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EXPERIMENTAL ARTICLES

The Effect of Transposons on the Expression of the Naphthalene Biodegradation Genes in *Pseudomonas putida* BS202(NPL-1) and Derivative Strains

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Abstract—NPL-1 and its derivative plasmid pBS106, which control the degradation of naphthalene and salicylate, were found to contain class II transposons of the *Tn*3 family. These transposons are involved in intraplasmid rearrangements, such as deletions and inversions, and can influence the expression of the catabolic and regulatory genes borne by biodegradation plasmids. The formation of a strong NahR-independent constitutive promoter by the inversion of a DNA fragment may be responsible for changing the character of naphthalene dioxygenase synthesis from inducible (in the case of plasmid NPL-1) to constitutive (in the case of plasmid NPL-41). The stability of plasmids NPL-1 and NPL-41 in *Pseudomonas putida* strains grown on different substrates depends on the expression of the *nah* and *tnp* genes.

Key words: biodegradation plasmids, transposons, *Pseudomonas.*

The mechanisms of the plasmid-controlled biodegradation of naphthalene by bacteria of the genus *Pseudomonas* are fairly well studied [1]. As a rule, the naphthalene biodegradation genes are organized in two operons, the upper *nah1* operon being responsible for the oxidation of naphthalene to salicylate and the lower *nah2* operon being responsible for the oxidation of the salicylate to catechol and the conversion of the latter to Krebs cycle intermediates through the *meta* cleavage pathway [2]. The regulation of the expression of the *nahR* gene, which is located between the operons, was comprehensively studied with reference to plasmid NAH7. The expression of the *nahR* gene was found to be induced by salicylate, the product of this gene being its positive regulator [3]. Simon *et al.* [4] determined the nucleotide sequences of the *nah1* and *nah2* operons in some plasmids and the complete sequence of the naphthalene biodegradation plasmid pDTG1.

The plasmid NPL-1 of *Pseudomonas putida* BS202 controls the degradation of naphthalene to salicylate. The further oxidation of the salicylate occurs through the *ortho* catechol cleavage pathway, which is controlled by chromosomal genes [5]. Plasmid NPL-1 bears the functionally active *nah1* operon and the silent genes of the *meta* catechol cleavage pathway. The plasmid-free and plasmid-bearing derivative strains of *P. putida* BS202(NPL-1), which were derived with the aid of elimination, transposon mutagenesis, and conjugal DNA transfer, have the Nah^+Sal^-, Nah^+Sal^+, and Nah^-Sal^+ phenotypes. These strains differ in the expression of the *nah* genes and in the stability of carried plasmids during long-term cultivation on various growth substrates.

The aim of this work was to study the mechanisms responsible for the formation of the derivatives of strain BS202 and the reasons for the different expression of the catabolic genes borne by plasmid NPL-1.

MATERIALS AND METHODS

The bacterial strains and plasmids used in this work are listed in Table 1.

Nutrient media and cultivation conditions. The bacterial strains were grown in LB broth [6] and in synthetic Evans medium [7], with 1 g/l naphthalene or salicylate as the carbon source. When required, the media were solidified by adding 1.5% Difco agar (United States). Ampicillin was added at concentrations of 50 and 100 μg/ml. The *Escherichia coli* strains were grown at 37°C, and the *Pseudomonas putida* strains were grown at 30°C.

The conjugal transfer of plasmids was carried out overnight on agar media with 0.45-μm Millipore nitrocellulose membrane filters. The membranes were washed in a mineral medium. The washings were diluted, and the appropriate dilutions were plated onto selective media.

Plasmid DNA was isolated by the alkaline lysis method [6]. The plasmid-bearing *Pseudomonas* strains were grown on a shaker at 30°C for 16 h in 200 ml of

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Table 1. The bacterial strains and plasmids used in this work

Strain (plasmid)	Relevant phenotype	Source
P. putida BS202 (NPL-1)	Nah^+Sal^+	Laboratory collection
P. putida BS203	Nah^+Sal^+	Ditto
P. putida BS261 (NPL-41)	$Nah^+Sal^+Ade^-$	″
P. putida BS394 (pBS7)	$Nah^+Sal^+Cys^-$	″
P. putida BS814 (NPL-1::*Tnl*)	$Nah^+Sal^+Km^r$	″
P. putida BS814 (pBS106)	$Nah^-Sal^+Km^r$	″
P. putida BS814 (pBS108)	$Nah^+Sal^+Km^r$	″
E. coli JM109	*rec*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, Δ(*lac-pro*AB), F'[*tra*D36, *pro*AB *lac*I^qZ ΔM15]	Promega, United States

Note: Nah^+ and Sal^+ indicate the ability to grow on naphthalene and salicylate, respectively. Cys^- and Ade^- stand for cysteine and adenine auxotrophy, respectively. Km^r is kanamycin resistance.

Evans medium with a carbon source. Cells were harvested by centrifugation at 4°C and resuspended in 4 ml of cold solution I (10 mM Tris–HCl + 5 mM EDTA, pH 8.0). The suspension was then mixed with 8 ml of solution II (1% SDS in 0.2 M NaOH) and incubated at room temperature until complete lysis occurred. The lysate was mixed with 6 ml of cold solution III (3 M K acetate, pH 5.0), incubated in an ice bath for 10 min, and centrifuged at 13000 rpm (J2-21, Beckman, United States) for 20 min. The supernatant was supplemented with 0.6 ml isopropanol, incubated at room temperature for 20 min, and centrifuged under the same conditions as above. The precipitate was dissolved in 0.6 ml of TE buffer (10 mM Tris–HCl + 1 mM EDTA, pH 8.0). The solution was supplemented with 200 μl 10 M LiCl, incubated in an ice bath for 10 min, and centrifuged at 15000 *g* for 5 min. To precipitate DNA from the supernatant, it was mixed with an equal volume of isopropanol, incubated at room temperature for 10 min, and centrifuged at 15000 *g* for 7 min. The precipitate was washed with 80% ethanol, dried, and dissolved in deionized water (20–100 μl). The amount and purity of the plasmid DNA prepared was evaluated by electrophoresis in 0.8% agarose in 0.5× Tris–borate buffer.

Cleavage of the plasmid DNA with restriction endonucleases was carried out by using buffer solutions and enzymes purchased from Amersham (United Kingdom). The total volume of the reaction mixture was 20–40 μl. To hydrolyze 100–300 ng of the plasmid DNA, the reaction mixture was supplemented with 20–30 U of a restriction enzyme and incubated at 37°C for 0.5–2 h as recommended by the manufacturer. The reaction was stopped by heating the mixture at 65°C for 15 min.

The electrophoresis of DNA samples was carried out in horizontal 0.8% agarose slabs in 0.5× Tris–borate buffer (89 mM H_3BO_3, 89 mM Tris–HCl, and 2 mM EDTA) in the presence of 0.5 μg/ml ethidium bromide. The DNA samples were applied in a solution containing 0.025% xylolcyanol, 0.025% bromophenol blue, and 2.5% phycol 400. The molecular weight marker was 1 kb DNA Ladder (Gibco BRL, United States).

The extraction of DNA fragments from agarose gel was performed by using a Qiaex II Agarose Gel Extraction System (Qiagen, Germany) as recommended by the manufacturer. DNA-containing gel strips were cut with a sterile scalpel. The strips were placed in 3 volumes of QX1 buffer in Eppendorf tubes. Each tube was supplemented with 10 μl of 3 M Na acetate and 10 μl Qiaex II resin and incubated at 50°C for 10 min with gentle stirring every 2 min. Then the tubes were centrifuged at 15000 *g* for 30 s, and the supernatant was collected. The precipitate was dissolved in 0.5 ml of QX1 buffer. The solution was centrifuged at 15000 *g* for 30 s, and the supernatant was also collected. The precipitate was washed with PE buffer. The resin was dried in the air for 10–15 min. The precipitate was dissolved in 20 μl of deionized water. The solution was centrifuged for 30 s, and the supernatant was transferred into clean tubes.

DNA ligation was conducted with the DNA ligase of bacteriophage T4 (USB, United Kingdom) according to the manufacturer's instructions. The ligation was carried out overnight at 12–14°C in 10 μl of 70 mM Tris–HCl buffer (pH 7.6) containing 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP, 0.05–0.1 μg of vector DNA, and a fivefold excess of the cloned DNA fragment.

The transformation of competent *E. coli* cells was carried out as described in the handbook [6]. A suspension (200 μl) of competent cells was supplemented with 5 μl of ligase solution and incubated in an ice bath for 30 min. The suspension was then heated in a water bath at 42°C for 90 s, cooled in the ice bath for 2 min, supplemented with 1 ml of LB broth, incubated at 37°C for 1 h, and plated onto selective media. To control the transformation frequency, the competent cells were transformed with 10 ng of plasmid pUC-18 DNA.

The polymerase chain reaction (PCR) was performed in a GeneAmp 2400 thermal cycler (Perkin-Elmer, United States). The reaction mixture contained

200 μM of each deoxyribonucleotide triphosphate; 1.5 or 2 mM $MgCl_2$; 0.5–1.5 U of *Taq* DNA polymerase (Amersham, United Kingdom); and, in some experiments, 5% dimethylsulfoxide.

DNA–DNA hybridization. DNA samples were purified by electrophoresis in horizontal agarose gel slabs and transferred onto Hybond N+ nylon membrane filters (Amersham) in 0.4 M NaOH for 3–4 h. The membranes were rinsed in 2× SSC (0.03 M sodium citrate + 0.3 M NaCl, pH 7.0) for 5 min. Prehybridization was carried out at 65°C for 2 h in 50 ml of a reaction mixture containing 5× Denhardt solution, 1% SDS, and 300 mg/ml denatured calf thymus DNA in 2× SSC. Hybridization was carried out at 65°C for 12–16 h in 1 ml of the prehybridization mixture supplemented with [^{32}P]dATP (1–2 million cpm) as the probe. The probe was labeled with the DECAprime II™ system (Ambion, United States) according to the manufacturer's instructions. After hybridization, the membrane was washed two times in 2× SSC with 0.1% SDS at room temperature for 5 min, two times in 0.2× SSC with 0.1% SDS at room temperature for 5 min, two times in 0.2× SSC with 0.1% SDS at 42°C for 15 min, one time in 0.1× SSC with 0.1% SDS at 65°C for 15 min, and one time in 2× SSC at room temperature for 1–2 min. Then the washed membrane was exposed to Hyperfilm ECL (Amersham) for 10–20 h to obtain a radioautograph.

DNA sequencing was performed by using the CEQ™ 2000XL DNA Analysis System (Beckman, United States) according to the manufacturer's instructions. The amount of DNA taken for analysis was 60–100 ng.

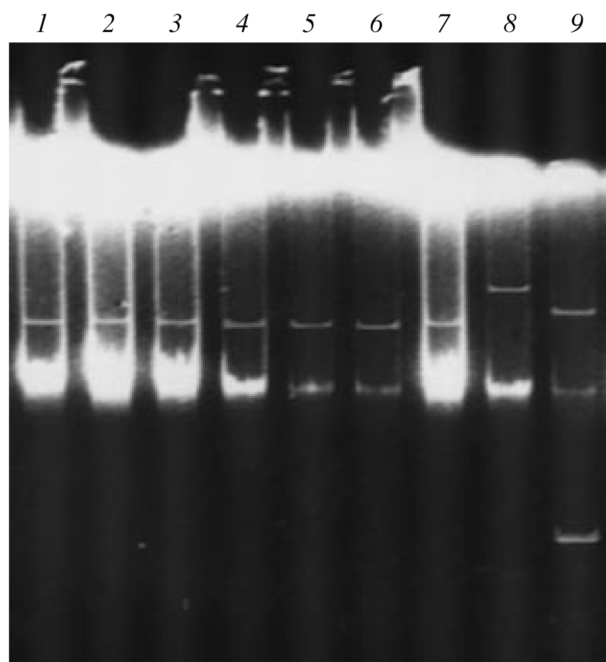

Fig. 1. Electrophoresis of plasmid DNA. Lanes: (*1–4*) the deletion variants of NPL-1; (*5–7*) the deletion variants of NPL-41; (*8*) NPL-1; (*9*) RP4 (54 kb) and RSF11010 (8.9 kb).

RESULTS

The derivative strains of *P. putida* BS202(NPL-1). The 100-kb IncP-9 plasmid NPL-1 of *P. putida* BS202 controls the oxidation of naphthalene to salicylate. The synthesis of the first enzyme of this pathway, naphthalene dioxygenase NahA, is strictly inducible, with salicylate as the inducer (as in the case of plasmid NAH7). Plasmid NPL-41, which is characterized by the constitutive synthesis of naphthalene dioxygenase, is a spontaneous mutant of plasmid NPL-1. Experiments on the elimination of plasmid NPL-1 showed that the chromosome of strain BS202 contains the functional gene of salicylate hydroxylase. Strain BS203 is a plasmid-free Sal^+ variant of strain BS202 [8].

The conjugal transfer of plasmid NPL-41 to the Sal^- strains of *P. putida* gave rise to Nah^+Sal^- transconjugants, the constitutive synthesis of NahA being preserved. Similar experiments with plasmid NPL-1 showed that, in this case, Nah^+Sal^- transconjugants are formed at a very low frequency (less than 10^{-8}). All of these transconjugants were found to contain a mutant plasmid with a constitutive synthesis of NahA [5].

The insertion of a *Tn1* transposon into plasmid NPL-1 [5] eliminated its Nah^+ characteristic and made it possible to select the transconjugants of Sal^- strains with plasmid NPL-1::*Tn1* by the ampicillin resistance characteristic. The long-term cultivation of one such transconjugant, BS814(NPL-1::*Tn1*), under nonselective conditions gave rise to clones with the Nah^+Sal^+ and Nah^-Sal^+ phenotypes (with a frequency of 10^{-6}–10^{-8}), which carried plasmids pBS108 and pBS106, respectively. At least in the case of pBS106, the restoration of the Sal^+ phenotype activated the silent plasmid genes of the *meta* catechol oxidation pathway.

The stability of plasmids NPL-1 and NPL-41 in *P. putida* strains during long-term cultivation. Our earlier studies showed that plasmids NPL-1 and NPL-41 are fairly stable in cells of the host strain BS202 cultivated on glucose [9]. However, plasmid NPL-1 tends to lose its stability during cultivation on salicylate as the sole source of carbon. Namely, after 65 generations, less than 1.5% of cells in a population had the Nah^+ phenotype. Some Nah^- clones, one of which was designated pBS7, carried a truncated plasmid about 40 kb in size.

Plasmid NPL-41 was fairly unstable during cultivation both on glucose and on salicylate (after 100 generations, the fraction of cells carrying NPL-41 diminished to about 1%). Some Nah^- clones also carried a truncated plasmid about 40 kb in size (Fig. 1).

The restriction and hybridization analyses of plasmid NPL-1 and its derivatives. The analysis of plasmids NPL-1 and NPL-41 with the aid of the *Eco*RI, *Hin*dIII, and *Bam*HI restriction endonucleases showed

Table 2. The size (in kb) of the *Eco*RI restriction fragments of the DNA of plasmid NPL-1 and its derivatives

NPL-1	NPL-41	pBS106	pBS108	pBS7
19.5	19.5	>23.0	>23.0	13.0
16.5	16.5	8.5	17.0	6.7
13.0	13.0	5.9	7.3	5.9
6.7	5.9	5.8	5.9 (2)	5.6
5.9	5.8	4.9	4.9	4.9
5.6	5.3	4.3	4.3	4.2
4.9	4.9	3.8	3.6 (2)	3.7
4.2	4.2	3.2 (2)	3.2	2.8
3.7	3.7	2.8	2.4	2.4
3.6	3.6 (2)	2.4	2.05	1.9
2.8	2.8	2.05	1.75	1.85
2.4	2.4	1.75	1.55	1.75
2.05	2.05	1.55		1.7
1.9	1.9			1.2
1.75	1.75			1.0
1.7	1.7			0.8
1.2	1.2			0.6
1.0	1.0			0.55
0.8	0.8			0.45
0.6	0.6			
0.55	0.55			
0.45	0.45			

their similarity except for a 4.2-kb region adjacent to the regulatory region of the *nah1* operon. Plasmid NPL-41 contains a 4.2-kb inversion as compared to plasmid NPL-1 [5]. The *Eco*RI restriction patterns of plasmids NPL-1 and NPL-41 are similar to those of the naphthalene biodegradation plasmids, including pDTG1 [10], pBS216, and pSN11 (unpublished data). The *Eco*RI restriction patterns of plasmids NPL-1 and NPL-41 differ mainly in two large fragments (19.5 and 16.5 kb). Like many other naphthalene biodegradation plasmids, the derivative plasmids pBS106 and pBS108 give only one large *Eco*RI fragment. The deletion mutants of plasmids NPL-1 and NPL-41 were similar to plasmid pBS7 in the restriction profiles (Table 2).

The hybridization analysis of plasmids NPL-1 and NPL-41 with the ^{32}P-labeled probes to the *nahA*, *nahH*, *nahR*, and *nahG* genes of the archetypal plasmid NAH7 showed that nearly all of the *nah1* operon genes localize within the 16.5-kb *Eco*RI fragment and that the 19.5-kb *Eco*RI fragment contains the nonfunctional genes of the *meta* catechol cleavage pathway and *nahG*. In the case of the *nahR* probe, neither plasmid produced a hybridization signal.

In contrast, plasmid pBS106 did not hybridize with the *nahA* probe but gave a marked hybridization signal (the large *Eco*RI fragment) with the *nahH, nahR*, and *nahG* probes.

None of the probes hybridized with the deletion variants of plasmids NPL-1 and NPL-41. Plasmid pBS7 did not give 19.5- and 16.5-kb *Eco*RI fragments. This is indicative of the absence of naphthalene catabolism genes in this plasmid and explains the loss of the Nah$^+$ phenotype.

The results of hybridization analysis were verified by PCR analysis with oligonucleotide primers to the same genes. If plasmid genes were able to hybridize with the aforementioned probes, these genes were found to be undergo PCR amplifications [11].

Cloning of the *nah* genes and adjacent regions of plasmids NPL-1 and pBS106. The hybridization of the *Hin*dIII-digested DNA of plasmid NPL-1 with the aforementioned probes showed that the *nah1* and *nah2* operon genes are localized within DNA fragments about 8 kb in size. These fragments were eluted from agarose gel, treated with *Eco*RI, and ligated with the cloning vector pBluescript II at the same sites. Recombinant plasmids were selected by hybridizing the clones with the respective probes. A comparison of the size of the fragments cloned and their partial sequencing made it possible to map the *nah* region of plasmid NPL-1 (Fig. 2). As is evident from this figure, the insertion of a class II *Tn3* transposon alters the regulatory regions of the *nah* operons and the *nahG* gene. The persistence of the inducible character of naphthalene dioxygenase synthesis in strain BS202 suggests that the *nahR* gene localizes on the chromosome but not on plasmid NPL-1.

The cloning of the *nahG* and *nahR* genes of plasmid pBS106 [12] and the sequencing of the cloned regions showed that there is another class II transposon in the vicinity of the *nahR* gene (Fig. 2). A comparison of the nucleotide sequences of the resolvase *tnpR* genes adjacent to the *nah2* operons of plasmids PBS106 and NPL-1 showed that both transposons belong to the *Tn3* family but are not identical (Fig. 3).

DISCUSSION

The presence of transposons and IS elements in biodegradation plasmids is well documented. The class II TOL plasmid pWW0 contains at least three transposons and belongs to the P-9 incompatibility group [13]. The basic replicon of this plasmid is similar, if not identical, to the replicon of plasmid NPL-1 [14]. The archetypal plasmid NAH7 contains a defective transposon, which can be restored [15]. Transposons, together with plasmids, play an important part in the horizontal transfer of biodegradation genes inside and between bacterial populations.

The results presented here show that transposons and IS elements considerably influence the genetic control over naphthalene biodegradation. The study of plasmid NPL-1 showed an important role of transpo-

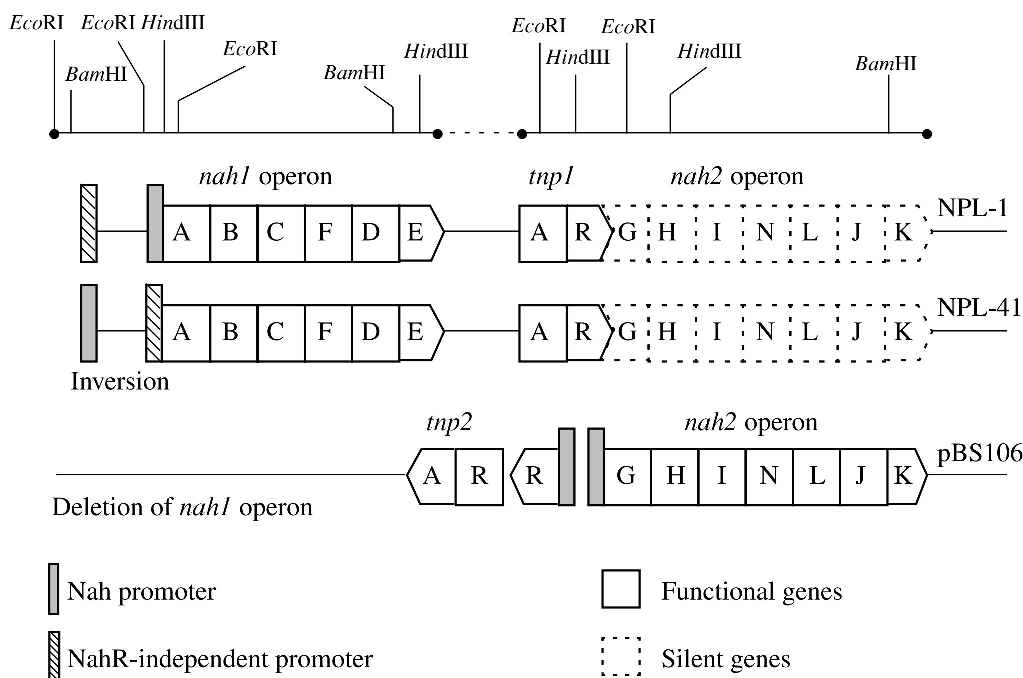

Fig. 2. The map of the *nah* regions of plasmids NPL-1, NPL-41, and pBS106.

son-induced plasmid rearrangements in the expression of catabolic operons. The insertion of a transposon into the starting region of the *nah2* operon of plasmid NPL-1 induces the loss of the regulatory *nahR* gene and switches off the *nah2* operon genes (including *nahG*), which are responsible for the *meta* catechol cleavage pathway (Fig. 2). It is known that the *xyl* and *nah* genes can control the degradation of substituted (methylated and halogenated) derivatives of benzene and naphthalene due to the broad substrate specificity of the encoded enzymes. The *meta* cleavage of halogenated catechols gives rise to toxic intermediates, whose accumulation induces cell death [16]. In contrast, the *ortho* cleavage pathway does not give rise to toxic intermediates. This may explain the triggering of the metabolic pathway in strain BS202(NPL-1). The study of plasmids pBS106 and pBS108, derived from NPL-1, showed that the absence of selective pressure toward switching off the *meta* cleavage genes may promote a recombination event, as a result of which the *nahR* and *nahG* genes are restored, *tnpA* and *tnpR* are deleted, and naphthalene metabolism switches over to the *meta* catechol cleavage pathway. In plasmid pBS106, the *nah1* operon is completely deleted. Bolognese *et al.* [17] described a similar switching over of the metabolic pathway in the *P. stutzeri* strains OX1 and M1, utilizing methylated benzenes.

The deletion of *tnpA* and *tnpR* and the restoration of the *nah2* operon in plasmid pBS106 eliminate the *Eco*RI site in the *tnpR* gene, due to which the restriction pattern of this plasmid becomes typical of most naphthalene biodegradation plasmids (one fragment of a larger size instead of two large *Eco*RI fragments). It should be noted, however, that the screening of the laboratory collection of the IncP-9 naphthalene biodegradation plasmids showed that some pseudomonads have plasmids which are similar to NPL-1 in size and *Eco*RI restriction profile (unpublished data).

The inversion of the 4.2-kb DNA region in plasmid NPL-41 changes the expression of the *nah* genes from inducible to constitutive. This makes such pseudomonads capable of degrading not only naphthalene but also higher molecular weight polycyclic aromatic hydrocarbons (PAHs), including phenanthrene [18]. It is possible that the mechanism of changing the expression type lies in the formation of a stronger NahR-independent constitutive promoter in the *nah1* operon, which results in an increase in the cellular content of naphthalene dioxygenase (the key enzyme of PAH degradation). Eventually, this widens the range of utilizable PAHs and enhances the adaptive capacity of pseudomonads occurring in PAH-contaminated environments.

The proposed organization of the *nah* genes in strain BS202(NPL-1) explains why the transconjugants of the Sal^- strains of *P. putida* are difficult to select on the medium with naphthalene as the carbon source in the case of plasmid NPL-1, whereas such transconjugants are readily produced in the case of plasmid NPL-41. Indeed, the *nah1* operon of plasmid NPL-1 is characterized by inducible expression, but neither this plasmid nor the chromosome of the host strain contains the regulatory *nahR* gene. As a result, the *nah* genes of plasmid NPL-1, when transferred to the recipient cells, are not expressed.

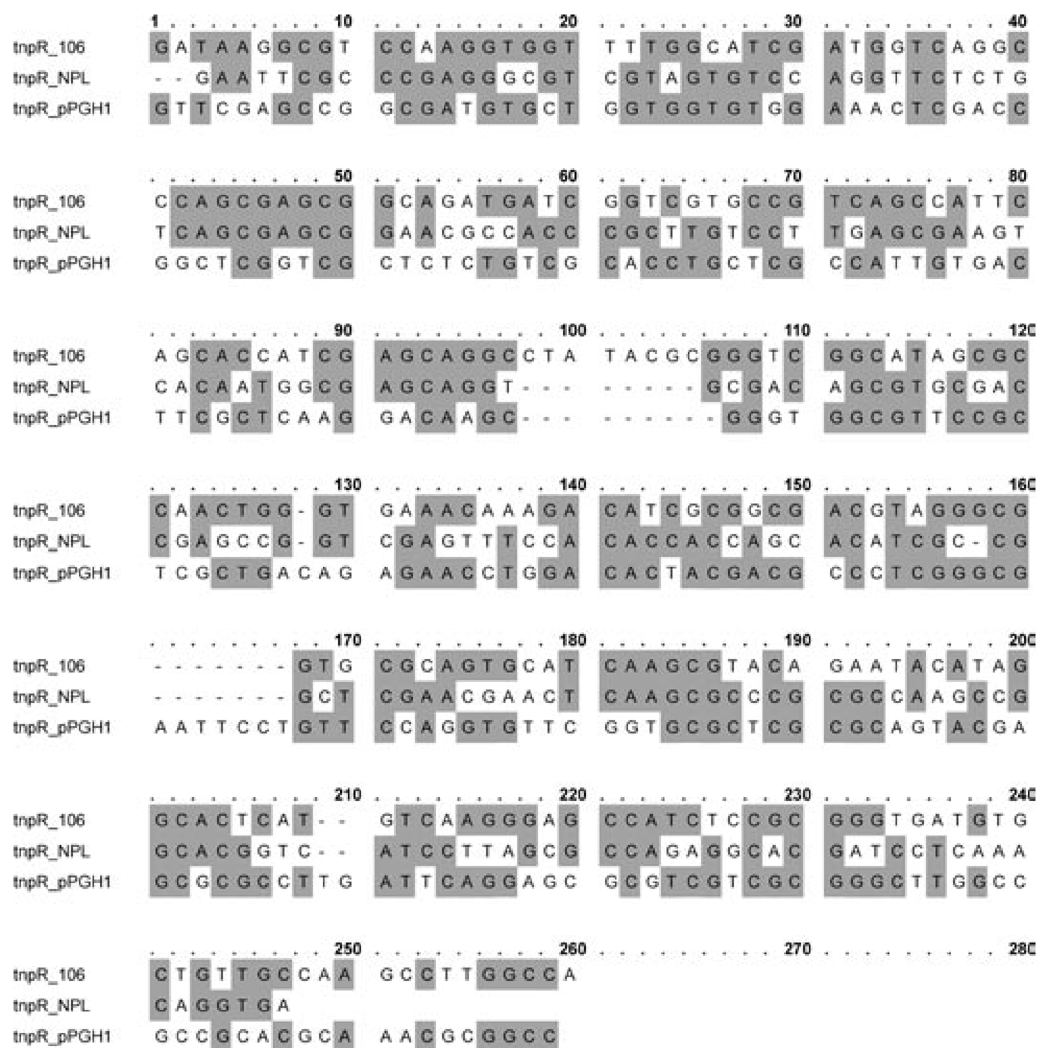

Fig. 3. Multiple alignments of the partial nucleotide sequences of the *tnpR* genes of plasmids pBS106 (tnpR_106), NPL-1 (tnpR_NPL), and pPGH1 (tnpR_pPGH1). The sequence of the *tnpR* gene of plasmid pPGH1 from *P. putida* H (GenBank accession no. Y09450) was taken as the reference standard.

The analysis of the cloned fragments of plasmids NPL-1 and NPL-41 failed to reveal the signals of transcription termination. The transposons and *nah* genes of plasmid pBS106 are localized on different DNA strands, whereas those of plasmid NPL-1 are localized on one DNA strand and are most likely to transcribe as a single transcript (Fig. 2). This suggestion is confirmed by experimental data indicating the persistence of plasmids NPL-1 and NPL-41 during cultivation on various substrates. The constitutive expression of the *nah* genes of plasmid NPL-41 provides for the continued expression of transposase. For this reason, plasmid NPL-41 is unstable during the cultivation of the host strain both on the rich medium and on the medium with the *nah* gene inducer salicylate. The instability of plasmid NPL-1 is directly related to the induction of the expression of the *nah* genes and, hence, is manifested only in salicylate-containing media. The concurrent expression of catabolic and transposon genes may provide for the rapid distribution of the catabolic genes in microbial populations due to the enhanced frequency of recombination events in the presence of PAHs in the environment.

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