Science of the Total Environment 441 (2012) 258–264

Contents lists available at SciVerse ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Technical Note

Culturable microbial groups and thallium-tolerant fungi in soils with high thallium contamination

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HIGHLIGHTS

- We reported the culturable microbial groups in Tl-polluted soils.
- The fungal population can grow in the presence of high Tl level up to 1000 mg kg^{-1}.
- We have isolated and identified nine Tl-tolerant fungal strains.
- The isolated Tl-tolerant fungi were potential sources for bioremediation.

ARTICLE INFO

Article history:
Received 29 May 2012
Received in revised form 22 September 2012
Accepted 22 September 2012
Available online 7 November 2012

Keywords:
Thallium
Microbial groups
Tolerance
Fungi

ABSTRACT

Thallium (Tl) contamination in soil exerts a significant threat to the ecosystem health due to its high toxicity. However, little is known about the effect of Tl on the microbial community in soil. The present study aimed at characterizing the culturable microbial groups in soils which experience for a long time high Tl contamination and elevated Hg and As. The contamination originates from As, Hg and Tl sulfide mineralization and the associated mining activities in the Guizhou Province, Southwest China. Our investigation showed the existence of culturable bacteria, filamentous fungi and actinomyces in long-term Tl-contaminated soils. Some fungal groups grow in the presence of high Tl level up to 1000 mg kg^{-1}. We have isolated and identified nine Tl-tolerant fungal strains based on the morphological traits and ITS analysis. The dominant genera identified were *Trichoderma*, *Penicillium* and *Paecilomyces*. Preliminary data obtained in this study suggested that certain microbes were able to face high Tl pollution in soil and maintain their metabolic activities and resistances. The highly Tl-tolerant fungi that we have isolated are potentially useful in the remediation of Tl-contaminated sites.

1. Introduction

The adverse effects of various heavy metals on microbial communities have been well documented (e.g. El-Sharouny et al., 1988; Arriagada et al., 2009; Haller et al., 2011; Margesin et al., 2011). Some studies have also shown that heavy metals can reduce the soil microbial biomass and the size of viable microbial population (e.g. Hu et al., 2007; Zhang et al., 2007, 2010), while certain microbial species can tolerate high metal exposures in soil (Wang et al., 2007a; Margesin et al., 2011; Sousa et al., 2012). Although the focus has been on bacteria (Chen et al., 2009; He et al., 2010; Pepi et al., 2011), some metal-tolerant fungi have been shown to be capable of immobilizing toxic metals by forming insoluble metal oxalates (Norris et al., 1976; Chen et al., 2009). These organisms can therefore successfully serve as efficient adsorbents for removal of heavy metals (Słaba and Długoński, 2011; Yin et al., 2011; Abd-Alla et al., 2012). Since the metal-tolerant fungal isolates can compete with the indigenous bacterial microflora in hostile situations (Lopez Errasquin and Vazquez, 2003; Arriagada et al., 2007), they have an advantage over bacteria for the bioremediation on polluted soils (Peter and Viraraghavan, 2008; Leung et al., 2010; Kalpana et al., 2011; Sharma and Adholeya, 2011).

Thallium (Tl), generally with low concentration in soil, has attracted increasing environmental concerns due to its high toxicity (Zitko, 1975; Tremel et al., 1997; Xiao et al., 2004a, 2007). Besides playing an important role in the dispersion of Tl compounds in the environment (Sklodowska and Matlakowska, 2004), microorganisms can oxidize Tl ion to Tl_2O_3 in their mitochondria (Lindegren and Lindegren, 1973). Studies on Tl uptake kinetics have indicated that Tl is rapidly bound presumably to cell surfaces, and is progressively accumulated by *Saccharomyces cerevisiae* and *Escherichia coli* through energy-dependent transport systems (Norris et al., 1976). However, little information is available about the toxicity of Tl to microbial groups

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http://dx.doi.org/10.1016/j.scitotenv.2012.09.053

in soil. Similarly, little is known about the occurrence of Tl-tolerant fungi in long-term Tl-polluted soils.

Some soils in Guizhou Province, Southwest China are highly contaminated with Tl, derived from the mineralization of Tl-rich sulfide and mining activity. As a result, crops grown in these soils accumulate high amounts of Tl (Xiao et al., 2004a), and Tl has been recognized as a major metal pollutant that causes the chronic poisoning on the local population, evidenced by epidemical symptoms of alopecia and high Tl concentrations in urine (Zhou and Liu, 1985; Xiao et al., 2007). In the present study, we aimed to characterize the culturable microbial groups isolated from the long-term Tl-polluted soils in Guizhou Province, Southwest China. In addition, we aimed to evaluate the in vivo tolerance of fungal isolates to Tl exposure with a view to using the isolates for the remediation of Tl-polluted soils. The results of this study would provide useful information for assessing the microbial susceptibility to Tl pollution, and also provide an insight into Tl remediation potential using such high tolerance fungal isolates.

2. Materials and methods

2.1. Study area

The study area is located on Lanmuchang (105°30′23″E, 25°31′28″N), a small town with approximately 1000 inhabitants, in the southwest Guizhou Province of Southwest China. The local residents suffer from chronic Tl poisoning and display some symptoms, such as weakness, muscle and joint pain, disturbance of vision, hair loss, and high Tl levels in urine, and these symptoms are all induced by high Tl contaminations in local soils, water and crops (Zhou and Liu, 1985; Xiao et al., 2007). The Tl source is the local sulfide mineralization of Tl, arsenic (As) and Hg, and mining activities. For example, Tl concentration is 100–35,000 mg kg^{-1} in sulfide minerals, 40–124 mg kg^{-1} in soils, 0.8–495 mg kg^{-1} in crops, 13–1966 μg L^{-1} in groundwater and 1.9–8.1 μg L^{-1} in stream water (Xiao et al., 2003; Xiao et al., 2004a,b,c). The elevated Tl contents in the local environment are prone to Tl pollution, thereby presenting a severe threat to public health of the local population (Xiao et al., 2007, 2012).

This area presents a karst topography, exhibiting a higher elevation in the northwest and a lower elevation in the southeast. The average altitude is 1400 m, and the relative relief is 100–200 m. The local outcropping rocks are composed of limestone, argillite and siltstone from Permian to Triassic in age. Previous studies have described the local geology and sulfide mineralization in details (Xiao et al., 2003, 2004c). The Lanmuchang area has been widely developed for agricultural and residential purposes.

2.2. Sampling and analysis

A total of 10 soil samples were collected from the Tl mineralized area within Lanmuchang and a background area without major Tl pollution (Fig. 1). The sampling patterns, regardless of sequential or random, corresponded to the characteristics of soils associated with pedogenesis, mining-related disturbance, and topographical characteristics (e.g. hill top, hill slopes and lowland areas) related to the Tl mineralization. These patterns served to delineate the variations of Tl in this study area, and were categorized into groups as follows: soils in the mining area (soils derived from mine wastes and arable soils in the mining areas), slope wash materials, undisturbed natural soils and background soils.

All the soil samples were kept in polyethylene bags and air-dried in the laboratory prior to final processing. The soils were passed through a 2-mm sieve for geochemical analysis. The sieved fractions were then ground in a Bico ceramic disk grinder, and they were further ground to 80-mesh (−180 μm) powder in a ceramic ball mill. A portion of each soil sample for microbiological analyses was immediately kept in sterile sacks at site and stored in coolers at 4 °C and then shipped to the laboratory where they were kept in coolers in the dark at the same temperature.

Soil samples for geochemical analysis were digested in a mixture of concentrated acids ($HF/HNO_3/HClO_4$), and the initial contents of Tl, Hg and As were determined using an inductively coupled plasma mass spectrometry (ICP-MS, ELAN DRC-e, Perkin-Elmer, USA). The soil pH was determined by a pH meter (AISI pHB9901, Taiwan; solid:de-ionized water = 1:5). Moreover, total carbon (TC) and total nitrogen (TN) were analyzed by dry combustion using an element analyzer (PE2400II, Perkin-Elmer, USA).

Standard references of soils GBW07403 and GBW07408 (National Institute of Standard Materials, China) were used to control the analysis quality. The analytical precision was determined by quality assurance/quality control procedures using duplicates, blanks, internal standards (Rh at 500 μg L^{-1}) and reference samples, and the result was better than ±10%.

2.3. Microbial assays in soil samples

The culturable microbial groups were determined from the fresh soil samples collected at the site. The culturable microbes in soils were enumerated for viable cells by the plate-count method. In order to obtain a soil suspension, 5 g fresh soil was added into 45 mL of sterile Milli-Q water, and the mixture was shaken at 180 rpm for 15 min. The serial dilutions of soil suspensions were arranged in Milli-Q water for the enumeration and isolation of the culturable microorganisms, and were prepared 3 min before use. Then, the dilutions of soil suspensions were surface spread onto agar plates. Colony-forming units (CFU g^{-1} dry soil) of culturable bacteria, actinomyces and filamentous fungi were determined on meat-peptone agar, Gause's starch agar and Martin agar, respectively (Huang, 1999). Bacterial colonies were counted at 28 °C after 48 h. Both actinomyces and fungal colonies were counted at 25 °C after 72 h, respectively.

2.4. Tolerance assay and isolation of Tl-tolerant strains

Tolerance assay was conducted in a 60-mm Petri dish test unit with agar medium containing Tl. To explore the tolerance of the isolated fungal strains, optimal culture conditions were employed with various initial Tl concentrations. Each test unit was supplemented with $TlNO_3$ (Merck, Germany) at concentrations of 200, 400, 600, 800 and 1000 mg kg^{-1}, respectively. After an incubation period of 24–48 h, CFUs were determined in each test unit. Agar medium without Tl addition was used as a control. All tests were performed in triplicate.

At a Tl level of 1000 mg kg^{-1}, all the fungal colonies were microscopically analyzed and transferred to Gause's starch agar. The fungal isolates were then purified and stored on potato dextrose agar slants at 4 °C until further analysis.

2.5. Identification of Tl-tolerant fungal isolates

The fungal isolates were identified by morphological traits and fungal ITS1-5.8S-ITS2 region sequence analysis. DNA was extracted from fungal isolates using a cetyltrimethylammonium bromide (CTAB) method (Stewart and Laura, 1993). The ITS1-5.8S-ITS2 region was amplified and sequenced using fungal-specific primers ITS1F (5′-CTTG GTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATT GATATGC-3′) as previously described (Martin and Rygiewicz, 2005). The sequences were searched against those already known at NCBI GenBank (http://www.ncbi.nih.gov/index.html) using BLAST search option. The sequences of ITS region were aligned with the sequences of similar fungi retrieved from databases using CLUSTAL X, and a phylogenetic tree was constructed using the neighbor-joining algorithm (MEGA version 4.0) with the bootstrap analysis of 1000 replicates (Kumar et al., 2004).

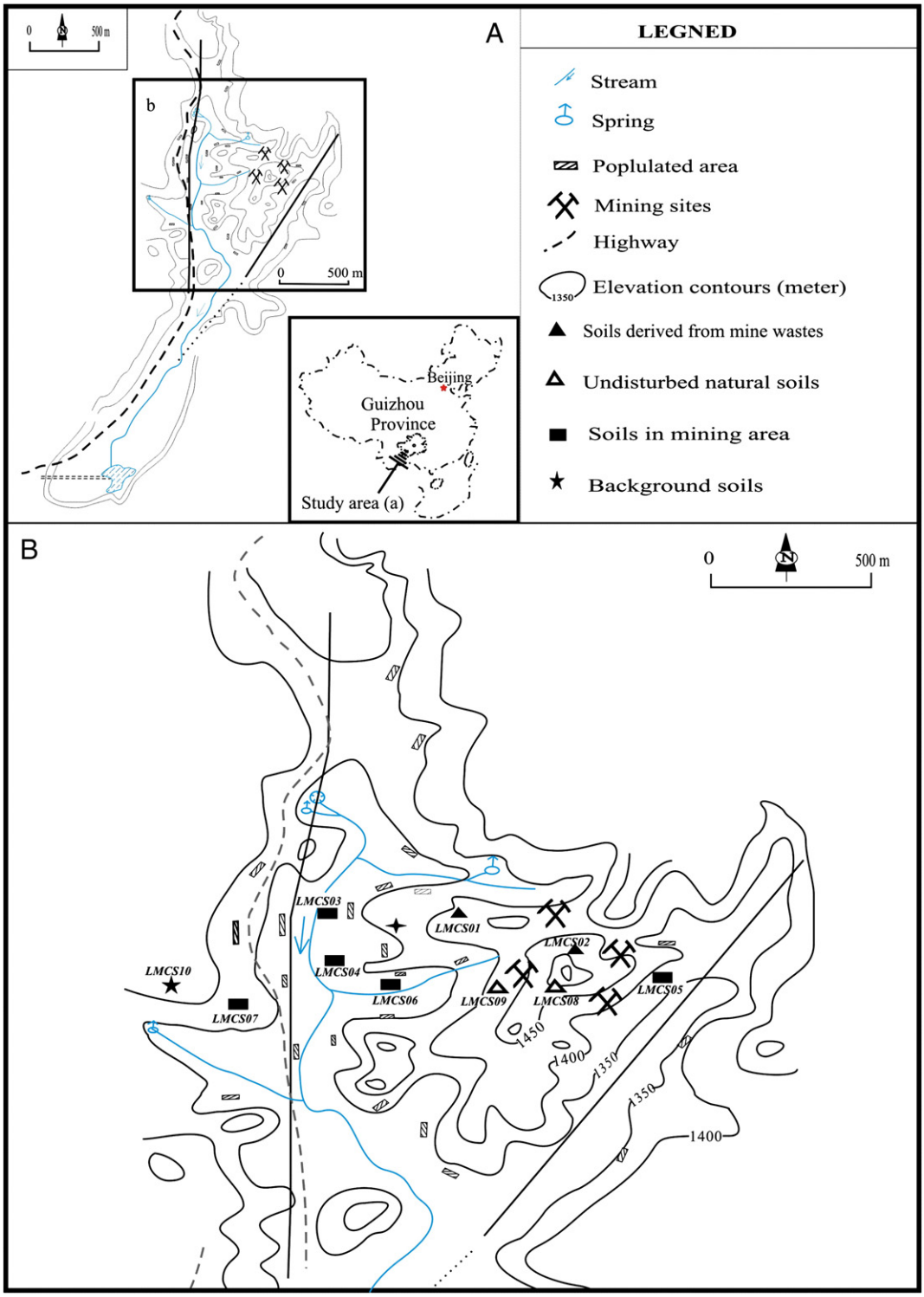

Fig. 1. Map showing the soil sampling sites.

2.6. Statistical analysis

Statistical analysis was carried out using the SPSS statistical package (version 16.0 for Windows, SPSS Inc., USA), and all the plots were analyzed by Origin (version 8.5 for Windows, Origin Lab Corp., USA). Each soil sample was analyzed with three replicate determinations, and the mean values were obtained. In the tolerance assay of Tl-tolerant strains, one-way analysis of variance (ANOVA) was performed for multiple comparisons, and significance analyses of different treatments were conducted using a least significant difference test (LSD). The *t*-test was used for two-way comparisons in microbial counts between the contaminated soils and the background soils.

3. Results and discussion

3.1. Soil properties and characteristics of the culturable microbial groups

Table 1 summarized the properties of the Tl-contaminated and background soils. In the study area, soils were characterized by low carbon and nitrogen contents, and the pH values ranged from 3.92 to 6.08.

Table 1
Soil properties and culturable microbial characteristics in the samples.

Samples	Soil properties						Microbial characteristics in soils			
	pH	TC (g kg^{-1})	TN (g kg^{-1})	Tl (mg kg^{-1})	As (mg kg^{-1})	Hg (mg kg^{-1})	Bacteria (CFU g^{-1})	Fungi (CFU g^{-1})	Actinomyces (CFU g^{-1})	Total (CFU g^{-1})
Soils derived from mining waste										
LMCS01	5.8	0.56	0.038	437	58.9	122	4.61×10^4	8.54×10^2	1.07×10^3	4.80×10^4
LMCS02	3.9	0.76	0.035	221	78.8	N/A	2.12×10^4	5.63×10^2	2.07×10^2	2.38×10^4
Soils in mining area										
LMCS03	6.1	4.55	0.26	144	47.1	135	2.22×10^5	4.39×10^3	7.29×10^3	2.34×10^5
LMCS04	5.1	0.55	0.040	34.5	16.8	659	8.64×10^4	1.77×10^3	1.36×10^3	8.95×10^4
LMCS05	5.2	0.47	0.040	77.3	292	116	6.66×10^4	1.43×10^3	4.99×10^3	7.30×10^4
LMCS06	5.2	0.91	0.048	269	55.3	130	1.09×10^5	2.49×10^3	1.58×10^3	1.13×10^5
LMCS07	5.8	0.46	0.027	64.3	62.5	N/A	1.91×10^4	3.68×10^2	5.12×10^2	2.00×10^4
Undisturbed natural soils										
LMCS08	3.9	0.85	0.041	164	19.7	N/A	9.57×10^4	2.74×10^3	3.05×10^3	1.01×10^5
LMCS09	4.8	0.98	0.044	232	37.9	243	3.61×10^5	8.59×10^3	4.11×10^3	3.74×10^5
Background soils										
LMCS10	4.9	0.27	0.030	5.3	8.1	0.8	4.06×10^5	5.71×10^3	1.65×10^4	4.28×10^5

Means of replicate determinations is given.
N/A = not available.

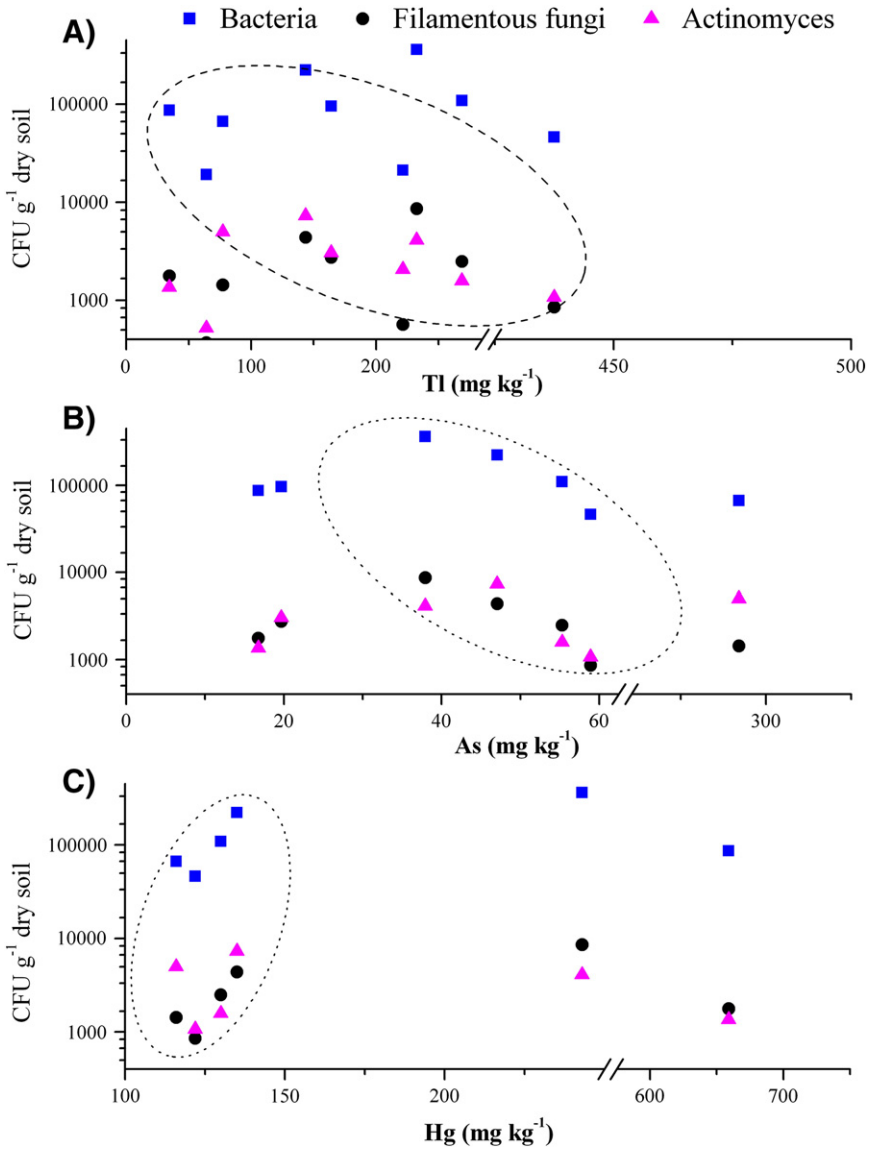

Fig. 2. Relationship between the microbial counts and the contents of Tl, As and Hg. Each data point represents an average of three replicates.

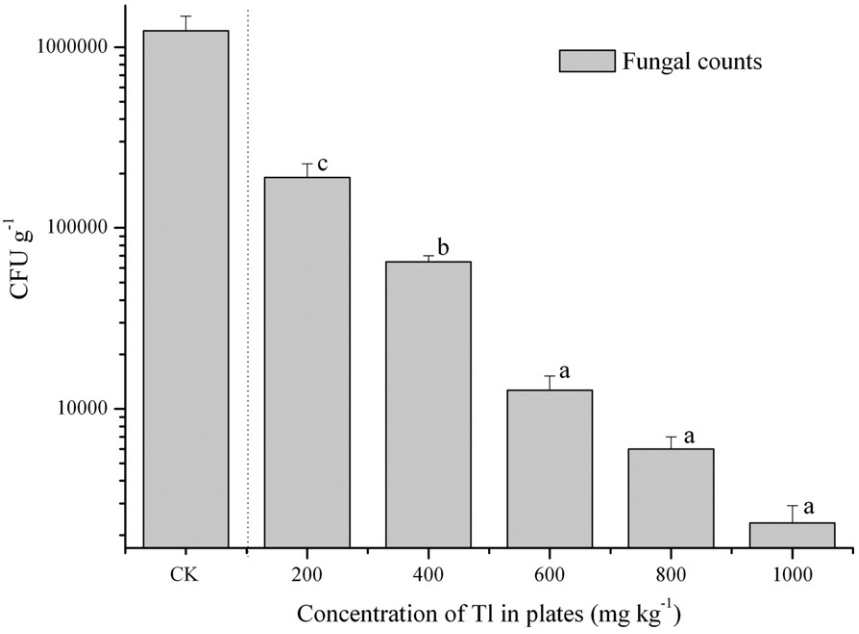

Fig. 3. Tolerance of the fungal isolates at various Tl concentrations. Data were expressed as mean ± SD. Letters above the bars indicate significant differences at $p<0.05$ within each treatment, and the vertical line on each bar shows the standard deviation.

The contents of Tl, Hg and As in soils were much higher than those in the background area, which was consistent with the intensive mineralization of Tl, Hg and As (Xiao et al., 2004c). The high concentrations of Tl, Hg and As indicated that these three toxic metals were enriched to different extents in the local soils, such as soils derived from the mining wastes, the soils in the mining area, the undisturbed natural soils, and the background soils. Table 1 showed Tl concentration ranging from 34.5 to 437 mg kg^{-1}, As concentration from 16.7 to 292 mg kg^{-1}, and Hg concentration from 116 to 659 mg kg^{-1} in the contaminated soils.

Enumeration of the soil microbial population in the specific medium clearly demonstrated the presence of considerable amounts of viable and culturable microorganisms in the contaminated soils. A large fraction of this population was able to grow in the presence of high Tl, together with Hg and As (Table 1, Fig. 2). For example, the CFUs were from 2.12×10^4 to 2.22×10^5 for heavy metal-tolerant bacteria, 3.48×10^2 to 8.59×10^3 for heavy metal-tolerant filamentous fungi, and 2.07×10^2 to 4.99×10^3 for heavy metal-tolerant actinomyces. The *t*-test suggested statistically significant difference in the microbial counts between the contaminated soils and the background soils ($p=0.001$).

Fig. 2 showed that heavy metals in the studied soils exerted strong threat on the culturable microbial groups. We observed that the actinomyces population was strongly suppressed following the elevated Tl level in the soils, and we also found the similar effects on bacterial and fungal counts. Arsenic demonstrated nearly the same effects on the bacteria, fungi and actinomyces. Mercury tended to stimulate microbial counts when Hg contents were below 150 mg kg^{-1}, but higher Hg contents still resulted in the reduction of microbial counts. However, considerable amounts of certain viable and culturable microorganisms were still observed in the metal-polluted soils, which indicated that such microorganisms may display high tolerance to metal menaces in the polluted soils.

3.2. Tl-tolerant fungal isolates in cultures

To characterize the fungal isolates, we tested the susceptibilities of the isolated strains to Tl at different concentrations (Fig. 3). In our present study, we found that the initial Tl content in soils played an important role in affecting the susceptibility. We also observed that the increasing Tl levels at interval of 200 mg kg^{-1} in the cultures resulted in a sharp reduction of the culturable microbial population, and the isolated strains showed a relatively higher resistance to Tl pollution. When the Tl concentration in the plate was at 200 mg kg^{-1}, the CFUs ranged from 1.5×10^4 to 2.2×10^4, exhibiting an 81% reduction compared with that of the control. Similarly, Tl level of 400 mg kg^{-1} resulted in a 93.5% reduction, whereas Tl levels of 600 and 800 mg kg^{-1} led to 98.7% and 99.4% reduction, respectively. We observed that the culturable microbial population was decreased by 99.8% when the Tl level was increased to 1000 mg kg^{-1}, but certain fungal isolates still survived in the cultures.

Interestingly, nine fungal isolates were successfully identified in the plate at Tl level of 1000 mg kg^{-1} (Table 2). This finding implied that these nine fungal isolates were highly Tl-tolerant. In order to identify the strains with maximum Tl-resistance, we performed further investigations on these nine isolates.

Fig. 3 demonstrated that higher levels of Tl (600–1000 mg kg^{-1}) in the culture media can dramatically decrease the culturable fungal population. The highest degree of difference or lowest degree of similarity was observed between the control and the groups with higher levels of Tl in the culture media. This suggested that Tl addition had only a limited effect above a certain threshold, a notion supported by the fact that 600 and 1000 ppm samples were the most similar ones.

Previous study reported the similar finding that the heavy metals affect microbial communities in a threshold-like manner (Ozbelge et al., 2007). The threshold-like effect of heavy metals could be explained by the potential presence of several groups of organisms with the similar

Table 2
Fungal isolates with GenBank no. of genes.

Strains	GenBank no. ITS	Similarity (%)	Taxon
T01	DQ379015.1	95%	*Trichoderma koningiopsis*
T02	DQ379015.1	99%	*Trichoderma koningiopsis*
T03	FJ441016.1	93%	*Mariannaea* sp.
T04	AJ230673.1	100%	*Trichoderma viride*
T05	AF368793.1	93%	*Paecilomyces farinosus*
T06	AJ509865.1	99%	*Penicillium ochrochloron*
T07	AJ509865.1	99%	*Penicillium ochrochloron*
T09	FJ004799.1	95%	*Trichoderma asperellum*

DNA sample of T08 strain was missing in this test.

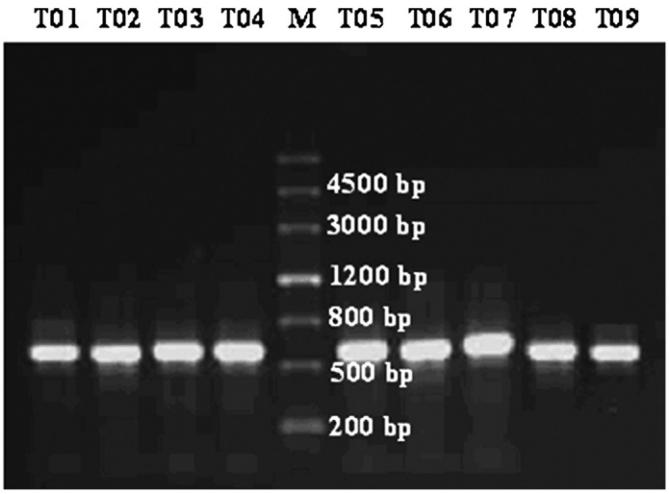

Fig. 4. Map showing the images of DNA analysis by AGE. T01–T09, represent the isolated fungal strains. M refers to DNA size marker.

type of metabolism, yet having various heavy metal tolerances (Sobolev and Begonia, 2008). In order to gain an insight into the microbial communities, it is necessary to combine different approaches, such as culture-dependent and culture-independent analyses. As well as the toxicological and biological assays should be used in parallel.

3.3. Identification of Tl-tolerant fungal isolates and bioremediation insights

To identify the high Tl-tolerant fungal isolates, we extracted DNA from the nine fungal isolates and then performed the ITS analysis. Fig. 4 showed that nine bands individually corresponding to the nine fungal isolates were clearly profiled through the agarose gel electrophoresis (AGE). The fragment length was approximately 600 bp, which also coincided with the expected size.

We identified these isolated fungal strains with high Tl-tolerance by their AGE profiles (Table 2), and BLAST analysis revealed that their sequence similarity in GenBank ranged from 93% to 100%. Furthermore, we constructed the euclidean distance dendogram based on ITS1-5.8S-ITS2 region sequence (Fig. 5). This revealed that these nine Tl-tolerant isolates could be classified into four main phylogenetic groups with multiple subclusters as follows. Group 1 consisted of *Trichoderma koningiopsis*, *Trichoderma viride* and *Trichoderma asperellum*, group 2 consisted of *Penicillium ochrochloron*, group 3 consisted of *Paecilomyces farinosus*, group 4 consisted of *Mariannaea* sp., and T08 strain belonged to *Trichoderma* according to its morphologic identification.

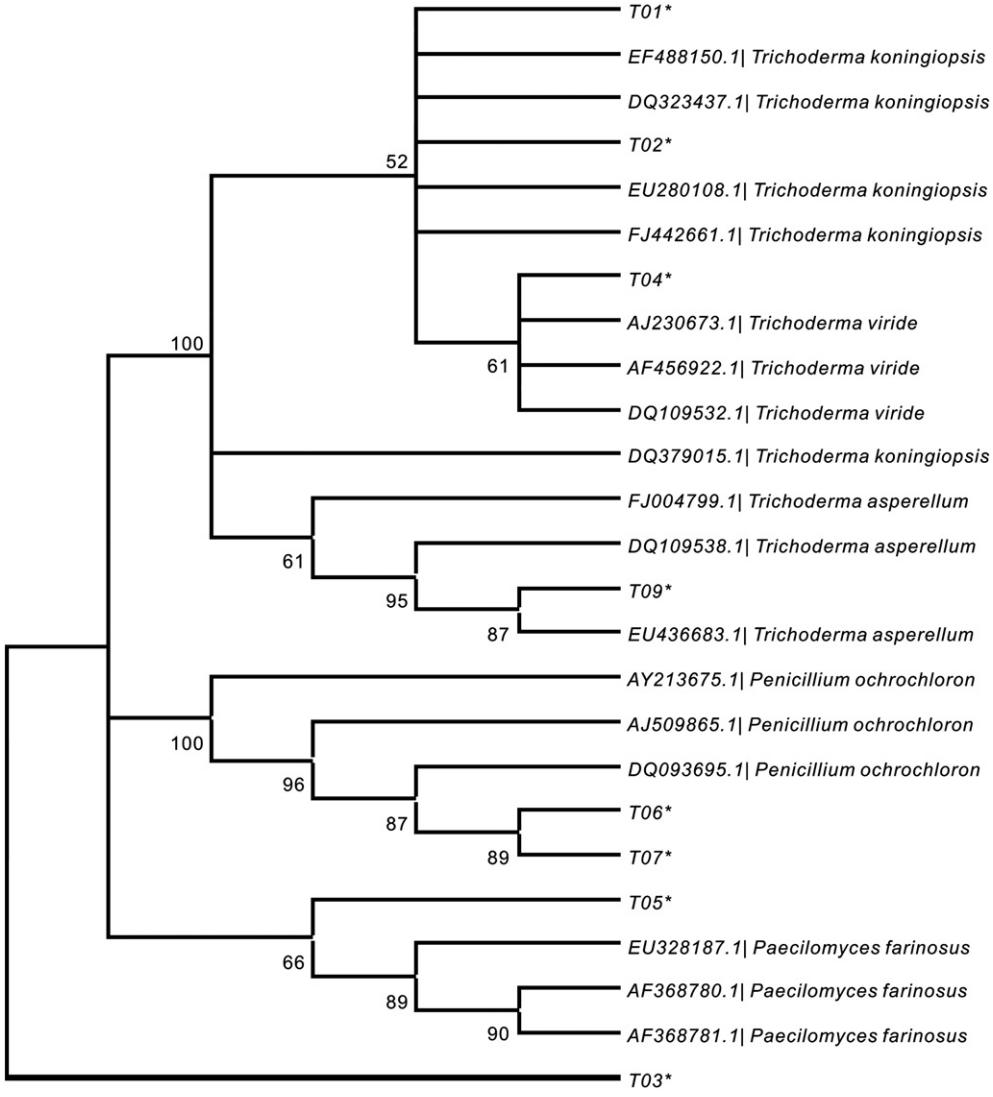

Fig. 5. Phylogenetic position of eight fungal strains. The number in each branch indicates the number of trees from 1000 bootstrap replications.

We found a high homology of the ITSs from the phylogenetic tree through the sequence comparison (Fig. 5). Moreover, *Trichoderma*, *Penicillium* and *Paecilomyces* are common metal-tolerant fungus as previously reported (Anand et al., 2006; Wang et al., 2007b; Słaba et al., 2009; Sharma and Adholeya, 2011).

Although heavy metals exert their toxic effects on microorganisms through various mechanisms, certain heavy metal-tolerant microbes can survive in the metal-polluted habitats, and are useful for bioremediation of the contaminated sites (Pepi et al., 2011). Previous experiments have shown that the isolated Tl-tolerant fungal strains have high potentials to bioadsorb and bioflocculate Tl in Tl-polluted soils and waters (Sun et al., 2010).

4. Conclusions

We reported the culturable microbial groups in Tl-polluted soils. Our work demonstrated that the culturable microbial groups were able to face with high Tl in long-term Tl-polluted soils. Taken together, we identified nine fungal strains with high Tl-tolerance based on the morphological traits and ITS analysis, and the phylogenetic tree exhibited a high homology. Our investigations provide a promising potential fungal source for bioremediation in Tl-polluted sites.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (41063005, 41173028) and the Key Knowledge Innovation Project of Chinese Academy of Sciences (KZCX2-YW-135). We are grateful to Dr. Zuoyi Liu, Dr. Guosheng Zhu, Mr. Zhengyu Zhu and Dr. Yongxiang Liu for their help in performing the microbial experiments at Guizhou Provincial Key Laboratory of Agricultural Biotechnology. The anonymous reviewers are acknowledged for their critical comments and suggestions which have considerably improved the manuscript. Thanks also go to Dr. Benny Theng at Landcare Research, New Zealand for a final reading of the text.

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