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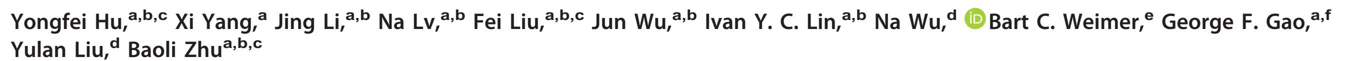

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ABSTRACT

Horizontally acquired antibiotic resistance genes (ARGs) in bacteria are highly mobile and have been ranked as principal risk resistance determinants. However, the transfer network of the mobile resistome and the forces driving mobile ARG transfer are largely unknown. Here, we present the whole profile of the mobile resistome in 23,425 bacterial genomes and explore the effects of phylogeny and ecology on the recent transfer (≥99% nucleotide identity) of mobile ARGs. We found that mobile ARGs are mainly present in four bacterial phyla and are significantly enriched in *Proteobacteria*. The recent mobile ARG transfer network, which comprises 703 bacterial species and 16,859 species pairs, is shaped by the bacterial phylogeny, while an ecological barrier also exists, especially when interrogating bacteria colonizing different human body sites. Phylogeny is still a driving force for the transfer of mobile ARGs between farm animals and the human gut, and, interestingly, the mobile ARGs that are shared between the human and animal gut microbiomes are also harbored by diverse human pathogens. Taking these results together, we suggest that phylogeny and ecology are complementary in shaping the bacterial mobile resistome and exert synergistic effects on the development of antibiotic resistance in human pathogens.

IMPORTANCE

The development of antibiotic resistance threatens our modern medical achievements. The dissemination of antibiotic resistance can be largely attributed to the transfer of bacterial mobile antibiotic resistance genes (ARGs). Revealing the transfer network of these genes in bacteria and the forces driving the gene flow is of great importance for controlling and predicting the emergence of antibiotic resistance in the clinic. Here, by analyzing tens of thousands of bacterial genomes and millions of human and animal gut bacterial genes, we reveal that the transfer of mobile ARGs is mainly controlled by bacterial phylogeny but under ecological constraints. We also found that dozens of ARGs are transferred between the human and animal gut and human pathogens. This work demonstrates the whole profile of mobile ARGs and their transfer network in bacteria and provides further insight into the evolution and spread of antibiotic resistance in nature.

Antibiotic resistance is a growing global threat to human public health, and the continuing emergence of drug-resistant bacteria has led to the potential for a postantibiotic era (1). Bacteria have evolved a dynamic array of antimicrobial resistance mechanisms to withstand antibiotic attacks, such as altering drug target(s), synthesizing drug-inactivating enzymes, changing cell membrane permeability, and exporting drugs via efflux pumps (2). A large number of antibiotic resistance genes (ARGs) involved in these resistance mechanisms have been discovered and are collected in various ARG databases, including ARG-ANOTT (3), CARD (4), ResFinder (5), and ARDB (6). Because antibiotic resistance can be endogenous or exogenous, ARGs are generally classified as intrinsic (chromosomally encoded in specific species or genera) and acquired (via specific point mutations or horizontal gene transfer [HGT]) ARGs (7). ResFinder is a repository of ARGs that covers only the horizontally acquired ARGs but not the resistance caused by mutations, according to public sources and published papers (5).

ARGs, including the cryptic resistance genes in bacteria, have been collectively referred to as the antibiotic resistome (8, 9), and, accordingly, intrinsic and horizontally acquired ARGs have been named the intrinsic resistome and the mobile resistome, respectively (10, 11). In recent years, the antibiotic resistome from both environmental and host-associated microbiomes has been explored (either function- or sequence-based), leading to the discovery of numerous ARGs (8, 12–15). However, the risk of these identified ARGs has been considered to be overestimated because most studies have failed to differentiate between intrinsic ARGs and mobile ARGs (16). The mobile ARGs that are frequently as-

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sociated with mobile genetic elements (MGEs) and disseminated by HGT have been considered to have the highest risk of developing resistance among the antibiotic resistome (16).

HGT is considered a very important mechanism that contributes to bacterial genome mutability and evolution (17, 18). Revealing associated factors, such as transfer barriers of HGT, has been a continued research interest because significant adaptive traits of bacteria such as virulence, beneficial metabolic properties, and antibiotic resistance can be rapidly disseminated through HGT. Phylogenetic, ecological, and functional barriers have all been highlighted to exert an influence on HGT (19). Regarding ARGs, bacterial phylogeny (20) and ecology (21, 22) both have been shown to be driving forces that shape the antibiotic resistome. Unfortunately, these studies targeted all ARGs but did not focus on the mobile ones. An in-depth understanding of the trends and barriers for HGT of the mobile resistome is undoubtedly essential for predicting the emergence of antibiotic resistance in human pathogens.

To provide a comprehensive view of the mobile resistome in bacteria and elucidate the forces shaping ARG transfer, we examined 23,425 bacterial genomes (including 2,768 complete genomes and 20,657 draft genomes available in August 2014 in GenBank) for known mobile ARGs and analyzed their recent HGT network and the roles of phylogeny and ecology in the transfer of the mobile resistome. We also sequenced the gut microbiome of three representative farm animals and compared the mobile ARGs we identified with those in the recently released human gut gene catalog (approximately 9.8 million genes) (23) to investigate the transfer of mobile ARGs at the bacterial community level.

MATERIALS AND METHODS

Data sets. A total of 23,425 bacterial genomes, including 2,768 complete and 20,657 draft genomes (as of August 2014) and their taxonomic affiliations were downloaded from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) GenBank. Concurrently, 1,680 mobile ARGs, including 88 *van* genes belonging to 19 vancomycin resistance operons with a total of 2,134 nucleotide sequences, were retrieved from the ResFinder database (5). Metadata defining the "Ecosystem Category" and "Disease" status, among others, of the strains were obtained from the Integrated Microbial Genomes (IMG) database (http://img.jgi.doe.gov). A data set consisting of the mobility genes of 180,787 integrase/recombinase and transposase was generated by searching the Universal Protein Resource (UniProt) (24) Uniref90 database using corresponding keywords; the data set was subsequently used to search the flanking regions of the mobile ARGs, which we termed the adjacent mobility genes. The human gut microbiome integrated gene catalog (IGC) (9,879,896 genes) and the separate European gene catalog (EGC) (8,096,991 genes), Chinese gene catalog (CGC) (3,547,396 genes), and American gene catalog (AGC) (2,681,342 genes), as well as the Gene Taxonomic Assignment Profiles, were downloaded from the GigaScience Database (http://dx.doi.org/10.5524/100064). The 889 reference bacterial genomes of different human body sites (airways, blood, gastrointestinal tract, oral cavity, skin, and urogenital tract) from the Human Microbiome Project (HMP) were downloaded from the Data Analysis and Coordination Center (DACC) (http://hmpdacc.org). The three animal gut gene data sets were generated as described below.

Searching for mobile ARGs and evaluating ARG diversity. To search for mobile ARGs, the bacterial genomes and the animal and human gut microbiome gene sets were subjected to BLAST using the mobile ARGs in the ResFinder database (5). To guarantee that the ARGs we annotated were accurate and homologous, we used a relatively strict cutoff value of ≥95% nucleotide identity and ≥90% coverage for the BLAST search, a criterion that has also been adopted to establish gene catalogues such as those for the human gut microbiome (23, 25). The Mann-Whitney U test was used for statistical comparisons of the median number of mobile ARGs contained in species from different phyla.

For the vancomycin resistance Van operons consisting of more than one structural gene, for example, the *vanB* operon consisting of the structural genes $vanA_B$, $vanH_B$, $vanR_B$, $vanS_B$, $vanW_B$, $vanX_B$, and $vanY_B$, only if at least three-fourths of the structural genes were presented simultaneously, was it deemed to be a real mobile *van* operon, and the operon was regarded as a single gene when the mobile ARG number was counted and in the subsequent analyses.

To evaluate the diversity of mobile ARGs in individual bacterial species, a rarefaction curve plotting the observed number of mobile ARGs against the number of genomes in each species was generated. Only those species with more than 50 genome sequences and ≥10 genomes containing mobile ARGs were used to generate the curve.

Analysis of the ARG cluster and the adjacent mobility genes. To determine which mobile ARGs were adjacent to each other in a specific region of a genome and to what extent they were associated with MGEs, we searched the flanking regions of each mobile ARG for adjacent ARGs or mobility genes such as integrase/recombinase and/or transposase. First, we selected fragments containing the mobile ARGs and both their upstream and downstream 5-kb flanking regions in each bacterial genome; then, if an ARG was adjacent to another ARG (≤5 kb), the overlapping regions were merged to generate a longer fragment harboring the two mobile ARGs, the two ARGs were considered to be clustered together (ARG cluster containing 2 mobile ARGs), and this process was repeated. The mobility genes were found with protein homology of ≥70% protein identity using the long fragments that contained clustered ARGs. If observed within this search parameter, they were considered to be adjacent to mobile ARGs.

Determination of the recent HGT of the mobile ARGs and the HGT frequency. A stringent cutoff of ≥99% nucleotide identity was used to determine the recent HGT of the mobile ARGs between species pairs (20, 21). That is, if a mobile ARG was shared between two species with ≥99% nucleotide identity, the ARG was considered to be a recent HGT. The HGT number of a species was defined as the number of ARGs shared between this species and all others. To calculate the intra- and intertaxon HGT frequencies of the mobile ARGs, the intra- and intertaxon HGT numbers of a species were counted separately, and the intra- and intertaxon HGT frequencies were calculated by dividing the HGT number of a species by the total number of species containing the mobile ARGs within and outside the taxon, respectively. For cross-ecology analysis, the strains were first assigned to different ecological categories according to the isolation source. Subsequently, the intracategory (eco_same) and intercategory (eco_diff) HGT frequencies in the same taxon (taxon_same) and in different taxa (taxon_diff) were calculated separately, generating the HGT frequencies in four combinations: taxon_same and eco_same, taxon_same and eco_diff, taxon_diff and eco_same, and taxon_diff and eco_diff. Cross-body site analysis was done using the intracategory and intercategory HGT frequencies, and results were calculated along the strain's phylogenetic distance with the maximum of 2% 16S rRNA gene sequence divergence. The Mann-Whitney U test was used to determine significant differences between the medians of the HGT frequencies among different groups.

Definition of environments/pathogens. The environment and pathogenicity information for all genomes were obtained from IMG. We generalized the environment information into four categories via the "Ecosystem" and "Ecosystem category" tokens: *env_aqua*, ecosystem "Environment" and ecosystem category "Aquatic"; *env_terr*, ecosystem "Environment" and ecosystem category "Terrestrial"; *host_homo*, ecosystem "Host-associated" and ecosystem category "Human"; and *host_anim*, ecosystem "Host-associated" and ecosystem category "Animal," "Mammals" or "Birds." The pathogenicity information of genomes was extracted via the "Diseases," "Phenotype," and "Relevance" tokens. If any

FIG 1 Heat map showing the distribution of mobile ARGs in different taxonomic ranks. Inner to outer circles indicate sequentially the phylum, class, order, family, genus, and species. Each bar in the outermost circle represents a species ($n = 790$), and the sizes of the other sectors representing different taxa are scaled according to the number of species assigned to that taxon. Scale bar, gene numbers. Species harboring more than 20 mobile ARGs are shown beside the species bars, and species with more than 40 ARGs are highlighted in bold. The phylogenetic affiliation is based on NCBI taxonomy.

token had a defined "patho-" as a defined keyword, the genome was considered to be pathogenic. The HGT frequencies of the mobile ARGs across phylogenetic hierarchies and across different environments were calculated as described above.

Sequencing animal gut microbiomes. Fresh stool samples from chickens, pigs, and cattle were collected from suburban farms in Beijing, China. The samples were collected and subsequently used for DNA extraction with the PSP Spin Stool DNA Plus kit according to the manufacturer's instructions. Paired-end Illumina sequencing DNA libraries with an insertion length of 350 to 400 bp (4 samples each for chickens and pigs and 5 samples for cattle) were constructed following the manufacturer's instructions (Illumina), followed by sequencing on an Illumina HiSeq 2000 platform to generate approximately 5 G of data for each sample (see Table S1 in the supplemental material). The raw reads were trimmed until three contiguous bases with a quality of $>$20 were found, and all read lengths shorter than 50 bp were discarded.

Sequence assembly and gene prediction. High-quality reads were assembled using the SOAPdenovo program (http://soap.genomics.org.cn) with automatic determination of the K-mer size; the resulting contigs shorter than 200 bp were discarded. Coding sequence (CDS) extraction was performed using MetaGeneAnnotator (26), and only those CDSs of $>$100 bp were retained for further analysis. The CDSs from chickens, pigs, and cattle were pooled, respectively, into nonredundant data sets using CD-HIT as described by Qin et al. (25).

Taxonomic classification of metagenomic sequences. The nonredundant CDS sequences were first aligned with the NCBI nonredundant protein database using NCBI BLAST. The tabular results were analyzed using MEGAN (27) for the assignment of taxonomy. Spearman correlations were used to test the similarity of community structures between gut microbiomes, and Pearson correlations were used to examine the relationship between the similarity of community structures and the number of mobile genes shared between the communities.

Accession number(s). All sequencing data were deposited in the NCBI Sequence Read Archive database under accession number SRP062937.

RESULTS

Mobile ARGs are enriched in *Proteobacteria*. Using a nucleotide identity cutoff value of $\geq$95% for the BLAST search against the ResFinder database, we identified a total of 515 mobile ARGs distributed across 790 species among 6,335 bacterial species (Fig. 1; see also Data Set S1A in the supplemental material). These mobile ARGs were mainly harbored by four bacterial phyla: *Proteobacteria* (399 genes), *Firmicutes* (86 genes), *Bacteroidetes* (46 genes), and *Actinobacteria* (40 genes); a small portion of the ARGs were shared among these phyla (see below). A comparison of the number of mobile ARGs harbored by each bacterial species showed that *Proteobacteria* species were highly enriched with mobile ARGs ($P = 2.9 \times 10^{-4}$, Mann-Whitney U test) compared with all species in the other three phyla. In detail, among 16 species that harbored more than 20 mobile ARGs, 10 species belonged to the phylum *Proteobacteria*, and, most importantly, 7 of these species harboring more than 40 mobile ARGs were all from *Proteobacteria*. In addition, the rarefaction curves showed that the ARG diversity in proteobacterial bacteria such as *Klebsiella pneumoniae* (119 mobile ARGs) and *Pseudomonas aeruginosa* (49 mobile ARGs) were far from saturation (see Fig. S1 in the supplemental material).

Mobile ARGs are frequently associated with MGEs. To determine which mobile ARGs clustered together in a specific region of a genome and to what extent they were associated with MGEs, we

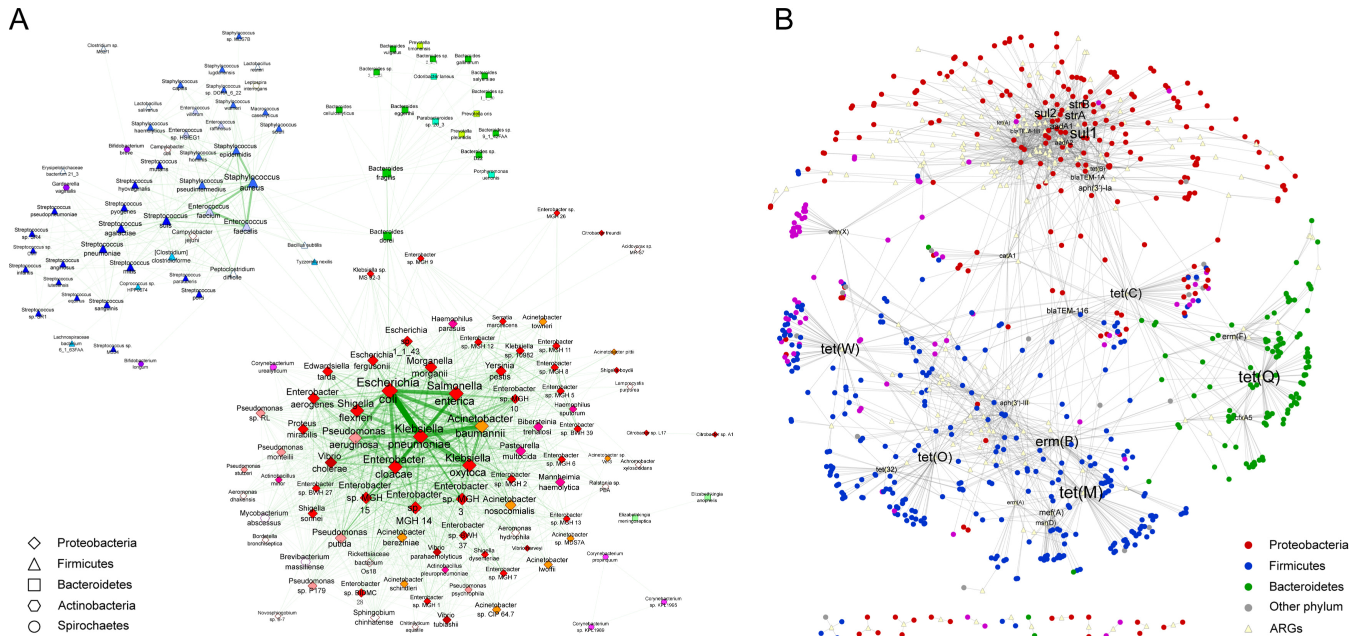


FIG 2 Recent HGT networks of the mobile resistome. (A) The network of species sharing mobile ARGs. Each node represents a species, and the node sizes and the labels are scaled according to the number of mobile ARGs detected in that species. The edge between any two nodes indicates that there are at least 3 shared mobile ARGs (≥99% nucleotide identity) by the species pair, with greater thickness and width of the edge for a larger number of shared mobile ARGs. Nodes of the same color indicate species belonging to the same family, and the same shape denotes the same phylum. The entire profile of this network can be found in Fig. S4A in the supplemental material. (B) The network of mobile ARGs shared between species. Dots and triangles represent species ($n = 703$) and ARGs ($n = 222$), respectively. Edges linking species and ARGs indicate that the ARGs were shared among those species. The sizes of the gene labels are scaled based on the number of species harboring the gene. The names of the ARGs shared among less than 20 species are not labeled.

searched the 5-kb flanking regions of each mobile ARG for adjacent mobile ARGs or integrase/recombinase and/or transposase (see Materials and Methods). We observed that ARG clusters containing 1, 2, and 3 mobile ARGs (19,569, 4,999, and 1,636 occurrences, respectively, in all genomes) accounted for the vast majority of the total ARG clusters, while ARG clusters containing more than 3 mobile ARGs accounted for a small proportion (656 occurrences) (see Fig. S2A in the supplemental material), suggesting that long multigene-containing ARG clusters are not frequently present in bacteria. Furthermore, 67.3% of the total ARG clusters (single gene or multigene) were associated with known mobility-associated genes such as integrase/recombinase and/or transposase, and most ARG clusters coexisted with both integrase/recombinase and transposase genes (see Fig. S2B in the supplemental material). In addition, the occurrence of mobility-associated genes increased with the number of mobile ARGs in the ARG cluster. These results emphasized the contribution of mobility-associated genes to the transfer of mobile ARGs.

ARGs that are clustered within an MGE in a bacterial genome are usually referred to as resistance islands (RIs) (28). We found a total of 656 ARG clusters, presumably potential RIs, each containing at least four different acquired ARGs (data not shown). The largest cluster containing 18 mobile ARGs was located in the largest resistance island known to date, AbaR1 (86-kb), which was discovered in *Acinetobacter baumannii* strain AYE (29); the second largest one harboring 12 ARGs was from the plasmid of a highly invasive and resistant zoonotic pathogen, *Salmonella enterica* serovar Choleraesuis (30); and the third was from *K. pneumoniae* MGH 43 (JCNT01000000). Among all ARG clusters, the sulfonamide resistance gene *sul1*, which is always associated with class 1 integrons, was observed in the neighborhood with the greatest number of different types of ARGs (99 different ARGs). This association reflects not only the widespread presence of class 1 integrons in resistance islands but also their important role in acquiring and disseminating ARGs in bacteria (31).

A recent HGT network of the mobile resistome. To determine recently horizontally transferred ARGs, we compared all 515 of the mobile ARGs obtained from the genomes using a cutoff of ≥99% nucleotide identity (20, 21), and an HGT network of these mobile ARGs at the species level (703 nodes, 16,859 species pairs) was generated (see Fig. S3A in the supplemental material). In general, the recent exchanges of the mobile ARGs were mainly detected in *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, and the exchanges were more active in *Proteobacteria* (see Fig. S3B in the supplemental material). We found a total of 11 species that each shared at least one mobile ARG with more than 200 other species. The top three species displaying the highest numbers of connections were *E. coli*, *Bacteroides fragilis*, and *Staphylococcus aureus*, which shared ARGs with 302, 266, and 260 species, respectively. Moreover, among 16,859 species pairs, 1,376 shared at least three different mobile ARGs (Fig. 2A). *E. coli* and *K. pneumoniae* shared the largest number of mobile ARGs (60 individual genes), followed by *E. coli* and *S. enterica* (38 genes), *A. baumannii* and *K. pneumoniae* (38 genes), and *Klebsiella oxytoca* and *K. pneumoniae* (36 genes) (see Data Set S1B in the supplemental material).

Next, we added to the network all 222 mobile ARGs that were transferred mutually between at least two different species (Fig. 2B). The tetracycline resistance genes, *tet*(M) and *tet*(Q), and the integron-associated sulfonamide resistance gene, *sul1*, were the

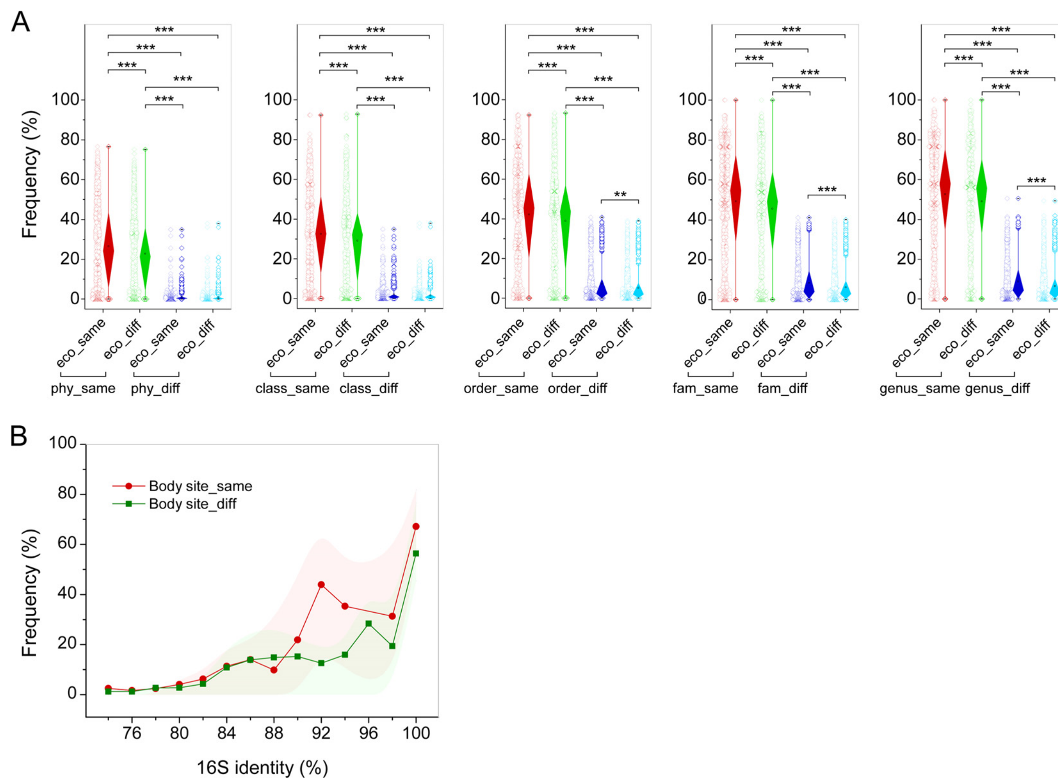


FIG 3 The HGT frequency of the mobile ARGs across phylogenetic hierarchies and ecologies. (A) Intra- and intertaxon HGT frequency of the mobile ARGs across human, animal, aquatic, and terrestrial environments. The phylogenetic affiliation was based on NCBI taxonomy. Statistical analysis was performed using the Mann-Whitney U test: **, $P \leq 0.01$; ***, $P \leq 0.001$. From left to right: phylum, class, order, family, and genus. (B) The 16S rRNA gene distance-based HGT frequency of the mobile ARGs across bacterial communities of different human body sites. The HGT frequency in panel B is calculated in bins of 2% 16S rRNA gene sequence divergence; the data shown are mean values, and light shading denotes the interquartile range (IQR) between the first and third quartiles. See Materials and Methods for the detailed calculation process.

top three widely transferred mobile ARGs at the species level (see Data Set S2 in the supplemental material), while *tet*(C), *tet*(W), and *sul1* were the top three at the genus level. Notably, a total of 37 mobile ARGs had been found transferred, even between different phyla. The exchanges of *tet*(C) and $bla_{\text{TEM-116}}$ genes spanned seven and six different phyla, respectively, and *aph*(3′)*-III*, *catA1*, and *erm*(B) were all transferred across five different phyla. These results revealed that mobile ARGs are frequently transferred between not only closely related bacteria but also phylogenetically distant species. However, a preferred exchange pattern of the recently exchanged mobile ARGs was found along the bacterial lineage (Fig. 2B). For examples, *tet*(Q), *erm*(F), and *cfxA5* were transferred solely within *Bacteroidetes*, *tet*(M), *tet*(O), and *erm*(B) were mainly transferred in *Firmicutes*, and *sul1*, *sul2*, *strA*, and *strB* were mainly transferred in *Proteobacteria*, suggesting that there is a potential phylogenetic barrier for HGT of the mobile resistome.

The recent HGT network of mobile ARGs is mainly shaped by bacterial phylogeny. To test the phylogenetic barrier prediction, we computed the HGT frequency of the mobile ARGs at different phylogenetic hierarchies (see Materials and Methods). As expected, the intrataxon HGT frequency of the mobile ARGs was significantly higher than the intertaxon HGT frequency at each phylogenetic level (see Fig. S4 in the supplemental material), which suggests the presence of a strong phylogenetic barrier. We further explored the ecological impact on mobile ARG transfer by comparing the HGT frequency across ecologies (human, animal, aquatic, and terrestrial) and across phylogenetic hierarchies. We found that at each phylogenetic level, the HGT frequencies of the mobile ARGs between strains with the same phylogenetic taxa (whether eco_same or eco_diff) were significantly higher than those with different phylogenetic taxa (whether eco_same or eco_diff); in contrast, the HGT frequencies in strains from the same ecology were only slightly higher than those from different ecologies (e.g., mean values of 26.4% versus 22.8% at the phylum level) (Fig. 3A). To avoid bias introduced by summarizing the ecological affiliation of bacteria according to the general descriptions of bacterial sources in the database, we next analyzed the HMP data set comprising 889 reference bacterial genomes with defined sources of different human body sites. We also analyzed the HGT frequency over a range of bacterial phylogenetic distances for these genomes. We found that phylogeny played a leading role in the recent ARG transfer; that is, the closer the 16S distance of the strains, the more frequently the transfer occurred (Fig. 3B). However, the HGT frequency was observably higher between strains from the same body site than for those from different body sites, especially when the strains reached approximately more than 90% 16S identity (Fig. 3B). Taking our results together, we suggest that bacterial phylogeny dominates the recent HGT network of the mobile resistome, but existing ecological barriers also supply a contribution.

Transfer of mobile ARGs between human and animal gut microbiomes. We compared the occurrence of mobile ARG

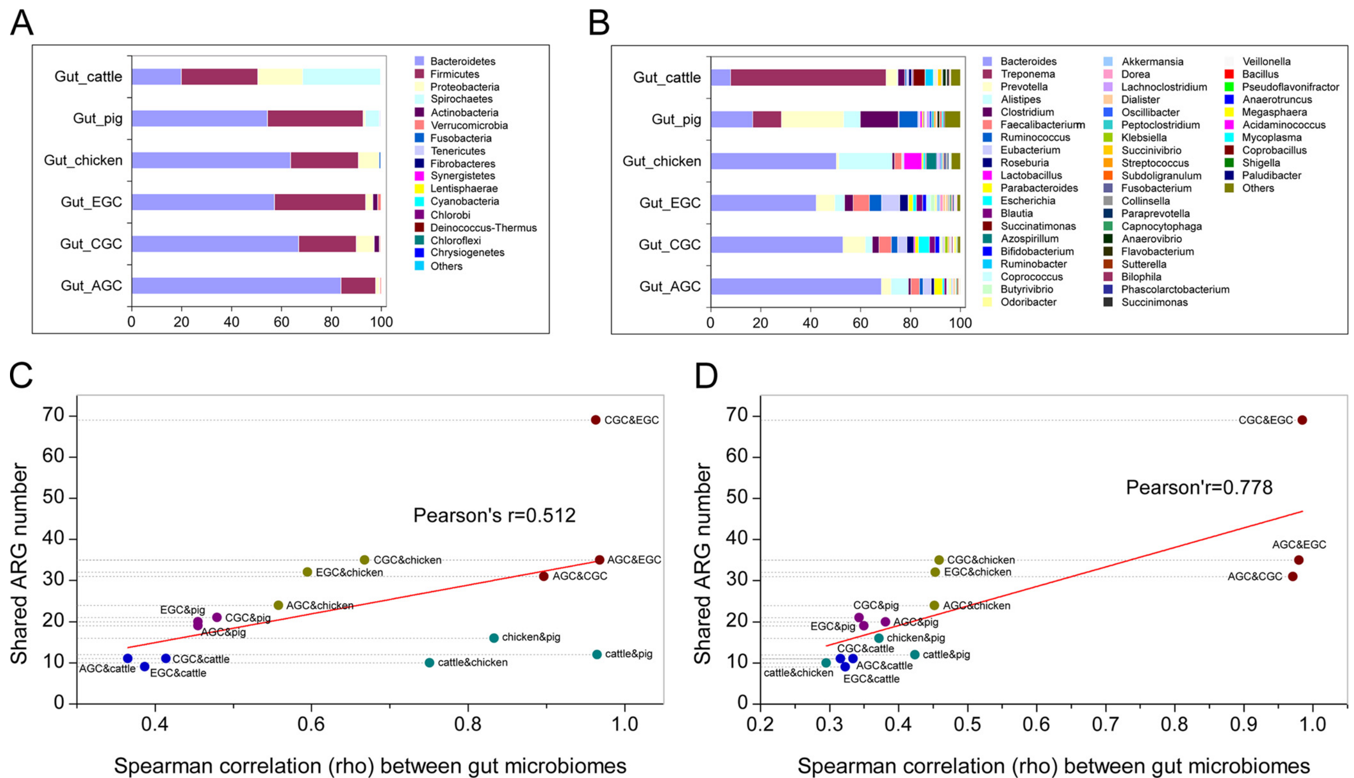


FIG 4 Structures of human and animal gut bacterial communities are correlated with mobile ARG transfer. Bacterial composition at the phylum level (A) and the genus level (B). Correlation of the shared mobile ARG number with the similarity of the bacterial community at the phylum level (C) and the genus level (D).

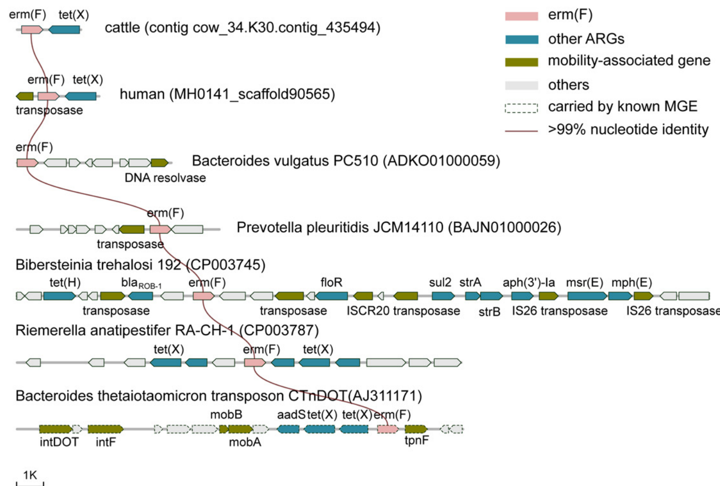
transfer in strains with different origins (restricted to those with defined isolation sources). Among strains that were either human- or animal-associated, approximately 35% carried mobile ARGs, while the ratio was relatively low for strains of aquatic or terrestrial origin at only approximately 5% (see Fig. S5A in the supplemental material). Furthermore, among the strains with human and animal origins carrying mobile ARGs, 98% and 95% shared mobile ARGs with strains from other sources, respectively, while the ratios were 88% and 64% for aquatic and terrestrial isolations, respectively. The HGT frequency of the mobile ARGs in animal-associated bacteria was the highest, followed by human, aquatic, and terrestrial bacteria (see Fig. S5B in the supplemental material). Interestingly, the mobile ARGs were exchanged most frequently between animal and human bacteria, followed by exchanges between animal and aquatic bacteria and then between animal and terrestrial bacteria (see Fig. S5C in the supplemental material). These findings may indicate that animal bacteria represent a more powerful mobile ARG pool that can impact both human and environmental mobile resistomes.

To further investigate the shared mobile ARGs between animals and humans at the bacterial community level, we focused on gut bacteria as an example and sequenced the gut microbiomes of three representative farm animals, including chickens, pigs, and cattle (see Table S1 in the supplemental material) and compared the mobile ARGs in these microbiomes with the integrated gene catalog (IGC) of the human gut microbiome (approximately 9.8 million genes) (23). We found that the recent mobile ARG transfer (≥99% nucleotide identity) indeed occurred between human and animal gut microbiomes and, most intriguingly, that the human IGC shared the most mobile ARGs with the chicken gut microbiome (36 genes) and the fewest with the cattle gut microbiome (10 genes) (see Fig. S6A in the supplemental material). Because the animals sequenced herein were all from China, to investigate whether a country-specific ARG transfer occurred between animals and humans, we constructed a network using separated data sets of the European gene catalog (EGC), Chinese gene catalog (CGC), and American gene catalog (AGC) and the animals. The results showed that the EGC and CGC shared the largest numbers of mobile ARGs (69 genes), but, unexpectedly, each of the three gene catalogs still shared more mobile ARGs with the chicken gut microbiome than with the gut microbiomes of the other animals (see Fig. S6B in the supplemental material).

We further computed the correlation between the similarity of the bacterial community structure (reflecting the phylogenetic similarity between bacterial communities) and the shared gene number. Regarding the relative abundances at both the phylum and genus levels, the microbiome profile of any of the three human gut microbiomes was more similar to that of chickens than of pigs or cattle (Fig. 4A and B), and positive correlations (Pearson's $r = 0.512$ [$P = 0.051$] and 0.778 [$P = 6.4 \times 10^{-4}$]) at the (phylum and genus levels, respectively) were observed for the similarities of community structures (Spearman correlation) and the numbers of shared genes (Fig. 4C and D). These results suggest that, at the levels of bacterial communities that are not closely ecologically connected, bacterial phylogeny may also contribute to the transfer of the mobile resistome, although there is a possibility that some of the shared mobile ARGs we observed were caused by the exchange of bacteria.

A

B



C

FIG 5 Shared mobile ARGs among human and animal gut microbiomes and human pathogens. (A) Network of the shared mobile ARGs. Edges linking species/gut and ARGs indicate that the ARGs were shared among those species/gut microbiomes. Number of human pathogen species: $n = 47$; number of shared mobile ARGs between humans and animals: $n = 41$; number of shared mobile ARGs among humans, animals, and human pathogens: $n = 33$. Comparison of representative animal and human gut assembled contigs harboring the macrolide-lincosamide-streptogramin B resistance gene *erm*(F) (B) and the aminoglycoside resistance gene *ant*(6)-*Ia* (C) with representative human pathogen genomes and known mobile genetic elements.

Mobile ARGs shared between the human and animal gut microbiomes are also harbored by human pathogens. We found a total of 84 mobile ARGs that were shared between at least two gut data sets (Fig. 5A). Among them, 41 genes were recently transferred between human and animal guts (human-animal-shared mobile ARGs). These human-animal-shared mobile ARGs covered six major antibiotic classes, including tetracyclines (11 genes), aminoglycosides (10 genes), macrolide-lincosamide-streptogramin B (MLSB) (9 genes), chloramphenicols (5 genes), beta-lactams (3 genes), and sulfonamides (3 genes) (see Data Set S3 in the supplemental material). In addition, 11 mobile ARGs were shared among all six gut data sets, among which 7 were tetracycline resistance genes, indicating that the transfer of tetracycline resistance genes is more common between human and animals.

We further examined 2,613 genomes of the human-associated pathogens (see Materials and Methods) for human-animal-shared mobile ARGs (≥99% nucleotide identity). Interestingly, we found that 33 of the 41 shared mobile ARGs were harbored by various human pathogens or opportunistic pathogens worldwide, spanning 47 species and 403 genomes (see Data Set S4 in the supplemental material). The tetracycline resistance gene *tet*(M) was the most widely distributed; it was detected in 13 species and was overrepresented in *Streptococcus agalactiae* (group B streptococcus) isolates (95 genomes). The latter is a causative agent for a wide range of human diseases, especially in newborns (32). *S. agalactiae*, *E. coli*, and *Streptococcus suis* were the top three species harboring the greatest number of ARGs (eight genes each) that were shared between human and animal guts. These pathogen-carried human-animal-shared mobile ARGs were moved via different mobile genetic elements, as reflected by the different regions surrounding the same genes and the nearby mobility-associated genes. For example, the MLSB resistance gene *erm*(F) shared between cattle and humans was also carried by pathogens such as *Bibersteinia trehalosi* 192 and by known MGEs such as the *Bacteroides thetaiotaomicron* transposon CTnDOT (Fig. 5B). The genetic environments of *erm*(F) differed between different bacterial hosts, and the gene was frequently flanked by other ARGs as well as different genes that facilitate the transfer process, such as integrase and transposase, indicating that the gene has been subjected to various recombination events. A similar phenomenon has been observed for the aminoglycoside resistance gene *ant*(6)-*Ia*, which is shared between pig and human guts (Fig. 5C), emphasizing the contributions of MGEs to the mobility of mobile ARGs. Taken together, our results provide evidence for the recent exchange of mobile ARGs among human, animal, and clinical pathogens, although we are uncertain about the directions of the transfers.

DISCUSSION

Our analysis showed that both the abundance and the HGT frequency of the mobile ARGs were overrepresented in *Proteobacteria*, and this finding was largely in accordance with our previous analysis showing that in the human gut microbiota, compared with other genes, ARGs are more enriched in *Proteobacteria* (13). An excessive number of ARGs carried by *Proteobacteria* may partially explain the expansion of this group in human and mouse gut microbiomes following exposure to antibiotics (33, 34). It is also interesting to note that a large majority of antibiotic-subsisting soil bacteria belong to the *Proteobacteria* (87% clonal isolates, each resistant to multiple antibiotics) (35). Taken together, these results suggest that *Proteobacteria* probably constitute a major ARG pool, potentially even a mobile ARG pool in nature, regardless of the origin of their ARGs. The enrichment of mobile ARGs in *Proteobacteria* can be interpreted in part by the fact that many human clinical pathogens are *Proteobacteria* (36), which presumably faced more antibiotic selection pressures due to clinical treatments.

It is reasonable to deduce that the recent transfer of the mobile resistome is generally under ecological constraint; the success of HGT depends on contact between the donor and recipient residing in the same niche (37). This trend is clearly demonstrated by our analysis targeting bacterial communities from different sites in the human body. However, the mobile ARG transfers are clearly controlled by bacterial phylogeny regardless of whether the ecology (or the body site) is the same or not (Fig. 3). This observation was consistent when we assessed mobile ARG exchange between animal and human gut microbiomes (Fig. 4). These findings suggest that mobile ARGs have a tendency to spread among phylogenetically closely related bacteria; i.e., phylogenetic evolutionary pressure may be a primary force that structures the bacterial mobile resistome. Therefore, we propose that antibiotic exposure is the source of selection, ecology provides a physical barrier, and phylogeny determines the HGT network of the mobile resistome. We cannot exclude the possibility that the sharing of mobile ARGs between different ecosystems (microbiomes) was caused by the exchange of bacteria harboring mobile ARGs; however, we propose that once the ecological barrier is overcome, the mobile ARGs were primarily expanded among phylogenetically similar populations.

Our analysis indicated that not only the number of bacteria harboring mobile ARGs but also the HGT frequency of the ARGs between animal-associated bacteria is higher than those for bacteria of human and environmental origins (see Fig. S5A and B in the supplemental material). One consideration, from the worldwide ecological view, is that bacteria of animal origin may face more antibiotic selection pressure because more antibiotics (nearly 80% in the United States [38]) are consumed by animals as growth promoters, infection prevention, and clinical treatments. The high exchange frequency of mobile ARGs between animals and humans or environmental bacteria is also noteworthy (see Fig. S5C in the supplemental material). Because we were unable to distinguish the direction of ARG transfer, we are not certain if this is a hint that the animal-associated bacteria have a larger contribution to the dissemination of ARGs to humans and the environment. However, evidence of the transfer of ARGs from animal bacteria to humans and the environment has been published elsewhere (39–41). It has also been suggested that the use of antibiotics in animals has contributed to the emergence of ARGs in human gut microbiomes (14, 42). However, if the use of antibiotics in animals leads to the dissemination of mobile ARGs to humans and the environment, we speculate that there would be fewer mobile ARGs in "organically" raised farm animals that are not subjected to routine antibiotic exposure than in traditionally raised farm animals. A future comparison of the resistome in animals with different husbandry with that of the human microbiome will provide more information regarding the transfer of ARGs among different ecological niches.

Previous studies investigating the bacterial resistome (not discriminating between intrinsic and mobile ARGs) in metagenomes

emphasized that the ARGs discovered represent a high risk for human pathogens because of HGT (8, 15, 43, 44). As suggested, however, if every gene indicates a risk, there are no differential risks and no workable interventions (16). Here, we focused on the mobile resistome and showed that identical mobile ARGs were shared between animal and human gut microbiomes and human pathogens, supporting the occurrence of real ARG transfers or bacterial exchange events, although we remain uncertain about the routes for spreading.

Very recently, a new mobile ARG, named *mcr-1*, encoding resistance to colistin, was found in animals and patients in China (45). We were surprised to find that this new, emerging mobile ARG has been transferred to the healthy Chinese human gut microbiota through a potential food chain dissemination pathway (46), which is more likely the case for several other mobile ARGs that are shared between the animals and humans we observed in this study. In addition, *mcr-1* is currently found in or circulated among *E. coli*, *K. pneumoniae*, and *S. enterica*, which supports our results that the mobile ARGs are exchanged most frequently among these three bacterial species (Fig. 2A; see also Data Set S1B in the supplemental material). As demonstrated in the ARG transfer network, a considerable amount of exchange of mobile ARGs occurs between *K. pneumoniae* and *A. baumannii*. We speculate that *A. baumannii* may become the next *mcr-1* carrier.

Finally, the HGT network of the mobile ARGs presented here was most likely formed by gene flow from a common mobile ARG reservoir and less likely by direct gene exchange, for the bacteria or microbiomes used for analysis were not in physical proximity to each other. Therefore, the observations point to the commonalities among different populations to be exposed to the source. Although statistical normalization was considered when we processed the genome data, we cannot exclude a potential bias in our results due to the database bias and lack of specific organisms within public resources. For example, more antibiotic resistance genes and more genomes from human and animal pathogens are included in the databases than are those from commensal or environmental bacteria. This may lead to, for example, an overrepresentation of mobile ARGs in *Proteobacteria* and an overestimation of the exchange of mobile ARGs between human and animal. However, we believe that the distribution of mobile ARGs in bacteria and the phylogeny-driven, ecology-constrained features of mobile ARG transfer largely reflect the real situation in nature. Because (i) all of the known genomes included herein, particularly the drug-resistant ones, are representative of current antibiotic resistance profiles, (ii) the mobile ARG transfer analysis is relatively less susceptible to database bias, as we showed that, at each phylogenetic level, the intrataxon HGT frequency of the mobile ARGs was significantly higher than the intertaxon HGT frequency, regardless of whether the ecology was the same, and (iii) the intention was to reduce bias from the unevenly sampled genomes in the different habitats, we investigated the ARG transfer within a single ecology (i.e., human body), and we obtained similar results that led to the same conclusions. Overall, large genome sequencing efforts are still needed to reduce the database bias and thus contribute to elucidating the detailed profiles of the mobile ARG transfer network among bacteria and microbiomes.

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