Functional Characterization of Bacteria Isolated from Ancient Arctic Soil Exposes Diverse Resistance Mechanisms to Modern Antibiotics

Functional characterization of bacteria isolated from ancient Arctic soil exposes diverse resistance mechanisms to modern antibiotics

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Abstract

Using functional metagenomics to study the resistomes of bacterial communities isolated from different layers of the Canadian high Arctic permafrost, we show that microbial communities harbored diverse resistance mechanisms at least 5,000 years ago. Among bacteria sampled from the ancient layers of a permafrost core, we isolated eight genes conferring clinical levels of resistance against aminoglycoside, β-lactam and tetracycline antibiotics that are naturally produced by microorganisms. Among these resistance genes, four also conferred resistance against amikacin, a modern semi-synthetic antibiotic that does not naturally occur in microorganisms. In bacteria sampled from the overlaying active layer, we isolated ten different genes conferring resistance to all six antibiotics tested in this study, including aminoglycoside, β-lactam and tetracycline variants that are naturally produced by microorganisms as well as semi-synthetic variants produced in the laboratory. On average, we found that resistance genes found in permafrost bacteria conferred lower levels of resistance against clinically relevant antibiotics than resistance genes sampled from the active layer. Our results demonstrate that antibiotic resistance genes were functionally diverse prior to the anthropogenic use of antibiotics, contributing to the evolution of natural reservoirs of resistance genes.

Introduction

The evolution and spread of antibiotic resistance in pathogenic bacteria is one of the most urgent challenges in public health today [1,2]. Although resistance genes are now widespread in most microbial communities [3,4,5,6,7], whether the extensive diversity of resistance in environmental reservoirs and pathogenic bacteria is the result of human activity is controversial [8,9]. Rare studies considering multiple clinical isolates that pre-date the anthropogenic use of antibiotics show that resistance was uncommon in pathogenic bacteria such as *Salmonella*, *Klebsiella*, and *Escherichia* [10,11]. Furthermore, the novel evolution of antibiotic resistance through genetic mutations is well documented in clinical [12,13] and laboratory [14,15] populations of bacteria [16]. Similarly, microbial communities found in human-impacted environments such as water streams surrounding hospitals tend to show high levels of resistance genes [17,18]. Thus, the impact of antibiotic pollution on microbial communities is undeniable [19].

However, most antibiotics used in medicine today are derived from biomolecules and secondary metabolites produced by soil-dwelling microorganisms [20]. While the biosynthesis and the role of antibiotics in microbial ecosystems are a matter of active investigation [21,22], even small concentrations of antibiotic substance can lead to the evolution of high-level resistance in laboratory environments [23]. Therefore, many specialized resistance genes likely evolved in response to the natural production of antibiotics in microorganisms [24]. Indeed, genomic and phylogenetic analyses of β-lactamases, a group of enzymes that degrade penicillin and other β-lactam antibiotics, predict that precursors of the enzymes originated and diversified in bacteria millions of years ago [25,26]. The presence of antibiotic-resistant phenotypes in populations of bacteria isolated from human activity strongly supports this hypothesis [6,27].

In attempt to directly study bacteria from the pre-antibiotic era, there has been an increased interest in studying microbial communities found in ancient glaciers and permafrost [28]. Under thick layers of ice and soil, bacteria found in permafrost have been unaffected by physical and biological factors experienced at the surface for thousands of years [29]. Given current coring and sampling methods, it is now possible to extract from such ancient milieus culturable cells or DNA free from surface contaminants [30,31,32]. Using an extensive series of PCR-based analyses, D'Costa and colleagues conducted a metagenomic survey of ancient Alaskan soil looking for the presence of resistance genes' molecular signatures [33]. The authors found multiple DNA fragments with similarity to genes associated with resistance against tetracyclines, β-lactams and vancomycin in modern bacteria, confirming that genes homologous to resistance genes existed in ancient bacteria [33].

DNA fragments, however, cannot confirm functional resistance against antibiotics, let alone whether they protect against the clinical use of antibiotics, for two main reasons [34]. First, the presence of DNA fragments similar to the sequence of known resistance genes is not sufficient to ensure the functional expression of a resistance phenotype [35].Second, the sequences of many resistance genes show high levels of similarity to genes that carry out other cellular functions [19]. For example, bacterial efflux pumps of the resistance-nodulation-division (RND) superfamily can confer resistance to antibiotics, transport hydrophobic proteins involved in cell division and nodulation, or both [36]. Likewise, activation of certain RND-type efflux pumps in Gram-negative bacteria can confer sub-clinical resistance against quinolones, a synthetic group of antibiotics, under stress conditions [37]. Furthermore, analyses based on DNA sequence identity solely are limited to known resistance genes [3], ignoring the wide array of other resistance mechanisms that could have existed in the past. Although unspecified resistance genes from ancient bacteria are less likely to be of immediate concern in medical practice, they may have

provided the necessary advantage for specific bacterial lineages to survive up to this day, contributing to the development of resistance reservoirs [6].

The functional characterization of resistance genes found in ancient permafrost environments would provide a unique window on the origins and evolution of antibiotic resistance in bacteria [8,9]. A previous study of ancient Siberian soils reported the presence of resistance to aminoglycoside, chloramphenicol and tetracyclines among Gram-positive and Gram-negative bacteria [38], but the results were met with serious criticisms [33]. Here, we use functional metagenomics to revive resistance genes associated with culturable bacteria collected from a single 14-m deep core of the Canadian high Arctic permafrost (Figure 1A). Even though limited in scope, the study of culturable bacteria from a single permafrost core provides information on the diversity and possible sources of antibiotic resistance genes prior to the introduction of antibiotics in modern medicine.

Materials and Methods

Isolation of Bacteria Strains. A single core of the Canadian high Arctic was collected in May 2003 at Eureka (80°0.029 N, 85°50.367 W85°50.367 W), Ellesmere Island, Nunavut, Canada (Figure 1A) [39]. The field research was done with the permission of the Government of Nunavut through a Territorial Scientific Research Licence to Prof. Whyte (McGill University) as issued on an annual basis by the Nunavut Research Institute from 2003 to 2012. Core Eur3 covers a depth of 14 meters and includes the active layer of the Arctic soil as well as permafrost (Figure 1B). The geology of the core is related to sediments from the late Meistocene and subsequent Holocene and was estimated to be between 5,000 and 6,000 years old [39]. Permafrost is ground that remains at or below 0ºC for at least two years [28]. During summer months, air temperatures

rise above 0°C producing thaw of a thin layer at the ground surface, called the active layer. The boundary between the active layer and permafrost is the permafrost table. The permafrost table acts as a physical and biogeochemical barrier that limits infiltration of both surface water and external environmental factors [29]. At the Ellesmere Island site where the permafrost samples were obtained, the active layer reaches a depth of ~ 50-60 cm (which has been and is readily measured with a permafrost probe) and where the underlying permafrost has an ambient and very stable temperature of -16ºC [39]. Hence, there would be extremely little if any migration of microorganisms from the active layer through the permafrost table into the underlying permafrost over the last 5000 years. On the other hand there would be movement of bacteria throughout the active layer when it is thawed.

Material was collected from each 1-m subsection of the core using the sterility controls and authenticity methods described previously [30]. Culturable heterotrophic bacteria were isolated from 5 g of each subcore using the serial plate dilution as described in [39]. Plates were incubated at 37, 25 and 5 °C until growth of new colonies was no longer detected. For each strain, we sequenced the first 800 base pairs of the16S rRNA gene amplified using primers 27F and 1492R (Table S1). The phylogenic profile of each strain was determined using SeqMatch from the Ribosomal Database Project release 10 [40]. The phylogenetic profiling of five strains was confirmed in two independent laboratories. The GenBank accession numbers of the 16S rRNA sequences are presented in Table S4 and S5 when available.

Construction of Metagenomic Libraries. All bacterial strains were grown in 200 μL of Tryptic Soil Broth for 72 hours at room temperature before cells were harvested by centrifugation and DNA was extracted using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories,

Carlsbad, CA USA). We then combined in equal proportion the DNA of all strains derived from the active layer into a single pool (hereafter referred to as "AL" for active layer) and all strains derived from the permafrost into another pool (hereafter referred to as "P" for permafrost). We constructed one metagenomics library for each pool according to the protocols described in [3]. Briefly, 10 μg of DNA were sheared using the automated Covaris S220 DNA shearer instrument using miniTUBE clear (Covaris, Woburn, MA USA) tubes and protocol for shearing of 1.5-2.5 kp fragments. Sheared DNA was end-repaired using the End-It repair kit (Epicentre, Madison, WI USA) and size-selected (1200-4000 bp) by electrophoresis through a 1% low melting point agarose gel in 1.0 X TE buffer. Size-selected and end-repaired DNA fragments were then ligated into the linearized pZE21 MCS 1 vector (Table S1) at the HincII site using the Fast Link Ligation kit (Epicentre). The ligation product was purified and resuspended in sterile deionized water before being transformed by electroporation into 20 μL of *E. coli* MegaX DH10B cells (Invitrogen, Grand Island, NY USA). Transformed cells were recovered in 1 mL of SOC medium (Invitrogen) and incubated with vigorous shaking for one hours at 37 °C. Libraries were diluted by plating out 1 μL, 0.1 and 0.01 μL of recovered cells onto LB agar plates containing 50 μg/mL kanamycin. For each library, insert size distribution was estimated by gel electrophoresis of PCR products obtained by amplifying the insert of twelve colonies using primers flanking the multiple cloning site of the pZE21 MCS1 vector (Table S1). The total size of each library was determined by multiplying the average PCR based insert size with the number of colony forming units (CFU) in a given library and varied between 2-5 X 10^9 base pairs. The rest of the recovered cells were inoculated into 9 mL of LB containing 50 μg/mL kanamycin and amplified over night at 37 °C. The overnight cultures were frozen with 20% glycerol and kept at -80 °C before subsequent analyses. We constructed a negative control library using the genomic DNA of the antibiotic-

182 sensitive *E. coli* strain MegaX DH10B (Invitrogen) to screen for the possibility of exogenous
183 antibiotic resistance gene contamination during our manipulations. No resistance was observed in
184 the negative control library at any time during this study.

185

186 **Screening for Antibiotic Resistance.** For each library, we plated 100 μL of library freezer stock,
187 corresponding to 1.0 x 10^7 CFU, onto LB agar plates containing binary combinations of
188 kanamycin (50 μg/mL) and one of the six antibiotics described in Table 1. Each antibiotic was
189 supplemented to the media at the minimal concentration required to completely inhibit the growth
190 of the control library (Table 1). We incubated the plates at 37 °C and recorded growth after 24
191 hours. Depending on library size, each unique clone in the libraries screened was plated out in 10-
192 100 copies, ensuring that every clone containing an insert conferring resistance was likely to be
193 sampled. When growth was observed, five colonies were randomly picked and streaked onto LB
194 plates containing kanamycin and the same antibiotic on which they were selected. We confirmed
195 that the inserts indeed conferred resistance by extracting and transforming each plasmid construct
196 into new cultures of *E. coli* MegaX DH10B cells (Invitrogen). For each resistance clone, a single
197 colony was picked and stored at -80 °C in 20% glycerol for later analyses.

198

199 **Measuring Resistance and Cross-Resistance.** To measure the level of resistance and cross-
200 resistance, we determined the minimal inhibitory concentration (MIC) of each clone against all
201 six antibiotics. For each antibiotic, MIC is defined as the lowest concentration of that antibiotic
202 that inhibits at least 90% of the bacteria's normal growth and was estimated from the mode of
203 four sets of replicates challenged with a dilution of twelve antibiotic concentrations: 0.0; 0.5, 1.0;
204 2.0; 4.0; 8.0; 16.0; 32.0; 64.0; 128.0; 256.0; 512.0; and 1024.0 μg/mL. After incubation at 37 °C

for 20 hours, we measured growth as optical density (OD_{600}) using the SpectraMax Plus 384 absorbance plate reader (Molecular Devices, Sunnvale, CA USA). As recommended by guideline protocols for microbroth dilution, all clones were grown to a similar density before being inoculated to the twelve antibiotic dilutions to control for initial population size (approx. 1000 cells) and variation in growth phase [41].

Sequencing Resistance Genes. All selected resistance clones were sequenced bi-directionally using primers flanking the *Hinc*II region of the extracted plasmid (Table S1). Sanger sequencing was performed by Genewiz (Cambridge, MA USA). Sequence corresponding to the cloning vector was removed using Geneious 5.5.4 (http://www.geneious.com). The GenBank accession number for each resistance gene can be found in Table 2. To estimate our sequencing error rate, the plasmids of five unique clones were sent for re-sequencing; error rate was estimated to be much lower than 0.5% over 5000 base pairs, meaning that sequencing error are negligible in our study.

Identifying Host Strains. We designed specific primers for every resistance insert (Table S2 & S3) and conducted diagnosis PCRs on the extracted DNA of every bacteria strain used in this study. We used the original amplicon of the insert conferring resistance as a positive control and the DNA of *E. coli* MegaX DH10B as a negative control. The presence of positive PCR products was verified through low melting agarose (1%) gel electrophoresis and sequencing. Confirmation PCR was considered positive when the sequences of the amplicon and the positive control were more than 99% identical at the nucleotide level. Primers were designed using MPprimers [42] and

their specificity was tested using the MFEprimer software and the *E. coli* genomic DNA database [43].

Bioinformatics. For each insert, open reading frames were identified and annoted using Glimmer 3.0, and compared to the GenBank non-redundant (nr) nucleotide database (February 20, 2012) using tblastx. For each query, the GenBank ID and the alignment coordinates for the top scoring hit as well as the top scoring hit derived from a clinical pathogenic isolate was obtained. Global sequence alignment and corresponding percentage identities between the query and the obtained sequences were computed using the clustalW algorithm at the nucleotide level and the amino acid level within the annotated frame. When multiple annotate features were obtained for a query sequence, only the sequence most similar to the query at the nucleotide level was retained (Table S7 and S8). We then constructed a phylogeny from the multiple alignment (clustalW) of every fourth sequence of the top 100 hits (for a total of 25 unique sequences). Genetic distances were estimate using the Juke-Cantor algorithm and an unrooted consensus tree (70% of 10,000 bootstraps) was constructed using the neighbor-joining algorithm. All analyses were computed in Geneious 5.5.4 [44].

Environmental Metagenomics. We downloaded the complete metagenomic sequences of predicted genes of eight environmental metagenomes available on MG-RAST (http://metagenomics.anl.gov/), including metagenomic survey of samples isolated from the 2-m subsection and the active layer of the same Eur3 core used in this study and twenty human intestinal gut microbiota (Table S9). The metagenomes were mounted as individual database in the Geneious 5.5.4 software to which the amino acid sequence of each resistant insert was compared using blastp. The number of significant hits (as defined by an *E*-value cutoff equal or

250 inferior to 10^{-5}) was recorded for each database. We also used ResFinder, a newly developed
251 web-based method that uses BLAST to identify acquired antimicrobial resistance genes from a
252 custom database [45]. We used the replicated DNA sequence file available on the MG-RAST
253 server for both the 2-m subsection and the active layer (Table S9).
254

255 # Results

256 **Isolating antibiotic resistance genes.**

257 In a previous study [39], nineteen bacteria strains were isolated from subsections of a
258 permafrost core that is estimated to be 5000-6000 years old, and twenty-one bacteria strains were
259 sampled from the overlaying active layer soil at the surface of the permafrost (Figure 1B). Using
260 a fragment of the 16S rRNA gene, we typed bacterial isolates to the genus level when possible.
261 We thus identified seven genera among bacteria isolated from ancient permafrost (Table S4),
262 including many isolates belonging to the Bacilli, a class of ubiquitous Gram-positive Firmicute
263 that includes free-living as well as pathogenic species. We identified eleven different genera
264 among active layer bacteria (Table S5), including isolates of *Arthrobacter*, a common soil Gram-
265 positive Actinobacteria, and *Stenotrophomonas*, a Gram-negative bacterium that includes soil and
266 pathogenic species. Despite differences in phylogenetic composition (Figure 1C), the two
267 communities are representative of culturable microbial communities normally associated with
268 permafrost environments [46].

269 We then screened for antibiotic resistance genes in two metagenomic libraries constructed
270 from the pooled DNA of the permafrost strains (Table S4) and another constructed from the DNA
271 of the active layer bacteria (Table S5). Antibiotic-resistant clones were selected by plating 1-7 x
272 10^7 unique constructs from each library on Luria broth agar containing one of six antibiotics

belonging to one of the three following antimicrobial classes: aminoglycosides, β-lactams and tetracyclines (Table 1). For each antibiotic class, we selected one molecule produced by microorganisms (referred to as native antibiotics), and another derived from synthetic modifications of a native antibiotic (referred to as semi-synthetic antibiotics). Positive inserts from resistant clones were functionally characterized, sequenced and annotated. In total, we found twenty unique inserts among the fifty inserts that we sequenced (Table S6). As we sequenced the inserts, the number of novel resistance genes quickly saturated within both libraries (Figure S1).

Among ancient bacteria, we found eight unique resistance genes, which conferred resistance against four different antibiotics (Table 2; see Table S7 for full description). More precisely, we observed resistance to all three native antibiotics, as well as resistance to one semi-synthetic antibiotic. By contrast, we found resistance to all six antibiotics among active layer bacteria, for a total of ten unique resistance genes (Table 2; see Table S8 for full description*).* On average, the similarity between each resistance gene isolated from the active layer and the gene most related to it on GenBank [47] was higher than similarities between permafrost genes and their closest relatives *($F_{(1,14)} = 25.154$; $P < 0.001$; Figure 3A).* This difference disappeared when nucleotide similarity was compared with the closest related resistance gene harbored by a pathogenic strain, 63.2% for active layer genes and 54.9% for permafrost genes ($F_{(1,14)} = 1.965$; $P > 0.10$: Figure 3B).

Identifying host strains.

Using diagnostic PCRs, we screened every bacterial strain for the presence of the resistance genes identified through our functional screen (Figure 1C). Positive results were confirmed via re-sequencing, and we did not detect any false positives. In other words, the sequence of every

297 amplified fragment corresponded to the predicted resistance genes. Among permafrost bacteria,
298 we found that nine strains out of nineteen (47.3%) harbored at least one resistance gene (Table
299 S4), and that eight strains out of twenty-five (32.0%) harbored resistance in the active layer
300 (Table S5)(Figure 1C). Among the active layer bacteria, we even found two isolates identified as
301 *Stenotrophomonas* sp. carrying resistance to all three antibiotic classes (Figure 1C; Table S5).
302 In bacteria associated with ancient permafrost, genes conferring resistance to tetracycline,
303 sisomicin and amikacin were distributed among five different strains of Bacillaceae. (Figure 1C;
304 Table S4), a family of Gram-positive bacteria that produces over 150 different antimicrobials,
305 including aminoglycosides [48]. In particular, resistance gene AMK_P_1 was most closely
306 related to aminotransferases (Table 2 & S7), a group of enzymes associated with aminoglycoside
307 biosynthesis in many bacterial species, including *Bacillus* [49]. *Bacillus* spp. are also known to
308 possess a large number of resistance mechanisms for self-protection [48]. One such mechanism is
309 a group of multidrug-efflux pumps that are related to TET_P_1, a tetracycline resistance gene
310 found in three ancient *Bacillus* strains (Table 2 & S4; Figure 1C). Therefore, resistance to
311 tetracycline and aminoglycosides in *Bacillus* may reflect an evolutionary response to
312 antimicrobials naturally produced within the genus.
313 We also found evidence for resistance evolution in response to environmental
314 production of antibiotics. Gene PEN_P_1, conferring resistance to penicillin, was
315 found in *Staphylococcus* sp. (Figure 1C), an organism that does not produce β-lactam
316 antibiotics [50]. Gene PEN_P_1 is most closely related to a penicillin acylase type II
317 found in *Bacillus* (Table 2 & S7), an enzyme that hydrolyzes penicillin G into 6-
318 aminopenicillanate [51]. The primary physiological role of the penicillin acylase in
319 bacteria is believed to involve the utilization of aromatic amides as carbon sources;
320 penicillins are in fact amidic compounds [52]. Even though penicillin acylases are used

in the industry to develop antibiotics, the enzyme is not part of the normal β-lactams biosynthesis in fungi or bacteria [53]. Therefore, bacteria from the genus *Staphylococcus* most likely acquired the gene either to use β-lactams as a carbon source or to protect itself in response to the production of the antibiotics by other microorganisms in its environments. Although many members of the genus are widely distributed in natural and artificial cold environments [54], *Staphylococcus* are only rarely isolated from permafrost soils [28]. To confirm that isolate Eur3 2.12 did not originate from contamination of our sample, we tested the growth of the isolate at different temperature. As observed in most bacteria isolated from permafrost soils, the isolate showed significant growth at 5°C and room temperature (Table S10). Crucially, the absence of growth at 37°C confirms that the properties of this *Staphylococcus* isolate are consistent with being an ancient constituent of an arctic environment (Table S10).

Resistance and cross-resistance.

Generally, resistance genes isolated from active layer bacteria conferred protection against higher concentrations of antibiotics than permafrost resistance genes (Figure 2). Resistance genes from the active layers were also more likely to confer cross-resistance to other antibiotics (Figure S2). We observed the largest difference in resistance levels between genes isolated from the active layer and the permafrost among β-lactam resistance genes (Figure 2A). The penicillin resistance gene isolated from the permafrost conferred a two-fold increase in resistance, while genes PEN_AL_1 and PEN_AL_2 from the active layer conferred resistance to the highest concentrations of penicillin and carbenicillin we tested (MIC > 1024 μg/mL) (Figure 2D). The

latter are related to β-lactamases found in pathogenic bacteria: gene PEN_AL_1 is related to β-lactamases of the pseudomonads, including the opportunistic pathogen *Pseudomonas aeruginosa* (73.7% protein identity; Figure S3A) while gene PEN_AL_2 is most related to the L2 β-lactamases of *Stenotrophomonas maltohpilia* (68.3% protein identity; Figure S3B), an emerging opportunistic pathogen and a common etiological agent of septicemia [55]. As discussed above, gene PEN_P_1 was most related to penicillin acylases found in *Bacillus*, including several pathogenic isolates (Figure S4).

We found a similar pattern for tetracycline resistance: genes isolated from the active layer conferred twice as much resistance as the genes isolated from the permafrost (Figure 2B). Also, while tetracycline resistance genes found in the permafrost and the active layer were related to transporter/efflux pumps of various types (Figure S7 & S8), resistance to the semi-synthetic doxycycline was observed only within the active layer (Figure 2B). DOX-AL_1 and DOX_AL_2 were most closely related to acyltransferases involved in the lipolipid biosynthesis of *Stenotrophomonas* sp. (Table 2 & S8), likely contributing to the bacterium's intrinsic resistance against multiple antibiotics [56,57]. Acyltransferases were previously associated with intrinsic resistance in pathogenic mycobacteria and with the inactivation of chloramphenicol in Gram-negative bacteria [58]. Again, cross-resistance between tetracycline and doxycycline was only observed in resistance genes isolated from the active layer (Figure 2E).

In contrast with β-lactams and tetracyclines, we did not find a general trend in resistance levels among aminoglycoside antibiotic resistance genes. The three sisomicin resistance genes found in ancient soil bacteria and the two genes found among contemporary bacteria showed similar levels of resistance (Figure 2C; Table 2 & S7). Genes SIS_P_2, found in the permafrost (Table 2; Table S7), and SIS_AL_2, found in the active layer (Table 2 & S8), were both related to

aminoglycoside N6'-acetyltransferase, or AAC(6'), one of the most studied families of resistance genes against aminoglycosides [59]. Both genes present a high degree of divergence at the amino acid level when compared to the most related genes found in GenBank, 51.8% and 52.3% global protein identity with the closest related gene respectively (Figure S7B & S8A), suggesting that this resistance mechanism had diversified long before the anthropogenic use of antibiotics [59]. In addition to two sisomicin resistance genes conferring cross-resistance to semi-synthetic antibiotic amikacin (Figure 2F), we isolated two genes conferring resistance uniquely to the antibiotic within the permafrost (Figure 2C & F; Table 2 & S7). While amikacin-resistance genes from the permafrost showed a lower level of similarity to their closest homologs (Figure S7) than the amikacin-resistance genes found in active layer bacteria (Figure S8), both genes conferred cross-resistance to sisomicin when tested in planktonic culture (Figure 2F).

Environmental distribution of resistance genes.

Finally, we used a comparative metagenomic strategy to examine the distribution of each resistance gene in microbial surveys of the active layer and the permafrost 2-m subsection (Table S9). Most resistance genes isolated from permafrost bacteria were in fact isolated from the latter subsection. We found homologous sequences to every resistance gene in both active layer and permafrost communities, except for the two β-lactamases, which were absent from the 2-m permafrost survey (Table 3). We also found that the vast majority of resistance genes isolated from permafrost and active layer shared significant identity to genes found in both soil and marine environments (Table S11), but were less frequent or absent in gut microbiomes (Table S12; Figure S9). Finally, every resistance gene isolated in our study also showed some levels of similarity to genes found in pathogenic bacteria (Figure S3-S8).

Discussion

In this study, we demonstrate that diverse functional antibiotic resistance mechanisms existed in bacteria at least 5,000 years ago. By conducting a functional metagenomics screen of bacteria isolated from ancient permafrost, we identified genes conferring resistance to four different antibiotics, covering three major classes of antimicrobials used in modern medicine. Many of the resistance genes isolated in our study were highly similar to resistance genes found in pathogenic bacteria today (Figure S3-S8). Furthermore, functional resistance genes were found both in bacterial genera known to produce antimicrobials as well as bacteria that are not normally associated with antimicrobial-production. Taken together these results support the hypothesis that a reservoir of resistance genes existed in a range of bacteria species prior to the discovery of antibiotics by Sir Alexander Fleming [24,25,26,33,60] and contribute to a growing body of evidence demonstrating that antibiotic resistance evolved alongside antibiotic production in the natural environment [6,33,35].

In a single sampling of the Canadian high Arctic permafrost, we found eight different resistance genes that encompass three broad classes of resistance mechanisms. More specifically, we found three resistance genes related to efflux pumps or transporters, two degrading enzymes and three genes related to membrane modification or synthesis. This diversity in functional resistance genes could help explain the rapid evolution of resistance against modern-day antibiotics, including semi-synthetic antibiotics developed and synthesized in the laboratory. Indeed, among the five genes conferring resistance to aminoglycosides, four provided resistance or cross-resistance against amikacin. This antibiotic was the first semi-synthetic aminoglycoside used in medicine and was specifically designed to counter resistance to native aminoglycosides such as streptomycin and sisomicin [61]. In fact, one of the amikacin resistance genes we isolated

was related to aminoglycoside-N6'-acetyltransferases found in modern *Citrobacter* spp. and *Salmonella enterica* (Figure S7B). Therefore, it is perhaps not surprising that amikacin resistance was quickly discovered in clinical isolates of *Salmonella* and other *Enterobacteriaceae* within a year of the antibiotic's introduction [62,63].

The above results suggest that exhaustive resistance screening strategies could help predicting the success of new antimicrobial molecules [64]. Information on the frequency and the diversity of functional resistance genes in natural microbial communities prior to the introduction of a new drug can tell us whether the drug has the potential to remain effective against pathogenic bacteria for significant periods of time. For example, the first acquired resistance gene against quinolones, a fully synthetic class of antibiotics for which distant analogues exist in the wild [65], was discovered only recently in clinical isolates [66,67]. Even though sequences homologous to the acquired resistance gene (*qrn)* were found in the genomes of many Gram-negative and Gram-positive bacteria [68], functional resistance to the synthetic antibiotic evolved in clinical populations mainly through the acquisition of point mutations in the genes encoding either of the two type IIA topoisomerases targeted, DNA gyrase and DNA topoisomerase IV [69]. Therefore, resistance to quinolones in clinical strains remained manageable for more than two decades after the introduction of the antibiotic class [70].

We also found that resistance genes associated with bacteria isolated in the active layer generally conferred higher levels of resistance than resistance genes isolated from the permafrost. Increases in resistance levels observed in soil microbiomes [71,72] or in clinical isolates [11] have been considered as evidence for the impact of antibiotic use on microbial communities. In our study, the difference in resistance levels is also associated with changes in community composition. For example, there seems to be a slight bias towards spore-forming bacteria in the permafrost community. Furthermore, there is growing evidence that subpopulations of

437 microorganisms in the permafrost constitute active microbial ecosystem rather than "ancient"
438 frozen microbial survivors [39,73]. Therefore, whether antibiotic resistance level changes in
439 permafrost result from changes in community composition, local ecological interactions or are the
440 consequences of anthropogenic antibiotic use remains to be tested.

441 Although the study of culturable bacteria enabled us to accurately identify the taxa
442 associated with antibiotic resistance in ancient permafrost, it likely underestimates the total
443 number of resistance genes in our samples. The use of a specific host and of a high copy number
444 plasmid also likely affected the identity of the resistance genes found in this study [74]. Indeed,
445 we expect that resistance genes that are distantly related to *E. coli* or more generally to Gram-
446 negative bacteria might be more difficult to detect. Still, the use of functional screens is a
447 powerful way to detect and confirm the phenotype of antibiotic resistance genes in microbial
448 communities [3,7,27]. While using a Gram-negative host enables us to detect resistance genes
449 that are more likely to be relevant in pathogenic bacteria such as *E. coli*, *P. aeruginosa* and
450 *Salmonella,* the amplification of bacterial genes on high copy number plasmids can inform us on
451 the possible effect of gene duplication or on the transfer of a gene to a plasmid [75].

452 The existence of a resistance reservoirs in the environment can greatly accelerate the
453 evolution of multidrug-resistant bacteria [7]. For this reason, it is crucial to take into account the
454 extensive diversity of antibiotic resistance genes found in microbial populations when developing
455 or deploying new antibiotic strategies. Future studies of ancient permafrost soils including total
456 metagenomics DNA and additional sampling will allow us to study the temporal and spatial
457 distribution of antibiotic resistance genes and the possible impacts of human activity on the
458 microbial world. For instance, it would be interesting to know whether the diversity of antibiotic
459 resistance genes as well as the prevalence of resistance genes followed similar trends over time.
460 While diversity can be measure as the total number of resistance genes identified in a library,

prevalence could be measured as the total number of growing colonies on selective plates given the total amount of DNA used to build the library. Finally, whole-genome analyses of bacteria isolated from ancient soils should shed a new light on the role of horizontal gene transfer in the evolution of antibiotic resistance and emerging diseases in general.

Acknowledgments We would like to thank M. Ackerman, C. MacLean, J. Moffitt, R. Popat, S. Simmons and members of the Desai lab for helpful discussions regarding this manuscript, and the technical assistance of Roli Wilhelm with microbial culturing, J. Colarco and L. Chubiz with cloning, M. Chafee and S. Simmons with DNA processing, and J. Goordial and H. Trigui for growth assays.

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652 # Figure Legends
653
654 **Figure 1.** Distribution of antibiotic resistance genes isolated from ancient permafrost bacteria and
655 its overlaying active layer. A) Samples were collected from Eureka on Ellesmere Island, Canada.
656 B) Antibiotic resistance genes isolated using functional metagenomics were traced back to
657 bacterial strains isolated at different depths of a single 14-m core. C) Resistance genes were
658 mapped onto the 16S rRNA gene phylogeny of all ancient (red) and modern (black) bacterial
659 isolates used in this study. Each resistance gene is represented by a unique color and shape
660 combination: resistance to β-lactams (red), tetracyclines (green), and aminoglycosides (blue) as
661 explained in **Table 2**. We could not identify the host strain for two resistance genes, most likely
662 because we could not revive three ancient strains.
663
664 **Figure 2.** Resistance (A-C) and cross-resistance (D-F) levels of resistance genes isolated from
665 ancient permafrost and its overlaying active layer. Each unique gene is depicted by a shape and
666 color combination based on sampling site and antibiotic on which it was isolated (shown on top
667 of panels): A) & D) β-lactams, penicillin (PEN) & carbenicillin (CAR); B) & E) tetracyclines,
668 tetracycline (TET) & doxycycline (DOX); and C) & F) aminoglycosides, sisomicin (SIS) &
669 amikacin (AMK). In panels A) to C), each point shows resistance to antibiotics indicated at left
670 (measured as minimum inhibitory concentration, MIC). Grey panels indicate resistance levels to
671 the drug in which genes were isolated, and white panels show cross-resistance to the other drug in
672 the same class. Dashed line indicates MIC of control libraries. Panels D) to F) show slopegraphs
673 of cross-resistance between antibiotics of a same family. The left axis represents relative
674 resistance (MIC of the isolated genes / MIC of the control *E. coli* library) in the antibiotics where
675 the gene was isolated. The right axis represents the relative fitness of the genes in the other
676 antibiotic of the same class. Any slope that doesn't go down to one on the right axis indicates
677 some degree of cross-resistance.
678
679 **Figure 3.** Nucleotide similarities of resistance genes isolated from ancient (red) and modern
680 (black) bacteria with the closest homologous genes found in A) any bacteria or in B) a pathogenic
681 bacteria. Add a new figure to this one to make it better.

682 **Table 1. List of antibiotics and minimal inhibitory concentration (MIC) used in this study.**
683 **Origin indicates whether the antibiotic is produced naturally by a microorganism or is**
684 **modified in the laboratory.**
685

Class	Antibiotic	Origin	MIC (mg/mL)	
			LB + agar	LB
Aminoglycoside	Sisomicin (SIS) [76]	*Micromonospora inyoensis*	4	1
	Amikacin (AMK) [61]	Semi-synthetic	100	32
Beta-lactamase	Penicillin (PEN) [77]	*Penicillium notatum*	50	32
	Carbenicillin (CAR) [78], [79]	Semi-synthetic	80	16
Tetracycline	Tetracycline (TET) [80]	*Streptomyces aureofaciens*	8	1
	Doxycycline (DOX) [81]	Semi-synthetic	4	0.5

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713 **Table 2. List of resistance genes and their predicted annotation.**

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Genes from permafrost				Genes from active layer			
Gene ID		Gen-Bank ID	Gene Annotation	Gene ID		Gen-Bank ID	Gene Annotation
AMK_P_1	◇	KC520481	Aminotransferase class V	AMK_AL_1	▼	KC520489	Putative acyl carrier protein phosphodiesterase
AMK_P_2	△	KC520482	Putative transporter	AMK_AL_2	■	KC520490	Putative dehydrogenase
PEN_P_1	●	KC520475	Penicllin acylase II	DOX_AL_1	▲	KC520491	Glycerol-3-phosphate O-acyltransferase
SIS_P_1	○	KC520478	Protoporphyrinogen oxidase	DOX_AL_2	⬢	KC520492	Acyl-CoA thioesterase I
SIS_P_2	▽	KC520479	Aminoglycoside N(6')-acetyltransferase (AAC(6')), putative	PEN_AL_1/ CAR_AL_1	▼	KC520483	Putative beta-lactamase family protein
SIS_P_3	□	KC520480	Porphobilinogen deaminase	PEN_AL_2/ CAR_AL_2	■	KC520484	L2 beta-lactamase
TET_P_1	●	KC520476	Permease of the major facilitator superfamily	SIS_AL_1	◆	KC520487	Aminoglycoside 6'-N-acetyltransferase Iz
TET_P_2	▽	KC520477	Putative drug antiporter (transporter)	SIS_AL_2	▲	KC520488	Multidrug ABC transporter ATPase and permease
				TET_AL_1	■	KC520485	drug resistance transporter, EmrB/QacA
				TET_AL_2	◆	KC520486	Putative transporter

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733 **Table 3. Number of sequences homologous to functional resistance genes in metagenomic**
734 **surveys of the Canadian high Arctic.**
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Samples	PEN_P_1	TET_P_1	TET_P_2	SIS_P_1	SIS_P_2	SIS_P_3	AMK_P_1	AMK_P_2	PEN_AL_1	PEN_AL_1	TET_AL_1	TET_AL_2	SIS_AL_1	SIS_AL_2	AMK_AL_1	AMK_AL_2	DOX_AL_1	DOX_AL_2
Active Layer	94	34	43	6	4	1	297	1	88	4	44	43	40	700	3	49	1	57
2-m permafrost	9	12	21	1	1	1	25	1	0	0	12	10	4	123	2	12	0	5

736 Metagenomic surveys data is described in Steven B, Pollard WH, Greer CW, & Whyte LG (2008) Microbial diversity and activity
737 through a permafrost/ground ice core profile from the Canadian high Arctic. *Environ Microbiol* 10(12):3388-3403.
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Supplementary Information

Figure S1: Sampling depth of resistance conferring inserts.

Figure S2: Resistance and cross-resistance levels conferred by inserts isolated from the permafrost and the active layer of single core collected from the Canadian high Arctic.

Figure S3: Phylogenetic distribution of full-length gene products encoding resistance to beta-lactams isolated from the Canadian high Artic active layer soil.

Figure S4: Phylogenetic distribution of full-length gene products encoding resistance to beta-lactams isolated from the Canadian high Artic permafrost.

Figure S5: Phylogenetic distribution of full-length gene products encoding resistance to tetracycline isolated from the Canadian high Arctic permafrost.

Figure S6: Phylogenetic distribution of full-length gene products encoding resistance to tetracycline isolated from the Canadian high Arctic active layer soil.

Figure S7: Phylogenetic distribution of full-length gene products encoding resistance to aminoglycoside isolated from the Canadian high Arctic permafrost.

Figure S8: Phylogenetic distribution of full-length gene products encoding resistance to aminoglycoside isolated from the Canadian high Arctic active layer soil.

Figure S9: Abundance of putative resistance genes and related proteins at the sampling sites and other metagenomes.

Table S1: List of strains, plasmids and primers used for library construction.

Table S2: Primers used to identify the permafrost bacteria strain(s) harboring each resistant inserts.

Table S3: Primers used to identify the active layer bacteria strain(s) harboring each resistant inserts.

Table S4: List of bacteria strains isolated from the permafrost and associated resistance genes.

Table S5: List of bacteria strains isolated from active layer and associated resistance genes.

Table S6: Numbers of antibiotic resistant clones sequenced and unique resistance genes found from a functional analysis of the permafrost and the active layer of the Canadian high Arctic.

Table S7: Resistance genes identified using metagenomic functional selections from Canadian High Arctic permafrost.

Table S8: Resistance genes identified using metagenomic functional selections from Canadian High Arctic active layer.

Table S9: List of environmental microbiomes used for studying the distribution of each resistant insert.

Table S10: Growth profile of isolate Eur3 2.12 at different temperatures.

Table S11: Number of significant BLASTP hits across environmental microbiomes.

Table S12: Number of significant BLASTP hits across gut microbiomes.

802 **Figure 1**

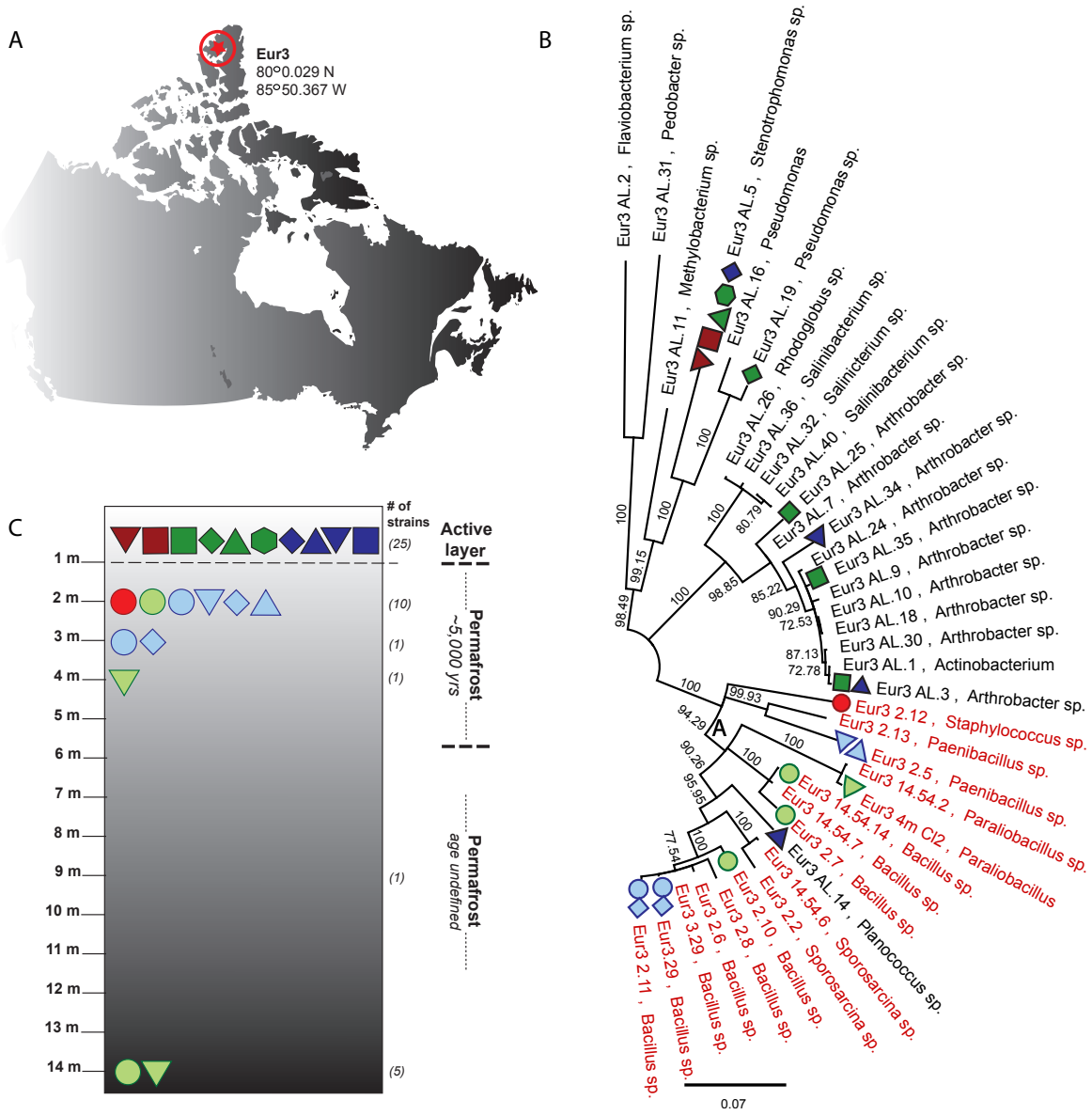

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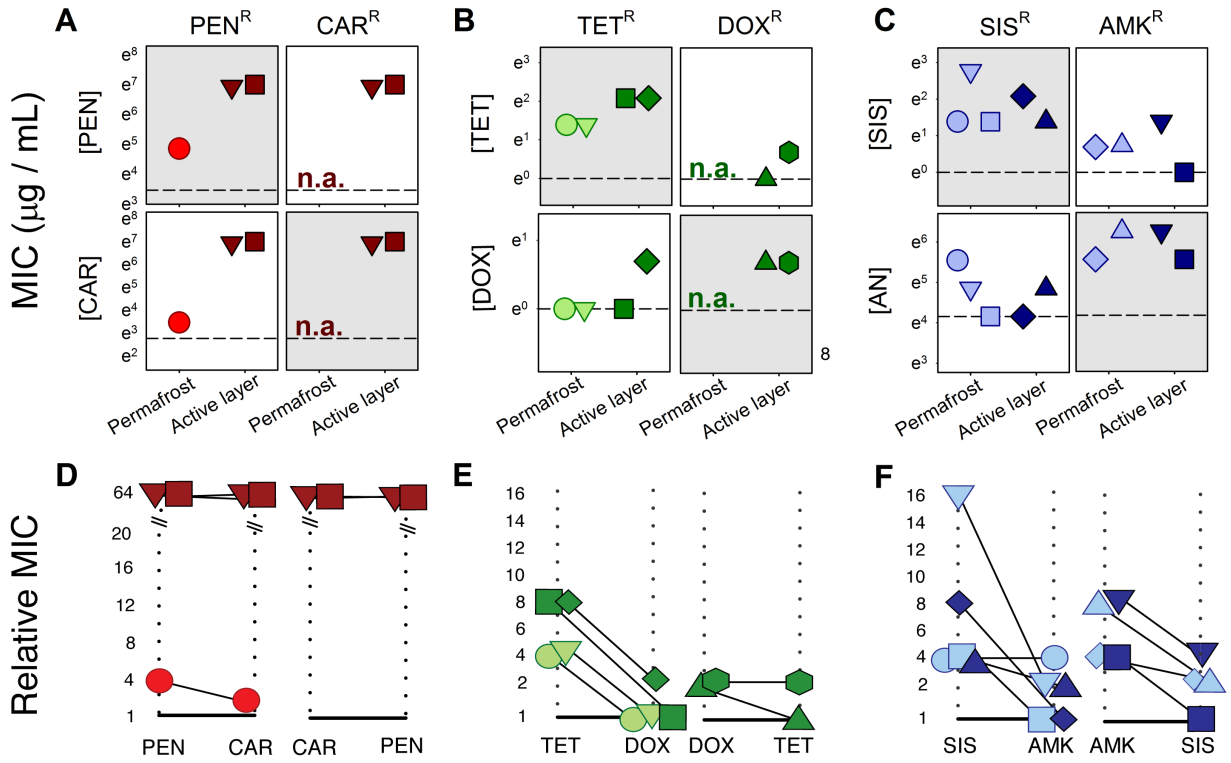
Figure 2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DOX^R
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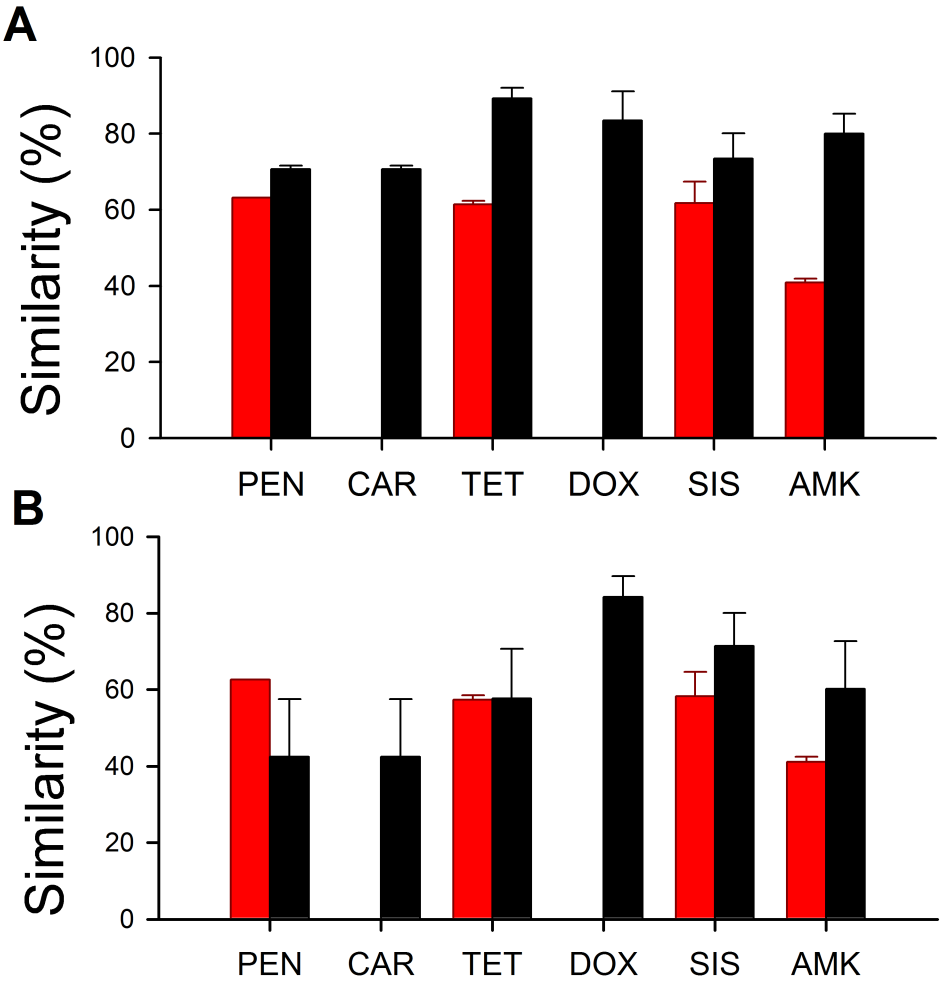
840 **Figure 3**



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