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Qualitative and Quantitative Detection of Agricultural Microorganisms Expressing Iturin and Mop Cyclase in Soils

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The environmental release of genetically engineered microorganisms (GEMs) to improve agriculture or remediate environmental hazards has raised concern over the fate of the organisms and their engineered genes. To detect the microorganisms released into the environment at the molecular level, *Bacillus subtilis* KB producing iturin and *Pseudomonas fluorescens* MX1 carrying the *moc* (mannityl opine catabolism) region from the *Agrobacterium tumefaciens* were employed as model microorganisms. Using specific fusion primers and the TaqMan probes, qualitative and quantitative detections of the model organisms by PCR and real-time PCR were conducted employing a small-scale soil-core device and pots during the six month period. The data indicate that the model bacteria can be easily detected by qualitative and quantitative methods in the test systems employed, and they do not give significant impacts on the other bacteria in soils on the Southern blotting analysis, although long-term observation may be needed.

KEYWORDS: Agricultural microorganism; genetically modified organism (GMO); iturin; PGPR; mop cyclase; soil-core device; real-time PCR; horizontal gene transfer

INTRODUCTION

The commercial use of genetically modified microorganisms (GEMs) as biological control agents and plant growth promoting rhizobacteria (PGPR) in the fields is still limited by injurious effects associated with public concerns, such as the perturbation of the soil ecosystems and detrimental risks to the human (*1*, *2*). However, global hectarage of transgenic crops has gradually increased every year since 1996 despite concerns for potential harmful impact on human health and the environment (*3*). In 2009 alone, 134 million hectares of transgenic crops were grown in 25 countries, with a market value of $10.5 billion U.S. dollars (*4*). In addition, as the use of synthetic agrochemicals is to be reduced in advanced countries, the necessity of biological control agents that can substitute for the synthetic chemicals is greater than ever before (*1*). In view of such increased hectarage of transgenic crops during the decade and need of biological control agents as alternatives to chemical pesticides, the frequency of GEM release to improve the productivity of crops in the near future is expected to rise as more sophisticated genetic constructions are produced that address particular traits (*5*, *6*).

Many *Bacillus* species, such as *B. amyloliquefaciens*, *B. megaterium*, and *B. subtilis*, produce a variety of nonribosomally synthesized cyclic lipopeptides with a long fatty moiety (*7*−*9*). Among them, the lipopeptide antibiotic iturin contains one β-amino fatty acid and seven α-amino acids with a D-configuration; it shows strong antifungal activity against various phytopathogenic fungi (*10*−*13*) and is used broadly as biological control agents (*12*−*15*). A recombinant *Pseudomonas fluorescens* MX1 strain has been used as a PGPR containing the mannityl opine catabolic (*moc*) genes integrated into the chromosome (*16*). The members of the mannityl opine, mannopine and agropine, are rare carbon compounds and found only in plant neoplasias induced by some pathogenic *Agrobacterium* strains, and the lactonization of mannopine to agropine is reversibly catalyzed by mop cyclase (*16*, *17*). It is suggested that this enzyme is a sensitive and highly specific marker for detection by PCR to identify the engineered *P. fluorescens* MX1 strain released into the environment.

Here, we describe the qualitative and quantitative detection methods for model microorganisms, such as *B. subtilis* KB producing iturin and *P. fluorescens* MX1 suitable for use as a biological control agent and PGPR, respectively, by using a small-scale soil-core device, designed to track drifting bacteria due to rain and surface water. Based on the data obtained, we investigated the applicability of pot experiments by real-time PCR and the possibility of gene transfer among bacteria in soils of soil-core device.

MATERIALS AND METHODS

Microorganisms and Culture Media. *Bacillus subtilis* KB producing iturin used in this study is a strain isolated from soils as described previously (*18*). The other bacteria were provided from Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea) and Seoul National University (Seoul, Korea). These included two *B. subtilis* 168 strains, one with spectinomycin resistance and the other with erythromycin and lincomycin resistance in chromosome. Also, three *Pseudomonas* species were used: *P. fluorescens* MX1 containing mop cyclase and

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kanamycin-resistance genes (*16*), *Pseudomonas fluorescens* with chitin synthase 1 derived from *Saccharomyces cerevisiae* and kanamycin-resistance genes, and *P. putida* containing the rifampicin-resistance gene. All of the *B. subtilis* species were incubated at 30 °C for 15 h using an LB broth without or with spectinomycin (100 μg/mL), erythromycin (0.5 μg/mL) and lincomycin (12.5 μg/mL), while *P. fluorescens* and *P. putida* were incubated at 30 °C for 15 h using an Pseudomonas Agar F medium (Difco) without or with kanamycin (50 μg/mL) or rifampicin (50 μg/mL). The culture broths of each strain were then centrifuged at 8000*g* for 10 min and the pellets suspended twice in sterilized distilled water to reach the absorbance of 1.0 at 550 nm wavelength. Thereafter, each suspension was used as an inoculum for small-scale soil-core devices and pots. *B. subtilis* KB producing iturin and *P. fluorescens* MX1 with mop cyclase were used as model microorganisms.

Small-Scale Soil-Core Device. To detect easily the model bacteria released into the environment qualitatively and quantitatively, a small-scale soil-core device was designed to track microorganisms, which can drift due to rain and surface water in the laboratory (*19*). The device set is very simple in construction and composed of commercially available acrylic. The dimension of each part is 10 cm in diameter × 4 cm in height and consisted of four detachable parts. Fine wire nettings are attached to the bottom of each of three columns to drain the water. The inside of three columns from the top was filled with sterilized field soils and the dripped water was collected in the bottom. Three types of bacteria, *B. subtilis* KB, spectinomycin-resistant *B. subtilis* 168, and erythromycin-resistant *B. subtilis* 168, were inoculated into each column of the soil-core devices in order from the top. *P. fluorescens* MX1, kanamycin-resistant *P. fluorescens*, and rifampicin-resistant *P. putida* were separately inoculated into the each column of soil-core device in order from the top. The cell density of *Bacillus* species was adjusted to $2.3-3.6 \times 10^7$ CFU/mL to reach the absorbance of 1.0 at 550 nm wavelength, while *Pseudomonas* species was adjusted to $1.7-4.9 \times 10^8$ CFU/mL. The soil cores were then sprinkled three times a week with 50 mL of sterilized water during the six month period and incubated at 25 °C and 65−70% relative humidity.

Extraction of Genomic DNA. Genomic DNAs from culture broth of each bacteria were extracted to perform qualitative detections by using Genomic DNA Extraction Mini Kit (RBC Bioscience, Taipei, Taiwan) according to the manufacturer's protocol. Soil genomic DNAs extracted from soil samples, which were collected at intervals of three weeks from small-scale soil-core devices with different markers in each column, were obtained by using FastDNA Spin Kit for Soil (MP Biomedicals, Solon, Ohio, USA). The extracted DNAs containing humic acid were further purified by using 0.8% agarose gel electrophoresis and QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and the purified DNAs were used in the following experiments. The concentrations of extracted DNAs were determined by measuring UV absorbance at 260 nm.

Sequence Analysis of Junction Regions by Genomic Library Screening. The sequence analyses of junction regions were performed by using the genomic library screening as follows. The genomic DNAs of *B. subtilis* and *P. fluorescens* cultivated at 30 °C overnight were extracted by using Genomic DNA Extraction Mini Kit (RBC Bioscience). The isolated genomic DNAs were purified with 10% 3 M sodium acetate and ethanol. Both ends of each genomic DNA were treated by using Copy-Control Fosmid Library Production Kit (Epicenter Biotechnologies, Madison, WI, USA) for 45 min at room temperature. The over 20 kb of DNAs containing both ends obtained from genomic DNAs were ligated into CopyControl pCC1Fos vector (Epicenter Biotechnologies) for 2 h at room temperature. In vitro packaging (MaxPlax Lambda Packaging Extract, Epicenter Biotechnologies) for 3 h at 30 °C was performed after ligation, and then diluted 100 times with buffer solution (10 mM Tris-HCl, pH 8.3, 100 mM NaCl, 10 mM $MgCl_2$). An aliquot of DNA was transformed into *Escherichia coli* EPI300 for 1 h at 30 °C and selected the colonies on the LB agar plates containing with or without antibiotic markers. The PCR products of each clone were confirmed, and then the DNA products obtained from PCR were analyzed by using ABI 3730XL Capillary DNA sequencer (SolGent, Daejeon, Korea).

Oligonucleotide Primers and TaqMan Probes. Fusion primers of the flanking regions specific to each marker and TaqMan probes were designed on the basis of the sequences of the junction region of inserted markers obtained from genomic libraries of each microorganism. The locations of primers and probes to detect the inserted marker DNA are shown in **Figure 1**. All primers were synthesized and purified by Bioneer Co. (Daejeon, Korea). PCR amplifications of the iturin biosynthetic gene were carried out by using specific fusion primers, B01-F 5′-GCG CAC ACA TAT GGA AAT AAC TTT A-3′ and B02-R 5′-ACT TGT GTT CGG ATA GAA TTT TTC TGT-3′ for iturin gene, and P01-F 5′-AGA CGC CGG GAC ATC AC-3′ and P02-R 5′-GCA TGC TCG CCC TAT TAC AC-3′ for mop cyclase gene. TaqMan probes on the basis of the sequence of the junction region of each marker were designed using Primer Express Software 3.0 (Applied Biosystems, Foster City, CA, USA) and labeled with 6-carboxy-fluorescein (FAM) and 6-carboxytetramethyl-rhodamine (TAMRA) at the 5′ and 3′ ends, respectively, and were synthesized by Applied Biosystems. The sequences of TaqMan probes for iturin and mop cyclase genes were TAA CAA ATG CTC AAA AAC GT and CTA TGC CGG AAT CTC T, respectively.

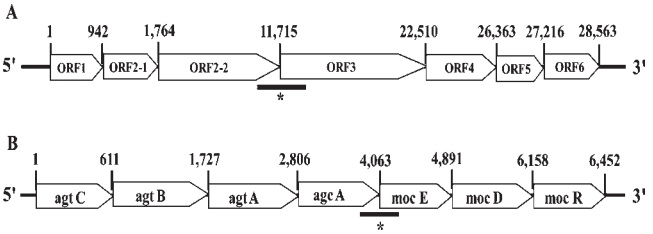

Figure 1. Schematic diagrams of PCR primers designed to detect the inserted DNA of *B. subtilis* KB producing iturin (**A**) and *P. fluorescens* MX1 with mop cyclase gene (**B**); * stars indicate specific probes for quantitative detection of each strain.

Qualitative Analysis by Conventional PCR. PCR amplifications of various selective markers integrated to chromosome of each microorganism were performed by using specific fusion primers to the sequences flanking each marker. PCR was performed on an i-cycler (Bio-Rad, Hercules, CA, USA). About 100 ng of isolated DNA was added to 20 μL of reaction mixture containing 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, 1.25 μM each of primer, 250 μM dNTP, and 1 unit of Taq polymerase (AccuPower PCR PreMix, Bioneer, Daejeon, Korea). Amplification was conducted under the following conditions: denaturation for 3 min at 94 °C, 40 cycles of PCR (denatured for 1 min at 94 °C, annealing for 1 min at 60 °C, extension for 2 min at 72 °C), and a final extension for 5 min at 72 °C. An aliquot (20 μL) of amplification products was separated using 1% agarose gels (SeaKem LE agarose, BMA) in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), and visualized under UV light after staining for 10 min in ethidium bromide solution. The sensitivities of specific fusion primer pairs designed for *B. subtilis* KB and *P. fluorescens* MX1 were determined by using the template mixing DNAs in the range of 200 ng to 0.1 pg.

Construction of the Reference Plasmids and Their Quantification. Two different reference plasmids were constructed on the basis of a T&A vector (RBC Bioscience, Taipei, Taiwan) integrated with each PCR product (500 bp), which was amplified from the specific primers, B03-F 5′-TAG GTA AAC CCT GTT GCT TAC A-3′ and B04-R 5′-ATA TAA TGC CAG ACC CTT GCT T-3′ for iturin gene, and P03-F 5′-AAG ACC GTT TAG ACC GGT GAA-3′ and P04-R 5′-ATC CGA TGC AAG CGA TGA TGA-3′ for mop cyclase gene. To construct the standard plasmid, about 50−100 ng of amplified DNA was added to 20 μL of reaction mixture containing 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, 1.25 μM each of primer, 250 μM dNTP, and 1 unit of Taq polymerase (AccuPower PCR PreMix, Bioneer, Daejeon, Korea). All PCR reactions were conducted under the following conditions: denaturation at 94 °C for 5 min, 30 cycles (denatured for 1 min at 94 °C, annealing for 1 min at 58 °C, extension for 1 min at 72 °C), and a final extension of 7 min at 72 °C. The final PCR products were purified by using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and then cloned into the T&A vector. The ligation reaction containing 2 μL of PCR product, 2 μL of vector, and 4 μL of enzyme solution was performed for 16 h at room temperature. The cloned plasmids were transformed into *Escherichia coli* DH5α and selected positive transformants by isopropyl β-D-1-thio-galactopyranoside (IPTG) induction. Transformed colonies were confirmed through DNA sequencing analysis by Bioneer Co. (Daejeon, Korea). The recombinant plasmids were extracted by the HiYield Plasmid

Mini Kit (RBC Bioscience), and then the plasmid DNAs were purified by using 1% agarose gel electrophoresis and the QIAquick Gel Extraction Kit (Qiagen). The concentration of plasmid DNA was measured using an Agilent model 8453 UV spectrophotometer (Agilent Technologies, Waldbronn, Germany). Each standard plasmid concentration was serially diluted 10-fold for use as a calibrant for quantification

Real-Time PCR for Quantitative Analysis. For quantification of bacteria DNA, a real-time PCR was conducted using the LightCycler 1.5 (Roche Applied Science, Mannheim, Germany). The reaction mixture (20 μL) for TaqMan assays consisted of 10 μL of Universal Master Mix (Applied Biosystems), 50 ng of sample DNA, 10 pM primer pair, and 0.5 μL of TaqMan probe. The PCR amplification was performed as follows: initial denaturation at 95 °C for 10 min; 50 cycles of 20 s at 95 °C, 20 s at 60 °C, 8 s at 72 °C, and a final extension of 5 min at 72 °C.

Five levels of standard plasmid as reference molecules were used for standard curve as calibrants. The standard curves for iturin or mop cyclase genes were confirmed for its linearity by real-time PCR. The sensitivities of the real-time PCR assays specific for *B. subtilis* KB and *P. fluorescens* MX1 were examined by using the specific fusion primers, TaqMan probes, and the standard plasmids in the range of 100 ng to 0.01 pg. The copy numbers of each marker, based on the calibration curve, were calculated by the equation as follows (*20*):

$$\frac{6.023 \times 10^{23}\ (\text{copies/mol}) \times \text{concn}\ (\text{g}/\mu\text{L})}{\text{molecular weight}\ (\text{g/mol})} = \text{amount}\ (\text{copies}/\mu\text{L})$$

Model Microorganisms Detection in Pots of the Greenhouse. The real-time PCR assays specific for *B. subtilis* KB with iturin and *P. fluorescens* MX1 with mop cyclase genes were performed for the practical detection from the soils of plants, using genomic DNAs extracted from the soils of cucumber and tomato plants, respectively, cultivated in pots during the six month period. Each pot has a dimension of 30 and 20 cm in upper and lower diameters, respectively, with 30 cm in height, which was filled with commercial horticulture nursery medium (Biomedia, Gyeongju, Korea). The drainage water was collected in the bottom tray. The model bacteria were cultivated at 30 °C overnight, and the cell density of bacteria was adjusted to reach the absorbance of 1.0 for *B. subtilis* and 1.3 for *P. fluorescens* at 550 nm wavelength. Then 1 L each of model microorganisms was inoculated into the soils in pots with cucumber or tomato plants, which were then grown in the greenhouse at 25 °C for six months.

Southern Hybridization. Soil DNAs extracted from each soil sample were also separated on 1.5% agarose gel (SeaKem LE agarose, BMA) in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and blotted to nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) for Southern hybridization (*21*). PCR amplification products of the iturin and mop cyclase genes were purified using the Qiaquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and labeled with [α-^{32}P]-dCTP using the Prime-a-Gene Labeling System (Promega, Madison, WI, USA). The modified Church buffer (1 mM EDTA, 250 mM $Na_2HPO_4 \cdot 7H_2O$, 1% casein hydrolysate, 7% SDS, 85% H_3PO_4, pH 7.4) was used as a hybridization solution, and hybridization was carried out at 65 °C for 16 h (*22*). The hybridized membrane was exposed to an X-ray film (Kodak X-Omat) at −70 °C for 24−48 h.

RESULTS AND DISCUSSION

Junction Region Analyses. To design the specific fusion primers to the sequences flanking ORF 2-2 and ORF 3 regions of *B. subtilis* KB or agcA and moc E genes of *P. fluorescens* MX1, the sequence analyses of the junction regions were carried out by using the genomic library screening. Full sequences of junction regions for each strain were confirmed repeatedly, and the specific fusion primers and TaqMan probes to the marker genes were designed on the basis of these sequences (**Figure 1**).

Qualitative Detection by Conventional PCR. Genomic DNAs from *B. subtilis* KB and *P. fluorescens* MX1 were subjected to conventional PCR using fusion primers of the flanking regions specific to iturin and mop cyclase genes. The fusion primer pairs for iturin and mop cyclase genes were B01-F/02-R and P01-F/02-R. When the DNAs of *B. subtilis* KB and *P. fluorescens* MX1 were amplified as templates, PCR products of 74 and 57 bp were observed, respectively. However, no amplified products were detected from the other antibiotic-resistant *B. subtilis* 168 and *P. fluorescens* strains. This specificity is caused by the specific fusion primer pairs designed to amplify the junction region of iturin and mop cyclase genes. Both iturin and mop cyclase genes were detected in samples containing 10 pg of genomic DNAs (equivalent to 3.68×10^3 and 1.83×10^4 copy numbers for iturin and mop cyclase, respectively) (**Figure 2**), although detection limits varied depending on the primers used. These results showed that the designed fusion PCR primers can be easily used for the detection of *B. subtilis* KB and *P. fluorescens* MX1.

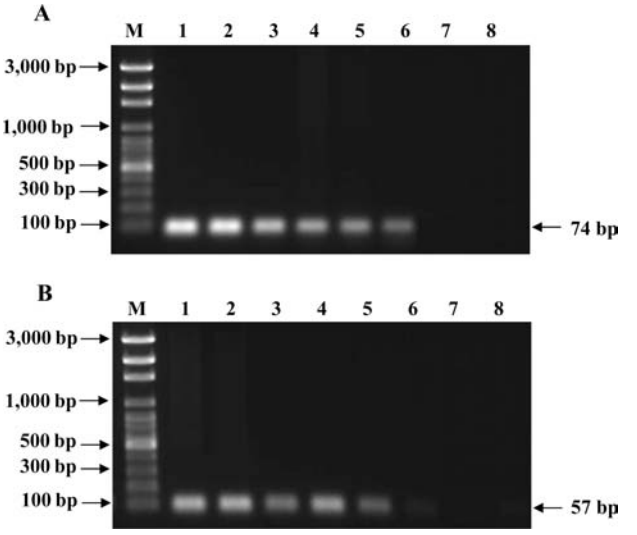

Figure 2. Sensitivity of primer pairs designed for *B. subtilis* KB (**A**) and *P. fluorescens* MX1 (**B**). The specific fusion primers for detection of *B. subtilis* and *P. fluorescens* were used. Template mixing DNAs for each lane as follows: lanes 1−8, 200, 100, 10.0, 1.0, 0.1, 0.01, 0.001, and 0.0001 ng, respectively; M, 100 bp size marker.

Standard Plasmid as a Reference Molecule for Quantitative Analysis. Plasmids pKR-KB and MX1 used as reference molecules were constructed by the integration of each PCR amplicon, which were derived from iturin biosynthetic gene and mop cyclase gene, respectively. The nucleotide sequences of standard plasmids are shown in **Figure 3**.

Five levels of each standard plasmid as reference molecules were arranged to be in a range of 0.026 pg to 0.26 ng for iturin and 1.02 pg to 10.2 ng for mop cyclase per reaction for real-time PCR. These levels were enough for quantitative detection of the genomic DNAs of each model bacteria corresponding to 7.19×10^3 to 7.19×10^7 copies for iturin and to 2.86×10^5 to 2.86×10^9 copies for mop cyclase. The standard curves for iturin or mop cyclase genes were confirmed for linearity by real-time PCR using the specific fusion primers, TaqMan probes, and the standard plasmids, which showed R^2 values of 0.999.

The repeatability of the copy numbers for each standard plasmid was confirmed by the data obtained from the triplicate reactions. The relative standard deviation in triplicate showed values ranging from 0.09 to 0.84% (**Table 1**). Each standard plasmid for iturin or mop cyclase genes was confirmed to be reproducible and reliable reference materials. From these results, it was suggested that this standard plasmid can be used as reference molecules for quantitative detection of model bacteria.

Sensitivities of the Quantitative Real-Time PCR. Serially diluted iturin and mop cyclase DNAs ranging from 100 ng to 0.01 pg were used to determine the limit of detection (LOD) of the real-time PCR assays specific for *B. subtilis* KB and *P. fluorescens* MX1.

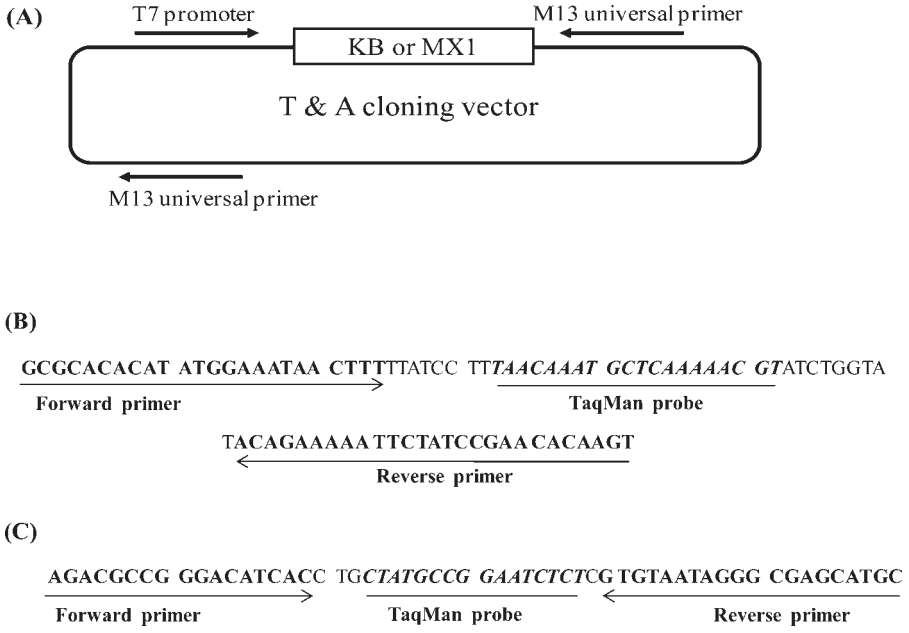

Figure 3. Schematic diagram of T&A cloning vector for the construction of standard plasmid (**A**); forward and reverse primer sequences of *B. subtilis* KB (**B**) and *P. fluorescens* MX1 (**C**), respectively. Capital letters in italics and bold indicate the sequence of TaqMan probes of *B. subtilis* and *P. fluorescens*.

Table 1. Repeatability of the Copy Numbers of Model Bacteria

			Cp[a]		
target organism	concn (ng)	copy no.	mean	SD[b]	RSD[c]
B. subtilis KB	2.6×10^{-1}	2.88×10^{9}	11.91	0.06	0.50
	2.6×10^{-2}	2.64×10^{8}	15.39	0.13	0.84
	2.6×10^{-3}	2.89×10^{7}	18.60	0.04	0.22
	2.6×10^{-4}	2.83×10^{6}	21.98	0.02	0.09
	2.6×10^{-5}	3.03×10^{5}	25.23	0.10	0.40
P. fluorescens MX 1	1.02×10^{1}	7.38×10^{8}	11.92	0.05	0.42
	1.02	7.11×10^{7}	15.40	0.08	0.52
	1.02×10^{-1}	6.98×10^{6}	18.85	0.07	0.37
	1.02×10^{-2}	7.31×10^{5}	22.20	0.10	0.45
	1.02×10^{-3}	7.44×10^{4}	26.01	0.15	0.58

[a] Crossing point. The Cp value (cycle number in a log-linear) region for each curve was calculated using the Lightcycler 1.5 software (version 4.1) program. The experiments were repeated three times each. [b] Standard deviation. [c] Relative standard deviation.

LOD was defined as lowest quantity of target DNA that can be reliably detected and quantified with a probability of ≥95% (*23*). Based on this definition, the LODs of the *B. subtilis* KB and *P. fluorescens* MX1 were determined to be 10 pg (12/12 positive signals; **Table 2**), corresponding to 3.68×10^{3} to 1.83×10^{4} copies per genome of *B. subtilis* and *P. fluorescens*. In addition, the absolute detection limit of each bacteria were determined to be 1.0 pg with 10−11/12 positive signals, depending on the strain. These sensitivities of real-time PCR were consistent with the those of *Brassica napus* published previously (*24*). The coefficient of variation (CV) of the concentrations of each diluted DNA was 0.03−1.45% (**Table 2**), although more variability is shown at lower concentrations. These CV values indicated the reliability, reproducibility, and accuracy of this real-time PCR assay. Estimated reaction efficiencies of twelve runs ranged from 0.996 to 0.998, as based on the slope of the standard curve generated in each reaction (**Table 2**).

Quantitative Analyses of Model Bacteria by Real-Time PCR. To monitor the microorganisms released into the environment, *B. subtilis* KB and *P. fluorescens* MX1, which have iturin biosynthetic and mop cyclase genes in the chromosome, respectively, were used as model bacteria in a small-scale soil-core device. The soil samples containing each model microorganism from separate soil-core devices were obtained at intervals of three weeks during the six month period. The genomic DNAs extracted from soil samples were purified by using QIAquick PCR purification kit (Qiagen) and analyzed by real-time PCR. As shown in **Figure 4A**, the copy numbers of *B. subtilis* KB inoculated at the top layer (core I) of the soil-core device were gradually decreased with the lapse of time, and then detected only 0.31% of the initial population on 24 weeks. However, the copy numbers of *B. subtilis* KB transferred to the medium layer (core II) and bottom layer (core III) were slightly increased from 3 to 9 weeks due to the movement of the strain by sprinkled water, and then exhibited a tendency of gentle decreases as time goes by. The copy numbers of the strain at each soil-core I, II, and III after six month incubation still remained 0.31, 0.16, and 0.02% of the initial population, respectively, though 50 mL of sterilized water was sprinkled from top to bottom every other day. In addition, the copy number of *P. fluorescens* MX1 inoculated at the top layer (core I) of the soil-core device separately was abruptly decreased as incubation time passes for 6 months, while those of *Pseudomonas* species transferred to medium layer (core II) and bottom layer (core III) shortly increased from 3 to 6 weeks due to the movement of sparkled water, and then decreased considerably until 12 weeks. Since then, the copy numbers of *Pseudomonas* species were varied slightly from 12 to 24 weeks (**Figure 4B**). The copy numbers of the strain at each soil-core I, II, and III after six month incubation were maintained as only 0.04, 0.02, and 0.05% of the initial population, respectively. It was found that the viability of *B. subtilis* KB incubated in soil-core device during the six month period is greater that that of *P. fluorescens* MX1. Collectively, these results indicated that model bacteria used could be easily detected by using the specific TaqMan probes and real-time PCR.

Detection of Model Microorganisms in Pots. The specific real-time PCR assays for the detection of model bacteria from the soils in pots as based on the specific fusion primers and TaqMan probes were performed using genomic DNAs extracted from the soils of cucumber and tomato plants cultivated in pots for the six month period. As shown in **Figure 5**, the copy numbers of

Table 2. Amplification Data Used To Determine the LOD[a] of the *B. subtilis*- and *P. fluorescens*-Specific Real-Time PCR Assays

(**A**) *B. subtilis*-Specific Real-Time PCR Assay

DNA amount (ng)	100	10	1	0.1	0.01	0.001	0.0001	0.00001
signal rate (no. of positives)	12/12	12/12	12/12	12/12	12/12	11/12	6/12	1/12
mean concn (ng)	1.26×10^{-1}	1.17×10^{-2}	1.04×10^{-3}	9.64×10^{-5}	1.33×10^{-5}	2.95×10^{-6}	1.56×10^{-6}	1.55×10^{-6}
SD[b] of concn (ng)	3.46×10^{-3}	5.12×10^{-4}	2.81×10^{-5}	2.35×10^{-6}	2.97×10^{-7}	8.32×10^{-8}	6.95×10^{-8}	7.09×10^{-8}
CV[c] of concn (%)	0.06	0.07	0.04	0.03	0.04	0.12	0.30	1.05

(**B**) *P. fluorescens*-Specific Real-Time PCR Assay

DNA amount (ng)	100	10	1	0.1	0.01	0.001	0.0001	0.00001
signal rate (no. of positives)	12/12	12/12	12/12	12/12	12/12	10/12	1/12	0/12
mean concn (ng)	3.58×10^{-1}	3.35×10^{-2}	3.25×10^{-3}	3.13×10^{-4}	6.55×10^{-5}	8.38×10^{-6}	3.22×10^{-7}	1.18×10^{-7}
SD of concn (ng)	1.51×10^{-2}	7.19×10^{-4}	8.31×10^{-5}	1.29×10^{-5}	6.23×10^{-5}	1.01×10^{-5}	1.00×10^{-7}	1.05×10^{-7}
CV of concn (%)	0.06	0.03	0.05	0.06	0.55	0.82	0.61	1.45

[a] Limit of detection. [b] Standard deviation. [c] Coefficient of variation.

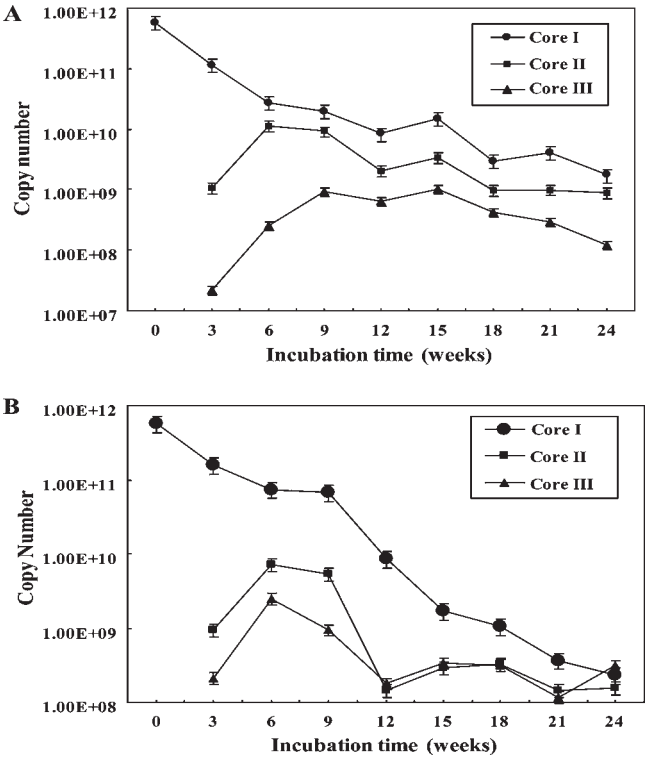

Figure 4. Copy numbers of *B. subtilis* KB (**A**) and *P. fluorescens* MX1 (**B**) amplified by real-time PCR using soil samples obtained from each soil-core at intervals of three weeks during the six month period.

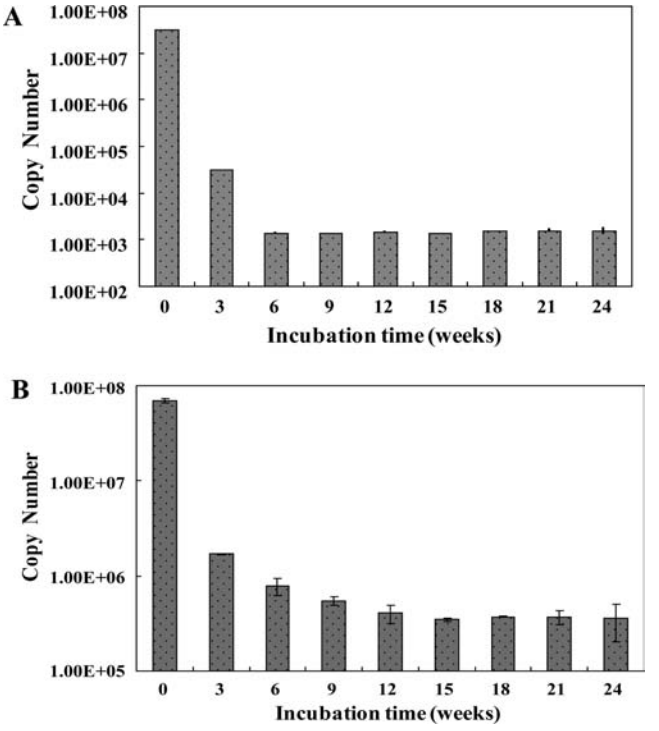

Figure 5. Quantitative changes of model bacteria from the soils of plants cultivated on pots for the six month period by real-time PCR. *B. subtilis* KB inoculated on cucumber plants (**A**); *P. fluorescens* MX1 inoculated on tomato plants (**B**).

B. subtilis KB were abruptly reduced during 3 weeks and decreased up to 6 weeks, but did not show significant changes from 6 to 24 weeks. The copy numbers of *B. subtilis* KB after 3 to 6 weeks remained as only 0.097 and 0.004% of the initial population, respectively. On the other hand, the copy numbers of *P. fluorescens* MX1 were considerably decreased by 15 weeks, reaching down to 0.49−2.46% of the initial population, with the time elapsed, and then exhibited little differences until 24 weeks. The copy numbers of *B. subtilis* KB and *P. fluorescens* MX1 at each pot after a six month period still remained 0.005 and 0.51% of the initial population, respectively. The observed difference of copy number between *B. subtilis* KB and *P. fluorescens* MX1 with the incubation time may be due to the different physiological characteristics of strain and the disparity of initial inoculum size, as the initial population of *P. fluorescens* MX1 is over 10 times greater than that of *B. subtilis* KB. Taken together, these outcomes suggested that the established real-time PCR method could be easily applied to detect specific model microorganisms from the soils of plants cultivated in the greenhouse.

Possibility of Horizontal Gene Transfer in a Small-Scale Soil-Core Device. To investigate the possibility of horizontal gene transfer released into the soil-core device from model bacteria to *B. subtilis* 168 or *Pseudomonas* species containing antibiotic resistance genes, genomic DNAs were extracted from the soils and drain waters collected from each soil-core device incubated during the six month period. The extracted genomic DNAs were digested with *Eco*RV or *Sph*I for *Bacillus* and *Eco*RI or *Sma*I for *Pseudomonas* to examine the possible gene transfer from model bacteria to other bacteria in soils and subjected to Southern blot analysis by using the purified PCR products, which are amplified fusion primers specific for iturin and mop cyclase genes, labeled with [α-^{32}P]-dCTP as probes. Genomic DNAs extracted from *B. subtilis* KB and *P. fluorescens* MX1 as controls showed positive bands by Southern hybridization (**Figure 6**). However, any

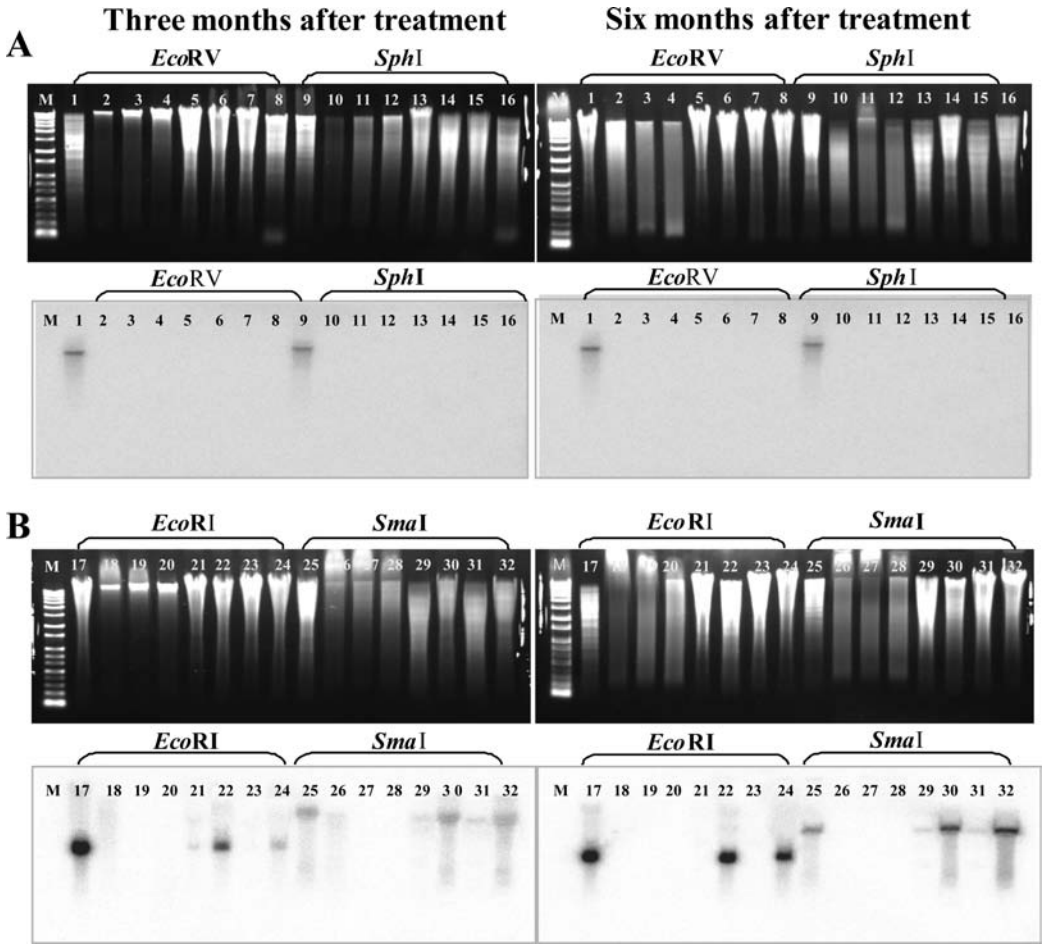

Figure 6. Southern hybridization of genomic DNAs extracted from soil-core and drained water samples for the detection of *B. subtilis* KB (**A**) and *P. fluorescens* MX1 (**B**). 1 and 9, genomic DNA of *B. subtilis* KB; 2 and 10, soil DNAs from core I; 3 and 11, soil DNAs from core II; 4 and 12, soil DNAs from core III; 5 and 13, DNAs from enriched culture of drained water with spectinomycin and erythromycin; 6 and 14, DNAs from enriched culture of drained water with spectinomycin; 7 and 15, DNAs from enriched culture of drained water with erythromycin; 8 and 16, DNAs from enriched culture of drained water without antibiotic; 17 and 25, genomic DNA of *P. fluorescens* MX1; 18 and 26, soil DNAs from core I; 19 and 27, soil DNAs from core II; 20 and 28: soil DNAs from core III; 21 and 29, DNAs from enriched culture of drained water with kanamycin and rifampicin; 22 and 30, DNAs from enriched culture of drained water with kanamycin; 23 and 31, DNAs from enriched culture of drained water with rifampicin; 24 and 32, DNAs from enriched culture of drained water without antibiotic.

positive signals for iturin probe of *B. subtilis* KB were not detected in soil and drain samples collected during the six month period, except for genomic DNAs of *B. subtilis* KB used as a positive control. The poor detection of iturin probe in Southern blot might be attributed to the excess of detection limit due to a very small amount of genomic DNAs, though detailed further study is required in the future. In addition, the genomic DNAs of *Pseudomonas* species from drained waters with or without kanamycin exhibited positive bands at the same position as genomic DNA of *P. fluorescens* MX1 on Southern blot analysis performed with a radiolabeled mop cyclase probe. Considering the facts that the *P. fluorescens* MX1 has also kanamycin resistance gene other than mop cyclase gene in the chromosome and the position of detected positive bands was lacking in variety as compared with model bacteria, it was thought that the positive bands from drained water with or without kanamycin were caused by transfer of the model bacterium. Based on these results, the possibility of horizontal gene transfer from model bacteria to other bacteria of the same genus did not occur during the six month period monitored, although long-term observation through continued research work may be needed.

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