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Identification and characterization of eight cadmium resistant bacterial isolates from a cadmium-contaminated sewage sludge

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Cadmium-resistant bacterial community, isolated from sewage sludge contaminated by cadmium ions, was characterized by biochemical reactions, amplified ribosomal DNA restriction analysis (ARDRA) and in physiological terms. Between bacteria from bacterial community short cadmium-resistant Gram-negative rods predominated. Eight of them were biochemically profiled using either API 20 E, API 20 NE systems or ENTEROtests, and by key conventional and confirmation tests. Biochemical tests assigned the eight isolates to six bacterial species, *Alcaligenes xylosoxidans*, *Comamonas testosteroni*, *Klebsiella planticola*, *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Serratia liquefaciens*. The ARDRA analysis of each of the eight isolates enabled five different ARDRA patterns to be recognized. *P. putida* and *P. fluorescens*, identified by biochemical tests as two different species, ARDRA analysis clustered these two strains to the same cluster indicating only one species. Differentiation among strains of the same ARDRA group was shown by analysis of whole cell protein patterns. Cadmium-resistant bacterial isolates were able to remove cadmium from solution and the efficiency of cadmium removal correlated with the amount of additionally synthesized proteins in the cell fractions. Analysis of plasmid content revealed that only two *K. planticola* strains harbored plasmids. The ability of biochemical and molecular methods to identify and characterize natural culturable bacterial community isolated from polluted environment, and the potential exploitation of cadmium-resistant bacterial strains in bioremediation processes aimed at heavy metal removal from contaminated environments, is discussed in this study.

Key words: ARDRA, biochemical tests, cadmium resistance, CDPs distribution, natural bacterial isolates, PCR, SDS-PAGE.

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Introduction

Among bacteria participating in polluted environment communities those genera predominate, which are known to be involved in biodegradation of organic pollutants. They often belong to the genus *Pseudomonas*, *Comamonas* or *Acinetobacter* (KUPKA & ŠEVČÍK, 1995; PROKŠOVÁ et al., 1997; BARBERIO & FANI, 1998; FERRERO et al., 1999); all of these being Gram-negative bacteria. However, in environments contaminated not only with organic pollutants but also with heavy metals, species diversity and metabolic activities of the microorganisms are reduced, and the metal-tolerant bacterial populations are developed (KNOTEK-SMITH et al., 2003) with species of *Pseudomonas* and/or acidophilic bacteria predominating (BABICH & STOTZKY, 1985; DOPSON et al., 2003). As a response to heavy metal challenge, either metal-induced adaptive cell protection evolved which requires newly synthesized proteins (BANJERDKIJ et al., 2003), or multiple-metal ion-resistant bacteria evolved which contain a variety of plasmid-encoded metal resistance determinants, e. g. *Staphylococcus aureus* (NOVICK & ROTH, 1968) and *Alcaligenes eutrophus* [*Ralstonia metallidurans*] strain CH34 (MERGEAY et al., 1985).

Biomass of algae, fungi and bacteria has been known to readily adsorb or accumulate metal ions (TSEZOS, 1985; GADD, 1988; VOLESKY & HOLAN, 1995). The ability of metal bioaccumulation by some Gram-negative bacterial species such as *Escherichia coli* (COHEN et al., 1991), *Pseudomonas putida* (HIGHAM et al., 1984), *Pseudomonas syringae* (CABRAL, 1992), *Pseudomonas aeruginosa* (HASSEN et al., 1998) was established on production of intracellular cadmium-binding proteins. Furthermore, another Gram-negative rod, *Alcaligenes eutrophus* [*Ralstonia metallidurans*] strain CH34 is known as biosorbent of heavy metals (DIELS & MERGEAY, 1990; NIES, 1992). Among heavy metals that are toxic, have long residence times, and have long biological half-lives, cadmium in particular constitutes a major problem in industrialized nations (FRIBERG, 1975), since its presence in environment mostly endangers the public health (DIELS, 1997). Cadmium is a potent oxidative agent (LAUŠOVÁ et al., 1999); it inhibits DNA replication (NYSTRÖM & KJELLEBERG, 1987) and appears to make the DNA more susceptible to nucleolytic attack resulting in single-strand DNA breaks (MITRA & BERNSTEIN, 1977). In addition, the observation that metal resistance determinants are located most frequently on plasmids and transposons (which are also likely to carry the genes for antibiotic resistance), has led to suggestions that the determinants have probably been spread by a horizontal transfer (NAKAHARA et al., 1977; BOGDANOVA et al., 1988). The identification of more bacterial strains that could uptake metals with high efficiency and specificity has attracted increasing attention from both medical and biotechnological points of view.

The aim of this work was to identify and characterize in physiological and molecular terms some cadmium-resistant bacterial strains from a culturable microbial community occupying a cadmium contaminated sewage sludge.

Material and methods

Isolation of bacteria

Bacterial strains were isolated from sewage sludge polluted by heavy metals - Cd-content: 7.5 μg cadmium per g (dry weight) of sludge. A portion 10 g (wet weight) of the sludge was mixed in a sterile 250 mL Erlenmeyer flask with 90 mL of liquid mineral medium containing (per litre): 0.1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 1.0 g NaCl, 1.0 g KCl, 1.0 g NH_4Cl, 5.0 g glucose, 0.67 g sodium β-glycerophosphate, 0.17 g alanine, 0.1 arginine, 0.1 g methionine, 0.9 g phenylalanine, 0.22 g serine, 0.12 g valine, and 50 mM Tris-Cl (pH 7.2) (HIGHAM et al., 1984) and incubated at 30 °C in a shaker incubator at 90 rpm for 2 h. The withdrawn subsamples (1.0 mL) were serially diluted (in range: 10^{-1}–10^{-6}) and each dilution plated in duplicate on mineral medium amended with agar and $CdCl_2$ (to a final concentration of 50 μg/mL Cd^{2+}). Plates were incubated aerobically at 30 °C for 24–48 h and independently growing colonies were repeatedly inoculated by sterile bacteriological loops on new same mineral medium. Several times pre-pured cultures were stained by Gram procedure and color development as well as size and the basic morphology of bacterial cells were followed by light microscopy.

Biochemical identification of bacterial isolates

Both, Gram-negative non-fermentative strains (GNNFR) and Gram-negative fermentative bacteria (GNFR) were incubated on nutrient agar (Imuna, Slovakia) at 30 °C or 37 °C, respectively, for 24 h. These isolates were tested and characterized by several physiological key conventional tests for basic differentiation of Gram-negative bacteria. Further, the isolates were identified on the basis of biochemical tests of commercial identification systems as follows: API 20 E and API 20 NE (bioMérieux, France), ENTEROtest 16 and ENTEROtest 24 (Lachema Brno, Czech Republic).

For determination of *Enterobacteriaceae* and other fermentative Gram-negative rods either API 20 E system (isolate marked as 11P), ENTEROtest 16 or ENTEROtest 24 (isolates marked as 1K and

10P) were applied. OF basal medium (HiMedia, India) was used for detection of the motility and oxidative/fermentative reaction. The ability of these cells to ferment lactose, sucrose, dextrose, to produce hydrogen sulfide and gas was observed on the TSI (Triple sugar iron agar) (HiMedia, India) during cultivation at 37 °C for 24 h. Additional conventional tests for fermentative bacteria identifications were used as follows: Oxitest, Colitest, Pyratest (Lachema Brno, Czech Republic), Tween 80, gelatine, DNA, VPtest, Mrtest, and SCI.

In addition, API 20 NE system was used for non-fermentative bacteria determinations (isolates marked as 2K, 3K, 3P, 4K, 5K). Besides, further 27 confirmation tests according to HOLMES et al. (1986) were used for the accurate non-fermentative bacteria determinations.

Bacterial identification was obtained by referring to the Analytical Profile Index and using software TNW 0.5 from Czech Collection of Microorganizms (CCM, Brno, Czech Republic).

Control CCM strains used:

For API 20 E: *Enterobacter cloacae CCM 1903*, *Proteus vulgaris CCM 1799*, *Pseudomonas aeruginosa CCM 1960*.

For API 20 NE: *Pseudomonas aeruginosa CCM 1960*, *Alcaligenes faecalis CCM*.

For ENTEROtest 16: *Serratia marcescens CCM 303*, *Proteus vulgaris CCM 1799*, *Edwarsiella tarda CCM 2238*, *Citrobacter koseri CCM 2535*.

For ENTEROtest 24: *Serratia marcescens CCM 303*, *Proteus vulgaris CCM 1799*.

For ARDRA: *Pseudomonas fluorescens* 3900 (A. Ternström 542), *Pseudomonas putida* 3423 (D. Halama).

Growth conditions of bacterial isolates

Isolates were grown in liquid mineral medium (HIGHAM et al., 1984) without (control sample) or with $CdCl_2$ amendment (to a final concentration of 50 μg/mL Cd^{2+}) in Erlenmeyer flasks placed in a rotary shaker (90 rpm) at 30 °C. Liquid mineral medium was inoculated 1:100 (v/v) with an over-night culture, and $CdCl_2$ was added immediately before growth commenced. In all following experiments growth of the isolates was monitored by measuring the optical density at 420 nm.

Extraction of total DNA

Total DNA was extracted from bacterial cells according to the protocol of AUSUBEL & FREDERICK (1995).

Amplification of 16S rDNA

Primers fD1 (5' AGA GTT TGA TCC TGG CTC AG 3') and rP2 (5' ACG GCT ACC TTG TTA CGA CTT 3') were used (LANE, 1991). The PCR reaction mixture (25 μL) contained bacterial DNA (10 ng), 1X Taq buffer, 1.0 U Taq polymerase (Promega, Madison, USA), 1.5 mM $MgCl_2$, 200 μM dNTPs, and 0.5 μM of each primer. PCR amplification was carried out in a Progene thermocycler. The tubes were subjected to the following thermal conditions: 5 min at 94 °C for one cycle, then 60 s at 94 °C, 60 s at 50 °C and 60 s at 72 °C for 35 cycles. After cycling, 10 μL of each reaction was analyzed for the presence of a 1,500 bp product on 1.5% (w/v) agarose gel containing ethidium bromide (0.5 μg/mL) in TAE buffer at 7 V/cm.

Amplified ribosomal DNA restriction analysis (ARDRA)

PCR products were digested with *Hae*III, *Msp*I and/or *Alu*I (New England BioLabs) restriction endonucleases. The products of digestion were analyzed by agarose gel (either 3.0% w/v (*Hae*III and *Msp*I) or 1.5% w/v (*Alu*I)) electrophoresis (Amresco agarose 3:1, Solon, Ohio, USA) in TAE buffer.

Analysis of plasmid content

Analytical amounts of plasmid DNA were obtained from 1.5-mL bacteria cultures using the alkaline lysis method (AUSUBEL & FREDERICK, 1995). Restriction analysis was performed by incubating 500 ng of plasmid DNA with 10 U of *Bam*HI and *Hin*dIII (Advanced Biotechnologies Ltd., U.K.) following the instructions of the supplier. The products of digestion were analyzed by 1.0% agarose gel (w/v) electrophoresis in TBE buffer containing 0.5 μg/mL of ethidium bromide.

Cadmium content measurement in liquid mineral medium

Isolates were grown in liquid mineral medium as described above. At the beginning of the experiment (control), and when cultures achieved a stationary phase, cells were harvested by centrifugation at 4,000 × *g* for 20 min at 4 °C. After centrifugation, supernatants were collected and cadmium content was measured by using an atomic absorption spectrometer (Perkin-Elmer model 403, USA).

Bacterial cell fractionation

Isolates were grown in liquid mineral medium without or with $CdCl_2$ as described above. When cultures reached an OD_{420} in the range 0.5–1.0, a portion (50 mL) of the culture was removed, harvested by centrifugation at 4,000 × *g* for 20 min at 4 °C, washed twice with 10 mM Tris-Cl (pH 8.0); the supernatant was removed and the cell pellet frozen at -20 °C until use. Cell pellets were fractionated for cytoplasmic, cell wall, inner- and outer membrane fractions according to the methods by ACHTMAN et al. (1983). After acetone precipitation, the sediments were analyzed by resolution of proteins in one-dimensional gels.

Sample preparation and SDS-PAGE analysis

Culture samples (withdrawn from growing cultures, when they reached an OD_{420} of 0.5, as described above) were centrifuged (4,000 × *g* for 20 min at 4 °C) and the pellets were frozen at −20 °C until processed. The cell pellets as well as sediments from fractionated cells were resuspended in SDS-PAGE sample buffer. The proteins were separated by electrophoresis on 12% SDS-polyacrylamide gels (LAEMMLI, 1970) using a BioRad Mini-Protean apparatus. The separated proteins were silver stained according to the method

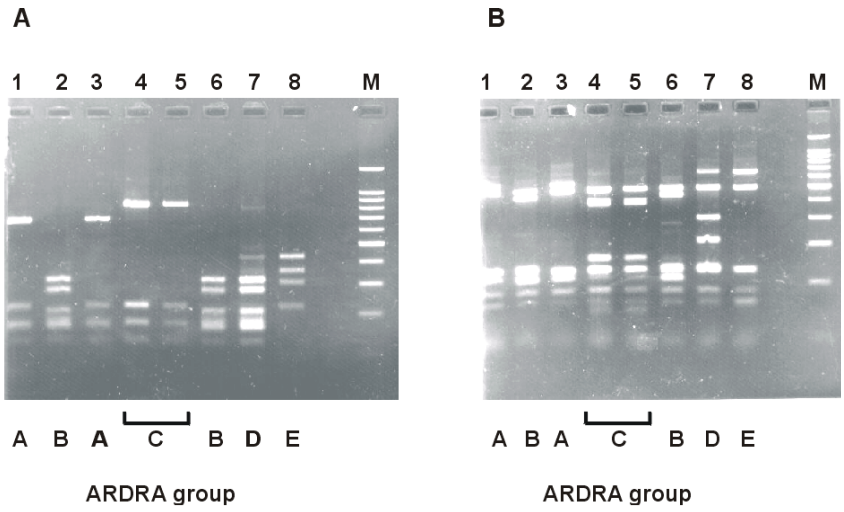

Fig. 1. ARDRA experiments: 3% agarose gel electrophoresis of amplified 16S rDNA digested with restriction endonucleases (**A**) *Hae*III and (**B**) *Msp*I of eight bacteria isolated from sewage sludge. Lanes: 1 = 3P (*P. putida*), 2 = 10P (*K. planticola*), 3 = 3K (*P. fluorescens*), 4 = 4K (*A. xylosoxidans*), 5 = 5K (*A. xylosoxidans*), 6 = 11P (*K. planticola*), 7 = 1K (*S. liquefaciens*), 8 = 2K (*C. testosteroni*), and M = 100 bp leader. ARDRA patterns are indicated by capital letters (A–E) under figures.

of Heukesloven & Dernick (1985). Comparison of gels of the untreated control and cadmium-treated cells were made by eye to identify proteins induced by cadmium exposure. Gels analyzed in two separate experiments resulted in identical protein patterns. All chemicals used for gel preparation were purchased from Bio-Rad (USA).

Results and discussion

Isolation of cadmium-resistant bacterial community

One of the goals of this study was to identify and characterize cadmium-resistant bacteria isolated from water environment contaminated by cadmium ions. For this purpose, in total, sixty-eight bacterial isolates were obtained from sewage sludge, which contained approximately 7.5 μg cadmium per g of dry weight. All isolates growing on mineral medium supplemented with cadmium ions were distinguished on the basis of color, size and morphology. The results revealed that fifty-four of the isolates were identified as Gram-negative bacteria with short rods predominating, and only fourteen of them were identified as Gram-positive bacteria with cocci predominating.

All fifty-four Gram-negative isolates were tested for their growth characteristics in liquid mineral medium (Higham et al., 1984) without $CdCl_2$ treatment (control sample) or supplemented with cadmium, and according to growth rate and length of lag-phase, only eight isolates, marked as 1K, 2K, 3K, 4K, 5K, 3P, 10P, and 11P, respectively, were chosen for further characterization. While five of the examined isolates (3P, 3K, 10P, 4K, 5K) showed similar growth curves, the growth curves of the remaining three isolates (11P, 1K and 2K) were completely different. The growth curve pattern of 3P, 3K, 10P, 4K, 5K represented twenty-two isolates, 11P twelve, 1K four, 2K nine, and the growth curve patterns with negligible growth rate represented remaining seven isolates, which were not characterized further.

All eight isolates retained their ability to grow in the presence of cadmium if they were grown previously in or on mineral medium in the absence of cadmium. We considered these isolates as cadmium resistant.

Biochemical and molecular identification of Gram-negative rods

When the 16S rDNA of each of the eight isolates was amplified by PCR, an amplification fragment of about 1,520 bp was observed. Restriction analysis of amplified DNA of each natural sample with *Hae*III and *Msp*I enabled five different ARDRA patterns to be recognized (Fig. 1), corresponding to five species similarly to *Alu*I (Grifoni et al., 1995; Di Cello & Fani, 1996). However, based on biochemical tests used, the previous same iso-

Table 1. Cadmium-resistant isolates and their characteristics.

Isolated strains	ARDRA clusters[a]	Species ID by commercial tests[b]	Commercial tests used for strain identification
3P	A	*Pseudomonas putida*[I]	API 20 NE
3K	A	*Pseudomonas fluorescens*	API 20 NE
10P	B	*Klebsiella planticola*	ENTEROtest 24
11P	B	*Klebsiella planticola*	ENTEROtest 24
4K	C	*Alcaligenes xylosoxidans*	API 20 NE
5K	C	*Alcaligenes xylosoxidans*	API 20 NE
1K	D	*Serratia liquefaciens*	ENTEROtest 16
2K	E	*Comamonas testosteroni*	API 20 NE

[a] Restriction analysis of the amplified ribosomal DNA with endonucleases *Hae*III and *Msp*I.
[b] Species assigned by best likelihood; other possibility indicated by superscript roman number I, *Pseudomonas fluorescens*.

lates were differentiated into six species in five clusters as follows: (i) isolates in cluster A (3P, 3K) were identified as *Pseudomonas putida* and *Pseudomonas fluorescens* by API 20 NE; (ii) cluster B isolates (10P, 11P) were characterized as *Klebsiella planticola* by ENTEROtest 24 or API 20 E, respectively; (iii) cluster C contains also two isolates (4K, 5K) identified as *Alcaligenes xylosoxidans* by API 20 NE; (iv) cluster D represents only one isolate 1K identified as *Serratia liquefaciens* by ENTEROtest 24; and (v) cluster E represents also one isolate 2K characterized as *Comamonas testosteroni* by API 20 NE (Table 1).

While four of the ARDRA groups (assigned as B-E) correspond with the results obtained from biochemical and physiological analysis, i. e. each of the four ARDRA clusters represents the isolates belonging to one species, one ARDRA cluster (assigned as A) involves two isolates identified as two different species, i. e. *P. fluorescens* and *P. putida*, respectively, which represent saprophytic fluorescent pseudomonads (Brosch et al., 1996; Grimont et al., 1996). Furthermore, both pseudomonad isolates differed from each other by additional features, predominantly by lipase, protease, and lecithinase activities. While the isolate identified as *P. putida* (3P) was negative for lipase, protease, and lecithinase activities, the isolate identified as *P. fluorescens* (3K) – also negative for lipase activity – appeared positive for protease and lecithinase activities. It has been previously shown that the isolates identified as *P. putida* by API 20 NE system were predominantly negative for lipase, protease and lecithinase activities, while the isolates identified by the same system as *P. fluorescens* were predominantly positive for the same enzymatic activities (Wiedmann et al., 2000). According to the same enzymatic activities evaluated by the same study, a few of the isolates identified by best likelihood as *P. putida* could possibly be identified also as *P. fluorescens*, although they were negative for all three enzymatic activities. On the other hand, none of the isolates identified by best likelihood as *P. fluorescens* with lecithinase activity, could be identified as *P. putida*. These results suggested that isolate 3P identified by API 20 NE as *P. putida*, probably represents *P. fluorescens* species (Table 1). Interestingly, an additional restriction analysis of the amplified DNA of

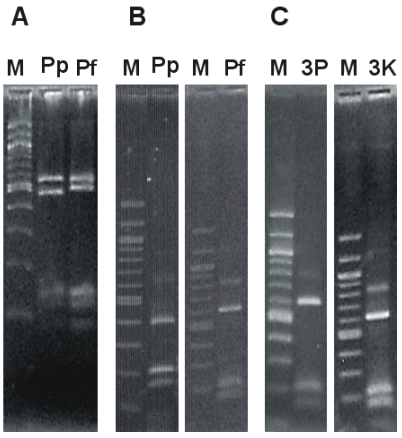

Fig. 2. (**A**) 3% and (**B, C**) 1.5% agarose gel electrophoresis of amplified 16S rDNA digested with restriction endonucleases (A) *Msp*I and (B, C) *Alu*I of (A, B) reference (control) bacterial strains and (C) of two bacteria (3P and 3K assigned as *P. putida* or *P. fluorescens*, respectively) isolated from sewage sludge. Lanes: (A, B) 1 = *Pseudomonas putida* 3423 (D. Halama), 2 = *Pseudomonas fluorescens* 3900 (A. Ternström 542), and M = 100 bp leader; (C) 1 = 3P, 2 = 3K, and M = 100 bp leader. Two reference (control) strains were purchased from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic).

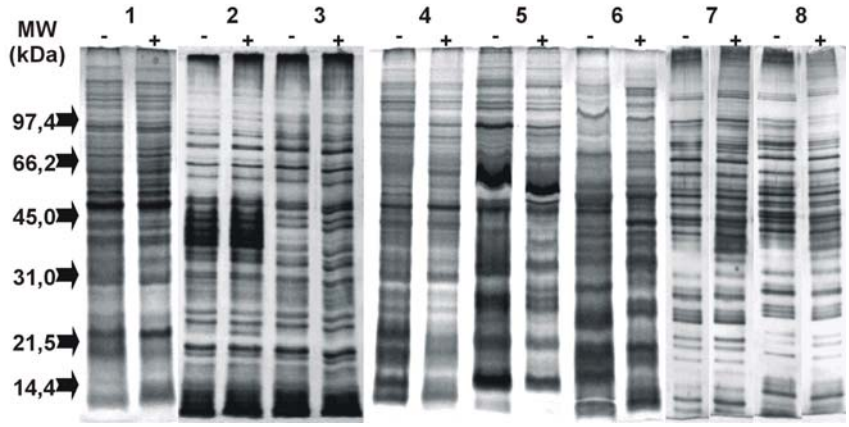

Fig. 3. 12% SDS-polyacrylamide gel electrophoresis of crude cell extract proteins of eight bacteria isolated from sewage sludge. The cells were grown aerobically at 30 °C in liquid mineral medium in the absence (–) or in the presence (+) of cadmium (50 μg/mL). Gels were silver stained to permit visualization of synthesized proteins. Lanes: 1 = 3P (*P. putida*), 2 = 10P (*K. planticola*), 3 = 11P (*K. planticola*), 4 = 1K (*S. liquefaciens*), 5 = 2K (*C. testosteroni*), 6 = 3K (*P. fluorescens*), 7 = 4K (*A. xylosoxidans*), and 8 = 5K (*A. xylosoxidans*). Standard molecular mass proteins are indicated. The experiment was repeated twice to confirm reproducibility; a representative result is shown.

two collection strains, e.g. *P. fluorescens* 3900 (A. Ternström 542) and *P. putida* 3423 (D. Halama) purchased from Czech Collection of Microorganisms (with *Msp*I and *Alu*I enzymes), differed these two strains to the two ARDRA patterns (Fig. 2). On the other hand, ARDRA pattern of both, 3P and 3K isolates was come up rather to collection *P. fluorescens* than to *P. putida* ARDRA pattern (Fig. 2) suggesting that both isolates belong to the same species. In addition, similarly to ARDRA patterns, whole cell protein pattern analysis of each of the eight isolates enabled rather five (not six) different protein patterns to be recognized (Fig. 3). In spite of the fact that identification of the bacterial isolates into species is shorted of further phylogenetic analysis of 16S rDNA sequences (Di Cello et al., 1997; Barberio and Fani, 1998), the results revealed that the microbial community consisted of some representatives belonging to the genus *Alcaligenes*, *Comamonas*, *Klebsiella*, *Pseudomonas* and *Serratia*. This suggests a relatively high interspecific variability in the culturable cadmium-resistant microbial community isolated from sewage sludge. The possible presence of representatives of *Pseudomonas*, *Comamonas* and *Alcaligenes* in this community is not surprising, since bacteria of these genera are often isolated from areas polluted by heavy metals (Diels & Mergeay, 1990; Godočíková et al., 1998; Hassen et al., 1998). Although representatives of the genus *Klebsiella* and *Serratia* are not directly linked with the presence of heavy metals, they were also isolated from the waste treatment systems (Fulthorpe et al., 1993). However, the used experimental protocol allows for only a limited sample of representatives of the real bacterial assemblage that occupy a cadmium-contaminated sewage sludge. Only a relatively small part of culturable community can thus be detected. The conditions of the isolation procedure can be advantageous for easily growing cells and can perhaps mask different, more important putative cells, originally present in the assemblage.

Characterization of bacterial isolates

In order to differentiate the strains within each ARDRA group, the whole cell protein patterns and presence of plasmid molecules of each of the eight isolates were analyzed either by one-dimensional gels or by agarose gel electrophoresis, respectively.

Analyses of whole cell protein patterns of the isolates growing in absence or presence of cadmium are presented in Figure 3. The individual isolates within the ARDRA group showed similar patterns with many matching bands, but not the same. Thus, protein patterns suggested the highest degree of intraspecific variability in ARDRA group A (3P and 3K, identified as *Pseudomonas* sp.), lower in ARDRA group B (10P and 11P, identified as *K. planticola*), and the lowest in ARDRA group C (4K and 5K, identified as *A. xylosoxidans*). Sim-

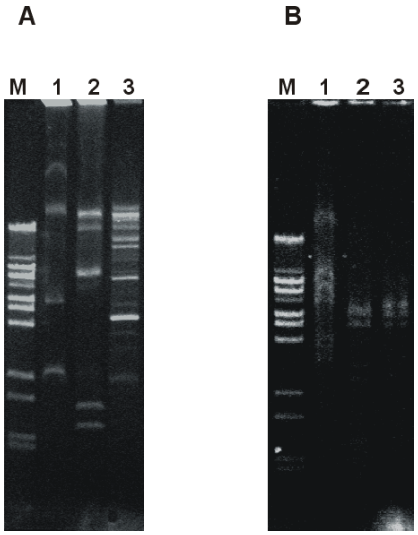

Fig. 4. Plasmid patterns of two bacterial isolates (**A**) 10P and (**B**) 11P assigned as *Klebsiella planticola*. Lanes: 1 = native plasmids, 2 = plasmids digested with restriction endonuclease *Hin*dIII, 3 = plasmids digested with restriction endonuclease *Kpn*I, M = λDNA-*Bst*E II digest.

ilarly, according to the protein patterns high intraspecific diversity was found between *Acinetobacter* isolates from activated sludge (MASZENAN et al., 1997). On the other hand, much smaller differences between protein pattern of the growing isolates in absence and presence of cadmium within the same ARDRA group were observed (Fig. 3).

Plasmid molecules of different size were detectable only in the two strains belonging to the ARDRA group B (10P and 11P, identified as *K. planticola*) (Fig. 4). These two strains did not give only different plasmid patterns, but they gave different restriction patterns with *Hin*dIII and *Kpn*I of plasmid mixtures as well (Fig. 4). Thus, this analysis suggested a certain degree of genetic variability between isolates belonging to ARDRA group B. The absence of plasmid molecules in remaining cadmium-resistant strains is surprising also from the point of view that bacterial resistance to heavy metals is often plasmid-encoded (MERGEAY et al. 1985; NIES, 1992; SCHMIDT & SCHLEGEL, 1994; BRUINS et al., 2003). Although our investigated cadmium-resistant isolates had no detectable plasmids by the extraction procedure of AUSUBEL & FREDERICK (1995), the possibility of a presence of large plasmids cannot be eliminated. The use of some additional methods suitable for isolation of larger plasmids (TAGHAVI et al., 1994) could reveal the large plasmid molecules in some of our strains.

Physiological properties of the bacterial isolates and distribution of cadmium-induced proteins (CDPs) in bacterial cells

Eight strains, representing the five ARDRA groups, were characterized physiologically in terms of their ability to grow in mineral media supplemented with cadmium and metal-accumulation with concomitant synthesis of CDPs.

Only five isolates (3P, 3K, 10P, 4K, 5K) with similar and relatively fast growth rate in the presence of cadmium were tested for their ability to remove cadmium from solution. When cultures achieved stationary phase (48 h after incubation) – in the same conditions described for growth rate investigations – samples were centrifuged to pellet bacterial cells and the supernatants were analyzed for cadmium content by atomic spectrophotometry. Bacterial removal of metal was expressed as a percentage distinction of the metal added initially to the medium and the metal found in the supernatant after experiment. Results showed that all five isolates were able to remove cadmium, but the isolates differed in their efficiency (Table 2). The highest percentage (47.6–49.4%) was observed with two isolates of the ARDRA group C (4K and 5K, identified as *A. xylosoxidans*), while the lowest percentage (10.8%) was obtained with isolate belonging to the ARDRA group B (10P, identified as *K. planticola*). Two representatives of ARDRA group A (3P and 3K, identified as *Pseudomonas* sp.) showed approximately twofold higher efficiency (19.0–22.5%) of cadmium removal compared to isolates of the ARDRA group B. In contrast, *Pseudomonas aeruginosa* cultured in nutrient broth in the presence of 100 μg/mL cadmium was able to adsorb 6.0 μg cadmium per mg of bacterial dry weight (HASSEN et al., 1998).

It is known that the resistance against cadmium is based in Gram-negative bacteria on the reduction of effective cadmium concentration in the cell, which is achieved predominantly by intracellular (HASSEN et al., 1998) or extracellular (NIES, 1992; SCHMIDT & SCHLEGEL, 1994; DIELS, 1997) metal-accumulation. It suggests that decrease of cadmium concentration in solution (Table 2) during growth of bacterial cells in the presence of cadmium could be connected to production of CDPs, and perhaps some of them could be potentially able to bind cadmium ions.

To study the protein synthesis and the distribution of the proteins in the cadmium-resistant

Table 2. Heavy metal removal by bacterial cells.

	Bacterial strains				
	3P	10P	3K	4K	5K
Wet weight of cells (mg/mL)[a]	21.2 ± 1.16	24.0 ± 0.78	19.8 ± 2.54	21.2 ± 0.94	17.9 ± 0.98
Calculated Cd-bound[b] (μg/mg of WW)	0.55 ± 0.08	0.23 ± 0.01	0.49 ± 0.14	1.15 ± 0.08	1.41 ± 0.14
Percentage of cadmium removal[c]	22.5 ± 1.47	10.8 ± 0.49	19.0 ± 3.02	47.6 ± 2.2	49.4 ± 3.43

[a] Bacteria were grown in liquid mineral medium supplemented with cadmium at 30 °C for two days. Calculated cadmium concentration yielded a final concentration of 50 μg/mL, whereas measured cadmium concentration yielded of 51 ± 1.63 μg/mL.
[b] Cd-bound expressed as a distinction of the metal added initially to the medium and the metal found in the supernatant after experiment.
[c] Removal expressed as a percentage distinction of the metal added initially to the medium and the metal found in the supernatant after experiment.
WW, wet weight; ±, standard deviation; $n = 4$.

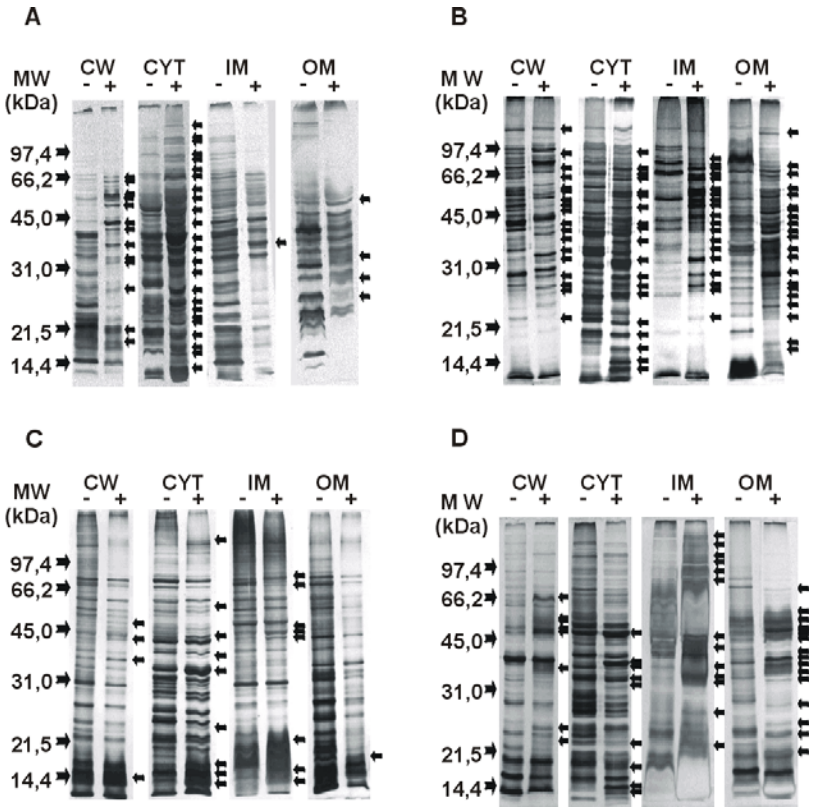

Fig. 5. 12% SDS-polyacrylamide gel electrophoresis of fractionated cell proteins of four bacterial strains isolated from sewage sludge. The isolates (**A**) 3P (*P. putida*), (**B**) 10P (*K. planticola*), (**C**) 3K (*P. fluorescens*), and (**D**) 4K (*A. xylosoxidans*) were grown aerobically at 30 °C in liquid mineral medium in the absence (–) or in the presence (+) of cadmium (50 μg/mL). Gels were silver stained to permit visualization of synthesized proteins. The arrows indicate the proteins, which shared increased synthesis in the presence of cadmium ions; these proteins are described in the text. Lanes: CW = cell wall, CYT = cytosol, IM = inner membrane, OM = outer membrane. Standard molecular mass proteins are indicated. The experiment was repeated twice to confirm reproducibility; a representative result is shown.

bacterial cells, samples were removed from the cultures growing in absence or presence of cadmium at specified times (when cultures achieved an OD_{420} of approximatelly 1.0), fractionated, and analyzed by one-dimensional gel electrophoresis. The proteins that increased in synthesis in the presence of cadmium were considered to be CDPs and marked (Fig. 5). The patterns of protein synthesis showed that cadmium induced several proteins in isolate 10P (*K. planticola*), slightly fewer in isolates 3P (*Pseudomonas* sp.) and 4K (*A. xylosoxidans*), and the lowest number in isolate 3K (*Pseudomonas* sp.) (Fig. 5). While *K. planticola* (10P) gave a more equal distribution of the CDPs between the cell fractions, the remaining isolates gave an unequal distribution of the CDPs. The isolates 3P and 3K (*Pseudomonas* sp.) had the highest number of CDPs in cytoplasmic fraction, whereas the majority of CDPs in the isolate 4K (*A. xylosoxidans*) was found in the cell membranes (Fig. 5).

In addition, the results also showed that the isolates differ in their efficiency to remove cadmium, as well as in their CDP distribution into bacterial cell fractions. The highest efficiency of cadmium removal was observed in the isolate belonging to the ARDRA group C (4K, identified as *Alcaligenes xylosoxidans*) and the majority of CDPs in this isolate was found in the cell membranes. The isolates belonging to the ARDRA group A (3P and 3K, identified as *Pseudomonas* sp.) showed lower efficiency of metal removal with the majority of CDPs in the cytoplasmic fraction (Fig. 5, Table 2). It appears that some relationship between these two traits exists, suggesting that bacterial cells expressing the CDPs within the cytosol are less efficient in removing cadmium from solution than the cells expressing these proteins in the cell membrane fractions. These suggestions are supported by other studies, which have previously been reported (Romeyer et al., 1990; Pazirandeh et al., 1995; Chen & Wilson, 1997). However, the isolate belonging to the ARDRA group B (10P, identified as *Klebsiella planticola*) is an exception, since it produced the highest number of proteins with their equal distribution into all cell fractions and removed the lowest amount of cadmium ions (Fig. 5, Table 2).

In conclusion, the bacterial strains described here, i.e. the representatives of ARDRA groups C (4K and 5K, identified as *Alcaligenes xylosoxidans*) and A (3P and 3K, identified as *Pseudomonas* sp.), could be used in bioremediation processes aimed at heavy metal removal from contaminated environments. Future studies will be focused on elucidating the role of the metal binding proteins in view of their potentially practical exploitation.

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