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Developing a Freeze-Thaw Assay to Quantify Antibiotic Resistance Gene Transfer in Bacterial Co-Cultures

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ABSTRACT

The hastening thaw of glaciers and permafrost due to climate change threatens to release archaic genetic material, including antibiotic resistance genes (ARGs), into aquatic environments. While the biological risks associated with this release have been recognized, it is vital to understand the effects that freeze-killed cells and their genetic material may have on living microbial communities.

This study aims to investigate whether DNA from freeze-killed cells can be taken up by living cells, specifically via transformation. Kanamycin-resistant MC1061 Escherichia coli, harboring the P3 plasmid, encoding the resistance gene aph(3')-Ia, were subjected to five freeze-thaw cycles (-20°C/37°C) to simulate glacial thawing and were then co-cultured with kanamycin-sensitive DH5α E. coli. Colony forming units on kanamycin-selective and non-selective LB agar were used to measure kanamycin resistance frequency (KRF) across experimental and control groups. Co-cultures containing freeze-thawed donors cells exhibited a substantially elevated KRF compared to co-cultures with donor cells that had not undergone freeze-thaw cycles and control group estimated, considering no interaction due to HGT, demonstrating that HGT of ARGs occurred at a significant rate despite the majority of donor cells being dead. These findings are consistent with gene transfer occurring via transformation, likely facilitated by cell lysis of frozen donor cells, though this was not explicitly confirmed and alternative explanations, such as resistance arising from surviving donor cells, cannot be excluded. This work highlights a way in which melting environments may contribute to the global spread of antibiotic resistance.

INTRODUCTION

Glaciers and other frozen environments such as permafrost and sea ice hold a wealth of biological information about our planet's past. These bodies of ice can preserve evidence of life from thousands of years ago (Zhong et al., 2021). From preserved humans to proteobacteria, valuable information about life and evolution can be gleaned from these ancient organisms. However, such environments also contain unseen dangers and are melting at rapid rates. Antimicrobials are the backbone of medicine today, used to

treat a variety of diseases and infections found in all living organisms. These drugs have shaped the development of medicinal science as a whole, but they are limited due to the difficulty involved in identifying new antibiotics. Furthermore, global trends of antimicrobial overuse and misuse are threatening the future of these valuable tools. Starting as early as the mid-20th century, cases of antimicrobial, particularly antibacterial, resistance have been identified, growing continually to the present day. According to a World Health Organization survey on antimicrobial resistance, there has been an alarming uptick in resistance rates worldwide. 1 in 5 cases of urinary tract infections exhibited at least partial resistance to common antibiotics in 2020, and in 76 countries. Both E. coli and Staphylococcus aureus showed incredibly high rates of resistance, averaging 42% and 35% respectively (Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report: 2022, n.d.). Thus the genetic material found inside glaciers and permafrost pose a serious risk to modern medicine. A 2024 study investigated a mountain glacier in Central Asia. Between glacier ice, cryoconite, and glacial streams, the researchers discovered a total of 944 distinct antibiotic resistance genes (ARGs), affecting over 22 groups of antibiotics (Ren & Gao, 2024). As frozen bodies melt, they release millennia old microbes trapped within. Although dead or in a state of stasis, these cells still contain genetic information. As shown in Frederick Griffith's influential 1928 study, non-virulent Streptococcus pneumoniae became virulent after being mixed with heat-killed virulent cells. This provides reasonable grounds to wonder whether long-frozen bacteria could still share their genetic material, specifically ARGs, with living cells. If this is the case, melting ice caps could pose more of a threat than just rising sea levels. This study aims to investigate the validity of these speculations by observing the rate of horizontal gene transfer (HGT) of antibiotic resistance genes between freeze-killed cells and living cells.

Horizontal gene transfer is the non-sexual transfer of genetic information between two distinct organisms. HGT can be split into 3 different processes: transduction, conjugation, and transformation (Arnold et al., 2022). However, transduction and conjugation require living donor and recipient cells, thus, this study predominantly focuses on the latter. In transformation, the most relevant form of HGT to this study since it doesn't require living donors, cells directly uptake genetic material from their surroundings, absorbing it through the cell membrane. The DNA does this by passing through channels in the cell membrane or Bayer's junctions, with each cell having an average of 400 of the zones, after which, the foreign DNA is then incorporated into the cell's genome (Johnston et al., 2014).

Once an ARG is present in the genome of a cell, it can function in multiple ways. The first of these mechanisms works by limiting the amount of drug that the cell absorbs. Secondly, some ARGs modify the protein that the drug targets. For example, a drug that works by inhibiting the construction of a cell wall could be countered by decreasing the number of proteins to which the drug can bind to. A third method of achieving antibiotic resistance is through β-lactamases. These enzymes work against β-lactam drugs by hydrolyzing a site on the ring, effectively deactivating the drug. The fourth and final category of ARGs function by inactivating the drug in question. These ARGs work by transferring chemical groups, such as acetyl, adenyl, and phosphoryl groups, to the drugs, rendering them non-functional (Reygaert, 2018).
The type of ARGs primarily investigated in this study fall into the fourth category, specifically being aphA-3 and NPTII. These genes translate into enzymes that phosphorylate aminoglycosides, the group of

antibiotics that includes drugs like kanamycin, tobramycin, and amikacin. The susceptible bacteria used in this study will be DH5α, a strain of E. coli, while the kanamycin resistant cells will harbor the P3 plasmid which contains the gene aph(3')-Ia, which is associated with kanamycin resistance. Results showed that freeze-thaw cycles did increase the kanamycin resistant frequency in co-cultured samples, thus exhibiting a greater rate of HGT.

METHODOLOGY

Materials & Reagents

Reagent / Product Name	Catalog Number	Supplier / Vendor
MC1061 E. coli (competent cells)	C66303	Thermo Fisher Scientific
DH5α E. coli	EC0112	Thermo Fisher Scientific
LB Broth	10855001	Thermo Fisher Scientific
LB Agar	CMLP-4	BioPioneer
LB Agar + Kanamycin 50μg/mL	CML-KA-2	BioPioneer
Sterile water	W0223	Teknova

Freeze-Thaw Cycles of Kanamycin Resistant E. coli

In order to prepare samples of kanamycin resistant bacteria, 100μL of MC1061 Escherichia coli were pipetted onto LB Agar with 50μg/mL of kanamycin. This culture was placed in an incubator to grow at 37°C for 24 hours. After proliferating, an inoculating loop was used to scrape bacterial growth off of the agar. This sample of bacteria was then suspended in 750μL of sterile water and vortexed to distribute bacteria properly. This solution was then placed in a freezer at -20°C for 4 hours, then thawed at 37°C until fully liquid after 5 minutes. The sample was then returned to the freezer, and the process was repeated until the sample had been frozen and thawed 5 times.

Creation of Secondary Samples

100µL of DH5α Escherichia coli (sensitive to kanamycin) was pipetted onto LB Agar. This sample was allowed to grow for 24 hours at 37°C. Following this, an inoculating loop was then used to collect colonies from the agar and suspend them in 750µL of sterile water, which was then vortexed. At the same time, a similar amount of bacterial growth was collected from the grown sample of MC1061 E. coli in section 3.1. Similarly, these bacterial colonies were suspended in 750µL of sterile water and then vortex.

Co-Culturing of Bacterial Samples

4 different combinations of samples were created and they will be referred to as A, B, C, & D respectively.
Sample A: 250µL of Freeze-Thawed MC1061 E. coli, 500µL of DH5α solution, 250µL of LB
Sample B: 250µL of MC1061 E. coli, 250µL of DH5α solution, 500µL of LB
Sample C: 500µL of Freeze-Thawed MC1061 E. coli, 500µL of LB
Sample D: 500µL of DH5α E. coli, 500µL of LB
Samples A, B, C, and D were each vortexed then placed in an incubator at 37°C for 4 hours.

Serial Dilutions & Final Plating on Agar

Following incubation, Samples A, B, C, and D were each reduced 7-fold to 1×10-7 of their original concentration by repeatedly adding 100µL of each previous sample to 900µL of sterile water (Reynolds, 2005). Following serial dilutions, 100µL of samples A×10-7, B×10-7, C×10-7, D×10-7 were independently pipetted onto LB Agar. Another 100µL of samples A×10-7, B×10-7, C×10-7 and D×10-7 were also respectively pipetted onto LB Agar with 50µg/mL of kanamycin. This adds to a total of 8 Agar Plates.

RESULTS

This study's primary focus was to identify a correlation between freeze-thaw cycles on MC1061 Escherichia coli, carrying kanamycin resistance, and the rate of horizontal gene transfer to susceptible cells of the same species through natural transformation of the gene. Freeze-killed cells were placed in contact with cells that are susceptible to the antibiotic of interest: kanamycin, allowing transformation to take place, causing the susceptible cells to integrate the ARG into their genome.

Colony-Forming Units and Kanamycin Resistance Frequency
Bacterial growth was quantified by counting colony-forming units (CFUs) on agar plates. Mean values were calculated for each of the 4 experimental groups and 3 biological replicates, conducted fully independent from one another (Table 1).

	LB	LB 95% CI	Kanamycin	Kanamycin 95% CI
(A) Frozen MC1061+DH5α	54±6.49	[43.7, 64.3]	37±6.47	[26.7, 47.3]
(B) MC1061+DH5α	103±11.02	[85.5, 120.5]	49±4.17	[42.4, 55.6]
(C) Frozen MC1061	9±2.97	[4.3, 13.7]	8±3.85	[1.9, 14.1]
(D) DH5α	54±9.12	[39.5, 68.5]	0±0.533	[0, 0.8]
(C+D)	64±12.011	[44.9, 83.1]	8±3.847	[1.9, 14.1]

Table 1: Mean CFUs ± SD of 4 experimental groups, rounded to the nearest whole number. Lower CI bounds truncate at 0, as negative CFU count is not biologically meaningful. (Source: Author)

When directly comparing samples A and B, it may appear as if the freeze-thaw cycles had no apparent effect on the number of CFUs that successfully grew on kanamycin. However, it is more important to consider the proportion of surviving colonies on kanamycin to the total growth on LB (Kanamycin Resistance Frequency). This is due to the fact that the Frozen MC1061 in sample A contributed far less CFUs than the MC1061 in sample B, equating to an uneven baseline for comparison. Comparing sample A with the calculated sum of samples C and D, it is clear that coculturing MC1061 and DH5α made a noticeable difference on kanamycin resistance frequency (37% vs. 8%). The fact that sample A far surpassed C+D in CFU count on kanamycin demonstrates that HGT occurred at a significant rate despite the fact that a majority of the frozen MC1061 was dead. However, while it is clear that the majority of MC1061 cells did not survive freeze-thaw cycles, the exact proportion of surviving cells was not determined. However, an estimate can be derived from existing data: Sample C (freeze-thawed MC1061 alone) had a mean of 9 CFUs on LB agar, compared to 103 CFUs for Sample B, suggesting a donor cell survival rate of around 8.7% following freeze-thaw cycles.Thus, surviving donor cells could account for a small portion of the kanamycin-resistant colonies observed in Sample A, though the substantially higher CFU count on kanamycin in Sample A compared to Sample C suggests that the majority of these colonies arose via HGT rather than from surviving donors.

	KRF
(A) Frozen MC1061+DH5α	63.872%
(B) MC1061+DH5α	47.452%

(D) DH5α	0.667%
(C+D)	12.70%

Table 2: Ratio of CFU count on kanamycin to CFU count of LB Agar. C+D is the sum of data from samples C and D. (Source: Author)

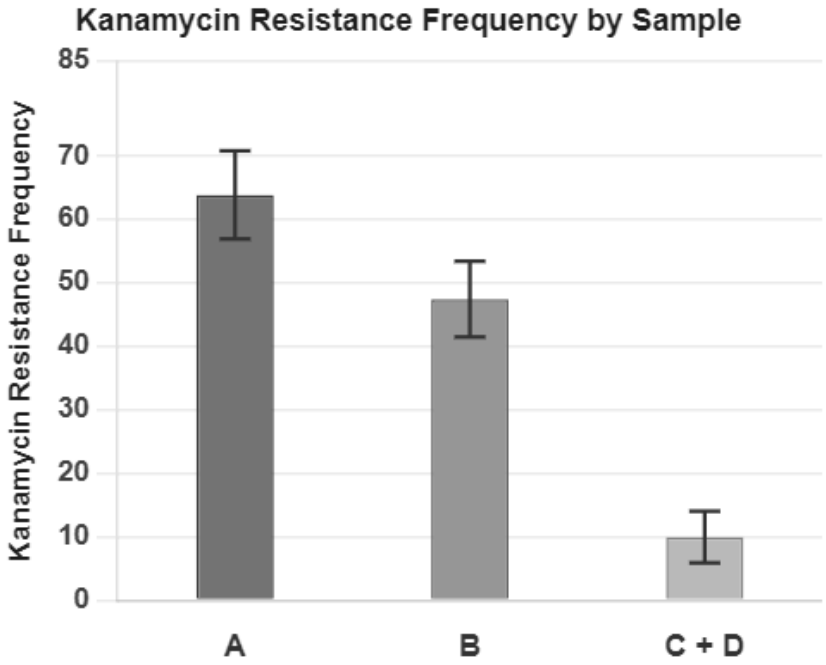

Figure 1: Kanamycin resistance frequency (KFR) by sample. Graphical representation of Table 2. (Source: Author)

As seen in both Table 2 and Figure 1, co-cultures containing frozen MC1061 exhibited a significantly greater proportion of kanamycin-resistant colonies compared to untreated cultures, suggesting that freeze-thaw treatments potentially elevate the rate of horizontal gene transfer. An unpaired t-test was used to compare the means of sample C+D and sample A across all 3 biological replicates ($p=0.0006$), which demonstrated a significant increase in kanamycin resistance frequency, which furthers the evidence that HGT did occur in sample A to a sizeable extent. In addition, this limits the claim that a significant proportion of kanamycin-resistant colonies in sample A survived from the frozen MC1061, rather than as a result of HGT. However, statistical testing relied on the assumption that the data within each group was approximately normal which cannot be guaranteed due to the small number of replicates.

Impact of Negative Controls

Samples C and D serve as negative controls to solidify the basis of previous results. Sample C, provides insight on the efficiency of freeze-thaw treatments whilst also acting as a baseline for comparison with sample A. On Kanamycin, sample A grew 37±6.47 CFUs, and given that sample C grew 8±3.85 under the same conditions, it is logical to assume that ~29 CFUs were a result of HGT, more specifically transformation. While a genetic mutation in DH5α cultures could invalidate any prior results, it is important to note that sample D grew nearly 0 CFUs on kanamycin, indicating that there was a low level of innate resistance.

DISCUSSION

The purpose of this study was to investigate the effects of repeated freeze-thaw cycles on the rate of horizontal gene transfer of antibiotic resistance genes between bacterial strains. Interpreting experimental results, it was concluded that HGT rate was increased by approximately 51.17 percentage points (from 12.70% in the C+D control to 63.87% in sample A), on average, following 5 freeze-thaw cycles. A 2024 environmental study on ARG transfer due to freeze-thaw cycles in soil on a dairy farm, reached similar conclusions, where the frequency of multiple ARGs increased by, in some cases, over 16% (Kong et al., 2024). Thus, it is reasonable to conclude that freeze-thaw cycles physiologically affect cells in a way that causes their DNA to be more available to other cells. The most likely of these causes is that the repeated formation of ice crystals triggered cell lysis or punctured the cell membrane, releasing genetic material into the environment. This theory accounts for the significant drop in viability of cultures of freeze-thawed cells alone. A 2014 study on HGT in the human oral microbiome concluded that genetically induced cell lysis caused a significant increase in HGT between cells (Roberts & Kreth, 2014). Thus it is reasonable to assume that the freeze-induced cell lysis significantly contributed to the higher rate of HGT in the presence of freeze-thawed cells. The uptick in kanamycin resistance frequency could also possibly, albeit minimally, be due to an SOS response. Certain stress factors, including freeze-thawing, can warrant an SOS response in *E. coli*, importantly freeze-thaw cycles can cause oxidative DNA damage resulting in double-strand breakage. After a critical accumulation of single-stranded DNA (ssDNA), RecA polymerizes onto the ssDNA, forming RecA-ssDNA nucleofilaments. This stimulates the LexA protein to cleave and degrade itself, meaning it can no longer bind to SOS box promoters which inhibit the transcription of ~40 SOS genes. Some of these genes in particular, conjugative transfer genes, are de-repressed along with the SOS genes which effectively increases the rate at which donor cells initiate conjugation (Len et al., 2019). While SOS responses more broadly apply to cells that would have survived in ice for a long time, they could potentially explain some of the increased HGT that occurred from surviving MC1061. Along a similar vein, oxidative stress can cause increased membrane permeability, potentially allowing for the movement of DNA out of the cell. Thus, plasmid release and HGT efficiency are enhanced, independent of the viability of the donor cell (Cheng et al., 2022).

Several aspects of this experimental setup could be refined in future iterations. Most notably, a better quantitative estimate of the survival frequency of sample C following freeze-thaw cycling would

strengthen the analysis. Future work could include a more rigorous quantification of donor cell survival following freeze-thaw cycles, for example, by plating freeze-thawed MC1061 on LB agar prior to co-culturing or by using staining techniques, to determine the viability of donor cells after freeze-thaw. Obtaining this value would allow for a more accurate assessment of whether the observed kanamycin-resistant colonies in sample A arose exclusively from HGT rather than from surviving colonies of MC1061.

Given the current state of climate change, freeze-thaw cycling and the thawing of permafrost and glaciers is to accelerate in high latitudes. With this, comes the release of a diversity of genetic and biological material that could eventually be carried into downstream environments, including soil, freshwater, and man-made food and water systems. At a microscopic and isolated scale, this study aimed to investigate the systems at work upon the transfer of genetic material from freeze-thawed cells. Provided the data and results previously presented, it is not unreasonable to state that freeze-thaw stress could priming bacteria to transfer ARGs, or other genes, at elevated rates upon thawing, had they not been frozen in the first place. Given the global trends of antibiotic resistance and its impact on the healthcare system, this is a concerning reality, however it is vital to not overstate these risks. This study functions as a simplified, small-scale, and entry-level laboratory model, and thus is not significantly representative of any broad environmental patterns. If given more resources, future studies could use metagenomic sequencing to track ARG transfer across microbial communities in simulated, or real, glacial environments, providing a more comprehensive picture of the real-world risk posed by thawing permafrost.

REFERENCES

Arnold, B. J., Huang, I.-T., & Hanage, W. P. (2022). Horizontal gene transfer and adaptive evolution in bacteria. *Nature Reviews Microbiology*, *20*(4), 206–218. https://doi.org/10.1038/s41579-021-00650-4

Cheng, Y.-Y., Zhou, Z., Papadopoulos, J. M., Zuke, J. D., Falbel, T. G., Anantharaman, K., Burton, B. M., & Venturelli, O. S. (2022). *Efficient plasmid transfer via natural competence in a synthetic microbial community* (p. 2020.10.19.342733). bioRxiv. https://doi.org/10.1101/2020.10.19.342733

Global antimicrobial resistance and use surveillance system (GLASS) report: 2022. (n.d.). Retrieved February 24, 2026, from https://www.who.int/publications/i/item/9789240062702

Johnston, C., Martin, B., Fichant, G., Polard, P., & Claverys, J.-P. (2014). Bacterial transformation: Distribution, shared mechanisms and divergent control. *Nature Reviews Microbiology*, *12*(3), 181–196. https://doi.org/10.1038/nrmicro3199

Kong, F., Qi, Z., Tong, H., Ren, N., & You, S. (2024). Case study on the relationship between transmission of antibiotic resistance genes and microbial community under freeze-thaw cycle on cold-region dairy farm. *The Science of the Total Environment*, *952*, 175989. https://doi.org/10.1016/j.scitotenv.2024.175989

Len, J. S., Koh, W. S. D., & Tan, S.-X. (2019). The roles of reactive oxygen species and antioxidants in cryopreservation. *Bioscience Reports*, *39*(8), BSR20191601. https://doi.org/10.1042/BSR20191601

Ren, Z., & Gao, H. (2024). Antibiotic resistance genes in integrated surface ice, cryoconite, and glacier-fed stream in a mountain glacier in Central Asia. *Environment International*, *184*, 108482. https://doi.org/10.1016/j.envint.2024.108482

Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiology*, *4*(3), 482–501. https://doi.org/10.3934/microbiol.2018.3.482

Roberts, A. P., & Kreth, J. (2014). The impact of horizontal gene transfer on the adaptive ability of the human oral microbiome. *Frontiers in Cellular and Infection Microbiology*, *4*. https://doi.org/10.3389/fcimb.2014.00124

Sleight, S. C., Wigginton, N. S., & Lenski, R. E. (2006). Increased susceptibility to repeated freeze-thaw cycles in Escherichia coli following long-term evolution in a benign environment. *BMC Evolutionary Biology*, *6*, 104. https://doi.org/10.1186/1471-2148-6-104

Zhong, Z.-P., Tian, F., Roux, S., Gazitúa, M. C., Solonenko, N. E., Li, Y.-F., Davis, M. E., Van Etten, J. L., Mosley-Thompson, E., Rich, V. I., Sullivan, M. B., & Thompson, L. G. (2021). Glacier ice archives nearly 15,000-year-old microbes and phages. *Microbiome*, *9*(1), 160. https://doi.org/10.1186/s40168-021-01106-w