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# Estimation of diversity, abundance and composition of bacterial population in tropical lake sediments using terminal restriction fragment technique

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## ABSTRACT

Soil bacteria carry out various ecological roles in the ecosystem. Diverse microbial community could be present in the soil. Very less knowledge is available regarding the association between soil structure and bacterial diversity. Soil bacteria are good indicators of soil health and soil fertility. The bacterial communities of soil/sediment of lake are affected by the environmental biotic, abiotic, natural and/or man made conditions. In the present study, bacterial diversity of soil/sediment of Sawanga Vithoba lake has been assessed by full form terminal restriction fragment length polymorphism in December and March. Bacterial diversity has been found to be rich, which ranged from 1 to 28 with an average number of restriction fragments 14.

**KEY WORDS:** TERMINAL RESTRICTION, FRAGMENT TECHNIQUE, MICROBIAL BIODIVERSITY, LAKE SEDIMENTS

## INTRODUCTION

Soil bacteria are vital components of the ecosystems and microbial community in the soil has been reported to be relatively diverse (Curtis *et al.*, 2002; Robe *et al.*, 2003), with the highest prokaryotic diversity compared to other environments (Van *et al.*, 2006; Roesch *et al.*, 2007). One gram of soil has been reported to contain up to 10 billion microorganisms and thousands of different species (Knietch *et al.*, 2003). Soil bacteria are genetically diverse and represent a major unexploited genetic resource (Whitman *et al.*, 1998).

Diversity, abundance and composition of microbial communities within soils are depth dependent. Changes in microbial community structure with soil depth are due to the response of microbes to the physico-chemical conditions (Holden and Fierer, 2005). Environmental factors such as pH (Eichorstet *et al.*, 2007), particle size (Sessitsch *et al.*, 2001), organic carbon content (Zhou *et al.*, 2002), nutrient availability (Fiereret *et al.*, 2003), water content (Treves *et al.*, 2003), and oxygen concentration (Ludemann *et al.*, 2000) also affect the microbial community composition and diversity. Thus, the physiology and metabolic potential of microbial communities may vary with location. In the soils/sediments mineral and organic matter components are organized into aggregates with varying size, porosity, pore size and continuity, and composition.

Microaggregates (2 to 20 µm) are considered to be the most favorable habitat for bacteria in most types of soil (Ranjard and Richaume, 2001), with a higher abundance of bacteria located in micropores (~2 µm) of the inner aggregate fraction (Hattori, 1988; Ranjard *et al.*, 2000). Little is known regarding

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the association between soil structure and bacterial diversity (Ranjard *et al*., 2000),

Microbial community composition may change with the location within (Jocteur-Monrozier *et al*., 1991, Ranjard *et al*., 1997) and size of (Kanazawa and Filip, 1986; Sessitsch *et al*., 2001; Vaisanen *et al*., 2005) soil aggregates. Aggregates of soil may support a diverse microbial community than the bulk soil by imposing size exclusion on selected biota from restricted pore domains (Bales *et al*., 1989; Champ and Schroeter, 1988), minimizing predation (Wright *et al*., 1995), and/or decreasing competition as a consequence of lower water tensions (Treves *et al*., 2003).

Anaerobic microsites within aggregates in aerobic soils may allow for a diverse range of aerobic- and anaerobic-based metabolisms that contribute to the bulk chemical fluxes of nutrients and metabolites within soils. Bacteria such as Crenarchaeota have been directly linked to chemoautotrophic nitrification (Konneke *et al*., 2005) and are reported to be involved in carbon metabolism (Kemnitz *et al*., 2007) and amino acid uptake/assimilation (Ouverney and Fuhrman, 2000; Teira *et al*., 2006 ). Bacteria play important roles in nitrogen cycling (Kowalchuk and Stephen, 2001; Tiedje and Stevens 1988), carbon cycling (Hogberg *et al*., 2001), transfor-mation of metals such as iron, manganese, and mercury (Paul and Clark, 1989), addition of organic humic content (Gobat *et al*., 2004) as well as soil formation (Rillig and Mummey, 2006). Bacteria are also required for nutrient acquisition in the soil ecosystem and therefore contribute to plant nutrition and health (George and Jakobsen, 1995; Timonen *et al*., 1996). Soil bacteria can exert positive or negative control over other organisms in the environment through the synthesis of antibiotics or growth factors like vitamins (Gobat *et al*., 2004). These bacteria are reported to involve in the biodegradation of human-made pollutants (Boubakri *et al*., 2006; Galvao *et al*., 2005).

Soil bacteria are good indicators of soil health, soil fertility (Yao *et al*., 2000; O'Donnell *et al*., 2001) and ecosystem status in much more comprehensive way than physical or chemical measures (Fierer and Jackson, 2006; Winding *et al*., 2005). Bacteria from specific taxa have been allied to definite ecological characteristics. For example, the presence of nitrogen fixers such as *Rhizobia* and *Azotobacter* and nitrifying bacteria (Nitroso-) indicates high N levels in the soil. Likewise, presence of copiotrophic bacteria such as *Acidobacteria* indicates low nutritional status, while the presence of oligotrophic bacteria such as ß-Proteobacteria and Bacteriodetes indicates high nutritional status (Fierer *et al*., 2007).

Bacteria are also responsive to man made activities such as agriculture, pesticide use and pollution (Deiglmayr *et al*., 2004; Klumpp *et al*., 2003). Soil microbial communities may alter their metabolic and genetic capability in response to changes in the environmental factors which result in rapid shifts in bacterial diversity within short period of time frame (Schmidt *et al*., 2007). Understanding soil microbial communities is therefore an ideal method to monitor the ecological changes occurring between seasons as well as over an extended period of time (Hill *et al*., 2000).

The variability of species richness and diversity index among enzyme digestions may be related to the fact that the same T-RF can be generated by different species of bacteria (Marsh *et al*., 2000). The species of bacteria producing a particular T-RF by one restriction enzyme digestion may produce more than one T-RF by another enzyme digestion, and vice versa. So, the peaks or 'genotypes' may have different compositions of species n different enzyme digestions. The number and area of T-RFs varied on the basis of different species compositions. Accordingly, the species richness and the diversity index changed. Further, the variation among observed and theoretical T-RFs depend on the restriction enzyme selected (Kaplan and Kitts, 2003). The 'precision' of enzyme digestion affected the definition of T-RFs and consequently determination of the number and area of T-RFs.

In the resent communication, it is hypothesized that soil/sediment bacterial communities of the Sawanga Vithoba lake do exhibit diversity and affect the ecological processes in the lake. The bacterial communities of soil/sediment of lake are affected by the environmental biotic, abiotic, natural and/or man made conditions. To my knowledge, no previous study has examined the bacterial diversity of the of soil/sediment of Sawanga Vithoba lake. It is hypothesized that the biogeographical patterns exhibited by soil/sediment bacterial communities of lake could be fundamentally similar to the patterns observed with plant and animal taxa. Soil/sediment bacterial communities of lake could be a good predictor of the status of the particular ecosystem, metabolic processes going on inside that ecosystem and energy balance. In this study, soil/sediment's bacterial diversity were studied to monitor the ecological system in this region by T-RFs.

## MATERIALS AND METHODS

### STUDY SITE:

For the present study, three stations were selected. Station two is west station, station three is north station and station four is south station. Station two is near the village (Sawanga Vithoba). Other stations of the lake are surrounded by vegetations. Soil(sediment) samples from the lake bottom were collected as described in the soil kit (make: Prerana), samples were transformed to sterilized plastic or glass containers and transported to the laboratory. The bacterial analysis was performed in the months of December and March.

### BACTERIAL ANALYSIS:

#### a. DNA isolation from environmental samples

For processing of soil samples 0.5g of soil was added to 2ml screw-capped microcentrifuge tubes containing 0.5 g each of 0.1 mm glass beads. STE buffer, lysozyme and proteinase K(10g/ml) were sequentially added for nucleic acid isolation. This method employed the process of NaCl and SDS lysis followed by Phenol: chloroform: iso-amyl alcohol based organic extraction for purification of nucleic acids.

The DNA was deproteinised thrice with Tris-satured phenol (phenol:$CHCl_3$: iso-amyl alcohol, 50:48:2), and then with $CHCl_3$:iso-amyl alcohol (24:1) and precipitated with 2% sodium

acetate and absolute ethanol. Dried DNA was dissolved in 30µl nuclease free water. Quality assessment of genomic DNA was performed by 1% agarose gel electrophoresis and estimated using aQubitˆTM Fluorometer(Invitrogen,USA). DNA extraction from positive control sample (E. Coli) was also performed to rule out the possibility of extraction failure. Similarly, a negative control (plain saline) was also subjected to extraction to establish the clean reagents.

### b. PCR amplification of the 16S gene

The bacterial 16 S rRNA gene was amplified by PCR using the primer set 8F:5'-AGAGTTTGATCATGGCT CAG-3' and 1491R; 5'-GGCTACCTTGCCACGACTTC-3' (Lane, 1991). The 8F primer was labeled at the 5'end with HEX fluorescent dye. The PCR mixture was 0.5µl of each primer (10mM), 5µl of the PCR buffer,1µl of dNTP (2.5mM), 0.5µl of Taq polymerase( 2.5 U/µl) and double-distilled water for final reaction volume of 50µl. PCR was performed at 95°C for 5 min; 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min; and 72°C for 10 min. PCR products were checked on 1% agarose gels and purified with the gene O-spin PCR purification kit(geneOmbio Technologies,India) according to the manufacturer's protocol.

### c. Restriction digestion and desalting of digested products

Following PCR, 10µl of PCR products were digested with 0.5 U of Taq I restriction enzymes (New England Biolabs) for 3 hours at 37°C in 20µl reaction volumes. Digests were separated on 3% agarose gels in 1X TBE buffer containing ethidium bromide, and visualized under UV light.

10µl of the digested DNA was desalted using the following procedure: 2.5µl of 125mM EDTA and 1/10 volume of 3M sodium Acetate pH 5.2 was added to 10µl of digested DNA. Further 2.5 volume of ice cold ethanol was added into the tubes and mixed well. Tubes were then centrifuged at 12000 RPM for 20 minutes at 18°C. Supernatant was removed being careful not to dislodge the pellet. Pellet was then washed with 60µl of 70% Ehtanol twice with centrifugation at 12000RPM for 20 minutes at 18°C temperature. Pellet was then dried at 37°C for 30 minutes.

### d. Sample preparation and loading

Hi-Di Formamide (9.7µl) from Applied Biosystems was added to the dried pellet. Each sample was added with 0.3µl GenScan 500 LIZ Internal size standard. This mixture was denatured at 95°C for 3 minutes and immediately chilled on ice before loading. The samples were then subjected to electrophoresis on the 3130 Genetic Analyzer using the FA_36_POP-7TM run module and G5 dye set.

### e. Gene Mapper data analysis

Gene Mapper software based analysis was performed for fragment analysis after completion of the capillary electrophoresis. Output from automated sequencers is in the form of an electropherogram, with peaks representing fluorescently labeled T-RFs detected over time in relation to the size standard. The duration and intensity of the fluorescent signal from T-RFs is reflected in the area and height of each peak detected, respectively. Software specific to each sequencing unit collects data from each run. The ABI 3730 capillary sequencer operates, Gene Mapper v3.5 (Applied Biosystem), which performs the functions of both GeneScan and Genotyper. Either data collection program provides researcher with several algorithms for sizing sample fragments by comparing their mobility with that of the size standard. Once data are processed and fragment lengths assigned, the data set is typically imported into a spreadsheet program, such as Microsoft Excel (Microsoft Corp., Redmond, WA). In the spread-sheet, sample identifiers can be added and presence/absence (1,0) matrices developed. Other manipulations, such as matrix inversions, can also be performed. The T-RFs for each sample run should be closely examined and the entire run evaluated for the average number of T-RFs detected per sample and the number of T-RFs contained in the various size classes.

## RESULTS AND DISCUSSION

After T-RFs analysis, different restriction fragments were observed. It was observed that all the sediment samples of the selected stations contain diversed bacterial community. It was also observed that the highest number of the restriction fragments were at station number three which is the north station of the lake. At this station total number of the restriction fragments was 28. Total numbers of restriction fragments were more in the month of March. At station two (east station, which is near village), on March 6 restriction fragments were observed. Station three is the north station which is surrounded by vegetation showed 03 restriction fragments in December while 28 in March. Station four is the south station which is also surrounded by vegetation shows 05 restriction fragments in December and 08 in March. The restriction fragments are depicted in table1. The average restriction fragments for the sediments of the lake was found to be 04 in December while in March it was 14 . Bacterial population was found to be more in the month of March.

## BACTERIAL RICHNESS:

TABLE 1: Total number of terminal Restriction Fragments(T-RFs) generated for each sample

| Station | Total No of T-RFs | |
|---|---|---|
| | December | March |
| Two | - | 6 |
| Three | 03 | 28 |
| Four | 05 | 8 |

The bacterial richness is the types of fragments obtained and elucidated in the T-RFLP technique, where each fragment is considered to arise from a different bacterial strain. Bacterial richness ranged from 1 to 05 in December with an average of 4 while range from 1 to 28 with an average of 14 in March. The highest richness was observed in the sample of station 3 which

is a north station of the lake. Total number of 50 different taxa (designated as allele in the supplementary data) were observed as per the size of the T-RFs.

## ELECTROPHEROGRAM:

The result of a T-RFLP profiling is a graph called Electropherogram,which is an intensity plot representation of an electrophoresis experiment(gel or capillary).In an electropherogram the X-axis marks the sizes of the fragments (basepairs) while the Y-axis marks the fluorescence intensity in fluorescent unit of each fragment. Thus, what appears on an electrophoresis gel as a band appears as a peak on the electropherogram whose integral is its total fluorescence. In a T-RFLP profile each peak assumingly corresponds to one genetic variant in the original sample while its height corresponds to its relative abundance in the specific community. Often, several different bacteria in a population might give a single peak on the electropherogram due to the presence of a restriction site for the particular restriction enzyme used in the experiment at the same position. T-RFs having size less than 40 bases were eliminated from the analysis as they might result from primer-dimers. Fluorescent signal threshold was set to 400 fluorescent units as per the standards to minimize the background signal and signals arising from ssDNA non-specific amplicons/fragments.

In the present study, bacterial diversity of soil/sediment of lake Sawanga (Vithoba) was assessed. The soil /sediment bacteria were tried to identify upto phylum level by 16S rDNA. Employing the 16S rDNA gene for this purpose is proper due to the ubiquitous nature of the gene and extremely low mutation rate and its phylogenetic significance (Ludwig1999; Ludwig and Schleifer1994). RFLP analysis of the 16S rDNA was used as an initial estimator of bacterial diversity. Indeed, it is well established that RFLP analysis of 16S rDNA can be used to study bacterial diversity (Moyer *et al*., 1994; Moyer, 1996).

In Table No. 4, the probable bacteria in the sediment/soil sample of the Sawanga Vithoba lake have been tested.

The composition and diversity of soil bacterial communities have a straight effect on many ecosystem processes (Schimel, 1995; Balser *et al*., 2002). Diversity of soil bacterial communities, soil microbial ecology, diversity of soil bacteria are affected by environmental biotic and abiotic factors (Buckley and Schmidt, 2002).

Ecological monitoring is crucial for assessing the environmental effects. There are previous studies on characterization of the dominant bacteria in environmental samples by using 16S rDNA molecular signatures . RFLP analysis of 16S rDNA can be used to study bacterial diversity. The bacterial diversity in soil is high (Dunbar *et al*., 2002; Tringe *et al*., 2005). More species could be found with greater sampling sizes. Thus, this study has been successful at establishing a baseline of bacterial diversity in soil/sediment of Sawanga (Vithoba) lake of Pune, India.

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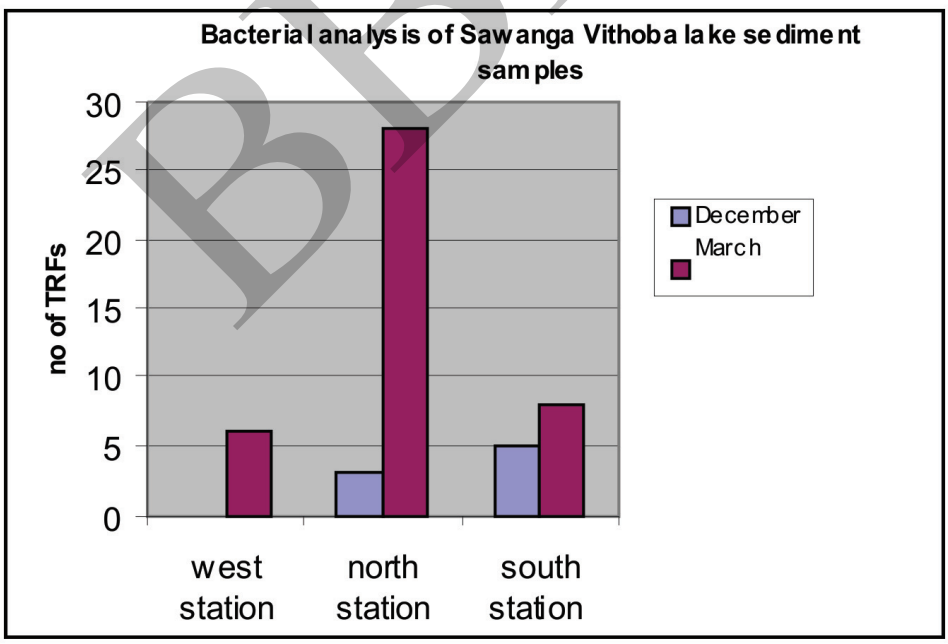


FIGURE 1: Bacterial analysis of Sawanga Vithoba lake sediment samples in ecember (winter) and in March (summer).

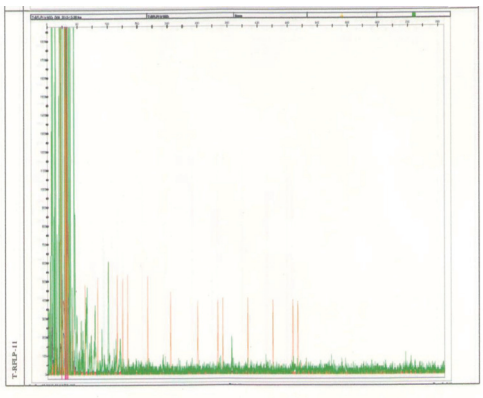

FIGURE 2: Electropherogram of the bacterial population for the North station in December.

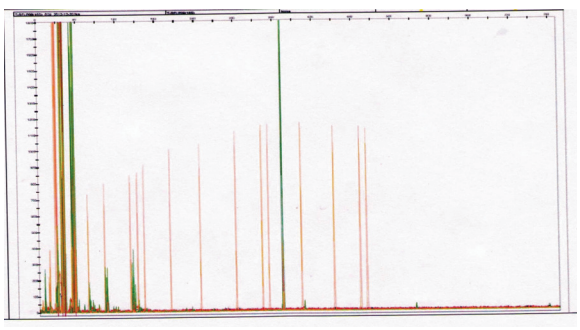

FIGURE 3: Electropherogram of the bacterial population for the South station in December.

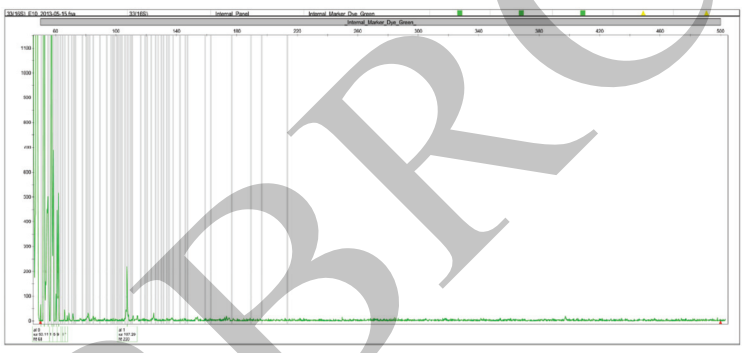

FIGURE 4: Electropherogram of the bacterial population for the station two (west station) in March.

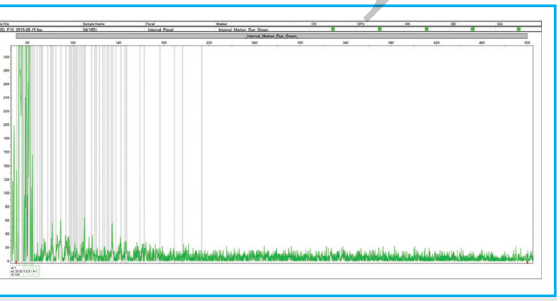

FIGURE 5: Electropherogram of the bacterial pulation for the station three (north station) in March.

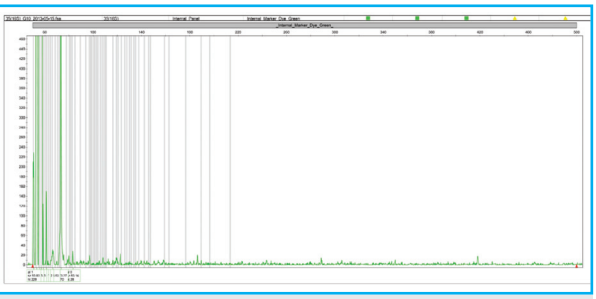

FIGURE 6: Electropherogram of the bacterial population for the station four(south station) in March.

TABLE 2: Probable bacterial communities in Sawanga-Vithoba lake soil

| S. No. | Name of bacteria | S. No. | Name of bacteria |
|---|---|---|---|
| 1 | *Anaerobacter polyendosporus* | 18 | *Salimicrobium halophilum* |
| 2 | *Bacillus subtilis subsp* | 19 | *Paenibacillus zanthoxyli* |
| 3 | *Bacillus licheniformis* | 20 | *Enterococcus faecium* |
| 4 | *Lactobacillus sp,rumen bacterium* | 21 | *Pseudoalteromonas* |
| 5 | *Streptomyces qinlingensis type strain* | 22 | *Antarctic seawater bacterium* |
| 6 | *Rhizobium sp.* | 23 | *Shewanella sp.* |
| 7 | *Ralstonia sp.* | 24 | *Shewanella frigidimarina* |
| 8 | *Vibrio sp* | 25 | *Shewanella waksmanii* |
| 9 | *Stenotrophomonas maltophilia* | 26 | *Gamma proteobacterium enrichment* |
| 10 | *Frateuria aurantia* | 27 | *Shewanella aquimarina* |
| 11 | *Acidobacteria bacterium* | 28 | *Allochromatium vinosum* |
| 12 | *Halothiobacillus halophilus* | 29 | *Citrobacter sedlakii* |
| 13 | *Chromatiales bacterium* | 30 | *Citrobacter amalonaticus* |
| 14 | *Citrobacter murliniae* | 31 | *Citrobacter werkmanii* |
| 15 | *Citrobacter braakii* | 32 | *Enterobacter sp.* |
| 16 | *Citrobacter gillenii* | 33 | *Cronobacter malonaticus* |
| 17 | *Grimontella senegalensis* | 34 | *Cronobacter sakazakii* |

TABLE 3: Probable bacterial communities in Sawanga-Vithoba lake soil of station 2

| S. No. | Accession No. | Name of bacteria |
|---|---|---|
| 1 | Am111092 | *Pseudomonas* |
| 2 | EF528288 | *Bacillus subtilis subsp.* |
| 3 | EF656455 | *Bacillus licheniformis* W-2 |
| 4 | GQ903382 | *Bacillus firmus* XJSL1-3. |
| 5 | GU097448 | *Rhizobium sp.* R5. |
| 6 | AY191846 | *Ralstonia sp.* 12D. |
| 7 | AB091196 | *Frateuria aurantia* IFO3249. |
| 8 | EF620455 | *Stenotrophomonas maltophilia.* |
| 9 | AB091201 | *Frateuria aurantia* |
| 10 | EU624430 | *Bacillus cereus* S6-08. |
| 11 | GQ903396 | *Bacillus firmus* XJSL2-7. |
| 12 | EU248957 | *Geobacillus sp.* H6a. |
| 13 | GQ903410 | *Salimicrobium halophilum* XJSL4-1. |
| 14 | AJ301830 | *Enterococcus faecium* (T) LMG 11423. |
| 15 | Ay685145 | *Selenomonas ruminantium* L6. |

TABLE 4: Probable bacterial communities in Sawanga-Vithoba lake soil of station 3

| S. No. | Accession No. | Name of bacteria |
|---|---|---|
| 1 | AF300975 | *Bacterium* C16S. |
| 2 | AM111055 | *Psychrobacter* sp. 7317. |
| 3 | AB188223 | *Isoptericola* sp. TUT1252. |
| 4 | AM111092 | *Pseudomonas* sp. 8058. |
| 5 | EF528288 | *Bacillus subtilis subsp. Subtilis* |
| 6 | EF656455 | *Bacillus licheniformis* W-2. |
| 7 | EF656456 | *Bacillus subtilis subsp. Subtilis* |
| 8 | GQ903382 | *Bacillus firmus* XJSL1-3. |
| 9 | AJ222546 | *Anaerobacter polyendosporus.* |
| 10 | Am167521 | *Streptomyces qinlingensis type* |
| 11 | GU097448 | *Rhizobium* sp. R5. |
| 12 | AY191846 | *Ralstonia* sp. 12D. |
| 13 | FM957478 | *Vibrio sp.* MY-2008-U64. |
| 14 | AB091196 | *Frateuria aurantia* IFO3249. |
| 15 | EF620455 | *Stenotrophomonas maltophilia* |
| 16 | AB353074 | *Stenotrophomonas maltophilia* MPU98. |
| 17 | AB091201 | *Frateuria aurantia* IFO13333. |
| 18 | EU685825 | *Bacillus* sp. PK-6. |
| 19 | EU624430 | *Bacillus cereus* S6-08. |
| 20 | EU248957 | *Geobacillus* sp. H6a. |
| 21 | GQ903410 | *Salimicrobium halophilum* XJSL4-1. |
| 22 | DQ358727 | *Paenibacillus zanthoxyli* Jh95. |
| 23 | AJ301830 | *Enterococcus faecium* (T) LMG 11423. |
| 24 | Ay685145 | *Selenomonas ruminantium* L6. |
| 25 | 68315145 | *Stridium botulinum* 468 toxin type |

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