ISSN 0026-8933, Molecular Biology, 2006, Vol. 40, No. 5, pp. 750–757. © Pleiades Publishing, Inc., 2006.
Original Russian Text © A.A. Leuchuk, I.M. Bulyha, T.Yu. Izmalkova, Ya.R. Sevastyanovich, I.A. Kosheleva, C.M. Thomas, M.A. Titok, 2006, published in Molekulyarnaya Biologiya, 2006, Vol. 40, No. 5, pp. 835–843.

GENOMICS. TRANSCRIPTOMICS. PROTEOMICS

UDC 576.851.132

Nah Plasmids of the IncP-9 Group in Natural *Pseudomonas* Strains

A. A. Leuchuk[a], I. M. Bulyha[a], T. Yu. Izmalkova[b], Ya. R. Sevastyanovich[c], I. A. Kosheleva[b], C. M. Thomas[c], and M. A. Titok[a]

[a] *Belarussian State University, Minsk, 220050 Belarus; e-mail: m_titok@yahoo.com*

[b] *Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow region, 142290 Russia*

[c] *University of Birmingham, Birmingham B15 2TT, United Kingdom*

Received January 10, 2006

Abstract—Polymerase chain reaction studies showed that naphthalene-utilizing bacteria isolated from various localities of Belarus most often contained Nah plasmids of the P-9 incompatibility group and plasmids of indefinite systematics. The conventional incompatibility test and restriction enzyme analysis revealed three new IncP-9 subgroups: ζ, η, and IncP-9-like. In addition to the known nucleotide sequences of *nah*G and *nah*Ac, two novel *nah*G variants were revealed by a restriction enzyme analysis of amplification products. An amplified rDNA restriction enzyme analysis (ARDRA) demonstrated that the native hosts of IncP-9 Nah plasmids were fluorescent bacteria of the genus *Pseudomonas* (*P. fluorescens, P. putida, P. aeruginosa*, and *Pseudomonas* sp.) and nonfluorescent bacteria of indefinite systematics.

DOI: 10.1134/S0026893306050098

Key words: Nah plasmids, *rep* region, *nah*Ac, *nah*G, polymorphism, *Pseudomonas*

INTRODUCTION

Plasmids of the IncP-9 group are very diverse. They include R plasmids, determining resistance to a wide range of antibiotics, heavy metal ions, and UV radiation [1–3], and D plasmids, responsible for degradation of various organic compounds [4, 5]. These plasmids are found mainly in fluorescent bacteria of the genus *Pseudomonas* (*P. putida, P. fluorescens*, and *P. aeruginosa*) and can be transferred via conjugation to other Gram-negative microorganisms [6]. Biodegradation systems of these plasmids are best studied [7], whereas less is known about their replication and conjugative transfer mechanisms. Recently, complete nucleotide sequences have been reported for D plasmids determining utilization of toluene and naphthalene (pWW0 and pDTG1) and the minireplicon of the R-plasmid pM3 [8–10]. Knowledge of the nucleotide sequences allows an analysis of the genetic organization of extrachromosomal elements of this group and provides grounds for comprehensive study of their replication and conjugative transfer.

In this study, we compared the *rep* regions and biodegradation determinants of IncP-9 Nah plasmids detected in bacteria identified as *Pseudomonas*.

EXPERIMENTAL

Experiments were performed with 61 naphthalene-utilizing bacterial strains isolated from various localities in Belarus (Table 1) and with plasmids stored in collections (Table 2).

Bacteria were grown in the complete and minimal liquid Evans media [15]. Agar media contained 1.5% agar. Glucose (0.2%) and naphthalene (100 μg/ml) were used as carbon sources.

Total bacterial DNA was isolated with sarcosyl [16]. Electrophoresis was performed as in [17].

Polymerase chain reaction (PCR). The oligonucleotide primers used in the analysis are listed in Table 3. The reaction was carried out with *Taq* polymerase purchased from Fermentas (Lithuania) or a Takara kit (Japan). The reaction mixture (50 μl) contained 1 unit of polymerase, 200 μM concentration of each primer, 200 μM dNTP, 1.5 mM $MgCl_2$, the standard buffer, and, in some cases, 5% dimethyl sulfoxide (Sigma). The PCR program was as follows: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at an appropriate temperature for 30 s, and elongation at 72°C for 1 min (or 2 min with 16S rDNA or 3 min with *rep-kor*A); and 10 cycles of 72°C for 5 min.

Digestion with restriction enzymes (*Hae*III, *Rsa*I, and *Msp*I) was carried out as recommended by Fermentas. Fragment sizes were determined with reference to 100-bp and 1-kb standard DNA marker ladders (Fermentas).

Table 1. Naphthalene-utilizing *Pseudomonas* strains isolated from natural sources

Strain	Source*
1–14	Minsk (territory of the enterprises AvtoVAZ, Kristall, Dialek, MAZ, Litmash, SPMK-127, and tractor works)
15–39, 61	Minsk (car parks, gas stations, and railway sides)
40–46	Polotsk (territory of the enterprises Polimir, Naphthan, and thermoelectric plant)
47–51	Brest (gas stations, gas storage tanks, and oil replacement station)
52–54	Vitebsk (gas station and car park)
55–58	Soligorsk (road-building enterprise and gas stations)
59	Bobruisk (hydrolysis plant)
60	Gomel (Tsentrolit enterprise)

* Naphthalene-utilizing bacteria were isolated from soil and water samples supposedly polluted with oil and oil products.

RESULTS AND DISCUSSION

The sequenced pMT2 minireplicon of the R-plasmid pM3 belonging to the IncP-9 group has ten open reading frames. Nine of them code for proteins Rep, ParA, ParB, KorA, TolA, MpfA, MpfB, MpfC, and TnpR. These proteins display significant similarity to proteins involved in replication initiation, in maintenance and conjugative transfer of known extrachromosomal genetic elements, and in site-specific recombination of the Tn*21* transposon [10]. Fourteen primers were designed on the basis of the pMT2 sequence for amplification of various fragments of the plasmid replicon containing the indicated functional domains. Primer pairs for identification and characterization of IncP-9 replicons were selected on the basis of an analysis of a collection of IncP-9 plasmids [22]. In particular, primers repF–repR allow amplification of the region containing *rep* (constructed on the basis of the pMT2 and pWW0 nucleotide sequences) [18] and can be applied to tentative identification of new plasmids of the IncP-9 group. In addition, PCR with primers korA3Fa–rep3Rc and mpfA1Fa–korA2Ra and a subsequent restriction enzyme analysis of the amplification products allows reliable detection of polymorphism of IncP-9 replicons. An analysis of the amplification products with *Hae*III has revealed three subgroups of IncP-9 plasmids: α, β, and γ [22]. An analysis of Nah plasmids isolated from natural bacterial strains in Russia has shown that some plasmids do not produce PCR products with primers korA3Fa–rep3Rc but allow amplification of a specific product with primers repF–repR. For this reason, a new IncP-9 subgroup has been established and named δ [23]. Its existence has been confirmed by sequencing [9, 24]. Moreover, it has been found that the R2 and pMG18 antibiotic resistance plasmids constitute a separate subgroup, ε. The existence of these subgroups has also been confirmed by sequencing of the *rep* regions [24].

Knowledge of the complete sequences of extrachromosomal genetic elements of the P-9 incompatibility group (pWW0, pDTG1, and pMT2) allowed us not only to characterize the plasmids of natural naphthalene-utilizing bacteria, but also to compare their *rep* regions with those of known IncP-9 plasmids by means of PCR and restriction enzyme analysis of the amplification products.

All known D plasmids furnishing naphthalene utilization belong to three incompatibility groups: IncP-2, IncP-7, and IncP-9 [25]. It should be noted that plasmids of the IncP-2 group are relatively large (>200 kb) and usually have two replicons, containing an additional *rep* region of IncP-9 or IncP-7 plasmids [25]. Therefore, we suggest that the natural naphthalene-utilizing strains must bear plasmids of one of these incompatibility groups.

PCR was conducted with primers directed to *rep* (repF–repR), replication origin *ori*V (orisF–orisR), and the *rep* regions of IncP-9 (3Fa–3Rc) and IncP-7 (rep7f–rep7r) plasmids. Total DNA or mix DNA isolated from natural naphthalene-utilizing bacteria served as a template. Control experiments were performed with DNA from strains with known plasmids of the IncP-9 (pM3, pBS101, R2, pBS267, and pBS216) and IncP-7 (Rms148) groups [22]. Positive PCR results with at least one IncP-9 primer pair were obtained for 30 of 61 natural naphthalene-utilizing strains examined (Table 4).

Table 2. Plasmids used in the study

Plasmid	Description	Reference
PM3	Tc^R, Sm^R, Tra^+, IncP-9 (α)	[3]
NPL-1	Nah^+, Tra^+, IncP-9 (β)	[11]
PBS101	Nah^+, Tra^+, IncP-9 (β)	[12]
PBS267	Cap^+, Tra^+, IncP-9 (γ)	[13]
PBS216	Nah^+, Tra^+, IncP-9 (δ)	[12]
R2	Ap^R, Km^R, Sm^R, Su^R, Uv^R, Tra^+, IncP-9 (ε)	[1]
Rms148	Sm^R, Tra^+ (IncP-7)	[14]

Table 3. PCR primers

Gene	Primer*	Nucleotide sequence	Annealing *T*, °C	Amplicon size, bp	Reference
IncP-9 *rep-ori*V	repF repR	5'-CCA GCG CGG TAC WTG GG-3' 5'-GTC GGC AGC TGC TTG AGC TT-3'	54	480	[18]
IncP-9 *rep-korA*	korA3Fa rep3Rc	5'-GCA GAC CCA TTC CAT GAC CAC C-3' 5'-CCA CCG ACA CTG ATG GTC TG-3'	58	2033	[18]
IncP-9 *ori*V	orisF orisR	5'-GCG GGA ATG RGT GAC TAG CG-3' 5'-GTC TGT ACC CAT GTR CCG-3'	50	540	*
IncP-7 *rep*	Forward Reverse	5'-CCC TAT CTC ACG ATG CTG TA-3' 5'-GCA CAA ACG GTC GTC AG-3'	52	524	*
16S rRNA	8f 1492r	5'-AGA GTT TGA TCM TGG CTC AG-3' 5'-TAC GGH TAC CTT GTT ACG ACT T-3'	52	1484	[19]
*nah*Ac	Ac149f Ac1014r	5'-CCC YGG CGA CTA TGT-3' 5'-CTC RGG CAT GTC TTT TTC-3'	43	865	[20]
*nah*G	shc1_up shc1_lo	5'-CGG CKT THG GTG ARG TCG GTG C-3' 5'-GGC GAG GAA RTA GGC GTC CTC AAG-3'	64	893	[31]
*nah*R	nahR_1f nahR_585r	5'-ATG GAA CTG CGT GAC CTG G-3' 5'-GCC GTA GGA ACA GAA GCG-3'	54	585	[31]
*nah*H	23OF 23OR	5'-ATG GAT DTD ATG GGD TTC AAG GT-3' 5'-ACD GTC ADG AAD CGD TCG TTG AG-3'	52	721	[21]

* The primers were provided by C.M. Thomas.

Table 4. Identification of plasmids of natural naphthalene-utilizing strains

Strain	Presence of amplification products with primers				Subgroup
	repF–repR	orisF–orisR	3Fa–3Rc	rep7f–rep7r	
1–6, 16, 17, 21, 29, 31, 33, 35, 38, 52–54, 57, 59	+	+	–	–	δ
19, 32, 60	+	+	+	–	ζ
15	+	+	+	+	η
22, 25, 40, 42, 45, 55	–	+	–	–	P-9-like
pM3	+	+	+	–	α*
NPL-1, pBS101	+	+	–	–	β*
pBS267	+	+	+	–	γ*
pBS216	+	+	–	–	δ*
R2	+	+	+	–	ε*
Rms148	–	–	–	+	IncP-7

* Subgroups were established by restriction enzyme analysis and sequencing of the amplification products [22, 24]. Plasmids were assigned to subgroups according to the restriction enzyme analysis of the amplification products (Figs. 1, 2).

With total DNA of strain 15 as a template, we obtained an additional 524-bp amplification product, characteristic of the *rep* regions of IncP-7 plasmids. These data allowed the plasmids detected in the 30 strains to be assigned to the IncP-9 group. The plasmid of strain 15 contained both IncP-9 and IncP-7 replicons. The other strains bore extrachromosomal elements of indefinite systematics.

The PCR data were verified by the conventional incompatibility test with bacteria of four strains (4,

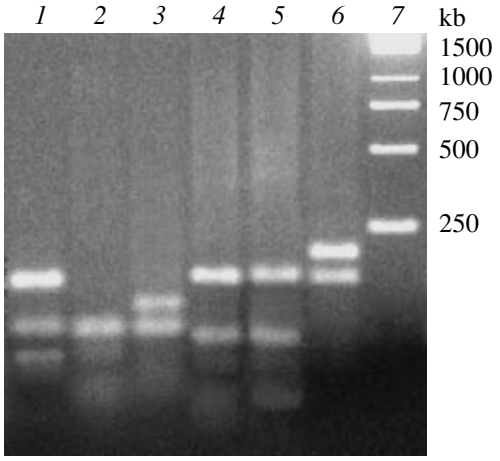

Fig. 1. Restriction enzyme analysis of the PCR products amplified from *rep* of IncP-9 plasmids. The amplification products were obtained with primers repF–repR and total DNAs of strains containing IncP-9 plasmids, and were digested with *Hae*III. Plasmids: (*1*) pM3 (subgroup α); (*2*) pBS101 (subgroup β); (*3*) pBS267 (subgroup γ); (*4*) pBS216 (subgroup δ); (*5*) plasmid of strain 4 (subgroup δ); (*6*) plasmid of strain 60 (subgroup ζ). Lane *7*, 1-kb ladder.

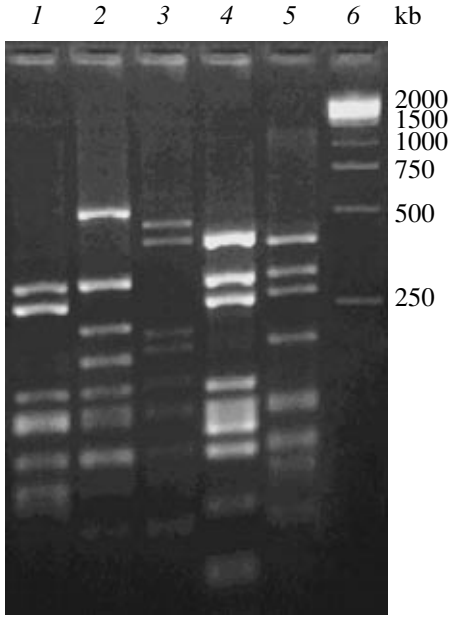

Fig. 2. Restriction enzyme analysis of the PCR products amplified from the *rep* region of IncP-9 plasmids. The amplification products were obtained with primers korA3Fa–rep3Rc and total DNAs of strains containing IncP-9 plasmids, and were digested with *Hae*III. Plasmids: (*1*) R2 (subgroup ε); (*2*) pBS267 (subgroup γ); (*3*) pBS101 (subgroup β); (*4*) plasmid of strain 15 (subgroup η); (*5*) pM3 (subgroup α). Lane *6*, 1-kb ladder.

15, 22, 25), presumably containing IncP-9 plasmids. In conjugative matings, the donors were bacteria bearing the Rms 148 (IncP-7), R2 (IncP-9), and pM3 (IncP-9) plasmids. Their transfer to cells of the natural strains was followed by tests for naphthalene utilization. Cells utilized naphthalene if they contained Nah plasmids. We found that bacteria of strains 4, 22, and 25 retained the ability to utilize naphthalene after receiving Rms 148, but lost it completely after the uptake of the R2 or pM3 plasmid. Bacteria of strain 15 lost the naphthalene degradation plasmid after reception of R2 or pM3, whereas the Nah character persisted in 41% of the clones tested after the uptake of Rms 148. It should be mentioned that this sort of inheritance is typical of plasmids containing two replicons [25].

Thus, the PCR data agree with conventional compatibility tests.

The results indicate that the examined naphthalene-utilizing strains possess extrachromosomal elements of three types. Plasmids of the IncP-9 group and plasmids of unknown systematics are predominant, and only one plasmid has two replicons, IncP-9 and IncP-7. Similar studies performed in Russia have revealed naphthalene degradation plasmids belonging mainly to the IncP-7 and IncP-9 groups [12, 25]. The absence of the IncP-7 group from our collection can be related to specific features of host bacteria, unable to maintain IncP-7 plasmids, known to have a narrow host range.

Polymorphism of the *rep* region in the IncP-9 plasmids was detected by restriction enzyme analysis of the PCR products obtained with two primer pairs, repF–repR and korA3Fa–rep3Rc. The PCR products of strains 1–6, 16, 17, 21, 29, 31, 33, 35, 38, 52–54, 57, and 59 showed *Hae*III patterns similar to those of plasmids of subgroup δ (Fig. 1). In addition, we found restriction patterns that did not correspond to any of the known IncP-9 plasmids. In particular, new *Hae*III sites were found in the products amplified from total DNAs from strains 19, 32, 60, and 15 (Figs. 1, 2). Therefore, the plasmids of these strains can be assigned to new subgroups, named ζ and η, respectively.

In strains 22 and 25, the conventional incompatibility test revealed IncP-9 extrachromosomal elements possessing new features. As mentioned above, these plasmids were eliminated at 100% frequency from parental bacterial cells after transformation with the pM3 and R2 plasmids of the IncP-9 group, and yielded specific amplification products only with primers orisF–orisR. This amplification pattern was also observed with strains 40, 42, 45, and 55 (Table 4). It is reasonable to suggest that the detected extrachromosomal elements are not typical members of the IncP-9 group but possess IncP-9-like replicons. The incompatibility of these plasmids can be controlled either by the replication origin (*ori*V), which binds with the Rep protein at each replication round, or by a centromere-like sequence, determining segregation of

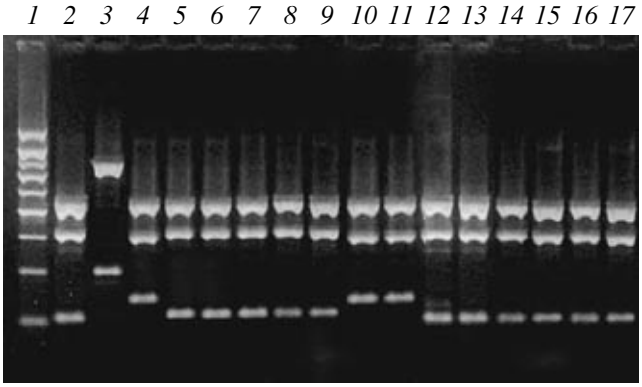

Fig. 3. Restriction enzyme analysis of the *nah*Ac amplification products with *Hae*III. Lane *1*, 100-bp ladder. Restriction patterns were obtained for (*2–4*) known *nah*Ac sequences of types C18, AN10, and A88, respectively, and (*5–17*) for the *nah*Ac amplification products synthesized with total DNAs of strains (*5*) 60 (ζ), (*6*) 32 (ζ), (*7*) 4 (δ), (*8*) 15 (η), (*9*) 25 (P-9-like), (*10*) 22 (P-9-like), (*11*) 19 (ζ), (*12*) 35 (δ), (*13*) 29 (δ), (*14*) 31 (δ), (*15*) 53 (δ), (*16*) 5 (δ), and (*17*) 6 (δ). Here and in Figs. 4 and 5, the IncP-9 subgroups are indicated in parentheses.

plasmid copies between daughter cells [26, 27]. The actual organization of these plasmids can be established only by cloning and sequencing of their minireplicons.

The differences among the *rep* regions of natural plasmids of naphthalene-utilizing bacteria were confirmed by sequencing of their *rep* and *ori*V [24].

Thus, the analysis of the collection of naphthalene-utilizing strains isolated in Belarus revealed two new subgroups of IncP-9 extrachromosomal elements and allowed the plasmids of strains 22, 25, 40, 42, 45, and 55 to be classified as having IncP-9-like replicons. None of the detected Nah plasmids belonged to subgroup β, which includes the majority of known biodegradation plasmids. This group includes, in particular, typical IncP-9 plasmids such as pWWO and NPL-1 [8, 11].

Our studies provide additional evidence that IncP-9 extrachromosomal elements are phenotypically different and highly diverse in the nucleotide sequences of replication origins.

Bacterial plasmids are complex systems consisting of various genetic elements. Thus, it was interesting to investigate the variation of the genetic organization of natural Nah plasmids. It is known that naphthalene catabolism genes of many replicons are highly conservative [28, 29]. The key enzymes of naphthalene, phenanthrene, and salicylate degradation are naphthalene 1,2-dioxygenase and salicylate 1-hydroxylase. They are encoded by *nah*A and *nah*G, respectively, which are positively regulated by NahR [30]. Catechol 2,3-dioxygenase performs *meta*-cleavage of catechol, an intermediate in degradation of polycyclic and monocyclic compounds. The presence of *nah*H, coding for catechol 2,3-dioxygenase, indirectly confirms the plasmid location of biodegradation operons [30]. The transposon location of catabolism operons suggests that their genes can migrate among bacterial populations and increase the adaptive potential of microorganisms. Recombination events, providing the transposition of mobile genetic elements, and also environmental factors and the genetic background can alter the genetic organization of elements responsible for degradation of organic compounds.

Indeed, PCR followed by the restriction enzyme analysis of the products revealed polymorphic loci containing genes for naphthalene biodegradation. In particular, it has been found that *nah*Ac, coding for the large naphthalene 1,2-dioxygenase subunit, occurs in three variants (types AN10, C18, and A88) [31]. A similar method has been applied to study of the diversity of *nah*G. Six variants of the *nah*G nucleotide sequence have been found in *P. fluorescens* strains: NAH7, pDTG1, AN10, KF715, A88, and NKNS [31].

All 14 naphthalene-utilizing strains that contained various IncP-9 plasmids possessed *nah*R and *nah*H. This fact additionally confirmed the extrachromosomal occurrence of the naphthalene biodegradation determinants [30] (Table 5). Amplification of *nah*Ac and *nah*G with specific primers and a subsequent restriction enzyme analysis revealed two known types of *nah*Ac nucleotide sequence (C18 and A88) and three of *nah*G (A88, NAH7, and pDTG1) (Table 5, Figs. 3, 4a, 4b).

We found no clear correlation between the type of degradation-related genetic elements and replicons of certain subgroups. For example, plasmids of subgroups δ and η were characterized by the C18 type of *nah*Ac and the pDTG1 type of *nah*G, whereas plasmids of subgroup ζ and IncP-9-like replicons contained various types and combinations of these genes. It is worth noting that strain 55, possessing an IncP-9-like replicon, did not yield the *nah*Ac-specific amplification product (865 bp), and the *nah*G nucleotide sequence differed from the known variants. For example, *nah*G lacked two *Rsa*I sites. As a result, a 430-bp fragment was observed in place of the 201-, 131-, and 101-bp fragments typical of type NAH7 determinants (Figs. 4a, 4b).

These data indicate that the organization of the *nah* genes in the plasmid of strain 55 differs from that of the known genes. In *nah*G, the difference is confined to single-nucleotide polymorphisms, whereas *nah*Ac may differ much more from its analogs, and our primers do not operate. However, the latter suggestion demands additional experimental proof.

Plasmids belonging to subgroup ζ possessed various combinations of *nah*Ac and *nah*G. In particular, the plasmids of strains 32 and 60 had *nah*Ac of the C18 type, whereas their *nah*G belonged to types

Table 5. Nah plasmids of the IncP-9 group isolated from natural *Pseudomonas* strains

Strain	Source (soil samples)	Subgroup*	Identification of *nah* genes,				Host species**
			*nah*Ac	*nah*G	*nah*R	*nah*H	
4	Minsk (Kristall enterprise)	δ	+/C18	+/pDTG1	+	+	*P. putida*
5	Minsk (tractor works)	δ	+/C18	+/pDTG1	+	+	*Pseudomonas* sp. III
6	Minsk (tractor works)	δ	+/C18	+/pDTG1	+	+	*Pseudomonas* sp. III
29	Minsk (car park)	δ	+/C18	+/pDTG1	+	+	*Pseudomonas* sp. II
31	Minsk (roadside)	δ	+/A88	+/pDTG1	+	+	*P. aeruginosa*
35	Minsk (gutter)	δ	+/C18	+/pDTG1	+	+	*P. fluorescens*
53	Vitebsk (dolomite works)	δ	+/C18	+/pDTG1	+	+	*Pseudomonas* sp. III
19	Minsk (bus station)	ζ	+/C18	+/A88^{++}	+	+	*P. fluorescens*
32	Minsk (railroad)	ζ	+/C18	+/pDTG1	+	+	*P. fluorescens*
60	Gomel (Tsentolit enterprise)	ζ	+/C18	+/NAH7	+	+	*P. fluorescens*
15	Minsk (gas station)	η***	+/C18	+/pDTG1	+	+	*P. fluorescens*
22	Minsk (gas station)	P-9-like	+/A88	+/Ä88	+	+	*P. fluorescens*
25	Minsk (railroad)	P-9-like	+/C18	+/pDTG1	+	+	*P. putida*
55	Soligorsk (road-building enterprise)	P-9-like	–	+/NAH7^{++}	+	+	*Pseudomonas* sp. I

Notes: The presence (+) or absence (–) of the amplification product is indicated. NAH7^{++} differs from NAH7 and A88^{++} differs from A88 in having additional *Rsa*I sites.

* Plasmids were assigned to a certain IncP-9 subgroup according to the *Hae*III restriction pattern of the amplification products obtained with primer pairs repF–repR and korA3Fa–rep3Rc.

** Host bacteria were identified according to the *Rsa*I and *Msp*I restriction patterns of the 16S rDNA amplification products. *Pseudomonas* sp. I and II belong to belong to the fluorescent group. Strains of *Pseudomonas* sp. III do not produce the fluorescent pigment.

*** The plasmid of strain 15 contains two replicons, IncP-9 (subgroup η) and IncP-7.

pDTG1 and NAH7. The subgroup ζ plasmid of strain 19 had type A88 *nah*Ac, whereas the similarity between its *nah*G and type A88 was limited to the presence of an *Msp*I site. The *nah*G locus of strain 19 had an additional *Rsa*I site in the 232-bp fragment, and its cleavage yielded two fragments, 131- and 101-bp (Figs. 4a, 4b). Thus, in addition to the known types of *nah*Ac and *nah*G, we revealed two new *nah*G variants, orthologous to the type NAH7 and A88 genes.

Biodegradation plasmids of the IncP-9 group generally occur in fluorescent bacteria of the genus *Pseudomonas* [4, 5]. The species of the natural strains were tentatively identified by amplified 16S rDNA restriction analysis (ARDRA). For this purpose, 16S rDNA was amplified and digested with *Rsa*I and *Msp*I. The type *Pseudomonas* strains included *P. putida* BS203, *P. fluorescens* 2-79, *P. chlororaphis* 17411, *P. aeruginosa* PAK NP1, and *P. aereofaciens* 3084. Although ARDRA cannot determine the species exactly, it allows differentiation of well-known species, such as *P. putida* and *P. fluorescens*. We found that the natural hosts of the plasmids of IncP-9 subgroups ζ and η belonged to *P. fluorescens*, and the IncP-9-like replicons of strains 25 and 22, as well as δ plasmids of strains 4 and 35, could be inherited by *P. putida* and *P. fluorescens*. In addition, plasmids of subgroup δ (strains 5, 6, and 53) were found in the

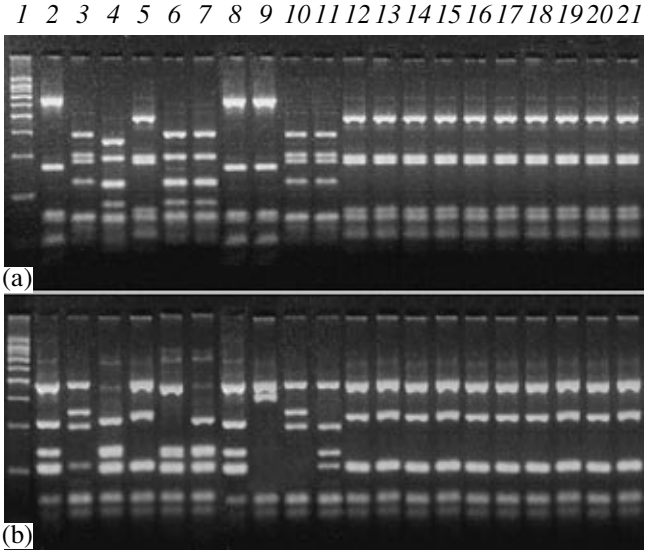

Fig. 4. Restriction enzyme analysis of the *nah*G amplification products with (a) *Msp*I and (b) *Rsa*I. Lane *1*, 100-bp ladder. Restriction patterns were obtained (*2–7*) for the known *nah*G sequences NAH7, A88, AN10, pDTG1, NKNS, and KF715, respectively, and (*8–21*) for the *nah*G amplification products synthesized with total DNAs of strains (*8*) 60 (ζ), (*9*) 55 (P-9-like), (*10*) 22 (P-9-like), (*11*) 19 (ζ), (*12*) 4 (δ), (*13*) 53 (δ), (*14*) 29 (δ), (*15*) 5 (δ), (*16*) 6 (δ), (*17*) 32 (ζ), (*18*) 15 (η), (*19*) 35 (δ), (*20*) 31 (δ), and (*21*) 25 (P-9-like).

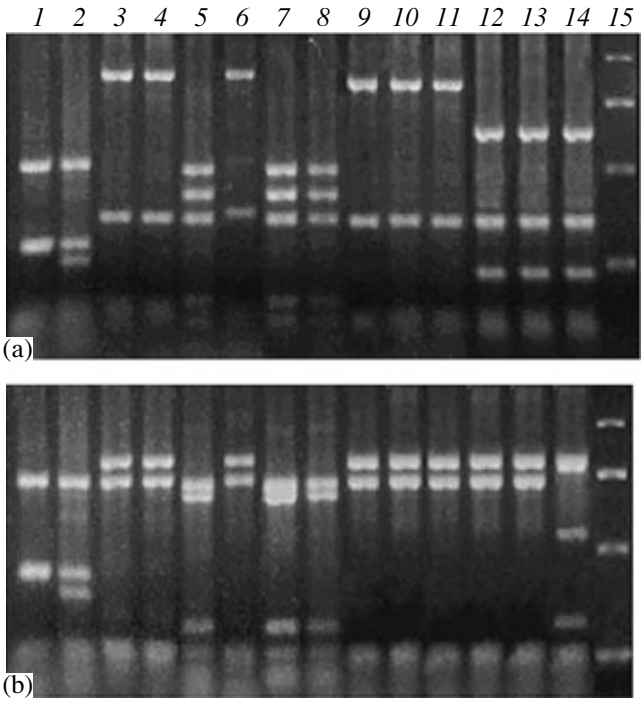

Fig. 5. Restriction enzyme analysis of the PCR products of the 16S rRNA genes with (a) *Msp*I and (b) *Rsa*I. The products were obtained with total DNAs of strains (*1*) 55 (P-9-like), (*2*) 29 (δ), (*3*) 35 (δ), (*4*) 15 (η), (*5*) 53 (δ), (*6*) 22 (P-9-like), (*7*) 5 (δ), (*8*) 6 (δ), (*9*) 60 (ζ), (*10*) 32 (ζ), (*11*) 19 (ζ), (*12*) 4 (δ), (*13*) 25 (P-9-like), and (*14*) 31 (δ). Lane *15*, 1-kb ladder.

same strains of an unidentified species. These strains do not belong to nonfluorescent *Pseudomonas*. Hence, these IncP-9 hosts are probably new and are of interest for studying the range of bacteria inheriting IncP-9 extrachromosomal elements. It is worth noting that one of the Nah plasmids of subgroup δ was found in *P. aeruginosa* strain 31. Bacteria of this species very seldom bear biodegradation plasmids. It is believed that they bear mainly antibiotic resistance IncP-9 plasmids [1, 2]. Another plasmid of subgroup δ (strain 29) and the IncP-9-like replicon of strain 55 were found in cells of different fluorescent strains of unknown species of the genus *Pseudomonas* (designated as *Pseudomonas* sp.; Table 5, Figs. 5a, 5b).

Thus, IncP-9 Nah plasmids found in the strains isolated in Belarus have diverse genetic systems controlling replication origin and naphthalene degradation. These plasmids can be inherited by cells of various fluorescent *Pseudomonas* species (*P. fluorescens, P. putida, P. aeruginosa*, and *P. species*) and by cells of nonfluorescent bacteria.

ACKNOWLEDGMENTS

This study was supported by an INTAS grant (no. 01-2383) and the Belarusian Ministry of Education (project no. 974).

REFERENCES

1. Kawakami Y., Mikoshiba E., Nagasaki S., et al. 1972. Prevelance of *Pseudomonas aeruginosa* strains prossesing R-factor in a hospital. *J. Antibiot*. **25**, 607–609.
2. Summers A.O., Jacoby G.A. 1978. Plasmid-determined reistance to boron and chromium compaunds in *Pseudomonas aeruginosa. Antemicrob. Agents Chemother.* **13**, 637–640.
3. Zheldakova R.A., Maksimova N.P., Kul'ba A.M., et al. 1985. Conjugative R plasmid of facultative methylotrophic bacteria *Pseudomonas* sp. *Mol. Genet. Mikrobiol. Virusol*. no. 1, 26–30.
4. Dunn N.W., Gunsalus I.C. 1973. Transmissible plasmids coding early enzymes of naphthaline oxidation in *Pseudomonas putida. J. Bacteriol*. **114**, 974–982.
5. Wong C.L., Dunn N.W. 1974. Transmissible plasmid coding for the degradation of benzoate and m-toluate in *Pseudomonas arvilla* mt-2. *Genet. Res*. **21**, 227.
6. Titok M.A., Maksimova N.P., Fomichev Yu.K. 1991. Characterization of R (IncP9) plasmid pM3 from a wide range of hosts. *Mol. Genet. Mikrobiol. Virusol*. no. 9, 9–13.
7. Yen K.M., Gunsalus I.C. 1982. Plasmid gene organization: Naphthalene/salicylate oxidation. *Proc. Natl. Acad. Sci. USA*. **79**, 874–878.
8. Greated A., Lambertsen L., Williams P.A., et al. 2002. Complete sequence of the IncP-9 TOL plasmid pWW0 from *Pseudomonas putida. Environ. Microbiol*. **4**, 856–871.
9. Dennis J.J., Zylstra G.J. 2004. Complete sequence and genetic organization of pDTG1, the 83-kb naphthalene degradation plasmid from *Pseudomonas putida* strain NCIB 9816-4. *J. Mol. Biol*. **341**, 753–768.
10. Greated A., Titok M.A., Krasowiak R. et al. 2000. Isolation and molecular analysis of the replication and stable maintenance functions of IncP-9 plasmid pM3. *Microbiology.* **146**, 2249–2258.
11. Boronin A.M., Starovoitov I.I., Borisoglebskaya A.N., et al. 1976. A *Pseudomonas putida* plasmid controlling initial stages of naphthalene oxidation. *Dokl. Akad. Nauk SSSR*. **228**, 962–965.
12. Kochetkov V.V., Boronin A.M. 1984. Comparative analysis of plasmids controlling naphthalene biodegradation by a *Pseudomonas* culture. *Mikrobiologiya*. **53**, 639–644.
13. Esikova T.Z., Grishchenko V.G., Kulakov L.A., et al. 1990. Incompatibility groups of plasmids providing for ε-caprolactam degradation by bacteria of the genus *Pseudomonas. Mol. Genet. Mikrobiol. Virusol*. no. 4, 25–28.
14. Sagai H., Krcmery V., Hasuda K. et al. 1974. R factor mediated resistance to aminoglyciside antibiotics in *Pseudomonas aeruginosa. Jpn. J. Microbiol*. **19**, 427–432.
15. Evans C.G.T., Herbert D., Tempest D.M. 1970. The continuous cultivation of microorganisms. In: *Methods in Microbiology.* Ed. Norris J.R. New York: Academic, pp. 277–327.
16. Te Riele H., Michel B., Ehrlich S.D. 1979. Single-stranded plasmid DNA in *Bacillus subtilis* and *Staphylo-*

coccus aureus. Proc. Natl. Acad. Sci. USA. **8**, 2541–2545.

17. Maniatis, T., Fritsch, E.F., Sambrook, J. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Lab. Pres.
18. Greated A., Thomas C.M. 1999. A pair of PCR primers for IncP-9 plasmids. *Microbiology.* **145**, 3003–3004.
19. Weisburg W.G., Barnes S.M., Pelletier D.A., et al. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol*. **73**, 697–703.
20. Ferrero M., Llobert-Brossa E., Lalucat J., et al. 2002. Coexistence of two distinct copies of naphthalene degradation genes in *Pseudomonas* strains isolated from the western Mediterranean region. *Appl. Environ. Microbiol*. **68**, 957–962.
21. Wilkstrom P., Wilklund A., Anderson A.C., et al. 1996. DNA recovery and PCR quantification of catechol-2,3-dioxygenase genes from different soil types. *J. Biotechnol*. **52**, 107–120.
22. Krasowiak R., Smalla K., Sokolov S., et al. 2002. PCR primers for detection and characterisation of IncP-9 plasmids. *FEMS Microbiol. Ecol*. **42**, 217–225.
23. Sokolov S.L., Kosheleva I.A., Kovalenko N.P., et al. 2003. Basic replicons of biodegradation plasmids of *Pseudomonas* strains. *Abstr. 1st FEMS Congress of European Microbiologists. 2003, Ljubljana, Slovenia*, p. 186.
24. Sevastyanovich Y.R., Krasowiak R., Bingle L. et al. 2004. Phylogeny and diversity of the IncP-9 plasmids of *Pseudomonas*. In: *Plasmid Biology.* Corfu, Greece, p. 134.
25. Kochetkov V.V., Boronin A.M. 1985. Naphthalene biodegradation plasmids incompatible with plasmids of groups IncP-2 and IncP-7. *Genetika*. **21**, 522–529.
26. Espinosa M., Cohen S., Couturier M., et al. 2000. Plasmid replication and copy number control. In: *The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread*. Ed. Thomas C.M. Amsterdam: Harwood, pp. 1–47.
27. Surtees J.A., Funnell B.E. 2003. Plasmid and chromosome traffic control: How ParA and ParB drive partition. *Curr. Top. Dev. Biol*. **56**, 145–180.
28. Senthilo V.S., Perebituk A.N., Zehnder A.J.B., et al. 2000. Molecular diversity of plasmid bearing genes that encode toluene and xylene metabolism in *Pseudomonas* strains isolated from different contaminated sites in Belarus. *Appl. Environ. Microbiol*. **66**, 2842–2852.
29. Stuart-Keil K.G., Hohnstock A.M., Drees K.P. et al. 1998. Plasmids responsible for horizontal transfer of naphthalene catabolism genes between bacterial at a coal tar contaminated site are homologous to pDTG1 from *Pseudomonas putida* NCIB 9618-4. *Appl. Environ. Microbiol*. **64**, 3633–3640.
30. Timmis K.N., Lechrbach P.R., Harayama S., et al. 1985. Analysis and manipulation of plasmid-encoded pathways for the catabolism of aromatic compounds by soil bacteria. In: *Plasmids in Bacteria*. Eds Helinski D.K., Clewel D.B., Cohen S.N., Jakson D.A., Hollander A. Plenum, pp. 156–167.
31. Izmalkova T.Yu., Sazonova O.I., Sokolov S.L., et al. 2005. Diversity of genetic systems of naphthalene biodegradation in *Pseudomonas fluorescens* strains. *Mikrobiologiya*. **74**, 60–68.