Metagenomic Analysis Revealing Antibiotic Resistance Genes (ARGs) and Their Genetic Compartments in the Tibetan Environment

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ABSTRACT

Comprehensive profiles of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in a minimally impacted environment are essential to understanding the evolution and dissemination of modern antibiotic resistance. Chemical analyses of the samples collected from Tibet demonstrated that the region under investigation was almost devoid of anthropogenic antibiotics. The soils, animal wastes, and sediments were different from each other in terms of bacterial community structures, and in the typical profiles of ARGs and MGEs. Diverse ARGs that encoded resistance to common antibiotics (e.g., beta-lactams, fluoroquinolones, etc.) were found mainly via an efflux mechanism completely distinct from modern antibiotic resistome. In addition, a very small fraction of ARGs in the Tibetan environment were carried by MGEs, indicating the low potential of these ARGs to be transferred among bacteria. In comparison to the ARG profiles in relatively pristine Tibet, contemporary ARGs and MGEs in human-impacted environments have evolved substantially since the broad use of anthropogenic antibiotics.

INTRODUCTION

In the realm of public health in the last century, antibiotics are a story of success. Once-high mortality rates from bacterial infectious diseases plunged. [1] Trust in the efficacy of antibiotics led to their widespread use in human therapies, husbandry, and aquaculture. However, antibiotic resistance is now prevalent in modern environmental and human commensal microbes, [2-4] while infectious diseases are still the second-leading cause of death worldwide. [5] The scientific community has begun to ponder why antibiotic resistance is so common and widespread in modern environments that some antibiotics are no longer effective in some clinical cases, and

treatment options for certain pathogens have become very limited. [6] Collections of microflora that predate the antibiotic age are highly sensitive to antibiotics, while their mobile genetic elements (MGEs) rarely harbor resistance genes. [7,8] In modern environments, antibiotics for anthropogenic use have served as the major selective pressure in the proliferation of antibiotic-resistant bacteria, as well as a driving force for bacteria to evolve and acquire antibiotic resistance. [9]

Antibiotic resistance inherently exists in natural environments. Indeed, a large variety of ARGs are carried by microbes that inhabit various niches where there is no anthropogenic impact, e.g., isolated caves, [10] deep oceans,11 and deep terrestrial subsurface.[12] In fact, microbes can secrete antibiotics as a competitive mode between microbes,[13] and the microbial synthetic pathways of antibiotics have evolved over millions of years.[14] Recently, a marine-derived actinomycete was shown to produce a novel natural antibiotic.[15] Soil bacteria have exhibited the ability to grow on antibiotics as the sole source of carbon.[16] Whether microbes are antibiotic producers or users, they must develop effective resistance mechanisms to protect themselves from antibiotics.[17,18] The genes associated with different antibiotic resistance mechanisms have evolved and been maintained in microbes over a long period of time.[19] Therefore, antibiotic resistance is a natural and ancient phenomenon.

In combination with a massive release of ARGs and ARB from significant sources of pollution into nearby environ- ments,20,21 MGEs (e.g., integrons, plasmids, etc.) facilitate horizontal gene shuttles of ARGs between gene locations or between bacterial hosts under external stress.[22,23] As a result, it could be especially difficult to retrieve the baseline profiles of ARGs and MGEs prior to the antibiotic era from such a complicated situation. Nevertheless, an alternative approach can be to study ARGs in different habitats of the pristine environment, which would provide good

insights into the provenance and evolution of modern resistome. The Tibetan environment is unique, as indigenous bacteria have never or have hardly been exposed to anthropogenic antibiotics and ARGs. The occurrence and prevalence of antibiotic resistance in this region is not yet well documented. Exploring these baseline resistance profiles of nearly intact environments can indicate from what initial state the evolution and development of contemporary antibiotic resistome began. Furthermore, although the role of MGEs in the spread of ARG has been established in human-impacted settings, the situation might be different in a pristine environment.[24] Characterizing MGEs and genetic compartments of ARGs in different environmental habitats of Tibet could be helpful in elucidating the potential transferability of ARGs in the minimally impacted environment. The aim of the present study is to compile comprehensive profiles of ARGs and their genetic locations in the soils, animal wastes, and sediments collected from the pristine Tibetan environment using a state-of-art metagenomic method, and to elucidate the baseline and primitive state of antibiotic resistance with minimal exposure of anthropogenic antibiotics.

MATERIALS AND METHODS

Study Area and Sampling

Study Area and Sampling. Lake Namco (N30°30′-30°35′, E90°16′-91°03′, 4718 m a.s.l.) is situated at the center of the Tibetan Plateau. The lake has a surface area of about 1982 km^2 and a depth exceeding 90 m. [25,26] It is mainly supplied from precipitation and glacier meltwater from the Nyainqen Tanglha Range in the southeast. The water loss is entirely via natural evaporation because the lake is enclosed. The mean values ofthe temperature, pH, dissolved oxygen, and electric con- ductivity of its surface water are approximately 11.43 °C, 9.21, 8.90 mg L^{-1}, and

1851 μS cm^{-1}, respectively. Only a few thousands of people live on the pastures around Lake Namco, and there is little industry in this region. Qiangyong Glacier Lake (N28°53.390″, E90°496′, 4855 m a.s.l.) is located in the southern part of the Tibetan Plateau, in the Indian monsoon climate region, and is comprised of a small lake (area, 0.03 km^2; maximum water depth, 3.5 m) and a large lake (area, 0.1 km^2; maximum water depth, 30 m). [27] It is less than 1 km from the tongue of the glacier. Most of its water comes from the meltwater from the upper part of the Qiangyong glacier. The temperature, pH, and electric conductivity of surface water are on average 6.89 °C, 8.32, and 136.4 μS cm^{-1}, respectively. No people inhabit the area near Qiangyong Glacier Lake are subjected to very little or no anthropogenic impacts.

Seven samples were collected from the Chinese Academy of Sciences' Namco Monitoring and Research Station for Multisphere Interactions (N30°46.44′, E90°59.31′, 4730 m a.s.l.) in August of 2013, including one sediment, two animal feces (yak and sheep), and two soil samples. As a comparison, two sediment samples were collected from Qiangyong Glacier Lake (N28°53.489′, E90°13.582′, 4796 m a.s.l.). Sediment samples were taken using a grab sampler, and the top 5 cm of soil samples were taken using a sampling shovel. Following the collection, all samples were stored in sterilized polyethylene plastic bags. All samples were immediately kept in a refrigerator at −20 °C until DNA was extracted and an analysis for common antibiotics was carried out in the laboratory.

Sewage treatment plants and husbandry are considered typical sources of ARGs pollution. [28,29] In the present study, an active sludge sample was collected from a sewage treatment plant in Shatin, Hong Kong, in July 2007; [28] and a swine feces sample was taken from a 7−8 month-old pig in a swine feedlot in Zhuhai, China in January 2013. [30] The two samples that represented as typical pollution sources of ARGs due to the use of anthropogenic antibiotics

were also compared with those collected from Tibet.

Analysis of Antibiotics.

Ten commonly used antibiotics, including norfloxacin and ofloxacin (fluoroquinolones), sulfacetamide, sulfadiazine, sulfamethazine, and sulfamethoxazole (sulfonamides), tetracycline (tetracyclines), and clarithromycin, erythromycin and roxithromycin (macrolides) were analyzed in this study. Detailed methods of pretreating and analyzing antibiotics have been described in our previous publica- tions.[21,31] In brief, the extraction and purification of antibiotics in the samples were performed through tandem solid-phase extraction (SPE) on SAX (6 mL, 200 mg, CNW, Germany) and HLB cartridges (6 mL, 500 mg, Waters, U.K.). The final extract was analyzed using an Agilent HP1100 liquid chromatography (Agilent, Palo Alto, CA) along with Applied Biosystems API 4000 tandem mass spectrometry (HPLC MS/ MS). The chromatographic separation of antibiotics was conducted using an Agilent ZORBAX C18 column (2.1 Å ~150 mm, 5 μm particle size). Following the separation, the elution was introduced into a mass spectrometer for the determination of antibiotics.

Potential losses during the analytical procedure were monitored using $^{13}C_3$-caffeine as a surrogate standard. Recoveries of $^{13}C_3$-caffeine from all samples were higher than 75%. Recoveries of target antibiotics from samples were determined at two spiking concentrations (5 and 10 ng/g for samples) with four replicates. The recoveries of macrolides, sulfonamides and tetracyclines were greater than 80%, and the recoveries of fluoroquinolones were approximately 50%. The relative standard deviations (RSDs) for all antibiotics were lower than 10%. The limits of quantification (LOQ) were determined as the minimum detectable concentration of an analyte with a signal-to-noise ratio (S/N) of 10. The LOQs of the target antibiotics in the samples were

135 0.04−4.50 ng/g.

136

137 **DNA Extraction and High-Throughput Sequencing.**

138 DNA was extracted from the samples using the PowerSoil DNA Isolation Kit (MOBIO, CA)
139 according to the manufacturer's instructions. DNA preparation was performed multiple times due
140 to the low content of DNA in some samples, and because combining DNA extractant can
141 minimize possible heterogeneity in the samples and avoid the potential bias arising from the
142 process of extracting a single DNA sample. The purity and concentration of the DNA were
143 measured using a Thermo Scientific NanoDrop1000 Spectrophotometer. DNA from each of the
144 samples was delivered to the Beijing Genomics Institute (BGI) (Shenzhen, China) for sequencing.
145 Approximately 5 μg of DNA was sheared into ~180 bp fragments. Then the overhangs resulting
146 from fragmentation were end-repaired using T4 DNA polymerase, Klenow Fragment and T4
147 Polynucleotide Kinase. After adding an 'A' base to the 3′ end of the blunt DNA fragments,
148 adapters were ligated to the ends of the DNA fragments. The desired fragments were puri*fi*ed
149 using gel-electrophoresis, and selectively enriched and amplified by PCR. The index tags were
150 introduced into the adapter to construct DNA libraries at the PCR stage. The quali*fi*ed DNA
151 libraries were used for sequencing by Illumina HiSeq 2000. The total data output of the 10 samples
152 exceeded 30 Gb. The size of the data for each sample was approximately 3.2 Gb. Raw
153 sequencing data was deposited on the GenBank and MGRAST, and accession number was
154 presented in Table S1 (Supporting Information (SI)).

155

156 **Bioinformatic Analysis**

157 The raw reads (100 bp in length) of each data set were first trimmed to discard low-quality reads

158 that contained ambiguous nucleotides or a quality value of lower than 20 using a customized
159 python script. Trimmed clean reads were overlapped into longer sequence tags according to the
160 following parameters: a length of at least 20 nt of overlapping region was required, and at most
161 two mismatches were allowed. The SILVA Small Subunit (SSU) database (version 10.4) was
162 used to characterize the structure of the bacterial community through a local BLASTN program
163 with an *E*-value cutoff of 10^{-20}. [32,33] The 16S rRNA gene-like sequences from the BLAST
164 results were then assigned to NCBI taxonomies with MEGAN (version 4.70.4) using the Lowest
165 Common Ancestor (LCA) algorithm, where the absolute cutoff was a BLAST bitscore of 50 and
166 the relative cutoff was 10% of the top 50 hits. [34] The BLAST program (Linux Release 2.2.29)
167 was downloaded from the NCBI, and run on the local sever to search target sequences from our
168 sequencing data sets. The local BLASTX program was used to align the trimmed clean tags
169 against an antibiotic resistance genes database (ARDB). [35] A tag was determined to have an
170 ARG-like sequence if the best BLASTX hit had a similarity of higher than 90% and an alignment
171 length of more than 25 amino acids (aa). [28] The local BLASTN program was employed to align
172 our sequencing data against the databases of integrons and plasmids for characterizing these two
173 kinds of MGEs in the samples. An integron database was constructed according to the
174 nucleotide sequences of all integrases available in the INTEGRALL database (1447 integrase
175 genes and 8053 gene cassettes). [36] Meanwhile, a plasmid database was developed on the basis
176 of the plasmid sequences of the NCBI RefSeq database (2465
177 sequences). [28] A tag was annotated as an integron-like sequence if the nucleotide sequence
178 identity of the best BLASTN hit was higher than 90% with an alignment length of at least 50 bp,
179 and the plasmid-like tags in all data sets were determined by the alignments with a nucleotide
180 sequence identity of above 95% over a length of at least 90 bp. [37]

Statistical Analysis

One-way analysis of variance (ANOVA), hierarchical cluster analysis (HCA) and principal component analysis (PCA) were conducted using SPSS for Windows Release 16.0 (SPSS Inc. U.S.). The ANOVA was used to analyze the differences in the abundance and profiles of ARGs and MGEs between samples. Differences at the $p < 0.01$ level (99% con*fi*dence interval) were considered statistically significant. HCA was carried out to cluster samples, where paired group algorithm and Euclidean distance were used. The samples were also characterized and grouped according to relative percentages of resistance types using PCA methods, where all sequencing data sets were used as statistical cases (n = 12) and antibiotic resistance types were used as the variables (n = 15).

RESULT AND DISCUSSION

Bacterial Community Structures in the Soils, Animal Wastes, and Sediments collected from Tibet

The 16S rRNA genes were extracted by sequence alignment against the SSU database. The analysis of bacterial community structures was completed using MEGAN. The results demonstrated that bacteria in the soils, animal wastes, and sediments were assigned to 15, 15, and 19 phyla, respectively. Figure 1A showed that relative abundance of these phyla varied greatly among the soils, animal wastes, and sediments in Tibet. Actinobacteria was highly abundant in the animal wastes, while its relative abundance was lower in the soils than in the animal wastes. In contrast, Proteobacteria was more enriched in the sediments than in other samples. The samples were further characterized using HCA, where relative percentages of bacterial phyla in each of the

204 samples were employed as data input (Figure 1B). In general, the samples were significantly
205 separated and clustered according to the structure of the bacterial community, which was in good
206 accordance with the sample types.
207 The Tibetan Plateau is considered a unique and important permafrost environment. [38] However,
208 the knowledge of bacterial communities in various environmental habitats of Tibet is limited. The
209 454 pyrosequencing-based approaches used in previous studies showed that *Actinobacteria* and
210 *Proteobacteria* phyla were dominant in the permafrost soils of the Tibetan Plateau, which was
211 completely consistent with the findings in the present study. [39,40] Most of the bacterial isolates
212 from the sediments in Tibet were affiliated with *Proteobacteria*. [38] Culture-independent
213 approaches also demonstrated that bacterial 16S rRNA gene clones in the sediments of the same
214 lake were mainly assigned to *Proteobacteria*. [25,41,42] To our knowledge, bacterial communities
215 in Tibetan animal wastes have not yet been explored. This study demonstrated the predominance
216 of Actinobacteria in animal wastes of Tibet, which was consistent with that ruminant feces often
217 harbored diverse symbiotic *Actinobacteria*. [43] The samples collected from Tibet could be
218 characterized and differentiated by typical patterns of composition and the relative abundance of
219 bacterial communities. Lake sediments, soils, and animal wastes were conceptualized as an
220 aquatic habitat, terrestrial habitat, and biological habitat, respectively.
221
222 **Occurrence of Common Antibiotics in Tibet.**
223 Ten common antibiotics belonging to four groups (sulfonamides, tetracyclines, fluoroquinolines,
224 and macrolides) in the soils, animal wastes, and sediments of Tibet were analyzed using HPLC-
225 MS/MS. The target antibiotics were not detectable in any of the samples collected from Tibet. The
226 results suggested that this region under investigation has minimally been exposed to detectable

anthropogenic antibiotics, and that the bacterial inhabitants of this region have also not been suffered from the significant impacts of the wide use of antibiotics.

Abundance and Resistance Types of ARGs in Tibet

The ARGs found in the soil, animal waste, and sediment samples of Tibet were sorted according to the types of antibiotics toward which they exhibited resistance (viz. resistance type), as shown in Figure 2A and Figure S1 (SI). In order to eliminate the bias caused by sequencing depth, the number of ARG-like tags in each of the samples was normalized to the total number of tags of the same sample. A duplicate analysis of S1, SW, and SD1 demonstrated good repeatability in the metagenomic profiling of ARGs, with a relative standard deviation (RSD) of less than 10%. ARGs were significantly more abundant in the sediments than in the soils or animal wastes ($p < 0.01$), and no significant difference was observed between the soils and animal wastes. The ARGs in the pristine environment of the Tibet were associated with more than 10 groups of antibiotics, including commonly used antibiotics (e.g., aminoglycosides, beta-lactams, fluoroquinolones, macrolides, etc.) and less popular antibiotics (e.g., bacitracin, polymyxin, etc.). A resistance gene specific to bacitracin was detectable in all of the samples, and was highly abundant in the sediments. In contrast, a larger part of ARGs in Tibet conferred simultaneous resistance to multiple antibiotics, and these accounted for at least 80% of the resistance genes related to common antibiotics, such as aminoglycosides, beta-lactams, fluoroquinolones, and macrolides. The ARGs in representative sources of pollution (e.g., a sewage treatment plant and swine feedlot) were also analyzed for their differences in ARG profiles from relatively pristine Tibet environment. Different from the ARGs in Tibet, the majority of the ARGs in the active sludge and swine feces samples were more specific to a single antibiotic (e.g., aminoglycosides, chloramphenicols, macrolides,

tetracyclines, etc.). In particular, the total abundance of ARG in the swine feces samples was at least 30 times higher than those in the animal waste samples from Tibet.

The existence of ARGs in environmental habitats subject to minimal anthropogenic impacts supports the view that antibiotic resistance originated naturally before the modern age of antibiotics. [10,12,19,44] Nevertheless, whole profiles of antibiotic resistome in the natural environment were seldom delineated, probably due to the enormous variety of ARGs. With rapid development of high throughput sequencing techniques, functional metagenomics was beneficial to the finding of novel ARGs in the environment, [45] and sequencing- based descriptive metagenomics used in this study was more employed to profile well-recognized ARGs in various samples. [30] The antibiotic resistance profile of Tibet showed that ARGs in pristine environments were frequently connected with the majority of the antibiotics currently in use or out of use, as observed in the South China Sea. [44] Furthermore, environ- mental microflora in the Tibetan environment tend to develop antibiotic resistance in an economic way, with most of the ARGs not restricted to resistance to a single antibiotic. It could not be denied that enzyme-mediated resistance to a single antibiotic (e.g., beta-lactams and macorlides) was found for culturable bacterial strains from isolated caves and the deep oceans. [10,11] However, resistance mechanisms specific to multiple antibiotics are dominant and abundant in the pristine environment.44 Assuming that energy is limited in unique environment, it is reasonable for microbes to select the most efficient mode of coping with various environmental stresses in terms of both maintenance cost and functional versatility. [46,47]

Figure 2B shows *beta*-lactam resistance gene subtypes in the Tibetan samples and in the representative sources of ARG pollution (e.g., swine feces and active sludge). Six subtypes related to *beta*-lactam resistance were identified in Tibet, with a preponderance of *mex*B and *acr*B.

The resistance to *beta*-lactam might be achieved mainly via an e*ffl*ux mechanism conferred by *acr*B, *mex*B, and *tol*C. In comparison to the Tibet samples, the lactamase-mediated inactivation mechanism was more prevalent than other mechanisms in the typical sources of ARG pollution. Almost 99% of the *beta*-lactam resistance in the swine feces was achieved via five subtypes of lactamases. Eight subtypes of lactamases in the active sludge accounted for approximately 54% of the resistance to *beta*-lactam.

Exploring baseline profiles of ARGs in the pristine environment could provide the clues to at what primitive state the evolution and development of contemporary antibiotic resistome began. Comparative studies have suggested that ARGs in the human-impacted environments have been diversifying in terms of both gene sequences and resistance mechanisms, as shown in Figure S2 (SI) . Therefore, microbes are surviving under much harsher conditions than in the preantibiotic era because a large amount of anthropogenic antibiotics are released into the ambient environment. [44, 48] For example, a significant fraction of lactamase-encoded genes in the ARDB database (a total of 259 subtypes) are the dominant resistance mechanism to anthropogenic *beta*-lactams. [28] Similarly, the sulfonamide and tetracycline resistance genes that were prevalent in typical sources of ARG pollution were seldom found in relatively pristine Tibet. [44] Although ARG profiles have changed substantially since the broad use of anthropogenic antibiotics, the efflux pump as a common way of clearing intracellular toxins is still important because of the essential role it plays in bacterial resistance to antibiotics [48, 50] as well as to pollutants with antimicrobial activity. [51-53]

Comparison of ARG Profiles among Different Environmental Habitats of Tibet

No significant differences in the total number of genotypes, sequence diversities, and resistance

296 mechanisms were observed among the three environmental habitats in Tibet (Figure S2 in the SI).
297 However, about 60% of the ARG subtypes that were identified in Tibet (a total of 47) were present
298 only in a single environmental habitat (Figure 3A). Thirteen subtypes of ARGs were concurrently
299 detected in the soils, animal wastes, and sediments of Tibet. Here, four subtypes (*bac*A, *mex*B,
300 *mex*F, and *mex*W) were the most popular and were predominant over other subtypes, which were
301 linked to different types of resistance. The protein product of the *bac*A gene can bypass the
302 inhibition of the isoprenyl pyrophosphate dephosphorylation caused by bacitracin (polypeptide).
303 [54] Interestingly, in spite of substantial differences in essential properties (e.g., salinity) between
304 Lake Namco of Tibet and the SCS, the *bac*A gene was prevalent and dominant in the sediments
305 of both pristine aquatic environ- ments. [44] A great difference in ARG subtype was also observed
306 between the samples of Tibet and typical sources of ARG pollution. More than 80% of ARG
307 subtypes identified in the SF and AS were not found in the samples of Tibet. It suggests that
308 antibiotic resistome has been substantially changed since anthropogenic antibiotics are widely
309 used.
310 Furthermore, the relationships between the samples were further explored using a PCA approach
311 according to the relative abundance of resistance types (Figure 3B). The first two principal
312 components accounted for approximately 66.8% of the total variance in antibiotic resistance types
313 among all samples. The samples collected from representative sources of ARG pollution were
314 significantly separated from those from the relatively pristine Tibetan environment. The samples
315 from the same environmental habitats were usually grouped together due to the similarity of their
316 antibiotic resistance profiles.
317 The ARGs of different environmental habitats in Tibet evolved in the absence of substantial
318 anthropogenic impacts. Natural gene flows, in the form of both bacterial hosts and/or gene material,

undoubtedly exist between different environ- mental habitats. They probably contributed to the copresence of some ARG subtypes in all of the samples from different habitats. However, bacterial communities residing in different environmental habitats in Tibet also maintained their respective traits of antibiotic resistance profiles. Different from the Tibetan environment, natural gene flows between different habitats in the human-impacted environments were always considerably disturbed by intensive human activities. Composting and fertilizing operations accelerated the flows of ARGs from animal intestines to the soils. [29,55] Huge amounts of ARGs and ARB were released from urban settings to nearby aquatic environments via the discharges of sewage treatment plants. [28,56]

As a consequence of anthropogenic impacts, accelerating gene flows could diminish differences among the antibiotic resistance profiles of various environmental habitats; thus, it is almost impossible to retrieve original resistance pro*fi*le of each of habitats in a highly human-impacted environment.

Occurrence and Abundance of MGEs in Tibet

Sequencing results were aligned against the databases of integrons and plasmids to investigate their occurrence and abundance in Tibet (Figure 4A). A large variety of integrons and plasmids were found in Tibet, but there were no great variations in total abundance among different sample types. The total quantity of plasmid-like tags was substantially greater than that of integron- ike ones. A large number of integron-like tags were annotated as various recombinase-encoded genes (e.g., *int*I, *tni*, etc.). The relative abundance of recombinase- related tags over the total number of integron-like tags was considerably higher in the soils and sediments (e.g., 22.5% and 33.8%) than in the animal wastes (e.g., 6.1%). The plasmids that were concurrently found in all

environmental habitats accounted for about 75% of the total plasmid-like tags in the Tibetan samples. Approximately 70% of integrons were shared by all samples of Tibet. The abundance of both plasmids and integrons in representative pollution sources (SW and AS) was significantly higher than those in the samples collected from Tibet ($p < 0.01$). Moreover, the plasmids and integrons coexisting in all samples of Tibet only took up less than 36% of those in the SW and AS.

A heat map of the abundance of the top nine major plasmids demonstrated that distinct patterns were observed among different environmental habitats in Tibet (Figure 4B). Samples were classified according to the trait of the plasmid genome, which was in good agreement with different environmental habitats. Representative profiles of the plasmid genome in the soils, animal wastes, and sediments of Tibet are shown in Figure S3 of the SI. The pNDAS01 and pMRAD01 plasmids were predominant in the soils, whereas the pGMI1000MP plasmid and CH34 megaplasmid were preponderant in the sediments. The fact that whole profiles of MGEs were present in the pristine environments has rarely been explored, as well as the exact factors that led to the formation of typical profiles of MGEs in different environmental habitats. [57] However, bacterial communities often bene*fi*t from MGE-carrying genes function- ing in harsh conditions. [58, 59] Consequently, the distribution patterns of these MGE-carrying genes among bacteria hosts became an important in*fl*uencing factor to the composition and structure of bacterial community, vice versa.

Horizontal Transferability of ARGs in Tibet

In order to evaluate the potential for ARGs to be transferred among bacteria in Tibet, ARG-like tags were extracted from all sequencing data sets and aligned against the databases of

integrons and plasmids. The results demonstrated that a tiny fraction of ARG-like tags in Tibet were also assigned to the integrons and plasmids. Three tags of ARGs (e.g., *aad*A, *sul*I, and *tet*G) were identified in the gene cassettes of the integrons, and approximately 2.0% of all ARG-like tags in all samples were carried by the plasmids (Figure 5). In case of plasmid-carrying ARGs being missed because of inadequate sequencing depth, the full-length sequences of the plasmids that had been concurrently identified in all of the Tibet samples were extracted from the plasmid database, and then were aligned against the ARDB database. These plasmids only carried complete sequences of *mex*B and *mex*C. By contrast, relative percentages of MEGs-related ARGs in the SF and AS were significantly higher than those in the samples from Tibet ($p < 0.01$). For instance, approximately half of the ARGs in the AS were potentially associated with the plasmids.

ARGs are disseminated in the human-impacted environments mainly via promiscuous gene transfers between genetic locations or between bacterial hosts under elevated antibiotic stresses, *viz*., horizontal gene transfers. [22,23] Such transfers of ARGs required MGEs as the carriers. As a result, significant relationships between ARGs and MGEs in terms of diversity and abundance were frequently observed in different environ- mental niches. [44,60,61] Integrons consist of a site-specific recombinase gene and a shuffled gene cassette. Class I integrons are ubiquitous in environmental microflora, [62-65] as was detectable in all samples from Tibet. It has been highlighted that gene cassettes in human-impacted settings are recombinant with diverse and abundant ARGs responding to anthropogenic antibiotics. [66,67] yet this is not the case in pristine environments. The present study showed that ARG-like genes were sparse in the integron genomes. As a MGE with larger size, the plasmids identified in the Tibetan samples were also quite devoid of ARGs. An increasing number of studies have demonstrated that ARB in the niches unaffected by human activities probably possess a wide array of clinically associated and

388 novel ARGs. [68] Nevertheless, transformation assays often showed that antibiotic resistance of
389 these ARB was not transferable to susceptible *Escherichia coli*. [69] Our results revealed that
390 the MGEs common found in the Tibetan environment lack ARGs, indicating that most of the
391 ARGs in Tibet could have a low potential to transfer between bacterial hosts. A substantial
392 number of potentially MGEs-carrying ARGs in the swine feces and active sludge could be the
393 consequence of the horizontal transfer of genes from the heavy use of anthropogenic antibiotics,
394 and would facilitate the continual transfer of ARGs between bacterial hosts.
395
396 **Environmental Implications**
397 Diverse ARGs originating from natural environments were found in the remote Tibetan
398 environment. The whole profiles of ARGs in Tibet differed significantly from those of
399 representative ARG pollution sources in terms of both major subtypes and resistance
400 mechanisms. It suggests that the modern resistome has evolved significantly from the preantibiotic
401 age or minimally human- impacted environment since bacterial communities in the human-
402 impacted environment endure a wide occurrence of anthropogenic antibiotics. ARGs found in the
403 Tibetan environment are seldom harbored in the MEGs, which implies that ARGs can hardly be
404 transferred horizontally between bacterial hosts in the absence of antibiotic stress in relatively
405 pristine environments. In the human-impacted settings, MGEs enriched with diverse ARGs can
406 greatly facilitate the acquisition of antibiotic resistance by bacteria.
407
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417

418 **ASSOCIATED CONTENT**

419 The Supporting Information is available free of charge on the ACS Publications website at DOI:
420 10.1021/acs.est.6b00619.

421

422 **LIST OF FIGURES**

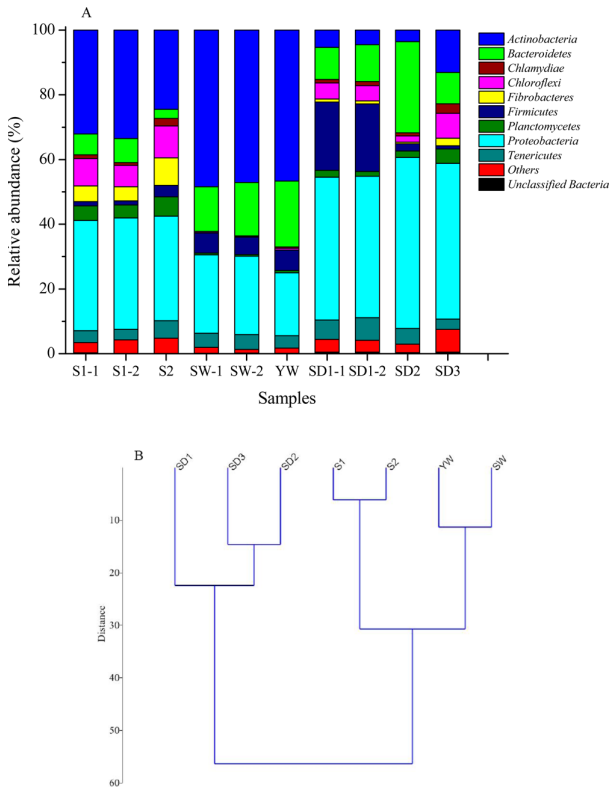


Figure 1. Bacterial community structures at the phylum level in Tibet (A) and a sample classification using hierarchical cluster analysis (B). The S, SW, YW, and SD represent soils, sheep waste, yak waste, and sediments, respectively. The number (−1 or −2) at the end of sample identities indicates that a duplicate sequencing analysis was performed for these samples. “Others” indicates the sum of the relative percentages of phyla that were lower than 2% in each sample.

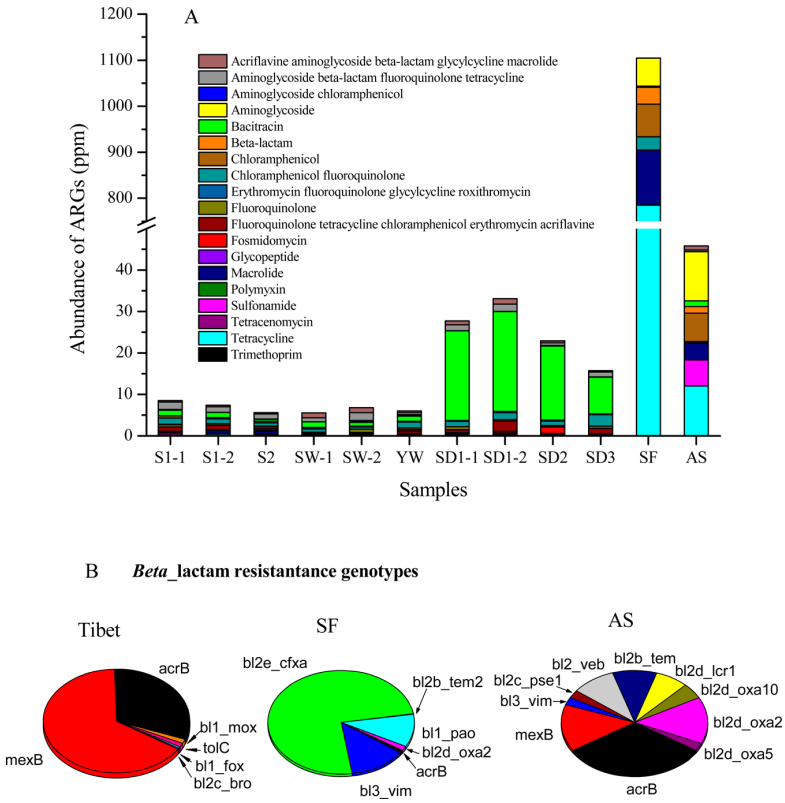
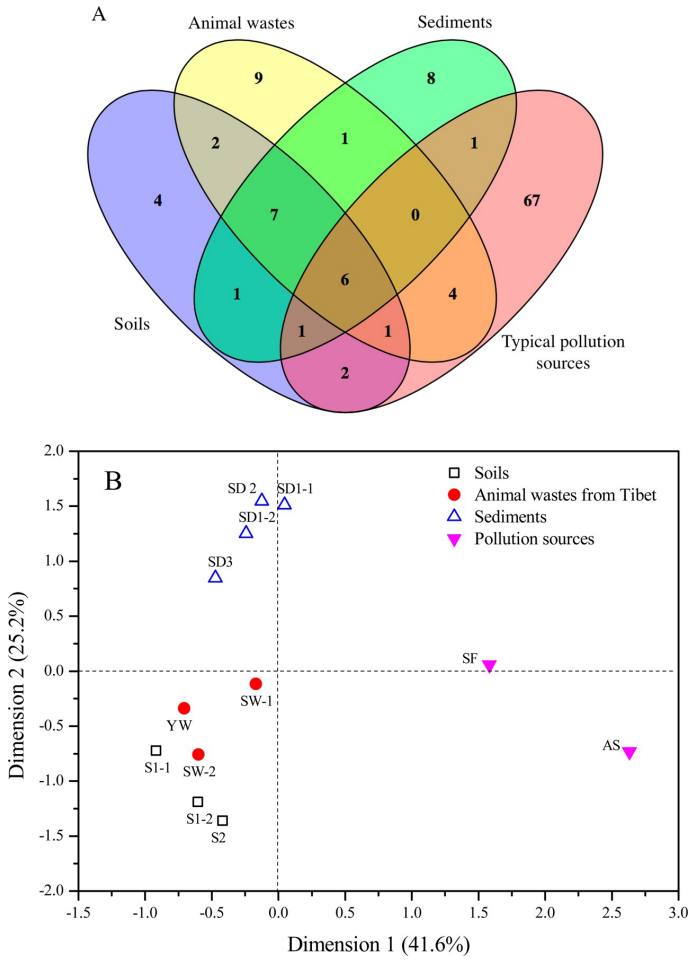


Figure 2. Comparisons of ARG abundance and resistance types (A) and the beta-lactam resistance gene profile (B) among samples of Tibet and representative sources of ARG pollution. Abundance of ARGs was normalized to the size of the sequencing data, and "ppm" refers to one ARGs-like tag in one million metagenomic sequencing tags. SF and AS represent swine feces and active sludge, respectively. Other sample identities are the same as those in Figure 1. In Figure 2B, subtypes represent the types of antibiotic resistance genes described in the ARDB database.



438

439 Figure 3. Venn diagram of antibiotic resistance subtypes (A) and PCA analysis of ARG profiles
440 (B) in different environmental habitats of Tibet and representative sources of pollution. Number
441 in each of the color blocks indicates the number of subtypes of ARGs that could be identified in
442 single or multiple types of samples. Relative percentages of resistance types in the samples were
443 used for the PCA analysis. Typical pollution sources represent swine feces and active sludge that
444 were collected from husbandry operations and sewage treatment plants, respectively. The sample
445 identities are the same as those in Figure 1 and 2

446

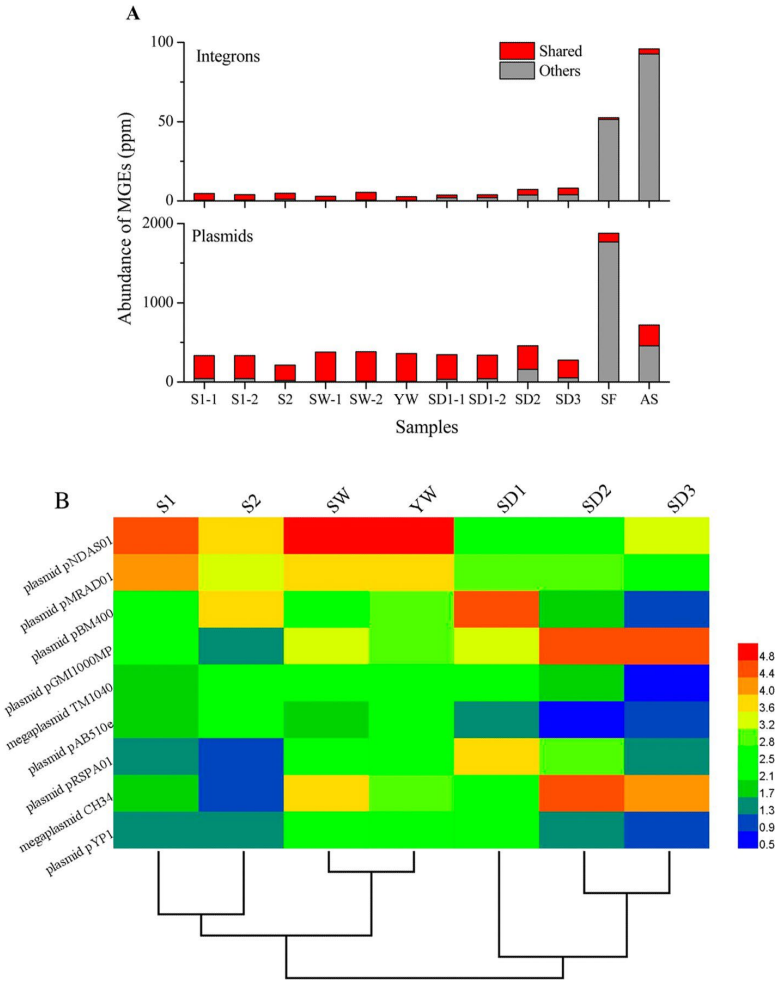


Figure 4. Abundance of MGEs in the soils, animal wastes, and sediments of Tibet and representative sources of pollution (A) and heat map of plasmids in the samples of Tibet (B). Sample identities are the same as those in Figure 1. "Shared" represents the MGEs that were found in all three types of samples, and "Others" refers to the MGEs that were found in only one or two types of samples. The abundance of plasmid-like tags in each of the samples was used for the HCA analysis. The natural logarithm of the number of plasmid-like tags was used to depict the heat map. The accession number of the plasmids in the NCBI was NC_014621.1 (plasmid pYP1), NC_007974.2 (CH34 megaplasmid), NC_009429.1 (plasmid pRSPA01), NC_013859.1 (plasmid pAB510e), NC_008043.1 (TM1040 megaplasmid), NC_003296.1 (plasmid pGMI1000MP), NC_004604.2 (plasmid pBM400), NC_010510.1 (plasmid pMRAD01), and NC_014211.1 (plasmid pNDAS01)

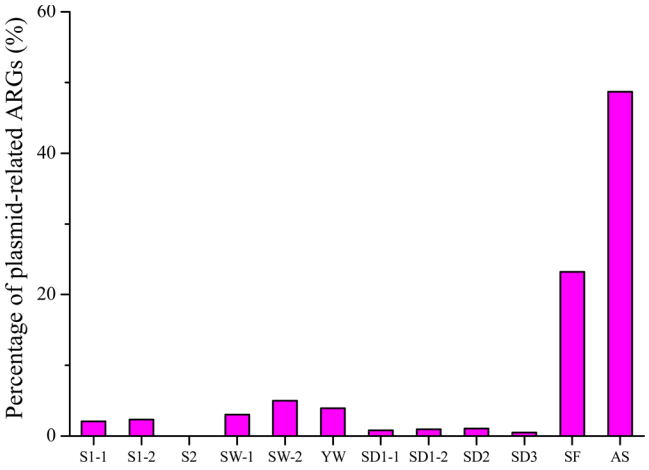


Figure 5. Relative percentages of ARGs potential related to plasmids over the total ARGs in the Tibetan samples and representative sources of ARG pollution.

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