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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 10^5 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 NO_3^{-1} leaching, which is suspected to be polluting underground aquifers[5]. 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 NO_3^{-1} leached to subsurface soil had been monitored[6] in order to find out regulation methods to decrease NO_3^{-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)

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was re-edited from DNA sequence files in DDBJ, EMBL, GenBank and RDPII[18]. 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[10,11]. 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[2], or the other phenotypic characterizations[10,11] using 25 reference strains, proteolytic bacteria (73 strains)[10,11], 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[17]. Within one to

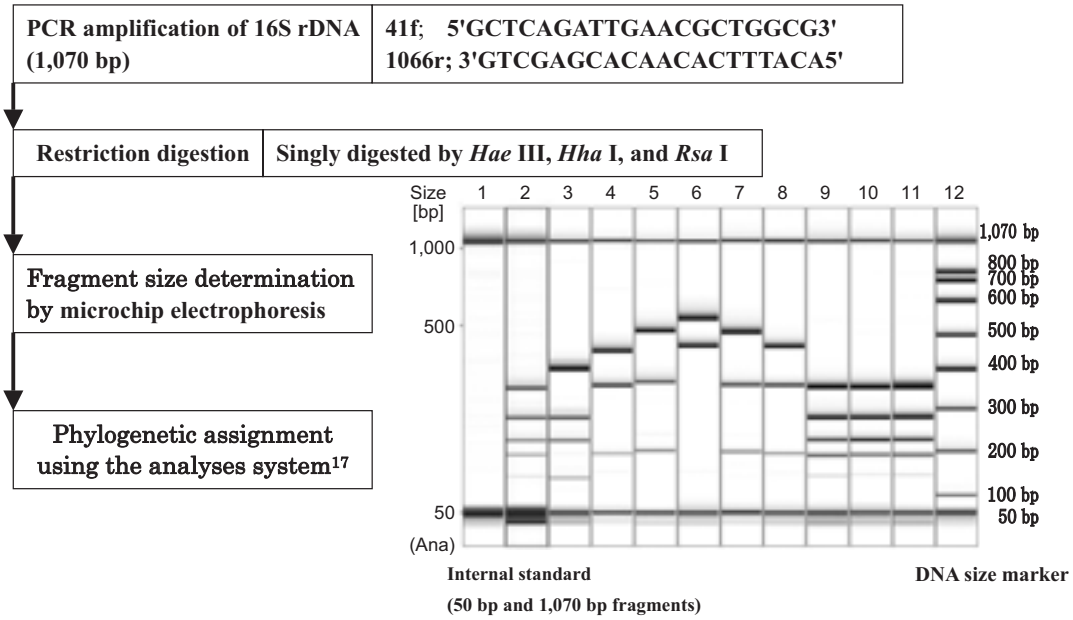

Fig. 1. Identification of soil bacteria using microchip electrophoresis system and system for searching for relationships between sequences in genes[17]

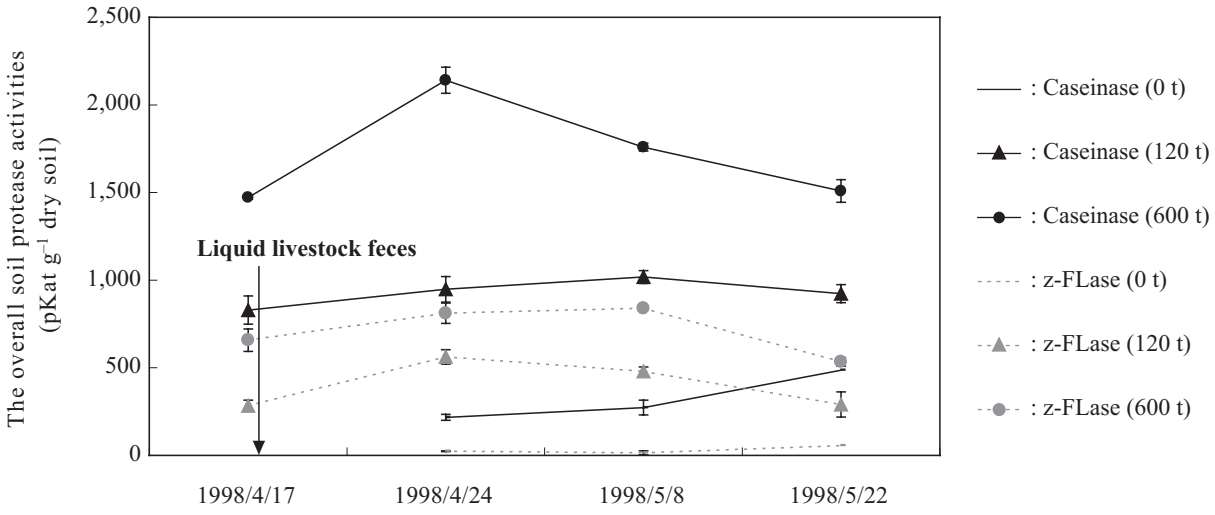

Fig. 2. Change of protease activities in the 600 t application field (●), the 120 t application field (▲), and the 0 t area (-)
Solid line indicates the overall soil caseinase activity and dashed line indicates the overall soil z-FLase activity. Bars shows 95% confidence limit.

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[11]. 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., *Stemptrophomonas 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

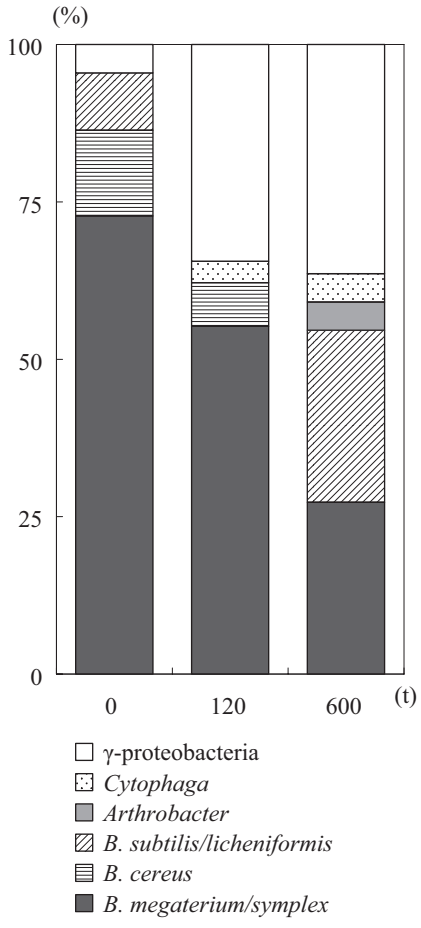

Fig. 3. Change of proteolytic bacterial flora in relation to the amount of liquid livestock feces applied

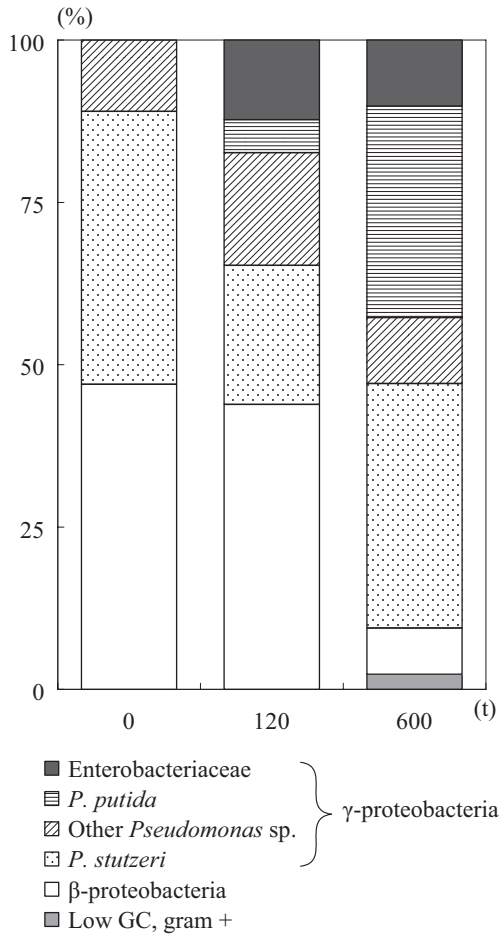

Fig. 4. Change of denitrifying bacterial flora in relation to the amount of liquid livestock feces applied

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 plate[1,17]. This is a selective medium for *Bacillus* spp.[16]. The phylogenetic assignments of bacteria isolated from PP agar plates are summarized in Fig. 5. All the isolates from the field without the feces were affiliated with members of *Bacillus* spp., while the ratios of *Bacillus* spp. became lower and those of the other bacterial 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 B[4], the PP agar was selective to *Bacillus* spp. Therefore, the non-*Bacillus* bacterial isolates were suggested to be antibiotic (polymyxin B)-resistant bacteria.

6. Enumeration of the non- indigenous soil bacteria

Numbers of proteolytic *S. marcescens* were estimated by PCR using DNA primer designed from extracellular metalloprotease of proteolytic *Serratia marcescens* isolated from the field soils[19]. 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, 4.2×10^4 cell g^{-1} soil; 120 t, 15.0×10^4 cell g^{-1} soil; 600 t, 60.0×10^4 cell g^{-1} soil)[8]. 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 2.6×10^6 CFU g^{-1} dry soil in the 600 t field, and 1.6×10^6 CFU in the 120 t field by MacConkey agar, and 4.5×10^5 CFU in the 600 t field, and 0.8×10^5 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, 3.71×10^7 CFU g^{-1} dry soil; April 24, 25.8×10^7 CFU; May 8, 20.93×10^7 CFU; and May 22, 6.98×10^7 CFU), while the numbers in most of the other soils were negative values, or under 1.0×10^7 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 following reasons[17]; (1) the denitrifying *E. coli/Salmonella* sp., are typical inhabitants in human or animal feces and were only isolated from 120 t and 600 t field soils, (2)

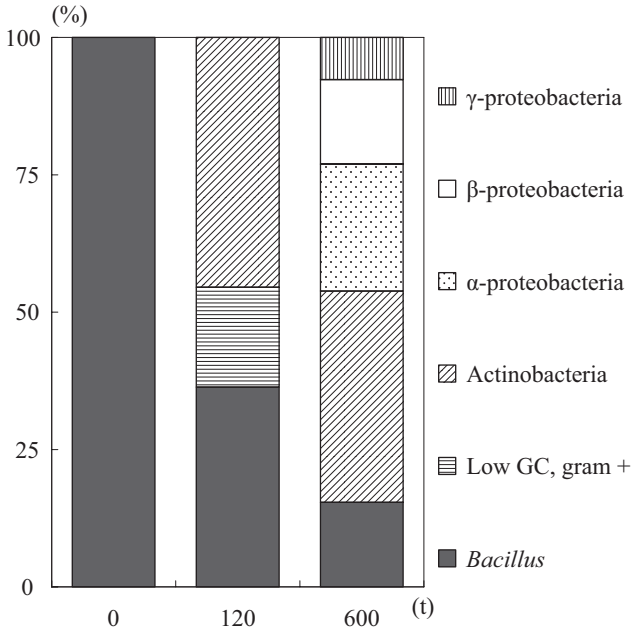

Fig. 5. Change of polymyxin B resistant bacterial flora in relation to the amount of liquid livestock feces applied

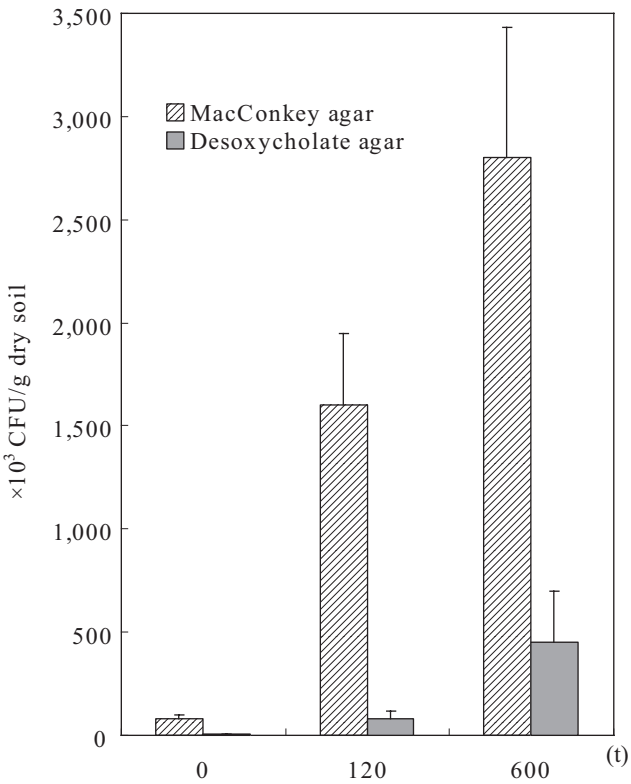

Fig. 6. Estimated numbers of the fecal coliform bacteria in the fields 2 month after application of liquid livestock feces

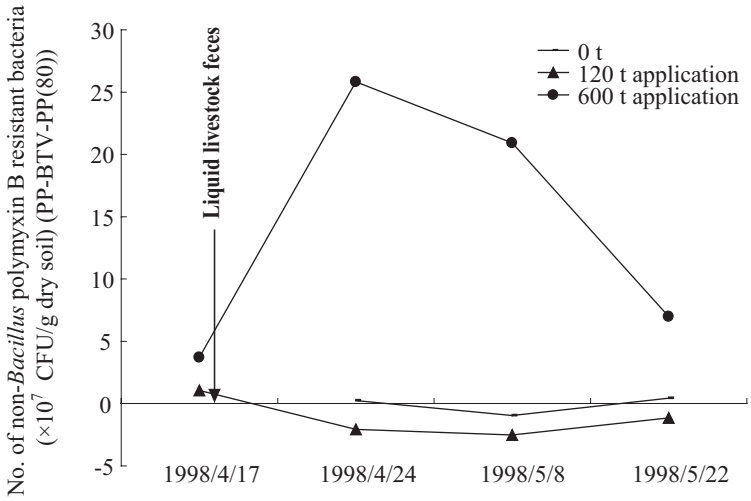

Fig. 7. The estimated number of non-*Bacillus* polymyxin B resistant bacteria in field soils with liquid livestock feces applied

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 cultureablity 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[18], diverse kinds of these cultureable non- indigenous bacteria can be qualified and quantified without isolation[9].

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