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 manteilii* 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 development 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.
bacterial isolates were selected and employed for further
stored for further analysis. A total of 252 oligotrophic
agar (Difco Inc., Detroit, MI). After 5 days of incubation on
sterile water, then plated directly onto 1/100 Luria Bertani
ed from various sources, including red pepper cultivated
soils in Jeollanamdo province, beach soils in Buan, and
natural low-nutrient conditions. Soil samples were collect-
effective from both a cost and survival standpoint under
screen” approach in order to develop micropesti-cides
ful colonization on plant surfaces, we isolated oligotrophic
field conditions (Kim et al., 2008; 2010).

SOIL SAMPLES COLLECTION AND CULTIVATION
Approximately 1 g of soil samples were placed in 10 ml of
sterile water, briefly vortexed, and serially diluted with
beach soils in Taean, Jeollanamdo province in year 2007.

One week after root treatment, 2 µl of the
leaves. Two days after pathogen inoculation, the severity of
activities against viral, bacterial and fungal disease, and
trophic bacterial strains evidenced a broad spectrum of ISR
pathogen (data not shown). Similar ISR-eliciting activities
inoculum (TMV-U1 strain), which was obtained from the
consisted of systemically infected Samsun leaf tissue
grown in 50 mM potassium phosphate buffer, at a of pH
water. The bacterial suspensions (10 µl) were inoculated on
roots of tobacco grown for 3 weeks in a growth chamber.
One week after root treatment, 2 µl of the P. carotovorum
suspending activity systemic individual tobacco leaves. Two days after pathogen inoculation, the severity of
soft-rot disease was measured as previously described (Han
 Freddie, 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 µl) were inoculated on
the roots of tobacco grown for 3 weeks in a growth chamber.

To assess the ISR spectrum of the selected isolates, we
employed the gray mold 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 18-22 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 µmol photons
m⁻²s⁻¹). The temperature was maintained at 25 ± 1 °C with a
relative humidity of 50 to 60%. The bacterial suspensions
were applied by root drenching seven weeks after planting.

Initial screening of each bacterial isolate for ISR was
conducted against the soft-rot disease caused by Pecto-
bacterium carotovorum in tobacco plants (Nicotiana tabacum
cv. Xanthi-nc) grown in MS microtiter plates as previously
reported (Kim et al., 2008; 2010). Five days after pathogen inoculation, the percentage
of infected area of each leaf was measured using Image

Additional screening of the selected isolates was
conducted against the soft-rot disease caused by Pecto-
bacterium carotovorum in tobacco plants (Nicotiana tabacum
cv. Xanthi-nc) grown in MS microtiter plates as previously
reported (Han et al., 2006). In brief, the bacterial strains
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One week after root treatment, 2 µl of the P. carotovorum
suspending activity systemic individual tobacco leaves. Two days after pathogen inoculation, the severity of
soft-rot disease was measured as previously described (Han
Freddie, 2006). In brief, the bacterial strains
grown for 48 hr in 1/100 LB broth, were harvested by
irrigating. The temperature was maintained at 25 ± 1 °C with a
relative humidity of 50 to 60%. The bacterial suspensions
were applied by root drenching seven weeks after planting.

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 exten-
sive 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 cm³ 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 µmol photons
m⁻²s⁻¹). The temperature was maintained at 25 ± 1 °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
grown in 50 mM potassium phosphate buffer, at a of 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

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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 oligo-
thropic 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soft-rot disease</th>
<th>Gray mold</th>
<th>Tobacco Mosaic Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Disease index*</td>
<td>Infected area (%)</td>
<td>Number of spots/leaf*</td>
</tr>
<tr>
<td>B001</td>
<td>1.65 ± 0.37 a</td>
<td>8.6 ± 0.38 a</td>
<td>84.0 ± 8.15 a</td>
</tr>
<tr>
<td>C003</td>
<td>3.01 ± 0.41 b</td>
<td>8.6 ± 1.01 a</td>
<td>83.8 ± 16.34 a</td>
</tr>
<tr>
<td>Control</td>
<td>4.06 ± 0.30 b</td>
<td>31.9 ± 3.45 b</td>
<td>126.8 ± 7.10 b</td>
</tr>
</tbody>
</table>

*Bacterial 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.

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

*Infected 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.

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

*Means and standard errors followed by different letters differed significantly according to Duncan’s multiple test at P<0.05 using SPSS14.0 software.

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.
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 ABI3101 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 parameters optimized for rRNA alignments (Castresana, 2000). 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 suspend- ed 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. montelli 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
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 Vleesschauwer 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 for its use in plant health promotion by plant-associated bacteria. 

Draft genome sequence of the biocontrol bacterium P. monteilii B001 was 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. This work was supported by a grant from Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (#311019-3), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

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