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# Bacterial Flora of Endophytes in the Maturing Seed of Cultivated Rice (*Oryza sativa*)

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Endophytic bacteria in the seeds of rice plants (*Oryza sativa*, cultivar Nipponbare) were studied during the rice maturation process by comparing them with the bacteria at the surface of rice seeds. The endophytic and surface bacteria were isolated from cultivated rice seeds by using a nutrient broth (NB) and a diluted nutrient broth (DNB) agar medium. The endophytes at the early stages of maturation were relatively diverse, consisting of strains closely related to the genera *Bacillus*, *Sphingomonas* and *Pantoea*. In contrast, the endophytic and surface bacteria at the middle and late stages of the maturation process (39 strains altogether) were all closely related only to the genus *Bacillus* with the exception of one isolate, and almost all of these strains were motile and spore-forming. It was deduced that these bacteria might have migrated into the rice seeds before they hardened that is, during the early stage (the endophytes at the early stage were also all motile and about half of them were spore-forming), subsisting as endospores during the middle and late stages of the maturation process. Furthermore, most of the isolates (19 of 23) from the surface bacteria and the endophytic bacteria grew better when sucrose (1.2 M) was added to the NB liquid medium. These bacteria may be able to adapt to a high osmotic pressure.

**Key words:** rice seed, bacterial flora, maturation process, 16S rRNA gene sequence, high osmotic pressure

Various kinds of microorganisms have been found in plants. These microbes (endophytes) include fungi[7], actinomycetes[3] and other bacteria.[16] Microbial endophytes are defined as those microorganisms detected in surface-sterilized plants[9]. Endophytes are known to be either pathogenic or nonpathogenic to host plants. In addition, recent studies indicate that some endophytic microbes are beneficial to host plants. Coombs[4] has shown that endophytic filamentous actinobacteria strengthened the resistance of wheat plants against pathogens. Furthermore, it has been suggested that the endophytic bacteria in the seed tubers of potato play an important role in seed-piece decay, tuberization and plant growth[18].

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Although rice is one of the most important crops agriculturally and economically, the study of the endophytic bacteria of rice plants has been limited. Elbeltagy *et al.*[5] have revealed by using GFP-tagged bacteria that, in the stem of wild rice, endophytes mainly colonize the intercellular spaces of the leaves. *Herbaspirillum* sp. has been revealed to have nitrogen fixation activity as an endophyte in young seedlings of wild rice[6]. Mukhopadhyay *et al.*[14] have isolated several endophytic bacteria from surface-sterilized rice seedlings, and showed that the isolates, *Enterobacter cloacae* and *Bacillus polymyxa*, protect the seedlings from pathogens. These studies focused on the endophytes in the stem and root of the plant. Little is known about endophytic bacteria in rice seeds.

A rice plant usually takes about 60 days to mature after flowering. During the maturation process, the amount and

concentration of water and sugar in the seed change greatly[11]. The endophytes seem to be exposed to the drastic changes taking place inside the seed. We aimed to clarify the nature of the bacterial flora in the seed during the maturation process. So, we divided the maturation process into three stages, *i.e.*, early, middle and late, and isolated bacteria at each of the stages.

We used a nutrient broth (NB) medium and a 100-fold diluted NB medium to isolate bacteria from various environments such as grassland[12], paddy field[8] and lake sediment[15]. By observing the total number of colonies on these agar media, we were able to provisionally determine whether the environment from which the isolates were obtained is suitable for copiotrophs or oligotrophs. Based on the pattern of increase in the number of colonies during incubation (colony forming curve; CFC), we were able to divide the isolates into several groups, according to when the colonies first appeared. It has been revealed previously that isolates taking a longer time to form visible colonies have lower growth rates[12]. In the present study, we employed the same technique to analyze the bacterial flora in the maturing rice seed.

Our study revealed a great change in the bacterial flora within the seed during its maturation, in contrast to the slight change at the seed's surface.

## Materials and Methods

### *Preparation of the rice seeds*

*Oryza sativa* (cultivars Nipponbare) was cultivated in a plastic garden pot (45 cm × 110 cm, depth 25 cm) containing 80 liters of commercial garden soil (Ueda Ringyo, Japan) on an experimental field located at Ritsumeikan University (Kusatsu city, Shiga Prefecture, Japan). Usually, the rice plant flowers about 100 days after seeding. The rice seed matures ca. 60 days after the flowering. We divided the maturation process into three stages, an early, a middle and a late stage. The rice seeds were sampled aseptically at each stage, *i.e.*, on days 10, 30 and 60 after the flowering. The samples were brought back to the laboratory in a sterilized box to prevent contamination, and used in the experiments within 1 h.

### *Plate count of surface and endophytic bacteria*

Bacteria were isolated from the rice-seed surface as follows. The hulls were removed from the rice seeds with sterilized forceps, and the rice seeds (0.1 g; about 5–8 seeds) were put into a test tube containing 10 mL of sterilized water, after which they were ultrasonically washed with a BRANSONIC 2510 J-MT sonicator (YAMATO, Tokyo, Japan) for 3 min (output power, 100 W). A portion of the supernatant (0.1 mL) was mixed with ca. 20 mL of a nutrient broth (NB) agar (1.5 wt %) medium (containing 10 g of polypepton, 10 g of nutrient broth, and 5 g of NaCl in 1000 mL of tap water with the pH adjusted to 7.2) or mixed with a 100-fold diluted nutrient broth (DNB) agar (1.5 wt %) medium. These plates were incubated at 27°C. Surface bacteria could not be obtained at the early stage of the maturation process because the rice seeds were too soft to be washed.

For the isolation of the rice seed endophytes, the surface of the rice seeds (1.0 g) was sterilized in 100% of ethanol for 10 sec. The seeds were washed with sterilized distilled water, and then with a 1% sodium hypochlorite solution (Nacalai Tesque, Kyoto, Japan) for 20 min. After another wash with sterilized water, the surface-sterilized rice seeds were crushed with a sterilized mortar and pestle. The subsamples were ultrasonically washed with a BRANSONIC 2510 J-MT sonicator (YAMATO) for 15 sec (output power, 100 W) and then serially diluted with sterilized water. Surface sterilization was confirmed by culturing sterilized rice seeds on NB agar media. The diluted suspension was mixed with NB and DNB agar media. The plates were incubated at 27°C.

The colonies appearing on the plates were counted everyday for 30 days. After 30 days, the strains were isolated at random from the colonies that appeared on the plates.

### *DNA extraction*

We isolated the surface and endophytic bacteria from colonies as follows. Five to seven strains were isolated at random from each sample, either from the surface or the inside of the rice seed, with differences in the stage of maturation and the medium used. All of the isolates were identified based on the analysis of the 16S rRNA gene sequences. The cells, which were at the exponential growth-phase (1.5 mL) in the NB liquid medium, were harvested by centrifugation and resuspended in 40 μL of TE buffer. Then, 10 μL of Proteinase K (10 mg/mL) and 50 μL of BL buffer[10] (containing 40 mM Tris aminomethane, 1 mM of EDTA•2Na, 1% of Tween 20 and 0.5% of Nonidet P40) were added to the suspension. After incubating at 60°C for 30 min, the suspension was centrifuged and the supernatant was used for amplification by PCR.

### *PCR amplification and sequencing of the 16S rRNA gene*

The PCR mixture contained 0.75 units of ExTaq (Takara, Shiga, Japan), 1x *Taq* polymerase buffer, 200 μM dNTPs, 50 pmol of each primer and the extracted DNA (50-100 ng)

in a 20-μL reaction mixture. The PCR primers used for amplifying the 16S rRNA gene of the isolated bacteria were 20F (5'-AGTTTGATCCTGGCTC-3') as the forward primer and 1510R (5'-GGCTACCTTGTTACGA-3') as the reverse primer, corresponding to positions 10-26 and 1495-1510, respectively, in the 16S rRNA gene sequences of *Escherichia coli.*

The thermal cycling program used was as follows: initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 1 min, 52°C for 2 min, and 72°C for 2 min, and a final extension at 72°C for 10 min. The amplified PCR products were analyzed by electrophoresis on 1% Agarose LO3 (Takara) gels in 1 × TAE. A 200-bp DNA Marker (BEXEL Biotechnology, California, USA) was used as the molecular weight standard, and ethidium bromide (1 μg/mL with 1 × TAE Buffer) was used for visualization. The PCR products were purified with a PCR-M Clean Up System (VIOGENE, Taipei, Taiwan) in accordance with the manufacturer's instructions.

The sequences were determined by using the primers 25F (5'-AGTTTGATCCTGGCTC-3') and 1115R (5'-AGGGTTGCGCTCGTTG-3') with a genetic analyzer, the ABI PRISM AVANT 3100 (PE Biosystems, Foster City, USA). The BigDye Terminator Cycle Sequencing Ready Reaction Kit ver. 3.1 (PE Biosystems) was used in accordance with the manufacturer's directions.

*Phylogenetic analysis*

Approximately 1000 bp were used for the phylogenetic analysis. The phylogenetic positions of the isolates were determined by searching the DDBJ (http://www.ddbj.nig.ac.jp/Welcome-j.html) database with the BLAST program, and the sequences were then aligned by using the CLUSTAL X program (version 1.83)[19]. A phylogenetic tree was constructed by the neighbor-joining method[17] with 1000 bootstrap replicates in CLUSTAL X.

*Accession numbers*

All of the partial sequences of the 16S rRNA gene determined in this study have been submitted to the DDBJ database under the accession numbers AB178169-AB178218.

*Morphological and physiological characterization of the isolates*

All of the isolates were characterized by examining their morphological traits. The cell shape, motility and Gram staining reaction were examined for the cells cultured in NB or DNB liquid medium for 1 to 2 days at 100 rpm and 27°C. When a strain was isolated from the NB (DNB) agar medium, the isolate was cultured in NB (DNB) liquid medium. It was checked with an optical microscope after culturing for one month in a liquid medium, in order to determine whether endospores had formed. The isolates were also characterized using the catalase and oxidase tests.

*Growth of isolates under high osmotic pressure*

In order to determine whether the isolates could grow under high osmotic pressure, they were cultured in NB medium containing 1.2 M sucrose. *Escherichia coli* strain IAM 12119, *Bacillus subtilis* strain 168, *Micrococcus luteus* strain IFO 03763 and *Pseudomonas syringae* strain NIAES 1309 were also examined for comparison. The strains examined were cultured in NB medium with and without 1.2 M sucrose for 3 days and the optical density at 530 nm was measured.

## Results

Samples from the different maturation stages (early, middle and late), obtained from the surface and the inside of the rice seeds, were poured into NB or DNB agar medium, and incubated for 30 days (with the exception of the surface samples from the early stage, as explained in "Materials and Methods"). The bacteria obtained from the washing of the rice seeds after the removal of the hulls were designated the surface bacteria, and the bacteria from the inside of the rice seeds, the endophytic bacteria.

*The formation of colonies on the NB and DNB agar media*

The increase in the number of colonies for the surface bacteria is shown in Fig. 1. At the early stage, the rice seeds were very soft, so we could not obtain surface samples. For the middle stage, almost all of the colonies (ca. 94% of the total number of colonies) appeared within 10 days after the initiation of incubation. After a further 10 days, the increase in the number of colonies was small on the NB and DNB plates. This tendency, whereby almost all of the colonies appeared early in the incubation period, was also apparent in the late stage.

The colony formation curve for the endophytic bacteria of the rice seeds is shown in Fig. 2. For the middle-stage sample, almost all of the colonies (96% and 86% of the total number on the NB and DNB agar plates, respectively) appeared within 10 days. In contrast, for the early-stage sample, the number of colonies increased stepwise throughout the entire incubation period. For the late-stage sample, the number of colonies was low (less than $3 \times 10^3$ colonies/g of

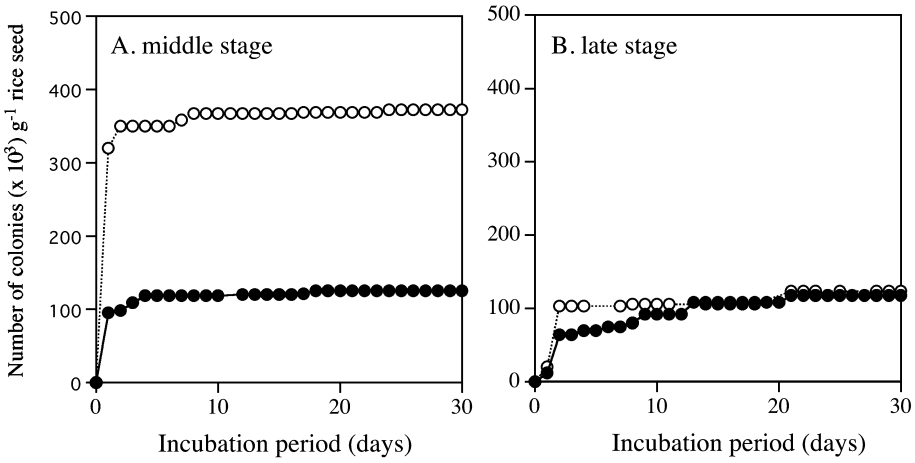


Fig. 1. Increase in the number of colonies on NB (●) and DNB (○) agar media with incubation for the surface bacteria of the rice seed. The plates were incubated at 27°C. The average of five plates was plotted as the colony number. The difference in the number of colonies formed on NB or DNB plates was usually less than 10–20% of the average colony number.

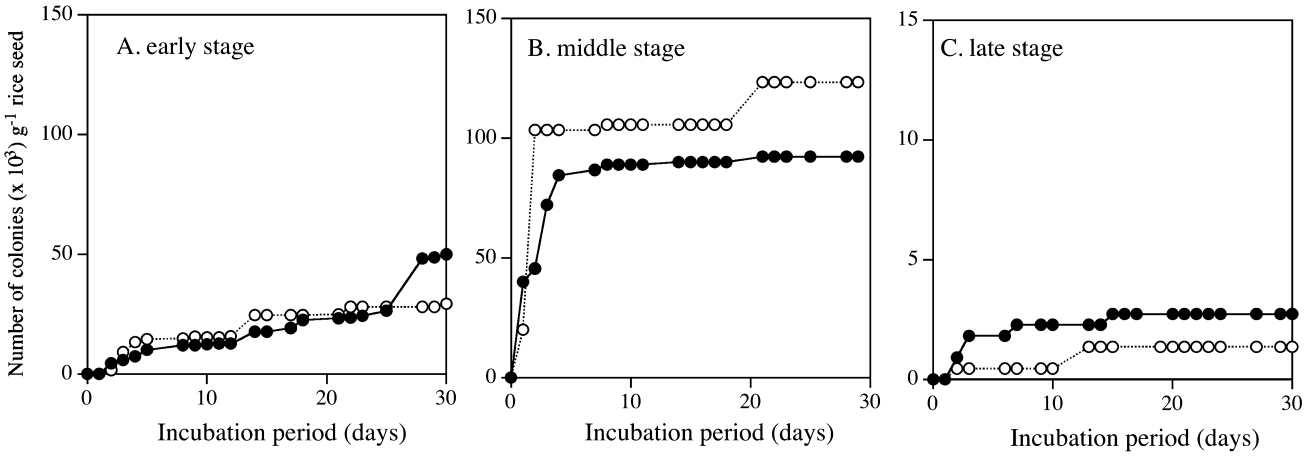


Fig. 2. Increase in the number of colonies on NB (●) and DNB (○) agar media with incubation for the endophytic bacteria of the rice seed. The plates were incubated at 27°C. The average of five plates was plotted as the colony number. The scale of the y-axis in Fig. 2-C is different from that in the other two figures, Fig. 2-A and B. The difference in the number of colonies formed on NB or DNB plates was usually less than 10–20% of the average colony number.

rice seeds), hence the colony formation curve showed no clear pattern.

The total number of colonies (the number of colonies counted at 30 days after incubation) for the middle-stage surface-sample on the NB medium was ca. $1.2 \times 10^5$/g of rice. The number on the DNB medium was about 3-fold greater, *i.e.,* ca. $3.7 \times 10^5$/g of rice. The difference between the NB agar medium and the DNB agar medium was small for the late-stage surface sample: the total number of colonies was ca. $1.1 \times 10^5$/g of rice on either medium.

For the sample taken from the inside of the rice seeds, the difference in the total number of colonies between the NB and the DNB agar medium was fairly small. The sample for the middle-stage inside of the rice seed formed the greatest number of colonies (ca. $1.0 \times 10^5$ colonies/g of rice); the numbers for the early and late stages were ca. $5.0 \times 10^4$ and ca. $3.0 \times 10^3$/g of rice, respectively.

### *Analysis of the isolates based on the 16S rRNA gene sequence*

The isolates from the surface and the inside of the rice seed were analyzed based on the 16S rRNA gene sequence. The closest relatives of the isolates are shown in Table 1, and phylogenetic trees of these isolates are shown with

Table 1. The morphological and physiological characteristics of the isolates from the surface and inside of the rice seed

| Isolate[a] | Gram staining | Cell shape | Motility | Spore formation | Catalase | Oxidase | Closest related strain[b] (accession no.) | Similarity |
|---|---|---|---|---|---|---|---|---|
| Early stage | | | | | | | | |
| Endophyte | | | | | | | | |
| E-(s)-e-N-1(1)* | − | Rod | + | − | + | − | *Pantoea ananatis* (AF364847) | 932/935 (99.7) |
| E-(s)-e-N-2(2) | − | Rod | + | − | + | − | *Pantoea ananatis* (AF364844) | 936/939 (99.7) |
| E-(s)-e-N-3(2) | + | Rod | + | + | + | − | *Bacillus cereus* (AY138270) | 848/850 (99.8) |
| E-(s)-e-N-4(4) | − | Rod | + | − | + | − | *Pantoea ananatis* (AF364844) | 1032/1036(99.6) |
| E-(s)-e-N-5(29)* | + | Rod | + | + | + | − | *Bacillus cereus* (AY138270) | 911/911 (100) |
| E-(s)-e-D-1(2) | − | Rod | + | − | + | + | *Sphingomonas echinoides* (AB033944) | 911/919 (99.1) |
| E-(s)-e-D-2(2) | − | Rod | + | − | − | + | *Sphingomonas echinoides* (AB033944) | 867/872 (99.4) |
| E-(s)-e-D-3(2)* | − | Rod | + | − | + | + | *Sphingomonas echinoides* (AB033944) | 816/819(99.6) |
| E-(s)-e-D-4(2) | − | Rod | + | − | − | + | *Sphingomonas parapaucimobilis* (D84525) | 913/919 (99.3) |
| E-(s)-e-D-5(4) | + | Rod Chain | + | + | + | + | *Bacillus cereus* (AY138270) | 883/885 (99.8) |
| E-(s)-e-D-6(4) | + | Rod | + | + | + | + | *Bacillus cereus* (AE016877) | 913/914 (99.9) |
| Middle stage | | | | | | | | |
| Surface | | | | | | | | |
| S-(s)-m-N-1(1)* | + | Rod Chain | + | + | + | + | *Bacillus cereus* (AY138270) | 959/961 (99.8) |
| S-(s)-m-N-2(1) | + | Rod Chain | + | + | + | + | *Bacillus cereus* (AY138270) | 955/959 (99.6) |
| S-(s)-m-N-3(1)* | + | Rod | + | + | + | − | *Bacillus cereus* (AY138270) | 969/969 (100) |
| S-(s)-m-N-4(4)* | + | Rod Chain | + | + | + | − | *Bacillus cereus* (AY138270) | 972/976 (99.6) |
| S-(s)-m-N-5(13)* | + | Rod Chain | + | + | + | + | *Bacillus cereus* (AY138270) | 957/960 (99.7) |
| S-(s)-m-N-6(13) | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 956/961 (99.5) |
| S-(s)-m-N-7(4) | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 943/943 (100) |
| S-(s)-m-D-1(1)* | + | Rod | + | + | + | − | *Bacillus cereus* (AY138270) | 939/942 (99.7) |
| S-(s)-m-D-2(1)* | + | Rod | + | + | + | − | *Bacillus cereus* (AY138270) | 944/944 (100) |
| S-(s)-m-D-3(1) | + | Rod | + | + | + | − | *Bacillus cereus* (AY138270) | 943/943 (100) |
| S-(s)-m-D-4(8) | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 943/943 (100) |
| S-(s)-m-D-5(16) | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 942/942 (100) |
| Endophyte | | | | | | | | |
| E-(s)-m-N-1(1)* | + | Rod Chain | + | + | + | + | *Bacillus cereus* (AY138270) | 888/889 (99.9) |
| E-(s)-m-N-2(1)* | + | Rod Chain | + | + | + | + | *Bacillus cereus* (AY138270) | 880/883 (99.7) |
| E-(s)-m-N-3(3)* | + | Rod Chain | + | + | + | + | *Bacillus cereus* (AY138270) | 899/899 (100) |
| E-(s)-m-N-4(7)* | + | Rod Chain | + | + | + | + | *Bacillus cereus* (AY138270) | 896/899 (99.7) |
| E-(s)-m-N-5(14)* | + | Rod Chain | + | + | + | + | *Bacillus cereus* (AY138270) | 929/931 (99.8) |
| E-(s)-m-D-1(1) | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 927/927 (100) |
| E-(s)-m-D-2(1)* | + | Rod Chain | + | + | + | − | *Bacillus cereus* (AY138270) | 938/938 (100) |
| E-(s)-m-D-3(1)* | + | Rod Chain | + | + | + | − | *Bacillus cereus* (AY138270) | 925/925 (100) |
| E-(s)-m-D-4(1)* | + | Rod | + | + | + | − | *Bacillus cereus* (AY138270) | 918/918 (100) |
| E-(s)-m-D-5(3)* | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 921/921 (100) |
| Late stage | | | | | | | | |
| Surface | | | | | | | | |
| S-(s)-l-N-1(1)* | + | Rod Chain | + | + | + | + | *Bacillus cereus* (AY138270) | 943/943 (100) |
| S-(s)-l-N-2(1)* | + | Rod Chain | + | + | + | + | *Bacillus cereus* (AY138270) | 948/960 (98.8) |
| S-(s)-l-N-3(26) | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 943/943 (100) |
| S-(s)-l-N-4(1) | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 943/943 (100) |
| S-(s)-l-N-5(12) | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 959/960 (99.9) |
| S-(s)-l-D-1(1)* | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 929/929 (100) |
| S-(s)-l-D-2(1)* | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 962/982 (98.0) |
| S-(s)-l-D-3(1)* | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 888/890 (99.8) |
| S-(s)-l-D-4(1) | + | Rod | + | + | + | − | *Bacillus cereus* (AY138270) | 891/895 (99.6) |
| S-(s)-l-D-5(1)* | + | Rod | + | + | + | − | *Bacillus cereus* (AY138270) | 885/889 (99.6) |
| S-(s)-l-D-6(20) | + | Rod | + | − | + | − | *Microbacterium testaceum* (AF474325) | 845/850 (99.4) |
| S-(s)-l-D-7(27) | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 897/900 (99.7) |
| Endophyte | | | | | | | | |
| E-(s)-l-D-1(9)* | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 925/925 (100) |
| E-(s)-l-D-2(9) | + | Rod Chain | + | + | + | + | *Bacillus cereus* (AY138270) | 937/937 (100) |
| E-(s)-l-D-3(9) | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 925/925 (100) |
| E-(s)-l-D-4(9) | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 923/923 (100) |
| E-(s)-l-D-5(14)* | + | Rod | + | + | + | + | *Bacillus cereus* (AY138270) | 925/925 (100) |

[a] The first letter of the name indicates Endophytic (E) or Surface (S) bacteria. The second letter in brackets means 'Seed'. The third letter, e, m and l means early, middle and late stage of maturing process, respectively. The forth letter means NB (N) or DNB (D) medium. The fifth letter means serial number of strain. The sixth letter means the day when the colony appeared on the agar plate."

[b] The closest relative by sequence comparison

* The strain tested in NB medium containing 1.2 M sucrose

some reference strains in Fig. 3 and 4.

All of the isolates from the surface, with the exception of S-(s)-I-D-6 (20), were closely related to the genus *Bacillus cereus* or *Bacillus thuringiensis*, regardless of the maturation stage of the rice seed. This result indicates that these surface isolates were genetically identical as regards their 16S rRNA gene sequences. However, there may be two physiologically different types of bacteria, one oxidase-positive and the other oxidase-negative, as shown in Table 1.

The isolates from the inside of the rice seed could be divided into 3 clusters, as shown in Fig. 4. Two of the 3 clusters, which consisted only of isolates from the early-stage

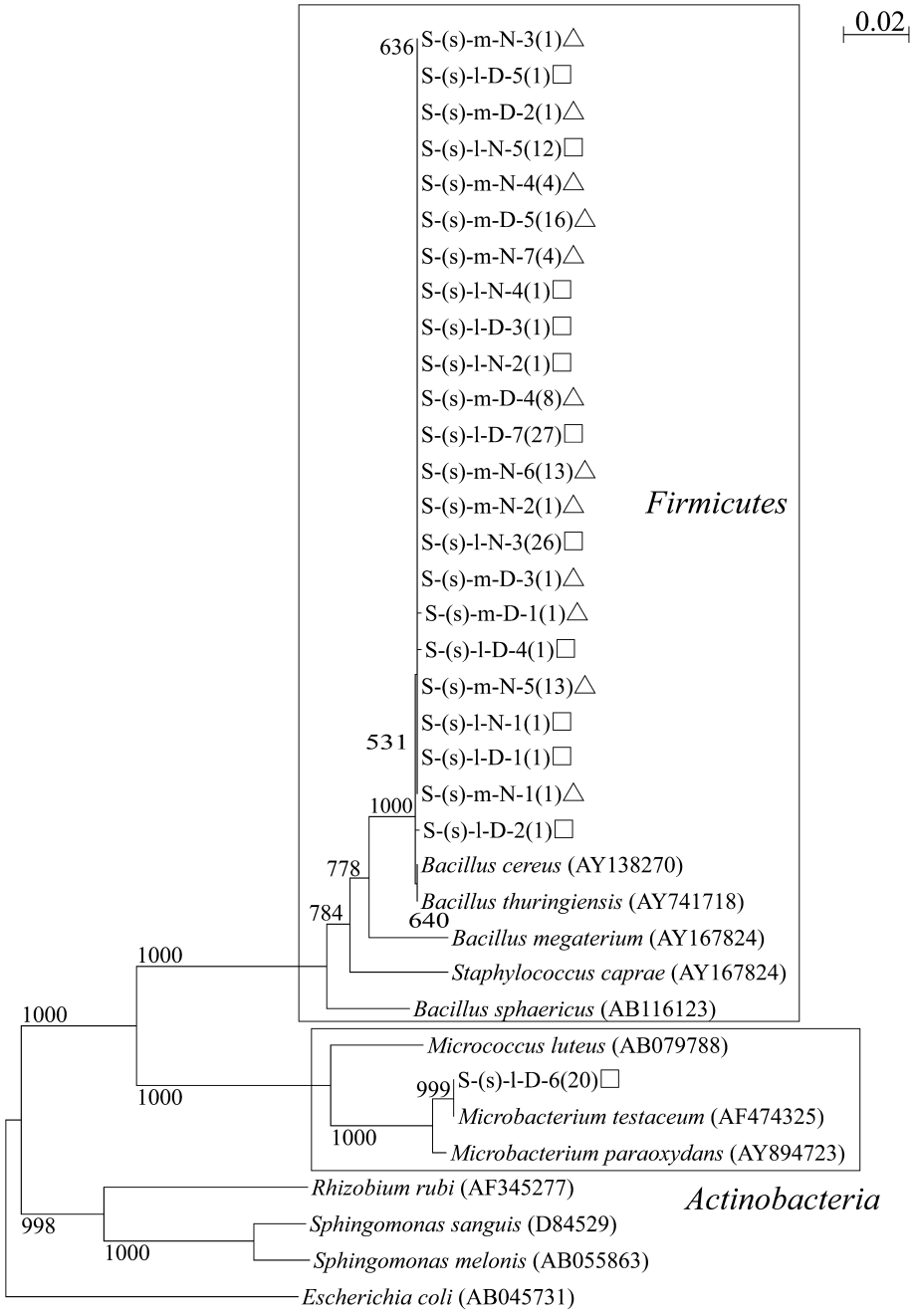


Fig. 3. Phylogenetic tree for the isolates from the surface of the rice seed based on the 16S rRNA gene sequences. The phylogenetic tree was calculated based on approximately 1000 nucleotides by the neighbor-joining method. The results of 1000 bootstrap trials are shown at the nodes. Symbols: △, isolates from the middle stage of the maturation process; □, isolates from the late stage of the maturation process.

sample, contained *Pantoea ananatis* and *Sphingomonas* spp. At the middle and late stages of the maturation process, all strains from the inside of the rice seed were similar to *Bacillus cereus or Bacillus thuringiensis*. These results coincide well with the findings shown in Table 1, according to which all isolates from the middle- and late-stage samples were able to produce endospores, while the isolates from the early-stage sample consisted of non-spore forming bacteria.

### *Morphological characteristics of the isolates from the early stage of maturation*

All of the isolates from the early-stage samples were rod or rod-chain in cell-shape. Interestingly, all of these isolates were motile, despite being endophytes (the early-stage sam-

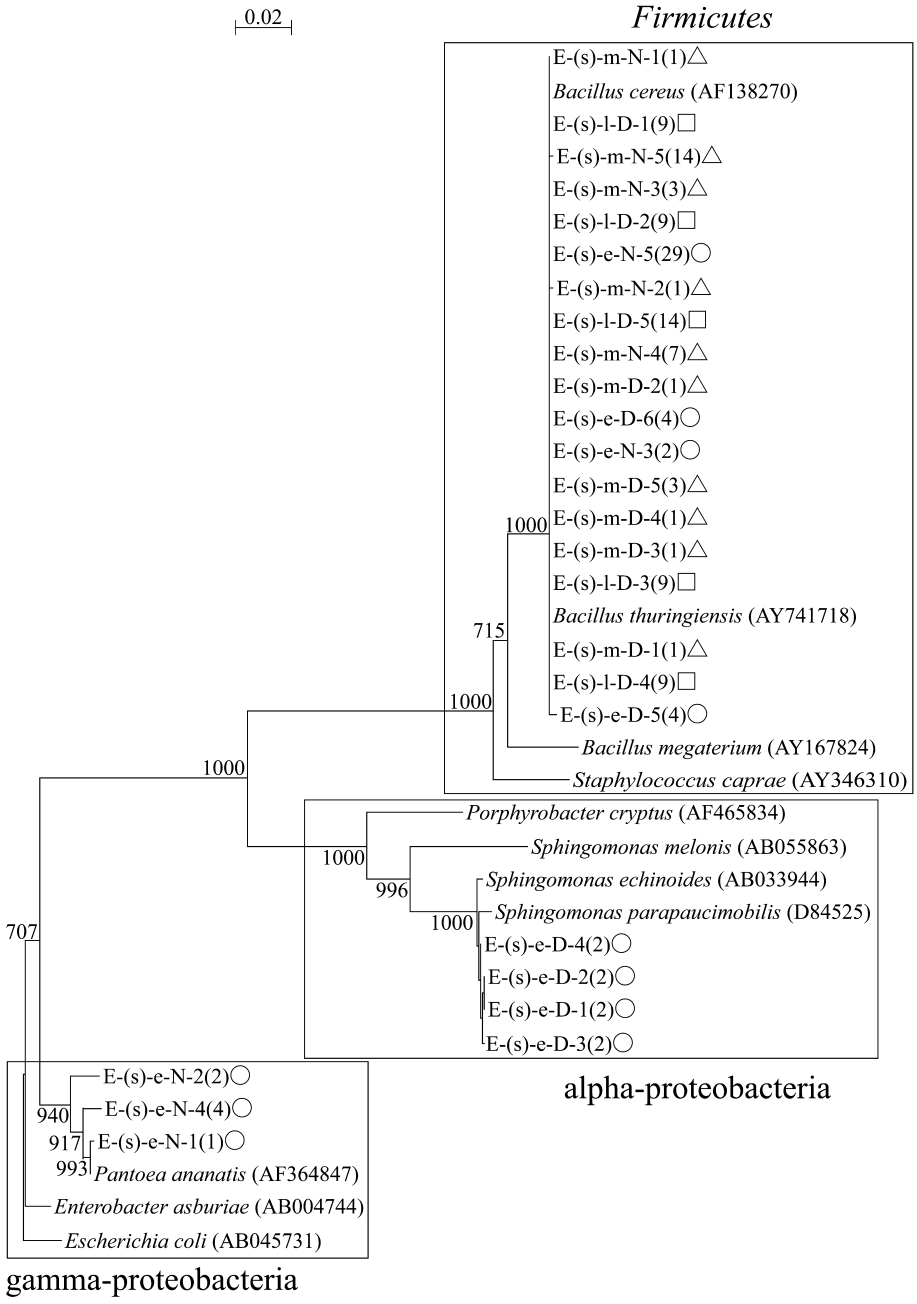


Fig. 4. Phylogenetic tree for the isolates from the inside of the rice seed based on the 16S rRNA gene sequences. The phylogenetic tree was calculated based on approximately 1000 nucleotides by the neighbor-joining method. The results of 1000 bootstrap trials are shown at the nodes. Symbols: ○, isolates from the early stage; △, isolates from the middle stage; □, isolates from the late stage of the maturation process.

ples contained only endophytes, as mentioned in "Materials and Methods"). These isolates consisted of three genera, *Bacillus*, *Sphingomonas* and *Pantoea*, according to the 16S rRNA gene sequence analysis. Four strains of the genus *Bacillus* were all similar to the same species, *B. cereus*. These four strains, spore-forming, Gram-positive and catalase-positive, differed in terms of the oxidase test, with half positive and half negative. Three out of four strains closely related to the genus *Sphingomonas* were similar to *S. echinoides* and the remaining strain was similar to *S. parapaucimobilis*. Two strains of *S. echinoids* were catalase-positive, whereas one strain was negative and one strain was closely related to *S. parapaucimobilis*. Three strains of the genus *Pantoea* were all closely related to *P. ananatis* and showed the same physiological traits. Thus, these endophytes in the early-stage sample seemed to have relatively diverse physiological traits.

### *Morphological characteristics of the isolates from the middle and late stages*

Contrary to the above results, the morphological and physiological traits of the isolates from the middle and late stages were extremely low in diversity; *i.e.,* all of the isolates were Gram-positive, rod or rod-chain in cell-shape, and catalase-positive. All of these isolates were spore-forming, with the exception of a single strain, S-(s)-I-D (20). The low diversity in morphological and physiological traits seems to reflect the result of the 16 S rRNA gene sequence analysis indicating that all the isolates were closely related to *B. cereus* except the strain S-(s)-I-D (20). It is noteworthy that all of the isolates from both the surface and the inside of the rice seed were motile. Sixty four percent of the isolates from the middle stage and 82 percent of the isolates from the late stage were oxidase-positive.

### *The effect of high osmotic pressure on the growth of the isolates*

The growth of the isolates under two different conditions, in NB and in NB containing 1.2 M sucrose, was examined to see whether the isolates could tolerate high osmotic pressure. As shown in Fig. 5, the reference strains (closed symbols) were plotted in the region below the diagonal line. This means that these strains grew less when sucrose was added. In contrast, most of the isolates (19 of 23) from the surface and the inside of the rice seed grew more when sucrose was added as indicated by the plots for these isolates (open symbols) shown above the diagonal line.

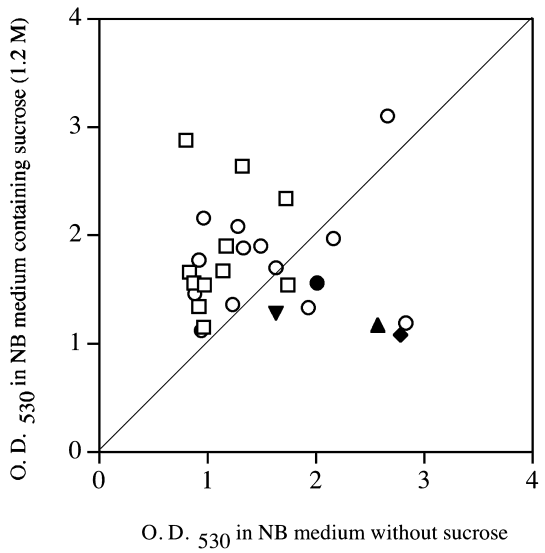


Fig. 5. The effect of osmotic pressure on the growth of the isolates. The isolates from the rice seed were cultured in NB medium with and without sucrose (1.2 M). □: isolates from the surface bacteria; ○: isolates from the endophytic bacteria; ◆: *E. coli* (IAM 12119); ●: *B. subtilis* (168 strain); ▲: *M. luteus* (IFO 3763); ▼: *P. syringae* (NIAES 1309)

## Discussion

The surface and endophytic bacteria were cultured in a conventional medium for bacteria, nutrient broth (NB). One-hundred-fold diluted nutrient broth (DNB) medium was also used for culturing. It has been reported that bacteria in natural environments, such as grassland soil, paddy field soil and lake sediment, form more colonies on DNB agar medium than on NB medium, due to the preponderance of oligotrophic bacteria relative to copiotrophic ones. In the present study, the total number of colonies on the NB and DNB agar media was almost the same, except for the surface sample from the middle stage, as shown in Fig. 1 and 2. This result differs greatly from those for various soil samples, as discussed above. The isolates from the surface and the inside of the rice seed seem to be copiotrophic, suited to higher nutrient conditions. The colony forming curve (CFC) of the isolates from the surface of the rice seed is rather simple; *i.e.*, the colony number increased during the early period of incubation and reached a plateau after that. This was also the case for the isolates from the inside of the rice seed at the middle stage. However, the endophytes at the early stage showed a more complex CFC; the colony number increased stepwise during the incubation. The endophytes at the early stage seemed to consist of various groups of bacteria differing in their growth rate, in contrast to the simple

composition dominated by fast-growing bacteria at the middle and the late stages of rice-seed maturation, both at the surface and on the inside.

At the early stage of maturation, the isolates from the inside of the rice seed contained several kinds of bacteria, according to the 16S rRNA gene analysis. On the other hand, all of the isolates at the middle and late stages were closely related only to *B. cereus* or *B. thuringiensis,* with the exception of the isolate S-(s)-I-D-6 (20), which was similar to *Microbacterium testaceum*. These results indicate that the bacterial flora of rice seed is relatively diverse early on, becoming less diverse as the seed matures. The morphological and physiological characteristics also support this finding. The change in the bacterial flora may be caused by the reduction in the water content of the rice seed as it matures[11]. Although we could not obtain surface samples at the early stage because the seeds were too soft, the surface bacteria at this stage may show greater diversity than at later stages; we are planning to examine early-stage samples in the future.

Quite surprisingly, all of the isolates were motile regardless of the stage of maturation and the location from which the bacteria were isolated (the surface or inside of the rice seed) (Table 1). It seems that motility is useless in the hard rice seed at the middle and late stages. Motility may enable the cells to migrate into the rice seed before it hardens. There were no bacteria in middle- and late-stage samples, with the exception of the strains closely related to the genus *Bacillus*, which can produce endospores. These bacteria may migrate into the rice seed using their motility when the seed is soft, and then may subsist in the hardened seed in an endospore-bearing form. The process by which the bacteria reach the inside of the rice seed and the state of the bacteria after this migration are the subjects of another project.

The bacterial flora in the rice seed changed with the maturation process; the restricted isolates, which were related only to *B. cereus* or *B. thuringiensis*, were able to subsist through the middle and late stages. During the later stages, the rice seed becomes hard, so the endophytes seem to be dormant, forming endospores. The strains lacking the ability to form endospores may migrate to other portions, as indicated by Turnbull *et al.*[20], owing to their motility. Mukhopadhyay *et al*.[14] have isolated several strains closely related to the genus *Enterobacter* from rice seedlings germinated from surface sterilized seeds. We also isolated a strain similar to *Pantoea ananatis*, which is phylogenetically very closely related to the genus *Enterobacter*. *Pantoea* and *Enterobacter* are members of the Enterobacteriaceae family which have been isolated as endophytes and epiphytes from a wide variety of crops[2, 5, 13]. Asis and Adachi have found an endophytic diazotroph *Pantoea agglomerans,* and a nondiazotroph *Enterobactoer asburiae,* in the stem of the sweet potato[1]. Although the genus *Pantoea* is a common endophyte among various plants as discussed above, the present study clarified for the first time that strains closely related to the genus *Pantoea* existed in the cultivated rice seed and that their existence may be limited to the early stage in the maturation of the rice seed.

Most of the endophytes and surface bacteria from the rice seed were able to grow in NB medium containing 1.2 M sucrose. Elbeltagy *et al*. have also reported that endophytic isolates from the stem, leaf and seed of wild and cultivated rice were able to grow on NB media with 0.6 and 1.2 M sucrose[6]. In the present study, we found that the isolates from the surface and the inside of rice seeds grew better in NB medium containing 1.2 M sucrose than in NB medium containing no sucrose. The isolates from the rice seed may not only adapt to sucrose-rich environments, but also utilize sucrose as a source of carbon. Further study is necessary to test this hypothesis.

Although several studies on endophytes have been reported, they were mainly concerned with nitrogen-fixing at a specific stage of the host plant's maturation. In the present study, we examined the endophytes throughout the maturation process of the host plant, rice. This study is the first report revealing the great change in bacterial flora inside the rice seed during the growth process of the host plant. As a next step, we plan to investigate non-culturable bacteria by using a molecular biological technique such as PCR-DGGE, in order to achieve a better understanding of endophytes.

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