Note
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 rhapsontici, 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 metabolite 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-cafeic acids to release 4-hydroxystryrenes, 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(4) derivable from gallic acid (1) and protocatechuic acid (2), respectively, as shown in Fig. 1 (Hashidoko, unpublished data).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

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Abbreviation: GD, gallate decarboxylase

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Fig. 1. Chemical Structures of Gallic Acid, Protocatechuic Acid, and Their Decarboxylation Products.
portions of the stock culture broth previously diluted
vestigation of the bacterial composition, 100-
cosity of the colonies (Table 1).

distinguishable by their phenotypic and physiological
characteristics, but clearly distinguishable from the vis-
tively named YL-1 and ML-1, respectively) were in-
the lea‰ets in developing and mature stages (tenta-
bacterial isolates that were accordingly puri‰ed from
colonies per plate of emerged on all of the plates. The
broth agar plate, respectively, and then kept at 20
potato-dextrose (PD) agar plate and on a nutrient-

were separately soaked, and the tubes were left at
water and then kept at room temperature overnight.
The aqueous leaf extract thus obtained was first
decanted and then centrifuged at 8000
z
decanted and then centrifuged at 8000

As the source of epiphytes, four lea‰ets, 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 lea‰ets
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 in-
vestigation 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 puri‰ed from
the lea‰ets in developing and mature stages (tentative-
vially named YL-1 and ML-1, respectively) were in-
distinguishable by their phenotypic and physiological
characters, but clearly distinguishable from the vis-
cosity of the colonies (Table 1).

On the other hand, senescing lea‰ets 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 differentia-
tion (Table 1) and a homology search for partial 16S
rDNA sequences on the BLASTN database, these
ﬁve phyllosphere bacteria were identiﬁed to be two
Ra. aquatilis (YL-1 and ML-1), Pantoea agglomer-
trans (SL-1), Klebsiella terrigena (tentative, SL-2),
and Erwinia rhapontici (SL-3), respectively, all of
which had been reported as phylloplane/phyl-
losphere or rhizosphere bacteria. In a comparison
of phenotypic and physiological characteristics with
those of the standard species in Bergey’s Manual of
determinative bacteriology, 9th edition, ﬁve iso-
lates had certain divergences in their cell size, oxidase
activity, catalase activity, H2S production, and so on
(see footnotes in Table 1). However, these character-
stics showed clear convergence amongst the isolates
of four different species inhabiting the Ro. rugosa
phylosphere, and their representative ones were
most identical with those of Phyllobacterium myr-
sinacearum, which has originally been isolated from
a leaf nodule of Ardisia crenata (Myrsinaceae) as a
nitrogen-ﬁxing bacterium. Interestingly, we ob-
served 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 charac-
teristic of Ph. myrsinacearum. This fact en-
couraged us to conceive that the physiological con-
vergence 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 (I), a major constituent of Ro.
rugosa leaves. Using Klebsiella oxytoca JCM 1665
(origin: pharyngeal tonsil) as a reference bacteri-
um, 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 epi-
phyte to give a spot of a major product on TLC, we
judged it positive for the metabolic ability.

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

**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 solu-
tion, 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 plate,
with 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.
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 as before. PCR products from the second amplification from position 375 to position 1186, in which region 90

<table>
<thead>
<tr>
<th>Identifiable genus/species</th>
<th><em>RB</em></th>
<th><em>Psammospora</em></th>
<th><em>Klebsiella</em></th>
<th><em>Escherichia</em></th>
<th><em>Phyllobacterium</em></th>
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<tr>
<td></td>
<td>(YL-1)</td>
<td>(ML-1)</td>
<td>(SL-1)</td>
<td>(SL-2)</td>
<td>(SL-3)</td>
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<td>FA</td>
<td>FA</td>
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<td>Gram stain</td>
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<td>Cell diameter (mm)</td>
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<td>(0.3–0.5)</td>
<td>(0.5–1.0)</td>
<td>(0.3–0.5)</td>
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<td>Indole production</td>
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<tr>
<td>xylonate</td>
<td>+</td>
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<td><strong>malic</strong></td>
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<td>F</td>
<td>F</td>
<td>F</td>
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</table>

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) 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. *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).
rugosa had inducible GD activity but none of the isometabolisms of phenolic compounds. enzyme activities while they were divergent in the losphere bacteria showing convergence in some logical properties, it is rather natural that these phyllosphere bacteria showed some diversity. Because bacteria and other high convergence, their metabolic behaviors toward properties of the phyllosphere bacteria that displayed others did not.

leaves, showed a weak tannase activity, but the most predominant epiphyte on the senescing leaves that are rich in late from the senescing leaves which are rich in chlorogenic acid or 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.

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-solubilizer18 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.

References


