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Involvement of Growth-Promoting Rhizobacterium *Paenibacillus polymyxa* in Root Rot of Stored Korean Ginseng

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Abstract *Paenibacillus polymyxa* is a plant growth-promoting rhizobacterium (PGPR) which can be used for biological control of plant diseases. Several bacterial strains were isolated from rotten roots of Korean ginseng (*Panax ginseng* C. A. Meyer) that were in storage. These strains were identified as *P. polymyxa*, based on a RAPD analysis using a *P. polymyxa*-specific primer, cultural and physiological characteristics, an analysis utilizing the Biolog system, gas chromatography of fatty acid methyl esters (GC-FAME), and the 16S rDNA sequence analysis. These strains were found to cause the rot in stored ginseng roots. Twenty-six *P. polymyxa* strains, including twenty GBR strains, were phylogenetically classified into two groups according to the ERIC and BOX-PCR analyses and 16S rDNA sequencing, and the resulting groupings systematized to the degrees of virulence of each strain in causing root rot. In particular, highly virulent GBR strains clustered together, and this group may be considered as subspecies or biovar. The virulence of the strains seemed to be related to their starch hydrolysis enzyme activity, but not their cellulase or hemicellulase activity, since strains with reduced or no starch-hydrolytic activity showed little or no virulence. Artificial inoculation of the highly virulent strain GBR-1 onto the root surfaces of Korean ginseng resulted in small brown lesions which were sunken and confined to the outer portion of the root. Ginseng root discs inoculated *in vitro* or two-year-old roots grown in soil drenched with the inoculum developed significant rot only when the inoculum density was 10^6- 10^7 or more colony-forming units (CFU) per ml. These results suggest that *P. polymyxa* might induce ginseng root rot if their population levels are high. Based on these results, it is recommended that the concentration of *P. polymyxa* should be monitored, when it is used as a biocontrol agent of ginseng, especially in the treatment of stored roots.

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Biological control of plant disease using microorganisms has long been an effective alternative to chemical control, especially since the former is more environmentally-friendly and safer for humans than the latter. A number of antagonistic microorganisms, including fungi and bacteria, have been developed as biocontrol agents [7]. Microbial pesticides registered with the EPA in the United States include bacteria belonging to the *Agrobacterium*, *Bacillus*, *Pseudomonas*, and *Streptomyces* genera, and fungi belonging to the *Ampelomyces*, *Candida*, *Coniothyrium*, and *Trichoderma* genera.

Biocontrol agents have had limited commercial success, mostly due to the economic considerations, geographic variations in their potential use [40], and their reliability and cost [12, 16]. These economic constraints stem from the fact that biological agents are less toxic than chemical fungicides and have a much narrower spectrum of activity [16]. The antagonists, therefore, need to be present in large amounts to provide acceptable disease control [21]. Greater antagonistic microbe populations give greater and broader disease control [15, 43], but are more expensive. In addition, the commercial development of biological control agents necessitates a full understanding of the characteristics of these organisms, including their ecology and their interactions with pathogens, host plant, and soil microbe communities [6, 13, 17, 22]. These studies should not only be focused on the beneficial actions of these organisms, but should also be extended to possible unwanted and even detrimental effects on plants in specific as well as general surroundings.

Paenibacillus polymyxa, which was referred to as *Bacillus polymyxa* until 1993 [3], is a plant-growth-promoting rhizobacterium (PGPR) that is used for the biocontrol of plant diseases [8, 18, 20, 26, 32]. This species

also inhibits some human pathogenic microorganism [31] and produces antibiotic substances that act against fungi and bacteria [30]. As an endophyte, it induces changes in gene expression in *Arabidopsis thaliana*, rendering the plant more resistant to *Erwinia carotovora* and abiotic stress such as drought [39].

In a 1999-2001 study of rot in stored roots of Korean ginseng (*Panax ginseng* C. A. Meyer), several bacterial strains were isolated from decaying ginseng roots, one of which was the PGPR bacterium *P. polymyxa*. This bacterium was found to be linked to storage disease in several inoculation tests. The purpose of this study is to investigate potential involvement of *P. polymyxa* in rot in stored ginseng roots, thereby to improving the use of the bacterium as a biocontrol agent.

MATERIALS AND METHODS

Bacterial Strains

Four-year-old roots of Korean ginseng with rot symptoms were collected from commercial markets and storage facilities in Seoul, Suwon, and Geumsan, Korea, from 1999 through 2001. The bacterial strains isolated from rotten ginseng roots were designated as ginseng brown rot (GBR) strains. Decaying root tissues were cut into small pieces with a sterile scalpel, surface-sterilized with 1% sodium hypochlorite for 1 min, and then rinsed in sterile distilled water for 1 min. The root tissue was ground in 0.1 M $MgSO_4$ solution and streaked onto potato-dextrose agar (PDA) plates, which were then incubated at 28°C. A total of 345 bacterial isolates were obtained from the decaying ginseng roots, from which 81 isolates were selected because of their *Bacillus*-like colony morphology. These isolates were identified by RAPD using a *Paenibacillus*-specific primer (PAEN515F, 1377R; 0.9 kbp), and the positive isolates were then subjected to RAPD using a *P. polymyxa*-specific primer (BAC11, POL; 660 bp), following standard methods. *P. polymyxa* strain E681 was kindly provided by Dr. C. S. Park, Gyeongsang National University, and five KCTC (Korean Collection for Type Cultures) strains were kindly provided by the Korean Research Institute of Bioscience and Biotechnology. Transposon mutants were made by following the method of Steinmetz and Richter [37], using one of the GBR strains (GBR-1). Mutagenesis of plasmid pIC333 was performed with a 2.4-kb mini-Tn*10*. The plasmid was then introduced into *P. polymyxa* GBR-1 by electroporation. From the choice of a number of mutants, two virulent and two avirulent mutants were used for determining the biological characteristics of this study.

Identification of Bacterial Strains

The identities of the isolates and strains identified in the above tests as *P. polymyxa* were confirmed by physiological and culture characteristics, Biolog program analysis, gas chromatography of fatty acid methyl esters (GC-FAME), and 16S rDNA sequence analysis.

The physiological and culture properties of E681, the strains isolated from ginseng root, and some KCTC strains were characterized. To examine the morphology of the bacterial cells, a colony grown on PDA was picked with a spatula, placed in distilled water on a Formvar-coated copper grid, dried, and stained with 2% uranyl acetate for negative staining. The preparation was examined under a JEM 1010 electron microscope (JOEL, Japan). The average bacterial size was calculated from measurements of twenty bacterium from each of three preparations examined by electron microscopy. The motility, Gram staining, growth at 45°C, growth at pH 5.7, acid production in cultures containing arabinose, mannitol, and xylose, and pigment production [determined on yeast extract-dextrose-calcium carbonate agar (YDCA)] were evaluated by the methods described by Leary and Chun [24] and Sneath [34].

All putative *P. polymyxa* strains were tested for utilization of 95 carbon sources using the Biolog program, following standard methods. Briefly, bacterial cells cultured on BUG+M+T agar (0.25% maltose and 0.9% thioglycolate) at 28°C for 48 h were suspended in inoculating fluid (0.4% NaCl, 0.03% Pluronic F-68, and 0.01% gellan gum), inoculated onto microplates (Biolog GP MicroPlate™), and incubated at 28°C. After 24 or 48 h of incubation, the plates were read with a MicroLog™ 3-Automated Microstation system. The bacterium was identified based on the MicroLog Gram-positive database (version 4.0).

Gas chromatography of fatty acid methyl esters (GC-FAME) was conducted to confirm the bacterial identification. The bacteria were cultured on tryptic soy agar (TSA) at 28°C for 48 h. The colonies were harvested and placed in screw-cap culture tubes, and 1 ml of saponification reagent (NaOH aqueous methanol) was added. Methylation reagent (hydrochloric acid in aqueous methanol) was added after heat treatment, and fatty acids were extracted with extraction solvent (hexane/MTBE), mild base (10.8 g NaOH in 900 ml), and a saturated NaOH solution. The fatty acid composition was analyzed with the Sherlock system, followed by the generation of a similarity index for isolates that corresponded to a microorganism in the database (MIDI Library version, TSBA 4.0, Library Generation system software version 4.0).

Genomic DNAs of all putative *P. polymyxa* strains were prepared according to Pospiech and Neumann [29]. Using the 27mF and 1492mR primers, 16S rDNA was amplified by PCR. Amplified fragments (<1500 nucleotides) of 16S rDNA were ligated into pBluescript II SK. These plasmids were transformed into *Escherichia coli* strain DH5α. Purified plasmid was sequenced on an Applied Biosystems DNA sequencer (model ABI 3700). The resulting sequence was compared to the GenBank database, and sequence

similarities to the known *P. polymyxa* strains, including three strains very similar and one less similar to GBR-1, were identified using the BLAST program.

Nucleotide Sequence Accession Numbers

The GenBank accession numbers of 16S rDNA sequences in this study are AF515611 (GBR-1), AY359614-359632 (19 GBR strains), and AY359633-359637 (5 KTCT strains).

Inoculation of Stored Ginseng Root Tissues

Fresh four-year-old ginseng roots were purchased from a commercial market. A bacterial culture grown for 48 h on PDA medium supplemented with 0.5% peptone was placed in sterile distilled water and adjusted to approximately 5×10^8 CFU/ml. The cut surfaces of root discs of about 0.5 cm thick were inoculated at the center of the disc, near the secondary xylem, with a 20-μl drop of the bacterial suspension. Inoculated root and tuber discs were placed in Petri dishes with sufficient moisture, provided by the inclusion of water-soaked cotton swabs, and incubated at 23-25°C. This temperature range was chosen, because in a preliminary study, 23-25°C was found to be optimum for root rot induction with no root rot occurring at temperatures lower than 15°C and contamination frequently occurring at 30°C. Nine discs were used, and the experiments were repeated twice. Sterile distilled water treatment was used as the control. Roots were examined daily for development of root rot symptoms. Three days after inoculation, the degree of rotting was scored using the following disease severity rating system: ++, severe brown rot; +, mild brown rot; ±, yellowish discoloration or small lesion formation; and -, no symptoms.

Enzymatic Activities of *P. polymyxa* Strains

Enzyme activity tests were conducted to determine the mechanisms of tissue rotting by *P. polymyxa* strains. Ten μl of the bacterial cell suspension (5×10^8 cells/ml) were spotted on agar media amended with the following substrates, per liter: for starch hydrolysis, 3 g beef extract, 5 g peptone, 2 g soluble starch, and 15 g agar; for cellulase, 0.5 g NH_4SO_4, 0.5 g L-asparagine, 1 g KH_2PO_4, 0.2 g crystalline $MgSO_4$, 0.1 g $CaCl_2$, 0.5 g yeast extract, 10 g carboxyl methyl cellulose, and 20 g agar; for hemicellulase, 5 g gum guar, 5 g yeast extract, 4 g K_2HPO_4, 10 g casein, 0.0015 g crystal violet, and 18 g agar; and for pectinase, 10 g pectin, 2 g $NaNO_3$, 0.5 g KCl, 1 g K_2HPO_4, 0.5 g $MgSO_4\cdot7H_2O$, 0.01 g $FeSO_4$, and 20 g agar. After two days of incubation at 28°C, the agar media were stained with the following stains, except for the hemicellulose stain crystal violet which was included in the medium during its preparation: Grams iodine for starch, 0.1% Congo red for cellulose, and saturated copper acetate for pectin. The halos that formed around bacterial spots due to enzymatic activity were measured.

Genetic Grouping of *P. polymyxa* Strains and Relationship to Tissue Rot Activity

The *P. polymyxa* strains identified were grouped genetically based on their 16S rDNA sequences, which were used for the original identification of the bacterial strains, and from sequence data obtained using the BOX (5'-CTACGGCAA-GGCGACGCTGACG-3'), ERIC1R (5'-ATGTAAGCTC-CTGGGGATTCAC-3'), and ERIC2 (5'-AAGTAAGTG-ACTGGGGTGAGCG-3') primers. The PCR followed the method of Versalovic *et al.* [41]. Briefly, a 25-μl reaction mixture composed of 600 μM dNTPs, 1.5 mM $MgCl_2$, 0.17 mg/ml bovine serum albumin, 10% (v/v) dimethyl sulfoxide, 1 μM primer(s), 1.25 units *Taq* DNA polymerase, and 50 ng template DNA was subjected to PCR using an MJ Research PTC 100 Programmable Thermal Controller (Watertown, MS, U.S.A.). The temperature cycling for PCR was composed of an initial denaturation for 7 min at 95°C; 30 cycles of 1 min at 94°C, 1 min at 52°C, and 8 min at 65°C; and a final extension of 16 min at 65°C. The amplified products were electrophoresed in an 1.5% agarose gel and 0.5× Tris-borate EDTA buffer, and viewed under a UV illuminator following staining with ethidium bromide. Cluster analysis of *P. polymyxa* strains was performed using the BOX and ERIC fingerprints and the NTSYS program (Exeter Software, NY, U.S.A., version 1.80), and also using 16S ribosomal RNA gene sequences and the BioNumerics program (Applied Maths, Antwerp, Belgium). The reliability of the phylogenetic clustering for the PCR and 16S rDNA sequence analyses were assessed by the bootstrap method using the Unweighed Pair Grouping by Mathematical Average (UPGMA) trees (PAUP 4.0 beta 8) with 1,000 pseudoreplications [5, 38].

Characterization of Root Rot Caused by *P. polymyxa* GBR-1: Pathogenicity on Ginseng Following Root Surface or *in Planta* Inoculation

A bacterial culture grown for 48 h on PDA supplemented with 0.5% peptone was collected in sterile distilled water and adjusted to approximately 5×10^8 CFU/ml. Fresh 2- to 4-year-old ginseng roots purchased from a ginseng market and obtained from the KT & G Central Research Institute were inoculated with the bacterium. The root surface was inoculated with 20-μl of the bacterial suspension at three inoculation sites per root, at sites that had been either left intact or wounded by pricking the main root surface with a needle. Inoculated roots and root discs were placed in Petri dishes with sufficient moisture supplied by the inclusion of a water-soaked cotton swab, and were incubated at 23-25°C. Sterile distilled water treatment was used as the control. Development of root rot symptoms was monitored daily. Nine roots were used, and experiments were repeated twice. Rot and lesion development were noted in three to seven days after inoculation.

The pathogenicity of the bacterium was also tested using *in planta* inoculation. Two-year-old ginseng roots at a stage just before sprouting were provided by the KT & G Central Research Institute, Korea. The roots were soaked with bacterial suspension with or without prior wounding as described above and planted in sterilized sandy loam soil in plastic pots of 8 cm in diameter. Another set of fresh ginseng seedling roots was planted in soil, and the rhizosphere was drenched with a bacterial suspension of approximately 50 ml per pot. Roots were examined for symptoms of disease development ten days after inoculation. The disease severity was graded with the following scale: ++, severe brown lesions; +, mild brown lesions; ±, yellowish discoloration or small lesion formation; and -, no symptoms. Nine plants were used for each treatment.

Effect of Inoculum Density on Root Rot Development

Bacterial suspensions at varying densities were used to inoculate cut discs of four-year-old ginseng roots and roots of two-year-old ginseng seedlings to test the effect of bacterial inoculum density on root rot severity. Twenty µl of bacterial suspension at 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, or 10^9 CFU/ml were spotted at the center of root discs, in the region of the secondary xylem. The inoculated root discs were placed in Petri dishes on Whatman No. 1 filter paper soaked with sterile distilled water and incubated at 23-25°C. Three plates containing three root discs each were used per treatment. Root discs were examined for symptom development three days after inoculation. For ginseng seedlings, roots were pricked with sterile needles, soaked in each bacterial suspension, and planted in sterile soil in pots of 10 cm diameter. Ten days after inoculation, the roots were unearthed and the development of rot symptoms was noted. For each treatment, fifteen ginseng roots were used.

RESULTS

Isolation and Identification of Bacterial Strains

Out of 345 bacterial isolates, including 81 that formed *Bacillus*-like colonies, 36 and 20 seemed related to the genus *Paenibacillus* and the species *P. polymyxa*, respectively, based on their RAPD PCR products amplified from genomic DNA with primers specific to the genus and species (data not shown). All of the isolates that were classified as *P. polymyxa* were among the 36 isolates originally grouped into the genus *Paenibacillus*. The putative *P. polymyxa*

Table 1. Comparison of characteristics of putative *Paenibacillus polymyxa* strains isolated from ginseng root rots (20 GBR strains) with those of other known *P. polymyxa* strains (including KCTC strains and E681).

Characteristics	GBR and KCTC strains	*P. polymyxa* E681	*P. polymyxa*[a]
Yellow or orange colonies on NGA, YDC, or NBY media	-[b]	-	-
Fluorescent pigment on KB	-	-	-
Aerobic growth	+	+	+
Anaerobic growth	+	+	+
More than four peritrichous flagella	+	+	V+
Growth on D-1 agar	-	-	-
Gram-positive	+	+	V+
Aerial mycelium	-	-	-
Motility	+	+	+
Growth at 45°C	- (+)[c]	-	-
Growth at pH 5.7	+ (-)[d]	+	+
Growth in 7% NaCl	-	-	-
Utilization of citrate	-	-	-
Anaerobic growth in glucose broth	+	+	+
Acids produced from:			
Arabinose	+	+	+
Mannitol	+	+	+
Xylose	+	+	+
Starch hydrolysis	+	+	+

[a]Described by Leary and Chun [23] and in *Bergey's Manual of Systematic Bacteriology* [34].
[b]Symbols: +, positive reaction; -, negative reaction; V, variable.
[c]Five GBR strains (strains GBR-2, GBR-180, GBR-447, GBR-462, and GBR-477), KCTC1663, and KCTC3554 have a positive reaction.
[d]Two GBR strains (strains GBR-501 and GBR-540) and KCTC3554 have a negative reaction.

isolates from ginseng root rots, hereafter referred to as GBR (ginseng brown rot) isolates, were rod-shaped, and Gram-positive, had peritrichous flagella, and could grow at pH 5.7 but not in 7% NaCl or at 45°C (Table 1). The bacteria utilized various carbohydrates, including arabinose, mannitol, and xylose, to produce acids, but not citrate. These biochemical and physiological characteristics matched well with those of *P. polymyxa* E681 and the *P. polymyxa* characteristics described by Leary and Chun [24] and in *Bergey's Manual of Systemic Bacteriology* [34].

The Biolog results of the twenty GBR strains, E681, and the KCTC strains showed that they could all be classified as *P. polymyxa*, because their closest match was *P. polymyxa*. For example, *P. polymyxa* GBR-1 was able to utilize various carbohydrates, including dextrin, glycogen, inulin, arabinose, cellobiose, fructose, galactose, gentiobiose, gluconic acid, maltose, lactulose, lactose, maltotriose, mannitol, mannose, melibiose, glucoside, raffiose, stachyose, xylose, and glycerol. Comparing these traits to the Biolog database revealed this strain with a match probability of 100% to *P. polymyxa* (0.75). The levels of similarity among the 26 strains ranged from about 0.65 to 0.97, and E681 was the strain least similar to GBR-1.

Based on the results of GC-FAME analysis, the twenty GBR strains, the five KCTC strains (KCTC1663, 1761, 3554, 3627, and 3717), and *P. polymyxa* E681 were all identified as *P. polymyxa*, with similarities from 0.45 to 0.94. GBR-1 showed a similarity of 0.90 to the species in the database. All of the tested strains had more than 80% similarity among themselves.

The 16S rDNA sequences of all strains tested contained 1371–1456 bases, and BLAST analyses showed that, with the exception of KCTC3717, each had 98.5% or higher similarity to the corresponding sequences of GBR-1 (GenBank accession number AF515611) and three known *P. polymyxa* strains, DSM36T (AJ320493), CF43 (AJ2233989), and PMD230 (AJ223988) that had been deposited in the GenBank database. However, these strains had lower

Table 2. Severity of tissue rot on four-year-old ginseng root discs caused by inoculation with *Paenibacillus polymyxa* strains, and the enzymatic activity of these strains.

P. polymyxa strains	Severity of root tissue rot[a]	Degree of enzyme activity[b]			
		Starch hydrolysis	Cellulase	Hemicellulase	Pectinase
GBR-1	++	++	++	++	-
GBR-2	+	++	++	++	-
GBR-11	++	++	++	++	-
GBR-27	++	++	++	++	-
GBR-180	±	+	++	++	-
GBR-192	++	++	++	++	-
GBR-325	+	++	++	++	-
GBR-447	+	++	++	++	-
GBR-462	+	++	++	++	-
GBR-464	++	++	++	++	-
GBR-465	±	+	++	++	-
GBR-472	±	+	++	++	-
GBR-477	+	++	++	++	-
GBR-478	++	++	++	++	-
GBR-485	++	++	++	++	-
GBR-501	±	++	++	++	-
GBR-515	++	++	++	++	-
GBR-540	++	++	++	++	-
GBR-602	++	++	++	++	-
GBR-603	++	++	++	++	-
KCTC1663	±	++	++	++	-
KCTC1761	±	+	++	++	-
KCTC3554	±	++	++	++	-
KCTC3627	±	++	++	++	-
KCTC3717	-	-	++	++	-
E681	+	++	++	++	-
M177	++	++	++	++	-
M233	++	++	++	++	-
M3109	-	-	++	++	-
M3630	-	-	++	++	-

[a]Root tissue rot severity: ++, brown rot; +, mild brown rot; ±, yellowish discoloration; -, no symptoms.

[b]Size of halos formed around bacterial colonies on agar media. For starch hydrolysis: ++, larger than 4 mm; +, smaller than 4 mm; -, no halo formed. Halos of cellulase and hemicellulase for all strains were around 4–6 mm in size.

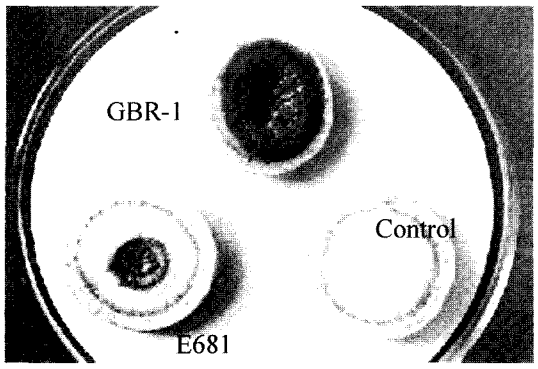

Fig. 1. Tissue rots on root discs of four-year-old ginseng four days after artificial inoculation with *Paenibacillus polymyxa* GBR-1.
The inner tissues of the root discs rotted completely after GBR-1 inoculation. *P. polymyxa* E681 inoculation induced rot symptoms that were similar to but milder than those caused by GBR-1.

similarities, around 93%, to *P. polymyxa* strain 6 (AF181573), to which KCTC3717 had 98.7% similarity.

Virulence for Ginseng Root Tissue Rot and Enzymatic Activity of *P. polymyxa* Strains

Wounded ginseng roots and ginseng root discs developed brown rot symptoms with variable severity within three days after inoculation with all *P. polymyxa* strains isolated from ginseng root rots, except for four strains that caused only yellowish discoloration symptoms (Table 2). All KCTC strains had low virulence, and one strain (KCTC3717) was not virulent. E681 induced rot symptoms milder than other severe GBR strains, including GBR-1 (Fig. 1).

A good cellulase and hemicellulase activity and no pectinase activity were detected in all the strains tested with little or no variation, however, the degree of starch hydrolysis activity varied among the strains (Table 2). Inoculation with strains with low or no starch hydrolysis activity always resulted in yellowish discoloration or a complete lack of symptoms, and highly virulent strains always had strong starch hydrolysis activity. Some bacteria with high starch hydrolysis activity were not virulent in producing ginseng root tissue rot. Transposon mutants of GBR-1 with no starch hydrolysis activity were less virulent than those with high starch hydrolysis activity (Table 2).

Genetic Grouping of *P. polymyxa* Strains and the Relationship to Tissue Rot Severity

Figure 2 shows ERIC and BOX fingerprints of 26 *P. polymyxa* strains, showing that several GBR strains are genetically closely related. Cluster analyses of the combined PCR (Fig. 3) and 16S rDNA sequences (Fig. 4) showed that the grouping of *P. polymyxa* based on genotypes revealed clearly differentiated subgroups. The clustering of these groups was highly related to the virulence levels of the individual strains. In particular, GBR strains with higher virulence were closely clustered. E681, GBR-462, and KCTC3717 were not closely clustered into either subgroup according to the combined ERIC and BOX-PCR results, and GBR-462 and KCTC3717 were the strains most remote from both subgroups when the 16S rDNA data were considered. The UPGMA trees exerted by the bootstrap method (with 1,000 replications) matched well with the above cluster analyses (data not shown). In the combined PCR, bootstrap values for subgroups were 99% for more virulent GBR strains, and 84% for less virulent GBR and other strains from which GBR-462, KCTC3717, and E681 were ruled out. For 16S rDNA sequence analysis, bootstrap values for both subgroups were 80% and 81%, respectively, and KCTC3717 and GBR-462 were not closely clustered into either subgroup (100%). However, E681 and GBR-501 were included in the more virulent subgroup (data not shown).

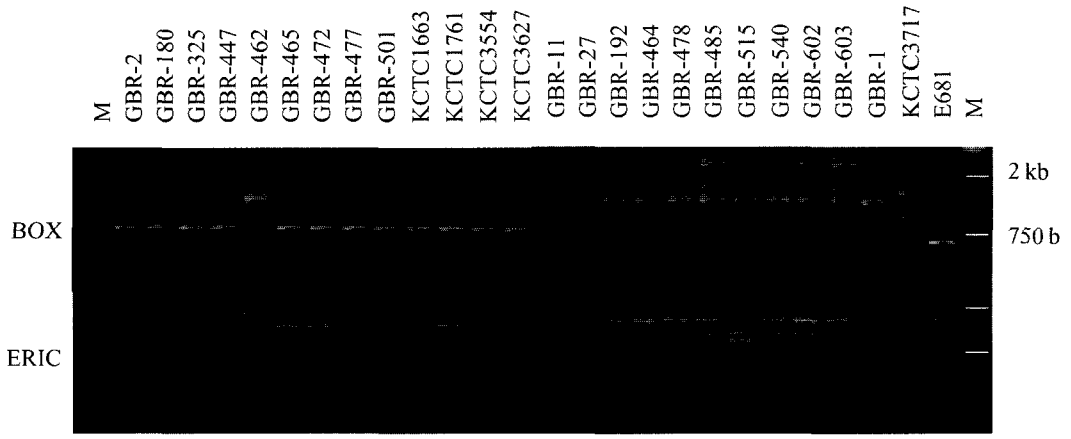

Fig. 2. BOX and ERIC fingerprints of PCR DNA fragments from 26 *Paenibacillus polymyxa* strains, including those from ginseng root rots (GBR strains). M: molecular marker.

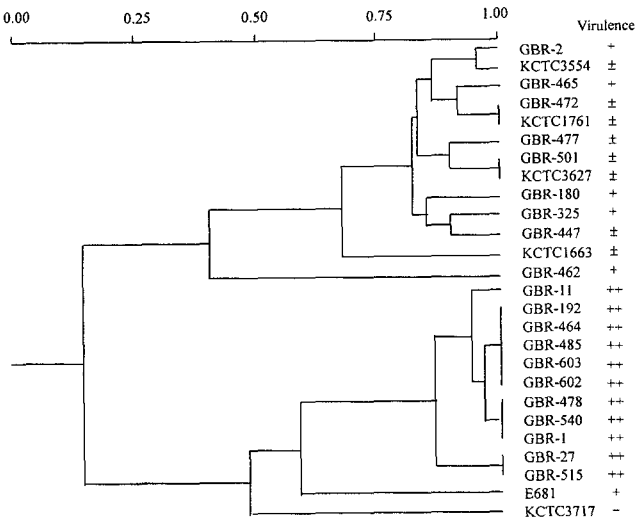

Fig. 3. Cluster analysis of 26 *Paenibacillus polymyxa* strains, including those from ginseng root rots (GBR strains), based on a combination of BOX and ERIC fingerprints.

Pathogenic Characteristics of *P. polymyxa*

The pathogenic characteristics of *P. polymyxa* were examined using the representative strain GBR-1. Symptoms developed in all roots after inoculation of wounded root surfaces. Surface inoculation of stored ginseng roots resulted in brown lesions that were somewhat sunken but rather superficial (Fig. 5). The lesion sizes ranged from a few millimeters to about 1 cm in diameter. No symptoms developed on unwounded root surfaces that had been inoculated.

Rot symptoms only rarely developed on unwounded ginseng root surfaces treated with either *in vitro* or *in planta* inoculations (Table 3). Surface inoculation of wounded roots or dipping of wounded roots in bacterial

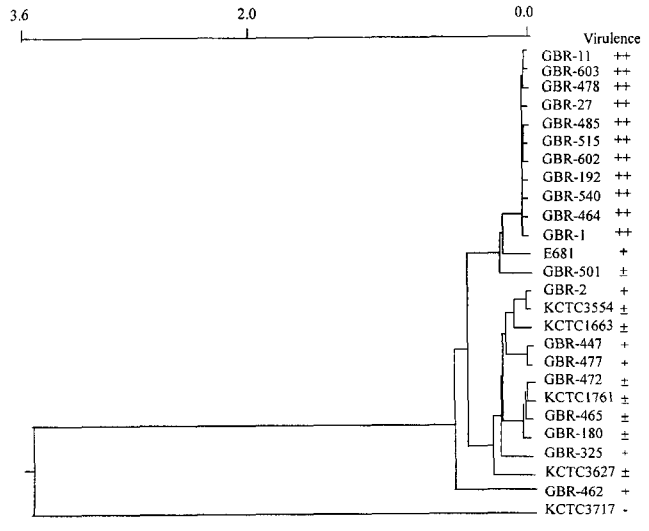

Fig. 4. Dendrogram based on 16S rDNA sequences of 26 *Paenibacillus polymyxa* strains, including those from ginseng root rots (GBR strains).

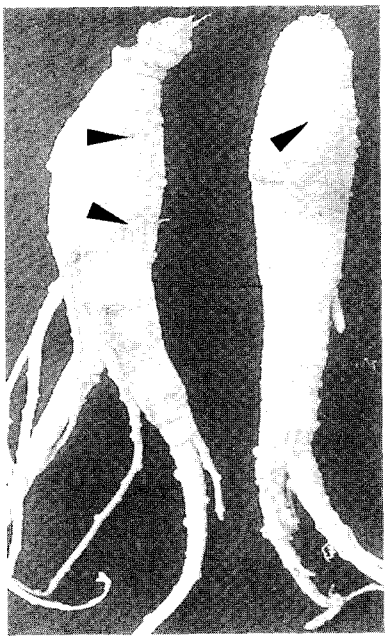

Fig. 5. Rot symptoms on the surface of three-year-old ginseng roots five days after wounding and inoculation with *Paenibacillus polymyxa* GBR-1.
Lesions (arrowheads) caused by the bacterium are somewhat sunken but superficial.

Table 3. Rot symptom development in ginseng roots following inoculation with different methods.

Inoculation method	Rot symptom development	
	Incidence (%)	Disease severity[a]
In vitro inoculation (root surface)		
2-year-old root		
Wounded	100.0	+
Unwounded	11.1	+
3-year-old root		
Wounded	100.0	+
Unwounded	0.0	-
4-year-old root		
Wounded	100.0	+
Unwounded	0.0	-
In planta inoculation		
2-year-old root		
Immersed after wounding	88.9	+
Immersed without wounding	22.2	+
Drenching of soil after planting	11.1	+

[a]Disease severity (counted only for roots showing symptoms) was examined seven days (for the root surface) and ten days (for *in planta*) after inoculation with 5×10^8 CFU/ml of GBR-1. ++, brown rot; +, mild brown rot; ±, yellowish discoloration or small lesions; -, no discoloration (no symptoms).

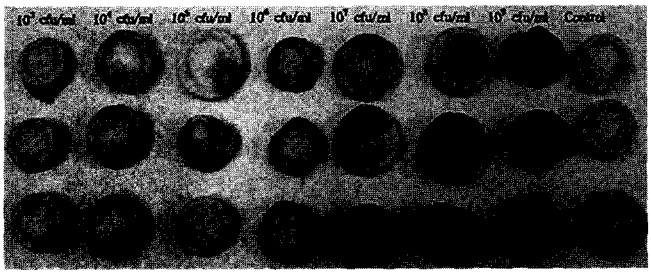

Fig. 6. Rot symptoms on four-year-old ginseng roots three days after inoculation with different densities of *Paenibacillus polymyxa* GBR-1.
Note brown rot symptoms developed following inoculation with densities higher than 10^7 CFU/ml.

suspensions caused about 89% rot incidence, whereas dipping unwounded roots or drenching the rhizosphere with a bacterial suspension induced only minimal root rot.

The Virulence of *P. polymyxa* is Related to the Density of the Inoculum

The concentration of the *P. polymyxa* GBR-1 inoculum affected the development of brown rot symptoms on root discs. Three days after treatment with inoculum of up to 10^5 CFU/ml, no root rot symptoms developed (Table 4). At an inoculum concentration of 10^6 CFU/ml, a yellowish or light brown discoloration developed on some discs, but no noticeable rot occurred. Brown rot symptoms developed only with inoculum concentrations of 10^7 CFU/ml and above (Fig. 6). With *in planta* inoculation of two-year-old ginseng roots, a significant incidence of brown rot was noted only at inoculum concentrations of 10^7 CFU/ml and above (Table 4).

Table 4. Occurrence of brown rot of ginseng roots caused by *Paenibacillus polymyxa* GBR-1 at different inoculum concentrations.

Inoculum conc. (CFU/ml)	Rot severity on root discs[a]	Rot on two-year-old ginseng roots[b]	
		Incidence (%)	Severity
Control	-	0.0	-
10^3	-	6.7	±
10^4	-	8.3	±
10^5	-	0.0	-
10^6	±	46.7	± or +
10^7	+	66.7	+
10^8	++	93.3	+
10^9	++	100.0	+

[a]Disease severity was determined three days after inoculation with GBR-1. ++, brown rot; +, mild brown rot; ±, yellowish discoloration; -, no discoloration (no symptoms).
[b]Disease incidence and severity were determined ten days after inoculation with GBR-1. ++, brown rot; +, mild brown rot or small lesions; ±, yellowish discoloration; -, no discoloration (no symptoms).

Discussion

Out of 345 bacterial strains isolated from rotting root tissues of Korean ginseng, 20 GBR strains were first selected by RAPD using a *P. polymyxa*-specific primer and were later confirmed to be *P. polymyxa* based on their physiological, morphological, and culture characteristics, and Biolog program, GC-FAME, and 16S rDNA sequence analyses. The ratio of *P. polymyxa* to the total bacterial strains isolated was 5.8%, suggesting that this species may be common in stored ginseng roots. This frequency of isolation is likely the result of bacterial multiplication in the root rot sites themselves, in which a myriad of microorganisms exists. Thus, *P. polymyxa* may not be a mere opportunistic contaminant of ginseng roots, but instead can probably cause root rot directly under certain storage conditions. In the artificial inoculation of root discs, only *P. polymyxa* could be isolated from the rotten tissues in a detectable amount, fulfilling Koch's postulates. The bacterial population increased remarkably in the inoculated tissues at the high inoculum level (10^8 CFU/ml), but not at the low inoculum level (10^6 CFU/ml). These results suggest that *P. polymyxa* may primarily act as a rotting agent for stored ginseng roots in the inoculation test.

Wounding was needed for lesion development on ginseng root surface. The few occurrences of root rot resulting from dipping of unwounded roots in a bacterial solution or drenching of the rhizosphere may have been due to accidental wounding of the root surface or infection through surface cracks formed by the emergence of lateral roots. The lesions that developed on the root surface after artificial inoculation were superficial, and tissue rot and root lesions could only be induced at high inoculum levels of not less than 10^6- 10^7 CFU/ml. Natural bacterial populations do not reach these levels under storage conditions [28] or in ginseng fields [19], indicating that the root rot disease may not readily occur under these conditions. Superficial rot symptoms in a whole ginseng root may not cause a serious problem during storage. Periderm-like layers, which are related to histological defense structures [1], were observed to form in tissues beneath surface inoculation sites (unpublished data), suggesting that the bacterium may not cause extensive root rot under normal conditions. Nevertheless, root surface lesions can lower the quality of the root, thus lowering market prices, especially for roots that have been stored. Natural bacterial populations can grow to high numbers during production of alfalfa and onion sprouts [28], suggesting that high relative humidity and temperature, together with nutrient-rich root exudates, can support rapid bacterial multiplication. In addition, severe injury of the ginseng root during harvest and transportation can occur, which may expose the inner root tissues to the bacterium, triggering root rot

development, especially when combined with storage conditions favorable for disease development.

Each of the *P. polymyxa* strains used in our study was able to induce root tissue rot or discoloration, except for KCTC3717 and two mutant strains, but the strains varied in their virulence in causing root tissue rotting. Clustering analyses of the *P. polymyxa* strains based on the results from the Biolog program and GC-FMAE analysis did not group the high-virulence strains together (data not shown). However, the strains were clustered with high reliability into two different subgroups depending on virulence in analyses based on ERIC and BOX-PCR and 16S rDNA sequencing, and on their bootstrap values obtained through the UPGMA method. These results suggest that the virulent GBR strains have similar genetic constituents. 16S rDNA analysis is valuable in identifying and classifying microbes [36, 44], and PCR genomic fingerprinting is a highly discriminatory and reproducible method for phylogenetic clustering [25, 41]. These methods assist in phylogenetic classifications up to the level of subspecies, biovars, or strains [25]. Therefore, the highly virulent GBR strains may be classified into a different subspecies (or biovar) from other strains. On the other hand, GBR-462 and KCTC3717 seemed not to be included in either of the subgroups (a bootstrap value of 100%) when the 16S rDNA data are considered (Fig. 4). In particular, KTCT3717 had the lowest (about 93%) sequence similarity to the other test strains in 16S rDNA analysis (data not shown). KTCT3717 and strain 6, which have a high sequence similarity to each other (more than 98%), may be designated as another species of *Paenibacillus*.

The virulence of these strains in causing ginseng root tissue rot may be closely related to their starch hydrolysis activities, since all strains with little or no starch hydrolysis activity showed low or no virulence, and highly- or medium-virulent strains always had high starch hydrolysis activity. Also, the transposon-mediated transgenic mutants of *P. polymyxa* GBR-1 displayed no pathogenicity and little or no starch hydrolytic activity, supporting the idea that the enzymatic activity may be involved in tissue rotting. However, some strains with high hydrolysis activity showed low virulence, suggesting that high hydrolysis activity does not necessarily control this phenomenon.

Starch hydrolysis has seldom been directly related to plant tissue rot. A hydrophobic amylase inhibitor from corn seeds inhibited the amylase of *Fusarium verticillioides*, and also its conidia germination [11]. This result indicated that starch hydrolytic activity affects microbial growth and metabolism. β-Amylase from *P. polymyxa* hydrolyzes raw starch granules [35], suggesting that starch hydrolytic activity may enhance the disintegration of the cellular components in ginseng. Ginseng root, like potato, contains plentiful amount of starch granules, therefore, starch hydrolytic activity of any kind (whether from the plant itself or from microbes) is needed for cellular disintegration in cells with a large amount of starch granules, although it is not the main factor in governing root rotting. Further studies are needed to confirm the relationships between starch hydrolytic activity and virulence of the bacterium.

Each of the strains in this study had high cellulase and hemicellulase activity, but none had pectinase activity which is related to soft rot. Ginseng root discs inoculated with virulent *P. polymyxa* strains always showed brown discoloration and dry rot, except for a few severe cases during later stages of the infection, which further supports the conclusion that pectinase is not involved in root tissue rot in this bacterium.

Bacillus (including *Paenibacillus*) species have seldom been reported to be plant pathogens, although they are well known as biocontrol agents. However, *Bacillus megaterium* pv. *cerealis* and *B. circulans* are known pathogens of wheat and date palm, respectively [14, 23]. Spotting of pepper fruit [42], partial decay of peanut kernels [27, 33], various minor diseases of soybean [9, 10], and potato tuber tissue rot [2] are caused by *Bacillus subtilis*. *P. polymyxa* (*Bacillus polymyxa*) causes rot of germinating seeds and seedling blight of tomatoes [4]. In this study, brown lesions developed when root discs or wounded roots were treated with high inoculum levels of *P. polymyxa* strains. When *P. polymyxa* E681 was used as a seed coating treatment for barley at a high inoculum density, some root damage occurred (Dr. C. S. Park, personal communication). However, inoculation with a high density of *P. polymyxa* GBR-1 did not cause any noticeable damage to tomato plants treated at the true leaf stages 5-6 (unpublished data). Therefore, the damage to plants caused by this bacterium may be dependent on the plant species, its organ and tissue, stage, and the inoculum density.

It is not surprising that a bacterium can be both beneficial and harmful to plants, because organisms cannot be naturally selected for either trait alone. *P. polymyxa* is a natural saprophyte, and it may thus harm seeds and fleshy roots in storage, especially if they have high starch contents and are stored under conditions of very high bacterial population densities. Therefore, the density of *P. polymyxa* cells should be controlled for the effective use of this species as a biocontrol agent, especially for seed coating and storage of roots, to avoid adverse affects on the host.

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