*J. Microbiol. Biotechnol.* (2002), **12**(3), 382–388

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY



# FAME Analysis to Monitor Impact of Organic Matter on Soil Bacterial Populations

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Received: October 8, 2001
Accepted: March 25, 2002

**Abstract** In order to assess the effects of organic fertilizer on soil microbial community structure and diversity in the greenhouse fields, fatty acid methyl ester (FAME) was analyzed by the MIDI (Microbial ID, Inc., Newark, DE, U.S.A.) system and enumerations were performed. In relation to bacterial division of each sample, low GC Gram-positive bacteria were predominant among bacteria cultured on aerobic bacteria media. On the other hand, alpha subdivision was predominant on proteobacteria of control and OM (organic matter) 1 treated plot, and *Flavobacterium* spp. existed in OM2 plot on crystal violet media of all samples. Shannon-Weaver index (H) of OM1 plot varied most by 1.9 and 5.0 among bacteria cultured on aerobic bacteria media and crystal violet media, respectively. Our results revealed that addition of the organic wastes to soil led to a highly diverse microbial community, but the excessive amounts of organic and mineral fertilizer applied in the greenhouse fields produced excess nutrients in soil and led to simplification on bacterial populations.

**Key words:** Soil bacterial population, organic matter, microbial community, FAME analysis, microbial diversity

The addition of organic wastes to soil can be used to reclaim degraded soils by supplying plant nutrients and improving soil physical properties such as the reduction of soil bulk density and increase in total porosity and aggregates [3, 9, 21]. The long-term management of soil with organic amendments has been shown to increase the soil organic matter content and microbial biomass, population, and activity [12, 22, 29, 31, 33]. Soils managed with organic fertilizers inputs generally have larger and more active microbial populations than those managed with mineral fertilizers [7, 33]. Organic wastes such as animal manures, sewage sludge, and city refuse are rich in nitrogen and other mineral nutrients and are of high agricultural value. Therefore, application of these organic wastes to soil is one method to dispose of these abundant environmental wastes and also maintain soil organic matter.

Recently, it has been recognized that repeated cultivation, fertilizer overdoses, and unsuitable crop management practices such as monoculture have caused deficiency of organic matter and nutrient, and salt accumulation. The answer to these problems, however, may involve managing soils with organic amendments, and understanding the soil microbial biomass, activity, and diversity. Community-level microbial interactions are complex, with individual species relying on the presence, function, and interaction of many other species. Therefore, the quantitative and qualitative changes in the composition of soil microbial communities may be important and sensitive indicators of both short- and long-term changes in soil health [10, 15, 16, 19, 33].

The microbial activity in soils managed with organic matter is higher than that in soils managed with fertilizers. However, there are few studies that have dealt with the application of both organic and mineral fertilizers and effect of soil salinity on microbial diversity. Also, a limited amount of information on microbial diversity and dynamics in agricultural soil exists. Fatty acid analysis is a well-established method for analyzing microbial diversity, because it is fast and a large number of conservative characteristics can be subjected to quantitative analysis [20, 27, 32].

Although a variety of media have been developed to optimize the number of heterotrophic microorganisms cultured from environmental samples, it has been estimated that fewer than 1% of the total bacteria in soil is typically recovered by such methods [1, 34]. All methods for enumeration of bacteria have inherent limitations, however, they are useful tools in soil microbiology as long as the

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user is aware of their limitations and how they may affect the interpretation of results [13, 20, 35]. Consequently, the main purpose of our present study was to determine the effect of several organic wastes on soil bacterial populations and diversity, using culture-dependent methods and fatty acid profile analysis.

## MATERIALS AND METHODS

### Experimental Plots and Soil Sampling

This study was conducted to determine the long-term effect of applications of organic and mineral fertilizers on the soil microbial community. A total of two test plots were utilized. The first plot (OM1) was a greenhouse soil amended with organic and mineral fertilizers. Lettuce (*Lactuca sativa*; cropped in 1996, 1997, 1998, and 1999) and cabbage (*Brassica oleracea*; cropped in 1992 and 1993) were raised in the OM1 plot which had been treated with fig manure for two years, composted food wastes for three years, and N, $P_2O_5$, and $K_2O$ for one year just before sampling. The second plot (OM2) was a greenhouse soil, which contained high levels of salts (Table 1). Cucumber (*Cucumis sativus* L.; cropped in 1990 and 1991), lettuce (*Lactuca sativa*; cropped in 1992, 1997, and 1999), and hot pepper (*Capsicum annuum* L.; cropped in 1993 and 1994) were previously grown in the OM2 plot which had been treated with phosphate and nitrogen for two years, humic acid for one year, popped rice hulls for two years, and sawdust and pig manure for three years. One year before sampling, the OM2 plot was treated with N, $P_2O_5$, and $K_2O$, and popped rice hulls for two years. The control plot was bare soil on which no crops were grown for this study.

Soil samples from each plot were taken from 10 cm below the soil surface. The samples were then preserved at 4°C and were analyzed within 24 h of storage. For chemical analysis, the soil samples were dried at room temperature and passed through a 2-mm sieve. The samples were analyzed for electrical conductivity (EC) and pH in a 1:5 water-soluble extract (w/v). The organic matter content [24], the total nitrogen [4], available phosphate [26], and cation-exchange capacities [30] were determined.

### Enumeration of Bacterial Populations

The microorganisms were cultured in temperature-controlled incubators at 25°C and spread dilution plates was used to determine microbial counts. After heat treatment for 10 min at 80°C, aerobic bacteria were cultured on yeast-glucose (YG) agar (yeast extract, 3.0 g; glucose, 1.0 g; $K_2HPO_4$, 0.3 g; $KH_2PO_4$, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; cycloheximide, 0.05 g; agar, 15 g; distilled water, 1 l; pH 6.8) and spore forming bacteria on YG agar. Gram-negative bacteria were cultured by adding 5 ml of 0.1% crystal violet to 1 l of YG. Actinomycetes were grown on Humic acid vitamin (HV) agar. Fungi were grown on Rose-Bengal agar [35]. The values averaged after counting triplicate plates from each treatment were used for microbial counts, which are indicated by the colony-forming unit (CFU).

### Fatty Acid Methyl Ester (FAME) Analysis

We used 241 bacterial isolates from cultures grown on YG and crystal violet media. Random samples of 30, 47, and 46 single colonies for aerobic bacteria of control, OM1, and OM2 plots and 28, 49, and 47 single colonies for Gram-negative, respectively, were transferred from YG and crystal violet media to 10% TSBA (Tryptic Soy Broth Agar) plates. Bacterial colonies were sampled from a single plate and the same dilution for all samples of each plot until all isolates became one colony over three times. The isolates were streaked on TSBA plates, and the plates were incubated at 28°C. After 24 h, a loopfull of cell material of late-log-phase cells was harvested. Fatty acids were extracted and methylated according to the procedure described by the manufacturer (Microbial ID, Inc., Newark, DE, U.S.A.). Briefly, procedures included (1) saponification of whole-cell preparations at 100°C with 1 ml of methanolic NaOH [15% (w/v) NaOH in 50% (v/v) methanol], (2) esterification of the fatty acids at 80°C with 2 ml of 3.25 N HCl in 46% (v/v) methanol, (3) extraction of the FAMEs into 1.25 ml of 1:1 (v/v) methyl-*tert*-butyl ether-hexane, (4) aqueous washing of the organic extract with 3 ml of 1.2% (w/v) NaOH, and (5) analysis of the washed extract by gas chromatography [5, 11, 14].

Samples were analyzed with Microbial Identification System on a Hewlett-Packard 6890A gas chromatograph (Palo Alto, CA, U.S.A.). Chromatograms were compared with a large reference database of cultures previously grown on TSBA. Names of bacteria species with the most similar chromatograms are given in Table 2.

### Bacterial Diversity Analysis

Three indices were used to calculate bacterial diversity, richness, and evenness. To describe the abundance of species distribution or species richness, the following equation was used [2, 32]:

$$D=S-1/\log N$$

in which N represents the total number of isolates and S is the number of different species.

To calculate diversity in relation to the sampling size, the Shannon-Weaver index (H) was used [2, 32]:

$$H=C/N\ (N\log_{10}N-\Sigma n_i \log n_i)$$

where C is 2.3 and $n_i$ is the number of individuals in *i*th species

To calculate the evenness of the species distribution, the following equation was used [2, 32]:

**Table 1.** Chemical properties of soils used.

| | pH 1:5 | EC* dS $m^{-1}$ | OM** g $kg^{-1}$ | $P_2O_5$ g $kg^{-1}$ | Ex. Cation K | Ca | Mg | CEC[c] | $NO_3$-N g $kg^{-1}$ | T-N*** g $kg^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Cmol$^+$ $kg^{-1}$ | | | | | |
| Control | 6.1 | 0.2 | 12.3 | 196 | 0.18 | 4.89 | 1.2 | 7.8 | 0.8 | 0.9 |
| OM1[a] | 6.0 | 6.0 | 20.3 | 674 | 1.22 | 11.5 | 3.1 | 17.1 | 189.0 | 1.5 |
| OM2[b] | 5.6 | 11.4 | 28.3 | 875 | 1.89 | 15.3 | 4.6 | 24.4 | 255.7 | 2.3 |

[a]Organic matter treatment 1 plot.
[b]Organic matter treatment 2 plot.
[c]Cation exchange capacity.
*Electrical conductivity, **Organic matter content, ***Total nitrogen content.

$$E=H/\log S$$

where S is number of species.

The bacterial division was analyzed according to Olsen *et al.* [25] and the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) taxonomy.

### Statistical Analysis

A one-way ANOVA test was used as a statistical analysis of bacterial populations in different treatments in order to elucidate the effect of organic matter. The 0.05 level of significance for probability was used as the criterion of statistical significance.

## Results and Discussion

As shown in Fig. 1, microbial population counts showed that aerobic bacteria were the most abundant in the OM1 plot, while fungi counts were the highest in the OM2 plot.

In Korea, accumulation of salts is due to overdose of chemical fertilizer, unsuitable soil management, and crop rotation. These trends of soil salinity are generally observed in greenhouse fields cultivated with vegetables, which may affect not only the microbial community in the soil-root system, but also the root exudation on which PGPR (plant growth-promoting rhizobacteria) migrated toward the root [17, 23, 28].

Chemical properties of the experimental soils are shown in Table 1. The EC and organic matter content of the control plot were 0.2 dS $m^{-1}$ and 12.3 g $kg^{-1}$, respectively. In OM1, the EC was 6.0 dS $m^{-1}$, organic matter content was 20.3 g $kg^{-1}$, available phosphate was 674 mg $kg^{-1}$, and cations such as K, Ca, and Mg were 1.22, 11.5, 3.1 cmol$^+$ $kg^{-1}$, respectively. In OM2, the EC was 11.4 dS $m^{-1}$, organic matter content was 28.3 g $kg^{-1}$, available phosphate was 875 mg $kg^{-1}$, and K, Ca, and Mg were 1.89, 15.3, 4.6 cmol$^+$ $kg^{-1}$, respectively. The organic matter content in the OM2 plot was about 2 times higher than in the control. The available phosphate was 196, 674, and 875 g $kg^{-1}$ in the control, OM1, and OM2 plots, respectively. The $NO_3$-N content in the control, OM1, and OM2 was 0.8, 189.0, and 255.7 g $kg^{-1}$, respectively. Overall, these results indicate that the salt level in the OM2 plot was much higher than in the control plot, which resulted from salt accumulation of overfertilizing. The carbon-to-nitrogen ratio (C:N) was 8.20, 8.12, and 7.38 for the control, OM1, and OM2 plots, respectively. Although differences between these values were relatively small, substantial amounts of organic matter and nitrogen accumulated in the OM2 plot, which affected the changes in the microbial community and distribution.

It was of significance to observe similar population change between Gram-negative bacteria and actinomycetes (Fig. 1). The effect of soil salinity on the microbial population has earlier been reported by several workers. The sensitivity to osmotic stress was a limiting factor for the root-colonization potential of certain fluorescent pseudomonads strains [18]. These results suggest that soil salinity stress has significant effect on Gram-negative bacteria and actinomycetes composition, and the popped rice hull applied sample is not affected by buffer. However, fungi and spore forming bacteria were the highest in the OM2 plot. These results suggest that the increase of fungi

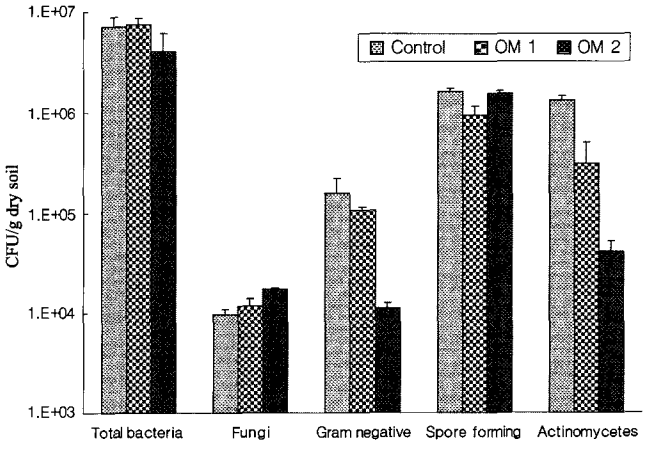


**Fig. 1.** Population changes of microorganism groups.
Total bacteria, total aerobic bacteria; Gram negative, Gram-negative bacteria; Spore forming, spore forming bacteria; OM1, 2, organic matter 1 plot and 2 plot.

**Table 2.** Numbers, identification, and diversity indices of the bacterial isolates obtained from soil samples[a].

| Isolate or index | Division | No. of aerobic bacteria isolates[c] | | | No. of Gram-negative bacteria isolates[d] | | |
|---|---|---|---|---|---|---|---|
| | | Control | OM 1 | OM 2 | Control | OM 1 | OM 2 |
| Isolates | | | | | | | |
| *Arthrobacter globiformis* | HighGC | | 2 | | | | |
| *Arthrobacter oxydans* | HighGC | 1 | | | | | |
| *Corynebacterium aquaticum* | HighGC | 1 | | | | | |
| *Cellulomonas fimi* | HighGC | 1 | 1 | 1 | | | |
| *Microbacterium liquefaciens* | HighGC | | | 1 | | | |
| *Brevibacillus agri* | LowGC | 1 | 4 | 2 | | | |
| *Brevibacillus brevis* | LowGC | 1 | 4 | 5 | | | |
| *Bacillus cereus* | LowGC | | 2 | | | | |
| *Bacillus flexus* | LowGC | 2 | | 2 | | | |
| *Brevibacillus laterosporus* | LowGC | | | 3 | | | |
| *Bacillus lentimorbus* | LowGC | | | 3 | 2 | 1 | |
| *Bacillus licheniformis* | LowGC | | 2 | | | 1 | |
| *Bacillus marinus* | LowGC | 1 | | | | | |
| *Bacillus megaterium* | LowGC | 16 | 19 | 17 | 5 | 2 | |
| *Bacillus pumilus* | LowGC | 1 | 4 | 3 | | | |
| *Bacillus psychrophilus* | LowGC | | | 1 | | | |
| *Bacillus sphaericus* | LowGC | 1 | | 2 | | | |
| *Kurthia gibosonii* | LowGC | 1 | | 1 | | | |
| *Paenibacillus gordonae* | LowGC | | 1 | 1 | | | |
| *Paenibacillus larvae* | LowGC | | 1 | | | | |
| *Paenibacillus macerans* | LowGC | | | 2 | | | |
| *Paenibacillus pabuli* | LowGC | | | | 1 | | |
| *Paenibacillus polymyxa* | LowGC | 1 | 2 | | | | |
| *Agrobacterium radiobacter* | α-Prot | | 1 | | 1 | | |
| *Brevundimonas diminuta* | α-Prot | | | | | 2 | |
| *Gluconobacter asaii* | α-Prot | | | | | 2 | |
| *Methylobacterium organophilum* | α-Prot | | 1 | | 4 | 12 | |
| *Methylobacterium zatmanii* | α-Prot | | | | 1 | 1 | |
| *Ochrobactrum anthropi* | α-Prot | | | | | 1 | |
| *Paracoccus denitrificans* | α-Prot | | | | 1 | | |
| *Phyllobacterium myrsinacearum* | α-Prot | | | | 1 | 2 | |
| *Phyllobacterium rubiacerium* | α-Prot | | | | 1 | 1 | |
| *Sphingomonas capsulata* | α-Prot | | | | 1 | | 33 |
| *Sphingomonas paucimobilis* | α-Prot | 1 | | 1 | | | |
| *Xanthobacter agilis* | α-Prot | 1 | | | 1 | 4 | |
| *Ralstonia eutropha* | β-Prot | | | | | 1 | |
| *Enterobacter cancerogenus* | γ-Prot | | | | | 1 | |
| *Pseudomonas chlororaphis* | γ-Prot | | | | 2 | | |
| *Pseudomonas fluorescens* | γ-Prot | | | | | 1 | |
| *Psychrobacter irmmobilis* | γ-Prot | | | | | 1 | |
| *Pseudomonas putida* | γ-Prot | | | | | 1 | |
| *Pseudomonas syringae* | γ-Prot | | | | | 1 | |
| *Yersinia pseudotuberculosis* | γ-Prot | | | | 1 | | |
| *Flavobacterium resinovorum* | CFB[b] | | | | | | 12 |
| NM | no match | | 2 | 1 | 6 | 14 | 2 |
| Diversity Indices | | | | | | | |
| D | | 13.3 | 13.4 | 15.4 | 13.3 | 17.4 | 2.4 |
| H | | 1.2 | 1.9 | 1.7 | 1.0 | 5.0 | 0.7 |
| E | | 1.0 | 1.7 | 1.4 | 0.9 | 4.0 | 1.5 |

[a]The bacterial division to which they belong is given. At the bottom, the diversity (D and H) and evenness (E) indices based on these data are presented (see Materials and Methods).
[b]CFB: *Cytophaga-Flexibacter-Bacteroides.*
[c]Bacterial isolates cultured on aerobic bacteria media.
[d]Bacterial isolates cultured on crystal violet media.

and spore forming bacteria density was caused by succession of populations.

The research on the effect of soil salinity on physiological activity and change of beneficial and harmful microorganism was not well carried out, and the effect of soil salinity on populations and composition of fluorescent *Pseudomonas* in plant rhizosphere has been studied [23], employing only the dilution plate counting method. Thus, in the present work, we attempted to elucidate in more detail using FAME analysis [27].

As shown in Table 2, results of FAME analysis indicate that *Bacillus* spp. were dominant among bacteria cultured on aerobic bacteria media. *Bacillus megaterium* was especially dominant in the control, OM1, and OM2 plots with 16, 17, and 18 isolates found, respectively. As for bacteria cultured on crystal violet media, the OM2 plot contained mostly *Sphingomonas* spp., while the control and OM1 plots were dominated by *Methylobacterium* spp.

The indices of species diversity relate to the number of species and the relative importance of individual species. Two major components of species diversity are species richness or variety, and evenness or equitability. A widely used measure of diversity is the Shannon-Weaver index [2]. This general diversity index is sensitive to both species richness and relative species abundance. Because the Shannon-Weaver index is sensitive to sample size, especially small sample sizes, it should be interpreted with discretion. Equitability, which can be calculated from the Shannon-Weaver index, is independent of sample size. Bacterial diversity, richness, and evenness indices of each experimental plot are shown in Table 2. The Shannon-Weaver index (*D*) and richness index (*H*) of the control, OM1, and OM2 plots among bacteria cultured on aerobic bacteria media had similar values: 13.3, 13.4, 15.4 and 1.2, 1.9, 1.7, respectively. The *D* and *H* among bacteria cultured on crystal violet media from the control, OM1, and OM2 plots were 13.3, 17.4, 2.4 and 1.0, 5.0, 0.7, respectively, and were the highest in OM1 plot. Although the EC value of the OM1 plot was 6.0 dS $m^{-1}$, the OM1 plot, which was applied animal manure and food wastes, rose in diversity. This result might have been due to addition of some organic matter. Even though the EC value of the control plot was 0.2 dS $m^{-1}$, the lower diversity might be due to the soil, to which carbon sources were not added, and did not give maximum growth, because carbon sources are necessary for microbial composition [8, 33]. The diversity indices of proteobacteria showed much difference, where an aberration with simplification of proteobacteria in the OM2 plot could occur. However, the aberration was few or nothing by three replications.

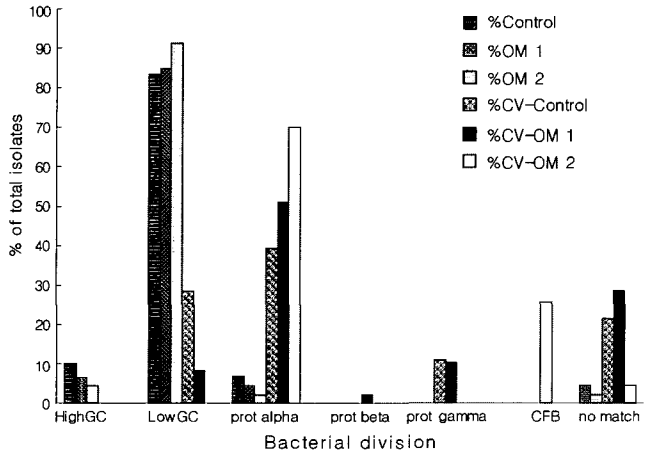


**Fig. 2.** Distribution of the isolates obtained from soil samples.
Prot alpha, α-Proteobacteria; prot beta, β-Proteobacteria; prot gamma, γ-Proteobacteria; CFB, *Cytophaga-Flexibacter-Bacteroides*; CV-, bacteria cultured in crystal violet media; OM1,2, organic matter 1 plot and 2 plot.

According to the results analyzed by ANOVA, no significant difference in aerobic bacteria was observed. However, because of lack of raw data on the normal distribution of Gram-negative bacteria, statistical analysis could not be attempted. As shown in Table 2, noticeable findings in OM2 were observed.

Figure 2 shows distribution percentages of the isolates obtained from various bacterial divisions of each sample. Here, low GC Gram-positive bacteria were predominant among total aerobic bacteria of all samples. On the other hand, alpha proteobacteria were predominant on proteobacteria of the control and OM1 plots. CFB just existed on proteobacteria of the OM2 plot. The bacterial division was carried out using 16S rDNA [6, 25].

Figure 2 shows that most of the microbial isolates are aerobic bacteria belonging to the low GC Gram-positive bacteria, which are representative of *Bacillus*, *Brevundimonas*, *Kurthia*, and *Paenibacillus*, and the *Methylobacterium* of the alpha subdivisions in proteobacteria, which are the representative groups of Gram-negative bacteria such as *Agrobacterium*, *Brevundimonas*, *Gluconobacter*, *Methylobacterium*, *Ochrobactrum*, *Paracoccus*, *Phyllobacterium*, *Sphingomonas*, and *Xanthobacter*. This result revealed that Gram-positive bacteria were predominant in the soil and *Bacillus* species were typical soil bacteria among them. Twelve bacterial isolates were related to the deeply rooted phyla such as the CFB (*Cytophaga-Flexibacter-Bacteroides)* group, but 25 bacterial isolates could not be assigned to any described taxa (no match).

The addition of the organic wastes to the soil led to an increase in microbial populations and microbial diversity, compared to soils that did not receive any treatment and received inorganic fertilizer. However, soils receiving overdose of organic fertilizer showed decreased microbial response. Soil salinity increased significantly with increased application of organic fertilizer, and high salinity in the soil is known to reduce the diversity of microbial organisms [23]. Thus, the addition of excess organic matter in the soil does not

necessarily influence microbial ecosystems. Such applications may have adverse effects on the microbial ecosystem in the soil.

## Acknowledgment

This paper was supported by Konkuk University in 2001.

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