Short Communication

Utilization of Phenylpropanoids by *Pseudomonas putida* Soil Isolates and Its Probable Taxonomic Significance

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Twenty seven soil bacterial isolates able to grow on cinnamic acid or ferulic acid as a sole carbon and energy source were examined for the ability to utilize other phenylpropanoids and phylogenetic interrelationships. A 16S rRNA gene sequence analysis revealed that 26 of the isolates were closely related to *Pseudomonas putida* biovar A and classified into 6 OTUs: cinnamic acid utilization-positive (OTUs, CN-1 to CN-3) and -negative groups (FR-1 to FR-3). *P. putida* NBRC 14164^T and JCM 13061 belonged to FR-3. A probable reclassification of *P. putida* was discussed in relation to the utilization of phenylpropanoids.

Key words: cinnamic acid, ferulic acid, aromatic degradation, *Pseudomonas putida*

Phenylpropanoids such as cinnamic acid (Fig. 1), 4-hydroxycinnamic acid (4-coumaric acid), 4-hydroxy-3-methoxycinnamic acid (ferulic acid) (Fig. 1), and 4-hydroxy-3,5-dimethoxycinnamic acid (sinapic acid) are plant secondary metabolites and constitute lignin, the most abundant natural polymer on earth after cellulose. In the biosynthesis of lignin, cinnamic acid is a precursor and transformed to 4-coumaric acid, ferulic acid, and sinapic acid before its incorporation into a lignin polymer²⁾. Phenylpropanoids are released into the environment through the biodegradation of lignin and other aromatic constituents of plants. Because of their abundance, the biodegradation of phenylpropanoids is important in the carbon cycle of the plant-soil ecosystem. In addition, many phenylpropanoids are pharmacologically active and thus of pharmaceutical interest²⁷⁾.

The biodegradation of ferulic acid and vanillic acid has been well studied and reported for not only many bacteria including soil oligotrophs^{9,10)} and bradyrhizobia⁷⁾ but also fungi³⁾. So far the proposed catabolic pathways of ferulic acid have been classified in to four routes according to the initial reactions (see reference²⁰⁾ for review): 1) side chain reduction forming saturated side chains, aldehydes and alcohols such as 4-hydroxy-3-methoxyphenylpropionic acid and vanillyl alcohol; 2) cleavage of acetate from the unsaturated side-chain flowing through vanillin and then oxidation of vanillin to vanillic acid; 3) *O*-dealkylation reactions giving caffeic acid; and 4) non-oxidative decarboxylation to form styrenes such as vinylguaiacol. Further oxidative degradation of the aromatic ring occurs through protocatechuic acid^{13,23)}. These initial degradation pathways are not the reverse of lignin biosynthesis and do not include cinnamic acid as a

breakdown intermediate. Therefore, most of the reported ferulic acid degraders did not degrade cinnamic acid and few bacteria have the ability to degrade both cinnamic acid and its *para*-hydroxylated derivatives¹⁵⁾. To our knowledge, several bacteria such as *Pseudomonas stutzeri* strain CINNS¹⁾, *Clostridium glycolicum* strains DSM 1288^T and CIN5⁴⁾, and *Bacillus* sp. strain B1¹⁵⁾ have the ability to attack both cinnamic acid and its *para*-hydroxylated cinnamic acids.

Previously, we have isolated soil bacteria able to grow on a mineral salt medium containing cinnamic acid or ferulic acid as a sole energy and carbon source. In this paper we report that most of our isolates are related to *Pseudomonas putida* biovar A and describe both the isolates and *P. putida* reference strains with the ability to utilize cinnamic acid and its hydroxylated derivatives. Because Yamamoto *et al.*²⁸⁾ demonstrated the heterogeneity of *P. putida* strains by a phylogenetic analysis using combined *gyrB* and *rpoD* nucleotide sequences, we have focused on the relationship between the phenylpropanoid utilization profile and the phylogenetic positions of the soil isolates and reference strains of *P. putida*.

Soil bacteria were isolated on Hutner's mineral basal medium⁵⁾ supplemented with either cinnamic acid (sodium salt) or ferulic acid (sodium salt) at a concentration of 0.2% (wt/vol) as the sole source of energy and carbon. The

Fig. 1. Chemical structure of cinnamic acid (A) and ferulic acid (B).

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medium was prepared essentially as described by Stanier *et al.*²⁴⁾ and contained per litre: 40 mL of Na₂HPO₄+KH₂PO₄ buffer (1 M, pH 6.8), 20 mL of Hutner's vitamin-free mineral base, and 1.0 g of (NH₄)₂SO₄. Soils were sampled from gardens of three parks at Nippori, Taito-ku, Tokyo. Approximately 1 g of the pooled soil sample was suspended in 3 mL of sterile water, and mixed vigorously. After the soil suspension was allowed to settle, 0.1- or 0.2-mL aliquots of the supernatant were taken and inoculated into the above two liquid media (3 mL). After incubation at 30°C until turbid growth, an aliquot of the culture was inoculated into a fresh liquid medium with the same carbon source. Each subculture was performed three times on the same liquid medium and terminal subculture on agar plates of the same medium. After three passages on the agar medium with a specific carbon source, 18 cinnamic acid-degrading isolates named C-n (n, number) and 32 ferulic acid-degrading isolates named F-n were obtained and maintained in Difco nutrient agar slants at room temperature. In this study, the successfully maintained C-n isolates (C-3, C-8, C-10, C-17, C-18, C-104, C-106, C-109, C-112, C-113, and C-115) and F-n isolates (F-1, F-12, F-28, F-102 to F-105, F-108, F-113 to F-115, F-118, F-119, and F-123 to F-125) were used. The following strains were used as references: *Pseudomonas putida* NBRC 14164^T, *Pseudomonas putida* NBRC 100650 and *Pseudomonas putida* (formally '*Pseudomonas ovalis*') NBRC 3738²⁹⁾ obtained from Biological Resource Center, National Institute of Technology and Evaluation (NBRC), Kisarazu, Chiba, Japan; *Pseudomonas japonica* IAM 15071^T (=JCM 21532^T) obtained from IAM Culture Collection, Institute of Molecular and Cellular Bioscience, The University of Tokyo, Bunkyo-ku, Tokyo, Japan (currently, the IAM Culture Collection has been transferred to RIKEN BRC-JCM); and *Pseudomonas putida* JCM 13061 (=ATCC 11172, biovar A)²⁸⁾ obtained from RIKEN BRC-JCM. All of these cultures were maintained in 10- or 100-fold diluted nutrient broth (NB)¹²⁾.

Bacterial isolates were examined for cell morphology, Gram staining, and catalase as previously described¹¹⁾. Representative isolates were phenotypically characterized using API 20 NE and 50 CH strips (bioMérieux Vitek, Inc., France) according to the manufacturer's instructions. Growth at 4°C and 41°C was determined after 7 days cultivation by the use of 10-fold diluted NB, and growth at 4°C was determined after 14 days. For the degradation profile of phenylpropanoids and several hydroxyl benzoic acids, cultures were grown at 30°C in Hutner's mineral medium supplemented with one of the following compounds (2 mM): cinnamic acid, ferulic acid, vanillic acid, 2-coumaric acid, 3-coumaric acid, 4-coumaric acid, 3-(3-hydroxyphenyl) propionic acid, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, and protocatechuic acid (organic acids were prepared as sodium salts). After 7 days, degradation of a compound was judged by a change in the UV absorption spectrum¹⁰⁾.

Partial nucleotide sequences of 16S rRNA genes from all isolates were obtained by PCR amplification of genomic DNA using a universal primer set as previously described²²⁾. The nucleotide sequence was determined with an ABI PRISMTM Big Dye Terminator Cycle Sequencing kit

(Applied Biosystems, Foster City, USA) and read on an Applied Biosystems 3130 DNA sequencer²²⁾. All sequences determined were compared with similar DNA sequences retrieved from the DDBJ/EMBL/GenBank databases using the BLAST program¹⁴⁾. The DNA sequences were analyzed using GENETYX-WIN (version 11.0) from the Genetyx Co. (Software Development, Tokyo, Japan). For the phylogenetic analysis of the sequence datasets, the CLUSTAL W program²⁵⁾ was utilized and a tree was constructed by the neighbor-joining method²¹⁾. The sequence data for bacterial isolates have been deposited under DDBJ accession numbers AB302395 to AB302402 and AB461490 to AB461508.

Partial nucleotide sequences (856 nucleotides) of 16S rRNA genes from isolates C-n and F-n were determined to know phylogenetic relationships. The 27 isolates tested shared ≥97.3% 16S rRNA gene sequence identity with each other. The numbers of OTUs calculated using various levels of sequence identity were as follows: ≥98.1%, 2 OTUs; ≥99.1%, 3 OTUs; ≥99.7%, 5 OTUs; ≥99.9%, 7 OTUs; 100%, 11 OTUs. The 11 OTUs (100% sequence identity) were subjected to NCBI nucleotide BLAST searches. The homology searches showed that all the OTUs belonged to the

Fig. 2. Neighbor-joining distance tree constructed using the aligned, partial 16S rRNA gene sequences (856 nucleotide positions) of cinnamic acid- and ferulic acid-utilizing soil isolates representing each of the 11 OTUs (generated using a sequence identity value of 100%; CNA-1 to CNA-6 and FRA-1 to FRA-5) and reference strains of the most closely related *Pseudomonas* species. The 11 OTUs in boldface are followed by brackets showing the representative strain identification, DDBJ accession numbers in parentheses, and the number of additional isolates: 5 isolates of **CNA-4**, C-104, C-109, C-112, C-113, and C-115; 2 isolates of **FRA-2**, F-105 and F-108; 3 isolates of **FRA-3**, F-102, F-103, and F-124; 1 isolate of **FRA-4**, F-115; 5 isolates of **FRA-5**, F-104, F-113, F-114, F-118, and F-119. '*P. putida*' NBRC 3738 is formally '*P. ovalis*' NBRC 3738. Bootstrap values (in percent) calculated from 1,000 resamplings using the neighbor-joining method are shown at the nodes for values of ≥60%. *P. aeruginosa* was used to position the root. The 6 OTUs (generated using a sequence identity value of ≥99.9%; CN-1 to CN-3 and FR-1 to FR-3) to which the strains belong are presented on the right. I.S., identical sequence; Bar, 0.01 substitutions per nucleotide position.

genus *Pseudomonas* and were closely related with *Pseudomonas putida* biovar A except for one OTU including isolate C-106: The isolate shared 100% 16S rRNA gene sequence identity with *Pseudomonas mendocina* strain PC19 (accession number, DQ178226)⁸. Figure 2 shows a neighbor-joining distance tree using the aligned, partial 16S rRNA gene sequences (856 nucleotide positions) of isolates representing each of the 11 OTUs (named CNA-1 to CNA-6 and FRA-1 to FRA-5) and the type or reference strains of the most closely related *Pseudomonas* spp. When the OTU was generated at a level of sequence identity of $\geq 99.9\%$, 26 isolates excluding strain C-106 were classified into 6 OTUs (CN-1 to CN-3 and FR-1 to FR-3). Additionally, Fig. 2 shows that *P. putida* ('*P. ovalis*') NBRC 3738 and *P. putida* NBRC 100650 were not clustered with the type strain of *P. putida* (NBRC 14164^T) at the 16S rRNA gene sequence identity level of $\geq 99.9\%$.

All of isolates C-n and F-n and 5 reference strains of *P. putida* (NBRC 14164^T, JCM 13061, and NBRC 100650), *P. putida* ('*P. ovalis*') NBRC 3738, and *P. japonica* IAM 15071^T (17) were examined for the utilization of vanillic acid, 2-coumaric acid, 3-coumaric acid, 4-coumaric acid, protocatechuic acid, and vanillin as well as cinnamic acid and ferulic acid (Table 1). None of the F-n isolates consumed cinnamic acid, while among the C-n isolates, 4 (C-3, C-17, C-18, and C-106) used ferulic acid. *P. putida* ('*P. ovalis*') NBRC 3738 and *P. japonica* IAM 15071^T used cinnamic acid but not ferulic acid. The other three reference strains, *P. putida* NBRC 100650, *P. putida* NBRC 14164^T (biovar A), and *P. putida* JCM 13061, were characterized as negative for the utilization of cinnamic acid (Table 1). Strains NBRC 14164^T and JCM 13061 did not use ferulic acid but used vanillin and vanillic acid and the utilization of these two compounds was common to the F-n isolates. All of the strains tested used protocatechuic acid but none used 2-coumaric acid.

All of the C-n and F-n isolates were Gram-negative, catalase-positive, rod-shaped bacteria. Nine isolates from 6 OTUs, CN-1 to CN-3 and FR-1 to FR-3, and *P. japonica* IAM 15071^T were examined for their phenotypic character-

istics using the API 20 NE and API 50 CH strips and the utilization of additional hydroxyl benzoic acids. Regarding the 63 API strip characteristics and an additional 6 characteristics including growth temperature, all the strains tested showed positive reactions for 17 characteristics and negative reactions for 41 characteristics: 11 characteristics were variable in different strains (Table 2). The isolates selected from FR-1 to FR-3 were highly analogous in their API strip characteristics and only one difference was found: Being positive for arginine dihydrolase except in isolate F-12 and the assimilation of D-lyxose except in strain NBRC 100650. In contrast, isolates from CN-1 to CN-3 showed several differences with each other in response to the 8 API strip characteristics (Table 2).

In the present study, the utilization of several phenylpropanoids was examined with the soil isolates and various *P. putida* reference strains (Table 1). The 26 soil isolates and 4 reference strains of *P. putida* were divided into two major categories: Cinnamic acid utilization-positive and -negative groups. This divergence was clearly correlated with the results of the phylogenetic characterization (Fig. 2). As shown in Table 1, the cinnamic acid utilization-negative strains of FR-1 to FR-3 were characterized as positive for the utilization of vanillic acid and vanillin, while the cinnamic acid utilization-positive strains were divided into 2 groups: Strains positive (CN-1 and isolate C-17 of CN-3) and negative (CN-2) for the utilization of ferulic acid, vanillic acid, and vanillin. According to the criterion of OTUs with $\geq 99.9\%$ 16S rRNA gene sequence identity, our 26 isolates and the 4 reference strains of *P. putida* seem to be classified into 6 species: CN-1 to CN-3 and FR-1 to FR-3. To examine this taxonomic notion and whether *P. putida sensu stricto* should be assigned to limited strains including NBRC-14164^T, further genetic studies including DNA-DNA relatedness tests are required.

P. putida NBRC 100650 (=KT2440) is a genetically well-characterized strain and belongs to our FR-1. This strain is a plasmid-cured, restriction-deficient derivative of *P. putida* mt-2⁶) which has been widely used in physiological and

Table 1. Utilization profile of several phenylpropanoids by the cinnamic acid-(C-n) and ferulic acid-(F-n) utilizing soil isolates and reference strains of *Pseudomonas*

Utilization of	OTU ^a /isolate and reference strain ^b													
	C-106	1	CN-1		CN-2		CN-3		FR-1	FR-2		FR-3		4, 5
			C-3	C-18	C-104	C-10, C-109	C-8	C-17	F-28, 3	F-1	F-102, F-103	F-12, F-104		
					C-112	C-113, C-115					F-105, F-108	F-113, F-114		
											F-123, F-124	F-115, F-118		
						2						F-119, F-125		
Cinnamic acid	+ ^c	+	+	+	+	+	+	+	−	−	−	−	−	
Ferulic acid	+	−	+	+	−	−	−	+	+	+	+	+	−	
Vanillin	+	−	+	+	−	−	−	+	+	+	+	+	+	
Vanillic acid	+	−	+	+	−	−	−	+	+	+	+	+	+	
3-Coumaric acid	−	+	−	+	−	−	+	−	−	+	−	−	−	
4-Coumaric acid	+	−	+	+	+	−	−	+	+	+	+	+	−	

^a OTU, generated at the level of a sequence (856 nucleotides) identity value of $\geq 99.9\%$.

^b Reference strains: 1, *P. japonica* IAM 15071^T; 2, *P. putida* ('*P. ovalis*') NBRC 3738; 3, *P. putida* NBRC 100650; 4, *P. putida* NBRC 14164^T; 5, *P. putida* JCM 13061.

^c Symbols: +, positive reaction; -, negative reaction. All the strains tested used protocatechuic acid. None of the strains used 2-coumaric acid.

Table 2. Comparison of phenotypic properties of cinnamate- or ferulate-utilizing soil isolates and their related *Pseudomonas* reference strains

Characteristic	OTU ^a /isolate and reference strain ^b									
	IAM	CN-1	CN-2	CN-3		FR-1		FR-2	FR-3	
	15071 ^T	C-18	C-10	C-8	C-17	F-28	NBRC 100650	F-1	F-12	NBRC 14164 ^T
Nitrate reduction	– ^c	+	–	–	–	–	–	–	–	–
Arginine dihydrolase	–	–	+	–	–	+	+	+	–	+
Hydrolysis of gelatin	–	–	–	+	+	–	–	–	–	–
Assimilation of										
L-Sorbose	–	–	–	+	–	–	–	–	–	–
Inositol	–	–	–	–	+	–	–	–	–	–
D-Lyxose	–	–	–	–	–	+ ^w	–	+	+	+ ^w
Potassium gluconate	–	–	+ ^w	+ ^w	+ ^w	+	+	+	+	+
Potassium 2-ketogluconate	–	–	–	–	+ ^w	+	+ ^w	+ ^w	+	+ ^w
2-hydroxy benzoic acid ^d	–	–	–	–	–	+	+	–	+	+
3-hydroxy benzoic acid ^d	–	+	–	–	–	+	+	–	–	+
3-(3-hydroxyphenyl) propionic acid ^d	–	+	–	–	–	+	+	+	+	–

^a OTU, generated at the level of a sequence (856 nucleotides) identity value of $\geq 99.9\%$.

^b Reference strains: IAM 15071^T, *P. japonica* IAM 15071^T; NBRC 100650, *P. putida* NBRC 100650; NBRC 14164^T, *P. putida* NBRC 14164^T.

^c Symbols: +, positive reaction; +^w, weakly positive reaction; –, negative reaction. All the strains tested showed positive reactions for growth at 4°C and the assimilation of the following compounds: capric acid, malic acid, citric acid, phenylacetate, 4-hydroxy benzoic acid, glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, D-melibiose, gentiobiose, and D-fucose. All the strains were negative for growth at 41°C, indole production, acid production from glucose, urease, β -galactosidase, and the assimilation of the following compounds: adipic acid, erythritol, D-arabinose, L-xylose, D-adonitol, methyl- β -D-xylopyranoside, L-rhamnose, dulcitol, D-mannitol, D-sorbitol, methyl- α -D-mannopyranoside, methyl- α -D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon (starch), glycogen, xylitol, D-turanose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate, and potassium 5-ketogluconate.

^d The compounds are not included in the API 20 NE and 50 CH strips. For the test procedure, see the text.

genetic studies^{18,19}). Analysis of its whole-genome sequence revealed the genetic organization of the genes *fcs*, *ech*, and *vdh*, encoding feruloyl-CoA synthetase, enoyl-CoA hydratase/aldolase, and vanillin dehydrogenase, respectively, which are essential for ferulic acid conversion to vanillic acid via vanillin¹⁶). This genetic organization applies to ferulic acid-utilizing *P. putida* WCS358²⁶) and *Pseudomonas* sp. strain HR199¹³), and thus can be expected to be present in our soil isolates of FR-1 to FR-3. As for the utilization of cinnamic acid, it was reported that *P. putida* strains CINNP and CINNW and *P. stutzeri* strain CINNS degraded cinnamic acid and 2 strains (CINNP and CINNS) harbored a catabolic plasmid of about 75 kbp encoding the machinery for the metabolism of cinnamic acid¹). It remains to be studied whether our CN-1 to CN-3 isolates harbor such a catabolic plasmid.

In conclusion, the isolation and characterization of cinnamic acid- and ferulic acid-utilizing soil bacteria in the present study resulted in the expansion of the *P. putida* clade and our results will be an aid to the reclassification of *P. putida*.

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