

## Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils

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### Abstract

A site in the former uranium mining area of Eastern Thuringia near Ronneburg was investigated with regard to effects of acid mine drainage (AMD) on reactive transport and bioattenuation. Processes involved in this attenuation might include physico-chemical reactions in reactive transport as well as activities of microorganisms for bioattenuation. In order to test the influence of the soil microbes, a mapping was carried out including both hydrogeochemical and microbiological parameters.

Mapping of contamination was performed along the banks of a creek in a 900 m stretch in 50 m steps by hydrogeochemical analysis of water extracts of soil samples, while general microbial activity was scored by examining soil respiration. The soil samples with high heavy metal load did show low soil respiration as a parameter for microbial activity and plating revealed minimal counts for spore producing bacteria at these contaminated locations. Actinobacteria strains isolated from adjacent locations revealed high levels of resistance as well as high numbers of resistant strains. Specific responses in actinobacteria were investigated after isolation from each of the 18 measuring points along the creek. Specific adaptation strategies and high yields of (intra)cellular heavy metal retention could be seen. Several

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strategies for coping with the high heavy metal contents are further discussed and genes for proteins expressed specifically under high nickel concentration were identified by two-dimensional gel electrophoresis.

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## 1. Introduction

Bacteria in soil consist of different taxa, among them aerobic, spore forming, Gram-positive bacteria of the *Bacillus* and actinobacteria groups. Spore formation is thought to be an adaptation to the differing conditions in soil with changing water contents due to dryness and rain (Vobis, 1997). Actinobacteria including streptomyces are known to be prominent representing up to 20% of the aerobic soil bacteria population in arable land, and their strong secondary metabolism makes them good candidates for identification of components altering soil chemistry. This capacity of formation of secondary metabolites is exemplified by production of geosmin, the compound responsible for the odor of tilled soil, as well as by production of antibiotics (Kieser et al., 2000). Other products of secondary metabolism may enable the bacteria to cope with stress factors including toxic levels of heavy metals (So et al., 2000).

While many heavy metals are essential micronutrients since they are incorporated into enzymes and cofactors (Fe, Zn, Mn, Co, Cu, Ni, V, Mo) they still are toxic in high concentrations because of adversary binding to enzymes and DNA, and by production of oxygen radicals through the Fenton reaction (Lopez-Maury et al., 2002). Therefore, the organisms must maintain a homeostasis within the cell that keeps the reactive heavy metals at an optimal, sub-toxic level. Resistance factors may allow them to maintain intracellular low levels of heavy metals or intracellular fractionation of the metal in non-harmful complexes (Eitinger and Mandrand-Berthelot, 2000). Thus, adapted microbial populations are prone to show higher resistance to heavy metals as compared to populations of non-contaminated sites.

Adaptive responses towards heavy metal stress may involve detoxification of oxygen radicals, as has been shown for superoxide dismutase overproducing strains of the bacterium *Escherichia coli* which are tolerant to higher heavy metal concentrations (Geslin et al., 2001). Streptomyces are known to possess two superoxide dismutases: one iron- and one nickel-containing enzyme which are regulated by nickel (Kim et al., 1998a,b). Other resistance mechanisms include sequestration of heavy metals and adsorption of generally positively charged heavy metal cations to the cells walls. The cell walls of the Gram-positive bacterium *Bacillus sphaericus* are commercially used for water treatment (Beveridge and Murray, 1976; Doyle et al., 1980; Raff et al., 2003). In addition, active uptake of radioisotopes has been known at least since the Chernobyl fall-out for basidiomycete fungi which led to extremely high enrichment of cesium in fruitbodies

(Haselwandter, 1978; Giovani et al., 2004). Heavy metal resistance has been investigated largely in Gram-negative bacteria, especially *Ralstonia metallidurans* where efflux transporters could be identified (Mergeay et al., 2003; Nies, 2003).

The former uranium mining site Wismut in Eastern Germany shows great promise to investigate adaptation of microorganisms to anthropogenic pollution with heavy metals. Uranium mining from this site has made the German Democratic Republic the third largest producer of uranium world-wide with more than 210,000 tons of uranium produced during the years 1949 through 1990 (Henningsen and Katzung, 2002). After German re-unification uranium mining stopped and remediation of the area was started by placing the waste rock material in the former open pit. The water table is allowed to rise, thereby re-establishing anoxic conditions in saturated zones. This will prevent further oxidation of the pyrite-rich material and prevent further production of acid mine drainage (AMD) waters. However, seepage waters that resulted from leaching of the former waste rock piles by AMD have infiltrated adjacent soils and surface waters. They contain large amounts of heavy metals including nickel, cadmium, copper, chromium and rare earth elements (Geletneky and Büchel, 2002; Russe et al., 1993).

A detailed hydrogeochemical and microbiological mapping of the surface waters was performed in order to establish the basis for the investigation of specific adaptive responses of microorganisms which in the future might lead to the development of advanced bioattenuation products. Nickel was investigated in particular for cellular adaptive responses in actinobacteria since mechanisms for coping with high nickel contents in the environment have already been analyzed in other microbes (McIlveen and Negusanti, 1994).

## 2. Material and methods

### 2.1. Soil sample collection

Soil samples were collected at 50 m intervals from the bank of the creek Gessenbach in a 900 m flow path after merging of the Badergraben with the Gessenbach. Control soils were taken in unpolluted sites within the city limits of Jena, Germany. In each case, mixed soil samples were used to even out heterogeneity which might persist on a very small scale.

Sterile Falcon tubes (50 ml) were punched into the soil at the bank of the creek approximately 15 cm above water level on 19 December 2003, for isolation of soil microbes. Soil for element analyses and soil parameters was collected from the same site in plastic bags. The place was weeded before sample collection if necessary and large roots were removed. The samples were immediately brought to the laboratory and dried (for abiotic tests) or stored in the cold (4 °C; for microbiological measurements).

### 2.2. Determination of pH, contamination, soil moisture and respiration

The pH was measured in a mixture of 10 g dried soil which was ground with a mortar and pestle. The sieved (1 mm) soil was incubated with 25 ml CaCl<sub>2</sub> (10 mM), thoroughly mixed and measured after 1 h (pH meter HydruS 300, Fisherbrand) (Alef, 1991).

Watery extracts were analyzed using inductively coupled plasma mass spectrometry (ICP-MS, PQ3-S, Thermo Elemental, Winsford, UK). The analyses included U, Al, a series of transition elements (Cd, Co, Cu, Mn, Ni), and alkaline earth elements (Mg, Sr).

Soil respiration was measured from incubation of 20 g soil of native moisture by capturing evolving CO<sub>2</sub> in NaOH and subsequent titration of phenolphthalein–NaOH with HCl (Alef, 1991). Thus, the amount of CO<sub>2</sub> produced could be calculated. The soil was then dried to constant weight and soil moisture and production of CO<sub>2</sub> per gram dry weight was calculated.

### 2.3. Isolation and growth of microbes

Air dried, ground soil samples were heat-treated at 80 °C for 45 min and suspended in *Aqua dest.* The emulsion was then plated on minimal medium (0.5 g/l L-asparagine, 0.5 g/l K<sub>2</sub>HPO<sub>4</sub>, 0.2 g/l MgSO<sub>4</sub>, 0.01 g/l FeSO<sub>4</sub>, 10 g/l D-glucose, 16 g/l agar). The plates were incubated for 5 days at 28 °C, colonies were microscopically analyzed and transferred to starch casein medium (10 g/l starch, 1 g/l casein dissolved in 0.3 M NaOH, 0.5 g/l K<sub>2</sub>HPO<sub>4</sub>, 15 g/l agar, pH 7.0–7.5). For supplementation sterile filtered stock solutions of the heavy metal salts (NiCl<sub>2</sub>, CoCl<sub>2</sub>, CuSO<sub>4</sub>, MnSO<sub>4</sub>, Cd(NO<sub>3</sub>)<sub>2</sub>) were used. Minimal medium has to be used in resistance determination in order to minimize complexation of the heavy metal ions. *Streptomyces coelicolor* A3(2) (DSM 40783) and *Streptomyces acidiscabies* E13 (Amoroso et al., 2000) were grown on minimal media. For two-dimensional (2D) gel electrophoresis the liquid growth media were supplied with 0.1 and 0.3 mM NiCl<sub>2</sub>.

### 2.4. Proteome analyses

The comparison of proteins expressed in a cell has become possible by high-resolution protein gel electrophoresis. At the same time, the identification of proteins extracted from a gel is possible for any organism, for which the entire genome encoding the proteins has been sequenced. If two gels are obtained from cultures grown under different conditions like metal stress versus normal media, proteins with higher or lower expression can be identified giving the differential expression pattern under the conditions used. 2D-gel electrophoresis (Lottspeich and Zorbas, 1998) was performed for identification of proteins regulated under heavy metal stress. First dimension separated isoelectric points, second dimension for size in SDS containing gels. Cytosol was prepared using polyvinylpyrrolidone (15.4 mg dithioerythritol and 2 g polyvinylpyrrolidone *ad* 100 ml with potassium buffer: 50 mM; 108 ml K<sub>2</sub>HPO<sub>4</sub>, 42 ml KH<sub>2</sub>PO<sub>4</sub>) as buffer for French Press (SLM Instruments) cell disruption followed by centrifugation at 140,000g to remove membrane and cell wall fragments. The cytosolic proteins were precipitated (20% trichloroacetic acid, 50% acetone, 20 mM dithiothreitol) for 30 min at –20 °C, incubated for 2 h at 4 °C and centrifuged at 11,000 rpm. After two steps of washing with acetone the pellet was dried (Speed Vac) and redissolved in rehydration buffer (8 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT). After ultracentrifugation at 75,000g, 500 µg protein (Bradford, 1976) were used for each IEF strip (Immobiline Dry Strip, 24 cm, pH 3–10, Amersham Biosciences) in 500 µl rehydration buffer adding 3 µl 4% bromophenol blue. IEF was performed at 150 V (2 h), 300 V (2 h), 600 V (1.5 h), 1200 V (1 h), 2400 V (1 h), and 3500 V (17 h) and 30/80 V, 16 h in 10% acrylamide (Rotiphorese, Roth) for the second dimension using IPGPHOR II and Ettan DALT twelve (Amersham Biosciences).

Analysis of the spots cut from the gel and extraction with acetonitrile and drying was performed by trypsin digestion and electron spray ionization mass spectroscopy (ESI-MS; Lottspeich and Zorbas, 1998). The system LCQ Deca XP (Thermo) was used with the S.

*coelicolor* genome (NCBI database) after reversed phase HPLC for purification for mass detection and in silico analysis with Sequest 3.1.

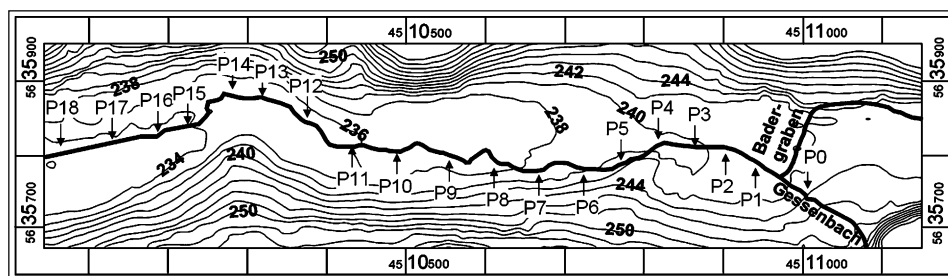
### 3. Results

#### 3.1. Characterization of the site

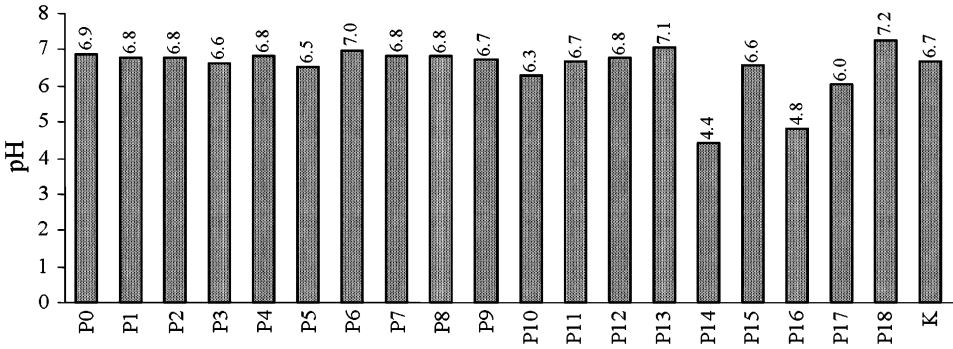
Soil samples were taken from the bank of creek Gessenbach near Ronneburg in Thuringia, Germany (Fig. 1). The distance between merging of the Badergraben with the Gessenbach and the next road connecting Kauern and Ronneburg of approx. 900 m was sampled every 50 m. At the first location in flow direction, P0, contamination was assumed to be highest due to passage of two former dump sites which release AMD waters. For control, five independent soil samples were taken from uncontaminated sites within the city of Jena in the Saale river valley.

The pH did not show the expected gradient of low pH at measuring point P0 with lowest pH increasing to point P18, but rather indicated additional, so far unknown, AMD influence. Additional acidification was observed at locations P14 and P16 (Fig. 2), while most of the other samples show circum-neutral pH comparable to control soils (sample K, Fig. 2). This interpretation was confirmed by analyses of heavy metals and other elements (Fig. 3). The effluence could be localized at two sources at point P14 and close to P16, with salt incrustations observed at the water sources.

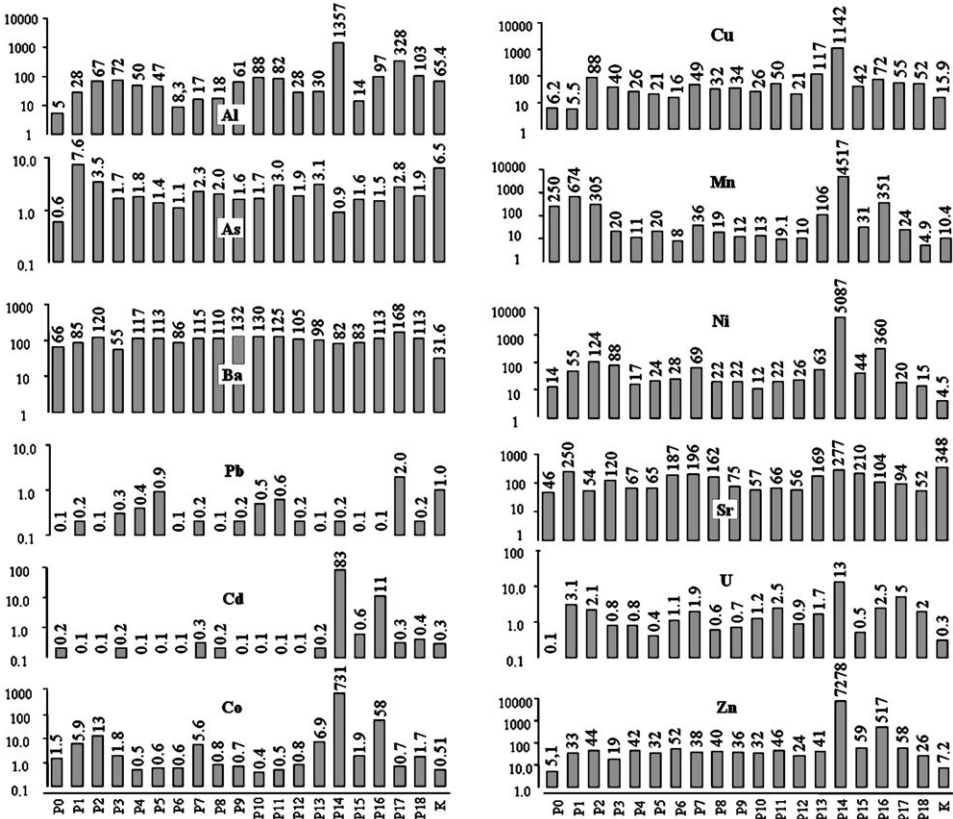
While some elements show clearly high enrichment (Al, Cd, Co, Cu, Mn, Ni, U, Zn), other elements proved to be of no clear ecotoxicological risk at the concentrations found (As, Pb, Sr) or can be viewed as markers for flow paths rather than influencing microbial life (Ba, most likely as  $\text{BaSO}_4$ ). The data provided are measurements of supernatant of roughly five-fold diluted soil (10 g soil plus 45 ml *A. dest.*). This represents a simple water extract which is equivalent to the lowest portion of bioavailable metals in the soil. Thus, the ecotoxicological risk is rather understated here.



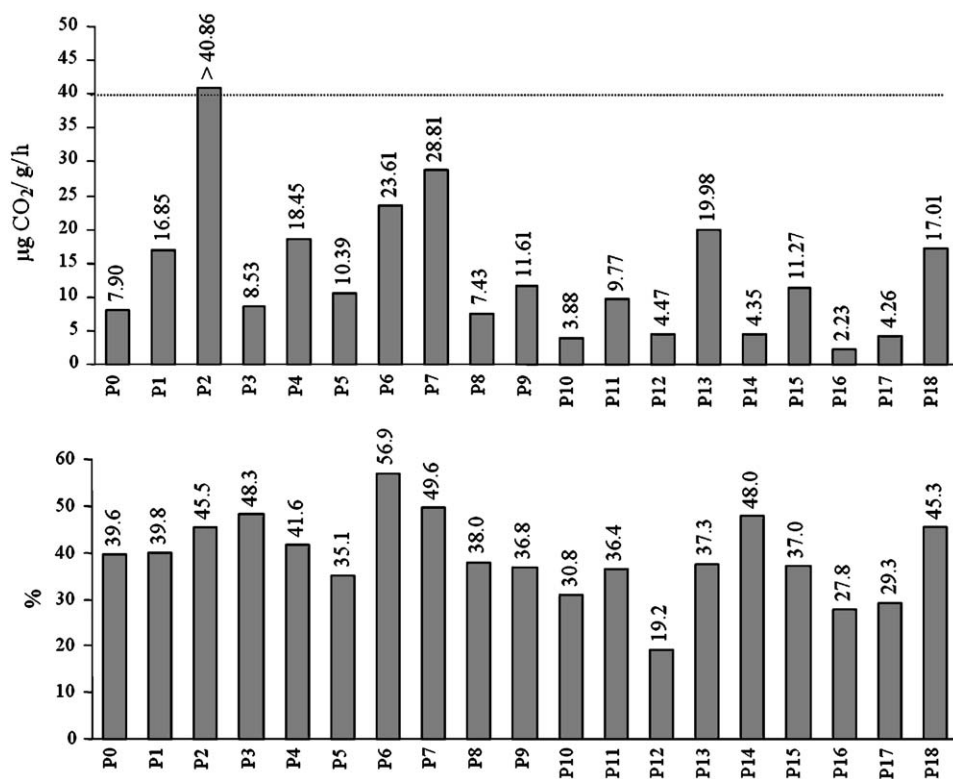
**Fig. 1.** Map of the area investigated with sites for soil sample collection along the bank of creek Gessenbach near Ronneburg in Thuringia, Germany (from the topographic map of Thuringia, Landesvermessungsamt, 1999).



**Fig. 2.** Determination of soil pH at sampling sites P0 through P18 along the Gessenbach. A control site (K) is included ( $n = 5$ ).



**Fig. 3.** Heavy metals contents of watery soil extracts at sampling sites P0 through P18 along the Gessenbach (ppb). A control site (K) is included ( $n = 5$ ).

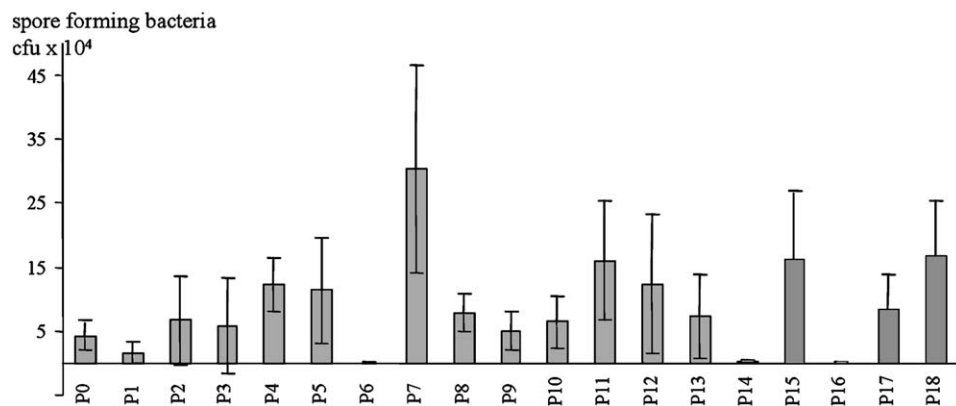


**Fig. 4.** Soil respiration (above) and soil moisture (below) at sampling points P0 through P18 along the Gessenbach. Soil respiration of control sites is indicated by a dotted line.

Soil respiration as a parameter for microbiological activities was examined at every sampling point (Fig. 4), since Cd, Co, Cu, Ni and at high concentrations Zn are known to threaten microbial life. While in control soil samples, respiration of about 40 µg CO<sub>2</sub> released per hour per gram dry weight of soil was found, five samples did show respiration below 5 µg CO<sub>2</sub> per hour per gram dry weight of soil. These included the two sites with high contamination, P14 and P16, but also encompassed P10, P12 and P17 for which contamination was intermediate. One of these sampling points, P12, did show very low soil moisture (19.2%, while all other samples had around 30–50%) which might explain low soil respiration for this site. Another site, P6, was very wet (57%) but did still show soil respiration at expected activities.

### 3.2. Isolation of spore forming bacteria

Colony forming units (cfu) for hyphal bacteria of the genus *Streptomyces* and other filamentous actinobacteria were analyzed in the samples after heat-inactivating



**Fig. 5.** Colony forming units (cfu) of bacteria on minimal media. cfu from heat resistant spores (80 °C).

vegetative cells. The plate counts clearly showed the toxic influence of contaminated sites as P14 and P16 had less than 10,000 cfu/g dry mass of soil (Fig. 5). Only one other sampling point (P6) showed such low levels of actinobacteria spore contents. In this case, the high water content of over 50% soil moisture can be assumed to cause the effect since actinobacteria are dependent on well-aerated dry habitats.

These isolation plates did not contain metals and thus allowed to determine the number of strains present in the sample. In order to assess the amount of strains which show adaptation to the contaminated environment, an isolation strategy was used including one heavy metal (Ni) into the plates. Subsequently heavy metal resistance towards an array of heavy metals was determined.

### 3.3. Heavy metal resistance

The soil samples were also used for isolation of heavy metal resistant strains on plates containing 5 mM NiCl<sub>2</sub> (Table 1). While soils samples P0, P1, P3, P6, P14, P15 and P16 yielded no nickel resistant colony, varying amounts of nickel resistant strains could be isolated from the other soil samples (0.01–29% compared to cfu on plates without nickel). In the five control soil samples, only six strains could be isolated on plates containing nickel which represents only  $5.3 \times 10^{-5}$  to  $2.8 \times 10^{-4}\%$  of the colonies found on plates lacking the heavy metal.

Four of the newly isolated strains and four control strains were tested more precisely for their heavy metal resistance. Plates containing the salt were inoculated with strains P4-3, P8-3, P9-9 and P17-6 from sampling points P4, P8, P9 and P17, respectively. These sampling points were comparable in their heavy metal content but slightly differ in contamination for single metals. Ni, Co, Cu and Mn were especially of interest since the contents are high. Control strains included *S. coelicolor* A3(2), since this is the genetically best-defined streptomycete with a known genome sequence which should not carry heavy metal resistances, as well as three

**Table 1.** Heavy metal resistance of spore forming actinobacteria colonies isolated with or without nickel

	cfu <sup>a</sup> /g–Ni	cfu/g + Ni	% resistant
P0	40988 ± 21485	0	—
P1	16676 ± 16438	0	—
P2	67046 ± 68668	19444	29.00
P3	57907 ± 74873	0	—
P4	122564 ± 41272	47	0.04
P5	115103 ± 81454	1024	0.89
P6	362 ± 724	0	—
P7	303758 ± 162874	1990	0.66
P8	78078 ± 28132	13367	17.12
P9	50607 ± 29837	52	0.10
P10	64393 ± 40614	2632	4.00
P11	160041 ± 93036	12	0.01
P12	123669 ± 108325	29	0.03
P13	73294 ± 65786	41	0.06
P14	2159 ± 2267	0	—
P15	162605 ± 107319	0	—
P16	616 ± 633	0	—
P17	83404 ± 54314	53	0.06
P18	166840 ± 86042	1895	1.14

<sup>a</sup>cfu/g dry soil are given.

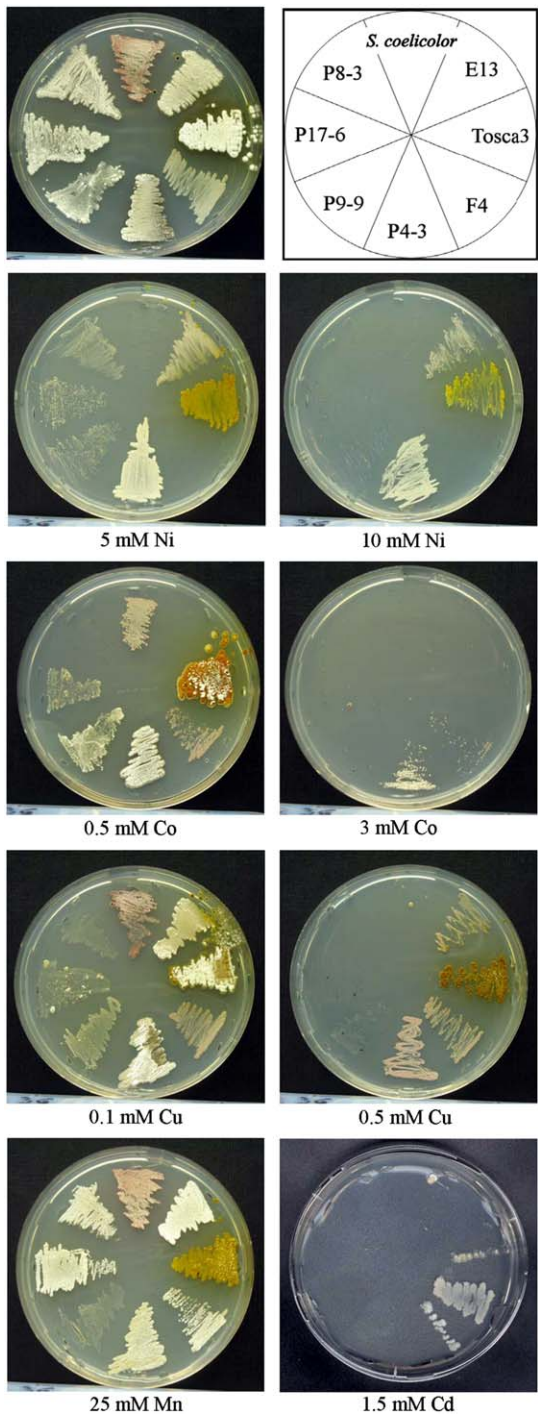
strains identified in previous studies which are known to be resistant to Ni (E13, Tosca3) or Cd (F4).

As can be seen from the plate assays (Fig. 6), strain P4-3 is highly nickel resistant. In contrast to other isolates, growth occurs still on 10 mM NiCl<sub>2</sub> (equivalent to 600 ppm) containing plates. In addition, this strain handles 3 mM cobalt well and is able to form spores on plates containing 2 mM CoCl<sub>2</sub>.

Strain P8-3 is the most sensitive of the tested strains against cobalt and copper, while strain P9-9 is manganese sensitive which is especially obvious if comparing spore formation (no spores even on 5 mM MnSO<sub>4</sub>).

Isolate 17-6 shows release of a diffusible substance which allows growth of strain P8-3 to form spores on manganese containing minimal medium just at the border line between both strains (Fig. 6). A similar phenotype, excreting chelating substances, could be observed for F4 on Cd containing media. This already showed the ability to form chelating, extracellular compounds.

Another feature associated with high heavy metal concentrations is the loss of capacity to form spores. Since this was observed here for the first time, the strategy for isolation of hyperresistant strains had to be reconsidered. During the first round of isolation, the soil samples were incubated at 80 °C before plating in order to reduce growth of vegetative soil bacteria. However, subsequently a new isolation protocol was followed by streaking soil samples directly on plates containing 15 mM



NiCl<sub>2</sub>. Indeed, with this isolation strategy six hyperresistant strains could be isolated from sampling points P13 through P18, growing with good growth rates on plates containing 0.3% or 3000 ppm nickel. Further analyses will be necessary to identify these actinobacteria to the genus and species level and to determine the maximum concentrations of heavy metals endured in solid and liquid minimal media.

### 3.4. Proteome analyses

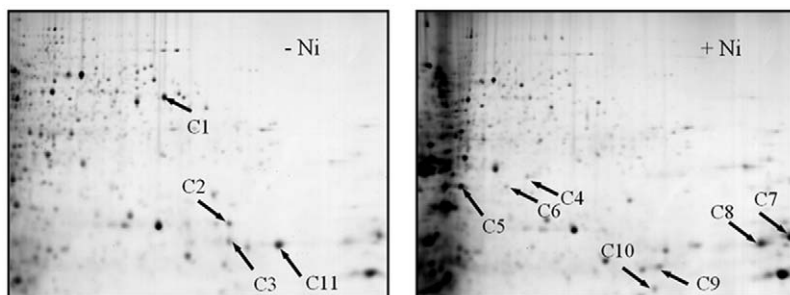
Other resistance mechanisms could involve intracellular sequestration. To identify intracellular proteins which are involved in heavy metal resistance, 2D gel electrophoresis was performed. Using *S. coelicolor* A3(2) was essential since its genome sequence allows identification of separated and extracted spots from the gel by ESI-MS and in silico comparison of fragments to fragments predicted for the annotated genes from the entire genome. In addition, first investigations with *S. acidiscabies* E13 were performed in order to assess the possibility to use a related, but heavy metal-resistant strain for analyses of the proteome.

First, the control strain *S. coelicolor* was grown with or without nickel in the medium and 2D gel electrophoresis (Fig. 7) indicated induction of some proteins. Of the proteins repressed by nickel, four proteins encoded on the actinorhodin biosynthesis gene cluster were identified while among the proteins induced with NiCl<sub>2</sub>, three ribosomal proteins (S8, S9 and L29) were identified, one protein could not be identified and the remaining two showed homology to a putative protein Lsr2 and a putative transcription factor of the *tetR*-like family (spot C6).

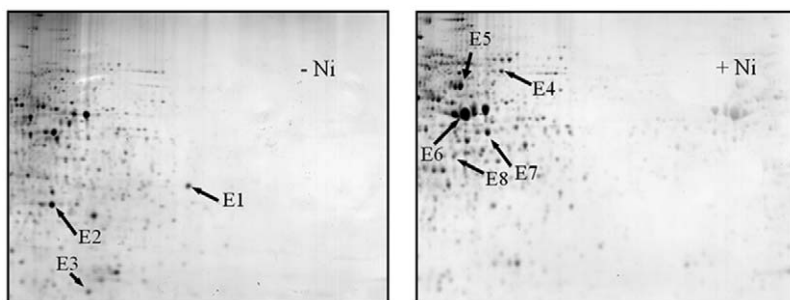
Thus, we could show that this approach is feasible for future, more thorough proteome analyses. However, the use of a strain that is heavy metal resistant promised more interesting results. Therefore it was investigated whether the use of *S. acidiscabies* E13 would allow the in silico comparison with the known genome of *S. coelicolor* for identification of heavy metal regulated proteins. In comparison, gels run from cells grown with and without nickel showed a substantial shift in physiology (Fig. 8). From the gels, eight spots were cut and analyzed. All could be identified in silico. From the three spots repressed under nickel, one served as an internal standard since the iron containing superoxide dismutase *sodF* is known to be repressed under nickel in streptomycetes. From the five spots induced in nickel grown cultures, three are of interest: induction of glycerin-3-phosphate dehydrogenase (spot E6) and fructose-1,6-bisphosphate aldolase (spot E7) were shown. This was unexpected as enzymes involved in glycolysis are generally assumed to be constitutively expressed. However, since more intracellular storage components could be visualized by electron microscopy (data not shown) in cells grown with nickel, enhanced glycolysis might be linked to biosynthesis of storage components. The third protein of interest is again, as seen with *S. coelicolor*, a homolog to the



**Fig. 6.** Plate assays for heavy metal resistance. Four strains from the isolation campaign and four control strains are shown without or with indicated concentrations of heavy metals.



**Fig. 7.** Two-dimensional gel electrophoresis of the control strain *Streptomyces coelicolor* A3(2) grown without or with 0.1 mM  $\text{NiCl}_2$  in the medium. The identified, differentially expressed protein spots C1 through C11 were used for identification of proteins.



**Fig. 8.** Two-dimensional gel electrophoresis of the control strain *Streptomyces acidiscabies* E13 grown without or with 0.3 mM  $\text{NiCl}_2$  in the medium. The identified, differentially expressed protein spots E1 through E8 were used for identification of proteins.

*tetR*-like transcription factor (spot E8). Further analysis defining the proteome answer in *S. acidiscabies* on heavy metal stress therefore is possible using 2D gel electrophoresis and ESI-MS.

#### 4. Discussion

The field site was characterized and two new sites of AMD influence on the Gessenbach could be determined by investigating soil samples along the creek's banks. It could be shown that adaptation has occurred within the past 40 years in that the amount of strains with the capacity to withstand high concentrations of heavy metals are enriched in soils which have been influenced by AMD during this period of time. The percentage of heavy metal resistant strains is significantly higher than in comparable control soils.

Four strains were examined in particular and different mechanisms can be assumed for protection of the cells from heavy metals. Strain P4-3 is capable of growth under high cobalt and nickel concentrations without losing the capacity to form spores at least on 0.5 mM Co. Since the loss of spore formation is due to altered secondary metabolism within the cell, it might be argued that the cells are able to exclude the detrimental heavy metals. This indicates an efflux transport system which has been investigated for Gram-negative bacteria where co-resistance against cobalt and nickel has been reported (Nies, 2003).

Strain P17-6 could be shown to produce a compound which is diffusible and provides a protection to some other strains (P8-3 could form spores at the side facing P17-6, while strain P9-9 did not profit from that same compound). Such a substance might become interesting in the future if water treatment is developed making use of biological complexation agents. A similar observation was made with one other strain, F4, which shows cadmium resistance and did produce diffusible compound(s) allowing sensitive strains to grow adjacent to the producer on cadmium. Diffusible components have been described for other heavy metal resistant bacteria (Raytapadar et al., 1995). Previous analyses had shown the ability to take up heavy metals (Merten et al., 2004).

For the two remaining strains, either intra- or extracellular binding of the heavy metals can be expected.

The observation that spore production ceases on media containing heavy metals led to a refined isolation procedure and indeed, so far six hyperresistant strains were identified. Their capacity to grow on solid media containing 0.3% NiCl<sub>2</sub> or 50 mM of nickel is a feature that makes them especially interesting in future studies.

The first, initial proteome analysis regarding soil bacteria and their heavy metal resistance could show that this approach is valuable for determining cellular responses and adaptive mechanisms in actinobacteria, especially within the genus *Streptomyces* since genome data are available and allow identification of up-regulated proteins from 2D gel electrophoresis. The analysis of a *tetR*-like transcription factor seems especially promising for future identification of a whole cascade of genes under transcriptional control by this protein. Such findings would allow addressing regulatory cascades in heavy metal resistance of soil microbes.

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