Prokaryotic microorganisms in uranium mining waste piles and their interactions with uranium and other heavy metals

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Man muss die Welt nicht verstehen,
man muss sich nur darin zurechtfinden.
Albert Einstein

To my grandmother Helga,
Marta and my parents

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CHAPTER 5

INTERACTIONS OF TWO *ARTHROBACTER* STRAINS ISOLATED FROM THE URANIUM MINING WASTE PILE HABERLAND WITH Pb AND U

Introduction

Migration of radionuclides from the radioactive waste repository sites, as well as from uranium mill tailings and mining piles, is of serious environmental concern. About 231000 tons of uranium were produced in Eastern Germany from 1946 to 1990 (Meinrath *et al*., 2003). More than 5 x 10^8 tons of radioactive wastes, a total of 3000 piles and about 20 tailings had to be remediated or decontaminated (Beleites, 1992). The fate and the transport of uranium are governed by the contrasting chemistry of U(IV) and U(VI). U(VI) generally forms soluble, and thus mobile, complexes with carbonate and hydroxide, while U(IV) precipitates as the highly insoluble mineral uraninite (Nyman *et al*., 2006). Abiotic factors such as ions and minerals strongly influence the migration process of uranium (Barnett *et al*., 2000; Arnold *et al*., 2001; Duff *et al*., 2002; Baik *et al*., 2004). In addition, microbial processes can influence the mobility of heavy metals and radionuclides and, thereby, their migration behaviour (Francis, 1998; Lloyd & Lovley, 2001; Merroun & Selenska-Pobell, 2001; Lloyd & Macaskie, 2002; Selenska-Pobell, 2002; Merroun *et al*., 2003, 2005, 2006; Suzuki *et al*., 2003, 2005; Kalinowski *et al*., 2004; Lloyd & Renshaw, 2005; Lloyd *et al*., 2005; Pedersen, 2005). These processes can act metal immobilising or mobilising and involve biotransformations as oxidation (DiSpirito & Tuovinen, 1982; Beller, 2005) and reduction (Lovley *et al*., 1991, 1993a; Lloyd, 2003; Khijniak *et al*., 2005; Wu *et al*., 2006), biosorption by cell surface polymers (Selenska-Pobell *et al*., 1999; Raff *et al*., 2003; Beveridge, 2005; Merroun *et al*., 2005), uptake of metals inside the cells (McLean & Beveridge, 2001; Merroun *et al*., 2003; Francis *et al*., 2004; Suzuki & Banfield, 2004), metal precipitation and generation of minerals (Macaskie *et al*., 2000; Merroun *et al*., 2006; Nedelkova *et al*., 2006) and chelation by siderophores and other microbial compounds (Kalinowski *et al*., 2004; Pedersen, 2005) (Fig. 1).

To better understand the microbial mechanisms which influence the radionuclide migration and in order to establish bioremediation strategies for the contaminated sites, information on the distribution and the activities of the microorganisms in these extreme habitats are required. Because more than 99% of the microorganisms in the environment are uncultivable by using standard cultivation techniques, culture-independent molecular approaches based on analyses of 16S rRNA genes were used to explore microbial diversity in nature (Pace, 1997; Pedersen, 1997; Hugenholtz, 2002; Selenska-Pobell, 2002; Torsvik & Øvreås, 2002).

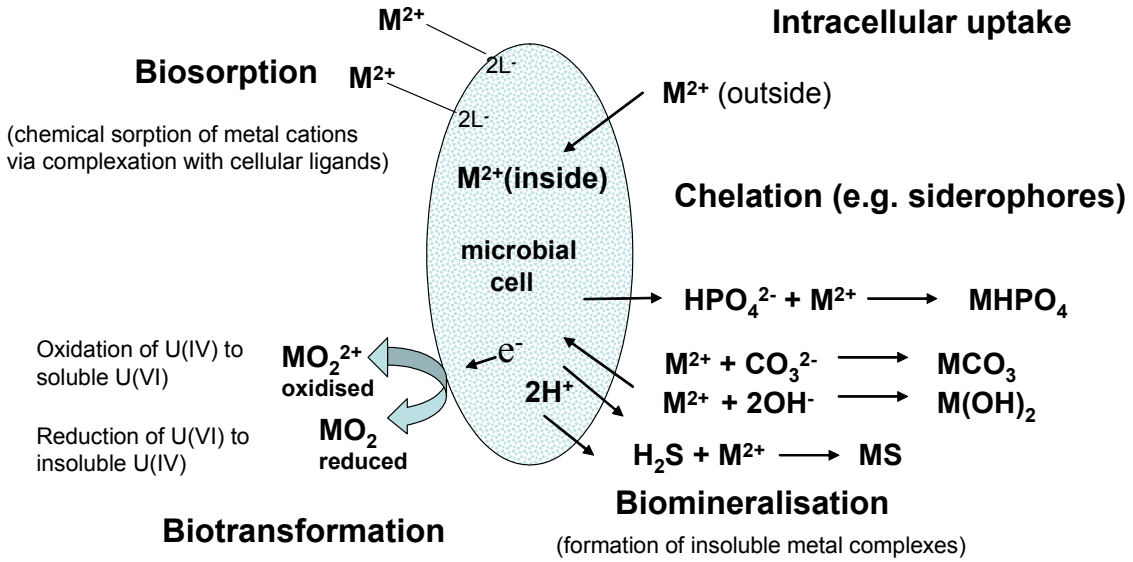


Fig. 1. Mechanisms of radionuclide-microbe interactions changed after Lloyd & Macaskie (2002).

During the last decade, a number of studies investigated microbial communities in radionuclide contaminated environments by using the 16S rRNA gene retrieval, in order to determine the phylogenetic placement of the microorganisms that are inhabiting these environments (Pedersen *et al*., 1996; Crozier *et al*., 1999; Selenska-Pobell *et al*., 2001; Selenska-Pobell, 2002; Selenska-Pobell *et al*., 2002; Suzuki *et al*., 2003, 2005; Fredrickson *et al*., 2004; Satchanska *et al*., 2004; Fields *et al*., 2005; Radeva & Selenska-Pobell, 2005; Satchanska & Selenska-Pobell, 2005).

Differences in the composition of the bacterial communities in contaminated environments were observed, which are site-specific and possibly connected to their different grade of contamination, to their different geographic and geologic origin as well as to their site history (Selenska-Pobell *et al*., 2002; Geissler, 2003). *Alphaproteobacteria* and *Acidobacteria* were found to be predominant applying the 16S rRNA gene retrieval with different primer sets in soil samples collected from different depths of the uranium mining waste pile Haberland, located near the town of Johanngeorgenstadt in Germany (Selenska-Pobell *et al*., 2002; Selenska-Pobell, 2002; Geissler, 2003; Satchanska *et al*., 2004). In soil samples from the uranium mill tailings Gittersee/Coschütz in Germany no *Acidobacteria* were identified and the *Alphaproteobacteria* were not predominant (Selenska-Pobell *et al*., 2002; Satchanska & Selenska-Pobell, 2005). Instead, the number of *Gammaproteobacteria* especially of *Pseudomonas* spp. as well as the number of representatives of the *Bacteroidetes* phylum was extremely high in these samples (Selenska-Pobell *et al*., 2002; Satchanska & Selenska-Pobell, 2005). *Gammaproteobacteria* were also predominant in a soil sample from the uranium

depository site Gunnison, Colorado, USA (Selenska-Pobell *et al*., 2002; Geissler, 2003). In contrast, the composition of the bacterial community in a soil sample from the uranium mill tailings Shiprock, New Mexico, USA was extremely complex and Gram-positive bacteria, especially *Bacillus* spp., green non-sulphur bacteria, and *Gammaproteobacteria* were found to be predominant (Selenska-Pobell *et al*., 2002; Geissler, 2003). Bacterial diversity was also investigated in water samples collected from several uranium mining wastes (Radeva & Selenska-Pobell, 2005). The analysis of the 16S rRNA gene retrieval revealed that *Nitrospina*-like bacteria are predominant in the uranium mill tailings Schlema/Alberoda, Germany, whereas *Pseudomonas* spp. and *Frateuria* spp. from *Gammaproteobacteria* were predominant in the uranium mill tailings Shiprock, New Mexico, USA (Radeva & Selenska-Pobell, 2005). Similarly to the soil samples, *Proteobacteria* and representatives of the *Bacteroidetes* phylum were also found in waters of the uranium mill tailings Gittersee/Coschütz (Radeva & Selenska-Pobell, 2005). However, in the Steinsee Deponie 1 in Germany mainly *Acinetobacter* spp. were identified (Radeva & Selenska-Pobell, 2005).

The bacterial community structure analyses based on 16S rRNA gene clone libraries of groundwater from the Field Research Centre (FRC), which is located at the Y-12 complex within the Security Oak Ridge National Laboratory in Oak Ridge, Tennessee, USA, demonstrated high diversity at the background site (Fields *et al*., 2005). However, three other studied acidic groundwater samples from the FRC site, with high concentrations of nitrate, nickel, aluminium, and uranium, had a decreased diversity and the majority of the clones were closely related to *Azoarcus* spp. and *Pseudomonas* spp. (Fields *et al*., 2005). From the same site, but from another sampling point, a large variety of 16S rRNA gene sequences was retrieved including species from the *Alpha*-, *Beta*-, *Delta*-, and *Gammaproteobacteria* as well as Gram-positive species with low- and high-G+C content (North *et al*., 2004). This part of the site was contaminated with uranium and nitrate as well as with other radionuclides such as plutonium, technetium, other heavy metals and a variety of organic contaminants (North *et al*., 2004).

In order to determine if the inhabiting microorganisms are capable of carrying out the processes of interest for bioremediation, functional genes or enzyme analyses were employed as well. In addition to the 16S rRNA gene retrieval, culture-independent approaches targeting specific metabolic key enzymes were used to obtain information about the activities and ecological impact of functional bacterial groups that are responsible for certain processes. The investigation of sulphate-reducing bacteria (SRB) in contaminated groundwaters from the uranium mill tailings disposal site at Shiprock, New Mexico, USA revealed a remarkable

diversity among the dissimilatory sulphite reductase (DSR) gene fragments, including sequences from *Deltaproteobacteria*, Gram-positive organisms, and the *Nitrospira* division (Chang *et al*., 2001). Dominance of ammonia-oxidising *Betaproteobacteria* was demonstrated by using both the 16S rRNA gene and ammonia monooxygenase gene (*amo*A) retrievals in the samples collected from the same site (Ivanova *et al*., 2000). Nitrate-contaminated groundwater samples from the already mentioned FRC site in Oak Ridge, Tennessee, USA were analysed for nitrite reductase gene (*nir*K and *nir*S) diversity (Yan *et al*., 2003). The results indicated that the contaminated groundwater contained novel, not previously described *nir*K and *nir*S sequences. The functional diversity of both genes changed in relation to contaminant gradients, but the *nir*K and the *nir*S functional diversity were differently affected by particular contamination fluctuations (Yan *et al*., 2003). Nitrate is a common cocontaminant in uranium mining wastes (Finneran *et al*., 2002; Istok *et al*., 2004; North *et al*., 2004) and can strongly influence the mobility of uranium. It was demonstrated that nitrate inhibits U(VI) bioreduction in acetate-amended aquifer sediments and that both U(VI) and Fe(III) were reduced concurrently only when nitrate was depleted (Finneran *et al*., 2002). The addition of nitrate can also result in reoxidation of U(IV) (Finneran *et al*., 2002; Senko *et al*., 2002; Istok *et al*., 2004). Nitrate-dependent U(IV) oxidation often proceeds through Fe(III) (Finneran *et al*., 2002; Senko *et al*., 2005a, 2005b). Fe(III) can be produced by nitrate-dependent Fe(II)-oxidising microorganisms (Straub *et al*., 1996, 2004; Straub & Buchholz-Cleven, 1998; Lack *et al*., 2002). Also possible is the oxidation of Fe(II) by nitrite, which accumulates during dissimilatory nitrate reduction (Senko *et al*., 2002, 2005a). Recently, it was demonstrated that the chemolithoautotrophic bacterium *Thiobacillus denitrificans* oxidatively dissolves synthetic and biogenic U(IV) oxides (uraninite) in a nitrate-dependent reaction under strictly anaerobic conditions (Beller, 2005). The reduction of nitrate to nitrite can be catalysed by three distinct nitrate reductases classes NAS, NAR and NAP in prokaryotes (Richardson *et al*., 2001). The membrane-bound nitrate reductase NAR was used as a molecular marker to target nitrate-reducing communities in different environments (Philippot *et al*., 2002; Chèneby *et al*., 2003; Deiglmayr *et al*., 2004; Mounier *et al*., 2004; Héry *et al*., 2005), but not yet in uranium mining wastes.

For developing cost-effective *in situ* bioremediation technologies, the microbial reduction of U(VI) has been intensively studied (Lovley *et al*., 1991, 1993a, 1993b). To reveal the occurrence of and pathways for U(VI) reduction in aquatic sediments containing naturally produced organic matter, sediments from the inactive open pit uranium mine Midnite, located in Stevens County, eastern Washington, were incubated for 30 days at room temperature

under anaerobic conditions and analysed (Suzuki *et al*., 2005). X-ray absorption near-edge structure (XANES) spectroscopy revealed that U(VI) was reduced to U(IV) in these sediments, which contained nitrate, Fe(III), and sulphate. The latter had also been reduced during the experiment (Suzuki *et al*., 2005). Analysis of the sediment particles and the microbial cells by scanning and transmission electron microscopy coupled with elemental analysis by energy dispersive spectroscopy revealed that the reduced uranium was concentrated at microbial cell surfaces. Because the U(IV) was not associated with framboidal pyrite or nanometre-scale iron sulphides, the authors suggested that U(VI) was reduced by the enzymatic activities of the microorganisms (Suzuki *et al*., 2005). Microbial populations in the original sediment were analysed by amplification and sequencing of the 16S rRNA and dissimilatory sulphite reductase genes, which demonstrated that organisms belonging to the families *Geobacteraceae* and *Desulfovibrionaceae* were present. Because cultivated members of these lineages are also able to reduce U(VI), the authors suggestion of biological U(VI) reduction was confirmed (Suzuki *et al*., 2005). In addition to the bacterial diversity, archaeal diversity was also investigated in these uranium contaminated sediments. Archaeal 16S rRNA gene sequences representing *Methanobacteria* of *Euryarchaeota* were found to be predominant (Suzuki *et al*., 2005). Methan-producing *Archaea* (*Methanobacterium subterraneum*) were also isolated from deep granitic groundwater from the Äspö hard rock laboratory located in the vicinity of the Simpevarp nuclear power plant north of Oskarshamm, South-East Sweden (Kotelnikova *et al*., 1998). Only a few additional studies investigated the archaeal diversity in heavy metal contaminated environments (Takai *et al*., 2001; Stein *et al*., 2002). Recently, it was demonstrated that the hyperthermophilic crenarchaeon *Pyrobaculum islandicum* is able to reduce U(VI) to the insoluble U(IV) mineral uraninite at ca. 100 °C (Kashefi & Lovley, 2000).

Different studies were performed by addition of nutrients to uranium contaminated groundwaters and soils to increase the number and activity of indigenous microorganisms prospective for bioremediation (Holmes *et al*., 2002; Anderson *et al*., 2003; Nevin *et al*., 2003; Suzuki *et al*., 2003; North *et al*., 2004; Brodie *et al*., 2006; Nyman *et al*., 2006). Changes in the microbial community were observed when U(VI) reduction was stimulated by addition of acetate in sediments from three different uranium-contaminated sites in the floodplain of the San Juan River in Shiprock, New Mexico, USA. The treatment resulted in a dramatic enrichment of microorganisms of the family *Geobacteraceae,* which are known as U(VI)- and Fe(III)-reducing microorganisms (Holmes *et al*., 2002). *Deltaproteobacteria*, including *Anaeromyxobacter dehalogenans*-related and also several *Geobacter*-related

species, were stimulated in acidic subsurface from the FRC in Oak Ridge, Tennessee, USA as well as by pH neutralisation and during *in situ* biostimulation with glucose or ethanol (North *et al*., 2004). These results were supported by the investigation of Fe(III)-reducing enrichment cultures initiated from sediments of the same site possessing low pH values as well as high uranium and nitrate contaminations (Petrie *et al*., 2003). These cultures were predominated by different Fe(III)-reducing *Anaeromyxobacter* spp., and by Gram-positive organisms previously not known to reduce Fe(III), such as *Paenibacillus* spp. and *Brevibacillus* spp. (Petrie *et al*., 2003).

Recently, interesting results were published from replicate batch microcosms containing contaminated sediment collected from a well within FRC in Oak Ridge, Tennessee, USA, added with an inoculum from a pilot-scale fluidised bed reactor representing the inoculum in the field experiment and then supplemented with ethanol and uranyl acetate (Nyman *et al*., 2006). After an initial reduction of nitrate, both sulphate and soluble U(VI) concentrations decreased, resulting in U(IV) formation, which was confirmed by XANES spectroscopy. Denitrifying organisms related to *Acidovorax* were predominant as demonstrated by terminal restriction fragment length polymorphism (T-RLFP) and cloning (Nyman *et al*., 2006). Interestingly, *Acidovorax* isolates from the inoculum were also shown to reduce U(VI). However, in some microcosms, the soluble U(VI) concentration increased after longer incubations due to reoxidation that was explained by the authors to be a result of the microbial and/or mineralogical heterogeneity among the samples (Nyman *et al*., 2006).

Uranium-contaminated sediment and water collected from the inactive uranium mine Midnite, located in Stevens County in eastern Washington, USA, were incubated anaerobically with a mixture of different organic substances (lactate, acetate, ethanol, benzoic acids, glucose, yeast extract and peptone) (Suzuki *et al*., 2003). The removal of U(VI) from the solution within one month was observed after this biostimulation. Bacterial community analysis, based on the 16S rRNA gene retrieval, revealed that the natural microbial populations were shifted from microaerophilic *Proteobacteria* to anaerobic low-G+C Gram-positive sporeforming bacteria, belonging to *Desulfosporosinus* spp. and *Clostridium* spp. (Suzuki *et al*., 2003). In the highly saline uranium-contaminated aquifer sediments at the uranium mill tailings site Shiprock, New Mexico, USA an enrichment of both *Desulfosporosinus* spp. and *Pseudomonas* spp. was observed by the addition of acetate, which was associated with the removal of U(VI) from the groundwater (Nevin *et al*., 2003).

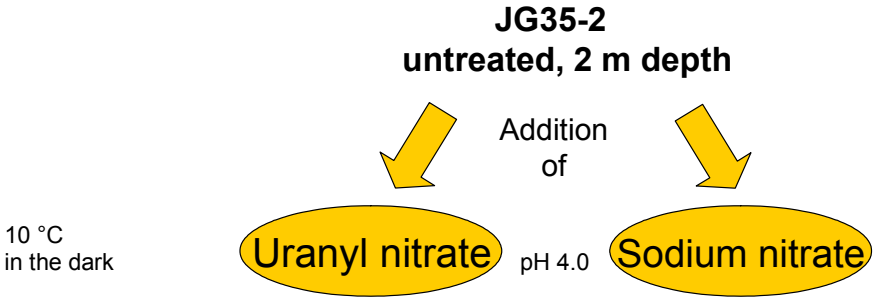
At the Uranium Mill Tailings Remedial Action site (UMTRA) in Rifle, Colorado, an *in situ* biostimulation with acetate was monitored over three months (Anderson *et al*., 2003). In this

case, the decrease in soluble U(VI) within 50 days was associated with an increase of Fe(II) and an enrichment of *Geobacter* spp. This indicated that the U(VI)-reducing microorganisms have the potential to immobilise uranium *in situ*. However, after 50 days of acetate injection, U(VI) began to increase within many of the field wells. These changes, after 50 days of injection, were accompanied by loss of sulphate from the groundwater and accumulation of sulphide. The composition of the microbial community was changed as well and sulphate reducers became predominant (Anderson *et al*., 2003). These results suggest that the long-term stability of reduced U(IV) is a major point for a successful bioremediation.

To monitor the long-term stability of bioreduced U(IV), flow through column incubations for more than 500 days, using soil from another area of the uranium contaminated FRC at Oak Ridge, Tennessee were performed by adding Na-lactate (Wan *et al*., 2005; Brodie *et al*., 2006). In this case, U(VI) reduction and immobilisation was successful within the first 100 days, followed by reoxidation and remobilisation of U(IV) under continuous reducing conditions (Wan *et al*., 2005). In order to determine if members of the microbial community are involved in the U(IV) reoxidation, a high-density oligonucleotide microarray-based approach was applied by Brodie *et al*. (2006). The amplicons of known metal-reducing bacteria, such as *Geothrix fermentans* and those within *Geobacteraceae*, were abundant during U(VI) reduction and U(IV) reoxidation. On the basis of these results, the authors suggest that observed reoxidation of uranium under reducing conditions occurred despite elevated microbial activity and the consistent presence of metal-reducing bacteria (Brodie *et al*., 2006). The terminal electron acceptor for U(IV) oxidation was not identified but the authors hypothesised that either Fe(III) or Mn(IV) were the most likely candidates (Wan *et al*., 2005; Brodie *et al*., 2006).

The fate of uranium in complex natural systems without the addition of organic substances is of great environmental importance in order to predict the potential risks of uranium migration within piles, tailings and depository sites and to prevent their spread out to groundwater flow. To our knowledge no experiments were performed including addition of uranyl nitrate to natural oligotrophic systems. For this purpose within the scope of this thesis, different microcosms experiments were performed to investigate the changes in the microbial community structure of a soil sample collected from the uranium mining waste pile Haberland after the addition of uranyl or sodium nitrate and incubations under aerobic or anaerobic conditions without supplementation with organic matter (Fig. 2). The work included the analysis of the bacterial as well as the archaeal diversity by using the 16S rRNA gene retrieval in the original untreated sample and in the treated samples (Fig. 2). Because uranium was

added in a form of uranyl nitrate to the soil samples and nitrate influences the mobility of uranium, the ability of the indigenous microorganisms to reduce nitrate was studied applying a culture-independent approach based on the nitrate reductase gene *nar*G as a functional marker. In addition, a spectroscopic method was used to determine the Fe(II)/Fe(III) ratio in the untreated sample and the uranyl nitrate treated samples incubated under anaerobic conditions.



Aeration conditions / Time of incubation	U content mg kg^{-1}	aerobic	anaerobic	aerobic	anaerobic	U content mg kg^{-1}
4 weeks	~100	JG35+U1	JG35+U4	JG35-K1	JG35-K4	26 - 40
14 weeks	~300	JG35+U2A	JG35+U3		JG35-K2	26 - 40
28 weeks	~300	JG35+U2B				

Fig. 2. Microcosms experiments performed.

Under aerobic conditions both treatments with uranyl and sodium nitrate resulted in the reduction of *Alphaproteobacteria* and *Acidobacteria* (Chapter 1), which were predominant in the untreated samples (Selenska-Pobell, 2002; Geissler, 2003; Satchanska *et al*., 2004). In the sub-sample treated with sodium nitrate for 4 weeks a strong proliferation of particular denitrifying and nitrate-reducing populations of *Arthrobacter* spp. and representatives of the *Bacteroidetes* phylum was observed. Stimulation of *Pseudomonas* spp. from *Gammaproteobacteria*, *Arthrobacter* spp. from *Actinobacteria* as well as of *Geobacter* spp. from *Deltaproteobacteria* was occurred in the parallel sub-sample incubated with uranyl nitrate for 4 weeks under the same conditions. However, by selective sequential extraction analyses and X-ray absorption spectroscopy no accumulation of U(IV) was observed in this sample even though the occurrence of some Fe(III)- and U(VI)-reducing *Geobacter* spp. (Geissler *et al*., 2005).

After a longer incubation for 14 weeks, even with higher amounts of uranyl nitrate different uranium-sensitive *Bacteroidetes* and alphaproteobacterial populations as well as

Arthrobacter spp. were stimulated. The strong proliferation of probably uranium sensitive representatives of the *Bacteroidetes* phylum indicated that at these late stages of the treatment the added U(VI) was no longer bioavailable. Moreover, it was noticed that after even longer incubation time of 28 weeks, of which half of the time was under anaerobic conditions, the bacterial community had a tendency to establish the initial structure of the natural sample JG35-2 (Chapter 1).

The treatment with uranyl or sodium nitrate under anaerobic conditions induced changes in the bacterial community structure as well, but as expected a proliferation of different bacterial groups occurred (Chapter 2). The anaerobic conditions correspond better to the natural conditions, because the sample studied was collected from a depth of 2 m. The addition of sodium nitrate and the incubation for 4 weeks resulted in a proliferation of representatives of *Firmicutes* and of *Betaproteobacteria*. By the treatment with uranyl nitrate under the same conditions only a small part of these populations was induced and established. Analogue to the untreated sample *Alphaproteobacteria* were predominant in the uranyl nitrate treated sample, although a shifting to probably uranium tolerant species was observed. In the latter sample *Gammaproteobacteria* especially *Rahnella* spp. were stimulated. Similarly to the sample treated with sodium nitrate for 4 weeks, *Betaproteobacteria* were stimulated after longer incubations for 14 weeks with sodium nitrate. However, after the addition of higher amounts of nitrate and a longer incubation for 14 weeks in contrast to the sample incubated for 4 weeks, the size of the *Firmicutes* was reduced and representatives of *Actinobacteria*, of the *Bacteroidetes* phylum as well as of *Gammaproteobacteria* were predominant. After the treatment with uranyl nitrate very diverse alphaproteobacterial populations and some uranium-sensitive betaproteobacterial and *Bacteroidetes* populations were established. The increased diversity, especially of U-sensitive populations in the latter sample indicated that at the late stages of the treatments the added U(VI) was no longer bioavailable. Interestingly, no or only a few representatives of *Deltaproteobacteria* were identified in the uranyl or sodium nitrate treated samples incubated under anaerobic conditions, whereas they represented the third predominant group in the untreated sample and most of them are known as sulphate- and metal-reducing bacteria (Lovley *et al*., 1991, 1993a, 1993b; Lovley & Phillips, 1992; Wu *et al*., 2006). Recently, biological U(VI) reduction was demonstrated for an increasing number of taxonomically diverse microorganisms such as *Clostridium* spp. (Francis *et al*., 1994; Suzuki *et al*., 2003), *Pseudomonas* sp. (McLean & Beveridge, 2001), *Desulfosporosinus* spp. (Suzuki *et al*., 2004), *Acidovorax* spp. (Nyman *et al*., 2006), *Salmonella subterranean* (Shelobolina *et al*., 2004), *Cellulomonas* spp. (Sani *et al*., 2002) and *Thermoanaerobacter*

spp. (Roh *et al*., 2002). For this reason we can not exclude U(VI) reduction in the studied uranyl nitrate treated samples.

By using Mössbauer spectroscopy it was revealed that the amount of Fe(II) increased in the samples treated with uranyl nitrate for 4 and for 14 weeks under anaerobic conditions, which indicated the reduction of Fe(III). Reduction of Fe(III) to Fe(II) was observed associated with the reduction of U(VI) after biostimulation with acetate (Anderson *et al*., 2003) and by the incubation of uranium contaminated sediments under anaerobic conditions (Suzuki *et al*., 2005). The reduction of Fe(III) can occur abiotically with hydrogen sulphide or U(IV) as the reductant (Luther *et al*., 2001; Senko *et al*., 2005a) or biologically by Fe(III)-reducing bacteria. The electron donor for the Fe(III) reduction in the samples treated with uranyl nitrate studied in this work are not yet identified. Further studies are necessary to characterise the fate of U(VI) in the samples studied as well.

The results presented in this thesis demonstrated that the indigenous bacterial community of the uranium mining waste pile Haberland possesses a high potential to adapt to changing nitrate and U(VI) concentrations under aerobic and anaerobic conditions. Most of the cultured members of the lineages stimulated by the treatments are known to interact with uranium and nitrate. However, the full impact of the metabolic capabilities of the bacteria stimulated by the treatment on the behaviour of U(VI) remain unknown.

The second objective of the thesis was to study if the inhabiting microorganisms are capable to reduce nitrate. For this propose the nitrate-reducing bacterial community was studied in the untreated sample from the uranium mining waste pile Haberland as well as in the samples treated with uranyl or sodium nitrate and incubated for 4 weeks under aerobic or anaerobic conditions by using the nitrate reductase gene *nar*G as a functional marker (Chapter 3, Fig. 2). Most of the sequences retrieved from the untreated sample were closely related to environmental NarG sequences of not yet cultured bacteria described in previous studies (Chèneby *et al*., 2003; Deiglmayr *et al*., 2004; Mounier *et al*., 2004; Héry *et al*., 2005). They were distantly related to the NarG sequence of the alphaproteobacterium *Brucella suis*. Only two additional small groups of sequences clustered with the NarG of the betaproteobacterial species *Thiobacillus denitrificans* and *Polaromonas naphthalenivorans*. In the sample treated with uranyl nitrate and incubated under aerobic conditions for 4 weeks, no NarG sequence related to *Brucella suis* was identified. Instead, sequences related to the NarG of *Geobacter metallireducens*, *Chromobacterium violaceum*, *Pseudomonas fluorescens*, as well as of *Arthrobacter* spp. were detected. This result confirms the presence of *Pseudomonas* spp., *Geobacter* spp. and *Arthrobacter* spp. identified by the 16S rRNA gene retrieval in this

sample (Chapter 1). By the treatment with sodium nitrate under aerobic conditions most of the retrieved NarG sequences were closely related to the NarG of *Arthrobacter* sp. FB24 or formed novel clusters within the "*Brucella*"- and "*Polaromonas*"-like clusters. Interestingly, proliferation of *Arthrobacter* spp. was observed by the 16S rRNA gene retrieval as well (Chapter 1). In contrast, by the treatment with sodium nitrate under anaerobic conditions most of the NarG sequences retrieved were distantly related to NarG of *Bacillus* spp. and *Chromobacterium violaceum*. Representatives of *Firmicutes* and *Betaproteobacteria* were found to be predominant in this sample by using the 16S rRNA gene retrieval. However, no 16S rRNA gene sequences related to *Chromobacterium* were detected and the *Firmicutes* were represented by *Bacillus* spp., and *Clostridium* spp. (Chapter 2). The latter could not be identified by the *nar*G gene retrieval. The treatment with uranyl nitrate under anaerobic conditions induced mostly NarG sequences distantly related to the NarG of *Polaromonas* spp. or to the NarG of particular *Alpha*- and *Betaproteobacteria* found in the untreated sample. The results revealed that the nitrate-reducing community was influenced by the treatments dependent on the nitrate solution used as well as on the aeration conditions. It was demonstrated that in consensus to the 16S rRNA gene retrieval some of the bacteria stimulated by the treatments possess the membrane-bound nitrate reductase NAR and are capable of nitrate reduction.

The third objective of the thesis was to investigate the archaeal diversity in the samples studied (Chapter 4). To our knowledge this is the first analysis of an archaeal response to treatments with uranyl nitrate under aerobic or anaerobic conditions. The archaeal diversity in the untreated sample was estimated to be lower in comparison to the bacterial diversity. All the sequences retrieved were related to only a few lineages of mesophilic *Crenarchaeota* and no representatives of *Euryarchaeota* were identified. 64% of the clones retrieved from the untreated sample represented the crenarchaeal group 1.1a and 36% of them represented the crenarchaeal group 1.1b. Independent on the addition of uranyl or sodium nitrate and the incubations under aerobic or anaerobic conditions as well as on the time of incubation a reduction of the archaeal diversity in comparison to the untreated sample and a shifting to the mesophilic *Crenarchaeota* of group 1.1b was observed. Most of the 16S rRNA gene sequences, retrieved from the treated samples were closely related to the 16S rRNA gene sequence of the fosmid clone 54d9. The fosmid clone 54d9 is a 43 kb genomic fragment with a ribosomal 16S rRNA gene, which was identified by PCR screening of archaeal operon-encoding fragments in a 1.2 Gb large-insert environmental fosmid library prepared from a soil sample collected from the upper 10 cm layer of a sandy ecosystem called "Am Rotböhl" near

Darmstadt, Germany (Treusch *et al*., 2005). The insert also encoded a homologue of a copper-containing nitrite reductase and two proteins related to subunits of ammonia monooxygenases (Treusch *et al*., 2005). Because of these results the authors suggested that the mesophilic *Crenarchaeota* might be capable of ammonia oxidation under aerobic and potentially also under anaerobic conditions (Treusch *et al*., 2005). On the basis of the close relatedness of our 16S rRNA sequences to the 16S rRNA gene found on the fosmid clone 54d9, a similar metabolism for the uncultured *Crenarchaeota* stimulated by the treatments performed in our study was suggested. Nitrate used in our treatments was most probably transformed to ammonium by nitrate reduction and denitrification or dissimilatory nitrate ammonification by the bacteria present in the samples. Indeed, the analysis of the bacterial diversity in the treated samples studied revealed a stimulation of nitrate-reducing, denitrifying and ammonifying populations (for instance *Geobacter* spp., *Pseudomonas* spp., *Arthrobacter* spp., and *Clostridium* spp.), which can supply the *Crenarchaeota* with different nitrogen oxide compounds (NO_2, NO) and ammonium (Chapters 1, 2, 3). Under anaerobic conditions, it is also possible that nitrite can be used as an electron acceptor and energy can be gained via nitrogen dioxide (NO_2)-dependent ammonia oxidation (Schmidt *et al*., 2004).

In line with the results of others (Vetriani *et al*., 1999; García-Martínez *&* Rodríguez-Valera, 2000; Benlloch *et al*., 2002), microdiversity among closely related archaeal 16S rRNA gene sequences was demonstrated in our samples. The microdiverse 16S rRNA genes might represent organismal genetic diversity, because up to date all cultured *Crenarchaeota* have only one rRNA operon (Vetriani *et al*., 1999; Ochsenreiter *et al*., 2003). The results presented in the thesis indicate dynamic and active archaeal populations that react to changes in environmental conditions in the studied uranium mining waste pile.

In addition to the description of the distribution of 16S rRNA and other gene sequences in the environment, it is necessary to have information on the physiology of pure microbial cultures related to the natural populations identified via molecular methods (Lovley, 2003). Thus, the fourth objective of the thesis was to isolate bacteria from the uranium mining waste pile and to study their interactions with uranium and other heavy metals (Chapter 5). A lot of efforts were put to culture microorganisms from radionuclide contaminated sites and to study their interactions with radionuclides and heavy metals (Selenska-Pobell *et al*., 1999; Selenska-Pobell, 2002; Benyehuda *et al*., 2003; Elias *et al*., 2003; Fredrickson *et al*., 2004; Nazina *et al*., 2004; Fields *et al*., 2005; Merroun *et al*., 2006; Nedelkova *et al*., 2006). The advantage of studying bacterial and archaeal cultures is that they can provide important information about the physiological and metabolical properties of the isolates. Lovley *et al*. (1991, 1993a)

demonstrated for the first time the dissimilatory U(VI) reduction by the Fe(III)-reducing bacteria *Geobacter metallireducens* and *Shewanella oneidensis* that can conserve energy for anaerobic growth via the reduction of U(VI). Other organisms as sulphate-reducing bacteria (*Desulfovibrio desulfuricans* (Lovley & Phillips, 1992), *Desulfovibrio vulgaris* (Lovley *et al*., 1993b), *Desulfosporosinus* sp. (Suzuki *et al*., 2004), *Clostridium* sp. ATCC 53464 (Francis *et al*., 1994), *Salmonella subterranean* (Shelobolina *et al*., 2004) and *Anaeromyxobacter dehalogenans* strain 2CP-C (Wu *et al*., 2006) are also able to reduce U(VI), however without conserving energy from this process. On the other hand, the oxidation of U(IV) to U(VI) by microorganisms was demonstrated (DiSpirito & Tuovinen, 1982; Beller, 2005).

The ability of different *Bacillus* spp. isolated from the uranium mining waste pile Haberland to remove toxic metals and uranium from solutions was also studied (Selenska-Pobell *et al*., 1999). The strain *Bacillus sphaericus* JG-A12 was used for construction of biological ceramics (biocers) via sol-gel immobilisation of its vegetative cells, spores or surface layer sheets. These biocers demonstrated high binding capacity of uranium and copper from contaminated waters (Raff *et al*., 2003).

Chemolithoautrophic bacteria, such as *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, as well as heterotrophic bacteria such as *Desulfovibrio* spp., *Pseudomonas* spp. as well as strains related to *Agrobacterium*, *Rhizobium* and *Sphingomonas* were isolated from different uranium mining waste piles (Merroun & Selenska-Pobell, 2001; Selenska-Pobell *et al*., 2002; Selenska-Pobell, 2002; Merroun *et al*., 2003).

Bacteria were also isolated from groundwater samples collected in the vicinity of radioactive waste repositories. From the S15 deep-well monitoring site, located at the Siberan radioactive subsurface depository Tomsk-7, Russia, *Alphaproteobacteria* and *Actinobacteria* from the genus *Microbacterium* were isolated, which accumulate high amounts of uranium (Nedelkova *et al*., 2006). Sulphate-reducing bacteria as for instance *Desulfovibrio aespoeensis* were isolated from the granitic groundwater of south-eastern Sweden, where the Äspö hard rock laboratory (HRL) has been constructed to study the function of underground repositories for long-lived nuclear fuel waste (Motamedi & Pedersen, 1998)

From the Department of Energy (DOE) Hanford site Gram-positive bacteria closely related to *Arthrobacter* spp. were most common isolates among all samples, but other phyla with high G+C content including *Rhodococcus* and *Nocardia* were also presented (Fredrickson *et al*., 2004). From another study from the Hanford site also mostly high G+C Gram-positives (*Arthrobacter* spp.), low G+C Gram-positive bacteria (*Bacillus* spp. and *Staphylococcus* spp.) and different *Proteobacteria* (*Caulobacter* spp., *Sphingomonas* sp., *Variovorax* spp.,

Pseudomonas spp. and *Acinetobacter* spp.) were isolated. Similarly, the DOE`s Savannah River site (SRS) isolates were mostly high G+C Gram-positive bacteria (mainly *Arthrobacter* spp.) and *Proteobacteria* (*Comamonas* spp. and *Acinetobacter* spp.) (Benyehuda *et al*., 2003). In another study where cultivation-based methods were used to isolate aerobic heterotrophs present in radionuclide and heavy-metal contaminated subsurface soils at the FRC in Oak Ridge, Tennessee, USA the majority of the isolates (392 of 400) recovered were Gram-positive and belonged to the high-G+C-content genus *Arthrobacter* and low-G+C-content genus *Bacillus* as well (Martinez *et al*., 2006). The authors could demonstrate that the *Arthrobacter* strains exhibited the greatest tolerance to low pH and U toxicity. The type strain *A. nicotianae* could remove more than 80% of the uranyl ions from an aqueous solution at pH 4.0, which was suggested to be due to physico-chemical binding to the cell components (Tsuruta, 2002). Recently, it was demonstrated that *Arthrobacter* sp. S3 closely related to *Arthrobacter ilicis* is able to accumulate uranium intracellularly (Suzuki & Banfield, 2004). The ubiquity of *Arthrobacter* spp. in contaminated soils stimulated our research interest.

Two isolates JG37-Iso2 and JG37-Iso3 were recovered from the uranium mining waste pile Haberland, which 16S rRNA gene sequences affiliated with *Actinobacteria* from the genus *Arthrobacter* (Chapter 5). Interestingly, an increased number of 16S rRNA gene sequences closely related to the 16S rRNA gene sequences of these two *Arthrobacter* strains were identified in the samples treated with uranyl or sodium nitrate (Chapters 1, 2, 5). In this thesis, a combination of wet chemistry, spectroscopic, microscopic and microbiological methods was used to elucidate the tolerance of the two bacterial strains to lead and uranium. The two strains tolerate different amounts of lead. Lead sorption studies demonstrated that *Arthrobacter* sp. JG37-Iso3 accumulates up to 110 mg Pb g^{-1} dry biomass at pH 4.5, whereas *Arthrobacter* sp. JG37-Iso2 accumulates lower amounts of this heavy metal (up to 76 mg Pb g^{-1} dry biomass). The time course of Pb sorption by non-growing (resting) cells of the two *Arthrobacter* strains at pH 4.5 was studied as well. It was demonstrated that in the first two hours, only a small portion of the Pb was accumulated and then continuously more Pb was removed from the solution. This is an indication that more than one process is involved in the removal of lead from the solution by the non-growing cells. X-ray diffraction (XRD) analysis revealed that a lead phosphate phase pyromorphite ($Pb_5(PO_4)_3Cl$) was precipitated. Transmission electron micrographs (TEM) demonstrated that the precipitates of pyromorphite were localised mainly at the cell surface. These results are in congruence with the results of Templeton *et al*. (2003), who demonstrated by Extended X-ray Absorption Fine

Structure (EXAFS) spectroscopy and TEM observations that the enhanced Pb accumulation is due to the formation of nanoscale crystals of pyromorphite ($Pb_5(PO_4)_3OH$) adjacent to the outer-membrane of a fraction of the total population of *Burkholderia cepacia*. Life/Dead images from our lead-treated *Arthrobacter* cells demonstrated that after incubation for 48 h at pH 4.5 more than 50% of the cells are still alive.

Changes in the colour of the colonies from milky-white to brownish black were observed on low phosphate agar containing lead. X-ray diffraction analysis revealed that lead sulphide (PbS, galena) was formed by these isolates in this case, in contrast to the above mentioned pyromorphite formation by non-growing cells. At this stage of investigation, the origin of sulphide implicated in the precipitation of Pb is unknown.

In Chapter 5 the interactions of the two *Arthrobacter* strains JG37-Iso2 and JG37-Iso3 with uranium were studied as well. The time course of uranium sorption for both strains was similar to the sorption kinetics of lead, which was relatively slow. It was demonstrated that *Arthrobacter* sp. JG37-Iso3 accumulates higher amounts of uranium (up to 162 ± 6 mg U g^{-1} dry biomass) in contrast to *Arthrobacter* sp. JG37-Iso2 (up to 108 mg U g^{-1} dry biomass). TEM demonstrated that both strains studied are able to accumulate uranium intracellularly. These results are in line with the results of Suzuki and Banfield (2004), who demonstrated that *Arthrobacter* sp. S3, related to *Arthrobacter ilicis*, is able to accumulate uranium intracellularly in precipitates closely associated with polyphosphate granules. X-ray absorption spectroscopy (XAS) studies showed that U(VI) formed complexes with organically bound phosphates of the cells of the both *Arthrobacter* strains in a monodentate binding mode with an average bond distance between U and P of 3.60 ± 0.02 Å. The structural parameters of these uranium complexes are similar to those of the uranium complexes formed by fructose-6-phosphate (Koban *et al*., 2004). The results of the minimal inhibitory concentration for the growth of the isolates for nickel, chromium, copper, cadmium, silver, lead and uranium determined on low phosphate solid medium also revealed high heavy metal tolerance.

The results presented in Chapter 5 demonstrated that the growing cells of the strains precipitated Pb as galena (PbS), whereas in non-growing conditions pyromorphite ($Pb_5(PO_4)_3Cl$) phase was produced, alleviating probably the toxicity of Pb. Uranium was accumulated intracellularly by both strains as uranyl organic phosphate complexes. Our results indicated that these strains are interesting for the remediation of radioactive and mixed-wastes sites.

In summary, the results of this thesis demonstrate that the complex and diverse microbial community in the soil sample of the uranium mining waste pile Haberland was influenced by the addition of uranyl or sodium nitrate. Some of the bacteria stimulated by the treatments possess a potential to influence the migration of uranium.

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Chapter 1

Addition of U(VI) to a uranium mining waste sample (under aerobic conditions) and resulting changes in the indigenous bacterial community

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ABSTRACT

Based on 16S rRNA gene sequence retrieval, changes in natural bacterial community structure induced by addition of uranyl or sodium nitrate to soil samples from a uranium mining waste pile were investigated. Our results demonstrate that both treatments cause drastic changes in bacterial composition of the studied samples, resulting in strongly reducing the originally predominant *Acidobacteria* and *Alphaproteobacteria*. The addition of sodium nitrate induced a strong propagation of particular denitrifying and nitrate-reducing populations belonging to *Actinobacteria* and *Bacteroidetes*. The treatment of the samples with uranyl nitrate demonstrated that most part of the mentioned *Bacteroidetes* and some of the actinobacterial populations do not tolerate high U(VI) concentrations. Instead a strong propagation of *Pseudomonas* spp. from the *Gammaproteobacteria* occurred. At the initial stages of incubation (4 weeks after the addition of uranyl nitrate) U(VI)-reducing *Geobacter* spp. appeared. However, at the later stages of incubation (14 weeks after the beginning of supplementation) no *Geobacter* populations were detected anymore. Interestingly, different U-sensitive *Bacteroidetes* and alphaproteobacterial populations propagated in the U(VI)-treated samples at these late stages of incubation. That indicated that the added U(VI) was no longer bioavailable.

The drastic changes in bacterial community structure of the soil samples from the depleted uranium mining waste caused by the addition of uranyl nitrate indicate that most of the established indigenous bacterial populations do not tolerate U(VI). By the treatment with uranyl nitrate they are replaced by particular uranium resistant nitrate-reducing and denitrifying populations which potentially interact with the added radionuclide. On the other hand, the large number of dead uranium-sensitive bacteria likely liberates phosphate-rich and other biopolymers capable of binding U(VI). On the basis of our results, we propose that bacteria along with the abiotic soil components such as minerals and humic acids may influence the behaviour of U(VI) in nature.

INTRODUCTION

The migration of uranium from uranium-mining and -processing operations as well as from the deep subsurface repositories of nuclear fuel and other radioactive wastes is of serious environmental concern. For this reason the biogeochemical composition of these extreme environments has been extensively studied during the last decade and it was demonstrated that they are occupied by a large variety of bacteria (Pedersen, 1997; Ehrlich, 1998; Francis, 1998; Selenska-Pobell, 2002; Fredrickson *et al*., 2004; Nazina *et al*., 2004; Nedelkova *et al*.,

2005; Selenska-Pobell, 2006). Due to their capability to tolerate radioactivity (Chicote *et al*., 2005), to bioaccumulate uranium (Selenska-Pobell *et al*., 1999; Merroun *et al*., 2003a,b, 2005; Francis *et al*., 2004; Suzuki & Banfield, 2004; Ohnuki *et al*., 2005), and to biotransform it (DiSpirito & Tuovinen, 1982; Lovley *et al*., 1991) bacteria are considered at present, along with minerals, as one of the most important factors influencing the transport of uranium in contaminated environments. Moreover, it was demonstrated that bacteria can be used for bioremediation of uranium-contaminated sites by *in situ* biostimulation (Holmes *et al*., 2002; Anderson *et al*., 2003; Nevin *et al*., 2003; Suzuki *et al*., 2003, 2005; Istok *et al*., 2004; North *et al*., 2004) or for construction of biologically coated ceramic filters for cleaning of waters polluted with uranium (Raff *et al*., 2003).

In their natural environments bacteria interact effectively with minerals (Hersman *et al*., 2001; Kim *et al*., 2004) and are even involved in the formation of some kinds of them (Lovley *et al*., 1991; González-Munoz *et al*., 1996, 2003). Most part of the natural bacterial populations is bound to the surfaces of the surrounding minerals which also possess U(VI)-binding ligands (Reich *et al*., 1996; 1998). These results require study into the behaviour of uranium in mixtures of minerals and bacteria. Studies of this kind were recently performed by adding U(VI) to bacterial cultures of *Pseudomonas fluorescens* mixed with goethite (Bencheikh-Latmani *et al*., 2003) and to cultures of *Bacillus subtilis* mixed with kaolinite clay (Ohnuki *et al*., 2005). The authors of the first study described an intriguing process of redistribution of U(VI) from goethite to the cells of *P. fluorescens* driven by a preferential uranyl sorption to the bacterial surfaces. They also described an additional precipitation of autunite due to the release of orthophosphate by the bacterial cells lysed due to starvation in the oligotrophic conditions of the experiment.

Concurring are the results of Ohnuki *et al*. (2005) who demonstrated that at acidic conditions, corresponding to those of the depleted uranium mining waste piles, U(VI) was preferentially bound by the cells of *B. subtilis* and not by kaolinite particles. Bearing in mind that the bacterial isolates recovered from uranium polluted-environments are already adapted to harsh oligotrophic conditions and that they are very effective in their interactions with uranium (Anderson & Lovley, 2002; Lovley, 2002; Raff *et al*., 2004; Suzuki & Banfield, 2004; Merroun *et al*., 2005), it is possible to expect that such isolates might possess even stronger U(VI)-binding capability in presence of minerals then the two strains mentioned above which originate from other environments.

In nature, however, bacteria are living in complex and synergetic communities, consisting of different species possessing very diverse physiological and biochemical properties (Minz *et*

al., 1999; Schäfer *et al*., 2001; Fields *et al*., 2005). These communities are able to adapt to and to survive in a large variety of unfavourable and sharply changing conditions which can be lethal for the members of one individual species (Pace, 1997; Hugenholtz *et al*., 1998a, b; Minz *et al*., 1999; Elias *et al*., 2003; Chicote *et al*., 2005). For this reason it is of great importance to study the behaviour of uranium directly in the complex natural systems such as uranium mining wastes, for instance. The latter consist of minerals, indigenous bacterial communities and their metabolites as well as of other organic compounds such as humic acids, for instance, which can also serve as electron donors for anaerobic respiration of some bacterial species (Coates *et al*., 2002). It is also important to consider that the size of the U-sensitive populations of the natural community will be strongly reduced at a sharp increase of the uranium concentration and that the lysed dead cells will liberate biological components possessing ligands with very strong affinity to uranium especially at acidic conditions, such as phosphate groups, for instance (Bencheikh-Latmani *et al*., 2003; Merroun *et al*., 2003a, b, 2005; Raff *et al*., 2004). Moreover, bacteria react to nutrient limitation, heavy metals, and other kinds of stress by accumulating highly phosphorylated proteins (Rosen *et al*., 2004). Most bacteria react to environmental stress by forming protective biofilms on the mineral surfaces (Allan *et al*., 2002; Chicote *et al*., 2005), and some of them are even able to release inorganic phosphate groups (Macaskie *et al*., 1992, 2000; Allan *et al*., 2002) or siderophores (John *et al*., 2001; Kalinowski *et al*., 2004; Ruggiero *et al*., 2005) interacting directly with uranium and other heavy metals and actinides.

The aim of this work was to study the influence of U(VI) on a natural bacterial community in a soil sample collected from a uranium mining waste pile in dependence on the time of incubation and the aeration conditions. To our knowledge this is the first effort to monitor bacterial community changes induced by addition of U(VI) directly to environmental samples and consequent incubation at conditions corresponding to their natural oligotrophic nutrient status.

MATERIALS AND METHODS

Site and samples description

The uranium mining waste pile Haberland is located north of the town of Johanngeorgenstadt, near the Schwarzenbach River in the south-western part of the province Saxony, Germany. In this area uranium was mined from 1946 until the beginning of the 1990s. The soil sample JG35-2 was collected from this pile under sterile conditions from a depth of 2 m by drilling in July 1997 and it was stored at -20 °C for further analysis. The sample had a pH of 4.5 and a

natural uranium content of 26 mg U kg^{-1}. The elemental composition of the soil sample was analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, ELAN 5000, Perkin Elmer, Shelton, Connecticut, USA) and is presented in Table 1.1.

Table 1.1. Element composition in mg kg^{-1} of the untreated samples JG35-2 (2 m depth) and JG36 (3 m depth, Satchanska *et al*., 2004)

Elements	**JG35-2**	**JG36**
	Conc. [mg kg^{-1}]	
	2 m depth	3 m depth
Al	18300	20400
Ca	968	918
Cr	63.7	71.3
Fe	56400	43500
Mn	863	2140
Co	28.9	130
Ni	41.8	110
Cu	140	249
Zn	427	1140
As	471	380
Sr	8.85	10.1
Ag	0.194	0.180
Cd	4.11	9.40
Sn	45.0	32.0
Hg	3.09	4.58
Pb	83.4	111
Th	1.97	2.09
U	26.3	108

The sample labelled JG35+U1 was prepared by adding 3.75 ml of a filter-sterilised 1 mM uranyl nitrate solution dissolved in ultrapure water (Seralpur Delta UV/UF, Seral, Ransbach-Baumbach, Germany) adjusted to pH 4.0 with NaOH in one step to 15 g of the JG35-2 sample to achieve a final concentration of 100 mg U kg^{-1}. The sample was then incubated in a sterile 50 ml centrifugation tube (Greiner, Frickenhausen, Germany) for 4 weeks without shaking at 10 °C in the dark. In parallel, under the same conditions 3.5 g of the sample were incubated with 0.9 ml of a filter-sterilised 2 mM sodium nitrate solution dissolved in ultrapure water adjusted to pH 4.0 (sample JG35-K1) in order to investigate the influence of nitrate on the bacterial community structure. In a further sample, JG35+U2, the amount of uranium was increased up to 300 mg kg^{-1}. In order to achieve a uranium concentration of 300 mg U kg^{-1} with an optimal distribution of the metal in the soil material, the uranyl nitrate solution was added in several steps. For this aim, the supernatant was six times taken and replaced by a new solution of uranyl nitrate. After incubation for 14 weeks without shaking in the dark at 10 °C, the sample JG35+U2 was split in two parts. One part of the sample designated further as JG35+U2A was analysed immediately as described below. In order to stimulate an establishment of anaerobic conditions the second part of the sample JG35+U2, called

JG35+U2B, was fumigated through a sterile filter with an anaerobic gas mixture containing N_2 and CO_2 (80:20). This soil sample was incubated additionally for 14 weeks under anaerobic conditions in an anaerobic jar with Anaerocult®-C (Merck, Darmstadt, Germany) at 10 °C in the dark.

DNA extraction

Total DNA was recovered from 3 g of the soil per sample by direct lysis according to Selenska-Pobell (1995), by which only high molecular DNA (>25 kb) from intact bacterial cells was recovered. The supernatant, after centrifugation of the samples, was finally purified by using Nucleobond® cartridges AXG-100 (Marcherey-Nagel, Düren, Germany) according to Selenska-Pobell *et al*. (2001). The resulting DNA pellet was dissolved in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8).

PCR and cloning

16S rRNA gene fragments were amplified in five parallel replicates for each sample by polymerase chain reaction (PCR) in a reaction mixture of 10 µl, containing 0.9 µl of DNA template, 2.5 mM of $MgCl_2$ (Applied Biosystems, Foster City, California, USA), 125 µM of each of the four deoxynucleoside triphosphates, 2 pmol each of the forward and reverse primers and 0.5 U Ampli*Taq* Gold® Polymerase with the corresponding GeneAmp10x PCR buffer II (Applied Biosystems, Foster City, California, USA). The forward primer was the degenerated primer $16S_{deg43F}$ (5'-HRKGCBTWABRCATGCAAGTC-3'), and the reverse primer was the bacteria-specific primer $16S_{1404R}$ (5'-GGGCGGWGTGT ACAAGGC-3') (Marchesi *et al*., 1998). The PCR amplifications were performed in a T3 thermocycler from Biometra (Göttingen, Germany) with a 'touch down' PCR. After an initial denaturation at 94 °C for 7 min, the annealing temperature was lowered from 59 °C to 55 °C over five cycles and then another 25 cycles followed with 94 °C for 1 min, 55 °C for 40 s, and 72 °C for 1.5 min, and completed with an extension period of 20 min at 72 °C. The amplified 16S rRNA gene fragments from the five replicates were combined and were cloned directly in *Escherichia coli* using a TOPO-TA Cloning® system (Invitrogen, Gröningen, The Netherlands), following the manufacturers instructions. A total of 150 single white colonies per sample were randomly selected and further analysed.

RFLP screening and sequencing

For restriction fragment length polymorphism (RFLP) screening, the inserted 16S rRNA gene fragments were amplified by PCR directly from the host cells with vector specific M13(-40) and M13rev primers (each 4 pmol) by using *Taq* DNA Polymerase (Promega, Mannheim, Germany) as described above. The presence and the correct size of the amplification products

were visualised by electrophoresis in 1.2% agarose gels. The clones with correct inserts were stored as glycerol cultures at -80 °C. Aliquots of the amplified rRNA gene PCR products were digested in parallel with 1 unit of each of the four-base-specific restriction endonucleases *Msp*I and *Hae*III in the corresponding buffers (Promega, Mannheim, Germany) overnight at 37 °C. The digests were separated in 3.5% Small DNA Low Melt Agarose (Biozym, Hessisch Oldenburg, Germany) gels in a 0.5xTBE buffer and visualised by staining with ethidium bromide and UV illumination. The resulting RFLP patterns were visually compared and grouped in RFLP-types. Representative clones from each of the RFLP groups considered to represent predominant bacterial populations were selected for sequencing.

The 16S rRNA gene products of the selected clones were purified by using an Edge BioSystems QuickStep®2 PCR Purification Kit (MoBiTec, Göttingen, Germany) and directly sequenced by using an ABI Prism® Big Dye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA), following the manufacturers instructions. Partial sequences were obtained by using the primers $16S_{802R}$ (5'-TACCAGGGTATCTAATC-3') and $16S_{1404R}$, and then assembled by using the Autoassembler 2.0 software (PE Applied Biosystems, Foster City, California, USA). RNA gene sequences were determined on an automated sequencer (Model 310 ABI PRISM®, PE Applied Biosystems, Foster City, California).

Phylogenetic analysis

The 16S rRNA gene fragments, flanked position 64 and 1386, retrieved were compared with 16S rRNA gene sequences available in the GenBank and the EMBL (European Molecular Biology Laboratory) Nucleotide Sequence Database by using BLAST (Basic Local Alignment Search Tool) analysis (Altschul *et al*., 1997). Sequences with less than 90% of similarity to their closest phylogenetic relatives were checked by the program Check_Chimera of the RDP (Ribosomal Database Project) (Maidak *et al*., 2000). The sequences were aligned with those corresponding to the closest phylogenetic relatives by using CLUSTALW version 1.7 (Thompson *et al*., 1994). Phylogenetic trees were generated using the neighbour-joining algorithm with distance analyses by using Jukes-Cantor corrections in the PHYLIP v. 3.5 package (Felsenstein, 1993).

Nucleotide sequence accession numbers

The nucleotide sequences reported here were deposited to the EMBL Nucleotide Sequence Database under accession numbers AM072412 to AM072427, AM071370 to AM071381, AM114425 to AM114452 and AM116720 to AM116745.

RESULTS

Analysis of bacterial community changes induced by added uranyl and sodium nitrate

Most part of the bacteria present in uranium mining wastes belong to not yet cultured species due to the limited knowledge about their nutrient and other life necessities (Selenska-Pobell, 2002; Fields *et al*., 2005). For this reason bacterial communities of the studied uranium mining waste pile samples and the changes in their composition, which occurred after the addition of uranyl nitrate or sodium nitrate, were investigated by using a culture-independent molecular approach, namely 16S rRNA gene retrieval (Pace, 1997; Hugenholtz *et al*., 1998a, b; Reysenbach *et al*., 2000). 16S rRNA gene clone libraries were constructed for the untreated sample JG35-2, for the control sample JG35-K1 (which was incubated with sodium nitrate four weeks at aerobic conditions), and for the samples supplemented with uranyl nitrate JG35+U1, incubated with uranyl nitrate under aerobic conditions for 4 weeks, JG35+U2A, incubated with uranyl nitrate for 14 weeks also under aerobic conditions, and JG35+U2B, which was initially incubated as JG35+U2A and then replaced to strict anaerobic conditions for additional 14 weeks (Table 1.2 and Materials and Methods).

Table 1.2. Soil samples used.

Samples	Treatment	Incubation time	Aeration conditions	Uranium content [mg kg^{-1}]
JG35-2	Untreated	-	-	26 - 40
JG36	Untreated	-	-	108
JG35-K1	Sodium nitrate	4 weeks	Aerobic	26 - 40
JG35+U1	Uranyl nitrate	4 weeks	Aerobic	~100
JG35+U2A	Uranyl nitrate	14 weeks	Aerobic	~300
JG35+U2B	Uranyl nitrate	28 weeks	14 aerobic + 14 anaerobic	~300

The comparative analysis of the 16S rRNA gene sequences of the clones representing the predominant bacterial groups in the samples studied is presented in Fig. 1.1.

As evident from the results presented in the figure the bacterial community of the untreated sample JG35-2 was predominated by representatives of *Alphaproteobacteria* and of the phylum *Acidobacteria*. The addition of both sodium nitrate (JG35-K1) and uranyl nitrate (JG35+U1) induced dramatic changes in these two bacterial groups. The alphaproteobacterial populations were reduced in both cases but more strongly in the case of JG35+U1. In the latter no acidobacterial sequences were retrieved whereas in the JG35+K1 sample only a few sequences were affiliated with this phylum. The addition of sodium nitrate induced strong propagation of actinobacterial and *Bacteroidetes* populations (see sample JG35-K1 in Fig. 1.1).

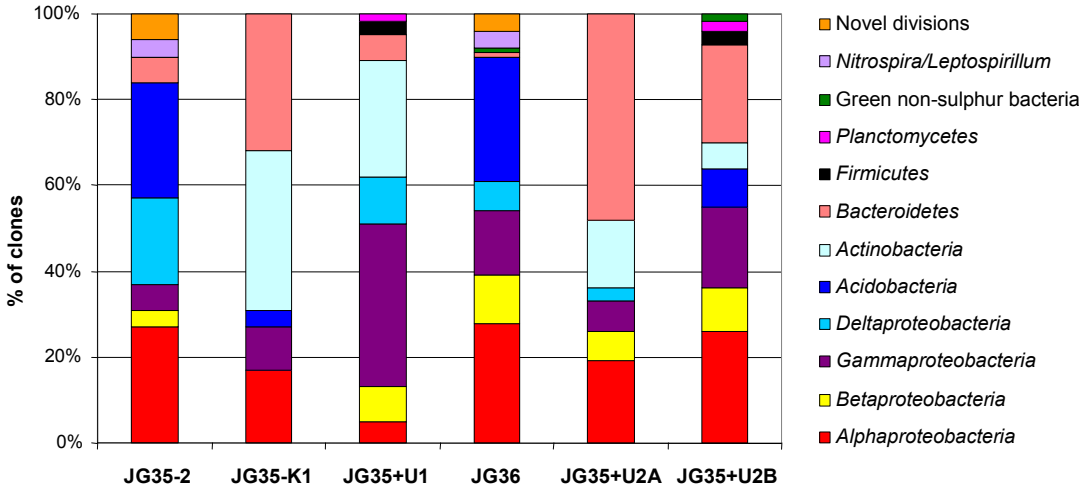

Fig. 1.1. Size of the predominant bacterial populations (given in percentages) in the untreated samples JG35-2 (2 m depth, containing 26 mg U kg^{-1}) and JG36 (3 m depth, containing 108 mg U kg^{-1}, Satchanska *et al*., 2004), the U(VI)-treated samples: JG35+U1 (uranium content increased by adding uranyl nitrate up to 100 mg U kg^{-1}, incubated under aerobic conditions for 4 weeks), JG35+U2A (uranium content of 300 mg U kg^{-1}, incubated for 14 weeks under aerobic conditions), JG35+U2B (uranium content of 300 mg U kg^{-1}, incubated for 14 weeks under aerobic conditions and then replaced to strict anaerobic conditions for additional 14 weeks), and the sodium nitrate treated sample JG35-K1, incubated under aerobic conditions for 4 weeks.

The actinobacterial sequences were affiliated with different *Arthrobacter* spp. (Fig. 1.2), which are potentially able to reduce nitrate (Crocker *et al*., 2000). Some of them were already found in low numbers in the soils of the uranium mill tailings Gittersee/Coschütz near the city of Dresden (sequence Gitt-KF-106 in the dendrogram) and in one of the previously studied samples of the JG waste pile (JG37-AG-83) (Selenska-Pobell *et al*., 2002; Geissler, 2003). Interestingly, from the latter sample two *Arthrobacter* isolates, *Arthrobacter* sp. JG37-Iso2 and *Arthrobacter* sp. JG37-Iso3 (Fig. 1.2) possessing high heavy metal tolerance and accumulating intracellularly U, were cultured (see Chapter 5). As seen in Fig. 1.2, closely related or even the same *Arthrobacter* spp. were identified in the three U-amended samples JG35+U1, JG35+U2A, and JG35+U2B, however in progressively decreasing numbers (see also Fig. 1.1).

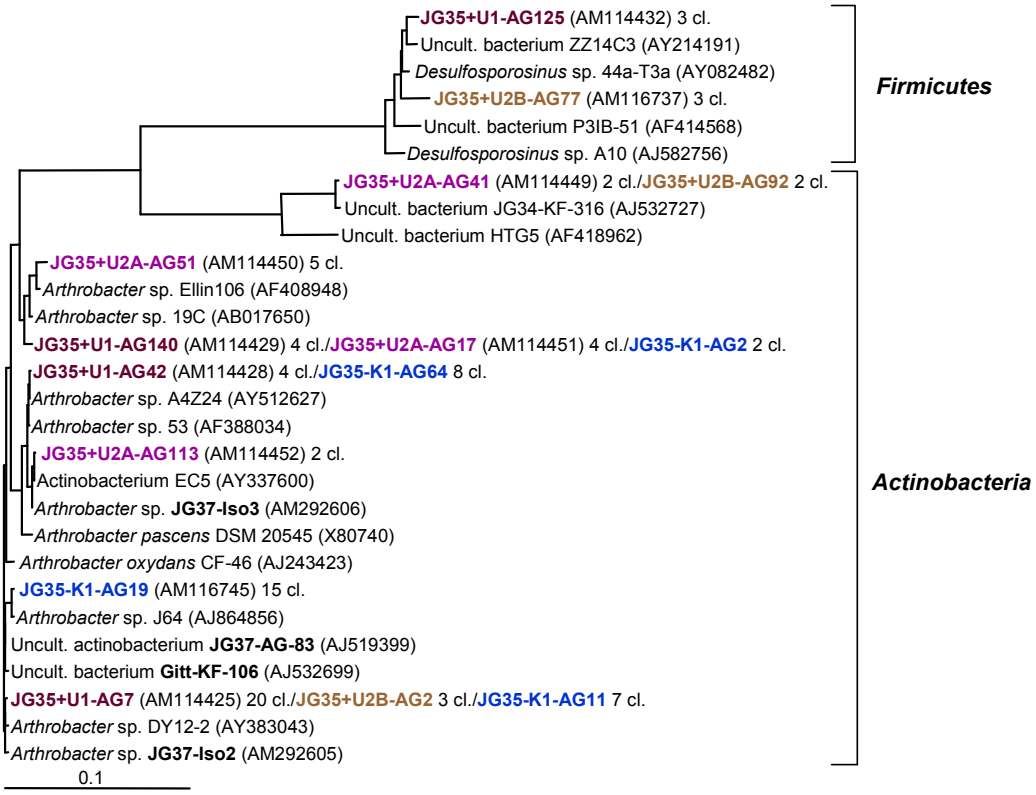

Fig. 1.2. Phylogenetic tree of the 16S rRNA gene sequences affiliated with *Actinobacteria* and *Firmicutes* retrieved from the samples **JG35+U1**, **JG35-K1**, **JG35+U2A** and **JG35+U2B**.

The behaviour of the *Bacteroidetes* populations, which were strongly induced by the addition of sodium nitrate, was very different (compare column JG35-K1 to JG35+U1, JG35+U2A and JG35+U2B in Fig. 1.1). The number of these bacteria was strongly reduced in the sample JG35+U1, which was incubated in the same way as JG35-K1 (at 10 °C in the dark under aerobic conditions, for 4 weeks after the amendment with uranyl nitrate). This indicates that representatives of this bacterial phylum do not tolerate high concentrations of U. However, after a longer incubation of 14 weeks at the same conditions even with higher amounts of uranium (Table 1.2) a strong propagation of *Bacteroidetes* was found (see JG35+U2A in Fig. 1.1). The latter is an indication that at this late stage of the treatment, uranium was possibly no longer bioavailable due to removal from the solution. In the parallel sample (JG35+U2B), which was additionally incubated for 14 weeks under strict anaerobic conditions, the populations of *Bacteroidetes* were also predominant but to a lesser extent. In this case stimulation of mostly *Alpha*-, *Gamma*- and *Betaproteobacteria* populations as well as of *Acidobacteria*, *Firmicutes, Planctomycetes* and green non-sulphur bacteria was observed.

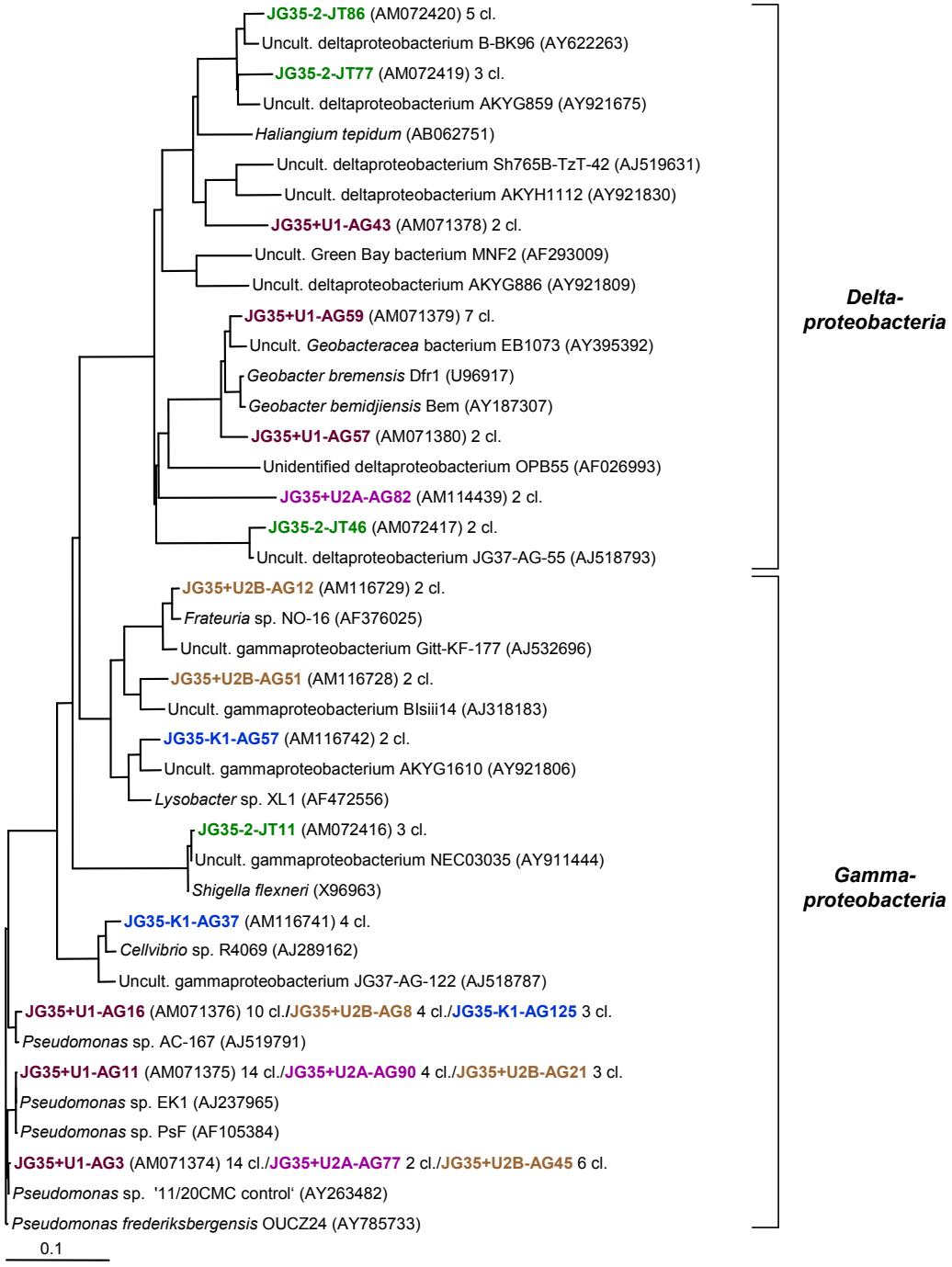

Fig. 1.3. Phylogenetic tree of the 16S rRNA gene sequences affiliated with *Gamma*- and *Deltaproteobacteria* retrieved from the samples **JG35+U1**, **JG35-K1**, **JG35+U2A** and **JG35+U2B**.

As shown in Fig. 1.1 the addition of U(VI) to the sample JG35+U1 induced during the first 4 weeks of incubation under aerobic conditions a strong proliferation of *Gammaproteobacteria*. The phylogenetic affiliation of the 16S rRNA gene sequences revealed that they represented mostly members of *Pseudomonas* spp. from *Gammaproteobacteria* (Fig. 1.3). As evident from the phylogenetic tree presented in Fig. 1.3,

16S rRNA gene sequences representing the same *Pseudomonas* spp.- but in lower numbers - were also retrieved from the samples JG35+U2A and JG35+U2B, which were monitored for longer periods of time (14 and 28 weeks, respectively) after the addition of U(VI). Interestingly, in sample JG35+U2B, which was kept during the last 14 weeks of its treatment under strict anaerobic conditions (as mentioned above), 16S rRNA gene sequences were affiliated with *Frateuria* sp. NO-16 isolate recovered from acidic drainage waters of a subarctic copper mine (Johnson *et al*., 2001).

The representatives of *Deltaproteobacteria*, which were completely suppressed by the addition of sodium nitrate, were still present in the sample treated with uranyl nitrate (compare columns JG35-2, JG35-K1, and JG35+U1 in Fig. 1.1). Moreover, in the sample JG35+U1 a significant shifting to populations of *Geobacter* spp. occurred (Fig. 1.3). This result is surprising bearing in mind the aerobic conditions of maintenance of the sample JG35+U1. Two 16S rRNA gene sequences (JG35+U1-AG125 and JG35+U2B-AG77), representing small groups of three clones, were retrieved from the samples JG35+U1 and JG35+U2B (Fig. 1.2). These sequences were affiliated with *Desulfosporosinus* spp., Gram-positive sulphate-reducing bacteria belonging to *Firmicutes*, which possess a potential for enzymatic reduction of U(VI) (Suzuki *et al*., 2004).

DISCUSSION

The main goal of the present work was to study the interactions of U(VI) with the members of the natural soil bacterial community of the depleted uranium mining waste pile Haberland near the town of Johanngeorgenstadt in Germany. For this purpose, bacterial community structures of one original and of several with uranyl nitrate amended parts of a sample collected from a depth of 2 m from the waste were studied and compared.

We demonstrated that the bacterial community of the untreated sample JG35-2 was predominated by representatives of *Alphaproteobacteria* and of the phylum *Acidobacteria*. This finding is concurrent with our previous studies on soil samples collected from the same site but from different depths, which were polluted with uranium and other metals in a various extent (Selenska-Pobell *et al*., 2002; Geissler, 2003; Satchanska *et al*., 2004; Selenska-Pobell, 2006; compare also JG35-2 to JG36 in Fig. 1.1 and in Table 1.1).

The aerobic incubation of the samples for 4 weeks with uranyl nitrate (JG35+U1) as well as with sodium nitrate (JG35-K1) resulted in strong structural changes in the indigenous bacterial community of the sample JG35-2. The alphaproteobacterial populations were strongly reduced, especially in the case of JG35+U1. In this sample no acidobacterial

sequences were retrieved whereas in the sample JG35-K1 only a few sequences of this phylum were found. Due to the fact that only high molecular DNA, extracted from intact bacterial cells according to Selenska-Pobell (1995) and Selenska-Pobell *et al*. (2001), was subjected to amplifications we conclude that the failure to detect acidobacterial sequences in the sample JG35+U1 indicates that a large part of these bacteria was killed by the treatment with uranium and that their number went below the limit of detection of the used method. We suggest that these dead bacterial populations release to the environment significant quantities of biopolymers such as siderophores, polypeptides, polysaccharides, phosphorylated peptides, alginates, nucleic acids, orthophosphate, amino acids etc. capable to bind uranium (Macaskie *et al*., 2000; Kalinowski *et al*., 2004; Rosen *et al*., 2004).

The strong propagation of Gamma-*Pseudomonas* spp. in the sample JG35+U1 is in agreement with the finding that many representatives of this proteobacterial subdivision can tolerate uranium and possess an ability to accumulate (Merroun *et al*., 2002; Francis *et al*., 2004) and/or to reduce U(VI) (McLean & Beveridge, 2001). Gamma-*Pseudomonas* are the most ubiquitous bacteria found in a large variety of extreme habitats (Yanagibayashi *et al*., 1999; McLean & Beveridge, 2001; Teitzel & Parsek, 2003). An enrichment of Gamma-*Pseudomonas* was also revealed in highly saline uranium-contaminated sediments after stimulation of U(VI) removal from groundwater by addition of acetate (Nevin *et al*., 2003). The extremely high survival capability of these bacteria is connected to their wide spectrum of metabolic features and also to their ability to form biofilms (Teitzel & Parsek, 2003; Harrison *et al*., 2004). Because biofilm formation is the key survival strategy for natural bacterial communities, the members of the *Pseudomonas* family are supposed to play a protective role for bacterial communities in many extreme environments (Characklis & Marshall, 1990; Fields *et al*., 2005). Because the sample JG35+U1 was supplemented with U(VI) in the form of uranyl nitrate and was afterwards incubated under aerobic conditions without agitation we suggest that most of the induced Gamma-*Pseudomonas* spp. in the sample were not only resistant to uranium but they were also able to reduce nitrate. The latter is in agreement with the finding that the denitrifying *Pseudomonas* spp. are characteristic for aerobic and microaerophilic conditions (Tiedje, 1998).

However, in the sample JG35-K1, which was treated with sodium nitrate under the same conditions as the sample JG35+U1, not Gamma-*Pseudomonas* spp. but *Arthrobacter* spp. and representatives of *Bacteroidetes* were predominant. The latter indicates that in the absence of uranium these two groups of bacteria are more competitive nitrate reducers then the *Pseudomonas* spp. In the above mentioned uranium supplemented sample JG35+U1 the

number of the retrieved *Arthrobacter* spp. 16S rRNA gene sequences was not so high as in the JG35-K1 sample but they were the second major bacterial group found whereas the *Bacteroidetes* represented only a minor group, obviously due to their sensitivity to U(VI). Interestingly, in the samples JG35+U2A and JG35+U2B, which were incubated for much longer periods of time (14 and 28 weeks, respectively) with higher amounts of uranium, related or even the same *Arthrobacter* spp. were identified as in the JG35+U1 sample, however, in progressively decreasing numbers. Because representatives of *Arthrobacter* were found in many heavy-metal or radionuclide-contaminated environments (Crocker *et al*., 2000; Fredrickson *et al*., 2004; Hanbo *et al*., 2004) and they can tolerate metals, we suggest that the declining of their populations in the mentioned two samples is possibly connected to the decrease of the nitrate concentration with increasing time of the incubation. In addition, it seems that at the discussed late stages of incubation the added U(VI) was also no longer bioavailable because a strong propagation of the uranium-sensitive representatives of the *Bacteroidetes* phylum occurred. Moreover, it was noticed that after the longer incubation time in the case of the sample JG35+U2B the bacterial community has a tendency to establish the initial structure of the natural sample JG35-2. In this respect, it was interesting to compare the community of the sample JG36, which contains originally 108 mg U kg^{-1}, to those of the sample JG35+U1, containing also about 100 mg U kg^{-1} after the supplementation with uranyl nitrate, and to those of the original JG35-2 sample (Fig. 1.1). One can see that the JG36 community looks much more similar to those of the less contaminated original sample JG35-2. This indicates that the changes in the community structure occur when the samples are freshly supplemented with U(VI). The community structure is, however, not dependent on the concentration of uranium if the latter is no longer bioavailable. It seems that other factors such as the availability of nutrients, oxygen etc. are more important for the community structure.

Surprising was the propagation of the obligate anaerobic *Geobacter* spp. in the sample JG35+U1 kept under aerobic conditions. We suppose that the incubation of the sample without agitation, certainly led to the formation of increasingly anaerobic subsurface conditions where the O_2-sensitive *Geobacter* spp. could survive. Members of the family *Geobacteracea* are known for their ability to reduce Fe(III) and U(VI) (Lovley *et al*., 1991, 1993) in presence of electron donors such as lactate and acetate. The conditions of our experiment (low pH and very limited nutrient sources) almost excluded that a large number of representatives of these species will survive for longer time. In several cases *Geobacter* populations were stimulated in contaminated sediments by addition of acetate for U(VI)

reduction (Holmes *et al*., 2002, North *et al*., 2004). In our case, however, possibly only in the first weeks after the addition of U(VI) the dead cells of the U-sensitive populations could provide some nutrients and electron donors to support to some extend the growth of the uranium-reducing bacteria, but this was not significant. The oligotrophic nutrient conditions were obviously the reason that no *Geobacter* spp. were retrieved (due to their low numbers) from the sample JG35+U2B incubated for 28 weeks of which 14 were under anaerobic conditions. The identification of the Gram-positive sulphate-reducing *Desulfosporosinus* spp., able to reduce U(VI) (Suzuki *et al*., 2004) in both JG35+U1 and JG35+U2B samples is an additional indication that these samples possess biological potential for U(VI) reduction. Interestingly, an enrichment with similar *Desulfosporosinus* spp. was revealed in a highly saline sediment at the uranium mill tailings at Shiprock, Mew Mexico, USA after stimulation of U(VI) removal from the groundwater by addition of acetate (Nevin *et al*., 2003).

Described dramatic changes in bacterial community induced by the addition of uranium raise the question how to connect them to the fate of uranium in the studied environment.

It is well known that about 60 - 70% of bacteria in soil environments are tightly bound to soil particles/minerals in a form of synergetic consortia or biofilms (Selenska-Pobell, 1995; Battin *et al*., 2001; Allan *et al*., 2002). In the biofilm matrix different large and small molecules are continuously liberated from the living as well as from the dead cells. The living cells are 'communicating' by exchanging signal molecules between each other, and the cells of the dead bacteria serve as nutrient source for the rest of the community. Bacterial cell surfaces as well as the released biomolecules are good candidates for complexation of U(VI). In addition, the bound U often serves as starting point for further biomineralisation (Macaskie *et al*., 1992; Ehrlich, 1998; Macaskie *et al*., 2000; Allan *et al*., 2002).

Interestingly, Bostick *et al*. (2002) performed EXAFS analyses of uranyl complexes formed in samples collected from several uranium waste disposals of the Department of Energy (DOE), USA. Some of their samples were collected from a depth of 1.5 m, pH 4.7 from acidic clay-rich geologic structure, i.e. they were very similar to those which we have analysed. According to these authors, their samples were most probably mixed complexes which were predominated by uranyl phosphates. Due to the observed dramatic changes in the bacterial communities induced by the addition of uranium to our samples we suggest that many functional groups including phosphate were released by the stressed and also by the dead bacteria. Some of these groups along with the ligands of the abiotic mineral particles are possibly involved in the complexation of the added U(VI).

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Chapter 2

Influence of uranyl and sodium nitrate on natural bacterial community of a uranium mining waste pile under strict anaerobic conditions

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ABSTRACT

The response of the subsurface soil bacterial community of a uranium mining waste pile to incubations with uranyl or sodium nitrate for different periods of time under anaerobic conditions was studied by using the 16S rRNA gene retrieval. Four portions of a sample, collected from 2 m depth of the pile, were dealt with. The incubation of the first sub-sample with sodium nitrate for 4 weeks resulted in strong proliferation of *Firmicutes* (*Bacillus* spp. and *Clostridium* spp.) and also of *Betaproteobacteria*, which had overgrown the populations of *Acidobacteria*, *Alpha*- and *Deltaproteobacteria* that predominated the untreated sample. Analysis of the second sub-sample, treated with uranyl nitrate for 4 weeks, revealed that only small parts of the nitrate-inducible *Firmicutes* and *Betaproteobacteria* are tolerant to the increased U(VI) concentration. Representatives of *Gammaproteobacteria*, especially *Rahnella* spp., were predominant in this sub-sample. The size of the initially predominant alphaproteobacterial populations was not reduced, although a shifting to other, possibly more U(VI) resistant species occurred. The *Acidobacteria* were reduced by only 50% and were shifted as well. The incubation of the third sub-sample with sodium nitrate for 14 weeks resulted in a strong propagation of *Betaproteobacteria* that was analogical to those which occurred in the first sub-sample. The size of the *Firmicutes* populations was, in contrast to the first sub-sample, strongly reduced. Instead, representatives of *Actinobacteria* and *Bacteroidetes* as well as *Pseudomonas* spp. from *Gammaproteobacteria* had propagated. In the fourth sub-sample, incubated with uranyl nitrate for 14 weeks, diverse alphaproteobacterial and also some U-sensitive betaproteobacterial and *Bacteroidetes* populations were established. The increased diversity, especially of U-sensitive populations, in the latter sample indicates that the added U(VI) was no longer bioavailable. Our results demonstrate that the natural bacterial community of the studied uranium mining waste pile possesses a high potential to adapt to changing nitrate and U(VI) concentrations under anaerobic conditions which correspond to their natural environment.

INTRODUCTION

The fate of uranium and other radionuclides and heavy metals in the depleted uranium mining and processing sites as well as in the nuclear fuel and municipal waste depositories and the prevention of its eventual release and long term contamination of the environment are of serious concern. Along with the well studied physicochemical processes (Means *et al*., 1978; Ticknor, 1994; Liger *et al*., 1999) many biotic factors affect the behaviour of radionuclides in nature (Ehrlich, 1998; Francis, 1998; Anderson & Lovley, 2002; Pedersen, 2005). It was

demonstrated, for instance, that the adsorption of aqueous U(VI) by Mn(IV) and Fe(III) oxides plays an important role in its immobilisation (Means *et al*., 1978; Arnold *et al*., 1998; Reich *et al*., 1998; Liger *et al*., 1999) and that part of these oxides can be produced by bacteria (Straub *et al.,*1996; Okazaki *et al.,* 1997; Francis & Tebo, 2002; Lack *et al*., 2002; Anderson *et al.,* 2006; Weber *et al.,* 2006a). Alternatively, the biotic and abiotic Fe(III) oxides can also be involved in abiotic redox interactions resulting in U(IV) solubilisation and production of U(VI) (Finneran *et al.,* 2002; Senko *et al*., 2005; Wan *et al*., 2005). Other biogenically produced, inorganic compounds, such as phosphate or hydrogen sulphide, can immobilise and induce biomineralisation of uranium by direct complexation of U(VI) to insoluble polycrystalline HUO_2PO_4 precipitates or to meta-autunite similar minerals (Macaskie *et al.*, 1992; Merroun *et al.*, 2006), as well as by chemical reduction to U(IV) as in the case of H_2S (Beyenal *et al*., 2004). On the other hand, many microorganisms release organic biopolymers to the environment, such as siderophores that can leach U(IV) and mobilise it from the contaminated solid wastes (Kalinowski *et al*., 2004).

Most impressive, however, is the ability of a large variety of microorganisms to interact directly with uranium and even to biotransform it. The following mechanisms of direct bacteria-uranium interactions are described: i) oxidation of U(IV) to U(VI) resulting in solubilisation (DiSpirito & Tuovinen, 1982; Beller, 2005); ii) reduction of U(VI) to insoluble U(IV) (Lovley *et al*., 1991, 1993; Lovley & Phillips, 1992; Francis *et al*., 1994; Tebo & Obraztzova, 1998; Lloyd, 2003; Khijniak *et al*., 2005; Wu *et al*., 2006); iii) bioaccumulation, which includes processes of biosorption by cell surface polymers (Friis & Myers-Keith, 1986; Selenska-Pobell *et al*., 1999; Fowle *et al.*, 2000; Francis *et al*., 2004; Raff *et al.*, 2004; Tsuruta, 2004; Merroun *et al*., 2005) and/or uptake inside the cells (Marqués *et al*., 1991; Merroun *et al.*, 2002; Francis *et al.*, 2004; Suzuki & Banfield, 2004). The U(VI) accumulated both on the bacterial surfaces or inside the cells as well as biologically reduced U(IV) precipitates can initiate biomineralisation processes, which result in immobilisation of additional amounts of uranium (Francis, 1998). On the other hand, transport and release of the bioaccumulated uranium by the migrating parts of the bacterial populations are also possible by decomposition processes after their dead (Francis, 1998).

The above described bacterial activities were studied in the laboratory and also observed in natural environments. Some of them were even exploited for uranium bio-leaching from ores in the production of uranium (Cerdá *et al*., 1993; Júnior, 1993). Many other studies focused on *in situ* bioremediative stimulation of U(VI)-reducing bacteria by addition of different organic electron donors such as acetate, lactate, glucose, and ethanol in the uranium mining

waste water and sediment samples (Holmes *et al*., 2002; Anderson *et al.*, 2003; Nevin *et al.,* 2003; Suzuki *et al.,* 2003; Istok *et al.,* 2004; North *et al.,* 2004). However, the fate of bioreduced and immobilised uranium remains unpredictable due to the very complex and rapidly changing biogeochemical factors in nature (Wan *et al*., 2005; Nyman *et al*., 2006). It was demonstrated that the bioreductive removal of U(VI) from contaminated groundwater aquifers can be followed by both biotic and abiotic reoxidation and resolubilisation (Anderson *et al.*, 2003; Wan *et al.*, 2005; Brodie *et al.,* 2006). The latter is not surprising, bearing in mind that the radioactively polluted mining soils and sediments often possess high amounts of biogenic and abiogenic Fe(III) oxides, which as above mentioned may oxidise uranium (Finneran *et al.,* 2002; Wan *et al.,* 2005). In addition, these environments are occupied by a large variety of very diverse and highly adaptive microorganisms, which are able to oxidise U(IV) in a direct (DiSpirito & Tuovinen, 1982; Berthelot *et al*., 1997; Beller, 2005) or in an indirect way by their inorganic metabolites (Ivanova *et al*., 2000; Finneran *et al.,* 2002; Fredrickson *et al.,* 2004). Moreover, it was demonstrated that natural bacterial communities of uranium contaminated wastes have various and site-specific structure, depending on their geographic origin and on the anthropologic history of the exploited sites (Selenska-Pobell, 2002; Radeva & Selenska-Pobell, 2005), which makes the prediction of the uranium behaviour even more difficult.

The above mentioned *in situ* stimulation experiments focused on activation of particular U(VI)-reducing bacterial groups by addition of organic substrates to water and sediment samples. However, changes in the concentration of the inorganic compounds, mostly uranium, nitrate, and sulphate are most frequent under the natural oligotrophic conditions of the uranium mining wastes (Finneran *et al*., 2002).

It was demonstrated that nitrate, a common co-contaminant of uranium, strongly influences natural bacterial community structure and activity (Finneran *et al*., 2002; Anderson *et al.*, 2003; Suzuki *et al.,* 2003; Istok *et al.,* 2004; Fields *et al.,* 2005). Nitrate is preferably used by microorganisms for anaerobic respiration because its reduction is thermodynamically more favourable than the reduction of U(VI), Fe(III) or sulphate that are also abundant in the waste milieu (Finneran *et al*., 2002; Cooper *et al.,* 2003; Suzuki *et al.,* 2003; Istok *et al.,* 2004). In addition, nitrate can serve as an electron acceptor in the biotic anaerobic oxidation of U(IV) to U(VI) (Beller, 2005). Additionally, it was also demonstrated that the intermediates of denitrification and of dissimilatory nitrate reduction to ammonium, namely nitrite, nitric oxide, and nitrous oxide are also capable to oxidise and mobilise U(IV) (Senko *et al.,* 2002).

Recently, we demonstrated that treatments with uranyl or sodium nitrate under aerobic conditions strongly influence natural bacterial community structure of soil samples collected from 2 m depth of the uranium mining waste pile Haberland, situated near the town of Johanngeorgenstadt in Germany (Chapter 1). The aim of this work was to study the influence of uranyl and sodium nitrate on the natural bacterial community of the same sample under strict anaerobic conditions which are identical to those of the natural habitat.

MATERIALS AND METHODS

Samples description

The soil samples studied in this work are listed in Table 2.1. The original sample JG35-2 was collected in July 1997 from a depth of 2 m from the uranium mining waste pile Haberland near the town of Johanngeorgenstadt in Germany as described previously (Chapter 1). The sub-sample JG35+U4 was prepared by adding 5.0 ml of a filter-sterilised 1 mM uranyl nitrate solution to 20 g of the original sample JG35-2 to achieve a final concentration of 100 mg U kg^{-1}. The uranyl nitrate was dissolved in ultra pure water (Seralpur Delta UV/UF, Seral, Ransbach-Baumbach, Germany) and pH 4.0 was adjusted with NaOH. The sample was fumigated through a sterile filter with an anaerobic gas mixture containing N_2 and CO_2 (80:20) and then incubated in a sterile 50 ml centrifugation tube (Greiner, Frickenhausen, Germany) for 4 weeks without shaking at 10 °C in the dark in an anaerobic jar with Anaerocult®-A (Merck, Darmstadt, Germany). In order to investigate the influence of the added nitrate on the bacterial community structure under anaerobic conditions, another 3.6 g of the original sample were incubated in the same way as JG35+U4 with 0.9 ml of a filter-sterilised 2 mM sodium nitrate solution dissolved in ultra pure water adjusted to pH 4.0 (sample JG35-K4, Table 2.1). In another sub-sample, JG35+U3, the amount of uranium was increased up to 300 mg U kg^{-1}. In order to achieve this high uranium concentration with an optimal metal distribution in the soil material, the uranyl nitrate solution was added in seven steps at one week intervals. For this aim, the supernatant was removed six times and replaced by a fresh solution of 1 mM uranyl nitrate. The sample was incubated for additional 7 weeks under the above described conditions. The influence of the higher nitrate concentration corresponding to those added to JG35+U3 on the natural bacterial community structure was studied in the sub-sample JG35-K2. For preparing the sample JG35-K2, altogether 4.4 ml of a 2 mM sodium nitrate solution were added in several steps to 3.5 g of the original sample JG35-2.

Table 2.1. Soil samples analysed.

Samples	Treatment	Incubation time	Uranium content [mg kg^{-1}]	Added nitrate [mg kg^{-1}]
JG35-2	Untreated	-	26 - 40	-
JG35-K4	Sodium nitrate	4 weeks	26 - 40	31
JG35+U4	Uranyl nitrate	4 weeks	~100	32
JG35-K2	Sodium nitrate	14 weeks	26 - 40	149
JG35+U3	Uranyl nitrate	14 weeks	~300	151

DNA extraction

Total DNA was recovered from 3.5 g of each of the sub-samples by direct lysis as described in Selenska-Pobell *et al*. (2001).

PCR and cloning

16S rRNA gene fragments were amplified in five parallel replicates for each sample as described in Chapter 1, by using the degenerated bacteria-specific forward primer $16S_{deg43F}$ (5'-HRKGCBTWABRCATGCAAGTC-3') and the reverse primer $16S_{1404R}$ (5'-GGGCGGW GTGTACAAGGC-3') (Marchesi *et al*., 1998). The PCR amplifications were performed in a T3 thermocycler from Biometra (Göttingen, Germany) with a "touch down" PCR. After an initial denaturation at 94 °C for 7 min, the annealing temperature was lowered from 59 °C to 55 °C over five cycles and then another 25 cycles followed with a profile of 94 °C for 1 min, 55 °C for 40 s, and 72 °C for 1.5 min. The amplification was completed with an extension step of 20 min at 72 °C. The amplified 16S rRNA gene fragments from the five replicates were combined and cloned directly in *Escherichia coli* using a TOPO-TA Cloning® system (Invitrogen, Gröningen, The Netherlands), following the manufacturers instructions. A total of 150 single white colonies per sample were randomly selected and further analysed.

RFLP screening and sequencing

For restriction fragment length polymorphism (RFLP) screening, the amplification of the inserted 16S rRNA gene fragments was performed according to Chapter 1. The resulting products were digested in parallel with 1 unit of each of the four-base-specific restriction endonucleases *Msp*I and *Hae*III in the corresponding buffers (Promega, Mannheim, Germany) overnight at 37 °C. The digests were separated in 3.5% Small DNA Low Melt Agarose (Biozym, Hessisch Oldenburg, Germany) gels in a 0.5xTBE buffer and visualised by staining with ethidium bromide and UV illumination. The resulting RFLP patterns were grouped in RFLP-types and one representative of every RFLP-type, representing two or more clones, was selected for sequencing.

The amplified 16S rRNA gene products of the selected clones were purified by using an Edge BioSystems QuickStep® 2 PCR Purification Kit (MoBiTec, Göttingen, Germany) and directly

sequenced by using an ABI Prism® Big Dye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA), following the manufacturers instructions. Partial sequences were obtained by using the primers $16S_{802R}$ (5'-TACCAGGGTATCTAATC-3') and $16S_{1404R}$ and then assembled by using the Autoassembler 2.0 software (PE Applied Biosystems, Foster City, California, USA). RNA gene sequences were determined on an automated sequencer (Model 310 ABI PRISM®, PE Applied Biosystems, Foster City, California, USA).

Phylogenetic analysis

The 16S rRNA gene fragments, flanked by positions 64 and 1386, were compared with the 16S rRNA gene sequences available in the public GeneBanks by using BLAST analysis (Basic Local Alignment Search Tool) (Altschul *et al*., 1997). Sequences with less than 90% of similarity to their closest phylogenetic relatives were checked for chimera formation by the program Check_Chimera of the RDP (Ribosomal Database Project) (Maidak *et al*., 2000). The sequences were aligned with those corresponding to the closest phylogenetic relatives by using CLUSTALW version 1.7 (Thompson *et al*., 1994). Phylogenetic trees were generated using the neighbour-joining algorithm with distance analyses by using Jukes-Cantor corrections in the PHYLIP v. 3.5 package (Felsenstein, 1993).

Nucleotide sequence accession numbers

The nucleotide sequences reported here were deposited to the EMBL Nucleotide Sequence Database under accession numbers AM072412 to AM072427, AM084881 to AM084897, AM116746 to AM116754, AM292605 to AM292627 and AM403298 to AM403326.

Mössbauer spectroscopy

0.5 g of the untreated sample, as well as the uranyl nitrate treated samples (JG35+U4 and JG35+U3) were dried at 60 °C for 2 h in a vacuum concentrator (Eppendorf AG, Hamburg, Germany) and then pulverised. Mössbauer spectra were measured at room temperature with a conventional constant acceleration spectrometer in transmission geometry with a ^{57}Co(Rh) source with an activity of nominally 3.7 GBq and a krypton filled proportional counter. The evaluation of the spectra was performed by using the NORMOS least square fitting program of Brand (1987).

RESULTS

Determination of the Fe(II)/Fe(III) ratio in the samples studied

The relative amount of Fe(III)- and Fe(II)-compounds in our samples, and in particular its change with uranyl nitrate treatment was determined via Mössbauer spectroscopy. The Mössbauer resonance absorption of the 14.4 keV γ-line of ^{57}Fe consists of a doublet both for Fe(III) as well as for Fe(II). However, the nuclear electric quadrupole splitting (about 0.8 mm s^{-1} for Fe(III), about 2.4 mm s^{-1} for Fe(II)) as well as their isomer shift (about 0.2 mm s^{-1} for Fe(III), about 1.2 mm s^{-1} for Fe(II)) are quite different for Fe in the two oxidation states. This usually allows a separation of the two doublets when they are superimposed in the Mössbauer absorption of a sample containing both Fe-species.

The Mössbauer absorption of the 14.4 keV γ-line, emitted from an α-Fe source, are shown for our 3 samples in Fig. 2.1. They represent an unresolved superposition of the two absorption doublets of the Fe(III)- and Fe(II)-compounds of our samples. We have fitted two doublets to these spectra with the quadrupole splitting Δ_i and the isomer shifts δ_i (relative to the α- Fe γ-source) as well as the ratio $A_{Fe(II)}/A_{Fe(III)}$ of the areas of the two doublets as fitting parametres. The area ratio $A_{Fe(II)}/A_{Fe(III)}$ is proportional to the relative amount of Fe(II)- to Fe(III)-compounds in our samples, amount Fe(II)/amount Fe(III). The results for these five parametres $\Delta_{Fe(II)}$, $\Delta_{Fe(III)}$, $\delta_{Fe(II)}$, $\delta_{Fe(III)}$, $\Delta_{Fe(II)}/\Delta_{Fe(III)}$ from the fits are given in Table 2.2. The results show that $\Delta_{Fe(II)}$, $\Delta_{Fe(III)}$, $\delta_{Fe(II)}$ and $\delta_{Fe(III)}$ are not changed by the uranyl nitrate treatment of our samples. This is the expected result because these parametres are determined by the oxidation state of Fe but not by the amount of the Fe species in the samples. The important result is the increase of the area $A_{Fe(II)}$ and the corresponding decrease of the area $A_{Fe(III)}$ with uranyl nitrate treatment. This clearly demonstrates that this treatment leads to an increase of the Fe(II)-compounds and corresponding decrease of the Fe(III)-compounds in our samples treated with uranyl nitrate.

Table 2.2. Hyperfine parameters and spectrum areas of the untreated sample JG35-2 and the samples treated with uranyl nitrate under anaerobic conditions for 4 weeks (JG35+U4) and for 14 weeks (JG35+U3).

Sample	Fe(II)			Fe(III)		
	δ mm s^{-1}	Δ mm s^{-1}	A %	δ mm s^{-1}	Δ mm s^{-1}	A %
JG35-2	1.211(6)	2.421(11)	56.1(1.5)	0.230(7)	0.867(14)	43.9(1.1)
JG35+U4	1.193(5)	2.473(9)	62.0(1.3)	0.236(8)	0.849(15)	48.0(9)
JG35+U3	1.145(3)	2.381(6)	70.3(1.2)	0.235(6)	0.850(12)	29.7(7)

δ isomer shift in reference to α-Fe, Δ quadrupole splitting, A spectrum area, value in brackets is the error of the last digit(s)

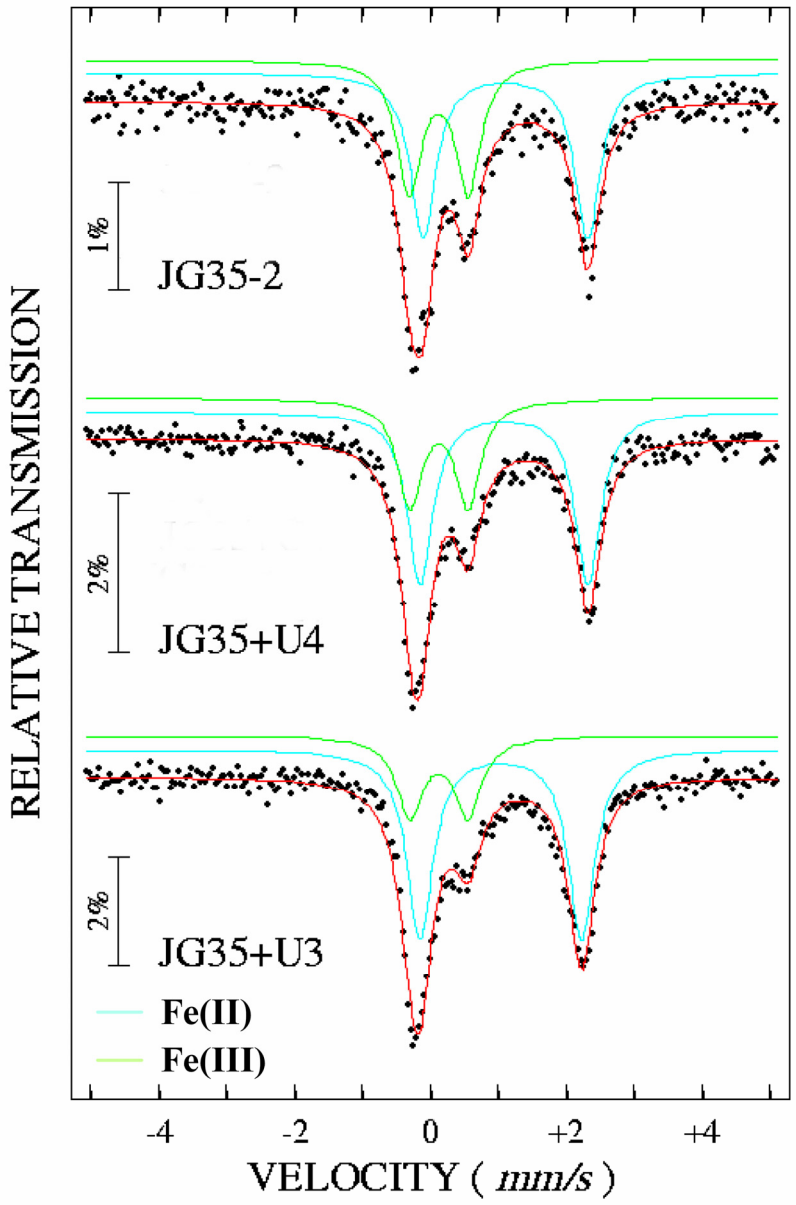

Fig. 2.1. Mössbauer spectra of the untreated sample JG35-2 and the samples treated with uranyl nitrate under anaerobic conditions for 4 weeks (JG35+U4) and for 14 weeks (JG35+U3).

Bacterial community structure

Bacterial community structure of the original untreated sample JG35-2 was compared to those established in the four sodium or uranyl nitrate supplemented sub-samples: JG35-K4, JG35+U4, JG35-K2, and JG35+U3 (Table 2.1, Materials and Methods). All treated samples were incubated under strict anaerobic conditions. The predominant 16S rRNA gene sequences retrieved from the samples, representing two or more clones, were involved in the analyses.

Changes in the JG35-2 bacterial community structure induced by sodium or uranyl nitrate treatments for 4 weeks

As evident from the results presented in Fig. 2.2, a strong propagation of representatives of *Firmicutes* and *Betaproteobacteria* was observed in the sub-sample JG35-K4, which was incubated with sodium nitrate under strict anaerobic conditions for 4 weeks. These two bacterial groups had overgrown the populations of *Acidobacteria, Alpha-* and *Deltaproteobacteria*, which were predominant in the untreated sample JG35-2 (compare also Tables S2.1 and S2.2).

65% of the clones analysed from sub-sample JG35-K4 were affiliated with *Firmicutes.* 37% of them were related to *Bacillus* spp. and 28% to *Clostridium* spp. (Table S2.2). The largest *Firmicutes* group, represented by the sequence JG35-K4-KF128, consisted of 35 clones (Fig. 2.3) and was affiliated with a 16S rRNA gene identity of 98.3% with the facultative anaerobic, nitrate-reducing grassland isolate *Bacillus bataviensis* LMG 21833 (Heyrman *et al*., 2004). A smaller group of 4 clones, represented by the sequence JG35-K4-KF16, was affiliated with a lower identity of 97.9% with *Bacillus drentensis* LMG 21831 (Heyrman *et al*., 2004). The smallest *Bacillus* group, consisting of 2 clones and represented by the sequence JG35-K4-KF86, formed a separate cluster with the sequences BSV06 and BSV05 retrieved from anoxic rice paddy soil (Hengstmann *et al*., 1999) (Fig. 2.3, Table S2.2).

To the same cluster also belong the sequences JG35-K2-AG49 and JG35+U3-JT7 (Fig. 2.3). These sequences were found in the sub-samples JG35-K2 and JG35+U3, which were treated with sodium or uranyl nitrate for 14 weeks (next paragraph).

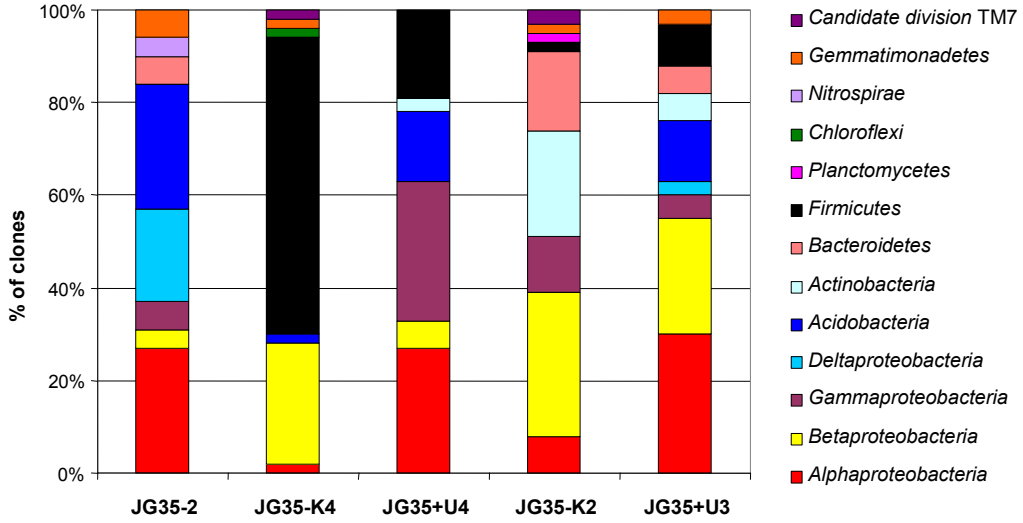

Fig. 2.2. Size of the predominant bacterial populations (given in percentages) in the untreated sample JG35-2 (2 m depth, containing 26 - 40 mg U kg^{-1}), the samples treated with uranyl nitrate (JG35+U4 (containing ~100 mg U kg^{-1}, 4 weeks) and JG35+U3 (containing ~300 mg U kg^{-1}, 14 weeks)), or treated with the sodium nitrate (JG35-K4 (4 weeks) and JG35-K2 (14 weeks)).

None of the mentioned *Bacillus* sequences were identified in the sub-sample JG35+U4 treated with uranyl nitrate for 4 weeks (Fig. 2.3, Table S2.3) where, as shown in Fig. 2.2, the size of the *Firmicutes* sequences was markedly reduced. Only one *Bacillus* group of 6 clones, represented by the sequence JG35+U4-KF48, was identified in that uranium treated sub-sample (Fig. 2.3, Table S2.3). This sequence was almost identical to the 16S rRNA gene of *Bacillus sphaericus* JG-A12. The latter strain was cultivated earlier from the same uranium mining waste pile but from another sampling point (Selenska-Pobell *et al*., 1999).

The *Clostridium* related 16S rRNA gene sequences retrieved from the sample JG35-K4 were organised in two closely related groups, including 19 and 8 clones (sequences JG35-K4-KF40 and JG35-K4-AG7 in Fig. 2.3 and Table S2.2) and one additional group of 4 clones represented by the sequence JG35-K4-KF36. The closest relatives of the two most predominant clostridial groups represented by the sequences JG35-K4-KF40 and JG35-K4-AG7 were retrieved from groundwater of trichlorethene (TCE)-contaminated deep fractured basalt aquifer (Macbeth *et al*., 2004) and from the sub-sample JG35+U3 (next paragraph) (sequences TANB115 and JG35+U3-JT4 in Fig. 2.3, Tables S2.2 and S2.5). Only one *Clostridium* group, which belongs to the same cluster, was found in the parallel sub-sample JG35+U4 treated with uranyl nitrate (sequence JG35+U4-KF21, Fig. 2.3 and Table S2.3).

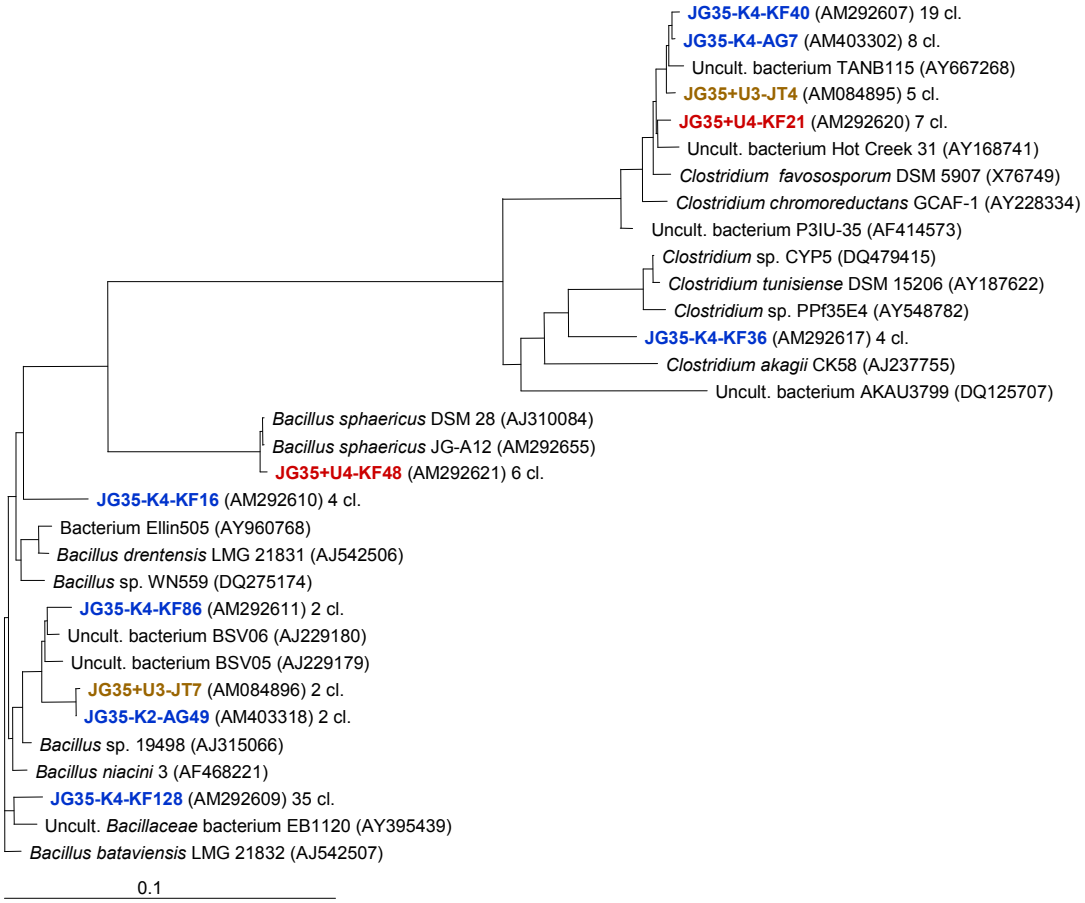

Fig. 2.3. Phylogenetic tree of the 16S rRNA gene sequences affiliated with *Firmicutes* retrieved from the samples treated with uranyl nitrate (**JG35+U4** (4 weeks) and **JG35+U3** (14 weeks)) or with sodium nitrate (**JG35-K4** (4 weeks) and **JG35-K2** (14 weeks)). The number of clones (cl.) is written after the accession numbers.

The closest cultured relatives to all these groups were *Clostridium favososporum* DSM 5907 (Collins *et al*., 1994) and *Clostridium chromoreductans* GCAF-1 (Fig. 2.3). Interestingly, the sequence P3IU-35, which was found in a nitrate rich uranium mine sediment sample supplemented with organic substrates and incubated anaerobically for one month (Suzuki *et al*., 2003), was also affiliated with the same cluster (Fig. 2.3, Table S2.2). The sequence JG35-K4-KF36 was related to the sequences *Clostridium* sp. PPf35E4, retrieved from sulphate-reducing fluidised-bed reactors treating acidic, metal-containing wastewater (Kaksonen *et al*., 2004) and AKAU3799, recently retrieved from a bacterial community stimulated for uranium reduction and reoxidation (Brodie *et al*., 2006).

Most of the betaproteobacterial clones (24 of 29) found in the clone library of the sample JG35-K4 were represented by the sequences JG35-K4-KF82 (20) and JG35-K4-AG145 (4), which shared an identity of about 99% with the 16S rRNA gene of the uncultured betaproteobacterium JG35+U1-AG61 (Fig. 2.4, Table S2.2). The latter was previously identified in another JG35-2 sub-sample, which was treated with uranyl nitrate for 4 weeks

under aerobic conditions, but in much lower numbers (Chapter 1). The three sequences mentioned formed a very tight cluster (Fig. 2.4). In addition, the sequences JG35-K2-AG7 and JG35+U3-AG5, representing highly predominant bacterial groups in the samples treated with sodium and uranyl nitrate for 14 week, were also allied to the same cluster (next paragraph). The closest cultured relative to this large cluster was the arsenic-oxidising strain *Cenibacterium arsenoxidans* ULPAs1 (Carapito *et al*., 2006) (Fig. 2.4, Table S2.2).

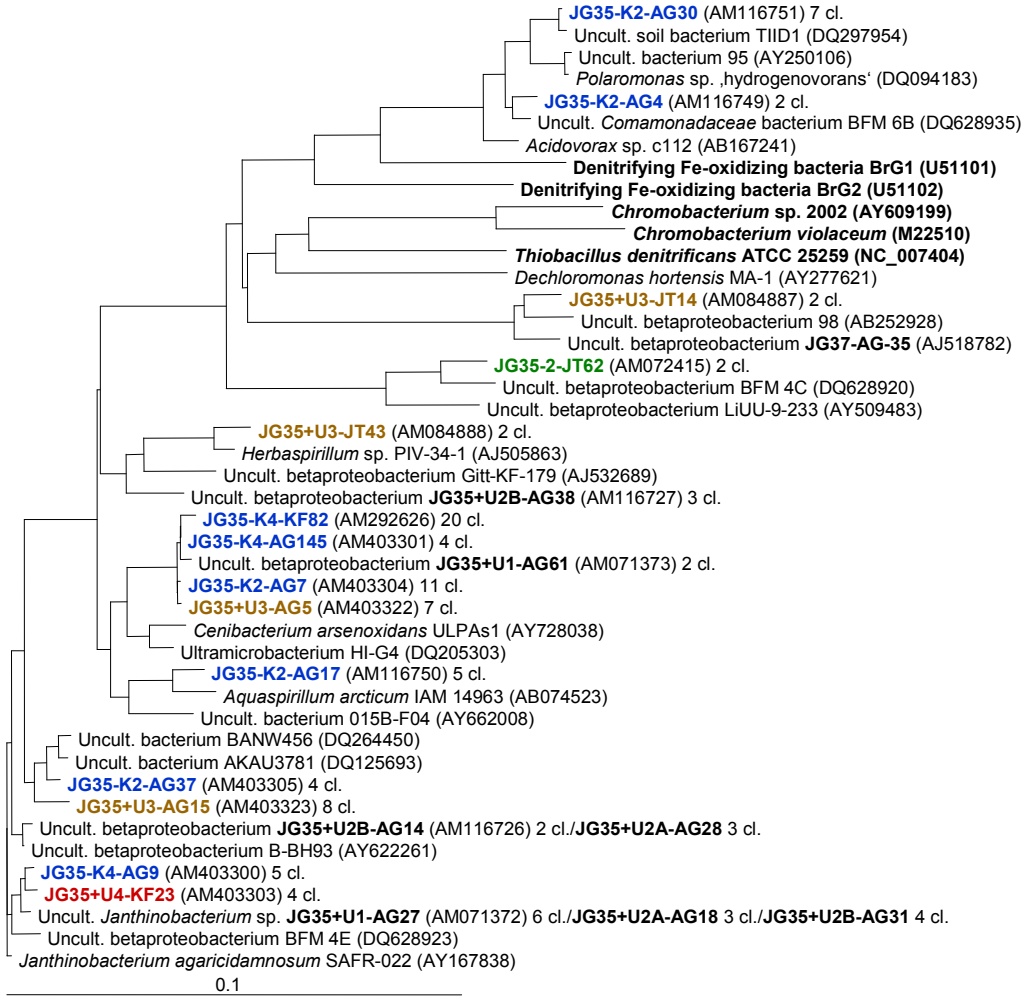

Fig. 2.4. Phylogenetic tree of the betaproteobacterial 16S rRNA gene sequences retrieved from the untreated sample **JG35-2** and the samples treated under anaerobic conditions with uranyl nitrate (**JG35+U4** (4 weeks), **JG35+U3** (14 weeks)) or sodium nitrate (**JG35-K4** (4 weeks), **JG35-K2** (14 weeks)) as well as in the samples incubated under aerobic conditions (Chapter 1) with uranyl nitrate (**JG35+U1** (4 weeks), **JG35+U2A** (14 weeks), **JG35+U2B** (28 weeks)) or with sodium nitrate (**JG35-K1** (4 weeks). The sequences of the Fe(II)-oxidising betaproteobacteria are given in bold. The number of clones (cl.) is written after the accession numbers.

The third betaproteobacterial group, represented by the sequence JG35-K4-AG9, was also affiliated with a very high 16S rRNA gene identity of 99.5% with another sequence (JG35+U1-AG27), which was retrieved from the same sub-sample as JG35+U1-AG61 (Chapter 1). More interesting, the only uranium resistant group found in the sub-sample treated with uranyl nitrate under anaerobic conditions was represented by the sequence JG35+U4-KF23 (Table S2.3). This sequence was almost identical to the sequence JG35+U1-AG27 and also to JG35-K4-AG9. The closest match of cultured organisms to these groups was *Janthinobacterium agaricidamnosum* SAFR-022 (Tables S2.2 and S2.3).

As shown in Fig. 2.2, no representatives of the deltaproteobacterial populations, initially predominant in the untreated JG35-2 sample, were found in the sub-samples JG35-K4 and JG35+U4. However, in contrast to JG35-K4, where mainly representatives of *Firmicutes* and *Betaproteobacteria* propagated, *Gammaproteobacteria* were the most predominant group in the sub-sample JG35+U4. As shown in the Table S2.3, the major gammaproteobacterial group included 17 clones and was represented by the sequence JG35+U4-KF30, which shared 99.6% of similarity with the 16S rRNA gene of the nitrate reducer *Rahnella* genosp. 3 DSM 30078 (Brenner *et al*., 1998). Two additional gammaproteobacterial clones, represented by the sequence JG35+U4-KF29, were affiliated with *Legionella gresilensis* ATCC 700509 (Lo Presti *et al*., 2001).

The effect of the treatment with uranyl nitrate on the initially predominant groups of *Alphaproteobacteria* and *Acidobacteria* was not as drastic as those of the treatment with sodium nitrate (compare JG35-K4 and JG35+U4 to JG35-2 in Fig. 2.2). In the sub-sample JG35-K4 *Alphaproteobacteria* were strongly reduced and limited to only one small group, represented by the sequence JG35-K4-KF54 related to *Rhodoplanes* sp. R2 (Table S2.2, Fig. 2.5). In contrast, the *Alphaproteobacteria* remained predominant in the sub-sample JG35+U4 (Table S2.3). However, the retrieved sequences were less diverse than in the case of the untreated sample and they were limited to only two groups (compare Table S2.1 and S2.3). The first group consisted of 10 clones and was represented by the sequence JG35+U4-KF36 (Fig. 2.5). This sequence shared an identity of 99.6% with the 16S rRNA gene of *Bradyrhizobium* sp. TM18_1 and of 99.4% with the sequence AKAU3720, which was recently retrieved from the already mentioned bacterial community stimulated for uranium reduction and reoxidation (Brodie *et al.,* 2006). The second group, represented by the sequence JG35+U4-KF11, was closely related to the most predominant alphaproteobacterial group in the original sample, represented by the sequence JG35-2-JT135 (Fig. 2.5, Tables S2.1 and S2.3).

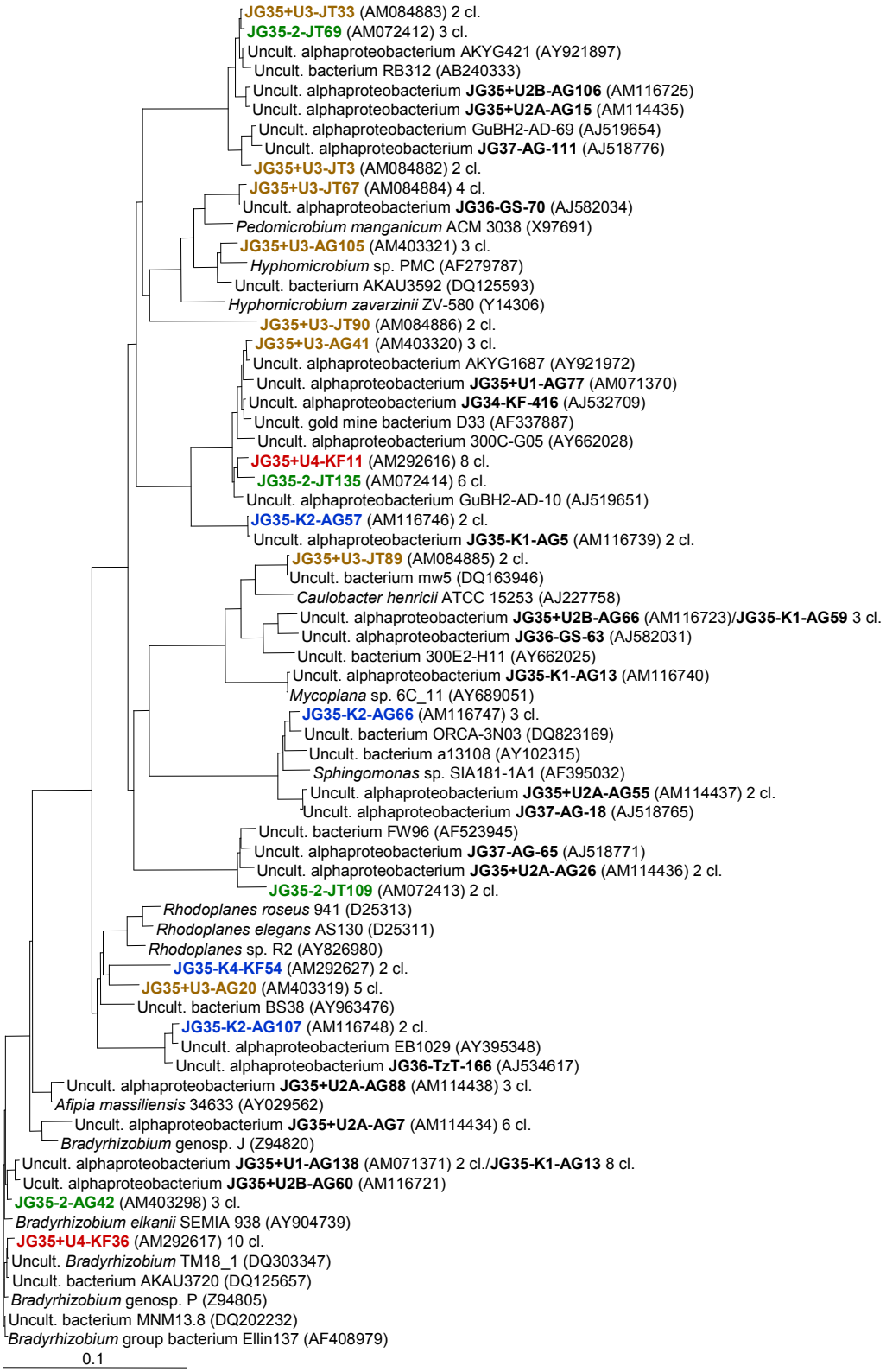

Fig. 2.5. Phylogenetic tree of alphaproteobacterial 16S rRNA gene sequences retrieved from the untreated sample **JG35-2** and the samples treated under anaerobic conditions with uranyl nitrate (**JG35+U4** (4-weeks), **JG35+U3** (14 weeks)) or with sodium nitrate (**JG35-K4** (4 weeks), **JG35-K2** (14 weeks)) as well as from the samples incubated under aerobic conditions (Chapter 1) with uranyl nitrate (**JG35+U1** (4 weeks), **JG35+U2A** (14 weeks), **JG35+U2B** (28 weeks)) or with sodium nitrate (**JG35-K1** (4 weeks). The number of clones (cl.) is written after the accession numbers.

Interestingly, the latter sequences were closely related to 16S rRNA gene sequences of other not yet cultured *Alphaproteobacteria* found in the vicinity of the uranium waste depository site Gunnison, Colorado, USA (sequence GuBH2-AD-10) and also in a sample of the here studied waste pile, but collected from another depth of 1 m (sequence JG34-KF-416, Fig. 2.5, Selenska-Pobell *et al*., 2002).

The uranyl nitrate treatment (sub-sample JG35+U4) resulted in shifting of the original acidobacterial populations found in the sample JG35-2, which was not such a drastic reduction as in sample JG35-K4 (Fig. 2.2, Tables S2.1, S2.2, S2.3). It is remarkable that the acidobacterial sequences found in all three samples were affiliated with diverse sequences previously retrieved from samples collected from different depths and sites of the same uranium mining waste pile (Selenska-Pobell *et al*., 2002; Geissler, 2003; Chapter 1).

Several small groups of clones, represented by the sequences JG35-K4-KF37, JG35-K4-KF12, and JG35-K4-KF44 were characteristic for the sample JG35-K4, which affiliated with *Chloroflexi*, *Gemmatimonadetes* and *Candidate division* TM7, respectively (Fig. 2.2, Table S2.2). Neither in the parallel, uranyl nitrate treated sub-sample, JG35+U4, nor in the original sample JG35-2 were such sequences found. In the sample JG35+U4 one sequence JG35+U4-KF8 was retrieved, representing a small group of two clones, which was affiliated with representatives of *Actinobacteria* (Table S2.3). No *Bacteroidetes* and *Nitrospira* 16S rRNA gene sequences, indigenous for the original sample JG35-2, were identified in the clone libraries of the sub-samples JG35+U4 and JG35-K4 (Fig. 2.2, Tables S2.1, S2.2, S2.3).

Changes in the bacterial community structure induced by sodium or uranyl nitrate treatments for 14 weeks

The 14 weeks treated sub-samples JG35-K2 and JG35+U3 were supplemented with higher amounts of sodium and uranyl nitrate, correspondingly. The end concentration of 300 mg U kg^{-1} in the sample JG35+U3 was three times higher than those of the already described sub-sample JG35+U4 (the preceding paragraph) and almost 12 times higher than in the untreated sample JG35-2 (Table 2.1). The sub-sample JG35-K2 was supplemented with five times higher amount of sodium nitrate, in contrast to sample JG35-K4, in order to achieve a nitrate concentration corresponding to those added to the sample JG35+U3 in form of uranyl nitrate. The changes induced in the community structure of the original sample by these treatments were significantly different than those observed after the shorter incubations of 4 weeks with lower amounts of sodium and uranyl nitrate (Fig. 2.2). Most remarkable in

the case of the sub-sample JG35-K2 is that only one small population of *Firmicutes* was established, in contrast to the sub-sample JG35-K4, which was very strongly predominated by this phylum. The only identified sequence, namely JG35-K2-AG49, representing two clones, was affiliated with *Bacillus* (Fig. 2.3, Table S2.4). The same *Bacillus* population, also represented by two clones, was found in the sub-sample JG35+U3 as well (sequence JG35+U3-JT7 in Fig. 2.3 and Table S2.5). The latter sub-sample contained also one population related to *Clostridium*, represented by the sequence JG35+U3-JT4 (Fig. 2.3, Table S2.5).

Another interesting matter in the community structures of the two longer time incubated samples was that *Betaproteobacteria* were predominant not only in the case of the sodium nitrate (JG35-K2) but also in the case of uranyl nitrate treated (JG35+U3) sub-sample. The latter is in strong contrast to the sub-sample JG35+U4 where the *Betaproteobacteria* represented only a minor part of the community (Fig. 2.2). Remarkable, in both longer incubated samples the betaproteobacterial populations were more diverse than in the sub-sample JG35-K4 (Fig. 2.4, Tables S2.2, S2.4, S2.5). The largest betaproteobacterial group found in the sub-sample JG35-K2 included 11 clones, represented by the sequence JG35-K2-AG7, and was affiliated with the second large group retrieved from the sub-sample JG35+U3, consisting of 7 clones and represented by the sequence JG35+U3-AG5 (Fig. 2.4). The most prominent betaproteobacterial group found in the sub-sample JG35-K4 (the proceeding paragraph) also belongs to the same cluster (Fig. 2.4). Additional JG35-K2 specific sequences were also affiliated with other betaproteobacterial genera such as *Polaromonas* (sequence JG35-K2-AG30), *Acidovorax* (sequence JG35-K2-AG4), and *Aquaspirillum* (sequence JG35-K2-AG17). The clone 015B-F04, which was identified in the groundwater of a nitric acid-bearing uranium waste (Fields *et al*., 2005), was also closely related to the sequence JG35-K2-AG17 (Fig. 2.4).

The largest group of *Betaproteobacteria* in the JG35+U3 clone library, represented by the sequence JG35+U3-AG15, included 8 clones and was allied with one additional JG35-K2 sequence, namely JG35-K2-AG37 representing 4 clones (Fig. 2.4). The latter two sequences formed a tight cluster with the sequences BANW456 and AKAU3781, retrieved by Brodie *et al*. (2006) from a community involved in reduction and reoxidation of uranium (Fig. 2.4). The sequence JG35+U3-JT43 of the uranium treated sub-sample was affiliated with *Herbaspirillum* sp. PIV-34-1 (Probian *et al*., 2003). One small group of two JG35+U3 clones (sequence JG35+U3-JT14) formed a cluster with the uncultured betaproteobacterium 98 identified in iron-oxidising biofilms (EMBL No. AB252928) and with the not yet cultured

bacterium JG37-AG-35 found in the same sampling point of the uranium mining waste pile Haberland but in a different depth (Geissler, 2003).

The second numerically predominant bacterial group in the sample JG35-K2 was affiliated with *Actinobacteria*, more specifically with different *Arthrobacter* spp., which were previously identified in the aerobically treated sub-samples as well (Table S2.4, Chapter 1). Most of these clones, represented by the sequences JG35-K2-AG26, JG35-K2-AG91 and JG35-K2-AG85 were affiliated with the already mentioned AKAU clones found by Brodie *et al*. (2006) (Table S2.4). The sequence JG35-K2-AG91 was identical with the 16S rRNA gene of the isolate *Arthrobacter* sp. JG37-Iso2, recovered from the uranium mining waste pile Haberland (Chapter 5). Only one related group, represented by the sequence JG35+U3-AG37 (Table S2.5), was found in the uranyl nitrate treated sample JG35+U3. Its representatives shared a high 16S rRNA gene identity with the already mentioned AKAU (Brodie *et al*., 2006) and JG35+U1 clones (Chapter 1).

The third predominant bacterial group in the sample JG35-K2 includes representatives of the *Bacteroidetes* phylum (Fig. 2.2, Table S2.4). Most of them were represented by 16S rRNA gene sequences which were affiliated with *Pedobacter* spp. and *Flavobacterium* spp. previously identified in the sub-samples JG35+U2A and JG35+U2B incubated for 14 weeks or longer with uranium (Table S2.4, Chapter 1). This is not surprising, because as demonstrated in the Chapter 1, at that late stage of the treatment the added uranium was no longer bioavailable. Surprising was, however, that the *Bacteroidetes* populations found in the parallel uranyl nitrate treated sub-sample JG35+U3 were only limited to two small groups of *Flavobacterium* spp. (Table S2.5).

Remarkable for the sample JG35+U3 is the establishment of a large number of very diverse *Alphaproteobacteria* (Fig. 2.2, Table S2.5). As shown in Fig. 2.5, some of the alphaproteobacterial sequences such as JG35+U3-JT33, JG35+U3-AG41 and JG35+U3-JT67 were affiliated with sequences found in natural samples of the same uranium waste pile, namely with JG35-2-JT69, JG34-KF-416, and JG36-GS-70 (Selenska-Pobell *et al*., 2002; Satchanska *et al*. 2004). The sequence JG35+U3-JT3 affiliated with the sequence GuBH2-AD-69, which was identified in another uranium contaminated waste (Geissler, 2003; Table S2.5). The sequence JG35+U3-JT89 was almost identical with the 16S rRNA gene of the alphaproteobacterial isolate mw5 and related to *Caulobacter henricii* ATCC 15253 (Abraham *et al*., 1999). The sequence JG35+U3-AG105 shared a high identity with the sequence AKAU3592, which was found in the uranium transforming community recently

studied by Brodie *et al*. (2006). Both sequences were related to *Hyphomicrobium* sp. LAT3 (Borodina *et al*., 2005) (Table S2.5).

The *Alphaproteobacteria* in the sample JG35-K2 were represented by three small groups (Table S2.4). The sequence JG35-K2-AG57 formed a tight cluster with the sequence JG35-K1-AG5, which was earlier retrieved from the sodium nitrate treated sub-sample incubated under aerobic conditions (Fig. 2.5, Chapter 1). The sequence JG35-K2-AG107 was related to sequence JG36-TzT-166, retrieved from a sample of the same site but collected from 1 m higher depth. The sequence JG35-K2-AG66 clustered with the 16S rRNA gene sequence a13108, which was identified in a heavy metal contaminated soil (Ellis *et al*., 2003). Both sequences were related to the 16S rRNA gene of *Sphingomonas* sp. SIA181-1A1 (Christner *et al*., 2001).

Distribution of *Acidobacteria* in the sub-samples JG35-K2 and JG35+U3 was similar to those found in the sub-samples JG35-K4 and JG35+U4 (previous paragraph). In analogy to the sub-sample JG35-K4, no acidobacterial sequences were retrieved from the sodium nitrate treated sub-sample JG35-K2. The same number of sequences was found in the uranyl nitrate treated sub-sample JG35+U3 as in the sample JG35+U4 and some of them were related to each other (Tables S2.3, S2.5).

Eleven clones represented *Gammaproteobacteria* in the sample JG35-K2 (Table S2.4). They were affiliated with different *Pseudomonas* spp. of which some were previously retrieved from the earlier studied sample JG35+U1, incubated with uranyl nitrate for 4 weeks under aerobic conditions (Table S2.4, Chapter 1). Only one small gammaproteobacterial group (sequence JG35+U3-AG1, Table S2.5) was retrieved from the uranyl nitrate treated sample, which affiliated with *Pseudomonas* spp. as well.

In analogy to the sub-samples JG35-K4 and JG35+U4 studied in the previous paragraph, no *Deltaproteobacteria* were also identified in the sub-sample JG35-K2. In contrast to that, a small deltaproteobacterial population was identified in the uranyl nitrate treated sample JG35+U3. This population was represented by the sequence JG35+U3-JT117, which was related to the sequences JG35-2-JT77 and JG35-2-JT67, found in the untreated sample (Tables S2.1, S2.5). These sequences form a novel lineage within the deltaproteobacterial sub-division consisting of not yet cultured members. The most closely related sequence B-BK96 (Tables S2.1, S2.5) was retrieved from a bacterial consortium formed on hematite, which was involved in reduction and oxidation of iron (Reardon *et al*. 2004).

Small groups of *Gemmatimonadetes* were found in both sub-samples, JG35-K2 and JG35+U3 (Tables S2.4, S2.5). Two additional small bacterial groups affiliated with *Planctomycetes* and

Candidate division TM7, which were only characteristic for the sodium nitrate treated sub-sample (Table S2.4). Concurrently to the previous paragraph, no sequences related to *Nitrospira* spp. were found in the two longer incubated samples, most probably due to the strict anaerobic conditions of the experiment.

DISCUSSION

In this work the influence of uranyl or sodium nitrate on the structure of the indigenous bacterial community of the uranium mining waste pile Haberland under strict anaerobic conditions was studied. The reducing conditions were identical to the natural conditions of the sample, which was collected from a depth of 2 m. Because the samples contained high concentrations of Fe, which can strongly influence the fate of both uranium and bacteria, we also monitored the changes in the Fe(II)/Fe(III) ratio in the samples treated with uranyl nitrate.

Changes in the Fe(II)/Fe(III) ratio in the samples treated with uranyl nitrate

By using Mössbauer spectroscopy a reduction of Fe(III) to Fe(II) was demonstrated in the uranyl nitrate treated samples. An important question arising from this result is, which role the members of the studied natural community in the observed Fe(III) reduction play.

It is increasingly becoming apparent that there is a wide phylogenetic diversity of *Bacteria* and *Archaea* capable of conserving energy to support growth from electron transport to Fe(III) (Lovley, 2000). The process results in the release of soluble Fe(II). Dissimilatory Fe(III) reduction may occur during the respiratory, fermentative or photosynthetic metabolism of a phylogenetically diverse range of microorganisms (Dobbin *et al*., 1999). A large variety of microorganisms capable to reduce Fe(III) such as *Geobacter* and *Desulfuromonas* spp. within the family *Geobacteraceae* of *Deltaproteobacteria* (Lovley, 2000), *Pantoea agglomerans* from *Gammaproteobacteria* (Francis *et al*., 2000), *Ferribacterium limneticum* and *Rhodoferax ferrireducens* (Finneran *et al*., 2003) from *Betaproteobacteria* (Cummings *et al*. 1999), *Geothrix fermentans* from *Acidobacteria* (Coates *et al*., 1999), *Clostridium beijerinckii* (Dobbin *et al*., 1999) and *Bacillus infernus* (Boone *et al*., 1995) from *Firmicutes*, *Acidiphilum cryptum* (Küsel *et al*., 1999) from *Alphaproteobacteria* and *Geovibrio ferrireducens* (Caccavo *et al*., 1996) were described. Our results in bacterial composition of the two samples (next paragraph) demonstrated that the more diverse bacterial composition of the sample JG35+U3 possessess a higher number of candidates able to reduce Fe(III). This is in agreement with the stronger Fe(III) reduction found in this sample than in the sample

JG35+U4. The reduction of Fe(III) to Fe(II) was also observed in iron-reducing enrichment cultures initiated from acidic subsurface sediments contaminated with U(VI) by the addition of amorphous Fe(III) and different organic substances (Petrie *et al*., 2003). A reduction of Fe(III) to Fe(II) was observed associated with the reduction of U(VI) after biostimulation with acetate (Anderson *et al*., 2003) and by the incubation of uranium contaminated sediments under anaerobic conditions (Suzuki *et al*., 2005). The reduction of Fe(III) can also occur abiotically with hydrogen sulphide or U(IV) as the reductant (Luther *et al*., 2001; Finneran *et al*., 2002; Senko *et al*., 2005; Wan *et al*., 2005). However, we do not have strong evidences for U(VI) reduction at the earlier stages of incubation.

Changes in bacterial community structure induced by sodium or uranyl nitrate treatments

The analysis of the 16S rRNA gene clone libraries provides indications of high microbial diversity and activity in the samples studied. It was demonstrated that the structure of the indigenous bacterial community from the uranium mining waste pile Haberland was differently influenced by treatments with uranyl or sodium nitrate under anaerobic conditions. In the sodium nitrate treated sample JG35-K4 representatives of *Firmicutes* were predominant after 4 weeks of incubation. In the absence of uranium a high number of sequences closely related to 16S rRNA gene sequences of the facultative anaerobic nitrate-reducing bacteria *Bacillus bataviensis* LMG 21832 (Heyrman *et al*., 2004) and *Bacillus drentensis* LMG 21831 was detected (sample JG35-K4, Fig. 2.3). None of these mentioned *Bacillus* sequences were identified in the uranyl nitrate treated sample JG35+U4. This indicates that these *Bacillus* spp. possibly do not tolerate uranium. In the uranyl nitrate treated sample JG35+U4 only one *Bacillus* population was identified, which affiliated with *B. sphaericus* JG-A12. The latter strain was recovered from the same uranium mining waste pile but from another sampling point (Selenska-Pobell *et al*., 1999). It was demonstrated that the isolate *B. sphaericus* JG-A12 accumulates selectively large amounts of uranium from contaminated waste water and that this capability is associated with the unusual structure of its surface protein layer (Raff *et al*., 2003; Merroun *et al*., 2005; Pollmann *et al*., 2005). However, the closest relative of this isolate *B. sphaericus* DSM 28 is not able to reduce nitrate (Reva *et al*., 2001). In addition, no *nar*G gene, encoding the membrane-bound nitrate reductase, could be amplified from the genomic DNA of JG-A12 (Chapter 3). On the basis of these results we suggest that the identified *Bacillus* population in the uranium treated sub-sample, in contrast to many other *Bacillus* spp. such as *B. bataviensis* (Heyrman *et al*., 2004) and *B. subtilis* (Hoffmann *et al.,*

1995), is possibly not capable to respire nitrate under anaerobic conditions. It is not excluded that this population is able to use alternative electron acceptors such as Fe(III) under anaerobic conditions, analogically to the obligate anaerobic species *Bacillus infernus* (Boone *et al*., 1995). In this way the mentioned *Bacillus* population might be involved in the Fe(III) reduction observed in this uranium treated sample. Such a function was not reported for the species *B. sphaericus*. However, it was demonstrated that the members of the highly heterogenic species *B. sphaericus* (Alexander & Priest, 1990) as well as of other *Bacillus* spp. often possess almost identical 16S rRNA genes but have low levels of total DNA similarity (Fox *et al.*, 1992). The latter is possibly due to the capability of *Bacillus* to be naturally transformed by DNA fragments released into environment by the death of the organisms (Lorenz & Wackernagel, 1994). It was suggested that *Bacillus* comprise fast diverging species and that the more conservative 16S rRNA genes in their case often do not reflect the fast changes occurring in their genomes (Fox *et al.*, 1992).

In addition to *Bacillus* spp. a high number of *Clostridium* spp. was found in the sub-sample JG35-K4. In the presence of uranium (sample JG35+U4) nearly the same *Clostridium* spp. were found but in lower numbers (Fig. 2.3). Different *Clostridium* spp. were also found in other uranium contaminated environments (Suzuki *et al*., 2003, 2005; North *et al*., 2004). Some *Clostridium* spp. are able to reduce fermentatively nitrate (Caskey & Tiedje, 1980; Tiedje, 1988; Fujinaga *et al.,* 1999; Kuhner *et al*., 2000), U(VI) (Francis *et al*., 1994; Suzuki *et al*., 2003) as well as Fe(III) (Dobbin *et al*., 1999; Kostka *et al*., 2002).

A proliferation of *Betaproteobacteria* was observed in both samples JG35-K4 and JG35-K2 treated with sodium nitrate. Oligotrophic or autotrophic *Betaproteobacteria* are the main denitrifiers under anaerobic conditions (Tiedje, 1988; Beller, 2005). In the sample JG35+U4 treated with uranyl nitrate for 4 weeks the betaproteobacterial populations represented only a small portion. This suggests that they possibly do not tolerate high concentrations of uranium. Interestingly, after 14 weeks of incubation (sample JG35+U3) under the same conditions even with higher amounts of uranium *Betaproteobacteria* were predominant. The increased number of probably uranium-sensitive *Betaproteobacteria* gave indications that the uranium in the sample was no longer available to the members of the bacterial community, analogically to the previously studied samples JG35+U2A and JG35+U2B (Chapter 1).

As demonstrated in Fig. 2.4, in nearly all treated samples sequences related to *Janthinobacterium agaricidamnosum* SAFR-022 were found. This suggests that these bacteria are able to reduce nitrate and that they are also resistant to uranium. None of the retrieved betaproteobacterial sequences from our work clustered with the 16S rRNA gene sequences of

the recently characterised anaerobic nitrate-reducing and ferrous-oxidising isolates BrG1 and BrG2 (Straub *et al.* 2004) or *Chromobacterium* sp. 2002 (Weber *et al.* 2006a) (Fig. 2.4). Moreover, in both uranyl nitrate treated samples a strong reduction of the natural Fe(III) was demonstrated by Mössbauer spectroscopic analyses. It cannot be excluded that some of the identified betaproteobacterial populations are able to reduce Fe(III) analogically to the members of the species *Ferribacterium limneticum* (Cummings *et al*., 1999) or *Rhodoferax ferrireducens* (Finneran *et al*., 2003). On the other hand, we cannot completely exclude Fe(II) oxidation to Fe(III) at the earlier stages of incubation, which might be rapidly reduced by other members of the community. Weber *et al*. (2006b) demonstrated that a variety of microorganisms is capable of both nitrate-dependent Fe(II) oxidation and Fe(III) reduction.

The proliferation of *Gammaproteobacteria* in the sample JG35+U4 is similar to those observed previously in the sample JG35+U1, which was also treated with uranyl nitrate but incubated under aerobic conditions for 4 weeks (Chapter 1). However, a stimulation of *Rahnella* spp. was observed under anaerobic conditions in the sample JG35+U4 (Fig. 2.4), whereas mostly *Pseudomonas* spp. from *Gammaproteobacteria* were identified in sample JG35+U1 (Chapter 1). *Rahnella* spp. are possibly involved in the nitrate reduction in the sample JG35+U4 (Brenner *et al*., 1998). Representatives of *Rahnella* were recovered from a contaminated subsurface soil sample of the saturated zone with elevated uranium and nitrate concentrations from the Department of Energy (DOE) Natural and Accelerated Bioremediation Field Research Centre located in the Oak Ridge National Laboratory Reservation at Oak Ridge, Tennessee, USA (Martinez *et al*., 2006) and were also induced in anaerobically treated microcosms amended with glucose, Fe(III) and Cr(VI) (Kourtev *et al*., 2006). It cannot be excluded that the *Rahnella* population induced in the sample JG35+U4 was also involved in the observed Fe(III) reduction. It was demonstrated that *Pantoea agglomerans* SP1, which belongs as well as *Rahnella* spp. to the family *Enterobacteriaceae*, is able to use Fe(III) as a terminal electron acceptor (Francis *et al*., 2000).

The *Alphaproteobacteria* remained predominant in the samples treated with different amounts of uranyl nitrate (Fig. 2.2). This result is in contrast to our previous observation in the sample JG35+U1, which was treated with uranyl nitrate but incubated under aerobic conditions for 4 weeks (Chapter 1). Sequences closely related (>98% similarity) to the 16S rRNA gene sequence of the uncultured bacterium JG34-KF-416 (Selenska-Pobell *et al*., 2002) were found in the untreated sample as well as in the two uranyl nitrate treated samples (Fig. 2.5, Tables S2.1, S2.3, S2.5). The sequence JG34-KF-416 was identified in a sample from the same site of the uranium mining waste pile Haberland but collected from another depth. So, it seems

that these groups of not yet cultured bacteria are able to tolerate uranium. Interestingly, the sequences JG35-K1-AG5 and JG35-K2-AG57, both detected in clone libraries from sodium nitrate treated samples, were related to the 16S rRNA gene sequence of the same bacterium, but with a lower similarity of only 94% (Fig. 2.5). This indicates that they represent another possibly not uranium tolerant species, which is induced only in the sodium nitrate treated samples.

In contrast to the aerobic samples previously studied (Chapter 1) and to the samples treated with sodium nitrate under anaerobic conditions, the acidobacterial populations were not very strongly reduced in both samples treated with uranyl nitrate and incubated under anaerobic conditions. So, they are maybe better adapted to anaerobic conditions and some of them can also tolerate uranium.

A longer incubation (14 weeks) of the sample JG35-K2 with higher nitrate concentrations under anaerobic conditions resulted in a stimulation of *Arthrobacter* spp. from *Actinobacteria* as well as of *Pedobacter* spp. and *Flavobacterium* spp. from the *Bacteroidetes* phylum.

This result is similar to that earlier obtained by the analysis of the sample JG35+U2A, which was treated with uranyl nitrate for 14 weeks but under aerobic conditions (Chapter 1). It was suggested that especially the strong propagation of possible uranium-sensitive *Bacteroidetes* in the sample JG35+U2A indicated that the added U(VI) was no longer bioavailable at these late stages of incubation (Chapter 1).

None or only a few sequences detected in the clone libraries from the uranyl or sodium nitrate treated samples incubated under anaerobic conditions were related to members of *Deltaproteobacteria*, which represented the third predominant group in the untreated sample (Fig. 2.2). This is surprising because representatives of *Deltaproteobacteria* are known for their ability to reduce Fe(III) and to immobilise uranium by reduction of soluble U(VI) to insoluble U(IV) (Lovley & Phillips, 1992; Lovley *et al*., 1993; Suzuki *et al*., 2004; Wu *et al*., 2006). However, not only the Fe(III) reduction as above mentioned but also U(VI) reduction, was recently demonstrated for an increasing number of taxonomically diverse microorganisms such as *Clostridium* spp. (Francis *et al*., 1994; Suzuki *et al*., 2003), *Pseudomonas* sp. (McLean & Beveridge, 2001), *Acidovorax* spp. (Nyman *et al*., 2006) and *Salmonella subterranean* (Shelobolina *et al*., 2004). For this reason we cannot exclude U(VI) reduction in our samples.

Despite the fact that bacteria were stimulated in the uranyl nitrate treated samples incubated under anaerobic conditions, which were probably involved in the reduction of nitrate, Fe(III) and U(VI), it cannot be excluded that also abiotic factors can influence the rates of these

processes in the studied environment. Additional studies are needed to determine the electron donors of the observed Fe(III) reduction.

We suggest that nitrate was reduced in the beginning of the incubations. When nitrate was depleted, the reduction of Fe(III) as demonstrated by Mössbauer spectroscopy could take place by Fe(III)-reducing bacteria. Previous research demonstrated that nitrate inhibits the microbial Fe(III) reduction (Arnold *et al*., 1986; Finneran *et al*., 2002; Cooper *et al*., 2003). Interestingly, in addition to the reduction of nitrate, Fe(III) and sulphate after the incubation of sediments from the Midnite mine for one month, the reduction of U(VI) was observed (Suzuki *et al*., 2003). The injection of acetate to a uranium contaminated aquifer located in Rifle, Colorado, USA resulted in an initial reduction of U(VI), which appeared to be associated with Fe(III) reduction (Anderson *et al*., 2003). It is also possible that U(IV), produced by biological reduction of U(VI), reduces ferric oxides (Sani *et al*., 2004; Senko *et al*., 2005; Wan *et al*., 2005). Sani *et al*. (2004) observed a reoxidation of U(IV) under lactate-limited sulphate-reducing conditions in cultures of *Desulfovibrio desulfuricans* G20 and Fe(III) (hydr)oxides (hematite, goethite, and ferrihydrite). Long-term experiments on microbial-mediated U(VI) reduction demonstrated that after 100 days the uranium was reoxidised (Wan *et al*., 2005). The authors proposed Fe(III) and Mn(IV) as terminal electron acceptors for the observed uranium reoxidation (Wan *et al*., 2005). However, it is also possible that the reduction of Fe(III) results from the reduction by microbially reduced humic acids or other microbially generated compounds such as hydroquinones (Nevin & Lovley, 2000).

On the other hand, despite the fact that we did not find 16S rRNA gene sequences of the known Fe(II) oxidisers in the studied stages of our study, we cannot exclude the biotic formation of Fe(III) oxides in the earlier stages of the performed treatments. These oxides along with a part of those present in the original sample were probably involved in processes such as dissimilatory microbial Fe(III) reduction or chemical oxidation of U(IV) at the later stages of the incubations. The accumulation of U(IV) at earlier stages of the incubations can be attributed not only to some of the identified bacteria such as the *Clostridium* spp., but also to chemical reduction (Francis *et al*., 1994; Liger *et al*., 1999; Suzuki *et al*., 2003, 2005).

Future studies are necessary to characterise the fate of the added U(VI) in the samples studied. For this reasons X-ray Absorption Spectroscopic (XAS) analysis and Time Resolved Laser-induced Fluorescence Spectroscopic (TRLFS) analysis of the uranyl nitrate treated samples are in progress.

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SUPPLEMENTAL MATERIAL

Table S2.1. Affiliation of 16S rRNA gene clones of the untreated sample JG35-2.

Clone name	Accession No. EMBL	No. of clones	Closest phylogenetic relative (EMBL No.)	BLAST % of similarity*
			Alphaproteobacteria	
JG35-2-AG42	AM403298	3	Uncult. bacterium MNM13.8 (DQ202232)	99.6
			Bradyrhizobium group bacterium Ellin137 (AF408979)	99.4
JG35-2-JT69	AM072412	3	Uncult. alphaproteobacterium JG35+U3-JT33 (AM084883)	99.7
			Uncult. alphaproteobacterium AKYG421 (AY921897)	99.3
			Uncult. alphaproteobacterium JG37-AG-111 (AJ518776)	99.3
JG35-2-JT109	AM072413	2	Uncult. forest soil bacterium DUNssu168 (AY913375)	97.4
			Uncult. bacterium FW96 (AF523945)	97.4
			Uncult. alphaproteobacterium JG37-AG-123 (AJ518778)	97.6
			Uncult. alphaproteobacterium JG35+U2A-AG26 (AM114436)	97.2
JG35-2-JT135	AM072414	6	Uncult. alphaproteobacterium JG35-U4-KF11 (AM292616)	98.5
			Uncult. alphaproteobacterium GuBH2-AD-10 (AJ519651)	98.1
			Uncult. alphaproteobacterium JG34-KF-416 (AJ532709)	97.9
			Betaproteobacteria	
JG35-2-JT62	AM072415	2	Uncult. betaproteobacterium BFM 4C (DQ628920)	97.7
			Uncult. betaproteobacterium LiUU-9-233 (AY509483)	95.9
			Gammaproteobacteria	
JG35-2-JT11	AM072416	3	Uncult. gammaproteobacterium NEC03035 (AY911444)	99.8
			Uncult. bacterium 22 (DQ413081)	99.8
			Shigella sp. BBDP15 (DQ337523)	99.8
			Escherichia coli UTI89 (CP000243)	99.7
			Shigella flexneri ATCC 29903 (X96963)	99.7
			Deltaproteobacteria	
JG35-2-JT46	AM072417	2	Uncult. deltaproteobacterium JG37-AG-55 (AJ518793)	98.1
			Uncult. bacterium RB203 (AB240314)	98.1
JG35-2-JT67	AM072418	5	Uncult. deltaproteobacterium B-BK96 (AY622263)	95.1
			Uncult. deltaproteobacterium JG35+U3-JT117 (AM084889)	95.1
JG35-2-JT77	AM072419	3	Uncult. deltaproteobacterium JG35+U3-JT117 (AM084889)	95.5
			Uncult. deltaproteobacterium AKYG859 (AY921675)	94.9
			Acidobacteria	
JG35-2-JT30	AM072421	4	Uncult. bacterium BS091 (AB240256)	93.9
			Uncult. bacterium CCU21 (AF485788)	93.9
			Uncult. bacterium S-Rwb_47 (DQ017940)	95.5
			Uncult. *Holophaga* sp. JG37-AG-29 (AJ519367)	95.3
JG35-2-JT72	AM072422	2	Uncult. *Holophaga* sp. JG37-AG-67 (AJ519378)	99.4
			Acidobacteria bacterium Ellin7137 (AY673303)	99.3
JG35-2-JT80	AM072423	4	Uncult. *Acidobacteria* bacterium JG35+U2B-AG54 (AM116732)	97.3
			Uncult. *Acidobacteria* AKYG857 (AY922071)	96.9
JG35-2-JT87	AM072424	2	Uncult. *Holophaga* sp. JG37-AG-81 (AJ519382)	98.6
			Uncult. bacterium S-Jos_44 (DQ017934)	98.6
JG35-2-AG139	AM403299	2	Uncult. bacterium BS118 (AB240262)	97.7
			Uncult. soil bacterium 894-1 (AY326576)	96.1
			Uncult. *Holophaga* sp. JG30a-KF-86 (AJ536874)	96.0
			Bacteroidetes	
JG35-2-JT27	AM084881	1	*Flavobacterium succinicans* DSM 4003 (AM230493)	98.4
			Flavobacterium limicola ST-82 (AB075230)	98.0
JG35-2-JT78	AM072425	2	*Flavobacterium* sp. WB 2.1-3 (AM167557)	98.1
			Agricultural soil bacterium SC-I-93 (AJ252668)	98.1
			Nitrospirae	
JG35-2-JT60	AM072426	2	Uncult. bacterium CCU23 (AY221079)	99.1
			Uncult. *Nitrospirae* bacterium JG36-TzT-13 (AJ534638)	98.3
			Nitrospira sp. GC86 (Y14644)	96.9
			Gemmatimonadetes	
JG35-2-JT61	AM072427	3	Uncult. *Fibrobacteres* bacterium 05 (AB252948)	96.6
			Uncult. bacterium a13101 (AY102345)	94.3
			Uncult. *Gemmatimonadetes* bacterium W2a-1F (AY192277)	94.6

* - taking those parts of the gene which were considered by the BLAST search.

Table S2.2. Affiliation of the 16S rRNA gene clones of soil sample JG35-K4.

Clone name	Accession No. EMBL	No. of clones	Closest phylogenetic relative (EMBL No.)	BLAST % of similarity*
			Alphaproteobacteria	
JG35-K4-KF54	AM292627	2	*Rhodoplanes* sp. R2 (AY826980)	94.3
			Rhodoplanes roseus 941 (D25313)	93.8
			Betaproteobacteria	
JG35-K4-AG9	AM403300	5	Uncult. *Janthinobacterium* sp. JG35+U4-KF23 (AM403303)	99.5
			Uncult. *Janthinobacterium* sp. JG35+U1-AG27 (AM071372)	99.5
			Janthinobacterium agaricidamnosum SAFR-022 (AY167838)	99.1
			Uncult. betaproteobacterium BFM 4E (DQ628923)	98.9
JG35-K4-KF82	AM292626	20	Uncult. betaproteobacterium JG35-K4-AG145 (AM403301)	99.6
			Uncult. betaproteobacterium JG35+U3-AG5 (AM403322)	99.5
			Uncult. beta proteobacterium JG35-K2-AG7 (AM403304)	99.5
			Ultramicrobacterium HI-G4 (DQ205303)	98.3
			Cenibacterium arsenoxidans ULPAs1 (AY728038)	97.7
JG35-K4-AG145	AM403301	4	Uncult. betaproteobacterium JG35-K2-AG7 (AM403304)	99.8
			Uncult. betaproteobacterium JG35+U3-AG5 (AM403322)	99.8
			Uncult. betaproteobacterium JG35-K4-KF82 (AM292626)	99.6
			Uncult. betaproteobacterium JG35+U1-AG61 (AM071373)	99.3
			Ultramicrobact. HI-G4 (DQ205303)	98.6
			Cenibacterium arsenoxidans ULPAs1 (AY728038)	97.9
			Acidobacteria	
JG35-K4-KF18	AM292612	2	Uncult. *Acidobacteria* bacterium i5 (DQ453805)	97.7
			Uncult. *Acidobacteria* bacterium FAC6 (DQ451445)	97.3
			Uncult. Acidobacterium sp. KCM-C-150 (AJ581626)	96.9
			Uncult. *Holophaga* sp. JG37-AG-74 (AJ519380)	96.6
			Firmicutes	
JG35-K4-KF40	AM292607	19	Uncult. bacterium JG35+U3-JT4 (AM084895)	99.5
			Uncult. bacterium TANB115 (AY667268)	99.2
			Uncult. bacterium JG35-K4-AG7 (AM403302)	99.7
			Uncult. *Clostridium* sp. JG35-U4-KF21 (AM292620)	98.8
			Uncult. bacterium P3IU-35 (AF414573)	99 (837 bp)
JG35-K4-AG7	AM403302	8	Uncult. bacterium JG35-K4-KF40 (AM292607)	99.7
			Uncult. bacterium JG35+U3-JT4 (AM084895)	99.5
			Uncult. bacterium TANB115 (AY667268)	99.4
			Uncult. *Clostridium* sp. JG35-U4-KF21 (AM292620)	99.0
			Clostridium chromoreductans GCAF-1(AY228334)	98.8
JG35-K4-KF36	AM292608	4	*Clostridium* sp. CYP5 (DQ479415)	95.3
			Clostridium sp. PPf35E4 (AY548782)	95.2
			Clostridium tunisiense DSM 15206^T (AY187622)	95.2
			Uncult. bacterium AKAU3799 (DQ125707)	96 (1115 bp)
JG35-K4-KF128	AM292609	35	Uncult. *Bacillaceae* bacterium EB1120 (AY395439)	98.5
			Bacillus sp. LMG 19498 (AJ315066)	98.3
			Bacillus bataviensis LMG 21833 (AJ542508)	98.3
JG35-K4-KF16	AM292610	4	*Bacillus* sp. WN559 (DQ275174)	98.0
			Bacterium Ellin505 (AY960768)	98.0
			Bacillus drentensis LMG 21831 (AJ542506)	97.9
JG35-K4-KF86	AM292611	2	Unident. bacterium BSV05 (AJ229179)	98.2
			Bacillus sp. LMG 19498 (AJ315066)	98.0
			Uncult. bacterium JG35+U3-JT7 (AM084896)	97.9
			Gemmatimonadetes	
JG35-K4-KF12	AM292614	2	Uncult. bacterium N14.23WL (AF432635)	99.1
			Uncult. candidate division BD bacterium BDfull1 (AF545649)	97.7
			***Candidate division* TM7**	
JG35-K4-KF44	AM292615	2	Uncult. bacterium AKAU3587 (DQ25590)	96.5
			Uncult. bacterium NoosaAW16 (AF269015)	97.4
			Chloroflexi	
JG35-K4-KF37	AM292613	2	Uncult. bacterium BS047 (AB240237)	97.2
			Uncult. soil bacterium ABS-705 (AY289463)	96.7

* -taking those parts of the gene which were considered by the BLAST search.

Table S2.3. Affiliation of the 16S rRNA gene clones of soil sample JG35+U4.

Clone name	Accession No. EMBL	No. of clones	Closest phylogenetic relative (EMBL No.)	BLAST % of similarity*
			Alphaproteobacteria	
JG35+U4-KF11	AM292616	8	Uncult. alphaproteobacterium JG34-KF-416 (AJ532709)	98.8
			Uncult. alphaproteobacterium GuBH2-AD-10 (AJ519651)	99.1
			Uncult. alphaproteobacterium JG35-2-JT135 (AM072414)	98.5
			Uncult. alphaproteobacterium JG35+U1-AG77 (AM071370)	98.4
JG35+U4-KF36	AM292617	10	Uncult. *Bradyrhizobium* sp. TM18_1 (DQ303347)	99.6
			Uncult. bacterium AKAU3720 (DQ125657)	99.4
			Betaproteobacteria	
JG35+U4-KF23	AM403303	4	Uncult. *Janthinobacterium* sp. JG35-K4-AG9 (AM403300)	99.5
			Uncult. *Janthinobacterium* sp. JG35+U1AG27 (AM071372)	99.4
			Uncult. betaproteobacterium BFM 4E (DQ628923)	98.9
			Janthinobacterium agaricidamnosum SAFR-022 (AY167838)	99.0
			Gammaproteobacteria	
JG35+U4-KF29	AM292618	2	*Legionella gresilensis* ATCC 700509 (AF122883)	97.3
			Legionella beliardensis Montbeliard A1 (AF122884)	97.0
JG35+U4-KF30	AM292619	17	*Rahnella aquatilis* BS-1 (DQ440548)	99.8
			Rahnella sp. GH05 (DQ365560)	99.6
			Rahnella genosp. 3 DSM 30078 (U90758)	99.6
			Acidobacteria	
JG35+U4-KF12	AM292623	2	Bacterial species mb1228 (Z95733)	99.2
			Uncult. *Acidobacteria* bacterium JG35+U2B-AG75 (AM116733)	99.4
JG35+U4-KF19	AM292624	2	Uncult. *Acidobacteria* bacterium AKYG811 (AY921919)	98.4
			Uncult. *Holophaga* sp. JG30a-KF-145 (AJ536875)	98.8
JG35+U4-KF38	AM292625	6	Uncult. bacterium JG34-KF-27 (AJ532717)	97.7
			Uncult. *Acidobacteria* bacterium JG35+U3-JT17 (AM084891)	97.2
			Firmicutes	
JG35+U4-KF21	AM292620	7	Uncult. bacterium TANB115 (AY667268)	99.3
			Uncult. bacterium Hot Creek 31 (AY168741)	99.0
			Uncult. bacterium JG35-K4-KF40 (AM292607)	98.8
			Uncult. bacterium JG35+U3-JT4 (AM084895)	98.8
			Clostridium favososporum DSM 5907 (X76749)	98.7
JG35+U4-KF48	AM292621	6	*Bacillus sphaericus* JG-A12 (AM292655)	99.7
			Bacillus sp. AzoR-7 (DQ279754)	99.6
			Bacillus sphaericus DSM 28 (AJ310084)	99.6
			Actinobacteria	
JG35+U4-KF8	AM292622	2	Uncult. actinobacterium S4 (AJ575506)	97.5
			Uncult. actinobacterium JG35+U2A-AG41 (AM114449)	91.5
			Uncult. bacterium JG34-KF-316 (AJ532727)	91.4

* - taking those parts of the gene which were considered by the BLAST search.

Table S2.4. Affiliation of the 16S rRNA gene clones of soil sample JG35-K2.

Clone name	Accession No. EMBL	No. of clones	Closest phylogenetic relative (EMBL No.)	BLAST % of similarity*
			Alphaproteobacteria	
JG35-K2-AG57	AM116746	2	Uncult. alphaproteobacterium JG35-K1-AG5 (AM116739)	99.8
			Uncult. alphaproteobacterium JG34-KF-416 (AJ532709)	94.0
JG35-K2-AG66	AM116747	3	Uncult. bacterium ORCA-3N03 (DQ823169)	98.7
			Uncult. bacterium a13108 (AY102315)	98.1
JG35-K2-AG107	AM116748	2	Uncult. alphaproteobacterium EB1029 (AY395348)	99.6
			Uncult. alphaproteobacterium JG36-TzT-166 (AJ534617)	98.4
			Betaproteobacteria	
JG35-K2-AG4	AM116749	2	Uncult. *Comamonadaceae* bacterium BFM 6B (DQ628935)	98.8
			Acidovorax sp. c112 (AB167241)	98.6
JG35-K2-AG7	AM403304	11	Uncult. betaproteobacterium JG35-K4-AG145 (AM403301)	99.8
			Uncult. betaproteobacterium JG35+U3-AG5 (AM403322)	99.8
			Uncult. betaproteobacterium JG35-K4-KF82 (AM292626)	99.5
			Uncult. betaproteobacterium JG35+U1-AG61 (AM071373)	99.1
			Ultramicrobacterium HI-G4 (DQ205303)	98.6
			Cenibacterium arsenoxidans ULPAs1 (AY728038)	97.9
JG35-K2-AG17	AM116750	5	*Aquaspirillum arcticum* IAM 14963 (AB074523)	98.6
			Uncult. bacterium RS 8-Bact32 (AJ867670)	97.9
JG35-K2-AG30	AM116751	7	Uncult. soil bacterium TIID1 (DQ297954)	99.8
			Polaromonas sp. 'hydrogenovorans' (DQ094183)	99.0
JG35-K2-AG37	AM403305	4	Uncult. bacterium BANW456 (DQ264450)	99.3
			Uncult. bacterium AKAU3781 (DQ125693)	99.1
			Uncult. betaproteobacterium B-BH93 (AY622261)	99.0
			Uncult. betaproteobacterium JG35+U2B-AG14 (AM116726)	98.9
			Janthinobacterium agaricidamnosum SAFR-022 (AY167838)	98.7
			Gammaproteobacteria	
JG35-K2-AG1	AM403306	3	Uncult. bacterium pLW-23 (DQ066942)	99.7
			Pseudomonas sp. PD 22 (DQ377763)	99.7
			Uncult. *Pseudomonas* sp. JG35+U1-AG16 (AM071376)	99.4
JG35-K2-AG6	AM403307	6	*Pseudomonas mandelii* (AY179326)	99.7
			Pseudomonas fluorescens PC17 (AY538263)	99.7
			Pseudomonas sp. '11/20CMC control' (AY263482)	99.4
JG35-K2-AG10	AM403308	2	*Pseudomonas* sp. EK1 (AJ237965)	99.7
			Uncult. *Pseudomonas* sp. JG35+U1-AG53 (AM071377)	99.7
			Bacteroidetes	
JG35-K2-AG5	AM403309	4	Uncult. *Bacteroidetes* bacterium JG35-K2-AG25 (AM403311)	99.9
			Uncult. *Bacteroidetes* bacterium JG35+U2B-AG125 (AM116730)	99.8
			Uncult. *Bacteroidetes* bacterium JG35+U2A-AG1 (AM114440)	99.8
			Uncult. *Bacteroidetes* bacterium JG35+U2A-AG30 (AM114445)	99.7
			Pedobacter jejuensis JS11-06T (AM279218)	98.8
JG35-K2-AG13	AM403310	2	*Pedobacter* sp. Ellin108 (AF408950)	99.6
			Sphingobacteriaceae EP316 (AF493692)	99.4
			Uncult. *Bacteroidetes* bacterium JG35+U1-AG84 (AM114431)	98.8
			Pedobacter jejuensis JS11-06T (AM279218)	98.8
JG35-K2-AG25	AM403311	4	Uncult. *Bacteroidetes* bacterium JG35-K2-AG5 (AM403309)	99.9
			Uncult. *Bacteroidetes* bacterium JG35+U2B-AG125 (AM116730)	99.7
			Uncult. *Bacteroidetes* bacterium JG35+U2A-AG1 (AM114440)	99.6
			Uncult. *Bacteroidetes* bacterium JG35+U2A-AG30 (AM114445)	99.6
			Pedobacter jejuensis JS11-06T (AM279218)	98.8
JG35-K2-AG41	AM403312	3	Uncult. *Bacteroidetes* bacterium JG35+U2A-AG62 (AM114447)	100
			Uncult. *Bacteroidetes* bacterium JG35+U2A-AG102 (AM114448)	99.9
			Uncult. bacterium BANW642 (DQ264594)	98.1
JG35-K2-AG43	AM403313	3	Uncult. *Bacteroidetes* bacterium JG35-K1-AG34 (AM116744)	98.9
			Uncult. bacterium TLM11/TLMdgge04 (AF534435)	97.8
			Uncult. bacterium 69-27 (DQ833502)	97.2
			Uncult. bacterium CV24 (DQ4992283)	97.3
			Uncult. *Bacteroidetes* bacterium JG35+U2A-AG22 (AM114444)	97.0
			Firmicutes	
JG35-K2-AG49	AM403318	2	Uncult. bacterium JG35+U3-JT7 (AM084896)	99.8
			Unident. eubacterium BSV06 (AJ229180)	98.4
			Bacillus sp. 19498 (AJ315066)	98.1

* - taking those parts of the gene which were considered by the BLAST search.

Table S2.4. Affiliation of the 16S rRNA gene clones of soil sample JG35-K2. (continued)

Clone name	Accession No. EMBL	No. of clones	Closest phylogenetic relative (EMBL No.)	BLAST % of similarity*
			Actinobacteria	
JG35-K2-AG26	AM403314	6	Uncult. actinobacterium JG35-K1-AG19 (AM116745)	99.7
			Arthrobacter sp. J64 (AJ864856)	99.6
			Arthrobacter sp. CAB1 (AB039736)	99.8
			Uncult. bacterium AKAU3635 (DQ125616)	99.8
			Uncult. actinobacterium JG37-AG-83 (AJ519399)	99.8
JG35-K2-AG91	AM403315	6	*Arthrobacter* sp. JG37-Iso2 (AM2292605)	100
			Arthrobacter sp. Tibet-IIU21 (DQ177477)	99.8
			Uncult. bacterium AKAU3843 (DQ125735)	99.8
			Arthrobacter sp. CAB1 (AB039736)	99.6
			Uncult. actinobacterium JG37-AG-83 (AJ519399)	99.8
			Uncult. actinobacterium JG35+U1-AG21 (AM114426)	99.6
JG35-K2-AG82	AM403316	7	*Arthrobacter* sp. A1-1 (AY512628)	100
			Arthrobacter sp. A4Z24 (AY512627)	100
			Uncult. actinobacterium JG35+U1-AG42 (AM114428)	99.9
JG35-K2-AG85	AM403317	3	Uncult. bacterium AKAU3827 (DQ125727)	99.4
			Uncult. bacterium AKAU3690 (DQ125644)	99.4
			Arthrobacter sp. SIB M20(5) (DQ628957)	99.5
			Arthrobacter polychromogenes A1-69 (AY512631)	99.1
			Uncult. actinobacterium JG35-K1-AG19 (AM116745)	99.5
			Gemmatimonadetes	
JG35-K2-AG2	AM116754	2	Uncult. bacterium JG34-KF-314 (AJ532726)	99.7
			Uncult. bacterium #0319-7G21 (AF234148)	98.7
			***Candidate division* TM7**	
JG35-K2-AG47	AM116752	3	Uncult. soil bacterium TF7 (DQ248299)	97.6
			Uncult. candidate division TM7 bacterium SM1G12 (AF445701)	95.6
			Planctomycetes	
JG35-K2-AG64	AM116753	2	Uncult. bacterium 030B10_P_DI_P15 (BX294819)	95.1
			Pirellula sp. Schlesner 678 (X81947)	91.0

* - taking those parts of the gene which were considered by the BLAST search.

Table S2.5. Affiliation of the 16S rRNA gene clones of soil sample JG35-U3.

Clone name	Accession No. EMBL	No. of clones	Closest phylogenetic relative (EMBL No.)	BLAST % similarity*
			Alphaproteobacteria	
JG35+U3-JT3	AM084882	2	Uncult. alphaproteobacterium GuBH2-AD-69 (AJ519654)	98.4
			Uncult. alphaproteobacterium JG35+U2B-AG106 (AM116725)	98.4
JG35+U3-AG20	AM403319	5	Uncult. bacterium BS38 (AY963476)	96.5
			Uncult. bacterium JH-GY22 (DQ537531)	99.0
			Uncult. Green Bay ferromanganous micronodule MNF4 (AF292996)	96.0
			Rhodoplanes elegans AS130 (D25311)	95.6
JG35+U3-JT33	AM084883	2	Uncult. alphaproteobacterium JG35-2-JT69 (AM072412)	99.7
			Uncult. alphaproteobacterium AKYG421 (AY921897)	99.4
			Uncult. alphaproteobacterium JG37-AG-111 (AJ518776)	99.3
JG35+U3-AG41	AM403320	3	Uncult. alphaproteobacterium AKYG1687 (AY921972)	99.5
			Uncult. alphaproteobacterium JG34-KF-416 (AJ532709)	99.3
			Uncult. alphaproteobacterium JG35+U1-AG77 (AM071370)	99.1
JG35+U3-JT67	AM084884	4	Uncult. alphaproteobacterium JG36-GS -70 (AJ582034)	99.4
			Pedomicrobium manganicum ACM 3038 (X97691)	96.4
JG35+U3-JT89	AM084885	2	Uncult. bacterium mw5 (DQ163946)	99.8
			Caulobacter henricii ATCC 15253 ^{T} (AJ227758)	96.1
JG35+U3-JT90	AM084886	2	*Hyphomicrobium zavarzinii* ZV-580 (Y14306)	91.6
JG35+U3-AG105	AM403321	3	Uncult. bacterium AKAU3592 (DQ125593)	98.3
			Hyphomicrobium sp. LAT3 (AY934489)	97.7
			Hyphomicrobium sp. PMC (AF279787)	97.7
			Betaproteobacteria	
JG35+U3-AG5	AM403322	7	Uncult. betaproteobacterium JG35-K2-AG7 (AM403304)	99.8
			Uncult. betaproteobacterium JG35-K4-AG145 (AM403301)	99.8
			Uncult. betaproteobacterium JG35-K4-KF82 (AM292626)	99.5
			Uncult. betaproteobacterium JG35+U1-AG61 (AM071373)	99.1
			Ultramicrobacterium HI-G4 (DQ205303)	98.6

* - taking those parts of the gene which were considered by the BLAST search.

Table S2.5. Affiliation of the 16S rRNA gene clones of soil sample JG35-U3. (continued)

Clone name	Accession No. EMBL	No. of clones	Closest phylogenetic relative (EMBL No.)	BLAST % similarity*
			***Betaproteobacteria* (continued)**	
JG35+U3-JT14	AM084887	2	Uncult. betaproteobacterium 98 (AB252928)	98.1
			Uncult. betaproteobacterium JG37-AG-35 (AJ518782)	97.5
JG35+U3-AG15	AM403323	8	Uncult. betaproteobacterium B-BH93 (AY622261)	98.7
			Uncult. betaproteobacterium JG35-K2-AG37 (AM403305)	98.7
			Uncult. bacterium AKAU3781 (DQ125693)	98.6
			Uncult. betaproteobacterium JG35+U2B-AG14 (AM116726)	98.6
			Uncult. *Janthinobacterium* sp. JG35+U1-AG27 (AM071372)	98.4
JG35+U3-JT43	AM084888	2	*Herbaspirillum* sp. PIV-34-1 (AJ505863)	98.7
			Herbaspirillum sp. G8A1 (AJ012069)	98.6
			Gammaproteobacteria	
JG35+U3-AG1	AM403324	4	*Pseudomonas* sp. PR04 (DQ640008)	99.6
			Pseudomonas sp. BE3dil (AY263472)	99.6
			Deltaproteobacteria	
JG35+U3-JT117	AM084889	2	Uncult. deltaproteobacterium B-BK96 (AY622263)	96.5
			Uncult. deltaproteobacterium JG35-2-JT77 (AM072419)	95.5
			Acidobacteria	
JG35+U3-JT12	AM084890	2	Uncult. soil bacterium ABS-400 (AY289388)	97.2
			Uncult. *Holophaga* sp. JG34-KF-27 (AJ532717)	96.9
JG35+U3-JT17	AM084891	3	Uncult. soil bacterium 869-1 (AY326539)	97.2
			Uncult. *Acidobacteria* bacterium JG35-U4-KF38 (AM292625)	97.2
JG35+U3-JT35	AM084892	3	Uncult. bacterium Biofilm_1093d_c1 (DQ058683)	97.6
			Uncult. soil bacterium UC3 (DQ297989)	97.2
			Uncult. *Acidobacteria* bacterium JG35-U4-KF19 (AM292624)	96.8
			Uncult. *Acidobacteria* bacterium AKYG811 (AY921919)	96.6
JG35+U3-JT93	AM084893	2	Uncult. *Acidobacteria* bacterium 32d1 (AY281353)	97.4
			Bacteroidetes	
JG35+U3-JT96	AM084894	2	Metal-contaminated soil clone K20-54 (AF145849)	97.4
			Flavobacterium sp. WB 4.4-46 (AM177637)	96.6
JG35+U3-AG111	AM403325	3	*Flavobacterium* sp. WB 2.1-80 (AM167560)	98.1
			Flavobacterium sp. WB 4.1-14 (AM177623)	97.6
			Uncult. *Bacteroidetes* bacterium JG35-2-JT78 (AM072425)	97.2
			Firmicutes	
JG35+U3-JT4	AM084895	5	Uncult. bacterium JG35-K4-KF40 (AM292607)	99.5
			Uncult. bacterium JG35-K4-AG7 (AM403302)	99.5
			Uncult. bacterium TANB115 (AY667268)	99.1
			Uncult. *Clostridium* sp. JG35-U4-KF21 (AM292620)	98.8
			Uncult. bacterium Hot Creek 31 (AY168741)	98.6
			Clostridium chromoreductans GCAF-1 (AY228334)	98.5
JG35+U3-JT7	AM084896	2	Uncult. bacterium JG35-K2-AG49 (AM403318)	99.8
			Bacillus sp. 19498 (AJ315066)	98.0
			Uncult. bacterium JG35-K4-KF86 (AM292611)	97.9
			Actinobacteria	
JG35+U3-AG37	AM403326	5	Uncult. bacterium AKAU3635 (DQ125616)	99.8
			Uncult. bacterium AKAU3583 (DQ125587)	99.8
			Uncult. actinobacterium JG35+U1-AG21 (AM114426)	99.8
			Gemmatimonadetes	
JG35+U3-JT62	AM084897	2	*Gemmatimonadetes* bacterium Ellin7146 (AY673312)	98.5

* - taking those parts of the gene which were considered by the BLAST search.

Chapter 3

Changes in nitrate-reducing bacterial community of the uranium mining waste pile Haberland induced by uranyl or sodium nitrate treatments

Andrea Geissler and Sonja Selenska-Pobell

ABSTRACT

Changes in nitrate-reducing community of a soil sample, collected from a depth of 2 m of a uranium mining waste pile, induced by treatments with uranyl or sodium nitrate under aerobic and anaerobic conditions were studied by using the *nar*G gene, encoding membrane-bound nitrate reductase as a functional marker. Most of the sequences found in the original untreated sample were related to the NarG of the alphaproteobacterium *Brucella suis* and formed distinct clusters with other environmental sequences retrieved from not yet cultured bacteria. Two additional small groups of sequences clustered with NarG of the betaproteobacterial species *Thiobacillus denitrificans* and *Polaromonas naphthalenivorans*. The incubation of a sub-sample with uranyl nitrate under aerobic conditions induced a strong shifting in the pool of the *nar*G genes. No sequences related to the "*Brucella*"-like clusters were found after this treatment and only two small groups of sequences shared relatedness with the above mentioned "*Polaromonas*"-like NarG. Instead, several groups of sequences were retrieved which formed a compact cluster, distantly related to the NarG of another betaproteobacterial species, *Chromobacterium violaceum*. The largest group of sequences in this sub-sample was related to the NarG of *Geobacter metallireducens*. One additional group shared a high identity with the NarG of *Arthrobacter* spp. and another one was identical with the NarG of *Pseudomonas fluorescens*. The changes in the nitrate-reducing community induced by the treatment with sodium nitrate under aerobic conditions were less drastic but also significant. In this case most of the NarG sequences formed novel sub-clusters within the "*Brucella*"-like and in the "*Polaromonas*"-like clusters found in the untreated sample, or formed novel clusters distantly related to them. The rest of the clones formed a tight cluster with the "*Arthrobacter*"-like NarG sequences found in the already mentioned uranyl nitrate treated sub-sample. The changes in the *nar*G-gene harbouring bacterial populations in the sub-samples treated under anaerobic conditions were notably different. In a strong contrast to the above described treatment under aerobic conditions, no "*Brucella*"-like or "*Arthrobacter*"-like NarG sequences were found in the sub-sample treated anaerobically with sodium nitrate. This treatment led mainly to a propagation of bacterial groups carrying "*Bacillus*"-like NarG, not expressed in any of the other samples studied. NarG sequences were also predominant which allied with the "*Chromobacterium*"-like cluster retrieved from the above mentioned sub-sample treated with uranyl nitrate under aerobic conditions. Interestingly, in the anaerobically with uranyl nitrate treated sub-sample the most predominant group of NarG sequences was closely related to the above mentioned "*Polaromonas*"-like cluster. However, it was ten times larger then those found in the other samples. Remarkable for this sub-sample

was that more than 80% of the NarG sequences were closely related to the NarG of particular *Alpha-* or *Betaproteobacteria* found in small numbers in the original sample.

INTRODUCTION

Nitrate-reducing bacteria play a central role in the nitrogen cycle by reducing nitrate into nitrite to obtain energy. The nitrite can then be reduced into gaseous nitrogen compounds by denitrification or into ammonium by dissimilatory nitrate reduction (Philippot *et al*., 2002; Chèneby *et al*., 2003). The nitrate reduction as the first step of denitrification is common with nitrate reduction into ammonium and is not considered to be specific for denitrification (Goregues *et al*., 2005). The interest in denitrification is connected to the processes, which are influenced by it, for example, the depletion of nitrate from soils (loss of essential plant nutrient) and removal of nitrate from wastewaters. Nitrous oxides as intermediates of denitrification contribute to modify the global atmospheric chemistry, essentially through the greenhouse effect (Lashof & Ahuja, 1990). Dissimilatory nitrate reduction, which leads to nitrogen conservation, is of minor significance in soils (Chèneby *et al*., 2003). Bacteria, which are capable to reduce nitrate are widespread in the environment. Dissimilatory nitrate reduction into nitrite can be performed by taxonomically diverse microorganisms including members of the *Alpha-*, *Beta-*, *Gamma-*, and *Epsilonproteobacteria*, high- and low-GC Gram-positive *Bacteria* and also of *Archaea* (Tiedje, 1988; Afshar *et al*., 2001; Philippot, 2002, 2005).

Nitrate is often a cocontaminant in uranium mining wastes and can influence the mobility of uranium (Senko *et al*., 2002, 2005; North *et al*., 2004). Microorganisms can utilise other electron acceptors than oxygen for anaerobic respiration. It is considered that the use of nitrate as electron acceptor is thermodynamically more favourable than U(VI) (Finneran *et al*., 2002). In addition, the intermediates of denitrification are able to oxidise and mobilise U(IV), which affects the fate of uranium in anoxic subsurface environments (Senko *et al*., 2002; North *et al*., 2004). Some bacteria are able to use Fe(II) or even U(IV) as electron donors instead of organic compounds (Straub *et al*., 1996; Beller, 2005; Weber *et al*., 2006). The anaerobic nitrate-dependent Fe(II) oxidation by different microorganisms is already described (Straub *et al*., 1996, 2004; Straub & Buchholz-Cleven, 1998; Beller *et al*., 2006; Weber *et al*., 2006). Finneran *et al*., (2002) hypothesised that Fe(III) produced by the nitrate-dependent Fe(II)-oxidising bacteria can oxidise U(IV). Alternatively, Lack *et al*. (2002) demonstrated that Fe(III) oxides produced by the anaerobic nitrate-dependent Fe(II) oxidation are involved in a rapid absorption of U(VI) from solutions. Recently, the anaerobic, nitrate-dependent

oxidation of U(IV) oxide minerals by the chemolithoautotrophic bacterium *Thiobacillus denitrificans* was demonstrated (Beller, 2005).

Three distinct nitrate reductase classes have been identified in prokaryotes: NAS, NAR and NAP (Richardson *et al*., 2001). NAS is participating in nitrogen assimilation (Lin & Stewart, 1998). The membrane-bound nitrate reductase NAR is typically involved in nitrate respiration under anoxic conditions (Richardson *et al*., 2001) and is more widely spread among microorganisms than the periplasmic one, NAP, restricted to Gram-negative bacteria (Philippot *et al*., 2002). Some bacteria express both enzymes, NAR and NAP, which can have different physiological roles (Richardson *et al*., 2001). The membrane-bound nitrate reductase NAR has been studied in several species but is best characterized in *Escherichia coli* (Blasco *et al*., 1989, Richardson *et al*., 2001). The enzyme is composed of three subunits: a ~ 140-kDa bis-MGD catalytic subunit, encoded by *nar*G, a ~ 60-kDa electron transfer subunit, encoded by *nar*H, which binds four FeS clusters and a di-*b*-heme integral membrane quinol dehydrogenase subunit, encoded by *nar*I (Richardson *et al*., 2001; Philippot, 2005). To study the microbial community capable of reducing nitrate, a culture-independent approach using the membrane-bound nitrate reductase gene *nar*G as a molecular marker was performed (Gregory *et al*., 2000; Philippot *et al*., 2002). For this purpose degenerated primers based on sequence data from cultured organisms were developed. Different *nar*G primer sets were developed and used to detect genes of membrane-bound nitrate reductase in nitrate-respiring bacteria and in community DNA (Gregory *et al*., 2000; Philippot *et al*., 2002; Goregues *et al*., 2005). The nitrate-reducing communities were investigated in different environments by molecular methods as well as by cultivation approaches in freshwater sediments (Gregory *et al*., 2003), in unplanted and maize-planted soils (Philippot *et al*., 2002), in fertilised soils (Enwall *et al*., 2005) and in mine soils (Shirey & Sextone, 1989; Héry *et al*., 2005).

To our knowledge, this is the first analysis of the changes in nitrate-reducing bacterial community induced by sodium or uranyl nitrate treatments of uranium mining waste pile samples under aerobic and anaerobic conditions. Results of these analyses were compared with those obtained by the 16S rRNA gene retrieval of the same samples (Chapters 1 & 2).

MATERIALS AND METHODS

Soil samples

Several portions of a soil sample collected from 2 m depth of the uranium mining waste pile Haberland near the town of Johanngeorgenstadt in Germany were supplemented with uranyl nitrate (pH 4.0) (samples JG35+U1 and JG35+U4) or sodium nitrate (pH 4.0) (samples

JG35-K1 and JG35-K4) as described in Chapters 1 & 2 (see also Table 3.1). The samples were incubated for four weeks under aerobic or anaerobic conditions without shaking at 10 °C in the dark. The anaerobic conditions were established by fumigation through a sterile filter with an anaerobic gas mixture containing N_2 and CO_2 (80:20) and a further incubation in an anaerobic jar with Anaerocult® A (Merck, Darmstadt, Germany).

Table 3.1. Soil samples used.

Samples	Treatment	Aeration conditions	Uranium-content [mg kg^{-1}]	Added nitrate [mg kg^{-1}]
JG35-2	Untreated	-	26 - 40	-
JG35+U1	Uranyl nitrate	Aerobic	~100	31
JG35-K1	Sodium nitrate	Aerobic	26 - 40	32
JG35+U4	Uranyl nitrate	Anaerobic	~100	31
JG35-K4	Sodium nitrate	Anaerobic	26 - 40	32

DNA extraction

Total DNA was recovered from 3.5 g of each of the soil samples studied as described in Chapters 1 & 2.

Construction of *nar*G gene clone libraries

*Nar*G gene fragments were amplified by PCR in a reaction mixture with a volume of 10 µl, containing 0.9 µl of diluted DNA template (1:20, 1:30, 1:50), 2.5 mM of $MgCl_2$ (Applied Biosystems, Foster City, California, USA), 200 nM of each of the four deoxynucleoside triphosphates, 5 pmol of each of the forward and the reverse primers, 0.5 U Ampli*Taq* Gold® Polymerase with the corresponding GeneAmp 10x PCR Puffer II (Applied Biosystems, Foster City, California, USA), 0.08 µl bovine serum albumin (10 mg ml^{-1}, Promega, Madison, Wisconsin, USA) and 0.5 µl dimethyl sulphoxide (Sigma, St. Louis, Missouri, USA). The forward primer was na3F (5'-TGGGCICAYTAYGTIGGICA-3'), and the reverse primer was narG5'R (5'-ANRTCRTSYTTYTCRTACCAIGTIGC-3') (Goregues *et al*., 2005). The PCR amplifications were performed in a T3 thermocycler from Biometra (Göttingen, Germany) with an initial denaturation at 94 °C for 7 min, followed by 30 cycles consisting of a 30 s denaturing step at 94 °C, an annealing step at 48 °C for 30 s, and a 40 s elongation step at 72 °C. The amplification was completed with an extension period of 3 min at 72 °C. The presence and the correct size of the amplification products were visualised by electrophoresis in 1.2% agarose gels. The amplified *nar*G gene fragments of three parallel reactions performed with DNA of different dilutions were mixed and 4 µl were used for cloning in *E. coli* by using the TOPO-TA Cloning® system of Invitrogen (Gröningen, the

Netherlands), following the manufacturers instructions. A total of nearly 50 single white colonies per sample were randomly selected and further analysed.

RFLP screening and sequencing

For the restriction fragment length polymorphism (RFLP) screening the inserted *nar*G gene fragments were amplified by PCR directly from the host *E. coli* cells with vector specific M13(-40) and M13rev primers by using *Taq* DNA polymerase (Promega, Mannheim, Germany) as described in Chapter 1. The clones containing correct inserts were stored as glycerol cultures at -80 °C. Aliquots of the PCR amplified *nar*G gene products were digested with 1 unit of the four-base-specific restriction endonuclease *Msp*I in the corresponding buffer (Promega, Mannheim, Germany) overnight at 37 °C. The digests were separated in 3.5% Small DNA Low Melt Agarose (Biozym, Hessisch Oldenburg, Germany) gels in a 0.5xTBE buffer and visualised by staining with ethidium bromide and UV illumination. The resulting RFLP patterns were visually compared and grouped in RFLP-types. Representative clones from each of the RFLP types were selected for sequencing. The *nar*G gene products of the selected clones were purified by using an Edge BioSystems QuickStep®2 PCR Purification Kit (MoBiTec, Göttingen, Germany) and directly sequenced by using an ABI Prism® Big Dye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA), following the manufacturers instructions. Sequences were obtained by using the plasmid specific primers PE-Sp6 and/or PE-T7. DNA sequences were determined on an automated sequencer (ABI PRISM®, PE Applied Biosystems, Foster City, California, USA).

Phylogenetic analysis

The *nar*G gene sequences were translated into amino acid sequences (www.expasy.ch) and the structure of the deduced proteins were compared with those available in the GenBanks by using BLAST analysis (Basic Local Alignment Search Tool) (Altschul *et al*., 1997). The CLUSTALW alignment program version 1.7 was used for DNA and amino acid sequence alignments (Thompson *et al*., 1994). Phylogenetic trees were generated by using PRODIST version 3.5c for the protein matrix and DNADIST version 3.5c for the DNA matrix (Felsenstein, 1993). The resulting *nar*G gene sequences were deposited to the EMBL Nucleotide Sequence Database under accession numbers AM412320 to AM412362.

RESULTS

Clone libraries with almost 50 clones containing *nar*G gene fragments were constructed for each of the samples studied. As shown in Table 3.2, almost half of the clones in the clone library of the untreated sample JG35-2 had an individual RFLP profile. The treatment with uranyl or sodium nitrate under aerobic or anaerobic conditions resulted in reduction of the number of the RFLP groups and of the individual clones. This is an indication that the diversity in the treated samples was lower in comparison to the untreated sample (Table 3.2). One clone of each RFLP group was subjected to sequence analysis. Altogether 43 *nar*G gene sequences were phylogenetically affiliated.

Table 3.2. Description of the clone libraries constructed for the soil samples used and their RFLP analysis.

Soil samples	Number of clones	RFLP-grouping	Number of individual RFLP-profiles
JG35-2	50	29 (4,4,3,2,2,2,2,2,2,2,2,2)	21
JG35+U1	47	33 (13,5,4,3,2,2,2,2)	14
JG35-K1	45	28 (5,5,5,3,2,2,2,2,2)	17
JG35+U4	52	40 (22,7,3,2,2,2,2)	12
JG35-K4	49	38 (11,8,7,3,3,2,2,2)	11

Nitrate-reducing community identified in the untreated soil sample

Most of the deduced amino acid sequences retrieved from the untreated sample JG35-2 made tight clusters with NarG sequences of not yet cultured bacteria (Table 3.3). Most of them were distantly related to the NarG of the alphaproteobacterium *Brucella suis* 1330 (Paulsen *et al*., 2002) (Fig. 3.1). As evident from the results presented in Fig. 3.1, the sequences JG35-2-AG-narG2, JG35-2-AG-narG22 and JG35-2-AG-narG44 clustered with the NarG sequence of the uncultured bacteria C67 and F89, identified in a soil sampled under *Lolium perenne* with low nitrogen fertilisation (Deiglmayr *et al*., 2004). The sequences JG35-2-AG-narG20 and JG35-2-AG-narG36 were closely related to the NarG sequence of the unidentified bacterium 7g31 (Dambreville *et al*., 2006) (Table 3.3, Fig. 3.1). As shown in Fig. 3.1, the sequences JG35-K1-AG-narG27 and JG35+U4-AG-narG9 also belong to the same cluster (next paragraph). The sequences JG35-2-AG-narG3 and JG35-2-AG-narG4 shared an identity of 93% and 95% with the NarG sequence of the uncultured bacterium NCSD62, which was found in nickel mine spoils revegetated with the plant *Serianthes calycina* (Héry *et al*., 2005). The sequence JG35-2-AG-narG15 was closely related to the NarG sequence of the uncultured bacterium GA31, which was identified in soil amended with mucilage (Mounier *et al*., 2004).

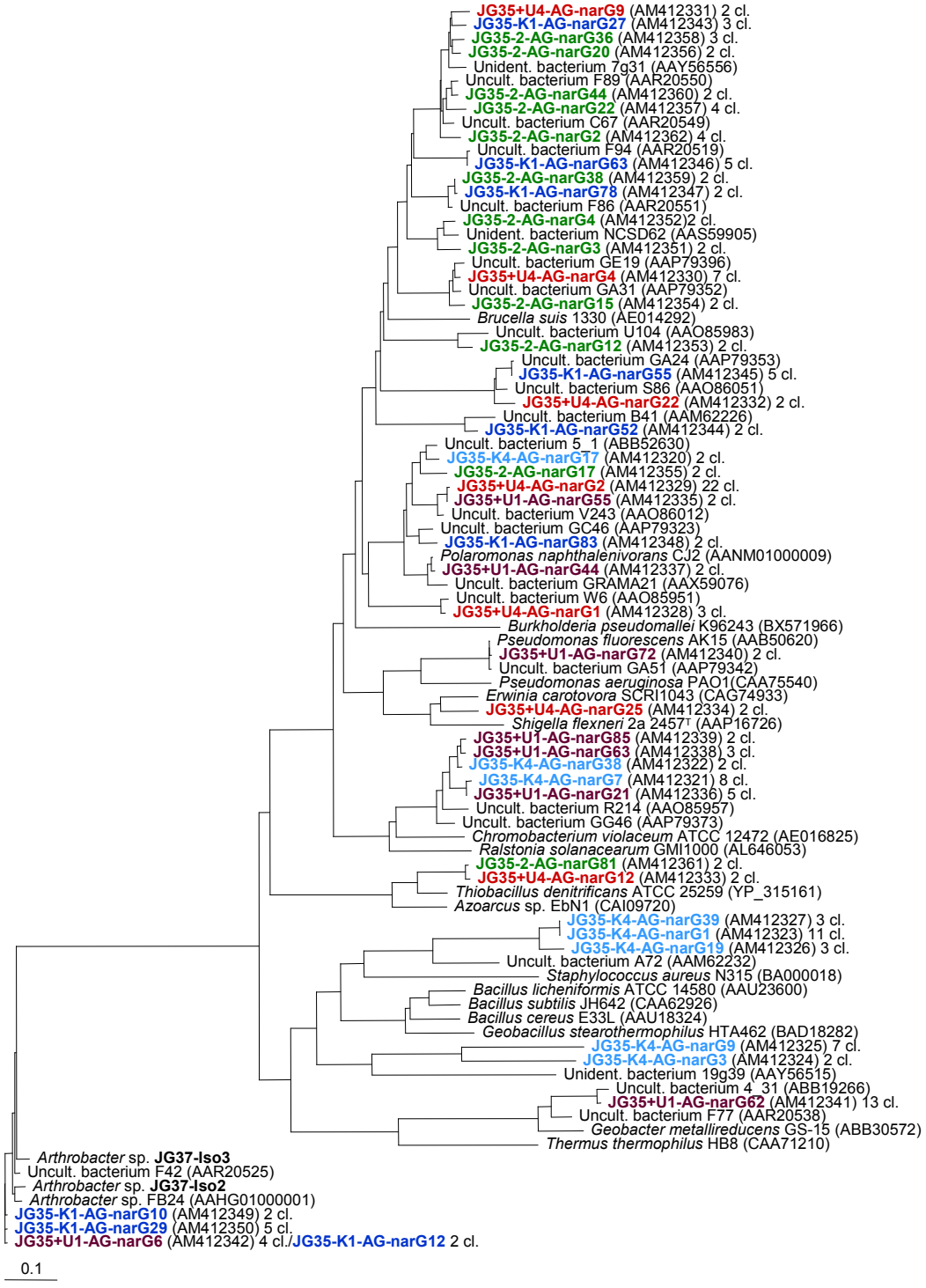

Fig. 3.1. Phylogentic dendrogram of the deduced protein sequences from the studied *nar*G clones retrieved from the untreated sample **JG35-2** of the uranium mining waste pile Haberland, the uranyl nitrate treated samples **JG35+U1** (aerobic) and **JG35+U4** (anaerobic) and the sodium nitrate treated samples **JG35-K1** (aerobic) and **JG35-K4** (anaerobic) and their closest amino acid sequences. The accession numbers of the NarG sequences are given in brackets and after them the numbers of the clones retrieved are written. The dendrogram was generated using distance-matrix and neighbour-joining methods.

A group of two clones, represented by the sequence JG35-2-AG-narG38, clustered with the sequence JG35-K1-AG-narG78, which was identified in the sodium nitrate treated sample incubated for 4 weeks under aerobic conditions (next paragraph) and shared 98% of similarity with the clone F86 (Deiglmayr *et al*., 2004).

Table 3.3. NarG sequences retrieved from the sample JG35-2 and their closest matches in the GenBank.

Clone name	Accession No. EMBL	No. of clones	Closest amino acid sequences (EMBL No.)	BLAST % of similarity*	References
JG35-2-AG-narG2	AM412362	4	Uncult. bacterium C67 (AAR20549)	94	Deiglmayr *et al*., 2004
			Unident. bacterium 7g31 (AAY56556)	92	Dambreville *et al*., 2006
JG35-2-AG-narG3	AM412351	2	Unident. bacterium NCSD62 (AAS59905)	93	Héry *et al*., 2005
			Unident. bacterium NCSF48 (AAS59891)	94	Héry *et al*., 2005
JG35-2-AG-narG4	AM412352	2	Unident. bacterium NCSD62 (AAS59905)	95	Héry *et al*., 2005
			Unident. bacterium NCSF48 (AAS59891)	94	Héry *et al*., 2005
JG35-2-AG-narG12	AM412353	2	Uncult. bacterium U104 (AAO85983)	91	Chèneby *et al*., 2003
			Unident. bacterium NCGA78 (AAS59883)	90	Héry *et al*., 2005
JG35-2-AG-narG15	AM412354	2	Uncult. bacterium GA31 (AAP79352)	95	Mounier *et al*., 2004
			Uncult. bacterium GE19 (AAP79396)	94	Mounier *et al*., 2004
JG35-2-AG-narG17	AM412355	2	Uncult. bacterium 5_1 (ABB52630)	92	unpublished
			Unident. bacterium 19g24 (AAY56509)	91	Dambreville *et al*., 2006
JG35-2-AG-narG20	AM412356	2	Unident. bacterium 7g31 (AAY56556)	94	Dambreville *et al*., 2006
			Uncult. bacterium F89 (AAR20550)	94	Deiglmayr *et al*., 2004
JG35-2-AG-narG22	AM412357	4	Uncult. bacterium C67 (AAR20549)	94	Deiglmayr *et a*l., 2004
			Uncult. bacterium F89 (AAR20550)	94	Deiglmayr *et al*., 2004
JG35-2-AG-narG36	AM412358	3	Uncult. bacterium V25 (AAO85944)	95	Chèneby *et al*., 2003
			Unident. bacterium 7g31 (AAY56556)	94	Dambreville *et al*., 2006
JG35-2-AG-narG38	AM412359	2	Uncult. bacterium F86 (AAR20551)	98	Deiglmayr *et a*l., 2004
			Uncult. bacterium F38 (AAM62260)	94	Philippot *et al*., 2002
JG35-2-AG-narG44	AM412360	2	Uncult. bacterium F89 (AAR20550)	97	Deiglmayr *et a*l., 2004
			Uncult. bacterium LT-075_12 (ABF20966)	96	unpublished
JG35-2-AG-narG81	AM412361	2	*Thiobacillus denitrificans* ATCC 25259 (YP_315161)	84	Beller *et al*., 2006
			Uncult. bacterium GG11 (AAP79370)	81	Mounier *et al*., 2004

* - taking those parts of the gene which were considered by the BLAST search.

Three other NarG sequences, JG35-2-AG-narG12, JG35-2-AG-narG17 and JG35-2-AG-narG81 retrieved from the untreated sample were affiliated with NarG sequences without the above described cluster. The sequence JG35-2-AG-narG12 shared 91% of similarity with the NarG of the uncultured bacterium U104, encountered in unplanted soil from INRA experimental station (Chèneby *et al*., 2003). The sequence JG35-2-AG-narG17 was with 92% similar to the NarG sequence of the uncultured bacterium 5_1 (Table 3.3). As evident from the results presented in Fig. 3.1, the sequence JG35-K4-AG-narG17, which was identified in the sample JG35-K4, also belongs to the same sub-cluster (see below). Both sequences and their references were distantly related to the NarG of the betaproteobacterium *Polaromonas naphthalenivorans* CJ2 (Fig. 3.1).

As shown in Table 3.3 and Fig. 3.1, the sequence JG35-2-AG-narG81 affiliated with a rather low identity of 84% with the NarG of the betaproteobacterium *Thiobacillus denitrificans* ATCC 25259, an obligate chemolithoautotrophic facultative anaerobic bacterium, which is

capable of anaerobic, nitrate-dependent oxidation of U(IV) oxide minerals (Beller, 2005; Beller *et al*., 2006).

Nitrate-reducing community in the soil samples treated with uranyl or sodium nitrate under aerobic conditions

The treatments of the sub-samples JG35-K1 and JG35+U1 with sodium or uranyl nitrate under aerobic conditions for 4 weeks induced shiftings in the nitrate-reducing community. As shown in Fig. 3.1, no sequences related to the „*Brucella suis*"-like NarG cluster were found after the treatment with uranyl nitrate. Only two small groups of sequences (see sequences JG35+U1-AG-narG44 and JG35+U1-AG-narG55 in Fig. 3.1 and Table 3.4) clustered with different similarity with the NarG of *Polaromonas naphthalenivorans* CJ2. The sub-sample JG35+U1 was strongly predominated by a group of 13 clones, represented by the sequence JG35+U1-AG-narG62. This sequence shared a similarity of 97% with the NarG of another uncultured bacterium 4_31 and still 83% with the NarG of the deltaproteobacterium *Geobacter metallireducens* GS-15 (EMBL No. ABB30572) (Table 3.4, Fig. 3.1).

Table 3.4. NarG sequences retrieved from the sample JG35+U1 and their closest matches in the GenBank.

Clone name	Accession No. EMBL	No. of clones	Closest amino acid sequences (EMBL No.)	BLAST % of similarity*	References
JG35+U1-AG-narG55	AM412335	2	Uncult. bacterium V243 (AAO86012)	97	Chèneby *et al*., 2003
			Uncult. bacterium GB14 (AAP79379)	95	Mounier *et al*., 2004
JG35+U1-AG-narG21	AM412336	5	Uncult. bacterium R214 (AAO85957)	90	Chèneby *et al*., 2003
			Chromobacterium violaceum ATCC 12472 (AE016825)	75	Vasconcelos *et al*., 2003
JG35+U1-AG-narG44	AM412337	2	*Polaromonas naphthalenivorans* CJ2 (AANM01000009)	98	unpublished
			Uncult. bacterium GRAMA46 (AAX59081)	96	Enwall *et al*., 2005
JG35+U1-AG-narG63	AM412338	3	Uncult. bacterium GG46 (AAP79373)	91	Mounier *et al*., 2004
			Uncult. bacterium R214 (AAO85957)	91	Chèneby *et al*., 2003
			Chromobacterium violaceum ATCC 12472 (AE016825)	77	Vasconcelos *et al*., 2003
JG35+U1-AG-narG85	AM412339	2	Uncult. bacterium GG46 (AAP79373)	91	Mounier *et al*., 2004
			Uncult. bacterium R214 (AAO85957)	90	Chèneby *et al*., 2003
			Chromobacterium violaceum ATCC 12472 (AE016825)	77	Vasconcelos *et al*., 2003
JG35+U1-AG-narG72	AM412340	2	*Pseudomonas fluorescens* AK15 (AAB50620)	100	Philipot *et al*., 1997
			Uncult. bacterium GA51 (AAP79342)	99	Mounier *et al*., 2004
JG35+U1-AG-narG62	AM412341	13	Uncult. bacterium 4_31 (ABB19266)	97	unpublished
			Geobacter metallireducens GS-15 (ABB30572)	83	unpublished
JG35+U1-AG-narG6	AM412342	4	Uncult. bacterium F42 (AAR20525)	96	Deiglmayr *et al*., 2004
			Arthrobacter sp. FB24 (AAHG01000001)	96	unpublished

* - taking those parts of the gene which were considered by the BLAST search.

The two almost identical sequences JG35+U1-AG-narG63 and JG35+U1-AG-narG85 as well as the sequence JG35+U1-AG-narG21 clustered with the NarG of the uncultured bacterium R214, identified in maize planted rhizospheric soil (Chèneby *et al*., 2003). As shown in Fig. 3.1, these sequences were distantly related to the NarG sequence of the betaproteobacterium *Chromobacterium violaceum* ATCC 12472, which uses nitrate and fumarate as final electron acceptors in a total absence of oxygen (Vasconcelos *et al*., 2003). The sequences JG35-K4-AG-narG7 and JG35-K4-AG-narG38, which were identified in the sodium nitrate treated sample JG35-K4 incubated under anaerobic conditions, also belong to the same cluster (see next paragraph). Interestingly, the sequence JG35+U1-AG-narG72, representing two clones, was identical to the NarG of the gammaproteobacterium *Pseudomonas fluorescens* AK15 (Philippot *et al*., 1997; Table 3.4). The sequence JG35+U1-AG-narG6 was related to the NarG of *Arthrobacter* sp. FB24 (EMBL No. AAHG01000001) (Table 3.4, Fig. 3.1).

Interestingly, closely related NarG harbouring *Arthrobacter* populations were predominat in the sodium nitrate treated sub-sample JG35-K1. They were represented by the sequences JG35-K1-AG-narG10, JG35-K1-AG-narG12 and JG35-K1-AG-narG29, which shared a high similarity with the NarG sequence of the uncultured bacterium F42 (Deiglmayr *et al*., 2004) and with the NarG deduced from the genome analysis of the *Arthrobacter* sp. FB24 (EMBL No. AAHG01000001) (Table 3.5).

Table 3.5. NarG sequences retrieved from the sample JG35-K1 and their closest matches in the GenBank.

Clone name	Accession No. EMBL	No. of clones	Closest amino acid sequences (EMBL No.)	BLAST % of similarity*	References
JG35-K1-AG-narG27	AM412343	3	Unident. bacterium 7g31 (AAY56556)	93	Dambreville *et al*., 2006
			Uncult. bacterium C67 (AAR20549)	93	Deiglmayr *et al*., 2004
JG35-K1-AG-narG52	AM412344	2	Uncult. bacterium B41 (AAM62226)	91	Philippot *et al*., 2002
			Uncult. bacterium B58 (AAM62222)	90	Philippot *et al*., 2002
JG35-K1-AG-narG55	AM412345	5	Uncult. bacterium GA24 (AAP79353)	99	Mounier *et al*., 2004
			Uncult. bacterium GA30 (AAP79336)	99	Mounier *et al*., 2004
JG35-K1-AG-narG63	AM412346	5	Uncult. bacterium F94 (AAR20519)	99	Deiglmayr *et al*., 2004
			Uncult. bacterium GRAMJ1 (AAX59106)	94	Enwall *et al*., 2005
			Uncult. bacterium C67 (AAR20549)	85	Deiglmayr *et al*., 2004
JG35-K1-AG-narG78	AM412347	2	Uncult. bacterium F86 (AAR20551)	97	Deiglmayr *et al*., 2004
			Uncult. bacterium F38 (AAM62260)	93	Philippot *et al*., 2002
JG35-K1-AG-narG83	AM412348	2	Uncult. bacterium GC46 (AAP79323)	93	Mounier *et al*., 2004
			Uncult. bacterium S20 (AAO86052)	92	Chèneby *et al*., 2003
JG35-K1-AG-narG10	AM412349	2	Uncult. bacterium F42 (AAR20525)	96	Deiglmayr *et al*., 2004
			Arthrobacter sp. FB24 (AAHG01000001)	96	unpublished
JG35-K1-AG-narG12		2	⇒ JG35+U1-AG-narG6		
			Uncult. bacterium F42 (AAR20525)	94	Deiglmayr *et al*., 2004
			Arthrobacter sp. FB24 (AAHG01000001)	96	unpublished
JG35-K1-AG-narG29	AM412350	5	Uncult. bacterium F42 (AAR20525)	96	Deiglmayr *et al*., 2004
			Arthrobacter sp. FB24 (AAHG01000001)	96	unpublished

* - taking those parts of the gene which were considered by the BLAST search.

However, the rest of the sequences retrieved from the sample JG35-K1 was in contrast to the sub-sample JG35+U1 closely related to NarG sequences of uncultured bacteria (Table 3.5). As shown in Fig. 3.1, the sequence JG35-K1-AG-narG27 was similar to the sequences JG35-2-AG-narG20 and JG35-2-AG-narG36 retrieved from the untreated sample. The sequence JG35-K1-AG-narG63, representing 5 clones, was closely related to the NarG sequence of the uncultured bacterium F94. The sequence JG35-K1-AG-narG78 clustered with the sequence JG35-2-AG-narG38, retrieved from the untreated sample, and was 97% similar to the NarG sequence of the uncultured bacterium F86. Both of the referent clones F94 and F86 were identified in soil sampled under *L. perenne* with low nitrogen fertilisation (Deiglmayr *et al*., 2004). All these sequences were distantly related to *Brucella suis* 1330 (Paulsen *et al*., 2002). The sequence JG35-K1-AG-narG83 clustered with the NarG sequence of the uncultured bacterium GC46 and was distantly related to the NarG of *Polaromonas naphthalenivorans* CJ2. As evident from Fig. 3.1, the sequences JG35-K1-AG-narG52 as well as JG35-K1-AG-narG55 together with JG35+U4-AG-narG22 (next paragraph) formed distinct novel clusters with their closest phylogenetic NarG sequences of uncultured bacteria (Table 3.5).

Nitrate-reducing community in the soil samples treated with uranyl or sodium nitrate under anaerobic conditions

The changes induced in the nitrate-reducing community by the incubations of the samples under anaerobic conditions were significantly different. In contrast to the aerobically treated samples no "*Brucella*"-like and "*Arthrobacter*"-like NarG sequences were detected in the sample JG35-K4, treated with sodium nitrate. More than 68% of the sequences retrieved from this sample were distantly related to the NarG of different *Bacillus* spp. (Table 3.6, Fig. 3.1). As evident from the results presented in Fig. 3.1, the sequences JG35-K4-AG-narG1, JG35-K4-AG-narG19 and JG35-K4-AG-narG39 were closely related to each other and shared a similarity of about 70% with the NarG sequence of the uncultured bacterium A72, which was identified in unplanted soil from the 20 cm deep top layer of an experimental field of the ENSAIA domain of La Bouzule (Nancy, France) (Philippot *et al*., 2002, Table 3.6). All these sequences affiliated with about 50% with the NarG sequence of *Bacillus cereus* E33L or *Bacillus licheniformis* ATCC 14580 (Table 3.6). The sequences JG35-K4-AG-narG3 and JG35-K4-AG-narG9 were with 49% and 55% similar to the NarG of the uncultured bacterium 19g39 (Dambreville *et al*., 2006) and with about 50% similar to the NarG of *Bacillus subtilis* JH642 or *Geobacillus stearothermophilus* HTA462 (Table 3.6). As shown in Fig. 3.1, the

sequences JG35-K4-AG-narG7 and JG35-K4-AG-narG38 represented the second predominant group in the sample JG35-K4 and formed together with the above mentioned sequences JG35+U1-AG-narG21, JG35+U1-AG-narG63 and JG35+U1-AG-narG85, retrieved from the aerobically uranyl nitrate treated sample JG35+U1, a tight cluster related to the NarG of the betaproteobacterium *Chromobacterium violaceum* ATCC 12472 (Vasconcelos *et al*., 2003). The sequence JG35-K4-AG-narG17 clustered with the sequence JG35-2-AG-narG17 encountered in the untreated sample and shared an identity of 95% with the NarG sequence of the uncultured bacterium 5_1. Both sequences were distantly related to the NarG of *Polaromonas naphthalenivorans* CJ2 (Table 3.6, Fig. 3.1).

Table 3.6. NarG sequences retrieved from the sample JG35-K4 and their closest matches in the GenBank.

Clone name	Accession No. EMBL	No. of clones	Closest amino acid sequences (EMBL No.)	BLAST % of similarity*	References
JG35-K4-AG-narG17	AM412320	2	Uncult. bacterium 5_1 (ABB52630)	95	unpublished
			Unident. bacterium 19g24 (AAY56509)	94	Dambreville *et al*., 2006
JG35-K4-AG-narG7	AM412321	8	Uncult. bacterium R214 (AAO85957)	90	Chèneby *et al*., 2003
			Uncult. bacterium GG46 (AAP79373)	89	Mounier *et al*., 2004
			Chromobacterium violaceum ATCC 12472 (AE016825)	74	Vasconcelos *et al*., 2003
JG35-K4-AG-narG38	AM412322	2	Uncult. bacterium GG46 (AAP79373)	92	Mounier *et a*l., 2004
			Uncult. bacterium R214 (AAO85957)	90	Chèneby *et al*., 2003
			Chromobacterium violaceum ATCC 12472 (AE016825)	78	Vasconcelos *et al*., 2003
JG35-K4-AG-narG1	AM412323	11	Uncult. bacterium A72 (AAM62232)	70	Philippot *et al*., 2002
			Bacillus cereus E33L (AAU18324)	56	Unpublished
JG35-K4-AG-narG3	AM412324	2	Unident. bacterium 19g39 (AAY56515)	49	Dambreville *et al*., 2006
			Bacillus subtilis JH642 (CAA62926)	49	Hoffmann *et al*., 1995
JG35-K4-AG-narG9	AM412325	7	Unident. bacterium 19g39 (AAY56515)	55	Dambreville *et al*., 2006
			Geobacillus stearothermophilus HTA462 (BAD18282)	51	Takami *et al*., 2004
JG35-K4-AG-narG19	AM412326	3	Uncult. bacterium A72 (AAM62232)	70	Philippot *et al*., 2002
			Bacillus licheniformis ATCC 14580 (AAU23600)	54	Rey *et al*., 2004
JG35-K4-AG-narG39	AM412327	3	Uncult. bacterium A72 (AAM62232)	70	Philippot *et al*., 2002
			Bacillus cereus E33L (AAU18324)	56	Unpublished

* - taking those parts of the gene which were considered by the BLAST search.

In the uranyl nitrate treated sub-sample JG35+U4, the most predominant group consisted of 22 clones and was represented by sequence JG35+U4-AG-narG2. This sequence was closely related to the already mentioned sequence JG35+U1-AG-narG55, which represented a small group of 2 clones in the sample treated with uranyl nitrate under aerobic conditions. Both sequences were closely related to the NarG sequence of the uncultured bacterium V243, identified in unplanted soil from the INRA experimental station (Chèneby *et al*., 2003), and clustered together in the NarG cluster of *Polaromonas naphthalenivorans* CJ2 (Fig. 3.1). The major part of the rest of the sequences retrieved from the sample JG35+U4 was related to sequences found in the original untreated sample. As shown in Fig. 3.1, the sequence JG35+U4-AG-narG4, representing 7 clones, clustered with the sequence JG35-2-AG-narG15

retrieved from the untreated sample. The sequence was with 97% similar to the uncultured bacteria GA31 and GE19 (Mounier *et al*., 2004). The sequence JG35+U4-AG-narG9 affiliated with the sequences JG35-2-AG-narG20 and JG35-2-AG-narG36 (Fig. 3.1). The already mentioned sequence JG35-K1-AG-narG27 also belongs to this cluster. As shown in Fig. 3.1, the sequence JG35+U4-AG-narG12 clustered with the sequence JG35-2-AG-narG81 and was with 85% similar to the NarG of the betaproteobacterium *Thiobacillus denitrificans* ATCC 25259 (Beller *et al*., 2006). The sequence JG35+U4-AG-narG22 clustered with the sequence JG35-K1-AG-narG55, which was identified in the sample aerobically treated with sodium nitrate (Fig. 3.1). The sequence JG35+U4-AG-narG1 formed with the NarG sequence of the uncultured bacterium W6 a distinct cluster. One NarG sequence (JG35+U4-AG-narG25) was with 86% similar to the NarG of the gammaproteobacterium *Erwinia carotovora* SCRI1043 (Table 3.7).

Table 3.7. NarG sequences retrieved from the sample JG35+U4 and their closest matches in the GenBank.

Clone name	Accession No. EMBL	No. of clones	Closest amino acid sequence (EMBL No.)	BLAST % of similarity*	References
JG35+U4-AG-narG1	AM412328	3	Uncult. bacterium W6 (AAO85951)	97	Chèneby *et al*., 2003
			Uncult. bacterium 323 (ABA39209)	97	unpublished
JG35+U4-AG-narG2	AM412329	22	Uncult. bacterium V243 (AAO86012)	96	Chèneby *et al*., 2003
			Uncult. bacterium GB14 (AAP79379)	94	Mounier *et al*., 2004
JG35+U4-AG-narG4	AM412330	7	Uncult. bacterium GA31 (AAP79352)	97	Mounier *et al*., 2004
			Uncult. bacterium GE19 (AAP79396)	97	Mounier *et al*., 2004
JG35+U4-AG-narG9	AM412331	2	Uncult. bacterium C67 (AAR20549)	92	Deiglmayr *et al*., 2004
			Unident. bacterium 7g31 (AAY56556)	92	Dambreville *et al*., 2006
JG35+U4-AG-narG22	AM412332	2	Uncult. bacterium S86 (AAO86051)	93	Chèneby *et al*., 2003
			Uncult. bacterium 4711 (ABA39219)	93	unpublished
JG35+U4-AG-narG12	AM412333	2	*Thiobacillus denitrificans* ATCC 25259 (YP_315161)	85	Beller *et al*., 2006
			Uncult. bacterium GG11 (AAP79370)	81	Mounier *et al*., 2004
JG35+U4-AG-narG25	AM412334	2	*Erwinia carotovora* SCRI1043 (CAG74933)	86	unpublished

* - taking those parts of the gene which were considered by the BLAST search.

DISCUSSION

The aim of this work was to study the influence of uranyl or sodium nitrate under aerobic or anaerobic conditions on the nitrate-reducing community of a soil sample collected from the uranium mining waste pile Haberland situated near the town of Johanngeorgenstadt in Germany. For this purpose the membrane-bound nitrate reductase gene *nar*G was used as a functional marker. The *nar*G gene was chosen because the membrane-bound nitrate reductase NAR is widespread among the Gram-positive and Gram-negative nitrate-amonifying and denitrifying species (Chèneby *et al*., 2003; Enwall *et al*., 2005). The nitrate-reducing bacterial populations were previously investigated in nickel mine spoils (Héry *et al*., 2005) and abandoned and reclaimed mine soils (Shirey & Sextone, 1989). Until now denitrifying

communities were studied by the use of nitrite reductase gene fragments (*nir*K and *nir*S) in nitrate- and uranium-contaminated groundwaters (Yan *et al*., 2003). To our knowledge, this study provides for the first time an insight in the microbial community capable of reducing nitrate in soil samples of uranium mining wastes by targeting the *nar*G gene. Phylogenetic analyses of NarG amino acid sequences showed that almost all of the NarG sequences retrieved from the untreated sample of the uranium mining waste were closely related to environmental NarG sequences retrieved from previous studies (Philippot *et al*., 2002; Chèneby *et al*., 2003; Deiglmayr *et al*., 2004; Héry *et al*., 2005). The majority of these environmental clones could not be affiliated with NarG of known bacteria and were distantly related to the NarG sequence of the pathogenic alphaproteobacterium *Brucella suis* 1330 (Paulsen *et al*., 2002). Interestingly, the majority of all *nar*G gene sequences found in mine spoils of an unexploited open cut nickel mine were also closely related to the NarG of environmental clones and clustered with the NarG of *Brucella suis* 1330 (Héry *et al*., 2005). Concurrently, by using 16S rRNA genes as a phylogenetic marker, uncultured *Alphaproteobacteria* were found to be predominant in the untreated sample (Chapters 1 & 2). Only two sequences representing each two clones found in the untreated sample clustered with the NarG of *Polaromonas naphthalenivorans* CJ2 and *Thiobacillus denitrificans* ATCC 25259. It seems that in the untreated sample an important portion of not yet characterised dissimilatory nitrate reducers are present. However, it is not excluded that some of the sequences represent silent unfunctional genes especially those of them which were not found in the nitrate treated samples as for instance JG35-2-AG-narG12 (Fig. 3.1).

Because nitrate is often a cocontaminant in uranium mining wastes and can influence the mobility of uranium (Finneran *et al*., 2002; Lack *et al*., 2002; Senko *et al*., 2002, 2005; North *et al*., 2004; Beller, 2005), we investigated the changes in nitrate-reducing bacterial community in the studied soil sample induced by addition of uranyl or sodium nitrate and incubations under aerobic or anaerobic conditions for 4 weeks. We expected that nitrate-reducing bacterial populations might be induced upon addition of nitrate solutions and that in the samples treated with uranyl nitrate differences in the nitrate-reducing community may occur due to the uranium toxicity.

Our analysis demonstrated that the induced changes in the nitrate-reducing bacterial community were dependent on the treatment with uranyl or sodium nitrate as well as on the aeration conditions. In the sample JG35+U1, incubated with uranyl nitrate under aerobic conditions, no NarG sequences related to the above mentioned environmental sequences belonging to the "*Brucella*"-like cluster were found. Instead of them, NarG sequences related

to the NarG of *Geobacter metallireducens* GS-15, *Chromobacterium violaceum* ATCC 12472, *Polaromonas naphthalenivorans* CJ2, *Pseudomonas fluorescens* AK15 and *Arthrobacter* sp. FB24 were identified. Most of the NarG sequences retrieved from this sample were distantly related to the NarG of *Geobacter metallireducens* GS-15, which is able to connect the oxidation of acetate with the reduction of nitrate, Mn(IV), and U(VI) (Lovley *et al*., 1993). However, it was also demonstrated that suspensions of *Geobacter metallireducens* ATCC 53774 oxidise Fe(II) and U(IV) with nitrate as an electron acceptor, which results in uranium mobilisation (Finneran *et al*., 2002). In our previous studies based on the 16S rRNA gene retrieval, 16S rRNA gene sequences closely related to *Geobacter bremensis* Dfr1 and *Geobacter bemidjiensis* Bem were identified in the sample JG35+U1 (Chapter 1). The affiliation of the 16S rRNA genes of bacterial strains related to those carrying the encountered NarG sequences is demonstrated in Fig. 3.2. As shown in this figure, a close relation between the species retrieved by the 16S rRNA and *nar*G gene retrievals exists. Thus, the presence of the *Geobacter* spp. found by the 16S rRNA gene retrieval in the sample JG35+U1 was confirmed by using the *nar*G gene retrieval.

The second predominant group of NarG sequences identified in the sample JG35+U1 was organised in sub-clusters distantly related to the NarG of the betaproteobacterium *Chromobacterium violaceum*. However, the *Betaproteobacteria* represented only a minor part of the bacterial community in this sample as estimated by the 16S rRNA gene retrieval (Chapter 1). Similar NarG sequences were also predominant in the sodium nitrate treated sample JG35-K4, which was incubated under anaerobic conditions. In the sample JG35-K4 the *Betaproteobacteria* were also shown to be predominant by the 16S rRNA gene retrieval. However, by the 16S rRNA gene retrieval of the studied samples no sequences closely related to the 16S rRNA gene of *Chromobacterium violaceum* were detected (Fig. 3.2). Instead, 16S rRNA gene sequences closely related to *Janthinobacterium agaricidamnosum* SAFR-022 and *Cenibacterium arsenoxidans* ULPAs1 were found in the samples JG35+U1 and JG35-K4.

In the sample JG35+U1 a proliferation of *Pseudomonas* spp. from *Gammaproteobacteria* was demonstrated by the 16S rRNA gene retrieval (Fig. 3.2, Chapter 1). By using degenerated primers for amplification of *nar*G genes one sequence, representing two clones, was detected in the sample JG35+U1, which was identical to the NarG sequence of *Pseudomonas fluorescens* AK15 (Philippot *et al*., 1997). As shown in Fig. 3.2, sequence JG35+U1-AG3, which was identified in the sample JG35+U1 by using 16S rRNA gene retrieval, was closely related to *Pseudomonas fluorescens* PC17.

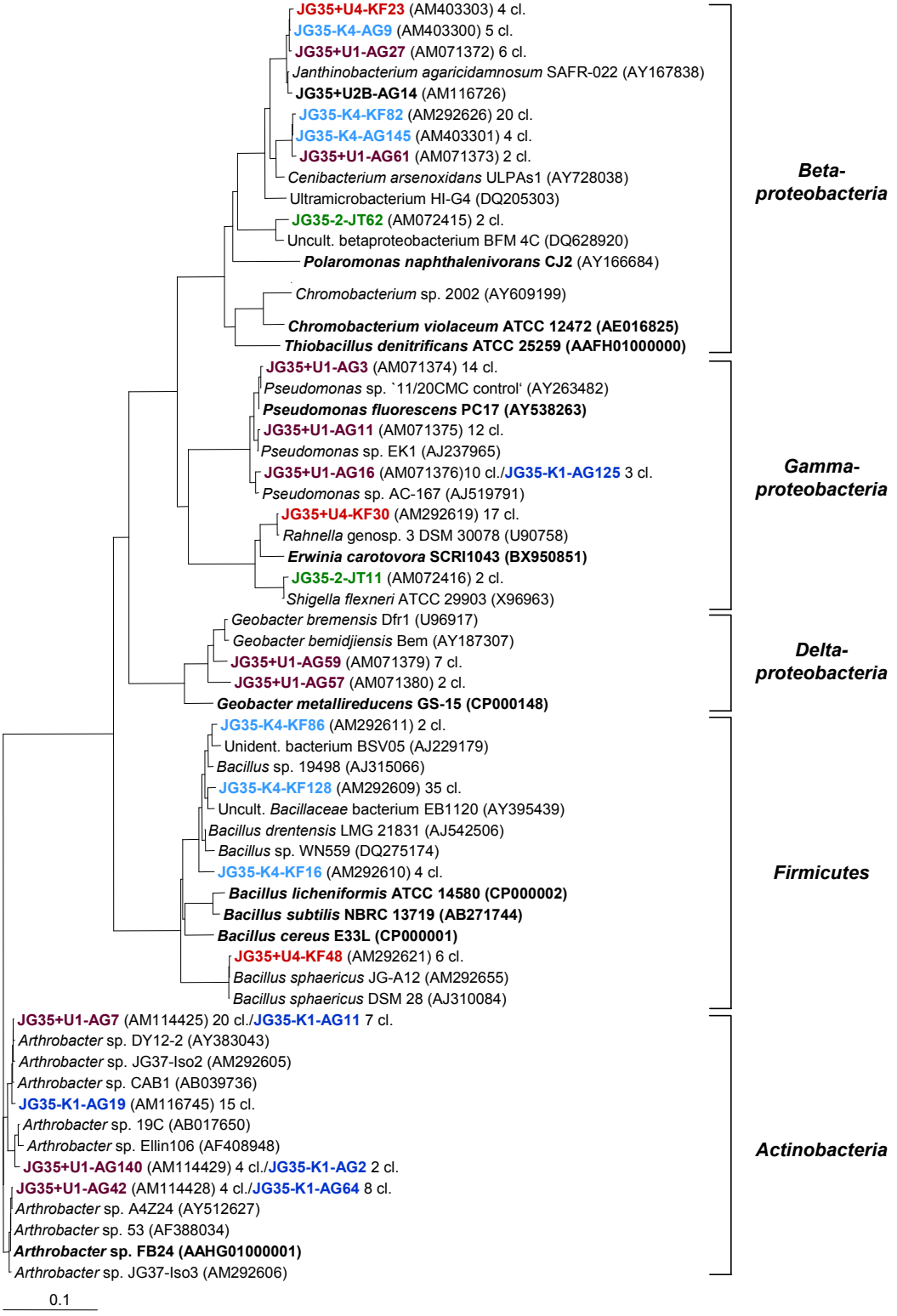

Fig. 3.2. Affiliation of 16S rRNA gene sequences of cultivated bacteria related to the encountered NarG sequences from the untreated sample **JG35-2**, the samples incubated under aerobic conditions with sodium nitrate **JG35-K1** or uranyl nitrate **JG35+U1** as well as under anaerobic conditions with sodium nitrate **JG35-K4** or uranyl nitrate **JG35+U4**. The 16S rRNA gene sequences retrieved from the samples studied by using 16S rRNA gene retrieval were compared with them. The number of clones (cl.) is written after the accession numbers. The dendrogram was generated using distance-matrix and neighbour-joining methods, and rooted with *Actinobacteria*.

Pseudomonas spp. are also known to interact effectively with uranium (Merroun *et al*., 2002; Renninger *et al*., 2004; Fields *et al*., 2005).

Additionally, a population carrying a NarG sequence closely related to the NarG of *Arthrobacter* sp. FB24 was identified in the sample JG35+U1. Concurrently, by using 16S rRNA gene retrieval in the analysis of the sample JG35+U1 a proliferation of *Actinobacteria* especially of *Arthrobacter* spp. was observed (Chapter 1). A large number of clones, which were closely related to the NarG sequence revealed by draft genome assembly based on sequencing of *Arthrobacter* sp. FB24 (EMBL No. AAHG01000001), was also found in the sample JG35-K1. *Arthrobacter* spp. were also found to be predominant in the sample JG35-K1 by the use of 16S rRNA gene retrieval (Chapter 1). As shown in Fig. 3.2, the 16S rRNA gene sequence of the *Arthrobacter* sp. FB24 is almost identical to the 16S rRNA gene sequence JG35+U1-AG42, which was identified in the uranyl nitrate treated sample incubated under the same conditions as JG35-K1 and to the sequence JG35-K1-AG64, which was identified in the sodium nitrate treated sample JG35-K1 by using the 16S rRNA gene retrieval (Chapter 1). In contrast, no NarG sequences of the *Arthrobacter* spp. were identified in the samples treated under anaerobic conditions. This is not surprising, bearing in mind that *Arthrobacter* sp. S2.26 is incapable of anaerobic denitrification, but it is able to respire nitrate aerobically (Gregory *et al*., 2000). The mentioned authors could not amplify the membrane-bound nitrate reductase genes and they suggested that this species does not possess the NAR (Carter *et al*., 1995; Gregory *et al*., 2000). However, we were able to amplify the *nar*G gene from genomic DNA of two *Arthrobacter* strains JG37-Iso2 and JG37-Iso3 isolated from another soil sample of the uranium mining waste pile Haberland by using our pair of primers (Fig. 3.1, Fig. 3.2). This indicates in agreement with the results from the draft genome assembly of *Arthrobacter* sp. FB24, that some *Arthrobacter* spp. possess membrane-bound nitrate reductase genes. It was demonstrated by others that different *Arthrobacter* spp. are present in heavy metal and radionuclide contaminated environments and that they are able to tolerate uranium as well as acidic pH and to accumulate uranium intracellularely (Fredrickson *et al*., 2004; Hanbo *et al*., 2004; Suzuki & Banfield, 2004; Martinez *et al*., 2006; Chapter 5).

All other sequences retrieved from sample JG35-K1 were closely related to the NarG sequences of environmental clones retrieved in other studies (Chèneby *et al*., 2003; Deiglmayr *et al*., 2004; Mounier *et al*., 2004). Some of them were similar to the sequences detected in the untreated sample or formed novel sub-clusters within the "*Brucella*"-like and the "*Polaromonas*"-like clusters.

As expected, under anaerobic conditions the nitrate-reducing community was changed as well but in a different way. Most of the *nar*G gene sequences, retrieved from the sample JG35-K4 treated with sodium nitrate, were distantly related to the NarG of *Bacillus cereus*-E33L, *Bacillus licheniformis* ATCC 14580 or *Bacillus subtilis* JH642. The ability of *Bacillus subtilis* to grow under strict anaerobic conditions using nitrate as an electron acceptor was demonstrated (Hoffmann *et al*., 1995). Interestingly, *Bacillus subtilis* has two distinct nitrate reductases, encoded by *nar*GHI and *nas*BC, which function in respiratory and assimilatory nitrogen metabolism (Richardson *et al*., 2001). In two abandoned mine sites in Mineral County, West Virginia, USA and Monongalia County, West Virginia, USA Gram-positive *Bacillus* spp. were the most common nitrate-reducing isolates as well (Shirey & Sextone, 1989). Adsorption of uranyl and aqueous uranyl complexes by functional groups of the bacterial cell wall of *Bacillus subtilis* was intensively studied (Fowle *et al*., 2000; Gorman-Lewis *et al*., 2005). By using the 16S rRNA gene retrieval it was demonstrated that the *Firmicutes* were represented by *Bacillus* spp. and *Clostridium* spp. in the sample JG35-K4 (Chapter 2). However, because most *Clostridium* spp. possess an alternative nitrate reductase system, which is involved in the so called nitrate fermentation (Fujinaga *et al*., 1999; Hasan & Hall, 1975; Caskey & Tiedje, 1980), the *nar*G approach was inapplicable for their identification.

By treatment with uranyl nitrate under anaerobic conditions the most predominant NarG group clustered with sequences related to the NarG of *Polaromonas naphthalenivorans* CJ2, which were found in significant lower numbers in the untreated as well as in all other treated samples. Most of the other sequences retrieved from this sample could not be affiliated with any NarG sequence of known bacteria and clustered with sequences identified in small groups in the untreated sample, which were distantly related to *Brucella suis* 1330. An explanation for this might be the fact that this treatment corresponds to the natural events in the original anaerobic environment from which the samples were collected, where fluctuations of the soluble uranium concentrations often occur. In addition two smaller groups of sequences retrieved from the sample JG35+U4 were distantly related to the NarG of cultivated strains. One to the already mentioned betaproteobacterium *Thiobacillus denitrificans* ATCC 25259 was also found in the untreated sample. However, no 16S rRNA gene sequences closely related to *Thiobacillus denitrificans* were identified in the uranyl nitrate treated sample JG35+U4 as well as in the untreated sample. The second retrieved group was affiliated with the NarG of the gammaproteobacterium *Erwinia carotovora* SCRI1043. As evident from Fig. 3.2, the 16S rRNA gene sequence of *Erwinia carotovora* forms a cluster with the 16S

rRNA gene sequence JG35+U4-KF30, which was identified in this sample by using the 16S rRNA gene retrieval (Chapter 2) and with *Rahnella* genosp. 3. In the 16S rRNA gene clone library from the same sample sequences were found, which were closely related to uncultured *Alphaproteobacteria*, gammaproteobacterial *Rahnella* spp. as well as *Clostridium* spp. and *Bacillus* spp. (Chapter 2). However, no *nar*G gene sequences closely related to the *nar*G gene sequences of *Bacillus* spp. were found in this sample by the *nar*G gene retrieval. As shown in Fig. 3.2, the 16S rRNA gene sequence JG35+U4-KF48 and their closest phylogenetic relatives *Bacillus sphaericus* JG-A12 and *Bacillus sphaericus* DSM 28 form a distinct cluster. However, we were not able to amplify the *nar*G gene fragment from genomic DNA of *B. sphaericus* JG-A12 (data not shown). It was also demonstrated that *B. sphaericus* DSM 28 is not able to reduce nitrate (Reva *et al*., 2001). On the basis of these results, we suggest that *B. sphaericus* JG-A12 most probably possesses no membrane-bound nitrate reductase.

Phylogenetic analysis of the complete NarG subunit from cultivated strains and from prokaryotic genome-sequencing projects showed some correlations with the 16S rRNA gene-based taxonomy (Philippot, 2005). However, recent analysis of both the 16S rRNA gene sequences and NarG of nitrate-reducing bacteria isolated from freshwater sediment showed that NarG sequences cannot be used in all cases with high confidence to predict the taxonomic position of organisms as defined by 16S rRNA gene sequences (Gregory *et al*., 2003). It was demonstrated, for instance, that NarG sequences of *Pseudomonas* isolates (according to 16S rRNA gene phylogeny) were involved in the NarG cluster of low G+C Gram-positive bacteria (Gregory *et al*., 2003). Horizontal transfer of *nar*G genes was suggested as an explanation for the occurrence of very similar *nar*G alleles in distantly related bacteria (Gregory *et al*., 2003). In our study, the results of the *nar*G functional gene approach are only partially congruent with the results of the 16S rRNA gene approach. On the basis of our results, we suggest the following explanations for the observed differences between the *nar*G and 16S rRNA gene approaches in the samples studied. First of all, not all bacteria identified by 16S rRNA gene retrieval are able to reduce nitrate and possess a nitrate reductase. By using the NarG as a functional marker, the diversity of only a part of the nitrate-reducing community was studied because nitrate-reducing bacteria having the periplasmic nitrate reductase or having not-yet-identified nitrate reductases were not taken into account. Additionally, some of the NarG sequences retrieved could only affiliate with NarG sequences of uncultured bacteria, so that their taxonomic position cannot be predicted.

Our work demonstrates that the bacterial community of the studied uranium mining waste pile responds to the addition of nitrate compounds in different ways depending on the treatment

with uranyl or sodium nitrate as well as on the aeration conditions. We suggest that by the treatment with uranyl nitrate, nitrate-reducing bacteria were stimulated, which have also a potential to interact with and to tolerate uranium. The exact processes such as denitrification or ammonification can be further studied by using approaches based on the enzymes involved in the consequent processes, for instance such as NIR, NOR or NOS. To obtain better quantitative information of the relative distribution of nitrate-reducing bacteria, hybridisation and real-time PCR should be performed with DNA extracted from the samples.

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Chapter 4

Changes in archaeal community structure of a soil sample collected from the uranium mining waste pile Haberland induced by treatments with uranyl or sodium nitrate

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ABSTRACT

The influence of uranyl or sodium nitrate under aerobic and anaerobic conditions on the archaeal community of a soil sample collected from the uranium mining waste pile Haberland, near the town of Johanngeorgenstadt in Germany, was investigated by the culture-independent 16S rRNA gene retrieval. The archaeal community in the uranium mining waste pile was less diverse than the previously studied bacterial community in the sample studied and was restricted to only a few lineages of mesophilic *Crenarchaeota*. 64% of the clones retrieved from the untreated sample represent the crenarchaeal group 1.1a and 36% of them represent the crenarchaeal group 1.1b. Almost all of the sequences retrieved from the treated samples independently on the way of incubation were, however, closely related to the 16S rRNA genes of one particular group of uncultured *Crenarchaeota* of the mesophilic group 1.1b. This group was affiliated with the fosmid clone 54d9 carrying also genes encoding enzymes responsible for nitrite reduction and ammonia oxidation. Characteristic for the treated and untreated samples was the presence of different clusters of almost identical 16S rRNA gene sequences, which indicates a considerable degree of microdiversity. Our results demonstrate the presence of dynamic and active archaeal populations in the studied uranium mining waste pile, which are able to react to changes in their environments.

INTRODUCTION

Archaea represent the third domain in the tree of life in addition to *Bacteria* and *Eucarya*. The domain *Archaea* consists of two major phyla *Crenarchaeota* and *Euryarchaeota* (Chaban *et al*., 2006). Recently, *Korarchaeota* (Barns *et al*., 1996; Auchtung *et al*., 2006) and *Nanoarchaeota* (Huber *et al*., 2002) were identified. More than half of the archaeal lineages at the phylum-level have none cultivated representatives (Hugenholtz, 2002). In former times the *Archaea* had been considered to be restricted to extreme environments, as those with high temperature, high salinity or extremes of pH. Several acidophilic archaea such as *Ferroplasma acidarmanus*, *Metallosphaera sedula*, *Sulfolobus acidocaldarius*, *Sulfolobus metallicus*, and *Sulfolobus solfataricus* have been identified in acidic, metal-rich environments resulted from biotechnological processes or mining operations, which have created extreme environments for archaea (Bond *et al*., 2000; Edwards *et al*., 2000; Norris *et al*., 2000; Dopson *et al*., 2003, 2004; Okibe *et al*., 2003; Chaban *et al*., 2006).

Recent molecular studies based on the phylogenetic analysis of environmental 16S rRNA genes revealed that *Archaea* also occupy moderate aquatic and terrestrial habitats (Bintrim *et*

al., 1997; MacGregor *et al*., 1997; Schleper *et al*., 1997; Buckley *et al*., 1998; Ochsenreiter *et al*., 2003; Sliwinski & Goodman, 2004). Archaeal diversity was investigated in different soil samples (Ochsenreiter *et al*., 2003, Bintrim *et al*., 1997; Kasai *et al*., 2005), in freshwater ferromanganous micronodules and sediments (Stein *et al*., 2001), in uranium-contaminated sediments (Suzuki *et al*., 2005), in ultra-high-pressure rocks (Zhang *et al*., 2005), in ultra deep mines (Onstott *et al*., 2003), in metal-rich particles from a freshwater reservoir (Stein *et al*., 2002), in hot (Kvist *et al*., 2005) and in cold sulphidic springs (Rudolph *et al*., 2004), and in waters from Deep South African gold mines (Takai *et al*., 2001).

Compared to the huge bacterial diversity that has been described in soils, the *Crenarchaeota* diversity seems to be restricted to a few specific lineages (Ochsenreiter *et al*., 2003). The relative number of *Crenarchaeota* in different soil samples was roughly estimated to be from 1.7 to 10% (Ochsenreiter *et al*., 2003).

So far, only two mesophilic *Crenarchaeota* are cultured. One of them, *Cenarchaeon symbiosum* is not yet completely physically separated from the tissues of its host, a single sponge species related to *Axinella mexicana* (Preston *et al*., 1996). The second one, the marine crenarchaeon *Nitrosopumilus maritimus* was isolated recently from a tropical tank at the Seattle Aquarium (Könneke *et al*., 2005). This strain grows chemolithoautotrophically by aerobic oxidation of ammonia to nitrite and represents the first example of nitrification in the *Archaea* domain (Könneke *et al*., 2005). Both cultured mesophilic *Crenarchaeota* belong to the group 1.1a, determined by DeLong (1998) and Jurgens *et al*. (2000).

By using cultivation-independent genomic studies a considerable number of archaeal 16S rRNA genes as well as other genes were assigned (Béjà *et al*., 2002; Tyson *et al*., 2004; Schleper *et al*., 2005; Treusch *et al*., 2005). From a 1.2 Gb large-insert environmental fosmid library from calcareous grassland soil for instance, a 43 kb genomic fragment was found carrying a 16S rRNA gene that affiliates with the group 1.1b of *Crenarchaeota*, possessing no cultured representatives up to date.

Interactions of some cultivated archaeal isolates with heavy metals and radionuclides were studied. A mobilisation of uranium was observed by *Sulfolobus metallicus* Kra 23 (Huber & Stetter, 1991) and by *Metallosphaera sedula* TH2 from an ore mixture (Huber *et al*., 1989). It was demonstrated that cell suspensions of the hyperthermophilic crenarchaeon *Pyrobaculum islandicum* can reduce U(VI), Tc(VII), Cr(VI), Co(III), Mn(IV) and Au(III) with hydrogen as an electron donor at ca. 100 °C (Kashefi & Lovley, 2000, Kashefi *et al*., 2001). It was also observed that the euryarchaeon *Halobacterium halobium* accumulates uranium extracellularly as dense deposits (Francis *et al*., 2004). However, only a few studies described the archaeal

diversity in uranium (Suzuki *et al*., 2005) or in heavy metal contaminated (Takai *et al*., 2001; Stein *et al*., 2002) environments.

The aim of the present study was to investigate the changes in the archaeal community structure of a soil sample collected from the uranium mining waste pile Haberland induced by treatments with uranyl or sodium nitrate and incubations for different periods of time under aerobic or anaerobic conditions.

MATERIALS AND METHODS

Soil samples

Soil samples from the uranium mining waste pile Haberland near the town of Johanngeorgenstadt in Germany were supplemented with different amounts of uranyl or sodium nitrate and incubated for different periods of time under aerobic and anaerobic conditions as described in Chapters 1 & 2. The samples analysed in this work are presented in Table 4.1.

Table 4.1. Soil samples used.

Soil samples	Treatment	Incubation time	Aeration conditions	Uranium content [mg kg^{-1}]	Added nitrate [mg kg^{-1}]
JG35-2	Untreated	-	-	26 - 40	-
JG35+U1	Uranyl nitrate	4 weeks	Aerobic	~100	31
JG35-K1	Sodium nitrate	4 weeks	Aerobic	26 - 40	32
JG35+U4	Uranyl nitrate	4 weeks	Anaerobic	~100	31
JG35-K4	Sodium nitrate	4 weeks	Anaerobic	26 - 40	32
JG35+U2A	Uranyl nitrate	14 weeks	Aerobic	~300	149
JG35+U3	Uranyl nitrate	14 weeks	Anaerobic	~300	149
JG35-K2	Sodium nitrate	14 weeks	Anaerobic	26 - 40	151

DNA extraction

Total DNA was recovered from 3.5 g soil of the samples studied by direct lysis according to Selenska-Pobell *et al*. (2001). The resulting DNA pellet was dissolved in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8).

PCR and cloning

Archaeal 16S rRNA gene fragments were amplified by semi-nested PCR in a reaction mixture with a volume of 10 µl, containing 0.9 µl of DNA template, 2.5 mM of $MgCl_2$ (Applied Biosystems, Foster City, California, USA), 125 µM of each of the four deoxynucleoside triphosphates, 4 pmol each of the forward and reverse primers and 0.5 U Ampli*Taq* Gold® Polymerase with the corresponding GeneAmp 10x PCR Puffer II (Applied Biosystems, Foster City, California, USA). For the first PCR step the *Archaea*-specific primer

$16S_{Ar21F}$ (5'-TTCCGGTTGATCCYGCCGGA-3') (DeLong, 1992) and the universal primer $16S_{1513r}$ (Lane, 1991) were used. The PCR amplifications were performed in a T3 thermocycler from Biometra (Göttingen, Germany) with a "touch down" PCR. After an initial denaturation at 94 °C for 7 min, the annealing temperature was lowered from 59°C to 55 °C over five cycles and then another 25 cycles followed with 94 °C for 1 min, 55 °C for 40 s, and 72 °C for 1.5 min, and completed with an extension period of 20 min at 72 °C. The presence and the correct size of the amplification products of the first PCR step were visualised electrophoretically in 1.2% agarose gels. The PCR products of the right size were diluted (1:50, 1:100, 1:200) and used as templates for the second PCR step. The second PCR step was performed by using the *Archaea*-specific primer $16S_{Ar958r}$ (5'-YCCGGCGTTGAMTCC AATT-3') (DeLong, 1992) as a reverse primer instead of the universal primer $16S_{1513r}$. Only 1 pmol of the two archaeal primers was used in this step. The second reaction was performed with an initial denaturation at 95 °C for 3 min, followed by 35 cycles with 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min, and completed with an extension period of 10 min at 72 °C. The amplified archaeal 16S rRNA gene fragments were cloned directly in *Escherichia coli* using a TOPO-TA Cloning® system (Invitrogen, Gröningen, The Netherlands), following the manufacturers instructions. A total of 150 single white colonies per sample were randomly selected and further analysed.

RFLP screening and sequencing

For restriction fragment length polymorphism (RFLP) screening, the inserted 16S rRNA gene fragments were amplified by PCR directly from the host *E. coli* cells with vector specific M13(-40) and M13rev primers as described above for the first step of the semi-nested PCR reaction. The presence and the correct size of the amplification products were visualised by electrophoresis in 1.2% agarose gels. The clones with correct inserts were stored as glycerol cultures at -80 °C. Aliquots of the amplified rRNA gene fragments were digested with 1 unit of the four-base-specific restriction endonuclease *Hae*III in the corresponding buffer (Promega, Mannheim, Germany) overnight at 37 °C. The digests were separated in 3.5% Small DNA Low Melt Agarose (Biozym, Hessisch Oldenburg, Germany) gels in a 0.5xTBE buffer and visualised by staining with ethidium bromide and UV illumination. The resulting RFLP patterns of all samples studied were grouped in RFLP-types. Representative clones from each RFLP group considered to represent predominant populations, as well as clones representing individual RFLP profiles, were selected for sequencing.

The 16S rRNA gene fragments of the selected clones were purified by using an Edge BioSystems QuickStep®2 PCR Purification Kit (MoBiTec, Göttingen, Germany) and directly

sequenced by using an ABI Prism® Big Dye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA), following the manufacturers instructions. Partial 16S rRNA gene sequences were obtained by using *Archaea*-specific primers, $16S_{Ar21F}$ and $16S_{Ar958r}$, and then assembled by using the Autoassembler 2.0 software (PE Applied Biosystems, Foster City, California, USA). The RNA gene sequences were determined on an automated sequencer (Model 310 ABI PRISM®, PE Applied Biosystems, Foster City, California).

Phylogenetic analysis

The archaeal 16S rRNA gene fragments retrieved were compared with 16S rRNA gene sequences available in the GenBanks and the EMBL Nucleotide Sequence Database by using BLAST analysis (Basic Local Alignment Search Tool) (Altschul *et al*., 1997). Sequences with less than 90% of similarity to their closest phylogenetic relatives were checked for chimera formation by the program Check_Chimera of the RDP (Ribosomal Database Project) (Maidak *et al*., 2000). The sequences were aligned with those corresponding to the closest phylogenetic relatives by using CLUSTALW version 1.7 (Thompson *et al*., 1994). Phylogenetic trees were generated using the neighbour-joining algorithm with distance analyses by using Jukes-Cantor corrections in the PHYLIP v. 3.5 package (Felsenstein, 1993).

RESULTS

Archaeal 16S rRNA gene clone libraries with nearly 150 clones were constructed for the untreated sample JG35-2 of the studied uranium mining waste pile and for each of the treated samples shown in Table 4.1 (Materials and Methods).

Archaeal diversity in the untreated sample JG35-2

By screening the clone library of the untreated sample JG35-2 using RFLP with the enzyme *Hae*III and sequencing of the clones representing distinct RFLP patterns, three major groups were found (Fig. 4.1). All the sequences retrieved were closely related to uncultured, non-thermophilic *Crenarchaeota*. The G/C-content of the analysed sequences was between 49.9% and 54.9% with a mean of 52.7%. This value is similar to the value calculated for non-thermophilic *Crenarchaeota* found in other studies (DeLong, 1992; Bintrim *et al*., 1997) and clearly lower than 60 to 66% G/C-content of their thermophilic relatives (Galtier *et al*., 1999). 64% of all clones retrieved from sample JG35-2 were affiliated with sequences of group 1.1a termed by DeLong (1998) and Jurgens *et al*. (2000) (Fig. 4.1). As shown in Fig. 4.2, the sequences JG35-2-TR-Ar2, JG35-2-TR-Ar8, JG35-2-TR-Ar68 and JG35-2-TR-Ar100 formed

a tight cluster with the sequences ARCHDBF10, identified in the Snake River plain aquifer (EMBL No. DQ190060) and 44a-A1-1, found in a ZnS-producing biofilm of a subsurface acid mine drainage system (EMBL No. AY082452). The sequences Gitt-GR-27 and Sh765B-AG-A33 retrieved from the uranium mill tailings Gittersee/Coschütz (Radeva & Selenska-Pobell, 2002) and from the Shiprock uranium mill tailings also belong to the same cluster (Fig. 4.2). The sequence JG35-2-TR-Ar14 shared only 97% of similarity with the uncultured archaeon ARCHDBF10 and formed a separate lineage (Fig. 4.2).

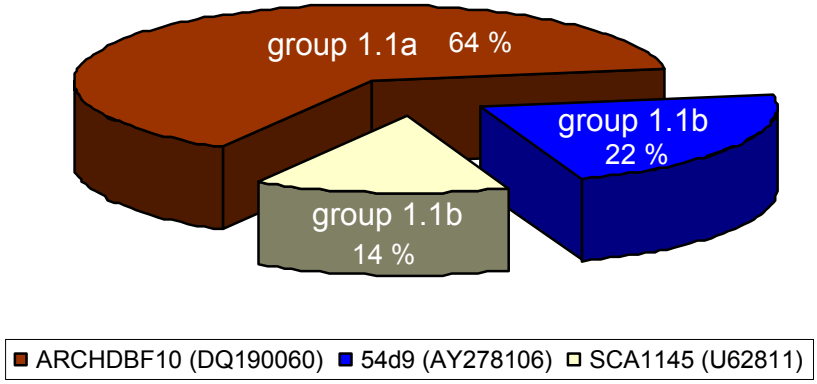

Fig. 4.1. Predominant archaeal RFLP-groups, their percentage in the clone library of the untreated sample JG35-2 and their crenarchaeal classification.

The rest of the clones (36%) were affiliated with sequences from the group 1.1b as defined by DeLong (1998) and Jurgens *et al*. (2000) and formed two sub-clusters (Fig. 4.1). The first of them represents 22% of the clones and included the sequences JG35-2-TR-Ar1, JG35-2-TR-Ar6, JG35-2-TR-Ar58, JG35-2-TR-Ar71 and JG35-2-TR-Ar150. These sequences had the same RFLP-profiles as the clones JG35+U1-TR-Ar1, JG35+U1-TR-Ar5, JG35-K2-TR-Ar62, JG35+U3-TR-Ar62 and JG35-K4-TR-Ar29 retrieved from the treated samples (Fig. 4.2). All these sequences were closely related to the 16S rRNA gene sequence of the uncultured crenarchaeote 54d9, which was identified in the upper 10 cm layer of a sandy ecosystem (‘Am Rotböhl’) near Darmstadt, Germany (Ochsenreiter *et al*., 2003; Treusch *et al*., 2005) (Fig. 4.2).

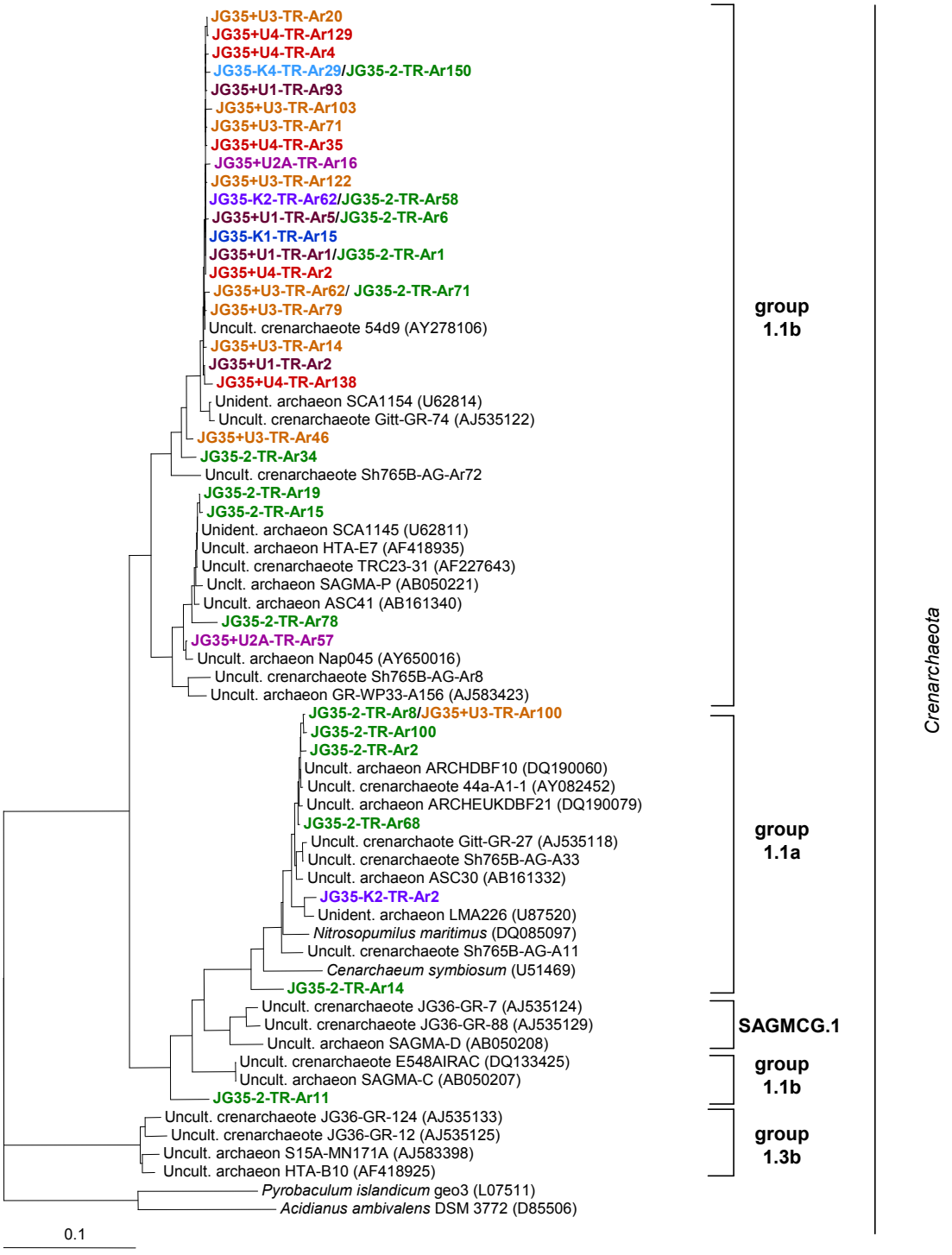

Fig. 4.2. Phylogenetic dendrogram of the archaeal 16S rRNA gene sequences retrieved from the untreated sample **JG35-2**, the uranyl nitrate treated samples **JG35+U1** (aerobic, 4 weeks), **JG35+U2A** (aerobic, 14 weeks), **JG35+U4** (anaerobic, 4 weeks) and **JG35+U3** (anaerobic, 14 weeks) and the sodium nitrate treated samples **JG35-K1** (aerobic, 4 weeks), **JG35-K4** (anaerobic, 4 weeks) and **JG35-K2** (anaerobic, 14 weeks) as well as the sequences of their closest relatives. The dendrogram was generated using distance-matrix and neighbour-joining methods and rooted with the hyperthermophilic crenarchaeon *Pyrobaculum islandicum* geo3 and *Acidianus ambivalens* DSM 3772. Group numbering is in accordance with DeLong (1998) and Jurgens *et al*. (2000). The absolute frequency of the clones represented by the sequences in the samples studied is given in Table 4.2.

As shown in Fig. 4.2, the sequence of the unidentified archaeon SCA1154, which was identified in subsurface (2-10 cm) soil sample collected from the West Madison Agricultural Research Station (Madison, Wisconsin, USA) (Bintrim *et al*., 1997), was also very closely related to the mentioned sequences. The uncultured crenarchaeon Gitt-GR-74 belongs to the same cluster as well (Radeva & Selenska-Pobell, 2002). The individual sequence JG35-2-TR-Ar34 shared a lower identity of 97% with the 16S rRNA gene sequence of the uncultured crenarchaeote 54d9.

The second 1.1b sub-cluster contained 14% of the clones, which are presented by the sequences JG35-2-TR-Ar15 and JG35-2-TR-Ar19. They were closely related to the 16S rRNA gene of the uncultured archaeon SCA1145, which was identified in the above mentioned subsurface soil sample collected from the West Madison Agricultural Research Station (Bintrim *et al*., 1997) and to the 16S rRNA gene sequence of the uncultured archaeon HTA-E7, identified in microbial communities associated with metal-rich particles formed in the Horsetooth Reservoir in Fort Collins, Colorado, USA (Stein *et al*., 2002) (Fig. 4.2). The sequence JG35-2-TR-Ar78, representing two clones, also belongs to the same 1.1b sub-cluster. This sequence was 97.7% similar to the sequence TRC23-31 (Simon *et al*., 2005) and to the sequence ASC41, identified in a petroleum contaminated soil (Kasai *et al*., 2005). As shown in Fig. 4.2, the sequence SAGMA-P, which was identified in waters from Deep South African gold mines (Takai *et al*., 2001), also belongs to the same cluster.

The individual sequence JG35-2-TR-Ar11 was distantly related to the above mentioned cluster but still belongs to the group 1.1b and shared an identity of 92% with the 16S rRNA gene sequence of the uncultured crenarchaeote E548AIRAC, identified in ultra deep mines (Onstott *et al*., 2003), and with the 16S rRNA gene sequence of the uncultured archaeon SAGMA-C, identified in waters from Deep South African gold mines (Takai *et al*., 2001).

Changes in archaeal community structure by treatments with uranyl or sodium nitrate

The treatment of the sub-samples with uranyl or sodium nitrate and their subsequent incubations for different periods of time under aerobic or anaerobic conditions resulted in a strong shifting to the above mentioned group of the sub-cluster 1.1b affiliated with the 16S rRNA gene of the fosmid clone 54d9 (Table 4.2). The number of the clones represented by sequences retrieved from the samples studied is given in Table 4.2. The phylogenetic affiliation of all sequences listed in Table 4.2 is shown in Fig. 4.2.

Table 4.2. Archaeal 16S rRNA gene sequences retrieved from the untreated sample JG35-2, the uranyl nitrate treated samples (**JG35+U1**, **JG35+U2A**, **JG35+U4**, **JG35+U3**) and the sodium nitrate treated samples (**JG35-K1**, **JG35-K4**, **JG35-K2**,), their closest phylogenetic relatives and the number of clones represented by them in all samples studied. The phylogenetic affiliation of the sequences is shown in the dendrogram presented in Fig. 4.2.

Group	Sequences	Closest phylogenetic relative	Number of clones							
			JG35-2	U1	K1	U2A	U4	K4	U3	K2
1.1a	JG35-2-TR-Ar2 (8/68/100)	Uncult. archaeon ARCHDBF10 (DQ190060)	89						1	
	JG35-2-TR-Ar14		2							
	JG35-K2-TR-Ar2	Uncult. archaeon LMA226 (U87520)								4
1.1b	JG35-2-TR-Ar11	Uncult. crenarchaeote E548AIRAC (DQ133425)	1							
1.1b	JG35-2-TR-Ar15 (19)	Unident. archaeon SCA1145 (U62814)	19		5		2	3	4	8
	JG35-2-TR-Ar78	Uncult. crenarchaeote TRC23-31 (AF227643)	2							
	JG35+U2A-TR-Ar57	Uncult. archaeon Nap045 (AY650016)				2				
	JG35-2-TR-Ar34	Uncult. crenarchaeote 54d9 (AY278106)	1							
	JG35+U1-TR-Ar1 (2/5)	Uncult. crenarchaeote 54d9 (AY278106)	24	145	140	140	137	141	113	130
	JG35-K1-TR-Ar15	Uncult. crenarchaeote 54d9 (AY278106)			2				1	
	JG35+U4-TR-Ar2	Uncult. crenarchaeote 54d9 (AY278106)					1	1		
	JG35+U4-TR-Ar4 JG35+U3-TR-Ar62	Uncult. crenarchaeote 54d9 (AY278106)	2		1		1	1	1	
	JG35+U4-TR-Ar35	Uncult. crenarchaeote 54d9 (AY278106)					1	1	1	
	JG35+U4-TR-Ar129	Uncult. crenarchaeote 54d9 (AY278106)					1		1	
	JG35+U4-TR-Ar138	Uncult. crenarchaeote 54d9 (AY278106)					2		1	1
1.1b	JG35-K4-TR-Ar29	Uncult. crenarchaeote 54d9 (AY278106)	2			1	1	2	1	
	JG35+U2A-TR-Ar16	Uncult. crenarchaeote 54d9 (AY278106)			1	4			1	
	JG35+U3-TR-Ar14	Uncult. crenarchaeote 54d9 (AY278106)							3	
	JG35+U3-TR-Ar20	Uncult. crenarchaeote 54d9 (AY278106)			1	1			2	
	JG35+U3-TR-Ar46	Uncult. crenarchaeote 54d9 (AY278106)			1	2	2	1	4	2
	JG35+U3-TR-Ar71	Uncult. crenarchaeote 54d9 (AY278106)						1	1	
	JG35+U3-TR-Ar79	Uncult. crenarchaeote 54d9 (AY278106)						1	1	1
	JG35+U3-TR-Ar103	Uncult. crenarchaeote 54d9 (AY278106)		1			1		1	
	JG35+U3-TR-Ar122	Uncult. crenarchaeote 54d9 (AY278106)			1				2	
	JG35-K2-TR-Ar62	Uncult. crenarchaeote 54d9 (AY278106)	2				1			1

Most of the clones retrieved from the treated samples studied have the same RFLP-profiles as the sequences JG35+U1-TR-Ar1, JG35+U1-TR-Ar2 and JG35+U1-TR-Ar5 (Table 4.2).

As shown in Table 4.2, all samples studied especially the samples treated with uranyl nitrate and incubated for 4 weeks or 14 weeks under anaerobic conditions (samples JG35+U4 and JG35+U3) contained clones possessing individual RFLP patterns. By sequencing it was demonstrated that all these sequences were very closely related to each other and to the uncultured crenarchaeote 54d9, which demonstrates a considerable degree of microdiversity (Fig. 4.2, Table 4.2).

The sequences JG35+U3-TR-Ar14 and JG35+U3-TR-Ar103 both closely related to the 16S rRNA gene of the fosmid clone 54d9, were only detected in uranyl nitrate treated samples (Table 4.2).

In all samples studied except those treated with uranyl nitrate under aerobic conditions for 4 weeks (sample JG35+U1) and for 14 weeks (sample JG35+U2A), clones with RFLP profiles identical to those of the sequences JG35-2-TR-Ar15 and JG35-2-Tr-Ar19, which affiliated with SCA1145 sub-cluster within the 1.1b group, were found but in lower numbers in comparison to the untreated sample (Table 4.2). In the sample JG35+U2A one sequence (JG35-U2A-Ar57) was detected, which was closely related to the uncultured archaeon Nap045, identified in a hot spring in the solfataric field Pisciarelli, Italy (Kvist *et al*., 2005). Both sequences were also affiliated with the SCA1145 sub-cluster.

Only 1 clone (JG35+U3-TR-Ar100) in the sample JG35+U3 and a group of 4 clones in the sample JG35-K2 represented by sequence JG35-K2-TR-Ar2 affiliated with the crenarchaeal group 1.1a (Table 4.2, Fig. 4.2). The sequence JG35-K2-TR-Ar2 was closely related to the unidentified archaeon LMA226, identified in Lake Michigan sediment (MacGregor *et al*., 1997).

DISCUSSION

In contrast to the extremely complex composition of the bacterial community in the studied samples of the uranium mining waste pile (Chapters 1 & 2), the archaeal populations in the uranium mining waste pile sample are only limited to a few lineages of non-thermophilic *Crenarchaeota*. The archaeal community in the untreated sample JG35-2 of the uranium mining waste pile Haberland includes members of the crenarchaeal group 1.1a (64%) and 1.1b (36%) (Fig. 4.1). Interestingly, in the sample JG36, collected from the same uranium mining waste pile Haberland but from a one meter deeper level at the same sampling point the composition of the archaeal community was different in comparison to the sample analysed in this study (Radeva & Selenska-Pobell, 2002). In that case most of the archaeal sequences retrieved were closely related to the uncultured South African Gold Mine crenarchaeote SAGMA-D, which belongs to group SAGMCG.1 (Fig. 4.2, Radeva & Selenska-Pobell, 2002). Some sequences retrieved from the sample JG36 belonged to the mesophilic crenarchaeal group 1.3b. To this group belong sequences identified in forest soils and freshwater habitats (Ochsenreiter *et al*., 2003). In contrast to other uranium contaminated environments (Suzuki *et al*., 2005), no representatives of *Euryarchaeota* could be found in the studied uranium mining waste pile.

Because all the sequences retrieved from the untreated sample are closely related to uncultured *Crenarchaeota*, it is difficult to predict the phenotypic and the ecological role of the corresponding organisms. Remarkable is that, the closest phylogenetic relatives of the sequences retrieved were identified in environments with similar ecological properties such as acid mine drainage systems, gold mine waters or metal-rich particles (Takai *et al*., 2001; Stein *et al*., 2002).

The addition of uranyl or sodium nitrate and subsequent incubations under aerobic or anaerobic conditions resulted in a very strong shifting in the crenarchaeal community. In the samples JG35+U1, JG35-K1, JG35+U4, JG35-K4 and JG35+U2A no sequences related to the group 1.1a were found, which were predominant in the untreated sample. Only a few sequences related to group 1.1a were found in the samples JG35+U3 and JG35-K2, incubated for a longer time under anaerobic conditions.

The number of clones related to the uncultured crenarchaeote SCA1145, which represented 14% of the clones in the untreated sample and belong to group 1.1b, were found in lower numbers (samples JG35-K1, JG35+U4, JG35-K4, JG35+U3 and JG35-K2) or were not detected (sample JG35+U1 and JG35+U2A) in the treated samples. One explanation for the reduction of the mentioned groups in the treated samples can be that they were overgrown by other members of the archaeal community stimulated by the treatment. In addition, it might be that they cannot tolerate high concentrations of U(VI) and nitrate.

Most sequences retrieved from the treated samples were closely related to the uncultured crenarchaeote 54d9, which 16S rRNA also affiliated with group 1.1b of *Crenarchaeota*. Representatives of the group 1.1b were found in different soils and were often the only lineage of archaea found (Ochsenreiter *et al*., 2003; Treusch *et al*., 2005). It was postulated that the group 1.1b of *Crenarchaeota* seems to inhabit virtually any soil on our planet (Ochsenreiter *et al*., 2003). Members within this group of *Archaea* have been detected in different environmental samples as for example in heavy-metal contaminated soils (Sandaa *et al*., 1999), in unsaturated petroleum-contaminated soils (Kasai *et al*., 2005), in hot springs (Kvist *et al*., 2005), and in waters from Deep South African gold mines (Takai *et al*., 2001).

A functional and genomic study was used to uncover the metabolism and activity of mesophilic *Crenarchaeota* (Treusch *et al*., 2005). The fosmid clone 54d9 was identified by PCR screening for archaeal operon-encoding fragments in a 1.2 Gb large-insert environmental fosmid library prepared from a soil sample collected from the upper 10 cm layer of a sandy ecosystem (‘Am Rotböhl’) near Darmstadt, Germany (Treusch *et al*., 2005). The insert encoded a homologue of a copper-containing nitrite reductase with an unusual C-terminus

that encoded a potential amicyanin-like electron transfer domain (Treusch *et al*., 2005). The copper-containing nitrite reductase (NirK) catalyses the important step of denitrification, the reduction of NO_2 to NO (Philippot, 2002). Sequences which varied with up to 27% over 190 amino acid residues to this novel archaeal nitrite reductase were found in soil and other environments, which suggests that this archaeal nitrite reductase is represented in more variants within the group of *Crenarchaeota* (Treusch *et al*., 2005).

Additionally, genes for two proteins related to subunits of ammonia monooxygenases (AmoAB) were localised on the fosmid clone 54d9 (Treusch *et al*., 2005). The predicted amino acid sequences of putative AMO encoding genes from the marine crenarchaeon *Nitrosopumilus maritimus* (Könneke *et al*., 2005) are very similar to those of soil *Crenarchaeota* found on the fosmid clone 54d9 (Treusch *et al*., 2005) as well as to sequences from Sargasso Sea (Venter *et al*., 2004). Additionally, the ubiquity and diversity of ammonia-oxidising archaea was demonstrated in water columns and sediments of ocean as well as soil aggregates from a non-contaminated "background" site near Oak Ridge, Tennessee, USA (Francis *et al*., 2005). The presence of putative AMO-encoding genes in different marine and soil *Crenarchaeota* implies a broad distribution of nitrifying physiology in these organisms (Könneke *et al*., 2005; Schleper *et al*., 2005). Recently, it was demonstrated that *Crenarchaeota* may be the most abundant ammonia-oxidising organisms in the soil ecosystems on the Earth (Leininger *et al*., 2006). On the basis of the close relation of the sequences retrieved in this work to the 16S rRNA gene of the fosmid clone 54d9, a similar metabolism for the uncultured crenarchaeal populations stimulated by our treatments is possible. Nitrate, which was used by the treatments, can be transformed to ammonium by nitrate reduction and denitrification or dissimilatory nitrate ammonification by the bacteria present in the samples. Interestingly, the analysis of the bacterial diversity in the treated samples studied revealed a stimulation of nitrate-reducing, denitrifying and ammonifying populations, which can supply the *Crenarchaeota* with different nitrogen oxide compounds (NO_2, NO) and ammonium (Chapters 1, 2, 3). Under anaerobic conditions, it is also possible that nitrite can be used as an electron acceptor and energy can be gained via nitrogen dioxide (NO_2)-dependent ammonia oxidation (Schmidt *et al*., 2004). A similar adaptation of the metabolism in dependence on the availability of oxygen is described for the bacterium *Nitrosomonas europaea* (Schmidt *et al*., 2004).

Because of these results a stimulation of ammonia-oxidising *Crenarchaeota* in the studied samples is suggested, but the phylogenetic relation on the basis of 16S rRNA gene sequencing is not sufficient for prediction of identical physiological properties (Chaban *et al*., 2006).

Interestingly, as shown in Table 4.2, in nearly all of the samples studied but especially in the samples treated with uranyl nitrate under anaerobic conditions a few clones represented individual RFLP patterns. By sequencing it was demonstrated that all these sequences were very closely related to each other and to the uncultured crenarchaeote 54d9. Such heterogeneity often attributes to multiple, not identical rRNA operons occurring within the same species. Because all members of *Crenarchaeota* are characterised to contain only one rRNA operon (Garret *et al*., 1991; Ochsenreiter *et al*., 2003), this possibility is excluded in our case. Two closely related but distinct variants of *Cenarchaeon symbiosum* exhibited <0.7% sequence divergence in their rRNA genes, which suggests that microdiversity among crenarchaeal 16S rRNA genes reflects organismal genetic diversity (Schleper *et al*., 1998). Microdiversity among closely related archaeal 16S rRNA sequences was demonstrated in other environmental samples by culture-independent analysis as well (Vetriani *et al*., 1999; García-Martínez & Rodríguez-Valera, 2000; Benlloch *et al*., 2002; Nicol *et al*., 2006). An adaptive radiation of species is also possible (Fuhrman & Campbell, 1998; Moore *et al*., 1998). It is also not excluded that some of the microdiverse sequences can be due to biases of the PCR amplifications (Qiu *et al*., 2001).

The observed shifting in the archaeal community of the uranium mining waste pile to probably nitrite-reducing and ammonia-oxidising *Crenarchaeota* by the treatments with uranyl or sodium nitrate under aerobic and anaerobic conditions indicates that these *Crenarchaeota* are able to tolerate nitrate and possibly to participate in denitrifying processes via nitrite reduction. In addition, it seems that these populations are very adaptive. The strong proliferation of the archaea from the 1.1b group in the uranyl nitrate treated samples is also an indication that they can tolerate higher amounts of U(VI). Our results indicate dynamic and active archaeal populations that react to changes in environmental conditions in the studied uranium mining waste pile. Finally, it is necessary to try to culture representatives of *Crenarchaeota* from these samples and to study their interactions with uranium and nitrate in pure cultures.

By using different media and aeration conditions an enrichment of relatives of the uncultured crenarchaeote 54d9 was demonstrated by Reitz (2006). By using the 16S rRNA gene retrieval it was found that the enrichment culture was mixed and consisted of 1.1b *Crenarchaeota* and *Clostridium* spp. (Reitz, 2006). The *Clostridium* spp., which are able to reduce fermentatively nitrate to ammonium (Caskey & Tiedje, 1980, Fujinaga *et al*., 1999), can possibly provide the latter to the stimulated ammonia-oxidising *Crenarchaeota*. As shown in Chapter 2,

Clostridium spp. were identified in the sample JG35+U4, which was used as inoculum for the enrichment cultures.

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Chapter 5

Interactions of two *Arthrobacter* strains isolated from the uranium mining waste pile Haberland with Pb and U

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ABSTRACT

Two bacterial strains, JG37-Iso2 and JG37-Iso3, were cultured from a soil sample of a uranium mining waste pile near the town of Johanngeorgenstadt (JG) in Germany. The JG site represents an extreme environment consisting of acidic soils with co-occurring heavy metals (Cu, Cr, Ni, Pb, etc.) and radionuclides (U, Th, etc.). By using the 16S rRNA gene sequence analysis the two strains were affiliated with *Actinobacteria* from the genus *Arthrobacter*. In this work, a combination of wet chemistry, spectroscopic, microscopic, and microbiological methods was used to elucidate the tolerance mechanisms of these two isolates to heavy metals, particularly to Pb and U, which occur at the contaminated site.

The cells of the two strains differ in their ability to accumulate and to tolerate Pb. X-ray diffraction (XRD) analysis indicated that the growing cells of the strains precipitated Pb as galena (PbS), whereas in non-growing conditions pyromorphite ($Pb_5(PO_4)_3Cl$) phase was produced by the cells, alleviating probably the toxicity of Pb. Transmission electron microscopy (TEM) showed that the pyromorphite precipitates were mainly located at the cell surface. Live/Dead cell staining indicated that 60% of the Pb-treated cells were viable.

The two strains accumulated different amounts of U but tolerate the same concentrations of this radionuclide. TEM analyses showed that both *Arthrobacter* strains accumulated uranium intracellularly. X-ray absorption spectroscopy (XAS) studies demonstrated that this radionuclide was mainly coordinated to bacterial organic phosphate groups in a monodentate binding mode.

The two isolates were capable to tolerate equal amounts of U, Cr, Cu, and Ag, but they differed in their tolerance to Ni, Cd, and Pb.

INTRODUCTION

The increasing contamination of soil, sediment, and water with heavy metals by natural and industrial processes is a worldwide problem. Mining processes produced million tons of material contaminated with radionuclides such as U and different heavy metals as, for instance, Cd, Ni, and Pb (Suzuki *et al*., 2003; Li *et al*., 2005; Wan *et al*., 2005; Chapter 1). For the prevention of an eventual release and migration of these contaminants into the environment all factors which can influence it such as ions, minerals and microorganisms must be considered (Stroes-Gascoyne & West, 1997; Anderson & Pedersen, 2003). Several studies showed that bacteria isolated from heavy metal and radionuclide contaminated environments are able to interact with metals in different ways (McLean & Beveridge, 2001; Lloyd & Lovley, 2001; Selenska-Pobell *et al*., 2001; Selenska-Pobell, 2002; Spain, 2003;

Merroun *et al*., 2005, 2006; Martinez *et al*., 2006). Interactions of microorganisms with radionuclides and metals that promote their precipitation and immobilisation *in situ* are promising strategies for treatment and cleanup of the contaminated subsurface (Anderson *et al*., 2003; Raff *et al*., 2003; Fredrickson *et al*., 2004; Martinez *et al*., 2006). At mixed waste sites where the concentrations of metal contaminants can reach toxic levels, the low resistance of indigenous microbial populations to some metals could be critical for the success of *in situ* biostimulation efforts (Martinez *et al*., 2006). While a number of microbes are able to immobilise particular heavy metals and radionuclides, the sensitivity of these organisms to other heavy metals could possibly limit their *in situ* activities (Martinez *et al*., 2006). Therefore, it is necessary to study the tolerance of the indigenous bacteria to different heavy metals occurring simultaneously at the site, as well as the interaction mechanisms of the bacteria with them.

In the present study, we examined the interaction mechanisms with and the tolerance against Pb and U of two *Arthrobacter* isolates recovered from a soil sample of the uranium mining waste pile Haberland situated near the town of Johanngeorgenstadt in Germany.

Studies of prokaryotic tolerance and resistance to soluble Pb have revealed two general mechanisms. The first of them is an active efflux mechanism (Silver & Walderhaug, 1992; Borremans *et al*., 2001), a frequent resistance strategy employed by bacteria against heavy metals (Nies, 2003; Mire *et al*., 2004). The second mechanism appears to be precipitation of Pb in an insoluble form, mainly as lead phosphate (Aickin *et al*., 1979; Aiking *et al*., 1985; Templeton *et al*., 2003; Mire *et al*., 2004). Very few reports described the bacterial precipitation of this metal as lead sulphide as, for instance, by a *Klebsiella* strain cultured in phosphate-limited medium (Aiking *et al*., 1985). Studies on Pb tolerance have been performed with growing (Aiking *et al*., 1985; Levinson *et al*., 1996; Mire *et al*., 2004) or non-growing (Templeton *et al*., 2003) microbial cells. However, little has been done to investigate comparatively the fate of Pb in both physiological states (Aickin *et al*., 1979). Therefore, one of the objectives of this work was to investigate the effect of the physiological state (growing and non-growing cells) on the interactions of the two *Arthrobacter* isolates with Pb.

In the case of U, the bacterial cells cope with toxicity of this radionuclide by different mechanisms such as the biosorption by cell surface polymers (Selenska-Pobell *et al*., 1999; Raff *et al*., 2003; Merroun *et al*., 2005), biotransformations such as oxidation (DiSpirito & Tuovinen, 1982; Beller, 2005) and reduction (Lovley & Phillips, 1992; Lovley *et al*., 1993; Francis *et al*., 1994; Suzuki *et al*., 2004; Wu *et al*., 2006), stimulation of metal precipitation and generation of minerals (Macaskie *et al*., 1992, 2000; Renninger *et al*., 2004), mobilisation

of U by chelation (Kalinowski *et al*., 2004) and intracellular accumulation of U (McLean & Beveridge, 2001; Francis *et al*., 2004; Suzuki & Banfield, 2004). The latter process has been suggested to be the main mechanism promoting the U tolerance of *Arthrobacter* spp. (Martinez *et al*., 2006). By using TEM in combination with Energy-dispersive X-ray spectroscopic (EDX) analysis Suzuki and Banfield (2004) demonstrated that the U accumulated by a bacterial isolate, closely related to *Arthrobacter ilicis*, is localised in the intracellular space as precipitates closely associated with polyphosphate granules. No molecular-scale studies have been performed to determine the structure of the bacterial uranium-bearing precipitates. The second aim of the present work was to examine the way how the two *Arthrobacter* strains cope with toxicity of U by using a combination of accumulation studies, microscopic (TEM) and spectroscopic (XAS) techniques.

MATERIALS AND METHODS

Soil material

The sample JG37 was collected from the uranium mining waste pile Haberland situated near the town of Johanngeorgenstadt in Germany in July 1997 from a depth of 4 m (same sampling point as sample JG35-2) by drilling under sterile conditions. The soil sample was handled aseptically and kept frozen at - 20 °C for further analyses. The sample had a pH of 4.5 and the total content of heavy metals and radionuclides was determined by using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) on spectrometer ELAN 5000 (Perkin Elmer, Shelton, Connecticut, USA). The element composition of the sample is presented in Table 5.1.

Table 5.1. Element composition in mg kg^{-1} of the sample JG37 from the uranium mining waste pile Haberland.

Al	**Ca**	**Cr**	**Fe**	**Mn**	**Co**	**Ni**	**Cu**	**Zn**
18700	1050	79.4	52000	1030	39.9	72.4	110.0	476
As	**Sr**	**Ag**	**Cd**	**Sn**	**Hg**	**Pb**	**Th**	**U**
418	12.2	0.223	4.11	24.0	1.450	46.7	1.96	69.7

Isolation of the soil bacteria and cultivation conditions

To isolate soil bacteria from the sample JG37, diluted nutrient broth (DNB) was used. This medium consisted of Difco NB (Becton Dickinson and Company, Sparks, Maryland, USA) at a concentration of 0.08 g l^{-1} in distilled water according to Janssen *et al*., 2002. One gram of the soil was added to 100 ml aliquot of sterile autoclaved and filtered (0.2 µm pore size) water in sterile 150 ml conical flasks and shacked for 15 min at 200 rpm. One millilitre of this soil suspension was used for dilution series in DNB. 200 µl aliquots of different dilutions were transferred to DNB plates. Triplicate samples were used to obtain as many culturable isolates as possible. Stains were purified by repeated streaking onto the same agar that was used for

initial isolation. One millilitre from the cell cultures was stored in 1:1 mixture with glycerol at - 80 °C. The bacterial strains were grown in nutrient broth (3 g meat extract per litre, 5 g peptone per litre) up to the mid-exponential growth phase for further investigations.

Phylogenetic analysis

DNA was extracted from 2 ml cultures, grown in liquid DNB medium up to the late exponential growth phase, by using NucleoSpin® Tissue (Machery-Nagel, Düren, Germany) according to instructions of the manufacturers. The 16S rRNA genes of the two bacterial strains JG37-Iso2 and JG37-Iso3 were amplified by using the primer pair $16S_{7F}$ and $16S_{1513R}$ (Lane, 1991) applying the PCR master mix and parameters as previously described (Chapters 1 & 2). The 16S rRNA gene products were purified by using an Edge Biosystems QuickStep®2 PCR Purification Kit (MoBiTec, Göttingen, Germany) and directly sequenced by using an ABI Prism® Big Dye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA), following the manufacturers instructions. The sequences were determined on an automated sequencer (Model 310 ABI PRISM, PE Applied Biosystems, Foster City, California, USA). The 16S rRNA gene sequences of the strains were obtained using the primers $16S_{7F}$ and $16S_{1513R}$ and additionally the forward primer $16S_{342F}$ (Muyzer *et al*., 1993). They were assembled by using the Autoassembler 2.0 software (PE Applied Biosystems, Foster City, California, USA). The sequences were compared with those available in the GenBanks by BLAST (Basic Local Alignment Search Tool) analysis (Altschul *et al*., 1997). For the sequence alignment the CLUSTALW version 1.7 was used (Thompson *et al*., 1994). Phylogenetic tree was generated using the neighbour-joining algorithm with distance analyses by using Jukes-Cantor corrections in the PHYLIP v. 3.5 package (Felsenstein, 1993). The almost complete 16S rRNA gene sequences of the *Arthrobacter* strains, JG37-Iso2 and JG37-Iso3, were deposited to the EMBL Nucleotide Sequence Database under accession numbers AM292605 and AM292606.

Carbon sources utilised by the strains studied

The substrate utilisation patterns were determined in duplicate. Cells grown to the mid-exponential phase were harvested by centrifugation at 8600 x g for 10 min at 4 °C and washed three times with 0.9% NaCl to remove residual substrates of the medium. For each 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 2 weeks of incubation at 30 °C and shaking at 110 rpm the growth was assessed by determining culture turbidity.

To test for growth on different carbon sources, sterile stock solutions of glucose, fructose, L- and D-arabinose, D-sorbitol, sucrose, and maltose were added to inorganic basal medium at a final concentration of 5 mM. The ability to utilise potential electron donors was analysed by addition of the following compounds to the medium: sodium acetate and sodium pyruvate (5 mM). Growth with alcohol such as methanol (2 mM) and glycerol (5 mM) was also tested. In addition, citric acid (5 mM), aspartic acid (5 mM) and casamino acid (0.05% vol/vol) were tested.

Heavy metal solutions

1 M stock solutions of $Pb(NO_3)_2$ (Fluka), $Cd(NO_3)_2*4H_2O$ (Merck), $Cr(NO_3)_2*9H_2O$ (Merck), $Cu(NO_3)_2*3H_2O$ (Merck), $Ni(NO_3)*6H_2O$ (Merck) and $UO_2(NO_3)_2*6H_2O$ (Chemapol) were prepared by dissolving appropriate quantities of the metals in 0.1 M $NaClO_4$. 1 M stock solution of $AgNO_3$ was prepared by dissolving the appropriate quantity of the metal in 0.1 M HNO_3 to prevent the precipitation of silver chloride. The pH value of the different metal solutions was adjusted by using NaOH or $HClO_4$ and then sterilised by filtration through 0.22 µm nitrocellulose filters.

Metal binding experiments

Metal sorption kinetics

Bacterial cells growing to mid-exponential growth phase were harvested by centrifugation at 10000 x g for 15 min at 4 °C and washed twice with 0.9% NaCl. The collected pellets (between 0.15 and 0.18 mg ml^{-1} dry weight) were resuspended in 10 ml $Pb(NO_3)_2$ or $UO_2(NO_3)_2$ solution (0.5 mM, pH 4.5) and incubated for 5, 10, 20, 30 min; 1, 2, 14, 19 or 24, 48 and 72 h at 30 °C by shaking at 110 rpm for each time in triplicate. Additionally, an experimental control sample without biomass was treated identically. After the metal contact, the cells were harvested and the metal content of the supernatant was analysed by ICP-MS (ELAN 6000, Perkin Elmer, Shelton, Connecticut, USA). Accumulation of the metal ions (q) by the biomass was calculated from the metal biomass balance yielding: q (mg metal per g dry biomass) = $V*(C_i-C_f)/M$ where V is the volume of the metal solution [l], C_i and C_f are the initial and the finial metal concentrations [mg l^{-1}] and M is the amount of the dry biomass [g] (Volesky, 2003). To measure the dry weight, the cell pellets were dried at 70 °C for 24 h and their weight was determined using a Sartorius LA 120 S (Göttingen, Germany) (±0.1 mg).

Effect of metal concentrations on the binding process

To determine the metal binding capacities at different metal concentrations, the cell pellets prepared as described above were resuspended in 10 ml of solutions possessing different concentrations (0.01, 0.05, 0.1, 0.25 and 0.5 mM) of Pb or U at pH 4.5, in triplicates and

shaked for 48 h. The shaked cells were harvested and the metal content of the supernatant was analysed with ICP-MS as described above.

TEM/EDX and electron diffraction analysis

This method was used for the localisation of accumulated Pb and U. Bacterial cells grown to mid-exponential phase were harvested by centrifugation at 10000 x g for 15 min at 4 °C and washed twice with 0.9% NaCl to remove the interfering ingredients of the growth medium. The pellet of the washed cells was resuspended in 30 ml $Pb(NO_3)_2$ or $UO_2(NO_3)_2$ solutions (0.5 mM, pH 4.5) and incubated for 48 h under the same conditions as described for metal binding studies. The metal treated cells were harvested by centrifugation and washed with 0.9% NaCl. The washed bacterial cell pellet was 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 buffer. The cell pellet was fixed for 60 min at 4 °C in 1% OsO_4 in cacodylate buffer before being hydrated 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. Samples were examined with high-resolution Philips CM 200 transmission electron microscope at an acceleration voltage of 200 kV under standard operating conditions with the liquid nitrogen anticontaminator in place. EDX analysis, which provides elemental information via the analysis of X-ray emissions caused by a high-energy electron beam, was also performed at 200 kV using a spot size of 70 Å and a live counting time of 200 s. For selected-area electron diffraction was performed as well. We used the Philips CM 200 transmission electron microscope in the diffraction mode with a camera length of 1000 mm and an exposure time between 15 and 20 s.

Life/Dead staining

After incubation with $Pb(NO_3)_2$ or $UO_2(NO_3)_2$ solutions (0.5 mM, pH 4.5) for 1 and 48 h, one millilitre of the cell suspensions was centrifuged at 10000 x g for 10 min at 4 °C. Cells treated only with 0.1 M $NaClO_4$ solutions at pH 4.5 and incubated for the same time, were used as controls. 3 µl of the dye (LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit L-7012, Molecular Probes, Inc., Eugene, Oregon, USA) were added to 1 ml cell suspension previously washed and resuspended in one millilitre 1xPBS (NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 1.4g, KH_2PO_4 0.2 g per liter pH 7.4), mixed thoroughly and examined with an Olympus BX-61 in combination with Olympus BX-UCB and Olympus U-RFL-T (Olympus, Hamburg, Germany) at a magnification of 1000 under UV illumination (filter U-MNU2). The LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit L-7012 contains a mixture of two fluorescing nucleic acid

stains with different membrane permeation characteristics. The green-fluorescing SYTO® 9 detects all cells, whereas the red-fluorescing propidium iodide can only pass through compromised or damaged membranes. Thus, red fluorescing cells are dead and green fluorescing cells are viable.

Preparation of samples for XAS analysis

For XAS analysis, the cells were harvested at mid-exponential growth phase by centrifugation at 10000 x g for 15 min at 4 °C and washed twice with 0.9% NaCl to remove the interfering ingredients of the growth medium. After contact of the cells with $UO_2(NO_3)_2$ solution (0.5 mM, pH 4.5, 48 h), the pellet was recovered by centrifugation, washed and dried at 70 °C for 48 h and finally powdered.

XAS measurement

Uranium L_{III}-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 flourescence mode using a 13-element Ge detector. The energy was calibrated by measuring the yttrium (Y) K-edge transmission spectrum of an Y foil and defining the first inflection point as 17038 eV.

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 μ_0-removal via spline fitting techniques and normalisation using a Victoreen function. Dead-time correction was applied. The ionisation energy for U L_{III} electron, E_0, was arbitrarily defined as 17185 eV for all averaged spectra. The EXAFS spectra were analysed according to standard procedures using the program EXAFSPAK (George & Pickering, 1995). The theoretical scattering phase and amplitude functions used in data analysis were calculated using FEFF8 (Ankudinov *et al*., 1998) using the model that contains fragments of two molecules, meta-autunite and uranyl triacetate (Merroun *et al*., 2005). All fits included the four-legged multiple scattering (MS) path of the uranyl group, U-O_{ax}-U-O_{ax}. The coordination number (N) of this MS path was linked to N of the single-scattering (SS) path U-O_{ax}. The radial distance (R) and Debye-Waller (σ^2) factor of the MS path were linked at twice the R and σ^2 of the SS path U-O_{ax}, respectively (Hudson *et al.*, 1996). During the fitting procedure, N of the U-O_{ax} SS path was held constant at two. The amplitude reduction factor was held constant at 1.0 for FEFF8 calculation and EXAFS fits. The shift in treshold energy, ΔE_0 was varied as a global parameter in the fits.

XRD of metal precipitates

To characterise the lead precipitates formed by the growing and non-growing cells of the two strains, the Pb-treated biomass harvested by scratching (growing) and centrifugation (resting cells) were dried at 70 °C for 24 h and pulverised. Samples for X-ray diffraction analysis were prepared by smearing the powder on a glass slide precoated with an ultrathin layer of petroleum jelly.Powder X-ray diffraction data were collected with a D8 diffractometer (Bruker-AXS, Karlsruhe, Germany) by using Cu Kα radiation. Data were collected at a speed between 18 and 60 s per 0.05°. Phase identification was carried out with the Library supplied by the Joint Commission on Powder Diffraction Software (JCPDS).

Heavy metal tolerance

To determine the tolerance of the two *Arthrobacter* strains JG37-Iso2 and JG37-Iso3 to six heavy metals (Cr, Ni, Cu, Cd, Pb, Ag) and U, inhibition growth experiments on low phosphate solid medium (LMP) were performed (Rossbach *et al*., 2000). Cells were grown to mid-exponential phase and washed twice with 0.9% NaCl. Ten µl of the cell suspension were transferred to LMP agar containing the following concentration of the metals: for Cu, Ni, and U increasing concentrations from 0.25 to 20 mM; for Cr from 0.5 mM to 16 mM; for Cd and Ag from 0.00375 mM to 0.5 mM; for Pb from 0.06 mM to 2 mM. Additionally, the Pb tolerance of the bacterial strains was studied using following media: modified LMP with a very low concentration of sulphate (16.7 µM) in form of $ZnSO_4$, modified LMP without sulphate and NB medium. After spreading the inoculums, the plates were incubated at room temperature for two weeks and the minimum inhibitory concentrations (MIC`s) were determined. MIC is defined as the lowest concentration of each of the metals at which complete inhibition of colony formation was observed (Rossbach *et al*., 2000).

RESULTS

Phylogenetic affiliation of the bacterial isolates

The phylogenetic affiliation of the bacterial strains isolated from the soil sample JG37 of the uranium mining waste pile Haberland near the town of Johanngeorgenstadt in Germany was based on their 16S rRNA gene sequence analysis. As evident from the results presented in Fig. 5.1, both isolates were affiliated with the actinobacterial genus *Arthrobacter*.

The 16S rRNA gene sequence of the strain JG37-Iso2 was identical to the 16S rRNA gene sequence of clone JG35-K2-AG91, retrieved from a sample of the same uranium mining waste pile but collected from lower depth and treated with sodium nitrate for 14 weeks under anaerobic conditions (Chapter 2). To the same cluster belongs the sequence AKAU3843, identified in soil heavily contaminated with U (206 mg U kg^{-1}), obtained from the NABIR Field Research Centre (FRC) at Oak Ridge National Laboratory, Tennessee, USA (Brodie *et al*., 2006). The phylogenetically closest cultured relative was *Arthrobacter* sp. Tibet-IIU21, which was isolated from the Qinghai-Tibet Plateau permafrost in China (EMBL No. DQ177477).

The 16S rRNA gene sequence of the strain JG37-Iso3 was almost identical to the 16S rRNA gene sequences of the Actinobacterium EC5 and the uncultured bacterium AKAU4199. The latter was identified in the above mentioned uranium contaminated soil of the NABIR FRC (Brodie *et al*., 2006). Interestingly, the sequence JG35+U2A-AG113, which was identified in a sample supplemented with uranyl nitrate up to 300 mg U kg^{-1} and incubated for 14 weeks under aerobic conditions, belongs to the same sub-cluster (Chapter 1). *Arthrobacter* populations were found in the untreated as well as in the samples treated with sodium nitrate (JG35-K1, JG35-K2) or uranyl nitrate (JG35+U1, JG35+U2A, JG35+U3), which were all collected from the uranium mining waste pile Haberland (Chapters 1 & 2, Fig. 5.1).

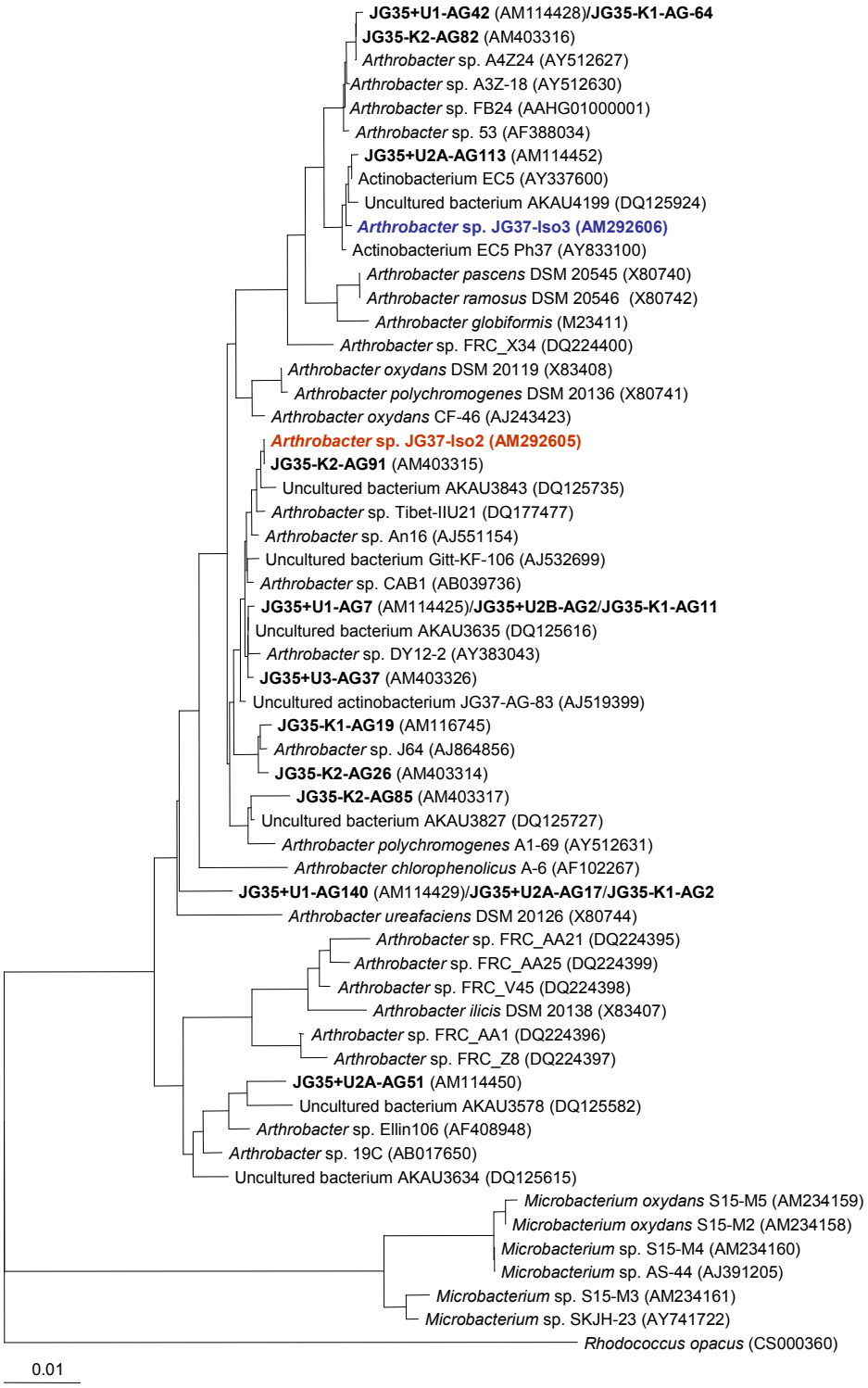

Fig. 5.1. 16S rRNA gene based phylogenetic affiliation of the bacterial strains **JG37-Iso2** and **JG37-Iso3** with members of *Actinobacteria*. The phylogenetic dendrogram was rooted with *Rhodococcus opacus*.

Carbon sources utilised by the strains studied

Both strains utilise four of the tested substrates, namely glucose, fructose, sucrose and maltose (Table 5.2). In addition, weak growth was observed in the presence of L- and D-arabinose.

Table 5.2. Substrate utilisation patterns of *Arthrobacter* sp. JG37-Iso2 and JG37-Iso3.

Substrate	***Arthrobacter* sp. JG37-Iso2**	***Arthrobacter* sp. JG37-Iso3**
Glucose	+	+
Fructose	+	+
L-arabinose	w	w
D-arabinose	w	w
D-sorbitol	-	-
Sucrose	+	+
Maltose	+	+
Sodium acetate	-	-
Sodium pyruvate	-	-
Methanol	-	-
Glycerol	-	-
Citric acid	-	-
Aspartic acid	-	-
Casamino acid	-	-

- no growth, + growth, w weak growth

Accumulation of and tolerance to Pb

Pb accumulation kinetics

The time course of Pb binding by the two isolates studied from a solution of $Pb(NO_3)_2$ containing 126 mg Pb l^{-1} at pH 4.5 is shown in Fig. 5.2. The ability of *Arthrobacter* sp. JG37-Iso2 to accumulate Pb increased rapidly with the exposure time up to 14 h (90% of the initial Pb) and remains constant between 24 and 72 h. The Pb accumulation by the second strain *Arthrobacter* sp. JG37-Iso3 increased slower than in the case of JG37-Iso2, reaching equilibrium after 48 h of contact with the metal.

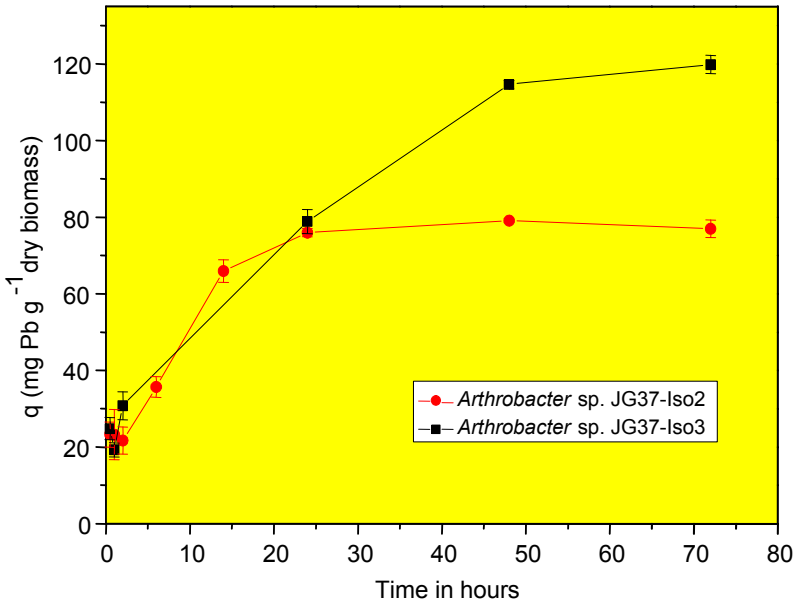

Fig. 5.2. Time-dependent accumulation of Pb by *Arthrobacter* strains JG37-Iso2 and JG37-Iso3.

Effect of metal concentration on the Pb accumulation

The removal of Pb from different solutions at pH 4.5 by both *Arthrobacter* strains, JG37-Iso2 and JG37-Iso3, is presented in Fig. 5.3. The results indicated that the amount of accumulated Pb increased with the increasing of the initial concentration of Pb (ranging from 2.53 mg l^{-1} to 122 mg l^{-1}) at pH 4.5. In addition, the cells of *Arthrobacter* sp. JG37-Iso3 bind Pb more effectively (110 ± 1 mg Pb g^{-1} dry biomass) than the strain *Arthrobacter* sp. JG37-Iso2 (76 ± 3 mg Pb g^{-1} dry biomass).

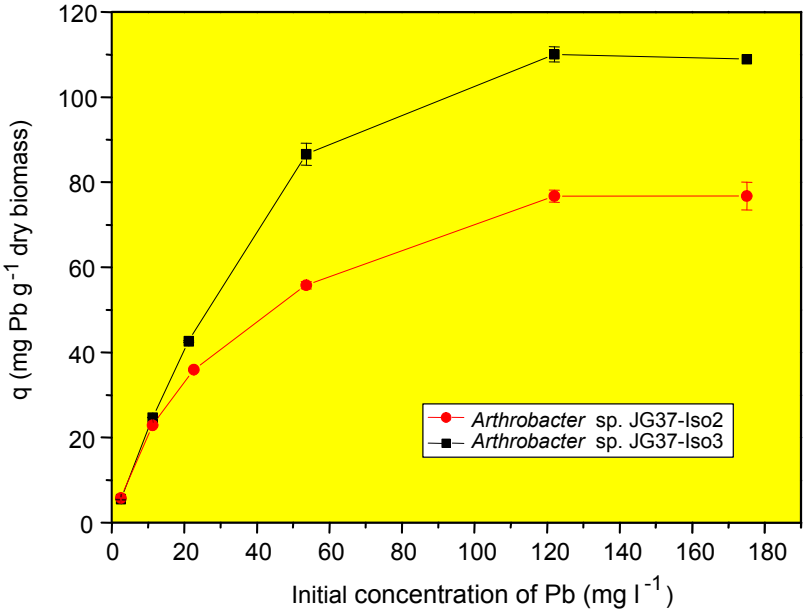

Fig. 5.3. Effect of initial metal concentration on the accumulation of Pb by the *Arthrobacter* strains JG37-Iso2 and JG37-Iso3.

TEM/EDX and electron diffraction analysis

Analysis of the cells of the *Arthrobacter* strains JG37-Iso2 and JG37-Iso3 treated with 0.5 mM solution of $Pb(NO_3)_2$ at pH 4.5 by using TEM demonstrated that the precipitation of Pb was mainly located at the cell surface (Fig. 5.4A and 5.5A). Additionally, a few Pb precipitates were localised intracellularly. The EDX spectra derived from the Pb precipitates (Fig. 5.4B and 5.5B) showed that they were composed of oxygen (O), phosphorus (P), and lead (Pb). The copper (Cu) peak resulted from the copper grid used to support the specimen. The uranium (U) peak originated from the uranyl acetate solution which was used to improve the contrast of the Pb treated cell thin sections. The presence of the silicon (Si) peak can be attributed to impurities in the culture medium and/or from the glass material of the flasks in which the cells were grown. The electron diffraction (Fig. 4C and 5C) showed the crystalline nature of the precipitates. The electron diffractogram showed a strong reflection with a spacing 4.3 and 2.94 Å, which are in fairly good agreement with the spacing 4.33 and 2.959 Å, reflections for pyromorphite ($Pb_5(PO_4)_3Cl$) (JCPDS, File 19-0701).

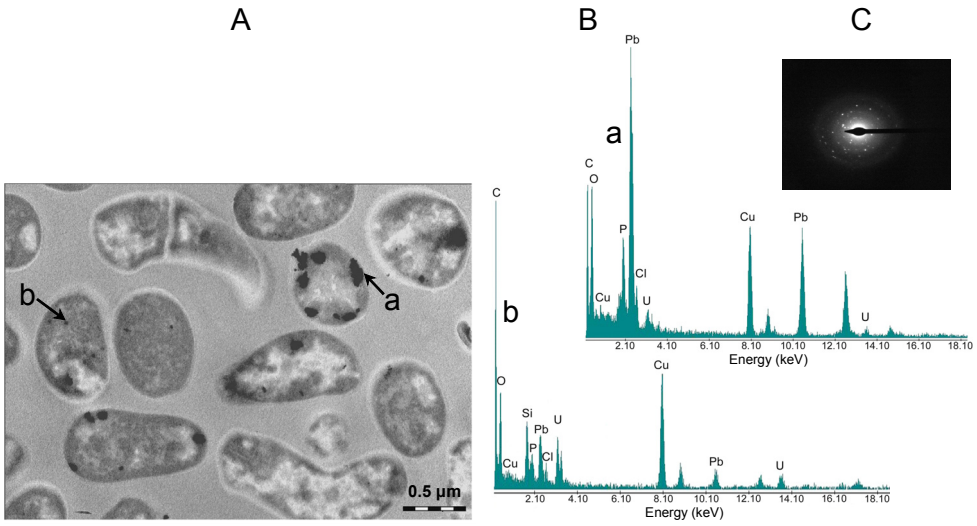

Fig. 5.4. Transmission electron micrograph (A) of a thin section of *Arthrobacter* sp. JG37-Iso2 cells treated with 0.5 mM $Pb(NO_3)_2$ at pH 4.5 and energy-dispersive X-ray spectra (B) of the points marked with arrowheads. The metal accumulated was localised at the cell surface (a) and intracellularly (b). Electron diffraction of the Pb precipitates marked with arrowhead (a) is shown in (C).

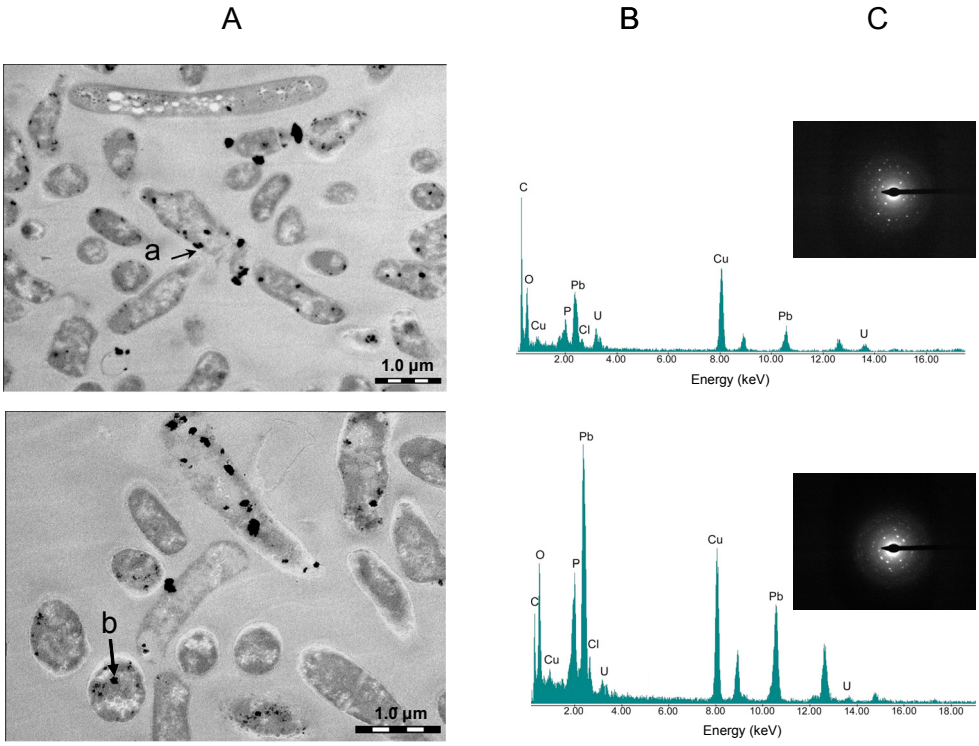

Fig. 5.5. Transmission electron micrographs (A) of a thin section of *Arthrobacter* sp. JG37-Iso3 cells treated with 0.5 mM $Pb(NO_3)_2$ at pH 4.5 and energy-dispersive X-ray spectra (B) of the points marked with arrowheads. The metal accumulated was localised at the cell surface (a) and intracellularly (b). Electron diffractions of the Pb precipitates are shown in (C).

Life/Dead staining

The viability of the *Arthrobacter* sp. JG37-Iso2 cells was not drastically affected by long-time exposure to 0.5 mM $Pb(NO_3)_2$ dissolved in 0.1M $NaClO_4$ at pH 4.5. More than 80% of the cells were viable (green fluorescing) after 1 h, and 60% were alive after 48 h (Fig. 5.6). In control samples, where the cells were incubated in a 0.1 M $NaClO_4$ solution at pH 4.5, 90% of the cells were alive even after 48 h.

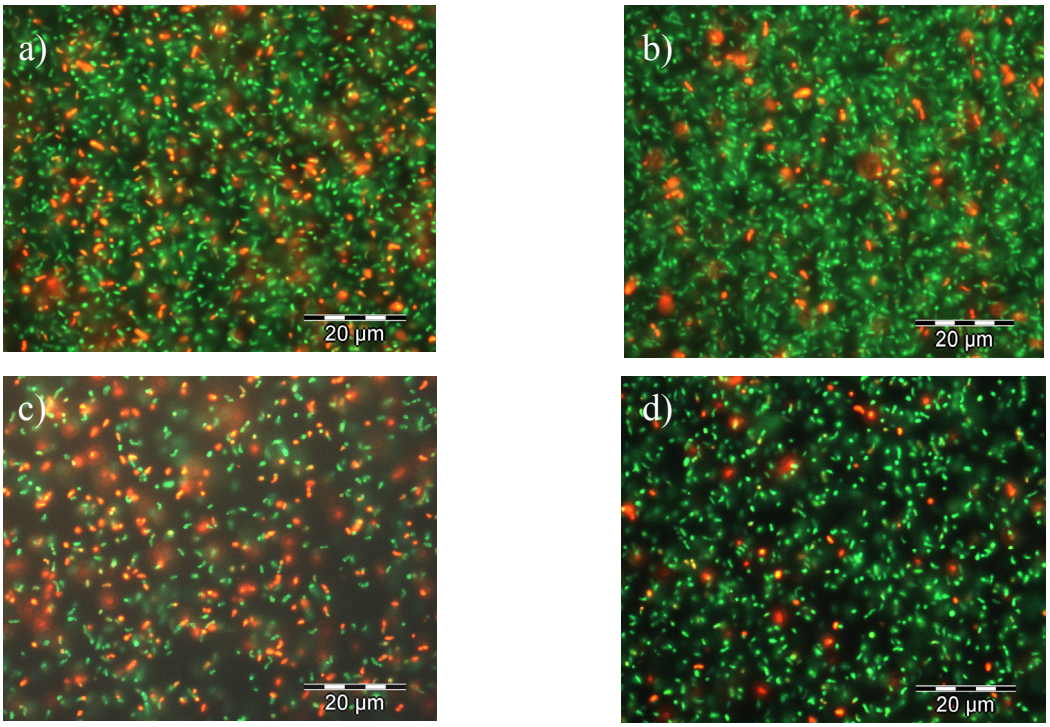

Fig. 5.6. Life/Dead micrographs of *Arthrobacter* sp. JG37-Iso2 cells treated with 0.5 mM $Pb(NO_3)_2$ dissolved in 0.1 M $NaClO_4$ pH 4.5 for 1 h (a) and for 48 h (c), and control sample treated only with 0.1 M $NaClO_4$ pH 4.5 for 1 h (b), and for 48 h (d). Red fluorescing cells are dead and green fluorescing cells are viable.

XRD

Non-growing conditions

The resting cells of the two strains studied, which were treated with 0.5 mM $Pb(NO_3)_2$ pH 4.5 for 48 h and prepared as described in Materials and Methods, were investigated by using XRD. In Fig. 5.7 an exemplary X-ray diffraction pattern of the Pb precipitate formed by resting cells of *Arthrobacter* sp. JG37-Iso2 is shown, which was identified by using JGPDS library of compounds. The close match between the pattern reported for pyromorphite ($Pb_5(PO_4)_3Cl$) (JCPDS card no. 19-0701) and our compound gave an indication that Pb was precipitated as a lead phosphate compound (pyromorphite ($Pb_5(PO_4)_3Cl$)).

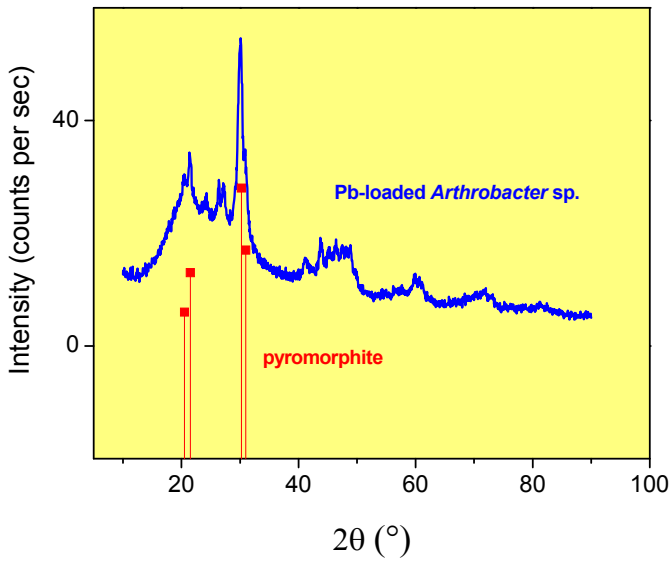

Fig. 5.7. X-ray diffraction pattern of resting cells of *Arthrobacter* sp. JG37-Iso2 treated with 0.5 mM $Pb(NO_3)_2$ pH 4.5 for 48 h, super imposed with vertical drop-down line corresponding to the expected positions of pyromorphite (($Pb_5(PO_4)_3Cl$), JCPDS pattern number 19-0701).

Growing conditions

In growing conditions, when the strain JG37-Iso2 was grown for 2 weeks at room temperature on LPM and NB supplemented with 0.5 mM $Pb(NO_3)_2$ it produced a brownish-black precipitate (Fig. 5.8a). Cells of the strain JG37-Iso2 growing on the LMP without Pb (controls) produced no dark compound, confirming that the presence of Pb(II) in the medium was necessary for appearance of the brown compound (Fig. 5.8b).

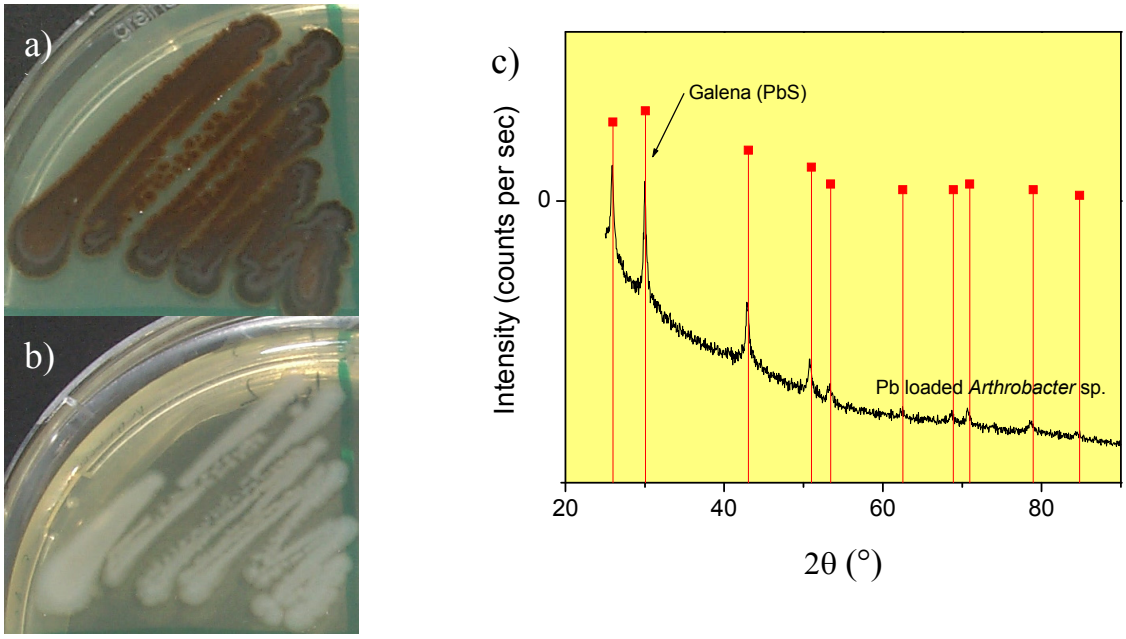

Fig. 5.8. Cells of *Arthrobacter* sp. JG37-Iso2 incubated for 2 weeks on low phosphate solid medium (LMP) containing 0.5 mM $Pb(NO_3)_2$ (a) and LMP without metal (b). X-ray diffraction pattern of the brown precipitate obtained from cells of *Arthrobacter* sp. JG37-Iso2 growing on LMP containing 0.5 mM $Pb(NO_3)_2$ (c), super imposed with vertical drop-down line corresponding to the expected positions of galena (PbS) (JCPDS pattern number 5-0592).

The brown precipitate was purified as described in Materials and Methods and investigated by using XRD. The close match between the pattern reported for PbS (JCPDS card no. 5-0592) and our compound gave an indication that the crystalline phase in the precipitate is a complex lead sulphide (PbS, galena).

Changes in the colour of the colonies could not be observed on modified LPM (lower sulphate concentration or without sulphate), indicating that Pb was not precipitated under these conditions. In addition, the Pb MIC`s for the growth of the studied strains on the modified LMP were lower than on LMP and NB solid media (Table 5.3).

Table 5.3. Minimum inhibitory concentrations (MIC's) of Pb (in mM) for the growth of the two *Arthrobacter* strains JG37-Iso2 and JG37-Iso3 on different media.

Strains \ Media	NB	LMP	LMP with only 16.7 µM SO_4	LMP without sulphate
Arthrobacter sp. JG37-Iso2	1	1	0.5	0.5
Arthrobacter sp. JG37-Iso3	2	0.5	0.5	0.5

NB - nutrient broth solid medium, LMP - low phosphate solid medium, LMP without SO_4 – all sulphate compounds were displaced by chloride compounds

Accumulation of and tolerance to U

U accumulation kinetics

Fig. 5.9 represents the time-dependent U accumulation by the two bacterial strains at pH 4.5. The U accumulation by *Arthrobacter* sp. JG37-Iso3 increased rapidly with exposure time up to 20 h, followed by gradual increase reaching equilibrium after 48 h contact time. Comparable accumulation profile was observed for the *Arthrobacter* strain JG37-Iso2: rapid U accumulation increase until 20 h exposure time, followed by gradual increase. However equilibrium was not attained within 72 h.

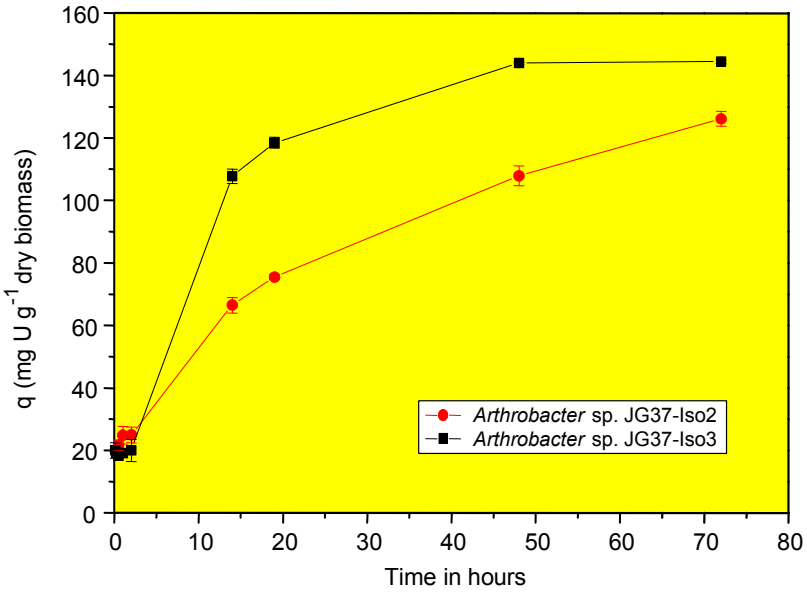

Fig. 5.9. Time-dependent accumulation of U by the *Arthrobacter* strains JG37-Iso2 and JG37-Iso3.

Effect of metal concentration on the U accumulation

The removal of U from solutions at different initial uranium concentrations (from 1.88 mg U l^{-1} to 125 mg U l^{-1}) at pH 4.5 for the *Arthrobacter* strains JG37-Iso2 and JG37-Iso3 are presented in Fig. 5.10. The results indicated that the amount of U accumulated by the two *Arthrobacter* strains increased with increasing U concentrations at pH 4.5. By an initial concentration of 119 mg U l^{-1} the strains JG37-Iso2 and JG37-Iso3 accumulated up to 108 ± 3 and 162 ± 6 mg U g^{-1} dry biomass, respectively (Fig. 5.10).

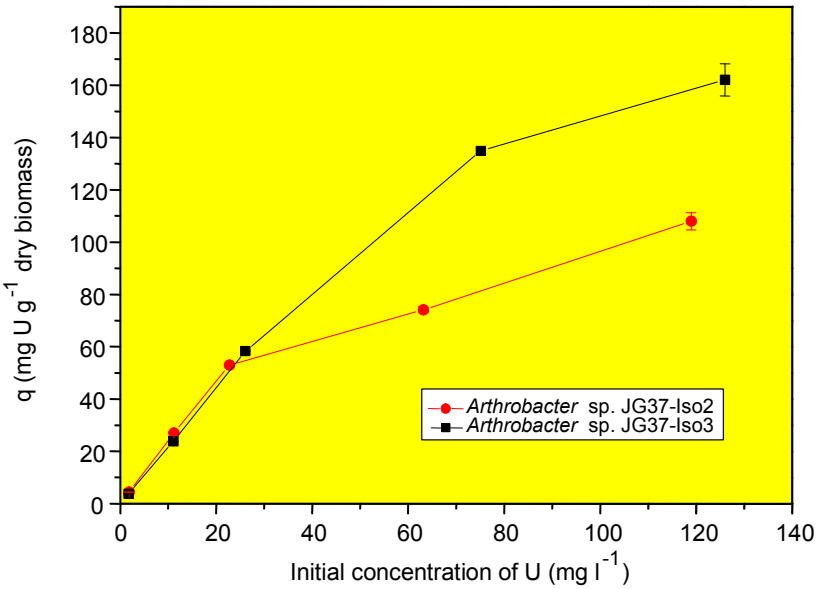

Fig. 5.10. Effect of the initial metal concentration on the U accumulation by the *Arthrobacter* strains JG37-Iso2 and JG37-Iso3.

TEM/EDX and electron diffraction analysis

TEM analysis of the *Arthrobacter* strains JG37-Iso2 and JG37-Iso3 cells treated with 0.5 mM $UO_2(NO_3)_2$ at pH 4.5 revealed intracellular accumulated needle-like fibrilles (Fig. 5.11).

The EDX spectra of the U deposits showed that they are composed of oxygen (O), phosphorus (P), and uranium (U). The high copper 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 visualisation of the uranium-treated cell thin sections.

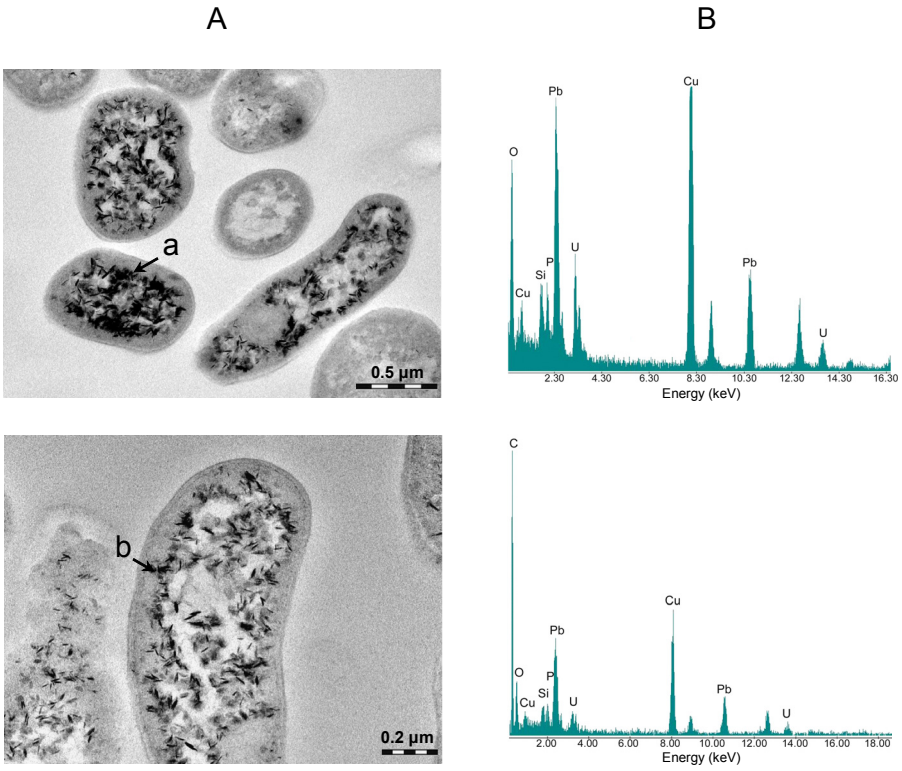

Fig. 5.11. Transmission electron micrographs (A) of a thin section of *Arthrobacter* sp. JG37-Iso2 (a) and *Arthrobacter* sp. JG37-Iso3 (b) cells treated with 0.5 mM $UO_2(NO_3)_2$ at pH 4.5 and energy-dispersive X-ray spectra (B) of the points marked with arrowheads. The metal accumulated was localised intracellularly (a, b).

XAS spectroscopy

XANES analysis showed that the oxidation state of the uranyl ions added to the cells was unchanged indicating that the bacteria do not reduce U(VI) (data not shown).

Information on the local environment of uranium atoms in the uranium bacterial samples was provided by analysis of the EXAFS data.

The k^3-weighted χ spectra determined from EXAFS analyses of the uranium species formed at pH 4.5 by the cells of the *Arthrobacter* strains JG37-Iso2 and JG37-Iso3, are presented in Fig. 5.12 along with the best fits obtained from the fitting procedure. Solely on the basis of the raw data, the spectra showed a strong similarity. There was close agreement between the two samples in the phases as well in the amplitude of all the features in *k* space. Similarly, the *R*-space plots of the Fourier-transformed EXAFS spectra shown were also in close agreement. The Fourier transforms (FTs) represent a pseudo-radial distribution function of the uranium near-neighbour environment. The peaks appeared at lower *R*-values relative to the true near-neighbour distances as a result of the EXAFS phase shift, which is different for each neighbouring atom (ϕ = 0.2-0.5 Å).

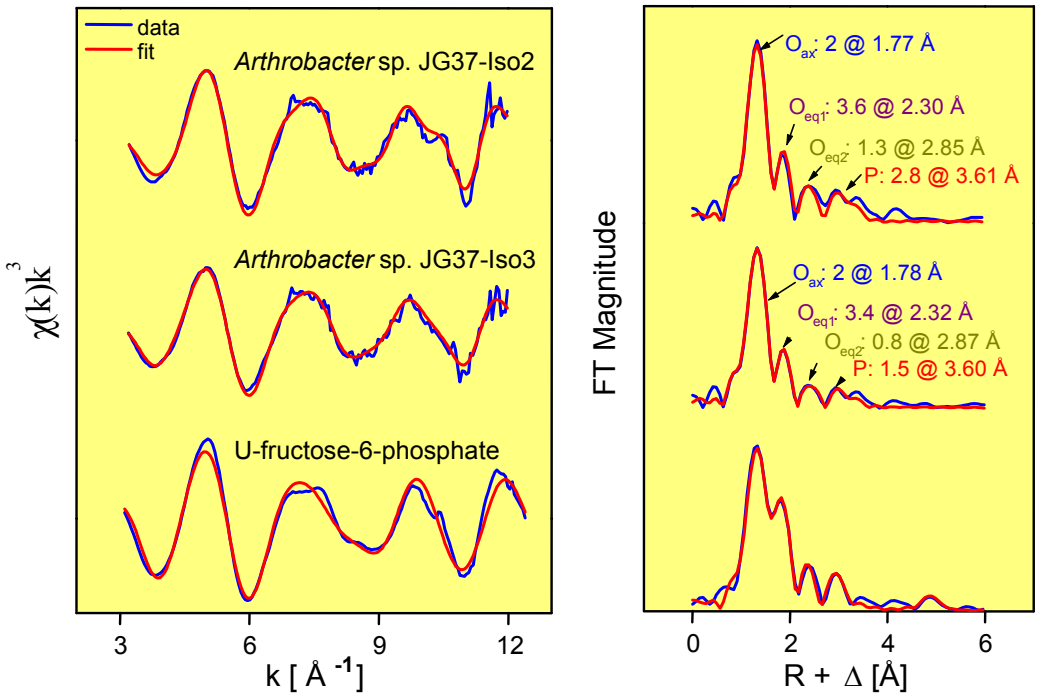

Fig. 5.12. Uranium L_{III}-edge k^3-weighted EXAFS spectra (left) and the corresponding fourier transforms (FT) (right) of the uranium complexes formed by the cells of *Arthrobacter* sp. JG37-Iso2 and *Arthrobacter* sp. JG37-Iso3 at pH 4.5, as well as of the reference compound U-fructose-6-phosphate at pH 4.0 (Koban *et al*., 2004).

In all samples, fitting showed that U(VI) has about two oxygen atoms in the axial plane (O_{ax}) at a distance of 1.77-1.78 Å and four oxygen atoms in the equatorial plane (O_{eq1}) at 2.30-2.32 Å (Table 5.4). The distance between U(VI) and the equatorial oxygen atoms is generally characteristic for both the type of ligand and the coordination polyhedra of the uranyl cation. The U-O_{eq1} bond distance is within the range of previously reported values for the oxygen atom of the phosphate bound to uranyl (Hennig *et al*., 2001; Kelly *et al*., 2002; Merroun *et al*., 2003a).

The high debey-waller factors of the U-O_{eq1} shells (Table 5.4) suggest that the equatorial shell is splitted into more than one oxygen distance. This splitting may involve two distances, a shorter U-O_{eq1} bond distance from the backscattering contribution of the phosphate oxygen(s) (monodentate) and a longer U-O_{eq1} bond distance from coordinated water molecules and/or from oxygen(s) of non-monodentate bound phosphate group(s). The two shells cannot be separated from each other in the analysis conveniently, because of the restricted maximum resolution of 0.18 Å in the radial distance.

Table 5.4. Structural parameters of the uranium complexes formed by *Arthrobacter* isolates at pH 4.5.

Sample	Shell	N[a]	R(Å)[b]	σ^2 (Å^2)[c]	ΔE (eV)
JG37-Iso2	U-O_{ax}	2[d]	1.77	0.0030	-15.1
	U-O_{eq1}	3.6(4)	2.30	0.011	
	U-O_{eq2}	1.3(2)	2.85	0.0040[d]	
	U-P	2.8(3)	3.61	0.0040[d]	
	U- O_{eq1}-P (MS)	5.6	3.74	0.0040[d]	
JG37-Iso3	U-O_{ax}	2[d]	1.78	0.0036	-13.7
	U-O_{eq1}	3.4(4)	2.32	0.013	
	U- O_{eq2}	0.8(2)	2.87	0.0040[d]	
	U-P	1.5(3)	3.60	0.0040[d]	
	U- O_{eq1}-P (MS)	3.0	3.73	0.0040[d]	

[a] Errors in coordination numbers are ±25%, and standard deviations, as estimated by EXAFSPAK are given in parentheses
[b] Errors in distance are ±0.02 Å
[c] Debye-Waller factor
[d] Value fixed for calculation

All FTs contain a peak at about 2.3 Å as a minor component. After correcting for the scattering phase shift, this distance is typical for carbonate groups coordinated to U(VI) in a bidentate fashion. However, further investigations (data not shown) excluded the contribution of carbon atoms in the coordination of uranium. Oxygen neighbours (O_{eq2}) provide a good fit to the EXAFS spectrum corresponding to this shell.

Approximately 20-30% of the 3 Å FT peak amplitude can be accounted for as linear MS in the uranyl transdioxo unit. In all samples, an additional P shell at the later FT peak (3 Å) was required to fit the spectra. This U-P distance is consistent with monodentate coordination of phosphate to the uranyl equatorial plane as it was found in U-fructose-6-phosphate (Koban *et al*., 2004).

Heavy metal tolerance

The MIC`s of Cr, Ni, Cu, Cd, Pb, Ag and U were determined on low phosphate solid medium for the growth of *Arthrobacter* strains JG37-Iso2 and JG37-Iso3 (Table 5.5).

The two bacterial strains differ in their tolerance to Ni, Cd and Pb. *Arthrobacter* sp. JG37-Iso3 tolerates lower concentrations of Ni, Cd and Pb in comparison to JG37-Iso2. Interestingly, *Arthrobacter* sp. JG37-Iso2 could grow at higher concentrations of Ni up to 18 mM in comparison to JG37-Iso3. No differences were observed in tolerance to Cr, Cu, Ag and U.

Table 5.5. Minimum inhibitory concentrations (in mM) for the growth of the *Arthrobacter* strains JG37-Iso2 and JG37-Iso3.

Strains	Cr	Ni	Cu	Cd	Pb	Ag	U
Arthrobacter sp. JG37-Iso2	4	20	8	1	1	0.125	2
Arthrobacter sp. JG37-Iso3	4	8	8	0.03	0.5	0.125	2

DISCUSSION

In this study, the interactions of two bacterial strains isolated from the soil sample JG37 of the uranium mining waste pile Haberland, with Pb and U were described. The isolated strains were affiliated with members of the actinobacterial genus *Arthrobacter*. A large variety of *Arthrobacter* spp. were found in different uranium or heavy metal contaminated environments (Balkwill *et al*., 1997; Tranjanovska *et al*., 1997; Konopka & Zakharova, 1999; Beneyhuda *et al*., 2003; Fredrickson *et al*., 2004; Hanbo *et al*., 2004; Fields *et al*., 2005; Martinez *et al*., 2006). The direct molecular 16S rRNA gene sequence analysis of the sample JG37 performed earlier retrieved only one individual sequence related to an *Arthrobacter* sp. (Geissler, 2003). However, a strong stimulation of *Arthrobacter* populations was observed by addition of uranyl or sodium nitrate to a sample collected from the same site, which was incubated under aerobic or anaerobic conditions (Chapters 1 & 2).

The bacteria in the soil sample JG37 are subjected to different contaminants including radionuclides, U and Th for instance, and heavy metals such as Pb, Fe, Al, Cu etc. In order to investigate how the JG37 bacterial isolates were able to cope with the toxicity of Pb and U, a combination of spectroscopic, microscopic, microbiological and sorption studies was performed.

Interations with Pb

The two JG37 isolates precipitated Pb as metal phosphates or sulphides depending on the physiological state of the bacterial cells (non-growing or growing conditions).

Non-growing conditions: XRD and electron diffraction studies indicated that the resting cells of the two strains deposited Pb as lead phosphate mineral phase, identified as pyromorphite ($Pb_5(PO_4)_3Cl$). Electron diffraction analysis demonstrated that the dominant form of the lead accumulates is pyromorphite, indicating that the precipitation of lead is the main mechanism implicated in the interaction of the two bacterial isolates with this toxic heavy metal. This finding is supported by the results of Pb sorption kinetic studies, which demonstrated that the binding of this metal by the two isolates is very slow. Only 25% of the total Pb was accumulated after a contact time of 2 h, and the accumulation equilibrium was reached between 24 and 48 h. TEM analysis indicated that not all cells of the *Arthrobacter* strains are able to form pyromorphite, which was mainly localised at the cell surface closely associated to cell membranes. Moreover, Live/Dead staining showed that only 60% of the Pb-treated *Arthrobacter* sp. cells are alive after 48 h of contact time. This indicates that the fact that only a part of the cells is mineralised can be due to the viability of the cells.

In many other studies the bacterial precipitation of lead phosphates of different composition is well documented (Aickin *et al*., 1979; Aiking *et al*., 1985; Templeton *et al*., 2003; Mire *et al*., 2004). Cells of *Citrobacter* sp. (Aickin *et al*., 1979) and *Vibrio harveyi* (Mire *et al*., 2004) deposited this heavy metal as $PbHPO_4$ and $Pb_9(PO_4)_6$, respectively. In addition, congruently to our studies, pyromorphite ($Pb_5(PO_4)_3OH$) was formed by cell suspensions and biofilms of *Burkholderia cepacia* (Templeton *et al*., 2003). The latter authors reported, however, that the formation of pyromorphite by cell suspensions and biofilms of *B. cepacia* requires metabolic activity, hypothesising that it may be due to the distribution of microbes at variables stages in the cell cycle or differences in the regulation of key enzymes within individual microcolonies.
A source of phosphate is required for the pyromorphite formation. The release of phosphate via the hydrolysis of an organic phosphate has been shown to be an effective method for precipitation of metals on cell membranes. Macaskie and Dean (1982) isolated a cadmium-resistant strain of *Citrobacter* that precipitates numerous metals, as metal phosphates through the use of a membrane-bound acid phosphatase. Using glycerol-2-phosphate as a phosphate source, the organisms cleaved phosphate from the source in the periplasmic space, leaving it to bind with metals that have been complexed in solution. In this study, no organic phosphate source was added to the metal-bacteria mixture**.** A possible source of phosphate could be intracellular or possibly from components of the cell membranes as suggested by Templeton *et al*. (2003) in the case of the precipitation of pyromorphite by cell suspensions and biofilms of *B. cepacia.*
Growing conditions: *Arthrobacter* sp. JG37-Iso2 cells growing on low phosphate and nutrient broth solid media containing 0.5 mM $Pb(NO_3)_2$ precipitated Pb as metal sulphides phase identified as galena, due to production of sulphide. For removal of heavy metals from wastewater, addition of hydrogen sulphide (biologically or nonbiologically) can be especially effective because the metal sulphide precipitates are extremely insoluble and stable (Peters *et al*., 1985). Biological hydrogen sulphide production could also be used to precipitate and stabilise heavy metals *in situ* (Janssen & Temminghoff, 2004). Previous research on bioprecipiation has predominantly focused on using sulphate-reducing bacteria to produce sulphide and precipitate heavy metals as metal sulphides (Fortin *et al*., 1994; Labrenz *et al*., 2000; White & Gadd, 2000; Weijma *et al*., 2002; Janssen & Temminghoff, 2004). However, sulphate-reducing bacteria are obligate anaerobes and their application is limited to anaerobic environments (Wang *et al*., 2000).
In this study, the source of sulphide responsible for lead precipitation is unknown. However, by using modified LMP with lower concentration of sulphate or without sulphate to study the

interactions of the two *Arthrobacter* strains with Pb, it was demonstrated that no brown colonies were observed and the MIC´s for Pb were in this case lower than on LMP and NB. This indicates that the presence of sulphate in the low phosphate medium influences the tolerance of the bacteria to Pb and the formation of PbS. Another mechanism was described for explaining the production of sulphide (Cunningham & Lundie, 1993; Wang *et al*., 2000). Cunningham and Lundie (1993) demonstrated that desulfhydration of cysteine, added to the growth medium as a reducing agent, was the likely source of sulphide for cadmium precipitation by *Clostridium thermoaceticum*. However, we have no indication that this mechanisms occurs in our case.

Interactions with U(VI)

TEM images of the *Arthrobacter* cells exposed to U(VI) solution showed that this radionuclide is accumulated intracellularly. In addition, accumulation kinetics studies indicated that the time dependent U accumulation by *Arthrobacter* strains JG37-Iso2 and JG37-Iso3 appears to be a two-step process. The first step is fast, within several hours. The second step proceeds more slowly and equilibrium is not attained within 48 h. This indicates that behind surface complexation another process possibly intracellular accumulation is implicated in the interactions of these strains with U. The intracellular U accumulation could be seen as a tolerance mechanism of the genus *Arthrobacter* against this metal.

The tolerance to U exhibited by Gram-positive and Gram-negative microorganisms may be explained by several different cellular mechanisms, as previously reported by other investigators (Macaskie *et al*., 1992; Renninger *et al*., 2004; Suzuki & Banfield, 2004; Merroun *et al*., 2005; Martinez *et al*. 2006). One mechanism is biosorption at the cell surface, which was hypothesised for *Arthrobacter nicotianae*, where no cellular localisation of uranium was determined (Tsuruta, 2002). In addition, Krueger *et al*. (1993) have shown that surface-bound uranium in *Pseudomonas fluorescens* was spread over the entire cell envelope, in an outer membrane-peptidoglycan-plasma membrane complex as fine-grained, platy uranium minerals (10 nm to 1 μm). Similar to our results an intracellular accumulation of uranium by a high G+C Gram-positive isolate, closely related to *Arthrobacter ilicis*, was observed and it was demonstrated that the precipitates were closely associated with polyphosphate granules (Suzuki & Banfield, 2004). Other microorganisms such as *Acidithiobacillus ferrooxidans* (Merroun *et al*., 2003a) can accumulate uranium intracellularly *via* passive transport mechanisms, resulting in the formation of dense uranium deposits inside the cells. An extremely rapid intracellular accumulation of uranium by *Pseudomonas*

aeruginosa was demonstrated for which metabolism was not required (Strandberg *et al*., 1981).

The mechanisms by which uranium enters the cells are not yet known. So far, no transport systems for this radionuclide were described. TEM analysis demonstrated that most of the cells bearing intracellular accumulates possess intact cell membranes, but congruently to others (Strandberg *et al*., 1981; Suzuki & Banfield, 2004), not all cells possessed visible intracellular uranium deposits. The intracellular accumulation of uranium could be due only to the changes in the permeability of the cell membranes of the U exposed cells (Suzuki & Banfield, 1999).

To our knowledge, this is the first study that investigated the coordination of U to arthrobacterial strains at molecular scale using XAS. The synchrotron-based technique (XANES) has been used to determine the oxidation state and to identify the number of atoms, and their distances in the local structural environment of U (EXAFS) within a variety of microbial samples (Hennig *et al*., 2001; Kelly *et al*., 2002; Merroun *et al*., 2003a, 2005, 2006; Nedelkova *et al*., 2006).

In the present study, EXAFS analysis indicated that U(VI) is mainly coordinated in the *Arthrobacter* accumulates by organic phosphate groups in a monodentate binding mode. A survey of structural parameters of the uranium complexes formed by different bacterial cells and their components at pH 4.5 found in the literature demonstrated that this radionuclide is coordinated differently to microbial cells: i) by organic phosphate groups as for instance by cells of *A. ferrooxidans* (Merroun *et al*., 2003b); ii) by carboxylic and phosphate groups of the cells and S-layer sheets of *Bacillus sphaericus* JG-A12, a bacterium isolated from the same uranium mining waste pile (Merroun *et al*., 2005), and iii) by inorganic phosphate groups forming a *m*-autunite-like phases by the cells of *Microbacterium oxydans* S15-M2 and *Sphingomonas* sp. S15-1 isolated from the S15 deep-well monitoring site near the Siberian radioactive subsurface depository Tomsk-7, Russia (Merroun *et al*., 2006; Nedelkova *et al*., 2006). Interestingly, in the case of the *Sphingomonas* sp. S15-1 cells, electron-dense intracellular granules and uranium-bearing precipitates at the cell membrane were localised by TEM. It was suggested that intracellular granules correspond to the cell polyphosphate bodies (Merroun *et al*., 2006). An intracellular accumulation of uranium closely associated with polyphosphate granules was also suggested for a high G+C Gram-positive isolate, closely related to *Arthrobacter ilicis*, by using TEM and EDX (Suzuki & Banfield, 2004). However in the case of the *Arthrobacter* strains studied in the present work no indication of U(VI) complexation by inorganic polyphosphates was found but organic phosphates were shown to

be involved in the U(VI) complexation. The origin of the organic phosphates complexing U(VI) was not defined. However, it was demonstrated that bacteria react to nutrient limitations, heavy metals, and other kinds of stress by accumulating highly phosphorylated proteins (Rosen *et al*., 2004). The latter are good candidates for binding U(VI). Studies on cells of *Thiobacillus ferrooxidans* grown in the presence of different heavy metals (Cu, Ni etc.) revealed that specific proteins were induced by copper and cadmium, and that in the presence of the latter metal the level of the protein phosphorylation was increased (Novo *et al*., 2000). Changes in the total protein synthesis pattern of the *Arthrobacter* strains studied in the present work were observed by treatment with uranyl nitrate dissolved in sodium perchloric acid in comparison to the control treated only with sodium perchloric acid (data not shown). Efforts to identify the up- and down-regulated proteins influenced by the addition of U(VI) to the cells are in progress in our laboratory.

Tolerance to other heavy metals

While some microbes are able to immobilise particular heavy metals the sensitivity of these organism to other heavy metals could possibly limit their *in situ* activities (Martinez *et al*., 2006). Therefore, tolerance studies of the *Arthrobacter* strains against different heavy metals have been performed. A comparison of the results from these investigations with other studies is often difficult because different conditions were used (Hanbo *et al*., 2004; Suzuki & Banfield, 2004). Our results can be compared with those obtained by the analysis of three other *Actinobacteria* strains of the genus *Microbacterium*, which were investigated under the same conditions in our laboratory (Nedelkova *et al*., 2006). The two *Arthrobacter* spp. studied in this work could tolerate higher concentrations of nickel on low-phosphate solid medium than the *Microbacterium* spp.. Interestingly, *Arthrobacter* sp. JG37-Iso2 and the three *Microbacterium* strains tolerate high concentrations of lead (Nedelkova *et al*., 2006). On the other hand, *B. sphaericus* JG-7B, which also belongs to the indigenous bacterial community of the uranium mining waste pile Haberland (Brottka, 2003), and *Arthrobacter* sp. JG37-Iso3 have only a MIC of 0.5 mM for lead nitrate. The cells of our two *arthrobacterial* isolates tolerated a U concentration of 1 mM, whereas the *Microbacterium* strains tolerates up to 2 mM (Nedelkova *et al*., 2006).

CONCLUSIONS

Our results demonstrate that the two *Arthrobacter* strains isolated from the uranium mining waste pile Haberland near Johanngeorgenstadt in Germany tolerate relatively high concentrations of different heavy metals and uranium in a strain specific way. Lead, a toxic

heavy metal, was precipitated as lead phosphates or lead sulphides depending on their physiological state of the bacterial cells. The U was immobilised intracellularly as uranyl organic phosphate complexes. Because of these properties the recovered *Arthrobacter* strains are of interest for *in situ* bioremediation of contaminated mixed waste sites.

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Outlook

The studies demonstrated that the microbial community structure changed differently after the addition of uranyl or sodium nitrate and a subsequent incubation for different periods of time under aerobic or anaerobic conditions by using the 16S rRNA gene retrieval. Real-time PCR based on 16S rRNA genes should be performed to obtain better quantitative information of the distribution of specific bacterial groups.

By using Mössbauer spectroscopy a reduction of Fe(III) to Fe(II) was demonstrated in the samples treated with uranyl nitrate and incubated under anaerobic conditions. Future studies are necessary to characterise the fate of the added U(VI) in the samples treated with uranyl nitrate under anaerobic conditions. For this reasons X-ray absorption spectroscopic (XAS) analysis and Time Resolved Laser-induced Fluorescence spectroscopic (TRLFS) analysis of these samples are in progress.

Further studies which focus on the interplay between abiological and biological processes influencing the fate of uranium in complex natural systems should be performed as column experiments with a combination of geochemical and spectroscopic methods together with molecular biological methods.

In addition, changes in the nitrate-reducing community were observed by using the membrane-bound nitrate reductase gene (*nar*G) as a functional marker. Specific primers for other nitrate reductases or enzymes involved in nitrogen cycle can be used to further study the influence of addition of different nitrate solutions on the microbial communities. Hybridization and real-time PCR should be performed with DNA extracted from the samples studied to obtain better quantitative information of the relative distribution of the nitrate-reducing bacteria.

Changes in the archaeal community were observed as well. The *Archaea*-specific primers used in this study were not capable to amplify the 16S rRNA genes of *Nanoarchaeota* and *Korarchaeota*. Primers specific for these archaeal divisions should be used for the identification of these lineages. It is important to try to culture representatives of the crenarchaeal group 1.1b from the uranium mining waste pile and to study their interactions with uranium, to get more information about the role of *Archaea* in the migration of uranium. So far, in our laboratory, mesophilic *Crenarchaeota* of group 1.1b were enriched in a mixture with *Clostridium* spp. The members of the mixed cultures could be separated, for instance in a density gradient (Percholl-gradient). Then the archaeal cells of the crenarchaeal group 1.1b

should be inoculate to specific media for ammonia oxidising organisms, as we suggested that metabolic feature for the identified *Crenarchaeota* of group 1.1b, and incubated under aerobic and anaerobic conditions.

Growing cells of two *Arthrobacter* strains JG37-Iso2 and JG37-Iso3 recovered from the uranium mining waste pile Haberland were able to precipitate Pb as lead sulphide (galena), whereas non-growing cells of these strains precipitate Pb as lead phosphate minerals (pyromorphite). Further studies are necessary to identify the source of sulphide and phosphate to precipitate lead.

The interactions of these strains with uranium were characterised as intracellular accumulation. The mechanisms of intracellular U accumulation are not yet fully understood and further information are necessary about the physiological state of the cells after accumulation of high amounts of uranium.

Experiments are running in our laboratory to examine the response of *Arthrobacter* isolate JG37-Iso2 cells to U(VI) and Pb(II) by using the genome expression profiling through one-dimensional gel electrophoresis. The aim is to identify proteins, which were down- or up-regulated in response to the addition of U(VI) and Pb(II) to the cells.

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