Environmental Science & Technology

Article

pubs.acs.org/est

Influence of Soil Characteristics and Proximity to Antarctic Research Stations on Abundance of Antibiotic Resistance Genes in Soils

Fang Wang,[†,‡,◇,⊥] Robert D. Stedtfeld,[§,⊥] Ok-Sun Kim,[¶,⊥] Benli Chai,[†,‡] Luxi Yang,[†,‡,§] Tiffany M. Stedtfeld,[§] Soon Gyu Hong,[¶] Dockyu Kim,[¶] Hyoun Soo Lim,[Δ] Syed A. Hashsham,[†,‡,§] James M. Tiedje,[†,‡] and Woo Jun Sul*[,∥]

[†]Department of Plant, Soil and Microbial Sciences, [‡]Center for Microbial Ecology, [§]Department of Civil and Environmental Engineering, Michigan State University, East Lansing, Michigan 48824, United States

[¶]Division of Life Sciences, Korea Polar Research Institute, Incheon 21990, Republic of Korea

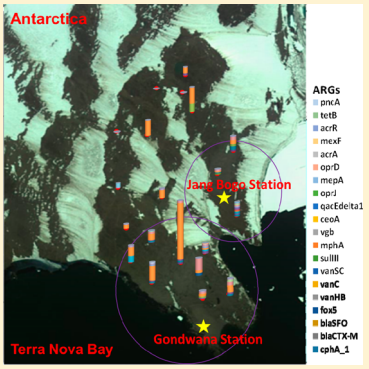
[∥]Department of Systems Biotechnology, Chung-Ang University, Anseong 17546, Republic of Korea

[◇]Key Laboratory of Soil Environment and Pollution Remediation, Institute of Soil Science, Chinese Academy of Sciences, Nanjing, Jiangsu 210008, PR China

[Δ]Department of Geological Sciences, Pusan National University, Busan 46241, Republic of Korea

Supporting Information

ABSTRACT: Soil is an important environmental reservoir of antibiotic resistance genes (ARGs), which are increasingly recognized as environmental contaminants. Methods to assess the risks associated with the acquisition or transfer of resistance mechanisms are still underdeveloped. Quantification of background levels of antibiotic resistance genes and what alters those is a first step in understanding our environmental resistome. Toward this goal, 62 samples were collected over 3 years from soils near the 30-year old Gondwana Research Station and for 4 years before and during development of the new Jang Bogo Research Station, both at Terra Nova Bay in Antarctica. These sites reflect limited and more extensive human impact, respectively. A qPCR array with 384 primer sets targeting antibiotic resistance genes and mobile genetic elements (MGEs) was used to detect and quantify these genes. A total of 73 ARGs and MGEs encompassing eight major antibiotic resistance gene categories were detected, but most at very low levels. Antarctic soil appeared to be a common reservoir for seven ARGs since they were present in most samples (42%−88%). If the seven widespread genes were removed, there was a correlation between the relative abundance of MGEs and ARGs, more typical of contaminated sites. There was a relationship between ARG content and distance from both research stations, with a significant effect at the Jang Bogo Station especially when excluding the seven widespread genes; however, the relative abundance of ARGs did not increase over the 4 year period. Silt, clay, total organic carbon, and SiO_2 were the top edaphic factors that correlated with ARG abundance. Overall, this study identifies that human activity and certain soil characteristics correlate with antibiotic resistance genes in these oligotrophic Antarctic soils and provides a baseline of ARGs and MGEs for future comparisons.



INTRODUCTION

The recent emergence of multidrug-resistant bacteria has motivated proposed strategies toward containment of antimicrobial resistance.[1,2] Recommendations include monitoring antibiotic resistance to examine pathways in which the natural resistome serves as a potential source of resistance in human and animal pathogens.[3−5] Recent studies have demonstrated anthropogenic influences on the levels of antibiotic resistance genes (ARGs) in agriculture soils,[6,7] food,[8,9] and water habitats.[10−12] However, the diversity and abundance of antibiotic resistance in regions less affected by anthropogenic influences may offer more insight into mechanisms for containment. Harsh environments such as the Antarctic may select for or against microorganisms with ARGs, in the former case by using antibiotic production and resistance to compete for very limited resources.[13,14]

Previous studies have reported antibiotic resistance in a number of environments thought to be less impacted by anthropogenic influence including some soils, water, sediments, glaciers, and the deep ocean.[15−19] ARGs have also been detected in remote human communities,[20] wild rodents, and porcine not exposed to antibiotics or external influences.[21] Antimicrobial resistance determinants have been observed in *Escherichia coli* isolates originating from Arctic birds.[22] However, studies examining ARGs in Antarctic samples are limited. A survey of six surface snow samples from Antarctica found only a single ARG from a site with 25 years of regular

Received: June 8, 2016
Revised: September 18, 2016
Accepted: October 31, 2016
Published: October 31, 2016

DOI: 10.1021/acs.est.6b02863
Environ. Sci. Technol. 2016, 50, 12621−12629

expeditions.[17] Contrary to this, multiple gentamicin resistance isolates were observed from three Antarctic sponge samples.[23]

Most antibiotics are produced by microorganisms that occur naturally in soil.[5,24] For example, soil is a natural habitat for *Streptomyces*, whose species account for a majority of naturally produced antibiotics.[4,25,26] Soil is also one of the most diverse and largest microbial habitats on earth and is recognized as a great repository of ARGs,[4,15,26,27] which may also serve as a reservoir due to anthropogenic influenced selection.[5] As such, soil is a well-suited matrix for capturing the selective influences of human activity.

ARGs can be dispersed by physical and biological forces such as wind, water, animals, and humans, resulting in their presence in many environments.[5,22,28] ARGs can also be widely disseminated by birds;[22] e.g., the Arctic tern travels to up to six continents and migrates to Antarctica for winter. From a microview, ARGs may be mobilized and transferred by mobile genetic elements (MGEs), including transposons, integrons, and plasmids. Many of the known ARGs are found on MGEs, which can be transferred to other bacteria of the same or different species.[5]

Our main hypothesis was that ARGs and MGEs in Antarctic soil would reflect human impact caused by construction of the new Jang Bogo Research Station. A secondary aim was to assess the types and distribution of ARGs in Antarctic soil with no or low human activity. To examine long and short-term influence of human activity on the distribution of ARGs, soil samples were collected at different proximities from the 30-year old Gondwana Research Station and the newly developed Jang Bogo Research Station, both in the Terra Nova Bay area of southwest Antarctica. A highly parallel qPCR array with 384 primer sets targeting 285 ARGs and 35 MGEs was used. Assays targeted three major resistance mechanisms: cellular protection, antibiotic deactivation, and efflux pumps and conferred resistance to most major antibiotics.

MATERIALS AND METHODS

Sites and Soil Sampling. A total of 62 soil samples were collected from the Terra Nova Bay (east side of the Ross Sea) in southeast Antarctica in the summers of 2011, 2012, 2013, and 2014, which is before and during construction of the Korean Antarctic Research Station, Jang Bogo (Figure S1, Table S1). A wide variety of life forms, such as bryophytes, lichens, sea birds, marine mammals, and invertebrates have been observed in this area. This site is mainly composed of exposed bedrock and glacial moraines,[29] with extreme low levels of organic matter for soil (total organic carbon contents are mainly lower than 0.08%) (Table S3). Samples were collected near the Jang Bogo Research Station to investigate the background level of ARGs before human activity and then followed by samples collected during facility construction where the soil nearby was heavily plowed, hence reflecting short-term human activity. Samples were also collected near the 30-year old German Antarctic Research Station (Gondwana) to investigate long-term but minimal impact of human activity on ARGs in soil. The sites from the coast to the research station and partially up the mountain were selected for investigating the background level of ARGs in soil further from the research stations. At each sampling site, soil samples were collected from a 0 to 3 cm layer, mixed inside a plastic zip lock bag to homogenize, and immediately frozen on dry ice. All soil samples were stored at −80 °C until DNA extraction. The environmental DNA was extracted from 0.5 g wet weight soil, using the Powersoil kit (MO BIO, Carsbad, CA, USA). The DNA quality and concentration were measured with a Qubit Fluorometer (Life Technology, Eugene, OR, USA).

Primer Design. A total of 384 primer sets (Table S2) were assayed, which included 356 primers that were designed, validated, and used in previous studies.[18,30] For the present study, 28 additional primer sets were designed targeting additional MGEs, β-lactamase, and multidrug resistance genes. The same design parameters and protocol were used as previously described[31] with alleles collected using the Fungene Pipeline Repository.[32]

QPCR Array. All quantitative PCR reactions were performed using a Wafergen SmartChip Real-time PCR system (Fremont, CA, USA) as reported previously.[19] Briefly, the Wafergen array allows for 5184 qPCR reactions each with a 100 nL volume to be run in parallel. Sample and primers were dispensed into the SmartChip using a Multisample Nanodispenser (Fremont, CA, USA). PCR cycling conditions and initial data processing were performed as described.[19] As previously described, ∼50% of tested assays had a detection limit lower than 10 genomic copies per reaction, and ∼85% of tested assays had a detection limit below 100 genomic copies per reaction, based on low volume qPCR and the primer design strategy.[31] Translation to absolute detection limit will vary based on sample matrices and the method of DNA extraction. Validation of ARG primer specificity has also been described previously,[18] including sequencing amplicons from 35 primer sets that commonly show positive amplification on the array.[33] In detail, over 98% of assembled amplicon reads (mapped to targeted references using FrameBot) were >90% identical to a reference sequence. A more detailed discussion on specificity, sensitivity, and limitations of the array is provided in the Supplemental Methods.

The mass of template DNA varied among samples (Table S1). To normalize for differences in DNA template quantities and PCR inhibitors that could influence amplification efficiency, estimated quantities were calculated as the relative abundance. This was calculated by dividing the estimated gene copies of the specific gene by estimated copies of the 16S rRNA gene. Gene copy numbers were estimated using the equation $10^{(30 - C_t)}/(10/3)$, where C_t equals the threshold cycle as described previously.[30] A threshold cycle of 30 was used as a cutoff to differentiate between true positive amplification and primer-dimers, which is more conservative than that previously used.[19]

The relative abundance and standard deviations were calculated using Microsoft Excel 2010 as previously described.[30] Genes detected in only one of the three technical replicates in each sample were considered false positives and were removed from analysis. Negative controls (no added DNA template) were also run on the array and those amplified were excluded.

Data Analysis. Multivariate analysis of the difference between ARG profiles in different samples was performed in R using the Vegan package.[34] Log_2 transformed percentage values of the gene abundances relative to the 16S rRNA gene were used for Redundancy Analysis. A Mantel test was conducted to test the spatial effect on the distribution of ARGs in soil with the Bray method in Vegan package. Canonical correspondence analysis (CCA) between the relative abundance of ARGs and environmental variables, analyzed as previously reported,[29] (Table S3) were performed followed by the Mantel test using Vegan package. Environmental variables were chosen on the basis of significance calculated from individual CCA results and variance inflation factors (VIFs)

DOI: 10.1021/acs.est.6b02863
Environ. Sci. Technol. 2016, 50, 12621−12629

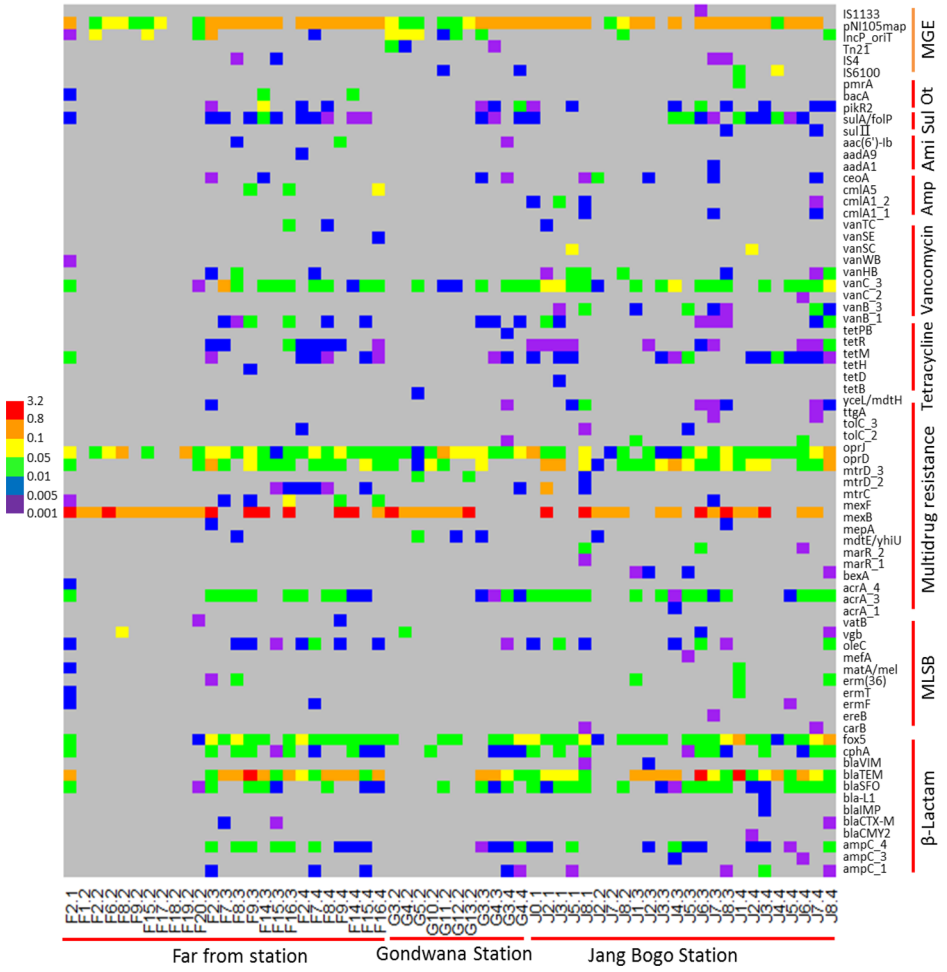


Figure 1. Gene profiles of 68 antibiotic resistance genes (ARGs) and six mobile genetic elements (MGE) detected in the Antarctic soils sampled near the Gondwana Station (1000 m radius) and Jang Bogo Station (500 m radius) and soils farther away (further than 1350 and 750 m, respectively) from the two stations. The heatmap shows soil samples as columns and detected ARGs as rows. The color scale indicates percent of ARG abundance values relative to the 16S rRNA gene. Gray indicates below the detection limit. MLSB, Macrolide-Lincosamide-Streptogramin B resistance; Amp, Amphenicol; Ami, Aminoglycoside; Sul, Sulfonamide; Ot, Others.

calculated during CCA. While microbial data (Table S3) were not available for the fourth year, correlation analysis between the Shannon index of bacteria and of ARGs was performed for the 3 years' samples. The Venn map was built in R with Vennerable package. Spearman correlation between abundances of ARG and MGE was performed with Sigmaplot 10.0. QPCR results from swine farms, park soils, sewage, and river and lake samples[18,19,35,36] (Table S4) were included to compare ARG occurrence among sample types and to assess validity of the qPCR array data. RDA ordination was performed using the Log transformed summarization of relative abundance for all primers targeting a given gene. All analyses were performed using R Studio v.3.1.3,[37] and the threshold for significance was selected at $p < 0.05$.

RESULTS AND DISCUSSION

ARGs in Antarctic Soils. ARGs were detected in all the Antarctic soil samples with the most frequently detected gene being *blaFOX (also fox5)* (Figure 1). The gene with the highest relative gene copy number as well as relative abundance was the *mexF* gene (Table S5). Overall, 67 ARGs were detected covering the eight major types of antibiotic resistances with the main ones conferring multiple drug resistances (Figures 1 and S2a). ARGs detected include four main resistance mechanisms, with the highest number classified as multidrug efflux pumps (Figure S2b). Contrary to a previous report in which only the *mefA/E* gene was detected in one of the Antarctic glacial samples,[17] the soil samples had a greater abundance of ARGs (Figure 1). The study by Segawa and coauthors[17] used only 96 TaqMan-based primer sets while our study used 384 SYBR-based primers. The different number of targets, coverage of primers, and sample matrices (surface snow versus underlying soil) confounds the comparison of results between the two studies.

Among the genes detected, seven ARGs (*blaTEM*, *blaSFO*, *blaFOX*, *cphA*, *mexF*, *oprD*, and *oprJ*) were frequently observed (Figure 1) and evenly represented in all soil samples (Figure S1), indicating that this Antarctic region is a reservoir for these genes or gene fragments. The *blaTEM* gene is one of the most widespread ARGs in the environment and is usually associated with *Enterobacteriaceae*.[38] The *blaFOX* gene is associated with *β*-lactam resistance[39] and is typically observed in Gram-negative enteric organisms (e.g., *E. coli* and *Klebsiella*). The *cphA* gene is intrinsic in the environmental isolates of *Aeromonas hydrophila* and *A. jandaei*.[40] The *mexF*, *oprD*, and *oprJ* genes are components of two operons (*mexC-mexD-oprJ* and *mexE-mexF-oprN*) of *Pseudomonas aeruginosa*, a clinically significant

DOI: 10.1021/acs.est.6b02863
Environ. Sci. Technol. 2016, 50, 12621−12629

pathogen but also in some soils. Overexpression of the two operons confers to cells resistance to fluoroquinolones, the "fourth-generation" cephems, tetracycline, imipenem, and chloramphenicol and hypersusceptibility to most other β-lactams.[41,42] *MexCD-OprJ* plays an important role in intrinsic multidrug resistance in wild-type *P. aeruginosa* which has resistance to many antimicrobial agents mediated by multidrug efflux pumps.[43] The *mexF* gene has previously been observed in many different environments, such as soil, sediments, river water, and drinking water.[44] It is also prevalent in pigs not exposed to antibiotics but, interestingly, is not found in pigs given antibiotic treatment.[45] The *mexF* and *oprD* genes are also proposed to naturally exist in water.[35]

Validity of the ARG qPCR array was demonstrated in comparing ARGs numbers detected in Antarctic soil samples with other samples types. As expected, lower detection frequency (proportion of qPCR positive assays to the total number of targeted assays) was found in the Antarctic soil than park soil, pig farms, sewage, and river water samples but was similar to those found in the Michigan lake water samples (Figure 2).[18,19,35,36] Furthermore, the number of ARGs

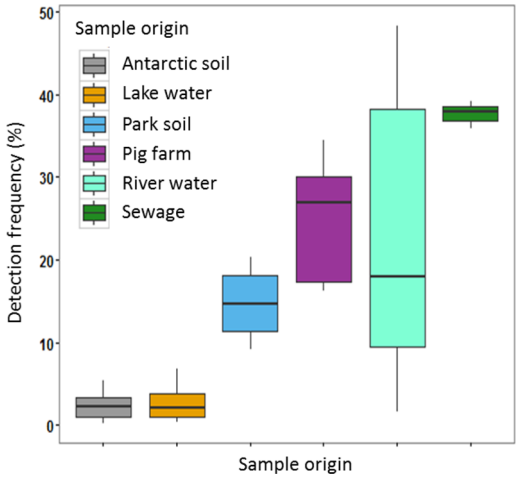


Figure 2. Comparison of detection frequency (proportion of qPCR positive assays to the total number of targeted assays) of antibiotic resistance genes detected in the Antarctic soils compared to published data from other sample types.[18,19,35,36]

detected in the Antarctic soils is similar to numbers detected in oligotrophic sample types using a different method, i.e., shotgun metagenomics. For example, between 9 and 38 ARGs were detected by this method in seven influent and effluent drinking water samples.[46] Regarding correspondence of individual genes, ARGs such as *mexF* (detected in 41 of 62 Antarctica soil samples using qPCR) were also detected in all seven drinking water samples via shotgun metagenomics.

MGEs in Antarctic Soil. MGEs were also detected in all of the tested soil samples. Overall, six out of 35 targeted MGEs were detected (Figure 1). Among these genes, the *pNI105* gene was detected in most of the soil samples (Figure 1). The *pNI105* gene has been described as a small plasmid, which is nontransmissible, but confers high-level resistance to kanamycin and neomycin.[47] However, ARGs associated with resistance to these antibiotics were not detected. The *Inc.P-1 oriT* genes were also found in some soil samples. The *Inc.P-1* plasmids harbor determinants for resistance to several groups of antibiotics, i.e., tetracyclines, quinolones, aminoglycosides, sulfonamides, β-lactams, and chemotherapeutics.[48−50] These plasmids have also been described as key plasmids in the horizontal transfer of ARGs of all classes in the environment, i.e., efflux pumps, enzymatic modification, or hydrolysis of the antibiotic molecule.[48] No correlation was observed between the relative abundance of MGEs and ARGs (Figure 3a); however,

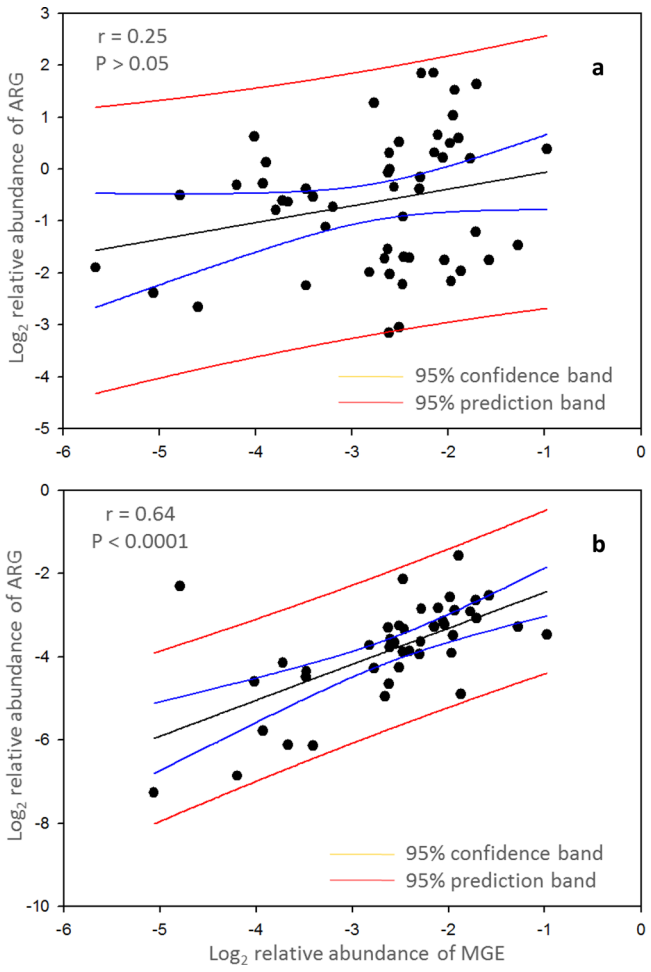


Figure 3. Relationship between the Log_2 transformed relative abundance of the antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in soil for (a) all the ARGs and (b) excluding the widely distributed genes: *blaTEM*, *blaSFO*, *blaFOX*, *cphA*, *mexF*, *oprD*, and *oprJ*.

exclusion of the seven ARGs present in most samples resulted in a significant positive correlation (Figure 3b). A high correlation between abundance of ARGs and levels of MGEs has been reported in farm samples,[18] which suggests that horizontal gene transfer may have aided dissemination of ARGs and, hence, may also have occurred at some point in their history for those genes currently in Antarctic soils.

Proximity of ARGs to Antarctic Stations. A redundancy analysis of relative abundance of detected ARGs in the soils based on geography was performed. Detected ARGs fell into three groups: soils near the Gondwana Station (1000 m radius) (GS), soils near the Jang Bogo Station (500 m radius) (JS), and soils farther away (further than 1350 and 750 m, respectively) from the two stations (FS) (Figure 4a). Redundancy analysis showed significant grouping; however, some soil samples were outliers, showing that other factors also affect the ARG distribution. Therefore, a CCA analysis was performed using

DOI: 10.1021/acs.est.6b02863
Environ. Sci. Technol. 2016, 50, 12621−12629

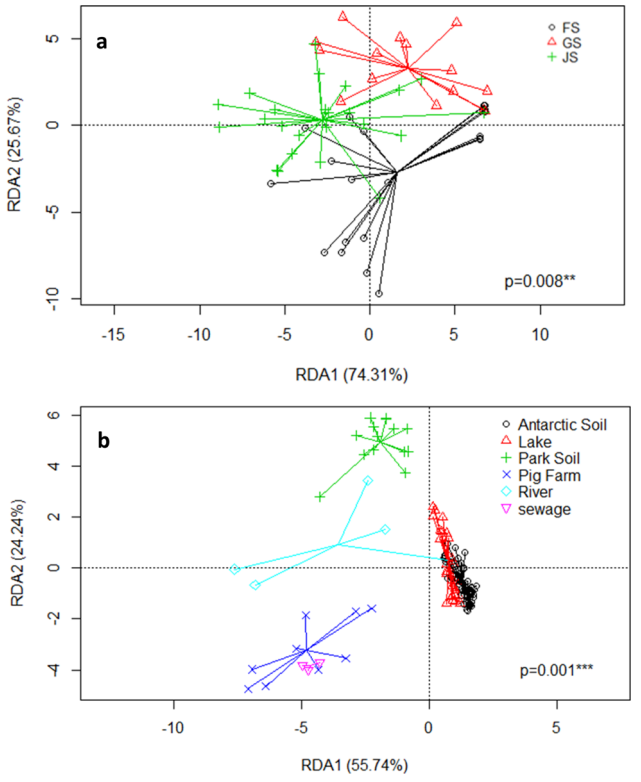


Figure 4. Redundancy analysis (RDA) of Log_2 transformed relative abundance of antibiotic resistance genes in the soil. (a) Soils sampled far from the stations (FS) and near the Gondwana Station (GS) and Jang Bogo Station (JS); *p* values indicate that ordination of ARGs based on proximity to stations is significant. (b) All the Antarctic soils and other samples from swine farms, park soils, sewage, and river and lake samples.[18,19,35,36] *p* values indicate that ordination of ARGs based on sample type is significant.

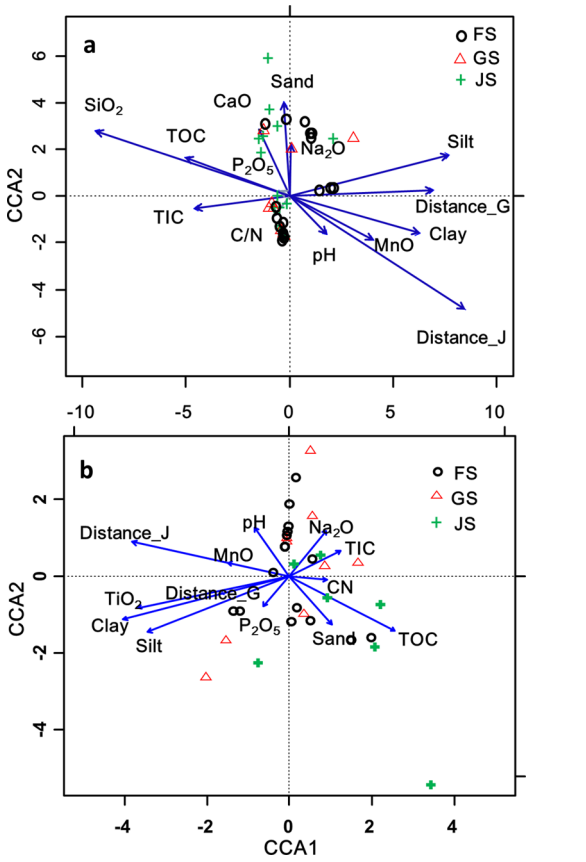


Figure 5. Canonical correspondence analysis (CCA) of sampling site distance to Gondwana Station (Distance_G) or Jang Bogo Station (Distance_J) and soil characteristics and the relative abundance of antibiotic resistance genes in soil (symbols) (a) excluding those with relatively high total organic contents (>0.9%) (b). Symbols are for soils.

25 soil parameters (Table S3), proximity to stations, and microbial community data (Figure 5). From the CCA analysis, distance to the research stations had two of the four highest correlations with relative abundance to ARGs (Figure 5a). A stronger correlation between relative abundance of ARGs and the proximity of the sampling site to Jang Bogo Station was observed compared to the proximity to Gondwana Station (Figure 5a), suggesting construction-based activities had a stronger effect on the distribution of ARGs in soil. A Mantel test showed that the correlation of proximity to both stations was not significant; however, when the seven ARGs presented in most of the samples were excluded, a significant correlation was observed between distance to the Jang Bogo Station and the relative abundance of ARGs in soil (Table S6). Redistribution of microbes containing ARGs might be attributed to increased dust as a result of plowing soil during construction of the Jang Bogo Station.

In total, 22 ARGs were shared within the three groups with seven and two ARGs exclusively found in FS and GS groups, respectively, whereas 23 ARGs were exclusively in the JS group (Figure S3). This also indicates that activity related to construction might contribute to ARGs solely observed near the Jang Bogo Station. More sites were sampled near the Jang Bogo Station (Figure 1), which may, however, influence this result. The seven ARGs exclusively observed in FS samples may be the result of a higher number of FS samples tested compared to GS samples or may be due to human activity resulting in organisms that do not harbor these genes.[51] A redundancy analysis of ARGs relative abundance showed that ARGs in the Antarctic soils are closer to those in the Michigan lake water sample[35] but are very different from park soil, pig farms, sewage, and river water samples (Figure 4b), which is expected due to level of anthropogenic influence on latter sample sets.

Influence of Soil Characteristics and Bacterial Diversity on ARGs. Soil physiochemical properties were also measured to examine links between ARG prevalence, edaphic factors, and bacterial community composition (Table S3). A positive correlation (R^2 = 0.39, p = 0.0001) was observed between bacterial Shannon index, measured via sequencing of 16S rRNA gene as previously described,[29] and Shannon index of ARGs in soil (Figure S4). This result implies that a more diverse bacterial population contributes to diversity of ARGs, an expected result.[52]

Soil properties also influenced the relative abundance of ARGs. Silt, clay, and SiO_2 were the top three soil attributes that influenced the relative abundance of ARGs (Figure 5a). Among them, silt showed a significant negative correlation with the relative abundance of ARGs in soils, whereas SiO_2 and clay only showed significant positive and negative correlations, respectively, when the seven widely distributed ARGs were excluded (Table S6). These results agree with previous reports that SiO_2 and soil texture are main factors shaping the soil bacterial community structure in the Terra Nova Bay.[29] Soil metals

DOI: 10.1021/acs.est.6b02863
Environ. Sci. Technol. 2016, 50, 12621−12629

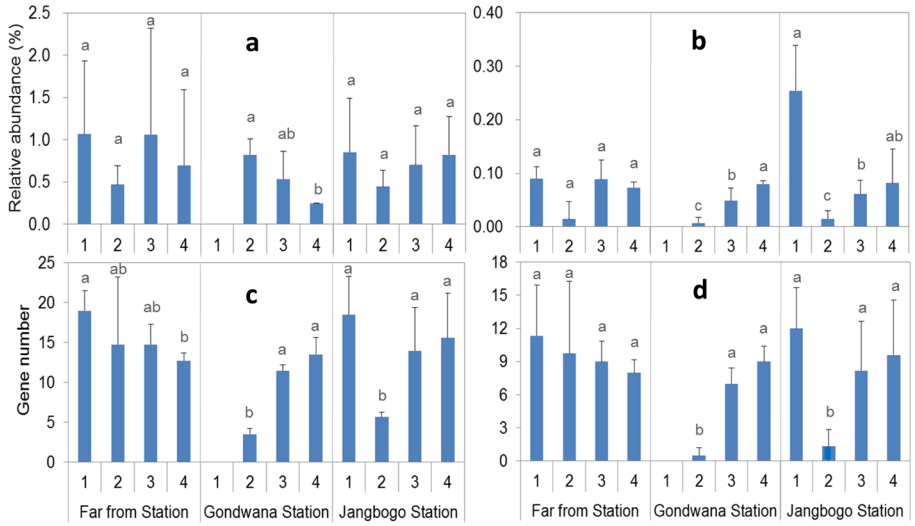


Figure 6. Relative abundance (a, b) and detected gene number (c, d) of antibiotic resistance genes (a, c) excluding the widely distributed *blaTEM*, *blaSFO*, *blaFOX*, *cphA*, *mexF*, *oprD* and *oprJ* (b, d) in soil sampled far from and near the Gondwana Station and Jang Bogo Station in the years of 2011 (1), 2012 (2), 2013 (3), and 2014 (4). The values, within one group, with different lower case letters were significantly different at $p < 0.05$.

(MnO) and pH also affected the distribution of ARGs (Figure 5a) with a significant correlation between pH and relative abundance of ARGs without the seven widely distributed ARGs (Table S6), which is expected as soil pH is a dominant factor affecting microbial community structure in this location.[29] Total organic carbon (TOC) may have an effect on the distribution of ARGs (Figure 5a); however, the Mantel test only showed a weak positive correlation when excluding the seven widely distributed ARGs (Table S6), which may be due to the wide variation of the soil TOC content in a few samples (Table S3). When the samples with relatively high TOC (>0.9%) were excluded, a very significant positive correlation was noted between TOC and relative abundance of ARGs without the seven widely distributed ARGs (Table S6) and TOC became the top soil attribute that influenced the relative abundance of ARGs (Figure 5b). Distance to Jangbogo Station, clay, and silt remained the main factors affecting the distribution of ARGs in soil (Figure 5b).

Temporal Abundance of ARGs in Antarctic Soil. The relative abundance of ARGs in soils sampled was also compared over 4 years. Due to the lower DNA concentrations in soils sampled in the second year (Table S1), lower relative ARG abundance and detected gene number were observed in almost all soils (Figure 6, Table S7). If the second year samples are excluded, both the average relative ARG abundance and detected gene numbers in the three groups of soils (FS, GS, and JS) were nearly constant during four years regardless of whether the seven ARGs present in most samples were excluded, indicating that bacteria harboring ARGs in the Antarctic soil are quite stable. Nevertheless, seven genes, *blaCMY2*, *blaIMP*, *bla-L1*, *mefA*, *marR_1*, *mexB*, and *sulII*, were exclusively found in soils sampled near and during the construction of the Jang Bogo station. Among these genes, *blaCMY2*, *blaIMP*, *marR_1*, and *mexB* are multidrug-resistant[53−56] and *blaCMY2*, *blaIMP*, and *bla-L1* are related to *β*-lactams.[54,57,58] The other genes are related to antibiotics that are critical for human medicine[59] including macrolide (*mefA*) and sulfonamide (*sulII*), implying that the emergence of these genes might be a consequence of the human activity during the construction of the Jang Bogo station (Figure S1).

Emergence and Spread of ARGs in Soil. The detected ARGs in Antarctic soil might come from several origins including (i) very long-term residence in Antarctic soils, i.e., (ii) a reservoir distributed by animals or birds, (iii) atmospheric transport, and (iv) human activity. Seven out of 67 ARGs were detected in most of the soil samples (Figure 1), indicating that Antarctic soils may be a reservoir for these genes. These findings support the growing body of evidence that ARGs are widespread in relatively pristine habitats which have not been exposed to antibiotic resistant pathogens or commercial sources of antibiotics.[13,15] In the Terra Nova Bay, the terrestrial surface is mainly composed of exposed bedrock and glacial moraines[29] and the microbial growth resources are limited. To survive in such a harsh environment, bacteria might produce antibiotics to inhibit or kill neighboring bacteria to acquire more resources.[5,13,14] On the other hand, ARGs might result from coselection by metals. Some ARGs have functions independent of resistance, such as efflux and electron transport, and will not always be associated with anthropogenic selective pressures.[60] It should also be noted that detection does not always represent activity in that (1) assays only detect gene fragments, not complete genes, (2) fragments might be from nonviable organisms or extracellular DNA, (3) resistance may only be conveyed if other required genes or other mechanisms are present, and (4) the gene(s) must be expressed and translated into a functional protein. Nonetheless, detection and quantitation of ARGs with gene specific primers is currently the most feasible way to assess ARG ecology and a first level to assess reservoirs and potential risk.

For dissemination related to animals or birds, a wide variety of life forms, such as sea birds, marine mammals, and invertebrates, have been observed in the Terra Nova Bay.[29] Antibiotic resistance was found in the Antarctic Indian ocean seawater,[34] and amphibians might transfer resistance genes from the seawater to the terrestrial surface. Birds might also transmit resistance genes from great distances, as Arctic terns may travel up to six continents and do spend winter in the Antarctic.[22]

The atmospheric transport and deposition might also contribute to the dissemination of ARGs to the Antarctic soil.

DOI: 10.1021/acs.est.6b02863
Environ. Sci. Technol. 2016, 50, 12621−12629

Airborne particulate matter facilitated dispersal of veterinary antibiotics and microbial communities containing ARGs.[28] Dissemination of ARGs through airborne particulate matter by atmospheric circulation from other continents to Antarctica might occur as evidenced by long-range transport of persistent organic pollutants from temperate to polar regions.[61]

Antarctic terrestrial ecosystem shifts in response to human activity and climate change have been reported.[62,63] Our results showed that the intensive human activity related to the construction of the Jang Bogo Research Station appears to have influenced the distribution of resistance genes in Antarctic soil (Figure 5a). However, this human activity did not significantly increase the relative abundance of ARGs in the soil over time (Figure 6). Previous studies have shown that limited contact (e.g., sporadic travelers, wild animals) produced an influx of resistant isolates and resistance genes in remote communities.[20] Resistance to quinolones was not detected in the Arctic bird isolates,[22] deep ocean isolates,[13] or the Antarctic soils tested in the present study (Figure 1); however, the presence of this resistance has been observed in urban environments.[64] Resistance can be developed by the endogenous microorganism through natural selection and adaptation to the harsh environment in the Antarctic and introduced from animals, birds, dust, or humans. Both long- and short-term human activity could also influence dissemination of ARGs in the Antarctic. As the Antarctic attracts more travelers,[63] the influence of human activity on ARGs in the Antarctic may increase.

Environmental Implications. Taken together, results show the presence of ARGs in Antarctic soils, which correlated with proximity to the research stations. Relative abundance of ARGs in the Antarctic soils also correlated with microbial biodiversity and select soil characteristics including silt, clay, TOC, and SiO_2. The natural resistome also serves as a reservoir for ARGs, which can be altered via anthropogenic activities. Intense human activity during construction of the Jang Bogo Research Station appears to influence ARGs in soil; however, temporal results showed that this activity did not significantly increase the overall level of ARGs in soil. Overall, this study documents the resistome of Antarctic soils and should serve as a global baseline data for future comparisons and understating the impacts of humans on the environmental resistome.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b02863.

Soil sampling locations, primer sets, physiochemical property of soil and biodiversity of bacteria, resistance genes classification, shared number of ARGs, maximum, median and average of relative abundance of 16S rRNA genes, ARGs and MGEs, information for different sources of samples, and relative abundance of ARGs in different years (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +82-31-670-4707; fax: +82-31-675-3108; e-mail: woojunsul@gmail.com.

Author Contributions

⊥F.W., R.D.S., and O.-S.K. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Dr. Ji Hee Kim, Dr. Joohan Lee, and Ms. Hyunjoo Noh for soil sampling and Dr. Teotonio Soares de Carvalho for help on data analysis. Soil sampling was permitted by the Ministry of Foreign Affairs, Republic of Korea. The research was funded in part by the Center for Health Impacts of Agriculture (CHIA) at Michigan State University and by the Korea Polar Research Institute (Grants PE16020, PE16070, and PP15101). F.W. was partially supported by the Outstanding Youth Fund of Natural Science Foundation of Jiangsu (BK20150050), National Natural Science Foundation of China (21677149), and the Institute of Soil Science, Chinese Academy of Sciences (ISSASIP1616). R.D.S. was partially supported by the Superfund Research Program (2 P42 ES004911-22A1) from the National Institute for Environmental Health Sciences.

■ REFERENCES

(1) McKenna, M. Antibiotic resistance: The last resort. *Nature* **2013**, *499* (7459), 394−396.

(2) WHO (World Health Organization). *The evolving threat of antimicrobial resistance: Options for action*; WHO: Geneva, Switzerland, 2012.

(3) Smillie, C. S.; Smith, M. B.; Friedman, J.; Cordero, O. X.; David, L. A.; Alm, E. J. Ecology drives a global network of gene exchange connecting the human microbiome. *Nature* **2011**, *480* (7376), 241−244.

(4) Forsberg, K. J.; Reyes, A.; Wang, B.; Selleck, E. M.; Sommer, M. O. A.; Dantas, G. The shared antibiotic resistome of soil bacteria and human pathogens. *Science* **2012**, *337* (6098), 1107−1111.

(5) Allen, H. K.; Donato, J.; Wang, H. H.; Cloud-Hansen, K. A.; Davies, J.; Handelsman, J. Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* **2010**, *8* (4), 251−259.

(6) Forsberg, K. J.; Patel, S.; Gibson, M. K.; Lauber, C. L.; Knight, R.; Fierer, N.; Dantas, G. Bacterial phylogeny structures soil resistomes across habitats. *Nature* **2014**, *509* (7502), 612−616.

(7) Fahrenfeld, N.; Knowlton, K.; Krometis, L. A.; Hession, W. C.; Xia, K.; Lipscomb, E.; Libuit, K.; Green, B. L.; Pruden, A. Effect of manure application on abundance of antibiotic resistance genes and their attenuation rates in soil: field-scale mass balance approach. *Environ. Sci. Technol.* **2014**, *48* (5), 2643−2650.

(8) Gündoğan, N.; Citak, S.; Turan, E. Slime production, DNase activity and antibiotic resistance of *Staphylococcus aureus* isolated from raw milk, pasteurised milk and ice cream samples. *Food Control* **2006**, *17* (5), 389−392.

(9) Jadhav, A.; Shanmugham, B.; Rajendiran, A.; Pan, A. Unraveling novel broad-spectrum antibacterial targets in food and waterborne pathogens using comparative genomics and protein interaction network analysis. *Infect., Genet. Evol.* **2014**, *27*, 300−308.

(10) Xi, C.; Zhang, Y.; Marrs, C. F.; Ye, W.; Simon, C.; Foxman, B.; Nriagu, J. Prevalence of antibiotic resistance in drinking water treatment and distribution systems. *Appl. Environ. Microbiol.* **2009**, *75* (17), 5714−5718.

(11) Amos, G. C.; Zhang, L.; Hawkey, P. M.; Gaze, W. H.; Wellington, E. Functional metagenomic analysis reveals rivers are a reservoir for diverse antibiotic resistance genes. *Vet. Microbiol.* **2014**, *171* (3), 441−447.

(12) Li, B.; Vellidis, G.; Liu, H.; Jay-Russell, M.; Zhao, S.; Hu, Z.; Wright, A.; Elkins, C. A. Diversity and antimicrobial resistance of *Salmonella enterica* isolated from surface water in southeastern United States. *Appl. Environ. Microbiol.* **2014**, *80* (20), 6355−6365.

(13) Brown, M.; Balkwill, D. Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface. *Microb. Ecol.* **2009**, *57* (3), 484−493.

DOI: 10.1021/acs.est.6b02863
Environ. Sci. Technol. 2016, 50, 12621−12629

(14) D'Costa, V. M.; Griffiths, E.; Wright, G. D. Expanding the soil antibiotic resistome: exploring environmental diversity. *Curr. Opin. Microbiol.* **2007**, *10* (5), 481−489.
(15) Nonaka, L.; Isshiki, T.; Suzuki, S. Distribution of the oxytetracycline resistance determinant Tet 34 among bacteria isolated from diseased fish. *Microbes Environ.* **2002**, *17* (1), 26−31.
(16) Wouters, B.; Martin-Español, A.; Helm, V.; Flament, T.; van Wessem, J.; Ligtenberg, S.; van den Broeke, M.; Bamber, J. Dynamic thinning of glaciers on the Southern Antarctic Peninsula. *Science* **2015**, *348* (6237), 899−903.
(17) Segawa, T.; Takeuchi, N.; Rivera, A.; Yamada, A.; Yoshimura, Y.; Barcaza, G.; Shinbori, K.; Motoyama, H.; Kohshima, S.; Ushida, K. Distribution of antibiotic resistance genes in glacier environments. *Environ. Microbiol. Rep.* **2013**, *5* (1), 127−134.
(18) Zhu, Y.; Johnson, T. A.; Su, J.; Qiao, M.; Guo, G.; Stedtfeld, R. D.; Hashsham, S. A.; Tiedje, J. M. Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110* (9), 3435−3440.
(19) Wang, F. H.; Qiao, M.; Su, J. Q.; Chen, Z.; Zhou, X.; Zhu, Y. G. High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation. *Environ. Sci. Technol.* **2014**, *48* (16), 9079−9085.
(20) Bartoloni, A.; Pallecchi, L.; Rodríguez, H.; Fernandez, C.; Mantella, A.; Bartalesi, F.; Strohmeyer, M.; Kristiansson, C.; Gotuzzo, E.; Paradisi, F.; Rossolini, G. M. Antibiotic resistance in a very remote Amazonas community. *Int. J. Antimicrob. Agents* **2009**, *33* (2), 125−129.
(21) Gilliver, M. A.; Bennett, M.; Begon, M.; Hazel, S. M.; Hart, C. A. Enterobacteria: Antibiotic resistance found in wild rodents. *Nature* **1999**, *401* (6750), 233−234.
(22) Sjölund, M.; Bonnedahl, J.; Hernandez, J.; Bengtsson, S.; Cederbrant, G.; Pinhassi, J.; Kahlmeter, G.; Olsen, B. Dissemination of multidrug-resistant bacteria into the Arctic. *Emerging Infect. Dis.* **2008**, *14* (1), 70−72.
(23) Mangano, S.; Michaud, L.; Caruso, C.; Giudice, A. L. Metal and antibiotic resistance in psychrotrophic bacteria associated with the Antarctic sponge *Hemigellius pilosus* (Kirkpatrick, 1907). *Polar Biol.* **2014**, *37* (2), 227−235.
(24) Chen, B.; Yang, Y.; Liang, X.; Yu, K.; Zhang, T.; Li, X. Metagenomic profiles of antibiotic resistance genes (ARGs) between human impacted estuary and deep ocean sediments. *Environ. Sci. Technol.* **2013**, *47* (22), 12753−12760.
(25) Kieser, T.; Bibb, M.; Buttner, M.; Chater, K.; Hopwood, D. *Practical Streptomyces Genetics*; The John Innes Foundation: Norwich, UK, 2000.
(26) D'Costa, V. M.; McGrann, K. M.; Hughes, D. W.; Wright, G. D. Sampling the antibiotic resistome. *Science* **2006**, *311* (5759), 374−377.
(27) D'Costa, V. M.; King, C. E.; Kalan, L.; Morar, M.; Sung, W. W.; Schwarz, C.; Froese, D.; Zazula, G.; Calmels, F.; Debruyne, R.; et al. Antibiotic resistance is ancient. *Nature* **2011**, *477* (7365), 457−461.
(28) McEachran, A. D.; Blackwell, B. R.; Hanson, J. D.; Wooten, K. J.; Mayer, G. D.; Cox, S. B.; Smith, P. N. Antibiotics, bacteria, and antibiotic resistance genes: aerial transport from cattle feed yards via particulate matter. *Environ. Health Perspect.* **2015**, *123* (4), 337−343.
(29) Kim, M.; Cho, A.; Lim, H. S.; Hong, S. G.; Kim, J. H.; Lee, J.; Choi, T.; Ahn, T. S.; Kim, O. S. Highly heterogeneous soil bacterial communities around Terra Nova Bay of Northern Victoria Land, Antarctica. *PLoS One* **2015**, *10* (3), e0119966.
(30) Looft, T.; Johnson, T. A.; Allen, H. K.; Bayles, D. O.; Alt, D. P.; Stedtfeld, R. D.; Sul, W. J.; Stedtfeld, T. M.; Chai, B.; Cole, J. R. In-feed antibiotic effects on the swine intestinal microbiome. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (5), 1691−1696.
(31) Stedtfeld, R. D.; Baushke, S. W.; Tourlousse, D. M.; Miller, S. M.; Stedtfeld, T. M.; Gulari, E.; Tiedje, J. M.; Hashsham, S. A. Development and experimental validation of a predictive threshold cycle equation for quantification of virulence and marker genes by high-throughput nanoliter-volume PCR on the openarray platform. *Appl. Environ. Microbiol.* **2008**, *74* (12), 3831−3838.
(32) Fish, J. A.; Chai, B.; Wang, Q.; Sun, Y.; Brown, C. T.; Tiedje, J. M.; Cole, J. R. FunGene: the functional gene pipeline and repository. *Front. Microbiol.* **2013**, *4* (4), 291.
(33) Johnson, T. A.; Stedtfeld, R. D.; Wang, Q.; Cole, J. R.; Hashsham, S. A.; Looft, T.; Zhu, Y.-G.; Tiedje, J. M. Clusters of antibiotic resistance genes enriched together stay together in swine agriculture. *mBio* **2016**, *7* (2), 2214−2215.
(34) Oksanen, J.; Blanchet, F.; Kindt, R.; Legendre, P.; Minchin, P.; O'Hara, R. *vegan: Community Ecology Package*; R package version 3.1.3; 2014; http://CRAN.R-project.org/package=vegan.
(35) Stedtfeld, R. D.; Williams, M.; Fakher, U.; Johnson, T. A.; Stedtfeld, T. M.; Wang, F.; Khalife, W. T.; Hughes, M.; Etchebarne, B.; Tiedje, J. M. Antimicrobial resistance (AR) dashboard application for mapping environmental occurrence and resistant pathogens. *FEMS Microbiol. Ecol.* **2016**, *92* (3), fiw020.
(36) Ouyang, W. Y.; Huang, F. Y.; Zhao, Y.; Li, H.; Su, J. Q. Increased levels of antibiotic resistance in urban stream of Jiulongjiang River, China. *Appl. Microbiol. Biotechnol.* **2015**, *99* (13), 5697−5707.
(37) R. Team *RStudio: integrated development for R*; RStudio, Inc.: Boston, MA, 2014; http://www.RStudio.com/ide.
(38) Narciso-da-Rocha, C.; Varela, A. R.; Schwartz, T.; Nunes, O. C.; Manaia, C. M. bla_{TEM} and *vanA* as indicator genes of antibiotic resistance contamination in a hospital−urban wastewater treatment plant system. *J. Glob. Antimicrob. Re.* **2014**, *2* (4), 309−315.
(39) Gonzalez Leiza, M.; Perez-Diaz, J. C.; Ayala, J.; Casellas, J. M.; Martinez-Beltran, J.; Bush, K.; Baquero, F. Gene sequence and biochemical characterization of FOX-1 from *Klebsiella pneumoniae*, a new AmpC-type plasmid-mediated beta-lactamase with two molecular variants. *Antimicrob. Agents Chemother.* **1994**, *38* (9), 2150−2157.
(40) Balsalobre, L. C.; Dropa, M.; Lincopan, N.; Mamizuka, E. M.; Matté, G. R.; Matté, M. H. Detection of metallo-β-lactamases-encoding genes in environmental isolates of *Aeromonas hydrophila* and *Aeromonas jandaei*. *Lett. Appl. Microbiol.* **2009**, *49* (1), 142−145.
(41) Gotoh, N.; Tsujimoto, H.; Tsuda, M.; Okamoto, K.; Nomura, A.; Wada, T.; Nakahashi, M.; Nishino, T. Characterization of the *MexC-MexD-OprJ* multidrug efflux system in Δ*mexA-mexB-oprM* mutants of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **1998**, *42* (8), 1938−1943.
(42) Piddock, L. J. Multidrug-resistance efflux pumps-not just for resistance. *Nat. Rev. Microbiol.* **2006**, *4* (8), 629−636.
(43) Morita, Y.; Murata, T.; Mima, T.; Shiota, S.; Kuroda, T.; Mizushima, T.; Gotoh, N.; Nishino, T.; Tsuchiya, T. Induction of *mexCD-oprJ* operon for a multidrug efflux pump by disinfectants in wild-type *Pseudomonas aeruginosa* PAO1. *J. Antimicrob. Chemother.* **2003**, *51* (4), 991−994.
(44) De Souza, M.-J.; Nair, S.; Bharathi, P. L.; Chandramohan, D. Metal and antibiotic-resistance in psychrotrophic bacteria from Antarctic marine waters. *Ecotoxicology* **2006**, *15* (4), 379−384.
(45) Götz, A.; Pukall, R.; Smit, E.; Tietze, E.; Prager, R.; Tschäpe, H.; van Elsas, J. D.; Smalla, K. Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. *Appl. Environ. Microbiol.* **1996**, *62* (7), 2621−2628.
(46) Li, B.; Yang, Y.; Ma, L.; Ju, F.; Guo, F.; Tiedje, J. M.; Zhang, T. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. *ISME J.* **2015**, *9* (11), 2490−2502.
(47) San Millan, A.; Heilbron, K.; MacLean, R. C. Positive epistasis between co-infecting plasmids promotes plasmid survival in bacterial populations. *ISME J.* **2014**, *8* (3), 601−612.
(48) Popowska, M.; Krawczyk-Balska, A. Broad-host-range IncP-1 plasmids and their resistance potential. *Front. Microbiol.* **2013**, *4*, 1−8.
(49) Sen, D.; Van der Auwera, G. A.; Rogers, L. M.; Thomas, C. M.; Brown, C. J.; Top, E. M. Broad-Host-Range plasmids from agricultural soils have IncP-1 backbones with diverse accessory genes. *Appl. Environ. Microbiol.* **2011**, *77* (22), 7975−7983.
(50) Heuer, H.; Krögerrecklenfort, E.; Wellington, E.; Egan, S.; van Elsas, J.; van Overbeek, L.; Collard, J.-M.; Guillaume, G.; Karagouni, A.; Nikolakopoulou, T.; et al. Gentamicin resistance genes in

DOI: 10.1021/acs.est.6b02863
Environ. Sci. Technol. 2016, 50, 12621−12629

environmental bacteria: prevalence and transfer. *FEMS Microbiol. Ecol.* **2002**, *42* (2), 289−302.
(51) Narciso-da-Rocha, C.; Manaia, C. M. Multidrug resistance phenotypes are widespread over different bacterial taxonomic groups thriving in surface water. *Sci. Total Environ.* **2016**, *563*−*564*, 1−9.
(52) Di Cesare, A.; Eckert, E. M.; D'Urso, S.; Bertoni, R.; Gillan, D. C.; Wattiez, R.; Corno, G. Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. *Water Res.* **2016**, *94*, 208−214.
(53) Li, X.-Z.; Nikaido, H.; Poole, K. Role of MexA-MexB-OprM in Antibiotic Efflux in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **1995**, *39* (9), 1948−1953.
(54) Senda, K.; Arakawa, Y.; Ichiyama, S.; Nakashima, K.; Ito, H.; Ohsuka, S.; Shimokata, K.; Kato, N.; Ohta, M. PCR detection of Metallo-β-Lactamase gene (bla_{IMP}) in gram-negative rods resistant to Broad-Spectrum β-Lactams. *J. Clin. Microbiol.* **1996**, *34* (12), 2909−2913.
(55) Terán, W.; Felipe, A.; Segura, A.; Rojas, A.; Ramos, J.-L.; Gallegos, M.-T. Antibiotic-dependent induction of *Pseudomonas putida* DOT-T1E TtgABC efflux pump is mediated by the drug binding repressor TtgR. *Antimicrob. Agents Chemother.* **2003**, *47* (10), 3067−3072.
(56) Perera, I. C.; Grove, A. Molecular mechanisms of ligand-mediated attenuation of DNA binding by MarR family transcriptional regulators. *J. Mol. Cell. Biol.* **2010**, *2* (5), 243−254.
(57) Zaidi, M. B.; Leon, V.; Canche, C.; Perez, C.; Zhao, S.; Hubert, S. K.; Abbott, J.; Blickenstaff, K.; McDermott, P. F. Rapid and widespread dissemination of multidrug-resistant bla_{CMY-2} *Salmonella* Typhimurium in Mexico. *J. Antimicrob. Chemother.* **2007**, *60* (2), 398−401.
(58) Okazaki, A.; Avison, M. B. Induction of L1 and L2 β-Lactamase production in *Stenotrophomonas maltophilia* is dependent on an AmpR-type regulator. *Antimicrob. Agents Chemother.* **2008**, *52* (4), 1525−1528.
(59) CIAHM. *Report of the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance*; WHO: Geneva, 2011.
(60) Dietrich, L. E.; Teal, T. K.; Price-Whelan, A.; Newman, D. K. Redox-active antibiotics control gene expression and community behavior in divergent bacteria. *Science* **2008**, *321* (5893), 1203−1206.
(61) Cabrerizo, A.; Dachs, J.; Barceló, D.; Jones, K. C. Influence of organic matter content and human activities on the occurrence of organic pollutants in Antarctic soils, lichens, grass, and mosses. *Environ. Sci. Technol.* **2012**, *46* (3), 1396−1405.
(62) Convey, P.; Smith, R. I. L. Responses of terrestrial Antarctic ecosystems to climate change. *Plant Ecol.* **2006**, *182* (2), 1−10.
(63) Kennicutt, M. C., II; Klein, A.; Montagna, P.; Sweet, S.; Wade, T.; Palmer, T.; Sericano, J.; Denoux, G. Temporal and spatial patterns of anthropogenic disturbance at McMurdo Station, Antarctica. *Environ. Res. Lett.* **2010**, *5* (3), 533−534.
(64) Bartoloni, A.; Pallecchi, L.; Fiorelli, C.; Di Maggio, T.; Fernandez, C.; Villagran, A. L.; Mantella, A.; Bartalesi, F.; Strohmeyer, M.; Bechini, A.; Gamboa, H.; Rodriguez, H.; Kristiansson, C.; Kronvall, G.; Gotuzzo, E.; Paradisi, F.; Rossolini, G. M. Increasing resistance in commensal *Escherichia coli*, Bolivia and Peru. *Emerging Infect. Dis.* **2008**, *14* (2), 338−340.

DOI: 10.1021/acs.est.6b02863
Environ. Sci. Technol. 2016, 50, 12621−12629