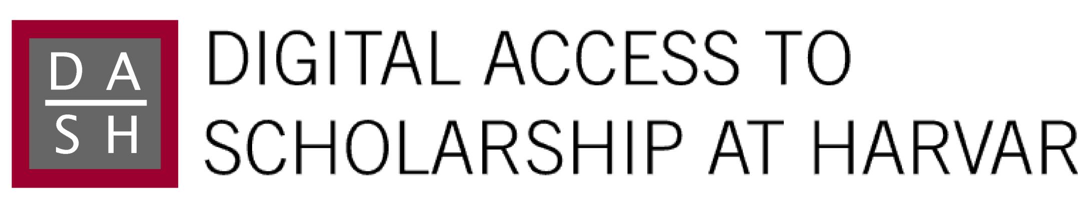
View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Harvard University - DASH



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

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters.

Citation	Perron, Gabriel, Lyle Whyte, Peter Turnbaugh, William P. Hanage, Gautam Dantas, and Michael M. Desai. Forthcoming. Functional characterization of bacteria isolated from ancient Arctic soil exposes diverse resistance mechanisms to modern antibiotics. PLoS ONE.
Accessed	February 19, 2015 12:08:39 PM EST
Citable Link	http://nrs.harvard.edu/urn-3:HUL.InstRepos:10859496
Terms of Use	This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Open Access Policy Articles, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#OAP

(Article begins on next page)

1
2
3
4
5 # Functional characterization of bacteria isolated from ancient Arctic soil
6 exposes diverse resistance mechanisms to modern antibiotics
7
8
9
10 Gabriel G. Perron[1,2,3*], Lyle Whyte[4], Peter J. Turnbaugh[1], William P. Hanage[5]
11 Gautam Dantas[6,7] & Michael M. Desai[1,2,8*]
12
13
14 1-FAS Center for Systems Biology, Harvard University, 52 Oxford Street, Cambridge, Massachusetts,
15 USA 02138.
16 2-Department of Evolutionary and Organismic Biology, Harvard University, 52 Oxford Street,
17 Cambridge, Massachusetts, USA 02138.
18 3-Center for Advanced Research in Environmental Genomics, Department of Biology, University of
19 Ottawa, Ottawa, Ontario, Canada KN5 5N6
20 4-Department of Natural Resource Sciences, McGill University, Macdonald Campus, 21,111 Lakeshore,
21 Ste-Anne-de-Bellevue, Quebec, Canada H9X 3V9.
22 5-Department of Epidemiology, Harvard School of Public School, 677 Huntington Avenue, Boston,
23 Massachusetts, USA 02115.
24 6-Center for Genome Sciences and Systems Biology, Washington University School of Medicine, 4444
25 Forest Park Avenue, St. Louis, Missouri, USA 63108.
26 7-Department of Pathology and Immunology, Washington University School of Medicine, 4444 Park
27 Forest Avenue, St. Louis, Missouri, USA 63108
28 8-Department of Physics, Harvard University, Cambridge Massachusetts, USA 02138.
29
30 *To whom correspondence should be addressed: (GGP) gperro@uOttawa.ca / 617-866-0464; (MMD)
31 mmdesai@fas.harvard.edu / 617-496-3613.
32
33
34
35
36
37
38
39
40
41
42

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.

66 ## Introduction

67 The evolution and spread of antibiotic resistance in pathogenic bacteria is one of the most
68 urgent challenges in public health today [1,2]. Although resistance genes are now widespread in
69 most microbial communities [3,4,5,6,7], whether the extensive diversity of resistance in
70 environmental reservoirs and pathogenic bacteria is the result of human activity is controversial
71 [8,9]. Rare studies considering multiple clinical isolates that pre-date the anthropogenic use of
72 antibiotics show that resistance was uncommon in pathogenic bacteria such as *Salmonella*,
73 *Klebsiella*, and *Escherichia* [10,11]. Furthermore, the novel evolution of antibiotic resistance
74 through genetic mutations is well documented in clinical [12,13] and laboratory [14,15]
75 populations of bacteria [16]. Similarly, microbial communities found in human-impacted
76 environments such as water streams surrounding hospitals tend to show high levels of resistance
77 genes [17,18]. Thus, the impact of antibiotic pollution on microbial communities is undeniable
78 [19].

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

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

100 DNA fragments, however, cannot confirm functional resistance against antibiotics, let alone
101 whether they protect against the clinical use of antibiotics, for two main reasons [34]. First, the
102 presence of DNA fragments similar to the sequence of known resistance genes is not sufficient to
103 ensure the functional expression of a resistance phenotype [35].Second, the sequences of many
104 resistance genes show high levels of similarity to genes that carry out other cellular functions
105 [19]. For example, bacterial efflux pumps of the resistance-nodulation-division (RND)
106 superfamily can confer resistance to antibiotics, transport hydrophobic proteins involved in cell
107 division and nodulation, or both [36]. Likewise, activation of certain RND-type efflux pumps in
108 Gram-negative bacteria can confer sub-clinical resistance against quinolones, a synthetic group of
109 antibiotics, under stress conditions [37]. Furthermore, analyses based on DNA sequence identity
110 solely are limited to known resistance genes [3], ignoring the wide array of other resistance
111 mechanisms that could have existed in the past. Although unspecified resistance genes from
112 ancient bacteria are less likely to be of immediate concern in medical practice, they may have

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

115 The functional characterization of resistance genes found in ancient permafrost environments
116 would provide a unique window on the origins and evolution of antibiotic resistance in bacteria
117 [8,9]. A previous study of ancient Siberian soils reported the presence of resistance to
118 aminoglycoside, chloramphenicol and tetracyclines among Gram-positive and Gram-negative
119 bacteria [38], but the results were met with serious criticisms [33]. Here, we use functional
120 metagenomics to revive resistance genes associated with culturable bacteria collected from a
121 single 14-m deep core of the Canadian high Arctic permafrost (Figure 1A). Even though limited
122 in scope, the study of culturable bacteria from a single permafrost core provides information on
123 the diversity and possible sources of antibiotic resistance genes prior to the introduction of
124 antibiotics in modern medicine.

125

126 Materials and Methods

127 **Isolation of Bacteria Strains.** A single core of the Canadian high Arctic was collected in May
128 2003 at Eureka (80°0.029 N, 85°50.367 W85°50.367 W), Ellesmere Island, Nunavut, Canada
129 (Figure 1A) [39]. The field research was done with the permission of the Government of Nunavut
130 through a Territorial Scientific Research Licence to Prof. Whyte (McGill University) as issued on
131 an annual basis by the Nunavut Research Institute from 2003 to 2012. Core Eur3 covers a depth
132 of 14 meters and includes the active layer of the Arctic soil as well as permafrost (Figure 1B).
133 The geology of the core is related to sediments from the late Meistocene and subsequent
134 Holocene and was estimated to be between 5,000 and 6,000 years old [39]. Permafrost is ground
135 that remains at or below 0ºC for at least two years [28]. During summer months, air temperatures

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

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

155

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

159 Carlsbad, CA USA). We then combined in equal proportion the DNA of all strains derived from
160 the active layer into a single pool (hereafter referred to as "AL" for active layer) and all strains
161 derived from the permafrost into another pool (hereafter referred to as "P" for permafrost). We
162 constructed one metagenomics library for each pool according to the protocols described in [3].
163 Briefly, 10 μg of DNA were sheared using the automated Covaris S220 DNA shearer instrument
164 using miniTUBE clear (Covaris, Woburn, MA USA) tubes and protocol for shearing of 1.5-2.5
165 kp fragments. Sheared DNA was end-repaired using the End-It repair kit (Epicentre, Madison,
166 WI USA) and size-selected (1200-4000 bp) by electrophoresis through a 1% low melting point
167 agarose gel in 1.0 X TE buffer. Size-selected and end-repaired DNA fragments were then ligated
168 into the linearized pZE21 MCS 1 vector (Table S1) at the HincII site using the Fast Link Ligation
169 kit (Epicentre). The ligation product was purified and resuspended in sterile deionized water
170 before being transformed by electroporation into 20 μL of *E. coli* MegaX DH10B cells
171 (Invitrogen, Grand Island, NY USA). Transformed cells were recovered in 1 mL of SOC medium
172 (Invitrogen) and incubated with vigorous shaking for one hours at 37 °C. Libraries were diluted
173 by plating out 1 μL, 0.1 and 0.01 μL of recovered cells onto LB agar plates containing 50 μg/mL
174 kanamycin. For each library, insert size distribution was estimated by gel electrophoresis of PCR
175 products obtained by amplifying the insert of twelve colonies using primers flanking the multiple
176 cloning site of the pZE21 MCS1 vector (Table S1). The total size of each library was determined
177 by multiplying the average PCR based insert size with the number of colony forming units (CFU)
178 in a given library and varied between 2-5 X 10^9 base pairs. The rest of the recovered cells were
179 inoculated into 9 mL of LB containing 50 μg/mL kanamycin and amplified over night at 37 °C.
180 The overnight cultures were frozen with 20% glycerol and kept at -80 °C before subsequent
181 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

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

210

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

219

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

227 their specificity was tested using the MFEprimer software and the *E. coli* genomic DNA database
228 [43].

229 **Bioinformatics.** For each insert, open reading frames were identified and annoted using Glimmer
230 3.0, and compared to the GenBank non-redundant (nr) nucleotide database (February 20, 2012)
231 using tblastx. For each query, the GenBank ID and the alignment coordinates for the top scoring
232 hit as well as the top scoring hit derived from a clinical pathogenic isolate was obtained. Global
233 sequence alignment and corresponding percentage identities between the query and the obtained
234 sequences were computed using the clustalW algorithm at the nucleotide level and the amino acid
235 level within the annotated frame. When multiple annotate features were obtained for a query
236 sequence, only the sequence most similar to the query at the nucleotide level was retained (Table
237 S7 and S8). We then constructed a phylogeny from the multiple alignment (clustalW) of every
238 fourth sequence of the top 100 hits (for a total of 25 unique sequences). Genetic distances were
239 estimate using the Juke-Cantor algorithm and an unrooted consensus tree (70% of 10,000
240 bootstraps) was constructed using the neighbor-joining algorithm. All analyses were computed in
241 Geneious 5.5.4 [44].

242

243 **Environmental Metagenomics.** We downloaded the complete metagenomic sequences of
244 predicted genes of eight environmental metagenomes available on MG-RAST
245 (http://metagenomics.anl.gov/), including metagenomic survey of samples isolated from the 2-m
246 subsection and the active layer of the same Eur3 core used in this study and twenty human
247 intestinal gut microbiota (Table S9). The metagenomes were mounted as individual database in
248 the Geneious 5.5.4 software to which the amino acid sequence of each resistant insert was
249 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

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

281 Among ancient bacteria, we found eight unique resistance genes, which conferred resistance
282 against four different antibiotics (Table 2; see Table S7 for full description). More precisely, we
283 observed resistance to all three native antibiotics, as well as resistance to one semi-synthetic
284 antibiotic. By contrast, we found resistance to all six antibiotics among active layer bacteria, for a
285 total of ten unique resistance genes (Table 2; see Table S8 for full description*)*. On average, the
286 similarity between each resistance gene isolated from the active layer and the gene most related to
287 it on GenBank [47] was higher than similarities between permafrost genes and their closest
288 relatives *(*$F_{(1,14)} = 25.154$*;* $P < 0.001$*; Figure 3A).* This difference disappeared when nucleotide
289 similarity was compared with the closest related resistance gene harbored by a pathogenic strain,
290 63.2% for active layer genes and 54.9% for permafrost genes ($F_{(1,14)} = 1.965$; $P > 0.10$: Figure
291 3B).
292

293 **Identifying host strains.**

294 Using diagnostic PCRs, we screened every bacterial strain for the presence of the resistance
295 genes identified through our functional screen (Figure 1C). Positive results were confirmed via
296 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

321 in the industry to develop antibiotics, the enzyme is not part of the normal β-lactams
322 biosynthesis in fungi or bacteria [53]. Therefore, bacteria from the genus
323 *Staphylococcus* most likely acquired the gene either to use β-lactams as a carbon
324 source or to protect itself in response to the production of the antibiotics by other
325 microorganisms in its environments. Although many members of the genus are widely
326 distributed in natural and artificial cold environments [54], *Staphylococcus* are only
327 rarely isolated from permafrost soils [28]. To confirm that isolate Eur3 2.12 did not
328 originate from contamination of our sample, we tested the growth of the isolate at
329 different temperature. As observed in most bacteria isolated from permafrost soils, the
330 isolate showed significant growth at 5°C and room temperature (Table S10). Crucially,
331 the absence of growth at 37°C confirms that the properties of this *Staphylococcus*
332 isolate are consistent with being an ancient constituent of an arctic environment (Table
333 S10).
334

335 **Resistance and cross-resistance.**

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

344 latter are related to β-lactamases found in pathogenic bacteria: gene PEN_AL_1 is related to β-
345 lactamases of the pseudomonads, including the opportunistic pathogen *Pseudomonas aeruginosa*
346 (73.7% protein identity; Figure S3A) while gene PEN_AL_2 is most related to the L2 β-
347 lactamases of *Stenotrophomonas maltohpilia* (68.3% protein identity; Figure S3B), an emerging
348 opportunistic pathogen and a common etiological agent of septicemia [55]. As discussed above,
349 gene PEN_P_1 was most related to penicillin acylases found in *Bacillus*, including several
350 pathogenic isolates (Figure S4).

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

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

367 aminoglycoside N6'-acetyltransferase, or AAC(6'), one of the most studied families of resistance
368 genes against aminoglycosides [59]. Both genes present a high degree of divergence at the amino
369 acid level when compared to the most related genes found in GenBank, 51.8% and 52.3% global
370 protein identity with the closest related gene respectively (Figure S7B & S8A), suggesting that
371 this resistance mechanism had diversified long before the anthropogenic use of antibiotics [59].
372 In addition to two sisomicin resistance genes conferring cross-resistance to semi-synthetic
373 antibiotic amikacin (Figure 2F), we isolated two genes conferring resistance uniquely to the
374 antibiotic within the permafrost (Figure 2C & F; Table 2 & S7). While amikacin-resistance genes
375 from the permafrost showed a lower level of similarity to their closest homologs (Figure S7) than
376 the amikacin-resistance genes found in active layer bacteria (Figure S8), both genes conferred
377 cross-resistance to sisomicin when tested in planktonic culture (Figure 2F).
378

379 **Environmental distribution of resistance genes.**

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

390 **Discussion**

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

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

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

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

430 We also found that resistance genes associated with bacteria isolated in the active layer
431 generally conferred higher levels of resistance than resistance genes isolated from the permafrost.
432 Increases in resistance levels observed in soil microbiomes [71,72] or in clinical isolates [11]
433 have been considered as evidence for the impact of antibiotic use on microbial communities. In
434 our study, the difference in resistance levels is also associated with changes in community
435 composition. For example, there seems to be a slight bias towards spore-forming bacteria in the
436 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,

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

465

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

471

472 References

473

474 1. Bush K, Courvalin P, Dantas G, Davies J, Eisenstein B, et al. (2011) Tackling antibiotic resistance. Nat Rev
475 Microbiol 9: 894-896.
476 2. Levy SB, Marshall B (2004) Antibacterial resistance worldwide: Causes, challenges and responses. Nat Med 10
477 (12 Suppl): S122-129.
478 3. Sommer MO, Dantas G, Church GM (2009) Functional characterization of the antibiotic resistance reservoir in the
479 human microflora. Science 325: 1128-1131.
480 4. D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006) Sampling the antibiotic resistome. Science 311: 374-
481 377.
482 5. Perron GG, Quessy S, Bell G (2008) A reservoir of drug-resistant pathogenic bacteria in asymptomatic hosts.
483 PLoS ONE 3: e3749.
484 6. Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, et al. (2012) Antibiotic resistance is prevalent in an
485 isolated cave microbiome. PLoS ONE 7: e34953.
486 7. Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MO, et al. (2012) The shared antibiotic resistome of soil
487 bacteria and human pathogens. Science 337: 1107-1111.
488 8. Davies J, Davies D (2010) Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev 74: 417-433.
489 9. Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, et al. (2010) Call of the wild: antibiotic resistance
490 genes in natural environments. Nat Rev Micro 8: 251-259.
491 10. Hughes VM, Datta N (1983) Conjugative plasmids in bacteria of the `pre-antibiotic' era. Nature 302: 725-726.
492 11. Houndt T, Ochman H (2000) Long-term shifts in patterns of antibiotic resistance in enteric bacteria. Appl
493 Environ Microbiol 66: 5406-5409.
494 12. Comas I, Borrell S, Roetzer A, Rose G, Malla B, et al. (2012) Whole-genome sequencing of rifampicin-resistant
495 *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. Nat
496 Genet 44: 106-110.
497 13. Lieberman TD, Michel JB, Aingaran M, Potter-Bynoe G, Roux D, et al. (2011) Parallel bacterial evolution within
498 multiple patients identifies candidate pathogenicity genes. Nat Genet 43: 1275-1280.
499 14. Hegreness M, Shoresh N, Hartl D, Kishony R (2006) An equivalence principle for the incorporation of favorable
500 mutations in asexual populations. Science 311: 1615-1617.

501 15. Perron GG, Gonzalez A, Buckling A (2007) Source-sink dynamics shape the evolution of antibiotic resistance
502 and its pleiotropic fitness cost. Proc Biol Sci 274: 2351-2356.
503 16. MacLean RC, Hall AR, Perron GG, Buckling A (2010) The population genetics of antibiotic resistance:
504 integrating molecular mechanisms and treatment contexts. Nat Rev Genet 11: 405-414.
505 17. Slekovec Cl, Plantin J, Cholley P, Thouverez M, Talon D, et al. (2012) Tracking down antibiotic-resistant
506 *Pseudomonas aeruginosa* isolates in a wastewater network. PLoS ONE 7: e49300.
507 18. Kristiansson E, Fick J, Janzon A, Grabic R, Rutgersson C, et al. (2011) Pyrosequencing of antibiotic-
508 contaminated river sediments reveals high levels of resistance and gene transfer elements. PLoS ONE 6:
509 e17038.
510 19. Martinez JL (2008) Antibiotics and antibiotic resistance genes in natural environments. Science 321: 365-367.
511 20. Rokem JS, Lantz AE, Nielsen J (2007) Systems biology of antibiotic production by microorganisms. Nat Prod
512 Rep 24: 1262-1287.
513 21. Yim G, Wang HH, Davies J (2007) Antibiotics as signalling molecules. Philos Trans R Soc Lond B Biol Sci 362:
514 1195-1200.
515 22. Aminov RI (2009) The role of antibiotics and antibiotic resistance in nature. Environ Microbiol 11: 2970-2988.
516 23. Gullberg E, Cao S, Berg OG, Ilback C, Sandegren L, et al. (2011) Selection of resistant bacteria at very low
517 antibiotic concentrations. PLoS Pathog 7: e1002158.
518 24. Davies J (1994) Inactivation of antibiotics and the dissemination of resistance genes. Science 264: 375-382.
519 25. Hall BG, Barlow M (2004) Evolution of the serine beta-lactamases: past, present and future. Drug Resist Updat
520 7: 111-123.
521 26. Aminov RI, Mackie RI (2007) Evolution and ecology of antibiotic resistance genes. FEMS Microbiol Lett 271:
522 147-161.
523 27. Allen HK, Moe LA, Rodbumrer J, Gaarder A, Handelsman J (2009) Functional metagenomics reveals diverse
524 beta-lactamases in a remote Alaskan soil. Isme Journal 3: 243-251.
525 28. Miller RV, Whyte LG (2011) Polar Microbiology. Washington, DC.: ASM Press. 398 p.
526 29. Steven B, Leveille R, Pollard WH, Whyte LG (2006) Microbial ecology and biodiversity in permafrost.
527 Extremophiles 10: 259-267.
528 30. Juck DF, Whissell G, Steven B, Pollard W, McKay CP, et al. (2005) Utilization of fluorescent microspheres and
529 a green fluorescent protein-marked strain for assessment of microbiological contamination of permafrost
530 and ground ice core samples from the Canadian High Arctic. Appl Environ Microbiol 71: 1035-1041.
531 31. Steven B, Briggs G, McKay CP, Pollard WH, Greer CW, et al. (2007) Characterization of the microbial diversity
532 in a permafrost sample from the Canadian high Arctic using culture-dependent and culture-independent
533 methods. FEMS Microbiol Ecol 59: 513-523.
534 32. Willerslev E, Hansen AJ, Poinar HN (2004) Isolation of nucleic acids and cultures from fossil ice and permafrost.
535 Trends Ecol Evol 19: 141-147.
536 33. D'Costa VM, King CE, Kalan L, Morar M, Sung WW, et al. (2011) Antibiotic resistance is ancient. Nature 477:
537 457-461.
538 34. Dantas G, Sommer MO (2012) Context matters - the complex interplay between resistome genotypes and
539 resistance phenotypes. Curr Opin Microbiol 15: 577-582.
540 35. Fuste E, Galisteo GJ, Jover L, Vinuesa T, Villa TG, et al. (2012) Comparison of antibiotic susceptibility of old
541 and current Serratia. Future Microbiol 7: 781-786.
542 36. Su CC, Long F, Zimmermann MT, Rajashankar KR, Jernigan RL, et al. (2011) Crystal structure of the CusBA
543 heavy-metal efflux complex of Escherichia coli. Nature 470: 558-562.
544 37. Piddock LJ (2006) Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol 4: 629-636.
545 38. Mindlin S, Soina V, Petrova M, Gorlenko Z (2008) Isolation of antibiotic resistance bacterial strains from Eastern
546 Siberia permafrost sediments. Russ J Genet 44: 27-34.
547 39. Steven B, Pollard WH, Greer CW, Whyte LG (2008) Microbial diversity and activity through a
548 permafrost/ground ice core profile from the Canadian high Arctic. Environ Microbiol 10: 3388-3403.
549 40. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA
550 sequences into the new bacterial taxonomy. Appl Environ Microbiol 73: 5261-5267.
551 41. Wiegand I, Hilpert K, Hancock RE (2008) Agar and broth dilution methods to determine the minimal inhibitory
552 concentration (MIC) of antimicrobial substances. Nat Protoc 3: 163-175.
553 42. Shen Z, Qu W, Wang W, Lu Y, Wu Y, et al. (2010) MPprimer: a program for reliable multiplex PCR primer
554 design. BMC Bioinformatics 11: 143.
555 43. Qu W, Shen Z, Zhao D, Yang Y, Zhang C (2009) MFEprimer: multiple factor evaluation of the specificity of
556 PCR primers. Bioinformatics 25: 276-278.

557 44. Drummund AJ, Ashton B, Buxton S, Cheung M, Cooper A, et al. (2011) Geneious v5.4. pp. Available from
558 http://www.geneious.com/.
559 45. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, et al. (2012) Identification of acquired
560 antimicrobial resistance genes. J Antimicrob Chemother 67: 2640-2644.
561 46. Yergeau E, Hogues H, Whyte LG, Greer CW (2010) The functional potential of high Arctic permafrost revealed
562 by metagenomic sequencing, qPCR and microarray analyses. ISME J 4: 1206-1214.
563 47. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW (2011) GenBank. Nucleic Acids Res 39: D32-
564 37.
565 48. Stein T (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol Microbiol 56: 845-
566 857.
567 49. Llewellyn NM, Spencer JB (2006) Biosynthesis of 2-deoxystreptamine-containing aminoglycoside antibiotics.
568 Nat Prod Rep 23: 864-874.
569 50. Brakhage AA (1998) Molecular regulation of beta-lactam biosynthesis in filamentous fungi. Microbiol Mol Biol
570 Rev 62: 547-585.
571 51. Claridge CA, Gourevitch A, Lein J (1960) Bacterial penicillin amidase. Nature 187: 237-238.
572 52. Tishkov VI, Savin SS, Yasnaya AS (2010) Protein engineering of penicillin acylase. Acta Naturae 2: 47-61.
573 53. Brakhage AA, Thön M, Spröte P, Scharf DH, Al-Abdallah Q, et al. (2009) Aspects on evolution of fungal beta-
574 lactam biosynthesis gene clusters and recruitment of trans-acting factors. Phytochemistry 70: 1801-1811.
575 54. Yumoto I, Yamazaki K (2013) Ecology and taxonomy of psycrhotolerant bacteria in artificial cold environments.
576 In: Yumoto I, editor. Cold-adapted microorganisms. Norfolk, UK: Caister Academic Press. pp. 220.
577 55. Hu RM, Chiang KH, Lin CW, Yang TC (2008) Modified nitrocefin-EDTA method to differentially quantify the
578 induced L1 and L2 beta-lactamases in Stenotrophomonas maltophilia. Lett Appl Microbiol 47: 457-461.
579 56. Magnuson K, Jackowski S, Rock CO, Cronan JE (1993) Regulation of fatty-acid biosynthesis in *Escherichia coli*.
580 Microbiological Reviews 57: 522-542.
581 57. Looney WJ, Narita M, Muhlemann K (2009) *Stenotrophomonas maltophilia*: an emerging opportunist human
582 pathogen. Lancet Infect Dis 9: 312-323.
583 58. Röttig A, Steinbüchel A (2013) Acyltransferases in bacteria. Microbiol Mol Biol Rev 77: 277-321.
584 59. Davies J, Wright GD (1997) Bacterial resistance to aminoglycoside antibiotics. Trends Microbiol 5: 234-240.
585 60. Benveniste R, Davies J (1973) Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those
586 present in clinical isolates of antibiotic-resistant bacteria. Proc Natl Acad Sci U S A 70: 2276-2280.
587 61. Kawaguchi H (1976) Discovery, chemistry, and activity of amikacin. J Infect Dis 134 SUPPL: S242-248.
588 62. Meyer RD (1977) Patterns and mechanisms of emergence of resistance to amikacin. J Infect Dis 136: 449-452.
589 63. Mazzei T, Paradiso M, Nicoletti I, Periti P (1976) Amikacin in obstetric, gynecologic, and neonatal infections:
590 laboratory and clinical studies. J Infect Dis 134 SUPPL: S374-379.
591 64. Wright GD, Poinar H (2012) Antibiotic resistance is ancient: implications for drug discovery. Trends Microbiol.
592 65. Heeb S, Fletcher MP, Chhabra SR, Diggle SP, Williams P, et al. (2011) Quinolones: from antibiotics to
593 autoinducers. FEMS Microbiol Rev 35: 247-274.
594 66. Poirel L, Rodriguez-Martinez JM, Mammeri H, Liard A, Nordmann P (2005) Origin of plasmid-mediated
595 quinolone resistance determinant QnrA. Antimicrob Agents Chemother 49: 3523-3525.
596 67. Martinez-Martinez L, Pascual A, Jacoby GA (1998) Quinolone resistance from a transferable plasmid. Lancet
597 351: 797-799.
598 68. Jacoby GA, Hooper DC (2013) Phylogenetic analysis of chromosomally determined qnr and related proteins.
599 Antimicrob Agents Chemother 57: 1930-1934.
600 69. Hooper DC (2001) Emerging mechanisms of fluoroquinolone resistance. Emerg Infect Dis 7: 337-341.
601 70. Robicsek A, Jacoby GA, Hooper DC (2006) The worldwide emergence of plasmid-mediated quinolone
602 resistance. Lancet Infect Dis 6: 629-640.
603 71. Knapp CW, Dolfing J, Ehlert PA, Graham DW (2010) Evidence of increasing antibiotic resistance gene
604 abundances in archived soils since 1940. Environ Sci Technol 44: 580-587.
605 72. Popowska M, Rzeczycka M, Miernik A, Krawczyk-Balska A, Walsh F, et al. (2012) Influence of soil use on
606 prevalence of tetracycline, streptomycin, and erythromycin resistance and associated resistance genes.
607 Antimicrob Agents Chemother 56: 1434-1443.
608 73. Mykytczuk NC, Wilhelm RC, Whyte LG (2012) *Planococcus halocryophilus* sp. nov., an extreme sub-zero
609 species from high Arctic permafrost. Int J Syst Evol Microbiol 62: 1937-1944.
610 74. Craig JW, Chang FY, Kim JH, Obiajulu SC, Brady SF (2010) Expanding small-molecule functional
611 metagenomics through parallel screening of broad-host-range cosmid environmental DNA libraries in
612 diverse proteobacteria. Appl Environ Microbiol 76: 1633-1641.

613 75. Sandegren L, Andersson DI (2009) Bacterial gene amplification: implications for the evolution of antibiotic
614 resistance. Nat Rev Microbiol 7: 578-588.
615 76. Reimann H, Cooper DJ, Mallams AK, Jaret RS, Yehaskel A, et al. (1974) The structure of sisomicin, a novel
616 unsaturated aminocyclitol antibiotic from *Micromonospora inyoensis*. J Org Chem 39: 1451-1457.
617 77. Fleming A (1929) On the antibacterial action of cultures of a penicillium with special reference to their use in the
618 isolation of *B. influenzae*. Br J Exp Pathol 10: 226-236.
619 78. Knudsen ET, Rolinson GN, Sutherland R (1967) Carbenicillin: a new semisynthetic penicillin active against
620 *Pseudomonas pyocyanea*. Br Med J 2: 75-78.
621 79. Acred P, Brown DM, Knudsen ET, Rolinson GN, Sutherland R (1967) New semi-synthetic penicillin active
622 against *Pseudomonas pyocyanea*. Nature 215: 25-30.
623 80. Duggar BM (1948) Aureomycin; a product of the continuing search for new antibiotics. Ann N Y Acad Sci 51:
624 177-181.
625 81. von Wittenau MS, Beereboom JJ, Blackwood RK, Stephens CR (1962) 6-Deoxytetracyclines. III.
626 Stereochemistry at C.6. J Am Chem Soc 84: 2645-2647.
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651

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

686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712

713 **Table 2. List of resistance genes and their predicted annotation.**
714

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 lz
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

715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732

733 **Table 3. Number of sequences homologous to functional resistance genes in metagenomic**
734 **surveys of the Canadian high Arctic.**
735

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.
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762

763 # Supplementary Information

764

765 **Figure S1:** Sampling depth of resistance conferring inserts.

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

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

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

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

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

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

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

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

782

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

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

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

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

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

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

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

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

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

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

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

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

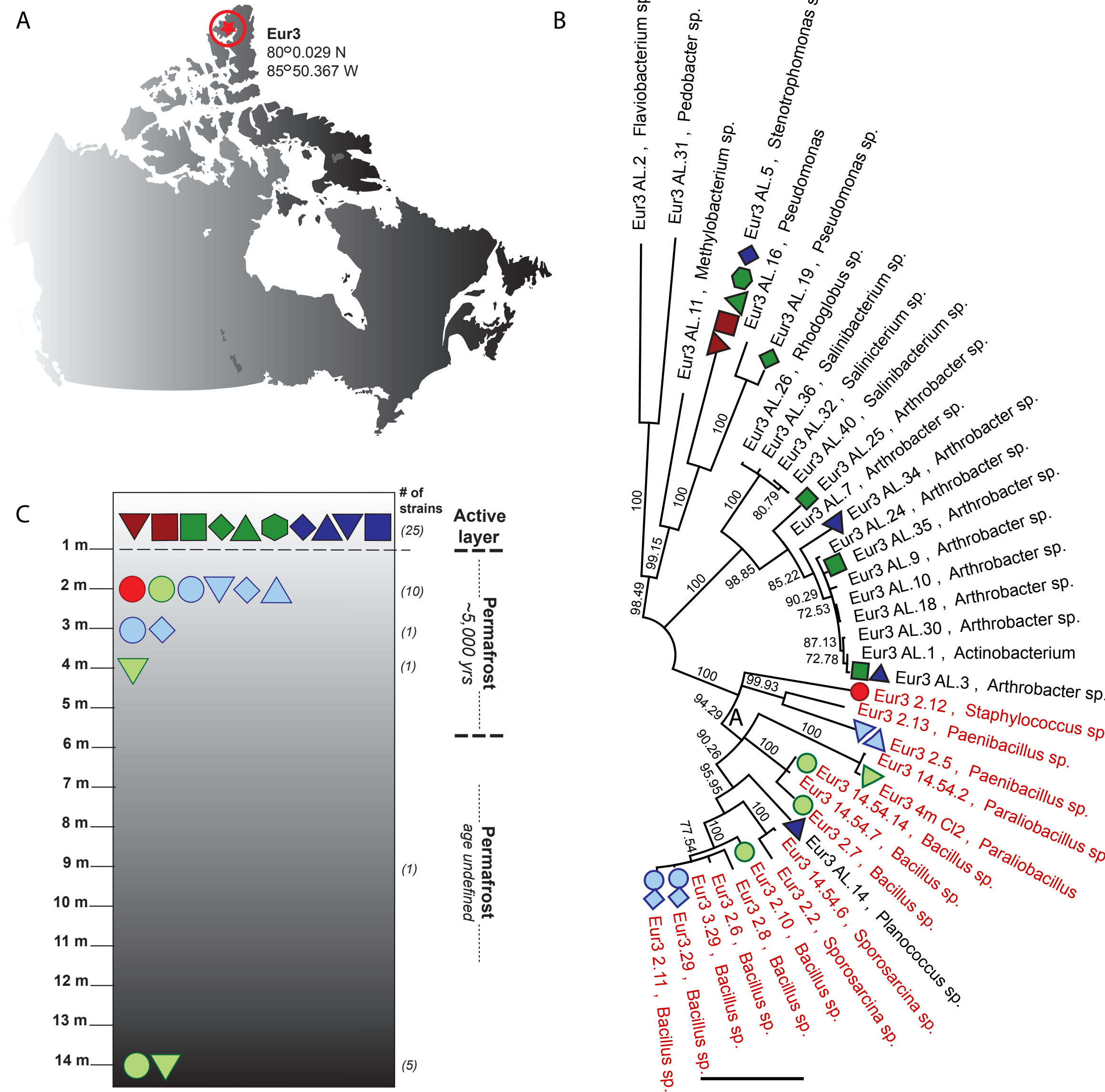
798

799

800

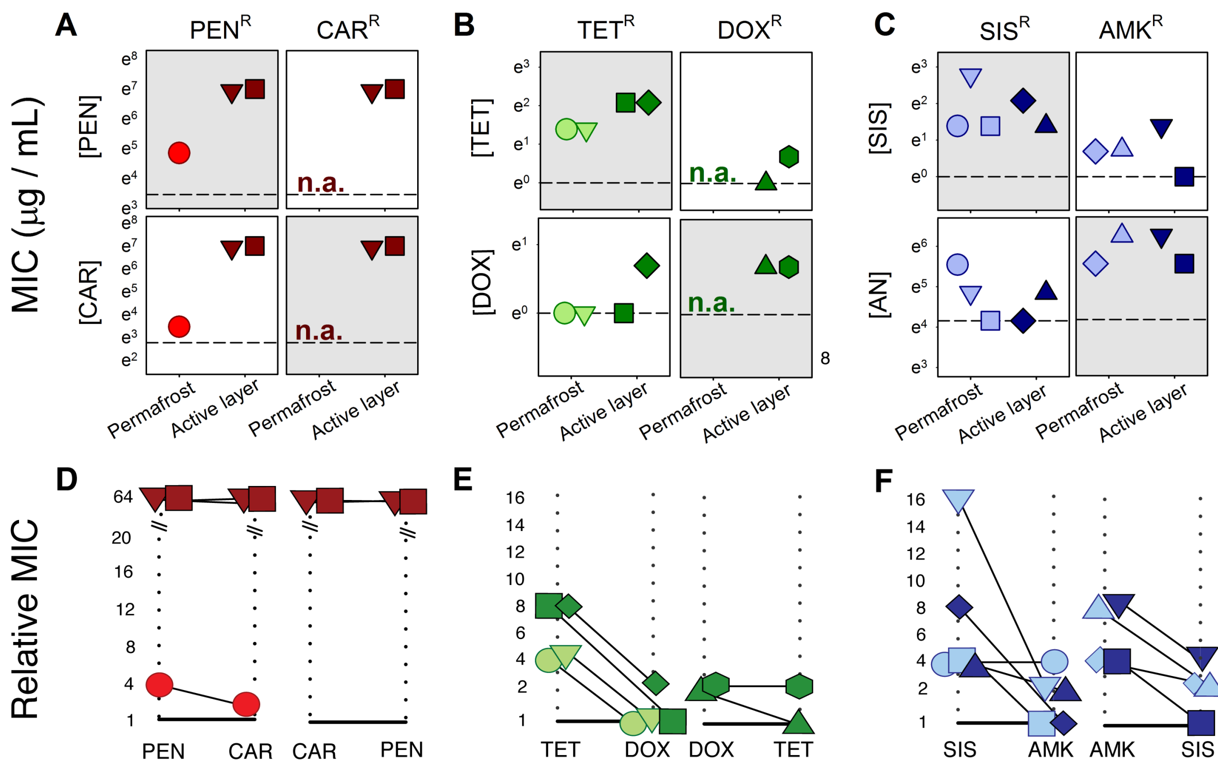
801

802 **Figure 1**



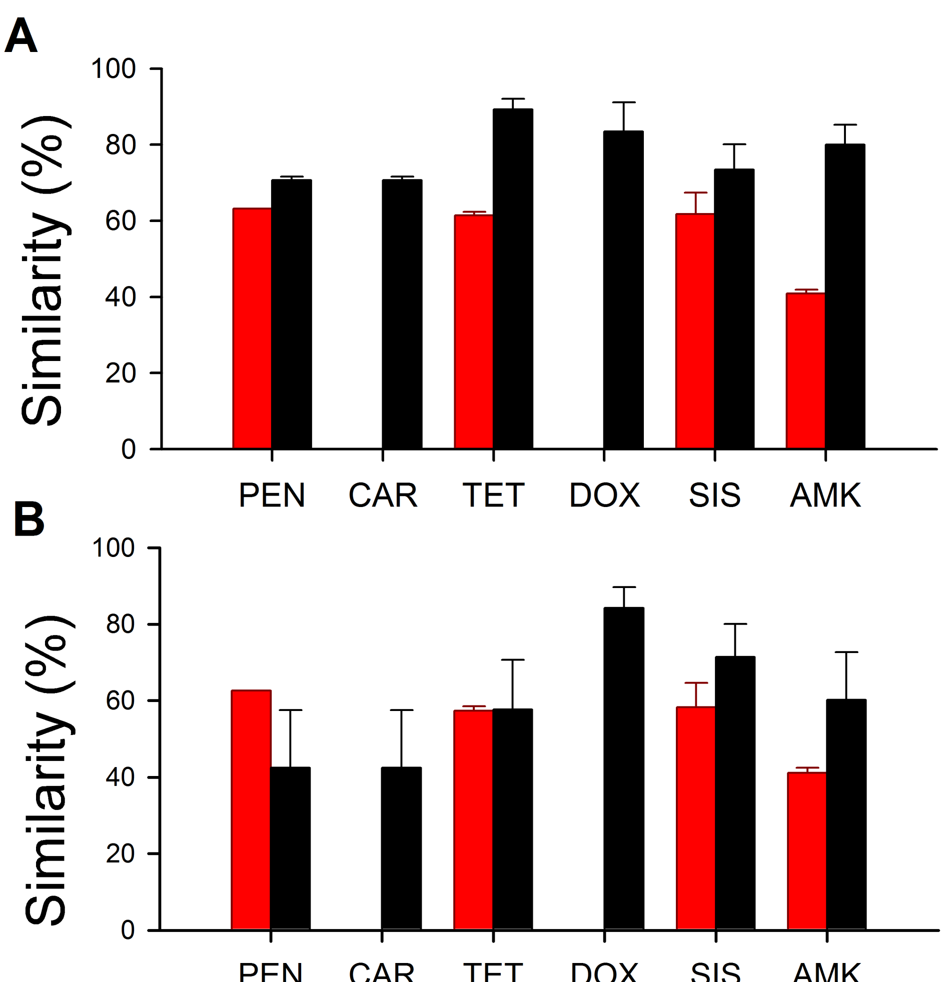
803
804
805
806
807
808
809
810
811
812
813
814
815

816 **Figure 2**
817



818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839

840 **Figure 3**



841