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Acquisition of a Deliberately Introduced Phenol Degradation Operon, *pheBA*, by Different Indigenous *Pseudomonas* Species

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Horizontal transfer of genes of selective value in an environment 6 years after their introduction into a watershed has been observed. Expression of the gene *pheA*, which encodes phenol monooxygenase and is linked to the *pheBA* operon (A. Nurk, L. Kasak, and M. Kivisaar, Gene 102:13–18, 1991), allows pseudomonads to use phenol as a growth substrate. *Pseudomonas putida* strains carrying this operon on a plasmid were used for bioremediation after an accidental fire in the Estonia oil shale mine in Estonia in 1988. The water samples used for studying the fate of the genes introduced were collected in 1994. The same gene cluster was also detected in *Pseudomonas* strains isolated from water samples of a nearby watershed which has been continuously polluted with phenols due to oil shale industry leachate. Together with the more frequently existing counterparts of the *dmp* genes (V. Shingler, J. Powlowski, and U. Marklund, J. Bacteriol. 174:711–724, 1992), the *pheA* gene was also represented in the phenol-degrading strains. The area where the strains containing the *pheA* gene were found was restricted to the regular route of phenolic leachate to the Baltic Sea. Nine *Pseudomonas* strains belonging to four different species (*P. corrugata*, *P. fragi*, *P. stutzeri*, and *P. fluorescens* biotypes B, C, and F) and harboring horizontally transferred *pheBA* operons were investigated. The *phe* genes were clustered in the same manner in these nine *phe* operons and were connected to the same promoter as in the case of the original *pheBA* operon. One 10.6-kb plasmid carrying a *pheBA* gene cluster was sequenced, and the structure of the rearranged *pheBA* operon was described. This data indicates that introduced genetic material could, if it encodes a beneficial capability, enrich the natural genetic variety for biodegradation.

Most open-water studies to evaluate the breakdown of organic contaminants have been carried out in laboratory mesocosm systems or small in situ enclosures (41). The majority of these studies have focused on risk assessment rather than on the potential for bioaugmentation, i.e., the introduction of microbes that contain the necessary genes for biodegradation (9). In practice, the released laboratory strain itself often survives only a few weeks as a result of abiotic and biotic stress (2, 26, 40). However, several studies indicate that significant transfer of a large catabolic plasmid from a released organism to an indigenous microorganism can occur (9, 45). The occurrence of highly similar chromosomal 2,4-dichlorophenoxyacetic acid-degradative genes (24), as well as the phylogenetic distribution of the transposon-encoded gene (28), in different bacterial species has been reported. Thus, gene transfer from introduced organisms to the autochthonous microflora could be an effective means of bioaugmentation, and survival of the introduced organism is not a prerequisite for biodegradation (9). The ecological impact of such gene flow should depend on the selective value of the introduced genetic material. However, little is known about the dissemination and persistence of introduced catabolic genes in real natural microbial communities during a prolonged period.

The present study has focused on the acquisition of a deliberately released phenol degradation operon by the indigenous microflora, after a 6-year period, from river water continuously polluted by phenolic compounds. The objectives were to verify the persistence and horizontal transfer of this operon. The release of phenol-degrading laboratory bacteria into the open environment was performed in March 1989, in accordance with Soviet regulations, due to the great need to decontaminate thousands of tonnes of polluted water which was pumped out of a mine and flowed through rivers to Lake Peipus and to the Baltic Sea (see Fig. 1). The phenol-polluted water resulted from a major subterranean fire in the Estonia oil shale mine which burned from November 1988 to February 1989 in northeastern Estonia. To reduce the produced pollution in situ, two nonpathogenic phenol-degradative strains of *Pseudomonas putida* PaW85 were used.

These deliberately released phenol-degrading bacterial strains harbored the *pheBA* operon encoding catechol 1,2-dioxygenase and phenol monooxygenase (PMO) (18, 32). This operon, which originated from the multiplasmid strain *Pseudomonas* sp. strain EST1001 (17), was transferred by conjugation into *P. putida* PaW85. The *pheB* and *pheA* genes are cotranscribed in the order *pheB* to *pheA*. The *pheBA* cluster (GenBank accession no. M57500) is flanked by two IS elements. The transposase genes of these IS elements are designated open reading frames 1 and 2 (ORF1 and ORF2). The promoter of the operon has been mapped upstream of the *pheB* gene and one of the above-mentioned transposase genes and shows extensive homology to the chromosomal *catBC* promoter region which is recognized by CatR. The regulatory protein CatR also activates transcription from the *pheBA* promoter (13, 33). The organization of the *pheBA* operon (13) (see Fig. 4) was the same in the strains which were released into the contaminated water. One of the strains released also harbored TOL plasmid pWW0 (44).

The first problem with redetection of the *pheBA* operon is how frequently either this operon and/or it counterparts occur in the environment. Unfortunately, there were no direct investigations on the distribution of the *pheBA* genes in the environment before the fire in the Estonia mine. The characteristic

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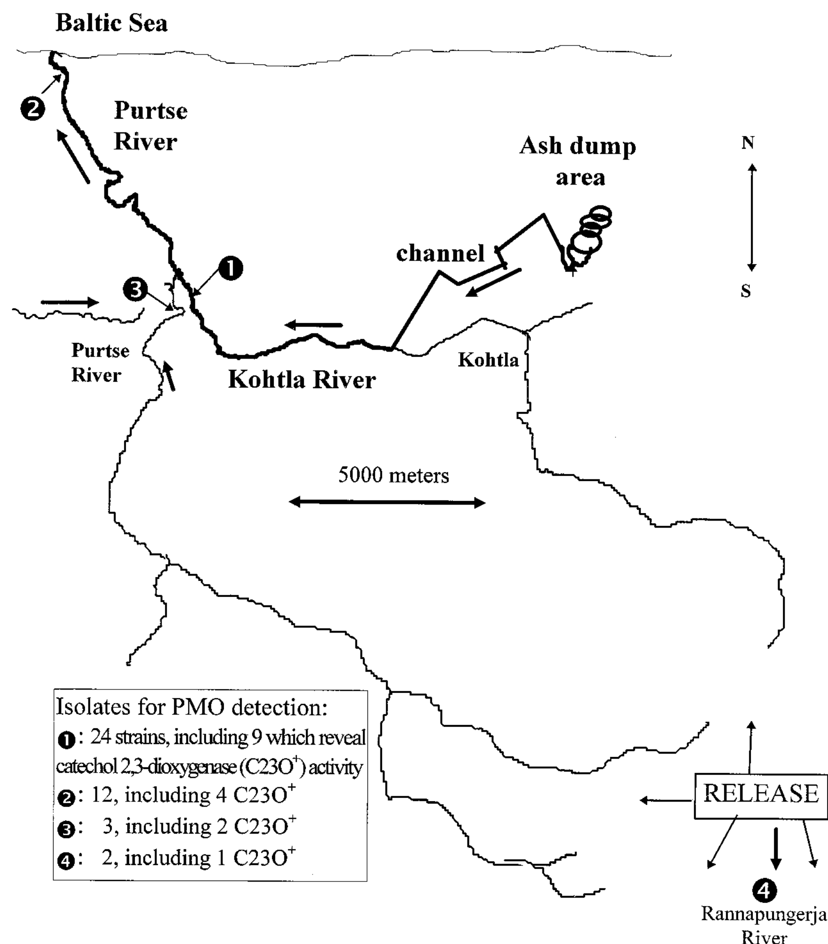

FIG. 1. Map of the study site, the watershed of the Purtse River, in northeastern Estonia. The heavy line indicates the route of phenolic leachate to the Baltic Sea. White numbers on a black background indicate water sampling points. The rectangle with "RELEASE" marks the site of the reservoir at the oil shale enrichment plant, where water enriched with the *pheBA* operon-carrying bacteria was released.

gene of the *pheBA* operon is *pheA*. Several other genes for phenol hydroxylases (PH) have been described (15, 20, 30, 34). There is no data on the detection of *pheA* counterpart genes from elsewhere. The closest similarity (46%) to the *pheA*-encoded PMO (32) has been found with 2,4-dichlorophenol hydroxylase, encoded by *tfdB* (34). The homology between *pheA* and *tfdB* is too low to detect by DNA-DNA hybridization under standard experimental conditions. Moreover, the source of the *phe* genes in released strains was the laboratory strain EST1001, the spontaneous Xyl^- derivative of *Pseudomonas* sp. strain S13. This parental strain S13, isolated in 1976, was a generous gift from P. A. Williams and was therefore not of local origin (17).

One area with potential selective value for phenol degradation genes is located 15 km from the enrichment plant reservoir, where the bacterial biomass was produced which was later used for the decontamination of the water containing the phenols produced during the fire. This area contains ash dumps, where the solid by-products formed in the semi-coking processing of oil shale are disposed. These ash dumps are compacted by the drainage waters from the chemical industry processes and are exposed to natural precipitation. The leachate from the ash dumps contains aromatic compounds, e.g., phenol, dimethylphenols, cresols, and resorcinols (14). This leachate, a continuous source of phenols, is discharged, without being treated, via rivers into the Baltic Sea (See Fig. 1).

We found that the *pheBA* operon was extant in the environment for 6 years and had been transferred into various aquatic bacteria isolated from the second half of this 25-km-long route. This operon allows these bacteria to utilize phenol as a growth substrate.

MATERIALS AND METHODS

Study site. The study area is situated in northeastern Estonia (Fig. 1). Aquatic bacterial strains were isolated from upper layers of watersheds of the Purtse River and the Rannapungerja River during 1994 (2 March, 2 June, and 15 September 1994) and plated onto minimal M9 solid medium containing either benzoate (5 mM), *m*-toluate (10 mM) or phenol (2.5 mM). Samples were collected at a depth of 15 cm in sterile bottles, kept on ice, and analyzed on the same day. Before being processed further, the samples were vortexed for 20 min. The sampling points relevant to this study are shown in Fig. 1. These were the sampling points via which the wastewater was discharged through the Kohtla River (site 1) and the Purtse River (site 2) to the Baltic Sea. Control waters were collected from sampling sites not polluted with the leachate: in the Purtse River 0.5 km upstream (site 3) from the Kohtla River and in the Rannapungerja River (site 4), whose source is also near the above-mentioned release area but which flows south (Fig. 1). The distance traveled by the leachate from the ash dump area to the sampling site 2 is 22.1 km.

Bacterial strains. *P. putida* PaW85 (4) and *Escherichia coli* DH5α (37) were used as DNA recipient strains, and *P. putida* EST1026 (19) was used as a reference strain for measuring the activities of the PMO.

The 41 selected original aquatic isolates grew well on solid and liquid media with phenol as the sole carbon and energy source. The strains were isolated repeatedly into pure culture and subsequently identified by the BIOLOG GN identification system (35).

Media and culture conditions. All pseudomonads were cultured at 30°C, and *E. coli* was cultured at 37°C. Besides selective minimal M9-salt (1) plates with the trace elements described by Bauchop and Elsden (3), King's B medium (16) was used for common tests to check for the *Pseudomonas* species and R2A agar (Difco) was used to cultivate the strains for identification by the BIOLOG system. Transformants harboring plasmid pAM10.6 were selected on 2.5 mM phenol plates. The same concentration of phenol was used in liquid media. Luria-Bertani medium was used for the cultivation of *E. coli.*

Colony rep-PCR. The colony rep-PCR fingerprinting patterns from bacterial genomic DNA were generated with BOXA1R primer under conditions described by Louws et al. (23), but instead of extracted DNA, a small number of cells was resuspended in the PCR mixture.

DNA manipulations and sequencing. Transformations of *E. coli* and *P. putida* cells with different plasmid DNAs by chemical methods were done as previously described (13). The alkali lysis conditions used by Connors and Barnsley (8) or described in the Qiagen Plasmid Handbook (Qiagen GmbH) were used for plasmid DNA isolation. DNA subcloning was done as described by Sambrook et al. (37). The nucleotide sequence was determined by dideoxy sequencing (38) with commercial kits involving *Taq* and T7 DNA polymerases (Promega; U.S. Biochemicals) with synthesized internal primers or standard primers supplied in the kits.

Hybridizations and DNA probes. For hybridization, colonies were grown directly on nitrocellulose disks (Schleicher & Schuell) placed on the surfaces of minimal phenol plates. DNA fixation, hybridization, and washing were done as recommended in the supplemented protocol. Southern hybridization analyses were performed on a Nytran-N nylon membrane filter (Schleicher & Schuell). For blotting, washing, and stripping, the original method described by the manufacturer was used. Hybridization was carried out in an aqueous hybridization solution at 68°C as described by Sambrook et al. (37).

DNA sequences and origins of specific radioactively labeled gene probes were used as follows: *tbuD*, 3.1-kb *Hin*dIII-digested DNA fragment from pGEM3Z: pRO1957 (21); *dmpKLMNOP*, 5.2-kb *Eco*RI-*Hin*dIII fragment from pVI261 (39); promoter region of *pheBA* operon, 158-bp *Avr*II/*Bgl*II fragment from pAT1163 (13); ORF1, 930-bp *Eco*47III-*Hin*dIII fragment from pEST1141 (13); overlapped junction of ORF1 and *pheB*, 780-bp *Bss*HII fragment from pEST1412; *pheB*, 670-bp *Hin*dIII-*Pst*I fragment from pEST1412 (18); *pheA*, 1830-bp *Eco*47III fragment from pEST1412; ORF2, 900-bp *Hin*dIII-*Kpn*I fragment from pEST1412 (18). The locations of the fragments on the *pheBA* operon are also shown in Fig. 4. Double-stranded DNA restriction fragments were purified from agarose gels and subsequently random-prime labeled with [α-^{32}P]dCTP (DuPont) and DNA labeling kit version 2 (MBI Fermentas). Autoradiograms were generated with a PhosphorImager IS (Molecular Dynamics).

Enzyme assays. Cells used for crude extracts were cultured in M9-phenol minimal medium at 30°C until early stationary phase (10 to 18 h). PMO was assayed by the procedure of Beadle and Smith (5). Catechol-1,2-dioxygenase and catechol-2,3-dioxygenase activities were measured spectrophotometrically at 25°C as described by Sala-Trepat et al. (36). Protein concentrations were determined by the Bradford method (7) with bovine serum albumin as the standard.

RESULTS

Selection of isolates. This study is part of prolonged monitoring of the bacterioplankton from the watershed of the Purtse River during the period from November 1993 to September 1995 (for more details, see reference 42). Water samples relevant for the present study were collected during 1994. We took samples from phenol-contaminated and uncontaminated areas. The colony-forming bacteria with biodegradative properties were plated out without further enrichment from different water samples onto phenol-minimal plates. For possible redetection of the *pheBA* operon, we picked out 41 strains that grew well on phenol. Interestingly, all the strains which originated from the phenol-contaminated water system (36 of 41 strains) were isolates from downstream areas. The distribution of different phenol-degrading strains between sampling points is shown in Fig. 1. While the *pheBA* operon for phenol degradation leads to *ortho* fission of catechol, we tested the proportion of phenol degraders via the *meta* pathway in this collection. The strains expressing catechol 2,3-dioxygenase activity accounted for 16 strains of the 41 selected.

All the strains were cultivated under standard laboratory conditions (30°C in M9 minimal medium), and therefore the members of the well-grown phenol-degrading bacterial strains isolated were somewhat limited by such growth conditions.

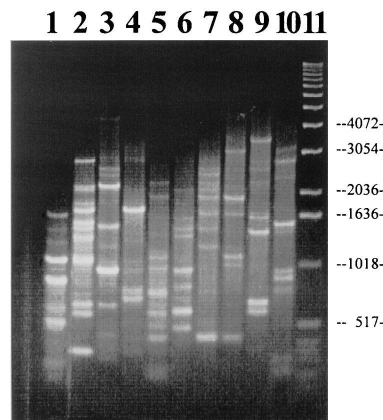

FIG. 2. rep-PCR fingerprinting patterns of isolates. The primer BOXAIR (23), corresponding to the BOXA subunit of the BOX element, was used for amplification. Lanes: 2, *P. corrugata* MB2/12; 3, *P. fragi* B1/25; 4, *P. fluorescens* B MT4/4; 5, *P. fluorescens* B 2.67; 6, *P. fluorescens* B MB3/26; 7, *P. fluorescens* F Cb36; 8, *P. fluorescens* F MB3/29; 9, *P. fluorescens* C 5F6; 10, *P. stutzeri* JP1/5. Lane 1 shows the original pattern of the released laboratory strain *P. putida* PaW85. Lane 11 shows the DNA molecular size marker (1-kb ladder [Gibco-BRL]).

Diversity of phenol hydroxylases. At the first stage, the origin of phenol degradation genes in 41 selected strains was investigated by a colony hybridization technique. We used three probes: the *dmpKLMNOP* genes from *Pseudomonas* sp. strain CF600 (39), the *tbuD* gene probe (21), and the *pheA* gene probe described by us (32). The most frequent genetic pathway for phenol degradation was related to the *dmpKLM NOP* genes. Of the 41 strains investigated, isolated DNA from 18 exhibited homology to these genes to different degrees. Eight of these DNAs revealed strong hybridization. All 8 of these isolates were also strains which expressed catechol 2,3-dioxygenase activity. Four of these strict *dmpKLMNOP*-hybridization-positive isolates also hybridized with *tbuD* at a low level. In addition, we found 13 isolates (seven from sampling point 1 and six from sampling point 2), DNA of which hybridized strongly with the *pheA* gene probe but not with the other two probes (data not shown). The strains that hybridized with the *pheA* probe were represented only in water samples which were contaminated with ash dump leachate. Of the 41 isolates, 10 revealed no hybridization with the probes used.

All these isolates were gram-negative bacterial strains belonging to the genus *Pseudomonas* as identified by using the BIOLOG GN system.

Proof of the *phe* operon derivates. We selected 9 of the 13 *pheA*-positive isolates for further investigation: *P. corrugata* MB2/12, *P. fragi* B1/25, *P. fluorescens* biotype B MT4/4, *P. fluorescens* biotype B 2.67, *P. fluorescens* biotype B MB3/26, *P. fluorescens* biotype F Cb36, *P. fluorescens* biotype F MB3/29, *P. fluorescens* biotype C 5F6, and *P. stutzeri* JP1/5. Although six of the nine strains studied belong to *P. fluorescens*, all these strains revealed different chromosomal fingerprints in rep-PCR (Fig. 2), and therefore they cannot be siblings. These strains harbored different plasmid DNA replicons (Fig. 3A), and the *pheA* gene was localized in plasmid DNA in all the strains except MB3/29 (Fig. 3B). Total DNA isolated from these strains was cleaved with *Hin*dIII, which gives a specific

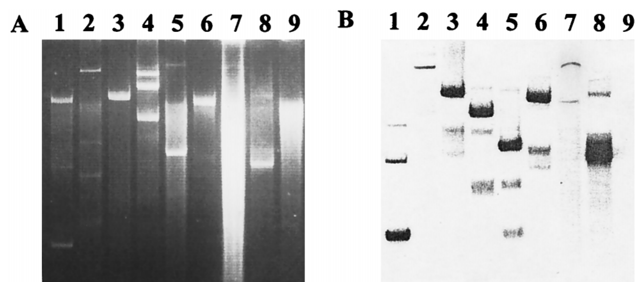

FIG. 3. (A) Agarose gel electrophoresis of plasmid DNA from the *pheA*-positive isolates; (B) autoradiogram of the gel after hybridization with the *pheA* gene probe. Lanes: 1, Cb36; 2, MB3/26; 3, 2.67; 4, MT4/4; 5, JP1/5; 6, B1/25; 7, 5F6; 8, MB2/12; 9, MB3/29.

pattern of restriction fragments from the *pheBA* operon (Fig. 4).

Data from serial hybridizations with specific gene probes from the original *pheBA* operon is shown and interpreted in Fig. 4. All nine strains gave hybridization with the *pheA* and *pheB* gene probes, revealing the same restriction pattern as that for the original *phe* genes from *P. putida* EST1412 (Fig. 4, lanes K), and are therefore considered to be linked in the same way as in the original *pheBA* operon. In six cases, ORF2 was absent, and in only two cases, ORF1 was deleted. Strain B1/25 lacked both ORF1 and ORF2. However, all nine DNAs hybridized with the DNA probe of the promoter region of the operon. Four strains (JP1/5, MT4/4, MB3/26, and MB3/29) contain the *pheA* and *pheB* genes and both ORFs in the same order as that in which they were organized in strains released into the environment 6 years earlier. Two different variants of the *phe* operon were found in strain MB3/26 (Fig. 4). In strain MB3/29, the *phe* DNA is definitely integrated into the chromosome (compare the data in Fig. 3 and 4). To summarize, eight operons include one or both IS-like elements in the same order (ORF1 upstream of *phe* genes and ORF2 downstream from these) as in the original *pheBA* operon.

None of the strains characterized in this study were established as a derivative of the released *P. putida* PaW85 strains, and we do not have data that allows us to suggest that this laboratory strain has survived under environmental conditions.

Characterization of one de novo-generated PHE plasmid, pAM10.6. We identified a small *pheA*-containing plasmid replicon among several plasmid DNAs of strain Cb36 (Fig. 3). We transformed *P. putida* PaW85 cells with total DNA isolated from strain Cb36 and selected transformants on phenol-minimal plates. All the transformants harbored the above-mentioned small (10.6-kb) PHE plasmid, which originated from strain Cb36. We named this plasmid pAM10.6. Analysis of the plasmid DNA of one such transformant is shown in Fig. 4, lanes M. We sequenced the DNA from pAM10.6 to verify the authenticity of this *phe* operon and to investigate the method of integration of the *pheBA* operon into a novel plasmid replicon. We did not detect any differences between the sequences of the original *pheBA* operon and the redetected *pheBA* genes found in plasmid pAM10.6. However, in the case of pAM10.6, the ORF2 part was absent.

Some attributes of the pAM10.6 structure, characteristic of this *phe* operon, are summarized in Fig. 5. This shows the *pheBA* promoter, ORF1, *pheB* and *pheA*, and part of ORF2 for transposase IS*1411* containing 80 nucleotides (nt) from its start.

A 240-bp DNA segment, which breaks the ORF2, is duplicated at the beginning of the *phe* operon. There is 60 nt between the −35 sequence of the *pheBA* promoter and the 3′ end of the upstream repeat of this DNA segment. The 240-bp DNA segment, designated ARM*phe*, has an IS-like structure with 39-nt inverted repeats (IRs) at both ends. IRs of ARM*phe* reveal remarkable sequence homology to the left-hand IR of the class II transposon Tn*4654* (43), with some minor differences between them (Fig. 5). The rest of ARM*phe*, excluding both IRs, is 162 nt long. Thus, the *phe* operon on pAM10.6 flanked by IS-like DNA elements looks like a composite transposon.

Study of expression of the PMO in *pheA*-positive strains. For pEST1226 in *P. putida* PaW85, which has the same regulatory protein, CatR, for control of expression of chromosomal catechol degradation genes and the *pheBA* operon, the expression levels of the *pheA* gene in the presence of phenol and benzoate as inducers in culture grown in Luria-Bertani broth have been described as being equal (13). To study how these *pheBA* operons containing bacterial strains are connected with the *ortho*-pathway metabolism, we tested PMO activities comparatively in cell cultures grown in the presence of phenol or benzoate. *P. putida* EST1026 (19), which is a parent strain of *P. putida* EST1226, was used as a control. Cells were grown on minimal M9 medium supplemented with phenol or benzoate in the presence or absence of glucose (Table 1).

Testing of seven newly described strains and EST1026 revealed a higher level of activity of PMO in the cells grown on phenol than in the cells grown on benzoate. However, two strains, MB2/12 and B1/25, expressed PMO at a higher level in the presence of benzoate in the growth medium (Table 1). When glucose was also added to the growth medium, about half the strains expressed PMO at a lower level than that at which it was expressed without the glucose. Strain 2.67 was not able to utilize benzoate as the sole carbon source, and strain 5F6 lost this ability during passages on phenol plates. Testing with the latter strain revealed constitutive expression of PMO. Therefore, this strain is a good example to illustrate how laboratory cultivation conditions via one-way selection could stimulate some regression of regulatory responses and catabolic abilities.

This PMO induction data (Table 1) shows that the expression of the *phe* genes depends on the genetic background of the hosts. Gene regulation in cells growing on benzoate ought to be controlled by different regulatory systems. However, in seven of nine cases investigated, both phenol and benzoate induce the *phe* genes. Therefore, we suggest that a regulatory protein resembling CatR is involved in the regulation of the *phe* operons in these strains.

DISCUSSION

The biggest oil shale industry in the world based on a local deposit is located in a narrow area in northeastern Estonia on the coast of the Baltic Sea. Oil shale has been used for 70 years as an important fuel for electricity production and as a raw material for the local chemical industry in producing crude shale oil and various chemical products such as coke, aromatic hydrocarbons, and resins. Processed oil shale and solid remnants containing several organic and inorganic compounds have been accumulated into ash dumps, forming ash mounds. The leachate from the ash mounds, water formed as a by-product of the oil shale-processing chemical industry (up to 7,200 to 8,000 m^3 day^{-1}), is discharged without treatment via channels and the Kohtla and Purtse rivers into the Baltic Sea. The concentration of phenolic compounds in the leachate ranges between 80 and 230 mg $liter^{-1}$, and phenol and cresols are the major components. The pH of the leachate is usually

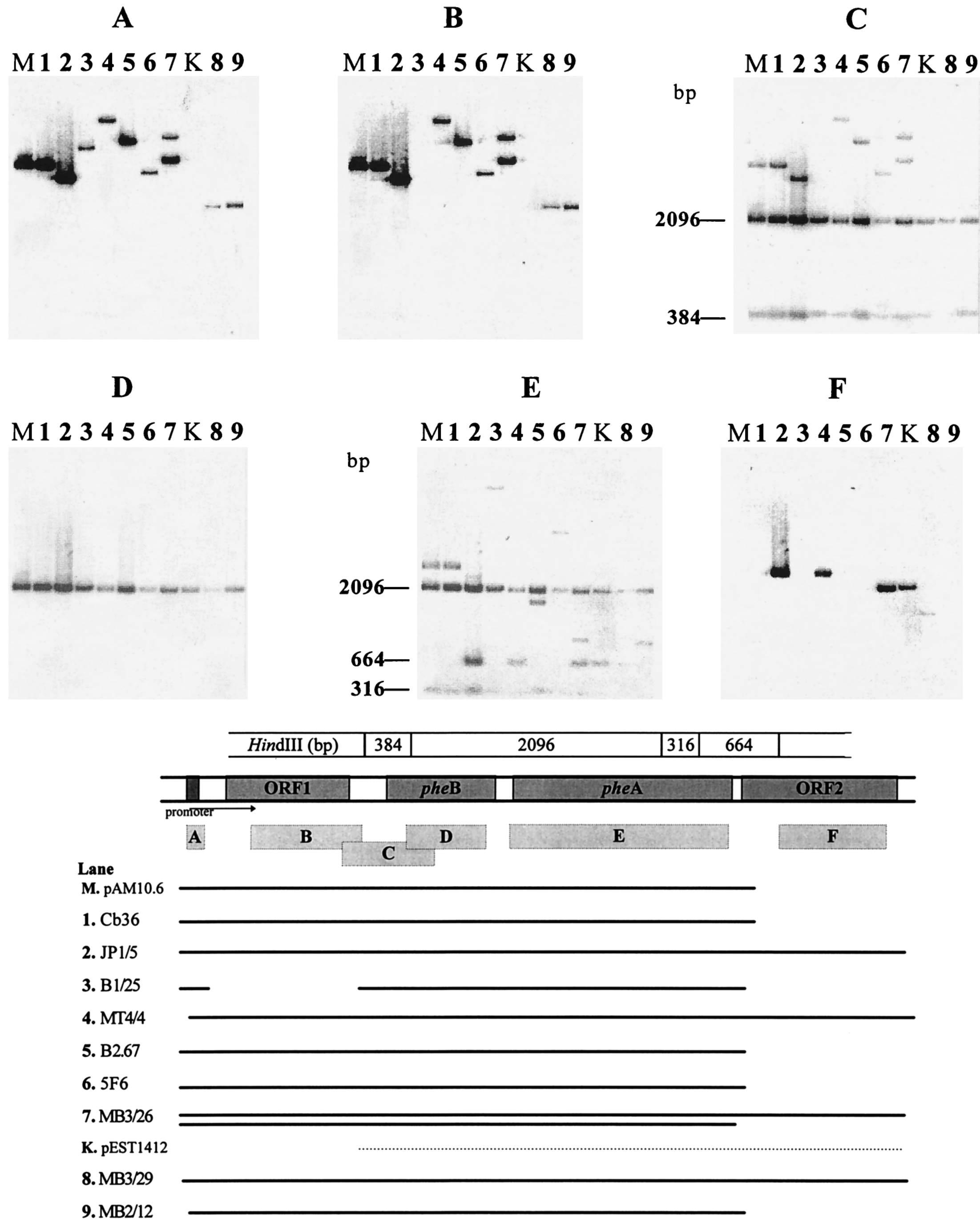

FIG. 4. Autoradiographs of the Southern analysis of total *Hin*dIII-digested DNA of bacterial strains hybridized with radiolabeled probes specific to the promoter region of the *pheBA* operon (A), ORF1 (B), the junction of ORF1 and *pheB* (C), *pheB* (D), *pheA* (E), and ORF2 (F). The locations of DNA fragments used for labeling are shown below the physical map of the *pheBA* operon and its *Hin*dIII restriction map. The sizes of corresponding *Hin*dIII fragments are given in base pairs beside the hybridizing bands. Rows M through 9 in the lower section, where data on hybridizations is summarized, correspond to the lane numbers on all six autoradiographs. Heavy lines indicate the area of *pheBA* DNA represented. In strain MB3/26, two variants of the *phe* operon are assumed.

12.0 to 12.5, but depending on the processing technology, it is drastically lower on some days (pH 2.5 to 3.5).

The large-scale release of phenol-degrading bacteria in 1989 was not in contradiction with Soviet regulations. The underground fire area (at a depth of 65 m) was enclosed by concrete sarcophagi. However, due to the geological conditions, a considerable amount of polluted water was leaching out into the neighboring gangways. According to mining technology principles, accumulated gangway water is pumped out; therefore, in the Estonia mine, this water was discharged via the Rannapungerja River to Lake Peipus. The use of bacterial biomass was probably the only possible way to prevent potential disaster to the ecosystem of the lake.

After bioaugmentation, the treated underground water (en-

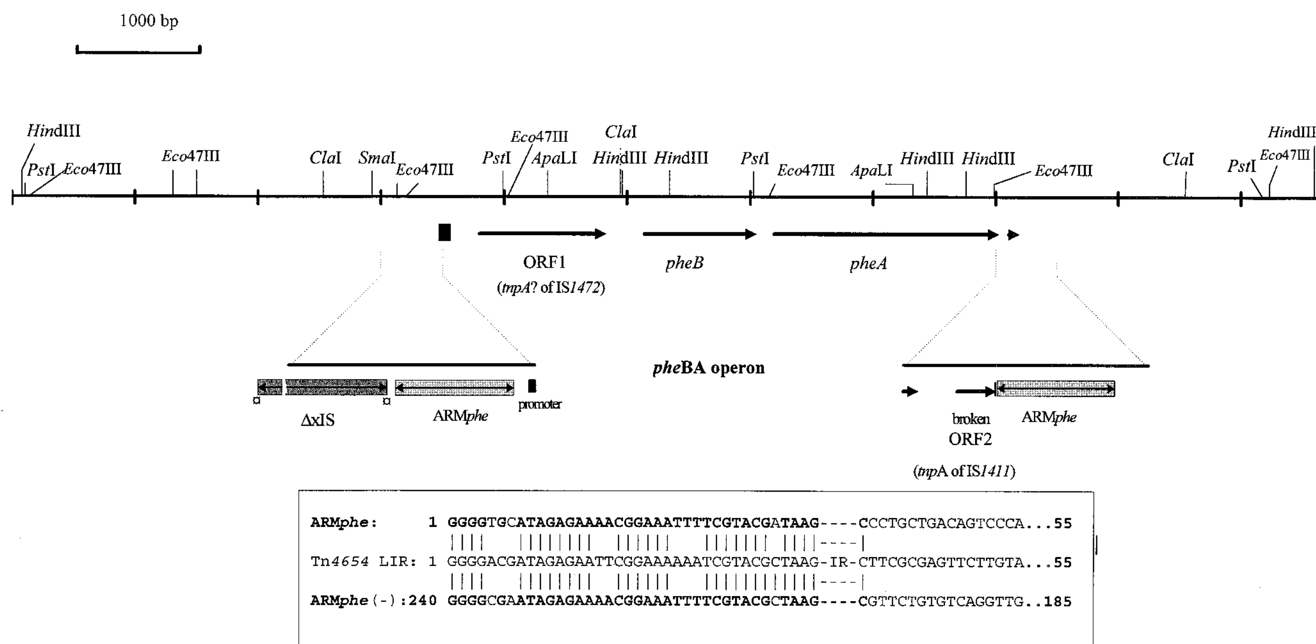

FIG. 5. Structure of pAM10.6. Arrows indicate ORFs. The small solid box marks the promoter of the *pheBA* operon; □, duplicated target sequence. The sequence of the IS-like element ΔxIS has been assigned GenBank accession no. AF020724. The comparison of the inverted repeats of the duplicated DNA segment ARM*phe* (GenBank accession no. AF020723) with the left-hand IR of transposon Tn*4654* (43) is shown in the box below the structures.

riched with phenol-degrading bacteria) was pumped out via a reservoir (1,500 tonnes) in the enrichment plant to the watershed in a northerly direction. This explains how released bacteria were discharged into the Purtse River (Fig. 1).

The Purtse River watershed is being continuously polluted by phenolic leachate. Hence, it could give a selective value for the persistence of phenol-degrading bacteria with the *pheBA* operon. We found that although the amount and concentration of toxic organic compounds in the leachate depend on the weather conditions (i.e., rainfall) and also on chemical industry production, the average concentration of volatile phenolic compounds in the Purtse River was 600 to 700 times lower than in the initial section of the channel (data not shown). Therefore, the natural biodegradation efficiency is high, because dilution of the leachate water with river water causes only a 200-fold decrease in the concentration of phenolic compounds.

The two phenol-degrading bacterial strains, derivatives of *P. putida* PaW85, released in 1989 are genetically well characterized. The molecular structure and genetic regulation of the *pheBA* operon in these strains have been thoroughly studied (13, 17–19, 32, 33).

The *pheBA* operon contains four ORFs (Fig. 4). ORF1 is located between the promoter region of the *pheBA* operon and the *pheB* and *pheA* genes. ORF1 is actually part of the IS element IS*1472* (16a), which is almost identical to the IS element described by Lauf et al. (22). ORF2 is located after the *pheB* and *pheA* genes and is a transposase gene of IS*1411*, currently under investigation by Kivisaar's group. The remaining two ORFs are for *pheB* (catechol-1,2-dioxygenase) and *pheA* (PMO). *pheB* is analogous to the chromosomally encoded catechol-1,2-dioxygenase genes (18, 33).

Our study on the diversity of phenol hydroxylases shows that 10 isolates did not reveal any detectable hybridization with the DNA probes used, i.e., the *dmpKLMNOP* (39), *tbuD* (21), and *pheA* gene probes (32). The most frequent genetic pathway for phenol degradation was related to the *dmpKLMNOP* genes, since 18 of 41 strains investigated revealed homology to those genes. Thus, our data supports findings that a multicomponent PH, as from *Pseudomonas* sp. strain CF600 (39), is common in phenol-degrading bacteria. By using gene probing techniques, analogous PH have also been identified in phenol-catabolizing *Pseudomonas* sp. strain U and in isolates from Norwegian seawater samples (31). Similar PH have been reported from *Acinetobacter calcoaceticus* NCIB8250 (10) and *P. putida* P35X (29). Moreover, all those PH genes determine multicomponent PH, and they are linked with the catechol-2,3-dioxygenase gene (22, 29, 30, 31).

It is important to underline that the *pheA* gene determines PMO, which is a single protein, and that the gene is linked with

TABLE 1. Study of the expression of PMO in *pheA*-positive strains

Strain	Sp act of PMO with substrate(s) of growth[a]:				
	Phenol	Glucose-phenol	Benzoate	Glucose-benzoate	Glucose
EST1026 (control)	0.25	0.20	0.14	0.18	<0.005
Cb36	0.32	0.15	0.18	0.11	<0.005
MB3/26	0.27	0.29	0.21	0.07	<0.005
MB3/29	0.28	0.07	0.14	0.07	<0.005
MT4/4	0.11	0.07	0.02	<0.01	<0.005
5F6	0.25	0.21	—(1/2)[b]	0.13	0.24
JP1/5	0.42	0.14	0.23	0.06	<0.005
B1/25	0.29	0.19	0.56	0.21	<0.005
2.67	0.28	0.28	—	—	<0.005
MB2/12	0.04	0.04	0.07	0.07	<0.005

[a] Specific activities (micromoles per minute per milligram of protein) are expressed as the NADPH decrease in cell extracts. All values are means of four repeats.

[b] —, default of the growth; (l/2), shift of the ability for growth during the series of measurements.

the catechol-1,2-dioxygenase gene (32). Although Shingler and coworkers also used the *pheA* gene probe, they did not detect hybridization with this gene in new isolates (31, 38a). Our finding that 13 isolates from the Purtse River watershed have identical or at least highly homologous counterparts of the *pheA* gene is the first report of capture of the *pheA* gene from environmental isolates.

We suggest that the *pheAB* operon is an artificial structure evolved under laboratory conditions at prolonged selective pressure by cultivating bacteria on phenol-containing media. In terms of structure, this means that the end of the *pheA* gene is located in IS*1411*, because the left IR of IS*1411* overlaps with 21 nt of the 3′ coding sequence of the *pheA* gene. The regulatory shortcut between the chromosomally encoded *ortho* pathway and PMO from *Pseudomonas* sp. strain EST1001 allows the use of the same regulatory protein, CatR, for activation of the transcription of the chromosomal *ortho*-pathway *cat* genes and the *pheBA* operon. We consider those features of the *pheBA* operon to be unique and the probability of such an operon occurring in nature to be extremely small. On the other hand, this operon could undergo particular deletions without inactivation of the *pheB* and *pheA* genes. A gene block homologous to *pheBA*, revealing an identical restriction pattern to that of the same vicinity of the *IS* elements and the *pheBA* promoter, could be derived from the original *pheBA* operon. The six strains reisolated by us from nature contain such deletion variants of the *pheBA* operon (Fig. 4).

We did not obtain any data on the survival of the released *P. putida* PaW85 laboratory strains under environmental conditions after a 6-year-period. The 13 isolated strains harboring *phe* operons belong to four different species, whereas the 6 *P. fluorescens* strains belong to three different biotypes. The leachate is a mixture of phenol and its numerous methyl derivatives. Bacterioplankton from the Purtse River watershed revealed both the functional dynamics of the physiological groups of the biodegradative bacteria and the biodegradation spectra at different points of the flow (42). In various polluted regions of the watershed, different *P. fluorescens* biotypes are predominant, and they harbor characteristic genetic traits (12). This is probably due to the higher concentration of methylated phenol isolates, which are able to degrade phenols and cresols through the *meta*-fission pathway or the protocatechuate *ortho*-fission pathway. These isolates dominate near the influx of contaminated water (12). This data explains, although indirectly, why the released *pheBA* operon is probably transferred horizontally to the different bacterial species and selectively maintained for a long time in the environment under strong selective pressure. It is understandable that the specific traits of catabolic pathways in different bacterial species may favor particular genetic rearrangements in an introduced new genetic material. Indeed, the PMO induction data shows that the expression of the *pheA* gene depends on the genetic background of the hosts (Table 1). We propose that the genetic background of the host bacteria could be the reason why *pheA* and *pheB* are linked in the same manner in the nine new isolates as in the original *pheBA* operon but six of them have a specific deletion in the regions of the IS-like elements of the *pheBA* operon. In eight operons, the vicinity of the *pheB* and *pheA* genes is, according to our data, the same as in the original *pheBA* operon, including one or both IS-like elements (Fig. 4).

For verification of redetection of the released *pheBA* operon, we sequenced the 10.6-kb plasmid pAM10.6, which originated from the new isolate, Cb36. This revealed that the *pheBA* operon of plasmid pAM10.6 is identical to the *pheB* and *pheA* genes and the ORF1 region (Fig. 5). In the downstream region, the first 80 nt of the ORF2, i.e., from the transposase gene of IS*1411*, is maintained. There is a 240-bp element, which we named ARM*phe*, flanking the *pheBA* operon in plasmid pAM10.6 (Fig. 5). A similar composition of the degradative operon is described for chlorobenzoate degradation genes, where two IS*1071* elements flank the catabolic genes (27). However, the ARM*phe* element is not a functional IS element, since it contains IRs typical of the Tn*3* family but not functional transposase and resolvase genes of class II transposons and the duplication of the target DNA sequence.

The ARM*phe* element reveals homology to Tn*4654* (Fig. 5). That type of element could be common among soil bacteria. There is a 5.4-kb DNA region with extensive homology to Tn*4654* in the OCT plasmid of *P. putida* PpS5 and both reveal homology to specific regions of the SAL and NAH plasmids (reference 11 and our unpublished data). It is known that efficient *recA*-independent DNA rearrangements could be mediated only by inverted repeats (6). Therefore, we argue that at the formation of plasmid pAM10.6, the ARM*phe* element plays the same role.

Another small (262-bp) IS-like DNA element, with inverted repeats typical of the Tn*3* family, was found 26 nt left of the left end of ARM*phe* (Fig. 5). This element, designated by us ΔxIS, is certainly too small to contain enough genetic information for transposition. However, transposition of this DNA element has probably taken place, since there is a typical 5-nt target DNA duplication of the flanking sequence of ΔxIS (Fig. 5). It could be that if we exclude the possibility of complementation of transposition function, some undefined deletion events within the original longer element took place after the transposition of ΔxIS into plasmid pAM10.6.

In conclusion, even though we are unable to define a probability for the appearance of a counterpart operon to the *pheBA* operon in nature before the release of phenol-degrading bacteria, we confirm that we have redetected, after 6 years, the released genetic material in the environmental isolates. The fact that the redetected *pheBA* operon was characterized in four different bacterial species could be seen as an example of horizontal gene transfer in nature. Alternatively, if the *pheBA* operon was really distributed in nature before the release, our investigation shows wide distribution and high-frequency rearrangements occurring in this operon, as needed for the persistence of the most adapted microbes in a continuously polluted watershed.

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