Research Paper

Effects of long-term fertilization on the diversity of bacterial mercuric reductase gene in a Chinese upland soil

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Soil mercury (Hg) pollution has received considerable attention due to its neurotoxin effects and its potential risk to food safety. The microbial transformation of Hg plays a key role in reducing Hg toxicity by the mercuric reductase (MerA) conferred by genes arranged in the *mer* operon. This study investigated the effects of long-term fertilization on the diversity of bacterial mercuric reductase gene (*merA*), which specify the reduction of ionic Hg^{2+} to the volatile elemental form Hg^0 , in an agricultural soil with relatively high Hg content. The soil samples were collected from different treatments, including control without fertilizer (CK), fertilizer nitrogen (N), combined fertilizers (NPK) of N, phosphorus (P) and potassium (K), and NPK plus organic manure (NPK + OM). The *merA* gene diversity patterns were analyzed based on the *merA* clone libraries and sequencing measurements. Results showed that the *merA* gene diversity was influenced by soil variables depending on the fertilization practices. In particular, NH_4^+ and NO_3^- contents had strong effect on the *merA* gene diversity pattern both in the N and NPK treatments, whereas the *merA* gene diversity pattern in NPK + OM treatment was distinctly influenced by the contents of organic matter, available P and K. These results suggested that long-term fertilization had significant influences on *merA* gene diversity, which could be helpful to understand the Hg reduction process and potentially serve microbial remediation of Hg contaminated soil.

Keywords: Soil / Mercury / *merA* gene / Diversity / Fertilization

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Introduction

For decades, mercury (Hg) pollution has received considerable attention due to its neurotoxin effects and its ability to biomagnify in the food chains [1–2]. The fate of Hg in soil depends upon lots of environmental factors related to physical, chemical and biological processes. Among them, the microbial transformation plays a critical role in modulating the toxicity of Hg through reducing ion Hg^{2+} into the volatile and less toxic element Hg^0 [3–5]. Indeed, this biochemical process is usually controlled by the mercuric reductase (MerA)

conferred by genes arranged in the *mer* operon as a resistance mechanism to Hg [6–8]. It was a critically important detoxification process as evidenced by the resumption of microbial growth after the removal of gaseous form of element Hg^0 [9, 10]. And thus, the *merA* gene has been studied as a relevant functional gene for understanding the microbial transformation of Hg speciation in environments.

Mercury resistance genes (*merA*) are often carried on plasmids or other mobile genetic elements [11, 12], which are widely spread in bacteria from diverse environments [13–17]. The bacteria that exposed to the long-term selective stress would probably be resistant, and thus leading to high *merA* diversity [18]. A high diversity of *merA* gene sequences was detected in an anaerobic mercury-contaminated sediment [19], which exhibited distinct differences of the *merA* sequences from other aerobic environments. Various *merA* diver-

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sity patterns were also detected along floodplain soil profiles [18], and even in the high Arctic area [10], suggesting the potential of Hg reduction by Hg-resistance microorganisms in very diverse environments. The *merA* genes were showing high diversities along with the variation of the environment. Additionally, Ramond *et al.* [20] reported that human activity could be a determining factor in the occurrence of the gram-negative *merA* gene in mudflat sediments. As we know, soil is a complex medium attributed with complicated physical and chemical properties, as well as strong disturbance from human activities, but little is known about the effects of anthropogenic factors, especially long-term fertilization practices, on *merA* gene diversity in agricultural soils.

Fertilization, as a common and important agricultural management practice, can provide essential nutrients for crop growth, and support a diverse and active microbial community [21]. Some studies have provided general appreciation of effects of fertilization on soil bacteria [18, 22]. Consequently, long-term fertilization practices could result in significant shifts in the abundance and community composition of soil functional microorganisms, such as ammonia oxidizers, methanotrophs and sulfate reducing prokaryotes [22–24]. However, knowledge of the effect of long-term fertilization on the *merA* gene diversity is particularly scarce. Previous study has reported the potential Hg pollution soil with long-term fertilization in the Qiyang experimental station, Hunan Province, China [25]. The Hg in this soil was mainly originated from soil parent materials but not an anthropogenic input. And the crop grown in this soil accumulated higher concentration of Hg beyond the hygienic standard for grains (0.02 mg kg^{-1}) indicating food chain health risk. The purpose of the present study was to examine the effects of long-term different fertilization practices on the *merA* gene diversity, and the obtained data would be helpful to the microbial remediation of Hg contaminated.

Materials and methods

Study site and sampling

The site was located at Qiyang Red Soil Experimental Station ($26^{\circ}45' \text{ N}$, $111^{\circ}52' \text{ E}$), Hunan Province, China. A long-term fertilizer experiment was established in 1990 with a wheat-maize rotation system. The top soil (0–20 cm) samples were collected from the experimental plots, including four treatments with three replicates: control without fertilizers (CK); chemical fertilizer nitrogen (N); combined chemical fertilizers (NPK) of N, phosphate (P) and potassium (K) and NPK plus organic manure (NPK + OM). The soil had high background concentration of Hg and potential environmental risk according to the previous study [25]. Basic soil characteristics are listed in Table 1. Soil samples were passed through a 2.0 mm sieve, and stored at -80° C for DNA extraction.

Soil DNA extraction and purification

DNA was extracted from 0.5 g fresh soil sample using Ultra-cleanTM soil DNA Isolation Kits (MoBio Laboratory, USA) according to the manufacturer's protocol. The quality of extracted DNA was evaluated on a 1% agarose gel and the concentration and purity of the extracts were estimated by a spectrophotometer (NanoDrop, USA).

PCR application and construction of *merA* library

The presence of *merA* gene in the DNA extracts was detected by a nested PCR approach as attempts to directly amplify the *merA* gene from the soil DNA extracts had been unsuccessful. A 1,250 bp fragment was amplified as follows: 15 cycles of 10 s at 94° C , 45 s at 54° C , and 30 s at 72° C , using the forward primer A2-n.F (5'-CCATCGGCGGCWCTGCGTSAA-3') and the reverse primer A5-n.R (5'-ACCATCGTCAGRTARGGRAAVA-3') [10]. The obtained PCR products were separated by 1% agarose gel electrophoresis, excised, purified using a gel

Table 1. Chemical properties of the Chinese upland red soil under different fertilization regimes^a.

	pH ^b (H ₂ O)	Organic matter ^b (%)	NH ₄ ⁺ -N (mg kg ⁻¹)	NO ₃ ⁻ -N (mg kg ⁻¹)	Available P (mg kg ⁻¹)	Available K (mg kg ⁻¹)	Total Hg ^c (mg kg ⁻¹)
CK	5.47 ± 0.18	1.36 ± 0.06	19.1 ± 0.61	5.1 ± 1.54	30.1 ± 2.3	29.4 ± 2.1	0.64 ± 0.13
N	3.71 ± 0.15	1.52 ± 0.29	21.0 ± 5.84	43.6 ± 7.7	4.4 ± 3.1	16.9 ± 3.3	0.60 ± 0.09
NPK	4.04 ± 0.06	1.69 ± 0.08	28.8 ± 2.40	8.7 ± 2.7	51.3 ± 4.2	85.7 ± 2.5	0.54 ± 0.07
NPK + OM	5.81 ± 0.15	2.13 ± 0.08	17.7 ± 1.24	18.7 ± 4.0	137.0 ± 3.5	19.0 ± 3.8	0.51 ± 0.09

^a treatments include: control without fertilizers (CK); chemical fertilizer nitrogen (N); combined chemical fertilizers (NPK) of N, phosphate (P) and potassium (K) and NPK plus organic manure (NPK + OM).

^b data is from reference 23.

^c data is from reference 25.

purification kit (Promega), and diluted as templates for the second PCR with the forward primer A7s-n.F (5'-CGATCCGCAAGTGGCIACBGT-3') and the reverse primer A5-n.R (5'-ACCATCGTCAGRTARGGAAVA-3') [4]. In this reaction, a 291 bp fragment internal to the 1,250 bp template was amplified as follows: 40 cycles of 10 s at 94 °C, 45 s at 54 °C, and 30 s at 72 °C. The final PCR products (291 bp) were confirmed by a 1% agarose gel electrophoresis and purified with a gel purification kit (Promega). Afterward, twelve *merA* clone libraries, consisting of the replicates samples from each of the four soil treatments, were constructed.

Sequencing and phylogenetic analysis

A total of 408 clones in the twelve libraries were single strand sequencing. Representative *merA* gene sequences were deposited in the GenBank nucleotide sequence database under Accession No. GU367623–GU367653. The *merA* nucleotide sequences were trimmed and assembled using SeqMan (DNASTar, Madison, WI). Homology analysis of the *merA* sequences was performed using the software DNAMAN version 4.0 (Lynnon Bio-soft, USA). Sequences displaying more than 95% identity with each other were grouped into the same operational taxonomic units (OTUs). Phylogenetic analysis was performed using MEGA version 4.0 and the neighbor-joining tree was constructed using the Poisson correction model with Bootstrap value of 1000.

Data analysis

Evaluation of the clone libraries was performed by a rarefaction analysis using software aRarefactWin Version 1.3 (Steven Holland, Stratigraphy Lab, University of Georgia [http://www.uga.edu/strata/software/Software.html]). The Shannon index was calculated based on the following equation: $H' = -\sum p_i \ln(p_i)$, where H' = Shannon-Wiener diversity index, $p_i = ni/N$, ni is the abundance of the OTU, N is the total abundance of all OTUs in the sample. Non-parametric richness estimates (Chao 1) were calculated according to the previous literature [26]. The similarity of phylogenetic compositions of *merA* clone libraries between different treatments were

compared using the Sorensen similarity index, $C_s = 2j/(a + b)$, in which j is the number of common OTUs in two *merA* clone libraries of different treatments, a and b are the numbers of OTUs in the two libraries, respectively [27].

The differences of *merA* gene diversity patterns among the four fertilization regimes were statistically analyzed using one-way analysis of variance (ANOVA). All these statistics were conducted using SPSS software. Canonical correspondence analysis (CCA) (Canoco 4.5 for Windows) was performed to explore the relationship between environmental variables and the *merA* gene diversity in the twelve clone libraries [28]. Variables to be included in the model were chosen by forward selection at 0.05 baseline. Test significance of the constrained ordination process was tested with the Monte Carlo permutation test.

Results

Analysis of *merA* clone libraries

Estimates of *merA* gene diversity in the four treatments, based on the sequenced clones, are shown in Table 2. All of the *merA* gene libraries were sampled adequately as the rarefaction curves approached a plateau (Fig. 1). The relatively high OTU number was observed in the N treatment, in which the value of richness (Chao 1) was significantly higher than those in the other treatments. No significant difference was found in Shannon index among the four treatments.

The Sorensen similarity index was used to identify the similarity of *merA* library compositions between the treatments, ranging from 0 to 75% in the four treatments (Table 3). The relatively high Sorensen similarity index was found between the N and NPK treatments, indicating that they had very similar *merA* clone library composition. The NPK + OM treatment had relatively lower values of 0, 27% and 15% compared with CK, N and NPK treatments, suggesting its library composition was distinctly different from the compositions of other *merA* clone libraries.

Table 2. Diversity analysis of *merA* gene fragment libraries.*

	No. of sequences	OTU (95%)	Chao 1	H'
CK	102	14	8.4 (7.1–9.1) ^a	1.1 (0.9–1.2) ^a
N	102	16	11.4 (10.9–11.8) ^b	1.6 (1.1–1.9) ^a
NPK	102	12	8.6 (6.1–11.1) ^a	1.2 (0.7–0.6) ^a
NPK + OM	102	10	7.4 (6.2–9.1) ^a	1.2 (1.2–1.3) ^a

* Chao1, Chao1 nonparametric richness estimate; H', Shannon-Weaver diversity index; Values within the same column followed by the same letter do not differ at $P < 0.05$.

Figure 1. Rarefaction analysis of the *merA* clones obtained from soils with the different fertilization treatments using software aRarefactWin Version 1.3. Treatment: control without fertilizers (CK); chemical fertilizer nitrogen (N); combined chemical fertilizers (NPK) of N, phosphate (P) and potassium (K) and NPK plus organic manure (NPK + OM).

Effects of soil environmental variables on the *merA* gene diversity

The Canonical Correspondence Analysis (CCA) was used to determine the environmental factors that appeared to have the strongest influence on *merA* composition (Fig. 2). The symbols were mostly grouped according to fertilization practice, indicating that fertilization regimes influenced the *merA* gene diversity. In the CK treatment, it is likely that total Hg content had strong effect on the *merA* gene diversity pattern. The NH_4^+ and NO_3^- contents had strongest effects on the *merA* gene diversity pattern in the N and NPK treatments, while the contents of organic matter, available P and K posed a strongest influence on the *merA* gene diversity pattern in the NPK + OM treatment.

Phylogenetic analysis in the different treatments

The phylogeny of *merA* sequences from the different treatments along with reference sequences from Genbank is shown in Fig. 3. The obtained 408 *merA* sequences yielded 31 *merA* phylotypes using a phylotype definition of 95% sequence identity. All the detected

Table 3. Comparison of the of *merA* library compositions between different treatments of a Chinese upland red soil.

	Sorensen similarity index (%) for the fertilization library		
	N	NPK	NPK + OM
CK	43	33	0
N		75	27
NPK			15

Figure 2. Canonical Correspondence Analysis (CCA) between the *merA* community and environmental variables of pH value, organic matter, total Hg content, NH_4^+ , NO_3^- and available P and K in the soil. The meaning of symbols: circle represents CK treatment; box represents N treatment; diamond represents NPK treatment; left-triangle represents NPK + OM treatment.

merA sequences were grouped into 7 distinct clusters. A total of 190 sequences in the twelve clone libraries were related to cultured mercury-resistance bacteria, and 167 sequences affiliated to the uncultured *merA* clone clusters from soil and sediment. Moreover, 51 *merA* sequences were grouped into unknown clusters.

The *merA* phylotypes in the four treatments were distinctly different. The proportion of sequence types representing various clusters in each treatment was calculated (Fig. 4). In the CK treatment, the *merA* gene was dominated by representatives of cluster 2 and 3, accounting for 83.3% (85/102) of the total phylotypes. All of detected sequences in the N treatment fell into cluster 1, 4 and 7, in which 52.9% (64/102) of sequences were grouped into uncultured bacterial clones. Sequences in the NPK + OM treatment were dominated by cluster 1 and the proportion reached 96.1% (98/102). The N and NPK treatments had several shared clusters, including mainly the *merA* gene representatives of cultured cluster 1 and uncultured cluster 7 related to the clones from sediment. No sequence affiliated with cluster 4 could be detected in the NPK and NPK + OM treatments.

Discussion

It is essential to investigate the diversity of Hg resistance gene that potentially modulates the toxicity of Hg

Figure 3. Neighbour-joining tree of *merA* DNA sequences. Bootstrap values (>50%) are indicated at branch points. Designation of the clones in bold includes the following information: accession number in the GenBank with fertilization treatments in the parentheses. The number of clones in a particular phylotype is indicated in parentheses. The scale bar represents 5% estimated sequence divergence.

in the soils [5, 29], because it could provide a better understanding of soil environmental factors related to the Hg resistance microbes. In this study, twelve clone libraries were constructed for examining the *merA* gene diversity in the upland soils under long-term fertiliza-

tion. Our results showed that some bacteria in these agricultural soils could have adapted to the long-term Hg stress.

This adaptation could partially involve the mechanisms: induction of enzymes related with the detoxifi-

cation of Hg and the enrichment of populations carrying Hg resistance trait [8, 30].

According to the results of this study, the soil environmental variables depending on the fertilization practices would play key roles in affecting the *merA* gene diversity pattern in these soils. The different environmental factors in the soils resulted from the different fertilizer applications. In the experimental plot, the CK treatment was considered to have not been disturbed by human activity and total soil Hg content in the soil could be regarded as a natural factor since its parent material source [25]. This non-anthropogenic factor was affecting the *merA* gene diversity in the CK treatment (Fig. 2), while only minor effect from it could be found in the other treatments with complex fertilizer applications. These results demonstrated that total Hg content, as a non-anthropogenic factor, should be important in impacting the *merA* gene diversity in these agricultural soils. However, anthropogenic fertilization effects were introduced and difference in *merA* gene diversity pattern was observed in the other fertilization treatments.

In general, long-term fertilizer applications could lead to a shift in soil physical and chemical property, thus the diversity pattern of soil microorganisms would be impacted. The results of CCA profile demonstrated that the *merA* gene diversity patterns were substantially influenced by the contents of NH_4^+ and NO_3^- in positive effects and pH in negative effect. The effect was different in different fertilization treatments. So in Fig. 2 the symbols of *merA* gene diversity from the NPK + OM treatment were around organic matter, available P and available K content. This suggested that the *merA* gene diversity in the soil from the NPK + OM treatment mainly depended on those environmental factors. In particular, the organic matter is a key impact factor in influencing the soil microorganisms [23, 24]. The relatively lower Sorensen similarity index of *merA* library compositions between the NPK + OM treatment and the others indicated that it was the most different treatment from the others. This treatment did not have shared OTU with the CK treatment, highlighting the fact that long-term application of chemical fertilizers plus manures had sharply changed the *merA* gene diversity pattern probably involved with the much higher contents of organic matter, available P and K (Table 1). However, the *merA* gene diversity pattern in the soils from the N and NPK treatments could be controlled by the content of NH_4^+ and NO_3^- according to the CCA profile, although the mechanism for this remained unclear. Furthermore, the *merA* gene diversity patterns in the N and NPK treatments exhibited similar trend,

which was also supported by the high Sorensen similarity index (75%) of the *merA* library compositions between them. Therefore, the status of *merA* gene diversity pattern in the soil was influenced by anthropogenic and non-anthropogenic factors together, and the different effects were found in the different treatments.

Many of the *merA* phylotypes reported here were related to those that thrived in other soils or sediments (Fig. 3). The 190 constituted *merA* sequences were closely to the phylotypes from Tn502, Tn5041 and *Pseudomonas aeruginosa* LESB58. The sequences related to Tn5041 *merA* phylotype has been detected not only in floodplain soil but also in an anaerobic enrichment of sediment [17, 18]. Therefore, the Hg resistance bacteria carrying *merA* gene are broadly distributed in diverse environments. The 167 detected sequences affiliated to uncultured *merA* clone clusters, highlighting that the actual *merA* gene diversity in natural soil was vast compared with those recognized in culture collections. The remain 51 sequences were most closely related to putative *merA* gene of uncultured clones from floodplain soil and anaerobic sediment, suggesting that many *merA* genes were present in microbial community though they were difficult to isolate in laboratory. The shifts of *merA* gene diversity pattern were observed as a response to the different fertilizer practices (Fig. 4). The sequences of cluster 1 could be detected in all samples except the CK treatment, indicating the fertilizer application could be helpful to the growth of cultured mercury-resistance microorganisms. Moreover, the proportion of cluster 1 in the NPK + OM treatment is 97.3%, which further demonstrated that balanced application of chemical fertilizers and organic manure could enhance the amount of culturable mercury-resistance microorganisms.

Figure 4. Proportions of *merA* phylotypes in the soils of four different fertilization treatments.

Concluding remarks

The analysis of soil *merA* gene described here examined the effects of long-term fertilization on *merA* gene for the first time. As claimed by Zheng *et al.* [25], parent material was still the main factor affecting the total Hg content in the soil. Combined with results in this study, it could be inferred that total Hg content should be major factor contributing to the *merA* gene diversity in the soils without anthropogenic disturbance. When the long-term fertilization was performed in the field, the *merA* gene diversity in soil would be mainly influenced by environmental variables depending on the fertilizer application. The various *merA* gene diversity patterns between treatments solely indicated the potential reduction process of Hg by a group of bacteria which can thrive in this specifically treated agricultural soil. Considering the removal of crop biomass might cause minor change of total Hg in the soil, how these factors combined together to influence the Hg concentrations in the soil? Both *in situ* investigations and lab-incubation should be conducted further.

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