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Bacteria and Antibiotic Resistance Genes (ARGs) in $PM_{2.5}$ from China: Implications for Human Exposure

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ABSTRACT

Airborne transmission is one of the environmental dissemination pathways of antibiotic resistance genes (ARGs), and has critical implications for human exposure through inhalation. In this study, we focused on three regions of China to reveal some unique spatiotemporal features of airborne bacteria and ARGs in fine aerosols ($PM_{2.5}$): (1) greater seasonal variations in the abundance of bacteria and ARGs in temperate urban Beijing than in the subtropical urban areas of the Yangtze River Delta (YRD) and Pearl River Delta (PRD) regions, with regional disparities in bacterial communities; (2) geographical fingerprints of ARG profiles independent of seasonal cycles and land-use gradients within each region; (3) region-independent associations between the targeted ARGs and limited bacterial genera; (4) common correlations between ARGs and mobile genetic elements (MGEs) across regions; and (5) $PM_{2.5}$ at the higher end of ARG enrichment across various environmental and human media. The spatiotemporally differentiated bacterial communities and ARG abundances, and spatiotemporally conserved profiles, mobility, and potential hosts of ARGs in the atmosphere have strong implications for human inhalational exposure over spatiotemporal scales. By comparing other contributing pathways for the intake of ARGs (*e.g.*, drinking water and food ingestion) in China and the U.S., we identified the region-specific importance of inhalation in China as well as country-specific exposure scenarios. Our study thus

highlights the significance of inhalation as an integral part of the aggregate exposure pathways of environmentally disseminated ARGs, which, in turn, may help in the formulation of adaptive strategies to mitigate the exposure risks in China and beyond.

INTRODUCTION

As a critical atmospheric component, airborne fine particulate matter ($PM_{2.5}$) influences air quality, regional climates, and human health.[1-3] With its ability to penetrate deep in our respiratory systems, $PM_{2.5}$ has been extensively associated with a range of health issues.[4-6] This prompted the developed world and, at a later stage, developing countries to formulate health-oriented measures to control air quality. In the past few years, China, for example, has seen a steady decline in $PM_{2.5}$ mass concentrations,[7] owing to the expanding knowledge on physicochemical compositions that has guided source apportionment for target emission reductions.[8] Our understanding of its (micro)biological compositions, an integral dimension of the multi-faceted complex mixtures of $PM_{2.5}$, remains disproportionately limited.[9] The biological particles (collectively known as bioaerosols) include bacteria, fungi, viruses, pollens, and cell debris, and constitute about 5–10% of atmospheric PM.[10] Inhalable biological particles, particularly the fine fraction associated with $PM_{2.5}$, may have a significant impact on human health.[11-12] $PM_{2.5}$ is becoming recognized as an important vector for the transmission of potential microbial hazards (e.g., allergens, pathogens, toxins, antibiotic resistance genes).[13-17] Among them is the issue of antimicrobial resistance, a 21st century public health challenge.[18]

In recent years, the overuse and misuse of antibiotics across the globe has led to the emergence of antibiotic-resistant pathogens and “superbugs”, rendering ineffective the

medications used to cure infections.[19] The selective pressure imposed by antibiotics and other anthropogenic stressors in the environment accelerates the spread of antibiotic resistance genes (ARGs) within and between bacterial species. China, for example, has recorded some of the world's highest levels of antimicrobial resistance in both Gram-positive and Gram-negative bacteria.[20] In response to the rising threat of antimicrobial resistance, major international agencies as well as national/local governments have launched concerted strategic action plans to tackle this public health challenge.[21]

Driven by the "One Health" concept, which emphasizes the interdependence of human, animal, and environmental health,[22] a comprehensive framework integrating the environmental and clinical settings has been set up to tackle antimicrobial resistance.[23-24] ARGs have thus attracted increasing attention in terms of their environmental dissemination pathways, since they were recognized as a class of emerging contaminants.[25] In addition to medication-induced ARGs, ARGs can be disseminated from the environment to humans through external exposure pathways, namely, through drinking water, food, skin contact, and inhalation.[26-27] It has also recently been recognized from a biogeochemical perspective that surface bacteria enter the atmosphere via soil resuspension or water spray, and undergo long-range transport driven by wind.[28] Correspondingly, air circulation is one of the driving mechanisms for the global mass movement of microbial life.[29] Likewise, bacteria and associated ARGs ubiquitous in soil and water can be aerosolized from these surface environments into the atmosphere. The size of airborne particles associated with bacteria at continental sites is about 4 μm, which is larger than the typical size of such bacteria (~1 μm).[30] This finding establishes the overlooked role of airborne PM in the environmental dissemination of ARGs, while $PM_{2.5}$ has particular relevance to consequent human

exposure to ARGs.

Constituents in drinking water and food may be transformed in treatment or processing systems, during which ARGs may be eliminated or "altered" to some extent.[31] By contrast, inhaled air undergoes virtually no treatment. The deep alveolar region of the lung is the relevant exposure receptor for $PM_{2.5}$-associated ARGs, which is distinguished from the gastrointestinal tract, which receives water- and food-borne ARGs. The aerodynamics of $PM_{2.5}$-associated ARGs favors their cross-boundary dissemination. However, the high degree of dispersion may subject airborne bacterial populations to physicochemical stressors that are not often experienced by their terrestrial counterparts. All of these factors make airborne $PM_{2.5}$ an important, albeit unique, vector in the dissemination of environmental ARGs. Therefore, inhalation needs to be incorporated as an integral part of all human exposure-relevant pathways of ARGs in order to assess their respective contributions. A better understanding of these issues would contribute to informed mitigation strategies concerning the sources and human exposure risks of ARGs, and help to address the public health challenges related to both antimicrobial resistance, as well as to the influence of bioaerosols on air quality.

Previous studies have indicated that environmental resistomes can be regulated by host bacterial communities, environmental factors, and anthropogenic impacts, resulting in site-specific ARG profiles.[32-33] Considering the highly dynamic nature of the atmosphere, the diversity and abundance of airborne bacteria and the associated ARG profiles could evolve more frequently over time and space gradients,[34-35] leaving specific spatiotemporal fingerprints with implications for site-specific human

exposures. Past investigations often provided a snapshot of airborne bacteria and associated ARGs in $PM_{2.5}$;[14, 36] however, spatiotemporally resolved dynamics are required to assess long-term human exposures via inhalation.

The abovementioned knowledge gaps motivated us to conduct an extensive investigation in China to characterize the spatiotemporal features of airborne bacteria and associated ARGs, with a particular focus on three typical regions with distinct geographical features and dense populations. By quantifying the targeted genes using real-time quantitative polymerase chain reactions (qPCRs) and elucidating the bacterial communities based on 16S rRNA amplicon sequencing, we aimed to reveal the natural cycle of the bacterial population and associated ARGs, the anthropogenic drives that alter natural rhythms, and the key implications for region-specific human exposure scenarios.

MATERIALS AND METHODS

$PM_{2.5}$ Sampling. The sampling campaign covered three regions of China, namely the Capital city of Beijing in northern China, the Yangtze River Delta (YRD) in eastern China, and the Pearl River Delta in southern China. There are a total of eight sampling sites in the three study regions, including two urban sites in Beijing, three sites (industrial, urban, and rural) in the YRD, and three sites (urban, suburban, and semi-rural) in the PRD (Figure S1). The site classification was based on the land-use type, population density, economic development and industrial activities of the district where each sampling site is located (Tables S1 and S2). $PM_{2.5}$ samples were collected at each

site over an annual cycle from spring 2016 to spring 2017, with the exception of one of the two urban sites in Beijing, where samples were collected from winter 2016 to autumn 2017. The seasons were demarcated as follows: March-May as spring, June-August as summer, September-November as autumn, and December-February as winter, in accordance with the China Meteorological Administration. $PM_{2.5}$ samples were collected on quartz microfibre filters (8 × 10 inches in size; prebaked at 500 °C for 5 h) using high-volume samplers at a flow rate of 1 m^3 min^{-1}. The sampling frequency was around one 24-h $PM_{2.5}$ sample every week at all sites, except for the YRD rural site and the PRD urban site with one 24-h sample every month and every three days, respectively. A total of 456 field $PM_{2.5}$ samples (Table S3) were collected plus ~5% field blanks. The concentrations of $PM_{2.5}$ in season at each site were plotted in Figure S2. All of the filter samples were stored at -20 °C before subsequent treatment.

DNA Extraction. A quarter of each filter sample was cut out for all the sites, except for the PRD urban site (one-eighth of each) and the YRD rural site (half of each). Each subsample was sonicated with sterilized 1× phosphate-buffered saline. Each monthly combined extract was then filtered through a 0.2-μm PES membrane disc filter (47 mm, Pall). A FastDNA SPIN Kit for Soil (MP Biomedicals) was used to extract DNA from disc filters following the manufacturer's instructions, with a modified purification step involving the use of Agencourt AMPure XP beads (Beckman Coulter).[14, 35] All of the DNA extracts were kept at -80 °C until analysis.

Real-time qPCR Quantification of Targeted Genes. Due to the limited amount of DNA extracted from airborne $PM_{2.5}$, we quantified ten target genes, including the 16S rRNA gene, six ARGs (*erm*B, *tet*W, *qnr*S, *lnu*A, bla_{TEM-1}, and *sul*1), and three mobile genetic elements (MGEs; *int*I1, *tnp*A-02, and *tnp*A-04) using the StepOnePlus Real-Time PCR System (Applied Biosystems, CA). The 16S rRNA gene served as an indicator of total bacterial loadings. The choice of the six ARGs encoding resistance to different classes of antibiotics was based on their prevalence in the surface and atmospheric environments.[35, 37-38] The choice of the three MGEs was based on their dominance as important genetic compartments for ARGs.[39-41] To minimize inhibition, a 10-fold dilution was applied to all of the DNA extracts to quantify the ARGs. The dilution factor was determined by testing a number of randomly selected samples. All of the samples, standards, and negative controls (procedural blanks and field blanks) were run in triplicate, with the efficiency of the amplification ranging from 90% to 105%. Detailed information about the standard construction, primer sets, and qPCR conditions used in this study is given in SI, Section S1 and Table S4. Note that the abundance of the 16S rRNA gene, *int*I1, *erm*B, *tet*W, and *qnr*S was reported in our recent work[31] and incorporated into the current study for a comprehensive comparison. All of the qPCR results were summarized in Table S5.

16S rRNA Gene Amplicon Sequencing. The V3-V4 hypervariable region of the 16S rRNA gene was amplified using KAPA HiFi HotStart ReadyMix (Kapa Biosystems) with primer pair 341F (ACTCCTACGGGAGGCAGCAG) / 806R

187 (GGACTACHVGGGTWTCTAAT). The targeted amplicons were then purified using
188 the MEGAquick-spin™ Total Fragment DNA Purification Kit (iNtRON Biotechnology,
189 Korea) and quantified with the Qubit™ dsDNA HS Assay Kit (Thermo Fisher) after
190 electrophoretic separation in 1.5% agarose gel. Purified amplicons from the same
191 season at the same sampling site were pooled on an equal mole basis, and sequenced
192 on the Illumina Miseq PE300 platform in the Beijing Genomics Institute (Wuhan,
193 China). The data were deposited in the NCBI BioProject database
194 (https://www.ncbi.nlm.nih.gov/bioproject/) with the accession number of
195 PRJNA485473.
196
197 **Data Analysis.** The structure of the bacterial community in the samples was
198 characterized by BLASTn[42] against the Silva SSU database (version 111) with an E-
199 value cutoff of 1e-20.[43] The sequences from the BLAST results were assigned to NCBI
200 taxonomies via MEGAN (version 4.67.5)[44] using the Lowest Common Ancestor (LCA)
201 algorithm and the default cutoff of BLAST bitscore 50, and 10% of the top 50 hits.
202
203 Principal component analysis (PCA) of regional differentiations in airborne bacterial
204 communities was performed using CANOCO (Version 4.5). The 16S rRNA gene copies
205 (copy m^{-3}) assigned to each bacterial genus were used as input data, which were
206 obtained by multiplying the total number of copies of 16S rRNA genes by the
207 percentage of each bacterial genus. Bacterial genera were excluded from this analysis
208 if their relative percentages were lower than 5%. The data were subjected to a square

root transformation and further checked to meet the normal distribution before undergoing a PCA analysis. One-way ANOSIM and non-metric multidimensional scaling (NMDS) analyses were performed in Past 3 to visualize the regional differentiations of $PM_{2.5}$-associated ARG profiles. Redundancy analysis (RDA) was performed using CANOCO (Version 4.5) to explore the correlations between bacterial genera and the analyzed ARGs. The total number of gene copies (copy m^{-3}) was used for the redundancy analysis, with the square root transformation of the data being carried out prior to the analysis. Other statistical analyses were conducted in R (version 3.2.2) and GraphPad Prism 7.

To assess the relative importance of inhalation to total human exposure to external ARGs, we estimated the human daily intake (DI) of the targeted genes via $PM_{2.5}$, drinking water (DW), and food items in urban populations in China and the U.S. as a comparison (eq. 1-3).

$$DI_{PM_{2.5}}(copy\ d^{-1}) = Concentration\ (copy\ m^{-3}) \times inhalation\ rate\ (m^3\ d^{-1}) \quad (1)$$

$$DI_{dw}(copy\ d^{-1}) = Concentration\ (copy\ L^{-1}) \times ingestion\ rate\ (L\ d^{-1}) \quad (2)$$

$$DI_{food}(copy\ d^{-1}) = Concentration\ (copy\ g^{-1}) \times ingestion\ rate\ (g\ d^{-1}) \quad (3)$$

The concentrations of the target genes in urban aerosols are from this study (China) and Refs [45-46] (U.S.), and those in other intake matrices (e.g., drinking water, aquatic products, and vegetables) are from refs [46-52]. The daily inhalation rate was commonly set as 20 m^3 d^{-1}.[53] The ingestion rates for drinking water, aquaculture products, and vegetables in China were 1.6 L d^{-1} (1.5-1.7 L d^{-1}), 57.5 g d^{-1} (40-75 g d^{-1}), and 400 g d^{-}

231 [1] (300-500 g d^{-1}), respectively, according to the Dietary Guidelines for Chinese
232 Residents.[54] For U.S. adults, the ingestion rates for drinking water and aquaculture
233 products (finfish) were set as 2 L d^{-1} and 12 g d^{-1}, respectively, as recommended by the
234 USEPA.[53] These comparisons were based on the assumption of equal DNA extraction
235 efficiency between matrices across studies.
236
237 **RESULTS AND DISCUSSION**
238 **Regional differences in $PM_{2.5}$-associated bacterial loadings and community**
239 **structure.** For lack of a rural site in Beijing, we first compared the total bacteria
240 loadings between the urban sites of the three studied regions. Seasonality of the total
241 bacterial loading was most apparent in the temperate urban Beijing, where the absolute
242 concentration of the 16S rRNA gene declined from spring to winter by nearly three
243 orders of magnitude and recovered in the following spring (Figure 1a). A similar
244 seasonal trend was also observed previously with the concentrations of bacteria in total
245 suspended particulate from urban Beijing.[55] In contrast, concentrations of the 16S
246 rRNA gene at the urban sites of the YRD and PRD regions varied only within an order
247 of magnitude (10^3-10^4 copy m^{-3}) over the annual cycle. The annual average
248 concentration of the 16S rRNA gene in Beijing were the highest of all the three studied
249 regions (Figure S3), which was consistent with the broader geographical comparison[56-
250 58] (Figure S4). $PM_{2.5}$ concentrations may not be a critical factor regulating the seasonal
251 dynamics of the airborne bacteria therein, as the abundance of the 16S rRNA gene did
252 not correlate with the concentration of $PM_{2.5}$ across the three studied regions (Figure

253 S5). Local sources such as soil and vegetation may be more influential factors driving
254 the abundance of ambient airborne bacteria.[59-60] For example, the *Cyanobacteria*
255 phylum, which is partially derived from plants, dropped from 24% of total bacteria in
256 spring (8.5×10^4 copy m^{-3}) to less than 1% in winter (3 copy m^{-3}) and revived to 47% in
257 spring (6.3×10^4 copy m^{-3}) (Figure 2). Similar trends were also found in Beijing
258 previously that the relative abundance of *Cyanobacteria_Chloroplast* dropped from
259 spring (32%) to winter (2%) in $PM_{2.5}$-associated bacteria.[34] In contrast, the relative
260 abundance of the phylum *Cyanobacteria* varied less among seasons in the subtropical
261 YRD and PRD regions (Figures 2 and S6). These observations provided evidence of
262 the seasonal dependence of vegetative contributions to airborne bacteria, typically in
263 the temperate regions due to the distinct climate features. This may partially account
264 for the larger seasonal variations in total airborne bacterial loadings in Beijing than in
265 the YRD and PRD regions.
266
267 Intra-regional comparisons of total bacterial loadings associated with $PM_{2.5}$ were
268 conducted across land-use transects between the YRD and PRD regions (Figure 1b).
269 Significant seasonal variations in the absolute concentrations of 16S rRNA genes were
270 observed at the rural site of the YRD region in our previous study,[35] with an obvious
271 decrease in the winter season and a rapid recovery in the following spring, in contrast
272 to the less distinct seasonal fluctuations in the urban and industrial sites. Similar to the
273 situation in the YRD region, the most significant variations in airborne bacterial
274 loadings were seen in the semi-rural site of the PRD region, but the differences

decreased along the semirural-suburban-urban gradient. With intense anthropogenic activities in urban areas, contributions of natural sources (e.g., vegetation) to the airborne microbes, were likely replaced by those from anthropogenic sources that are more stable across seasons, thus flattening the seasonal rhythms of bacterial loadings observed in (sub)urban and industrial areas. Compared with all of the sampling sites in the YRD and PRD, seasonal variations in the crucial phyla, which explained most of the structural variations over seasons, were most significant in the rural site of the YRD (Figure S7). This corresponded to the seasonal changes in the total $PM_{2.5}$-borne bacterial loading under natural regulation in this area. At other sites impacted by human activities, seasonal variations in the bacterial community structure were likely subject to the combined influences of multiple dominant phyla (Figure S7).

Regional fingerprints of ARG profiles. The overall annual median concentrations of all of the $PM_{2.5}$-associated ARGs that were analyzed were comparable in the three study regions, with no statistical differences (Figure S8a), indicating that levels of airborne ARGs are relatively consistent across different regions in China in an annual average base. Echoing the disparities in airborne bacterial communities at the genus level (Figure S9), the corresponding ARG profiles exhibited different regional patterns between northern (Beijing), eastern (the YRD), and southern (the PRD) China (Figure 3). *lnu*A dominated in the $PM_{2.5}$-associated ARGs in northern China, while *lnu*A and *sul*1 collectively played a leading role in the PRD region. By contrast, the most dominant position was transferred to *erm*B in the YRD region, except in the urban area,

where *bla*$_{\text{TEM-1}}$ played as dominant a role as *erm*B. Regional differentiations in airborne ARG profiles relating to air fine particles were also demonstrated by three clusters in a Bray-Curtis based the NMDS plot (Figure S10; R=0.6258, p<0.01). Recently, the terrestrial resistome in natural environments has been found to differ between temperate and subtropical regions and to be regulated by plants and soil bacterial communities.[32] As an important source of atmospheric bacteria and ARGs, soil with different resistomes among the regions might play a noticeable role in the formation of regionally different airborne ARG profiles. In contrast to the regional disparities, the annual average ARG profile within a region was generally less diverse, which was indicative of regional impacts overwriting local influences.

The seasonality of the relative abundance of most of the predominant airborne ARGs was most remarkable in the rural site of the YRD region among all the sampling sites (Figure S11), evolving from spring to winter along a seasonal cycle. For other sites within the same region, the seasonal contrast seemed to be dimmed gradually. Since all of the sampling sites in this study are more or less affected by strong anthropogenic activities, with the exception of the rural site in the YRD region, which is closer to the natural state (Table S1), the relative consistency in the relative abundance of the targeted ARGs suggests that urbanization and industrialization are having a key effect on seasonal variations in the community structure of naturally occurring bacteria and in the resultant airborne ARGs.

To further explore the relationships between airborne bacterial genera and ARGs, a redundancy analysis (RDA) was conducted in this study. Despite the regionally divergent bacterial communities, there may be a limited number of core taxa members that could be the true ARG hosts, as suggested by the association between ARGs and specific bacterial genera independent of seasons and land-use types (Figure S12). The relative compositions of these potential hosts within the airborne bacterial community of each region may explain the regionally specific signature of ARG profiles as discussed above. Additionally, significant positive correlations were found between most of the ARGs and between most of the bacterial genera (Figure S13), suggesting the co-abundance relationship between the ARGs and between the bacterial genera. Therefore, the RDA result should be interpreted with caution due to potential co-correlation between ARGs and bacterial genera. Nevertheless, it is still worth noting that some of the identified bacterial genera significantly associated with ARGs, such as *Acinetobacter*, *Burkholderia, Clostridium, Sphingomonas*, and *Staphylococcus*, include certain clinically important pathogenic species. However, some environmental bacteria harbor intrinsic resistance to endogenous or naturally-occurring antibiotics.[61] For example, *Pseudomonas aeruginosa* was found with high intrinsic resistance in the presence of β-lactamase encoding gene on chromosome, in addition to its outer-membrane barrier and the efflux pumps[62-63]. These natural resistance mechanisms have also been discovered in other species like *Burkholderia pseudomallei*, *Burkholderia cepacia*, and *Stenotrophomonas maltophilia*.[64] It is therefore imperative in future studies to distinguish intrinsic and acquired resistance and ascertain the mobility of

ARGs as well as the identities of ARG-carrying bacteria (pathogen or not) in $PM_{2.5}$, using a combination of whole genome sequencing and culture-dependent methods. These efforts would help address the health relevance of airborne ARGs.

Enrichment of ARGs and MGEs in fine aerosols. Integrons and transposons are important MGEs responsible for the dissemination of ARGs in the environment. In this study, *int*I1 and *tnp*A belonging to the IS4 group and IS6 group were widely detected in all of the sampling sites. The absolute abundance of the total analyzed MGEs generally varied by two orders of magnitude in all of the regions, with the annual median being lowest in the YRD region (Figure S8b), possibly implying spatially and temporally different prospects for the potential of horizontal gene transfers in the atmospheric environment. In addition, significant correlations between the relative abundance of the total analyzed ARGs and *int*I1 normalized to the 16S rRNA gene were found across all land-use types, with the only exception of insignificance at the industrial site of the YRD region (Figure 5). Strong correlations for ARGs with *tnp*A also occurred in most of the sampling sites. These findings point to a co-occurrence pattern of ARGs and MGEs prevalent in the atmosphere. It should, however, be noted that the co-abundance of MGEs and ARGs does not necessarily mean the occurrence of horizontal gene transfer. Future studies are warranted to verify the mobility and transferability of these ARGs and, if so, elucidate the genetic context of ARGs (e.g., types of MGEs) underlying the transfer mechanism (e.g., plasmid-mediated conjugative transfer).

363

364 The relative abundance of ARGs normalized to the 16S rRNA gene and *int*I1
365 normalized to cell numbers based on metagenomic sequencing was previously found to
366 increase from natural environments to engineered systems and human and animal
367 excreta samples (Figures 4 and S14). This trend coincides with an increase in the level
368 of anthropogenic impacts to exert selective pressure for the development of antibiotic
369 resistance.[65-66] However, the relative abundance of ARGs and MGEs in outdoor $PM_{2.5}$
370 seemed to break this trend. The median relative abundance of ARGs detected in $PM_{2.5}$
371 samples is comparable to that in wastewater systems, with variability covering the
372 entire span of all of the other types of samples (Figure 4). This was also the case for the
373 $PM_{2.5}$-associated *int*I1 in the present study (Figure S14). The qPCR analysis used in
374 this study only targeted a limited set of ARGs, whereas all of the identifiable ARG or
375 ARG-like sequences in the other three groups of matrices based on metagenomics were
376 included in this comparison. Therefore, the relative abundance of total airborne ARGs
377 could be at an even higher level. A broader-spectrum analysis of ARGs are thus desired
378 using either high-throughput qPCR profiling or shotgun metagenomic sequencing. To
379 this end, upscaling of the DNA amount from composite $PM_{2.5}$ samples would be
380 required. In addition, considering soil, wastewater, and feces to be potential sources of
381 airborne bacteria and ARGs,[60, 67-69] the large variability in the relative abundance of
382 ARGs in the $PM_{2.5}$ samples is an indication of the mixing effects of these surface
383 sources. The current findings reaffirm the importance of the atmosphere as a potential
384 gateway for surface sources of ARGs, and as a key transmission route for human

385 exposure. Future investigations of airborne ARGs in vicinity of typical surface sources
386 coupled with atmospheric dispersion modeling are warranted to identify their
387 contribution to the ambient airborne ARGs.
388
389 **Relative importance of inhalation to human exposure to environmental ARGs.** The
390 contribution of $PM_{2.5}$ exposure to the total daily intake of ARGs from various pathways
391 varied between the studied regions in China (Figure 6), because of regional
392 differentiations in airborne ARG profiles. Furthermore, the daily intake of ARGs via
393 ingestion (drinking water and the intestines of aquatic products) exceeded that of
394 inhalation in most circumstances, with the exception of a comparable level for *erm*B
395 and bla_{TEM} between drinking water and $PM_{2.5}$ occurring in the YRD region indicating
396 the unequal roles of these pathways for human exposure. This is similar to the situation
397 in the U.S. $PM_{2.5}$ exposure plays a non-negligible role compared to other pathways,
398 based on the limited data on 16S rRNA gene and ARGs in urban airborne particles and
399 other intake-relevant matrices. It is stressed, however, that the intestines of aquaculture
400 species are rarely consumed directly. Including this ingestion pathway represents a
401 worst-case exposure scenario, subject to the availability of data. The processing of
402 drinking water (often boiled in China) and food (normally well-cooked around the
403 world) might further modify the magnitude of exposure to bacteria and ARGs through
404 ingestion, in contrast to the direct inhalation of $PM_{2.5}$-associated bacteria and ARGs
405 without any prior treatment. Therefore, it is imperative to perform a daily basket survey
406 of waterborne and foodborne bacteria and ARGs, taking into consideration the effects

of cooking/boiling, in order to conduct a more comprehensive analysis of exposure pathways. On the other hand, bacteria and associated ARGs inhaled through the respiratory tract may have different fates from those of their ingested counterparts traveling through the gastrointestinal tract. The ultimate effects of these external uptakes, which may complicate medication-induced ARGs, warrant more systematic research in the future. Despite these uncertainties, the comparisons of daily intake provide a first-tier screening of the relevance of the respective pathways to human aggregate exposure to environmental ARGs in region-specific scenarios. The inclusion of inhalable and ingestible ARGs in the full range of sources of exposure would make the environmental framework of ARGs more relevant to human health, and eventually make possible a communication to the clinical framework of ARGs.

Environmental implications. The current study, along with other studies,[16] reaffirms the ubiquity of ARGs in the atmosphere across spatiotemporal scales, including the inhalation-relevant fine particle fraction. The Earth's atmosphere serves as a habitat for bacteria where ARGs can be highly enriched, and fine aerosols act as a transmission vector for human exposure to environmentally disseminated ARGs. This contributes to our understanding of the link between bioaerosols and human health. We noticed that the evolution of airborne ARGs does not necessarily follow the trend of urbanization that is often observed in the surface environment. The higher abundance and enrichment of $PM_{2.5}$-associated ARGs in rural areas, as well as the common relationship between ARGs and MGEs across land-use gradients, suggest more complex interactions

between natural and anthropogenic influences.

Few studies have been conducted to assess whether various surface anthropogenic activities are potential sources of ARG emissions. These studies often generate a static snapshot of the airborne ARGs above the related systems, while the dynamic mechanisms of the dissemination of ARGs via the aerosolization of surface biological particles into the ambient air remain to be elucidated. Aerodynamics and transport models, coupled with cluster analysis, may help to resolve the flux and contribution of ARGs from urban functional spots to ambient airborne ARGs. Once emitted from the surface to the air, bacteria and associated ARGs will further evolve along atmospheric processes. The airborne bacterial communities are unlikely to experience co-occurring anthropogenic stressors as selective pressure for antibiotic resistance. Instead, they are subjected to a high degree of dispersion, as well as to high oxidation capacity, particularly in urban air. This may determine the alternative strategies of airborne bacteria in adaptation to stressors, such as oxidative gases, free radicals, strong irradiation, and nutrient limitations. As our findings provide evidence of the heterogeneity of the airborne dissemination of ARGs, it is reasonable to hypothesize that the horizontal gene transfers that occur in the atmosphere may differ from those that occur in terrestrial and aquatic environments. Differentiating between natural and anthropogenic sources of airborne ARGs and between prevailing weather conditions and physicochemical pollution status is an essential, a challenging, task in assessing spatiotemporal scenarios of human inhalational exposures.

Our results also suggest that there may be few core taxa members that harbor ARGs independent of spatiotemporal scales, despite the diverse airborne bacterial communities across regions and seasons. A "what's next" question would follow logically – the identification of ARG-carrying bacteria at the species level, whether they be pathogenic or non-pathogenic, via the integrated use of both culture-dependent and culture-independent techniques.[38, 70-72] Answering this question would definitely transform the current exploratory investigations on airborne bacteria and associated ARGs into more tailored, hypothesis-driven efforts to examine issues related to respiratory infections. An elevated mechanistic understanding of the interactive behaviors between inhaled bacteria from ambient air and innate bacteria in the lungs would, in a broad sense, help to decipher the fate and effects of the bacteria in human airways,[73] thus addressing the relevance to health of inhalation exposure over spatiotemporal scales. In past years, the emphasis was on the environmental processes of clinically important ARGs and bacterial hosts; now it is time to examine the clinical relevance of environmentally disseminated ARGs and bacterial hosts, *i.e.*, to distinguish between the "good," "bad," and "ugly" in future investigations.

As mentioned earlier, inhalation is among the few pathways of transmission that are directly relevant to the human intake of environmental ARGs and bacterial hosts, which together form a framework on aggregate pathways to exposure.[74-75] To identify the control checkpoint for mitigating the risks posed by environmental ARGs, it is

imperative to determine the relative contributions of respective pathways to total exposure for humans. A first-tier assessment of exposure would be required to quantify the flow of clinically relevant ARGs and pathogenic hosts via these intake routes in balance with exhalation and excretion, and to identify the route-specific transmission of these microbial hazards. For risk estimates, it is vital to elucidate the internal fate of inhaled and ingested ARGs in the human body system and the potential influences of these environmental resistomes on medication-induced clinical resistomes. Filling these knowledge gaps in sequence is essential to charting a path to an eventual dialogue between the environmental and clinical research communities concerning antimicrobial resistance, in order to combat the challenge threat it poses to public health.

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ASSOCIATED CONTENTS

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXX.

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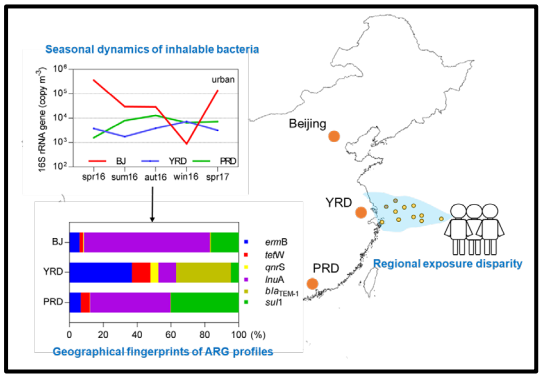
Author Contributions

Jiawen Xie, Ling Jin, and Xiangdong Li designed the study. The laboratory experiments on air PM bacteria and ARGs were performed by Jiawen Xie and Tangtian He. Data analysis was conducted by Jiawen Xie, Ling Jin, Baowei Chen, and Xiangdong Li. The manuscript was written with contributions from all of the co-authors. All of the authors gave their approval to the final version of the manuscript.

Notes

The authors declare that they have no competing financial interests.

TOC Art



List of figures

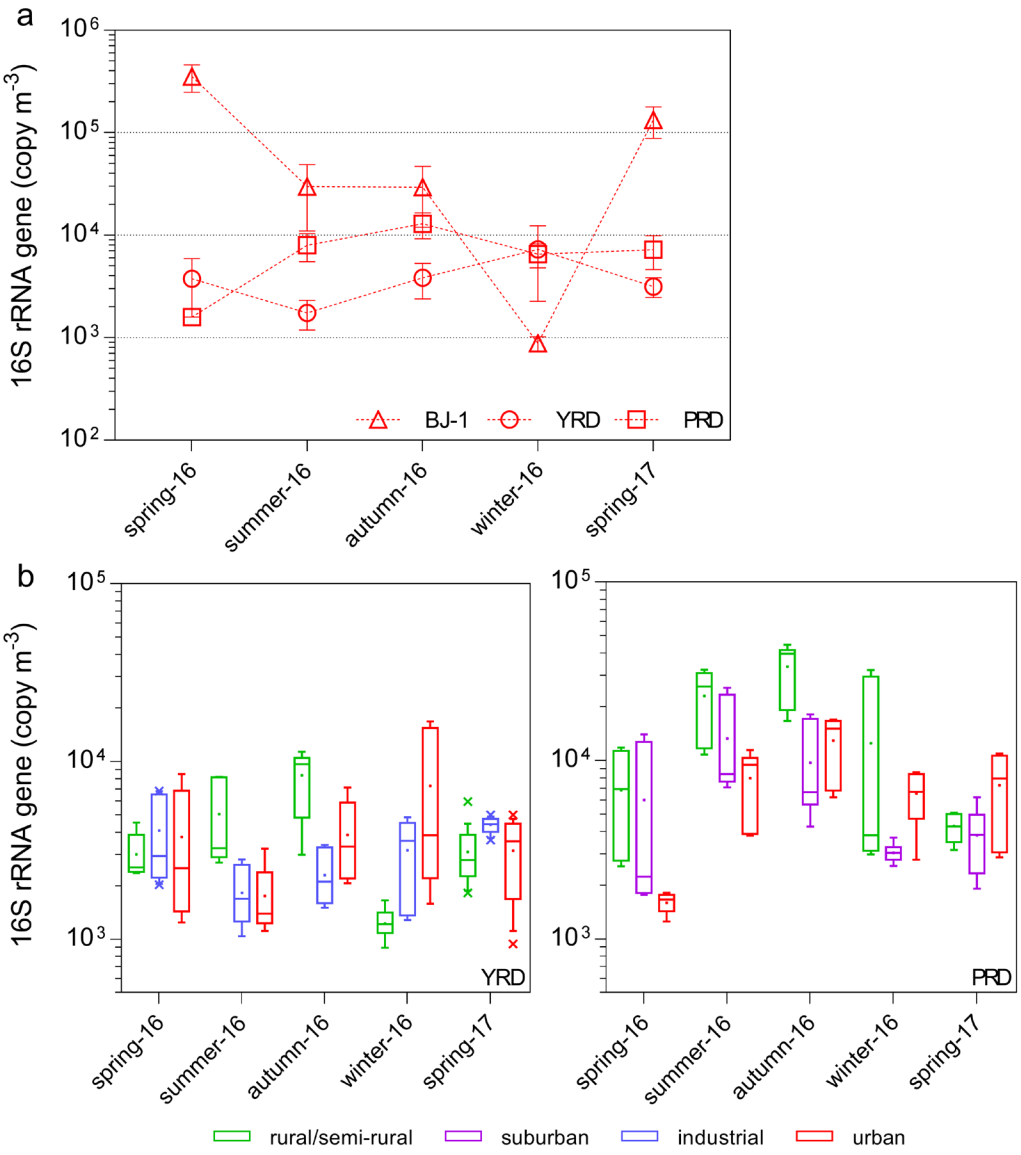
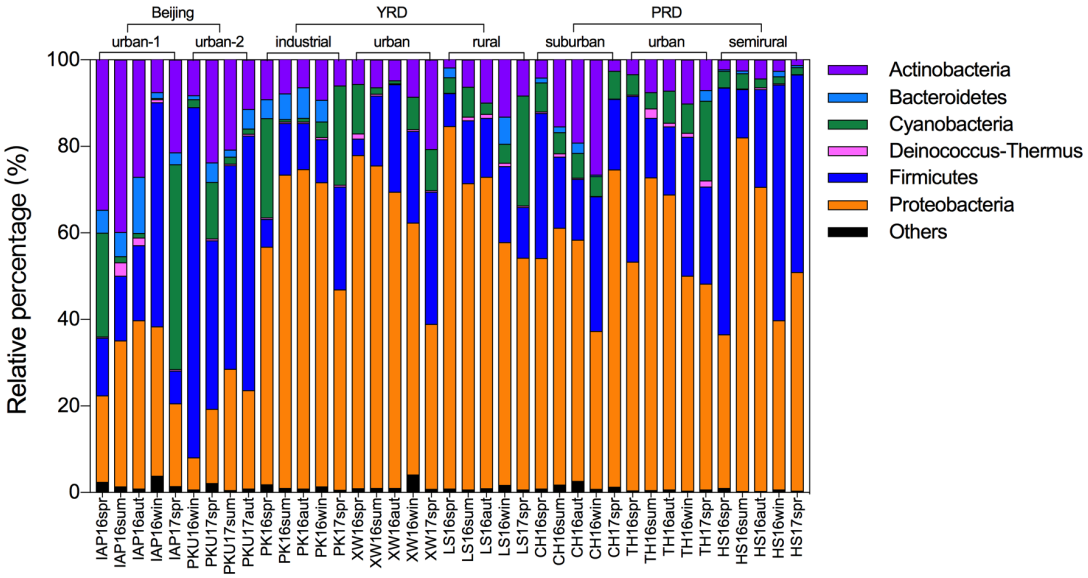


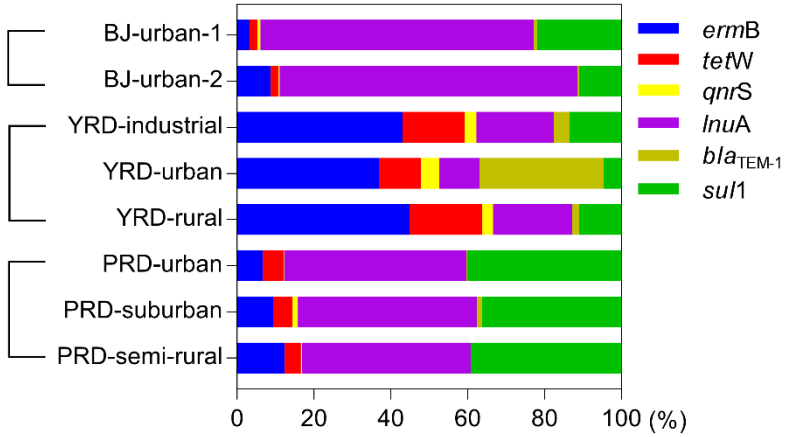
Figure 1. (a) Inter-regional comparisons of $PM_{2.5}$-associated 16S rRNA genes in urban areas across China and intra-regional rural-urban contrasts in the YRD region and PRD region; (b) Seasonal variations in the second urban site in Beijing are not shown here because of the non-parallel sampling period.



529

530 **Figure 2.** Spatiotemporal patterns of $PM_{2.5}$-associated bacterial communities at the

531 phylum level in the three studied regions.

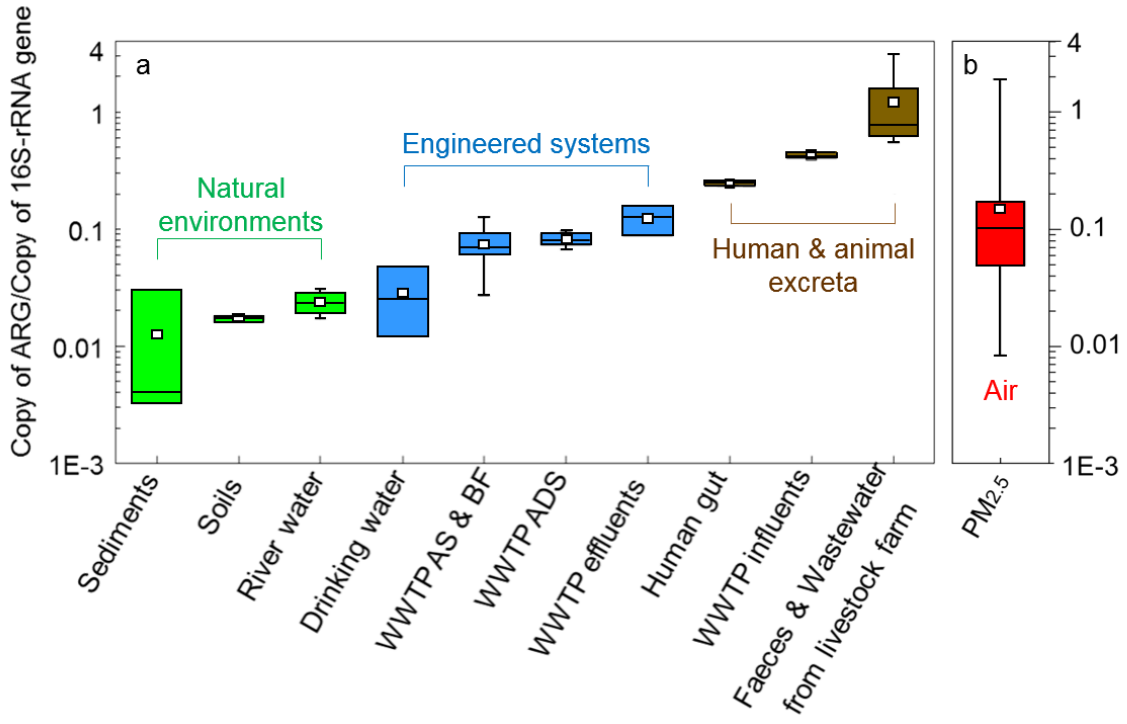


532

533 **Figure 3.** Regional signatures of airborne ARG profiles. The relative percentage of each gene

534 was an annual average of all of the samples.

535



536

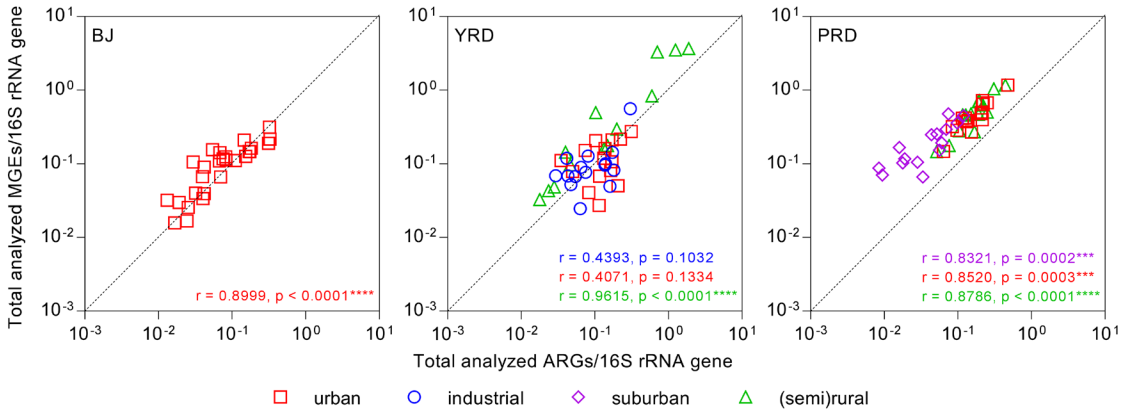
537 **Figure 4.** Relative abundance of ARGs normalized to the 16S rRNA gene in environmental

538 matrices. Part (a) was adapted with permission from Li et al.[65] Copyright 2015 Springer Nature.

539 Note that the data in part (a) were based on metagenomic sequencing, while those in part (b)

540 were based on qPCR analyses from this study.

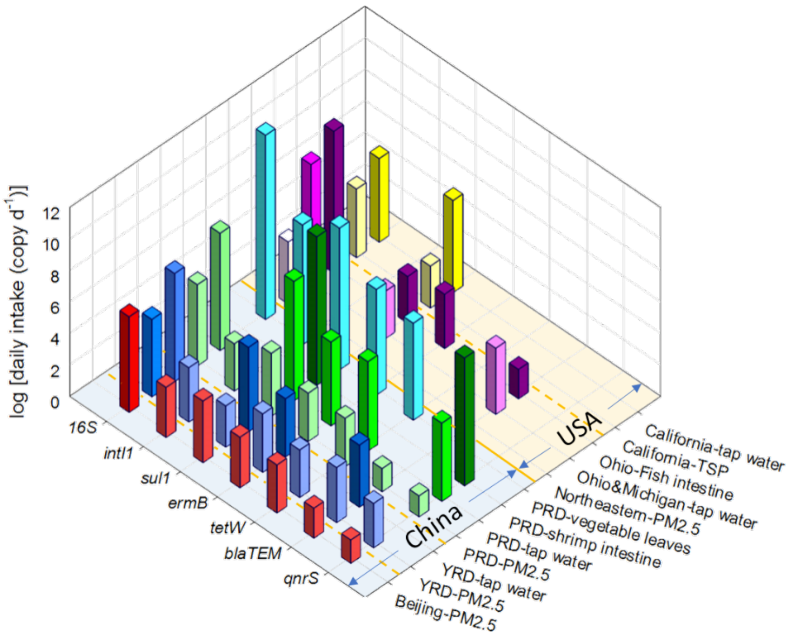
541



542

543 **Figure 5.** Relationships between ARGs and MGEs in $PM_{2.5}$ across the studied sites.

544



545

546 **Figure 6.** Regional comparisons of the human daily intake of ARGs and 16S rRNA genes
547 between inhalation and ingestion, coupled with a contrast with the U.S. situation. The
548 calculation was based on eq. 1-3. Annual average concentrations of target genes in China
549 generated in this study and in our previous study[35] were used to estimate daily intake via
550 inhalation. The raw data on the concentrations of the targeted genes in tap water in the YRD
551 and PRD regions were from Shi et al.[47] and Su et al.,[48] respectively. The raw data for samples
552 of shrimp intestines and washed vegetable leaves in the PRD region were also from Su et al.[49]
553 and He et al.[50] The U.S. data were obtained from Hospodsky, et al. [45] Xi, et al. [51] Huang [52] and
554 Echeverria-Palencia, et al. [46]

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