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Antibiotic resistance profile and genetic traits of soil isolated bacteria and their subsistence on Penicillin or Tetracycline

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Research Article

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ABSTRACT

It is evident nowadays that soil-dwelling bacteria can develop resistance against certain antibiotics. In this perspective, the main objectives of this study were to evaluate the soils from a Chinese tea plantations and forests, which had not previously been exposed to antibiotics, but contained bacterial strains, which could actually subsist on the antibiotics for energy and nutrients, and to study the genetic traits for antibiotic resistance in these strains. Five bacterial isolates belonging to the *Lysobacter, Variovorax*, *Pseudomonas, Chitinophaga*, and *Bradyrhizobium* genera were identified. It was observed that high-level multiple antibiotic resistances were common among these bacteria both before and after plasmid curing. The p4 and p5 isolates were remarkably resistant to β-lactams, whereas the t1, t5, and t9 isolates were resistant to tetracycline at both the concentrations used. The resistance genes t*etC* and *blaPSE-1* were relatively widely distributed in these bacteria, whereas integrons were not found in any of the isolated strains. The antibiotic resistance of these five bacterial isolates may have been derived from plasmids or chromosomes.

INTRODUCTION

The ability of antibiotics to kill a broad spectrum of bacteria has revolutionized medicine industry and world. However, the improper use of antibiotics has also resulted in an alarming dispersal of multiple-antibiotic resistant bacteria in the clinical as well as agricultural setup. There have been identified a number of antibiotic-resistant bacteria in clinical and veterinary settings. Even, the distribution of veterinary antibiotics has been detected in soil, surface water, and groundwater recently and the development of multidrug resistance has been investigated [1-3] through horizontal gene transfer mechanisms [4].

The concept that antibiotic resistance originates from the soil environment is not new and has been around for decades [5]. Burgos et al. [6] found gram-negative enteric bacteria associated with the topsoil of dairy farms in New Mexico. Sengupta et al. [7] isolated a soil sample containing diverse anaerobic bacteria and detected resistance for 16–25 antibiotics. Several soil bacteria possessing and showing resistant genes are not because of they have been exposed to antibiotics, but because they live in an environment full of antibiotic producers. And most of these genes are constitutive. For example, Dantas et al. [8] showed that some soil bacteria could actually utilize carbon from antibiotic substrates even if they have never been exposed to these antibiotics previously. The phylogenetic diversity of these bacteria is well known, however, it remains unclear that how these bacteria are

widely spread in the environment. Soil bacteria play a key role in the soil ecology while considered as a potential reservoir of antimicrobial resistance genes. Liu and Pop [9] reported more than 20,000 potential resistance genes belonging to ac. 400 different types in the ecological environment. Chromosomes usually encode antibiotic sensitivity and are becoming code for antimicrobial resistance due to mutations with the change of the environment [10,11]; however, these mutations occur at a rate of approximately 1 in 108 per chromosomal replication. Many antimicrobial resistance genes exist in plasmids or transposons, which are small pieces of circular DNA and are only 1% of the total size of chromosomes and can be transferred among bacterial species. Over the last few years, integron, a novel DNA element, that can integrate antibiotic resistance genes has been investigated and explored well [12], which was found to be a part of the transposons from the Tn21 family or was independently found in several groups of broad-host-range plasmids.

In our previous study, soil bacteria were isolated and identified based on their ability to subsist on penicillin or neomycin without previous exposure to synthetic antibiotics, using the methods described by Dantas et al. [8]. The isolates were found to have high levels of resistance and they surprisingly showed a great phylogenetic diversity and were closely related to human pathogens [13]. Hence, we continued this line of research by measuring soil bacterial growth in the presence of penicillin or tetracycline in soil samples collected from China. Penicillin and tetracycline were selected for trials keeping in view that they belong to structural classes, and they are widely used not only in clinical settings but also in livestock production sector in China. The genetic traits for antibiotic resistance in the isolated bacteria were also investigated to determine how the soil environment influences the spread of antibiotic resistance. Our study and findings would provide deeper insights into the mechanism of transfer and translocation of resistance genes within and among bacterial communities.

MATERIALS AND METHODS

Soil samples

Samples of topsoil (0-15 cm) were collected from a tea plantation (120°06'41", 30°13'36") and a pristine forest (120°07'12", 30°14'20"), without any anthropogenic disturbance, each located in Hangzhou, China. Based on the preliminary investigations it was assumed that none of these two sites has previously been exposed to penicillin or tetracycline. At each site, 10-12 soil samples were collected using a sterile spade. Samples were mixed thoroughly and a composite sample was obtained by placing this homogenize mixture into a plastic bag. The composite samples were transported to the laboratory on ice within 2-6 h and stored at 4°C. Selected soil characteristics **(Table 1)** were analyzed according to the methods described by Sparks et al. [14].

Table 1. Brief description of the soils from which bacteria were isolated.

Soil	Actual location	pH	Salinity	Avail[a] N	Avail[a] K	Avail[a] P	Total N	OM[b]	CEC[c]
			$g \cdot kg^{-1}$	$mg \cdot kg^{-1}$	$mg \cdot kg^{-1}$	$mg \cdot kg^{-1}$	$g \cdot kg^{-1}$	$g \cdot kg^{-1}$	$cmol \cdot kg^{-1}$
Tea	Tea plantation, Hangzhou, China	5.56	0.05	105.32	154	21.12	0.96	13.46	14.18
Forest	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

Isolation and identification of soil bacterial growth with penicillin or tetracycline

Soil bacteria were isolated and cultured with antibiotics using already reported methodology [8]. Minimal medium was utilized with a single carbon source (SCS); bacteria were serially diluted and spread-plated onto SCS agar, supplemented with the appropriate corresponding antibiotic. Two antibiotics, penicillin G and tetracycline hydrochloride, with the purities >99% were purchased from Sango Biotech; their chemical structures are displayed in **Figure S1.** Bacteria cultures on SCS agar, without any antibiotics, were selected as negative control. The phylogenetic profiles of the bacteria were determined as previously described by Zhang et al. [15]. Briefly, the 16S rRNA gene sequence (nucleotides 27-1492) was amplified by PCR and sequenced. The bacterial phylogeny was determined based on the variation of 16S rRNA gene sequences, identified using the BLAST program. A phylogenetic tree of the bacterial species based on the 16S rRNA sequences was constructed using the neighbor-joining tree method by (MEGA5.05) software. Bootstrap analysis was used to find out the statistical significance of branching order, which involved the construction of 1000 trees by re sampling the original data.

Detection of resistance genes and integron genes in isolates and two soils (forest and tea)

The presence of ten different genes both in the bacterial isolates and soils were determined by using PCR and real-time quantitative PCR (qRT-PCR). Primers and PCR, qRT-PCR conditions used in this study are shown in **Tables S1 and S2.** The bacterial DNA used as template was extracted by harsh lysis using the Power Soil DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA), following the manufacturer's instructions. The concentration and quality of the extracted DNA were determined by spectrophotometer (NanoDrop-2000) and agarose gel electrophoresis, respectively.

PCR procedure: The PCR mixture (total volume, 20 µL) was consisted of 10 µL of 2x Easy Tap PCR SuperMix (TransGen Biotech), 1 µL of each primer (10 µM), 1 µL of template, and 7 µL of sterile double-distilled water. PCR was initiated by denaturation

at a temperature of 94°C for 4 min; followed by 35 cycles of 45 s at 94°C, 45 s at different annealing temperatures, and 72°C for 1 min; and then a final extension step for 6 min at 72°C. PCR products were analyzed by electrophoresis on a 2% agarose gel in 1x TAE buffer. All bands were excised from the agarose gel, and purified using a QIA quick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA), and then 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 determined from the GenBank database using the NCBI-BLAST program. For all reactions in this study, PCR assays were performed in triplicate for each sample. Standard PCR mixtures without DNA template were used as negative controls, and positive bacterial strains carrying genes or integrons verified by sequencing were used as positive controls.

qRT-PCR procedure: The abundance of ARGs and 16S rRNA genes [16] in soil was quantified using a LightCycler® 480 Real-Time PCR System (Roche Applied Science, CA) for qPCR. Cloning vectors were transformed into *E.coli* DH5α, and the transformant colonies were observed and selected by blue-white screening system on Luria-Bertani agar plates, containing 100 g·mL^{-1} ampicillin, 1 mg·plate^{-1} 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-Gal), and 2.38 mg·plate^{-1} isopropyl-beta-D-thiogalactopyranoside (IPTG). PCR amplification with a pair of universal primers (M13F and M13R), targeting both sides of the cloning vector, was used to determine the appropriate length of inserted DNA. The inserted DNA fragments were sequenced by Sangon Biotech (Shanghai) Co., Ltd. The qPCR mixture (total volume, 20 μL) consisted of 10 μL of 2x SYBR qPCR SuperMix (TransGen Biotech), 1 μL of each primer (10 μM), 1 μL of template, and 7 μL of sterile double-distilled water. The thermal program included an initial denaturation at 94°C for 30 s; followed by 40 cycles of 5 s at 94°C, 15 s at different annealing temperatures, and 72°C for 10 s; followed by a final melting curve stage where temperature raised from 60°C to 95°C. Plasmid carrying the target gene was serially diluted (up to 10 dilutions) and was used to generate the calibration curves (Ct value versus log value of initial target gene copy number per reaction) with seven points for each qRT-PCR. Calibration curves were run together with each measurement. The PCR efficiency varied and was in the range of 87.38 to 100.24%, and R2 values were >0.993 for all calibration curves. Based on the calibration curves, the copy number of target genes was determined using the Ct value of a test sample with unknown concentration, which was then normalized against the volume (mL) of the original samples and the mass (ng) of the extracted DNA.

Resistance profiling of isolates grown on antibiotics before and after plasmid curing

Sodium dodecyl sulfate (SDS) was used in the protocol according to Stephen et al. [17], with some modifications for plasmid curing. Bacterial isolates were incubated in water bath at 45ºC for 18 h in Luria Broth (LB) containing 0.5% SDS and then incubated again with constant shaking at 37ºC for 18 h in LB to eliminate plasmids. To select the strains that lost antibiotic resistance, the colonies were transferred to corresponding agar plates supplemented with the respective antibiotics at 200 mg·L^{-1} using the replica plating method. Colonies arising from cells that had lost the plasmid were detected by electrophoresis.

Resistance profiling was performed for bacterial isolates before and after plasmid is cured. Resistance profiling for isolates was carried out by using liquid media (rich media), prepared by dissolving the relevant antibiotics at a concentration 200 mg·L^{-1} and 1000 mg·L^{-1} into cooled but previously autoclaved Luria Broth as previously described [13]. The antibiotics tested included aminoglycosides (streptomycin and neomycin), β-lactams (amoxicillin and ampicillin), macrolides (azithromycin and erythromycin), and tetracycline's (chlortetracycline and oxytetracycline).

Statistical analyses

Data preparation, calculation, and statistical analyses were performed using Microsoft Office Excel and SPSS 16.0 for Windows. Analysis of variance (ANOVA) was used to determine treatment effects. Comparisons of means were performed using Duncan's studentized range test at P<0.05. The 16S rRNA gene sequences of the 5 strains in this study were deposited in GenBank database with accession number KF898093-KF898097, and the nucleotide sequences greater than 200 bp for genes can be accessed under accession numbers KM594534, KM594536-KM594538, and KM594543-KM594546.

RESULTS AND DISCUSSION

Isolation and identification of isolates

Eight bacterial isolates were identified that were capable of using penicillin or tetracycline at 200 mg·L^{-1} as their sole carbon source. The morphologies were categorized according to Pollack et al. [18]. After isolation, two isolates were not viable during storage, and one of the viable isolates could not be produced at a high yield. The rest of the experiments were conducted with the remaining five isolates. The five bacterial isolates were grown on penicillin or tetracycline at 200 mg·L^{-1}. Two isolates (p4 and p5) grown on penicillin were obtained from the forest, whereas the other three isolates grown on tetracycline were from the tea plantation (t5 and t9) and forest (t1). All of the isolates thrived and reached a plateau within 30 to 50 h in Luria Broth with the appropriate corresponding antibiotic at a concentration of 200 mg·L^{-1} **(Figure 1).** The 16S rRNA genes of the five isolates were sequenced, and the sequence data were used to develop phylogenetic relationships **(Figure 2).** The 16S rRNA gene sequences of five strains were deposited in the GenBank database under the accession numbers KF898093-KF898097. Based on the phylogenetic analysis it became clear that the bacteria isolated from the two soils belong to a diverse set of genera. The two isolates p4 and p5 were grown on penicillin and belonged to Lysobacter enzymogenes and *Variovorax paradoxus*, respectively. The species Lysobacter enzymogenes belongs to the family *Xanthomonadaceaewithin* the *Gammaproteo* bacteria.

The *L. enzymogenes* strain has recently been declared as a biological control agent for plant several diseases [19,20], as it produces numerous extracellular enzymes, including multiple forms of β-1, 3-glucanases, and chitinases, which contribute to biocontrol activity [2,15]. The strain has also shown an ability to induce systemic resistance in certain plants to protect them from pathogenic infection [21,22]. *Variovorax paradoxus* is a ubiquitous, gram-negative, and aerobic bacterium from the *Variovorax* genus belonging to the *Comamonadaceae* family. *Variovorax paradoxus* plays an important role in biodegradation in nature and also promotes plant growth [23].

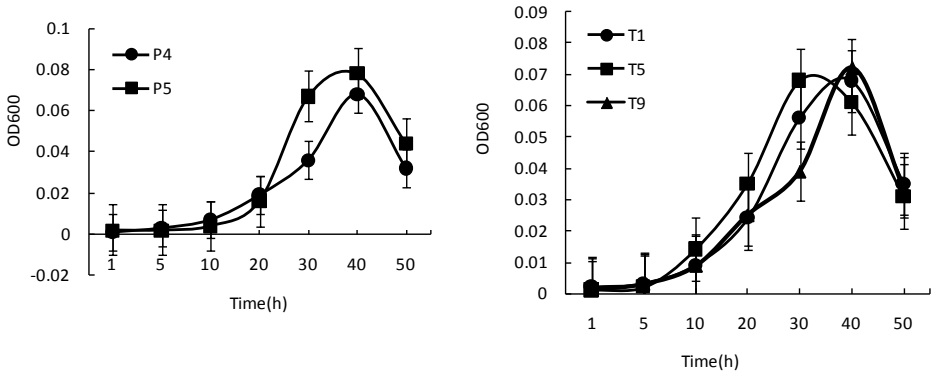

Figure 1. Growth curves of isolates supplemented with (a) penicillin or (b) tetracycline at a concentration of 200 mg/L in Luria Broth.

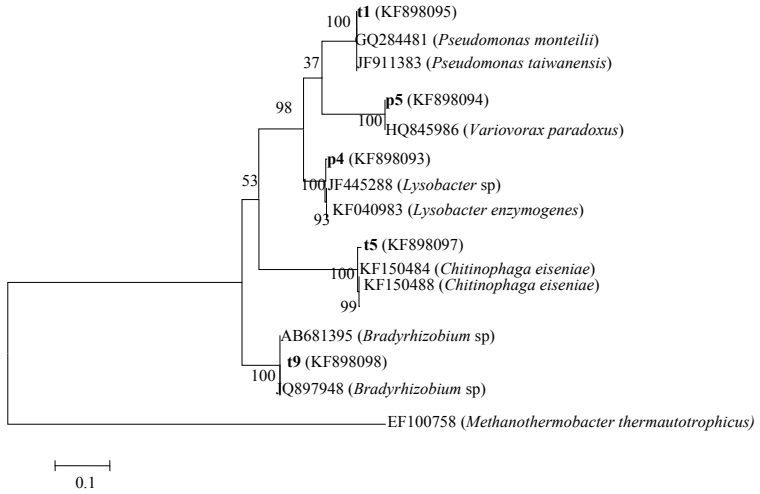

Figure 2. Neighbor-joining tree showing the phylogenetic relationships of soil bacterial isolates capable of subsisting on penicillin and tetracycline (shown in bold type). Sequences from GenBank are identified by accession numbers shown in parentheses. The scale bar indicates 1% sequence divergence.

Three isolates (t1, t5, and t9) that were grown on tetracycline were classified into three different genera: *Pseudomonas, Chitinophaga, and Bradyrhizobium*, respectively. The isolate t1 was most closely related to *Pseudomonas monteilii* (99%16S rRNA gene sequence similarity). *Pseudomonas monteilii*, a gram-negative, rod-shaped, and motile bacterium isolated from clinical specimens, is a rare opportunistic pathogen or colonizer [24]. It was detected and isolated from bronchial aspirate in Lyon, France, and the value of its G+C content was 60 mol% [24]. The isolate t5 was most closely related to *Chitinophaga eiseniae*, which belongs to the genus *Chitinophaga* in the family *Chitinophagaceae* and is a gram-negative, rod-shaped bacterial strain that was detected and identified from vermicompost collected at Masan, Korea [25]. Isolate t9 was most closely related to *Bradyrhizobium* sp. (99% 16S rRNA gene sequence similarity), which is capable of using penicillin as its carbon source, and was isolated from a cornfield in the US [14]. *Bradyrhizobium* is a genus of gram-negative bacteria associated with soil, many of which fix nitrogen. Although a lesser number of strains was isolated and identified from the Chinese tea plantation and forest soil as compared to the previous research carried out in the US [14], these studies are consistent with respect to the identification of microbial community members.

Diversity of antibiotic resistance genes and integron genes in bacterial isolates and in soils

The presence of tetracycline genes, β-lactam-resistance genes, and integron genes in the five soil isolates and two soils (forest and tea) were studied after amplification of genomic DNA by PCR, qRT-PCR and also confirmed by DNA sequencing. To ascertain the distribution of antibiotic resistance genes for tetracyclines, we evaluated the presence of tetM, tetO, tetT, tetW, and tetC. Although the resistance to β-lactams has been mediated by several mechanisms, the focus of the study was β-lactamases, which can hydrolyze the β-lactam ring. Hundreds of β-lactamases have been found, and the most clinically important ones are primarily the derivatives of several existingβ-lactamase genes such as *blaPSE-1* (AMP) and *blaTEM*. The tetracycline gene *tetC*, which encodes a tetracycline efflux pump, and the β-lactamase gene *blaPSE-1* (AMP), which encodes β-lactamase, were

widespread in the isolates even though these five strains belong to different species isolated from two soil types **(Table 2).** These two genes are responsible for encoding factors that lead to bacterial resistance to several antibiotics, including penicillin and tetracycline, and can be transferred from non-pathogenic bacteria to pathogenic bacteria, thus can lead to clinically important antibiotic resistance [26]. The spreading of antibiotic resistance genes among bacterial strains is becoming a frequently occurring problem in infectious diseases. For example, the detection rate of the gene that encodes PSE was found to be high not only in Pseudomonas aeruginosa but also in Salmonella [27,28].

Table 2. PCR-based assay to detect the presence/absence of various ARGs in different isolates.

ARG[b]	Strain identification[a]				
	p4	p5	t1	t5	t9
tetM	-[c]	-	-	-	-
tetO	-	-	-	-	-
tetT	-	-	-	-	-
tetC	+	+	+	+	-
tetW	-	-	-	-	-
bla_{PSE-1}*(AMP)*	+	+	+	+	+
blaTEM	-	-	-	-	-
intI	-	-	-	-	-
intII	-	-	-	-	-

[a]Information regarding strains is provided in Table 2.
[b]ARG = Antibiotic resistance genes. The primers used to test for their presence are provided in Table 2.
[c]The "-" symbol indicates that the gene was not detected in the isolate, and the "+" symbol indicates that the gene was detected in the isolate.

Many genes resistant to antibiotics are found on plasmids and transposons, which enable their transfer among different bacterial species. In recent studies, a direct relationship was found between the increase in antibiotic resistance and integrons that collect resistance determinants embedded in the form of one or more gene cassettes by site-specific recombination [29,30]. To date, more than 100 different classes of integrons have already been identified [31]. However, class 1 and class 2 integron genes (*int1* and *intII*) have been identified as a primary source of antimicrobial resistance genes and are thought to serve as a potential gene pool for resistance genes in a variety of Gram-negative and, to a lesser extent, Gram-positive bacteria [32]. However, in the present study, both classes of integron genes were not detected in the isolates, indicating that integron genes were not widely disseminated in soil bacterial strains that were not only resistant to antibiotics but could also utilize the antibiotics for energy and nutrients. The lack of integrons in resistant strains isolated from the clinical environment has previously been reported in a study [33]. Notably, some of the bacteria may have been refractory to the isolation procedures, or there may have been trace copies of integrons that we could not detect. Our findings do not account for the potential presence of integrons that fall below our detection limits.

The qRT-PCR method serves as an important tool to quantify ARGs in the environment and to study how environmental factors determine the fates of ARGs. The forest sample from a pristine area without anthropogenic influence contained a significant number of genes, of *tet(T)*(105 copies·g^{-1}), i (105 copies·g^{-1}), *tet(C)* (103 copies·g^{-1}) and 16S (1010 copies·g^{-1}) as shown in **Table 3**, suggesting that the forest soil contains a certain degree of ARGs. However, this was usual, as ARGs are expected to be present in all natural environments [3]. The level of resistance in relation to the size of overall population was determined by using the copy numbers that were normalized to bacterial 16S rRNA genes relatively; the expression of these genes in forest samples was lower than that in samples from collected from the soil associated with tea plantation **(Figure 3).** Tea plantations are influenced by agricultural activities such as application of organic fertilizers, given that 30-90% of the parent antibiotics are excreted as animal feces used to make organic fertilizer [34]. Among these genes, *blaTEM* and *int1* showed higher expressions in the soil samples.

Table 3. Absolute copies of target genes and standard deviation in soils.

Copies·g^{-1}	bla_{PSE-1}*(AMP)*	*blaTEM*	*tet(W)*	*tet(T)*	*tet(M)*	*tet(O)*	*tet(C)*	*int1*	*int2*	*16S*
Tea	0	1.57E+06a	1.32E+05a	1.61E+05b	3.85E+04a	1.34E+05b	2.93E+03b	1.19E+06a	3.79E+04a	5.69E+09b
SD	0	6.27E+01	6.20E+02	2.10E+02	2.00E+01	5.60E+01	1.20E+01	5.58E+03	2.30E+01	6.81E+05
Forest	0	1.58E+06a	4.90E+03b	1.94E+05a	4.64E+04a	1.61E+05a	7.49E+03a	4.75E+05b	0.00E+00b	4.85E+10a
SD	0	2.77E+02	8.26E+00	4.50E+02	4.50E+01	4.12E+02	1.56E+01	3.70E+02	0.00E+00	1.14E+06

Antibiotic resistance prevalence and levels before and after plasmid curing

Antibiotic resistance of each of the five isolates was determined against eight antibiotics that belonged to four different classes (aminoglycosides, macrolides, β-lactams, and tetracycline's) before and after the curing of plasmid. All of the antibiotics were used at a concentration of 200 mg·L^{-1} and 1000 mg·L^{-1} and prepared using a rich carbon source (Luria Broth). The isolate p4 was found resistant to all of the antibiotics tested at a concentration of 200 mg·L^{-1} and also showed resistance to most of the antibiotics at a concentration of 1000 mg·L^{-1}, but before plasmid curing **(Table 4).** Out of the remaining isolates, most were resistant to β-lactams and macrolides. The p4 and p5 isolates were resistant to β-lactams, whereas, t1, t5, and t9 isolates were

resistant to tetracycline at both concentrations. These results were similar to the findings of Dantas et al. [8], who reported that if a bacterial isolate was found resistant to one antibiotic; it was also resistant to other antibiotics belonging to the same class. From an ecological point of view, we have presented how the antibiotic resistance may prevail across the soil environment. An increasing number of bacterial pathogens, resistant to multidrug, are spreading in clinical as well as community settings. Emergence of serious infections resulted by these resistant bacteria is becoming alarmingly common worldwide [35,36]. In our study, a high level of resistance was found among the soil-dwelling isolates from forest soil where there was no history of anthropogenic activity had been reported. Overall, our study highlights the importance of analyzing bacterial growth in the presence of antibiotics, as high antibiotic resistance was observed in normal flora rather than in individual pathogenic species.

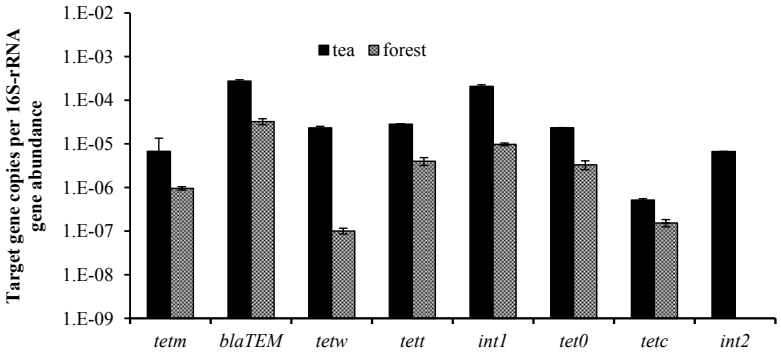

Figure 3. Detected levels of target genes normalized to copies of the ambient 16S rRNA gene.

Table 4. Resistance patterns of the resistance strains before and after plasmid elimination treatment.

Classes of antibiotics	Antibiotics	Concentration mg·L^{-1}	Before plasmid elimination					After plasmid elimination				
			p4	p5	t1	t5	t9	p4	p5	t1	t5	t9
Aminoglycosides	Streptomycin	200	R[a]	S[b]	R	S	S	S	S	S	S	S
		1000	R	S	R	S	S	S	S	S	S	S
	Neomycin	200	R	S	R	S	S	S	S	S	S	S
		1000	S	S	S	S	S	S	S	S	S	S
β-lactams	Amoxicillin	200	R	R	R	R	R	S	R	R	S	R
		1000	R	R	R	S	S	S	R	S	S	S
	Ampicillin	200	R	R	R	R	R	S	R	R	S	R
		1000	R	R	S	S	S	R	S	S	S	S
Macrolides	Azithromycin	200	R	R	R	R	R	S	S	S	S	S
		1000	R	S	S	R	S	S	S	S	S	S
	Erythromycin	200	R	R	R	R	R	S	S	S	S	S
		1000	R	S	S	S	S	S	S	S	S	S
Tetracyclines	Chlorotetracycline	200	R	S	R	R	R	S	S	S	S	S
		1000	S	S	R	R	R	S	S	S	S	S
	Oxytetracycline	200	R	S	R	R	R	S	S	S	S	S
		1000	S	S	R	R	R	S	S	S	S	S

[a]R: resistance; [b]S: sensitivity

Antibiotic resistance may be a result of horizontal gene transfer or it may be due to unrelated point mutations that may occur in the pathogen genome at a rate of approximately 1 in 108 per chromosomal replication. It is well documented that the genes responsible for the production of penicillinase in most strains is plasmid-borne. Plasmid curing experiments were performed to determine whether the resistance genes are originated from chromosomes or plasmids in these bacteria. The plasmids of a strain were eliminated by growing the bacteria in the presence of, sodium dodecyl sulfate, an anionic, surface-active agent. The sensitivity of p4 and t5 isolates was observed to all eight antibiotics at both tested concentrations used after the elimination of plasmid. These findings suggest that the aminoglycoside, tetracycline, β-lactams, and macrolide, resistance was mediated by plasmid-associated functions. The rest of the isolates (p5, t1, and t9) were sensitive to aminoglycosides, macrolides, and tetracycline's after plasmid elimination; however, they were still found resistant to amoxicillin and ampicillin at a lower concentration of 200 mg·L^{-1}, but not at higher concentration, 1000 mg·L^{-1}. The resistance to β-lactams appeared to be mediated by chromosome-associated function in the above three isolates. Ash et al. [37] sampled gram-negative bacteria from different rivers in the United States and found that over 40% of bacteria, resistant to more than one antibiotic, harbor at least one plasmid, ranging in size from 2 kb to >23 kb. Our results suggest that the antibiotic resistance of these bacteria may be associated with plasmids or chromosomes. However, we did not perform analyses to find the truth of this hypothesis. The mechanism of resistance, observed in our work, could further be clarified by the amplification of plasmid and chromosome sequences and comparing the sequences with the known antibiotic resistance-carrying elements.

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CONCLUSION

Soils are rich reservoirs of a very large and diverse microbial community. Five bacterial isolates that could thrive on penicillin or tetracycline were isolated belonging to the Lysobacter, Variovorax, Pseudomonas, Chitinophaga, and *Bradyrhizobium* genera. High-level multiple antibiotic resistance was observed as a common phenotype among these bacteria both before and after plasmid curing. Antibiotic resistance genes were also found in these bacterial isolates. Integrons were not detected in any of the isolates but copies were detected in the soil, and the antibiotic resistance of these five bacterial isolates perhaps is associated with plasmids or chromosomes. The presence of antibiotic-resistant bacterial strains and genes in soil may provide both hurdles and future prospects for research in antimicrobial drug discovery and microbial ecology.

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REFERENCES

1. Nwosu VC. Antibiotic resistance with particular reference to soil microorganisms. Res Microbiol 2001; 152: 421-430.
2. Palumbo JD, et al. Mutagenesis of beta-1,3-glucanase genes in Lysobacter enzymogenes strain C3 results in reduced biological control activity toward Bipolaris leaf spot of tall fescue and Pythium damping-off of sugar beet. Phytopathology 2005; 95: 701-707.
3. Cytryn E. The soil resistome: The anthropogenic, the native, and the unknown. Soil Biol Biochem 2013; p 63: 18-23.
4. Nelson DL and Cox MM (2008) Lehninger Principles of Biochemistry, 5th Editon, W.H. Freeman, New York.
5. D'Costa VM, et al. Expanding the soil antibiotic resistome: exploring environmental diversity. Curr Opin Microbiol 2007; 10: 481-489.
6. Burgos JM, et al. Presence of multidrug-resistant enteric bacteria in dairy farm topsoil. J Dairy Sci 88: 2005; 1391-1398.
7. Sengupta N, et al. Diversity and antibiotic susceptibility pattern of cultivable anaerobic bacteria from soil and sewage samples of India. Infect Genet Evol 2011; 11: 64-77.
8. Dantas G, et al. Bacteria subsisting on antibiotics. Science 2008; 320: 100-103.
9. Liu B and Pop M. ARDB-Antibiotic Resistance Genes Database. Nucleic Acids Res 2009; 37: D443-D447.
10. Rodriguez-Verdugo A, et al. Evolution of Escherichia coli rifampicin resistance in an antibiotic-free environment during thermal stress. Bmc Evol Biol 2013; 13.
11. Hsu JT, et al. Prevalence of sulfonamide-resistant bacteria, resistance genes and integron-associated horizontal gene transfer in natural water bodies and soils adjacent to a swine feedlot in northern Taiwan. J Hazard Mater 2014; 277: 34-43.
12. Stokes HW and Hall RM. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol Microbiol 1989; 3: 1669-1683.
13. Zhang Q and Dick WA. Growth of soil bacteria, on penicillin and neomycin, not previously exposed to these antibiotics. Sci Total Environ 2014; 493: 445-453.
14. Sparks DL, et al. Methods of Soil Analysis: Part 3-Chemical Methods. Soil Sci Soc Am, Madison, WI 1996.
15. Zhang Z, et al. Chitinases from the plant disease biocontrol agent, Stenotrophomonas maltophilia C3, Phytopathology 1996; 91: 204-211.
16. Muyzer G, et al. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 1993; 59: 695-700.
17. Sonstein SA and Baldwin JN. Loss of the Penicillinase plasmid after treatment of staphylococcus aureus with sodium dodecyl sulphate. J Bacteriol 1972; 109: 262.
18. Pollack AR, et al. Laboratory Exercises in Microbiology, John Wiley & Sons. Inc., New York 2002; p. 32.
19. Folman LB et al. Characterisation of Lysobacter enzymogenes (Christensen and Cook 1978) strain 3.1T8, a powerful antagonist of fungal diseases of cucumber. Microbiol Res 2003; 158: 107-115.
20. Sullivan RF, et al. Taxonomic positioning of two biological control agents for plant diseases as Lysobacter enzymogenes based on phylogenetic analysis of 16S rDNA, fatty acid composition and phenotypic characteristics. J Appl Microbiol 2000; 94: 1079-1086.

21. Kilic-Ekici O and Yuen GY. Induced resistance as a mechanism of biological control by Lysobacter enzymogenes strain C3. Phytopathology 2003; 93: 1103-1110.

22. Kilic-Ekici O and Yuen GY. Comparison of strains of Lysobacter enzymogenes and PGPR for induction of resistance against Bipolaris sorokiniana in tall fescue. Biol Control 2004; 30: 446-455.

23. Jamieson WD, et al. Coordinated surface activities in Variovorax paradoxus EPS. Bmc Microbiol 2009; 9.

24. Elomari M, et al. Pseudomonas monteilii sp. nov, isolated from clinical specimens. Int J Syst Bacteriol 1997; 47: 846-852.

25. Yasir M, et al. Chitinophaga eiseniae sp nov., isolated from vermicompost. Int J Syst Evol Micr 2011; 61: 2373-2378.

26. Wright GD. Antibiotic resistance in the environment: a link to the clinic?. Curr Opin Microbiol 2010; 13: 589-594.

27. Bert F, et al. Identification of PSE and OXA beta-lactamase genes in Pseudomonas aeruginosa using PCR-restriction fragment length polymorphism. J Antimicrob Chemother 2002; 50: 11-18.

28. Cambray G, et al. Integrons. Annu Rev Genet 2010; 44: 141–156.

29. de Toro M, et al. Genetic characterization of the mechanisms of resistance to amoxicillin/clavulanate and third-generation cephalosporins in Salmonella enterica from three Spanish hospitals. Int Microbiol 2011; 14: 173-181.

30. Moura A, et al. Diversity of Gene Cassette Promoters in Class 1 Integrons from Wastewater Environments. Appl Environ Microbiol 2012; 78: 5413-5416.

31. Rapa RA and Labbate M. The function of integron-associated gene cassettes in Vibrio species: the tip of the iceberg. Front Microbiol 2013; 4.

32. Partridge SR, et al. Gene cassettes and cassette arrays in mobile resistance integrons. Fems Microbiol Rev 2009; 33: 757-784.

33. Levesque C, et al. PCR mapping of integrons reveals several novel combinations of resistance genes, Antimicrob. Agents Chemother 1995; 39: 185-191.

34. Alcock RE, et al. Assessment of organic contaminant fate in waste water treatment plants I: Selected compounds and physicochemical properties. Chemosphere 1999; 38: 2247-2262.

35. Levy SB and Marshall B. Antibacterial resistance worldwide: causes, challenges and responses. Nat Med 2004; 10: S122-S129.

36. Alanis AJ. Resistance to antibiotics: Are we in the post-antibiotic era?. Arch Med Res 2005; 36: 697-705.

37. Ash RJ, et al. Antibiotic resistance of gram-negative bacteria in rivers, United States. Emerg Infect Dis 2002; 8: 713-716.