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EXPERIMENTAL ARTICLES

Salicylate Degradation by *Pseudomonas putida* Strains not Involving the "Classical" *nah2* Operon

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Abstract—Genetic systems for salicylate catabolism were analyzed in 12 strains of *Pseudomonas putida*, isolated from polluted soil samples collected in the Murmansk and Tula oblasts. All of the studied *P. putida* strains utilize salicylate in the *ortho*-pathway of catechol cleavage without employing the enzymes of the "classical" *nah2* operon. The data demonstrates that salicylate degradation in the studied strains is performed with the involvement of the salicylate hydroxylase gene analogous to the *nahU* gene of strain *P. putida* ND6. New variants of salicylate hydroxylase genes *nahG1* and *nahU* were found.

Key words: fluorescent pseudomonads, biodegradation, salicylate catabolism genes.

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Salicylate is widespread in nature, being an important part of metabolism in animals, plants, and microorganisms. Salicylate is the key intermediate in the biodegradation pathways of naphthalene, phenanthrene, and naphthoquinones and plays an important role in regulating the expression of some bacterial genes [1–4]. At this time, the biochemical pathways of salicylate degradation in microorganisms have been well studied. Bacteria of the genus *Pseudomonas* can utilize salicylate in two ways, transforming it either into catechol, using salicylate hydroxylase [5] or into gentisic acid, using salicylate 5-hydroxylase [6, 7]; the former variant of salicylate transformation is more widespread (Fig. 1). Genetic control of salicylate degradation through the *meta*-pathway of catechol degradation was studied in detail on the *nah2*-operon of *P. putida* G7 plasmid NAH7 [8]. The catabolic genes of plasmid NAH7 are arranged into two operons: *nah1* (*nahAaAbAcAdBFCED*) controls naphthalene transformation into salicylate and *nah2* (*nahGTHINLOMKJ*) controls salicylate utilization to the intermediates of the tricarboxylic acid cycle. The expression of both operons is under the positive control of the regulatory gene *nahR* [9]. Genetic control of salicylate catabolism in fluorescent pseudomonads is very conservative. At present, the data on the distribution of the genes for salicylate catabolism in this group of microorganisms, concerns mainly the genes controlling salicylate degradation by the catechol cleavage *meta*-pathway.

The goal of the present work was to search for and study the genes of salicylate degradation, different from the archetype of plasmid NAH7 in the strains of fluorescent pseudomonads.

MATERIALS AND METHODS

Salicylate-degrading strains isolated by the method of enrichment cultures from polluted soils as described

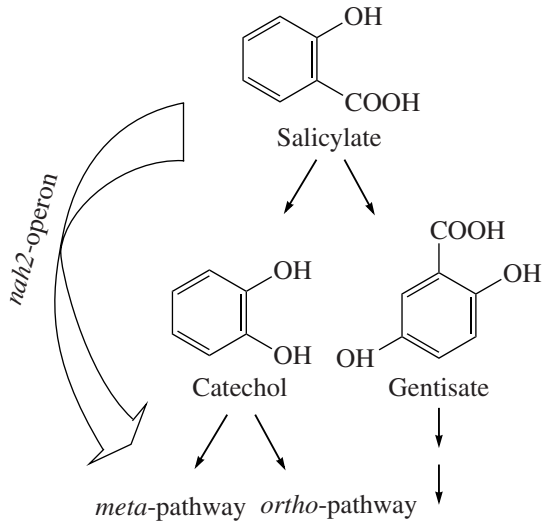

Fig. 1. Biochemical pathways of salicylate degradation by *Pseudomonas* bacteria.

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Table 1. Bacterial strains and plasmids used in the work

Strain	Characteristics*	Plasmid	Source
P. putida PpG7	Nah^+Sal^+	NAH7, IncP-9β (83 kb)	I.C. Gunsalus,USA
P. putida g15 F	$Sal^+Gen^+Tol^+$	pG15	Soil samples from the highway shoulder, Kandalaksha, Murmansk oblast
P. putida g20 F	Sal^+	pG20	
P. putida g24 F	Sal^+Tol^+	pG24	
P. putida NS7	$Sal^+Cap^+BTEX^+Tol^+Rif^R$	pNS7-1, pNS7-2	Soil samples from the territory of the Shchekinoazot Company, Shchekino, Tula oblast
P. putida NS11	$Sal^+Cap^+Ben^+Gen^+Tol^+Rif^R$	pNS11-1, pNS11-2	″
P. putida NS12	$Sal^+Cap^+BTEX^+Ben^+Tol^+Rif^R$	pNS12-1, pNS12-2	″
P. putida NS15	$Sal^+Cap^+Gen^+Rif^R$	pNS15	″
P. putida NS17	$Sal^+Cap^+Rif^R$	pNS17	″
P. putida NS18	Sal^+Cap^+	pNS18	″
P. putida NS20	$Sal^+Cap^+Gen^+Rif^RSm^R$	pNS20-1, pNS20-2	″
P. putida NS22	$Sal^+Cap^+Ben^+Tol^+Rif^R$	pNS22-1, pNS22-2	″
P. putida NS24	$Sal^+Cap^+Ben^+Tol^+Rif^RSm^R$	pNS24	″
P. putida BS202	$Nah^+Sal^+Gen^+$	pNPL-1, IncP-9β (100 kb)	Laboratory collection, isolated from coal mine soil samples, Makeevka, Ukarine
P. putida KT2442	Nah^-Sal^-*gfpKm*^{R}	–	Kindly provided by K. Smalla (Germany)

* The ability to utilize, as the sole carbon and energy source: naphthalene (Nah^+), salicylate (Sal^+), caprolactam (Cap^+), gentisate (Gen^+), toluene (Tol^+), benzene (Ben^+), and BTEX ($BTEX^+$); BTEX is a mixture of monocyclic hydrocarbons of benezene, toluene, ethylbenzene, and xylene; Rif^R, Sm^R, and Km^R: resistance to rifampicin, streptomycin, and kanamycin, respectively.

previously [10], were used in the work. The bacterial strains and plasmids used are presented in Table 1. The strains were grown overnight in a shaker, in an LB medium [11] and in an Evans mineral medium [12] at 28°C. Salicylate or succinate (1 g/l) was used as a carbon source.

Total bacterial DNA was isolated according to [13]. DNA concentration was determined in a TKO-100 fluorimeter (Hoefer Scientific Instruments, United States) with Hoechst 33258 dye (Bio-Rad, United States) according to the manufacturer's protocol.

Plasmid DNA was visualized by the method of field inverted gel electrophoresis (FIGE). Plasmid DNA was prepared as agarose-block inserts according to [14]. Inverted electrophoresis was performed according to the protocol of the manufacturer (Bio-Rad, United States).

Conjugation transfer of the plasmids into a recipient strain *P. putida* KT2442 was carried out according to [15]. Transconjugants were tested for the presence of plasmids and the *gfp* gene.

Enzymes and buffer solutions used in the work were produced by Fermentas (Lithuania). All procedures were performed in accordance with the recommendations of the manufacturer.

Polymerase chain reaction (PCR) was performed in a Mastercycler Gradient device (Eppendorf, Germany). The reaction was conducted under standard conditions, with the final concentration of deoxyribonucleotide triphosphates, 200 μM; $MgCl_2$, 1.5 mM; and, in some cases, 5% dimethyl sulfoxide (DMSO) (Sigma, United States). Oligonucleotide primers used are presented in Table 2.

DNA electrophoresis was performed in 0.8% agarose in a 0.5× Tris–borate buffer by the standard procedure [11]. DNA was visualized by gel staining in an ethidium bromide solution.

Cell-free extracts were obtained by the destruction of frozen biomass in an IBPM press (Russia). Cell debris was precipitated by centrifugation (11000 g, 10 min). The activities were measured as follows: salicylate hydroxylase as in [4], catechol-1,2-dioxygenase as in [20], and cat-

Table 2. PCR primers used in the work

Gene	Primer	Nucleotide sequence	PCR product size, bp	Reference
	BOXA1R	5'-CTA CGG CAA GGC GAC GCT GAC G-3'	–	16
16S pPHK	8f 1492r	5'-AGA GTT TGA TCM TGG CTC AG-3' 5'-TAC GGH TAC CTT GTT ACG ACT T-3'	1484	17
nahG	shc1_up shc1_lo	5'-CGG CKT THG GTG ARG TCG GTG C-3' 5'-GGC GAG GAA RTA GGC GTC CTC AAG 3'	893	18
nahG1	KT_136f KT_1129r	5'-ATT CAT ATC GGC CCT AAC G-3' 5'-CAA GCTGCT GCC CAT AGA G-3'	994	Present work
nagG	458f 1224r	5'-CCT GAC CAA GCT SAA GGT-3' 5'-CGT YTC GGT SAC CAT GTG-3'	766	15
nahR	nahR_1f nahR_585r	5'-ATG GAA CTG CGT GAC CTG G 3' 5'-GCC GTA GGA ACA GAA GCG 3'	585	18
nahH	23OF 23OR	5'-ATG GAT DTD ATG GGD TTC AAG GT-3' 5'-ACD GTC ADG AAD CGD TCG TTG AG-3'	721	19
nahU	nahGU_244f nahU_898r	5'-GACATCTGGTTCGAATGGCG-3' 5'-CAAGATCATGCAGCGCCC-3'	654	Present work
catA	C12O_UP C12O_LOW2	5'-GCG HAC VAT CGA AGG NCC RYT GTA-3' 5'-TCR CGS GTN GCA WAN GCA AAG TC-3'	462	15

echol-2,3-dioxygenase as in [21]. Protein concentration was assayed spectrophotometrically [22].

RESULTS

Isolation and characterization of salicylate-degrading strains. Strains of salicylate destructors isolated by the method of enrichment cultures, from two samples of polluted soils from the Murmansk and Tula oblasts, were used in the work. As a result of the inoculation of 60 obtained salicylate destructors in a King B medium, 45 strains of the fluorescent group were selected. According to their morphological and physiological characteristics, the studied strains were assigned to the genus *Pseudomonas* according to [23]. The species affiliation of the strains of fluorescent pseudomonads were determined on the basis of an amplified ribosomal DNA restriction analysis (ARDRA) of the 16S rRNA gene amplification products according to [10]. Among the salicylate destructors, 25 were tentatively classified as *P. putida.* In the present work, 12 *P. putida* strains, different from each other, were used (Table 1). All the selected strains could grow on salicylate, seven of them also grew on toluene, four strains grew on benzene, and two strains grew on BTEX. In addition, four of the 12 strains were able to degrade gentisate and nine strains could degrade caprolactam (Table 1).

Genotypic analysis of *P. putida* strains. The phylogenetic differences between the strains of the same species were established by genome fingerprinting (REP-PCR), using the BOXA1R primer [16]. REP-PCR revealed that the studied *P. putida* strains belonged to four groups of genome fingerprints by the degree of similarity (Fig. 2). The first group is formed by strains *P. putida* g15f, g20f, and g24f isolated from soils of Kandalaksha (Table 1). The second group includes strains *P. putida* NS7, NS15, NS17, NS18, and NS24 (Shchekino, Tula oblast) and the third group includes *P. putida* NS11, NS12, and NS20 (Shchekino). Strain *P. putida* NS22 occupies a separate position, forming its own group. Strains *P. putida* NS11, NS12, and NS20 proved to be closely related to the previously described naphthalene-degrading strain, *P. putida* BS202 [18], although they were isolated from geographically distant regions (Fig. 2, Table 1).

The method of field inverted gel electrophoresis showed that all strains possessed plasmids of more than 180 kb in size and, besides, five of them had a second plasmid of a much larger size; these findings were confirmed by processing of the block inserts by restriction endonucleases *Eco*RI and *Hin*dIII (Fig. 3). Nevertheless, the experiments on conjugal transfer into the recipient strain *P. putida* KT2442 in a selective medium with salicylate gave no positive results.

Amplification of the key genes of salicylate degradation. For the study of genetic control of salicylate degradation in the relevant strains, salicylate hydroxylase gene *nahG,* catechol-2,3-dioxygenase gene *nahH*, and regulatory gene *nahR* were used as the marker genes of the "classical" *nah2*-operon. Additionally, the strains were tested for the presence of catechol-1,2-dioxygenase gene (*catA*), "non-classical" salicylate hydroxylase gene *nahU* of strain *P. putida* ND6 (GenBank Acc. No. AY208917) not included in the *nah2* operon, chromosomal salicylate hydroxylase pseudogene *nahG1* of strain *P. putida* KT2440 (AE016788) (the so-called "KT-like" gene), and the gene of the large subunit of salicylate-5-hydroxylase (*nagG*). The results of the amplification of salicylate catabolism genes are shown in Table 3.

Specific amplification of the genes of catechol-1,2-dioxygenase, *catA*, and salicylate hydroxylase, *nahU*, was observed in all of the analyzed samples. As a result of amplification of the *nahG1* pseudogene, PCR products of 994 bp were obtained only in strains *P. putida* g15f, g20f, g24f, NS11, NS12, NS20, and NS22. None of the studied samples showed amplification of the selected marker genes of the "classical" *nah2* operon or the *nagG* gene of salicylate-5-hydroxylase (Table 3).

Analysis of the polymorphism of the *nahU* and *nahG1* genes of *P. putida* strains. The polymorphism of the *nahU* and *nahG1* genes of salicylate hydroxylases in the studied strains was analyzed by a restriction fragment length polymorphism (RFLP) analysis, using restriction endonucleases. The *nahU* gene amplification products (654 bp), were treated with restriction endonucleases *Rsa*I, *Hae*III, and *Msp*I. RFLP analysis of amplicons showed the presence of the salicylate hydroxylase gene in eight of the studied strains, and the pattern of its hydrolysis by these restriction endonucleases was identical to the hydrolysis pattern of the *nahU* gene from *P. putida* ND6 (Fig. 4a). In three of the studied strains (*P. putida* g15f, g20f, g24f), the hydrolysis patterns of the amplicons of this gene differed from the profiles of the *nahU* gene and were absolutely identical to each other (Fig. 4a).

The products of amplification of the "KT-like" salicylate hydroxylase gene *nahG1* (994 bp) were treated with restriction endonucleases *Hae*III and *Msp*I (Fig. 4b). The obtained hydrolysis patterns were compared with the restriction profiles of the amplicons of the *nahG1* gene of marker strains: *P. putida* KT2440 and strain *P. putida* BS3790 from the laboratory collection (unpublished data).

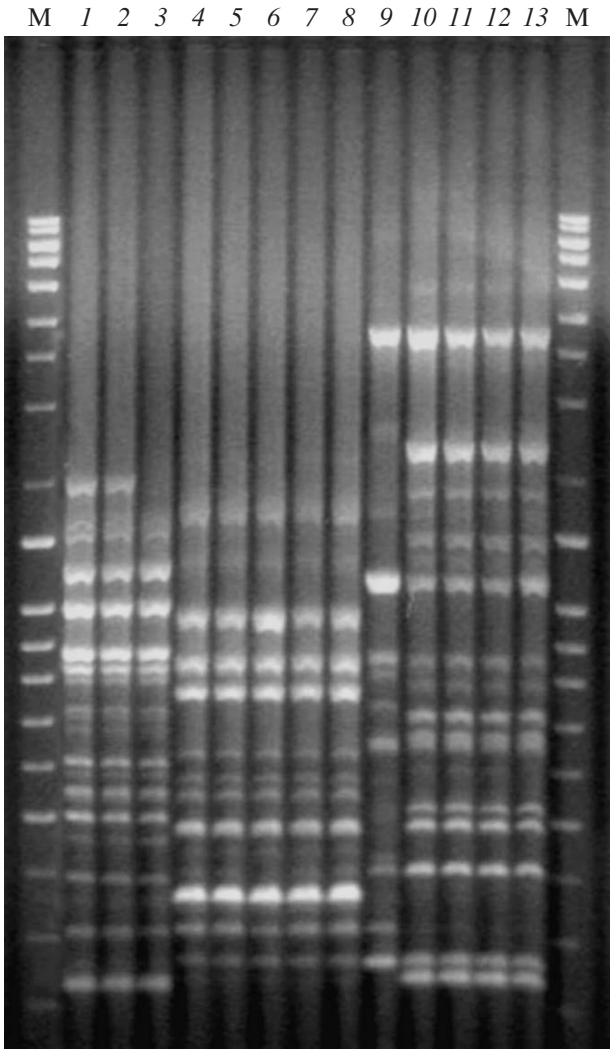

Fig. 2. Genomic fingerprint (REP-PCR) of *P. putida* strains with the BOXA1R primer. M, M1 (Ekobiotekhnologiya); *1*, *P. putida* g15f; *2*, *P. putida* g20f; *3*, *P. putida* g24f; *4*, *P. putida* NS7; *5*, *P. putida* NS15; *6*, *P. putida* NS17; *7*, *P. putida* NS18; *8*, *P. putida* NS24; *9*, *P. putida* NS22; *10*, *P. putida* NS11; *11*, *P. putida* NS12; *12*, *P. putida* NS20; *13*, *P. putida* BS202.

Restriction analysis of the amplicons showed that the strains *P. putida* NS11, NS12, NS20, and NS22 had the *nahG1* gene and the patterns of its digestion with *Hae*III and *Msp*I were similar to hydrolysis patterns of the *nahG1* gene from *P. putida* BS3790 (Fig. 4b). Besides, the patterns of hydrolysis of *nahG1* amplicons in three of the studied strains (*P. putida* g15f, g20f, and

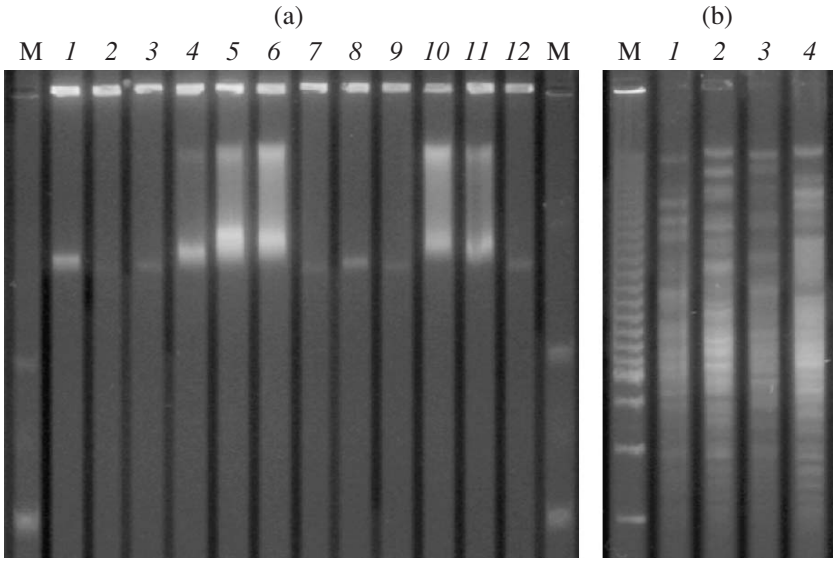

Fig. 3. Plasmid profiles of the studied strains obtained by the method of field inverted gel electrophoresis. a, electrophoregram of native plasmids: M, λ co-integrates; *1*, *P. putida* g15f; *2*, *P. putida* g20f; *3*, *P. putida* g24f; *4*, *P. putida* NS7; *5*, *P. putida* NS11; *6*, *P. putida* NS12; *7*, *P. putida* NS15; *8*, *P. putida* NS17; *9*, *P. putida* NS18; *10*, *P. putida* NS20; *11*, *P. putida* NS22; *12*, *P. putida* NS24; b, electrophoregram of restriction of block inserts by restriction endonuclease *Hin*dIII: M, 2.5 Kb Ladder (BioRad); *1*, *P. putida* g24f; *2*, *P. putida* NS12; *3*, *P. putida* NS17; *4*, *P. putida* NS22.

g24f) were absolutely identical to each other and differed from restriction profiles of both marker strains (Fig. 4b).

The analysis of specific activities of the salicylate catabolism enzyme in *P. putida* strains. Specific activities of the key enzymes of salicylate biodegradation (salicylate hydroxylase, catechol-2,3-dioxygenase, and catechol-1,2-dioxygenase) were measured in all of the studied *P. putida* strains. Analysis of enzyme activities revealed the activities of salicylate hydroxylase

Table 3. Amplification of the key genes of PAH biodegradation

Strain	*nahG*	*nahH*	*nahR*	*nahG1*	*nagG*	*nahU*	*catA*
P. putida g15 F	–	–	–	++	–	++	++
P. putida g20 F	–	–	–	++	–	++	++
P. putida g24 F	–	–	–	++	–	++	++
P. putida NS7	–	–	–	–	–	++	++
P. putida NS11	–	–	–	++	–	++	++
P. putida NS12	–	–	–	++	–	++	++
P. putida NS15	–	–	–	–	–	++	++
P. putida NS17	–	–	–	–	–	++	++
P. putida NS18	–	–	–	–	–	++	++
P. putida NS20	–	–	–	++	–	++	++
P. putida NS22	–	–	–	++	–	++	++
P. putida NS24	–	–	–	–	–	++	++

Note: Amplification efficiency: (++), very good; (–), no amplification product.

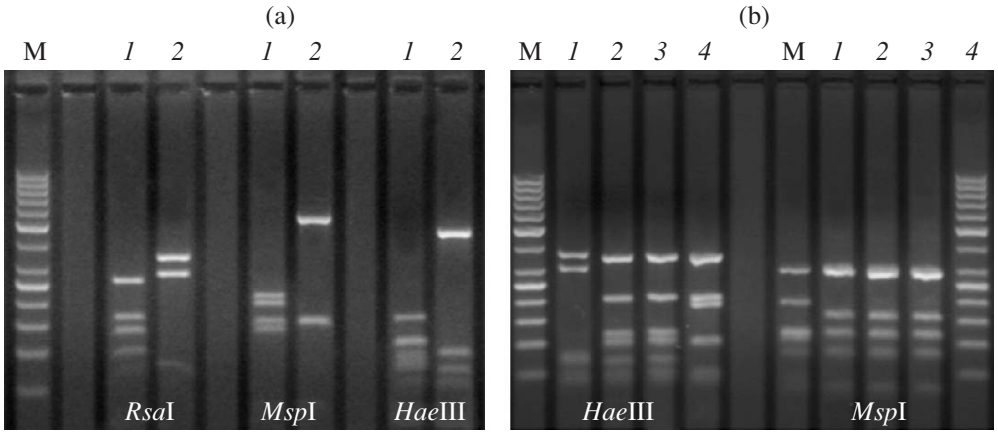

Fig. 4. Restriction of amplification products: a, two variants of the *nahU* gene, 654 bp, treated with restriction endonucleases *Rsa*I, *Msp*I, and *Hae*III: M, 50 bp Ladder (Fermentas, Lithuania); *1*, *P. putida* g20f; *2*, *P. putida* NS12; b, two variants of the *nahG1* gene, 994 bp, treated with restriction endonucleases *Hae*III and *Msp*I: M, 50 bp Ladder (Fermentas, Lithuania); *1*, *P. putida* g15f; *2*, *P. putida* NS11; *3*, *P. putida* BS3790; *4*, *P. putida* KT2442.

and catechol-1,2-dioxygenase in all strains. These activities are induced by salicylate and are absent at cultivation of the strains in succinate. All of the studied strains, with the exception of *P. putida* NS22 and g24f, showed the high level of salicylate hydroxylase activity (204–484 nmol/min per mg protein). The activity of catechol-2,3-dioxygenase was not revealed.

DISCUSSION

Salicylate is a representative of a heterogeneous group of plant phenols, and plants are the main suppliers of salicylates into the environment. Salicylate is also an intermediate compound in microbial degradation of different aromatic hydrocarbons; it can be used by microorganisms as a growth substrate, and plays an important role in the regulation of metabolic processes [24]. However, accumulation of salicylate in the medium may inhibit growth of many microorganisms.

The 12 *P. putida* strains analyzed in the present work were isolated from polluted soils and were capable of salicylate utilization as a sole carbon and energy source. Seven of these strains were also capable of utilizing monocyclic aromatic hydrocarbons such as benzene and/or toluene (Table 1). Microorganisms utilize benzene and toluene, just as salicylate, with formation of the same intermediate product, catechol, which is further degraded either by the *ortho*-pathway (β-ketoadipate) or by the *meta*-pathway to the Krebs cycle intermediates [1, 25]. It provides destructor strains with additional adaptive capabilities at the minimal set of genes.

In fluorescent pseudomonades, the genes responsible for the catabolism of various xenobiotics are often located on large, conjugative plasmids. Although all of the studied strains were shown to have plasmids, their role in the genetic control of salicylate degradation was not confirmed. The presence of the catechol-2,3-dioxygenase gene (*nahH*) encoding the key enzyme of the *meta*-pathway of catechol cleavage is indirect evidence of plasmid localization of biodegradation operons [26]. This work has demonstrated that all of the studied strains contain neither the *nahH* gene nor the other "marker" gene of the *nah2*-operon, i.e. the "classical" salicylate hydroxylase gene *nahG*. The analysis of specific enzyme activities also confirms that salicylate, probably similar to benzene and toluene, is utilized by the *ortho*-pathway of catechol cleavage without employing the *meta*-pathway. In all of the studied *P. putida* strains, salicylate degradation was supposed to involve the salicylate hydroxylase gene analogous to the *nahU* gene of strain *P. putida* ND6. Some strains were shown to contain the sequence of the "KT-like" salicylate hydroxylase gene. However, it is quite possible that in the studied destructors, like in the initial strain *P. putida* 2440, this sequence is a pseudogene. The *nahU* gene of strain *P. putida* ND6, just as the salicylate hydroxylase gene *nahW* of strain *P. stutzeri* AN10 (AF039534), is located apart from the *nah2* operon and functionally duplicates the "classical" salicylate hydroxylase gene *nahG* in these strains. The "classical" *nahG* gene was not found in the *P. putida* strains studied in this work. It seems that the sequences like *nahU* and *nahW*, which are not a part of catabolic operons, being shifted and included into the metabolic pathway of a host strain, extend the range of utilized substrates and thus contribute to the adaptation of microorganisms to environmental conditions.

ACKNOWLEDGMENTS

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REFERENCES

1. Davies, J.I. and Evans, W.C., Oxidative Metabolism of Naphthalene by Soil Pseudomonas: The Ring-Fission Mechanism, *J. Biochem.*, 1964, vol. 91, pp. 251–261.
2. Evans, W.C., Fernley, H.N., and Griffiths, E., Oxidative Metabolism of Phenanthrene and Anthracene by Soil Pseudomonads: the Ring-Fission Mechanism, *J. Biochem.*, 1965, vol. 95, pp. 819–831.
3. Müller, U. and Lingens, F., Degradation of 1,4-Naphthoquinones by Pseudomonas putida, *Biol. Chem. Hoppe Seyler*, 1988, vol. 369, pp. 1031–1043.
4. Shamsuzzaman, K.M. and Barnsley, E.A., The Regulation of Naphthalene Metabolism in Pseudomonads, *Biochem. Biophys. Res. Commun.*, 1974, vol. 60, pp. 582–587.
5. Katagiri, M., Maeno, H., Yamamoto, S., Hayaishi, O., Kitao, T., and Oae, S., Salicylate Hydroxylase, a Monooxygenase Requiring Flavin Adenine Dinucleotide. II. The Mechanism of Salicylate Hydroxylation to Catechol, *J. Biol. Chem.*, 1965, vol. 240, pp. 3414–3417.
6. Starovoitov, I.I., Nefedova, M.Y., Yakovlev, G.I., Zyakun, A.M., and Adanin, V.M., Gentisic Acid as a Microbial Oxidation Product of Naphthalene, *Izv. Akad. Nauk. SSSR Ser. Khim*, 1975, vol. 9, pp. 2091–2092.
7. Buswell, J.A., Paterson, A., and Salkinoja-Salonen, M.S., Hydroxylation of Salicylic Acid to Gentisate by a Bacterial Enzyme, *FEMS Microbiol. Letts.*, 1980, vol. 8, pp. 135–137.
8. Dunn, N.W. and Gunsalus, I.C., Transmissible Plasmid Coding Early Enzymes of Naphthalene Oxidation in *Pseudomonas putida, J. Bacteriol.*, 1973, vol. 114, pp. 974–979.
9. Habe, H. and Omori, T., Genetics of Polycyclic Aromatic Hydrocarbons Metabolism in Diverse Aerobic Strains, *Boisci. Biotechnol. Biochem.*, 2003, vol. 67, pp. 225–243.
10. Izmalkova, T.Yu., Sazonova, O.I., Sokolov, S.L., Kosheleva, I.A., and Boronin, A.M., Diversity of Genetic Systems Responsible for Naphthalene Biodegradation in *Pseudomonas fluorescens* Strains, *Mikrobiologiya,* 2005, vol. 74, no. 1, pp. 70–78 [*Microbiology* (Engl. Transl.), vol. 74, no. 1, pp. 60–68].
11. Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: a Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor*, New York, 1989.
12. Evans, C.G.T., Herbert, D., and Tempest, D.W., The Continuous Cultivation of Microorganisms: II. Construction of a Chemostat, *Meth. Microbiol.*, 1970, vol. 2, pp. 277–327.
13. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K.Eds., *Shot Protocols in Molecular Biology*, Wiley, 1999, p. 4.
14. Alonso, R., Martín, A., Peláez, T., Marin, M., Rodríguez-Creixéms, M., and Bouza, E., An Improved Protocol for Pulsed-Field Gel Electrophoresis Typing of *Clostridium difficile, J. Med. Microbiol.*, 2005, vol. 54, pp. 155–157.
15. Izmalkova, T.Yu., Sazonova, O.I., Sokolov, S.L., Kosheleva, I.A., and Boronin, A.M., The P-7 Incompatibility Group Plasmids Responsible for Biodegradation of Naphthalene and Salicylate in Fluorescent Pseudomonads, *Mikrobiologiya*, 2005, vol. 74, no. 3, pp. 342–348 [*Microbiology* (Engl. Transl.), vol. 74, no. 3, pp. 290–295].
16. Dombek, P.E., Johnson, L.K., Zimmerley, S.T., and Sadowsky, M.J., Use of Repetitive DNA Sequences and the PCR to Differentiate *Escherichia coli* Isoletes from Human and Animal Sources, *Appl. Environ. Microbiol.*, 2000, vol. 66, pp. 2572–2577.
17. Weisburg, W.G., Barnes, S.M., Pelletier, D.A., and Lane, D.J., 16S Ribosomal DNA Amplification for Phylogenetic Study, *J. Bacteriol.*, 1991, vol. 73, pp. 697–703.
18. Izmalkova, T.Yu., Mavrodi, D.V., Sokolov, S.L., Kosheleva, I.A., Smalla, K., Thomas, C.M., and Boronin, A.M., Molecular Classification of IncP-9 Naphthalene Degradation Plasmids, *Plasmid*, 2006, vol. 56, pp. 1–10.
19. Wilkstrom, P., Wilklund, A., Anderson, A.C., and Forman, M., DNA Recovery and PCR Quantification of Catechol-2,3-Dioxygenase Genes from Different Soil Types, *J. Biotechnol.,* 1996, vol. 52, pp. 107–120.
20. Hegeman, G.D., Synthesis of the Enzymes of the Mandelate Pathway by *Pseudomonas putida* I. Synthesis of Enzymes by the Wild Type, *J. Bacteriol.*, 1966, vol. 91, pp. 1140–1154.
21. Feist, C.F. and Hegeman, G.D., Phenol and Benzoate Metabolism by *Pseudomonas putida* of Tangential Pathways, *J. Bacteriol.*, 1969, vol. 100, pp. 869–877.
22. Kalb, V.F. and Bernlohr, R.W., A New Spectrophotometric Assay for Protein in Cell Extract, *Anal. Biochem.*, 1977, vol. 82, pp. 362–366.
23. *Bergey'S Manual of Systematic Bacteriology*, Krieg, N.J. and Holt, J.G., Eds., Baltimore: Williams & Wilkins, 1984.
24. Raskin, I., Salicylate, a New Plant Hormone, *Plant Physiol.*, 1992, pp. 799–803.
25. Reiner, A.M., Metabolism of Benzoic Acid by Bacteria: 3,5-Cyclohexadiene-1,2-Diol-1-Carboxilic Acid Is an Intermediate in the Formation of Catechol, *J. Bacteriol.*, 1971, vol. 108, pp. 89–94.
26. Timmis, K.N., Lehrbach, P.R., Harayama, S., Don, R.H., Mermod, N., Bas, S., Leppick, R., Weightman, A.J., Reineke, W., and Knackmuss, H.-J., Analysis and Manipulation of Plasmid-Encoded Pathways for the Catabolism of Aromatic Compounds by Soil Bacteria, in *Plasmids in Bacteria*, Helinski D.R., Cohen C.N., Clewell D.B., Jackson D.A., and Hollaender A, Eds., New York: Plenum Press, 1985, pp. 719–739.