinated-solvent-contaminated aquifer (Dojka et al., 1998). The sequence S15A-MN128 was related to CFB 16S rDNA sequence 8-1, found in coal tar waste-contaminated groundwater (Bakermans and Madsen, 2002). S15A-MN27 is the only 16S rDNA sequence in the library related to a 16S rRNA gene of a cultured CFB bacterium - *Flavobacterium aquatile*. As mentioned above, in contrast to the S15A library, the S15D library was dominated by clones containing CFB inserts (see Table 2). These inserts shared a very high identity on the level of species with several cultured Flavobacteria related strains (Nedelkova and Selenska-Pobell, 2004, and to be published). The members of the CFB group possess a fascinating ability to change their size according to the nutrient conditions of their environment. In oligotrophic environments they were found in the form of small-sized ultra-microbacteria (Giuliano et al., 1999; Lebaron et al., 2001). Interestingly, the representatives of CFB as well as some  $\alpha$ -Proteobacteria are able to rapidly increase their cell size and their growth rates in relation to the enrichment of

the environment with nutrient sources (Lebaron et al., 2001). The results presented in Table 2 indicate that at the S15 site, populations of small-sized bacteria occur and that they are most probably dominated by the members of the CFB group.

#### Actinobacteria

The number of sequences of the S15A clone library related to 16S rRNA genes of Gram-positive bacteria with high G+C was relatively high. Most of the retrieved rDNA sequences matched 16S rDNA sequences in the Gene Bank of not-yet-cultured bacteria (see Figure 1). The S15A-MN25 and S15A-MN100 sequences shared about a 94% identity with the rDNA sequence KB20, which is found in oil-contaminated groundwater (Watanabe et. al., 2002). The S15A-MN99 sequence was almost identical to the 16S rRNA genes of *Propionibacterium acnes*, whereas S15A-MN29 was related to the ARFS-5 sequence retrieved from rice paddy soils (Lüdemann and Conrad, 2000). S15A-MN4 was related to the 16S rRNA genes of an oral plaque bacterial isolate *Microbacterium* sp. C24KA, but shared high identity also with those of nonpathogenic actinobacterial strains found in wastewaters of a sugar-beet factory (Matsuyama et al., 1999) and in soil environmental samples (Valinsky et al., 2002). Interestingly, we were able to culture from the biomass collected on one of the 0.22  $\mu\text{m}$  filters three microdiverse isolates - S15D-Iso2, S15D-Iso4 and S15D-Iso5, which were affiliated by more than a 99.5% identity with the species *Microbacterium oxydans* (see Figure 2).

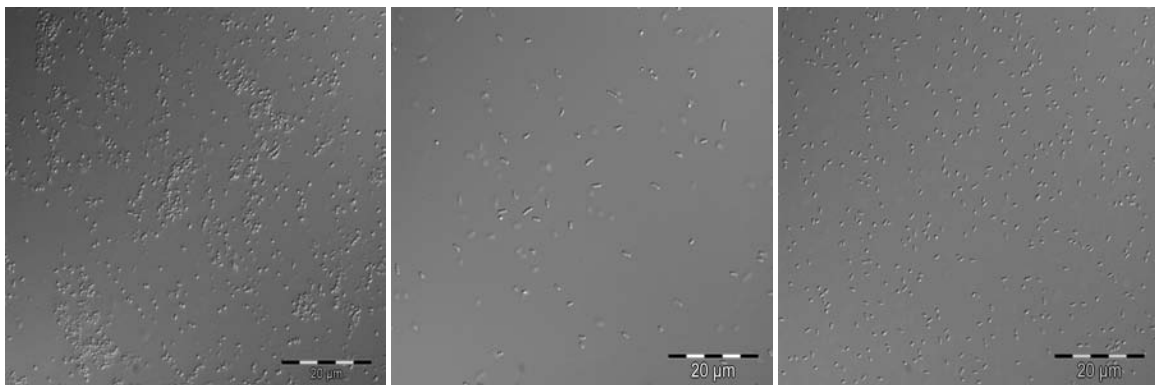


Fig. 2. Bacterial isolates S15D-Iso2, S15D-Iso4, and S15D-Iso5

As shown in the figure, all three isolates are extremely small but have slight morphologic differences. Their 16S rRNA genes possess only a few mismatches, in particular in the regions that may be connected to the observed higher growth rates of the isolates S15D-Iso2 and S15D-Iso5, when compared to those of the S15D-Iso4 isolate (to be published). Such examples of microdiversity among strains of the same species were demonstrated for many

environmental bacterial populations (Moore et al., 1998; Prüß et al., 1990; Ruis et al., 2001; Selenska-Pobell et al., 2001). The 16S rDNA genes of such strains possess specific short sequence stretches that are characteristic for genetically distinct populations, which were adapted for optimal growth under different environmental conditions. It seems that in environments such as the one studied here, a Siberian radioactive waste repository, which has been subjected to extreme environmental changes, the phenomenon of microdiversity is widespread and it indicates that the bacterial populations there possess extremely high plasticity and adaptability.

One sequence from the S15A library, S15A-MN66, shared an identity of over 98% with the 16S rRNA genes of the species *Gemella haemolysans*, which is a Gram-positive bacterium with low G+C content.

Two individual sequences, S15A-MN131 and S15A-MN30, were distantly related to the 16S rRNA genes of the chemolithoautotrophs of the *Nitrospira* group. The closest matching sequence in the Gene Bank, called *Nitrospira* sp. 4-1, was retrieved from the uncontaminated area of the already mentioned coal tar waste-contaminated groundwater (Bakermans and Madsen, 2002).

The insert of the clone S15A-MN55 was related with 96% identity to the 16S rDNA of *Geothrix fermentans*, from the *Holophaga/Acidobacterium* phylum. This bacterium was isolated from petroleum-contaminated aquifers and was characterized as Fe(III) reducing organism (Coates et al., 1999a). The low number of *Holophaga/Acidobacterium* specific sequences was somewhat surprising. We suppose, however, that this finding was not a result of biases of our method because, using the same approach, we were able to find highly abundant representatives of this phylum in soil and sediment samples from other extreme environments, such as some uranium mining waste piles (Selenska-Pobell et al., 2001 and 2002).

Three of the sequences in the S15A library share a low identity of 89% with the 16S rDNA of the uncultured Planctomycete bacterium MB-C2-147, retrieved at a depth of about 300 m in the deep-marine sediments in a Forearc basin (Reed et al., 2002).

Two sequences, S15A-MN-46 and S15A-MN56, seem to represent novel 16S rRNA genes or chimera formations (see Table 1). At the moment, we are not able to decide their status and for this reason they have not yet been submitted to the Gene Bank. Bearing in mind that only a few percent of the terrestrial bacteria have been cultured and characterized under laboratory conditions and also the fact that horizontal transfer of 16S rDNA-specific sequences has been demonstrated for several groups of bacteria (Eardly et al., 1995; Martinez-Murcia et al., 1992; Sneath, 1993), it is sometimes difficult to discriminate between artifacts and “genetically

rearranged" 16S rRNA genes that possibly represent novel bacterial lineages. A nice example is the 16S rRNA gene structure of the ferro-oxidizing chemolithoautotroph *Leprospirillum ferrooxidans* (Lane et al., 1992). These genes, containing stretches related to 16S rDNA of  $\delta$ -Proteobacteria and of Gram-positive bacteria, possess a chimera-like structure. If they were only found in an environmental 16S rDNA clone library, they would probably be considered as chimera up to date.

## Conclusions

Results presented in this study indicate that the structure of the bacterial communities at the S15 monitoring site is rather complex and diverse. Interestingly, about 75% of the sequences of the S15A 16S rDNA library shared extremely high identity at the level of species with the 16S rRNA genes of already cultured bacterial strains. About 8% of the sequences were not so closely related but shared more than 92% of identity with genes of cultured bacteria. About 17% of the retrieved 16S rDNA sequences in the S15A library were related to sequences of not-yet cultured bacteria.

The presence of extremely large numbers of  $\beta$ -proteobacterial 16S rDNA sequences in this library that were highly related to the 16S rDNA genes of *Dechlorosoma* sp. is an indication that a *Dechlorosoma*-like population was predominating the water of the S15 monitoring well. The latter, however, masked the presence of a significant part of the members of the natural bacterial community when total DNA was analyzed, as was the case with the S15A library. This problem can be overcome by parallel analyzes of DNA samples recovered separately from different particles fractionated from the water sample via subsequent filtration through filters with different pore sizes.

Many of the retrieved 16S rDNA sequences were related to the 16S rRNA genes of bacterial strains that are involved in different interactions with metals, such as *Geothrix fermentans* H5, *Sinorhizobium* sp. 9702-M4, *Geobacter* sp. JW-3, the isolate BEN-5, etc. We suppose that these sequences represent strains that might also be involved in metal/radionuclide migration processes. However, one should not overestimate the results obtained via direct molecular analysis as the 16S rRNA genes in some bacterial groups might not always reflect the evolution of their genomes (Fox et al. 1992, Stackebrandt and Goebel, 1994). On the other hand, even if some bacterial isolates are successfully cultured and studied under laboratory conditions, one should be careful when making conclusions about their behavior in the much more complex natural environments (Minz et al., 1999). Culturing bacteria from heavy-metal-polluted and other extreme environments is, however, very important because it gives one the opportunity to study novel, not-yet-described metabolic pathways and properties of life that

can help in understanding the biogeochemical processes on the Earth (Lovley 2002, Straus 1999). In addition, some of the isolates can serve as templates for developing in situ bioremediation procedures, as in the case of the uranium mining waste isolate *Bacillus sphaericus* JG-A12, which was used for construction of biological ceramics for cleaning the drain waters of uranium wastes (Raff et al., 2003).

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## Chapter II

### **Microbial diversity in ground water at the deep-well monitoring site S15 of the radioactive waste depository Tomsk-7, Siberia, Russia**

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## Abstract

Recently, bacterial diversity was studied by using the 16S rDNA retrieval in a groundwater sample, collected at a depth of 290 to 324 m below the land surface from the S15 monitoring well, located near the nuclear waste depository Tomsk-7 in Siberia, Russia (Nedelkova et al., 2005, see Chapter I). In the present work the archaeal diversity in the same sample was analysed. We demonstrate that all of the identified archaea clustered with not yet cultured representatives of the Euryarchaeota and Crenarchaeota phyla.

In addition to that, in this work bacterial diversity was studied in a parallel sample collected from the same site but handled differently. The biomass of this second sample was fractionated like the previously studied sample on 1.2, 0.45 and 0.22  $\mu\text{m}$  pore-size filters. However, in contrast to the earlier sample, where one total DNA, recovered from the biomass of all the three filters together, was analysed, here we extracted and analysed individually the DNA from each, the 0.45 and the 0.22  $\mu\text{m}$  filters.

Our analyses demonstrate that the estimation of bacterial diversity strongly depends on the way of handling the biomass. The 0.45  $\mu\text{m}$  fraction was predominated by representatives of *Dechlorosoma* spp., but not so strongly as earlier published for the whole biomass. In addition to that of the *Dechlorosoma* spp., other 16S rDNA sequences representing  $\delta$ -subclass of Proteobacteria and Gram positive bacteria with low G+C, *Firmicutes*, were identified in the 0.45  $\mu\text{m}$  fraction as well. Only in this fraction members of the bacterial clusters *Deinococcus-Thermus*, Candidate divisions OP8 and TM7 were identified. The 0.22  $\mu\text{m}$  fraction was strongly predominated by representatives of the class *Flavobacteria* of the *Bacteroidetes* phylum. No *Dechlorosoma* spp. 16S rDNA were retrieved from this fraction however other  $\beta$ -Proteobacteria like *Ralstonia* spp. and *Acidovorax* sp. were identified. Interestingly, relatively high number of sequences deeply branching within Cyanobacteria was encountered in both the 0.45 and the 0.22  $\mu\text{m}$  fractions, but not in the previously studied "unfractionated" sample.

## Introduction

Deep-subsurface environments have been used for disposal of radioactive and other wastes since decades (Bachofen et al., 1998; Cho and Kim, 2000; Fredrickson et al., 2004; Nazina et al., 2004) with the primary objective to permanently isolate the injected wastes from the biosphere. The presence of viable and active microbial populations within the deep biosphere strongly influences the geochemical processes there (Ehrlich, 1998; Pedersen, 1997; Murakami et al., 2002; Jain et al., 1997). Moreover microorganisms may effectively interact with the introduced radionuclides (Douglas and Beveridge, 1998; Lovley, 2002; Lack et al., 2002; Macaskie et al., 1992; Merroun et al., 2003; Shelobina et al., 2004) and influence their

fate within the environment (Francis, 1998; Pedersen, 1996, 2000; Selenska-Pobell, 2002). In this aspect the knowledge about the structure and diversity of the indigenous microbial communities in the deep-subsurface environments is very important for predicting a possible biodeterioration, monitoring the natural degradation and attenuation processes (Bachofen et al., 1998; Pedersen, 2000).

During the last years increasing number of studies focused on investigation of the microbial communities associated with different ground waters and subsurface sediments (Bakermans and Madsen, 2002; Chandler et al., 1998, Fields et al., 2005; Frederikson et al., 2004; Kormas et al., 2003; Nazina et al., 2004; North et al., 2004; Takai et al., 2001; Tiago et al., 2004). However, less attention has been paid on the small-sized microorganisms within the subsurface habitats so far (Miyoshi et al., 2005; Ross et al., 2001). They were shown to be frequently occurring in the other components of the biosphere (Hahn et al., 2003; Haller et al., 1999). Some of the small-sized bacteria are starvation-survival forms, which are able to increase rapidly when nutrients become available (Haller et al., 1999; Lebaron et al., 2001; Ross et al., 2001; Torrella and Morita, 1981). Therefore they can serve as potential consumers of different introduced substrates.

Recently, applying the 16S rDNA approach, we analysed the bacterial diversity of the whole biomass of a groundwater sample, collected at a depth of 290 to 324 m below the land surface from the S15 monitoring well (Nedelkova et al., 2005, see Chapter I). We identified members from different bacterial lineages, however *Dechlorosoma* spp. were the most predominant bacteria in that sample.

In this study we analysed the archaeal populations in the same sample. We investigated also a parallel sample from the S15 monitoring well, in which the diversity was characterized individually for the biomasses, collected on the 0.45 and 0.22  $\mu\text{m}$  pore-sized filters. The ground waters from the S15 well, located near the nuclear waste repository Tomsk-7 in Siberia, Russia, were characterized as oligotrophic with insignificant content of organic matter (Wickham et al., 2003). Oligotrophic environments like the studied one include small-sized bacteria, which are able to adapt and develop when changes in the environmental conditions occur, such as variations of the nutrients concentration. It has been demonstrated that even a small change in the latter has a strong effect on the community composition in the oligotrophic systems, in contrary to the case of the richer ones, where the bacterial communities are more stable and resistant (Eiler et al., 2003).

The analyses of the two different biomass fractions collected on 0.45 and 0.22  $\mu\text{m}$  filters, gave us an opportunity to obtain more profound information about the planktonic and also about the small-sized bacterial fractions in the S15 ground waters. These populations seem to

be masked by the *Dechlorosoma* spp. populations which were found to be predominant and mainly associated with the particles larger than 1.2  $\mu\text{m}$ .

## Materials and Methods

### Site description and sample collection

The S15 monitoring well is located near to the borehole radioactive waste injection site Tomsk-7 in Siberia, Russia. The well corresponds to the sandstone horizon II at 290-324 m below the land surface (EC Project: FIKW-CT-2000-00105; <http://www.galson-sciences.co.uk/BORIS>). The formation water from the well was described as oligotrophic with insignificant content of organic matter (Wickham et al., 2003). The dominant ions were bicarbonate, Na, Ca, Mg and merely natural radionuclides were found. At the collection time (Juli 2002) the water had the following *in situ* characteristics: temperature around 14°C, pH between 6.9 and 7.2, Eh -95 mV. Sampling from the well was performed after pumping the underground water out by an electric pump. Three parallel water samples each with a volume of 1 L were poured into sterile glass vessels filled with nitrogen gas in order to keep them isolated from contact with the atmosphere. The biomass from each of the samples was concentrated via subsequent filtration on one glass fibre filter with a pore size of 1.2  $\mu\text{m}$  and on two nitrocellulose filters with pore-sizes of 0.45 and 0.22  $\mu\text{m}$ . The 1.2  $\mu\text{m}$  filter was used to separate the bigger colloidal particles, which might be covered with biofilms of the planktonic microbial fraction of the samples. The filters were kept frozen at -20°C for further analyses. The biomasses collected on the filters from two of the water samples were used for characterization of the microbial diversity applying the 16S rDNA molecular approach. From the filters of the third sample, which is an object of another study, bacteria were isolated under aerobic and anaerobic conditions and their capability to interact with different heavy metals was investigated (see Chapter IV).

### DNA extraction

Total DNA was extracted using the method described by Selenska-Pobell et al. (2001). This procedure includes direct bacterial lysis followed by purification and concentration using AXG-100 Nucleobond-type anion exchange cartridges (Machery-Nagel, Düren, Germany). Recently, we have extracted total DNA, called S15A, from the biomass of one of the water samples, concentrated on the three types of filters (1.2  $\mu\text{m}$  + 0.45  $\mu\text{m}$  + 0.22  $\mu\text{m}$ ) simultaneously and analyzed bacterial diversity (Nedelkova et al., 2005, see Chapter I). In the present study the same whole or "unfractioned" DNA was used for construction of an archaeal 16S rDNA clone library.

In addition, using one of the other water samples, DNA was recovered individually from the biomasses collected on the 0.45  $\mu\text{m}$  (designated S15B) and 0.22  $\mu\text{m}$  (designated S15D)



filters, applying the above mentioned protocol. Each of the filters was shaken for 1h at 37°C in 10 ml of buffer G3 with a pH of 8 (50mM EDTA, 50mM Tris-HCl, 0.5 % Tween 20, 0.5 % Triton X100, lysozyme 4 mg/ml) and then centrifuged. The supernatant was purified following the protocol of Machery-Nagel for extraction of bacterial genomic DNA. The obtained DNA pellets were washed with 70 % ethanol, dried at room temperature and each of them was dissolved in 20 µl TE buffer (10mM Tris, 1mM EDTA, pH 8).

### PCR amplification

The PCR amplifications of the 16S rDNA fragments from the studied DNA samples were carried out in a Biometra thermal cycler (Göttingen, Germany). Reaction mixtures had a final volume of 20 µl and contained 200 µM deoxynucleotide triphosphates, 1.25 mM MgCl<sub>2</sub>, 10 pmol of each primer, 1-5 ng of template DNA, and 1 U AmpliTaq Gold polymerase with the corresponding 10x buffer (Perkin Elmer, Foster City, California, USA). The archaeal 16S rDNA fragments were amplified from the S15A total DNA, applying a semi-nested PCR reaction. In the first step of this reaction the archaeal specific 16S<sub>21-40:F</sub> (5'-TTCCGGTTGATCCYGCCGGA-3') and the universal 16S<sub>1492-1513:R</sub> (5'-TACGGYTACCTTGTTACGACTT-3') primers, *E. coli* numbering, were used. Prior to the amplification, the DNA was denaturated at 95°C for 7 min. The first 5 polymerization cycles were performed under the following conditions: 90 s at 95°C, 40 s at 59°C, and 90 s at 72°C. At each of the 5 cycles the annealing temperature decreased with 1°C, until reaching 55°C. The parameters for the following 25 cycles were: 60 s at 94°C, 40 s at 55°C, and 90 s at 72°C. At the end of the reaction an extension for 20 min at 72°C was performed. The products of the first reaction were used as template for the second round of PCR, where the archaeal specific primer pair 16S<sub>21-40:F</sub> and 16S<sub>940-958:R</sub> (5'-YCCGGCGTTGAMTCCAATT-3') were applied (DeLong, 1992). The cycling parameters used for the archaeal specific primers were: initial denaturation at 95°C (7min), followed by 25 cycles of 94°C for 60 s, annealing at 60°C for 60 s, 72°C for 60 s with a 10 min final extension at 72°C. The PCR primers used to amplify the 16S rRNA bacterial genes from the S15B and S15D DNA samples were as follows: bacterial specific primer 16S<sub>7-27:F</sub> (5'-AAGAGTTTGTATYMTGGCTCAG-3') and the above mentioned universal primer 16S<sub>1492-1513:R</sub>, *E. coli* numbering. The amplification parameters were the same as those for the first step of the above described semi-nested amplification of the 16S rDNA of archaea.

### Construction of the archaeal and bacterial 16S rDNA libraries

The amplified 16S rDNA fragments were directly cloned in *E. coli* using a TOPO<sup>TM</sup>-TA cloning vector (Invitrogen, Karlsruhe, Germany) following the instructions of the manufacturer. The obtained white colonies were randomly picked and cultured overnight at

37°C in 2 ml Luria Broth (LB) medium with kanamycin 12.5 mg/ml. The size of the 16S rDNA inserts was checked by *in situ* PCR using the forward M13(-40) (5'-GTTTTCCCAGTCACGA-3') and the reverse M13 (5'-CAGGAAACAGCTATGAC-3') plasmid specific primers, followed by agarose gel electrophoresis with subsequent ethidium bromide staining. 118 archaeal clones possessing correct archaeal 16S rDNA inserts, as well as 137 and 184 bacterial 16S rDNA clones from the S15B and S15D libraries, respectively, were stored as glycerol cultures at -80°C for further analysis.

### **RFLP-typing**

For screening the 16S rDNA diversity of the constructed archaeal and bacterial clone libraries the PCR products were digested in parallel with two frequently cutting endonucleases *MspI* and *HaeIII* (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA). The digests were analysed electrophoretically in 3.5% Small DNA Low Melt Agarose gels (Biozyme, Hessisch Oldenburg, Germany). The RFLP patterns obtained were compared and grouped in 16S rDNA RFLP types.

### **Sequence analysis**

For the three clone libraries, one representative of each RFLP group as well as all the individual clones were sequenced. Some of the clones within the two bacterial libraries possessed identical RFLP patterns, therefore only representatives from one of the library were sequenced. Direct sequencing of the PCR products was performed using standard bacterial or archaeal 16S rDNA primers on an ABI PRISM 310 Genetic Analyser (Perkin Elmer, Foster City, California, USA). The following program was applied: denaturation at 96°C for 2 min followed by 25 cycles of 45 s at 96°C, 15 s at 55°C, and 4 min at 60°C. The 16S rDNA sequences were compared with those available in the GenBanks by BLAST analysis. The CLUSTAL W alignment program was used for sequence alignment. Phylogenetic trees were generated based on the results of the neighbour-joining algorithm with distance analysis with Jukes-Cantor corrections according to the PHYLIP v.3.5 package (Felsenstein, 1993). The sequences were checked for the presence of chimeras by using the RDP CHECK\_CHIMERA program.

### **16S rDNA sequences accession numbers**

The 16S rDNA sequences retrieved in this study have been deposited in the EMBL database under accession numbers from AJ583163 to AJ583211 for the bacterial sequences and from AJ583380 to AJ583406 for the archaea sequences.

## Results

### Archaeal diversity of the whole “unfractionated” biomass

The phylogenetic analysis of the archaeal 16S rDNA sequences is presented in Fig. 1 and Table 1. This analysis revealed that 26% of the retrieved clones, represented by the sequence S15A-Ar-66, possess relatively high similarity (97%) with the *Methanosaeta* related 16S rDNA sequence ARC3 encountered in freshwater ferromanganous micronodules (Stein et al., 2001). Another 28% of the clones, represented by the sequence S15A-Ar-67, were affiliated with low identity with the environmental 16S rDNA clone WCA5 from the Euryarchaeota cluster as well (Fig. 1 and Table 1). The individual clones S15A-Ar175 and S15A-Ar136 shared lower similarities of 94% and 91% with the euryarchaeota sequences LH-06 and SAGMA-F, retrieved from northern peat land and deep gold mine (Takai et al., 2001), respectively.

Fortyfour per cent of the clones from the S15A-Ar library clustered within the Crenarchaeota phylum. Groups of similar clones, represented by the sequences S15A-Ar68 and S15A-Ar95 (Fig. 1 and Table 1), were closely related to the 16S rDNA sequence HTA-B10 retrieved from metal-rich particles (Stein et al., 2002). These groups of clones possess also high identity to the environmental clones JG36-GR-124 encountered in a uranium mining waste pile (Radeva and Selenska-Pobell, 2003) and GFS5-9500ii retrieved from glacier foreland (Nicol et al., 2005). In addition, a group of two clones represented by S15A-Ar89, and two individual clones, S15A-Ar-185 and S15A-Ar-93, (see Fig. 1) were closely related to the environmental clone Gitt-GR-78, retrieved from an uranium mill tailing (Radeva and Selenska-Pobell, 2003). This cluster includes also the environmental clone GFS4-135ii from the already mentioned mature archaeal community at the glacier foreland (Nicol et al., 2005). Two groups of clones, represented by the sequences S15A-Ar69 and S15A-Ar101, and the individual clone S15A-Ar155 show low levels of similarity (~90%) with environmental clones FS142-24A-02 and Napoli-2A-25, identified in the oceanic crust and deep sea, respectively (Fig. 1 and Table 1).

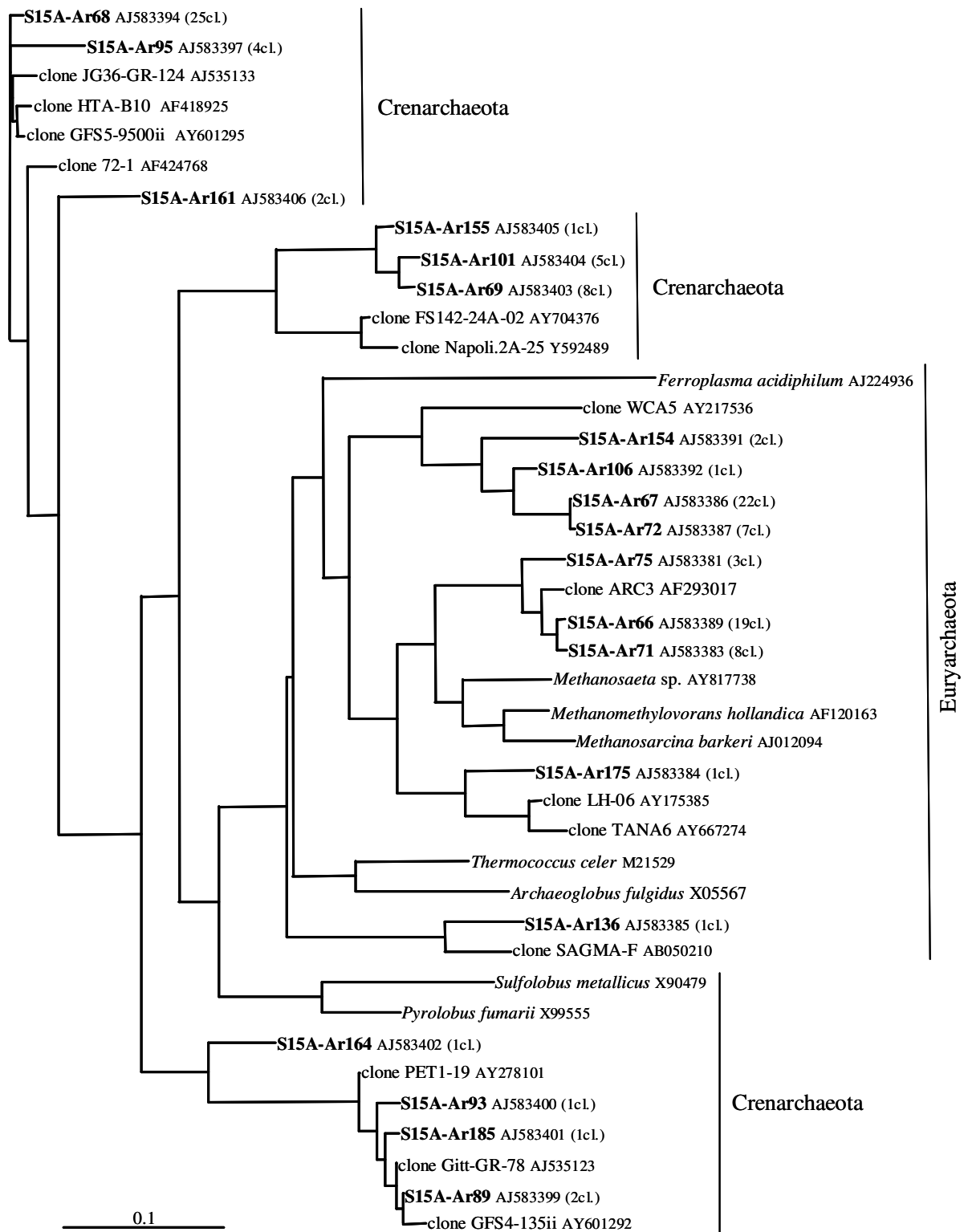


Fig. 1. Phylogenetic affiliation of the 16S rDNA archaeal clones (given in bold), encountered in the “unfractioned” biomass. The number of clones is given in brackets.

Table 1. Affiliation of the 16S rDNA clones from the S15A-Ar archaeal library

Clone name (Accession number)	Number of clones	Closest phylogenetic relative (EMBL No.), origin	BLAST % of similarity
		<b>Euryarchaeota</b>	
S15A-Ar-66 (AJ583380)	18+1	clone ARC3 (AF293017) ferromanganous micronodules <i>Methanomethylovorans hollandica</i> (AF120163)	97.5 89.2
S15A-Ar-75 (AJ583381)	3	clone ARC3 (AF293017) ferromanganous micronodules <i>Methanosaeta</i> sp. 8Ac (AY817738)	96.8 86.7
S15A-Ar-71 (AJ583383)	8	clone ARC3 (AF293017) ferromanganous micronodules	97.7
S15A-Ar-175 (AJ583384)	1	clone LH-06 (AY175385) northern peatland	94.2
S15A-Ar-136 (AJ583385)	1	clone SAGMA-F (AB050210) deep gold mines	91.2
S15A-Ar-67 (AJ583386)	17+6	clone WCA5 (AY217536) sediment	88.4
S15A-Ar-72 (AJ583387)	7	clone WCA5 (AY217536) sediment	88.4
S15A-Ar-154 (AJ583391)	2	clone WCA5 (AY217536) sediment	89.2
		<b>Crenarchaeota</b>	
S15A-Ar-68 (AJ583394)	16+8+1	clone HTA-B10 (AF418925) metal-rich particles clone JG36-GR-124 (AJ535133) uranium mining wastes clone GFS5-9500ii/i (AY601295) northern peatland	98.0 97.4 98.4
S15A-Ar-95 (AJ583397)	3+1	clone GFS5-9500iii (AY601296) northern peatland clone HTA-B10 (AF418925) metal-rich particles clone JG36-GR-124 (AJ535133) uranium mining wastes	98.1 97.2 96.6
S15A-Ar-89 (AJ583399)	2	clone Gitt-GR-78 (AJ535123) uranium mill tailing clone GFS4-135ii (AY601292) northern peatland	99.1 98.6
S15A-Ar-93 (AJ583400)	1	clone Gitt-GR-78 (AJ535123) uranium mill tailing clone GFS4-135ii (AY601292) northern peatland	97.0 96.9
S15A-Ar-185 (AJ583401)	1	clone Gitt-GR-78 (AJ535123) uranium mill tailing clone GFS4-135ii (AY601292) northern peatland	98.6 98.0
S15A-Ar-164 (AJ583402)	1	clone PET1-19 (AY278101) soil clone Gitt-GR-78 (AJ535123) uranium mill tailing	96.9 97.2
S15A-Ar-69 (AJ583403)	8	clone FS142-24A-02 (AY704376) oceanic crust clone OHKA1.27 (AB094524) subseafloor sediment	90.1 88.2
S15A-Ar-101 (AJ583404)	5	clone FS142-24A-02 (AY704376) oceanic crust clone OHKA1.27 (AB094524) subseafloor sediment	88.6 87.7
S15A-Ar-155 (AJ583405)	1	clone pGrfB286 (U59984) sediment clone OHKA1.27 (AB094524) subseafloor sediment	98.0 88.8
S15A-Ar-161 (AJ583406)	2	clone 72-1 (AF424768) wastewater sludge clone MRR6 (AY125681) rice root clone GR-WP33-A11 (AJ583414) uranium mill tailing	96.8 98.5 97.4

### Comparative analysis of the bacterial composition estimated in the 0.45 and 0.22 $\mu\text{m}$ fractions and in the whole “unfractionated” biomass

The comparative analysis of the S15B (0.45  $\mu\text{m}$  fraction) and the S15D (0.22  $\mu\text{m}$  fraction) libraries as well as the S15A library of the whole “unfractionated” biomass, characterized in our previous study (Nedelkova et al., 2005, see Chapter I), is shown in Fig. 2.

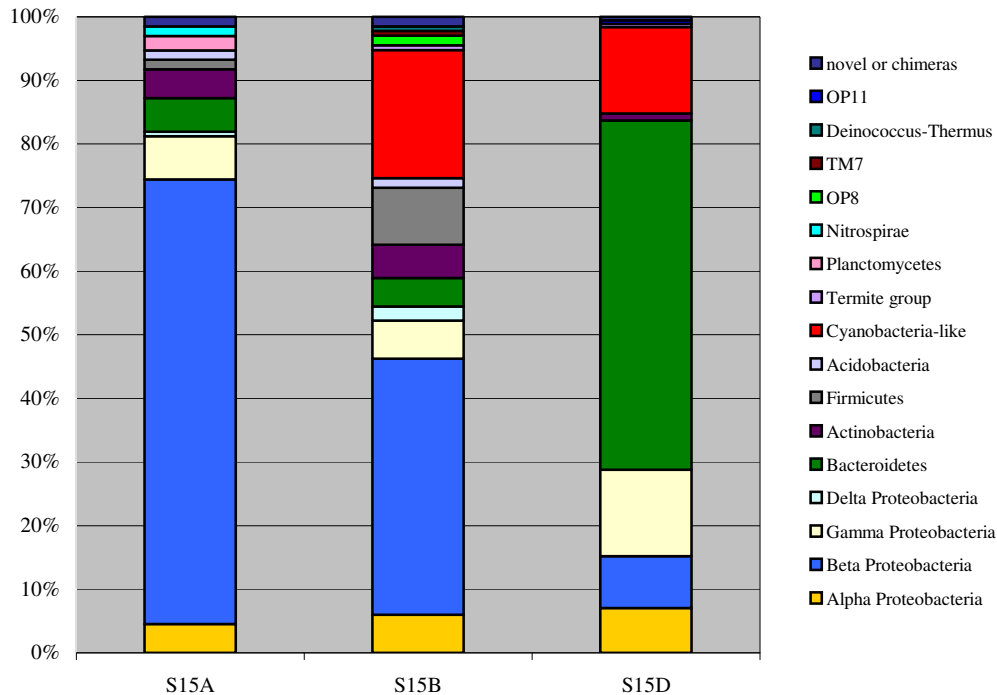


Fig. 2. Distribution of the bacterial groups (given in %) identified in the S15A (“unfractionated” biomass), S15B (0.45  $\mu\text{m}$  biomass fraction) and S15D (0.22  $\mu\text{m}$  biomass fraction).

The results demonstrate that the S15B library indicates for greater overall diversity in comparison to the S15D and S15A libraries. Proteobacteria was the most frequently identified group in the S15A and S15B libraries and accounted for 81% and 53% of the clones, respectively. In the S15D library, only 29% of the clones were related to members from this group. In this library 55% of the retrieved clones were affiliated with representatives of class *Flavobacteria* of the *Bacteroidetes* phylum (formerly *Cytophaga/Flavobacterium/Bacteroides*). The clusters of  $\alpha$ -,  $\beta$ - and  $\gamma$ - subclasses of Proteobacteria, *Bacteroidetes* and Actinobacteria were identified in all the three libraries. Members of the  $\delta$ -subclass of Proteobacteria, Acidobacteria and of Gram positive bacteria with low G+C, *Firmicutes* were encountered in the S15A and the S15B libraries (see Fig. 2 and Chapter I). Representatives of a novel bacterial lineage, deeply branching in Cyanobacteria and also of the Termite group were found in the 0.45 and 0.22  $\mu\text{m}$  biomass fractions, but not in the formerly studied “unfractionated” S15A DNA sample. The *Deinococcus-Thermus* group, Candidate divisions OP8 and TM7 were represented as small groups in the S15B library. A single clone related to the Candidate division OP11 was identified in the S15D library. In addition no representatives

of the *Nitrospirae* and *Planctomycetes* encountered earlier in the S15A library were identified in the 0.45 and 0.22  $\mu\text{m}$  biomass fractions.

### **Proteobacteria**

As evident from the results presented in Figs. 2, 3 and in Table 2,  $\beta$ -subclass of Proteobacteria was the most dominant group identified in the S15B library and accounted for 39% of the clones. The main part of them, represented by the sequence S15B-MN110, shared more than 99% identity with the 16S rRNA gene of the *Dechlorosoma* sp. PCC (recently renamed to *Azospira*, Tan and Reinhold-Hurek, 2003) from the *Rhodocyclus-Azoarcus* group. This result confirms the predominance of these bacteria in the studied ground waters which was found earlier by analysing the S15A library (see Chapter I). Interestingly a group of ten clones, represented by the sequence S15B-MN104, was almost identical with the individual clone S15A-MN107 retrieved from the above mentioned library. These sequences shared 97.1% similarity with the 16S rDNA of the denitrifying *Herbaspirillum* sp. PIV-34-1 (Probian et al., 2002). The rest of the clones from the  $\beta$ -proteobacterial cluster in the S15B library possessed individual RFLP patterns and were also sequenced. The clones S15B-MN8 and S15B-MN114 were almost identical to other environmental clones but show also high similarity (98 and 99% respectively) to the 16S rRNA gene of *Pseudomonas saccharophila* 5-1, isolated from ultrapure water (Fig. 3 and Table 2) (Kulakov et al., 2002). The clone S15B-MN125 was closely related to the uranium-mining waste clone Gitt-KF-149 (98.3%) and to the environmental clone MIZ16 retrieved from a sedimentary rock (98.2%). The individual clone S15B-MN57 possesses 98.3% similarity with the 16S rDNA clone 8-5 identified in coal-tar-waste-contaminated ground waters (Bakermans and Madsen, 2002). As shown in Fig. 3, these two environmental clones are closely related to the chemolithoautotrophic iron-oxidizing *Gallionella ferruginea* (see also Table 2).

In contrary to the S15A and S15B libraries which were predominated by the *Dechlorosoma* spp.-related 16S rDNA sequences, the S15D library contains no such sequences. As shown in Fig. 3 and Table 2, the most frequently identified  $\beta$ -Proteobacteria in this library, represented by the clones S15D-MN99 and S15D-MN15 were highly related to the 16S rDNA of the chemolithoautotrophic *Ralstonia* spp. identified in a spent nuclear fuel pool (Chicote et al., 2005) and in soil, respectively. An individual clone from the S15B library possesses identical RFLP patterns with these clones as well. A group of two clones from the S15D library, represented by the clone S15D-MN120, shows 95.2% of identity to the 16S rDNA clone FukuN108 identified in a forest lake (Glöckner et al., 2000). The individual clone S15D-MN220 shared 98.4% similarity with the 16S rRNA gene of *Acidovorax* spp.

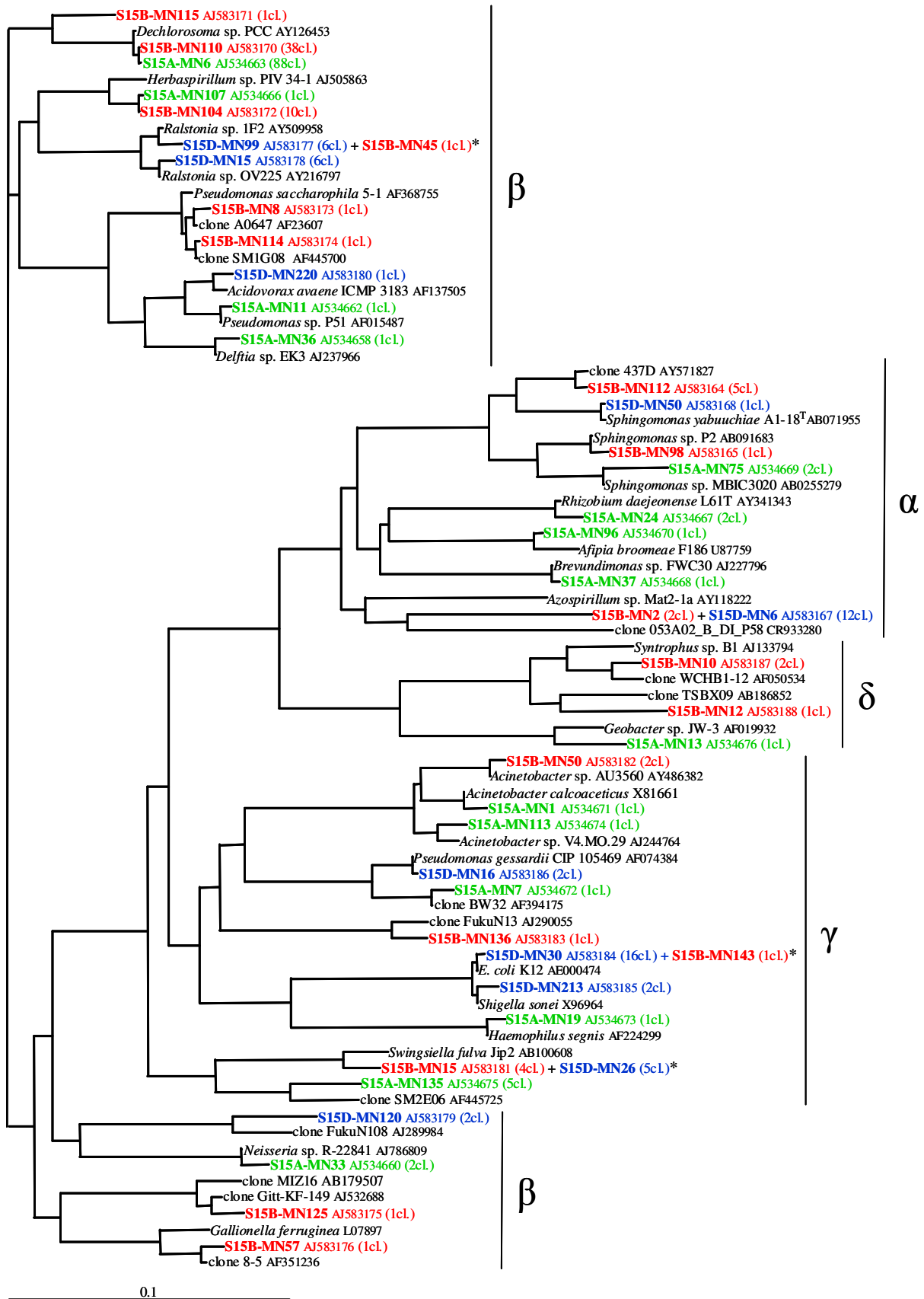


Fig. 3. Phylogenetic affiliation of the 16S rDNA proteobacterial sequences, retrieved from the S15 samples. The green, red and blue colors indicate the clones identified in the "unfractionated" biomass and in the 0.45 and 0.22 μm fractions, respectively. In brackets the number of clons is given. The asterisk indicates that the correlation is based upon RFLP-typing.



As shown in Fig. 3 and Table 2, the  $\alpha$ -proteobacterial cluster of the S15B and S15D libraries was represented mainly by members of *Sphingomonadaceae* and also by a group of sequences branching deeply in this subclass of Proteobacteria. In the S15B library five from the clones, represented by the sequence S15B-MN112, were 99.1% identical to the uncultured *Sphingomonas* sp. 437D. The individual clone S15B-MN98 was 99.2% similar to 16S rDNA of different *Sphingomonas* spp. The clone S15D-MN50 was related to the 16S rRNA gene of *Sphingomonas yabuuchiae* A1-18<sup>T</sup> isolated from the space laboratory Mir (Li et al., 2004) and to the environmental clone SSM263-NB12 identified in 0.2  $\mu$ m groundwater filtrate (Miyoshi et al., 2005). Two clones from the S15B library, represented by the sequence S15B-MN2, and twelve clones from the S15D library (see S15D-MN6) have almost identical 16S rDNA sequences with a difference of one nucleotide and shared about 90% similarity with the 16S rDNA of *Azospirillum* sp. Mat2-1a and clone 053A02\_b\_DI\_P58. *Azospirillum* sp. was not identified in the S15A library, where, however, species from the family *Rhizobiaceae*, the genera *Afipia* and *Brevundimonas* as well as another *Sphingomonas* sp. were encountered (see Chapter I).

Members of the  $\gamma$ -subclass of Proteobacteria comprised 5.8% of the clones from the S15B library and 13.6% of the S15D clones and some of them were identical between the two libraries. The sequence S15B-MN15 represents a group of four clones, which shared identical RFLP patterns with five clones of the S15D library (see S15D-MN26 in Table 2). As shown in Fig. 3 and Table 2, the sequence analysis of the clone S15B-MN15 revealed 97.7% identity with the 16S rDNA of the soil bacterium *Swingsiella fulva* Jip2. The individual clone S15B-MN143 has also identical RFLP pattern with a group of sixteen clones from the S15D library, represented by the sequence S15D-MN30. They shared 99.8% similarity with the 16S rDNA of *E. coli* K12. Group of two clones from the S15D library (see sequence S15D-MN213) were 99.7% identical to the 16S rRNA gene of another member of the *Enterobacteriaceae* - *Shigella sonnei*. Two other clones (see sequence S15D-MN16) from the same library were closely related to the 16S rDNA of *Pseudomonas gessardii* CIP 105469, isolated from mineral water (Verhille et al., 1999). A *Pseudomonas* - related sequence was identified also in the S15A library, constructed for the "unfractionated" biomass (see Chapter I). Within the S15B library a group of two clones, represented by the sequence S15B-MN50 were almost identical to 16S rDNA of the clinical isolate *Acinetobacter* sp. AU1523. Clones affiliated with environmental representatives of the *Acinetobacter* genus were encountered in the S15A library as well (see Fig. 3 and Chapter I).

Table 2. Affiliation of the 16S rDNA clones from the S15B and S15D bacterial clone libraries. The asterisk indicates that the correlation is based upon RFLP-typing

Clone name (Accession number)	Number of clones	Closest phylogenetic relative (EMBL No.), source	BLAST % of similarity
		<b><math>\alpha</math>-Proteobacteria</b>	
S15B-MN112 (AJ583164)	5	clone 437D (AY571827) contaminated soil <i>Sphingomonas faenia</i> MA-olki (AJ429239) air	99.1 98.9
S15B-MN98 (AJ583165)	1	<i>Sphingomonas</i> sp. P2 (AB091683) <i>Sphingomonas yanoikuyae</i> Q1 (U37525) PAH oxidizer	99.2 99.2
S15B-MN2 (AJ583166)	2	clone 053A02_B_DI_P58 (CR933280) anaerobic digester <i>Azospirillum</i> sp. Mat2-1a (AY118222) river	89.4 90.4
S15D-MN6 (AJ583167)	12	clone 053A02_B_DI_P58 (CR933280) anaerobic digester <i>Azospirillum</i> sp. Mat2-1a (AY118222) river	89.4 90.4
S15D-MN50 (AJ583168)	1	<i>Sphingomonas yabuuchiae</i> A1-18T (AB071955) space laboratory clone SSM263-NB12 (AB193921) ground water	99.2 98.4
		<b><math>\beta</math>-Proteobacteria</b>	
S15B-MN110 (AJ583170)	38	<i>Dechlorosoma</i> sp. PCC (AY126453) activated sludge	99.7
S15B-MN115 (AJ583171)	1	clone cclsm36 (AY133096) TCE-contaminated site	94.9
S15B-MN104 (AJ583172)	10	clone S15A-MN107 (AJ534666) ground water <i>Herbaspirillum</i> sp. PIV-34-1 (AJ505863) soil <i>Herbaspirillum</i> sp. G8A1 (AJ012069) denitrifying	99.7 97.1 96.6
S15B-MN8 (AJ583173)	1	clone A0647 (AF236007) <i>Pseudomonas saccharophila</i> 5-1 (AF368755) ultrapure water	99.0 98.0
S15B-MN114 (AJ583174)	1	clone SM1G08 (AF445700) hot spring <i>Pseudomonas saccharophila</i> 5-1 (AF368755) ultrapure water	99.7 99.0
S15B-MN125 (AJ583175)	1	clone MIZ16 (AB179507) sedimentary rock clone Gitt-KF-149 (AJ532688) uranium mill tailing	98.2 98.3
S15B-MN57 (AJ583176)	1	clone 8-5 (AF351236) ground water <i>Gallionella ferruginea</i> (L07897)	98.3 96.2
S15B-MN45*	1	<i>Ralstonia</i> sp. 1F2 (AY509958) spent nuclear fuel pool	
S15D-MN99 (AJ583177)	6	<i>Ralstonia</i> sp. 1F2 (AY509958) spent nuclear fuel pool	98.2
S15D-MN15 (AJ583178)	6	<i>Ralstonia</i> sp. OV225 (AY216797) soil	99.1
S15D-MN120(AJ583179)	2	clone FukuN108 (AJ289984) lake	95.2
S15D-MN220(AJ583180)	1	<i>Acidovorax avenae</i> subsp. <i>avenae</i> ICMP 3183 (AF137505)	98.4
		<b><math>\gamma</math>-Proteobacteria</b>	
S15B-MN15 (AJ583181)	4	<i>Swingsiella fulva</i> Jip2 (AB100608) soil	97.7
S15B-MN50 (AJ583182)	2	<i>Acinetobacter</i> sp. AU1523 (AY043369) clinical	99.7
S15B-MN136 (AJ583183)	1	clone FukuN13 (AJ290055) lake <i>Methylobacter</i> sp. SV96 (AJ414655) arctic wetland soil	97.3 96.9
S15B-MN143*	1	<i>Escherichia coli</i> K12 (AE000474)	
S15D-MN26*	5	<i>Swingsiella fulva</i> Jip2 (AB100608) soil	
S15D- MN30 (AJ583184)	16	<i>Escherichia coli</i> K12 (AE000474)	99.8
S15D-MN213(AJ583185)	2	<i>Shigella sonnei</i> (X96964)	99.7
S15D- MN16 (AJ583186)	2	<i>Pseudomonas gessardii</i> CIP 105469 (AF074384) mineral water	99.7
		<b><math>\delta</math>-Proteobacteria</b>	
S15B-MN10 (AJ583187)	2	clone WCHB1-12 (AF050534) contaminated aquifer <i>Syntrophus</i> sp. B1 (AJ133794)	97.4 95.0
S15B-MN12 (AJ583188)	1	clone TSBX09 (AB186852) microcosm <i>Syntropus gentianae</i> HQgoe1 (X85132)	93.1 93.7

Table 2. Continued ...

Clone name (Accession number)	Number of clones	Closest phylogenetic relative (EMBL No.), source	BLAST % of similarity
		<b><i>Bacteroidetes</i> (formerly <i>Cytophaga/Flavobacterium/Bacteroides</i>)</b>	
S15B-MN138 (AJ583189)	2	clone WCHB1-53 (AF050539) contaminated aquifer	97.3
S15B-MN34 (AJ583190)	2	<i>Porphyromonas</i> sp. DP023 (AF385516) oral clone	98.4
S15B-MN17 (AJ583191)	2	clone CLi112 (AF529321) PCE-contaminated site	92.4
S15D-MN4 (AJ583192)	31	<i>Chr. meningosepticum</i> LDVH 337.01 (AY468482) diseased fish <i>Chr. meningosepticum</i> 1F4 (AY509953) spent nuclear fuel pool	99.6 100
S15D-MN7 (AJ583193)	69	<i>Chryseobacterium</i> sp. JIP 105/82 (AY468457) diseased fish <i>Chryseobacterium</i> sp. H20 (AY345551) Hawaiian archipelago <i>Chryseobacterium indolgenes</i> SB1 (AY050493) biofilm reactor	98.3 98.0 98.1
S15D-MN101 (AJ583194)	1	<i>Chryseobacterium</i> sp. LDVH 42/00 (AY468475) diseased fish	98.8
		<b>Gram positive bacteria High G+C, Actinobacteria</b>	
S15B-MN4 (AJ583195)	6	<i>Propionibacterium acnes</i> W1392 (AY642051) clinical clone PH-B24 (AF513962) lake	99.5 99.5
S15B-MN49 (AJ583196)	1	clone S15A-MN100 (AJ534681) ground water	94.0
S15D-MN206*	1	<i>Propionibacterium acnes</i> W1392 (AY642051) clinical clone PH-B24 (AF513962) lake	99.5 99.5
S15D-MN233 (AJ583197)	1	<i>Rothia mucilaginosa</i> DSM (X87758)	99.0
		<b>Gram positive bacteria Low G+C, Firmicutes</b>	
S15B-MN35 (AJ583198)	4	clone AC036 (AY330126) methanogenic reactor clone LJ3 (AY756592) sediment clone AA02 (AF275914) anaerobic digester clone adhufec236 (AF132250) human gut	92.6 92.9 92.3 93.6
S15B-MN18 (AJ583199)	2	<i>Staphylococcus</i> sp. J33 (AB167056)	99.3
S15B-MN30 (AJ583201)	5	<i>Streptococcus oralis</i> ATCC 35037 (AY485602) clinical	99.5
S15B-MN51 (AJ583202)	1	<i>Streptococcus sanguinis</i> ChDC YSA1 (AY691542) clinical clone Ma-NB08 (AB193888) ground water	99.7 99.4
		<b>Acidobacteria</b>	
S15B-MN21 (AJ583203)	2	<i>Geothrix fermentans</i> H5 (U41563)	98.1
		<b>Cyanobacteria</b>	
S15B-MN24 (AJ583204) S15D-MN45 (AJ583205)	27 25	clone 30 (AY853673) contaminated soil clone RCP2-5 (AF523901) forested wetland clone DSSD30 (AY328729) drinking water	92.2 91.0 88.6
		<b><i>Deinococcus-Thermus</i> group</b>	
S15B-MN92 (AJ583206)	1	<i>Deinococcus radiodurans</i> (M21413)	98.2
		<b>Termite group 1</b>	
S15B-MN11 (AJ583207)	1	clone ccs37 (AY133074) TCE - contaminated site	90.8
S15D-MN48 (AJ583208)	1	clone vadinHB76 (U81754) anaerobic digester	92.5
		<b>Candidate division OP8</b>	
S15B-MN72 (AJ583209)	2	clone SHA-124 (AJ306781)	95.3
		<b>Candidate division OP11</b>	
S15D-MN64 (AJ583210)	1	clone LGd10 (AF047567) sediment	89.4
		<b>Candidate division TM7</b>	
S15B-MN121 (AJ583163)	1	clone 734 (AF513103) activated sludge	97.0
		<b>Novel or chimeras</b>	
S15B-MN6	2	clone FW25 (AF524027) forested wetland	85.9

The individual clone S15B-MN136 shared 97.3% similarity with the 16S rDNA of an uncultured bacterium FukuN13, identified in the above mentioned forest lake (Glöckner et al., 2000) and with the methanotrophic *Methylobacter* sp. SV96 (see Table 2). All the above described clones retrieved from the two different biomass fractions were sufficiently closely related to 16S rRNA genes of cultured  $\gamma$ -Proteobacteria. In the S15A library, however, the most frequently encountered  $\gamma$ -proteobacterial sequence, which represented the second numerically predominant RFLP group in that library, possessed 94.8% identity to the environmental clone SM2E06 (see Fig. 3 and Chapter I).

The results, presented in Fig. 3 and Table 2 demonstrate that three clones from  $\delta$ -subclass of Proteobacteria were identified in the S15B library. Two of them were with 97.4% similar to the environmental clone WCHB1-12, retrieved from a methanogenic subsurface zone (Dojka et al., 1998). The clone S15B-MN12 was related with 93.1% to the 16S rDNA sequence TSBX09. As shown on the dendrogram, the three clones clustered with cultured representatives of the genus *Syntrophus* (Zengler et al., 1999) with relatively low identity. One clone from the S15A library was earlier affiliated to the 16S rRNA gene of *Geobacter* sp. JW-3, whereas in the S15D library no sequences representing this subclass of Proteobacteria were identified.

### ***Bacteroidetes***

The comparative analysis of the clones of the three bacterial libraries, presented in Fig. 2, demonstrate that about 55% of the S15D clones retrieved from the 0.22  $\mu\text{m}$  biomass fraction were affiliated with the members of the class *Flavobacteria* of the *Bacteroidetes* phylum. In contrast to that only 5.4% of the S15A and 4.4% of the S15B clones represented this phylum. All of the *Flavobacteria*-related clones of the S15D library shared more than 98% identity with the 16S rRNA genes of different *Chryseobacterium* isolates. The clone S15D-MN7 represented a group of 69 clones, which have almost identical RFLP patterns. This clone was closely related to the 16S rRNA gene of *Chryseobacterium* sp. JIP 105/82, isolated from diseased fish. As evident from the dendrogram, presented in Fig. 4, S15D-MN7 possesses also very high identity to the 16S rDNA of the strains H20 and SB1, isolated from a Hawaiian archipelago and from a biofilm reactor, respectively (see also Table 2). The individual clone S15D-MN101, clustered within this group of sequences as well. The clone S15D-MN4 represents a group of 31 clones, which were almost identical to the 16S rDNA of *Chryseobacterium meningosepticum* LDVH 337.01, isolated also from diseased fish. In addition, this clone has identical 16S rRNA gene to the *Chryseobacterium* sp. 1F4 obtained recently from a spent nuclear fuel pool (Chicote et al., 2005). As demonstrated earlier and also now presented in Fig. 4 only one 16S rDNA clone from the S15A library was affiliated

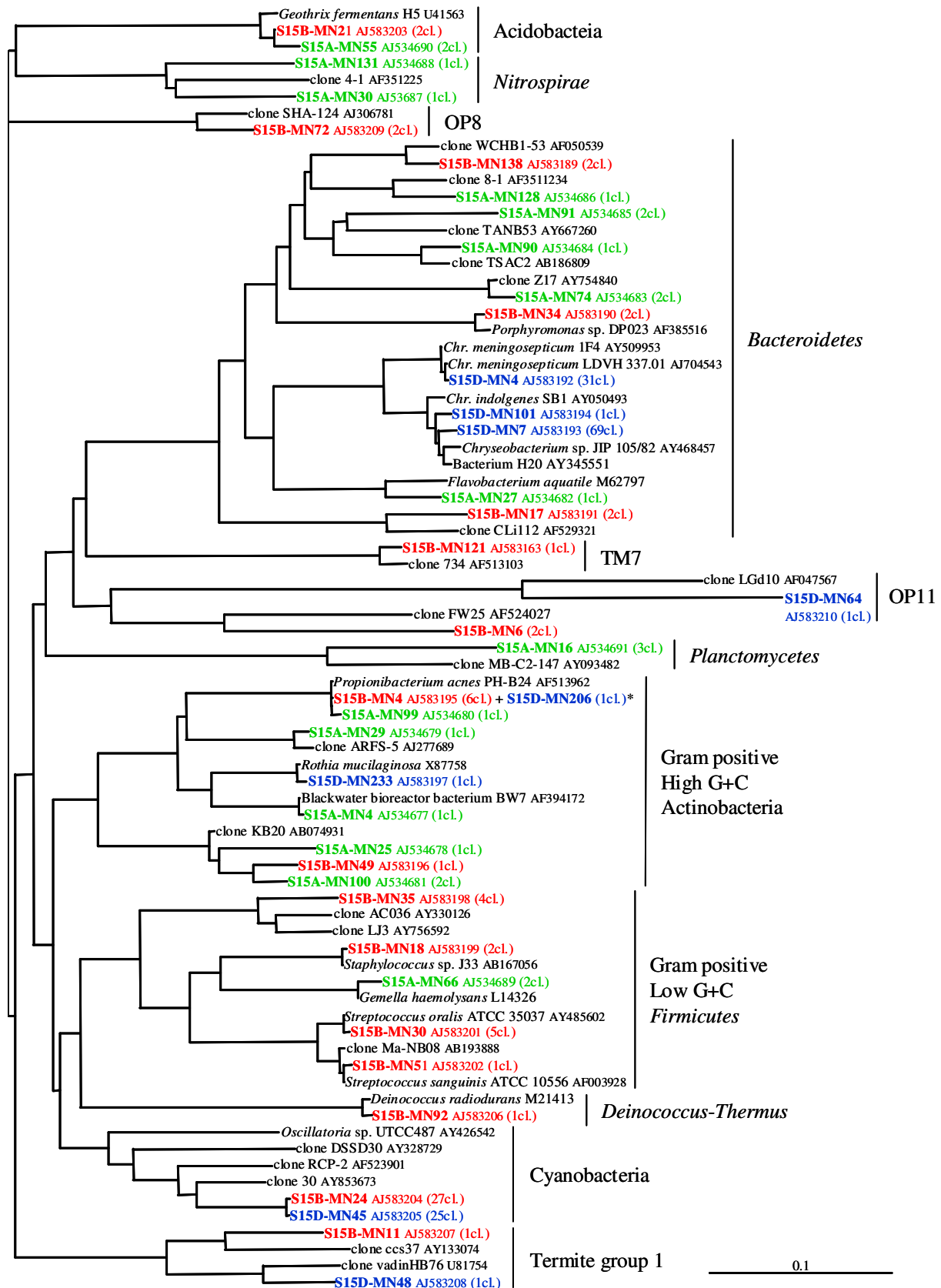


Fig. 4. Phylogenetic affiliation of the 16S rDNA sequences retrieved from the S15 samples with bacterial groups other than Proteobacteria. The green, red and blue colors indicate the clones identified in the "unfractionated" biomass and in the 0.45 and 0.22 μm fractions, respectively. In brackets the number of clons is given. The asterisk indicates that the correlation is based upon RFLP-typing.

with member of the class *Flavobacteria*, *Bacteroidetes* phylum. The other sequences of this group from the S15A and S15B libraries clustered mainly within the class *Bacteroidetes* (see Fig. 4 and Chapter I). The clone S15B-MN138 shared 97% similarity with the environmental 16S rDNA clone WCHB1-53, identified in the above mentioned methanogenic subsurface zone (Dojka et al., 1998). The sequence S15B-MN34 was 98% related to the 16S rRNA gene of the oral strain *Porphyromonas* sp. DP023. Another clone, S15B-MN17, possesses lower identity of 92% with an environmental clone Cli112, retrieved from a PCE contaminated site. The three mentioned sequences from the S15B library, represented groups of two clones with identical RFLP patterns.

### **Gram positive bacteria**

Gram positive bacteria with high G+C content, Actinobacteria, were identified in all the three libraries. Clone S15B-MN4 represents a group of six clones highly related to the 16S rDNA of the clinical isolate *Propionibacterium acnes* W1392 and to the environmental clone PH-B24 identified in a Hawaiian lake (see Fig. 4 and Table 2). The clone S15D-MN206 from the S15D library possesses identical RFLP patterns with this group. As evident from the dendrogram in Fig. 4, the individual clone S15A-MN99 retrieved formerly from the "unfractionated" biomass (S15A library) was also highly related to the mentioned *Propionibacterium acnes* – cluster. The individual clone S15B-MN49 shared 94% similarity with the 16S rDNA sequence S15A-MN100, identified previously in the S15A library. The individual clone S15D-MN233 from the S15D library was 99% identical to the 16S rDNA of *Rothia mucilaginosa*.

Gram positive bacteria with low G+C content, *Firmicutes*, were presented mainly in the S15B library. As previously published, in the S15A library a group of two clones clustered within this group (see Fig. 4 and Chapter I), whereas in the S15D library no sequences representing it were found. As shown in Fig. 4 the clone S15B-MN18 represented group of two clones almost identical to the 16S rDNA gene of the euryhaline *Staphylococcus* sp. J33. The sequence S15B-MN30 related to *Streptococcus oralis* 16S rDNA, represented group of five clones. The individual clone S15B-MN51 clustered with high similarity to 16S rRNA genes of clinical and groundwater (Miyoshi et al., 2005) members of the *Streptococcus* genus. In addition, a group of four clones (see clone S15B-MN35) shared an identity of 92% with environmental clones identified in a methanogenic reactor (Burrell et al., 2004) and sediment.

### **Cyanobacteria-like cluster**

The second numerically abundant cluster in the S15B and S15D libraries was a group of microdiverse sequences deeply branching within the Cyanobacteria phylum. As shown in Fig. 4, the clones S15B-MN24 and S15D-MN45, which represented two groups of 27 and 25

clones, were affiliated with an identity of 92% and 91% to the environmental clones 30 and RCP2-5 which were retrieved from a contaminated soil and wetland, respectively (Brofft et al., 2002). However, the sequences of the clones 30 and RCP2-5 were shorter than ours. Another clone DSSD30 identified recently in a distribution system simulator (Williams et al., 2004) has almost the same length as the S15B-MN24 and S15D-MN45 sequences but possesses 88% identity with them. The closest cultured referent, *Oscillatoria* sp. UTCC487 from the Cyanobacteria phylum, possesses rRNA gene with less than 88% similarity to the encountered S15 sequences. The relatively low identities to other environmental clones, as well as the very low identity to cultured representatives, indicates that the identified group of 16S rDNA sequences possibly represents a novel distantly related subgroup within the Cyanobacteria or even a new bacterial division. No sequences from the above described cluster were identified in the whole "unfractionated" biomass, which might be explained by the strong predominance of the *Dechlorosoma* spp. populations there.

#### **Bacterial groups represented by small number of clones**

Some of the encountered bacterial clusters (see Fig. 4 and Table 2) were represented by three or less clones, which contribute to the overall bacterial diversity. The Acidobacteria phylum was represented in the S15B library by a group of two clones, 98% similar to the 16S rRNA gene of the Fe(III)-reducing *Geothrix fermentans* H5 (Coates et al., 1999a). As earlier published and now shown in Fig. 4, two clones, encountered in the "unfractionated" microbial biomass clustered with the *Geothrix fermentans* 16S rRNA genes. The Termite group 1 was represented in the S15B and S15D libraries by the individual clones S15B-MN11 and S15D-MN48, respectively. Several other bacterial groups were retrieved only in one of the three constructed libraries. Members of the *Planctomycetes* and *Nitrospirae* were identified only in the S15A library (see Chapter I). The Candidate division OP8 was represented by two clones in the S15B library, which clustered with the 16S rDNA sequence SHA-124 (see clone S15B-MN72 in Fig. 4 and Table 2). The individual clone S15B-MN121 was 97% identical to another environmental clone 734 affiliated with the candidate division TM7. The clone S15B-MN92 was related to the radiation-resistant bacterium *Deinococcus radiodurans* from the *Deinococcus-Thermus* group. The clone S15D-MN64 encountered in the S15D library shows 89% similarity with clone LGd10 of the Candidate division OP11. A group of two clones from the S15B library (see clone S15B-MN6 in Fig. 4 and Table 2) was related to clone FW25 (86% of similarity), retrieved from the uncontaminated zone of the above mentioned wetland (Brofft et al., 2002).

## Discussion

The phylogenetic characterization of the archaeal populations identified in the whole "unfractionated" biomass of a water sample from the S15 subsurface monitoring well, showed the presence of methanogenic Euryarchaeota, as well as three distinct clusters of the Crenarchaeota phylum. The encountered 16S rDNA sequences were most closely related to environmental clones. Some of them were identified previously by other authors in metal-rich habitats (Stein et al., 2001, 2002), in uranium mining wastes (Radeva and Selenska-Pobell, 2003) and in a deep gold mine (Takai et al., 2001).

The phylogenetic analysis of the bacterial 16S rDNA clone libraries demonstrated that the estimation of the diversity strongly depends on the way of dealing with the biomass. The analysis of the biomass, passed through the 1.2  $\mu\text{m}$  filter and retained on the filter with a pore size of 0.45  $\mu\text{m}$  (S15B library) revealed the greatest overall diversity, compared to the analyses of the fraction of the small-sized cells captured on the 0.22  $\mu\text{m}$  filter (S15D library) and of the above mentioned whole "unfractionated" microbial biomass (collected on 1.2, 0.45 and 0.22  $\mu\text{m}$  filters), represented in the S15A library (see Chapter I). The comparative analysis demonstrated dominance of clones almost identical to the 16S rDNA of *Dechlorosoma* spp. in the S15A and S15B libraries. These two libraries shared also several other similar groups of sequences. On the other hand, more than one fourth of the clones from the S15B library were almost identical to clones encountered in the S15D library. They were affiliated with 16S rRNA genes of species from Proteobacteria, Actinobacteria and Cyanobacteria-like clusters and could comprise bacteria from different physiological states, distinct ecological subpopulations, as well as smaller cells, retained on bigger colloidal particles. One can not exclude that some of them are even slightly larger than 0.45  $\mu\text{m}$  but flexible enough to pass through the filter. In contrast, only one clone from the 0.22  $\mu\text{m}$  fraction (S15D library) was similar with a 16S rDNA sequence retrieved from the S15A clone library, suggesting "under-representation" of the small-sized cells in the whole "unfractionated" biomass. In agreement with our results, no overlapping between the analyses of the whole bacterial biomass, captured on 0.22  $\mu\text{m}$  filter and the 0.1  $\mu\text{m}$  fraction was demonstrated recently for groundwater samples by other authors (Myoshi et al., 2005). In another study, however, which was performed on microorganisms from marine habitat, most of the members from the total bacterial population retained on the 0.1  $\mu\text{m}$  filter, were identified also in the studied 0.2  $\mu\text{m}$  filtrate (Haller et al., 1999).

The dominance of sequences almost identical to the 16S rDNA of the hydrogen-oxidizing *Dechlorosoma* sp. PCC in the S15B and the S15A clone libraries, suggests that these organisms were abundant in the studied groundwater samples. Members of the *Dechlorosoma*



genus were primarily described as nitrate and per-chlorate reducing bacteria (Coates et al., 1999b). Some of them can couple the reducing processes with anaerobic Fe(II) oxidation (Lack et al., 2002). Recently, a *Dechlorosoma*-related strain TW2, isolated from wetland sediments, was demonstrated to grow lithoautotrophically via Fe(II) oxidation and it was suggested that lateral gene transfer from e.g. *Gallionella*-like organism could possibly explain this capability (Sobolev and Roden, 2004). Other studies described chemolithoautotrophic *Dechloromonas* spp. which are closely related to members of the *Dechlorosoma* genus (Shrout et al., 2004; Zhang et al., 2002). It should be noted that the genus *Dechlorosoma* was recently renamed as *Azospira* (Tan and Reinhold-Hurek, 2003), previously known to belong to the *Azoarcus* spp. group D (Reinhold-Hurek and Hurek, 2000). Dominance of denitrifying bacteria from the *Azoarcus* genus was demonstrated in pristine (Röling et al., 2001) and in contaminated ground waters, applying the 16S rDNA approach (Bakermans and Madsen, 2002; Fields et al., 2005). Members of the closely related *Dechloromonas* genus were identified also in uncontaminated subsurface sediments, where the  $\beta$ -subclass of Proteobacteria was found to be predominant (North et al., 2004). The above mentioned sites represent different and not related environments. The presence of identical genera and even species in habitats with distinct hydrogeochemical characteristics demonstrates the enormous metabolic versatility and adaptive capabilities of these bacteria and underlines their important role in nature. However, it should be noted, that the identification of microorganisms, applying the 16S rDNA molecular retrieval, does not necessary implies their *in situ* activity (Dojka et al., 1998). The 16S rDNA approach expanded greatly our knowledge about the microbial diversity and composition in the environment, but only coupled with culture-dependent and other techniques, as well as with the physico-chemical characteristics of the habitat it can give insights into the possible role of the identified microorganisms in a given habitat.

The oligotrophic characteristic of the water from the S15 monitoring well is similar to the nutrient-poor nature of most ground waters (Smith, 2002). Such conditions favour development of chemolithotrophic, as well as of oligotrophic subsurface communities, which could predominate there (Anderson et al., 1998; Chapelle et al., 2002; Stevens and McKinley, 1995). A relatively high number of the encountered S15 clones were sufficiently closely related to 16S rDNA genes of nitrate reducing  $\beta$ -Proteobacteria, like *Dechlorosoma*, *Herbaspirillum* and *Ralstonia* spp. Applying traditional culturing methods, significant quantities of denitrifying auto- and heterotrophic bacteria were identified in the waters from the same site (Wickham et al., 2003), as well as in other oligotrophic ground waters (Jain et al., 1997; Nazina et al., 2004). This could be expected taking into account that after the

depletion of oxygen the nitrate-reduction is thermodynamically more favorable process (Dojka et al., 1998; Smith, 2002; Suzuki et al., 2003). Considering the insignificant content of organic matter one cannot exclude that part of the populations at the S15 subsurface monitoring well derives their energy via chemolithoautotrophic growth. Low potential rates of lithotrophic and acetoclastic methanogenesis, as well as sulfate-reduction were previously demonstrated in the waters from the S15 well (Wickham et al., 2003). The identification of archaeal clones of methanogens from the suborder *Methanosarcinales* together with 16S rDNA sequences of the  $\delta$ -proteobacterial *Syntrophus* spp. is not surprising, bearing in mind the ability of these two organisms to live in syntrophic-methanogenic associations (Chauhan et al., 2004; Dojka et al., 1998; Zengler et al., 1999). No sulfate-reducing bacteria were identified in the Tomsk-7 groundwater samples studied in the present work. The absence of 16S rDNA sequences related to sulfate-reducing bacteria despite the presence of sulfate-reducing activity was demonstrated also by others for deep sea sediments (Marchesi et al., 2001). Interestingly, small number of 16S rDNA clones affiliated with Fe(III)-reducing bacteria was presented. Previously it was demonstrated that metal oxide-reducing bacteria may dominate environments where Fe(III) is abundant, because of their ability to out compete the sulfate-reducing bacteria for electron donors (Chapelle and Lovley, 1992).

The dominance of members from the  $\beta$ -subclass of Proteobacteria in the S15A and S15B libraries is not surprising, taking into consideration that this subclass constitutes the main bacterial fraction in freshwater systems (Battin et al., 2001; Glöckner et al., 1999). However, our results suggested, that significant bias in the community structure analysis could be introduced through neglecting the small-sized cell bacterial fraction. Some of these bacterial cells represent physiologically starvation forms (Haller et al., 1999; Lebaron et al., 2001; Ross et al., 2001), which are able to increase their size and numbers rapidly by nutrient availability. Therefore they can serve as potential consumers of different introduced substrates, thus inducing changes in the composition of the bacterial community. Interestingly, starved cells of *Shewanella alga* and *Dechloromonas agitata* were able to recover to a normal size rapidly and still retained their metal- and perchlorate-reducing capabilities, which is important in the context of the *in situ* bioremediation (Caccavo et al., 1996; Coates et al., 1999c). Other bacteria remain small even under nutrient-rich conditions like previously described by other authors members of Proteobacteria, Actinobacteria and *Bacteroidetes* (Elsaied et al., 2001; Hahn et al., 2003; Iizuka et al., 1998; Schut et al., 1997; Torrella and Morita, 1981). Unfortunately, only few studies provide information on the small-sized bacterial fraction in groundwater environments so far (Miyoshi et al., 2005; Ross et al., 2001). Our results demonstrate predominance of sequences similar to 16S rDNA of *Chryso bacterium* spp. from

the class *Flavobacteria* of the *Bacteroidetes* phylum. Groundwater isolates from 0.22  $\mu\text{m}$  filter, affiliated with members of the *Bacteroidetes* group were reported previously by others (Lills and Bissonnette, 2001; Shirey and Bissonnette, 1991). It was suggested that representatives of this group are adapted to low nutrient and substrate concentrations (Battin et al., 2001). However, cultured bacteria from the *Bacteroidetes* cluster are generally known for their ability to degrade complex substrates (Eiler et al., 2003; Lipson and Schmidt, 2004). According to some authors, the *Bacteroidetes* group appears to be under-represented in the 16S rRNA clone libraries (Glöckner et al., 1999; Kirchmann, 2002), whereas other authors demonstrated overestimation of the group in the latter in comparison to the FISH analysis (Liu et al., 2001). No *Bacteroidetes*-related sequences were retrieved from the total bacterial biomass captured on the 0.2  $\mu\text{m}$  filter as well as from the 0.1  $\mu\text{m}$  fraction of groundwater samples by Miyoshi et al. (2005). Instead, members of  $\beta$ -Proteobacteria and different Candidate divisions were found to dominate the two biomass fractions.

The identification of novel bacterial lineages, widely distributed within different habitats, applying molecular methods is no longer surprising, although it is still very exciting (Hugenholtz, 1998; Webster et al., 2004). The second numerically predominant group of clones in the S15B and S15D libraries clustered within a possible novel taxonomic lineage, which is distantly related to the Cyanobacteria phylum. Since members of this group were described as one of the first photosynthetic organisms on the earth (Awramik, 1992), we cannot exclude that the retrieved sequences represent early evolved not photosynthetically dependent branch, which is a good survival strategy within the deep-subsurface habitat. Despite the frequent abundance in the S15B and S15D libraries this type of clones was not identified in the whole biomass of the parallel sample (S15A library), which might be explained by the masking effect of the predominant *Dechlorosoma* spp. populations there. However, members of the groups *Planctomycetes* and *Nitrospirae* were encountered only in the whole "unfractionated" biomass (see Chapter I) and we suggest that the cells of these organisms are possibly associated with biofilm formations on the colloidal particles, bigger than 1.2  $\mu\text{m}$ .

The identification of sequences in environmental clone libraries similar to commensally or pathogenic species is a controversial matter. In this study relatively big population of *E. coli* K12 related sequences was identified in the S15D library and an individual clone was encountered in the 0.45 $\mu\text{m}$  fraction. We cannot exclude that some of the derived sequences might be sample contaminants, like already described (Chandler et al., 1998; Kormas et al., 2003; Tanner et al., 1998). However, as evident from the dendrograms in Figs. 3 and 4 these sequences were affiliated also with other environmental clones. Different *Staphylococcus*

strains were isolated from this and other groundwater habitats (Balkwill et al., 1997; Tiago et al., 2004; Wickham et al., 2003). Previously, *Azoarcus* and *Rhodocyclus* related sequences from a deep-subsurface clay formation were described as possible contaminants (Boivin-Jahns et al., 1996) which is surprising, bearing in mind the wide distribution of these genera in different subsurface environments (Bakermans and Madson, 2002; Fields et al., 2005; Röling et al., 2001). Environmental isolates of *Escherichia hermannii* and *Enterobacter cloacae* was shown to accumulate toxic metals (Hernandez et al., 1998). Recently, it was reported U(VI) reduction for the environmental *Salmonella* strain, isolated from subsurface sediment (Shelobolina et al., 2004). The authors suggested that a branch of *Salmonella* spp. is adapted to live in soils in contrast to the rest of their enteric relatives.

## Conclusions

The phylogenetic analysis of the 454 bacterial and 118 archaeal full-length 16S rDNA clones indicated that the microbial community structure of the ground water from the S15 monitoring well is rather complex and diverse. Methanogenic Euryarchaeota and three distinct clusters of Crenarchaeota were identified. Representatives of twelve major bacterial lineages as well as a possible novel bacterial division, deeply branching within Cyanobacteria, were encountered. A significant part of the clones was closely related to 16S rDNA of denitrifying bacteria. The lack of organic electron donors in the waters from the S15 monitoring well suggests that part of the populations could possibly obtain their energy via lithoautotrophic growth. The small-sized bacterial cell fraction of the S15 samples was masked when the diversity was estimated in the whole biomass.

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## Chapter III

### **Microbial diversity in ground water at the deep-well monitoring site S15 of the radioactive waste depository Tomsk-7, Siberia, Russia. Autotrophic bacteria, RubisCO approach**

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## Abstract

Autotrophic bacterial diversity was studied in groundwater samples collected at a depth of 290 to 324 m below the land surface from the S15 monitoring well, located near the nuclear waste depository Tomsk-7 in Siberia, Russia. For this purpose, genes were targeted, encoding the form I (*cbbL*) and form II (*cbbM*) of the large subunit of the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), involved in the CO<sub>2</sub> fixation. The sequence analyses of the retrieved form I RubisCO genes demonstrated presence of relatively high number of clones closely related or even identical to *cbbL* genes of cultured autotrophic  $\beta$ -Proteobacteria, such as *Hydrogenophilus thermolitheolus* TH-1, *Nitrosospira* sp. 40KI, and *Ralstonia* spp. This result confirms the dominance of  $\beta$ -Proteobacteria in the studied groundwater samples, which was demonstrated previously via the 16S rDNA retrieval (see Chapters I and II). However, in contrast to the RubisCO approach, the 16S rDNA retrieval revealed a strong dominance of *Dechlorosoma* spp. in the samples, although some other bacteria such as *Herbaspirillum* spp. and *Ralstonia* spp. were also identified.

In addition to the  $\beta$ -proteobacterial RubisCO form I, a lower number of clones related to  $\alpha$ -proteobacterial RubisCO *cbbL* genes were identified in the studied samples as well. The number of the retrieved RubisCO form II *cbbM* genes was lower, and the sequences were affiliated with environmental clones, encountered in deep-sea hydrothermal vents and with magnetotactic bacteria.

## Introduction

In the last decade, a number of studies reported the presence of lithoautotrophic microbial populations in different subsurface environments (Anderson et al., 1998; Bach and Edwards, 2003; Chapelle et al., 2002; Kotelnikova and Pedersen, 1997; Selenska-Pobell, 2002; Stevens and McKinley, 1995; Vlasceanu et al., 1997). These microorganisms are capable to derive energy via oxidation of different inorganic substrates and can use CO<sub>2</sub> as a sole carbon source. In order to reveal information about these bacterial populations, it is advantageous to focus directly on phylogeny of the genes involved in the CO<sub>2</sub> fixation. There are six known carbon fixation pathways found within the three domains of life - Bacteria, Archaea and Eucarya (Macalady and Banfields, 2004). The Calvin-Bassham-Benson (cbb) cycle is the major and most abundant one described for members belonging to all the three kingdoms. The key enzyme catalyzing the assimilation of carbon dioxide to organic carbon in this cycle is ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), which occurs in several forms. The most widely distributed and studied is form I which is divided in the phylogenetically distinct green- and red-like groups, each consisting of two subgroups, IA and IB, plus IC and

ID, respectively. RubisCO *cbbL* genes from the IA cluster were found in different Proteobacteria and in some Cyanobacteria such as *Synechococcus* and *Prochlorococcus* strains (Watson and Tabita, 1997). Most of the Cyanobacteria, algae and higher plants carry *cbbL* genes from the IB green-like subgroup. The red-like radiation encompasses members of the bacterial (IC) and diatom and other algae (ID) branches. The form II of the RubisCO enzyme, identified in some Proteobacteria (Liu et al., 2004) and dinoflagellates (Morse et al., 1995), functions mainly at microaerophilic conditions (Haygood, 1996). It is assumed that the form II represents the common ancestor of all RubisCO forms (Jordan and Ogren, 1981). The form III RubisCO is distributed among some members of Archaea (Watson, et al., 1999). Recently, proteins closely related to RubisCO but not involved in the Calvin cycle were described for *Bacillus subtilis* (Ashida et al., 2003), *Chlorobium tepidum* (Hanson et al., 2001) and *Archaeoglobus fulgidus* (Klenk et al., 1997). These proteins lack several amino acid residues required for the catalytic activity of RubisCO and are considered as form IV RubisCO-like proteins (Ashida et al., 2003).

A number of studies on microbial diversity and activity in subsurface and other environments investigated the diversity of functional genes, involved in fundamental metabolic processes like ammonia-oxidation (Ivanova et al., 2000; Jordan, et al., 2005; Purkhold et al.; 2000), sulphate-reduction (Baker et al., 2003, Klein et al., 2001; Nercessian et al., 2005; Pérez-Jiménez and Kerkhof, 2005), nitrogen-fixation (Rösch et al., 2002; Ueda et al., 1995), methanogenesis (Nercessian et al., 2004). The organisms harbouring these genes play a central role in the global natural cycling of elements, and their diversity, distribution and activity has vast environmental significance. However, microbial diversity based on the genes of the functional protein RubisCO, involved in the CO<sub>2</sub> fixation, is not yet extensively studied. Considering the lack of organic electron donors and acceptors in the ground waters, which indicates about abundance of autotrophic microorganisms there, it is surprising that almost no information exists about the organisms harbouring the RubisCO genes in ground waters as well as in other subsurface habitats so far (Alfreider et al., 2003; Lawrence et al., 2000). Some authors investigated the autotrophic microbial populations in different hydrothermal vents applying molecular approaches based on functional enzymes, responsible for CO<sub>2</sub> fixations (Campbell and Craig Cary, 2004; Elsaied and Naganuma, 2001).

Recently, applying the 16S rDNA approach, we have demonstrated a significant microbial diversity in groundwater samples, collected at a depth of 290 to 324 m below the land surface from the S15 monitoring well of the Siberian radioactive waste depository Tomsk-7 (Nedelkova et al., 2005, see Chapters I and II). The waters from the S15 well were characterized as oligotrophic with insignificant content of organic matter (Wickham et al.,



2003). The most frequently abundant phylogenetic group there identified by the 16S rDNA retrieval was represented by members of the  $\beta$ -subclass of Proteobacteria. In addition, relatively high number of clones clustered with the *Bacteroidetes* group and with a novel bacterial lineage, deeply branching within the Cyanobacteria phylum. The hydrogeochemical characteristics, as well as the previous studies, based on cultivation and 16S rDNA analyses, suggested the presence of autotrophic organisms at the S15 monitoring well (see Chapter II, Wickham et al., 2003). In order to obtain more detailed information about the autotrophic bacterial community there, the diversity of the large subunit gene of the form I and II RubisCO enzyme, involved in the CO<sub>2</sub> fixation, was studied. The results were compared with the 16S rDNA analyses, performed recently on the same samples.

## **Material and Methods**

### **Site and sample description**

The S15 monitoring well is located near to the borehole radioactive waste injection depository Tomsk-7 in Siberia, Russia. The well corresponds to the sandstone horizon II at 290-324 m below the land surface (EC Project: FIKW-CT-2000-00105; <http://www.galson-sciences.co.uk/BORIS>). An extensive hydrogeochemical characterization of the waters from the S15 monitoring well and the description of the sample collection can be found elsewhere (Wickham et al., 2003). For molecular analyses, the biomass of the samples was concentrated via subsequent filtration on one glass fibre filter with a pore size of 1.2  $\mu\text{m}$  and on two nitrocellulose filters with pore-sizes of 0.45 and 0.22  $\mu\text{m}$ . The 1.2  $\mu\text{m}$  filter was used to separate the bigger colloidal particles of the samples from the planctonic and small-sized bacteria. The filters were kept frozen at -20°C for further analyses.

For the molecular analysis performed in this study we have used total DNA extracted previously from two parallel samples (see Chapters I and II) applying the method described by Selenska-Pobell et al. (2001). The whole "unfractionated" DNA (designated S15A) from one of the sample was recovered simultaneously from the three above mentioned filters, and represents the whole microbial community within the studied sample. From the parallel sample, total DNA (named as S15B) was recovered from the biomass collected at the filter with pore-sizes of 0.45  $\mu\text{m}$ .

### **PCR amplification of the *cbbL* and *cbbM* genes**

The amplification of the genes, coding for the large subunit of the form I and form II of the RubisCO enzyme was carried out in a Biometra thermal cycler (Göttingen, Germany). Reaction mixtures had a final volume of 20  $\mu\text{l}$  and contained 200  $\mu\text{M}$  deoxynucleotide triphosphates, 1.25 mM MgCl<sub>2</sub>, 10 pmol of each primer (see Table 1), 1-5 ng of template DNA, and 1 U AmpliTaq Gold polymerase with the corresponding 10x buffer (Perkin Elmer,

Foster City, California, USA). For targeting the RubisCO form I *cbbL* gene primer pairs developed by Alfreider (Alfreider et al., 2003) and Nanba (Nanba et al., 2004) were applied (see Table 1). The *cbbM* gene coding for the large subunit of RubisCO form II was amplified with the primer set constructed by Elsaied and Naganuma (2001).

Table 1. RubisCO primers used in this study

Primer, binding position, reference	Sequence (5'-3')
<i>cbbL</i> K12F (168-192)* Alfreider et al., 2003	CGGCACSTGGACCACSGTSTGGAC
<i>cbbL</i> K12R (766-786)* Alfreider et al., 2003	GTARTCGTGTCATGATGATSGG
<i>cbbL</i> K2F (496-515)* Nanba et al., 2004	ACCAYCAAGCCSAAGCTSGG
<i>cbbL</i> V2R (970-990)* Nanba et al., 2004	GCCTTCSAGCTTGCCSACCRC
<i>cbbM</i> ( <i>Riftia</i> F) (663-693)° Elsaied and Naganuma, 2001	ATCATCAARCCSAARCTSGGCCTGCGTCCC
<i>cbbM</i> ( <i>Riftia</i> R) (1033-1063)° Elsaied and Naganuma, 2001	MGAGGTGACSGCRCCGTGRCCRGCMCGRTG

\* *Thiobacillus intermedius* K12 position (RubisCO-*cbbL*)

° *Riftia pachyptila* endosymbiont position (RubisCO-*cbbM*)

A touch-down PCR reaction was performed for the Alfreider and Nanba primers. The cycling parameters for the two primer pairs consisted of initial denaturation step at 95°C for 7 min, a primer annealing step for 45 s, and an extension step at 72°C for 45 s. After 30 cycles, final 10-min incubation was performed at 72°C. During the first 5 cycles, the annealing temperature was decreased by 1°C each cycle, starting at 59°C for the Alfreider primers until reaching a temperature of 55°C. For the Nanba primer pair the starting annealing temperature was 64°C, and after 5 touchdown cycles, the annealing temperature of 60°C was reached. The thermal profile for the Elsaied primer pair *cbbM* (*Riftia*F) and *cbbM* (*Riftia*R) consisted of initial denaturation step at 95°C for 7 min, followed by 30 cycles 45 s at 95°C, 40 s at 69°C and 45 s at 72°C and a final extension for 10 min at 72°C.

### Construction of RubisCO gene clone libraries

The amplified RubisCO fragments were directly cloned in *E. coli* using a TOPO™-TA cloning vector (Invitrogen, Karlsruhe, Germany) following the instructions of the manufacturer. White colonies were randomly picked and cultured overnight at 37°C in 2 ml Luria Broth (LB) medium with kanamycin 12.5 mg/ml. The size of the RubisCO inserts was checked by *in situ* PCR using the forward M13 (-40) (5'-GTTTTCCCAGTCACGA-3') and the reverse M13 (5'-CAGGAAACAGCTATGAC-3') plasmid specific primers, followed by agarose gel electrophoresis with subsequent ethidium bromide staining. A total of 90 RubisCO clones, with *cbbL* inserts, as well as 5 clones, which possessed *cbbM* genes, were stored as glycerol cultures at -80°C for further analysis.

### RFLP-typing

For screening the RubisCO diversity of the constructed clone libraries the PCR products were

digested in parallel with two frequently cutting endonucleases *MspI* and *HaeIII* (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA). The digests were analysed electrophoretically in 3.5% Small DNA Low Melt Agarose gels (Biozyme, Hessisch Oldenburg, Germany). The RFLP patterns obtained were compared and grouped in RubisCO RFLP types.

### Sequence analysis

One representative of each RFLP group as well as all the individual clones were sequenced. Direct sequencing of the PCR products was performed using the appropriate RubisCO primers on an ABI PRISM 310 Genetic Analyser (Perkin Elmer, Foster City, California, USA). The following program was applied: denaturation at 96°C for 2 min followed by 25 cycles of 45 s at 96°C, 15 s at 55°C, and 4 min at 60°C. The RubisCO nucleotide sequences were translated into amino acid sequences. The deduced amino acid sequences were compared with those available in the GenBanks by BLAST analysis. The CLUSTAL W alignment program was used for sequence alignment. Phylogenetic trees were generated based on the results of the neighbour-joining algorithm with distance analysis with Jukes-Cantor corrections according to the PHYLIP v.3.5 package (Felsenstein, 1993).

## Results and Discussion

Ninety clones containing the RubisCO form I *cbbL* gene were encountered in the S15A and S15B DNA applying the primer pairs developed by Alfreider (Alfreider et al., 2003) and Nanba (Nanba et al., 2004). In addition five clones, which possess RubisCO form II *cbbM* gene inserts, were retrieved from the S15A DNA as well. This DNA was recovered from the whole "unfractionated" microbial biomass of one sample, whereas the S15B DNA was extracted from the 0.45 µm biomass fraction of a parallel sample (see Material and Methods). The results from the affiliation of the deduced RubisCO *cbbL* amino acid sequences are presented in Fig. 1 and Table 2. As evident from the dendrogram in Fig. 1 the clones clustered within the green-like and red-like subgroups of the RubisCO form I.

Almost all of the green-like sequences were identified with the primer pair developed by Alfreider (Alfreider et al., 2003). These primers were described as specific for the RubisCO green-like IA cluster whereas the Nanba primers encountered sequences affiliated with RubisCO genes from both the green-like IA and red-like IC types (Nanba et al., 2004). More than 50% of the encountered RubisCO form I clones were obtained from the 0.45 µm biomass fraction (see Table 2). The sequence S15B-A1, which represents a group of 45 clones, was identical with the *cbbL* gene of the facultative chemolithoautotrophic, hydrogen-oxidizing *Hydrogenophilus thermolitheolus* TH-1 (Hayashi et al., 1999). Two other clones, S15B-A3 and S15B-A40, show high similarity to this gene as well.

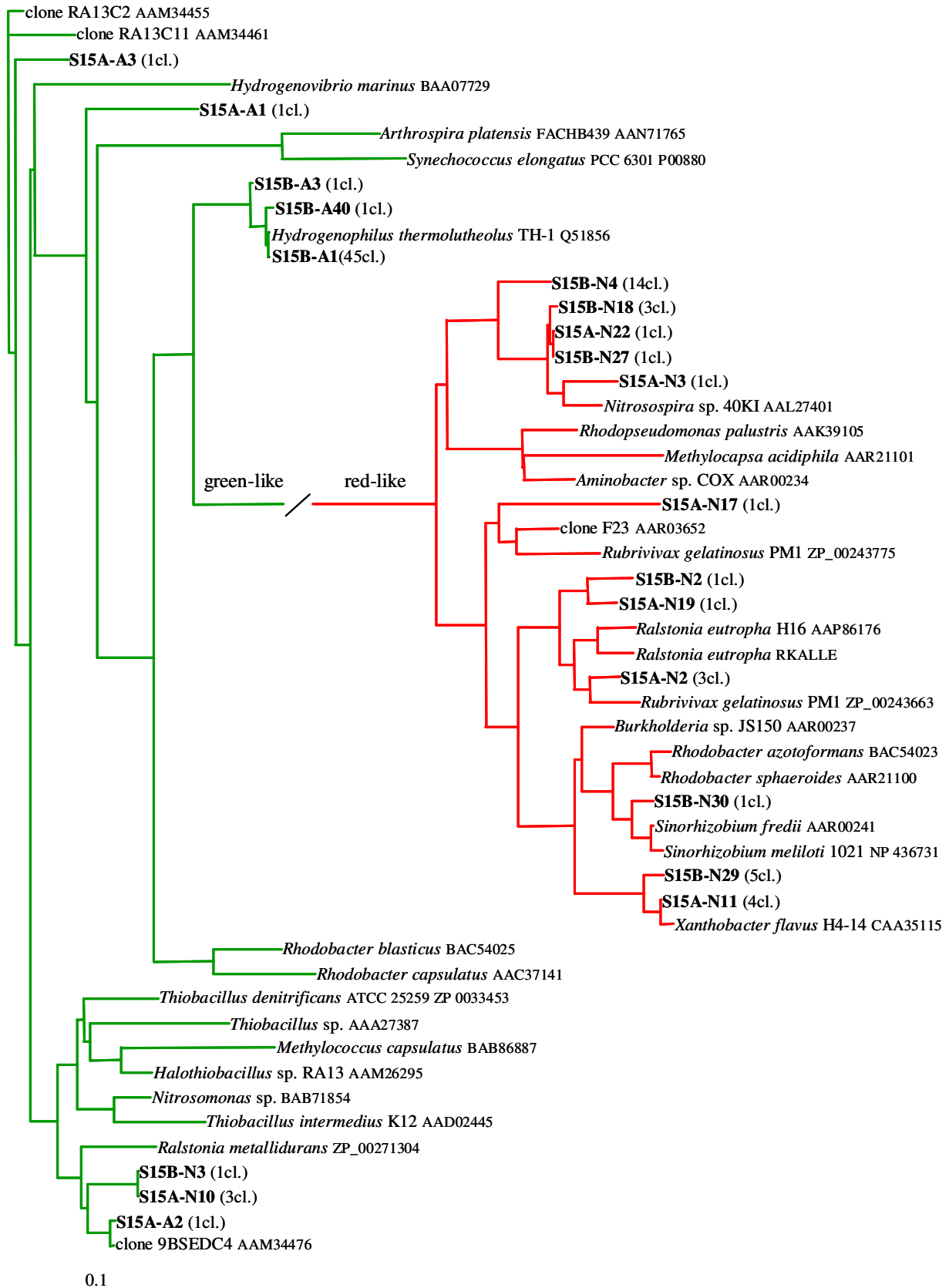


Fig. 1. Phylogenetic affiliation of the deduced amino acid sequences of the *cbhL* genes retrieved from the S15 site. The clones, designated as S15A and S15B, were encountered in the whole biomass of one sample and the 0.45  $\mu\text{m}$  fraction of a parallel sample, respectively. The primer pairs applied are abbreviated as A (Alfreider et al., 2003) and N (Nanba et al., 2004). In brackets the number of clones is given.

As shown in Fig. 1 and Table 2, only three clones within the green-like cluster were obtained with the Alfreider primer pair from the whole biomass. They show similarities to *cbbL* RubisCO genes encountered in subsurface environments applying the same primer pair (Alfreider et al., 2003). The sequence S15A-A1 possesses the lowest amino acid identity among the retrieved clones in this study and shared 81.3% similarity with the RubisCO clone RA13C11. The clone S15A-A3 was 96.1% similar to the clone RA13C2. The two referent clones, RA13C11 and RA13C2 were identified in significant number in organic polluted underground *in situ* reactor (Alfreider et al., 2003). The clone S15A-A2 was almost identical (99.4%) with the clone 9BSEDC4 that was obtained from subsurface aquifer, located near to an organic contaminated site (Alfreider et al., 2003). Intriguing is the presence of highly related *cbbL* clones in subsurface environments, which are rather different in their geographical and physicochemical characteristics, like the S15 deep monitoring well and the above described sites (<30 m below the land surface), studied by Alfreider and colleagues.

As shown in Fig. 1, similar to the cluster of S15A-A2 and 9BSEDC4 were the sequence S15A-N10, which represents a group of three clones retrieved from the whole biomass and the identical to this group individual clone S15B-N3, encountered in the 0.45  $\mu$ m biomass fraction. These sequences were identified by using the primer pair developed by Nanba (Nanba et al., 2004). Interestingly, the S15A-N10 and S15B-N3 sequences possess the additional glycine codon, described previously for the *cbbL* genes from the IA green-like radiation, encountered using the same primers (Nanba et al., 2004). As shown in Fig. 1 and Table 2 the clone S15A-N10 shared 93% similarity with the *in silico* predicted *cbbL* gene from the genome of *Ralstonia metallidurans* CH34. Interestingly, the related to it chemolithoautotroph *Ralstonia eutropha* H16 harbours dual *cbbL* gene copies (chromosomal and megaplasmid), which however belong to the red-like radiation (Uchino and Yokota, 2003). In accordance to this is the reported presence of green-like *cbbL* in *Rhodobacter capsulatus* and red-like *cbbL* in the related to it *Rhodobacter sphaeroides* (Paoli et al., 1998). This case was taken as a clear proof of a horizontal gene transfer event in the evolution of the form I of the RubisCO gene. Recently, it was demonstrated for the first time the presence of both the green-like and red-like *cbbL* genes even in one organism, namely *Rhodobacter azotoformans* (Uchino and Yokota, 2003). The clone S15A-N10 was affiliated with a slightly lower identity of 91.1% with the *cbbL* of *Thiobacillus denitrificans*. This bacterium was described as obligate autotroph and harbours both the form I and form II of the enzyme (Hernandez et al., 1996). In addition, the clone shared 89.9% of identity, still very high for proteins, with *cbbL* gene of *Nitrosomonas* sp. ENI-11. The high relatedness between the sequences of the S15A-N10 cluster and *cbbL* genes from  $\beta$ -proteobacterial species of

different genera like *Ralstonia*, *Thiobacillus* and *Nitrosomonas* represents a nice example for the phylogenetic inconsistency based on the *cbb* genes. Recently, a cluster of closely related *cbbL* sequences from *Bacillus*, *Streptomyces* and *Arthrobacter* strains, which belong to different bacterial groups like *Firmicutes* and *Actinobacteria*, was described (Slesi et al., 2005).

Table 2. The closest amino acid sequences of the encountered *cbbL* and *cbbM* genes

Clone name	Number of clones	Closest amino acid sequences (EMBL No.)	BLAST % of similarity
<b><i>cbbL</i> Alfreider primers</b>			
S15A-A1	1	clone RA13C11 (AAM34461)	81.3
S15A-A2	1	clone 9BSEDC4 (AAM34476)	99.4
S15A-A3	1	clone RA13C2 (AAM34455)	96.1
S15B-A1	45	<i>Hydrogenophilus thermoluteolus</i> TH-1 (Q51856)	100
S15B-A3	1	<i>Hydrogenophilus thermoluteolus</i> TH-1 (Q51856)	98.9
S15B-A40	1	<i>Hydrogenophilus thermoluteolus</i> TH-1 (Q51856)	99.4
<b><i>cbbL</i> Nanba primers</b>			
S15A-N10	3	<i>Ralstonia metallidurans</i> CH34 (ZP_00271304) <i>Thiobacillus denitrificans</i> ATCC 25259 (ZP_00334530) <i>Nitrosomonas</i> sp. ENI-11 (BAB71854)	93.0 91.1 89.9
S15B-N3	1	<i>Ralstonia metallidurans</i> CH34 (ZP_00271304) <i>Thiobacillus denitrificans</i> ATCC 25259 (ZP_00334530) <i>Nitrosomonas</i> sp. ENI-11 (BAB71854)	93.3 91.5 90.0
S15A-N11	3	<i>Xanthobacter flavus</i> H4-14 (CAA35115)	98.7
S15A-N4	1	<i>Xanthobacter flavus</i> H4-14 (CAA35115)	98.0
S15B-N29	5	<i>Xanthobacter flavus</i> H4-14 (CAA35115)	96.3
S15A-N2	3	<i>Ralstonia eutropha</i> H16 (AAP86176) <i>Rubrivivax gelatinosus</i> PM1 (ZP_00243663)	95.2 95.2
S15A-N19	1	<i>Ralstonia eutropha</i> (RKALLE) <i>Rubrivivax gelatinosus</i> PM1 (ZP_00243663)	92.5 92.5
S15A-N17	1	clone F23 (AAR03652) <i>Rubrivivax gelatinosus</i> PM1 (ZP_00243775)	87.2 85.2
S15B-N2	1	<i>Ralstonia eutropha</i> (RKALLE) <i>Rubrivivax gelatinosus</i> PM1 (ZP_00243663)	93.4 90.5
S15A-N22	1	<i>Nitrosospira</i> sp. 40KI (AAL27401) clone F36 (AAR03663)	96.6 96.0
S15A-N3	1	<i>Nitrosospira</i> sp. 40KI (AAL27401) clone Mariana (IC)-4 (BAD22661)	94.5 93.1
S15B-N18	3	<i>Nitrosospira</i> sp. 40KI (AAL27401) clone F38 (AAR03665)	96.3 93.9
S15B-N4	14	<i>Nitrosospira</i> sp. 40KI (AAL27401) clone F30 (AAR03659)	91.4 91.4
S15B-N27	1	<i>Nitrosospira</i> sp. 40KI (AAL27401) clone F38 (AAR03665)	96.8 94.9
S15B-N30	1	<i>Sinorhizobium meliloti</i> 1021 (NP_436731)	97.5
<b><i>cbbM</i> Elsaied primers</b>			
S15A-E1	2	clone Fryer(II)-8 (BAD90717) clone Fryer(II)-3 (BAD90712) <i>Magnetospirillum magnetotacticum</i> AAR21103	88.3 88.3 82.5
S15A-E2	3	clone 820-E10 (AAS02069) magnetite-containing magnetic vibrio MV-1 (AAL76921)	89.2 86.1

A total of thirty-six clones retrieved from the S15A and S15B DNA clustered within the red-like group of form I RubisCO. All of them were encountered with the Nanba primer pair (Nanba et al., 2004). Interestingly, applying these primers, Nanba and colleagues identified in microbial mats *cbbL* genes that fell only within form IA (green-like), whereas those derived from the volcanic deposit clustered exclusively with form IC (red-like) *cbbL* sequences. In this study, *cbbL* genes from the IA (see above), as well as the IC subgroups, were encountered in one and the same sample, applying the Nanba primer pair.

In contrary to the green-like sequences almost all from the retrieved clones of the red-like type were similar between the whole biomass from the one sample and the 0.45  $\mu\text{m}$  microbial fraction of the parallel sample. As shown in Fig. 1 and Table 2, the two identical between each other clones S15A-N22 and S15B-N27 were highly related (>96%) to *cbbL* gene of the obligate autotrophic *Nitrosospira* sp. 40KI. The clone S15B-N18, which represents a group of three clones, differed in one amino acid position from these clones. The individual clone S15A-N3 and the clone S15B-N4, which represents a group of 14 clones, clustered within the *Nitrosospira* sp. 40KI related clones as well. Interestingly, this cluster of sequences was closely related also to the environmental clones F30, F36 and F38 (see Table 2), retrieved from volcanic deposits by others, where low levels of ammonium oxidation has been detected (Nanba et al., 2004). It was discussed that the phylogeny based on the RubisCO *cbbL* genes from ammonia oxidizers is comparable to the phylogeny of the 16S rRNA genes (Utåker et al., 2002). In accordance to this statement, the retrieved S15A and S15B clones with *cbbL* inserts could represent ammonia oxidizers of the *Nitrosospira* group. Members from this genus were not identified within the studied samples applying the 16S rDNA approach (see Chapters I and II). However, one representative from the cluster of *Nitrosomonadales*, which was affiliated with 16S rDNA of *Gallionella ferruginea*, a well known autotrophic Fe(II)-oxidizing bacterium, was encountered (see below Fig. 3B and Chapter II). The identification of organisms via the 16S rDNA and RubisCO approaches, does not necessary imply their *in situ* activity. Potential for ammonia- or Fe(II)-oxidation possess also other bacteria, identified in the S15 samples via the 16S rDNA approach, like members of the *Planctomycetes* group or *Dechlorosoma* spp., respectively.

As shown in Fig. 1 and Table 2, a group of three clones, represented by S15A-N2 shared 95.2% identity with RubisCO gene from *Ralstonia eutropha* and to one of the two *cbbL* genes predicted *in silico* from the genome of *Rubrivivax gelatinosus* PM1 (EMBL No. ZP\_00243663). The individual clones S15A-N19 and S15B-N2 were affiliated with lower identity with these *cbbL* genes as well. Relatively high number of 16S rDNA clones obtained from the 0.22  $\mu\text{m}$  biomass fraction and an individual clone, retrieved from the 0.45  $\mu\text{m}$

fraction in our previous study were affiliated with 16S rRNA genes of *Ralstonia* spp. (see Chapter II). However, no such sequences were identified in the whole biomass of the parallel sample, which could be explained by the masking effect of the predominant *Dechlorosoma* spp. populations there (see Chapter I).

As shown in Fig. 1, the individual clone S15A-N17 shared 87.2% similarity with the environmental clone F23, identified in the above mentioned volcanic deposits (Nanba et al., 2004). Interestingly, the S15A-N17 clone was related with 85.2% to the second *cbbL* gene predicted *in silico* from the *Rubrivivax gelatinosus* PM1 genome (EMBL No. ZP\_00243775). As evident from Fig. 1 and Table 2 the two *cbbL* genes of *Rubrivivax gelatinosus* PM1 (EMBL No. ZP\_00243663 and ZP\_00243775) possess different amino acid sequences. Dual red-like *cbbL* forms have been reported also for *Ralstonia eutropha* (Kusian et al., 1995), which together with *Rubrivivax gelatinosus* PM1 belong to the order *Burkholderiales* from the  $\beta$ -subclass of Proteobacteria. Dual green-like *cbbL* forms were identified in *Acidithiobacillus ferrooxidans*, *Allochromatium vinosum*, *Hydrogenovibrio marinus* (Kusano et al., 1991; Viale et al., 1989; Nishihara et al., 1998). Studying the similarities between the *cbbL* genes from these bacteria, it was hypothesized that gene duplication, rather than lateral gene transfer, was the possible past event, responsible for the acquisition of the dual genes (Uchino and Yokota, 2003).

A group of four clones, represented by S15A-N11, encountered in the whole biomass and five clones (see S15B-N29) retrieved from the 0.45  $\mu$ m microbial fraction differed in two positions of their *cbbL* amino acid sequences. They shared 98% identity with the RubisCO gene of *Xanthobacter flavus* H4-14. The individual clone S15B-N30 was related to RubisCO sequence of the *Agrobacterium-Rhizobium* branch of the  $\alpha$ -Proteobacteria. A 16S rDNA clone, S15A-MN24, identified in our previous study, was affiliated to this group as well (Chapter I and see below Fig. 3A). This sequence possesses high levels of similarity with 16S rDNA genes of the chemolithoautotrophic arsenite-oxidizing bacterium BEN-5 and the strain *Sinorhizobium* sp. SA-2 (Janecka et al., 2002; Santini et al., 2000). According to Selesi and colleagues, the *Xanthobacter* and *Sinorhizobium* spp. are example of congruency between the 16S rDNA and RubisCO *cbbL* gene phylogeny (Selesi et al., 2005).

As demonstrated in Fig. 2 and Table 2 the five clones obtained applying a primer pair specific for the *cbbM* gene, encoding the second form of the large subunit of RubisCO, were affiliated with other environmental clones identified in deep-sea hydrothermal vents and with magnetotactic bacteria. The clone S15A-E1 represents a group of two clones and possesses identity of 88.3% to the clones Fryer (II)-8 and Fryer (II)-3. This clone shared 82.5% similarity with the *cbbM* gene of *Magnetospirillum magnetotacticum*. The other three *cbbM*



clones (see S15A-E2) were similar to the environmental clone 820-E10 (Campbell and Craig Cary, 2004). These clones were affiliated also with the *cbbM* gene of a magnetite-producing bacterium MV-1 (see Table 2) (Bazylinski et al., 2004). This strain was recently isolated from the oxic-anoxic transition zone of a chemically stratified marine environment.

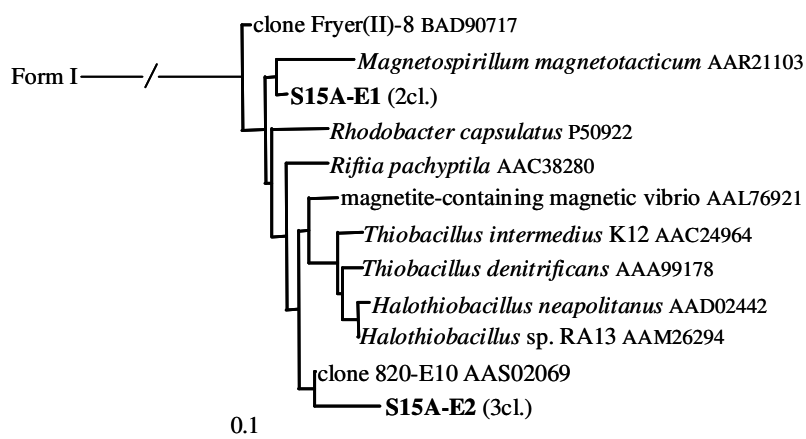


Fig. 2. Phylogenetic affiliation of the deduced amino acid sequences (given in bold) of the *cbbM* genes retrieved from the whole biomass of one sample. The primer pair applied is abbreviated as E (Elsaied and Naganuma, 2001). The number of clones is indicated in brackets.

Clones, with *cbbM* gene inserts, retrieved from groundwater samples by others, were related to *Halothiobacillus* sp. (Alfreider et al., 2003). The presence of *cbbM* genes of *Thiobacillus denitrificans* was reported for different depths of a clay-rich aquitard subsurface habitat (Lawrence et al., 2000). Relatively high phylogenetic diversity of *cbbM* genes was presented for hydrothermal vent environment (Elsaied and Naganuma, 2001). However, bacteria, possessing the second form of the RubisCO enzyme were not encountered in environment such as the hypersaline alkaline Mono lake (Giri et al., 2004). The identification of organisms, harboring this form of RubisCO is very intriguing, because it was assumed, that the common ancestor of RubisCO was similar to the form II of the enzyme (Jordan and Ogren, 1981).

The microbial *cbbL* gene diversity was previously studied in deep-sea hydrothermal vent (Elsaied and Naganuma, 2001), shallow subsurface environments (Alfreider et al., 2003), volcanic deposits (Nanba et al., 2004), alkaline Mono lake (Giri et al., 2004), agricultural soils (Selesi et al., 2005). In this study we have identified bacteria, harbouring *cbbL* and *cbbM* genes, in oligotrophic groundwater samples, collected from about 300 m below the land surface in Siberia, Russia. Our results demonstrate that the majority of the identified RubisCO form I clones were closely related (amino acid similarities >92%) or even identical with *cbbL* genes of known chemolithoautotrophic bacteria.

The dendrograms in Fig. 3 represent the affiliation of the 16S rDNA sequences of the bacterial strains encountered via the RubisCO approach in this work. These sequences were

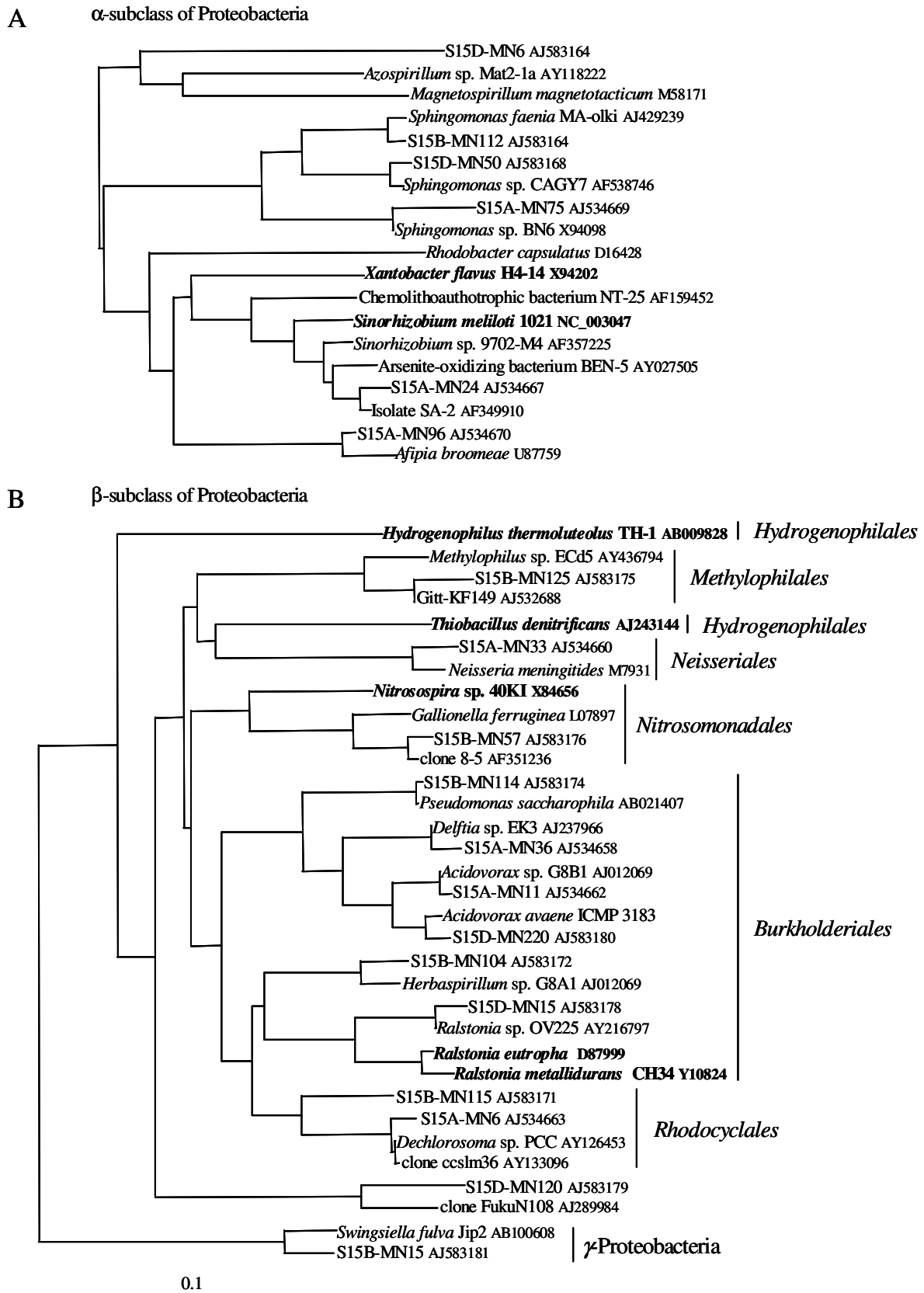


Fig. 3. Affiliation of the 16S rDNA sequences of the  $\alpha$ - (A) and  $\beta$ -proteobacterial (B) strains (given in bold), related to the encountered S15 sequences applying the RubisCO approach. The sequences were compared with 16S rDNA clones, designated as S15A-MN, S15B-MN and S15D-MN, identified in the S15 samples via the 16S rDNA retrieval.

compared with related clones, retrieved applying the 16S rDNA approach from the same samples in our previous study (see Chapters I and II). The RubisCO relevant clones clustered with members within the  $\alpha$ - and  $\beta$ -subclasses of Proteobacteria which is not surprising, considering the widely distribution of the *cbb* operon among these groups from the Bacteria domain. The largest part of the retrieved RubisCO form I clones were affiliated with *cbbL* genes of  $\beta$ -Proteobacteria which confirms the predominance of this bacterial group in the studied groundwater samples. However, the most abundant  $\beta$ -Proteobacteria found in our previous studies via the 16S rDNA approach, showed 99.7% identity with the 16S rRNA gene of the hydrogen-oxidizing and perchlorate-reducing *Dechlorosoma* sp. PCC, recently renamed to *Azospira* sp. from the *Rhodocyclus-Azoarcus* group (Tan and Reinhold-Hurek, 2003). Autotrophic growth for a strain, related to *Dechlorosoma* sp. was already described (Sobolev and Roden, 2004), but RubisCO sequence was not reported for the chemolithoautotrophic Fe(II)-oxidizing bacterium so far. Recently, the *Dechloromonas aromatica* genome has been found to contain RubisCO (Coates et al., 2005). However, the environmental conditions required for the expression of this phenotype have not been identified yet. This suggests that the identification of the RubisCO enzyme does not necessary imply capability for carbon dioxide fixation.

Relatively high number of other  $\beta$ -Proteobacteria like *Herbaspirillum* and *Ralstonia* spp. were encountered in our previous studies, based on the 16S rDNA retrieval, as well (see Chapter II). *Ralstonia*-related sequences were retrieved also in this work applying the RubisCO approach. The other autotrophic  $\beta$ -Proteobacteria were highly related to the small subunit *cbbL* genes of *Hydrogenophilus thermoluteolus* TH-1 and *Nitrosospira* sp. 40KI. Few studies reported on comparison between the RubisCO and 16S rDNA sequences from environmental samples so far, probably because of the disagreements between the *cbb* and rDNA phylogenies. Such inconsistencies were described for higher taxonomic level between the groups of Cyanobacteria and Proteobacteria, as well as among the different subclasses of Proteobacteria (Delwiche and Palmer, 1996). The recent studies on *cbb* genes from different *Rhodobacter* species demonstrated a clear proof of a horizontal gene transfer event in the evolution of RubisCO gene (Paoli et al. 1998; Uchino and Yokota, 2003). The phylogenetic similarity between the *cbbL* genes, retrieved from deep-sea chemoautotrophic bacteria and the *cbbL* genes of photosynthetic organisms was suggested to indicate a lateral transfer of RubisCO genes among deep-sea and surface water organisms (Elsaied and Naganuma, 2001). On the basis of our results we suggest the following explanations for the above described differences between the RubisCO and 16S rDNA approaches in the S15 samples. From one side the high identity of the retrieved RubisCO genes with already known autotrophic bacteria

indicates that these organisms were presented in the studied samples and were not encountered via the 16S rDNA retrieval. From the other, the identification of a very high number of *cbbL* genes (50% of the retrieved clones) which were affiliated with the *Hydrogenophilus thermolitheolus* RubisCO enzyme looks surprising. According to the Bergy's manual of systematic bacteriology  $\beta$ -subclass of Proteobacteria consist of six orders: *Burkholderiales*, *Hydrogenophilales*, *Methylophilales*, *Neisseriales*, *Nitrosomonadales* and *Rhodocyclales* (Garrity, 2001). As evident from the dendrogram in Fig. 3B, in our previous studies on the same samples applying the 16S rDNA approach, we have identified clones sufficiently closely related to members from five of them. In addition clones affiliated to environmental sequences, deeply branching within the cluster of  $\beta$ -Proteobacteria were encountered as well. Members of the genus *Hydrogenophilus*, order *Hydrogenophilales*, identified in this study via the RubisCO approach, were not encountered applying the 16S rDNA retrieval. The genus *Hydrogenophilus* harbours thermophilic bacteria, and form a distinct lineage among the  $\beta$ -Proteobacteria. Representatives of this genus were retrieved in thermal environments so far (Anitori et al., 2002; Hayashi et al., 1999; Smith et al., 2003; Mountain et al., 2003; Nakagawa et al., 2002), where the conditions are rather different from those described for the groundwaters at the S15 monitoring well (Wickham et al., 2003). A study on the *cbb* operon in *Rhodobacter capsulatus* has demonstrated that the genes encoding form I RubisCO and the neighbouring genes were acquired by a horizontal gene transfer (Paoli et al., 1998). Interestingly, the RubisCO of this bacterium was most closely related to that of the *Hydrogenophilus thermolitheolus*. It is not excluded, that the retrieved RubisCO genes in our case are harboured not by *Hydrogenophilus thermolitheolus* exclusively, but also by other phylogenetically distant bacteria.

Incongruence between the phylogeny based on 16S rDNA and functional gene like *dsrAB* (Chang et al., 2001; Klein et al., 2001) was reported, but this gene is widely applied for detection of the organisms involved in the sulphate-reduction (Baker et al., 2003; Lawrence et al., 2000; Nakagawa et al., 2004; Pérez-Jiménez and Kerkhof, 2005). The process of CO<sub>2</sub> fixation is of great environmental significance nevertheless the contribution of the microorganisms to it is still not well understood. The diversity and distribution of bacteria and archaea utilizing CO<sub>2</sub> via fixation pathways like rTCA, 3-hydroxypropionate, acetyl-coA, RuMP and serine, are largely unknown (Macalady and Banfield 2003). The identification of RubisCO in widely distributed heterotrophic bacteria like *Bacillus*, *Arthrobacter* (Selesi et al., 2005), *Dechloromonas* (Coates et al., 2005) suggested a potential for autotrophic growth under appropriate environmental conditions. It should be pointed out, that CO<sub>2</sub> assimilation via the *cbb* pathway requires a large amount of energy (Alfreider et al., 2003), which could be

used for other purposes, if CO<sub>2</sub> fixation is not essential. However, in oligotrophic environments, like most of the ground waters, the presence of microorganisms, able to grow autotrophically will be an advantage and can contribute to the widely discussed carbon dioxide sequestration in different subsurface environments.

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## Chapter IV

### **Microbial diversity in ground water at the deep-well monitoring site S15 of the radioactive waste depository Tomsk-7, Siberia, Russia. Cultivation approach, interactions of the indigenous bacteria with uranium and other heavy metals**

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## Abstract

Oligotrophic bacteria were isolated from the S15 deep-well monitoring site, located at the Siberian radioactive subsurface depository Tomsk-7, Russia. They were affiliated with  $\alpha$ -proteobacterial species like *Sphingomonas*, *Brevundimonas* and *Methylobacter* spp. and with Actinobacteria from the genus *Microbacterium*. The minimal inhibitory concentrations (MIC's) of cadmium-, copper-, lead-, nickel-, silver-, chromium- and uranium for the growth of four *Microbacterium* spp. strains, namely S15-M2, S15-M3, S15-M4 and S15-M5 and of *Sphingomonas* sp. S15-S1 isolate were determined. The results showed relatively high heavy-metal tolerance of the S15 isolates that was species and even strain specific. The full sequenced 16S rDNA of three of the *Microbacterium* strains, S15-M2, S15-M4 and S15-M5, differ only at four positions. However, the isolates S15-M2 and S15-M5 based on further physiological analyses were affiliated with *Microbacterium oxydans*, whereas S15-M4 represents another *Microbacterium* species. All *Microbacterium* spp. strains accumulate higher amounts of uranium than the *Sphingomonas* isolate. The influence of the radionuclide on the growth of the *Microbacterium* spp. was examined by using flow cytometry. The results showed presence of two subpopulations of cells with different surface properties. After longer exposure to uranium the percentage of one of them increased. The X-ray absorption spectroscopy (XAS) studies indicated that both the cells of *Microbacterium oxydans* S15M-2 and of *Sphingomonas* sp. S15-S1 precipitate at pH 4.5 U(VI) as inorganic meta-autunite like uranyl phosphates. Transmission electron microscopy (TEM) and energy dispersive X-ray (EDX) analyses revealed that *Microbacterium* spp. accumulate uranium mainly as extracellular precipitates. In the cells of *Sphingomonas* sp. S15-S1 both intra- and extracellular uranium deposits were found. At pH 2, U(VI) was complexed by organically bound phosphates on the cell surfaces of the studied strains.

## Introduction

The concept of underground storage of radioactive wastes was applied with the primary objective to permanently isolate the injected wastes from the biosphere (Bachofen et al., 1998; Christofi and Philip, 1997; Stroes-Gascoyne and West, 1996). A number of countries is involved in research programs that examine this option of repository for the future. The main concern for this kind of disposal is the prevention of eventual release and migration of the radioactive wastes into the sediments and the ground waters. For this reason, multidisciplinary monitoring studies were undertaken in order to evaluate the impacts of such wastes to the subsurface environments and the potential for their remediation (Anderson and Pedersen, 2003; Nazina et al., 2004; Stroes-Gascoyne and West, 1996). It was demonstrated, that these

extreme habitats are occupied with viable and metabolically active microorganisms, which exploit a wide range of redox reactions and thus influence the geochemical conditions (Ehrlich, 1998; Lovley and Chapelle, 1995; Pedersen, 2000).

A number of studies has shown that natural microorganisms, isolated from heavy metal-contaminated habitats (McLean and Beveridge, 2001; Merroun and Selenska-Pobell, 2001; Selenska-Pobell, 2002; Selenska-Pobell et al., 2001; Shelobolina et al., 2004) or from other extreme environments (Benyehuda et al., 2003; Duxbury and Bicknell, 1983), as well as some laboratory strains (Ben Omar et al., 1997; Merroun et al., 2001) effectively interact with toxic metals and radionuclides via direct and indirect mechanisms. These interactions cause mobilization and/or immobilization of the metals (Banaszak et al., 1999; Francis, 1998; Lloyd and Lovley, 2001; Nies, 1999; Selenska-Pobell, 2002). They encompass biotransformations as oxidation and reduction of metals (Beller, 2005; Lack et al., 2002; Lovley, 1993; Shelobolina et al., 2004); biosorption by cell surface polymers (Hafez et al., 2002; Selenska-Pobell et al., 1999; Vieira and Volesky, 2000); uptake of metals inside the cells (Francis et al., 2004; McLean and Beveridge, 2001; Merroun et al., 2003c; Suzuki and Banfield, 2004); induction of metal precipitation and generation of minerals (Douglas and Beveridge, 1998; Macaskie et al., 1992; Merroun et al., 2005b; Renninger et al., 2001, 2004); or alteration of metal speciation caused by microbially induced redox changes in the environment (Bosecker, 1997). The above described microbial activities strongly influence the fate of the toxic metals in and outside the habitat where the radioactive waste has been deposited (Banaszak et al., 1999).

Investigation of the microbe-metal interactions gives insight into the potential of the organisms to sustain the radionuclide and heavy metal-toxicity and to influence their further behavior within the environment. Therefore, the understanding of the underlying mechanisms is of great importance for the development of bioremediation strategies (Finneran et al., 2002; Raff et al., 2003). The optimization of the latter requires extensive studies on the speciation and the localization of the toxic metals associated with the microorganisms. The speciation and bonding environment of uranium in bacteria has been examined by using variety of spectroscopic techniques such as Fourier-transform infrared (FT-IR) and time-resolved laser-induced fluorescence spectroscopy (TRLFS) (Merroun et al., 2003a; Panak et al., 1998). Synchrotron-based techniques such as XAS have been used to determine the oxidation state of uranium [X-ray absorption near edge spectroscopy (XANES)] and the fine atomic structure of the uranium complexes [Extended X-ray absorption fine structure (EXAFS)] formed by various microorganisms (Francis et al., 2004, Kelly et al., 2001; Merroun et al., 2003c; 2005a, b, Panak et al., 1998). In addition by using TEM and EDX techniques the metal deposits can be localized and identified (McLean and Beveridge, 2001; Merroun et al., 2003c; van

Hullebusch et al., 2003).

The aim of this work is to characterize several oligotrophic bacterial strains recovered from the water of the S15 subsurface monitoring well, located in close proximity to the radioactive disposal site Tomsk-7, Siberia, Russia. Of special interest was to study the interactions of these isolates with uranium and other toxic metals. The results can serve for risk assessments of the studied Siberian subsurface environment, where deposition of radioactive wastes is still performed.

## **Materials and Methods**

### **Site description**

The S15 monitoring well is located about 2 km apart from the radioactive waste injection site Tomsk-7, and uncovers an aquifer at 290 to 324 m below the land surface, which corresponds to one of the radioactive disposal horizons (Wickham et al., 2003). The ground water from the well was determined as oligotrophic with merely concentrations of natural radionuclides. Extensive geochemical characterization of the S15 monitoring well, as well as site and sample descriptions can be found elsewhere (see Chapters I and II; Wickham et al., 2003).

### **Bacterial strains, isolation and cultivation conditions**

Bacterial strains were isolated under aerobic and anaerobic conditions from the biomasses, collected on 0.22  $\mu\text{m}$  and 0.45  $\mu\text{m}$  nitrocellulose filters. For the cultivation in aerobic conditions, respectively, oligotrophic R2A medium (Reasoner and Geldreich, 1985) was used. The medium contained 0.5 g yeast extract, 0.5 g peptone, 0.5 g casamino acid, 0.5 g glucose, 0.5 g soluble starch, 0.3 g  $\text{K}_2\text{HPO}_4$ , 0.05 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.3 g sodium pyruvate per liter of distilled water ( $\text{dH}_2\text{O}$ ). The pH was adjusted to 7.2 by addition of  $\text{KH}_2\text{PO}_4$ . For solid medium, 15 g agar per liter was added. Parts from the 0.22  $\mu\text{m}$  filter were put in Erlenmeyer flasks, containing 50 ml R2A medium and incubated at 25°C, by shaking at 110 rpm in a water bath. After three days of incubation, serial dilutions from  $10^{-1}$  to  $10^{-5}$  were made and 10  $\mu\text{l}$  aliquots were inoculated on R2A agar. Colonies with different morphology and colors were picked up from the  $10^{-4}$  and  $10^{-5}$  dilutions and transferred in liquid R2A medium. In order to obtain pure cultures the isolates were transferred two times to liquid / agar R2A medium and after that 1 ml from the cultures were stored in a 1:1 mixture with glycerol at -80°C. The seven isolated strains were affiliated according to their 16S rDNA (see below) to *Microbacterium oxydans* strains S15-M2 and S15-M5, *Microbacterium* sp. S15-M3, *Microbacterium* sp. S15-M4, *Sphingomonas* sp. S15-S1, *Methylobacter* sp. S15-IsoA and *Brevundimonas mediterranea* S15-IsoB.

For the anaerobic enrichments an anoxic medium, described by (Bruce et al., 1999), with acetate as a sole electron donor was used. Instead of sodium chlorate ( $\text{NaClO}_3$ ), sodium

nitrate  $\text{NaNO}_3$  (10 mM final concentration) was used in this study as a sole electron acceptor. The pH was adjusted to 7.2 with 10 M HCl and the medium was autoclaved for 15 min at 121°C. Vitamins and trace elements were added (10 ml l<sup>-1</sup>) from stock solutions (Bruce et al., 1999) to the cool medium. 20 ml from the medium was poured in Schott glass vessels and stirred under  $\text{N}_2/\text{CO}_2$  (80/20% v/v) atmosphere for 20 min. Parts from the 0.45 µm nitrocellulose filter were added to the medium under sterile conditions. After two weeks of incubation under anaerobic conditions, serial dilutions from 10<sup>-1</sup> to 10<sup>-5</sup> were made and 10 µl aliquots were plated on R2A agar under aerobic conditions. After that the cultures were handled in the same way as described above for the aerobic cultivation.

### **Molecular characterization of the isolates**

DNA extraction was performed from 2 ml bacterial cultures, grown in liquid R2A medium up to the late exponential growth phase. The cells were centrifuged (9000 rpm, 10°C, 5 min) and DNA was isolated from the pellet by using the NucleoBond® AXG 100 Kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. The 16S rRNA gene fragments were amplified using the primer pair 16S<sub>7-27:F</sub> (5'-AAGAGTTTGATYMTGGCTCAG-3') and 16S<sub>1492-1513:R</sub> (5'-TACGGYTACCTTGTTACGACTT-3'), *E. coli* numbering, applying the PCR master mix and parameters, as previously described (see Chapters I and II). The PCR products were digested with three frequently cutting endonucleases *MspI*, *HaeIII* and *RsaI* (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA). The digests were analysed electrophoretically in 3.5% Small DNA Low Melt agarose gels (Biozyme, Hessisch Oldenburg, Germany). The 16S rDNA products were sequenced on an ABI PRISM 310 Genetic Analyser (Perkin Elmer, Foster City, California, USA).

### **Physiological analysis**

The substrate utilization patterns were determined in duplicate for *Microbacterium* spp. S15-M2, S15-M4 and S15-M5 and *Sphingomonas* sp. S15-S1. The bacterial cells were initially grown in R2A medium. The optical density (OD) of the cultures was measured at 600 nm with a Spektrometer Ultrospec® (Amersham Pharmacia Biotech). The cells, grown to the beginning of the exponential growth phase (OD<sub>600</sub> of 0.2) were harvested by centrifugation (9000 rpm, 4°C, 10 min) and washed three times with 0.9% NaCl, to remove the residual substrates from the medium. For the growth test, 200 µl aliquots of the cell suspension were inoculated in inorganic basal medium (Hahn et al., 2003), which was distributed in Cellstar® 24 well plates (Greiner Bio-one GmbH, Frickenhausen, Germany). The wells with an end volume of 2 ml were supplemented with different substrates to the required final concentrations. After 4 weeks of incubation at room temperature and shaking at 110 rpm, the



growth was monitored by determining the turbidity.

The sugar derivatives used were glucose, fructose, L- and D-arabinose, D-sorbitol, sucrose and maltose (final concentration of each, 5 mM). The acids were sodium acetate (5 mM), sodium pyruvate (5 mM), citric acid (2 mM), maleate (5 mM), Na-L-lactic acid (5 mM), tartaric acid (2 mM), nicotinic acid (2 mM). The alcohols tested were methanol (2 mM), ethanol (5 mM), 2-propanol (5 mM). In addition, L-arginine (2 mM), L-glutamine (2 mM), and Tween 80 (0.001% v/v) were tested.

### **Scanning electron microscopy (SEM)**

The examination of the bacterial cells was performed using a low voltage scanning electron microscope LEO 982 Gemini (LEO, Elektronenmikroskopie GmbH, Oberkochen, Germany). The cell suspensions were fixed in 4% glutaraldehyde solution in 25 mM  $\text{KH}_2\text{PO}_4$  buffer (pH 7) for 24 h at 4°C. The cells were harvested by centrifugation (8000 rpm, 4°C, 5 min) and dehydrated with increasing ethanol concentrations (from 10 to 99%). The dehydrated samples were fixed on a carbon tape covered aluminum sample holder, followed by shadow casting with carbon (MED 010, Baltec AG, Lichtenstein).

**Heavy metal solutions.** 1 M stock solutions of  $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ ,  $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ ,  $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{Pb}(\text{NO}_3)_2$  and 0.1 M stock solution of  $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  were prepared by dissolving appropriate quantities of the metals in 0.1 M sodium perchloric acid ( $\text{NaClO}_4$ ). The required final concentration of uranium solutions for the biosorption, flow cytometric, EXAFS and TEM analyses were obtained by dilution of the stock solution with 0.1 M  $\text{NaClO}_4$ . After the adjustment to the appropriate pH, the solutions were filtered through 0.22  $\mu\text{m}$  nitrocellulose filters.

### **Heavy metal tolerance**

The Minimum Inhibitory Concentrations (MIC's) of seven heavy metals were determined in duplicate for the strains *Sphingomonas* sp. S15-S1, *Microbacterium oxydans* S15-M2 and S15-M5, *Microbacterium* sp. S15-M3 and *Microbacterium* sp. S15-M4. The cells were grown to  $\text{OD}_{600}$  of 0.4 in low phosphate medium (LPM) (Rossbach et al., 2000), washed twice with 0.9% NaCl and 10  $\mu\text{l}$  of the cell suspension was transferred in LPM agar, containing the following concentrations of the metals: for copper, nickel and uranium increasing concentrations from 0.25 to 16 mM; for chromium from 0.5 to 16 mM; for cadmium and silver from 0.03 to 1 mM; for lead from 0.06 to 2 mM. After spreading, the plates were incubated at room temperature for one week. The MIC was defined as the lowest concentration at which complete inhibition of colony formation was observed (Rossbach et al., 2000).

The metal solutions, as well as the other components of the LPM medium were added to the

agar at 50°C. The calculation of the metal ions concentrations was performed using the following equation:

$$x = \frac{V * C_f}{C_s}$$

where  $x$  and  $C_s$  are the volume (in  $\mu\text{l}$ ) and the concentration (in M) of the metal stock solutions respectively (see heavy metal solutions),  $V$  the volume of the solid medium in ml and  $C_f$  the final metal concentration in mM

### **Uranium biosorption**

Bacterial cells grown to  $\text{OD}_{600}$  of 0.6 (late-exponential growth phase) in Nutrient Broth (NB) medium (8 g/l, BD Difco™, Detroit, USA) or R2A medium were harvested by centrifugation (9000 rpm, 4°C, 20 min) and washed twice with 0.1 M  $\text{NaClO}_4$ . Equal amounts of bacterial cells (between 0.2 and 0.3 mg/ml dry weight) were resuspended in 10 ml solutions possessing different concentrations of uranium (from 2.38 to 144 mg/l) adjusted with  $\text{HClO}_4$  to pH values of 2, 3, and 4.5. After 48 h of shaking, (110 rpm, 30°C) the cells were removed from the solution by centrifugation and 1 ml of the supernatant was collected for metal content analysis by using an ELAN 9000 Inductively Coupled Plasma- Mass Spectrometer (ICP-MS) (Perkin-Elmer, USA). Accumulation of the metal ions ( $q$ ) by the biomass was calculated from a metal biomass balance yielding:  $q$  (mg U per g dry biomass) =  $V(C_i - C_f) / m$  where  $V$  is the sample volume (l),  $C_i$  and  $C_f$  are the initial and the final metal concentrations (mg/l), respectively and  $m$  is the amount of dry biomass (g). Three replicates were prepared for each concentration. In addition experimental control samples without biomass were treated identically. For determination of the dry weight, the cell pellets were dried at 70°C for 48 h and their weights were measured using Sartorius LA 120 S (Göttingen, Germany) ( $\pm 0.1$  mg).

### **Uranium biosorption kinetic**

The bacterial cells were treated as described above for the uranium biosorption. They were distributed in the appropriate number of flasks and resuspended in 10 ml uranium solution (23 mg/l, pH 4.5). The cells were incubated for 30 min, 2 h, 16 h, 24 h, 48 h, and 72 h at 30°C, and shaken (110 rpm) for each time in triplicate. After that they were harvested and the supernatants were analysed as already described for the biosorption experiments.

### **Flow Cytometry**

The *Microbacterium oxydans* strains S15-M2 and S15-M5 and *Microbacterium* sp. S15-M4 were cultured in LPM medium to  $\text{OD}_{600}$  between 0.3 and 0.55. The cultures were diluted (1:10) in LPM medium (pH 4.5) containing 0.01 and 0.1 mM uranium and incubated for 14, 24 and 48 h (30°C, 110 rpm). The cells were harvested by centrifugation (9000 rpm, 4°C, 10 min), washed twice with 1 x PBS buffer (NaCl - 8 g; KCl - 0.2 g;  $\text{Na}_2\text{HPO}_4$  - 1.4 g;  $\text{KH}_2\text{PO}_4$  -

0.2 g, per 1 L, pH 7.4). To determine the toxicity of uranium, 1 ml cell suspensions were incubated for 10 to 30 min, with 1  $\mu$ l from the Live/Dead® *BacLight*<sup>TM</sup> bacterial viability kit (Molecular Probes, Inc., Eugene, Oregon, USA). *BacLight* is composed of two nucleic-acid binding stains: SYTO 9<sup>TM</sup> and propidium iodide. SYTO 9<sup>TM</sup> penetrates all bacterial membranes and stains the cells in green, while propidium iodide penetrates only cells with damaged membranes, and the combination of the two stains produces red fluorescing cells (Boulos et al., 1999). Flow cytometric measurements were performed on FacsCalibur (Becton Dickinson, Heidelberg, Germany).

### **X-ray absorption spectroscopy measurements**

Uranium L<sub>III</sub>-edge X-ray absorption spectra were collected at the Rossendorf Beamline at the European Synchrotron Radiation Facility (ESRF), Grenoble (France) (Matz et al., 1999) using a Si(111) double-crystal monochromator, and Si-coated mirrors for focusing and rejection of higher harmonics. The data were collected in fluorescence mode using a 13-element Ge detector. The energy was calibrated by measuring the Y K-edge transmission spectrum of an yttrium foil and defining the first inflection point as 17038 eV. The biological/uranyl samples were measured as dry samples. Eight spectra for each sample were recorded. The EXAFS oscillations were isolated from the raw, averaged data by removal of the pre-edge background, approximated by a first-order polynomial, followed by  $\mu_0$ -removal *via* spline fitting techniques and normalization using a Victoreen function. Dead-time correction was applied. The ionization energy for the U L<sub>III</sub> electron,  $E_0$ , was arbitrarily defined as 17185 eV for all averaged spectra. The EXAFS spectra were analyzed according to standard procedures using the program EXAFSPAK (George and Pickering, 1995). The theoretical scattering phase and amplitude functions used in data analysis were calculated using FEFF8 (Ankudinov et al., 1998). All fits included the four-legged multiple scattering (MS) path of the uranyl group, U-O<sub>ax</sub>-U-O<sub>ax</sub>. The coordination number (N) of this MS path was linked to N of the single-scattering (SS) path U-O<sub>ax</sub>. The radial distance (R) and Debye-Waller ( $\sigma^2$ ) factor of the MS path were linked at twice the R and  $\sigma^2$  of the SS path U-O<sub>ax</sub>, respectively (Hudson et al., 1996). During the fitting procedure, N of the U-O<sub>ax</sub> SS path was held constant at two. The amplitude reduction factor was held constant at 1.0 for the FEFF8 calculation and EXAFS fits. The shift in threshold energy,  $\Delta E_0$ , was varied as a global parameter in the fits.

### **Transmission electron microscopy (TEM) and energy dispersive X-ray (EDX)**

Bacterial cells were examined with a high resolution transmission electron microscope Philips CM 200 (Philips, Eindhoven, Nederland) at an acceleration voltage of 200 kV under standard operating conditions with the liquid nitrogen anticontaminator in place. After incubation with 0.5 mM uranium solution at pH 2, 3 and 4.5, the cells were fixed in 2.5% glutaraldehyde in

0.1 M cacodylate buffer (pH 7.2) for 2 h at 4°C and then washed three times with the same cacodylate buffer. The cell pellets were fixed for 60 min at 4°C in 1% OsO<sub>4</sub> in cacodylate buffer before being dehydrated with ethanol and embedded in Spurr resin. The samples were thin-sectioned (0.25 µm) using a diamond knife on a Reichert Ultracut S ultramicrotome and the sections were supported on copper grids and coated with carbon. EDX analysis was performed at 200 kV using a spot size of 70 Å, and a live counting time of 200 s. For selected area electron diffraction, we used the Philips CM 200 in the diffraction mode with a camera length of 1000 mm and an exposure time between 15 and 20 seconds.

Chemicals used in this work were supplied by Fluka (Sigma-Aldrich, Deisenhofen, Germany), Sigma (Sigma-Aldrich, Deisenhofen, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) with the following exceptions: Agar, Bacto® yeast extract, Bacto® peptone, Bacto® casamino acid (Difco Laboratories, Detroit, USA) and AgNO<sub>3</sub> (VEB, Feinchemie, Sebnitz, Germany).

## Results

### Phylogenetic affiliation of the S15 isolates

The phylogenetic affiliation of the bacterial strains, isolated from the S15 monitoring well was based on the 16S rDNA analysis. The results are presented in Figs.1 and 2 and in Table 1.

Table 1. Affiliation of the 16S rDNA of the S15 bacterial strains

Source	Strain	Closest relative (accession No.)	BLAST % of similarity
0.22 µm filter (aerobic conditions)	S15-S1	<i>Sphingomonas</i> sp. SKJH-30 (AY749436) clone S15D-MN50 (AJ583168)	100 97.1
	S15-IsoA	clone C-CF16 (AY622227) <i>Methylobacterium</i> sp. A1 (AJ416978)	94.4 94.4
	S15-IsoB	<i>Brevundimonas mediterranea</i> V4.BO.18 (AJ244706) <i>Brevundimonas</i> sp. LMG 11070 (AJ244648) clone S15A-MN37 (AJ534668)	100 100 98.3
	S15-M2	<i>Microbacterium oxydans</i> CV71a (AJ717358) <i>Microbacterium oxydans</i> AC44 (AJ717357) <i>Microbacterium oxydans</i> SW366-KB-3	100 100 100
	S15-M5	<i>Microbacterium oxydans</i> AC94 (AJ717356)	100
	S15-M3	<i>Microbacterium</i> sp. SKJH-23 (AY741722)	99.5
	S15-M4	<i>Microbacterium</i> sp. AS-44 (AJ391205)	100
0.45 µm filter (anaerobic enrichment)	S15-NaNO <sub>3</sub> -B	<i>Microbacterium schleiferi</i> DSM 20489 (Y17237)	97.3
	S15-NaNO <sub>3</sub> -C	Blackwater bioreactor bacterium BW7 (AF394172) clone S15A-MN4 (AJ534677)	100 99.9

The strains S15-S1, S15-IsoA and S15-IsoB, cultured under aerobic conditions, were affiliated with representatives of α-subclass of Proteobacteria. The strain S15-S1 possesses 16S rDNA identical to the one of *Sphingomonas* sp. SKJH-30, isolated from liquid nitrogen vessel. The 16S rRNA gene of the strain S15-IsoA shared 94.4% identity with clone C-CF16,

encountered in uranium contaminated aquifer (Reardon et al., 2004) and with *Methylobacterium* sp. A1, isolated from algal crusts. The 16S rDNA of the isolate S15-IsoB was identical to the ones of the *Brevundimonas mediterranea* V4.BO.18 and the *Brevundimonas* sp. LMG 11070.

As shown on the dendrogram in Fig. 1 closely related to the S15 strains *Sphingomonas* and *Brevundimonas* spp. were identified in parallel samples from the same site via the 16S rDNA retrieval (see Chapters I and II). Clone S15D-MN50, encountered in the 0.22  $\mu\text{m}$  biomass fraction of one sample, was affiliated with 99.2% with the 16S rRNA gene of *Sphingomonas yabuuchiae* A1-18<sup>T</sup>, isolated from the space laboratory Mir (Li et al., 2004) (see Fig. 1). The 16S rDNA of the strain S15-S1 has identity of 97.1% with the S15D-MN50 clone. Clone S15A-MN37 was retrieved from the whole biomass of another sample and possesses similarity of 99.4% to the 16S rDNA of *Brevundimonas* sp. FWC30 (see Fig. 1). The strain S15-IsoB shared 98.3% identity with the 16S rDNA clone S15A-MN37.

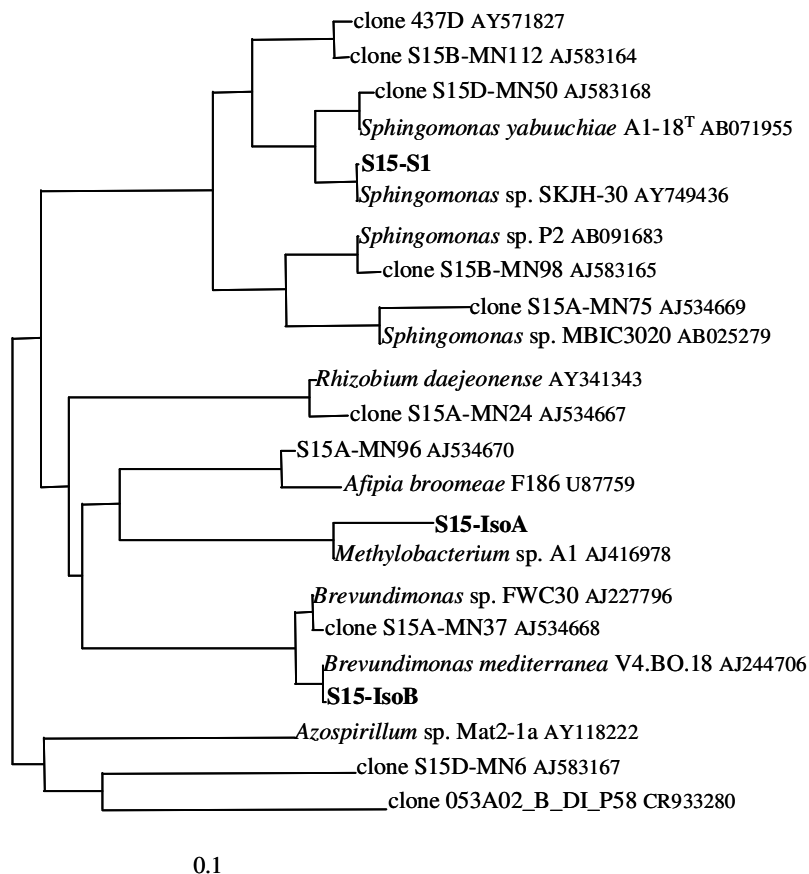


Fig. 1. 16S rDNA based affiliation of the cultured S15 bacterial strains with members of  $\alpha$ -subclass of Proteobacteria.

As shown in Fig. 2 six strains from the group of Actinobacteria, *Microbacterium* genus, were isolated from the S15 monitoring well. The strains S15-NaNO<sub>3</sub>-B and S15-NaNO<sub>3</sub>-C were

enriched under anaerobic conditions with acetate and nitrate as sole electron donor and acceptor, respectively, and after that further grown under aerobic conditions (see Material and Methods). The 16S rDNA of the isolate S15-NaNO<sub>3</sub>-B was similar at the level of species to the 16S rRNA gene of *Microbacterium schleiferi* DSM 20489. The isolate S15-NaNO<sub>3</sub>-C possesses almost identical 16S rDNA (one nucleotide difference) with the clone S15A-MN4 retrieved from a parallel sample of the same site applying the 16S rDNA approach (Nedelkova et al. 2005, see Chapter I). They both are identical (see Table 1) to the 16S rDNA of bacterium BW7, isolated from substrate-limited reactor (Morgan et al., 2002). The aerobically cultured strain S15-M3 was phylogenetically most closely related to *Microbacterium* sp. SKJH-23.

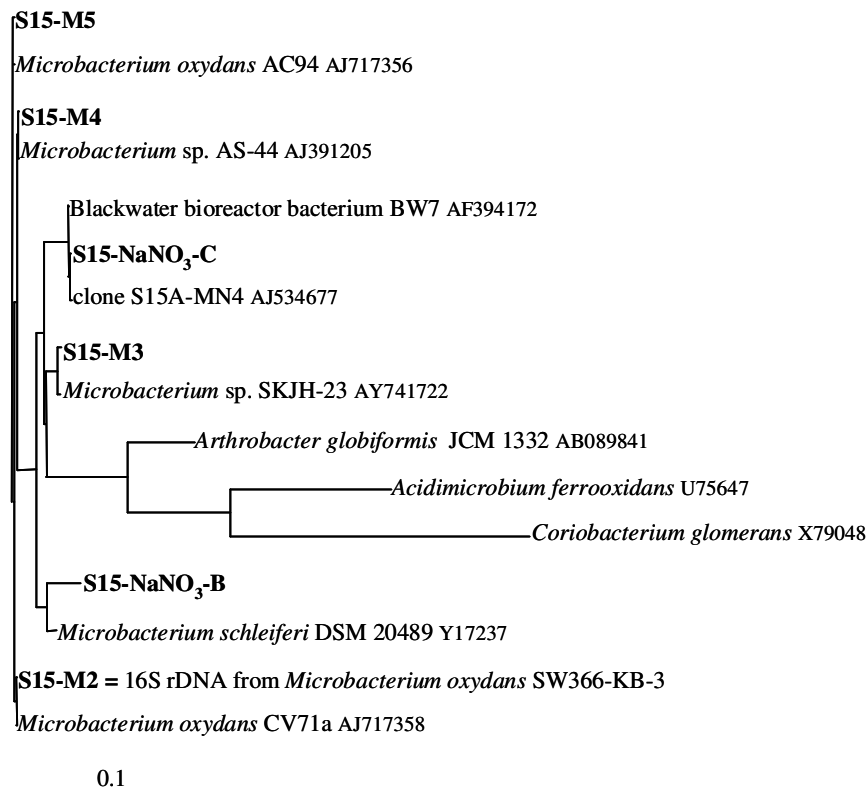


Fig. 2. 16S rDNA based affiliation of the S15 bacterial strains with members of Actinobacteria

The strains S15-M2, S15-M4 and S15-M5, which were also isolated under aerobic conditions, possess almost identical 16S rDNA sequences with only four mismatches. As shown in Fig. 3, there are two mismatches, at positions 191 and 412 (*E. coli* numbering), between the S15-M2 and S15-M5 strains. At the same positions the strain S15-M4 has the nucleotide C like strain S15-M2 and the nucleotide T like strain S15-M5, respectively. At positions 613 and 627, S15-M4 differs from the other two, S15-M2 and S15-M5, strains. Sequence variability among closely related Actinobacteria isolates was demonstrated previously within the 16S rDNA

nucleotide fragment flanked by positions 75 to 412, *E. coli* numbering (Hahn et al., 2003).

	<i>E. coli</i> 191	<i>E. coli</i> 412	<i>E. coli</i> 613	<i>E. coli</i> 627
S15-M2	GTGACC GCAT	GGAC GCACG	CGGAGGCTCAACCTCCG	
S15-M5	GTGATC GCAT	GGAT GCACG	CGGAGGCTCAACCTCCG	
S15-M4	GTGACC GCAT	GGAT GCACG	CCGAGGCTCAACCTCCG	

Fig. 3. 16S rDNA stretches of the *Microbacterium oxydans* S15-M2 and S15-M5 and *Microbacterium* sp. S15-M4, possessing mismatches.

The differences in the position 412 within the 16S rRNA gene of these isolates were the same as those found in the three *Microbacterium* strains analysed here, where T or C occur. The phylogenetic analysis represented in Fig. 2 and Table 1 revealed that the two strains S15-M2 and S15-M5 belong to the same species and are microdiverse. Their 16S rDNA is identical to that of *Microbacterium oxydans* strains CV71a and AC94, respectively, which were recently isolated from alkaline ground waters (Tiago et al., 2004). In contrast, the strain S15-M4 possesses identical 16S rRNA gene to the sea isolate *Microbacterium* sp. AS-44, and as shown further represents another *Microbacterium* species. Interestingly, the strain S15-M2 has identical 16S rDNA to *Microbacterium oxydans* SW366-KB-3 (see Table 1 and Fig. 2). This strain was recently isolated in our laboratory from waters with high uranium (3.62 mg/l) and other heavy metals concentrations.

#### Morphological and physiological characterization of the bacterial S15 isolates

The SEM micrographs depicted in Fig. 4 indicate that the cells of the strain *Microbacterium* sp. S15-M4 are smaller and morphologically different than those of the microdiverse *Microbacterium oxydans* strains S15-M2 and S15-M5.

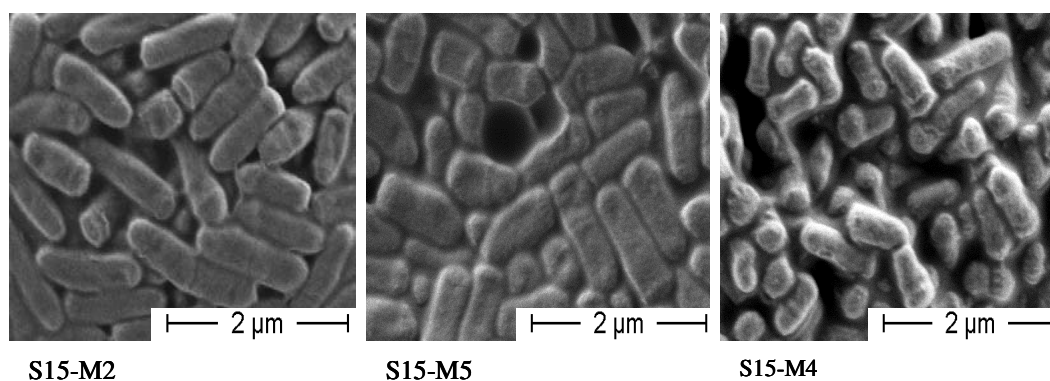


Fig. 4. SEM images of the strains *Microbacterium oxydans* S15-M2 and S15-M5, and *Microbacterium* sp. S15-M4

The results from the physiological analyses of the recovered *Sphingomonas* sp. S15-S1,

*Microbacterium oxydans* strains S15-MN2 and S15-MN5 and *Microbacterium* sp. S15-MN4 are presented in Table 2. The *Sphingomonas* sp. strain S15-S1 was able to utilize almost all of the twenty tested substrates, with the exception of citric acid and maleate. Weak growth was observed in the presence of tartaric and nicotinic acid. In comparison with the *Sphingomonas* S15-S1 strain, the *Microbacterium* isolates were able to utilize a lower number of substrates. The strain S15-M4 utilizes eight of the tested substrates, namely glucose, fructose, L-arabinose, maltose and sucrose. In addition, weak growth was observed in the presence of sodium acetate, sodium pyruvate and L-arginine. In contrast to the *Microbacterium* sp. S15-M4, the two microdiverse *Microbacterium oxydans* strains S15-M2 and S15-M5 utilize three and two of the tested substrates, respectively. They both grow in the presence of sucrose and sodium pyruvate, whereas the S15A-MN2 strain utilizes also L-arabinose.

Table 2. Substrate utilization patterns of *Sphingomonas* sp. S15-S1, *Microbacterium oxydans* S15-M2 and S15-M5, and *Microbacterium* sp. S15-M4. Results are indicated as: +, growth; w, weak growth; -, no growth.

Substrate	S15 strains			
	<i>Sphingomonas</i> sp. S15-S1	<i>M. oxydans</i> S15-M2	<i>M. oxydans</i> S15-M5	<i>Microbacterium</i> sp. S15-M4
glucose	+	-	-	+
fructose	+	-	-	+
L-arabinose	+	+	-	+
D-arabinose	+	-	-	-
maltose	+	-	-	+
sucrose	+	+	+	+
D-sorbitol	+	-	-	-
sodium acetate	+	-	-	w
sodium pyruvate	+	w	w	w
citric acid	-	-	-	-
maleate	-	-	-	-
Na-L-lactic acid	+	-	-	-
tartaric acid	w	-	-	-
nicotinic acid	w	-	-	-
methanol	+	-	-	-
ethanol	+	-	-	-
2-propanol	+	-	-	-
tween-80	+	-	-	-
L-arginine	+	-	-	w
L-glutamine	+	-	-	-

### Heavy metals tolerance

The minimum inhibitory concentrations (MIC's) of cadmium-, copper-, lead-, nickel-, silver-, chromium- and uranium, determined on LPM solid medium for the growth of *Sphingomonas* sp. S15-S1, *Microbacterium oxydans* S15-M2 and S15-M5, *Microbacterium* sp. S15-M3 and *Microbacterium* sp. S15-M4 are presented in Table 3. The Gram negative *Sphingomonas* sp.



S15-S1 strain was more sensitive to cadmium, copper and chromium in comparison to the Gram positive *Microbacterium* isolates. The S15-S1 strain tolerates lead in higher, nickel, cadmium and uranium in the same, whereas copper, chromium and silver in lower concentration than *Sphingomonas* sp. SW366, isolated previously in our laboratory from heavy metals contaminated water. The growth of all the S15 isolates was inhibited at 2 mM lead concentration. The MIC's of copper and chromium were identical for the *Microbacterium* strains. The two microdiverse *Microbacterium oxydans* strains S15-M2 and S15-M5 tolerate identical concentrations of the tested metals, with the exception of cadmium. The strain S15-M2 grows at concentration of 1 mM cadmium, whereas S15-M5 tolerates only 0.25 mM. Interestingly, the *Microbacterium oxydans* SW366-KB-3, which possesses identical 16S rDNA with S15-M2, could tolerate 0.5 mM concentration of cadmium. The strain SW366-KB-3 has the same MIC's for the other metals, as S15-M2 and S15-M5. The strains S15-M3 and S15-M4 possess identical resistant patterns for cadmium and nickel. The *Microbacterium* strains S15-M2, S15-M4 and S15-M5 were more tolerant to silver and uranium in comparison to the *Sphingomonas* sp. S15-S1 and *Microbacterium* sp. S15-M3.

Table 3. Minimum inhibitory concentrations (mM) for the growth of the S15 isolates

Strain	cadmium	copper	chromium	lead	nickel	silver	uranium
<i>Sphingomonas</i> sp. S15-S1	0.25	2	2	2	4	< 0.03	2
<i>Sphingomonas</i> sp. SW366	0.25	4	8	0.5	4	0.06	2
<i>M. oxydans</i> S15-M2	2	8	16	2	4	0.06	4
<i>M. oxydans</i> SW366-KB-3	1	8	16	2	4	0.06	4
<i>M. oxydans</i> S15-M5	0.5	8	16	2	4	0.06	4
<i>Microbacterium</i> sp. S15-M4	1	8	16	2	2	0.06	4
<i>Microbacterium</i> sp. S15-M3	1	8	16	2	2	< 0.03	2

### Accumulation of uranium

The uranium sorption data for the *M. oxydans* strains S15-M2 and S15-M5, for *Microbacterium* sp. S15-M4, and *Sphingomonas* sp. S15-S1 are presented in Figs. 5 and 6. The accumulation of uranium by the bacteria was dependent on the initial uranium concentration and the pH of the metal solution. As shown in Fig. 5, the amount of the uranium bound by the *Microbacterium* strains increases with the increase of the metal concentration (from 10 to 160 mg/l) at pH 2, 3 and 4.5. At pH 4.5 the strains were able to accumulate higher amounts of uranium (up to 240 mg/g dry biomass), in comparison with pH 2 and 3. In addition, the capacity to bind uranium by *Microbacterium oxydans* S15-M2 and S15-M5 and *Microbacterium* sp. S15-M4 was very similar under the studied conditions and comparable

with that of the above mentioned *Microbacetrium oxydans* SW366-KB-3 (not shown). At an initial uranium concentration of 60 mg/l, pH 4.5, SW366-KB-3 accumulated up to 120 mg/g dry biomass and the *Microbacterium* spp. S15-M2, S15-M4 and S15-M5 bound up to 140 mg/g dry biomass. As shown in Fig. 6, *Sphingomonas* sp. S15-S1, removes lower amount of uranium from the solution than the *Microbacterium* isolates. The S15-S1 strain already reached the biosorption saturation at 130 mg/l at pH 4.5 whereas the *Microbacterium* strains have still not reached it at 240 mg/l.

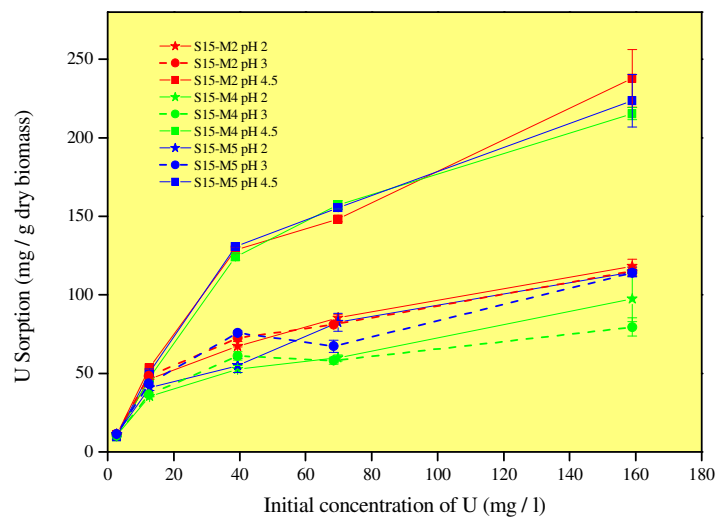


Fig. 5. Biosorption of uranium by the strains *Microbacterium oxydans* S15-M2 and S15-M5 and *Microbacterium* sp. S15-M4

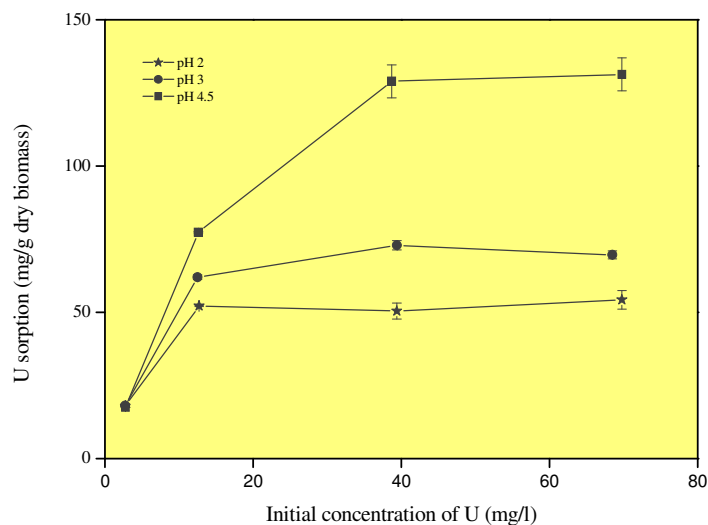


Fig. 6. Biosorption of uranium by the *Sphingomonas* sp. S15-S1 strain

The time course of uranium sorption by the *Microbacterium oxydans* S15-M2 is shown in Fig. 7. The saturation was reached about 48 hours after the exposure of the cells to the uranium solution (23 mg/l, pH 4.5). After 24 h of exposure to uranium, the strains S15-M4 and S15-M5 have also not reached saturation (data not shown). In contrary to biosorption kinetics experiments of uranium and other heavy metals performed by others (Malekzadeh et al., 2002a and 2002b; Nakajima and Tsuruta, 2004; Sar and D'Souza, 2001; Shuttleworth and Unz, 1993), the present results suggest relatively slow uranium saturation by the cells of the *Microbacterium* strains. This indicates that another process in addition to the sorption is responsible for the uranium removal.

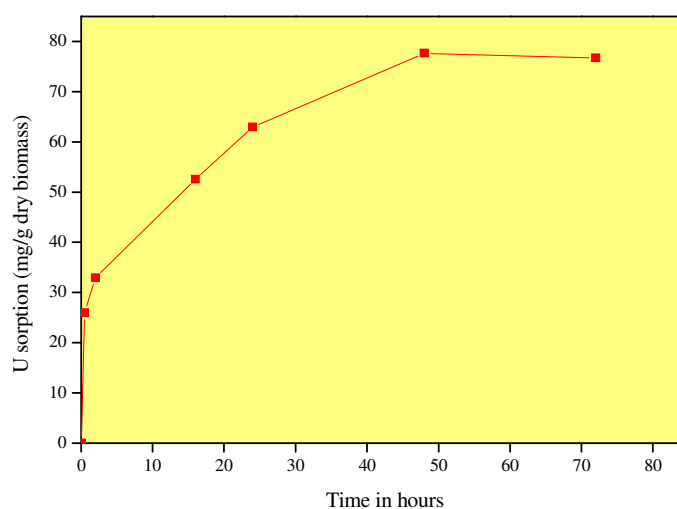


Fig. 7. Time course of the uranium biosorption by the strain *Microbacterium oxydans* S15-M2

### Flow cytometry

The percentages of viable cells of the *Microbacterium oxydans* S15-M2 and S15-M5 and *Microbacterium* sp. S15-M4, detected after exposure for 14, 24 and 48 h to different concentrations of uranium, are presented in Table 4. Cells with intact membrane according to the staining with SYTO 9™ were counted as viable (Alonso et al., 2002). The number of viable cells decreased with increasing the uranium concentration and the exposure time for the S15-M2 and S15-M5 populations. After incubation with uranium for 48 h the number of the viable cells of the strain S15-M4 increased, in comparison with that after 14 and 24 h, indicating a possible adaptation. Similar effects were observed for other bacteria in presence of metals other than uranium. For instance, the cells of *Rhodobacter capsulatus* overcome the initial tellurite-induced stress and the percentage of dead cells decreased with the time (Borghese et al., 2004).

Table 4. Percentage of viable cells in the presence of uranium according to the SYTO-9-staining

Strain	Uranium concentration (mM)	% viable cells SD < / = 1%		
		14 hours	24 hours	48 hours
<i>M. oxydans</i> S15-M2	control	69.6	82.4	74.2
	0.01	59.5	58	38.4
	0.1	38.7	34.8	24.7
<i>M. oxydans</i> S15-M5	control	76.2	84.4	45
	0.01	55.1	66.8	not measured
	0.1	50	38.3	18.7
<i>Microbacterium</i> sp. S15-M4	control	77.3	85.8	78.1
	0.01	64.1	60.6	82.8
	0.1	29.3	14.3	57

Reversible or nonpermanent toxic effects of lead were demonstrated by others for *Desulfovibrio desulfuricans* G20 via measurement of the membrane integrity and cell protein concentrations (Sani et al., 2003).

In Fig. 8 the presence of two subpopulations of cells, R1 and R4, for cultures of the three *Microbacterium* strains is shown. The cells of these subpopulations have the same size (see forward scatter) but possess different surface properties (see side scatter). Interestingly, the percentage of the R4 subpopulations increases for the cultures of the three strains, after exposure for 48 h to 0.1 mM uranium, which indicates that possibly uranium induces changes of some surface compounds.

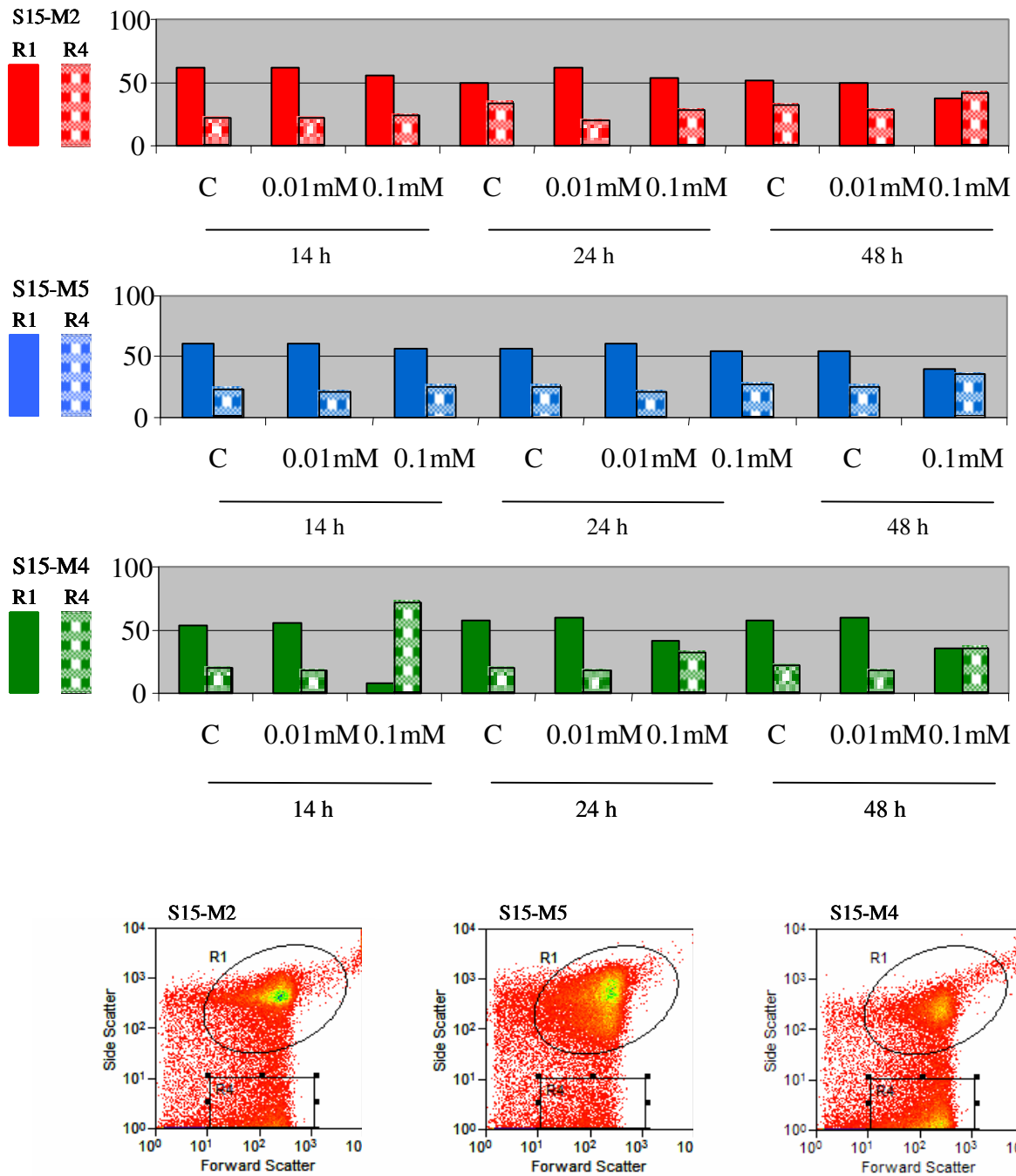


Fig. 8. Distinct subpopulations within the cultures of the *Microbacterium oxydans* S15-M2 and S15-M5 and *Microbacterium* sp. S15-M4 incubated for 14, 24 and 48 h in LP medium without and with 0.01 and 0.1 mM uranium

## XANES

XANES spectra (data not shown) of the uranium complexes formed by *Microbacterium oxydans* S15-M2 and *Sphingomonas* sp. S15-S1 at pH values 2, 3 and 4.5 contain a XANES peak at 17188 eV which has previously been attributed to uranium in the 6+ oxidation state (Hudson et al. 1996). In addition, the intensity maximum for the absorption edge occurred at the position characteristic for U(VI). Both observations indicate that uranium is present in the samples as U(VI).

## EXAFS

Information on the local environment of uranium atoms in the uranium treated bacterial samples was provided by analysis of the EXAFS data. Uranium L<sub>III</sub>- edge EXAFS spectra of the uranium species formed at pH 2, 3 and 4.5 by the cells of *Microbacterium oxydans* S15-M2 and *Sphingomonas* sp. S15-S1 and their corresponding Fourier transforms (FT's) are presented in Figs. 9 and 10, respectively. The Fourier transforms (FTs) represent a pseudo-radial distribution function of the uranium near-neighbor environment.

The peaks appeared at lower *R*-values relative to the true near-neighbor distances as a result of the EXAFS phase shift, that is different for each neighboring atom (= 0.2-0.5 Å). The theoretical phase and amplitude functions used in data analysis were calculated with FEFF 8 using the model described in Fig. 11, which contains fragments of two molecules- meta-autunite and uranyl triacetate (Merroun et al. 2005a). This model includes two oxygen axial (O<sub>ax</sub>) atoms, two shells of equatorial oxygen atoms (O<sub>eq1</sub> and O<sub>eq2</sub>), a shell of carbon (C) atoms, a U-O<sub>ax</sub>-U-O<sub>ax</sub> MS path from the two tightly bound O<sub>ax</sub> atoms, a shell of phosphorus (P) atoms, an MS path from the O<sub>eq</sub> atoms to the P atoms, and a shell of uranium atoms at approximately 3.65 Å.

Quantitative fit results (see Table 5 and 6) indicate that the adsorbed U(VI) has the common linear trans-dioxo structure: two axial oxygens at about 1.75-1.77 Å, and an equatorial shell of 4 to 5 oxygens at 2.27-2.33 Å. The U-O<sub>eq1</sub> bond distance is within the range of previously reported values for phosphate bound to uranyl (Hennig et al., 2001; Merroun et al., 2002, 2003, 2005).

The FT spectra of all samples contain an FT peak at about 2.3 Å. After correcting for the scattering phase shift, this distance is typical for carbonate groups coordinated to U(VI) in a bidentate fashion (Coda et al., 1981). Indeed, carbon atoms at 2.86 to 2.91 Å provide a good fit to the 2.3 Å FT peak. If this FT peak originates from bidentate carboxyl ligands, the U-O<sub>eq1</sub> bond distance should be approximately 2.46-2.49 Å (Denecke et al., 1997), which is not the case in this work (2.27 to 2.33 ± 0.02 Å). For these two reasons, we exclude the possibility of interpreting this shell as a contribution of carbon atoms. Moreover, this shell is too short to be

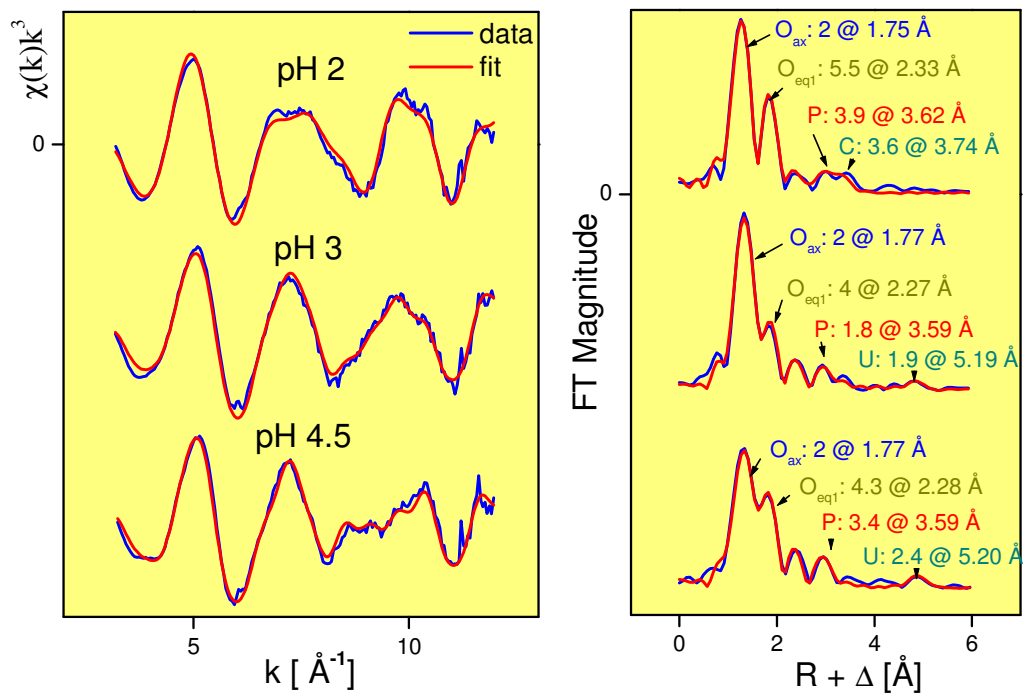


Fig. 9. Uranium L<sub>III</sub>-edge  $k^3$ -weighted EXAFS spectra (left) and the corresponding fourier transforms (FT) (right) of the uranium complexes formed by the cells of *Microbacterium oxydans* S15-M2 at pH values 2, 3 and 4.5.

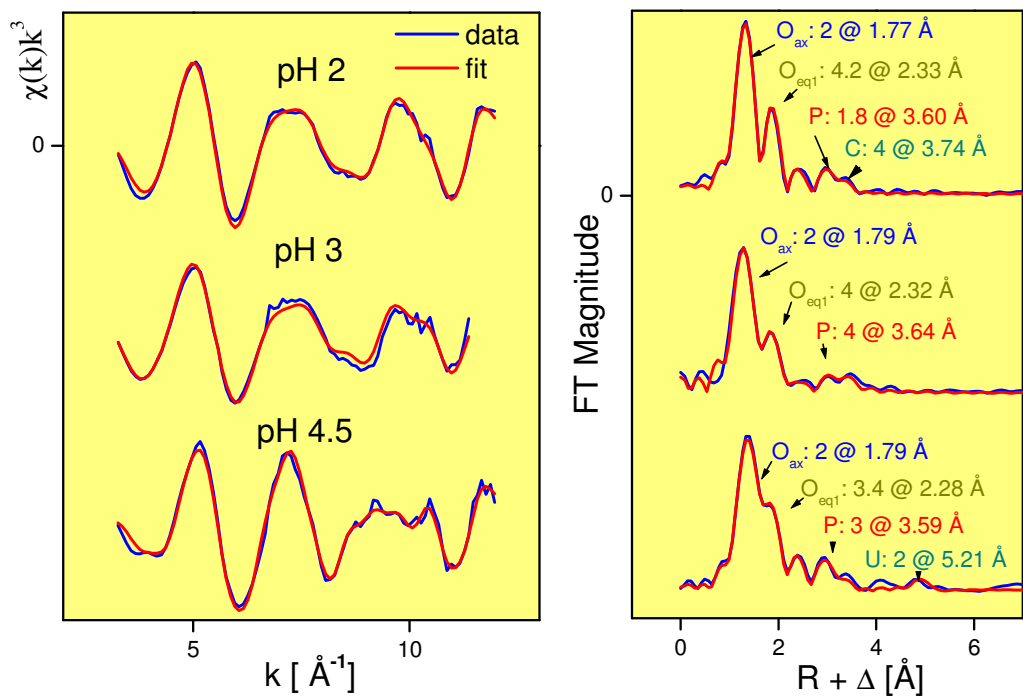


Fig. 10. Uranium L<sub>III</sub>-edge  $k^3$ -weighted EXAFS spectra (left) and the corresponding fourier transforms (FT) (right) of the uranium complexes formed by the cells of *Sphingomonas* sp. S15-S1 at pH values 2, 3 and 4.5.

attributed to MS pathways. Oxygen neighbours ( $O_{eq2}$ ) provide a good fit to the residual EXAFS spectrum corresponding to this shell. Thus, we interpret this peak in the FT as oxygen neighbours. The fourth FT peak, which appears at 3 Å (radial distance of 3.59-3.64 Å) is a result of the back-scattering from phosphorous atoms. This distance is typical for a monodentate coordination of U(VI) by phosphate (Hennig et al., 2001; Merroun et al., 2002, 2003c, 2005a).

For both kinds of bacteria, at pH 4.5, a sixth shell corresponds to U at distance of 5.20-5.21 Å. This distance is also present in meta-autunite (Hennig et al., 2001), suggesting that a similar inorganic uranyl phosphate phase was precipitated by the bacterial cells at pH 4.5, probably due to the release of inorganic phosphates from the cells as result of the acid phosphatase activity. At pH 2, however, this U-U peak is absent, but we could fit a contribution of U-C at a distance of 3.74 Å. This C atom most probably originates from the organic phosphate molecules of the cell surface which are implicated in the binding of uranium. In addition, the  $U-O_{eq1}$  bond distance of  $2.33 \text{ Å} \pm 0.02$  and the coordination number of  $5.3 \pm 0.4$  found for the U/bacterial complexes at pH 2 are similar to those found in the uranium complexes formed by fructose 6-phosphate at pH 4 (Koban et al., 2004).

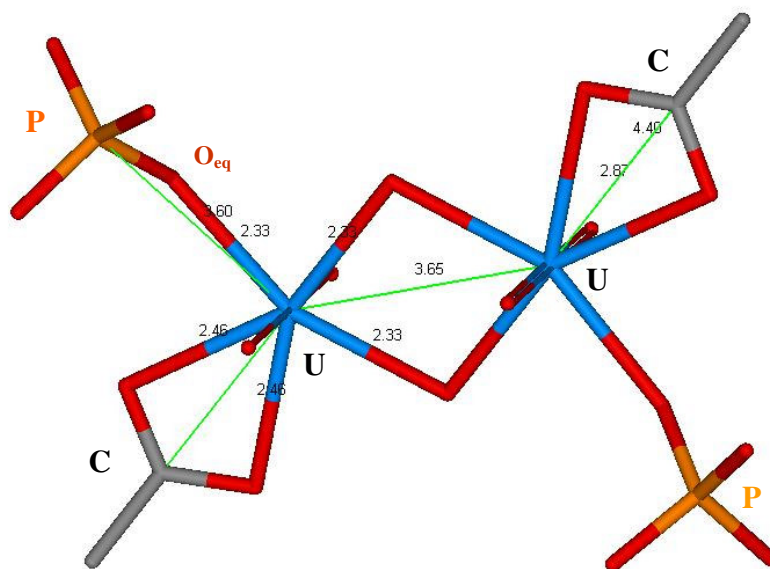


Fig. 11. Model used for the fit of the bacterial spectra (fragments of 2 molecules: meta-autunite and uranyl triacetate). In this model, the uranium has two oxygen axial ( $O_{ax}$ ) at distance of 1.77 Å and two types of equatorial oxygen atoms ( $O_{eq1}$  at distance of 2.33 Å and  $O_{eq2}$  distance of 2.46 Å). The uranium is coordinated to carboxyl groups in a bidentate fashion with an average distance between the U atom and the C atom of 2.87 Å, and to phosphate groups in a monodentate fashion with an average distance between the U atom and the P atom of 3.60 Å. In addition, there are a U-U bond distance of 3.65 Å.



Sample	Shell	N <sup>a</sup>	R[Å] <sup>b</sup>	σ <sup>2</sup> [Å <sup>2</sup> ] <sup>c</sup>
<b>pH 2</b>	U-O <sub>ax</sub>	2 <sup>d</sup>	1.75	0.0022
	U-O <sub>eq1</sub>	5.5(5)	2.33	0.0110
	U-O <sub>eq2</sub>	1.0(2)	2.82	0.0040 <sup>d</sup>
	U-P	3.9(9)	3.62	0.0040 <sup>d</sup>
	U-C	4 <sup>d</sup>	3.74	0.0040 <sup>d</sup>
<b>pH 3</b>	U-O <sub>ax</sub>	2 <sup>d</sup>	1.77	0.0021
	U-O <sub>eq1</sub>	4.0(2)	2.27	0.0080
	U-O <sub>eq2</sub>	0.9(1)	2.86	0.0040 <sup>d</sup>
	U-P	1.8(2)	3.59	0.0040 <sup>d</sup>
	U-U	1.9(6)	5.19	0.0080 <sup>d</sup>
<b>pH 4.5</b>	U-O <sub>ax</sub>	2 <sup>d</sup>	1.77	0.0046
	U-O <sub>eq1</sub>	4.3(2)	2.28	0.0061
	U-O <sub>eq2</sub>	1.0(2)	2.89	0.0040 <sup>d</sup>
	U-P	3.4(5)	3.59	0.0040 <sup>d</sup>
	U-U	2.4(5)	5.20	0.0080 <sup>d</sup>

Table 5. Structural parameters of the uranium complexes formed by *Microbacterium oxydans* S15-S2  
a: Errors in coordination numbers are ±25% and standard deviations as estimated by EXAFSPAK are given in brackets

b: errors in distance are ±0.02 Å

c: Debye-Waller factor

d: value fixed for calculation

Sample	Shell	N <sup>a</sup>	R[Å] <sup>b</sup>	σ <sup>2</sup> [Å <sup>2</sup> ] <sup>c</sup>
<b>pH 2</b>	U-O <sub>ax</sub>	2 <sup>d</sup>	1.77	0.0023
	U-O <sub>eq1</sub>	4.2(5)	2.33	0.0095
	U-O <sub>eq2</sub>	0.8(2)	2.86	0.0040 <sup>d</sup>
	U-P	1.8(4)	3.59	0.0040 <sup>d</sup>
	U-C	4 <sup>d</sup>	3.72	0.0040 <sup>d</sup>
<b>pH 3</b>	U-O <sub>ax</sub>	2 <sup>d</sup>	1.76	0.0042
	U-O <sub>eq1</sub>	4.1(3)	2.32	0.0120
	U-O <sub>eq2</sub>	0.6(3)	2.83	0.0040 <sup>d</sup>
	U-P	4.1(6)	3.64	0.0040 <sup>d</sup>
	U-C	4 <sup>d</sup>	3.65	0.0040 <sup>d</sup>
<b>pH 4.5</b>	U-O <sub>ax</sub>	2 <sup>d</sup>	1.79	0.0032
	U-O <sub>eq1</sub>	3.3(6)	2.28	0.0040
	U-O <sub>eq2</sub>	1.0(2)	2.87	0.0040 <sup>d</sup>
	U-P	3.0(5)	3.62	0.0040 <sup>d</sup>
	U-U	2.0(3)	5.21	0.0080 <sup>d</sup>

Table 6. Structural parameters of the uranium complexes formed by *Sphingomonas sp.* S15-S1

a: Errors in coordination numbers are ±25% and standard deviations as estimated by EXAFSPAK are given in brackets

b: errors in distance are ±0.02 Å

c: Debye-Waller factor

d: value fixed for calculation

## TEM

TEM observations of *Sphingomonas* sp. S15-S1, *Microbacterium oxydans* S15-M2 and S15-M5, and *Microbacterium* sp. S15-M4 cells exposed to 0.5 mM uranium solution at pH 2 and 4.5 (Figs. 12, 13 and 14) revealed significant differences on the uranium accumulation profile of the two types of bacteria. The cells of *Sphingomonas* sp. S15-S1 accumulate uranium on the cell surface (Fig. 12A, red arrow) and inside the cell as precipitates bound to polyphosphate-like bodies (Fig.12B, blue arrow) and as other precipitates (Fig.12C, green arrow) at pH 4.5. As shown in Fig. 13, at pH 4.5 the cells of the three different *Microbacterium* strains accumulate uranium at the cell surface (cytoplasmic membranes, peptidoglycan) and also extracellularly. However, at pH 2, only small amounts of uranium were bound to the surfaces of both bacterial cell types (see Fig. 14B). There was no extracellular precipitation of uranium (see Figs. 12D and 14) and these results are confirmed by those found using EXAFS spectroscopy (see above).

Energy Dispersive X-ray (EDX) analysis provides elemental information via the analysis of X-ray emissions caused by a high energy electron beam. The EDX spectra derived from the uranium deposits on Figs. 12, 13, and 14 showed that they are composed of oxygen (O), phosphorus (P) and uranium (U). The latter indicates binding of uranium to phosphate groups. The high copper (Cu) peak is from the grid used to support the specimen. The lead (Pb) peak originated from the lead citrate solution which was used to improve the visualization of the uranium-treated cell thin sections. The presence of silicium (Si) peak can be attributed to impurities from the nutritional culture medium and/or from the glass material of the flasks where the cells were grown.

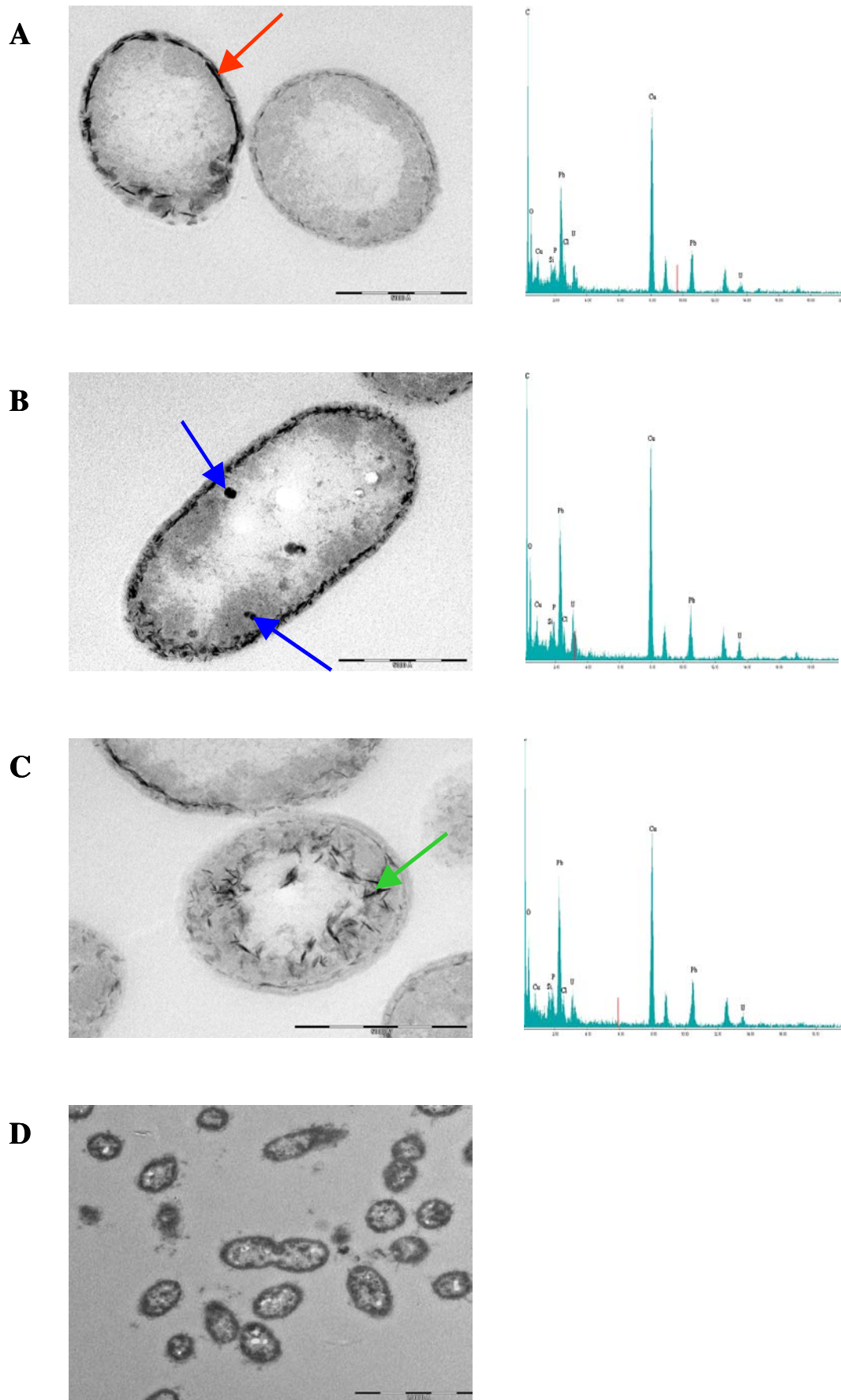


Fig. 12. TEM images, coupled with EDX analyses for the interactions of uranium with the strain *Sphingomonas* sp. S15-S1 at pH 4.5 (Fig. 12 A, B, C) and pH 2 (Fig. 12 D)

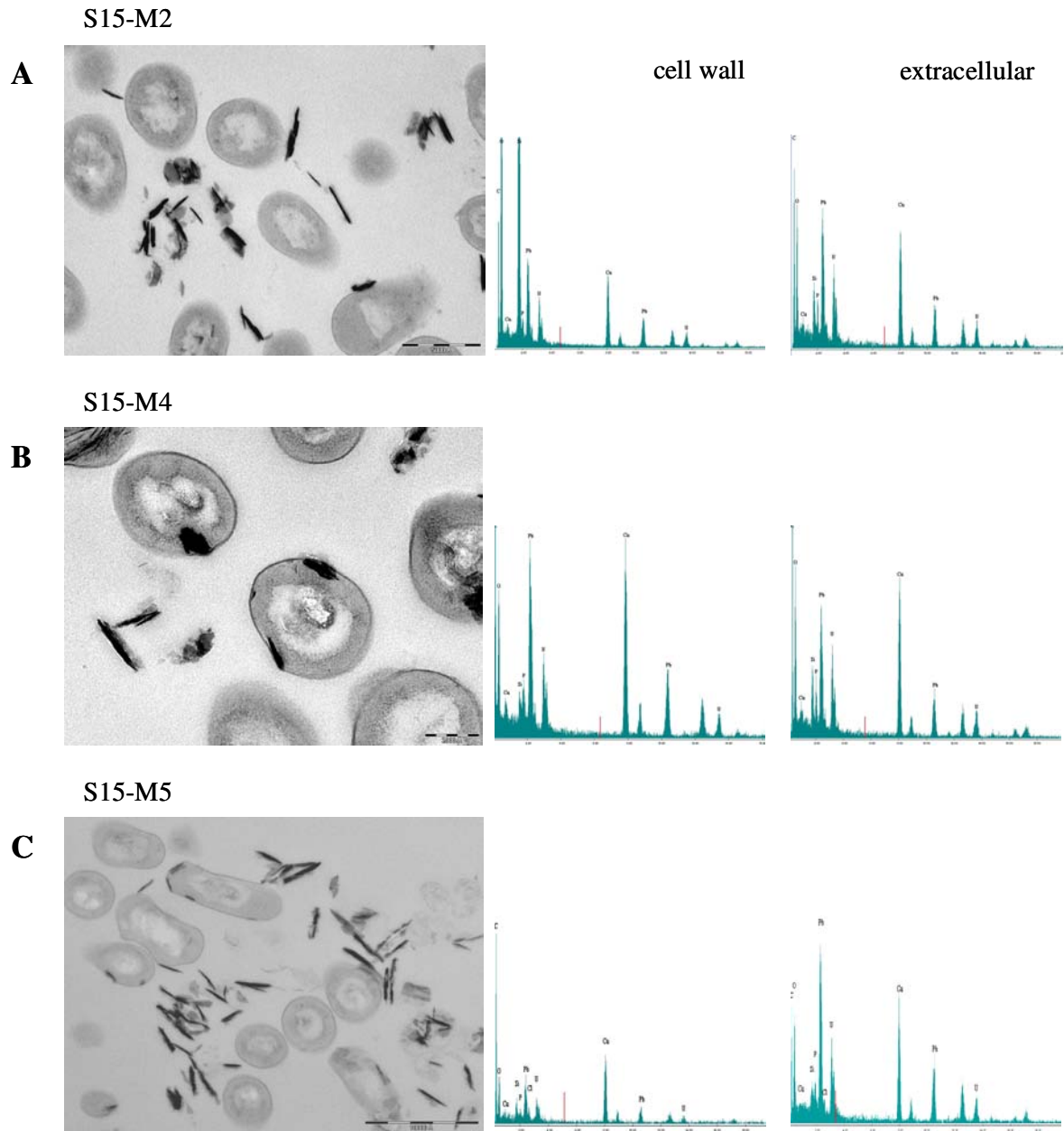


Fig. 13. TEM images, coupled with EDX analyses for the interactions of uranium with the *Microbacterium* strains: A) S15-M2; B) S15-M4, and C) S15-M5 at pH 4.5

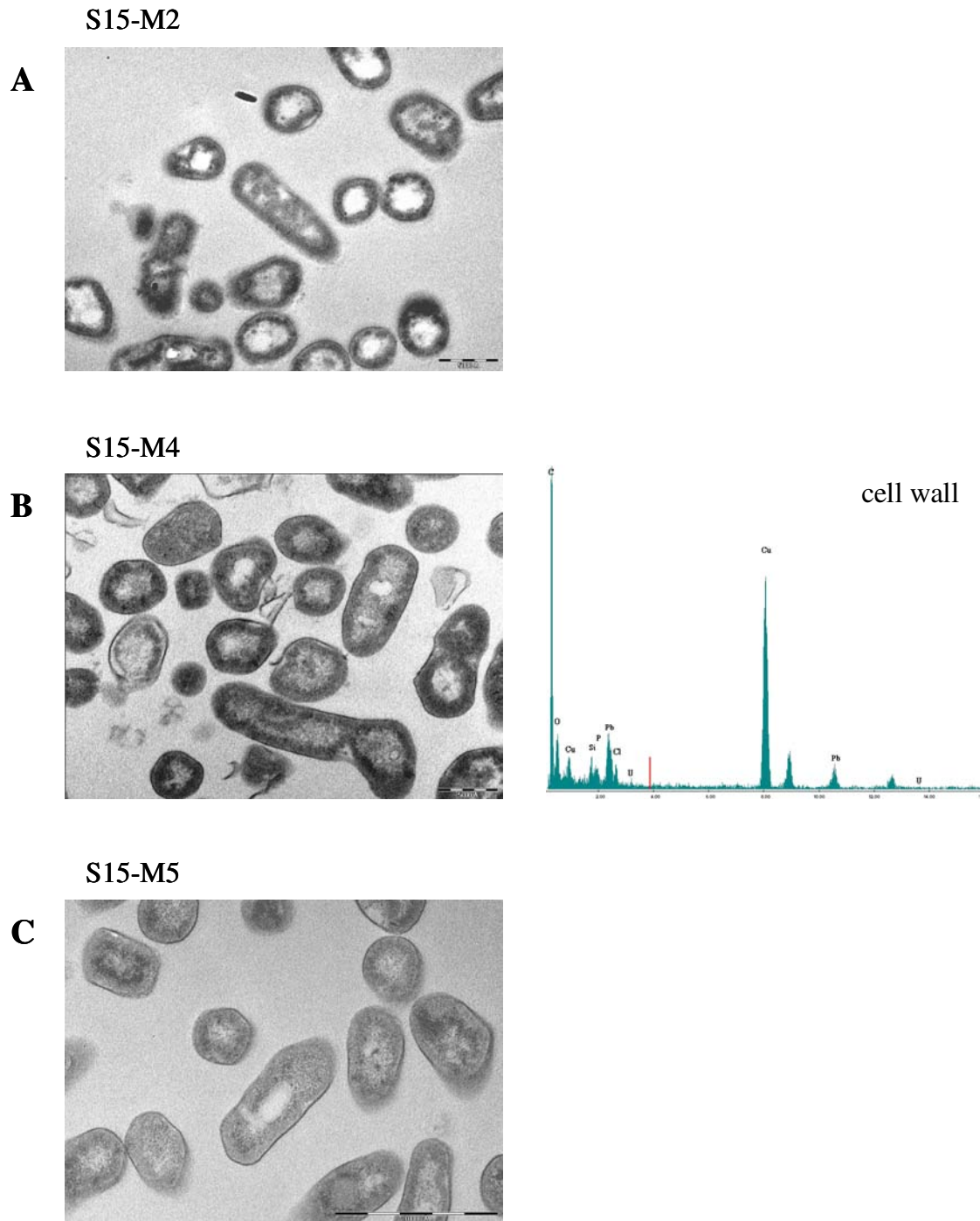


Fig. 14. TEM images, coupled with EDX analyses for the interactions of uranium with the *Microbacterium* strains: A) S15-M2; B) S15-M4, and C) S15-M5 at pH 2

## Discussion

The present study describes oligotrophic bacteria, isolated from a groundwater sample, collected from the S15 deep monitoring well of the radioactive waste depository Tomsk-7, and characterizes their interactions with uranium and other heavy metals. The isolated strains were affiliated with members of  $\alpha$ -subclass of Proteobacteria, like *Brevundimonas*, *Methylobacter* and *Sphingomonas* spp. and with members of Actinobacteria, especially genus *Microbacterium*. Representatives of these two bacterial groups were isolated previously from the same (Wickham et al., 2003) and other subsurface habitats (Balkwill et al., 1997; Boivin-Jahns et al., 1995; Chandler et al., 1997; Fields et al., 2005; Johnson and Hill, 2003). The 16S rRNA genes of some of the strains were closely related to clones, encountered in parallel samples from the same site, applying the direct 16S rDNA molecular approach (see Figs. 1 and 2 and Chapters I and II). However, the molecular retrieval indicates that members of  $\beta$ -subclass of Proteobacteria, *Bacteroidetes* and of a novel "Cyanobacteria-like" cluster represent the most abundant bacterial groups in the S15 samples (see Chapters I and II). The discrepancy between the cultivation-dependent and -independent approaches in bacterial studies is a common phenomenon (Dunbar et al., 1999). Some of the bacteria from the above mentioned dominant groups, detected via the 16S rDNA retrieval were probably not able to growth under the given laboratory conditions. Recently it was shown by other authors that most of the bacteria from ground water of a municipal water supply, which were not cultured, but identified via the 16S rDNA approach, were  $\beta$ -Proteobacteria (Ultee et al., 2004). Strains from the groups of  $\beta$ -Proteobacteria and *Bacteroidetes* (formerly *Cytophaga/Flavobacterium/Bacteroides*) were isolated under aerobic conditions on complex media from spent nuclear fuel pools (Chicote et al., 2005). Interestingly, some of them are closely related to the dominant 16S rDNA clones, identified in the S15 samples (see Chapter II). It was suggested that culturability of bacteria is related to their physiological status and is an ecologically relevant parameter (Ellis et al., 2003). It should be noted that the studied extreme habitat is characterized as oligotrophic and the microbial populations there are possibly in dormant or starvation-survival state. Organisms called "*r* strategists" that are able to rapidly adjust their growth rate to the new conditions, could dominate situations where nutrient resources are available (Lebaron et al., 2001). Such bacteria are reported for representatives of the  $\alpha$ -subclass of Proteobacteria and *Bacteroidetes* groups (Lebaron et al., 2001). It seems that members of Actinobacteria belong also to this type of rapidly adapting organisms. Mainly Actinobacteria, but also  $\alpha$ -subclass of Proteobacteria and *Bacteroidetes* were obtained from other extreme oligotrophic environments, like the deep Greenland glacier ice core (Miteva et

al., 2004) and alkaline ground waters (Tiago et al., 2004). The most common isolates from radioactive waste contaminated sediments were members of Actinobacteria as well (Fredrickson et al., 2004). Applying gas chromatography-mass spectrometry analysis, Wickham and colleagues demonstrated previously, that the group of Actinobacteria was highly abundant in samples from the S15 monitoring well (Wickham et al., 2003). In this study two from the Actinobacteria strains, *Microbacterium oxydans* S15-M2 and S15-M5, possess microdiverse 16S rDNA stretches. Distinct niche adaptations and physiological differences were reported by other authors for environmental microbial populations with microdiverse as well as with identical 16S rRNA genes (Casamayor et al., 2002; Hahn and Pöckl, 2005; Jaspers and Overmann, 2004; Moore et al., 1998). This suggests that the microdiverse bacterial populations possess high plasticity and adaptability. Interestingly, the strain S15-M4 has 16S rDNA, which differs from each of the two microdiverse strains in only three positions. However, the strain S15-M4 has distinct physiological requirements and was affiliated with different *Microbacterium* species. High level of 16S rDNA homology was reported for distinct *Aeromonas* (Martinez-Murcia et al., 1992) and *Bacillus* species (Fox et al., 1992), which have low levels of DNA similarity. It was suggested, that they comprise fastly diverged species. The presence of populations of microdiverse and fast evolving species that can adapt rapidly to the new environmental conditions is important in the context of subsurface disposal of radioactive wastes and bioremediation of such habitats. Often these wastes contain organic compounds, which can serve as energy and carbon source for the microorganisms (Nazina et al., 2004). Therefore the organisms, which could response fast to the new growth conditions, will dominate the situation and will possibly interact with the introduced radionuclides.

A number of studies demonstrated resistance to and sorption of heavy metals by bacteria, isolated from heavy metal contaminated and pristine habitats, as well as of laboratory strains (Benyehuda et al., 2003; Ben Omar et al., 1997; Hafez et al., 2002; Hernandez, 1998; McLean and Beveridge, 2001; Shelobolina, et al., 2004; Tsuruta, 2004). Strains, obtained from heavy metal contaminated sites have adapted and can resist high concentrations of heavy metals, because of the continuous selective pressure (Hanbo et al., 2004; Suzuki and Banfield, 2004; McLean and Beveridge, 2001). However, in nature, the toxicity of metals to microorganisms depends also on the site-specific physico-chemical conditions, which could determine the metal bioavailability. Recently it was demonstrated, that in hydrothermal vent environment, metal-sulfide complexes most likely decreased the metal bioavailability, thus protecting the organisms against toxic concentrations (Edgcomb et al., 2004). It is well established, that under laboratory conditions factors like medium composition, growth phase and others could

strongly influence the resistance of the microorganisms to metals (Borghese et al., 2004; Sani et al., 2001). High heavy metal tolerance was demonstrated for a number of bacteria, like *Klebsiella* sp., (Choudhury and Kumar, 1998), *Enterobacter cloace* and *Escherichia hermannii* strains (Hernandez et al., 1998), *Frankia* isolates (Richards et al., 2002) and many other (Malik, 2004). In order to minimize the above mentioned factors, we compare the heavy metal resistance patterns of the S15 strains with those of bacterial isolates studied previously under the same conditions in our laboratory. The resistance of the *Sphingomonas* sp. S15-S1 and the four *Microbacterium* spp. strains to different heavy metals is similar to the tolerance of *Sphingomonas* and *Microbacterium* strains, obtained from heavy metal contaminated sites. This suggests that the studied bacteria represent populations, which are flexible, adapt rapidly to stress conditions and could outcompete the other members. Strains, isolated from other unimpacted environments, have been shown to resist also against high concentrations of heavy metals (Benyehuda et al., 2003, Duxbury and Bicknell, 1983). Studying the development of metal tolerance in soil communities under laboratory conditions, Diaz-Ravina and Bääth have demonstrated immediate and later effects on the bacterial population. The immediate effect causes death of the sensitive species, whereas the later was due to the different competitive abilities and adaptation of the surviving bacteria (Diaz-Ravina and Bääth, 1996).

The microdiverse strains *Microbacterium oxydans* S15-M2 and S15-M5, and the strain *Microbacterium* sp. S15-M4 tolerate identical concentrations of the studied metals, with the exception of cadmium and sorbed relatively high and very similar amounts of uranium (see Table 3 and Fig. 5). In comparison to the studied *Microbacterium* spp. strains the Gram negative strain *Sphingomonas* sp. S15-S1 was less tolerant to uranium and immobilized relatively smaller amount of the radionuclide. We can not exclude, that under other growth conditions, the strains may behave differently. However, the identical tolerance to and the similar sorption of uranium by the *Microbacterium* spp. could be explained by the fact that they were isolated from a site not contaminated with uranium and the strains respond similarly to the new conditions. This was not the case for previously described *Acidithiobacillus ferrooxidans* eco-types I, II and III (Flemming et al., 2000; Selenska-Pobell et al., 2001), isolated from areas, containing different amounts of uranium. It was shown that types I and III, which dominated in more contaminated samples, tolerate higher concentrations and accumulate lower amounts of uranium, in contrast to the type II, distributed in less contaminated sites (Merroun and Selesnka-Pobell, 2001). From another point of view *Microbacterium oxydans* SW366-KB-3, obtained from a heavy metal contaminated site, interacts with heavy metals and especially with uranium similarly to the



two *Microbacterium oxydans* S15 strains. This suggests that such organisms could possibly sustain an eventual change in the habitat, induced by the introduction of heavy metals. However, the same results under laboratory conditions do not automatically imply identical *in situ* behavior, because of the complexity of the environmental habitats.

Interestingly, the flow cytometric measurements showed the existence of two distinct subpopulations of cells with different surface properties in the populations of the *Microbacterium oxydans* S15-M2 and S15-M5 and *Microbacterium* sp. S15-M4 (see Fig. 8). The amount of one of them increased after longer incubation with higher uranium concentration in the cultures of the different strains and at the end was numerically almost the same as the other subpopulation. It was suggested, that the presence of discrete subpopulations of cells of another bacterial species, *Acinetobacter johnsonii*, demonstrated by flow cytometry analysis, may represent the divergence of the type cells within the population, which could be induced under selective pressure (Boswell et al., 2001).

In order to reveal more detailed information about the cellular localization of the accumulated uranium as well as to understand the molecular and atomic level of the metal complexes formed by the bacterial strains at different pH values, microscopic and spectroscopic analyses were performed. We infer from the EXAFS data that the cells of all studied strains precipitate U(VI) as meta-autunite-like phase (inorganic uranyl phosphate) at pH 4.5. At pH 2 uranium formed complexes with organically bound phosphate of the cell surface of the *Microbacterium oxydans* S15-M2 and *Sphingomonas* sp. S15-S1. These results are in accordance with those, demonstrated for the interaction of uranium with other *Sphingomonas* and *Microbacterium* strains (Merroun et al., 2005b). We postulate two hypotheses to explain the differences of the local complexation of uranium with the bacterial strains at different pH values.

The first hypothesis is connected with the activity of nonspecific microbial acidic phosphatase which in presence of U at pH 4.5 causes release of inorganic phosphates precipitating this radionuclide as a meta-autunite-like phase. The activity of this enzyme is minimal at very low pH values (pH 2 in this work). Therefore at pH 2, uranium was found to be bound mainly to organic phosphate groups of the cells. The acidic phosphatase of members of *Enterobacteriaceae* was shown to possess pH optimum in the range of 5.5 – 6.5 (Rossolini et al., 1998). Precipitation of uranium and other heavy metals, through the use of membrane-bound acidic phosphatase was reported for *Citrobacter* sp. (Macaskie et al., 1992). Recently it was suggested, that subsurface microorganisms that exhibit (acidic) phosphatase activity and are resistant to heavy metals have the potential to immobilize uranium via a biomineralization process (Sobecky et al., 2005).

According to the second hypothesis, the activity of the above mentioned acid phosphatase occurs at both pH values resulting in the precipitation of U as meta-autunite, which is insoluble at pH 4.5 and soluble at pH 2. Factors like available phosphate and other anionic groups at the cell surface, which depend on type of the organism and the pH, play also an important role. At pH 3, by *Sphingomonas* sp. S15-S1 dominated the organic uranyl complexes, whereas by *Microbacterium oxydans* S15-M2 uranium was associated with both the inorganic and organic substances.

The distinct localization of uranium at pH 4.5 for the *Microbacterium* spp. and *Sphingomonas* sp. S15-S1 demonstrated by TEM analysis (see Figs. 12 and 13) suggested that different processes were possibly involved in the uranium immobilization by the two types of bacteria. The strains were able to precipitate uranium as meta-autunite-like phase at pH 4.5 as was demonstrated by EXAFS spectra, however, the localization of these deposits was different. Uranium was associated with the cell surface of *Sphingomonas* sp. S15-S1, whereas extracellular as well as cell surface precipitates were identified for the *Microbacterium* spp. Cell surface associated uranium was shown for some *Pseudomonas*, *Bacillus* and *Deinococcus* strains (Francis et al., 2004; Hafez et al., 2002; Raff et al., 2003; Selenska-Pobell et al., 1999; Suzuki and Banfield, 2004). Recently, precipitation of uranyl phosphate on the cell membrane of *Pseudomonas aeruginosa* was demonstrated via controlled polyphosphate metabolism (Renninger et al., 2004). Large amounts of extracellular uranium precipitates were found for biomass from biological phosphorus removal reactor. According to the authors, the latter begin their formation at the cell membrane, grow to a very large size and fall off or get separated from the cells during preparation (Renninger et al., 2001). It was suggested, that cell-surface associated mineralization results in limitations of nutrient transport and disruption of the proton motive force (Southam, 2000). Therefore, the extracellular complexation of uranium by the *Microbacterium* spp. strains could be a good survival strategy especially in oligotrophic environments. Moreover, the ability of the *Microbacterium* spp. to immobilize relatively high amounts of uranium as extracellular meta-autunite phase is important in the context of the radionuclide mobility. It was shown, that stable assemblages of U(VI) as meta-autunite, were responsible for uranium retention for hundreds of years, within slightly acidic oxidizing bedrock aquifer (Jerden and Sinha, 2003). Interestingly, intracellular accumulation of uranium as two types of precipitates was identified for the *Sphingomonas* sp. S15-S1, but not for the *Microbacterium* spp. For instance, different types of uranium depositions for the *Sphingomonas* sp. S15-S1 cells may depend on distinct mechanisms of interactions with uranium. Different internal distribution of the metalloid tellurium in the cells of *Rhodobacter capsulatus* was shown to be related with the growth

mode of the cultures (Borghese et al., 2004). However, in the case of *Sphingomonas* sp. S15-S1, the cells were from the same growth phase. Both intra- and extracellular disposal of uranium was reported for strains of *Hallomonas*, *Pseudomonas*, *Acidithiobacillus ferrooxidans* (Francis et al., 2004; McLean and Beveridge, 2001; Merroun et al., 2002, 2003c), whereas intracellular accumulation was reported for different *Arthrobacter* isolates (Suzuki and Banfield, 2004).

## Conclusions

In this study, oligotrophic bacteria, from the groups of  $\alpha$ -subclass of Proteobacteria and Actinobacteria, were isolated from the S15 deep monitoring well of the radioactive waste depository Tomsk-7 and characterized. The strains tolerate relatively high concentrations of different heavy metals and interact effectively with uranium. The *Microbacterium* spp. tolerate identical and sorbe similar amounts of uranium, which were higher than those found for the *Sphingomonas* isolate. The EXAFS analysis showed that the studied S15 strains precipitate U(VI) as inorganic meta-autunite-like phase at pH 4.5. This could be connected to the liberation of inorganic phosphate due to the activity of acidic phosphatase. TEM and EDX analysis revealed bacterium-specific extracellular and/or intracellular uranium accumulations to varying degrees, which suggest that different processes were possibly involved in the uranium accumulation by the *Sphingomonas* and *Microbacterium* strains. At pH 2, uranium forms complexes with organically bound phosphate of the cell surface of the studied strains. The results indicate the presence of rapidly adapting bacterial populations in the subsurface environment around the radioactive waste depository site Tomsk-7, which can influence the migration of uranium and other heavy metals.

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## Summary

In the present work microbial diversity was studied in groundwater samples, collected from the S15 monitoring well located near the radioactive depository Tomsk-7, Siberia, Russia. The well uncovers aquifer at 290 to 324 m below the land surface, which corresponds to one of the radioactive disposal horizons. In order to overcome the inherent problems of microbial culturing, direct molecular approaches based on PCR amplification and cloning of 16S rRNA and RubisCO genes of the members of the natural microbial communities were applied.

The phylogenetic analyses indicate that the structure of the microbial community at the S15 monitoring site is rather complex and diverse. The presence of extremely large populations of *Dechlorosoma* spp. from the  $\beta$ -subclass of Proteobacteria was identified when the analysis was performed on the whole biomass of one water sample, which was collected on 1.2, 0.45 and 0.22  $\mu\text{m}$  pore-size filters. Members of different bacterial lineages like  $\alpha$ -,  $\gamma$ - and  $\delta$ -subclass of Proteobacteria, *Bacteroidetes*, Actinobacteria, *Planctomycetes*, *Nitrospirae* and Gram positive bacteria with low G+C were identified in the sample in lower numbers as well. The archaeal populations in the same sample were represented by methanogens and three distinct clusters of Crenarchaeota.

In order to obtain more profound information about the other bacterial groups, which could be masked by the dominant *Dechlorosoma* spp. populations, a parallel sample was studied, collected from the same site but handled differently. The biomass of this second sample was fractioned analogically to the above mentioned sample on 1.2, 0.45 and 0.22  $\mu\text{m}$  pore-size filters. However, in contrast to that sample, where one total DNA, recovered from the biomass of all the three filters together, was analysed, in this parallel sample DNA was extracted and analysed individually from each, the 0.45 and the 0.22  $\mu\text{m}$  filters.

In the 0.45  $\mu\text{m}$  fraction the dominance of *Dechlorosoma* spp. from the  $\beta$ -subclass of Proteobacteria was not as strong as in the whole biomass. Other sequences representing the  $\delta$ -subclass of Proteobacteria and Gram positive bacteria with low G+C were identified as well. Members of the bacterial clusters *Deinococcus-Thermus*, Candidate divisions OP8 and TM7 were retrieved only in the 0.45  $\mu\text{m}$  fraction.

The 0.22  $\mu\text{m}$  fraction of the biomass was strongly predominated by representatives of the CFB group. The *Dechlorosoma* sp. population was not identified in this fraction, while other  $\beta$  - Proteobacteria like *Ralstonia* spp. were encountered. Interestingly, relatively high number of sequences deeply branching within Cyanobacteria was retrieved in both the 0.45 and the 0.22  $\mu\text{m}$  fractions, but not in the whole biomass of the parallel sample. Hence, the analyses demonstrate that the estimation of the bacterial diversity strongly depends on the way of

dealing with the biomass.

The hydrogeochemical characteristics, the 16S rDNA analysis as well as the previous studies, based on cultivation approaches, suggested the presence of autotrophic organisms in the S15 monitoring well. In order to characterize the autotrophic bacterial community there, genes were targeted, encoding the form I (*cbbL*) and form II (*cbbM*) of the large subunit of the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), involved in the CO<sub>2</sub> fixation. The results confirm the dominance of the members from the  $\beta$ -subclass of Proteobacteria, identified via the 16S rDNA retrieval in the studied samples. However, the RubisCO approach encountered only autotrophic or mixotrophic  $\beta$ -Proteobacteria, such as *Hydrogenophylus*, *Nitrospira* and *Ralstonia* spp.

Of special interest was to isolate and to characterize bacteria from the S15 monitoring well in order to determine their resistance to heavy metals and in particular to study their interactions with uranium. The isolated oligotrophic strains were affiliated to  $\alpha$ -proteobacterial species like *Sphingomonas*, *Brevundimonas* and *Methylobacter* spp. and to Actinobacteria from the genus *Microbacterium*. The full sequenced 16S rDNA of three of the *Microbacterium* strains S15-M2, S15-M4 and S15-M5, differ only at four positions. However, the isolates S15-M2 and S15-M5, based on further physiological analysis were affiliated with *Microbacterium oxydans*, whereas S15-M4 represents another *Microbacterium* species.

The minimal inhibitory concentrations (MIC's) of cadmium-, copper-, lead-, nickel-, silver-, chromium- and uranium for the growth of the four *Microbacterium* spp. strains, S15-M2, S15-M3, S15-M4 and S15-M5 and of the isolate *Sphingomonas* sp. S15-S1 were determined. The results showed high heavy-metal tolerance of the S15 isolates, which were species and even strain specific. The uranium sorption analysis demonstrated that the *Microbacterium* spp. sorbed uranium up to 240 mg/g dry biomass, whereas the *Sphingomonas* isolate accumulated lower amounts of the radionuclide (up to 130 mg/g dry biomass).

The influence of uranium on the growth of the *Microbacterium* spp. strains was examined using flow cytometry. The results demonstrated the presence of two subpopulations of cells, with different surface properties and the percentage of one of them increased after longer exposure to uranium. This indicates that uranium possibly induces changes of some surface compounds.

The X-ray absorption spectroscopy (XAS) studies showed that the cells of the strains *Microbacterium oxydans* S15M-2 and *Sphingomonas* sp. S15-S1 both precipitate U(VI) as inorganic uranyl phosphates like meta-autunite at pH 4.5. This could be connected to the liberation of inorganic phosphate due to the activity of acidic phosphatases.

Transmission electron microscopy (TEM) and energy dispersive X-ray (EDX) analysis

revealed that *Microbacterium* spp. precipitated uranium mainly extracellularly. Both intra- and extracellular uranium accumulations were found for the *Sphingomonas* sp. S15-S1. These results suggest that possibly different processes are involved in the uranium accumulation by the *Sphingomonas* sp. and *Microbacterium* spp. strains. At pH 2 uranium formed complexes with organically bound phosphate groups of the cell surface of the studied strains.

In summary, the analyses indicated the presence of rather complex and diverse microbial communities in the subsurface environment around the radioactive depository Tomsk-7 and the presence of a variety of bacterial populations which have the potential to bind and transport radionuclides.