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# Improvement for Isolation of Soil Bacteria by Using Common Culture Media

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**Traditional microbiological cultivation strategies are often not suitable for majority of microbial species, and new approaches to the cultivation of bacteria are in demand. This study introduces a novel cultivation method for soil bacteria based on using a polycarbonate permeable membrane of transwell plates in liquid cultures with common synthetic media. The method mimics natural conditions in that the membrane acts as a growth support while natural nutrients from soil environment can pass through 0.4-$\mu$m membrane pores to reach microorganisms in culture. Analysis of 16S rRNA genes revealed that 68 strains were isolated from forest soil using both the new and traditional methods: 67 strains through the new and 21 strains through the old method including 20 strains overlapped in both methods. Among the 68 strains 14 were potentially new species isolated by the new method with 5 of them also isolated by the traditional method. The novel modified cultivation method proved a valuable tool in isolation and cultivation of soil bacteria including new species that are difficult to culture by conventional methods.**

**Key words:** not-yet isolated bacteria; novel modified cultivation method; permeable membrane; transwell plate; soil; synthetic common media.

To date, microbial cultivation has provided unprecedented advances in understanding of microbial diversity. Still the progress is insufficient considering that only less than 1% of the total microbial species can be recovered by traditional cultivation methods[1]. Development of novel approaches to isolation and cultivation of yet unculturalable bacteria is therefore an important target and considerable challenge for microbiological research The main aim of modern methodology is finding the best way to artificially reproduce growth conditions similar to those in natural microbial habitats including complex interactions between bacterial species. Species interdependence, which exists in complex bacterial communities, can be an obstacle for bacterial growth in pure cultures, which is why species should not be separated as we cannot observe at the initial step of cultivation.

Since Grobstein introduced the transfilter culture of metanephric mesenchymal cells in 1953[2], membrane filters have proven to be an invaluable tool in experimental cell biology. They have been widely used as cell growth substrates to study cellular transport, absorption and secretion; for example porous transwell membranes were used for cultivation of human bronchial epithelial cells at the air/liquid interface[3] and for adhesion, invasion, and migration of SK-Hep1 human hepatoma cells[4]. Successful application of transwell membranes in mammalian cell culture prompted

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their utilization as substrates for growth of bacteria from diverse habitats with the notion that transwell membranes would promote isolation of new microbes recalcitrant to traditional cultivation.

A previous study showed that utilization of soil substrate membrane system (SSMS) allowed for selection of previously uncultured soil bacteria[5]. As a result, 8 dominant microcolonies were isolated after 7-day incubation using SSMS; only 1 of them could grow in subculture, confirming that SSMS-microcultivated organisms preferred a slow growth K-strategy[6]. This observation was further substantiated in another study showing benefits of a polycarbonate membrane for recovery of microcolony-forming bacteria resistant to traditional growth conditions[7].

Based on these data, the current study presents a novel cultivation method for previously uncultured soil bacteria that is not only very simple but also productive for subsequent sub-cultivation. This method is based on utilization of a transwell permeable membrane as a growth support, soil as microbial source, and soil extract as the substrate, enabling isolation of previously uncharacterized bacterial species.

## MATERIALS AND METHODS

### Soil sampling and transwell plates

Soil was collected from the surface around plant roots in the forest at Kyonggi University, Suwon, Gyeonggi-do, South Korea. Samples were dried at room temperature, passed through a 2-mm-mesh sieve to remove soil aggregates, gravels, and debris of plant materials, analysed for physic-chemical properties (Table 1), and used as a source of soil nutrients in cultivation medium. A subsample of soil was used as an inoculum, which was added to media in a transwell insert– this strategy ensured recovery of all bacteria present in the environment, as some microorganisms could be lost to sample preparation methods such as sieving. Note that we added medium to each transwell insert when it sank down, which ensured sufficient supply of nutrients for microbial growth, preventing membrane drying and subsequent bacterial death.

Transwell plates (Corning, Lowell, NY, USA) originally designed for cell culture, in this study were used for cultivating soil bacteria, especially strains resistant to traditional cultivation. Each 6-well plate was supplied with 6 24 mm-diameter transwell inserts that at the bottom had a 0.4-µm porous membrane permeable only for water-soluble soil nutrients, but not for soil particles or bacteria (Fig. 1A). Figures 1B and 1C show the schematic diagram and actual setting of transwell experiments, respectively.

### Cultivation and isolation of soil bacteria

In the novel cultivation method, 3 g of soil were added to each well of a 6-well plate and covered with an insert; then 3 mL of each medium supplemented with 100 µL of soil suspension (1 g-soil in 10 mL DW) was added to each insert. Plates were incubated in a shaking incubator (Hanbaek Science Co., Bucheon, Korea) at 120 rpm and 28°C for 2 weeks. During the incubation, the level of medium in each well was monitored every 2 days and restored to original volume as needed. After 2 weeks we collected the culture media and performed a 10-fold serial dilutions up to $10^{-6}$ using 15 mL test tubes; 100 µL of each dilution was spread on an agar plate, and incubated at 28°C in an incubator (Vision Scientific, Daejeon, Korea) until colonies appeared. The number of colonies was counted and all different types of colonies were picked up and streaked on a plate for isolation of pure culture. As a control conventional cultivation method was used in which 3 grams of soil were added to 30 mL of each medium in 50 mL Erlenmeyer flask (the most popular liquid culture method in laboratory); incubation and isolation conditions and processes were the same as for the new method.

For cultivation we used 5 traditional media such as nutrient broth (NB), Luria Bertani (LB), tryptic soy broth (TSB), mineral salts medium (MSM), and R2A; soil extract (SE) and its mixtures MSMSE (500 mL MSM plus 500 mL SE) and R2ASE (500 mL R2A plus 500 mL SE); and distilled water (DW). SE was completed through the following steps: mixing soil (500 g) and DW (1 L) by stirring overnight, filtration, and centrifugation. Chemical composition of each medium is shown in Table 2.

Among isolates, the potential new species were stored as glycerol stocks and freeze-dry amples, resulting in regrowth afterward.

### DNA extraction and 16S rRNA gene amplification

Bacterial genomic DNA was extracted from bacterial cells grown on agar plates using an InstaGene™ Matrix (BIO-RAD). Primers 518F – 52 - CCA GCA GCC GCG GTA ATA CG-32 and 800R –

52 -TAC CAG GGT ATC TAA TCC-32 were used for the PCR amplification (EF-Taq, SolGent, Daejeon, Korea). Reactions were performed using 20 ng of genomic DNA as a template in 30-µl reaction mixture in following conditions: activation of Taq polymerase at 95°C for 2 min; 35 cycles of 95°C, 55°C, and 72°C for 1 min each; and a final elongation step at 72°C for 10 min.

Amplified products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA) and sequenced using the PRISM BigDye Terminator v3.1 Cycle sequencing Kit. DNA samples were added to Hi-Di formamide (Applied Biosystems, Foster City, CA), and mixtures were incubated at 95°C for 5 min, followed by 5 min on ice and analysed using the ABI Prism 3730 XL DNA analyzer (Applied Biosystems, Foster City, CA).

**Phylogenetic tree construction**

The EzTaxon server was used to identify phylogenetic neighbours and to calculate the pairwise 16S rRNA gene sequence similarities[8]. The related 16S rRNA sequences were obtained from GenBank database and edited with the BioEdit program[9]. Multiple alignments were performed with the CLUSTAL_X program[10]. A phylogenetic tree was constructed by applying evolutionary distance, parsimony, and bootstrapped parsimony using the neighbour-joining algorithm[11], maximum likelihood, and maximum parsimony method[12] in the MEGA5.03 program[13] with bootstrap value on 1000 replications[14].

## RESULTS AND DISCUSSION

**Isolation properties of the novel cultivation method**

In this study, 68 soil-bacterial strains among total 151 bacterial isolates including many same strains were obtained from the same soil through the new and traditional cultivation methods using several conventional media and distilled water. Among the 68 strains (100%), 20 (29.4%) overlapped in both methods, i.e., only 1 strain (NHI-5) (1.5%) was obtained through the traditional while 47 strains (69.1%) through the new method (Table 3 and Fig. 2A). Thus, the new cultivation method appeared to be much more effective in isolation of soil bacteria. However, strain NHI-5 probably grew well in only synthetic media without the addition of soil nutrients as the new method.

Furthermore, only 5 potentially new species were isolated by the conventional method versus 14 new species obtained by the new method (Table 3). The 5 conventionally isolated species were also selected by the new method, indicating that the additional 9 isolates could be obtained only by using the new method. The potentially new species were identified based on the 16S rRNA gene sequence similarity of less than 98.5% with the closest phylogenetic neighbour. Thus, the new method appeared to be over 3 times more efficient for isolation of new microbial species than the traditional cultivation technique.

Among the 39 strains (including overlapped) obtained through the traditional method, the majority (12 strains) were isolated from the R2A cultures, 8 from LB, 7 from NB, 6 from TSB, 4 from MSM, and 1 from each of MSMSE and SE cultures (Fig. 2B). Among the 10 potentially new species (including overlapped) 3 were isolated from R2A, 2 from each of NB and TSB, and 1 from each of LB, MSMSE, and SE media. Among total of 112 strains (including overlapped) detected by the new method, the largest number of isolates, 31, was similarly obtained from the R2A cultures, while 17 were isolated from NB, 15 from DW, 13 from TSB, 12 from LB, 8 from MSM, 7 from MSMSE, 5 from SE, and 4 from R2ASE cultures (Fig. 2B). Among the 24 potentially new species (including overlapped) 7 were isolated from R2A, 4 from each of NB, TSB and DW, 2 from LB, and 1 from each of MSMSE, R2ASE, and SE media. These results indicate that among all the media tested R2A was the best for cultivation of diverse strains including potentially new species, either by the traditional or by the new method. The reason that R2A provides more diverse colonies seems to be the low concentration of carbon and energy sources (Table 2) as mentioned in the previous study regarding cultivation of uncultivable bacteria[1].

Otherwise, the total colony number was not distinguishable between two methods, but it depended on the kinds of media: 50-80 cfu in 100 µL at $10^{-4}$ dilution in TSB, LB, NB and R2A; 30-60 cfu at $10^{-2}$ dilution in MSM, MSMSE and SE. This indicates that the new method influence only the diversity of colonies, not the number.

The modified new method developed in this study differs from the techniques used in previous studies[5,7] in that it promotes adaptation

of cultivation-resistant soil bacteria to traditional culture media with continuous supply of soil micronutrients thereby facilitating continuous cultivation. An increased number of different strains successfully isolated by the new method using traditional media proved that this novel cultivation approach can be a useful tool for isolation and characterization of diverse soil bacteria.

**Community structure analysis**

All 68 strains are shown in a phylogenetic tree with their closest neighbours and are grouped according to phylum or class level (Fig. 3). They are affiliated with 4 phyla: *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes*. All isolated strains in *Proteobacteria* fall into three classes: *á-Proteobacteria*, *α-Proteobacteria*, and *ã-*

**Table 1.** Physico-chemical properties of the soil sample used in this study

| Anions (mg/kg) | | Moisture | Temperature | Soil Texture (%) | | | Texture Class |
|---|---|---|---|---|---|---|---|
| Cl | $SO_4$ | content (%) | (°C) | Sand | Silt | Clay | |
| 19 | 9 | 14.5 | 20 | 76 | 20 | 4 | Loamy Sand |

**Table 2.** Compositions of media used in the new cultivation method

| No. | Components | Medium type (g/L distilled water-DW) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | NB | LB | TSB | R2A | MSM | SE | MSMSE | R2ASE |
| 1 | Beef extract | 3 | | | | | | 500 mL of MSM | 500 mL of R2A |
| 2 | Peptone | | | | | | | | |
| 3 | Proteose Peptone | | | | 0.5 | | | | |
| 4 | Tryptone | | 10 | | | | | | |
| 5 | Acid digest of Casein | | | | 0.5 | | | | |
| 6 | Yeast Extract | | 5 | | 0.5 | | | | |
| 7 | Enzymatic Digest of Gelatin | 5 | | | | | | | |
| 8 | Enzymatic Digest of Casein | | | 17 | | | | | |
| 9 | Enzymatic Digest of Soybean Meal | | | 3 | | | | | |
| 10 | Soluble Starch | | | | 0.5 | | | | |
| 11 | Sodium Pyruvate | | | | 0.3 | | | | |
| 12 | Dextrose | | | 2.5 | 0.5 | | | | |
| 13 | $CaCl_2 \cdot 2H_2O$ | | | | | 0.02 | | | |
| 14 | $KH_2PO_4$ | | | | | 2 | | | |
| 15 | $K_2HPO_4$ | | | 2.5 | 0.3 | 2 | | | |
| 16 | $MgSO_4 \cdot 7H_2O$ | | | | 0.05 | 0.2 | | | |
| 17 | NaCl | | 10 | 5 | | 0.4 | | | |
| 18 | $KNO_3$ | | | | | 1 | | | |
| 19 | $FeCl_3 \cdot 7H_2O$ | | | | | 0.01 | | | |
| 20 | $(NH_4)_2SO_4$ | | | | | 2 | | | |
| 21 | Trace element solution SL-6[a] | | | | | 1 mL | | | |
| 22 | Vitamin solution[b] | | | | | 1 mL | | | |
| 23 | Soil extract | | | | | | 1 L | 500 mL | 500 mL |

[a]$ZnSO_4$•$7H_2O$, 0.1 g; $MnCl_2$•$4H_2O$, 0.03 g; $H_3BO_3$, 0.3 g; $CoCl_2$•$6H_2O$, 0.2 g; $CuCl_2$•$2H_2O$, 0.01 g; $NiCl_2$•$6H_2O$, 0.02 g; $Na_2MoO_4$•$2H_2O$, 0.03 g; DW, 1000 ml.

[b]biotin, 10 mg; nicotiamide, 35 mg; thiamine dicloride, 30 mg; p-aminobenzoic acid, 20 mg; pyridoxal chloride, 10 mg; Ca-pantothenate, 10 mg; vitamin B12, 5 mg; DW, 100 ml.

**Table 3.** Bacterial strains isolated from forest soil by using the traditional and new cultivation methods using various traditional media.

| No | Name of isolates | Closest type strains | Similarity (%) | Accession number | Used Media Traditional culture method | New culture method |
|---|---|---|---|---|---|---|
| 1 | TSB11 | *Achromobacter spanius* LMG 5911$^T$ | 99.93 | AY170848 | LB, R2A | LB, R2A, MSM |
| 2 | EU-2 | *Arthrobacter oxydans* DSM 20119$^T$ | 99.32 | X83408 | | R2A |
| 3 | NHI-27 | *Arthrobacter parietis* LMG 22281$^T$ | 99.93 | AJ639830 | | LB, NB, SE |
| 4 | NHI-19 | *Arthrobacter ramosus* CCM 1646$^T$ | 98.86 | AM039435 | | MSM |
| 5 | EU-8 | *Arthrobacter ramosus* CCM 1646$^T$ | 99.06 | AM039435 | | SE, MSM |
| 6 | NHI-15 | *Bacillus anthracis* ATCC 14578$^T$ | 98.69 | AB190217 | LB, TSB | TSB, LB, MSM, NB, R2A |
| 7 | R2ASE9 | *Bacillus anthracis* ATCC 14578$^T$ | 100.00 | AB190217 | | R2ASE |
| 8 | R2A1 | *Bacillus aryabhattai* B8W22$^T$ | 100.00 | EF114313 | | MSMSE |
| 9 | NHI-1 | *Bacillus cereus* ATCC 14579$^T$ | 99.86 | AE017333 | LB, TSB | TSB, LB, DW(NA) |
| 10 | SE2 | *Bacillus horikoshii* DSM 8719$^T$ | 98.99 | X76443 | | MSM |
| 11 | TSB1 | *Bacillus fordii* R-7190T | 98.29 | AY443039 | | TSB |
| 12 | Aii-TSB | *Bacillus fordii* R-7190$^T$ | 99.48 | AY443039 | | TSB |
| 13 | LB1 | *Bacillus fortis* R-6514$^T$ | 98.38 | AY443038 | | TSB |
| 14 | NHI-37 | *Bacillus licheniformis* DSM 13$^T$ | 100.00 | AE017333 | | R2A, MSMSE |
| 15 | NHI-38 | *Bacillus methanolicus* NCIMB 13113$^T$ | 96.15 | AB112727 | | R2A |
| 16 | AR-II-1 | *Bacillus methylotrophicus* CBMB205$^T$ | 99.90 | EU194897 | | R2A |
| 17 | NHI-10 | *Bacillus mycoides* DSM 12442T | 99.02 | ACMX01000133 | NB, R2A | DW(R2A) |
| 18 | NHI-16 | *Bacillus thuringiensis* ATCC 10792T | 99.78 | ACNF010000156 | R2A | R2A |
| 19 | NHI-5 | *Bosea robiniae* LMG2638$^T$ | 99.64 | FR774994 | LB, NB, TSB, R2A, MSM | |
| 20 | NHI-8 | *Bosea thiooxidans* AJ25079$^T$ | 93.19 | AJ250796 | NB, R2A | NB, DW(R2A) |
| 21 | R2A2 | *Brevibacillus reuszeri* NRRL NRS-1206$^T$ | 99.71 | D78464 | | LB |
| 22 | EU-1 | *Burkhoideria stabils* LMG 14294$^T$ | 100 | AF148554 | | R2A |
| 23 | NB2 | *Citrobacter farmeri* CDC 2991-81$^T$ | 98.45 | AF025371 | | R2A |
| 24 | NB1 | *Citrobacter farmeri* CDC 2991-81$^T$ | 99.32 | AF025371 | | R2A |
| 25 | NHI-6 | *Cupriavidus basilensis* CCUG 49340$^T$ | 99.93 | FN597608 | LB | DW(LB), TSB |
| 26 | NHI-14 | *Cupriavidus necator* N-1$^T$ | 98.89 | CP002878 | R2A | R2A, NB |

| | | | | | | |
|---|---|---|---|---|---|---|
| 27 | NHI-48 | *Dyella japonica* XD53$^T$ | 99.86 | AB110498 | | R2A |
| 28 | NHI-12 | *Dyella terrae* JS14-6$^T$ | 99.02 | EU604273 | R2A | MSM, R2A |
| 29 | R2A | *Enterobacter asburiae* JCM 6051$^T$ | 99.78 | AB004744 | | R2A |
| 30 | NHI-20 | *Kitasatospora saccharophila* SK15$^T$ | 99.58 | AB278568 | MSM | DW(MSM), SE |
| 31 | TSB3 | *Leucobacter iarius* 40$^T$ | 99.79 | AM040493 | | LB |
| 32 | NHI-46 | *Lysinibacillus boronitolerans* 10a$^T$ | 99.53 | AB199591 | | NB, R2A |
| 33 | NB6 | *Lysinibacillus fusiformis* NBRC 15717$^T$ | 99.32 | AB271743 | | NB, R2A |
| 34 | TSB13 | *Lysinibacillus macroides* LMG 18474$^T$ | 99.05 | AJ628749 | | NB |
| 35 | NB9 | *Lysinibacillus sphaericus* C3-41$^T$ | 100 | CP000817 | | NB |
| 36 | NB11 | *Lysinibacillus xylanilyticus* XDB9$^T$ | 98.44 | FJ477040 | LB, TSB, R2A, NB | TSB, LB, NB, R2A |
| 37 | TSB12 | *Lysinibacillus xylanilyticus* XDB9$^T$ | 99.26 | FJ477040 | LB, R2A, NB | TSB, LB, NB, R2A |
| 38 | NHI-23 | *Mesorhizobium chacoense* Pr5$^T$ | 97.79 | AJ278249 | MSMSE, SE | MSMSE, SE, R2A, NBLB |
| 39 | NB4 | *Mesorhizobium robiniae* CCNWYC 115$^T$ | 97.34 | EU849582 | | R2A |
| 40 | LB10 | *Mesorhizobium shangrilense* CCBAU 65327$^T$ | 98.06 | EU074203 | | TSB |
| 41 | NHI-34 | *Methylobacterium komagatae* 002-079$^T$ | 100 | AB252201 | | DW(MSM), SE |
| 42 | NHI-33 | *Methylobacterium oryzae* CBMB20$^T$ | 99.36 | AY683045 | | DW(MSM), R2A |
| 43 | EU-7 | *Microbacterium natoriense* TNJL143-2$^T$ | 99.93 | AY566291 | | R2A |
| 44 | SEM-I-3 | *Microbacterium oleivorans* DSM16091$^T$ | 99.85 | AJ698725 | | MSMSE |
| 45 | SEM-II-5 | *Micrococcus yunnanensis* YIM 65004$^T$ | 99.76 | FJ214355 | | MSMSE, R2A |
| 46 | SEM-II-6 | *Mycobacterium obuense* ATCC 27023$^T$ | 99.76 | X55597 | | MSMSE |
| 47 | NHI-24 | *Niabella tibetensis* 15-4$^T$ | 96.74 | GU291295 | TSB | DW(TSA), DW(R2A) |
| 48 | ET-1 | *Nitrobacter alkalicus* AN1$^T$ | 96.66 | AF069956 | | R2A |
| 49 | NHI-39 | *Paenibacillus alvei* DSM 29$^T$ | 99.13 | AJ320491 | | TSB |
| 50 | NB5 | *Paenibacillus nanensis* MX2-3$^T$ | 96.85 | AB265206 | | R2A |
| 51 | NHI-28 | *Paenibacillus pabuli* JCM 9074$^T$ | 100.00 | AB073191 | | NB, R2A |
| 52 | R2ASE5 | *Paenibacillus terrae* AM141$^T$ | 99.73 | AF391124 | | MSM |
| 53 | NHI-13 | *Pedobacter panaciterrae* Gsoil 042$^T$ | 98.26 | AB245368 | R2A | NB, DW(R2A) |
| 54 | NHI-21 | *Pelomonas puraquae* CCUG 52769$^T$ | 100.00 | AM501439 | MSM | MSM |
| 55 | TSB5 | *Pseudomonas beteli* ATCC 19861$^T$ | 99.79 | AB021406 | | TSB |
| 56 | R2A7 | *Pseudomonas geniculata* ATCC 19374$^T$ | 99.08 | AB021404 | R2A | R2A, NB |
| 57 | NHI-42 | *Pseudomonas koreensis* Ps 9-14$^T$ | 99.66 | AF468452 | | R2A, NB |
| 58 | EU-6 | *Rhodococcus equi* DSM 20307$^T$ | 98.88 | X80614 | | R2A |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 59 | NHI-47 | *Rhodococcus erythropolis* PR4$^T$ | 100.00 | AP008957 | | | R2ASE |
| 60 | TSB8 | *Serratia marcescens subsp. sakuensis* KRED$^T$ | 99.87 | AB061685 | | | LB |
| 61 | TSB7 | *Serratia nematodiphila* DZ0503SBS1$^T$ | 99.66 | EU036987 | | | LB |
| 62 | NHI-3 | *Sporosarcina koreensis* F73$^T$ | 99.72 | DQ073393 | LB, NB, TSB, R2A | | TSB, LB, NB, R2A |
| 63 | NHI-11 | *Staphylococcus warneri* ATCC 27836$^T$ | 100.00 | L37603 | NB | | TSB, NB, DW(R2A), DW(LB) |
| 64 | II-5-3 | *Staphylococcus epidemidis* ATCC 14990$^T$ | 99.86 | L37605 | | | MSMSE |
| 65 | NHI-22 | *Stenotrophomonas maltophilia* ATCC 13637$^T$ | 99.29 | AB008509 | MSM | | R2ASE |
| 66 | NHI-49 | *Streptomyces althioticus* NRRL B-3981$^T$ | 98.39 | AY999791 | | | R2ASE |
| 67 | NHI-35 | *Streptomyces xanthocidicus* NBRC 13469$^T$ | 99.71 | AB184427 | | | R2A |
| 68 | NHI-25 | *Tsukamurella pulmonis* DSM 44142$^T$ | 99.65 | X92981 | | | DW(TSA),DW(LB), DW(MSM) |

Note: distilled water as a medium was added into one transwell insert, and soil samples were cultured for 2 weeks as described in Materials and Methods. The cultivated inoculum was transferred to agar plates containing TSA (tryptic soy agar), NA (nutrient agar), LB, MSM, and R2A media. Visible colonies obtained on these plates were named as DW(TSA), DW(NA), DW(LB), DW(MSM), and DW(R2A), respectively. The potential new species are bolded to be distinguished.

*Proteobacteria*. Most of the strains – 27 out of 68 – belong to *Firmicutes*, including 18 of 45 strains isolated exclusively by the new cultivation method. Other 24 strains (including 14 isolated by the new method) belong to *Proteobacteria* as the second most populated phylum; among them *α-Proteobacteria* comprises $11^8$, *α-Proteobacteria* $8^5$, and *γ-Proteobacteria* $5^1$, respectively. Fifteen strains were identified as members of *Actinobacteria*, among them 13 (87%) were isolated exclusively by the new method constituting the highest proportion among the 4 phyla. The last 2 strains belong to *Bacteroidetes*; they were isolated by both methods.

The distribution of potentially new species among *Proteobacteria, Firmicutes, Bacteroidetes*, and *Actinobacteria,* was 6, 5, 2, and 1, respectively. Among the 6 *Proteobacteria* strains, 5 belong to *α-Proteobacteria* and 1 to *γ-Proteobacteria*. The 9 potentially new species isolated exclusively by the new method belong to *Proteobacteria*$^4$, *Firmicutes*$^4$, and *Actinobacteria*$^1$. Among the 4 *Proteobacteria* species, 3 belong to *α-Proteobacteria*, and 1 to *γ-Proteobacteria.* These results indicate that the new method may be particularly useful for isolation of new species of soil bacteria related to *Firmicutes* and *α-Proteobacteria*.

According to distribution of the strains on the genus-level, the new cultivation method appears to be selective for specific genera (Fig. 4). It may improve those members to adapt synthetic media more than other genera by supply of soil nutrients in the beginning. The conventional method detected isolates of the following 16 genera: *Bacillus* (17.4%), *Lysinibacillus* (13.0%), *Bosea* (8.7%), *Cupriavidus* (8.7%), *Achromobacter* (4.3%), *Arthrobacter* (4.3%), *Dyella* (4.3%), *Kitasatospora* (4.3%), *Mesorhizobium* (4.3%), *Niabella* (4.3%), *Pedobacter* (4.3%), *Pelomonas* (4.3%), *Pseudomonas* (4.3%), *Sporosarcina* (4.3%), *Staphylococcus* (4.3%), and *Stenotrophomonas* (4.3%). The new method identified isolates belonging to *Bacillus* (19.7%), *Lysinibacillus* (7.6%), *Arthrobacter* (6.1%), *Paenibacillus* (6.1%), *Mesorhizobium* (4.5%), *Pseudomonas* (4.5%), *Citrobacter* (3.0%), *Cupriavidus* (3.0%), *Dyella* (3.0%), *Methylobacterium* (3.0%), *Microbacterium* (3.0%), *Rhodococcus* (3.0%), *Serratia* (3.0%),

*Staphylococcus* (3.0%), *Streptomyces* (3.0%), *Achromobacter* (1.5%), *Bosea* (1.5%), *Brevibacillus* (1.5%), *Burkholderia* (1.5%), *Enterobacter* (1.5%), *Kitasatospora* (1.5%), *Leucobacter* (1.5%), *Micrococcus* (1.5%), *Mycobacterium* (1.5%), *Niabella* (1.5%), *Nitrobacter* (1.5%), *Pedobacter* (1.5%), *Pelomonas* (1.5%), *Sporosarcina* (1.5%), *Stenotrophomonas* (1.5%), and *Tsukamurella* (1.5%).

The strains of *Bacillus* and *Lysinibacillus* were isolated in relatively high proportion by both methods, while 4 strains of *Paenibacillus* and 1 or 2 strains in each of other 14 genera: *Brevibacillus*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Leucobacter*, *Methylobacterium*, *Microbacterium*, *Micrococcus*, *Mycobacterium*, *Rhodococcus*, *Serratia*, *Streptomyces*, *Nitrobacter*, and *Tsukamurella* could be obtained exclusively by the new method.

Many microbiologists have successfully cultivated many uncultivable bacteria by using modified or diluted media, and long incubation times; strains thus isolated belonged to 11 phyla such as *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Rubrobacteridae*, *Chloroflexus*, *Firmucutes*, *Fusobacteria*, *Gemmatimonadetes*, *Planctomydetes*, *Proteobacteria*, and *Verrucomicrobia* (1). Here, we succeeded in isolation representatives of only 4 of those phyla, suggesting that our new method can be complemented by incorporation of the aforementioned approaches in future investigations of phylogenetic diversity in soil bacterial communities.

## CONCLUSION

This study was based on application of transwell membranes previously used in mammalian cell culture, for isolation and growth of soil bacteria resistant to traditional cultivation. This novel method proved efficient for cultivation of uncultivable and identification of new, bacterial species through adaptation to laboratory culturing conditions. The transwell membrane liquid culture technique provided isolation of higher number of strains including potentially new species than the traditional method suggesting that the novel method can be successfully utilized to study phylogenetic diversity of complex bacterial populations. This study also showed that R2A medium was more suitable for isolation of soil bacteria than other media such as LB, NB, TSB, MSM, and SE whether by the conventional or the new method. We believe that the use of modified and diluted media with the transwell membrane

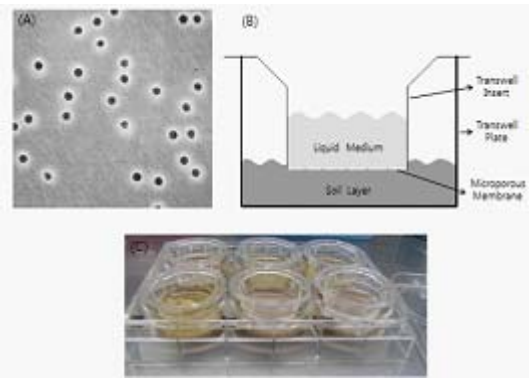



**Fig. 1.** Transwell plate system.
(A) Scanning electron micrograph of the surface of a 0.4 µm-pore polycarbonate membrane. (B) Schematic diagram of the transwell plate system. (C) A transwell plate with 6 wells

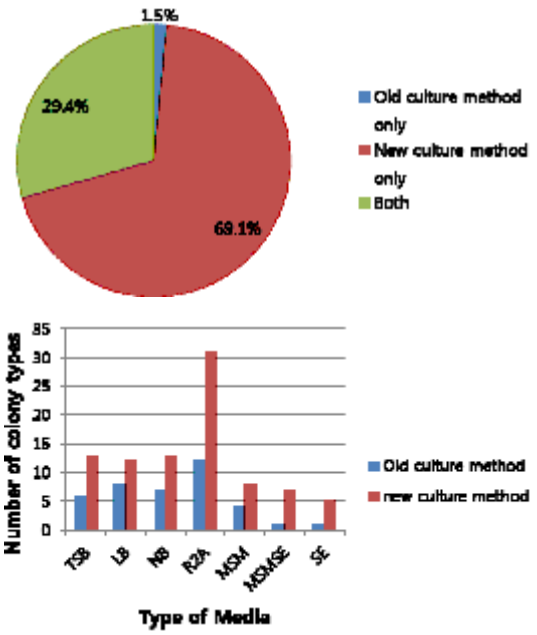
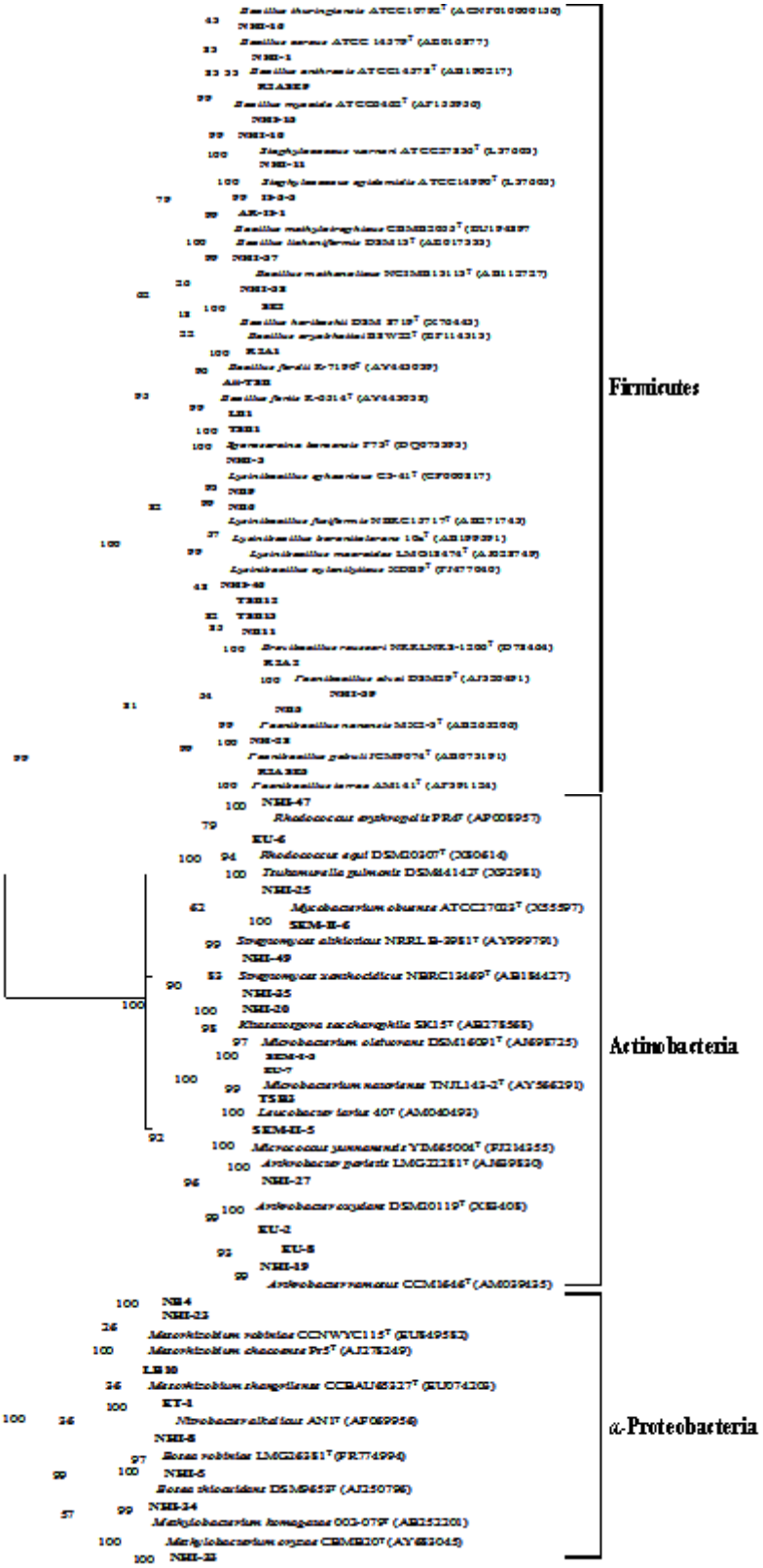



**Fig. 2.** Comparison of bacteria isolation efficiency between the new and traditional cultivation methods entirely (A) and by each medium (B).


Firmicutes
Actinobacteria
α-Proteobacteria

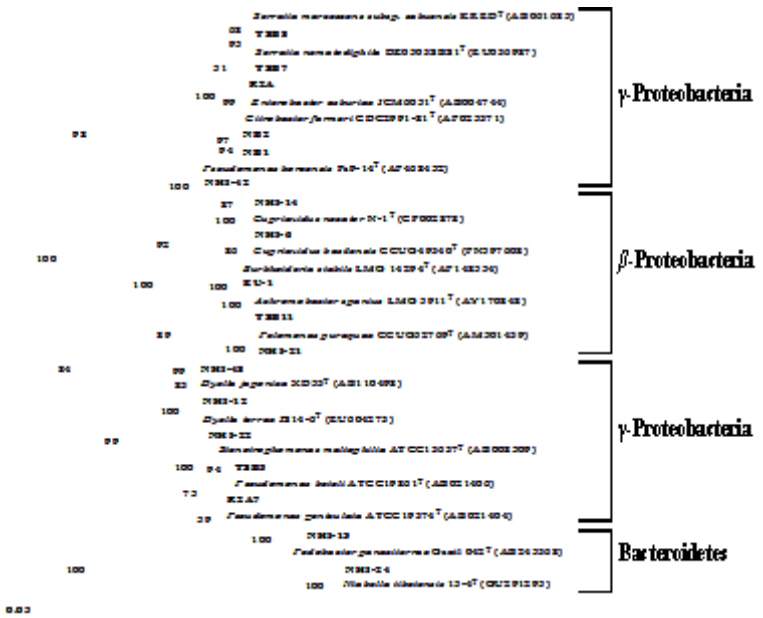



**Fig. 3.** Evolutionary phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic distribution of bacteria isolated from forest soil and their closest neighbors. Bootstrap percentages were based on 1000 replications and are shown at the branch points. Bar, 0.05 substitutions per nucleotide.

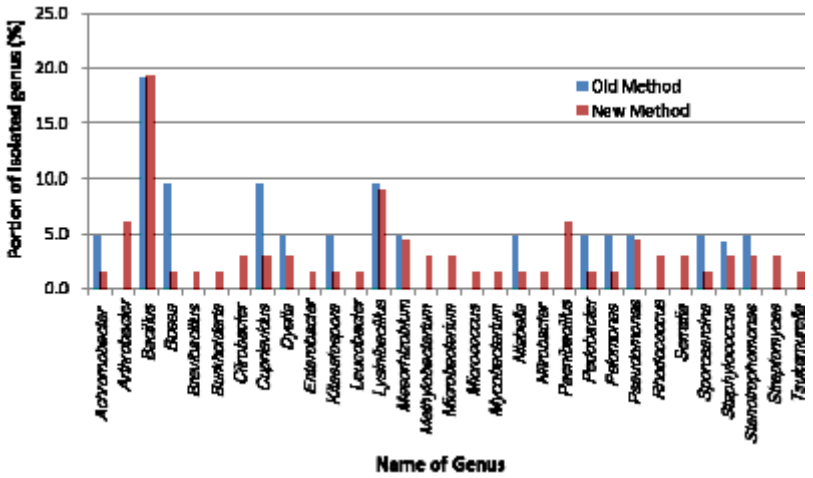



**Fig. 4.** Genetic diversity of microorganisms isolated from forest soil by the old and new cultivation methods.

system would pave the way for more diverse bacterial isolates and new species in the future.

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