Monitoring of non-genetically engineered *Pseudomonas putida* strains after release in an aquatic ecosystem

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Abstract

Monitoring strategies were developed to track non-genetically engineered *Pseudomonas putida* strains in an aquatic ecosystem. The strain E1 was used for four years for the biodegradation of phenolic compounds in industrial waste water in Põlva, Estonia. In this study the strain E2 was used which is a non-carbenicillin-resistant variant of the strain E1. Both strains have a deletion of approximately 34 kb in the TOL plasmid pWW0 which served for discrimination from indigenous bacteria by molecular techniques. Other targets used for PCR and hybridization were the *xylE* gene and a sequence located in the left hand of the transposon Tn*4652* of the TOL plasmid. Detection of the released strain was possible only to 32 hours after release. It is assumed that the released strains did not survive in the aquatic ecosystem mainly due to the high dilution rate. The combination of cultivation on selective media and molecular techniques proved useful for tracking *Pseudomonas putida* strain E2 in an aquatic environment.

1. Introduction

Specific methods for detection and monitoring of microorganisms used for bioremediation and in agriculture are needed to investigate the fate of the released microorganism or to study its possible impact on the ecosystem [3, 4, 18]. The majority of studies performed to date deal with the monitoring of genetically engineered microorganisms (GEMs) [7, 15]. Consequently, due to the strong restrictions existing for working with recombinant organisms, nearly all of these studies have been undertaken in contained conditions, mostly in microcosms [7, 10]. In contrast, studying non-recombinant microorganisms is not restricted to laboratory microcosms, and hence can be performed under natural conditions. However, monitoring of non-recombinant organisms, which have no artificial marker, may prove to be more difficult [16]. Concerning the specific detection of non-recombinant bacteria used for bioremediation, immunological methods with monoclonal antibodies [3] as well as molecular, i.e., genetic, techniques [16], have been applied.

In a timber processing plant at Põlva, Estonia, 50 km from Tartu, the non-genetically engineered *Pseudomonas putida* E1 was used for degradation of phenolic waste waters over a period of four years. After decontamination, the treated water together with the biomass was then transferred to the municipal sewage plant and hence released into the aquatic ecosystem. Because it is also the intention to use this strain for biodegradation of phenolic contaminations at a different site in the north-east region of Estonia, at Kohtla-Järve, there existed a need to study the survival of the released bacteria in the aquatic ecosystem.

The objective of this study was to develop detection methods for this non-engineered strain with particular regard to molecular techniques. To track the released bacteria we applied a combination of cultivation on selective media and genotypic methods. Concerning the latter methods, naturally occurring sequences were chosen as targets for gene probes and PCR. Due to the fact that the release of the biomass occurred at irregular intervals (depending on the effluent of waste water), a controlled release experiment was performed to look for the strain immediately after release.

2. Material and Methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used are listed in Table 1. *Pseudomonas putida* E1 represents a segregant of the strain E0 which was generated by co-cultivation of the strains *Pseudomonas putida* AC783 and *Pseudomonas putida* PaW160 at the Institute of Molecular and Cell Biology in Tartu, Estonia. It harbours two plasmids, a larger one of about 83 kb which carries the *meta*-cleavage operon, and a smaller one of about 13 kb on which the carbenicillin resistance is thought to be encoded. It grows well on phenol, *m*-toluate, and Na-benzoate and exhibits good decontamination capabilities of phenolic waste water under non-optimized conditions. The strain *Pseudomonas putida* E2 used in this study is a non-carbenicillin-resistant variant of the strain *Pseudomonas putida* E1 lacking the small (13 kb) plasmid. The strains were maintained on M9 medium [8] supplemented with trace elements [1] and with 5 mM phenol. To evaluate the antibiotic resistances, Luria Broth agar (Sigma, Deisenhofen, Germany) supplemented with streptomycin 1000 µg/ml (LB/Sm) or carbenicillin 1000 µg/ml (LB/Cb) was used. Strain *Pseudomonas putida* PaW160 [18] was used as a positive control for the TOL deletion amplification product.

2.2. Target-sequences for gene probes and primers

Due to the fact that the strains *P.putida* E1 and E2 respectively harbour a plasmid something similar to the TOL plasmid pWW0, three sequences, known as components of the pWW0, were chosen as targets for both PCR amplification and gene probe hybridization. Of these, one was the *xylE* sequence (EMBL Data Library, accession number J01845), and the others were situated at the right-hand (*RH*) sequence (accession number X83686), and the left-hand (*LH*) sequences (not known to date, see *Results and Discussion*) of the transposon Tn*4652*. On the basis of the known sequences of *xylE* and RH of Tn*4652*, the following primers for PCR amplification of the target-se-

quences were selected using the PC Gene program (IntelliGenetics, Inc., USA): *xylE* primers: E1: 5'-TCAAGGTTGTGGATG-AGGATGC-3', E2: 5'-AGAACACTTCGTTGCGGTTACC-3'; *RH* primers of Tn*4652*: RH1: 5'-TAGCGGAGGCATTGCCGGACATGAC-3', RH2: 5'-TATTCGA-GAGGCCGTGGCTTGCTGG-3'. By using these primer pairs the PCR amplification should result in products of 594 bp (*xylE*) and 329 bp (*RH*).

Table 1. *Pseudomonas putida* strains used in this study

Strain	Plasmid	Characteristics[a]	Notes	Reference	Source
PaW85	none	$Phe^-Xyl^-Tol^-$	Tn*4652* in chromosome	[2]	P. Broda (UMIST, Manchester, UK)
PaW160	pWW0-160	$Phe^-Xyl^+Tol^+$		[18]	P. Broda
AC783	none	$Phe^-Xyl^-Tol^-$ Sm^rAde^-*catA*$^-$	Strain of A.M.Chakrabarty	[14]	I Starovoitov (Puschino, Russia)
E0	pWW0-160	$Phe^+Xyl^+Tol^+$ Sm^r*catA*	Spontaneous prototrophic transconjugant of AC783 from the cross PaW160 x AC783		This study
E1	$pWW0^{mut90}$	$Phe^+Xyl^-Tol^+$ Sm^rCb^r*catA*$^-$	Segregant of E0 having 34-kb deletion in plasmid		This study
E2	$pWW0^{mut90}$	$Phe^+Xyl^-Tol^+$ Sm^rCb^r*catA*$^-$	Non-carbenicillin resistant derivative of E1		This study

[a] $Phe^+Xyl^+Tol^+$, ability to grow on phenol, *m*-xylene, *m*-toluate as sole carbon sources; Sm^rCb^r, resistance to streptomycin and carbenicillin; Ade, requirement for adenine; *catA*$^-$, mutation in catechol 1,2-dioxygenase gene

2.3. PCR conditions and colony hybridization

Amplification was performed in a total volume of 50 ml using the UNO-Thermoblock (Biometra, Göttingen, Germany). The concentrations of the components in the reaction mixture were as follows: 10mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100, 0.4 mM each of the primers, and 0.2 mM each of the four nucleotides. The temperature program started with lysis at 98°C for 10 min, followed by addition of 0.5 U of polymerase (Primezyme, Biometra, Göttingen, Germany) at 72°C. Thermal cycling began with denaturation at 93°C for 1 min, followed by annealing at a temperature which depended on the primer pairs (60°C for *xylE*, 53°C for LH, and 68°C for RH) for 1 min, and extension at 72°C for 1 min. 35 cycles were performed. We applied a final extension step at 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

To perform colony hybridization, cells from colonies grown on different selective media were dotted with a toothpick onto the uncharged nylon membranes Nytran NY 13 N (pore size 0.45 mm; Schleicher & Schuell, Dassel, Germany) which were placed on R2A (Difco) agar media followed by 24 hours growth at 30°C. Labeling of the PCR-generated gene probes with DIG DNA labeling and detection kit (Boehringer, Mannheim, Germany) and the subsequent hybridization procedure were performed in accordance with the Boehringer protocol.

2.4. Membrane filtration

To simulate the tracking of the released bacteria under natural conditions, a membrane filtration experiment was performed. Fixed numbers of the strain E2 were added to 20 ml river water samples from the River Elster in Leipzig. The cell number of the suspension of the strain E2 used for inoculation was determined by the AODC (Acridine Orange Direct Count) method [5] and by determination of the CFUs on different media (R2A-agar; Luria Broth agar supplemented with streptomycin 1000 µg/ml; M9 medium [8] containing 5 mM phenol). The inoculated samples were filtered through nylon membranes (Nytran NY 13 N, pore size 0,45 mm) which were subsequently placed on selective agar media. After 24 hours' growth on LB/Sm or 48 hours growth on minimal medium containing 5 mM phenol at 30°C, a replica was made. Colony hybridization was performed according to some protocols given in literature.

2.5. Release experiment

Due to the fact that the strain E1 had been used for the degradation of phenolic wastes by the factory for four years, it might be expected that these bacteria could be isolated at or near the point of discharge. However, E1 cells could not be detected in water samples taken from the aquatic ecosystem at different points. Consequently, we decided to perform a controlled release experiment after using the strain E2 for the decontamination of phenolic waste waters in the tank at the timber processing plant.

The fermentation of the biomass which was needed for decontamination was performed separately. In total, 220 l of biomass with an OD_{580}=1.0 was prepared. The biomass was added to 6000 liters of phenolic waste water which were contained in a subterranean fermentation tank.
The temperature in the tank was 11.5°C and the pH 7.3. The content of the tank was weakly aerated (approximately 20 l/min) for 5 days and transferred through the municipal sewage pipes system to the sewage station. The average length of time the waste water spent in the cleaning station was approximately 4 hours. To monitor the bacteria, samples were taken from the tank before and after addition of biomass both on the second and fifth day, immediately before release. After releasing the biomass into the sewage pipes system, samples were taken at 8, 32, and 104 hours at two different points of the aquatic ecosystem. Sampling sites were located at the sewage station, and on the River Orajõgi, 3 km downstream from the sewage cleaning station. Samples of 1 l were collected in sterile bottles and placed on ice for transport to the laboratory where analysis began not more than 4 hours after the samples had been taken. Different volumes of water samples were plated onto selective agar plates. Phenol, *m*-toluate, Na-benzoate, and LB/Sm were used as

selective media. Grown colonies were analyzed by colony hybridization using the *xylE* gene probe. Positive colonies were tested by PCR using the *xylE* primer set. Final verification was made using *LH* and *RH* primer sets.

3. Results and discussion

As verified by restriction analysis using restrictases *HindIII, EcoRI* and *XhoI*, the larger plasmid in the strains E1 and E2 respectively, designated by us as pWW0^{mut90}, is a 34-kb deletion derivative of the TOL plasmid pWW0 and encompasses the whole upper TOL plasmid operon *xylCAB*, the right hand (*RH*) of the transposon Tn*4652* and adjoining nucleotide sequences up to tra-genes in the original pWW0 plasmid. Due to the deletion of the transposase gene *tnpA*, the transposon Tn*4652* should not be able to act as a transposon and hence not detract from the stability of these strains. This deletion gives the strains E2 and E1 a unique "positive" marker making it distinguishable from other strains with homologous sequences.

Due to the fact the left hand nucleotide sequences were not available to date, we partially determined the sequences of this region by the dideoxy method [12]. On the basis of these results two 20-nucleotide sequences were chosen which were used as primer pairs for PCR amplification generating a DNA fragment approximately 720 bp in length: *LH* primers of Tn*4652*: LH1: 5'-AGTGTT-CGACGATGGTCTCG-3', LH2: 5'-GCTTTCCCTGTGTATCAACG-3'. The amplified fragment hybridized exclusively to *P. putida* strains with this Tn*4652* left hand sequence and not to any of the strains lacking this transposon.

To test the sensitivity and specificity of the detection methods in a model experiment, we combined membrane filtration with colony hybridization and the PCR. We were able to detect the number of cells that had been added to 20 ml of river water at concentrations as low as 2 cells/20 ml. However, this high sensitivity of detection was only possible due to the low numbers of phenol-degrading and streptomycin-resistant bacteria among the indigenous flora. The limitation is dictated by the number of colonies which can grow on the membrane placed on the selective agar media.

In the release experiment, we used the more conventional and laborious strategy of cultivation and subsequent application of molecular techniques to overcome the high abundance of indigenous bacteria and assumed low numbers of strain E2. This enabled us to ensure that rare events of released cells could be detected. From 2405 colonies, which were isolated from different sampling points using selective media, 51 hybridized with the *xylE* gene probe. However, only 2 isolates - one each in samples taken from the sewage station 8 and 32 h after release and no isolates from all other samples - could be verified as being identical with the released strain by the PCR amplification with *xylE*, *LH*, and *RH* primers (Table 2).

This was possible because the deletion of the *RH* fragment allowed us to differentiate between the released strain and indigenous strains that were supposed to have high homology to the *xylE* sequence of our strain. However, we did not find TOL plasmid carrying bacteria other than E2 in the aquatic ecosystem. From this we have no evidence of transfer of plasmid pWW0^{mut90} of the strains E1 and E2 in the environment to other bacteria in Põlva region.

In evaluating the results of tracking the released strains E1 and E2 in the aquatic ecosystem near Põlva, we have to assume that the strains do not persist in nature. This may be due to not only

physiological and biological but also technological reasons. Assuming no growth, the introduction of the tank water (6 m^3; 2.0 x 10^4 CFUs/ml) into the activated sludge reactor (250-m^3 capacity) the high throughput of waste water through the sewage station (250 m^3/h), and subsequent dilution by the river water (2,400 m^3/h) lead to the sharp reduction of population size.

Table 2. Analysis of isolates of the release experiment

Sampling sites	Postrelease sampling time (hours)	Number of analyzed isolates	No. Hybridizing to the *xylE* probe	No. verified as E2 by PCR
Sewage station	8	653	21	1
	32	662	17	1
	104	270	5	-
River Orajögi	8	349	3	-
	32	255	3	-
	104	216	2	-

Therefore, it is difficult to study survival of released bacteria in open aquatic ecosystems under these conditions. Our experiments confirm that the added bacteria do not persist in nature. However, it has been demonstrated that the molecular techniques that were used were appropriate for tracking the non-genetically engineered *Pseudomonas putida* strains in the open environment.

Acknowledgments

This project was financed by the Ministry of the Environment and Conservation of Saxony-Anhalt, Germany. We would like to thank M. Kivisaar from the Institute of Molecular and Cell biology, Tartu, Estonia, for supplying cloned fragments of the LH sequences of the transposon Tn4652 in vector pUC19. We also thank the officials from the A/S Liimpuit plant in Põlva, Estonia, for granting us permission to perform the biodegradation and release experiments.

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UFZ-Bericht Nr. 10/1998

Microbiology of Polluted Aquatic Ecosystems

Proceedings
of the Workshop
held on the 4th and 5th December 1997
at the
UFZ Centre for Environmental Research Leipzig-Halle

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