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EXPERIMENTAL ARTICLES

Inheritance of Biodegradation Plasmids in the Cells of Homo- and Heterologous Hosts

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Abstract—A systematic analysis of the inheritance of D plasmids of the IncP-9 group (α-, β-, γ-, δ-, ε-, ζ-, η-, and θ-subgroups), IncP-7, as well as of those of undefined systematic affiliation in the cells of homologous (*Pseudomonas putida*) and heterologous (*Escherichia coli*) hosts was performed for the first time. For this purpose, mini-Tn5 transposons determining resistance to kanamycin (or streptomycin) were introduced into all the D plasmids under study. It has been established that all IncP-9 plasmids can be transmitted to the cells of a heterologous host *E. coli* (with the exception of plasmid pSVS15 from θ-subgroup). IncP-7 plasmids and those of undefined systematic affiliation do not possess this property and can be transmitted and stably inherited only in *P. putida*. The distinctive feature of most IncP-9 plasmids (α-, β-, δ-, ε-, and ζ-subgroups) is strict dependence of their inheritance on the temperature factor. At 37°C, the plasmids of δ-, ζ-, and θ-subgroups are unstable in *P. putida* cells, while in *E. coli* nearly all plasmids of this systematic group are unstable. The exceptions are the plasmids of η- and γ-subgroups. Inheritance of these plasmids does not depend on temperature. At 28°C and 37°C, the η plasmid is not maintained stably (inheritance stability is 2%), while the γ plasmid has almost 100% stability.

Key words: plasmid, IncP-7, IncP-9, *Pseudomonas putida, Escherichia coli.*

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Horizontal and vertical transfer of genetic material is one of the most important factors facilitating adaptation of microbial communities to changing environmental conditions [1]. In this respect, D plasmids are of interest, as they transfer characteristics related to the biodegradation of organic compounds between bacteria of different taxonomic groups by way of conjugation [2, 3]. At the same time, the transposon location of biodegradation genes has been demonstrated for some D-plasmids (pWWO, NAH7, and NPL-1) [4, 5]. That feature contributes to the distribution of these determinants among environmental populations via incorporation into bacterial genomes or other plasmids and thus promotes bioremediation of polluted soils [6] and facilitates for the evolution of these genetic systems. The study of horizontal transfer of biodegradation genes requires marked plasmids for tracing their distribution in model and environmental microbial populations.

The goal of the present work was to study the ability of biodegradation plasmids carrying the marker of antibiotic resistance (mini-Tn5) to be maintained in the cells of homo- and heterologous hosts. This study is one of the initial steps of the search for the optimal bacterial host of biodegradation plasmids for creation of effective destructor strains. It provides a basis for various genetic manipulations in order to study the mechanisms of inheritance of D plasmids and their application in construction of vector systems for molecular cloning.

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MATERIALS AND METHODS

Bacterial strains and plasmids are characterized in Tables 1 and 2.

Media. Bacteria were grown in LB broth and M9 minimal salt medium [7]. The agarized media contained 1.5% agar. Glucose (0.2%) or naphthalene (100 μg/ml) were used as carbon sources. Amino acids, bases, and vitamins were added in concentrations of 20, 10, and 1 μg/ml, respectively. Commercial preparations of antibiotics (kanamycin, streptomycin, chloramphenicol, and nalidixic acid) were used in the final concentrations of 25, 50, 25, and 100 μg/ml, respectively.

Total DNA was isolated by the sarcosyl method [8].

Identification of plasmids. Plasmids of native strains were affiliated with certain classification groups by the classical incompatibility test [9].

IncP-9 and IncP-7 plasmids were searched among naphthalene-utilizing bacteria by polymerase chain reaction. Primers repF (5'-CCA GCG CGG TAC WTG GG-3') and repR (5'-GTC GGC AIC TGC TTG AGC TT-3') pro-

Table 1. Characterization of bacteria used in the work

Strain	Characterization	Source
*P. putida*KT2442	Prototroph, Rif^R	I. A. Kosheleva, IBPM RAS, Pushchino, Russia
P. putida BS394	*cys*$^-$, Nal^R, Sm^R	same
P. putida M2	*met*$^-$, Nal^R	Collection of the Department of Genetics, Belarusian State University
Pseudomonas sp. NL4	Environmental strain, prototroph, Nah^+	same
Pseudomonas sp. NL15	Environmental strain, prototroph, Nah^+	"
Pseudomonas sp. NL29	Environmental strain, prototroph, Nah^+	"
Pseudomonas sp. NL61	Environmental strain, prototroph, Nah^+	"
Pseudomonas sp. AL1	Environmental strain, prototroph, Nah^+	"
Pseudomonas sp. AL2	Environmental strain, prototroph, Nah^+	"
P. aeruginosa ML4262	*his*308, *trp*6, *ilv*309, *met*316, Rif^R	I. A. Kosheleva, IBPM RAS, Pushchino, Russia
P. aeruginosa PAO1	*cml*2	A. E. Filonov, IBPM RAS, Pushchino, Russia
E. coli K12 C600-2	*leu*B6, *thr*1, *thi*1, *lac*Y1, *gal*K2, *ton*A21, *sup*E44, Nal^R, Tc^R (Tn10)	Collection of Laboratory of Molecular Genetics of Bacteria, BSU
E. coli C2110	*his*$^-$, *pol*A	same
E. coli S17–1	*pro*$^-$, *thi*$^-$, λ-*pir*, *hsd*R, *rec*A	"

vided amplification of the *rep* gene of IncP-9 plasmids (480 bp) at: 94°C, 5 min (1 cycle); 94°C, 1 min, 54°C, 1 min, and 72°C, 1 min (30 cycles); and 72°C, 10 min (1 cycle) [10]. The *rep* region of IncP-7 plasmids (524 bp) was amplified using primers rep7f (5'-CCC TAT CTC ACG ATG CTG TA-3') and rep7r (5'-GCA CAA ACG GTC GTC AG-3') at: 94°C, 5 min (1 cycle); 94°C, 30 s, 54°C, 30 s, and 72°C, 1 min (30 cycles); and 72°C, 5 min (1 cycle) [11].

Restriction of amplification products was performed using the MspI enzyme under the conditions recommended by manufacturer (Fermentas, Lithuania). The DNA Ladder Mix standard reference (Fermentas, Lithuania) was used to determine fragment sizes.

Transposon mutagenesis was carried out by the method proposed in [12].

Replica technique matings. Donor bacteria were applied to the surface of rich agarized medium and grown for 18 h. Then they were transferred to a medium with the lawn of recipient cells by the method of replicas. After 18 h of incubation, the formed colonies were replicated on the selective media. The results were scored after 48–72 h.

Matings on membrane filters. The cultures of donor and recipient bacteria from the exponential growth phase were condensed 100-fold, mixed in a 1 : 1 ratio, and applied to nitrocellulose filters (Synpor 6, pore size 0.45 μm, Czech Republic) placed onto the surface of complete or minimal agarized media. Bacteria were incubated for 3–18 h, and then the cells were washed off with physiological saline and inoculations on selective media were made from appropriate dilutions. The results were scored after 48–72 h of incubation at 28°C. The frequency of transfer of plasmid markers was determined by the ratio of the number of transconjugates to the total number of donor cells.

Determination of plasmid inheritance stability. Plasmid inheritance stability was tested by cultivation of plasmid-bearing bacteria (initial quantity 10^3 cells/ml) under nonselective conditions (LB broth) to the stationary growth phase at different temperatures (28 and 37°C) followed by planting on rich agarized medium and testing the formed colonies for the integrity of plasmid markers.

RESULTS AND DISCUSSION

One hundred and three stains utilizing naphthalene as a sole carbon and energy source have been isolated from the sites of excessive pollution with organic compounds. The capability for naphthalene utilization is known to be partially or fully determined by extrachromosomal genetic elements of groups IncP-9, IncP-7, and IncP-2 [13]. In the cells of the isolated strains, the plasmids were identified by polymerase chain reaction with specific primers that provided amplification of the *rep* regions of IncP-7 and IncP-9 plasmids. Total DNA was isolated from the cells of environmental isolates and used as a PCR template. It was established that 62 strains carried plasmids belonging to the IncP-9 group, 4 strains had plasmids of the IncP-7 group, 3 strains had plasmids bearing the replicons of both IncP-9 and IncP-7 groups, and the remaining 34 strains carried plasmids of undefined affiliation. For a number of

Table 2. Characterization of plasmids used in the work

Plasmid	Characterization	Source
pM3	Tc^R, Sm^R (IncP-9, α-subgroup)	Collection of Laboratory of Molecular Genetics of Bacteria, BSU
pBS101	Nah^+ (IncP-9, β-subgroup)	I. A. Kosheleva, IBPM RAS, Pushchino, Russia
pBS267	Cap^+ (IncP-9, γ-subgroup)	same
pNL4	Nah^+ (IncP-9, δ-subgroup)	Collection of the Department of Genetics, BSU
R2	Km^R, Ap^R, Sm^R, Su^R, Cb^R, Uvr^R (IncP-9, ε-subgroup)	same
pNL29	Nah^+ (IncP-9, ζ-subgroup)	″
pNL15	Nah^+ (IncP-9, η-subgroup)	″
pSVS15	Tol^+ (IncP-9, θ-subgroup)	V. S. Senchilo, Institute of Genetic and Cytology, National Academy of Sciences, Republic of Belarus
Rms148	Sm^R (IncP-7)	Collection of Laboratory of Molecular Genetics of Bacteria, BSU
pAL1	Nah^+ (IncP-7)	same
pAL2	Nah^+ (IncP-7)	″
pBS33	Sm^R Hg Chr Ter Uv Tra^+ (IncP-2)	A. E. Filonov, IBPM RAS, Pushchino, Russia
pNL61	Nah^+ (undefined group)	Collection of the Department of Genetics, BSU
pUT/Km	Tra^+, miniTn5 (Km^R, xylE)	same
pUT/Sm	Tra^+, miniTn5 (Sm^R)	″
pBS101-2	pBS101::miniTn5 (Km^R)	Obtained in this work
pBS267-19	pBS267::miniTn5 (Km^R)	same
pNL4-1	pNL4::miniTn5 (Km^R)	″
pNL29-1	pNL29::miniTn5 (Km^R)	″
pNL15-13	pNL15::miniTn5 (Km^R)	″
pSVS15-29	pSVS15::miniTn5 (Km^R)	″
pNL61-1	pNL61::miniTn5 (Sm^R)	″
pAL1-2	pAL1::miniTn5 (Km^R)	″
pAL2-2	pAL2::miniTn5 (Km^R)	″

strains that contained plasmids of different classification groups, the results of polymerase chain reaction were confirmed by the classical incompatibility test. For this purpose, plasmids Rms148 (IncP-7), pM3 (IncP-9), and pBS33 (IncP-2) were used as the type ones (Table 3).

As seen in Table 3, plasmids pNL4, pNL15, and pNL29 were affiliated with the group IncP-9 according to PCR results and were incompatible with the plasmid pM3 of the same group. These plasmids, however, were retained with 98–100% frequency after transfer of plasmid Rms148 (incompatibility group P-7) into the above bacterial strains. Plasmids pAL1 and pAL2 referred to the group IncP-7 could be inherited along with plasmid pM3 (93–99%) and were almost completely lost upon introduction of plasmid Rms148 into the cells. Plasmids that were not referred to the P-7 or P-9 group according to PCR results remained in the cells with 100% frequency after transfer of plasmids Rms148, pM3, and pBS33. Thus, on the basis of the classical incompatibility test and polymerase chain reaction, it has been established that plasmids pNL4, pNL15, and pNL29 belong to group IncP-9, plasmids pAL1 and pAL2 belong to group IncP-7, and plasmid pNL61 does not belong to any of the known incompatibility groups typical of the naphthalene biodegradation plasmids.

Restriction and sequence analysis of amplification products for IncP-9 plasmids revealed polymorphisms within the *rep* gene and the site of replication initiation *ori*V. Based on these results, eight classification subgroups were isolated [11, 14, 15]. Restriction analysis of the amplification products of the *rep* regions of plasmids pAL1 and pAL2 (group IncP-7, environmental strains) revealed the difference in the nucleotide sequences of their replication initiation systems from that of the drug resistance plasmid Rms148, which is the type one for this classification group (figure). This data could indicate that polymorphisms of the DNA sequences within the systems of initiation of replication of extrachromosomal genetic elements comprising a single systematic group is a typical phenomenon associated with the divergence of vitally important systems in the course of evolutionary transformations of their hereditary material [16].

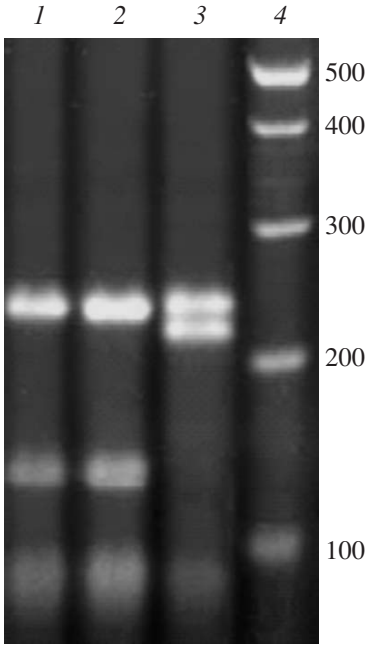
Table 3. Compatibility of plasmids of environmental strains with the type plasmids of *Pseudomonas* bacteria from groups IncP-7, IncP-9, and IncP-2

Donor	Analyzed plasmid of the recipient strain	Transfer frequency of donor plasmid	Preservation of analyzed plasmid in transconjugant cells, %
P. aeruginosa ML4662/Rms148 (IncP-7)	*Pseudomonas* sp./pNL4	1.1×10^{-3}	99
	Pseudomonas sp./pNL15	5.3×10^{-4}	98
	Pseudomonas sp./pNL29	7.8×10^{-6}	100
	Pseudomonas sp./pNL61	2.2×10^{-6}	100
	Pseudomonas sp./pAL1	4.5×10^{-6}	0
	Pseudomonas sp./pAL2	5.5×10^{-6}	3
E. coli C600/pM3 (IncP-9)	*Pseudomonas* sp./pNL4	1.8×10^{-3}	0
	Pseudomonas sp./pNL15	3.2×10^{-3}	0
	Pseudomonas sp./pNL29	8.1×10^{-6}	0
	Pseudomonas sp./pNL61	8.1×10^{-3}	100
	Pseudomonas sp./pAL1	1.5×10^{-2}	99
	Pseudomonas sp./pAL2	2.5×10^{-2}	93
P. aeruginosa PAO1/pBS33 (IncP-2)	*P. putida* KT2442/pNL61	1.2×10^{-6}	100

It is known that replication initiation systems (of the *rep* region) are the main functional units which determine the ability of any extrachromosomal genetic element to be inherited in a bacterial cell. For some plasmids (including IncP-1 and IncP-4), there are actually no genetic barriers. In many respects, this is due to the peculiar organization of the *rep* regions, which enables their maintenance in cells of various gram-negative and gram-positive bacteria and even in some eukaryotic organisms [17–19]. IncP-9 plasmids actually have not been characterized in this respect. The absence of data concerning the spectrum of IncP-9 plasmid hosts is primarily due to the fact that the most attention has been paid to the study of organization of D plasmids, namely, the molecular genetic mechanisms of biodegradation. However, utilization of certain organic compounds is known to be restricted to the plasmid-bearing strains of *Pseudomonas* bacteria [20]. The absence of selective markers in these extrachromosomal genetic elements (the genes of biodegradation are not expressed in heterologous systems) hinders the study of their inheritance in the cells of microorganisms of other taxonomic groups.

It has been shown that R-plasmid pM3 from group IncP-9 (the α-subgroup) can be transferred by conjugation to the cells of various gram-negative bacteria. The distinctive feature of plasmid pM3 was the temperature sensitivity of its inheritance in bacteria of the family *Enterobacteriaceae*. This was demonstrated by 100% elimination of pM3 after 20 generations under growth in nonselective conditions at 37°C [21].

As has been mentioned above, IncP-9 plasmids are characterized by the polymorphism of replication initiation regions. It seemed interesting to find out the differences in stability of inheritance of plasmids from different IncP-9 subgroups by the cells of homologous (*P. putida*) and heterologous (*E. coli*) hosts in comparison with plasmid pM3. The conjugation ability of D plasmids of group IncP-7 (pAL1, pAL2) and those of undefined systematic affiliation (pNL61) was to be



Results of restriction analysis of amplification products of the *rep* region of IncP-7 plasmids. Amplification products were treated with *Msp*I restriction endonuclease and were obtained with the use of the total DNA of plasmid-bearing strains as a template: (*1*) pAL1; (*2*) pAL2; and (*3*) Rms148. Lane *4* corresponds to the DNA ladder mix.

Table 4. Inheritance of plasmids of different incompatibility groups in *P. putida* and *E. coli*

Plasmid	Incompatibility group	Subgroup	Frequency of plasmid transfer between *P. putida* bacteria*	Stability of inheritance in *P. putida* KT2442, %		Frequency of plasmid transfer into *E. coli* K12 C600-2**	Stability of inheritance in *E. coli* K12 C600-2, %		Frequency of plasmid transfer between *E. coli* bacteria***
				28°C	37°C		28°C	37°C	
pM3	P-9	α	9.7×10^{-2}	100	100	5.0×10^{-7}	78	0	1.2×10^{-3}
pBS101-2		β	3.0×10^{-2}	100	100	2.8×10^{-3}	70	0	2.3×10^{-3}
pBS267-19		γ	4.8×10^{-3}	100	100	4.0×10^{-5}	100	98	4.8×10^{-4}
R2		ε	2.3×10^{-2}	100	100	2.0×10^{-9}	65	0	4.5×10^{-5}
pNL15-13		η	1.0×10^{-3}	100	100	3.9×10^{-4}	3	2	4.1×10^{-3}
pNL4-1		δ	7.0×10^{-4}	100	0.05	2.3×10^{-7}	68	0	1.3×10^{-5}
pNL29-1		ζ	9.9×10^{-5}	100	50	1.1×10^{-7}	97	0	9.8×10^{-6}
pSVS15-29		θ	1.2×10^{-9}	100	46	0	-	-	-
pAL1-2	P-7		4.9×10^{-2}	100	100	0	-	-	-
pAL2-2			8.2×10^{-3}	100	100	0	-	-	-
pNL61-1	not determined		4.4×10^{-3}	100	100	0	-	-	-

Notes: * The donor strain was *P. putida* KT2442 and the recipient strains were *P. putida* BS394 and *P. putida* M2 (for plasmid pNL61-1).
** The donor strain was *P. putida* KT2442.
*** The donor strain was *E. coli* K12 C600-2 and the recipient strain was *E. coli* C2110.

determined. For this purpose, at the first stage the mini-Tn5 transposon (determining resistance to kanamycin) was inserted into IncP-9 plasmids, which determined the biodegradation of naphthalene in pBS101 (β-subgroup), pNL4 (δ-subgroup), pNL29 (ζ-subgroup), and pNL15 (η-subgroup); caprolactam: pBS267 (γ-subgroup); toluene: pSVS15 (θ-subgroup); and Nah plasmids pAL1 and pAL2 of group IncP-7. Since the environmental strain bearing plasmid pNL61 was resistant to kanamycin, the mini-Tn5 transposon (determining resistance to streptomycin) was inserted into this plasmid. Introduction of additional phenotypic markers made it possible to select transconjugates that had assimilated the extrachromosomal elements under study. In addition to biodegradation plasmids, R-plasmid R2 (IncP-9, ε-subgroup) was used; plasmid pM3 (IncP-9, α-subgroup) was applied as a control.

In these experiments, the mini-Tn5 transposon determining resistance to kanamycin (or streptomycin, in case of the plasmid pNL61), as a part of suicidal vector *pUT*, was introduced into the cells with biodegradation plasmids. The frequency of transposition was 10^{-7}–10^{-8}. The obtained Km^R variants (or Sm^R variants) were mated with recipient bacteria *P. putida* KT2442, and the transposon-bearing plasmids were selected. All transposon-containing plasmids retained the ability to degrade organic compounds. Later on, plasmids of different systematic groups were transferred into cells of *E. coli* C600-2 by conjugation (Table 4). It was established that the transfer frequency of IncP-9 plasmids varied greatly from 10^{-3} to 10^{-9}. No transfer of plasmid pSVS15-29 (IncP-9, θ-subgroup), plasmids pAL1 and pAL2 (IncP-7), or plasmid pNL61 (undefined incompatibility group) into *E. coli* cells was registered. The inability of these plasmids to be transferred into heterologous host cells may be due to a peculiar organization of their replication systems, which do not provide inheritance of these replicons in a foreign genetic environment. This explanation is certainly valid for plasmids pAL1 and pAL2 (IncP-7), because the extrachromosomal elements of this classification group are known to have a narrow range of bacterial hosts. Plasmid pNL61 of an undefined systematic affiliation can also be referred to this type of plasmids. As for plasmid pSVS15, it can only be stated that this is the only representative of the IncP-9 group unable to be transferred into *E. coli* cells.

The presence in *E. coli* bacteria of the transferred extrachromosomal genetic elements of the IncP-9 group was additionally confirmed by restriction analysis of the products of amplification of their *rep* regions. It was obtained using a template of total DNA isolated from the cells of native hosts and from *E. coli* cells containing the initial and Tn variants of the abovementioned plasmids, respectively. The transfer of IncP-9 plasmids between *E. coli* cells was shown. The frequency of the conjugation transfer in these matings was 4.1×10^{-3} to 9.8×10^{-6} (Table 4).

Analysis of the preservation of the studied plasmids in *E. coli* cells revealed different degrees of their inher-

itance stability. It was shown that all the studied plasmids could be maintained in recipient bacteria to some extent for 20 generations when grown without antibiotics (at 28°C). The frequency of inheritance stability varied from 2% (for pNL15 of the η-subgroup) to 100% (for pBS267 of the γ-subgroup). Plasmids pM3 (α-subgroup), R2 (ε-subgroup), pBS101 (β-subgroup), pNL29 (ζ-subgroup), and pNL4 (δ-subgroup) were completely lost during cultivation of plasmid-bearing *E. coli* cells at 37°C, while plasmid pNL15 (η-subgroup) was maintained at 2% frequency and plasmid pBS267 (γ-subgroup) was inherited stably (98–100%) (Table 4).

It was shown that all of the plasmids studied were transferred via conjugation matings in the bacterial system of *P. putida* at a frequency of 9.7×10^{-2} to $1.2 - 10^{-9}$ (Table 4). In the type strain *P. putida* KT2442, all plasmids were inherited stably at 28°C. However, plasmids of δ-, ζ-, and θ-subgroups of IncP-9 were lost at a frequency of 50–99% when the cultivation temperature was increased to 37°C (Table 4).

These results demonstrate that the property of thermal instability manifested in the cells of homo- and heterologous hosts is not typical of all the plasmids of the P-9 incompatibility group. This may be the result of the revealed differences in the structure of the replication initiation regions of these extrachromosomal elements. However, the nucleotide sequence of the *rep* region of plasmid pM3 is more similar to that of plasmid pBS267 than to that of pNL4 [15] and, consequently, the inheritance of plasmids pM3 and pBS267 should have been less different. In fact, the opposite is true: the plasmids, which are closer in inheritance stability, differ to a greater extent. Nevertheless, the revealed differences demonstrate that the temperature instability is most likely caused by the peculiarities of the functioning of the plasmid replicons proper; this characteristic is only indirectly influenced by the effect of the host cell genome on certain stages of the replication of plasmid DNA and on the distribution of newly formed molecules between daughter cells. This process may also involve the genetic systems of the plasmid genome, which are only indirectly connected with the process of replication initiation.

The property of "thermal instability" revealed for some IncP-9 plasmids may be of interest in two respects. The first is the comparison of their replicative functions with those of the known native heat-sensitive plasmids, e.g., plasmid Rts1 of *Proteus vulgaris* [22] and pPS10 of *Pseudomonas savastanoi* [23]. It is probable that the nature of heat sensitivity in them is identical. Moreover, the revealed properties may be useful for the application of these extrachromosomal elements in genetic analysis of the members of the family *Enterobacteriaceae*, e.g., for creation of vectors of integrative suppression for the targeted introduction of mutations into bacterial chromosome on their basis. Besides, the transposon-containing variants of D plasmids obtained in this work can be used in the search for optimal bacterial hosts for the creation of effective destructor strains of organic compounds and can be used for the analysis of the transfer of biodegradation genes between bacterial populations in model and native soil systems.

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