ISSN 0026-8933, Molecular Biology, 2013, Vol. 47, No. 1, pp. 105–111. © Pleiades Publishing, Inc., 2013.
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GENOMICS.
TRANSCRIPTOMICS

UDC 575.13:577.21

scpA, a New Salicylate Hydroxylase Gene Localized in Salicylate/Caprolactam Degradation Plasmids

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Received June 25, 2012; in final form, July 5, 2012

Abstract—Both caprolactams and salicylate biodegradation by *Pseudomonas* salicylate/caprolactam degraders are controlled by large conjugative plasmids (SAL/CAP). Some of these plasmids have been assigned to the P-7 incompatibility group. The new salicylate 1-hydroxylase gene (*scpA*) has been detected in SAL/CAP plasmids and partially sequenced. The *scpA* gene was equally related to the closest homolog genes *nahG* (NAH7), *salA* (*P. reinekei* MT1), and *nahU* (pND6-1); however, the identity rate did not exceed 72–74%. The synthesis of salicylate 1-hydroxylase ScpA was not induced by salicylate. This enzyme had wide substrate specificity and exhibited the highest specific activity toward 4-methylsalicylate and nonsubstituted salicylate substrates. Furthermore, conjugative pseudomonads' plasmids of salicylate degradation without the classical *nah2* operon, which harbors only salicylate 1-hydroxylase gene *nahU* have been described for the first time.

DOI: 10.1134/S0026893313010147

Keywords: salicylate 1-hydroxylase, caprolactam, *Pseudomonas*, plasmid, *nahU* gene, and *scpA* gene

Salicylates (salicylic acid and its conjugates) are abundant aromatic hydrocarbons, which are typical plant metabolites involved in the induction of systemic resistance to phytopathogens [1]. Furthermore, salicylate is a key intermediate in the biodegradation pathways of naphthalene, phenanthrene, anthracene, and other toxic and carcinogenic compounds that contaminate the atmosphere [2]. In most cases, the accumulation of salicylates (particularly halogenated) in the medium suppresses microorganism growth; however, there are bacteria that utilize these aromatic compounds as the only carbon and energy source. There are two ways in which bacteria of the *Pseudomonas* genus may utilize salicylate. First, salicylate 1-hydroxylase can transform it into catechol, which is further degraded following *ortho*- or *meta*-pathways. Secondly, salicylate may be oxidized by salicylate 5-hydroxylase to gentisic acid. In *Pseudomonas* genus, genes of the aromatic hydrocarbon catabolism, including the catabolism of salicylate, are often located in large conjugative plasmids [3] and comprise various transposons [4]. Today, salicylate hydroxylase genes located in the plasmids of naphthalene and salicylate biodegradation have been described in fluorescent pseudomonads (*nahG, nahU*, and *nagG*) [5–7]. Chromosomal salicylate 1-hydroxylase genes are also known, both of which are silent (e.g., the *nahG1* pseudogene in a number of *P. putida* strains) [8] and involved in biochemical pathways that provide the host with serious competitive advantages.

Recently, the collection of the laboratory of plasmid biology (Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences) was enlarged by the *Pseudomonas* strains able to degrade salicylate and caprolactam, a xenobiotic widely used for capron (nylon-6) production. A combination of these metabolic activities has only been previously observed in the *P. putida* strain MCM V-408, contained a stable 32-kbp long plasmid [9]. Furthermore, several strains have been reported [10] that degraded salicylate, which employs the nonclassical (NahU) salicylate hydroxylase, were able to grow on caprolactam (but not naphthalene), and contained large plasmids. However, no clear correlation between biodegradation of salicylate and caprolactam and the presence of plasmids was revealed in these two works. A group of caprolactam biodegradation plasmids, CAP, has been isolated from bacteria living in waste waters of several caprolactam production factories and characterized [11]. However, no single plasmid-free strain of pseudomonads has been reported that could use caprolactam as the only carbon, nitrogen, and energy source. The biodegradation of salicylate may be controlled by both plasmid and chromosomal genes, while nucleotide sequences of salicylate hydroxylase genes, as well as their closest genetic surroundings, may differ considerably.

In our work, we found that the ability to degrade both caprolactam and salicylate in SAL/CAP *Pseudomonas* strains of the laboratory collection and

isolate from new soil samples is determined by the large conjugative plasmids; we also identified and characterized the new salicylate hydroxylase gene, which comprises the SAL/CAP plasmid.

MATERIALS AND METHODS

Plasmid-free *P. putida* strain KT2442 (*gfp*, Km^r, Rif^r) (K. Smalla, Germany) was used as a recipient of biodegradation plasmids. The bacteria were grown in an LB medium and in an Evans mineral salt medium at 28°C [12]. Sodium salicylate and caprolactam were introduced into the medium at concentrations of 1 g/L; naphthalene was added to the lid of an overturned Petri dish; and a BTEX mixture (benzene, toluene, ethylbenzene, and xylenes in equal volumes) was poured into silicone tubes, which were also placed on the lids of overturned Petri dishes after inoculation. Kanamycin was introduced to a final concentration of 100 μg/mL in the medium to select transconjugants. Plasmids, except pBS270 [13] and pS6f (isolated from *P. fluorescens* strain S6f, from the soil of JSC Shchekinoazot, Tula region), were isolated at various points in time from various soil samples that were arbitrarily clean or contaminated by autotransport, from Pushchino, Moscow region, by exogenous isolation in Evans medium with sodium salicylate.

Method of exogenous isolation of conjugative plasmids. Soil (2 g) was resuspended in 10 mL of LB medium and placed in a thermostat (28°C) for 12 h. Recipient strain *P. putida* KT2442 was grown in 4 mL of LB medium under the same conditions, then 200 μL of the medium were mixed into 1 mL of soil suspension and centrifuged for 5 min at 6000 *g*. A Millipore filter with 0.22–0.4-μm pores was placed onto a Petri dish with agarized LB medium, and 100 μL supernatant were placed onto the filter. The dish, without being turned over, was incubated in a thermostat for 24 h at 28°C. Then, the filter was washed with 10 mL of saline and 100 μL of suspension was inoculated into Evans medium with kanamycin and sodium salicylate. The dishes were incubated at 28°C and colonies glowing with green light under a UV lamp were selected. After clearance, the clones were transferred with a replicator onto dishes with Evans medium containing salicylate, caprolactam, naphthalene, or BTEX as sole sources of carbon and energy.

Plasmid DNA was isolated by alkaline lysis according to [12], also a ZR Plasmid Miniprep–*Classic* (ZymoResearch, United States) equipment was used.

Polymerase chain reaction (PCR) was performed on a Mastercycler Gradient (Eppendorf, Germany) equipment under standard conditions using Taq-DNA polymerase. The oligonucleotide primers used in the work are presented in Table 1. Electrophoresis of DNA was performed in 1.5% agarose gel in Tris–acetate buffer following standard procedure [12]. DNA was visualized by staining the gel with ethidium bromide and isolated using a Zymoclean Gel DNA Recovery Kit (ZymoResearch) according to the manufacturer's instructions.

DNA was sequenced on an automated ABI Prism 373 3130XL Genetic Analyzer (Perkin-Elmer) by Sintol research and production company (Moscow). Nucleotide sequences were analyzed using DNAStar, pDraw (AcaClone Software, http://www.acaclone.com), and BLAST N (NCBI, http://www.ncbi.nlm.nih.gov) software packages. TREECON software [19] was used to build the phylogenetic tree.

For restriction analysis, restriction endonucleases produced by Fermentas (Lithuania) were used. DNA was treated with enzymes at 37°C for 2–6 h according to the manufacturer's recommendations.

To induce salicylate hydroxylase activity, bacterial cells were grown to the middle of logarithmic phase in 250 mL of Evans medium supplemented with 1 g/L succinate as the source of carbon and energy. Then, an inductor of salicylate hydroxylase, 0.1 g/L sodium salicylate solution, was added and cells were grown for 2 h. Cells were isolated by centrifugation, washed twice with buffer [12], and destroyed using an MSE 150-W (England) ultrasonic disintegrator at 22 kHz for 1 min (2 × 30 s) at 4°C.

Protein concentration was determined according to Bradford [20].

Specific activity of salicylate hydroxylase [EC 1.14.13.1] was determined using a UV-160A (Shimadzu, Japan) spectrophotometer by decreasing the extinction of NADH in reaction mixture ($\lambda = 340$ nm, $\varepsilon = 6.220\ mM^{-1}\ cm^{-1}$) containing 100 μM NADH, 100 μM salicylate, cell-free extract, and 0.05 M phosphate buffer, pH 7.5, taking into account the consumption of endogenous NADH by cell-free extract at 30°C [21].

Activity of catechol 2,3-dioxygenase was determined by the rate of α-oxymuconic semialdehyde formation in the reaction mixture ($\lambda = 375$ nm, $\varepsilon = 33.4\ mM^{-1}\ cm^{-1}$) containing 0.5 mM catechol, cell-free extract, and 0.05 M Tris-HCl buffer (pH 7.5) [22].

The activity of catechol 1,2-dioxygenase was determined by the rate of *cis*,*cis*-muconate formation in the reaction mixture ($\lambda = 260$ nm, $\varepsilon = 16.9\ mM^{-1}\ cm^{-1}$) containing 5 mM Na-EDTA, 1 mM catechol, cell-free extract, and 0.05 M phosphate buffer (pH 7.0) [23].

The specific activity of the enzymes was expressed as micromoles of the consumed substrate (cofactor) or the product (cofactor) formed in 1 min per 1 mg total bacterial protein. Values of specific activities of the enzymes were calculated using the Enzyme software (Institute of Biochemistry and Physiology of Microorganisms, Russia).

Table 1. Primers used in work

Gene	Primer	Nucleotide sequence, (5' → 3')	Primer annealing *T*, °C	Size of the PCR product, bp	Reference
repA (IncP-7)	RepAP7F1 RepAP7R1	GCCCATGCCGAAAAAGGTGTC GAATCGTTGATAGGCATCCGAC	53	412	[14]
repAB (IncP-9)	repF repR	CCAGCGCGGTACWTGGG GTCGGCAICTGCTTGAGCTT	54	398	[15]
nahAc	Ac149f Ac1014r	CCCYGGCGACTATGT CTCRGGCATGTCTTTTTC	43	865	[16]
nahH	23DOF 23DOR	ATGGATDTDATGGGDTTCAAGGT ACDGTCADGAADCGDTCGTTGAG	50	721	[17]
nahR	nahR_1f nahR_585r	ATGGAACTGCGTGACCTGG GCCGTAGGAACAGAAGCG	52	585	[18]
nahG	shc1_up shc1_lo	CGGCKTTHGGTGARGTCGGTGC GGCGAGGAARTAGGCGTCCTCAAG	54	893	[18]
nahU	nahGU_244f nahU_898r	GACATCTGGTTCGAATGGCG CAAGATCATGCAGCGCCC	58	654	[10]
nagG	458f 1224r	CCTGACCAAGCTSAAGGT CGTYTCGGTSACCATGTG	56	766	[7]
salA	salAF salAR	CAAAGTCGAAGACCGCACC ACGCCACCACGTTGATAATG	58	522	Current work
catA	C12O_UP C12O_LOW2	GCGHACVATCGAAGGNCCRYTGTA TCRCGSGTNGCAWANGCAAAGTC	58	462	[7]

RESULTS AND DISCUSSION

Plasmids of Salicylate Biodegradation

For the exogenous isolation of conjugative plasmids of salicylate biodegradation from samples of forest soil collected close to Pushchino, plasmid-free *P. putida* strain KT2442, which functions as an efficient recipient of exogenous DNA due to the defective system of restriction–modification and *gfp* gene inserted in the chromosome [24]. The expression of the *gfp* gene, which codes green fluorescent protein, allows one to easily distinguish KT2442 colonies growing on solid media. As a result, 12 transconjugants capable of growing on salicylate were obtained, seven of which also used naphthalene as a single source of energy and carbon, while two strains, KT2442 (pScp1) and KT2442 (pScp2) used ε-caprolactam and three strains, KT2442 (pS1), KT2442 (pS2), and KT2442 (pS3), grew exclusively on salicylate. We isolated plasmids from all transconjugants; three of the plasmids were of the same size (over 50 kbp). An analysis of restriction fragment length polymorphism (RFLP) using EcoRI and BamHI restriction endonucleases showed that these plasmids are similar, if not identical. According to PCR with primers for groups of incompatibility IncP-7 and IncP-9, pS1, pS2, and pS3 did not belong to these two groups, which are abundant among catabolic plasmids. Seven NAH/SAL plasmids differed insignificantly by size (70–100 kbp) and belonged to the incompatibility group P-9. Plasmids of salicylate/caprolactam biodegradation differed significantly by size (pScp1, below 40 kbp, pScp2, over 70 kbp) and did not belong to IncP-7/IncP-9 groups. The acquisition of the ability to biodegrade salicylate and caprolactam together with the single plasmid DNA by the plasmid-free strain indicates that the genetic determinants of these features are localized in the plasmid.

It is not surprising that most plasmids isolated with the exogenous method by salicylate degradation characteristics turned out to be naphthalene degradation plasmids. This is the most abundant and well-studied type of polycyclic aromatic hydrocarbon (PAH) biodegradation plasmids. Plasmids of this type usually contain the classical gene of salicylate hydroxylase *nahG* in the low (*nah*2) operon of naphthalene biodegradation and control salicylate oxidation to catechol, which is further utilized in the *meta*-pathway. Plasmids pS1, pS2, and pS3, which control the catabolism of salicylate (but not naphthalene), as well as pScp1 and pScp2, which enables the biodegradation of both salicylate and caprolactam, seemed more interesting. In addition, we used five plasmids (also supported in the laboratory strain KT2442) isolated from soil previ-

ously, which determine the utilization of salicylate or caprolactam, i.e., pBS270, pS6f, pEx4, pNP6, and pNP7. It is known that most plasmids of naphthalene or salicylate biodegradation are assigned to the IncP-9 group (less frequently, IncP-7) and, as a rule, plasmids of caprolactam biodegradation belong to IncP-9 or IncP-2 [11]. Using PCR, we found that three of SAL/CAP plasmids (pBS270, pS6f, pEx4) belonged to the P-7 group of incompatibility and four plasmids (pScp1, pScp2, pNP6, and pNP7) could not be classified.

It should be noted that caprolactam is a toxic compound that causes dermatitis and chromosomal aberrations in mammals [25]. Only few decades ago, bacteria that destroy these compounds were merely isolated from soils in contact with waste waters of caprolactam and caprolactam-based polymer production [11]. In recent years, as in this work, bacteria that destroy caprolactam, although few in number, have been revealed in nonspecifically contaminated soils. Most likely, the expansion and selection of the ability to utilize caprolactam in bacteria of soils remote form chemical-production waste, was stimulated by the pollution of the environment with products of caprolactam polymerization (space frames of auto- and aviatreads, fishing nets, garments, etc.), followed by their abiotic and biotic erosion. For example, it is known that lignolytic fungi are able to cleave nylon-6 and nylon-66 to oligomers aggressively and rapidly [26]. It is likely that, under modern conditions, the combination of the salicylate biodegradation pathway, which is needed for soil and especially rhizosphere pseudomonads, with determinants of caprolactam utilization in a single mobile genetic element provides a certain competitive advantage in hosts of salicylate–caprolactam plasmids. Furthermore, the existence of cross points between the catabolism of salicylate and caprolactam (genes of caprolactam biodegradation have not been identified yet) or their common regulation in strains carrying SAL/CAP plasmids can not be excluded.

Identification of Salicylate Hydroxylase Gene Localized in Plasmids of Salicylate/Caprolactam Biodegradation

Genetic control of salicylate degradation in transconjugants and five strains from the laboratory collection were studied with PCR using primers specific to classical genes of plasmid operons of naphthalene and salicylate biodegradation, i.e., *nahAc*, *nahG*, *nahH*, and *nahR*. These genes code for the large subunit of naphthalene 1,2-dioxygenase, salicylate-1-hydroxylase, catechol 2,3-dioxygenase, and a regulatory protein of the LysR type, respectively (Table 1). The amplification of all these genes was only observed when seven NAH/SAL plasmids were used as templates. Other plasmids (SAL and SAL/CAP) encoded a nonclassical pathway of salicylate biodegradation, since PCR analysis did not reveal a single gene of the *nah*2 operon.

Previously, in our laboratory, salicylate hydroxylase genes were identified in a group of strains, degraders of salicylate; and in PCR negative for *nahG*, *nahH*, and *nahR* [10]. Primers specific to the unique sequence of the *nahU* salicylate hydroxylase gene of naphthalene biodegradation plasmid pND6-1 (IncP-7) (GenBank Acc. no. AY208917.2) located far from the *nah*2 operon [6] were designed. In contrast to pND6-1, which also carries the standard *nahG* gene, only *nahU* was amplified in the strains under study. However, the localization of this gene in all strains remained unclear.

Amplification products with primers specific to the *nahU* gene were obtained for three plasmids of salicylate degradation analyzed in our work, i.e., pS1, pS2, and pS3. Amplified fragments 654 bp long were isolated from agarose gel and subjected to hydrolysis by RsaI endonuclease. The restriction profile of all three amplicons resembled the restriction profile of pND6-1 *nahU*.

Therefore, for the first time, we describe plasmids of salicylate biodegradation that contain no operon *nah*2 and carry only the nonclassical gene of salicylate 1-hydroxylase *nahU*.

None of SAL/CAP plasmids contained the *nahU* gene. The remaining variant also could not be amplified; it is rather rare among pseudomonads' plasmids [7] gene that codes for the large subunit of salicylate 5-hydroxylase (*nagG*), which transfers salicylate not into catechol, but into gentisate.

An analysis of the nucleotide sequences of salicylate hydroxylase genes deposited in GenBank over the past 2–3 years resulted in the identification of the *salA* gene of the MT1 strain (AY944685.2) of *P. reinekei*, which is phylogenetically remote from all known variants and utilizes chlorinated salicylates in the modified *ortho*-pathway to prevent the formation of protoanemonin antibiotics [27, 28]. Based on the nucleotide sequence of *salA*, we developed salAF and salAR primers in order to search for the gene in SAL/CAP plasmids. Amplification with new primers was insufficiently specific; however, using pDraw software, it was found that, under certain conditions, salAF primer could be substituted with nahGU_244f primer designed for the *nahU* gene. Amplification products (fragments of ~500 bp) were obtained in all cases in which SAL/CAP plasmids were used as templates. At the same time, *nahU* and *nahG* genes (control variants) could not be amplified using this pair of primers (Fig. 1). Amplified fragments of plasmids pScp1, pScp2, and pBS270 were isolated from the gel and sequenced in two directions. The identified salicylate hydroxylase gene was called scpA (from the phrase "salicylate/caprolactam plasmid").

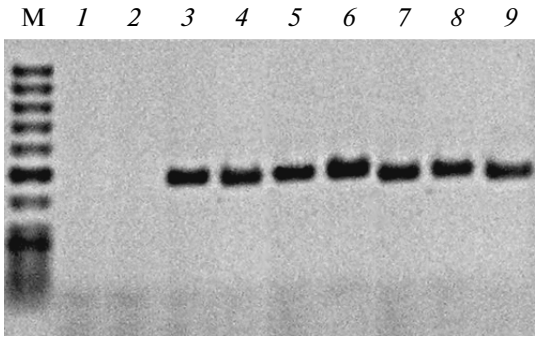

Fig. 1. Electrophoresis picture of PCR products obtained with primers specific to the *scpA* gene. M, marker, 50-bp DNA ladder (Fermentas, Lithuania): (*1*) pNO2 (negative control, contains *nahG* gene); (*2*) pS3 (negative control, contains *nahU* gene); (*3*) pScp1; (*4*) pScp2; (*5*) pBS270; (*6*) pS6f; (*7*) pEx4; (*8*) pNP6; and (*9*) pNP7.

Phylogenetic Analysis of the *scpA* Gene

The 440-bp long nucleotide sequence of salicylate hydroxylase gene located in three SAL/CAP plasmids pScp1, pScp2, and pBS270, was determined and deposited in GenBank under the number JQ926744. It turned out that, in all plasmids, the internal parts of the *scpA* gene were absolutely identical. The restriction profile of the amplified fragments of all SAL/CAP plasmids treated with RsaI was also identical. Interestingly, all analyzed plasmids were isolated at different points in time from microflora of soil samples collected in distant regions and pBS270 is an honorary member of the first group of CAP plasmids isolated by our laboratory over 20 years ago from soil contaminated with waste from caprolactam producing factories situated in Kemerovo and other territories of the Soviet Union. Therefore, the *scpA* gene, or at least its internal part, appears to be conserved in the whole group of SAL/CAP plasmids independently of the location or time of isolation of these extrachromosomal elements.

A comparison of nucleotide sequences of the internal parts of *scpA* and other genes of salicylate hydroxylases in BLAST N software yielded the following results: *scpA* is 73% identical to *salA* (*P. reinekei* MT1), *nahU* (pND6-1) is 72% identical, and the classical *nahG* (NAH7) is 74% identical. The calculated amino acid sequence of the ScpA fragment was identical to the relevant fragment of the known salicylate hydroxylases by no more than 77%. Dendrogram illustrating the evolutionary affinity of nucleotide sequences of *scpA* in plasmids pScp1, pScp2, and pBS270 to other genes of salicylate hydroxylases of other strains and plasmids, is presented in Fig. 2. The *scpA* gene forms a separate branch in the group of salicylate 1-hydroxylase genes that is roughly equally distant from the branches of *nahG, salA*, and *nahU*.

Activity of the ScpA Salicylate Hydroxylase Encoded by SAL/CAP Plasmids

An important characteristics of an enzyme is its activity toward various substrates and its demand for induction of gene expression. It is known that salicylate activates the transcription of *nah*-operons [29] containing the gene of the classical salicylate hydroxylase NahG. Addition of small amounts of salicylate into cultural medium in the middle of exponential growth phase of NAH/SAL degraders promotes the accumulation of considerable amounts of NahG, which is necessary to measure the specific activity of this enzyme.

To determine the specific activity of salicylate hydroxylase ScpA, we chose *P. putida* strains KT2442 (pScp1) and KT2442 (pBS270). We used salicylate, 3-, 4-, and 5-chlorosalicylates and 3-, 4-, and 5-methylsalicylates as substrates. The measurement results are presented in Table 2. It was found that activity of ScpA toward substituted salicylates depends on the type and

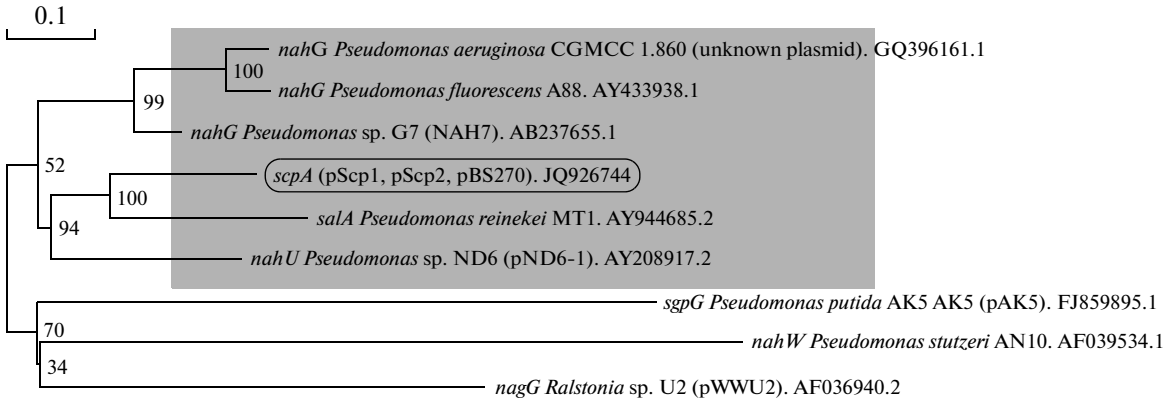

Fig. 2. Dendrogram illustrating evolutionary affinity between the nucleotide sequences of salicylate hydroxylase gene *scpA* of pScp1, pScp2, and pBS270 plasmids and genes of other strains and plasmids. Tree was built in TREECON [19] software. GenBank record numbers are reported after points. Dark background highlights the group of homologous salicylate 1-hydroxylases.

Table 2. Relative activity of SpcA salicylate 1-hydroxylase of *P. putida* KT2442 strain containing pScp1 or pBS270 plasmids

Substrate	Specific activity, nmol/(min mg) protein	
	pScp1	pBS270
Salicylate (without induction)	142	177
Salicylate	128 (100)	151 (100)
3-Chlorosalicylate	40 (31)	81 (54)
4-Chlorosalicylate	96 (75)	118 (78)
5-Chlorosalicylate	62 (48)	88 (58)
3-Methylsalicylate	70 (55)	83 (55)
4-Methylsalicylate	165 (128)	214 (142)
5-Methylsalicylate	117 (91)	135 (90)

Note: Values in parentheses were obtained using extracts of cells induced by salicylate and indicate the percent to the specific activity towards salicylate upon induction.

position of the substituent. The enzyme exerted the maximal activity toward salicylates with substituents at position 4, lower activity toward 5-substituted salicylates, and the lowest activity toward 3-substituted salicylates. On the whole, the enzyme activity was lower toward chlorosalicylates than methylsalicylates.

The specific activity of salicylate hydroxylases NahU and NahG of the naphthalene degrader strain *Pseudomonas* sp. ND6 (pND6-1) was measured using 3- and 5-methylsalicylates, 5-chlorosalicylate, 3- and 5-dinitrosalicylates, sulfosalicylates, and aspirin as substrates [6]. The NahG enzyme exerted higher activity toward 3- and 5-methyl-, and 5-chlorosalicylate than to the unsubstituted salicylate (activity to it is considered 100%), i.e., 128, 123, and 115% respectively. At the same time, in the case of NahU, these values were much lower, i.e., 45, 70, and 22% respectively.

In the *P. stutzeri* AN10 strain, two genes, i.e., *nahG* and *nahW*, were identified that code for two nonhomologous salicylate hydroxylases. It turned out that NahG and NahW are more active toward 4-methylsalicylate (224 and 175% activity to salicylate, respectively) than to other substituted derivatives [30].

The salicylate hydroxylase SalA of *P. reinekei* MT1 strain, which is capable of utilizing 4- and 5-chlorosalicylates also exerted wide substrate specificity toward various monosubstituted salicylates, i.e., compared to 4- and 5-chlorosalicylates, which were utilized less efficiently, 4- and 5-methylsalicylates were transformed at a higher rate than salicylate [27].

The profile of specific activities of ScpA enzyme encoded by SAL/CAP plasmids is more similar to the NahU profile. Unfortunately, it is not feasible to compare the absolute values of the specific activities of the enzymes from different strains, since the result is influenced by even tiny differences in the growth phase of the bacteria culture. It is assumed that the synthesis of salicylate hydroxylase ScpA does not require induction, since specific activities of ScpA in KT2442 (pScp1) and KT2442 (pBS270) cells grown on succinate both upon induction and without induction with salicylate (Table 2) were comparable.

Since the product of the reaction catalyzed by salicylate 1-hydroxylases is catechol, the specific activities of both catechol 1,2-dioxygenase and catechol 2,3-dioxygenase of the *P. putida* KT2442 strain that carry pScp1 or pBS270 plasmids were measured. The specific activity of catechol 2,3-dioxygenase was not detected, which supports the validity of the negative results on *nahH* gene amplification. Specific activity of catechol 1,2-dioxygenase of the *P. putida* KT2442 strain containing plasmids pScp1 or pBS270 was high, i.e., 553 nmol/(min mg) protein and 187 nmol/(min mg) protein, respectively. For all SAL/CAP plasmids, the PCR product of the widespread variant of catechol 1,2-dioxygenase gene *catA* was obtained. However, in preparations of plasmids isolated from pseudomonads, an admixture of the host chromosomal DNA is always present; chromosome of KT2442 contains two genes of catechol 1,2-dioxygenase, one of which is *catA*. PCR with genomic DNA of the natural host of the plasmid pS6f, i.e., the strain *P. fluorescens* S6f (pS6f), did not reveal any *nahH* (catechol 2,3-dioxygenase) or *catA* (catechol 1,2-dioxygenase) genes. This means that the chromosome of the S6f strain and the pS6f plasmid comprise the catechol dioxygenase gene, which is nonhomologous to *catA* and *nahH*; therefore, it is probable that the transformation of catechol in KT2442 strains that contain SAL/CAP plasmids is not controlled by chromosomal genes alone.

Summarizing the results of our work, we can note the following. SAL plasmids pS1, pS2, and pS3 isolated by the method of exogenous isolation do not carry classical genes of *nah*2-operon but contain a salicylate 1-hydroxylase gene *nahU* type pND6-1. It was found for the first time that the ability to degrade both caprolactam and salicylate in degrader of these compounds from the laboratory collection and new soil samples is determined by the large conjugative plasmids, some of which belong to the P-7 incompatibility group. In SAL/CAP plasmids, a new salicylate 1-hydroxylase gene *scpA* was identified and partially sequenced; it was found to be no more than 72–74% identical to the known sequences. The synthesis of ScpA is not induced by salicylate; furthermore, the enzyme has wide substrate specificity and exerts the highest activity toward 4-methylsalicylate and unsubstituted salicylate. The oxidation of catechol by KT2442 (pScp1) and KT2442 (pBS270) strains occurs through the *ortho*-pathway, which is rarely seen in plasmid that contains salicylate degraders.

ACKNOWLEDGMENTS

The work was supported by the Ministry of Education and Science of the Russian Federation (project no. RNP 2.1.1/1038), International Science and Technology Center (project no. 4033), and the Russian Foundation for Basic Research (project no. 11-04-97562r_tsentr_a).

REFERENCES

1. Raskin I. 1990. Role of salicylic acid in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **2**, 439–463.
2. Habe H., Omori T. 2003. Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Biosci. Biotechnol. Biochem*. **67**, 225–243.
3. Herrick J.B., Stuart-Keil K.G., Ghiorse W.C., Madsen E.L. 1997. Natural horizontal transfer of a naphthalene dioxygenase gene between bacteria native to a coal tar-contaminated field site. *Appl. Environm. Microbiol*. **63**, 2330–2337.
4. Tan H.-M. 1999. Bacterial catabolic transposons. *Appl. Microbiol. Biotechnol*. **51**, 1–12.
5. Tsuda M., Iino T. 1990. Naphthalene degrading genes on plasmid NAH7 are on a defective transposon. *Mol. Gen. Genet*. **223**, 33–39.
6. Zhao H., Chen D., Li Y., Cai B. 2005. Overexpression, purification and characterization of a new salicylate hydroxylase from naphthalene-degrading *Pseudomonas* sp. strain ND6. *Microbiol. Res*. **3**, 307–313.
7. Izmalkova T.Yu., Sazonova O.I., Sokolov S.L., Kosheleva I.A., Boronin A.M. 2005. The P-7 incompatibility group plasmids responsible for biodegradation of naphthalene and salicylate in fluorescent pseudomonads. *Microbiology* (Moscow). **74** (3), 290–295.
8. Kosheleva I.A., Izmalkova T.Yu., Sokolov S.L., Sazonova O.I., Boronin A.M. 2003. Structural and functional variation in genetic systems for catabolism of polycyclic aromatic hydrocarbons in *Pseudomonas putida* strains. *Russ. J. Genet*. **39** (9), 997–1003.
9. Kulkarni R.S., Kanekar P.P. 1998. Effects of some curing agents on phenotypic stability in *Pseudomonas putida* degrading ε-caprolactam. *W. J. Microbiol. Biotechnol*. **14**, 255–257.
10. Sazonova O.I., Izmalkova T.Yu., Kosheleva I.A., Boronin A.M. 2008. Salicylate degradation by *Pseudomonas putida* strains not involving the "classical" *nah2* operon. *Microbiology* (Moscow). **77** (6), 710–716.
11. Esikova T.Z., Grishchenkov V.G., Kulakov L.A., Morenkova M.A., Boronin A.M. 1990. Incompatibility groups of plasmids controlling ε-caprolactam bioodegradation in bacteria of the genus *Pseudomonas*. *Mol. Genet. Mikrobiol. Virusol*. **4**, 25–28.
12. Sambrook J., Fritsch E.F., Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Lab. Press.
13. Esikova T.Z., Grishchenkov V.G., Boronin A.M. 1990. Plasmids controlling ε-caprolactam biodegradation. *Mikrobiologiya*. **59** (4), 547–552.
14. Volkova O.V., Kosheleva I.A., Boronin A.M. 2012. Structure of replication initiation region in *Pseudomonas* IncP-7 streptomycin resistance plasmid Rms148. *Mol. Biol*. (Moscow). **46** (4), 542–547.
15. Greated A., Thomas C.M. 1999. A pair of PCR primers for IncP-9 plasmids. *Microbiology* (UK). **145**, 3003–3004.
16. Ferrero M., Llobet-Brossa E., Lalucat J., Garcia-Valdes E., Rosselo-Mora R., Bosch R. 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.
17. Wilkstrom P., Wilklund A., Anderson A.C., Forman M. 1996. DNA recovery and PCR quantification of catechol-2,3-dioxygenase genes from different soil types. *J. Biotechnol*. **52**, 107–120.
18. Izmalkova T.Yu., Mavrodi D.V., Sokolov S.L., Kosheleva I.A., Smalla K., Thomas C.M., Boronin A.M. 2006. Molecular classification of IncP-9 naphthalene degradation plasmids. *Plasmid*. **56**, 1–10.
19. Van de Peer Y., De Wachter R. 1994. TREECON for Windows: A software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput. Appl. Biosci*. **10**, 569–570.
20. Bredford M.M. 1976. Rapid and sensitive method for the quantitation of microgram quantity of protein utilizing the principle of protein–dye binding. *Ann. Biochem*. **72**, 248–254
21. Kiyohara H., Nagao K. 1978. The catabolism of phenantrene and naphthalene by bacteria. *Gen. Microbiol*. **105**, 69–75.
22. Feist C.F., Hegeman G.D. 1978. Phenol and benzoate metabolism by *Pseudomonas putida*: Regulation of tangential pathways. *J. Bacteriol*. **2**, 869–877.
23. Ornston L.N. 1966. The conversion of catechol and protocatechuate to beta-ketoadipate by *Pseudomonas putida*: 3. Enzymes of the catechol pathway. *J. Biol. Chem*. **16**, 3795–3799.
24. Regenhardt D., Heuer H., Heim S., Fernandez D.U., Strompl C., Moore E.R.B., Timmis K.N. 2002. Pedigree and taxonomic credentials of *Pseudomonas putida* strain KT2440. *Environ. Microbiol*. **4**, 912–915.
25. Kulkarni R.S., Kanekar P.P. 1998. Bioremediation of ε-caprolactam from nylon-6 waste water by use of *Pseudomonas aeruginosa* MCM B-407. *Curr. Microbiol*. **37**, 191–194.
26. Premraj R., Mukesh D. 2005. Biodegradation of polymers. *Indian J. Biotech*. **4**, 186–193.
27. Camara B., Bielecki P., Kaminski F., dos Santos V.M., Plumeier I., Nikodem P., Pieper D.H. 2007. A gene cluster involved in degradation of substituted salicylates via ortho cleavage in *Pseudomonas* sp. strain MT1 encodes enzymes specifically adapted for transformation of 4-methylcatechol and 3-methylmuconate. *J. Bacteriol*. **189**, 1664–1674.
28. Blasco R., Wittich R.-M., Mallavarapu M., Timmis K.M., Pieper D.H. 1995. From xenobiotic to antibiotic, formation of protoanemonin from 4-chlorocatechol by enzymes of the 3-oxoadipate pathway. *J. Biol. Chem*. **49**, 29229–29235.
29. Barnsley E.A. 1975. The induction of the enzymes of naphthalene metabolism in pseudomonads by salicylate and 2-aminobenzoate. *Gen. Microbiol*. **1**, 193–196.
30. Bosch R., Moore E.R.B., Garcia-Valdes E., Pieper D.H. 1999. NahW, a novel, inducible salicylate hydroxylase involved in mineralization of naphthalene by *Pseudomonas stutzeri* AN10. *J. Bacteriol*. **181**, 2315–2322.