# Original Article

# Application of multiple enzyme restriction fragment length polymorphism analysis and microchip electrophoresis for estimation of antibiotic-tolerant bacterial group

Katsuji Watanabe*

*National Agricultural Research Center for Kyushu Okinawa Region, Suya 2421, Koshi, Kumamoto, 861–1192 Japan*

A combined method of microchip electrophoresis and multiple enzyme restriction fragment length polymorphism analysis, by which bacterial phylogeny was estimated by comparing the measured multiple enzyme restriction fragment (MERF) to that of the theoretical MERF of various kinds of bacteria, was adapted to monitor an antibiotic-resistant bacterial group in field soils. One hundred and ninety bacteria showing polymyxin B tolerance were newly isolated from experimental field soils where a large amount of liquid livestock feces (600 t/ha/year, 120 t/ha/year) has been applied annually, and neighboring area where feces were not applied (0 t area). In the 0 t area, most bacteria grown on PP agar was *Bacillus* spp. (80%), which decreased to 21.7% in the 120 t field, and 15.6% in the 600 t field. As the percentages gram negative bacteria, Cytophagales, and Spirochaetales increased to 10.0% in the 120 t field, and 33.3% in the 600 t field, and 7 isolates from the 120 t field and 18 isolates from the 600 t field were ordinary bacteria inhabiting in animals and feces, they originated from livestock. As polymyxin B is bactericidal for gram-negative bacteria, some of the gram-negative bacteria isolated from both the feces-applied fields were supposed to be polymyxin B-resistant bacteria. © Pesticide Science Society of Japan

*Keywords*: polymyxin B resistant bacteria, field soil, livestock feces, microchip electrophoresis, multiple enzyme restriction fragment length polymorphism analysis.

## Introduction

The application of antimicrobial agents has inevitably produced resistant microorganisms, and the breakpoint based on $MIC_{50}$ (index of antibiotic resistance) for each hazardous clinical isolates has normally been used as an index for chemotherapy.[1,2] This method is insufficient to predict an outbreak of new resistant bacteria or to evaluate risk to the environment where various resistant microorganisms are freely transmitted.[3,4]

Bactericidal agents used for crop protection in agriculture are mainly converted from the same compounds used for chemotherapy in clinical use or chemotherapy for livestock, and used as antibiotic growth promoters for livestock (AGP). When bacteria, which have already acquired antibiotic resistance during usages, are transmitted to fields, the application

of bactericidal agents for crop protection might not only be effective, but might specifically promote the proliferation of resistant bacteria in the field.[3–6] A method to evaluate an outbreak of antibiotic resistance in the field is required not only to search for effective antibiotics for crop protection but also to evaluate the risk for antibiotic resistant bacteria.[3–6]

At present, various kinds of manure and compost originating from diverse biological waste, which sometimes includes antibiotic resistant bacteria,[3–7] *e.g.*, livestock feces, organic manure, and sewage sludge, are introduced into field soils to be used as organic fertilizers or under a governmental policy aiming to promote recycling and re-use limited organic waste.

In this manuscript, our newly developed phylogeny estimation system, based on multiple enzyme restriction fragment length polymorphism,[8,9] was used to confirm whethere the method is suitable to monitor antibiotic resistance in the field.

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* To whom correspondence should be addressed.
  E-mail: katsuji@affrc.go.jp

## Materials and Methods

### 1.  Field soil and isolation of bacteria showing resistance to polymyxin B

Bacteria having polymyxin B tolerance were newly isolated from two upland andosol experimental field soils in Miyakonojyo, Miyazaki, Japan, on April 17, 1998 and June 18, 2004; a field under 10-year monoculture of corn supplied with liquid livestock feces (600 t/ha/year), and the same field supplied with liquid livestock feces (120 t/ha/year) twice a year from 1985. The feces was applied to the soil surface and mixed to a 10 cm depth on April 18.[10] Bacteria were also isolated from an adjoining andosol grassland, and a neighboring area, which had been left fallow for over 10 years, where the application of feces had no influence on microbial flora.

The number of total *Bacillus* spp. was estimated by the dilution plate method using peptone-polymyxin medium (PP medium; 10 g proteose peptone (Difco, Sparks MD), 5 g NaCl, 15 g agar and 5 mg polymyxin B sulfate per liter, pH 7.0.[11] After 3 days incubation at 30°C, bacteria were isolated and re-isolated using the same PP medium. Culturable bacteria were also counted using MacConkey Agar (DAIGO, Tokyo Japan) plates, and DesoxyCholate Agar (DAIGO, Tokyo, Japan) plates.

### 2.  MERFLP of amplified 16S rDNA

Chromosomal DNA of isolate was prepared as described previously and purified by conventional methods.[12] Amplification of 16S rDNA was according to a previous study[8] using the V2 forward primer (41f; 5′GCTCAGATTGAACGCTG-GCG 3′, corresponding to 22–41 positions of the 16S rRNA gene of *E. coli*[13]), and the V6 reverse primer (1066r; 3′GTC-GAGCACAACA CTTTACA5′ corresponding to the 1066–1085 positions).[14,15] The PCR product (10 $\mu$l) was separately digested using 10 units of the restriction enzyme, *Hae*III or *Hha*I or *Rsa* I (Takara Bio Co., Ltd., Shiga, Japan) in Low salt buffer solution (10x Low salt buffer, Takara Bio Co., Ltd.).

### 3.  Fragment length measurement by microchip electrophoresis system

Fragment lengths of 12 samples were automatically measured using a microchip electrophoresis system within 7 min (Cosmo-i SV1200; Hitachi Electronics Engineering Co., Ltd., Tokyo, Japan). Measurement was performed basically according to the manufacturer's manual. Sample preparation was modified and internal standards were newly prepared in order to measure the sizes and intensities of all the restriction fragments as acuurately as possible. The sample was diluted using de-ionized water (10-folds Low salt buffer) before loading onto an i-tip DNA (IC-1000, Hitachi Chemical Industry Co., Ltd., Tokyo, Japan) disposable electrophoresis capillary filled with gel to decrease salt concentration (data not shown). A DNA fragment (65 bp; 5′GCTCAGATTGAACGCTGGCGA-CATTTCACAACACGAGCTGGCTCAGATTGAACGCTG-

GCGACATT3′) was used as the lower internal standard, and the PCR product amplified by 41f/1066r primers was used as the upper internal standard after separation by agarose gel electrophoresis and purification by a Qiaex II Gel extraction kit (Qiagen, Maryland USA), which was co-applied with samples. To calibrate fragment sizes, a 100 bp DNA Ladder marker (100 bp to 1500 bp; Promega, Madison WI) was used.

### 4.  Theoretical multiple enzyme restriction fragment length (MERFL) database used for estimation

The theoretical MERFL database was edited using a system developed by Watanabe and Okuda,[9] as described previously.[8] For 41f/1066r primers, 4,370 sequence files having a DNA region between the same reverse and forward primers used for sample analysis (post-amplification sequence files), which consisted of 576 bacterial genera, and 143 uncultured and 34 unidentified bacteria, were mainly re-edited from 20,952 small subunit rRNA files in RDP II release 7.01[16] under 5-base mismatches at both primer annealing sites. The theoretical MERFL database was constructed from 4,370 post-amplification sequence files for 41f/1066r primers. Restriction sites having unread bases were ignored and the data of the restriction enzymes, DNA primers, and each fragment length were automatically obtained from the post-amplification sequence files.

### 5.  Phylogenetic estimation and representation by the developed system[8,9]

The pairwise distance ($D_{AB}$) between the measured RFLP(A) and the theoretical RFLP(B) was calculated by the following equation; $D_{AB}=1-2N_{AB}/(N_A+N_B)$, where $N_A$ and $N_B$ were the numbers of fragments of each RFLP and $N_{AB}$ was the number of shared fragments of the same size within an error range, according to Nei and Li.[17] The pairwise distance of the MER-FLPs ($D_{ABME}$) was an average of all the $D_{ABS}$ for used restriction enzymes. Similarity (%) was $(1-D_{ABME})\times100$ (Tables 2–5).

In the similarity search process, the data processing described in the previous paper[6] was basically used and fragments smaller than 100 bp were eliminated from both theoretical and measured MERFLs, because there were many fragments smaller than 100 bp, which were misread over 10% allowance limit of the measuring error.[6] The following data processing was also used; fragments originating from a minor gene, unselectively amplified DNA or noise were differentiated from fragments originating from homogeneous 16S rDNA and eliminated as follows; until the sum of the fragment sizes did not exceed the original 16S rDNA length (1070 bp±measuring error), the length of the selected fragments, which had higher relative mole concentrations (ratio of fluorescent intensity to fragment size), was summarized.

The allowance limit for measuring error was set at 10% in the first analysis, and then gradually increased to 16% until completely identical to theoretical MERFL (100% similarity)

(Tables 2–5). If the completely identical theoretical MERFL could not be reached, the combinations of restriction enzymes used for analysis were changed (Tables 2–5). As to the measured MERFL which had no completely identical theoretical MERFL, the theoretical MERFL having the highest similarity to the measured MERFL is shown in Table 2–5.

## Results

### 1. Culturable bacterial numbers

Bacterial numbers counted on PP medium in all fields were higher than those counted on MacConkey Agar plates and Desoxycholate Agar plates (Table 1). The numbers in the 120 t field (17.3×10⁶ CFU/g dry soil) and in the 600 t field (17.9×10⁶ CFU) were higher than those in the uncultivated soil (0.2×10⁶ CFU),[18] cultivated upland soil (1.2×10⁶ CFU),[18] and paddy field soil (0.9×10⁶ CFU).[19]

### 2. Phylogenetic estimation by MERFLP

Thirty-nine bacterial isolates, which were affiliated to gram-negative bacteria consisting of $\alpha$ proteobacteria (10 isolates), $\beta$ proteobacteria (4 isolates), $\gamma$ proteobacteria (12 isolates), $\delta$ proteobacteria (4 isolates), Cytophagales (8 isolates), and Spirochaetales (1 isolate), are summarized in Table 2. Nine isolates (23.1%) were completely identical to the corresponding measured MERFL (100% similarity) by permitting a 10% allowance limit of the measuring error, and 13 isolates reached 100% identity by re-setting the allowance limit to a higher value (56.4%).

Eighty-one bacterial isolates, which were affiliated to be Actinobacteria, consisting of Actinomycetaceae (3 isolates), Bifidobacteriaceae (9 isolates), Cellulomonadaceae (3 isolates), Corynebacteriaceae (7 isolates), Kineococcus group (3 isolates), Microbacteriaceae (5 isolates), Micrococcaceae (19 isolates), Nocardioidaceae (3 isolates), Nocardiaceae (15 isolates), Streptomycetaceae (6 isolates), Pseudonocardiaceae (2 isolates), and other Actinobacteria (6 isolates), are summarized in Tables 3 and 4. Twenty-one isolates (25.9%) were completely identical to the corresponding measured MERFL (100% similarity) by permitting a 10% allowance limit of the measuring error, and 18 isolates reached 100% identity by re-setting the allowance limit to a higher value (48.2%).

Seventy isolates, which were affiliated to low GC content gram-positive bacteria consisting of *Bacillus* spp. (53 isolates), its related genera (6 isolates), and the other GC content gram-positive bacteria (11 isolate), are summarized in Tables 4 and 5. Thirty-six isolates (51.4%) were completely identical to the corresponding measured MERFL (100% similarity) by permitting a 10% allowance limit of the measuring error, and 12 isolates reached 100% identity by re-setting the allowance limit to a higher value (68.6%).

### 3. Analysis of bacterial flora

Bacterial isolates from the 0 t area comprised *Bacillus* spp. (80.0%), the other low GC content gram-positive bacteria

**Table 1.** Culturable microbial numbers counted on peptone polyxin medium (PP), MacConkey agar, and desoxycholate agar plates of upland field soils applied with liquid livestock feces (600 t field, 120 t field), and soil without feces (0 t area)

| | CFU/g dry soil±95% confidence limit | | |
| --- | --- | --- | --- |
| | PP medium (×10⁶) | MacConkey Agar (×10⁴) | Desoxycholate Agar (×10³) |
| 0 t area | 2.7±0.9 | 7.9±2.0 | 4.2±3.2 |
| 120 t field | 17.3±5.4 | 159.7±34.3 | 79.1±38.3 |
| 600 t field | 17.9±2.8 | 282.8±63.1 | 453.7±243.2 |

(Low GC+) (2.5%), Actinobacteria (12.5%), and Proteobacteria (5.0%) (Fig.1). The percentages of *Bacillus* spp. were higher than those isolated from the 120 t field (21.7%), and the 600 t field (15.6%) (Fig. 1). The percentages of Actinobacteria and proteobacteria were lower than those from the 120 t field (58.3%, 8.3%), and the 600 t field (45.6%, 25.6%) (Fig. 1). Cytophagales or Spirochaetales were only isolated from both feces-applied fields (Fig. 1).

Phylogenetic estimation by MERFLPs indicated that the following 7 isolates from the 600 t field were of animal origin: 60-2 26 *Brucella canis* (L37584), B6007 *Eikenella corrodens* (Eik.corro3), 60-2 37 *Prevotella bivia* (L16475), 60-2 46 *Prevotella buccalis* (L16476) (Table 2), and 60-1 34, 60-20 6, 60-20 2 *Rothia dentocariosa* (M59055) (Table 3), and the following 11 isolates were of feces origin: 60-2 09 *Bacteroides distasonis* (M86695), 60-2 20 *Spirochaeta stenostrepta* (M88724) (Table 2), 60-1 2, 60-1 35, 60- 2 33, 60-2 34, 60-2 32, 60-1 6 *Bifidobacterium adolescentis* (M58729) or *B. asteoides* (M58730) or *B. breve* (M58731) (Table 3), 60- 2 30,
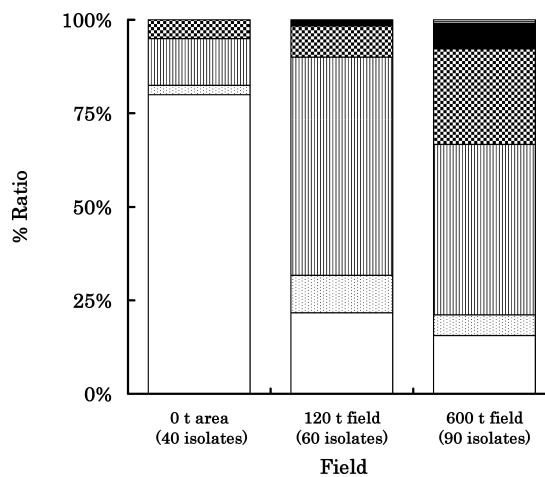


**Fig. 1.** Percentage (%) of bacterial groups isolated from the 0 t area, 120 t field, and 600 t fields. Percentage of the constituted groups of bacteria Bacillus spp. (□), Low GC content gram positive bacteria (▦), Actinobacteria, (▥) Proteobacteria (▨), Cytophagales (■), and Spirochaetales (▤).

**Table 2.**   Affiliation of 39 bacteria newly isolated using PP medium (gram-negative bacteria) by MERFLP

| | Strain No.[a] | Restriction enzymes[b] | Similarity (%)/ Allowance limit[c] | | Affiliation (Accession number, name)[d] and origin[e] |
|---|---|---|---|---|---|
| *α* Proteobacteria (10 isolates) | 60-2 26 | Ha, Hh, A | 100 | 10 | *Brucella canis* (L37584) (A) |
| | 12-1 11 | Ha, R | 100 | 10 | *Agrobacterium tumefaciens* (D14504); *A. rhizogenes*; (X67224); |
| | 12-1 18 | Ha, R | 100 | 10 | *Phyllobacterium rubiacearum* (D12790); *Chromatium okenii* (Y12376) |
| | 60-1 11 | Ha, Hh, A | 77 | 10 | *A. tumefaciens* (M11223) |
| | 60-2 03 | Ha, Hh, A | 85 | 10 | *Afipia broomeae* (U87760); *Bradyrhizobium lupini* (U69637) |
| | 60-1 10 | Ha, Hh, A | 75 | 10 | *Rhodopseudomonas palustris* (M59068) |
| | 60-1 20 | Ha, Hh, A | 72 | 10 | *Rhodomicrobium vannielii* (M34127) |
| | B60-01 | Ha, Hh | 73 | 10 | *Rhizobium huautlense* (Rhb.huautl) |
| | 60-2 08 | Ha, R | 75 | 10 | *Aquaspirillum itersonii* (AB000478) |
| | B60-012 | Ha, Hh | 90 | 10 | *Aminobacter niigataensi* (AJ011761); *Pseudaminobacter salicylatoxi* (Pab.slcylt); *Sinorhizobium fredii* (Srh.fredi5); *Rhizobium giardinii* (U86344); *Duganella zoogloeoides* (Dg.zooglo5) |
| *β* Proteobacteria (4 isolates) | 60-1 8 | Hh, R | 100 | 11 | *Acidovorax avenae* (AF078761, AF137504); *A. delafieldii* (AF078764) |
| | 60-1 32 | Ha, Hh | 100 | 10 | *Herbaspirillum rubrisubalbican* (AF137508); *H.frisingense* (AJ238359); *H. seripedicae* (Y10146); *Pediococcus urinaeequi* (D87677); *Kingella kingae* (M22517) |
| | 60-1 9 | Ha, R | 100 | 10 | *Bordetella avium* (AF177666, U4947); *Alcaligenes xylosoxidans*; (D88005, M22509) |
| | B6007 | Ha, Hh | 100 | 11 | *Eikenella corrodens* (Eik.corro3) (A) |
| *γ* Proteobacteria (12 isolates) | B60-03 | Ha, Hh | 100 | 15 | *Pseudomonas fluorescens* (D84013) |
| | B60-16 | Ha, Hh | 87 | 10 | |
| | B60-19 | Ha, Hh | 90 | 10 | *P. chlororaphis* (D84011); *P. ficuserectae* (Z84013) |
| | 0-2 121 | Ha, A | 83 | 10 | *P. ficuserectae* (Z76661) |
| | 60-2 38 | Ha, A | 100 | 11 | *Moraxella cuniculi* (AF005188) |
| | 12-2 1 | Ha, Hh | 100 | 10 | *Metylobacter luteus* (M95657) |
| | B12-19 | Ha, Hh | 100 | 12 | *Methylocaldum gracile* (Mca.gracil) |
| | B60-02 | Ha, Hh | 78 | 10 | *Methylomicrobium agile* (Mmb.agile) |
| | B60-20 | Ha, Hh | 90 | 10 | *Methylomicrobium album* (M95659) |
| | B60-12 | Ha, Hh | 83 | 10 | *Methylococcus capsulalus* (Mlc.capsu4, Mlc.capsu3); *Ectothiorhodospira vacuolata* (Ec.vacuola) |
| | 60-2 17 | Ha, Hh, A | 69 | 10 | *Halomonas metidiana* (M93356) |
| | 60-2 31 | Ha, A | 100 | 15 | *Halorhodospira halochloris* (M59152) |

**Table 2.** (Continued)

| | Strain No.[a] | Restriction enzymes[b] | Similarity (%)/ Allowance limit[c] | | Affiliation (Accession number, name)[d] and origin[e] |
|---|---|---|---|---|---|
| δ Proteobacteria (4 isolates) | 0-2 122 | Ha, R | 100 | 14 | *Desulfobacter curvatus* (M34413); *D. vidrioforme* (U12254) |
| | 12-1 25 | Ha, Hh | 80 | 10 | *Desulfovibrio africanus* (X99236) |
| | 60-1 16 | Ha, R | 100 | 10 | *Desulfomonas chloroethemic* (U49748) |
| | B60-14 | Ha, Hh | 100 | 16 | *Syntrophobotulus glycolicus* (Syp.glycol) |
| Cytophagales (8 isolates) | 60-1 3 | Hh, R | 100 | 10 | *Chryseobacterium balustinum* (M58771); |
| | 60-1 5 | Hh, R | 100 | 10 | *Flabobacterium indoltheticum* (M58774) |
| | 60-2 24 | Ha, Hh, A | 100 | 12 | |
| | 12-1 9 | Hh, R | 100 | 14 | *Flavobacterium hydatis* (M58764); *Sporocytophaga cauliformis* (M93151) |
| | 60-1 28 | Ha, A | 100 | 11 | *Taxeobacter gelupurpurascens* (Y18836) |
| | 60-2 37 | Ha, Hh | 100 | 15 | *Prevotella bivia* (L16475) (A) |
| | 60-2 46 | Ha, Hh | 100 | 15 | *Prevotella buccalis* (L16476) (A) |
| | 60-2 09 | Ha, R | 87 | 10 | *Bacteroides distasonis* (M86695) (F) |
| Spirochaetales (1 isolate) | 60-2 20 | Ha, Hh | 72 | 10 | *Spirochaeta stenostrepta* (M88724) (F) |

[a] The first number in the strain number represented the site of isolation; "0", "12", and "60" represent the 0 t area, 120 t field, and 600 t field. "B" indicates isolates in 1998, the others are in 2004. [b] Restriction enzymes used for similarity search; "Ha", "Hh", "R", and "A" represented *Hae* III, *Hha* I, *Rsa* I, and *Alu* I. [c] Allowance limit for measuring error was first set at 10%, and then increased as described in Materials and Methods. [d] Species name and accession number of the theoretical MERFL having the highest similarity with the measured MERFL indicated by "Strain No." [e] (A) indicates animal origin, (F) indicates feces origin.

**Table 3.** Affiliation of 49 bacteria newly isolated using PP medium (Actinobacteria) by MERFLP

| | Strain No.[a] | Restriction enzymes[b] | Similarity (%)/ Allowance limit[c] | | Affiliation (Accession number, name)[d] and origin[e] |
|---|---|---|---|---|---|
| Actinomycetaceae (3 isolates) | 12-2 4 | Ha, Hh, R | 75 | 10 | *Actinomyces europaeus* (Y08828) |
| | B60-13 | Ha, Hh | 86 | 10 | *Actinomyces hydrovaginalis* (X69616) |
| | 60-1 37 | Hh, A | 100 | 11 | *Dactylosporangium thailandeni* (D85481, X92630); *Acanobacterium pyogenes* (X79225) |
| Bifidobacteriaceae (9 isolates) | 12-1 16 | Ha, R | 100 | 10 | *Bifidobacterium adolescentis* (M58729) (F); *B. asteoides* (M58730) (F); |
| | 60-1 2 | Ha, R | 100 | 10 | *B. breve* (M58731) (F) |
| | 60-1 35 | Ha, R | 100 | 11 | |
| | 12-2 11 | Ha, R | 100 | 13 | |
| | 60- 2 33 | Ha, R | 100 | 13 | |
| | 60-2 34 | Ha, R | 100 | 13 | |
| | 60-2 32 | Ha, R | 100 | 14 | |
| | 12-1 15 | Ha, R | 100 | 16 | |
| | 60-1 6 | Ha, Hh, R | 76.7 | 10 | |

**Table 3.** (Continued)

| | Strain No.[a] | Restriction enzymes[b] | Similarity (%)/ Allowance limit[c] | | Affiliation (Accession number, name)[d] and origin[e] |
|---|---|---|---|---|---|
| Cellulomonadaceae (3 isolates) | 60-1 24 | Hh, A | 100 | 12 | *Cellulomonas pachnodae* (AF105422) |
| | 60-1 29 | Hh, A | 100 | 13 | |
| | 60-2 35 | Ha, A | 93 | 10 | *Oeskovia xanthineolytica* (X79453); *O. turbata* (X79454); *Promicromonospora enterophi* (X83807) |
| Corynebacteriaceae (7 isolates) | 60-2 18 | Ha, Hh, A | 100 | 10 | *Corynebacterium confusum* (Y15886) |
| | B12-20 | Ha, Hh | 100 | 12 | *C. genitalium* (U87819) |
| | 12-1 38 | Ha, Hh | 100 | 14 | *C. auriscani* (AJ243820) |
| | 12-1 8 | Ha, Hh, R | 81 | 10 | *C. pseudotuber* (D38578); *Saccharomonospora glauca* (Z38004); *S. caesia* (Z38019); *S. azurea*; (Z38017) |
| | 60- 2 21 | Ha, Hh, A | 87 | 10 | *C. xerosis* (AF145257) |
| | 60- 2 11 | Ha, R | 83 | 10 | *C. confusum* (Y15886) |
| | B60-18 | Ha, Hh | 87 | 10 | *C. renale* (D37803) |
| Kineococcus group (3 isolates) | 12-1 17 | Hh, R | 100 | 13 | *Kineosporia aurantiaca* (AF095336,D86937); |
| | 12-1 20 | Hh, R | 100 | 14 | *Clavibacter xyli* (M60935); *Agromyces ramosus* (X77447) |
| | 12-1 12 | Ha, R | 100 | 10 | *K. rhamnosa* (AB003934, AB003935); *Cellulomonas pachnodae* (AF105422) |
| Microbacteriaceae (5 isolates) | 0-2 212 | Ha, A | 100 | 10 | *Agromyces cerinus* (D45060); *A. ramosus* (X77447) |
| | 60-1 36 | Ha, Hh, A | 88 | 10 | *A.mediolanus* (D45052) |
| | 60- 2 45 | Ha, A | 100 | 10 | *Aureobacterium kitamiense* (AB013919) |
| | 60-1 12 | Ha, R | 100 | 10 | *Clavibacter xyli* (M60935) |
| | 12-1 36 | Ha, Hh, R | 81 | 10 | |
| Micrococcaceae (19 isolates) | 12-1 19 | Hh, R | 100 | 10 | *Arthtobacter chlorophenolicus* (AF102267); *Micrococcus luteus* (AF057289); |
| | 60-1 30 | Ha, A | 100 | 10 | *Corynebacterium glutamicu* (Z46753); *Rubrobacter radiotolerans* (U65647); |
| | 60-2 10 | Ha, R | 90 | 10 | *Rhodococcus coprophilus* (U93340) |
| | 60-2 25 | Hh, A | 88 | 10 | |
| | 12-2 3 | Ha, Hh | 87 | 10 | |
| | 12-2 10 | Ha, Hh | 87 | 10 | |
| | 60-1 31 | Ha, Hh, A | 85 | 10 | |
| | 12-1 10 | Ha, Hh | 80 | 10 | |
| | B60-15 | Ha, Hh | 93 | 10 | *A. globiformis* (Arb.globi2, Arb.globif ) |
| | 60-1 34 | Ha, Hh, A | 96 | 10 | *Rothia dentocariosa* (M59055) (A) |
| | 60-20 6 | Ha, Hh, | 87 | 10 | |
| | 60-20 2 | AHa, Hh, A | 81 | 10 | |
| | 12-1 2 | Ha, R | 100 | 10 | *Micrococcus luteus* (AF057289); *Arthrobacter chlorophenolicus* (AF102267, |
| | 12-2 9 | Ha, R | 100 | 10 | AF057289); *Rhodococcus coprophilus* (U93340); *R.coprophilus* (X80626); |

**Table 3.** (Continued)

|  | Strain No.[a] | Restriction enzymes[b] | Similarity (%)/ Allowance limit[c] | | Affiliation (Accession number, name)[d] and origin[e] |
|---|---|---|---|---|---|
| Micrococcaceae | 12-2 16 | Ha, R | 100 | 10 | *Corynebacterium glutamicum* (Z46753); *Rubrobacter radiotolerans*; |
| (19 isolates) | B60-122 | Ha, Hh | 95 | 10 | (U65647, X87134) |
|  | 60-2 27 | Ha, Hh | 94 | 10 |  |
|  | 12-2 14 | Ha, Hh, R | 58 | 10 |  |
|  | 60- 2 171 | Ha, Hh | 100 | 10 | *Micrococcus lylae* (X80750) |

[a–e] Remarks are represented as described in Table 2.

**Table 4.** Affiliation of 49 bacteria newly isolated using PP medium (Actinobacteria and low GC content gram-positive bacteria) by MERFLP

|  | Strain No.[a] | Restriction enzymes[b] | Similarity (%)/ Allowance limit[c] | | Affiliation (Accession number, name)[d] and origin[e] |
|---|---|---|---|---|---|
| Nocardioidaceae | B12-18 | Ha, Hh | 97 | 10 | *Aeromicrobium erythreum* (AF005021); |
| (3 isolates) | B12-62 | Ha, Hh | 93 | 10 | *A. fastidiosum* (AF005022, Armb.fasti); *Nocardioides* sp. (Ncr.sp3005) |
|  | B12-7 | Ha, Hh | 87 | 10 |  |
| Nocardiaceae | 60-1 33 | Ha, A | 93 | 10 | *Rhodococcus equi* (X80614) |
| (15 isolates) | 60-1 17 | Hh, A | 80 | 10 |  |
|  | 60-2 16 | Ha, Hh, A | 93 | 10 | *R. coprophilus* (U93340, X80626) |
|  | 12-2 17 | Ha, Hh, R | 80 | 10 |  |
|  | 12-1 24 | Hh, R | 100 | 10 | *R. opacus* (AB032565, X80631, AF095715, Y11892, Y11893); |
|  | 12-2 15 | Ha, Hh | 100 | 13 | *R. koreensis* (AF124342); *R. equii* (X80614, Rco.equi, Rco.equi2); |
|  | B12-4 | Ha, Hh | 93 | 10 | *Nocardioides* sp. (Ncr.sp3005) |
|  | B12-5 | Ha, Hh | 93 | 10 |  |
|  | 60-2 22 | Ha, R | 90 | 10 |  |
|  | 60-2 41 | Ha, Hh, A | 87 | 10 |  |
|  | 12-1 29 | Ha, R | 84 | 10 |  |
|  | 60-1 212 | Ha, Hh, A | 78 | 10 |  |
|  | 12-2 12 | Ha, Hh, R | 73 | 10 |  |
|  | 12-1 231 | Ha, R | 100 | 15 | *Rhodococcus ruber* (X80625); *R. rhodnii* (X80621) |
|  | 12-1 34 | Ha, Hh, R | 70 | 10 | *R. pyridinovorans* (AF173005) |
| *Streptomycetaceae* | 0-1 103 | Hh, R | 100 | 10 | *Kitasatospora setae* (M55220,U93332); *K. griseola* (M55221); |
| (6 isolates) | 60-2 36 | Hh, A | 100 | 10 | *K. azatica* (U93312); *K. paracochleate* (U93328); *K. mediocidica* (U93324) |
|  | 0-2 209 | Ha, Hh, R | 100 | 10 | *Streptomyces avermitilis* (AF145223); *Jonesia denitrificans* (X83811) |
|  | 0-2 2 | Ha, Hh | 100 | 10 |  |
|  | 60-1 1 | Ha, Hh, R | 100 | 10 |  |
|  | 60-1 18 | Ha, Hh, A | 100 | 10 |  |
| Pseudonocardiaceae | 0-2 124 | Hh, R | 100 | 12 | *Saccharothrix tangerinus* (AB020031) |
| (2 isolates) | 12-1 35 | Hh, R | 100 | 10 | *Saccharothrix syringae* (AF114812) |

**Table 4.**   (Continued)

| | Strain No.[a] | Restriction enzymes[b] | Similarity (%)/ Allowance limit[c] | | Affiliation (Accession number, name)[d] and origin[e] |
|---|---|---|---|---|---|
| Other Actinobacteria (6 isolates) | 12-1 32 | Ha, R | 100 | 15 | *Gordonia rubropertinctus* (X80632) (A) |
| | 60-1 23 | Ha, A | 93 | 10 | *Dermatophilus congolensis* (L40615); *Jonesia denitrificans* (X83811); *Kitasatospora mediocidica* (U93324) |
| | 60-2 14 | Hh, A | 100 | 11 | *Microbacterium keratanolytica* (Y14786); Microtetraspora glauca (X97891) |
| | 60-2 13 | Ha, Hh, A | 85 | 10 | *Promicromonospora citrea* (X83808) |
| | 12-1 1 | Ha, R | 83 | 10 | *Propionibacterium thoenii* (X53220) |
| | B12-202 | Ha, Hh | 94 | 10 | *Stomatococcus mucilaginosus* (Stt.muclag) (A) |
| Low GC gram-positive bacteria (17 isolates) | 60-2 30 | Ha, R | 100 | 11 | *Clostridium scatologenes* (M59104) (F) |
| | 60-1 7 | Ha, R | 87 | 10 | |
| | 60-1 14 | Ha, Hh, A | 100 | 10 | *Clostridium lituseburense* (M59107) (F) |
| | 60-1 15 | Hh, A | 100 | 10 | *Sarcina maxima* (X76650) |
| | B12-172 | Ha, Hh | 100 | 10 | *Brochothrix campestria* (X56156) (A) |
| | B12-17 | Ha, Hh | 87 | 10 | |
| | B12-2 | Ha, Hh | 100 | 10 | *Staphylococcus captis* (AB009937); *S. equorum* (AB009939); *S. lugdunensis* (AB009941) |
| | 12-2 7 | Hh, R | 100 | 12 | *Gemella morbillorum* (L14327) (A) |
| | 12-1 3 | Ha, R | 100 | 10 | *Desulfitobacterium* sp. (X95742) |
| | 12-1 37 | Ha, R | 100 | 13 | *Halobacteroides halobius* (U32595) |
| | 0-1 111 | Hh, R | 100 | 10 | *Mycoplasma imitans* (L24103) |
| | B60-142 | Ha, Hh | 93 | 19 | *Halobacillus litoralis* (Hb.litora); *Listeria seeligeri* (Lis.seelig) |
| | 60-2 29 | Ha, Hh, A | 75 | 10 | *Sporolactobacillus racemicus* (D16289) |
| | B12-08 | Ha, Hh | 97 | 10 | *Sporolactobacillus dextrus* (D16282) |
| | 0-2 3 | Ha, A | 100 | 13 | *S. laevis* (D16286); *S. racemicus* (D16289); *S. inulinus* (M58838) |
| | 60-1 27 | Hh, A | 100 | 11 | *Paenibacillus curdlanolyticus* (D88515) |
| | 0-1 101 | Ha, Hh, R | 82 | 10 | *Paenibacillus lautus* (D85394) |

[a–e] Remarks are represented as described in Table 2.

60-1 7 *Clostridium scatologenes* (M59104), and 60-1 14 *Clostridium lituseburense* (M59107) (Table 4). Similarly, the following 4 isolates from the 120 t field were of animal origin: 12-1 32 *Gordonia rubropertinctus* (X80632), B12-202 *Stomatococcus mucilaginosus* (Stt.muclag), B12-172 *Brochothrix campestria* (X56156), and 12-2 7 *Gemella morbillo-* *rum* (L14327) (Table 4), and 3 isolates were of feces origin: 12-1 16, 12-2 11, 12-1 15 *Bifidobacterium adolescentis* (M58729) or *B. asteoides* (M58730) or *B. breve* (M58731) (Table 3).

The present results suggested that some bacteria isolated from both feces-applied field soils were of feces origin, which

**Table 5.**   Affiliation of 53 bacteria newly isolated using PP medium (*Bacillus* spp.) by MERFLP

| Strain No.[a] | Restriction enzymes[b] | Similarity (%)/ | Allowance limit[c] | Affiliation (Accession number, name)[d] and origin[e] |
|---|---|---|---|---|
| 60-1 25 | Ha, Hh | 100 | 10 | *Bacillus amyloliquefaciens* (X60605); *B. poplliae* (X60633); |
| B0-6 | Ha, Hh | 100 | 10 | *B. pumilus* (X60637); *B. subtilis* (X60646) |
| 60-2 19 | Ha, A | 100 | 10 | |
| 60-1 26 | Ha, Hh | 100 | 12 | |
| 60-1 191 | Ha, Hh, A | 67.5 | 10 | |
| B0-14 | Ha, Hh | 100 | 10 | *B.cereus* (X55060); *B. mycoides* (X55061); *B. thuringiensis* (X55062); |
| B0-15 | Ha, Hh | 100 | 10 | *B. medusa* (X60628) |
| B0-5 | Ha, Hh | 100 | 10 | |
| 0-2 207 | Ha, Hh, R | 100 | 10 | |
| 0-1 105 | Hh, R | 100 | 10 | |
| B12-15 | Ha, Hh | 100 | 10 | |
| B12-61 | Ha, Hh | 100 | 10 | |
| 12-2 5 | Ha, Hh | 100 | 10 | |
| 60-2 42 | Ha, Hh, A | 100 | 10 | |
| 0-1 1 | Ha, Hh, R | 100 | 10 | *B. anthracis* (X55059) |
| 0-1 107 | Ha, A | 100 | 10 | |
| 0-1 108 | Ha, Hh, R | 100 | 10 | |
| 0-1 112 | Ha, Hh, R | 100 | 10 | |
| 0-2 210 | Ha, A | 100 | 10 | |
| 0-2 123 | Ha, Hh, R | 100 | 11 | |
| 0-2 119 | Ha, Hh, R | 100 | 12 | |
| 0-1 113 | Ha, Hh, R | 100 | 13 | |
| 0-2 118 | Ha, Hh, R | 100 | 14 | |
| 60-2 43 | Ha, A | 100 | 15 | |
| 0-2 211 | Ha, A | 79 | 10 | |
| 0-1 1071 | Ha, R | 100 | 10 | *B. anthracis* (X55059); *B. macerans* (X57306, X60624); |
| 0-1 106 | Ha, R | 100 | 10 | *Staphylococcus aureus* (L37598) |
| 0-1 109 | Ha, A | 100 | 10 | |
| 0-2 201 | Ha, Hh, R | 100 | 10 | *B. licheniformis* (X68416); *Lactobacillus amylophilus* (M58806) |
| B0-16 | Ha, Hh | 93 | 10 | *B. licheniformis* (X68416); *B. sphaericus* (L14011, L14012, L14014) |
| 60-1 4 | Ha, R | 100 | 15 | *B. pasteurii* (X60631); *Exiguobacterium* sp. (X86964) |
| 0-1 114 | Ha, Hh, R | 80 | 10 | |
| 0-2 115 | Ha, A | 100 | 10 | *B. gordonae* (X60617); *Paenibacillus glucanolyticus* (D78470); |
| B0-11 | Ha, Hh | 96 | 10 | *P. lautus* (D78473, D85394) |
| B0-8 | Ha, Hh | 96 | 10 | |
| 0-2 206 | Hh, R | 100 | 10 | *B. megaterium* (D16273); *Heliobacterium modestocaldun* (U14559); *H. mobilis* (U14560); *Desulfotomaculum rumin* (Y11572) |
| B12-13 | Ha, Hh | 100 | 10 | *B. megaterium* (X60629, B. megateri); *B. simplex* (D78478); *Listeria grayi* (Lis.grayi); *L. seeligeri (Lis.seelig)* |

**Table 5.**   (Continued)

| Strain No.[a] | Restriction enzymes[b] | Similarity (%)/ Allowance limit[c] | | Affiliation (Accession number, name)[d] and origin[e] |
|---|---|---|---|---|
| B0-13 | Ha, Hh | 87 | 10 | *B. smithii* (B.smithii2, Z26935) |
| B12-16 | Ha, Hh | 93 | 10 | *B. smithii* (X60643); *B. insolitus* (X60642); *B. sphaericus* (D16280); |
| B0-2 | Ha, Hh | 93 | 10 | *B. firumus* (B.firmus); *B. circulans* (B.circula3) |
| B0-20 | Ha, Hh | 93 | 10 | *B. thermoglucosadicus* (X60641); *B. sphaericus* (B.sphaeric); *B. lentus* (B.lentus); *B. insolitus* (B.insolitu); *B. firmus* (B.firmu3) |
| B0-3 | Ha, Hh | 87 | 10 | *B. fastidiosus* (B.fastidio) |
| 12-1 27 | Ha, Hh | 100 | 10 | *B. lentus* (X60601); *B. maroccanus* (X60626); *B. sphaericus* (X60639) |
| 12-1 23 | Ha, R | 100 | 10 | *B. thermoglucosadicus* (X60641); *B. circulans* (X60613); *B. azotoformans* (X60609) |
| 12-1 4 | Ha, R | 100 | 10 | *B. fusiformis* (L14013); *B. sphaericus* (L14014); *B. lentimorbus* (X60622); *B. popolliae* (X60633); *Clostridium acetobutylicum* (X81021) |
| 12-1 5 | Ha, R | 93 | 10 | |
| 12-1 26 | Ha, Hh, R | 75 | 10 | *B. aneurinolyticus* (X60645) |
| 60-2 47 | Ha, Hh, A | 66 | 10 | *B. spaericus* (L14016) |
| 60-1 21 | Ha, R | 100 | 10 | *B. sphaericus* (L14011, L14012); *B. cereus* (X55060); *C. botulinum* (L37585) |
| B12-192 | Ha, Hh | 100 | 10 | *B. sphaericus* (X60639); *B. firmus* (X60616, B.firmus); |
| B12-8 | Ha, Hh | 93 | 10 | *B. alcalophilus* (B.alcaloph); *B. psychrosaccharolyticas* (X60635) |
| 60-2 04 | Ha, Hh, A | 67 | 10 | *Bacillus lentimorbus* (X60622) |
| B60-05 | Ha, Hh | 100 | 10 | *B. circulans* (B.circula3); *B. firmus* (B.firmus, B.firmus3); *B. maroccanus* (B.maroccan) |

[a–e] Remarks are represented as described in Table 2.

survived for at least 2 months after introduction into field soils.

## Discussion

Compared to previous studies using agarose gel electrophoresis where 63 isolates (52.5%) of 120 protease-producing bacteria isolated from various field soils were completely identical to the corresponding theoretical MERFL,[8] the percentage of isolates completely identical to the corresponding theoretical MERFL were lower with a 10% allowance limit of the measuring error (39/190; 20.5%), and became similar by resetting the allowance limit to a higher value (110/190; 57.9%). The percentages were lower than those using a microchip electrophoresis system for $NO_3^-$-reducing bacteria isolated from the same fields: 60/132 (45.5%) were identical

under a 10% allowance limit and 121/132 (91.7%) were identical over the 10% allowance limit (Watanabe and Koga private communication 2008).

A lack of corresponding theoretical MERFLs was one reason for the lower percentage of complete identity. In terms of bacterial groups, the percentage of complete identity over the 10% allowance limit was lowest for Actinobacteria (48.2%), followed by that for gram-negative bacteria (56.4%), and that for low GC content gram-positive bacteria (68.6%). In terms of soils, the percentage of complete identity over 10% was lowest for those isolated from the 600 t field (51.1%), followed by those from the 120 t field (58.3%), and the 0 t area (72.5%). Actinobacteria isolated from the feces-applied fields, which had no remarkable characteristics and whose 16S rDNA sequences had not been determined and listed in the

public database, might mainly lower the percentage of complete identity.

Our previous studies of experimental fields have shown that flora of protease-producing bacteria[8] and $NO_3^-$-reducing bacteria[20] in field soils were changed by the annual application of liquid livestock feces, and bacteria grown on peptone polyxin B medium were markedly increased soon after application of the feces.[10] The results of plate counting indirectly suggested that a number of bacteria gained resistance to polymyxin B and survived for several months in the field soils,[10] and isolation of pathogenic bacteria suggested that some were of livestock origin.[21] The results of floral changes of polymyxin B-tolerant bacteria in both feces-applied field soils were quite similar to those of the protease-producing bacterial flora[8] and those of $NO_3^-$-reducing bacteria in these field soils.[20] The present results supported the suggestion of previous studies that rapidly increased bacterial number (7.7 times before application) counted on PP medium 1 week after feces application in the 600 t field[10] was caused by contamination of bacteria along with the applied feces.

Polymyxins (B and E), polycationic peptide anitibiotics produced by *Bacillus polymyxa*, were bactericidal to gram-negative bacteria ($MIC_{50}=1$ ppm for most gram-negative bacteria, except for *Burkholderia cepacia*[2]) due to crossing the bacterial outer membrane by competitive divalent cation displacement by bulky polycations and little to no effect on gram-positive bacteria. As a breakpoint for the reported resistant bacteria of clinical isolates, such as *Acinetobacter* sp.,[2] and *P. aeruginosa*,[22] was 4 ppm ($MIC_{50}>4$ ppm),[2] some of the gram-negative bacteria among isolated polymyxin B-tolerant bacteria (Table 2) were supposed to acquire antibiotic resistance. Although polymyxin B have mainly been used in hospitals and have never been used as antibiotic growth promoters (AGP), or applied for livestock, polymyxin E (colistin), which has a similar structure and has the same site of action, has been used as an AGP. As large numbers of polymyxin B (over $10^6$ CFU/g dry matter) and colistin-resistant bacteria (over $10^6$ MPN/g dry matter) are typically found in raw livestock feces,[23] polymyxin B-resistant bacteria isolated in this study were supposed to have been contaminated from liquid livestock feces.

A specific group of hazardous bacteria[24] or a specific group of antibiotic-resistant bacteria[25] could be monitored by using a specific gene primer for target bacteria[24] or inoculation experiment,[25–28] while no method available by which the whole risk of antibiotic resistant bacteria can be evaluated. ① Because antibiotic-resistant bacteria, including opportunistic pathogens mainly cause harmful effects by unexpectedly rapid proliferation under antibiotic treatment[5] rather than through the production of a specific toxin, a primer for producing gene of a specific toxin was not suitable to evaluate their risk; ② Because resistance to antibiotics can arise from mutations in the bacterial genome or through the acquisition of genes coding for resistance,[5] which only induce negligible variation in whole bacterial DNA,[1,29] taxonomical difference was not suitable to discriminate resistant bacteria from susceptible bacteria; ③ Because there exist various mechanisms for acquired resistance,[30] a primer for a specific resistant gene could not cover whole resistant bacteria but could be used for certain resistant bacteria.[31]

The method presented here was found to be effective and useful for the purpose of monitoring whole antibiotic-resistant bacteria in the environment and to evaluate their risk. A more convenient and secure method by which types of bacteria and their numbers can be identified and enumerated by the combined use of a most provable number method (MPN) and a MERFLP method without isolating bacteria have already been developed. As the method was useful not only to enumerate and identify the types of antibiotic-resistant bacteria, but also to search for effective antibiotics, further research in which the numbers and types of multidrug-resistant bacteria in livestock feces were estimated by MPN/ERRFLP will be presented in the next manuscript.

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