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Characterization of Five Phyllosphere Bacteria Isolated from *Rosa rugosa* Leaves, and Their Phenotypic and Metabolic Properties

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Five Gram-negative bacteria, all of which were Enterobacteriaceae, were isolated from the phyllosphere of green or senescing leaves of *Rosa rugosa*, and their phenotypic and physiological characteristics were examined. Partial 16S rDNA sequences led to identification of these isolates as *Pantoea agglomerans*, *Klebsiella terrigena*, *Erwinia rhapontici*, and two strains of *Rahnella aquatilis*. Interestingly, these phyllosphere bacteria had certain phenotypic and physiological convergences, while they showed their own metabolic properties toward phenolic compounds of plant origin. In particular, the two *Ra. aquatilis* isolates from the green leaves had a substrate-inducible gallate decarboxylase activity in the resting cells that had been cultured in 1 mM gallic acid- or protocatechuic acid-containing medium. The other three isolates from the senescing leaves did not have this enzyme activity. Simple phenolics that the *Ra. aquatilis* decarboxylatively produced from benzoic acid derivatives had better antimicrobial activities than those of the substrates.

Key words: leaf epiphytic bacteria; phyllosphere; gallate decarboxylase; *Rahnella aquatilis*; *Rosa rugosa*

Some leaf epiphytes can detoxify secondary metabolic compounds of plants, and the resulting metabolites may play further allelochemical roles on the phylloplane to eliminate their competitors. In fact, *Klebsiella oxytoca*, isolated as one of the predominant leaf epiphytic bacteria from damaged leaves of *Polymnia sonchifolia*, inducibly decarboxylated 4-hydroxycinnamates including *E*-4-hydroxycinnamic and *E*-caffeic acids to release 4-hydroxystyrenes, which were not toxic to *K. oxytoca* itself but were highly toxic to other pathogenic and saprophytic microorganisms.[1] Such leaf epiphytes that occupy phylloplane niches on the damaged leaves, likely to be cooperative, phylloplane-associating microorganisms, may be regarded as one of the primary defense factors of host plants.[2]

In the mature leaves of *Rosa rugosa*, we observed a similar phenomenon to that on *Po. sonchifolia*. The leaves, mechanically damaged and soaked in distilled water, released in the aqueous layer large amounts of pyrogallol (**3**) and pyrocatechol (**4**) derivable from gallic acid (**1**) and protocatechuic acid (**2**), respectively, as shown in Fig. 1 (Hashidoko, unpublished data). Those simple phenolics had significant antimicrobial activities against some saprophytic or phytopathogenic fungi, *Pythium ultimum* (Fig. 2), *Rhizopus* sp., and *Aspergillus flavus*, and also on Gram-positive and negative bacteria, indicating an increase of antimicrobial activities after the decarboxylation. Because hydrolyzable tannins are the dominant secondary metabolite in the leaves of *Ro. rugosa*,[3] accumulation of **3** and **4** was thought to be another example of regulating phylloplane or phyllospheric microflora by leaf epiphytic bacteria. We here report the isolation of leaf epiphytes from *Ro. rugosa* leaves of different stages, and further discuss their defensive roles and their phenotypic and physiological convergence in the host phyllosphere.

For primary screening of epiphytes on the *Ro. rugosa* leaves, we first collected 57.9 g of the fresh leaves (in early June, near Hokkaido University Farm) to prepare a leaf extract. The compound leaves were chopped, soaked in 115 ml of deionized

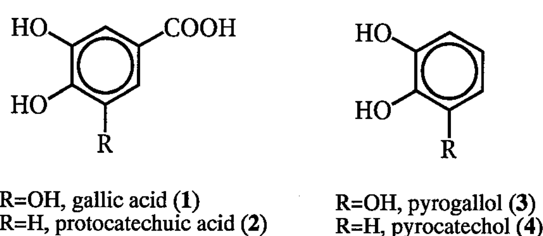

Fig. 1. Chemical Structures of Gallic Acid, Protocatechuic Acid, and Their Decarboxylation Products.

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Abbreviation: GD, gallate decarboxylase

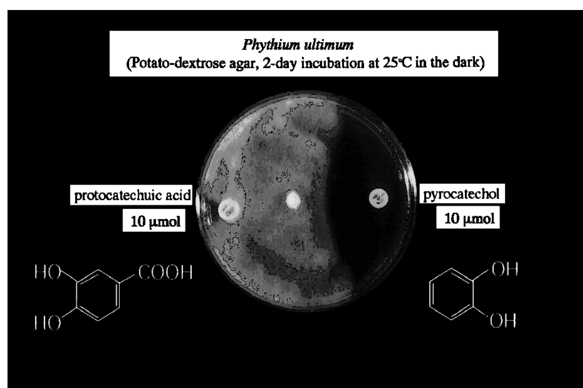

Fig. 2. Antifungal Test of Protocatechuic Acid and Pyrocatechol against *Pythium ultimum*.

For the bioassay, protocatechuic acid (**2**) and pyrocatechol (**4**) were separately dissolved in ethanol and prepared 500 mM solution, and 20 μl of each solution was put on a ϕ 8-mm paper disc. The amount of the test compound was 10 μmol/disc. After being air-dried, the discs were put on potato-dextrose agar plates, of which the center position had a mycelial disc taken from a mycelial front of a pre-incubated fungal plate. The test plate was incubated at 25°C in the dark for 2 days. *Escherichia coli* and *Bacillus subtilis* were, however, susceptible toward both **2** and **4**. Gallic acid (**1**) and pyrogallol (**3**) showed a similar tendency.

water and then kept at room temperature overnight. The aqueous leaf extract thus obtained was first decanted and then centrifuged at $8000 \times g$ for 15 min. The resulting supernatant was finally filtered through a 0.20 μm nitrocellulose membrane to produce a germ-free aqueous extract of the leaves.

As the source of epiphytes, four leaflets, two detached from newly developing and two from mature leaves, were separately washed several times with sterile water. In sterile Falcon tubes filled with 20 ml of the filtered leaf extract, the cleaned leaflets were separately soaked, and the tubes were left at 17°C in the dark.

After one week of incubation, the leaf-extract medium was turbid due to bacterial propagation. The cultured media were each diluted with an equal volume of 20% glycerol and kept at −80°C. In investigation of the bacterial composition, 100-μl portions of the stock culture broth previously diluted 1×10^{-3} times with water were spread on a ϕ 9 cm potato-dextrose (PD) agar plate and on a nutrient-broth agar plate, respectively, and then kept at 20°C for 3 days. Approximate 2,000 uniform bacterial colonies per plate of emerged on all of the plates. The bacterial isolates that were accordingly purified from the leaflets in developing and mature stages (tentatively named YL-1 and ML-1, respectively) were indistinguishable by their phenotypic and physiological characters, but clearly distinguishable from the viscosity of the colonies (Table 1).

On the other hand, senescing leaflets of *Ro. rugosa* (4.3 g) collected in mid-October at the same place were washed four times with sterile water and chopped under aseptic conditions to soak directly in 50 ml of sterile water. After overnight-incubation allowing epiphytes to grow in the aqueous extracts, 100 μl each of the aqueous layer was directly spread on nutrient-broth agar and PD agar plates. The most predominant (SL-1), second major (SL-2), and minor (SL-3, less than 3%) bacteria became apparent on the plates, so that in total five isolates were obtained as leaf epiphytes of *Ro. rugosa*.

By their phenotypic and physiological differentiation (Table 1) and a homology search for partial 16S rDNA sequences[4] on the BLASTN database,[5] these five phyllosphere bacteria were identified to be two *Ra. aquatilis* (YL-1 and ML-1), *Pantoea agglomerans* (SL-1), *Klebsiella terrigena* (tentative, SL-2), and *Erwinia rhapontici* (SL-3), respectively, all of which had been reported as phylloplane/phyllosphere or rhizoplane bacteria.[6–9] In a comparison of phenotypic and physiological characteristics with those of the standard species in Bergey's Manual of Determinative Bacteriology, 9th Edition,[10] five isolates had certain divergences in their cell size, oxidase activity, catalase activity, H_2S production, and so on (See footnotes in Table 1). However, these characteristics showed clear convergence amongst the isolates of four different species inhabiting the *Ro. rugosa* phyllosphere, and their representative ones were most identical with those of *Phyllobacterium myrsinacearum*, which has originally been isolated from a leaf nodule of *Ardisia crenata* (Myrsinaceae) as a nitrogen-fixing bacterium.[10–12] Interestingly, we observed a star-like cluster of *Ra. aquatilis* ML-1 that had been cultured in a nutrient broth medium, a property that has been reported as the unique characteristic of *Ph. myrsinacearum*.[13] This fact encouraged us to conceive that the physiological convergence observed amongst the phyllosphere bacteria of *Ro. rugosa* must be widely involved in bacterial flora on the phylloplane or phyllosphere of certain plants.

We examined metabolic abilities of the five isolates toward common phenolic compounds plant forms, including gallic acid (**1**), a major constituent of *Ro. rugosa* leaves.[3] Using *Klebsiella oxytoca* JCM 1665 (origin: pharyngeal tonsil) as a reference bacterium,[14] these isolates were separately inoculated into PD-medium containing 1 mM test compound, and shake-cultured at 23°C for 24 h. The resulting culture fluid was extracted with 1/5-volume of EtOAc, and 10 μl of the organic layer was put directly on TLC (Merck Kieselgel 60 F_{254}, 0.25 mm thick). When a large part of the substrate was converted by the epiphyte to give a spot of a major product on TLC, we judged it positive for the metabolic ability.[15]

According to the metabolic assays, gallic acid (**1**), protocatechuic acid (**2**), 4-hydroxybenzoic acid, 3,4-dihydroxycinnamic acid, and chlorogenic acid were

Table 1. Phenotypic and Physiological Characters of Phyllosphere Bacteria Isolated from *Ro. rugosa* Leaves

All isolates were eventually identified from their partial 16S rDNA sequences. Total DNA of each bacterial isolate used as PCR template was prepared with a DNA extraction kit (Isoplant II, Wako Pure Chemicals Industries, Ltd.) according to the manufacturer's instructions. The 16S rDNA region was amplified from the template DNA by PCR with *Taq* DNA polymerase (Nippon Gene). The first amplification with universal forward (5F) and reverse (1540R) primers for the 16S rDNA region was done with 35 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min. The PCR products were used directly as the template for the second amplification with different forward (357F) and reverse (1512R) primers as before. PCR products from the second amplification from position 375 to position 1186, in which region 90% of the sequence, with some 400 bases were conserved, were then searched for in a database. Phenotypic and physiological characters of two isolates, YL-1 and ML-1, were identical, but isolate YL-1 was distinguishable from ML-1 by its highly sticky colony on a solid medium of Winogradsky's mineral mixture with 0.005% yeast extract, 1.0% saccharose, and 2.0% agarose. The partial base sequence of the 16S rDNA of isolate YL-1 (DDBJ accession No.; AB087712) completely matched the sequences of *Ra. aquatilis* CDC 1327-79 and CDC 2989-79, except at position 449. Isolate ML-1 (DDBJ accession No.; AB087711) was also most easily aligned with these *Ra. aquatilis* strains. On the other hand, *Pa. agglomerans* DSM 3493 and *Er. rhapontici* ATCC 29283 were most easily aligned with strains SL-1 (DDBJ accession No.; AB087713) and SL-3 (DDBJ accession No.; AB087715), respectively. The sequence of SL-2 (DDBJ accession No.; AB087714) matched with the sequence of *Enterobacter agglomerans* A8 and *K. oxytoca* JCM 1665 well; however, we eventually identified isolate SL-2 as being *K. terrigena* because the isolate was not motile and did not produce indole, and several species in the genus *Klebsiella*, including *K. terrigena* ATCC 33257T, were readily aligned with the isolate. Among the phenotypic and physiological characters of the isolates, differences from reference data[10] are marked with asterisks. Data about the phenotypic and physiological differentiation of *Ph. myrsinacearum* are from Bergey's Manual.[13]

Identifiable genus/species	*Rahnella aquatilis*		*Pantoea agglomerans*	*Klebsiella terrigena*	*Erwinia rhapontici*	*Phyllobacterium myrsinacearum***
(Isolate)	(YL-1)	(ML-1)	(SL-1)	(SL-2)	(SL-3)	
Respiratory type	FA	FA	FA	FA	FA	A
Gram stain	—	—	—	—	—	—
Optimum pH	6–7	6–7	6–7	6–7	6–7	6–7
Optimum temperature	25°C	25°C	**25°C***	25°C	25°C	28–34°C
Colony	white, slimy	white	**yellow***	white	white	colorless or beige, slimy
Main shape of cells	rod	rod	rod	rod	rod	rod
Cell diameter, μm	**(0.3–0.5) × (0.5–1.0)***	**(0.3–0.5) × (0.5–1.0)***	**(0.3–0.5) × (0.5–1.0)***	**(0.3–0.5) × (0.5–1.0)***	**(0.3–0.5) × (0.5–1.0)***	(0.4–0.8) × (0.8–1.4)
Motility (swimming)	+	+	+	—	+	+
Flagellar arrangement:						
numbers:	2–3	2–3	2–3		peritrichous	several
attachment:	nonpolar	nonpolar	nonpolar		nonpolar	lateral
Oxidase	**d***	**d***	**+***	**+***	**d***	+
Catalase	**+***	**+***	**+***	+	+	+
Indole production	—	—	—	—	—	
Methyl red (MR) test	+	+	+	+	+	+
Voges-Proskauer (VP) test	+	+	+	+	+	d
H_2S production	**+***	**+***	+	**+***	**—***	
Growth in NaCl (5%)	+	+	+	+	+	—
Nitrate reduction to nitrite	+	+	+	+	+	+
Nitrate reduction to N_2	—	—	—	**—***	—	
Citrate (Simmon's)	+	+	+	+	+	+
Hydrolysis of:						
cellulose	—	—	—	—	—	—
gelatin	—	—	—	—	—	—
starch	—	—	—	—	—	—
pectin	—	—	—	—	**—***	—
Acid production from:						
ethanol	—	—	—	—	+	—
salicin	+	+	+	+	+	
sorbitol	**+***	**+***	+	**+***	+	+
rhamnose	+	+	+	+	+	
melibiose	+	+	+	—	+	
xylose	+	+	+	+	+	
raffinose	+	+	—	—	+	
mannose	+	+	+	+	+	
cellobiose	+	+	—	+	+	
Pigment	—	—	yellow	—	pink, diffusable	—
OF-test, glucose	F	F	F	F	F	

respectively tested on these five isolates. We also tested salicylic acid and tannic acid. *Ra. aquatilis* YL-1 and ML-1, which had been isolated from green leaves of *Ro. rugosa*, were able to decarboxylate **1** and **2** to accumulate pyrogallol (**3**)[16] and pyrocatechol (**4**) in the culture fluids, but not 4-hydroxybenzoic acid (in Table 2). Whereas, the other three from the senescing leaves did not show any abilities to decarboxylate those hydroxybenzoic acids. The gallate decarboxylase (GD) activities of two *Ra. aquatilis* isolates were substrate-inducible, and the strain YL-1 from newly developing leaves induced obviously higher GD activity than did ML-1 under the exposure to 1 mM **1** in the culture medium (see footnote in Table 2). In contrast, *Pa. agglomerans* SL-1 did not show any GD activity, although *Pa. agglomerans* T71, a different strain originally from soil, was the first successful enzyme source for purification of GD.[17] *Pa. agglomerans* SL-1, *Ra. aquatilis* YL-1, and ML-1 decarboxylated *E*-3,4-dihydroxycinnamic acid, and their decarboxylation activities were all constitutive, unlike that of *K. oxytoca* (see footnote in Table 2).[14]

Table 2. Ability of Phyllosphere Bacteria to Metabolize Phenolics of Plant Origins

All test bacteria were cultured in potato-dextrose medium with 1 mM substrate as a metabolic enzyme inducer or without any substrate. Cells incubated for 2 or 3 days were collected by centrifugation and washed several times with 50 mM Tris-HCl buffer (pH 7.2). The cell density was adjusted so that absorbance at 660 nm was about 1.0, and a test compound was added to the cell suspension in Tris-HCl buffer to a final concentration of 5 mM (with tannic acid, the final concentration was 0.2 mM). After 24 h of incubation at 25°C, the cell suspension was adjusted to a pH of 4.0 and extracted with 3 ml of EtOAc, and the resulting organic layer was developed on TLC in a 50:5:1 mixture of $CHCl_3$:MeOH:HCOOH. For chlorogenic acid and tannic acid, plus marks show that hydrolysis occurred; for other compounds, such marks show that decarboxylation occurred (+ + +, almost all was converted; + +, much was converted; +, a small or trace amount was converted; —, none was converted). Asterisks indicate substrate-inducible activity.

Substrate	Isolate	Degree of metabolism (hydrolysis or decarboxylation)					
		YL-1	ML-1	SL-1	SL-2	SL-3	JCM 1665
Gallic acid (**1**)		+ + +*	+ +*	—	—	—	+ + +*
Protocatechuic acid (**2**)		+ +*	+*	—	—	—	+ +*
4-Hydroxybenzoic acid		—	—	—	—	—	+ +*
3,4-Dihydroxycinnamic acid		+ + +	+ + +	+ + +	—	—	+ + +*
Salicylic acid		—	—	—	—	—	—
Chlorogenic acid		—	—	—	—	—	+ + +
Tannic acid		—	—	+*	—	—	—

None of the isolates hydrolyzed chlorogenic acid or decomposed salicylic acid. *Pa. agglomerans* SL-1, the most predominant epiphyte on the senescing leaves, showed a weak tannase activity, but the others did not.

In contrast to the phenotypic and physiological properties of the phyllosphere bacteria that displayed high convergence, their metabolic behaviors toward major secondary metabolites in the host leaves showed some diversity. Because bacteria and other microorganisms are highly versatile in their physiological properties, it is rather natural that these phyllosphere bacteria showing convergence in some enzyme activities while they were divergent in the metabolisms of phenolic compounds. *Pa. agglomerans* T71, with a GD activity,[17] is a typical example of such divergence of a bacterial species. So far we investigated in the literature, certain strains of *Ra. aquatilis* and *Pa. agglomerans* have been recorded as a phosphate-solubilizer[18] and competitive bacteria toward phytopathogens,[19] respectively. Moreover, both *Ra. aquatilis* and *Pa. agglomerans* isolated from pear leaves and fruits were reported as indole-3-acetic acid-producing bacteria.[20]

Regardless of the physiological convergence amongst these phyllosphere bacteria, two strains of *Ra. aquatilis* isolated from the green leaves of *Ro. rugosa* had inducible GD activity but none of the isolates from the senescing leaves that are rich in phenolic compounds as well as the green leaves had any GD activity. Because simple phenolics released by *Ra. aquatilis* isolates had significant anti-microbial activities *in vitro*, these GD-positive leaf bacteria may help regulate the phyllosphere microflora of the green leaves.

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