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Tetracycline resistance genes are more prevalent in wet soils than in dry soils

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ABSTRACT

This study aimed to reveal the effects of water content on the spread of tetracycline resistance genes (TRGs) in the soil. Amendments of four samples with different soil water contents, namely 16% (dry soil) and 25% (wet soil), and with or without pig manures (PM) were conducted under laboratory conditions. Quantitative polymerase chain reaction (q-PCR) results showed that the relative abundance of TRGs (*tet*B, *tet*C, *tet*M, *tet*O, *tet*T, and *tet*Z) in the wet soils was significantly higher than that in the dry soils whether under fertilization or non-fertilization conditions. Moreover, PM application enhanced the relative abundance of TRGs. The absolute copies of TRGs did not decline with the decrease in 16S rRNA genes in wet soils, implying that most TRGs were probably located in facultative anaerobic bacteria. However, cultivable tetracycline-resistant bacteria (TRB) in the wet soils were not in line with the q-PCR results, further indicating that aerobes might not account for the increases in the relative abundance of TRGs. Diversities of aerobic TRB were significantly higher in the wet soils than in the dry soils, especially on days 14 and 28. The patterns of community structures of aerobic TRB in the wet soils or dry soils containing PM were different from those in the dry soils. Together, this study showed that the variations in bacterial communities between the wet and dry soils, especially reflected in the diversity of aerobic TRB and/or community structure of facultative anaerobic TRB, might be an important reason behind the changes in the abundance of TRGs.

1. Introduction

The long-term use of tetracyclines (TCs) against a wide range of gram-positive and gram-negative bacteria in both humans and animals (Chopra and Roberts, 2001) results in the selection of resistant animal pathogens and of mutants in the bacterial genome, in which genes code for resistance through mobile genetic elements (Recchia and Hall, 1995; Roberts, 1996; Witte, 1998). Some experimental studies have shown that tetracycline resistance genes (TRGs) are predominant antibiotic resistance genes (ARGs) in agricultural soils or even in human individuals in China (Hu et al., 2013; Wu et al., 2010; Zhu et al., 2013). Besides the large-scale use of TCs, some co-selecting factors, including heavy metals, organic pollutants, and biocides, have already been discussed for their promoting effects in the spread of TRGs in the soil (Pal et al., 2015; Seiler and Berendonk, 2012; Sun et al., 2015). However, as important factors for many biological processes in the soil, the effects of chemical and physical properties of the soil, such as pH, organic matter, and water content, on the spread of ARGs in soil are largely unknown. Of these, the water content is of special interest because it is capable of affecting soil microbiota via regulation of soil nutrient bioavailability and oxygen concentration in the micro-environment (Dilly, 2003; Fierer et al., 2003). The changes in bacterial communities may lead to the spread of abundant ARGs in the soils (Su et al., 2015). Moreover, whether and how this factor affects the spread of ARGs in the soil is of special significance in Southeast Asia region, mainly in China, owing to the large-scale cultivation of rice (Khush, 1997), and the need to immerse the paddy soils in water for around 15 days.

Theoretically, the wet soil is conducive to the transfer of ARGs in the soil. A previous study has shown that low moisture may limit the multiplication of fecal bacteria (Byappanahalli and Fujioka, 2004). Furthermore, a water body with high moisture may accelerate the spread of ARGs via improving the accessibility for bacteria to achieve the exchange of genes (Ma et al., 2012; McDaniel et al., 2010). Many studies have pointed out the consequences of transmission of ARGs from topsoil to deeper soil and even to groundwater along with irrigation and rain-water (Kang et al., 2016a; Su et al., 2015). However, a few studies explored whether wet conditions could amplify the effects of the spread of ARGs in the soil. Hence, the wet and dry soils should be

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kept in a relatively sealed condition to avoid the potential underestimation of ARG abundance resulting from soil permeability.

In this study, the effects of soil moisture on the spread and abundance of TRGs and tetracycline-resistant bacteria (TRB) in soils were tested using soils of different water contents. Besides, TRB communities between treatments were compared to uncover the possible mechanisms underlying the differences in TRG abundance between the wet and dry soils.

2. Materials and methods

2.1. Collections of pig manure and soil

As in previous studies (Kang et al., 2016a, 2016b), PM (TC content 925.3 ± 78.7 μg kg^{-1}) was collected from a pig farm located in Qinfeng Town, Yangzhou City. Besides common feeds, fattening pan age (Loushi Co. Ltd.) with 21.57 ± 16.33 μg kg^{-1} of TCs was used as a growth promoter for pigs at a daily rate of 2.5 kg per pig. Fresh PMs were applied directly in 1 day.

The soil was collected from deep soil layers (> 60 cm) in the Yangzhou University. The characteristics of the soil samples were as follows: pH of 6.21, a soil water ratio of 1:1, organic matter of 11.07 g kg^{-1}, and cation exchange capacity of 9.34 cmol kg^{-1}. After routine treatments via pulverization and sieving (2 mm), about half of the soil was mixed evenly with fresh pig manure at a rate of 0.4% based on the traditional fertilization recommendation.

2.2. Experimental design

A total of 10 Petri dishes (150 × 33 mm^2), each containing 50 g soil, were prepared first. Using ddH_2O, soil water contents of half of the 10 Petri dishes were adjusted to 16% (dry soil) and 25% (wet soil), accounting for 70% and 100% of the maximum water-holding capacity, respectively. Additional 10 Petri dishes were used as control groups similarly except for using the non-fertilized soils. Thus, four treatments with five replicates each were performed in the following experiments. The soil water contents were calculated using the following formula: water weight (g)/dry soil weight (g) × 100; the dry soil weight was determined after drying to constant weight in an oven at 110 °C (Cambardella et al., 1994).

Each Petri dish was put in an incubator at 25 °C with 60% humidity for 28 days. On days 0, 14, and 28, the soils were sampled to determine the abundance of TRGs and TRB.

2.3. Enumeration of the populations of TRB

The method for enumeration of TRB was similar to that described in a previous study (Kang et al., 2016b). Briefly, 5 g soil samples (wet weight) were added to 45 mL of sterile distilled water (dH_2O) and shaken at 120 rpm for 20 min, followed by serial tenfold dilutions with dH_2O. Then, 100 mL of proper serial dilutions were transferred and plated on Luria-Bertani (LB)-TC agar media, which comprised 1/10-strength LB (Silby et al., 2011) agar supplemented with 16 μg mL^{-1} of TC, to grow cultivable TRB according to the Clinical and Laboratory Standards Institute document M100-S16 (Clinical and Laboratory Standards Institute, 2006). Dilutions were plated on 1/10-strength LB agar without TC to enumerate total culturable bacteria (TCB). Agar plates were incubated at 37 °C for 24 h, followed by counting and calculation of colony-forming units (cfu) (Sengeløv et al., 2003). The data were presented as the mean cfu g^{-1} of the dry soil of five replicate samples.

2.4. Quantitative polymerase chain reaction analysis of TRGs

Total microbial DNA was extracted from each soil sample using a Power-Soil DNA Isolation Kit (MO BIO Laboratories Inc., CA, USA) and amplified for *tet*B, *tet*C, *tet*M, *tet*O, *tet*T, and *tet*Z genes using the primers according to a previous study (Kang et al., 2016a). The quantitative polymerase chain reaction (qPCR) assay for detecting TRGs was the same as that described previously (Kang et al., 2016b). For normalization purposes, the copies of 16S rRNA genes were also quantified with the primer set 341F (5′-CCTACGGGNGGCWGCAG-3′) and 515R (5′-ATTCCGCGGCTGGCA-3′) (He et al., 2007).

2.5. PCR-denaturing gradient gel electrophoresis analysis

After TRB numeration, two plates per treatment were randomly selected for the molecular determination. For each plate, total bacterial colonies were washed off three times with 10-mM phosphate-buffered saline (pH 7.2). The total bacterial DNA was extracted using a Bacterial DNA Extraction Kit (Thermo Fisher Scientific, Inc., MA, USA). A 50-μL amplification reaction mixture consisted of 10 μL of DNA template, 0.2 mM of each dNTP, forward primer 338f with GC-clamp 5′-CGCCC GCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGA GGCAGCAG-3′ (0.5 μM), reverse primer 518r 5′-ATTACCGCGGCTGC TGG-3′ (0.5 μM) (Muyzer et al., 1993), 1.25 U PrimeSTAR HS DNA Polymerase (TaKaRa, Dalian, China), and 1 × buffer (including Mg^{2+} at 1.5 mM final concentration). The amplification was carried out under the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min (decreasing 0.1 °C per cycle to 52 °C), 72 °C for 1.5 min, and terminated by a final extension at 72 °C for 10 min. The amplification products were checked by agarose gel electrophoresis at 1% concentration.

A denaturing gradient gel electrophoresis (DGGE) analysis was performed on 8% (w/v) polyacrylamide gel with a denaturing gradient of 30–60% in 1× Tris-acetic acid-ethylenediaminetetraacetic acid buffer using a D-Code Mutation Detection System (Bio-Rad Laboratories, Inc., CA, USA) at 150 V and 60 °C for 7 h (Kang et al., 2016b). Following electrophoresis, the gel was stained with ethylene dibromide (0.5 μg mL^{-1}) for 25 min and photographed under ultraviolet light with a Gel Doc XR system (Bio-Rad Laboratories, Inc.). With Quantity One (Version 4.6.2, Bio-Rad Laboratories, Inc.), relative intensities of identified bands were calculated according to the percentage of the intensity of each band from the same lane. Shannon's diversity index (*H*') was calculated using the formula $H' = -\Sigma p_i \ln(p_i)$, where p_i is the ratio of the relative intensity of the band *i* to the relative intensity of the lane (Kang et al., 2016b). In view of the good repeatability (not shown), only one lane represented a treatment.

2.6. Statistical analysis

The data of culturable TRB were expressed as cfu g^{-1} of the dry soil over time. The proportion of TRB to TCB was also calculated for each treatment. Means and standard errors were imported into SPSS for Windows (SPSS, IBM, NY, USA), and differences between treatments were tested using analysis of variance with Duncan's multiple range tests. The significance level was set at a *P*-value of 0.05. The correlation analysis for 16S rRNA gene and TRGs copies was carried out using the curve-fitting model in SPSS for Windows.

3. Results

3.1. Comparison of relative abundance of TRGs between the wet and dry soils

*Tet*B, *tet*C, and *tet*Z are efflux pump proteins-coding genes; *tet*M, *tet*O, and *tet*T are ribosomal protection proteins-coding genes (Aminov et al., 2001). Of six TRGs, *tet*Z and *tet*O exhibited higher relative abundance compared with the others. The relatively low levels of relative abundance of TRGs were shown in *tet*C and *tet*T (Fig. 1).

Both in the fertilized and non-fertilized soils, the relative abundance of TRGs in the wet soils were higher than those in the dry soils (Fig. 1).