

## Minireview

# Endophytic Bacteria in the Rice Plant

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Endophytic bacteria are defined as bacteria detected inside surface-sterilized plants or extracted from inside plants and having no visibly harmful effects on the plants. Various kinds of endophytic bacteria, such as *Pantoea*, *Methylobacterium*, *Azospirillum*, *Herbaspirillum*, *Burkholderia* and *Rhizobium* etc., have been found inside rice plants. This minireview summarizes and discusses recent studies of endophytic bacteria residing in rice plants, focusing on flora, origin, movement, and interaction with plants/other microbes and referring to endophytes in other plants. The findings concerning bacterial flora obtained by cultural and non-cultural methods are also compared and discussed. Some attempts to apply endophytes to the rice plant and the resultant effects are introduced. The future perspective to deepen the study of endophytes in terms of both application and basic science is considered.

**Key words:** endophytic bacteria, rice, plant-microbe interaction

## Introduction

Various kinds of microorganisms, including fungi, actinomycetes and other bacteria, have been found inside plants and designated as endophytes. Generally, pathogens are not included in endophytes. The presence of endophytes was first recorded by Vogl in 1898<sup>84)</sup> who revealed a mycelium residing in grass seed (*Lolium temulentum*)<sup>75)</sup>. In the early years, studies of endophytes focused on filamentous fungi such as *Neotyphodium* living in the above-ground tissue of gramineous plants<sup>11,71)</sup>. With the finding that infecting by *Neotyphodium* increased yield and intensified tolerance to pests, disease, and dryness, the application of this endophyte to agriculture has been attempted<sup>30)</sup>. Fungal endophytes, including those inhabiting gramineous plants, have been intensively reviewed<sup>10,14,75)</sup>.

Research on the bacteria residing in the internal tissues of plants began in the 1870s with Pasteur and others<sup>33)</sup>. Although there was a general tendency to consider the presence of any kind of bacteria within plant tissues as symptomatic of a pathological condition, Perotti<sup>65)</sup> was the first to describe the occurrence of a nonpathogenic flora in root tissues, and Hennig and Villforth<sup>31)</sup> reported the presence of bacteria in the leaves, stems and roots of 28 apparently healthy plants. Since 1940, there have been numerous reports on indigenous endophytic bacteria in various plant tissues<sup>28)</sup>. In the 1980s, endophytic bacteria having nitrogen-fixing activity were found in gramineous plants<sup>61,68)</sup>. Ever since, the application of endophytes capable of biological nitrogen fixation to plants, presently non-nodulated cereals such as rice, has been a persistent goal.

Endophytes have been defined in several ways, and the definitions have been modified as the research has

advanced<sup>11,28)</sup>. Among the definitions given to endophytic bacteria, the following, by Hallmann *et al.*<sup>28)</sup>, seems to be the most adequate. Hallmann *et al.* defined endophytic bacteria as bacteria detected inside surface-sterilized plants or extracted from inside plants and having no visibly harmful effects on the plants. This definition includes internal colonists with apparently neutral behavior as well as symbionts. It would also include bacteria which migrate back and forth between the surface and inside of the plant during their endophytic phase. Some endophytic bacteria have been discovered to have beneficial effects on the host plant, such as plant growth promotion, the induction of increased resistance to pathogens, as well as the supply of fixed nitrogen to the host plant. To utilize these beneficial features in agriculture, a variety of experiments are currently being carried out. However, our knowledge of endophytic bacterial ecology is still limited.

Rice (*Oryza sativa*) is the most important cereal crop in the world, feeding more than 50% of the world's population<sup>27)</sup>, and its yield must be increased to match the rise in consumption. This must be achieved without the mass use of chemical fertilizers and pesticides, which may cause environmental pollution and negatively influence human health. Thus, the application of endophytic bacteria having beneficial characteristics to the cultivation of rice as well as other plants is crucial. Here, we would like to review recent studies on endophytic bacteria residing in the rice plant, referring to studies carried out for other plants.

## Endophytic bacterial flora in the rice plant

The endophytic bacteria isolated from various parts of the rice plant are listed in Table 1. Researchers have commonly isolated *Pantoea* from the seeds, *Methylobacterium* from the shoots, *Azospirillum* and *Herbaspirillum* from the stems and roots, and *Burkholderia* and *Rhizobium* from the roots. These

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**Table 1.** Endophytic bacteria isolated from various parts of rice plants

Rice part	Rice species	Bacterial taxa	Reference
Seed	<i>Oryza alta</i>	<i>Pantoea ananas</i>	17
Seed	<i>Oryza meridionalis</i>	<i>Herbaspirillum seropedicae</i> , <i>Methylobacterium</i> sp.	17
Seed	<i>Oryza sativa</i>	<i>Klebsiella oxytoca</i>	17
Seed	<i>Oryza sativa</i>	<i>Acidovorax</i> sp., <i>Bacillus pumilus</i> , <i>Bacillus subtilis</i> , <i>Curtobacterium</i> sp., <i>Methylobacterium aquaticum</i> , <i>Micrococcus luteus</i> , <i>Paenibacillus amylolyticus</i> , <i>Pantoea ananatis</i> , <i>Sphingomonas melonis</i> , <i>Sphingomonas yabuuchiae</i> , <i>Xanthomonas translucens</i>	47
Seed	<i>Oryza sativa</i>	<i>Bacillus cereus</i> , <i>Pantoea ananatis</i> , <i>Sphingomonas echinoides</i> , <i>Sphingomonas parapaucimobilis</i>	60
Seed	<i>Oryza sativa</i>	<i>Ochrobactrum anthropi</i> *, <i>Pantoea agglomerans</i> *, <i>Pseudomonas boreopolis</i> *, <i>Pseudomonas fulva</i> *	82
Leaf	<i>Oryza sativa</i>	<i>Aurantimonas altamirensis</i> , <i>Bacillus gibsonii</i> , <i>Bacillus pumilus</i> , <i>Curtobacterium</i> sp., <i>Diaphorobacter nitroreducens</i> , <i>Methylobacterium aquaticum</i> , <i>Methylobacterium</i> sp., <i>Pantoea ananatis</i> , <i>Sphingomonas echinoides</i> , <i>Sphingomonas melonis</i> , <i>Sphingomonas yabuuchiae</i> , <i>Stenotrophomonas maltophilia</i> , <i>Streptomyces</i> sp.	48
Leaf sheath	<i>Oryza sativa</i>	<i>Methylobacterium</i> sp.	17
Stem	<i>Oryza alta</i>	<i>Azospirillum amazonense</i> , <i>Flavobacterium gleum</i>	17
Stem	<i>Oryza barthii</i>	<i>Herbaspirillum rubrisubalbicans</i> *	18
Stem	<i>Oryza brachyantha</i>	<i>Methylobacterium</i> sp.	17
Stem	<i>Oryza glandiglumis</i>	<i>Azospirillum lipoferum</i> *	18
Stem	<i>Oryza latifolia</i>	<i>Methylobacterium</i> sp.	17
Stem	<i>Oryza longiglumis</i>	<i>Methylobacterium</i> sp.	17
Stem	<i>Oryza meridionalis</i>	<i>Methylobacterium</i> sp.	17
Stem	<i>Oryza minuta</i>	<i>Methylobacterium</i> sp.	17
Stem	<i>Oryza officinalis</i>	<i>Herbaspirillum seropedicae</i> *	18
Stem	<i>Oryza ridleyi</i>	<i>Rhodopseudomonas palustris</i>	17
Stem	<i>Oryza rufipogon</i>	<i>Methylobacterium</i> sp., <i>Sphingomonas adheasiva</i>	17
Stem	<i>Oryza rufipogon</i>	<i>Azospirillum brasilense</i> *, <i>Enterobacter cancerogenus</i> *, <i>Herbaspirillum seropedicae</i> *	18
Stem	<i>Oryza sativa</i>	<i>Cytophagales</i> str. MBIC4147, <i>Methylobacterium</i> sp.	17
Stem	<i>Oryza sativa</i>	<i>Azospirillum lipoferum</i> *, <i>Ideonella dechloratans</i> *	18
Stem	<i>Oryza sativa</i>	<i>Agrobacterium vitis</i> *, <i>Azorhizobium caulinodans</i> *, <i>Azospirillum</i> sp., <i>Bacillus megaterium</i> , <i>Bacillus subtilis</i> , <i>Pseudomonas cepacia</i> *	77
Leave, Stem, Root	<i>Oryza sativa</i>	<i>Herbaspirillum seropedicae</i> *	61
Stem, Root	<i>Oryza sativa</i>	<i>Serratia marcescens</i> *	27
Root	<i>Oryza granulata</i>	<i>Klebsiella</i> sp.*	20
Root	<i>Oryza minuta</i>	<i>Azoarcus</i> sp.*	20
Root	<i>Oryza nivara</i>	<i>Gallionella</i> sp.*	20
Root	<i>Oryza officinalis</i>	<i>Azoarcus</i> sp.*, <i>Sphingomonas paucimobilis</i> *	20
Root	<i>Oryza sativa</i>	<i>Azoarcus</i> sp.*, <i>Azoarcus indigenus</i> *, <i>Azorhizobium caulinodans</i> *, <i>Azospirillum brasilense</i> *, <i>Azospirillum lipoferum</i> *, <i>Burkholderia</i> sp.*, <i>Herbaspirillum</i> sp.*, <i>Klebsiella pneumoniae</i> *, <i>Ochrobactrum</i> sp.*, <i>Sphingomonas paucimobilis</i> *	20
Root	<i>Oryza sativa</i>	<i>Herbaspirillum seropedicae</i> *	5
Root	<i>Oryza sativa</i>	<i>Azospirillum irakense</i> , <i>Bacillus luciferensis</i> , <i>Bacillus megaterium</i> , <i>Bradyrhizobium elkanii</i> , <i>Bradyrhizobium japonicum</i> , <i>Brevibacillus agri</i> , <i>Burkholderia kururiensis</i> , <i>Caulobacter crescentus</i> , <i>Chryseobacterium taichungense</i> , <i>Enterobacter cloacae</i> , <i>Enterobacter ludwigii</i> , <i>Hyphomicrobium sulfonivorans</i> , <i>Methylocapsa acidiphila</i> , <i>Micrococcus luteus</i> , <i>Mycobacterium petroleophilum</i> , <i>Paenibacillus alvei</i> , <i>Rhizobium loti</i> , <i>Roseateles depolymerans</i>	48
Root	<i>Oryza sativa</i>	<i>Burkholderia cepacia</i> ***, <i>Rhizobium leguminosarum</i> **	76
Root	<i>Oryza sativa</i>	<i>Rhizobium leguminosarum</i> **	87

\*Nitrogen-fixing endophytes isolated from rice plants by using nitrogen free medium.

\*\*Nitrogen-fixing endophytes isolated from rice roots by using a legume trap.

endophytes have also been isolated from other plants: *Pantoea* from corn, cotton, potato, red clover, grapevine and pea<sup>8,19,50,78,79</sup>, *Methylobacterium* from corn, cotton and red clover<sup>50,79</sup>, *Azospirillum* from gramineous plants<sup>40</sup>, *Herbaspirillum* from maize, sorghum, sugar cane and several gramineous plants<sup>4,37,61,62</sup>, and *Burkholderia* from corn, cotton and cucumber<sup>44,50,79</sup>. The most common taxa among the endophytic bacteria isolated from the tissues of various plants including alfalfa, corn, cotton, cucumber, rough lemon, sugar beet, potato, grapevine, and tomato etc., are the

former *Pseudomonas* group (*Pseudomonas*, *Burkholderia*, *Phyllobacterium*) and *Enterobacteriaceae* (*Enterobacter*, *Erwinia*, *Klebsiella*)<sup>28</sup>.

The functional gene possessed by endophytes may relate to their distribution in rice plants. Many nitrogen-fixing endophytes have been isolated from seeds and stems as well as roots (Table 1). These beneficial endophytes may be preferentially received by the host plant. The endophytes in rice seeds seem to be exposed to high osmotic pressure. *Pantoea ananatis* which is frequently isolated from rice seeds was

**Table 2.** Bacterial diversity of rice plants as revealed by non-culture-based methods

Rice part	Rice species	Bacterial group	Bacterial taxa	Reference
Surface and inside of milled rice	<i>Oryza sativa</i>	Gamma proteobacteria	<i>Erwinia amylovora</i> , <i>Pseudomonas fluorescens</i> , <i>Xanthomonas sacchari</i>	34
		Firmicutes	<i>Staphylococcus</i> sp.	
		Actinobacteria	<i>Nocardia globerula</i>	
Inside of root	<i>Oryza sativa</i>	Alpha proteobacteria	<i>Brevundimonas diminuta</i> , <i>Caulobacter</i> sp., <i>Kaistina koreensis</i> , <i>Methylobacterium</i> sp., <i>Novosphingobium tardaugens</i> , <i>Sinorhizobium terangae</i>	78
		Beta proteobacteria	<i>Achromobacter xylosoxidans</i> , <i>Acidovorax facilis</i> , <i>Burkholderia fungorum</i> , <i>Burkholderia</i> sp., <i>Comamonas testosteroni</i> , <i>Curvibacter gracilis</i> , <i>Delftia acidovorans</i> , <i>Delftia tsuruhatensis</i> , <i>Duganella violaceinigra</i> , <i>Gallionella ferruginea</i> , <i>Herbaspirillum frisingense</i> , <i>Hydrogenophaga taeniospiralis</i> , <i>Methyloversatilis universalis</i> , <i>Sterolibacterium denitrificans</i> , <i>Variovorax</i> sp.	
		Gamma proteobacteria	<i>Acinetobacter baumannii</i> , <i>Alkanindiges illinoisensis</i> , <i>Enterobacter</i> sp., <i>Methylophaga marina</i> , <i>Pantoea</i> sp., <i>Plesiomonas shigelloides</i> , <i>Pseudomonas stutzeri</i> , <i>Stenotrophomonas maltophilia</i> , <i>Stenotrophomonas</i> sp.	
		Deltaproteobacteria	<i>Bdellovibrio bacteriovorus</i> , <i>Geobacter</i> sp.	
		Epsilonproteobacteria	<i>Sulfurospirillum multivorans</i>	
		Bacteroidetes	<i>Flavobacterium frigidum</i> , <i>Flavobacterium psychrophilum</i> , <i>Sphingobacterium</i> sp.	
		Firmicutes	<i>Acidaminobacter hydrogeniformans</i> , <i>Clostridium</i> sp., <i>Lachnospiraceae</i> bacterium, <i>Planomicrobium okeanokoites</i> , <i>Planomicrobium mcmeekinii</i>	
		Deinococcus-Thermus	<i>Deinococcus indicus</i>	
		Acidobacteria	<i>Holophaga foetida</i>	

reported to be tolerant of high osmotic pressure<sup>47</sup>). *Methylobacterium* which is frequently isolated from rice shoots is composed of a variety of pink-pigmented, facultatively methylotrophic (PPFM) bacteria which are tolerant to various stress factors such as gamma-ray radiation, dryness and lack of nutrients<sup>26</sup>). This may lead the PPFM to inhabit the surface<sup>15</sup>) and to invade inside the plant, or *vice versa*. PPFMs are known to produce cytokinin, a plant growth regulator, while they seem to utilize the methanol which is formed in the plant body through the demethylation of pectin etc., as a carbon source<sup>32,58</sup>). PPFMs may also utilize methanol produced from methane (generated in paddy soil and emitted from stomates of leaves through the rice plant body) by methane-oxidizing bacteria.

Traditional plating techniques usually result in underestimates of diversity; for example, in the case of soil, less than 10% of the total bacterial community present can be detected<sup>81</sup>). In the rhizosphere, this percentage is between 1% and 10%<sup>9</sup>). Therefore, molecular fingerprinting techniques independent of culturing and based on small-subunit (SSU) rRNA genes (rDNA) have become popular in addressing the problems related to the diversity, structural composition and dynamics of microbial communities. However, the investigation of endophytic bacterial communities using culture-independent techniques is hampered by the organelle SSU rDNA from the plant tissue, which can be amplified by the polymerase chain reaction with the universal primer for the endophytic SSU rDNA. Many researchers, including the authors, have encountered the same difficulty<sup>24,49,59,73,86</sup>). To overcome this obstacle, Chelius, Sakai, Sun and others<sup>12,70,80</sup>) have designed new primers that specifically amplify the SSU rDNA of various bacterial species without amplifying the SSU rDNA of the plant plastids. Sun *et al.* examined the endophytic bacterial diversity in rice roots by conducting an

amplified ribosomal DNA restriction analysis (ARDRA)<sup>80</sup>) using the new primers. They revealed diverse taxa of bacteria in the SSU rDNA library (Table 2). Some clones showed high similarity to uncultured bacteria and archaea. Ikeda *et al.*<sup>35</sup>) have shown that a ribosomal intergenic spacer analysis (RISA) can be used to generate profiles for plant-associated microbial communities from diverse agronomic products without any noticeable bias caused by the presence of excess amounts of plant DNA. They successfully examined the microbial diversity in milled rice seeds using RISA without distinguishing between the surface and the inside of the seeds and detected several sequences highly similar to *Pseudomonas fluorescens*, *Xanthomonas sacchari*, *Erwinia amylovora*, *Nocardia globerula* and *Staphylococcus* sp., as well as several uncultured bacteria (Table 2). The bacteria detected using non-culture-based methods by Ikeda, Sun and others<sup>35,80</sup>) (Table 2) are not found among the endophytic bacteria isolated using conventional culture methods (Table 1), except for *Stenotrophomonas maltophilia* isolated from leaves of rice plants (*Oryza sativa*)<sup>48</sup>). The evaluation of the findings obtained by both culture-based and non-culture-based methods and the consideration of the discrepancy between those findings will promote our understanding of endophytes.

### Origin of endophytic bacteria residing in the rice plant

Endophytic bacteria have been considered to originate from the outside environment and enter the plant through stomata, lenticles, wounds (including broken trichomes), areas of emergence of lateral roots and germinating radicles<sup>34</sup>). By comparing the internal and external root communities of cucumber<sup>44</sup>), cotton<sup>29</sup>) and potato<sup>78</sup>), it was revealed that almost all endophytic bacteria of those plants are also found

in the rhizosphere. Also, the endophytic bacterial communities in the roots of canola, wheat<sup>25,74</sup>, cotton, sweet corn<sup>50</sup> and maize<sup>72</sup> were reported to be a subset of the soil community. Marilley and Aragno<sup>49</sup> found that the bacterial diversity decreased from the bulk soil to the rhizosphere and further to the roots in *Lolium perenne* and *Trifolium repens*. Many endophytic bacteria residing not only in roots but also in seeds and leaves were found in the external environment. We investigated the endophytic and surface bacterial flora of the seeds (after removing hulls) and leaves of rice plants cultivated in a paddy field<sup>47,48</sup>. Among the endophytic bacteria of the seeds and leaves, 56% and 63% were also isolated from the surface of those parts, respectively.

The role of seeds as a source of endophytic bacteria is still controversial<sup>28</sup>. Sato<sup>71</sup> has not detected endophytic bacteria in rice seeds. On the other hand, we have detected culturable endophytic bacteria in rice seeds at a viable population density ranging from  $10^2$  to  $10^6$  CFU/g fresh weight<sup>47,60</sup>. Mundt and Hinkle<sup>57</sup> obtained endophytes only from the damaged seeds of 29 kinds of plants other than rice. Endophytic bacteria were detected in the seeds of cotton at a viable population density ranging from  $1 \times 10^3$  to  $1 \times 10^5$  CFU/g fresh weight, and in the seeds of sweet corn below  $1 \times 10$  CFU/g fresh weight<sup>51</sup>. Adams and Kloepper<sup>2</sup> detected endophytes in radicles 48 h after placing surface-disinfected cotton seeds on water agar, but not before incubation.

McInroy and Kloepper<sup>51</sup> have assessed the contribution of seeds as sources of endophytic bacteria using surface-disinfected seeds of sweet corn and cotton on water agar. The endophytic bacterial population of the seedlings was below  $10^2$  CFU/g fresh weight for sweet corn and  $10^5$  CFU/g fresh weight for cotton at 6 days after germination. They described that seeds may be one of the origins of the endophytes. The authors are now investigating the role of rice seeds as the origin of endophytic bacteria (unpublished) by examining the endophytic bacterial flora of rice seedlings aseptically cultivated *in vitro* using surface-disinfected seeds. The genera found in rice plants cultivated in a paddy field<sup>47,48</sup> have also been detected in the rice seedlings. These endophytes may exist in an embryo, or migrate from the endosperm to the radicle and plumule when they newly emerge through the internal tissue of the seed. On the other hand, these endophytes may invade the internal tissue from the surface of the seedling after they spread to the surrounding environment following germination. In either case, rice seeds appear to be one of the origins of endophytic bacteria.

In general, endophytic bacteria colonize the intercellular spaces and vascular system of the host plant, with only a few reports demonstrating intracellular colonization<sup>28</sup>. Since endophytic bacteria are considered to compete for the limited space provided by the host plant with each other and with other endophytic organisms such as fungi<sup>23,66</sup>, the endophytic bacteria residing in seeds may have the advantage of being able to immediately infect the next generation of plants after germination.

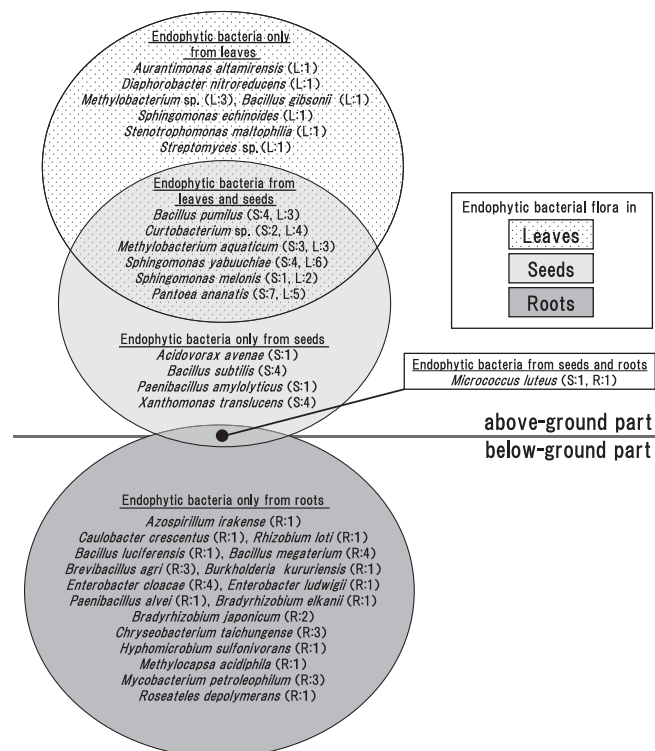
#### ***Invasion and movement of endophytic bacteria residing in the rice plant***

Endophytic bacteria have been considered to originate

from the outside environment and enter the plant through stomata, lenticles, wounds (including broken trichomes), areas of emergence of lateral roots and germinating radicles, as mentioned above<sup>34</sup>. However, endophytic bacteria appear mainly to invade the plant through the wounds which occur naturally at the points of lateral root emergence and at the root tips as a result of plant growth. The extensive colonization of the secondary root emergence zones (sites of root branching) by endophytic bacteria has been reported for several plants: *Brassica* species<sup>3</sup>, sugar beet<sup>36</sup>, *Phaseolus vulgaris*<sup>41,46</sup>, maize and other grasses<sup>64</sup>, kallar grass<sup>69</sup>, and *Lupinus albus* and *Pisum sativum*<sup>85</sup>. Because of the breaks in the endodermis at these points, the bacteria colonizing the cortex traverse the endodermis into the vascular tissue. In general, endophytic bacteria colonize the intercellular spaces and vascular system, with only a few reports demonstrating intracellular colonization. In addition, some endophytic bacteria colonize the host plant via the vascular system<sup>28</sup>.

The endophytic bacteria of the rice plant appear to enter the host plant and colonize it in the manner mentioned above for other plants. However, intracellular colonization has not been observed yet. A  $\beta$ -glucuronidase (GUS)-marked strain of *Herbaspirillum seropedicae* Z67 enters the root of the rice seedling via cracks at the points of lateral root emergence, and subsequently colonizes the root's intercellular spaces, aerenchyma and cortical cells, with a few penetrating the stele to enter the vascular tissue. Thereafter, the endophyte colonizes the epidermal cells, intercellular spaces and substomatal cavities of the leaves and stem<sup>38</sup>. *Serratia marcescens* IRBG500 marked with *gusA* appears to enter the root of the rice seedling via cracks at the points of lateral root emergence and at the root tips. Immunogold-marked IRBG500 bacteria endophytically establish themselves within the root, stem and leaves, and can be observed within the intercellular spaces, aerenchyma, xylem vessels and senescing root cortical cells whose cytoplasm is no longer intact and exists only as fragments, but they are not found within intact host cells<sup>27</sup>. Fluorescence stereomicroscopy has revealed that GFP-tagged *Herbaspirillum* sp. B501 colonizes the shoots and seeds of aseptically grown wild-rice seedlings (*Oryza officinalis*) after inoculation of the seeds. Observations by fluorescence and electron microscopy have demonstrated that *Herbaspirillum* sp. strain B501 colonizes mainly the intercellular spaces in the leaves of wild rice<sup>18</sup>.

The studies mentioned above have investigated bacteria which had been inoculated on rice seedlings aseptically cultivated *in vitro* from surface-disinfected seeds. In the field, how do endophytic bacteria migrate into/on the rice plant? We investigated the endophytic bacterial flora in the seeds (after removing hulls), leaves and roots of the same rice plant individual cultivated in a paddy field<sup>47,48</sup>. About 30 strains were isolated from each part of the rice plant and identified based on the SSU rRNA gene sequences. The isolates from inside the leaves, seeds and roots were close to 13, 11 and 18 species, respectively. Six species (*Bacillus pumilus*, *Curto-bacterium* sp., *Methylobacterium aquaticum*, *Sphingomonas yabuuchiae*, *Sphingomonas melonis* and *Pantoea ananatis*) were common to the leaves and seeds, and constituted over 60% of the endophytic bacterial floras colonizing each of these parts. On the other hand, the endophytic root bacteria



**Fig. 1.** Comparison of the culturable endophytic bacterial floras in leaves, seeds and roots of rice plants (*Oryza sativa*). Data from Mano et al., 2006, 2007. About 30 strains were isolated from each part of the plant and identified by 16S rRNA gene sequences. Abbreviations (L, S, R) and figures in brackets after the species name stand for the part of the plant (Leaves, Seeds, Roots) from which the species were isolated and the number of isolates identified as the species.

differed from the isolates in the leaves and seeds, with the exception of *Micrococcus luteus* (Fig. 1). The floras differed greatly according to the outside environment, i.e. the atmosphere in the case of the rice shoot and the soil in the case of the root. We also investigated the bacterial flora of the surface of the seeds and leaves. Among the 13 species isolated from inside the leaves, 7 species (*Aurantimonas altamirensis*, *Bacillus pumilus*, *Curtobacterium* sp., *Methylobacterium aquaticum*, *Methylobacterium* sp., *Sphingomonas yabuuchiae* and *Sphingomonas melonis*) were also detected on the surface of the leaves. Among the 11 species isolated from inside the seeds, 6 species (*Bacillus subtilis*, *Curtobacterium* sp., *Methylobacterium aquaticum*, *Micrococcus luteus*, *Sphingomonas yabuuchiae* and *Xanthomonas translucens*) were also detected on the surface of the seeds. These species constituted 69% and 56% of the endophytic bacterial flora in the leaves and seeds, respectively<sup>47,48</sup>. Since many endophytic bacteria in the rice plant appear to enter the tissue from the surface of each part, these bacteria may constitute a subset of the epiphytic bacterial population. The bacteria at the phyllosphere and spermosphere are exposed to stress factors, such as UV radiation, dryness, reactive oxygen and lack of nutrients<sup>42</sup>. In contrast, in the rhizosphere, organic compounds such as amino acids and saccharides are released from the roots. Also, some roots are necrotized with the growth of the plant, and can be utilized by the microbe as a nutrient source in the rhizosphere. These differences in the outside environment between the leaves, seeds and roots

seem to result in the specific epiphytic bacterial flora inhabiting each part. Some of the epiphytic bacteria may enter the rice plant through stomata, wounds, etc. Mocali et al.<sup>55</sup> revealed that the endophytic bacterial flora of the elm tree (*Ulmus* spp.) is influenced by temperature variations. This could mean that endophytic bacteria are related to epiphytic bacteria, which are affected by temperature changes.

There are endophytic bacteria which have been isolated so far only from inside the rice plant (leaves: *Bacillus gibsonii*, *Diaphorobacter nitroreducens*, *Pantoea ananatis*, *Sphingomonas echinoides*, *Stenotrophomonas maltophilia* and *Streptomyces* sp.; seeds: *Acidovorax avenae*, *Bacillus pumilus*, *Paenibacillus amylolyticus*, *Pantoea ananatis* and *Sphingomonas melonis* (Fig. 1)). Where do they originate from? One possibility is that they originate from the surface of the plant, but these bacteria may be so scarce on the surface that they may not be easy to detect there. Another possibility is that they originate from the seed and infect the tissue of the next generation of plants by migrating from the seed. A third possibility is that endophytic bacteria which are in very small numbers in the root are transported to the shoot through the vascular system. We have reported that the population diversity of endophytic bacteria in the rice roots is richer than that in the seeds and leaves<sup>47,48</sup>. According to McInroy and Kloepper<sup>50</sup> and Sturz et al.<sup>79</sup>, endophytic bacteria frequently appear to be transported from the root to the lower part of the stem in plants which grow in the field. Although the endophytic bacterial floras differ among the shoot (seed and leaves) and root in the rice plant, many endophytic bacteria were common to the stem and root of sweet corn and cotton<sup>50</sup>. Among the species isolated from inside the stems of sweet corn and cotton, 93% and 94% were also isolated from inside the roots, respectively. The height of the sampled plant part might have to be considered. We sampled the uppermost leaf (about 80 cm from the ground) and the seed of the rice plant<sup>48</sup>, whereas McInroy and Kloepper sampled the stem of sweet corn and cotton at a height of about 1~10 cm from the ground. The endophytic bacteria which migrate from the root may be able to reach the lower part of the stem at the farthest. Sturz et al.<sup>79</sup> have investigated the endophytic bacterial flora in the upper and lower foliage as well as the root and nodules of red clover. Of the species isolated from the lower foliage of red clover, 87% were also isolated from inside the root and nodules. In the case of the upper foliage, only 58% were isolated from inside the root and nodules.

#### Interaction of endophytic bacteria in the rice plant with other endogenous organisms and with the host plant

Endophytic bacteria interact with other endogenous organisms in the host plant. For example, an endophytic bacterium is considered to compete for the limited colonization space and nutrients provided by the host plant with other plant-colonizing microorganisms such as fungi and other bacteria. When *Enterobacter asburiae* JM22 is applied to cotton together with *Paenibacillus macerans*, the coexistence of these two endophytes results in reduced population densities for both endophytes as compared with the population densities resulting from their separate application<sup>66</sup>. GFP-tagged *Pantoea* sp. and *Ochrobactrum* sp. colonize the intercellular

spaces in the root cortex of the rice plant when inoculated separately. Colonization by *Ochrobactrum* sp. is severely inhibited when this endophyte is co-inoculated with an equal number ( $10^5$  CFU/ml) of *Pantoea* sp. But the extent of colonization by *Pantoea* sp. in the presence of *Ochrobactrum* sp. is as vigorous as it was in the case when only *Pantoea* sp. was used for inoculation<sup>83</sup>). *Pantoea* sp. is a more aggressive endophytic colonizer of its host than *Ochrobactrum* sp. Several endophytic bacteria are known to inhibit plant pathogens, and their application in agriculture has been attempted<sup>28</sup>). Mukhopadhyay *et al.*<sup>56</sup>) have reported that several endophytic bacteria isolated from rice seeds exhibit strong anti-fungal activity against *Rhizoctonia solani*, *Pythium myriotylum*, *Gaeumannomyces graminis* and *Heterobasidium annosum*. Also, the rice endophyte *Bacillus megaterium* has strong antagonistic activity against *Meloidogyne graminicola*, which is a pest affecting rice<sup>63</sup>). Recently, Minamisawa *et al.*<sup>52,53</sup>) have reported a new type of interaction among endophytic bacteria. They found anaerobic nitrogen-fixing consortia (ANFICOs) consisting of  $N_2$ -fixing clostridia and diverse nondiazotrophic bacteria in gramineous plants. The nitrogen fixation by the anaerobic clostridia is considered to be supported by the elimination of oxygen by the accompanying bacteria.

Endophytic bacteria appear to be influenced by the host's living conditions. Mahaffee and Kloepper<sup>45</sup>) have examined the culturable bacterial communities of three habitats (rhizosphere, endorhiza and soil) associated with field-grown cucumber roots for quantitative and qualitative changes in the community structure over time. They found that the bacterial flora in the soil remained relatively unchanged throughout the study, while the floras in the rhizosphere and endorhiza were dissimilar from that in the soil and from each other at particular sampling times. The endophytic bacterial flora of the rice plant changes with the growth of the plant<sup>47,48,60</sup>). Barraquio *et al.*<sup>7</sup>) have also reported that the ratio of diazotrophic endophytes in the root of a rice plant grown in an N-unfertilized field was higher at or near the heading. Some endophytic bacteria may be preferentially received by the host plant as rhizobia forming nodules at the roots of legume. A number of researchers have recently reported the induction of nodule-like structures on the roots of cereals primarily by rhizobia<sup>16</sup>). Also, Yanni *et al.*<sup>87</sup>) have found a previously unknown relationship among endophytic bacteria and two host species, rice and Egyptian berseem clover. For over 7 centuries, the production of rice (*Oryza sativa* L.) in Egypt has benefited from rotation with Egyptian berseem clover (*Trifolium alexandrinum*). The researchers posited the possibility that rice plants cultivated in this area can utilize the fixed N supplied by the clover symbiont, *Rhizobium leguminosarum* bv. *trifolii*, which colonizes the roots of the rice plants endophytically, as well as the mineralization products of N-rich clover crop residues.

#### **Application of endophytic bacteria to rice plants**

Some endophytic bacteria in rice plants have been reported to promote host growth. When the diazotrophic endophytes *Herbaspirillum seropedicae* Z67<sup>38</sup>), *Herbaspirillum* sp. B501<sup>89</sup>), *Serratia marcescens* IRBG500<sup>27</sup>), and some

strains of *Herbaspirillum seropedicae* and *Burkholderia* spp.<sup>6</sup>) are inoculated on rice seedlings, the inoculated plants show a significant increase in weight compared to the controls. A significant increase in biomass and grain yield has also been recorded in greenhouse-grown rice plants inoculated with *Rhizobium leguminosarum* bv. *phaseoli*<sup>76</sup>). *Pantoea agglomerans* YS19 shows nitrogen-fixing activity in N-free medium, produces four categories of phytohormones (indole-3-acetic acid, abscisic acid, gibberellic acid and cytokinin) in Luria-Bertani medium, and can enhance the biomass of the host rice seedlings<sup>22</sup>).

The biological control of plant pathogens using endophytic bacteria exhibiting anti-pathogen activity has been examined for rice plants as well as other plants<sup>56,63</sup>). Cho *et al.*<sup>13</sup>) have put forth the possibility that the virulence of *Burkholderia glumae*, which is a casual pathogen of seedling rot and grain rot in rice controlled by quorum sensing, is inhibited by *Burkholderia* sp. KJ006 possessing an *N*-acyl-homoserine lactonase (*aiiA*) gene and occupying the same ecological niche as *Burkholderia glumae*. Two rhizobial strains have been found to induce phenolic acids, which mediate systemic resistance and provide bioprotection to rice plants infected by *Rhizoctonia solani*, which is the causal agent of sheath blight disease in rice<sup>54</sup>). In addition, both rhizobial strains promote the growth and productivity of rice plants under greenhouse conditions. Li *et al.*<sup>43</sup>) have indicated that *Leifsonia xyli* subsp. *cynodontis* has the potential to be used as an endophyte for the expression of foreign genes in rice.

#### **Future**

The application of endophytes with nitrogen-fixation activity and inhibitory activity against pathogens to agriculture has been a long-standing goal. It has been revealed that endophytic bacteria are influenced by the host plant and other endogenous organisms; therefore, a way of ensuring the stable existence of endophytes must be developed by taking these influences into account. Using *dapB*-based *in vivo* expression technology (IVET), Rediers *et al.*<sup>67</sup>) have identified several candidate genes of the diazotrophic endophyte *Pseudomonas stutzeri* A15 that are involved in the colonization of the root of the rice plant. *Pantoea agglomerans* is a diazotrophic endophyte isolated most frequently and in large numbers from all surface disinfected tissues (root, stem, leaf and seeds) sampled from the rice plant at different stages of growth and development<sup>21</sup>). This endophyte forms multicellular structures called symplasmata<sup>1,21</sup>). The symplasmata may improve the stability of endophytic bacteria in the host plant by buffering the influences exerted by other endobiotic organisms and the changes in the inner environment during host plant growth.

The use of endophytic bacteria in agriculture may be enhanced if we understand the interaction between endophytic bacteria and the host plant. For example, the mechanism through which endophytes colonize the host plant and bypass the plant's defenses, and the substrate supplied by the host plant and utilized by the endophytic bacteria as a nutrient source, etc. can be pointed out. In addition, the combination of endophytic bacteria with other endobiotic organisms

such as ANFICOs may induce synergistic effects on as yet unknown functions. However, we must also take into account the safety of biocontrol agents. *Bacillus thuringiensis*, which is characterized by its ability to form parasporal crystalline inclusions toxic to the insect larvae of different orders, has been commercially used as an insecticide since the beginning of the 1950s. Recently, the gene for enterotoxin and its product have been found in a strain of *Bacillus thuringiensis*, which has led to a closer examination of the safety of this insecticide<sup>39</sup>.

In the past decade, molecular biological methods independent of culture, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP), ribosomal intergenic spacer analysis (RISA) and amplified ribosomal DNA restriction analysis (ARDRA), have been used to study the structural composition and diversity of endophytic bacterial communities<sup>12,24,35,49,59,70,73,80,86</sup>. The interference caused by organelle SSU rDNA from the plant tissue can now be bypassed using RISA<sup>35</sup> and the new primers designed by Chelius, Sakai, Sun and others<sup>12,70,80</sup>. You *et al.*<sup>88</sup> have investigated the expression of nitrogenase genes in *Herbaspirillum* sp. B501 localized in the shoot (leaves and stem) of wild rice, *Oryza officinalis*, by means of reverse transcription-PCR (RT-PCR) targeted at the *nifH* gene. Such molecular biological methods will be applied in many studies of endophytic bacteria in future, not only for community analyses but also for functional analyses. Conventional culture methods, on the other hand, enable us to study the inner environment of the plant through the analysis of the functions of isolates, and there will be efforts to obtain many more isolates by improving the procedures and media that mimic the inner environment of the plant. By combining the findings obtained by both culture-dependent and -independent methods, the frontier of our understanding of plant endophytes will be pushed forward.

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