Predominant Culturable *Bacillus* Species in Japanese Arable Soils and Their Potential as Biocontrol Agents

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To reveal predominant culturable *Bacillus* species in Japanese arable soils, aerobic spore-forming bacteria were isolated from different soils in Japan and their phylogenetic positions were estimated from the 16S rDNA sequences. *Bacillus* species were also isolated from tomato roots grown in soils for 4 weeks to estimate the difference in phylogenetic positions between soil and root isolates. The number of culturable *Bacillus* species ranged from 6.4 to 7.4 log cfu g^{−1} soil and 4.7 to 6.7 log cfu g^{−1} root in soils and tomato roots, respectively, and the proportion of *Bacillus* species among total culturable bacteria ranged from 3.8% to 17.9% and 0.1% to 6.7% in soils and roots, respectively. The differences in the number and percentage of culturable *Bacillus* species between the soils and roots were highly significant ($P=0.0000$ and 0.0023 , respectively), indicating that *Bacillus* species were more predominant in soil than in root. A total of 108 isolates were characterized using the restriction fragment length polymorphism (RFLP) method and partial 16S rDNA sequences. They affiliated to 22 species including the genus *Bacillus*, *Brevibacillus* and *Paenibacillus*. No nitrogen fixers were isolated. The predominant species was *Bacillus megaterium* to which 42 isolates (39%) belonged. Some of the species, such as *Bacillus luciferensis* and *Brevibacillus agri*, were isolated only from the tomato roots and considered to be more adapted to the rhizosphere than soil. Among 108 isolates, only one strain, N2S6, belonging to *Bacillus megaterium*, showed consistent antagonistic activity against the bacterial wilt of tomato caused by *Ralstonia solanacearum*.

Key words: *Bacillus*, *Bacillus megaterium*, *Brevibacillus*, *Paenibacillus*, rhizosphere

Spore-forming bacteria, typically *Bacillus* species, are one of the major types of soil bacteria²⁾. Recently, Felske *et al.*^{6,7)} reported that *Bacillus* species, especially *B. benzoovorans*, constitute a majority of the bacteria in Dutch soil using culture-independent and culture-dependent methods. It is known that *Bacillus* species produce at least 66 different antibiotics¹³⁾ and certain *Bacillus* strains are antagonistic to plant pathogenic fungi and bacteria and thereby function as a biocontrol agent^{3,4,9,19,24,27)}.

The biodiversity of the selected *Bacillus* species, such as culturable *Bacillus* species³⁰⁾, *B. subtilis*^{26,31)}, *B. polymyxa*¹⁷⁾,

*Paenibacillus azotofixans*²⁹⁾, and *Paenibacillus alvei*⁵⁾, has been reported. Recently, the biodiversity of *Bacillus* species in Dutch soil has also been studied using a molecular method¹⁰⁾, revealing the dominant species to be *B. mycoides*, *B. pumilus*, *B. benzoovorans* and *B. cereus*. Felske *et al.*⁸⁾ also found that *B. megaterium*, *B. benzoovorans*, and *Paenibacillus* were the major culturable *Bacillus* species in soil and more than half of the *Bacillus* isolates might belong to new species. However, few studies have revealed the biodiversity of *Bacillus* species in Japanese soils. Katoh and Suzuki¹⁴⁾ isolated ca. 300 bacterial isolates from Japanese soils amended with cow dung and found that 102 were spore-formers. They classified 22, 13, 7 and 5 strains into *B. megaterium*, *B. cereus*, *B. pumilus* and *B. sphaericus*, respectively, based on phenotypic characteristics such as the

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morphology of sporangia, VP-tests and the presence of nitrate reductases, but the classification of more than 50% of the spore-formers (57 strains) remained unclear. Shintani³²⁾ reported on the biodiversity of indigenous *Bacillus* species, but targeted only nitrogen-fixers which account for a fraction of *Bacillus* species²⁸⁾. Consequently, the biodiversity of *Bacillus* species in Japanese soils remains mostly unknown. The objective of this study was to isolate the predominant *Bacillus* species in different Japanese arable soils and estimate their biodiversity. In addition, the ability of isolates to control the bacterial wilt of tomato caused by *Ralstonia solanacearum*, a serious soil-borne disease in Japan, was evaluated since one of the major applications of *Bacillus* species in the soil environment is as a biocontrol agent.

Materials and Methods

Soils used

Arable soils (0 to 10 cm) were collected from the Higashi-Mikawa Agricultural Research Institute, Aichi Prefecture Agricultural Research Center (Toyohashi, Aichi, Japan), Department of Fruit Vegetables, National Institute of Vegetables and Tea Science (Taketoyo, Aichi, Japan), National Agricultural Research Center²²⁾ (Tsukuba, Ibaraki), National Agricultural Research Center for Western Region (Ayabe, Kyoto, Japan), Kanagawa Prefecture Agricultural Research Institute (Hiratsuka, Kanagawa, Japan), farmland in Esashi²⁰⁾ (Iwate, Japan) and Nakijin (Okinawa, Japan) and Tokyo University of Agriculture and Technology (Fuchu, Tokyo, Japan) (Table 1), and used for growing tomato plants within 3 months of the samplings.

Isolation of aerobic spore-forming bacteria from soil and tomato root

Tomato (*Lycopersicon esculentum* Mill. cv. Momotaro) seeds were pregerminated in a moist petri dish in the dark at 28°C for two days. Then, one tomato seedling was transplanted into a vinyl pot 9 cm in diameter containing 200 g of soil and grown in a climatron, adjusted to 80% relative humidity and 30°C with a light regime (day:night=12 h:12 h, light intensity=270 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Three pots were prepared for each soil. Hyponex fertilizer that contains NPK was added once a week to each pot to support plant growth throughout the period of incubation. Watering was performed twice a day to maintain 55% maximum water-holding capacity (MWHC). After 4 weeks of incubation, tomato roots were carefully removed from the pots and soaked in sterile water for 10 min. The roots were washed in sterile water three times, cut into two fragments, the upper and lower parts, and then macerated separately in a sterile mortar with pestle. Non-rhizosphere soil was collected from the pots after cultivation and 10 g of the soil was suspended in a 0.85% NaCl solution. The root and soil suspensions were heated at 80°C for 10 min and serially diluted. Portions of appropriate dilutions (10^{-2} to 10^{-4}) were spread onto 10^{-1} nutrient agar medium (1/10 NA). After 3 to 4 days of incubation at 30°C, the colonies were enumerated and those with a different appearance were isolated for further characterization. The formation of spores by the isolates was confirmed by microscopic observation and spore-positive isolates were regarded as *Bacillus*. The total culturable bacteria were similarly enumerated using the root and soil suspensions without the heat treatment, but colony counts were made after 2 weeks of incubation.

Table 1. Characteristics of the soils used

Site (soil name)	Soil texture	MWHC (g g ⁻¹)	pH (H ₂ O)	Total C (g kg ⁻¹)	Total N (g kg ⁻¹)	C/N ratio
Esashi, Iwate (Yellow soil)	Light clay	0.60	6.3	11.0	1.2	9.4
Tsukuba, Ibaraki (High-humic andosol)	Loam	0.97	5.7	78.1	4.8	16.4
Fuchu, Tokyo (High-humic andosol)	Loam	1.43	5.0	80.1	8.2	9.8
Hiratsuka (Gray lowland soil)	Sandy loam	0.95	5.6	14.1	1.6	9.1
Hiratsuka (Low-humic andosol)	Loam	1.30	5.5	24.5	2.8	8.9
Hiratsuka (High-humic andosol)	Loam	1.30	5.7	ND	ND	ND
Toyohashi (Yellow soil)	Light clay	0.56	5.3	9.0	1.1	8.5
Taketoyo (Yellow soil)	Light clay	0.55	5.9	8.5	0.9	9.9
Ayabe (Brown lowland soil)	Clay loam	0.48	5.3	6.9	1.0	7.3
Nakijin (Red soil)	Light clay	0.51	4.2	ND	ND	ND

ND: not determined.

Characterization of the isolates

DNA was extracted from the purified *Bacillus* strains with the conventional method²¹⁾ and the PCR primers 27f and L1401²³⁾ were used to amplify the segment of bacterial 16S rDNA from nucleotide 27 to 1401 (*Escherichia coli* numbering¹⁶⁾). The primer L1401 added with the M13 reverse sequence was used. PCR was carried out using a basic reaction mixture containing Ex Taq buffer (Takara, Otsu, Japan), 2.5 mM of each deoxyribonucleotide triphosphate, 2.5 U of Ex Taq (Takara), 0.2 mM of each oligonucleotide primer and 1 µl of template (purified DNA) in a total volume of 25 µl. The programme was 3 min at 94°C; amplification for 30 cycles, each consisting of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C; and a final extension for 8 min at 72°C. For the restriction fragment length polymorphism (RFLP) analysis, the PCR products (ca. 3 µg) were digested with 5 U of each endonuclease *Hinf*I, *Hae*III and *Msp*I (Takara, Otsu, Japan) and the digested products were electrophoresed in a 2% agarose gel with 0.5 µg ml⁻¹ of ethidium bromide. RFLP analysis was done for a total of 108 isolates and separated them into different patterns. Then, direct sequencing (800 b.p.) of the PCR products derived from 70 isolates with different RFLP patterns and different origins was done by Hitachi Science Systems, Ltd. (Tokyo, Japan). The phylogenetic position of the sequences obtained (AB111827 to AB111848) was examined using the DDBJ homology search system BLAST.

The nitrogen-fixing activity of the isolates was determined using the acetylene reduction assay¹⁵⁾. Individual isolates were inoculated into an air-tight vial containing semi-solid Rennie's medium²⁵⁾. After inoculation, 10% of the headspace was replaced with acetylene and the vials were incubated for 3 to 5 days at 28°C. The production of ethylene was measured with a Shimadzu model GC14B gas chromatograph with an FID detector (Shimadzu Co., Kyoto, Japan).

Biocontrol

The ability of the isolates to control the bacterial wilt of tomato caused by *Ralstonia solanacearum* was examined using the yellow soil (Esashi). Two hundreds gram of soil inoculated with *R. solanacearum* YU1Rif43³⁴⁾ at a density of 5×10^6 cfu g⁻¹ soil was put into a vinyl pot 9 cm in diameter. Then, six tomato seedlings were transplanted and 1 ml of the bacterial culture in 10⁻¹ nutrient broth was poured around the seedlings. Growth conditions for tomato plants were the same as above. A disease index was recorded periodically for 28 days on the following basis: 0; no wilting,

1; 0 to 25% of plant showing wilting, 2; 26 to 50%, 3; 51 to 75%, 4; 76 to 100%. After the first screening, the isolates which showed apparent suppressive effects were re-tested using duplicate pots. One isolate which showed consistent suppressive effects in the first and second screenings was used in further experiments.

Effect of the addition of poultry manure compost (PMC), the disease-suppressive effect of which was previously reported¹²⁾, on the suppressive effect of the *Bacillus* strain was examined. PMC was added to the soil at a ratio of 10% (v/v), corresponding to 5 g of dry weight compost in 1 kg of soil, and mixed thoroughly. Methods for inoculating the pathogen and growing the tomato plants were described previously. Two pots were prepared per treatment and the experiment was repeated three times.

Results and Discussion

Number of total culturable bacteria and *Bacillus* species in soil and root

The numbers of total culturable bacteria in the soils and roots ranged from 7.4 to 8.7 (8.0 ± 0.5) and 6.7 to 8.7 (7.6 ± 0.5) log cfu g⁻¹, respectively (Table 2). Those of spore-forming bacteria, regarded as *Bacillus* species, in the soils and roots were significantly ($P=0.0303$) different depending on the soils used, ranging from 6.3 to 7.4 (6.8 ± 0.3) and 4.7 to 6.7 (5.5 ± 0.5) log cfu g⁻¹, respectively. Numbers of *Bacillus* species were significantly larger in the root tip (5.7 ± 0.5 Log cfu g⁻¹ root) than root base (5.3 ± 0.3 Log cfu g⁻¹ root). The proportion of *Bacillus* as a percentage of the total number of culturable bacteria ranged from 3.8% to 27.8% (Mean $\pm$ SD= $8.7 \pm 8.6\%$) in the soils and 0.1% to 6.7% (Mean $\pm$ SD= $1.5 \pm 2.0\%$) in the root tip and base and the difference was highly significant ($P=0.0023$). There was no significant difference in this value between the root tip and base.

Garbeva *et al.*¹⁰⁾ reported that numbers of *Bacillus* species in the soil and rhizosphere of maize ranged from 4.7 to 5.7 and 5.2 to 5.9 log cfu g⁻¹, respectively. According to Seldin *et al.*²⁸⁾, numbers of aerobic spores in Brazilian soils ranged from 6.0 to 6.2 log cfu g⁻¹. The numbers of *Bacillus* species observed in the present study were similar to those in the previous studies, but were higher in the soils than in the roots, unlike in Garbeva *et al.*¹⁰⁾, and the reason for this remains unclear.

Characterization of predominant *Bacillus* species in soil and root

From the plates with the highest dilutions, several colo-

Table 2. The number of total culturable and spore-forming bacteria in soils and roots of tomato grown in the soils

Soil	Total culturable bacteria Log cfu g ⁻¹ root or soil	Spore-forming bacteria Log cfu g ⁻¹ root or soil	Ratio (%)
Yellow soil (Esashi)			
Tip of root	8.4	6.1	0.46
Base of root	7.8	5.6	0.68
Soil	8.6	7.4	5.2
High humic andosol (Tsukuba)			
Tip of root	8.0	5.2	0.1
Base of root	7.7	5.2	0.3
Soil	7.8	6.4	3.8
High humic andosol (Fuchu)			
Tip of root	7.4	5.8	2.6
Base of root	6.7	5.4	6.7
Soil	7.4	6.6	17.9
High humic andosol (Hiratsuka)			
Tip of root	8.4	5.8	0.33
Base of root	7.9	5.8	1.0
Soil	7.5	6.3	6.4
Low humic andosol (Hiratsuka)			
Tip of root	8.7	5.8	0.1
Base of root	7.3	5.3	1.1
Soil	8.0	6.7	4.3
Grey lowland soil (Hiratsuka)			
Tip of root	7.9	6.7	6.4
Base of root	6.9	5.4	2.9
Soil	7.6	7.1	27.8
Yellow soils (Taketoyo)			
Tip of root	7.3	5.6	2.0
Base of root	7.5	5.4	0.8
Soil	8.2	6.8	4.1
Brown lowland soil (Ayabe)			
Tip of root	7.0	5.1	1.2
Base of root	7.1	4.7	0.41
Soil	7.8	6.7	8.0
Red soil (Nakijin)			
Tip of root	7.8	5.1	0.21
Base of root	7.6	5.2	0.44
Soil	8.7	6.8	1.2

nies with different appearances were isolated. An RFLP analysis was conducted for a total of 108 isolates which were then divided based on different patterns (data not shown). Next, the 16S rDNA of 70 isolates with different RFLP patterns and different origins were partially sequenced. All the sequence results showed more than 98.9% identity to known species, except for the one closest to *Paenibacillus agarexedens* with 96.9% (Table 3). The closest species of 42 isolates were *B. megaterium*, recovered

from a wide range of soils and roots, indicating that *B. megaterium* may be the predominant *Bacillus* species in Japanese arable soils. Strains of *B. cereus* (14 isolates) and *B. niacini* (6 isolates) were also frequently isolated from both the soils and roots. Eight and six isolates belonging to *B. luciferensis* and *Brevibacillus agri*, respectively, were obtained and all were from the roots. Four *B. marisflavi* and three *B. firmus* isolates were also recovered from only the roots. Strains of several species, including *B. sphaericus*

Table 3. Phylogenetic position of *Bacillus* species, based on RFLP analysis and 16S rDNA sequencing, isolated from different Japanese soils and roots of tomato grown in the soils

Nearest species	Soil								Root								Total
	Yellow soil (Esashi)	High humic andosol (Tsukuba)	Grey lowland soil (Hira-tsuka)	Low humic andosol (Hira-tsuka)	Yellow soil (Toyo-hash)	Yellow soil (Take-toyo)	Brown lowland soil (Ayabe)	Red soil (Nakijin)	Yellow soil (Esashi)	High humic andosol (Tsukuba)	Grey lowland soil (Hira-tsuka)	Low humic andosol (Hira-tsuka)	Yellow soil (Toyo-hash)	Yellow soil (Take-toyo)	Brown lowland soil (Ayabe)	Red soil (Nakijin)	
<i>B. bataviensis</i>	1 (1)					2 (2)							2 (1)				5 (4)
<i>B. cereus</i>	2 (1)	1 (1)								3 (1)	5 (2)	2 (2)		1 (1)			14 (8)
<i>B. dretensis</i>				1 (1)				1 (1)									2 (2)
<i>B. firmus</i>											3 (3)						3 (3)
<i>B. fumarioli</i>														1 (1)			1 (1)
<i>B. gelatini</i>						3 (0)								1 (1)			4 (1)
<i>B. luciferensis</i>									1 (1)				4 (1)	2 (1)		1 (1)	8 (4)
<i>B. macroides</i>														1 (1)			1 (1)
<i>B. marisflavi</i>												3 (1)				1 (1)	4 (2)
<i>B. megaterium</i>	3 (0)	1 (1)		2 (1)		5 (3)	4 (1)	2 (1)	6 (2)	5 (5)	3 (3)	1 (1)	3 (3)	3 (1)	1 (0)	3 (0)	42 (22)
<i>B. mycoides</i>		2 (2)															2 (2)
<i>B. niacini</i>				1 (1)		1 (1)	1 (1)							3 (2)			6 (5)
<i>B. pumilus</i>								1 (1)		1 (1)				1 (1)			3 (3)
<i>B. simplex</i>											1 (1)						1 (1)
<i>B. sphaericus</i>										1 (1)				1 (1)			2 (2)
<i>B. subtilis</i>														1 (1)			1 (1)
<i>Brevib. agri</i>											3 (3)		2 (2)	1 (1)			6 (6)
<i>Brevib. choshinensis</i>				1 (1)													1 (1)
<i>P. agarexedens</i>											1 (1)						1 (1)
<i>P. pabli</i>														1 (1)			1 (1)
Total	6 (2)	4 (4)	ND	5 (4)	ND	11 (6)	5 (2)	4 (3)	7 (3)	10 (8)	16 (13)	6 (4)	9 (6)	19 (14)	1 (0)	5 (2)	108 (70)

Numbers in the table show total numbers of the isolates belonging to the respective species after the RFLP analysis. (): the number of isolates sequenced. ND: not determined. *B.*: *Bacillus*, *Brevib.*: *Brevibacillus*, *P.*: *Paenibacillus*

and *B. fumarioli*, were isolated from only the roots, but their isolation frequency was only one or two, making difficult a sound interpretation. χ^2 analysis ($P=0.05$) suggested that *B. luciferensis* were adapted to rhizosphere rather than soil, although Seldin *et al.*²⁹⁾ reported the presence of two subgroups, one in nonrhizosphere soil and one in rhizosphere, within a single species, *Paenibacillus azotofixans*.

Garbeva *et al.*¹⁰⁾ have revealed the diversity of *Bacillus* species in soil using the culture-independent method, i.e. to extract community DNA, to amplify the 16S rDNA specific to *Bacillus* species and then to sequence it. In their study, 8 different groups (*B. cereus*, *B. mycoides*, *B. megaterium*, *B. subtilis*, *B. pumilus*, *B. benzoovorans*, *B. niacini*/*B. lentus*, and *Paenibacillus* species) were identified from 3 kinds of soil managed differently. In spite that the present study targeted only culturable forms of *Bacillus* species in soil and root, the isolates obtained could be categorized into 7 out of the 8 groups (no *B. benzoovorans*). In addition, strains of

some other species, such as *B. bataviensis*, *B. dretensis* and *B. luciferensis*, were isolated in this study, but not in their study. *B. bataviensis* and *B. dretensis* are novel species reported by Heyrman *et al.*¹¹⁾ who successfully isolated these *Bacillus* lineages from soil using only a non-cultural method. *Brevibacillus* strains were not obtained in their study, but 7 isolates belonged to *Brevibacillus* species in the present study. This is because the specific primers, BacF and BacR, used by Garbeva *et al.* did not amplify the DNA of *Brevibacillus* species. The total of 108 isolates determined in this study was comparable to the 115 clones in Garbeva's study, indicating that even culturable *Bacillus* species possess quite a diverse species composition. This could be due to the result that more kinds of soils (8 soils) were used, or due to the different medium and different isolation conditions.

Felske *et al.*⁸⁾ tried to isolate novel *Bacillus*-related lineages from soil. They isolated 401 *Bacillus* stains and found

that ca. 40% belonged to known species with more than 97% sequence identity, but ca. 60% did not. In the present study, all the 70 isolates sequenced, except for one (96.9%), showed more than 98.9% sequence identity to one of the species listed in Table 3. In the present study, strains of *Bacillus* species were isolated after heat treatment of the soil suspension like in most studies, but heat treatment was not done in Felske's study, resulting in less bias in the isolation procedure. This could be significant, but the exact influence is not clear. They revealed that the major constituents of *Bacillus* detected were *B. niacini*-relatives (21.9%), unknown *Paenibacillus* species (16.0%), *Paenibacillus* species (12.5%) and *B. megaterium* (10.0%). The higher frequency of *B. megaterium* and *B. niacini*-relatives is consistent with our study.

McCaig *et al.*¹⁸⁾ isolated strains of *B. macroides* (sequence similarity 99.1%) and *B. insolitus* (92.5%) as the major culturable bacteria in Scottish soil. An isolate closely related to *B. macroides* was also obtained in the present study. Siciliano and Germida³³⁾ studied the taxonomic diversity of bacteria associated with the roots of *Brassica napus*, revealing that *Bacillus* species accounted for 43 of 437 isolates identified by FAME analysis and the dominant *Bacillus* species were *B. megaterium* (16 isolates), *B. pumilus* (10) and *B. brevis* (5). The fact that *B. megaterium* was the most frequently isolated species from roots was consistent with the present study.

The nitrogen-fixing activity of all the isolates was tested using the acetylene reduction assay, but none of them showed a positive reaction, suggesting that nitrogen-fixing *Bacillus* species like *B. polymyxa* and *B. azotificans* were not dominant *Bacillus* species in the soils. According to a study by Seldin *et al.*²⁸⁾, *B. polymyxa* constituted 0.2 to 2% of all *Bacillus* species. This relatively small ratio might result in no isolates of nitrogen-fixing *Bacillus* species. Several authors have isolated nitrogen-fixing *Bacillus* species using anaerobic culture and therefore, such species could have been isolated if anaerobic conditions had been used in this study.

Biocontrol ability of the *Bacillus* species

All the isolates (108) obtained in this study were tested for their ability to suppress the bacterial wilt of tomato in *in vitro* pot experiments. Ten isolates tended to decrease the disease incidence in the 1st screening, but 9 did not show any suppressive effects in the 2nd screening (data not shown). Therefore, a majority of *Bacillus* species in soil may not be antagonistic to the bacterial wilt caused by *R. solanacearum*. Only one isolate, designated *B. megaterium*

N2S6, consistently reduced the disease incidence in 7 independent experiments (Fig. 1a). Isolates belonging to *B. megaterium* were most frequently obtained (42 out of 108), but only strain N2S6 was antagonistic to bacterial wilt. Many antagonists, including *P. fluorescens* and *B. subtilis*, useful for suppressing soil-borne plant pathogens have been reported^{1,35)}, but this is the first report in Japan of a *B. megaterium* strain exhibiting biocontrol to our knowledge.

To enhance the biocontrol shown by the N2S6 strain, the use of poultry manure compost (PMC), the disease-suppressive effect of which was previously reported¹²⁾, was examined. The disease incidence decreased with the application of PMC and then further with strain N2S6 (Fig. 1b). Nakasaki *et al.*¹⁹⁾ found that compost from grass clippings suppressed *Rhizoctonia* patch disease on the inoculation of *B. subtilis* N4. This study demonstrated that the suppressive effect of PMC was enhanced by the inoculation of *B. megaterium* N2S6.

Fig. 1. Effect of inoculation of *B. megaterium* N2S6 and application of poultry manure compost (PMC) on the disease index of the bacterial wilt of tomato grown in yellow soil (Iwate). (a) a single inoculation of N2S6: mean $\pm$ SD (seven independent experiments), (b) a combination of N2S6 and PMC: mean $\pm$ SD (three independent experiments)

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