Diversity of Tetracycline Resistance Genes in Bacteria Isolated from Various Agricultural Environments

YURI KOBASHI^{1*}, AKIRA HASEBE^{2†}, MICHINORI NISHIO³ and HIROO UCHIYAMA¹

¹ Graduate School of Life and Environmental Sciences, University of Tsukuba, 1–1–1, Tennodai, Tsukuba, Ibaraki 305–8572, Japan

² National Institute for Agro-Environmental Sciences, 3–1–3, Kannondai, Tsukuba, Ibaraki 305–8604, Japan

³ Formerly of the Institute of Agricultural and Forest Engineering, University of Tsukuba, 1–1–1, Tennodai, Tsukuba, Ibaraki 305–8572, Japan

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A total of 350 tetracycline-resistant (Tc^r) bacteria were isolated from livestock feces, farmyard manure (FYM), and soil in Japan. The isolates were classified into 28 established genera of the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* by studying partial 16S rRNA gene sequences. PCR analysis revealed that 249 of the 350 Tc^r isolates contained at least 1 of 15 Tc^r genes (*tet* genes): 140 isolates contained an efflux pump gene and 109 isolates contained a ribosomal protection protein (RPP) gene. The predominant fecal isolates were identified as members of the genera *Enterococcus* and *Escherichia*, carrying *tet*(M) and *tet*(H), respectively. The predominant soil isolates were identified as members of the genera *Burkholderia* and *Afipia*, carrying *tet*(Z) and *tet*(B), respectively. This study is the first to describe the presence of *tet* genes in members of the genera *Bordetella*, *Bradyrhizobium*, *Burkholderia*, *Dyella*, *Flexibacter*, *Kurthia*, *Luteibacter*, *Lysobacter*, “*Nordella*”, *Ochrobactrum*, *Pediococcus*, *Rhodopseudomonas*, and *Vagococcus*. It has revealed that *tet* genes are distributed in much more diverse phylogenetic groups of bacteria in the environment than has been reported previously.

Key words: farmyard manure, livestock feces, soil, tetracycline-resistant bacteria, *tet* gene

An important issue with antibiotic resistance is the fact that the widespread use of antibiotics not only selects for drug-resistant pathogenic bacteria, but also exerts selective pressure on the normal commensal microbiota. It is thought that, in light of the ubiquitously demonstrated phenomenon of horizontal transfer of antibiotic resistance genes in the microbial world, the presence of such reservoirs may explain the rapid dissemination of antibiotic resistance from commensal organisms to pathogenic microbiota³.

Animal manure contains high numbers of resistant bacteria^{6,22}. Since animal manure is frequently spread on farmland, a risk exists not only for the spread of resistant

bacteria, but also for horizontal transfer of resistance genes to indigenous bacteria¹. In a recent study, soil bacteria isolated from soil and groundwater underlying swine production facilities contained tetracycline resistance genes that are identical to those found in bacteria isolated from manure⁷.

In our previous work, we examined the distribution of antibiotic-resistant bacteria in animal-soil systems by analyzing 19 samples of livestock feces, solid farmyard manure (FYM), soil from arable fields and orchards treated with FYM or not, and forest soils¹². In the fecal samples from livestock fed with antibiotics as feed additives, a high occurrence of antibiotic-resistant bacteria was observed. In contrast, in the 2 fecal samples from livestock fed without antibiotics as feed additives, a relatively low occurrence of antibiotic-resistant bacteria was observed. The results suggested that there might be a link between the use of in-feed antibiotics and the prevalence of antibiotic-resistant bacteria

* Corresponding author. E-mail address: kobashi@affrc.go.jp; Tel.: +81–29–838–8648; Fax: +81–29–838–8648.

† Present address: Agriculture, Forestry and Fisheries Research Council Secretariat, Ministry of Agriculture, Forestry and Fisheries of Japan, 1–2–1, Kasumigaseki, Chiyoda, Tokyo 100–8950, Japan

in animal feces. Furthermore, our study also showed that the occurrence of broad-range multi-drug resistance (MDR) was much higher in bacteria in the feces of swine fed with antibiotics than in bacteria in the natural forest soil, suggesting that the occurrence of broad-range MDR in the feces was enhanced by the use of antibiotics as feed additives.

Tetracyclines are broad-spectrum antimicrobial agents, exhibiting activity against a wide range of Gram-positive and Gram-negative bacteria, protozoan parasites, and atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae. The favorable antimicrobial properties of these agents and the absence of major adverse side effects have led to their extensive use in treating infections in humans, farmed fishes, and animals^{9,16,18}. In many countries, tetracyclines are added at subtherapeutic levels to animal feeds as growth promoters. As a result, tetracycline resistance genes have spread widely in clinical and natural environments²⁻⁷, and tetracycline-resistant bacteria, particularly those isolated from farming environments, show broad-range MDR¹². Although several studies have examined tetracycline resistance among bacteria, most have employed pathogenic and important epidemiologic isolates⁵. These studies, while useful, did not give enough information about the presence and the types of *tet* genes that are present naturally in the environment.

In the present study, for a better understanding of the resistance genes and their distribution in the environment, a total of 350 tetracycline-resistant bacterial isolates from 19 environmental samples were used. We also investigated the distribution of *tet* genes (11 genes encoding tetracycline efflux pumps: *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*(Y), *tet*(Z), and *tet*(30); and 8 genes encoding ribosomal protection proteins (RPP): *tet*(M), *tet*(S), *tet*(O), *tet*(W), *tet*(Q), *tet*(T), *tet*(P)(B), and *otr*(A)).

Materials and Methods

Isolation of *Tc^r* bacteria

In order to characterize *Tc^r* bacteria from various sources, *Tc^r* isolates were obtained from 6 fecal samples, 6 FYM samples, and 7 soil samples in Japan. Sources and preparation of the samples were described previously¹². To isolate *Tc^r* bacteria from fecal samples, the following 4 types of agar media each containing 50 µg ml⁻¹ of tetracycline (Sigma Chemical Co, St. Louis, MO, USA) were used: PTYG agar medium for detection of aerobic heterotrophic tetracycline resistant bacteria¹²; EF agar medium (Nissui, Tokyo, Japan) for detection of tetracycline resistant enterococci; MRS agar medium (Becton, Dickinson and Co.,

Franklin Lakes, NJ, USA) for detection of tetracycline resistant lactic acid bacteria; and Desoxycolate agar medium (Nissui) for detection of tetracycline resistant coliform bacteria. To isolate *Tc^r* bacteria from FYM samples, PTYG and Desoxycolate agar media each containing 50 µg ml⁻¹ of tetracycline were used. To isolate *Tc^r* bacteria from soil samples, PTYG agar medium containing 50 µg ml⁻¹ of tetracycline was used.

We used the dilution plate method to isolate *Tc^r* bacteria by making 10-fold dilutions with phosphate-buffered saline (pH 7.0). PTYG agar plates were incubated at 30°C for 7 days, EF agar plates and MRS agar plates were incubated at 37°C for 48 h, and Desoxycolate agar plates were incubated at 37°C for 24 h. After the incubation period, about ten bacterial strains were selected randomly. Purification of the bacterial isolates was carried out using PTYG agar medium or Luria-Bertani agar medium¹³ containing 50 µg ml⁻¹ of tetracycline (Sigma).

Characterization of the *Tc^r* bacterial isolates

Template DNA was extracted from the *Tc^r* isolates by the hexadecyltrimethylammonium bromide (CTAB) method²⁰. PCR primers 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGWGTGTACAAGGC-3')¹⁵ were used to amplify the segment of the bacterial 16S rRNA gene. PCR was carried out using a basic reaction mixture containing 1.0 U of Ex *Taq* polymerase (Takara, Shiga, Japan), 5 µl of 10×Ex *Taq* reaction buffer (Takara), 10 nmol of each deoxynucleoside triphosphate, 1 nmol of template DNA, and 25 pmol of each primer in a total volume of 50 µl. PCR amplification was conducted in an I-Cycler thermal cycler (Bio Rad, Hercules, CA, USA) for 30 cycles, each consisting of 30 sec at 94°C, 30 sec at 58°C, and 30 sec at 72°C, with a final extension for 10 min at 72°C. The PCR products were purified with the Ultra Clean PCR Clean-up DNA Purification Kit (Mo Bio Laboratories, Solana Beach, CA, USA). For the restriction fragment length polymorphism (RFLP) analysis¹⁷, the PCR products were digested with endonucleases *Hae*III and *Rsa*I (Toyobo, Osaka, Japan). Digested DNA fragments were analyzed by horizontal electrophoresis on 2.0% agarose gel in TAE (40 mM Tris, 20 mM acetate, and 2 mM EDTA) and staining with ethidium bromide. RFLP analysis was conducted for 350 isolates, which were classified into 69 groups on the basis of the band patterns. Then, direct sequencing of approximately 1,200 bp of PCR products of representative isolates from each of the 69 groups was performed with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The reaction products were analyzed with

an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). The phylogenetic positions of the isolates were determined by searching the DDBJ database (<http://www.ddbj.nig.ac.jp/Welcome-e.html>) with the BLAST program.

The sequences of bacterial strains generated in this study have been deposited in the DDBJ database under accession numbers AB272318 to AB272386.

PCR detection of *tet* genes

All isolates were tested for the presence of *tet* genes by PCR. The primers used for PCR amplification of the 19 *tet* genes were described by Aminov *et al.*^{3,4)}. The target *tet* genes were 11 genes encoding tetracycline efflux pumps: *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*(Y), *tet*(Z), and *tet*(30); and 8 genes encoding ribosomal protection proteins (RPP): *tet*(M), *tet*(S), *tet*(O), *tet*(W),

tet(Q), *tet*(T), *tet*P(B), and *otr*(A).

All PCRs for detecting the presence or absence of resistance genes were based on the method described by Aminov *et al.*^{3,4)}. The validity of the PCR system and the sizes of amplification products were ascertained by using positive control plasmids each carrying the *tet* gene^{3,4)}. Nucleotide sequences of the PCR products were determined with a Big-Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

Results

Isolation of bacteria resistant to tetracycline

A total of 350 Tc^r bacterial isolates were obtained from the various sources on the 4 types of agar media (Table 1).

RFLP analysis was conducted for each of the 350 isolates, which were subsequently classified into 69 groups on

Table 1. Tetracycline-resistant isolates, their origins, and types of *tet* gene

Origin	Media ^a	No. of isolates	<i>tet</i> genes		
			Efflux genes	RPP genes	Unknown
Swine feces	PTYG	25	10	6	9
	Desoxycolate	28	20	4	4
	EF	21	1	20	0
	Subtotal	74	31	30	13
Poultry feces	PTYG	28	22	5	1
	Desoxycolate	26	17	6	3
	EF	30	0	30	0
	MRS	18	6	10	2
	Subtotal	102	45	51	6
Swine FYM	PTYG	18	4	7	7
	Desoxycolate	3	3	0	0
	Subtotal	21	7	7	7
Poultry FYM	PTYG	13	2	2	9
	Desoxycolate	10	2	4	4
	Subtotal	23	4	6	13
Cattle FYM	PTYG	10	8	2	0
	Desoxycolate	8	6	0	2
	Subtotal	18	14	2	2
Soil (swine FYM applied)	PTYG	9	2	3	4
Soil (poultry FYM applied)	PTYG	27	20	2	5
Soil (FYM not applied)	PTYG	32	11	8	13
Forest soil	PTYG	44	6	0	38
Total	All media	350	140	109	101

^a PTYG: PTYG agar medium for detection of heterotrophic bacteria; Desoxycolate: Desoxycolate agar medium for detection of coliform bacteria; EF: EF agar medium for detection of enterococci; MRS: MRS agar medium for detection of lactic acid bacteria.

the basis of different band patterns. Then, direct sequencing (approximately 1,200 bp) of PCR products of representative isolates from each of the 69 groups was performed. All of the sequence results showed more than 94.5% similarity to known genera, and all isolates were classified with the same member as the nearest genera.

The 350 Tc^r isolates affiliated to 28 bacterial genera (20 genera of Gram-negative and 8 genera of Gram-positive Tc^r bacteria) (Table 2). From the 176 animal fecal isolates, 11 bacterial genera were classified. The predominant fecal isolates were identified as members of the genus *Enterococcus* to which 60 isolates belonged and the genus *Escherichia* to which 53 belonged. From the 62 FYM isolates, 9 bacterial genera were classified. The predominant FYM isolates were identified as members of the genus *Serratia* to which 16 isolates belonged. From the 102 soil isolates, 15 bacterial genera were classified. The predominant soil isolates were identified as members of the genus *Burkholderia* to which 47 isolates belonged.

Distribution of tet genes

1. Outlines from all isolates

The 350 Tc^r bacterial isolates were examined for the presence of 19 types of Tc^r determinant. PCR analysis indicated that 71.1% of the isolates contained at least 1 of 15 of those 19 Tc^r genes. Among all Tc^r bacterial isolates, tetracycline resistance genes encoding efflux pumps were detected in 40% (140 isolates), and those encoding RPP were detected in 31.1% (109 isolates) (Table 1).

2. Isolates from animal feces

In the swine feces, 31 isolates contained the efflux pump gene *tet(A)*, *tet(B)*, *tet(C)*, *tet(H)*, *tet(J)*, or *tet(Y)*, and 30 isolates contained the RPP gene *tet(M)*, *tet(Q)*, or *tet(W)* (Tables 3 and 4). The most common *tet* gene identified was *tet(M)*, which was found in 39.3% of all the isolates. Of the 24 *tet(M)* positive isolates, 20 were identified as members of the genus *Enterococcus*.

In the poultry feces, 45 isolates contained the efflux pump gene *tet(A)*, *tet(B)*, *tet(C)*, *tet(H)*, or *tet(J)*, and 51 isolates contained the RPP gene *tet(M)*, *tet(O)*, *tet(Q)*, or *tet(W)*. The most common *tet* gene identified was *tet(M)*, which was found in 43.8% of all the isolates. Of the 42 *tet(M)* positive isolates, 30 were identified as members of the genus *Enterococcus*. The second most common gene identified was *tet(H)*, which was found in 28.1% of all the isolates. Of the 29 *tet(H)* positive isolates, 19 were identified as members of the genus *Escherichia*.

3. Isolates from solid FYM

In the swine FYM, 7 isolates contained the efflux pump gene *tet(B)* or *tet(J)*, and 7 isolates contained the RPP gene *tet(M)*, *tet(S)*, or *tet(W)* (Tables 3 and 4). In the poultry FYM, 4 isolates contained the efflux pump gene *tet(B)*, and 6 isolates contained the RPP gene *tet(S)* or *tet(W)*. In the cattle FYM, 14 isolates contained the efflux pump gene *tet(B)*, *tet(J)*, or *tet(Z)*, and 2 isolates contained the RPP gene *tet(W)*.

4. Isolates from soils

In soil to which swine FYM had been applied, 2 Tc^r isolates carried efflux pump genes-1 carried *tet(B)* and 1 carried *tet(J)*-and 3 isolates contained the RPP gene *otr(A)* or *tet(W)* (Tables 3 and 4). In soil to which poultry FYM had been applied, 20 Tc^r isolates carried the efflux pump gene *tet(B)*, *tet(G)*, *tet(J)*, or *tet(Z)*, and 2 isolates contained the RPP gene *tet(Q)* or *tet(W)*. The most common *tet* gene was *tet(B)*, which was found in 9 isolates. In the soil to which FYM had not been applied, 11 isolates contained the efflux pump gene *tet(B)*, *tet(G)*, *tet(J)*, or *tet(Z)*, and 8 isolates contained the RPP gene *tet(M)*, *otr(A)*, or *tet(W)*. In the forest soil, all 6 Tc^r isolates carried efflux pump genes; no isolates contained RPP genes.

Discussion

Tc^r bacteria are more diverse than ever thought

In 2001, 39 genera of Gram-negative bacteria and 23 genera of Gram-positive bacteria and related genera had been described in which the mechanism of tetracycline resistance had been determined⁸⁾. In 2005, the number of Tc^r bacterial genera had increased from 62 to 115¹⁹⁾. Furthermore, in this study we found 13 genera in which *tet* genes had not been previously detected: identified as members of the genera *Kurthia*, *Pediococcus*, and *Vagococcus* in fecal isolates; *Bordetella*, *Bradyrhizobium*, and *Ochrobactrum* in FYM isolates; and *Burkholderia*, *Dyella*, *Flexibacter*, *Luteibacter*, *Lysobacter*, “*Nordella*”, and *Rhodopseudomonas* in soil isolates (Table 2).

There are two reasons why bacterial genera conferring the Tc^r phenotype have been found here for the first time. Firstly, we tried to isolate Tc^r bacteria from various sources, such as feces, FYM, and soils. Previous studies on Tc^r bacteria, on the other hand, mostly focused on enterobacterial pathogens of fecal origin, and these few studies determined the Tc^r phenotype among heterotrophic bacterial flora. Secondly, we used the non-selective PTYG medium, a well-

Table 2. Phylogenic affiliations of tetracycline-resistant bacteria isolated from different Japanese environmental samples

Genus assigned	16S rRNA gene sequences comparison			No. of isolates							
	Closest relative and accession number	Similarity (%)	Total	Feces		FYM			Soil		
				Swine	Poultry	Swine	Poultry	Cattle	Soil (+) ^a	Soil (–) ^b	F-soil ^c
<i>Acinetobacter</i>	<i>Acinetobacter</i> sp. (AJ007008)	98.6	2 (2)	2 (2)							
<i>Afipia</i>	<i>A. massiliensis</i> (AY029562)	98.5	6 (6)						6 (6)		
<i>Alcaligenes</i>	<i>Alcaligenes</i> sp. (AY346138)	98.8	6 (1)		1 (1)		5 (0)				
	<i>Alcaligenes</i> sp. (AY628412)	97.6	2 (2)		2 (2)						
	<i>Alcaligenes</i> sp. (AJ786800)	95.8	1 (1)				1 (1)				
<i>Bacillus</i>	<i>Bacillus</i> sp. (AF169535)	98.9	1 (1)			1 (1)					
<i>Bordetella</i> *	<i>Bordetella</i> sp. (DQ302159)	96.1–98.0	6 (3)				6 (3)				
<i>Bradyrhizobium</i> *	<i>B. elkanii</i> (AY904771)	99.1–99.8	3 (1)			1 (0)	1 (1)			1 (0)	
	<i>Bradyrhizobium</i> sp. (DQ100058)	100	1 (0)			1 (0)					
	<i>Bradyrhizobium</i> sp. (AY603956)	99.9–100	3 (0)								3 (0)
<i>Burkholderia</i> *	<i>B. cepacia</i> (AY677087)	99.4	10 (1)						1 (0)		9 (1)
	<i>B. cepacia</i> (AY769903)	99.3	5 (1)						3 (1)		2 (0)
	<i>Burkholderia</i> sp. (DQ847125)	98.8	32 (15)						8 (5)	14 (10)	10 (0)
<i>Clostridium</i>	<i>Clostridium</i> sp. (AF443595)	94.5	3 (2)				3 (2)				
<i>Dyella</i> *	<i>D. koreensis</i> (AY884571)	98.3	5 (1)								5 (1)
<i>Enterococcus</i>	<i>E. faecalis</i> (AY850358)	100	60 (58)	22 (22)	38 (36)						
<i>Escherichia</i>	<i>E. coli</i> (U00096)	99.5–100	43 (43)	13 (13)	29 (29)	1 (1)					
	<i>E. coli</i> (AM184233)	99.4	9 (8)	1 (1)		8 (7)					
	<i>E. coli</i> (AM184252)	99.0	1 (1)	1 (1)							
<i>Flexibacter</i> *	<i>Flexibacter</i> sp. (AY238335)	97.1–99.0	19 (12)						4 (3)	12 (6)	3 (3)
<i>Inquilinus</i>	<i>I. ginsengisoli</i> (AB245352)	99.4	1 (0)								1 (0)
<i>Kurthia</i> *	<i>K. gibsonii</i> (AM184261)	96.7–99.9	11 (11)	5 (5)	6 (6)						
	<i>K. gibsonii</i> (DQ071565)	99.1	1 (1)		1 (1)						
	<i>K. zopfii</i> (X70321)	99.5	1 (1)		1 (1)						
<i>Luteibacter</i> *	<i>L. rhizovicinus</i> (AY162032)	98.3	4 (0)								4 (0)
	<i>L. rhizovicinus</i> (AJ580499)	98.2	1 (1)								1 (1)
<i>Lysobacter</i> *	<i>L. antibioticus</i> (AB019582)	99.3	2 (2)							2 (2)	
	<i>L. gummosus</i> (AB161361)	99.5	2 (1)							2 (1)	
	<i>Lysobacter</i> sp. (AB083480)	100	1 (0)								1 (0)
<i>Nordella</i> *	<i>N. oligomobilis</i> (AF370880)	98.3	4 (4)						4 (4)		
<i>Ochrobactrum</i> *	<i>O. grignonense</i> (AJ242581)	97.3	3 (3)			3 (3)					
	<i>Ochrobactrum</i> sp. (DQ305290)	99.3	2 (0)					2 (0)			
<i>Pandoraea</i>	<i>Pandoraea</i> sp. (AF247699)	99.8	1 (0)								1 (0)
<i>Pediococcus</i> *	<i>Pediococcus</i> sp. (AB219053)	99.9	10 (7)		10 (7)						
<i>Proteus</i>	<i>P. mirabilis</i> (AF008582)	99.8–100	8 (8)	7 (7)	1 (1)						
	<i>Proteus</i> sp. (DQ512963)	98.2	1 (1)		1 (1)						
<i>Providencia</i>	<i>P. heimbachae</i> (AM040490)	99.5	6 (4)		6 (5)						
<i>Rhodopseudomonas</i> *	<i>Rhodopseudomonas</i> sp. (AB251406)	97.0	1 (1)						1 (1)		
<i>Serratia</i>	<i>S. marcescens</i> (AJ302156)	99.5–100	30 (22)	13 (5)	1 (0)			16 (16)			
	<i>S. marcescens</i> (AY514435)	99.1	5 (4)						4 (4)		1 (0)
	<i>Serratia</i> sp. (AM396490)	99.0	3 (2)	3 (2)							
<i>Staphylococcus</i>	<i>S. epidermidis</i> (CP000029)	99.4–99.8	6 (4)	1 (1)					5 (3)		
	<i>S. equorum</i> (AM237374)	99.6–99.8	3 (0)				3 (0)				
	<i>S. saprophyticus</i> (AP008934)	100	12 (7)	6 (2)	3 (3)	3 (2)					
	<i>S. xylosus</i> (AF515587)	99.0–99.5	7 (3)			3 (0)	4 (3)				
<i>Streptomyces</i>	<i>S. olivaceus</i> (AB184735)	99.5	1 (0)								1 (0)
<i>Vagococcus</i> *	<i>V. fluvialis</i> (Y18098)	100	1 (1)		1 (1)						
<i>Variovorax</i>	<i>V. paradoxus</i> (DQ256487)	99.1	1 (0)							1 (0)	
	<i>Variovorax</i> sp. (AY689027)	100	2 (0)								2 (0)
Total			350 (249)	74 (61)	102 (96)	21 (14)	23 (10)	18 (16)	36 (27)	32 (19)	44 (6)

Numbers in the table show numbers of isolates belonging to the respective genus after RFLP analysis.

^a: Soil treated with FYM. ^b: Soil not treated with FYM. ^c: Forest soil.

(): the number of isolates having *tet* genes, *: genus in which *tet* genes were found for the first time.

Table 3. Tetracycline efflux genes detected in bacterial isolates

Gene	Efflux genes		
	Genus assigned ^a	Number of isolates	Origin
<i>tet(A)</i>	<i>Alcaligenes</i>	2	Poultry feces
	<i>Escherichia</i>	4	Swine feces
<i>tet(B)</i>	<i>Afipia</i>	6	Poultry FYM applied soil
	<i>Alcaligenes</i>	1	Poultry FYM
	<i>Bordetella</i>	1	Poultry FYM
	<i>Bradyrhizobium</i>	1	Poultry FYM
	<i>Burkholderia</i>	1	Swine FYM applied soil
		1	Forest soil
	<i>Enterococcus</i>	2	Poultry feces
	<i>Escherichia</i>	4	Swine feces
		2	Swine FYM
	<i>Flexibacter</i>	3	Forest soil
	<i>Kurthia</i>	2	Swine feces
		3	Poultry feces
	<i>Ochrobactrum</i>	3	Swine FYM
	<i>Pediococcus</i>	2	Poultry feces
	<i>Providencia</i>	3	Poultry feces
	<i>Serratia</i>	5	Swine feces
		2	Cattle FYM
	<i>Staphylococcus</i>	1	Swine feces
		1	Poultry FYM
		3	Poultry FYM applied soil
	<i>Vagococcus</i>	1	Poultry feces
<i>tet(C)</i>	<i>Escherichia</i>	2	Poultry feces
	<i>Staphylococcus</i>	1	Swine feces
<i>tet(G)</i>	<i>Flexibacter</i>	1	FYM not applied soil
	<i>Rhodopseudomonas</i>	1	Poultry FYM applied soil
<i>tet(H)</i>	<i>Alcaligenes</i>	1	Poultry feces
	<i>Enterococcus</i>	1	Swine feces
	<i>Escherichia</i>	4	Swine feces
		19	Poultry feces
	<i>Kurthia</i>	1	Poultry feces
	<i>Pediococcus</i>	1	Poultry feces
	<i>Proteus</i>	5	Swine feces
	<i>Serratia</i>	1	Poultry feces
	<i>Staphylococcus</i>	3	Poultry feces
<i>tet(J)</i>	<i>Burkholderia</i>	1	Swine FYM applied soil
		1	FYM not applied soil
	<i>Escherichia</i>	2	Swine FYM
	<i>Lysobacter</i>	1	FYM not applied soil
	<i>Nordella</i>	3	Poultry FYM applied soil
	<i>Proteus</i>	2	Swine feces
	<i>Serratia</i>	1	Swine feces
	<i>Pediococcus</i>	2	Poultry feces
	<i>Providencia</i>	1	Poultry feces
	<i>Serratia</i>	10	Cattle FYM
<i>tet(Y)</i>	<i>Acinetobacter</i>	1	Swine feces
	<i>Dyella</i>	1	Forest soil
	<i>Luteibacter</i>	1	Forest soil
<i>tet(Z)</i>	<i>Burkholderia</i>	4	Poultry FYM applied soil
		6	FYM not applied soil
	<i>Serratia</i>	2	Cattle FYM
		2	Poultry FYM applied soil

^a Genus was assigned based on partial 16S rRNA gene sequences.

Table 4. Tetracycline RPP genes detected in bacterial isolates

Gene	RPP genes		
	Genus assigned ^a	Number of isolates	Origin
<i>tet(M)</i>	<i>Bacillus</i>	1	Swine FYM
	<i>Burkholderia</i>	1	FYM not applied soil
	<i>Enterococcus</i>	20	Swine feces
		30	Poultry feces
	<i>Escherichia</i>	1	Swine feces
		7	Poultry feces
		1	Swine FYM
	<i>Flexibacter</i>	2	FYM not applied soil
	<i>Kurthia</i>	3	Swine feces
		5	Poultry feces
	<i>Proteus</i>	1	Poultry feces
<i>tet(O)</i>	<i>Staphylococcus</i>	2	Swine FYM
	<i>Enterococcus</i>	2	Poultry feces
	<i>Pediococcus</i>	1	Poultry feces
<i>otr(A)</i>	<i>Burkholderia</i>	1	FYM not applied soil
	<i>Serratia</i>	1	Swine FYM applied soil
<i>tet(Q)</i>	<i>Enterococcus</i>	1	Swine feces
	<i>Flexibacter</i>	1	Poultry FYM applied soil
	<i>Providencia</i>	1	Poultry feces
	<i>Staphylococcus</i>	1	Swine feces
<i>tet(S)</i>	<i>Clostridium</i>	1	Poultry FYM
	<i>Escherichia</i>	1	Swine FYM
<i>tet(W)</i>	<i>Acinetobacter</i>	1	Swine feces
	<i>Bordetella</i>	1	Poultry FYM
	<i>Burkholderia</i>	1	FYM not applied soil
		1	Poultry FYM
	<i>Clostridium</i>	2	Poultry FYM
	<i>Enterococcus</i>	4	Poultry feces
	<i>Escherichia</i>	2	Swine feces
		2	Swine FYM
	<i>Flexibacter</i>	3	FYM not applied soil
		2	Swine FYM applied soil
	<i>Nordella</i>	1	Poultry FYM applied soil
	<i>Pediococcus</i>	1	Poultry feces
	<i>Serratia</i>	1	Swine feces
		2	Cattle FYM
	<i>Staphylococcus</i>	2	Poultry FYM

^a Genus was assigned based on partial 16S rRNA gene sequences.

known low-nutrient medium used for isolating oligotrophic soil bacteria. Although it is surprising that Tc^r bacteria were isolated from fecal and FYM samples using the PTYG medium, our results show that PTYG medium is well applicable to isolating oligotrophic bacteria, even from fecal and FYM samples.

Tc^r bacterial isolates seem to be more diverse than our study would suggest, because there is a report that CFU counts were about 10 times higher when a gellan gum-based medium rather than agar-based medium was used with freshwater sediment samples¹¹). If we were to use, for example, a gellan gum-based medium, we may obtain more diverse Tc^r isolates. Further studies are needed to isolate culturable bacteria.

A number of tet genes newly identified in Tc^r bacterial genera

In this study, we found a number of *tet* genes in the documented Tc^r bacterial genera as well as in some genera not previously identified as being tetracycline resistant. Of the 115 previously documented Tc^r bacterial genera, 11 were found in this study. In Gram-negative bacteria, we found the *tet*(Y) and *tet*(W) genes in *Acinetobacter*, the *tet*(B) gene in *Afipia*, the *tet*(B) and *tet*(H) genes in *Alcaligenes*, the *tet*(W) gene in *Escherichia*, the *tet*(H) and *tet*(M) genes in *Proteus*, the *tet*(J) and *tet*(Q) genes in *Providencia*, and the *tet*(H), *tet*(J), *tet*(Z), *otr*(A), and *tet*(W) genes in *Serratia*. In Gram-positive bacteria, we found the *tet*(S) and *tet*(W) genes in *Clostridium*, the *tet*(B), *tet*(H), *tet*(Q), and *tet*(W) genes in *Enterococcus*, and the *tet*(B), *tet*(C), *tet*(H), and *tet*(Q) genes in *Staphylococcus*. These combinations are new, although a variety of *tet* genes have been reported in these species up to now.

In addition, 13 genera were found for the first time in this study as carrying *tet* genes. In Gram-negative bacteria, the *Bordetella* isolates contained *tet*(B) or *tet*(W), *Bradyrhizobium* isolate contained *tet*(B), *Burkholderia* isolates contained *tet*(B), *tet*(J), *tet*(Z), *tet*(M), *tet*(W), or *otr*(A), *Dyella* isolate contained *tet*(Y), *Flexibacter* isolates contained 1 of 5 *tet* genes (*tet*(B), *tet*(G), *tet*(M), *tet*(Q), or *tet*(W)), *Luteibacter* isolate contained *tet*(Y), *Lysobacter* isolates contained *tet*(B) or *tet*(J), “*Nordella*” isolates contained *tet*(J) or *tet*(W), *Ochrobactrum* isolates contained *tet*(B), and *Rhodopseudomonas* isolate contained *tet*(G). In Gram-positive bacteria, the *Kurthia* isolates contained 1 of 3 *tet* genes (*tet*(B), *tet*(H), or *tet*(M)), *Pediococcus* isolates contained *tet*(B), *tet*(H), *tet*(J), *tet*(O), or *tet*(W), and *Vagococcus* isolate contained *tet*(B).

One reason for these new findings is that the scale of the experiment on the *tet* gene in the agricultural environment was small, and there is a possibility that the *tet* gene exists in various bacteria.

Broad or narrow host range of the tet genes

The *tet* genes are found in a variety of bacteria isolated

from humans, animals, and the environment. The majority of the *tet* genes are associated with either conjugative or mobilizable elements which may partially explain their wide distribution among bacterial species. The Gram-negative *tet* efflux genes are found as transposons inserted into plasmids of a diverse group from plasmids of a variety of incompatibility groups. Gram-positive efflux genes are associated with small plasmids. The ribosomal protection genes *tet*(S) and *tet*(O) can be found on conjugative plasmids, or in the chromosome, where they are not self-mobile. The *tet*(M) and *tet*(Q) genes are generally associated with conjugative chromosomal elements, which code for their own transfer⁸).

The *tet*(B) gene has the widest host range of any Gram-negative bacterium and often presents on a conjugative plasmid⁸). Also in our study, *tet*(B) was detected in a wide range of bacterial genera isolated from animal feces, FYM, and soil (Table 3). Similarly, *tet*(M), *tet*(W), and *tet*(J) were ubiquitously detected in various environments. It has been well established that bacteria carrying *tet*(M) and *tet*(W) belong to various genera¹⁹), and are often associated with conjugative transposons. In the case of the *tet*(J) gene, we do not yet have enough information, with only one report of its detection in *Proteus*⁸). The reason why *tet*(B), *tet*(M), and *tet*(W) were detected in numerous genera is thought to be because of such conjugative determinants²³).

On the other hand, some *tet* genes were detected only in limited environments. In this study, *tet*(A), *tet*(C), *tet*(H), and *tet*(O) were only detected in animal feces. These four genes have been frequently detected in isolates associated with the gastrointestinal tract of pigs or other farm animals^{1,6,10,13}). One reason why these *tet* genes were distributed in limited hosts is their lower mobility. Another explanation is that these *tet* genes are less well studied than other wide host range *tet* genes. Indeed, *tet*(H) was detected in animals and fish farm water¹⁶). A recent report also detected *tet*(H) in manure-applied soil²¹).

In this study, we isolated 350 tetracycline-resistant bacteria from 19 wide-ranging types of environmental samples and investigated the distribution of 19 *tet* genes. Our results suggested that even usually nonpathogenic and nonclinical bacteria had tetracycline resistance genes at a high rate and that Tc^r bacteria are more diverse than previously thought. The risk of antibiotic resistance genes diffusing from animal production sites to various environments is a serious public health problem. Further studies will be needed for a detailed understanding of gene transfer in various environments. Such knowledge may be useful for risk assessment in the future.

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