REVIEW
Bacterial Community Shift in Field Soil Caused by Annual Application of Liquid Livestock Feces

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Abstract
The microorganisms concerned with a higher ratio of biological nitrogen turnover in upland field soils applied with large amounts of raw liquid livestock feces were searched for using a newly developed genetic analysis system and method. In upland field soils, the flora of proteolytic bacteria, denitrifying bacteria, and bacteria isolated on peptone-polymyxin medium changed in relation to the amount of the applied liquid feces (120 t ha–1 y–1 and 600 t ha–1 y–1). The isolated proteolytic Serratia marcescens, denitrifying Salmonella/E. coli, and non-Bacillus polymyxin B-resistant bacteria were non-indigenous soil bacteria typically found in the soil applied with raw feces, and their numbers were estimated to be over 105 CFU g–1 dry soil after 2 months of the application. They were supposed to have contaminated the field soils from bacteria present in the raw feces that survived for a while in the fields.

Discipline: Soil, fertilizers and plant nutrition
Additional key words: denitrifying bacteria, fecal coliforms, polymyxin B-resistant bacteria, proteolytic bacteria

Introduction
In the southern region of Kyushu Island, where a number of livestock farms have occupied a considerably limited area, the application of large amounts of livestock feces to the considerably limited field area of forage crops has inevitably caused NO3–1 leaching, which is suspected to be polluting underground aquifers5. As the regulation of the soil nitrogen cycle is becoming a serious social demand, experimental fields had been founded in 1985 where large amounts of raw liquid livestock feces (600 t ha–1 y–1, 120 t ha–1 y–1) had been applied every year, and NO3–1 leached to subsurface soil had been monitored6 in order to find out regulation methods to decrease NO3–1 leaching.

When we had started microbial analyses of these field soils on 1994, the ecology of indigenous microorganisms in field soils remained not clearly demonstrated. Enormous microbial diversity (over 10,000 different species in g–1 dry soil) was one of the factors disturbing exact analysis in field soil as well as the higher ratio of unculturable microorganisms (over 99% of total)7. We had started to construct a system and method, by which phylogeny of diverse kinds of bacteria could easily be affiliated.

1. Development of a new system and method
An essential component of the system was a new program, by which a database having a DNA region between the same reverse and forward primers used for the sample analysis (post-amplification sequence files)
was re-edited from DNA sequence files in DDBJ, EMBL, GenBank and RDPII. The database for 41f/1066r primers included 4,370 post-amplification sequence files, consisting of 576 bacterial genera, 143 uncultured bacteria, and 34 unidentified bacteria. By using a new type of electrophoreses system (microchip electrophoresis system, Hitachi Chemical Co., Ltd., Hitachi Electronic Engineering Co., Ltd.), it became possible to assign microbial phylogeny in a short time and by relatively lower cost compared to DNA sequence determination (Fig. 1). The feasibility as a phylogenetic assignment method for bacteria was verified by comparing the results with those by comparative analyses of the 16S rDNA sequence, by BIOLOG, or the other phenotypic characterizations using 25 reference strains, proteolytic bacteria (73 strains), and denitrifying bacteria (102 strains).

2. Biochemical activity of field soils
The bacteria were isolated from three upland andosol fields at Miyakonojyo, Miyazaki, Japan; a field under a 10-year monoculture of corn applied with raw liquid livestock feces (600 t ha\(^{-1}\) y\(^{-1}\)), the same field applied with raw liquid livestock feces (120 t ha\(^{-1}\) y\(^{-1}\)), and a fallow land without liquid livestock feces. Within one to...
three weeks after application of the raw feces, the soil caseinase activity increased to the maximum (120 t, 1,018 pKat; 600 t, 2,141 pKat g\(^{-1}\) dry soil), and then decreased to the original level (Fig. 2)\(^{17}\). The soil caseinase activities of the 600 t application fields were several times higher than those of the other field soils\(^{17}\).

3. Community shift of proteolytic bacteria\(^{10,11}\)

Proteolytic bacteria were isolated on azocoll agar plates\(^3\) and selected by a gelatin liquefaction test\(^{13}\), which produced extracellular protease 10 times more than bacteria isolated from albumin agar plates\(^{12}\). The phylogenetic assignments of proteolytic bacteria in field soils are summarized in Fig. 3. Our previous results\(^{13-15}\) suggested that proliferation of Bacillus spp. might define the type of soil protease, and that these bacteria might be the source of soil proteases in paddy field soils.

The ratio of Bacillus spp. became lower while those of γ-proteobacteria (including Pseudomonas sp., Serratia marcescens, and Xanthomonas spp.) became higher in relation to the amount of the feces applied\(^1\). The present results suggested that Bacillus spp. might also be the major proteolytic bacteria in fields without liquid livestock feces, and suggested that Gram-negative bacteria, such as Pseudomonas sp., Xanthomonas sp., Stempthomonas maltophilia, and Serratia marcescens might be other candidates of the sources of soil protease in field soils applied with the feces (Fig. 3)\(^{11}\). The community difference of proteolytic bacteria in the applied fields might be the factor responsible for increasing soil caseinase activity.

4. Community shift of denitrifying bacteria\(^{10}\)

Denitrifying bacteria were isolated from the positive tubes in each serial dilution series of the most probable number (MPN) method. The phylogenetic assignments of denitrifying bacteria in field soils were summarized in Fig. 4. In the field without liquid livestock feces, β-proteobacteria (Burkholderia sp. and Ralstonia sp.) and γ-proteobacteria (Pseudomonas sp.) were the major isolated denitrifying bacteria. The ratio of β-proteobacteria (including Burkholderia sp. and Ralstonia sp.) became lower, while that of γ-proteobacteria (mainly including Pseudomonas sp.) became higher in relation to the

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![Fig. 3. Change of proteolytic bacterial flora in relation to the amount of liquid livestock feces applied](image-url)

![Fig. 4. Change of denitrifying bacterial flora in relation to the amount of liquid livestock feces applied](image-url)
amount of feces applied (Fig. 4). Especially, the ratios of  
*P. putida* and Enterobacteriaceae were high in the 600 t 
ha⁻¹ application field, and some of the Enterobacteriaceae 
were affiliated with *Salmonella* spp. or *E. coli*.

5. Community shift of bacteria isolated on peptone-
polymyxin medium

Bacteria were also isolated on peptone-polymyxin 
(PP) agar plate¹,¹⁷. This is a selective medium for *Bacillus* 
spp.¹⁶. The phylogenetic assignments of bacteria iso-
lated from PP agar plates are summarized in Fig. 5. All 
the isolates from the field without the feces were affili-
ated with members of *Bacillus* spp., while the ratios of *Bacillus* 
spp. became lower and those of the other bacte-
rial groups became higher in relation to the amount of the 
feces applied (Fig. 5)¹⁷. As most of the bacteria except 
for *Bacillus* spp. were susceptible to polymyxin B¹, the 
PP agar was selective to *Bacillus* spp. Therefore, the 
non-*Bacillus* bacterial isolates were suggested to be anti-
biotic (polymyxin B)-resistant bacteria.

6. Enumeration of the non-indigenous soil bacteria

Numbers of proteolytic *S. marcescens* were esti-
mated by PCR using DNA primer designed from extra-
cellular metalloprotease of proteolytic *Serratia marcescens* isolated from the field soils¹⁹. From an 
amount of the amplified protease gene in the mixed DNA 
extracted from the field soils, the numbers were estimated 
according to the calibration curves made by pure bacterial 
DNA of *S. marcescens* (0 t area, 4.2 × 10⁴ cell g⁻¹ soil; 
120 t, 15.0 × 10⁴ cell g⁻¹ soil; 600 t, 60.0 × 10⁴ cell g⁻¹ soil). MacConkey agar and Desoxycholate agar were 
used to indicate contamination of fecal coliform such as *Salmonella* sp., *Shigella* sp., *Proteus* sp., and *E. coli* from 
human or animal feces. Their numbers were estimated to 
be 2.6 × 10⁶ CFU g⁻¹ dry soil in the 600 t field, and 1.6 × 
10⁶ CFU in the 120 t field by MacConkey agar, and 4.5 × 
10⁶ CFU in the 600 t field, and 0.8 × 10⁵ CFU in the 120 t 
field by Desoxycholate agar (Fig. 6).

The number of non-*Bacillus* polymyxin B-resistant 
bacteria was estimated from the difference between the 
total number of polymyxin B-resistant bacteria and the 
sum of vegetative cells and the spores of *Bacillus* spp.¹⁷. 
They rapidly increased after the application of the feces 
in the 600 t field (April 17, 3.71 × 10⁷ CFU g⁻¹ dry soil; 
April 24, 25.8 × 10⁷ CFU; May 8, 20.93 × 10⁷ CFU; and 
May 22, 6.98 × 10⁷ CFU), while the numbers in most of 
the other soils were negative values, or under 1.0 × 10⁷ 
CFU (Fig. 7)¹⁷. They were supposed to be contaminated 
by bacteria from raw liquid livestock feces that colonized 
and survived for a while in these field soils for the fol-
lowing reasons¹⁷; (1) the denitrifying *E. coli/Salmonella* 
spp., are typical inhabitants in human or animal feces and 
were only isolated from 120 t and 600 t field soils, (2)
proteolytic *S. marcescens* is an opportunistic pathogen which was also typically isolated from these field soils and was occasionally detected in numbers irrelevant to the annual accumulation of applied feces, and (3) non-*Bacillus* polymyxin B-resistant bacteria were never detected in the other 0 t field soil\(^{15,16}\).

7. **Prospect for development of the system and method**

At present, various kinds of manures and composts originating from diverse biological wastes, e.g., livestock feces, and sewage sludge, are introduced into field soils under the expectation to be used as organic fertilizers. The field soils applied with these manures and composts, containing raw wastes, might not only become sources of fecal coliforms such as *Escherichia coli*, *Salmonella* sp., *Shigella* sp., and *Vibrio* sp., but also antibiotic resistant bacteria, which are suspected to gain antibiotic resistance during feeding.

As cultureability or higher proliferation rate are indispensable properties for antibiotic resistance or pathogenicity of the fecal coliforms, the culture independent approaches such as DGGE or TRFLP are not suitable to distinguish such a minority of culturable bacteria (under 1% of total) from the majority of unculturable bacteria (over 99% of total) in field soil. By direct counting approach such as FISH, culturable and unculturable microorganisms\(^7\) could not be differentiated.

By combination of the most probable number method (MPN) and our system and method\(^9\), diverse kinds of these cultureable non-indigenous bacteria can be qualified and quantified without isolation\(^9\).

**Reference**


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**Fig. 7.** The estimated number of non-*Bacillus* polymyxin B resistant bacteria in field soils with liquid livestock feces applied.


