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METABOLIC AND GENETIC DIVERSITY OF PHENOL-UTILIZING BACTERIA AS AN ENHANCER OF NATURAL BIODEGRADATION IN POLLUTED WATERS

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The metabolic and genetic diversity of phenol-utilizing 119 bacterial strains isolated from the rivers polluted by ash dump leachate in Kohtla-Järve, Estonia, has been studied. Of eighteen different bacterial species identified, 82 % belong to the genus Pseudomonas. Comparison of genetic (phenol monooxygenase /PMO/ and catechol 2,3-dioxygenase /C23O/ gene probes) and biochemical (PMO, C23O, catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase activities) data with species of isolated bacteria shows that in different polluted regions of the rivers different Pseudomonas fluorescens biotypes and Ps. mendocina are predominant and they harbor characteristic genetic traits. The incidence of plasmid-bearing bacteria is much higher in phenol-polluted waters (up to 81 % of the strains) than in nonpolluted river waters (38 %). We found three main types of induction of aromatic ring-fission pathways among the isolated phenol-growing bacteria which also grow on p-cresol. Some new bacterial isolates, such as Ps. fluorescens strains MT4/4 and I16 revealed even higher biodegradation capabilities of volatile phenols in leachate than laboratory-selected phenol-growing bacteria.

Introduction

Given the complexity of both the environmental and the microbial consortia that are involved in biodegradation in polluted rivers, the evaluation of the feasibility of both natural and *in situ* bioremediation must be made on a site-by-site basis [1]. Our limited understanding of microbial diversity results from methodological limitations and a basic lack of taxonomic information [2]. A quantitative approach to examine components of biodiversity gives information about functional diversity of microbial communities [3]. Natural consortia may be important for the functioning of catabolic bacteria in the environment [4]. The use of microorganisms for the degradation of toxic compounds, for *in situ* remediation, is mainly influenced by the ability to maintain high degradation rates and to mineralize all the pollutants completely. Before the application of manipulation of microbial metabolic activities to environmental detoxification it is important to monitor the indigenous populations. In particular, their genetic and biochemical potential, which determines the substrate range and metabolic efficiency of individual isolates.

The phenol-degrading bacteria possess a wide variety of enzymes for the initial attack of phenols. The central metabolic intermediate at the biodegradation of phenol, benzoate, salicylate, naphthalene and substituted phenolic compounds is catechol or its substitutions which is further metabolized either by enzymes of the so called *meta*- or of the *ortho*-cleavage pathway. Key enzymes of these pathways are the catechol 2,3-dioxygenase (C23O) and the catechol 1,2-dioxygenase (C12O). Typically unsubstituted phenolic compounds are metabolized by the chromosomally encoded *ortho*-pathway, while the *meta*-pathway is characteristic for biodegradative plasmids determining the break-down of methylated phenolic compounds.

The above mentioned catabolic pathways are genetically alternatively regulated due to the fact that *ortho*- pathway enzymes produce dead end intermediates from methylated phenols. In natural bacterial assemblages, mixtures of differently substituted phenolic compounds can cause induction of both catabolic pathways, resulting in misrouting of intermediates, poisoning of key enzymes, accumulation of detrimental products and possible death of the microbial community [5]. The second key intermediate in the metabolism of phenolic compounds is protocatechuate, which favors catabolism of methylated phenols by protocatechuate 3,4-dioxygenase (PCD). This cleavage route is an alternative branch of the *ortho*-pathway (also known as the β-ketoadipate pathway) determining *ortho*-fission of protocatechuate [6]. The use of one pathway or the other is dependent upon the microbial species and the nature of the growth substrate.

The principal aromatic pollutants in oil shale ash mound leachates are unsubstituted and methyl-substituted phenolic compounds. These compounds have become a serious environmental problem for the North-Eastern part of Estonia and for the Baltic Sea. The aim of this work was to investigate the natural biodegradation processes through monitoring isolates (such as identification of phenol-degraders, determining of biodegradative pathways by hybridization with specific gene probes and measuring the activities of key enzymes of phenols catabolism, occurrence of biodegradative plasmids and genes in differently polluted sites) in highly polluted river waters. We compared the biodegradative ability of several isolates with different catabolic pathways with laboratory-selected bacteria *Pseudomonas putida* E_2 in microcosms containing oil shale ash leachate in *in situ* conditions.

Material and Methods

Isolation and Identification of Strains

Bacterial strains were isolated from phenol-contaminated and noncontaminated river waters from the North-Eastern part of Estonia. Water samples were taken downstream of the discharge from a polluted area: the Channel, the rivers Kohtla and Purtse (sites *1-7*) and from different sites without contamination - control (sites *8-10*) employing aseptic procedures and with storage on ice prior to processing (usually within 10 hrs). The well mixed and diluted samples were immediately plated onto minimal M9-salts media containing different carbon sources - phenol (2.5 mM), benzoate, *m*-toluate, salicylate (5 mM), naphthalene, camphor and heptane (vapors). The single colonies were picked from the selective agar plates and purified by streaking on R2A or LB agar plates.

Prior to taxonomic identification, the isolates were screened for Gram reaction and then identified using BIOLOG GN microtiter plates (Biolog Inc., Hayward, Cal., USA). The plates were read automatically at 620 nm after 24 hr and 48 hr of incubation at 30 °C using a microplate reader (Labsystems Multiskan MCC/340).

Growth on Aromatic Compounds

The ability to utilize different aromatic compounds by isolates able to grow on phenol as a sole source of carbon and energy, was tested on minimal M9-salt plates supplemented with several aromatic substrates: benzoate, *m*-toluate and salicylate (5 mM). Degradation of *p*-cresol was detected with selective agar plates containing 1.3 mM *p*-cresol. The plates were incubated at 30 °C for 2 or 3 days, and bacterial growth was visually monitored.

Cell Growth Prior to Enzyme Assay and Plasmid Screening

150-ml Erlenmeyer flasks containing 50 ml of the basal medium M9 with phenol at a final concentration 2.5 mM were inoculated and incubated on a rotary shaker (30 °C, 150 rpm) until the late exponential growth phase was reached. The centrifuged cells were used for plasmid DNA detection by the procedures of Connors and Bransley [7] and for making crude extract preparations for enzyme assays. For this, cells were washed twice with cold K_2HPO_4-KH_2PO_4 buffer (100 mM; pH 7.5), resuspended in the same buffer and sonically disrupted (0.5 min, three times at 4 °C). Unbroken cells and cell debris were removed by centrifugation at 12,000 × g for 30 min at 4 °C and collected crude soluble supernatant fractions were used for enzyme assays.

Determination of Catabolic Pathways of Isolates Enzymatically and by Colony Hybridization Method

Assays of catabolic enzymes were performed spectrophotometrically at 25 °C. C23O was determined by Feist and Hegeman [8] and C120 by Hegeman [9]. The reaction rates were determined by monitoring the appearance of 2-hydroxymuconic semialdehyde at 375 nm and *cis-cis* muconate at 260 nm. Extracts used for C12O activity measurements were pre-treated for 5 min with H_2O_2 at a final concentration of 10 mM to inactivate enzymes and thus avoid interference by any C23O present in the protein extract [10].

PCD activity was measured at 290 nm by monitoring the disappearance of protocatechuate [11]. Phenol monooxygenase (PMO) was assayed by monitoring the disappearance of NADPH at 340 nm [12]. The protein contents of cell extracts were determined by the method of Bradford [13] using bovine serum albumin as a standard.

For colony hybridization, cells of isolates were dotted with a toothpick on to the non-charged nylon membranes Nytron NY 13 N (Schleicher & Schuell, Dassel, Germany) which were placed on LB agar media. The plates were incubated for 24 h at 30 °C. Colonies were then analyzed by hybridization using *xyl*E (C230 gene) and *phe*A (PMO gene) gene probes. Conditions for colony lysis, prehybridization, hybridizations with appropriate gene probes, washing and autoradiography have been previously described [14]. The radioactively labeled gene probes were used:

*xyl*E primers 5′-TCAAGGTTGTGGATGAGGATGC-3′
and 5′-AGAACACTTCGTTGCGGTTACC-3′;

*phe*A primers 5′-CAGGA TCGAATATCGGTGGCCTCG-3′
and 5′-CTTCACGCTGGCGTAACCAATCGC-3′.

The oligonucleotide primers were synthesized by Biometra (Göttingen, Germany). Autoradiograms were generated by Phosphorimager™ IS (Molecular Dynamics).

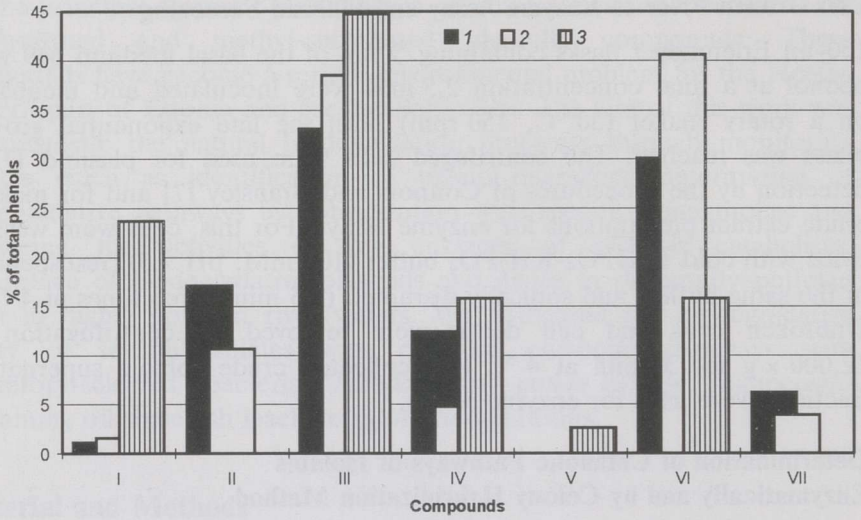

Fig. 1. Volatile phenolic compounds in oil shale ash leachate in different water samples from the beginning of the Channel. HPLC data are presented as percentages. Legend: I - resorcinol; II - 5-methylresorcinol, III - phenol; IV - 4,5- dimethylresorcinol; V - 5-methyl-2-ethylresorcinol; VI - *o*-, *m*-, *p*-cresols; VII - 2,3-. 2,6- and 3,4-dimethylphenols; *1* - March 1994, total phenols 137.3 mg/l; *2* - June 1994, total phenols 75.8 mg/l; *3* - September 1994, total phenols 3.8 mg/l

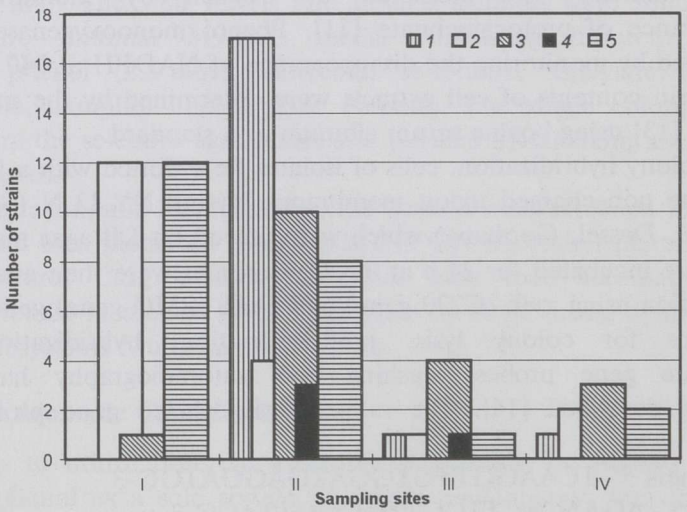

Fig. 2. The number of *Ps. fluorescens* biotypes B, C, G, F and *Ps. mendocina* strains at different sampling sites. The concentration of phenolic compounds is decreasing in the direction Channel (I) - River Kohtla (II) - River Purtse (III) - Control (IV). *1* - *Ps. fluorescens* B; *2* - *Ps. fluorescens* C; *3* - *Ps. fluorescens* G; *4* - *Ps. fluorescens* F; *5* - *Ps. mendocina*

Table 1. **Characterization of Phenol-Growing Bacterial Species**

Bacterial species	Number of strains isolated from				Total	Plasmids	PMO (*phe*A)	C23O (*xyl*E)	Growth on			
	Channel	Kohtla	Purtse	Control					benzoate	*p*-cresol	*m*-toluate	salicylate
Ps. fluorescens B	0	17	1	1	19	17	9	7	16	9	3	10
Ps. fluorescens C	12	4	1	2	19	11	13	4	15	9	2	3
Ps. fluorescens G	1	10	4	3	18	12	6	5	17	9	1	8
Ps. fluorescens F	0	3	1	0	4	2	4	0	4	0	0	0
Ps. mendocina	12	8	1	2	23	9	0	19	7	8	7	1
Ps. putida	0	2	3	1	6	2	1	1	5	2	2	2
Ps. stutzeri	1	0	3	0	4	1	1	3	1	1	0	1
Ps. corrugata	0	1	0	0	1	1	1	0	1	1	0	0
Ps. fragi	0	0	1	0	1	1	1	0	1	0	0	0
Ps. cichorii	0	1	0	0	1	1	1	0	1	1	0	0
Ps. vesicularis	1	0	0	0	1	0	1	0	0	0	0	0
Ac. lwoffii	1	0	0	0	1	1	0	0	0	0	0	0
Ac. genospecies	1	2	0	1	4	2	0	1	2	1	0	0
Ac. johnsonii	2	1	0	0	3	2	0	0	2	0	1	1
Ac. radioresistens	1	1	0	0	2	2	0	0	2	0	0	0
X. maltophilia	2	0	0	1	3	1	0	2	1	0	1	1
X. oryzae	1	0	0	1	2	1	0	0	2	0	1	1
Psychrobacter immobilis	2	0	0	0	2	2	0	0	2	0	0	0
Aeromonas media	2	0	0	1	3	0	0	0	0	0	0	0
Agrobacterium tumefaciens	0	0	0	1	1	1	1	0	0	1	0	0
Burkholderia cepacia	0	1	0	0	1	1	1	0	1	1	0	1
Total	39	51	15	14	119	70	40	42	80	43	18	29

Microcosm Experiments

Oil shale ash leachate was collected from the beginning of the Channel (site *2*). The basal salt solution M9 was used in shake flask experiments for adjustment of pH of leachate and for feeding media with nutrients. Distilled water was used to dilute the leachate. After sterilizing by filtration through a Millipore filter (0.22 μm), 100 ml was added to the autoclaved 250-ml Erlenmeyer flasks. These were inoculated with microbes and incubated on a rotary shaker operating at 60 rpm. Culture densities were measured at 580 nm and incubation at 10 °C continued for 44-76 hours until the densities had reached a constant level. A control was incubated with uninoculated leachate to determine chemical degradation of phenolic compounds. Samples of cell suspensions were filtered through a Millipore filter (0.22 μm) and frozen at -20 °C for substrate analyses by high pressure liquid chromatography (HPLC) using Chromatopac C-R3A (Shimadzu, Japan) equipped with an Ostion ABN column.

Results and Discussion

Chemical Composition of Oil Shale Ash Leachate

The ash dump leachate composition is not well defined. It depends on rainfall and chemical industry process waters, which are compacted the ash mounds [15]. We studied the chemical composition of leachate in three different water samples taken at the beginning of the Channel (Fig. 1). The HPLC data shows that the volatile phenols in the leachate contain mainly phenol (from 33.2 to 44.2 per cent) and cresols (from 16.1 to 40.6 per cent), while the concentrations of resorcinol, methylresorcinols and dimethylphenols do not exceed 22.4 %.

Data presented in the Fig. 1 reveals that despite the great differences in total concentrations of volatile phenols, the ratio between particular phenolic compounds remains quite similar. It does mean that the pressure of volatile phenols to the river waters investigated could be similar during the long period of time and it favors the development of characteristic microbial community.

Taxonomic Study of Phenol-Growing Bacteria

We have isolated, purified and identified from the Channel, the River Kohtla, the River Purtse and from the neighbourings watersheds of the rivers Kohtla and Purtse (Control) 119 phenol-utilizing bacterial strains. The majority of those (97 strains or 82 %) belong to the genera *Pseudomonas,* among them 60 strains to the species *Ps. fluorescens*, 23 *Ps. mendocina,* 6 *Ps. putida,* 4 *Ps. stutzeri* and 4 to other *Pseudomonas*

species (Table 1). It is generally known that pseudomonads are very common in many natural habitats and are able to use a wide variety of low molecular weight organic compounds as sole sources of carbon and energy. This is why *Pseudomonas* species, including *Ps. fluorescens*, have attracted increasing interest in relation to environmental biotechnology, e.g. as potential hosts for engineered pathways for degradation of xenobiotics [16].

Bacterial community structure may be influenced by the type of pollution. We examined the species diversity of phenol-degrading bacteria in the Channel, the River Kohtla, the River Purtse and in nonpolluted control waters where the concentration of phenolic pollutants is lower and species diversity tends to be greater. We determined 18 different bacterial species from 119 isolates. We found from the water samples of the Channel 13 different bacterial species, the River Kohtla 12, the River Purtse only 8 and 10 different bacterial species from the control waters (Table 1). The data presented in the Fig. 2 shows that the most frequent phenol-degraders in stressful conditions (the Channel) are *Ps. fluorescens* biotype C and *Ps. mendocina*. The other *Ps. fluorescens* biotypes (B, G, F) as well as *Ps. mendocina* are distributed in less toxic but, however, in strongly polluted conditions, in the River Kohtla. This species and biotype diversity reflects toxicity of the environment and survival of tolerant species. A limited number of physiologically specialized microorganisms with particular genetic characters (such as plasmids or particular metabolic gene clusters present in those strains) may be present.

Diversity of Key Genes and Enzymes of Catabolic Pathways

The results of hybridization experiments with *xyl*E and *phe*A gene probes were restudied with the determination of catechol 2,3-dioxygenase (*xyl*E product) and phenol monooxygenase (*phe*A gene product) enzyme activities. In both cases we got the same results. In addition, we determined the catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase activities of all strains investigated (data not shown). Our data shows that the *phe*A gene is more frequent among isolates from phenol-contaminated waters and the *xyl*E gene, in contrast, is more frequent in noncontaminated waters (Fig. 3). Moreover, the presence of both *phe*A and *xyl*E genes seems to be restricted to the same bacterial strains due to the possible metabolic conflict between the degradation of phenol and methylsubstituted phenols. It is known that *meta*- (via catechol 2,3-dioxygenase) and *ortho*-pathways (via catechol 1,2-dioxygenase) are alternative on the level of induction of corresponding metabolic routes [17]. In most cases, phenol would be degraded by the *ortho*-pathway [6], which enables the cells to degrade cresols via *meta*-fission of catechol by C23O. On the other hand the

degradation of cresols via *ortho*-pathway results in toxic dead end products [18]. This implies that if waters contain both phenol and cresols, the phenol-growing bacteria without C23O gene must resolve the problem of degradation of cresols by using another metabolic route. It is known that methylated phenols could be degraded via protocatechuate by the *ortho*-pathway where the ring fission enzyme is protocatechuate 3,4-dioxygenase [6].

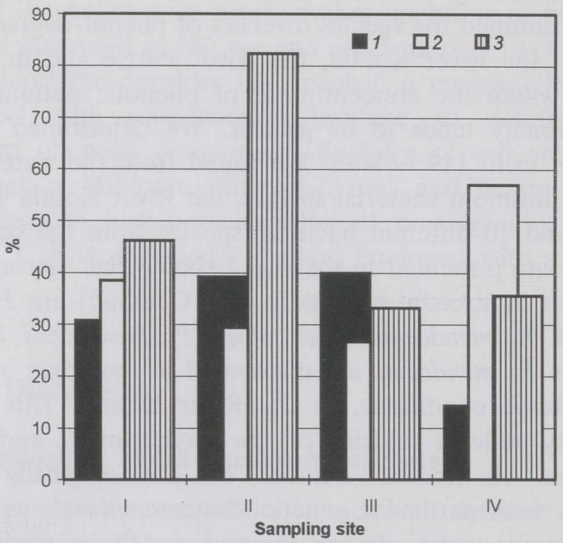

Fig. 3. The frequency of phenol monooxygenase (*phe*A) (*1*) and catechol 2,3-dioxygenase (*xyl*E) (*2*) genes and the presence of plasmids (*3*) among 119 bacterial strains at different sampling sites. The concentration of phenolic compounds is decreasing in the direction Channel (I) - River Kohtla (II) - River Purtse (III) - Control (IV)

Importance of Protocatechuate Pathway

We found the *phe*A gene mostly in the strains of *Ps. fluorescens* (79 % of the strains) (Fig. 4). The strains of *Ps. mendocina* did not harbor *phe*A gene, but in contrast to the pseudomonads, of 23 strains, 19 contained the *xyl*E gene (Fig. 4 and Table 1). The concentration of the chemical substrate for the expressed catabolic pathway is clearly one important selection factor [19]. Of 119 phenol-utilizing bacteria, 43 grew on *p*-cresol, of which only 14 have C23O activity. Even in this case, with some isolates, e.g. *Ps. fluorescens* biotype *G* strain 5.85 (Table 2), growth on *p*-cresol did not result in the induction of C230 but the protocatechuate 3,4-dioxygenase activity was found. We found three main types of aromatic ring-fission pathways among the isolated phenol-growing bacteria which also grow on *p*-cresol (Table 2). The first type is characterized by the degradation of phenol through C12O and *p*-cresol

through PCD (*Ps. fluorescens* C I 16). Thus, only the *ortho*-pathway is present. The second type of isolate (*Ps. fluorescens* G 5.85) degrade phenol via C230 and *p*-cresol via PCD. The third type of isolate (*Ps. mendocina* SB2/8) degrades both phenol and *p*-cresol via C230.

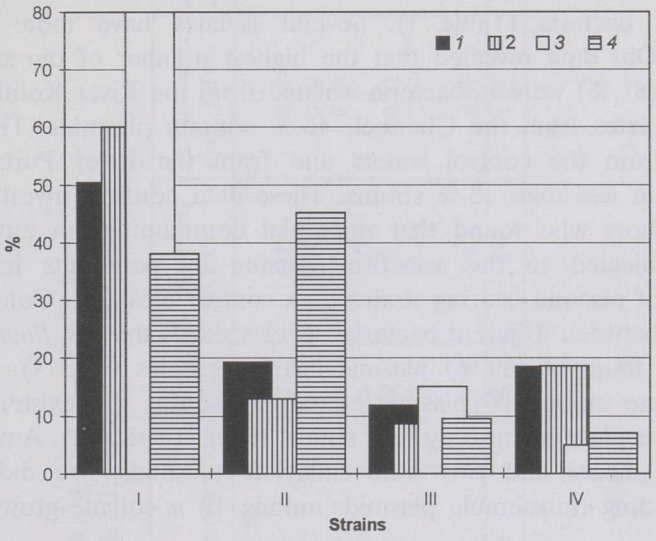

Fig. 4. The frequency of phenol monooxygenase (*phe*A) and catechol 2,3-dioxygenase (*xyl*E) genes and the presence of plasmids compared with the number of bacterial strains belonging to the pseudomonads and nonpseudomonads species. Altogether 119 strains were investigated. Legend: I - Ps. *fluorescens*; II - *Ps. mendocina*; III - other pseudomonads; IV - nonpseudomonas species; *1* - number; *2* - plasmids; *3* - *phe*A; *4* - *xyl*E

Table 2. **Specific Activities of Catabolic Enzymes (Micromoles per Minute per Milligram of Protein) in Cell Extracts of the Strains of *Ps. fluorescens* I 16 and 5.85 and *Ps. mendocina* SB2/8 Grown on Different Substrates**

Enzymes	*Ps. fluorescens* I 16		*Ps. fluorescens* 5.85		*Ps. mendocina* SB2/8	
	Growth substrate		Growth substrate		Growth substrate	
	Phenol	*p*-Cresol	Phenol	*p*-Cresol	Phenol	*p*-Cresol
Phenol monooxygenase	0.179	0	0	0	0	0
Catechol 1,2-dioxygenase	1.928	0.102	0	0	0	0
Catechol 2,3-dioxygenase	0	0	1.09	0	1.52	0.892
Protocatechuate 3,4-dioxygenase	0.2	0.62	0	0.456	0	0

N o t e : Mean values of three experiments are presented.

Occurrence of Degradative Plasmids

The occurrence of degradative plasmids in pseudomonads gives them more wide metabolic diversity in different environmental conditions [20]. In the present study, plasmids were observed in 59 % of phenol-degrading bacteria (Table 1). Several isolates have more than one plasmid. Our data revealed that the highest number of the strains with plasmids (81 %) were in bacteria isolated from the River Kohtla (Fig. 3). In the isolates from the Channel, 46 % contain plasmids. The bacteria isolated from the control waters and from the River Purtse contain plasmids in less than 38 % strains. These data confirm investigations of other authors who found that microbial communities in environments which subjected to the selective pressure by pollutants have higher numbers of plasmid-bearing strains than control sites [21]. Comparison of plasmids between different bacterial species shows that *Ps. fluorescens* was the most frequent (60 %) plasmid-bearing species (Fig. 4). We could demonstrate among 70 plasmid-containing strains the existence of the conjugative plasmids in only in 8 strains (data not shown). Among these, six were phenol and two were salicylate plasmids. We did not find corresponding transferable plasmids among 18 *m*-toluate-growing strains (Table 1).

Effectiveness of Laboratory Selected and Isolated Bacterial Strains to Use Phenolic Compounds in Leachate

We compared the degradation efficiency of phenolic compounds in leachate with 3 new isolates (*Ps. fluorescens* strains I16 and MT4/4, *Acinetobacter lwoffii* II19) and with the laboratory-selected strain *Ps. putida* E_2 at different pH values 7.3 and 9.3 (Fig. 5).

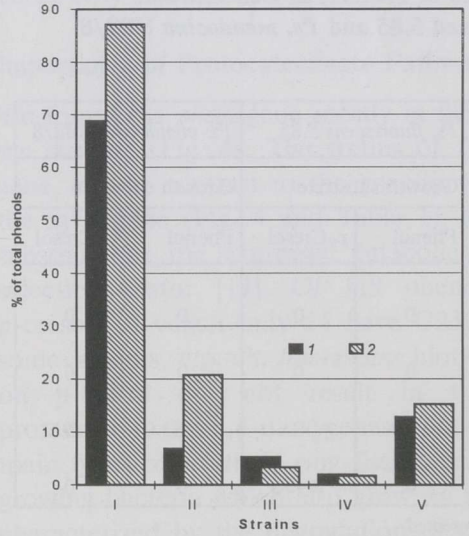

Fig. 5. Decomposition of volatile phenolic compounds in leachate without microbes (control) (I), by laboratory-selected *Ps. putida* strain E_2 (II) and new isolates *Ps. fluorescens* strains I 16 (III) and MT4/4 (IV) and *Ac. lwoffii* strain II 19 (V) at different pH values. *1* - pH 7.3, total phenols 50.66 mg/l; *2* - pH 9.3, total phenols 42.35 mg/l

Table 3. **Utilization of Phenolic Compounds from Oil-Shale Ash Leachate by Different Bacterial Strains (76 hr; 10 °C; pH 7.3; aeration 80 rpm), mg/l**

Substrates	Concentration of compound	Control without microbes	*Ps. putida* strain E2	*Ps. fluorescens* strain I 16	*Ps. fluorescens* strain MT4/4	*Ac. lwoffii* strain II 19
Resorcinol	0.77	0.38	0.14	0	0	0.15
5-Methylresorcinol	1.42	2.83	0.32	0.2	0.07	0.14
Phenol	21.19	16.22	0	0.09	0.02	0.51
4,5-Dimethylresorcinol	2	1.16	0.29	0	0.36	0.51
m-Cresol	7.71	2.58	0.54	0.23	0	1.31
p-Cresol	9.56	6.73	1.38	0.52	0.02	1.78
o-Cresol	4.89	3.02	0.53	0	0	1.5
2,3-, 2,6- and 3,4-Dimethylphenols	3.13	2.38	0.27	1.6	0.57	0.59
Total	50.66	34.81	3.46	2.65	1.03	6.48

The high pH (9.3), low speed of rotation (60 rpm) and low incubation temperature (10 °C) were selected for the better simulation of environmental conditions. The natural strains used differ in genetical and biochemical characteristics. The laboratory-selected strain E_2 uses during 76 hr experiments phenolic compounds much more effectively at pH 7.3 (93.2 %) than at pH 9.3 (79.2 %). The *Ps. fluorescens* strain MT4/4 was the most effective biodegrader in both pH values (98 %). The *Ps. fluorescens* strain I16 degrades 94.8-96.7 % of volatile phenols. The *Acinetobacter lwoffii* strain II19 has much lower efficiency (84.7-87.2 %) for removal of phenolic compounds. The multicomponent nature of wastewaters and the role of auxiliary substrates can facilitate the degradation process [22]. The control experiments (phenolic water without microbes) revealed that the concentration of phenolic compounds decreases only 13-30 % from the culture media after 76 hours of incubation at 10 °C.

The strains investigated show different efficiencies of removal of particular phenolic compounds from leachate (Table 3). The strain E_2 degrades phenol more quickly, the strain I16 and MT4/4 were more effective against resorcinols and cresols. Thus, in natural microbial communities bacteria could be specialized for degradation of particular phenolic compound. This is determined by genetic potential and gene induction of the bacteria. More detailed investigations of new isolates are in progress.

Acknowledgments

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