INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
15–317/2015/17–6–1133–1140
DOI: 10.17957/IJAB/15.0047
http://www.fspublishers.org

Full Length Article

Identification of Super Antibiotic-resistant Bacteria in Diverse Soils

Qichun Zhang[1], Changping Hou[1], Imran Haider Shamsi[2*], Essa Ali[2], Noor Muhammad[2], Jawad Munawar Shah[2] and Abbas Ali Abid[1]

[1]*MOE Key Laboratory of Environment Remediation and Ecosystem Health, College of Environment and Resource Sciences, Zhejiang University, Hangzhou, 310058, P.R. China*

[2]*Key Laboratory of Crop Germplasm Resource, Department of Agronomy, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, 310058, P.R. China*

[*]For correspondence: drimran@zju.edu.cn

Abstract

Environmental bacteria have been revealed to be a reservoir of antibiotic resistance genes and a potential pool of novel resistance genes in clinical pathogens. Recently, the soils, which have never been treated with antibiotics before have proved to contain the resistant bacterial strains. In this study, we assessed whether the soil that had not previously been influenced by antibiotics contained super-resistant bacteria or not. We identified super penicillin-resistant strains in four diverse soils containing up to 1000 mg/L penicillin compared to 100 mg/L, but none of super tetracycline resistant strains were detected in four soils containing up to 1000 mg/L tetracycline, which is the highest concentration of exogenous penicillin or tetracycline reported to date. Twenty bacterial isolates were selected, representing a diverse set of species in the genera *Pseudomonas* (85%) and *Variovorax* (15%); these bacteria showed multiple antibiotic resistance patterns for four or more antibiotics. Using polymerase chain reaction and sequence, we carefully examined the existence of antibiotic-resistance genes and integron genes in soils and strains. It was observed that the resistance genes *tet(C)* and *blaTEM* were fairly widely distributed in these super penicillin-resistant isolates, and 20%–50% of tested genes were found in the study soils. All the study sites provided great opportunities for horizontal resistance transfer. The antibiotic-resistant genes pool, which is potentially large and diverse, may have considerable implications for ecology and health. © 2015 Friends Science Publishers

Keywords: Antibiotic-resistant genes; Multiple antibiotic resistance; Penicillin; Soil-resistant bacteria

Introduction

The universal existence of antibiotic resistance, especially the resistance to different drugs, among bacterial pathogens has brought serious challenges to clinical therapy (Levy and Marshall, 2004). Environmental bacteria have been recognized as a reservoir of antibiotic resistance genes and have a great potential to be taken as a pool of novel resistance genes in clinical pathogens (D'Costa *et al.*, 2006). Recently, the soils, which have never received antibiotics have proved to contain the resistant bacterial strains (Dantas *et al.*, 2008; Rodríguez-Verdugo *et al.*, 2013). Dantas *et al.* (2008) observed that some of the soil bacteria can decompose antibiotic substrates and can use the resulting carbon and nutrients to facilitate their growth, even when the bacteria have not been exposed to these antibiotics previously. Those bacteria are startlingly phylogenetically diverse, their patterns of distribution and the prevalence are not yet known. These soil-dwelling antibiotic-resistant bacteria could be closely linked to human pathogens, if it is so they may be a potential threat to human health also. During a recent annual report on global health risk, the World Economic Forum concluded that antibiotic-resistant bacteria pose the greatest risk to human health (Spellberg *et al.*, 2013). Given the potential health risk, a number of studies have drawn attention on antibiotic-resistant bacteria isolated from various ecosystems (Wittwer *et al.*, 2005; Li *et al.*, 2010). However, the lack of fundamental knowledge concerning the roles of soils and soil bacterial communities in generating antibiotic resistant bacterial strains still hampers effective prevention and treatment of antibiotic-resistant bacterial infection.

Soil ecosystems are highly complex and contain many bacterial and fungal species (Torsvik *et al.*, 1990). Most soil microorganisms have not been identified, and the knowledge of community structure and its relationship with soil performance are still poorly understood. Moreover, most of the widespread of antibiotic-resistance genes (ARGs) (Pruden *et al.*, 2006) are discovered in the genes of bacteria (Liu and Pop, 2009), which could be resistant after exposure to sub lethal doses of antibiotics, even an antibiotic-free environment (Rodríguez-Verdugo *et al.*, 2013). The acquisition of those resistance genes through lateral transfer has been observed recently to be widespread

To cite this paper: Zhang, Q., C. Hou, I.H. Shamsi, E. Ali, N. Muhammad, J.M. Shah and A.A. Abid, 2015. Identification of super antibiotic-resistant bacteria in diverse soils. *Int. J. Agric. Biol*., 17: 1133–140

in clinical pathogens (Levy and Marshall, 2004). It was shown that the abundance of tetracycline resistance genes in soils rose considerably during the antibiotic era (Knapp *et al.,* 2010) It is not clear, however, whether an antibiotic-free environment likewise would increase tetracycline resistance genes and penicillin resistance genes or not. Thus, the control of antibiotic-resistant bacteria both in antibiotic-polluted environment and antibiotic-free environment is a critical concept that must be addressed in order to improve global health.

Therefore, the current study was conducted to isolate and identify bacteria that could survive in the presence of ultrahigh concentrations of penicillin or tetracycline despite lack of previous exposure to penicillin or tetracycline; to evaluate the occurrence of super resistant bacteria in diverse soils and study their characteristics; and to examine the extent of multiple antibiotic resistances in all isolates.

Materials and Methods

Soil Samples

Samples of topsoil (0–15 cm depth) were collected from a tea plantation (S1), swine feedlot (S2), hospital garden (S3), and forest (S4). S1 (120º06'41", 30º13'36") and S4 (120º07'12", 30º14'20") were located near the scenic area of the West Lake in Hangzhou, China, and S2 (120º18'82", 30º26'92") and S3 (120º20'11", 30º13'15") were located within Zhejiang University, China. The field studies did not relate to endangered or protected species, and specific permissions were not needed for these locations/activities. Preliminary investigations showed that none of these four sample sites had ever been contaminated by penicillin and tetracycline. At each site, 10–12 samples of soil were collected through a sterile spade, they were blended completely, and later were transferred to a plastic bag to get the composite sample. These soil samples were taken to the lab on ice (2–6 h) and after that were stored at 4°C. The soil parameters (Table 1) were later determined as the methods derived by Sparks *et al.* (1996).

Resistance against Penicillin and Tetracycline Treatments

The culturing of soil bacteria in antibiotic-selective media was initially done by the method of Pei *et al.* (2006) with some appropriate modifications as 1 g dry soil sample was suspended in 9 mL sterilized phosphate-buffered saline (PBS, pH 7.4) for 30 min, then 1 mL of this suspension was inoculated into fresh 9 mL PBS, resulting in a 10^{-1} dilution at each passage. Solid penicillin/tetracycline-selective media were prepared by adding 15 g/L agar in liquid beef extract peptone agar medium followed by autoclaving before adding antibiotic at concentrations of 0 (CK), 30 (T1), 60 (T2), 120 (T3) and 1000 (T4) mg/L. Subsequently, 200 µL diluted soil was directly applied onto the antibiotic-selective media and incubated at 28°C for 24 h. The colony forming units (CFU) of cultured bacteria were counted and the antibiotic resistance colony number was determined. The percentage resistance of each treatment was counted as the ratio between the number of colonies on beef extract peptone agar medium with and without penicillin, multiplied by 100 (Rho *et al.*, 2012).

Isolation and Identification of Penicillin/Tetracycline-resistant Bacteria

None of super tetracycline resistant strains was found in four soils containing up to 1000 mg/L tetracycline. Twenty single colonies were picked from 1000 mg/L penicillin-selective media and restreaked on other corresponding selective plates to obtain pure colonies. The predominant colony types resisting high concentrations penicillin were sub-cultured and identified by 16S rRNA gene sequencing. The 16SrRNA gene (rDNA) of each of the clonal isolates obtained in this study was amplified using universal bacterial 16S primers pairs 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GTTTACCTTGTTACGACTT-3′) to produce an amplicon of about 1400 bp. Colonies were suspended in 50 µL double-distilled water, boiled for 10 min, and centrifuged at 16,000 × g for 5 min. 1 µL of the supernatant was used as the template in each amplification. The DNA was amplified by PCR in 20-µL volumes with 1 µL of each primer (10 µM) and 10 µL of 2× Easy Tap PCR SuperMix (TransGen Biotech, Shanghai), and added right amount of sterile double-distilled water to finally get the desired volume. PCR amplification was carried out with the process as : 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min; with a single final extension at 72°C for 7 min, using a Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). An aliquot of 5 µL amplification products were analyzed on a 1% agarose gels containing ethidium bromide. The PCR products were purified, concentrated, and sequenced by Sangon Biotech Co., Ltd. (Shanghai, China).

Detection of Resistant and Integron Genes in Soils and Strains

The presence of 8 different genes and class I and II integrons in the soil samples and bacterial isolates was verified by the PCR. Total DNA in soil samples was extracted as a template by harsh lysis using a Power Soil DNA kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity and quality of the extracted DNA were analyzed by spectrophotometer analysis (NanoDrop-2000) and agarose gel electrophoresis, respectively. Bacteria DNA worked as template, while standard PCR mixture (20 µL) were the same as described above. The PCR primers and conditions for genes amplification are listed in Table 2. Amplified products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. All bands were separated from the agarose gel and purified using a QIAquick Gel

Extraction Kit (Qiagen Inc., Valencia, CA, USA), and sequenced by Sangon Biotech Co., Ltd (Shanghai, China) using a BigDye terminator cycle sequencing ready reaction kit on an ABI 3730 capillary sequencer. The nearest matches were provided by Gen Bank database using the BLASTn program. For all reactions in this work, duplicate PCR assays were performed for each sample. Negative controls were standard PCR mixtures without DNA template, and positive bacterial strains carrying genes or integrons verified by sequencing were used as positive controls.

Kirby-Bauer Disc Diffusion Susceptibility Test

The disc diffusion susceptibility test was performed according to the guidelines as described by Helena Rebouças *et al.* (2011). In short, the resistant isolates were grown in beef extract peptone liquid medium and incubated at 28°C for 8 h (the time was set according to the growth curve) under aerobic conditions, and bacterial cultures were then streaked evenly onto the entire surface of a beef extract peptone agar medium plate and incubated at 28°C for 24 h. The antibiotics tested included streptomycin, neomycin, amoxicillin, ampicillin, azithromycin, erythromycin, chlorotetracycline, and oxytetracycline. The classification of the antibiotics tested and the concentrations of antibiotics used per disk are presented in Table 3. Later, the inhibition zones were recorded, (S) and (R) interpreted as susceptible and resistant, respectively. All antibiotics used were purchased from Sangon Biotech Co., Ltd (Shanghai, China).

Statistical Analyses

Data statistical analyses were performed by the tool of Microsoft Office Excel and SPSS 16.0. Significant differences between treatments were analyzed by a one-way analysis of variance (ANOVA). Comparisons of means between different treatments were performed using Duncan's studentized range test. The significance level was set to 0.05. For bacterial identification, the 16S rRNA gene sequences were analyzed by GenBank database using the BLASTn program for the closest known relatives. Gene sequence alignment was done between the resulting 16S rRNA sequences and the corresponding sequences from representative species. Neighbor-joining trees were then constructed using MEGA5.05 software. The statistical significance of branching was observed and evaluated by bootstrap analysis involving the construction of 1000 trees from re-sampled data. Sequences within clusters were separately aligned and later was compared with each other. The 16S rRNA gene sequences of the 20 strains in this study were deposited in GenBank database with accession no. KF434522–KF434541 and the nucleotide sequences greater than 200 bp for genes can be accessed under accession no. KM594532-KM594533, KM594535, KM594547, KM594540-KM594542.

Results

Total and Antibiotics-resistant Cultivable Bacteria in Soils

Appropriate culture media with soil suspensions led to the recovery of cultivable bacteria, including antibiotics-resistant isolates. The total number and antibiotic resistance of cultivable bacteria, determined by calculation of the CFU, is shown for each soil type and for the different antibiotics concentrations in (Fig. 1a and 1b). The total number of cultivable bacteria was significantly different ($p<0.01$) between the four soils. In addition, the level of resistant isolates was significantly different among the different soil types. The number of tetracycline-resistant cultivable bacteria was considerably less at low concentrations of tetracycline treatment (Fig. 1b) and tetracycline-resistant strain couldn't grow at concentrations greater than 120 mg/L, thus we couldn't isolate any super tetracycline-resistant strains at the 1000 mg/L tetracycline treatment. Contrary to tetracycline treatments, penicillin-resistant bacteria from each of the four soils could grow even at the concentration of 1000 mg/L penicillin; this value was nearly double that reported in other studies. Moreover, the colonies exhibited different colors, including milky white, light yellow, and orange. The percentages of resistant bacteria decreased with the increase in antibiotic concentration, and these effects differed among the various types of soils (Fig. 1c). The widespread presence of penicillin-resistant bacteria was remarkably higher in forests, ranging from 1.75% to 25.30% for T4 and T1, respectively (Fig. 1c).

Isolation and Identification of Isolates

Twenty bacterial isolates were selected from four different soil samples that were resistant to 1000 mg/L penicillin (Table 4). All of the selected isolates were also tested for growth in liquid medium containing 1000 mg/L penicillin. The results showed that all isolates grew well and could reach a plateau within 8–10 h. The 16S rRNA genes sequence of the 20 isolates was determined, and phylogenetic relationships were evaluated (Fig. 2). All the resistance isolates were identified and classified into two different genera: Pseudom*onas* (85%, 17 of 20 isolates) and *Variovorax* (15%, 3 of 20 isolates).

Prevalence and Degree of Antibiotic Resistance

Eight strains were chosen for further studies according to the phylogenetic tree. The multiple antibiotic resistance (MAR) of each of the eight isolates against eight antibiotics covering four antibiotic families using the disc diffusion assay was detected (Table 3). All the resistant isolates from the four soil samples were susceptible to tetracycline antibiotics, including chlorotetracycline and oxytetracycline, but resistant to β-lactam and macrolide antibiotics, including

Table 1: Locations and selected properties of the tested soils

Soil	Actual locations	pH	Salinity (g/kg)	Avail[a] N (mg/kg)	Avail[a] K (mg/kg)	Avail[a] P (mg/kg)	Total N (g/kg)	OM[b] (g/kg)	CEC[c] (cmol/kg)
S1	Tea plantation, Hangzhou, China	5.56	0.05	105.32	154	21.12	0.96	13.46	14.18
S2	In the vicinity of a swine feedlot, Hangzhou, China	6.7	0.55	264.89	700	287.68	2.42	43.64	18.36
S3	In a garden of a hospital, Hangzhou, China	7.16	1.2	147.44	350	131.52	1.75	24.72	18.46
S4	Pristine Forest, Hangzhou, China	7.82	0.45	225.62	147	8.96	3.08	39.89	8.36

[a]Available N, Available P, Available K; [b]Organic matter; [c]CEC, cation exchange capacity

Table 2: Primers and PCR conditions used in this study

Class	Target	Primer	Sequence (5'-3')	Annealing temperature (ºC)	Amplicon size (bp)	Source
Tetracycline resistance genes	tet(M)[a]	tetM-FW[c]	ACAGAAAGCTTATTATATAAC	46	171	(Aminov et al., 2001)
		tetM-RV	TGGCGTGTCTATGATGTTCAC			
	tet(O)[a]	tetO-FW	ACGGARAGTTTATTGTATACC	58	171	(Aminov et al., 2001)
		tetO-RV	TGGCGTATCTATAATGTTGAC			
	tet(T)[a]	tetT-FW	AAGGTTTATTATATAAAAGTG	46	169	(Aminov et al., 2001)
		tetT-RV	AGGTGTATCTATGATATTTAC			
	tet(W)[a]	tetW-FW	GAGAGCCTGCTATATGCCAGC	60	168	(Aminov et al., 2001)
		tetW-RV	GGGCGTATCCACAATGTTAAC			
	tet(C)[b]	tetC-FW	GCGGGATATCGTCCATTCCG	68	207	(Aminov et al., 2001)
		tetC-RV	GCGTAGAGGATCCACAGGACG			
β-lactamase genes	bla_{OXA-1}(AMP)	oxa1-FW	AGCAGCGCCAGTGCATCA	66.5	708	(Guerra et al., 2001)
		oxa1-RV	ATTCGACCCCAAGTTTCC			
	bla_{PSE-1}(AMP)	pse1-FW	CGCTTCCCGTTAACAAGTAC	60	419	(Sandvang et al., 1998)
		pse1-RV	CTGGTTCATTTCAGATAGCG			
	blaTEM	TEM-FW	CTCACCCAGAAACGCTGGTG	54.5	569	(Colomer-Lluch et al., 2011)
		TEM-RV	ATCCGCCTCCATCCAGTCTA			
Integron genes	class 1 integron	int1-FW	CAGTGGACATAAGCCTGTTC	55	160	(Koeleman et al., 2001)
		int2-RV	CCCGAGGCATAGACTGTA			
	class 2 integron	int2-FW	GTTATTTTATTGCTGGGATTAGGC	55	166	(Luo et al., 2010)
		int2-RV	TTTTACGCTGCTGTATGGTGC			

Note: [a]: Gene coding for ribosomal protection protein; [b]: Gene coding for efflux pump; [c]: FW, forward; RV, reverse

Table 3: Resistance patterns of the resistances strains from different samples

Classes of antibiotics	Antibiotics (μg per disk)	s4p22	s1p23	s2p11	s4p23	s2p31	s2p32	s4p13	s3p12
	Streptomycin (10)	S	S	R	R	S	S	S	S
Aminoglycosides	Neomycin (10)	S	S	R	R	R	S	S	S
	Amoxicillin (10)	R	R	R	R	R	R	R	R
β-lactams	Ampicillin (10)	R	R	R	R	R	R	R	R
Macrolides	Azithromycin (10)	R	R	R	R	R	R	R	R
Antibiotics	Erythromycin (10)	R	R	R	R	R	R	R	R
	Chlorotetracycline (30)	S	S	S	S	S	S	S	S
Tetracyclines	Oxytetracycline (30)	S	S	S	S	S	S	S	S

R: resistances; S: sensitivity

amoxicillin, ampicillin, azithromycin and erythromycin. Notably, the strains s2p11 and s4p23 (*Variovorax paradoxus*) were resistant to six of the eight types of antibiotics screened, indicating a wide range of multiple resistance patterns.

Diversity of ARGs and Integron Genes in Bacterial Isolates and PCR-amplified DNA from Soil

The presence of tetracycline genes, β-lactam-resistance genes, and integron genes in the eight soil isolates according to the phylogenetic tree was investigated after amplification of genomic DNA by PCR and confirmed by DNA sequencing (Table 2). *Tet(C)*, which encodes a tetracycline efflux pump, and *blaTEM*, which encodes β-lactamase, were present in eight bacterial isolates; these strains belonged to different species isolated from different soil types. Neither of the two integron genes was found in the isolates.

Because cultivable bacteria represented less than 1% of all bacteria, the multiplicity of antibiotic genes in the soil bacterial community was further studied by a culture-independent method. Bacterial metagenomic DNA in soil samples was extracted and then submitted to PCR using gene specific primers (Table 2). Overall, 20%–50% of tested genes were present in the study soils. Five of 10 tested genes were identified in the swine feedlot (S2), indicating a huge antibiotic resistance gene pool in swine

feedlots. Fewer resistance genes detected in S1, S3 and S4 samples than in the S2 sample. Moreover, all soil samples contained the class 1 integron gene (*int1*), which provided great opportunity for the horizontal resistance gene transfer, while the class2 integron gene (*int2*) was not detected in the soils, indicating that *int2* was not as ubiquitously expressed as *int1*.

Discussion

Occurrence of Resistance in Four Diverse Soil Types

Many isolation procedures in the study of antibiotic-resistant bacteria are chosen to have relatively low concentrations of antibiotics in order to increase the number or type of bacteria obtained (Ozaktas *et al.*, 2012). In this study, the four diverse soils contained tetracycline-resistant bacteria at concentration of 60 mg/L and super penicillin-resistant strains at concentration of up to 1000 mg/L. The number of bacterial colonies ranged from 4.00×10^4 to 4.83×10^5 CFU/g at a high selective penicillin concentration of up to 1000 mg/L, which is the highest concentration reported to date and nearly two orders of magnitude higher than in other studies (Macauley *et al.*, 2007; Li *et al.*, 2010; Ozaktas *et al.*, 2012). Thus, these bacterial isolates appeared to be quite unique in their ability to survive even under such high concentrations of penicillin, which would generally be considered lethal, even to resistant cells. The widespread presence of cultivable bacteria, which were resistant to penicillin, exhibited some heterogeneity that associated not only with different types of soils, but also with the penicillin concentration of the selective agar media. However, this was not unexpected considering that resistant bacteria occur naturally in all environments (Cytryn, 2013).

The presence of ARGs in the different soil samples (Table 5) indicated that these soils represent an under appreciated reservoir of MAR machinery. Because we knew that these soils had never been treated with antibiotics especially at the four sites, we believe our results regarding the antibiotic resistance observed in bacteria from forest soils, which represented the most ecologically pristine sample among soils collected in our study, were observed as quite interesting. Indeed, soil from a pristine forest without anthropogenic influence showed a higher percentage of resistant bacteria than soils from the other three sites, thus eliminating any possibility of contamination by penicillin and tetracycline, which is the convincing evidence of the potentially omnipresent occurrence of ARGs in forests. This result would justify the presence of *tet* and *int1* in forest soils because these genes may already be common in environmental bacteria though tetracycline-resistant strain couldn't grow at concentration greater than 120 mg/L. Moreover, some studies have shown that indigenous bacteria exhibited antibiotic resistance even in unpolluted environments (Dantas *et al.*, 2008; D'Costa *et al.*, 2011). There are five classes of integrons and over 50 different

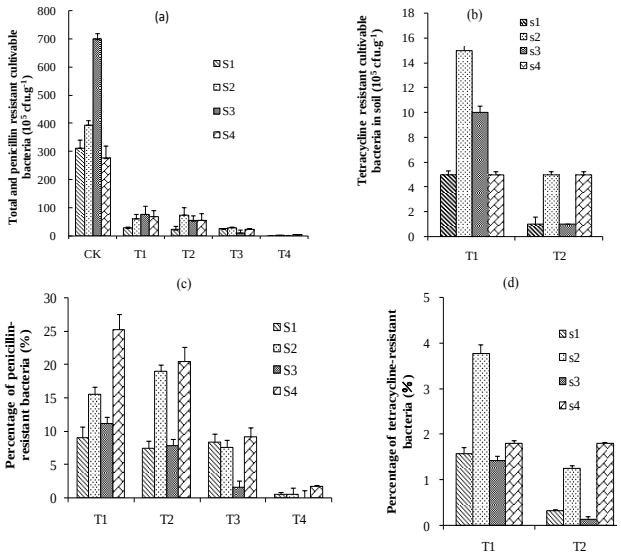

Fig. 1: The total number of bacterial colonies from four different soils in different treatments. Penicillin treatments (a) and tetracycline treatments (b) The incidences of bacteria resistant to penicillin (c) and the incidences of bacteria resistant to tetracycline (d)

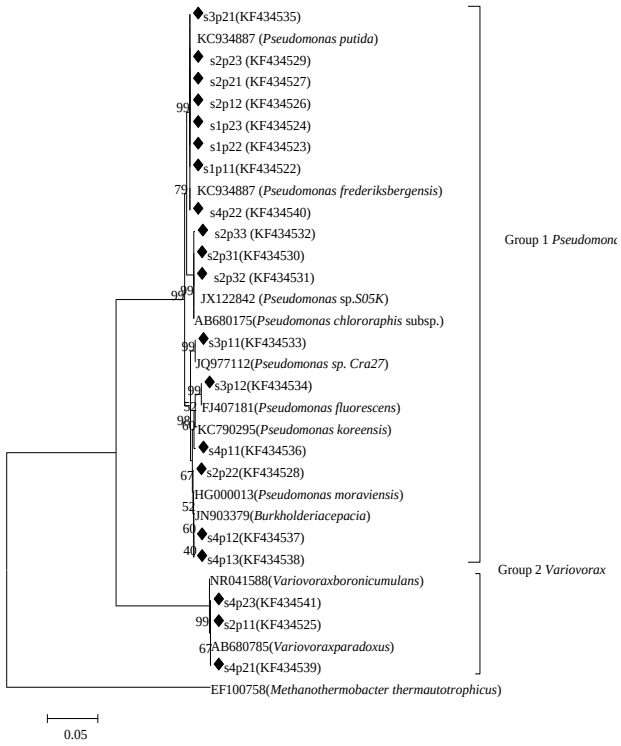

Fig. 2: Phylogenetic analysis of 20 strains of penicillin resistance bacteria. The tree was constructed on the bases of almost-complete 16s rRNA gene sequences using MEGA5.05; Bootstrap values are given for 1000 replicates with the outgroup *Methanothermobacter thermautotrophicus*

Table 4: Strain identification and summary information for the 20 isolates obtained from soil sample

Strain	Color	Gram character	Closest relative	Accession no.	From soil
S1p11	milky	negative	*Pseudomonas frederiksbergensis*	KF434522	S1
S1p22	light yellow	negative	*Pseudomonas frederiksbergensis*	KF434523	S1
S1p23	light yellow	negative	*Pseudomonas frederiksbergensis*	KF434524	S1
S2p11	milky	negative	*Variovoraxparadoxus*	KF434525	S2
S2p12	milky	negative	*Pseudomonas frederiksbergensis*	KF434526	S2
S2p21	light yellow	negative	*Pseudomonas frederiksbergensis*	KF434527	S2
S2p22	light yellow	negative	*Pseudomonas koreensis*	KF434528	S2
S2p23	light yellow	negative	*Pseudomonas frederiksbergensis*	KF434529	S2
S2p31	orange	negative	*Pseudomonas chlororaphissubsp*	KF434530	S2
S2p32	orange	negative	*Pseudomonas chlororaphissubsp*	KF434531	S2
S2p33	orange	negative	*Pseudomonas chlororaphissubsp*	KF434532	S2
S3p11	milky	negative	*Pseudomonas koreensis*	KF434533	S3
S3p12	milky	negative	*Pseudomonas koreensis*	KF434534	S3
S3p21	light yellow	negative	*Pseudomonas frederiksbergensis*	KF434535	S3
S4p11	milky	negative	*Pseudomonas koreensis*	KF434536	S4
S4p12	milky	negative	*Pseudomonas koreensis*	KF434537	S4
S4p13	milky	negative	*Pseudomonas koreensis*	KF434538	S4
S4p21	light yellow	negative	*Variovoraxparadoxus*	KF434539	S4
S4p22	light yellow	negative	*Pseudomonas frederiksbergensis*	KF434540	S4
S4p23	light yellow	negative	*Variovoraxparadoxus*	KF434541	S4

Table 5: Detection of resistance genes and integron genes in soil samples and resistance strains

	S1	S2	S3	S4	s4p22	s1p23	s2p11	s4p23	s2p31	s2p32	s4p13	s3p12	+control
tet(M)	-	-	-	-	-	-	-	-	-	-	-	-	+
tet(O)	-	+	+	+	-	-	-	-	-	-	-	-	+
tet(T)	-	+	-	-	-	-	-	-	-	-	-	-	+
tet(W)	+	+	+	+	-	-	-	-	-	-	-	-	+
tet(C)	-	+	-	-	+	+	+	+	+	+	+	+	+
bla_{OXA-1}(AMP)	-	-	-	-	-	-	-	-	-	-	-	-	+
bla_{PSE-1}(AMP)	-	-	-	-	-	-	-	-	-	-	-	-	+
blaTEM	-	-	+	+	+	+	+	+	+	+	+	+	+
int1	+	+	+	+	-	-	-	-	-	-	-	-	+
int2	-	-	-	-	-	-	-	-	-	-	-	-	+

+: present;-: absent. The positive control was *E. coli* DH5 with cloning vectors and cloned target genes

class 1 integrons have been identified (Tamang *et al.*, 2007). Integrons provide a gene-capture system to bacteria, threatening the multiple-antibiotic treatment regimen (Mazel, 2006). In our study, class 1 integrons were found predominantly in all soils, indicating that horizontal gene transfer occurred commonly in the soils (Gundogdu *et al.*, 2013). Therefore, prudent use of antibiotics in medicine and agriculture is needed; otherwise, the selective pressure triggered by these compounds may occur via horizontal gene transfer, affecting the treatment of human diseases (Colomer-Lluch *et al.*, 2011; Forsberg *et al.*, 2012). The horizontal transfer of genes related to the enzymatic machinery, which is responsible for subsistence on antibiotics, could bring novel antibiotic-resistance mechanisms not yet observed in the clinic actually. However, we should note that some resistance genes were not detected by traditional PCR detection, probably due to the low copy number of target genes. In contrast, real-time PCR arrays are more sensitive to resistance genes in the environment, even in glacier and permafrost sediments (Ushida *et al.*, 2010; D'Costa *et al.*, 2011). Thus, because ARGs exist naturally, the quantitative characteristics of the pristine area may be appropriate for setting background levels of resistance to compare with affected sites. Further studies are required; in particular, approaches based on real-time PCR arrays should be applied to environmental monitoring.

Of particular interest in our study was the observation that some soils harbored an ARG, but no isolates from that soil that also encoded by the same ARG. For example, *tetW* and *int1* were found in the soils but not in the bacterial isolates (Table 5), indicating that some other antibiotic-resistant bacteria existed in these soils could not be cultured in this study, most likely because cultivable bacteria represent less than 1% of the total bacteria. These results may have also been constrained by the limited efficiency of the investigation protocols.

Characteristics of Super Penicillin-resistant Bacteria

We couldn't isolate any super tetracycline-resistant strains at the1000 mg/L tetracycline treatment. However, twenty super penicillin-resistant bacterial strains were selected from four diverse soil types at a relatively high selective penicillin concentration up to 1000 mg/L. All the bacterial isolates in this study were gram-negative bacteria, which are notoriously difficult to kill as these bacteria have an extra

cell membrane to prevent the penetration of drugs, as well as other defense mechanisms that gram-positive bacteria lack, such as multidrug resistance efflux pumps, resistance mutations, and inactivation of the antibiotic (Taubes, 2008; Allen *et al.*, 2010).

The bacteria isolated in this research were classified into two different genera: *Pseudomonas*, belonging to the order *Pseudomonadales*, and *Variovorax*, belonging to the order *Burkholderiales*. Organisms in the orders *Pseudomonadales* and *Burkholderiales* typically have large genomes, which have been inferred to be linked to their metabolic diversity and MAR characteristics (Dantas *et al.*, 2008; Levy, 2008; Rho *et al.*, 2012). Bacteria belonging to the genera *Pseudomonas* and *Variovorax* are common indigenous flora in soil and water. Both of these genera are associated with a large variety of diverse and uncommon metabolic features, including the degradation of toxic and complex chemical compounds (Winsor *et al.*, 2011; Satola *et al.*, 2013). Ramos *et al.* (2002) found that several *Pseudomonas spp.* strains were resistant to high concentrations of many toxic organic solvents. Moreover, *Variovorax paradoxus* can accomplish a wide variety of catabolic pathways and has other characteristics related to the mediation of organic sulfur compounds, aromatic compounds, polymers, metal ions, and other compounds (Satola *et al.*, 2013). Both of these genera are thought to represent promising strains for bioremediation and other biotechnical applications by some researchers (Sniegowski *et al.*, 2011; Satola *et al.*, 2013).

Nevertheless, in this study, all isolates tested exhibited co-resistance to four or more types of antibiotics and had *tet(C)* and *blaTEM* resistance genes, which was sequenced and confirmed as the corresponding resistance genes using the BLAST alignment tool and the antibiotic resistance genes database, indicating that the use of penicillin could select strains harboring *blaTEM* efficiently or that these genes were present in antibiotic-resistant soil bacteria naturaly. The *TEM* subset of β-lactamases are high prevalence in the genomes of indigenous isolates, and also as gram-negative bacteria that are directly relevant to human infectious diseases (e.g., *Burkholderia pseudomallei* and *Delfitia acidovorans*) (Endimiani *et al.*, 2007; Nyberg *et al.*, 2007), suggesting that *BlaTEM* plays an important function in developing resistance to the β-lactams antibiotics in this study. Although isolates with *tet(C)* coding for a tetracycline efflux pump did not express resistance to tetracyclines in the disc diffusion susceptibility test (Table 3), this potential resistance risk cannot be ignored. All the resistant isolates from our four soil samples showed MAR patterns for four or more antibiotics, indicating that these bacteria harbored other antibiotic-resistance mechanisms besides bacteria encoded by tetracycline and β-lactam resistance genes.

Conclusion

In conclusion, different types of soils contained tetracycline and penicillin-resistant bacteria widely, despite the fact that those soils have not having been exposed to penicillin before. However, only super penicillin-resistant bacteria were isolated from four diverse soils with the concentrations up to 1000 mg/L. Resistant bacteria isolated were classified as either *Pseudomonas* or *Variovorax*, common indigenous flora in soil and water, but exhibited phenotypic and genotypic unique characteristics. No pathogens were isolated in this research, however, the high prevalence of indigenous antibiotic resistant bacteria encoded by *tet(C)* and *blaTEM* genes could trigger a potential health risk.

Acknowledgement

The authors gratefully acknowledge the financial support by NSFC (41401266), National Key Basic Research Program of China (2015CB150502) and the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry.

References

Allen, H.K., J. Donato, H.H. Wang, K.A. Cloud-Hansen, J. Davies and J. Handelsman, 2010. Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.*, 8: 251-259

Colomer-Lluch, M., L. Imamovic, J. Jofre and M. Muniesa, 2011. Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs and poultry. *Antimicrob. Agent. Chemother.*, 55: 4908–4911

Cytryn, E., 2013. The soil resistome: The anthropogenic, the native, and the unknown. *Soil Biol. Biochem.*, 63: 18–23

D'Costa, V.M., C.E. King, L. Kalan, M. Morar, W.W. Sung, C. Schwarz, D. Froese, G. Zazula, F. Calmels and R. Debruyne, 2011. Antibiotic resistance is ancient. *Nature*, 477: 457–461

D'Costa, V.M., K.M. McGram, D.W. Hughes and G.D. Wright, 2006. Sampling the antibiotic resistome. *Science*, 311: 374–377

Dantas, G., M.O. Sommer, R.D. Oluwasegun and G.M. Church, 2008. Bacteria subsisting on antibiotics. *Science*, 320: 100–103

Endimiani, A., F. Luzzaro, R. Migliavacca, E. Mantengoli, A.M. Hujer, K.M. Hujer, L. Pagani, R.A. Bonomo, G.M. Rossolini and A. Toniolo, 2007. Spread in an Italian hospital of a clonal *Acinetobacter baumannii* strain producing the TEM-92 extended-spectrum β-lactamase. *Antimicrob. Agent. Chemotherapy*, 51: 2211–2214

Forsberg, K.J., A. Reyes, B. Wang, E.M. Selleck, M.O. Sommer and G. Dantas, 2012. The shared antibiotic resistome of soil bacteria and human pathogens. *Science*, 337: 1107–1111

Gundogdu, A., A.V. Jennison, H.V. Smith, H. Stratton and M. Katouli, 2013. Extended-spectrum beta-lactamase producing Escherichia coli in hospital wastewaters and sewage treatment plants in Queensland, Australia. *Can. J. Microbiol.*, 59: 737–745

Helena Rebouças, R., O. Viana de Sousa, A. Sousa Lima, F. Roger Vasconcelos, P.B. de Carvalho and R.H.S. dos Fernandes Vieira, 2011. Antimicrobial resistance profile of Vibrio species isolated from marine shrimp farming environments Litopenaeus vannamei at Ceará, Brazil. *Environ. Res.*, 111: 21–24

Knapp, C.W., J. Dolfing, P.A. Ehlert and D.W. Graham, 2010. Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ. Sci. Technol.*, 44: 580–587

Levy, S.B., 2008. Genome size and antibiotic resistance. *Antimicrob. Agent. Chemotherapy*, 52: 2696–2696

Levy, S.B. and B. Marshall, 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nat. Med.*, 10: S122–S129

Li, D., T. Yu, Y. Zhang, M. Yang, Z. Li, M. Liu and R. Qi, 2010. Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river. *Appl. Environ. Microbiol.*, 76: 3444–3451

Liu, B. and M. Pop, 2009. ARDB—antibiotic resistance genes database. *Nucleic Acids Res.*, 37: 443–447

Macauley, J.J., C.D. Adams and M.R. Mormile, 2007. Diversity of tet resistance genes in tetracycline-resistant bacteria isolated from a swine lagoon with low antibiotic impact. *Can. J. Microbiol.*, 53: 1307–1315

Mazel, D., 2006. Integrons: agents of bacterial evolution. *Nat. Rev. Microbiol.*, 4: 608–620

Nyberg, S.D., M. Österblad, A.J. Hakanen, P. Huovinen, J. Jalava and F.S.G.F.A. Resistance, 2007. Detection and molecular genetics of extended-spectrum beta-lactamases among cefuroxime-resistant Escherichia coli and Klebsiella spp. isolates from Finland, 2002–2004. *Scand. J. Infectious Dis.*, 39: 417–424

Ozaktas, T., B. Taskin and A.G. Gozen, 2012. High level multiple antibiotic resistance among fish surface associated bacterial populations in non-aquaculture freshwater environment. *Water Res.*, 46: 6382–6390

Pei, R., S.C. Kim, K.H. Carlson and A. Pruden, 2006. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.*, 40: 2427–2435

Pruden, A., R. Pei, H. Storteboom and K.H. Carlson, 2006. Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. *Environ. Sci. Technol.*, 40: 7445–7450

Ramos, J.L., E. Duque, M.T. Gallegos, P. Godoy, M.I. Ramos-González, A. Rojas, W. Terán and A. Segura, 2002. Mechanisms of solvent tolerance in gram-negative bacteria. *Annu. Rev. Microbiol.*, 56: 743–768

Rho, H., B. Shin, O. Lee, Y.H. Choi, J. Rho and J. Lee, 2012. Antibiotic resistance profile of bacterial isolates from animal farming aquatic environments and meats in a peri-urban community in Daejeon, Korea. *J. Environ. Monitoring*, 14: 1616–1621

Rodríguez-Verdugo, A., B.S. Gaut and O. Tenaillon, 2013. Evolution of Escherichia coli rifampicin resistance in an antibiotic-free environment during thermal stress. *BMC Evol. Biol.*, 13: 50

Satola, B., J.H. Wübbeler and A. Steinbüchel, 2013. Metabolic characteristics of the species Variovorax paradoxus. *Appl. Microbiol. Biotechnol.*, 97: 541–560

Sniegowski, K., K. Bers, K. Van Goetem, J. Ryckeboer, P. Jaeken, P.. Spanoghe and D. Springael, 2011. Improvement of pesticide mineralization in on-farm biopurification systems by bioaugmentation with pesticide‐primed soil. *FEMS Microbiol. Ecol.*, 76: 64–73

Sparks, D.L., A. Page, P. Helmke, R. Loeppert, P. Soltanpour, M. Tabatabai, C. Johnston and M. Sumner, 1996. *Methods of Soil Analysis: Part 3-Chemical Methods*. Soil Science Society of America, Washington DC, USA

Spellberg, B., J.G. Bartlett and D.N. Gilbert, 2013. The future of antibiotics and resistance. *New Engl. J. Med.*, 368: 299–302

Tamang, M.D., J.Y. Oh, S.Y. Seol, H.Y. Kang, J.C. Lee, Y.C. Lee, D.T. Cho and J. Kim, 2007. Emergence of multidrug-resistant Salmonella enterica serovar Typhi associated with a class 1 integron carrying the dfrA7 gene cassette in Nepal. *Int. J. Antimicrob. Agent.*, 30: 330–335

Taubes, G., 2008. The bacteria fight back. *Science*, 321: 356-361

Torsvik, V., J. Goksøyr and F.L. Daae, 1990. High diversity in DNA of soil bacteria. *Appl. Environ. Microbiol.*, 56: 782–787

Ushida, K., T. Segawa, S. Kohshima, N. Takeuchi, K. Fukui, Z. Li and H. Kanda, 2010. Application of real-time PCR array to the multiple detection of antibiotic resistant genes in glacier ice samples. *The J. General Appl. Microbiol.*, 56: 43–52

Winsor, G.L., D.K. Lam, L. Fleming, R. Lo, M.D. Whiteside, Y.Y. Nancy, R.E. Hancock and F.S. Brinkman, 2011. Pseudomonas Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes. *Nucleic Acids Res.*, 39: D596–D600

Wittwer, M., J. Keller, T.M. Wassenaar, R. Stephan, D. Howald, G. Regula and B. Bissig-Choisat, 2005. Genetic diversity and antibiotic resistance patterns in a Campylobacter population isolated from poultry farms in Switzerland. *Appl. Environ. Microbiol.*, 71: 2840–2847

(Received 25 March 2015; Accepted 04 May 2015)