Isolation and Characterization of Oligotrophic Bacteria Possessing Induced Systemic Disease Resistance against Plant Pathogens

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Biocontrol microbes have mainly been screened among large collections of microorganisms *via* nutrient-rich *in vitro* assays to identify novel and effective isolates. However, thus far, isolates from only a few genera, mainly spore-forming bacilli, have been commercially developed. In order to isolate field-effective biocontrol microbes, we screened for more than 200 oligotrophic bacterial strains, isolated from rhizospheres of various soil samples in Korea, which induced systemic resistance against the soft-rot disease caused by *Pectobacterium carotovorum* SCC1; we subsequently conducted *in planta* bioassay screening. Two oligotrophic bacterial strains were selected for induced systemic disease resistance against the Tobacco Mosaic Virus and the gray mold disease caused by *Botrytis cinerea*. The oligotrophic bacterial strains were identified as *Pseudomonas mantelii* B001 and *Bacillus cereus* C003 by biochemical analysis and the phylogenetic analysis of the 16S rRNA sequence. These bacterial strains did not exhibit any antifungal activities against plant pathogenic fungi but evidenced several other beneficial biocontrol traits, including phosphate solubilization and gelatin utilization. Collectively, our results indicate that the isolated oligotrophic bacterial strains possessing induced systemic disease resistance could provide useful tools as effective biopesticides and might be successfully used as cost-effective and preventive biocontrol agents in the field.

Keywords : biocontrol, biopesticide, low-nutrient media, preventive disease control

Recent demand for organic and sustainable agriculture products has been a strong driving force toward the develop-

ment and use of biopesticides in the field. Additionally, biopesticides for the control of plant diseases represented a rapidly growing industry, because of their efficacy and environmentally-friendly qualities. Certain plant-associated bacteria can promote plant growth and suppress plant diseases by the expression of various mechanisms, including the production of antimicrobial secondary metabolites and the induction of systemic resistance against plant disease (Kim et al., 2011). The efficacy of biocontrol bacteria requires successful colonization on plant surfaces, and thus microbial biopesticides have generally been on isolated primarily from microbial collections derived from cultivated soils and plants.

Soils and plants are recognized as extremely rich reservoirs of microbial diversity, and are also profoundly rich natural resources for the isolation of many effective microbial pesticides (Sloan et al., 2008). The approach traditionally employed in the development of microbial inoculants has been to first isolate large collections of microorganisms, followed by systematic screening for activity using simple and cheap and then more complicated and expensive bioassays to identify those with the greatest activity (Fravel, 2005). Only certain species of *Agrobacterium*, *Bacillus*, *Burkholderia*, *Erwinia*, *Pantoea*, *Pseudomonas* and *Streptomyces* have been commercialized as microbial biopesticides for the control of plant diseases (Cropping, 2004).

Even though bioformulated products for gram-positive biocontrol bacteria, such as endospore-forming *Bacillus* spp. strains, have been developed and commercialized, only a few biopesticides have been developed from gram-negative bacteria, mainly because of short shelf-life (Cropping, 2004). An effective biocontrol bioformulation containing a complex of chitinase-producing biocontrol bacterial strains and chitin under large-batch conditions was previously developed and effectively used to control Phytophthora blight of pepper (Kim et al., 2008) as well as Alternaria

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blight and the anthracnose disease of Korean ginseng under field conditions (Kim et al., 2008; 2010).

Since the efficacy of biocontrol bacteria requires successful colonization on plant surfaces, we isolated oligotrophic potential biocontrol microbes possessing induced systemic resistance to plant pathogens by the traditional “isolate and screen” approach in order to develop micropesticides effective from both a cost and survival standpoint under natural low-nutrient conditions. Soil samples were collected from various sources, including red pepper cultivated soils in Jeollanamdo province, beach soils in Buan, and beach soils in Taean, Jeollanamdo province in year 2007. Approximately 1 g of soil samples were placed in 10 ml of sterile water, briefly vortexed, and serially diluted with sterile water, then plated directly onto 1/100 Luria Bertani agar (Difco Inc., Detroit, MI). After 5 days of incubation on 1/100 LB agar plates, individual colonies were picked and stored for further analysis. A total of 252 oligotrophic bacterial isolates were selected and employed for further *in vivo* bioassays for induced systemic resistance.

Initial screening of each bacterial isolate for ISR was conducted against the soft-rot disease caused by *Pectobacterium carotovorum* in tobacco plants (*Nicotiana tabacum* cv. Xanthi-nc) grown in MS microtiter plates as previously described (Han et al., 2006). In brief, the bacterial strains grown for 48 hr in 1/100 LB broth, were harvested by centrifugation, then re-suspended as equal volume of sterile water. The bacterial suspensions (10 μ l) were inoculated on the roots of tobacco grown for 3 weeks in a growth chamber. One week after root treatment, 2 μ l of the *P. carotovorum* suspension was inoculated onto systemic individual tobacco leaves. Two days after pathogen inoculation, the severity of soft-rot disease was measured as previously described (Han et al., 2006; Spencer et al., 2003). Through initial screening, two bacterial isolates, designated B001 and C001, evidenced strong ISR activity against soft-rot disease in the tobacco bioassay system.

To assess the ISR spectrum of the selected isolates, we employed the gray mold disease caused by *Botrytis cinerea* and Tobacco Mosaic Virus (TMV) in tobacco plants. To evaluate ISR activity against gray mold disease, *B. cinerea* KACC 40573 obtained from the Korean Agricultural Culture Collection (RDA, Suwon, Korea) was used. Approximately 1×10^5 conidia/ml of conidial suspension harvested from *B. cinerea* grown on potato dextrose agar plates in 30% tomato juice and 0.1 M KH_2PO_4 (pH 7.0) was inoculated onto systemic tobacco leaves, which were grown for 3 weeks on MS microplates and inoculated with the bacterial suspension into the root area as described above. Five days after pathogen inoculation, the percentage of infected area of each leaf was measured using Image Insicide software Ver. 2.32 (IHwa Inc., Seoul, Korea).

For TMV bioassays, tobacco (cv. Xanthi) seeds were surface-sterilized by 2 min of soaking in 70% ethanol and 20 min of soaking in 1% sodium hypochlorite. After extensive washing with sterile distilled water, the seeds were incubated in sterile water for 3 days in the dark at 4°C to promote germination. The sterilized seeds were subsequently planted in sterile soil-less medium (BioHorticulture soil: vermiculite, 7:3, vol/vol) using 500 cm^3 of medium for each 18-by-18-by-22 cm pot, at 1 seed/pot. These pots were watered with 20 ml of sterile water every 2 days. Seedlings were grown with a cycle of 16 h of light and 8 h of darkness under 40-W fluorescent lights (2,000 lux, 80 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). The temperature was maintained at $25 \pm 1^\circ\text{C}$ with a relative humidity of 50 to 60%. The bacterial suspensions were applied by root drenching seven weeks after planting. TMV inoculum (TMV-U1 strain), which was obtained from the Plant Virus Bank at Seoul Women's University, consisted of systemically infected Samsun leaf tissue ground in 50 mM potassium phosphate buffer, at a pH 7.0 and a ratio of 1 g of tissue per 10 ml of phosphate buffer. The tobacco plants were mechanically rub-inoculated with TMV and Carorundum as previously described (Park et al., 2012). Five days after pathogen inoculation, the number of spot was counted. In plant bioassay, three experiments were performed; in each experiment, 15 tobacco plants per treatment were used.

Initial study indicated no or much lower ISR-eliciting activities of both strains 1 to 2 days after pre-treatment with bacterial suspensions prior to inoculation with the soft-rot pathogen (data not shown). Similar ISR-eliciting activities upon the duration of ISR-inducing bacterial strains were noted in other bacterial strains. In *P. chlororaphis* O6, significant ISR-eliciting-activity against soft-rot disease was noted 3 days after root drenching of the strain and reached maximal levels after 7 days of application to the root with strain O6 (Cho et al., 2011). ISR-eliciting activities of B001 and C003 against gray mold and TMV were also observed. The greatest reduction in the disease severity of gray mold was achieved when the B001 or C003 bacterial suspension was applied to the rhizosphere of tobacco seedlings 7 days prior to fungal inoculation (Table 1 and Fig. 1). Total symptomatic spots were greatly reduced in the tobacco plants treated by root inoculation with B001 or C003 bacterial suspension compared to the water-treated control plants 7 days prior to the pathogen inoculation (Table 1 and Fig. 1). These results indicated that the selected oligotrophic bacterial strains evidenced a broad spectrum of ISR activities against viral, bacterial and fungal disease, and could be employed in the development of protective biocontrol agents.

Additionally, other phenotypic properties of the selected bacterial strains including antifungal, chitin degradation,

Table 1. Induced systemic resistance upon root colonization of the selected bacteria against various plant pathogens

Treatment ^a	Soft-rot disease	Gray mold	<i>Tobacco Mosaic Virus</i>
	Disease index ^a	Infected area (%) ^c	Number of spots/ leaf ^d
B001	1.65 ± 0.37 a ^e	8.6 ± 0.38 a ^e	84.0 ± 8.15 a ^e
C003	3.01 ± 0.41 b	8.6 ± 1.01 a	83.8 ± 16.34 a
Control	4.06 ± 0.30 b	31.9 ± 3.54 b	126.8 ± 7.06 b

^aBacterial strains B001 and C003 were applied to the tobacco root system to evaluate induced systemic resistance activity against *Pectobacterium carotovorum* subsp. *carotovorum* SCC1, *Botrytis cinerea*, and *Tobacco Mosaic Virus*. Control plants were treated with equal volumes of sterile water.

^bDisease index (0–5) was measured 2 days after *P. carotovorum* SCC1 inoculation. Twelve tobacco plants were evaluated with two trials.

^cInfected area (%) was measured 5 days after *B. cinerea* inoculation. The infected area was calculated using ImageInside Ver. 2.32 software program. Three selected tobacco plants, including 3 leaves from 12 plants, were evaluated in 4 trials.

^dNumber of systemic spots were counted 5 days after TMV inoculation. Three tobacco leaves from one plant were evaluated over 4 trials.

^eMeans and standard errors followed by different letters differed significantly according to Duncan's multiple test at $P < 0.05$ using SPSS14.0 software.

gelatin degradation, and solubilizing phosphate activity were assessed on the detection agar plates, because the microbial properties are known to involve in biocontrol of plant disease and insect (Han et al., 2008; Kim et al., 2008; 2011; Martin and Gundersen-Rindal, 2008). The *In vitro* antifungal activities of the selected strains were tested by dual inoculation on potato dextrose agar (Difco Inc., Detroit, MI) with plant pathogenic fungi *Fusarium oxysporum* (KACC NO. 41083) and *Magnaporthe grisea* (KACC NO.

40439), and the inhibition zone of the fungal pathogens was measured. The bacterial isolates were inoculated on hydroxyapatite medium ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g, NaCl 0.5 g, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 0.1 g, NH_4NO_3 0.75 g, glucose 10 g, Hydroxyapatite 4 g, yeast extract 0.5 g, agar 20 g per liter, adjust pH 7.0) to evaluate phosphate solubilizing activity, and the production of extracellular chitinase was determined on chitin medium (Colloidal chitin 2%, Na_2HPO_4 2 g, KH_2PO_4 1 g, NaCl 0.5 g, NH_4Cl 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 0.5 g, yeast extract 0.1 g, agar 20 g per liter, adjusted pH 7) and gelatin utilization on gelatin containing medium (1% gelatin, 0.5 g KH_2PO_4 , 0.5 g K_2HPO_4 , 1 g NaCl, 1.5 g NH_4NO_3 , 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g KCl, 0.2% yeast extract, 1.5% agar per liter, pH 7.0).

Both strains B001 and C003 were able to utilized gelatin as nutrient source and strongly solubilized insoluble phosphate on the detection plates, but did not produce extracellular chitinase on the chitin medium and did not exhibit anti-fungal activities against *F. oxysporum* and *M. grisea*. Additionally, both strains B001 and C003 proved capable of growth in both full-length and 1/100 diluted-LB broth. In the soil and rhizosphere, the limitation of microbial growth has been proposed to result in the release of compounds from roots, these released compounds are collectively referred to as rhizodeposits (Dennis et al., 2010). In the rhizodeposits, sugars and amino acids are believed to be released in the greatest quantities. Suwa and Hattori (1984) showed that two different oligotrophs were recovered from soils; one was able to grow in both full-length, whereas the other was able to grow only in the diluted nutrient broth. Many soil bacteria appeared to belong to the latter group. The bulk soil and rhizosphere are recognized as low-nutrient available environments, and nutrient levels in the

Fig. 1. Systemic disease resistance induced by root colonization of the oligotrophic bacterial strains against various plant diseases in tobacco plants. Images of typical symptomatic lesions evidencing induced systemic resistance activity of the individual bacterial strains were taken two days after treatments of the pathogens in tobacco plants. The tobacco plants were pre-treated with the selected oligotrophic bacterial strains B001 and C003, or sterile water as a negative control, 7 days prior to the pathogen treatments. The images are representative of three independent experiments.

rhizosphere may prove sufficient for oligotrophs but are prohibited low for many other microorganisms (Darrah, 1991). Oligotrophs are adapted for growth under low-nutrient available conditions. Additionally, oligotrophs may be important components of the bacterial communities observed in the rhizosphere and soil environments. The results of a recent study indicated that the ratio of copiotrophic and oligotrophic bacteria depends on growth and survival activity in the rhizosphere and bulk soils, both of which are known to be low-carbon flux habitats (Maloney et al., 1999). Both copiotrophic and oligotrophic bacteria existed at higher populations at the upper portions of the root than at the tip locations or in the bulk soils. The ratio of copiotrophs to oligotrophs was high at the root tip and low at the root base in the lettuce rhizosphere, but an opposite pattern was noted in the tomato rhizosphere.

Certain rhizobacteria are beneficial to plants because they promote plant growth and induce resistance against pathogenic attack or abiotic stresses. ISR involves an enhanced and accelerated ability to mount a defense response upon challenge (Van Loon et al., 1998). However, for induced resistance to be successful, colonization of the root surface by the inducing bacterium must be effective with regard to timing, cell density and the location of coverage (Raaijmakers et al., 1995; Han et al., 2006). In this study, the isolated B001 and C003 strains were found to be excellent candidates for biocontrol agents, as they remain competitive in soils in which nutrients are not readily available. However, in the future, the root colonization and competitiveness characteristics of the selected isolates must be carefully evaluated to determine the microbial community dynamics of the selected oligotrophs in relation to the induction of systemic resistance activities under various rhizosphere and soil conditions.

In order to identify two selected bacterial strains, at first we used biochemical profiles using the API 20 NE kit and API 50CHB test kit (BioMérieux, Marcy l'Etoile, France), following the manufacturer's instructions. API test kit results were interpreted using the Analytical Profile Index (API) database of the Apiweb software (version 4.0, BioMérieux, Marcy l'Etoile, France). The API test kit result of B001 showed 99.6% certainty with *Pseudomonas putida*. For B001 strain, the test using the API 20 NE strip shows that the strain was oxidase-positive and hydrolysis of arginine and esculin. The B001 strain assimilated glucose, arabinose, gluconate, caprate, malate, citrate, phenyl-acetate, but did not assimilate mannose, mannitol, N-acetyl-glucosamine, maltose, and adipate. The API 50 CHB test result of C003 showed 99.6% certainty with *Bacillus cereus*. The API result indicated that C003 utilized glycerol, ribose, D-glucose, D-fructose, D-mannose, inositol, N-acetyl glucosamine, arbutin, esculin, saucing, cellobiose, maltose, treha-

lose, starch, glycogen, and gluconate.

The isolated were also identified by phylogenetic analysis of 16S rRNA and morphological analysis. The 16S rRNA sequence was amplified using PCR with the universal primers, forward 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 5'-ACG GCT ACC TTG TTA CGA CTT-3'. The PCR reaction was performed with premix (Bioneer Inc., Daejeon, Korea) in a gradient thermo block (Bioneer Inc., Daejeon, Korea) with the following profile: 1 cycle of 5 min pre-denaturation at 94°C, 30 cycles consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 60°C, and 100 sec of extension at 72°C, and a final extension step at 72°C for 7 min. The 1.4 kb PCR product was purified from agarose gel using a PCR product purification kit (Bioneer Inc., Daejeon, Korea) and cloned into pGEM T-easy vector (Promega, Madison, WI) in accordance with the manufacturer's instructions. Recombinant plasmids harboring PCR inserts were isolated using a mini plasmid purification system purchased from Bioneer Inc. (Bioneer Inc., Daejeon, Korea). Nucleotide sequences analyses were conducted using an ABI1301 DNA sequencer (Applied Biosystems, Foster City, CA) at the Korea Basic Science Institute (KBSI), Gwangju Branch.

The multiple alignment file was edited using BioEdit version 5.0. The sequences were initially aligned using CLUSTAL_X software. The nucleotide sequence alignment of each gene was subjected to GBLOCKS analysis using parameters optimized for rRNA alignments (Castresana, 2000). The phylogenetic trees were constructed with the MEGA 3.0 software package using the Neighbor-joining method with the Kimura two-parameter model (Kumar et al., 2004; Kimura, 1980) using bootstrap values based on 1,000 replications (Fig. 2A and 2B).

For TEM observations, bacterial cells were cultured on Tryptic Soy Agar plates for 18 h at 28°C, and then suspended in sterile distilled water. Bacterial cells harvested from the plates were negatively stained with 0.1% uranyl acetate. The samples were examined under a Leo g12AB transmission electron microscope (Carl Zeiss, Germany) at 100 Kv.

Strain B001 was a rod-shaped gram-negative bacterium and lophotrichous flagella were detected under electron microscopic observation. Comparative 16S rRNA gene sequence analyses showed that strain B001 was most closely related to the *P. monteli* CIP 104883 type strain, with a sequence similarity of 99.6%, and was 99.0% similar to *P. putida* type strain IFO14164. Sequence similarities to other members, such as *P. chlororaphis* and *P. fluorescens*, used in the phylogenetic analysis were lower than 96% (Fig. 2A).

Strain C003 was identified as *Bacillus cereus* based on morphological and 16S rRNA analysis (Fig. 2B). C003 was

Fig. 2. Phylogenetic positions based on a neighbor-joining analysis of 16S rRNA gene sequences of the oligotrophic bacterial strain B001 (A) and C001 (B) possessing induced systemic disease resistance. The accession numbers of the 16S rRNA nucleotide sequences used are indicated in parentheses. The trees were constructed with the neighbor-joining method. Bootstrap values, which expressed as percentages of 1,000 replications, greater than 50% are indicated at the branches. Bar, 0.01 substitutions per nucleotide position. Transmission electron micrographs of each strain were observed from 18 h of culture.

a rod-shaped gram-positive bacterium and peritrichous flagella were detected under electron microscopic observation. Comparative 16S rRNA gene sequence analyses demonstrated that strain C003 was most closely related to

B. cereus type strain CCM2010, with a sequence similarity of 99.9%, and to *B. thuringiensis* type strain ATCC10792 with a sequence similarity of 99.8%. Sequence similarities to other members used in the phylogenetic analysis, such as

B. amyloliquefaciens, *B. licheniformis*, and *B. subtilis*, evidenced sequence similarities below 95%.

Since the 1980s, a number of papers have reported the beneficial effect of certain bacterial strains on the promotion of plant growth and the induction of systemic disease resistance (Kloepper et al., 1980; van Loon et al., 1998). Most of the ISR-triggering rhizobacterial strains were selected on nutrient-rich medium and screened for ISR-bioassay. However, we focused on the isolation of the oligotrophic bacterial strains evidencing ISR-triggering activities. It is not surprising that *B. cereus* C003 and *P. monteilii* B001 were selected in this study, because of the abundance of both of these genera as major groups in the microbial community occupying plants and soils, and also because numerous rhizobacteria, mainly those belonging to *Pseudomonas* and *Bacillus* species, are capable of eliciting ISR (de Vleeschauwer and Hofte, 2009; Kloepper et al., 2004). Whereas *B. cereus* strains are known to induce systemic resistance in plants (Kloepper et al., 2004; Liu et al., 2008), this report is the first, to the best of our knowledge, to demonstrate that *P. monteilii* B001 evidences a strong induced broad-spectrum systemic resistance. Recently, draft genome sequence of the *P. monteilii* B001 has been completed and published, owing primarily to vast potential as a protective biocontrol agent (Park et al., 2011). To the best of our knowledge, this is the first report in which the oligotrophic bacterial strains *P. moneilii* B001 and *B. cereus* C003, were isolated and identified, and shown to evidence a broad spectrum of induced systemic resistance.

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