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A Twist to the Kirby-Bauer Disk Diffusion Susceptibility Test: an Accessible Laboratory Experiment Comparing *Haloferax volcanii* and *Escherichia coli* Antibiotic Susceptibility to Highlight the Unique Cell Biology of Archaea

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INTRODUCTION

Undergraduate lab syllabi seldom include experiments involving one of the three domains of life, archaea. Their exclusion, however, undermines their importance: metagenomic studies have shown that representatives of this prokaryotic domain of life are found in highly diverse environments and are likely the ancestors of the first eukaryotic cells (1, 2). Although archaea appear morphologically similar to most bacteria, there are significant differences between these two domains in terms of structure, composition, and function (3, 4). For example, while most archaea and bacteria have a protective cell wall, the bacterial cell wall is composed of a peptidoglycan layer, whereas the most common archaeal cell wall, the S-layer, is composed of a single glycoprotein (5). A subset of archaea possesses a pseudopeptidoglycan layer that lacks D-amino acids and *N*-acetylmuramic acid, both of which are unique bacterial components that are targets of antimicrobial agents (5). Moreover, archaea more closely resemble eukaryotic cells in regard to the processing of genetic information and protein transport (3). Interestingly, even the archaeal ribosome, although similar in size to that of bacteria, differs in structure as well as biogenesis and shows resistance to drugs that inhibit the bacterial 70S and eukaryotic 80S ribosomes (6, 7).

The Kirby-Bauer disk diffusion susceptibility test is commonly used in biology classrooms to illustrate differences in antibiotic susceptibility between bacterial species based on distinct cellular structures as well as the development of antibiotic resistance in bacteria, one of the most serious current health threats (8, 9). The already ubiquitous presence of this test in classrooms provides an excellent opportunity to easily introduce archaea into the curriculum as well, such as by comparing the effects of various antibiotics on bacteria and archaea, which allows students to examine similarities and differences between the two domains. The beta-lactams, such as ampicillin, for example, are commonly used antibiotics in disk diffusion lab experiments, which prevent bacterial growth by targeting peptidoglycan synthesis at the cell wall (6) (Fig. 1). These antibiotics, however, do not affect archaea given their differential cell wall composition as discussed above (6, 10). Additionally, while archaea are not sensitive to antibiotics such as kanamycin or gentamicin, which inhibit the 30S subunit of the ribosome, bacteria generally are (6, 7). Conversely, other antibiotics such as novobiocin can in principle prevent growth in both domains since novobiocin targets the DNA gyrase, which is required for DNA replication in archaea as well as bacteria. However, while both haloarchaea and Gram-positive bacteria are sensitive to novobiocin, this antibiotic cannot effectively penetrate the outer membrane of Gram-negative bacteria, rendering them resistant to it (6, 10, 11) (Fig. 1).

As discussed in previous publications, *Haloferax volcanii*, an aerobic haloarchaeon, is ideal for use in an undergraduate curriculum (12, 13). *H. volcanii* is nonpathogenic and simple to grow and store, and the medium is easily prepared. Moreover, the halophilic nature of this organism eliminates the need for autoclaves or expensive sterile practices before or after experiments, as this organism thrives in concentrations of salts that are prohibitive for bacterial growth, reducing the risk of contamination. This ease of use is particularly critical since ill-equipped lab facilities often prevent successful implementation of engaging scientific curricula and present challenges to successful science, technology, engineering, and mathematics (STEM) exposure, despite the fact that early exposure to science is critical to promote retention in STEM (14, 15). This lack of opportunities and resources is predominantly found in areas with a higher percentage of students traditionally underrepresented in STEM than areas with higher STEM representation (16). A long-term effect of these inequalities is a lack of diversity in the STEM fields within academia and the workforce (15). Thus, there is a fundamental and immediate need for scientific experiments that offer immersive

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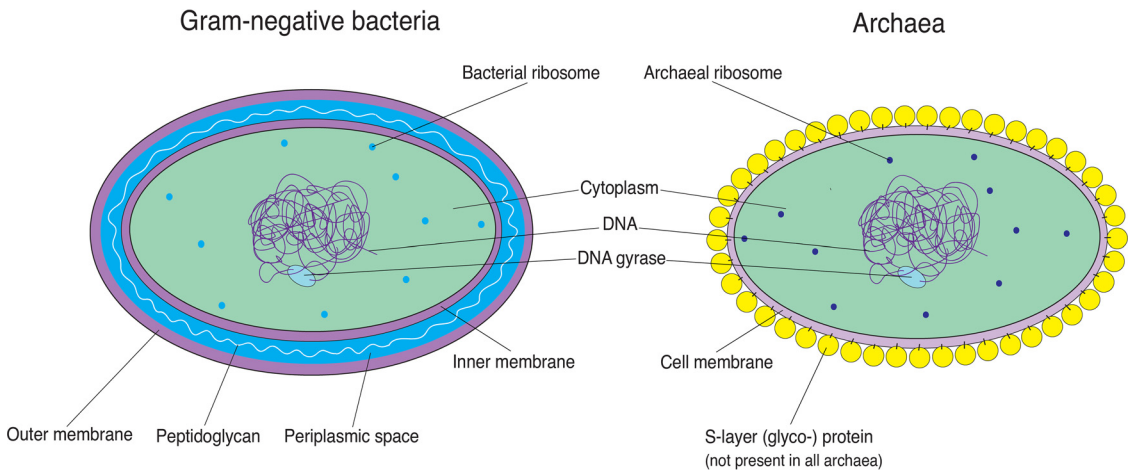

FIG 1. Schematic of bacterial and archaeal cells and their antibiotic targets. This cartoon highlights the distinct cellular structures between Gram-negative bacteria and archaea and allows students to visualize the different antibiotic targets. In addition to distinct cell walls, archaeal rRNA is more similar to that of eukaryotes than bacteria, while bacterial and eukaryotic lipid composition within the membrane, consisting of fatty acid chains linked to glycerol, differ from that of archaea, which is composed of isoprene chains linked to glycerol.

and effective scientific exposure while requiring few resources and funding. The experiment presented here can aid in overcoming such barriers, as it is both low-cost and accessible. Through comparing the antibiotic susceptibilities of *H. volcanii* and *E. coli*, this experiment provides an opportunity to discuss antibiotic susceptibility and a platform to explore the differences in cellular biology between archaea and bacteria. We also provide two different versions of this experiment suitable for both undergraduate and high school curricula (Appendix 1).

PROCEDURE

Activity overview

The activity presented in this paper requires two lab periods. The first lab will include both setting up and performing the experiment, and the second lab will consist of documenting, analyzing, and discussing the results. Instructors will have to account for prelab preparation time to grow the strains and prepare plates for the students. In the first lab period, students will streak *E. coli* and *H. volcanii* cells on their respective plates to create a lawn. Instructors have the option to just use *H. volcanii* and, during the second laboratory period, substitute comparison of an actual *E. coli* Kirby-Bauer plate with an image of an *E. coli* Kirby-Bauer plate. Subsequently, using forceps, the students will place the antibiotic filter disks on the plates. Teachers have the option to choose which antibiotics the students use based on which cellular targets they would like to be addressed (see Fig. 1 and Table S1 in Appendix 2). To help students with disk placement, we provide a plate template with optimal disk locations (see https://doi.org/10.5281/zenodo.5646561 for template download). The plates

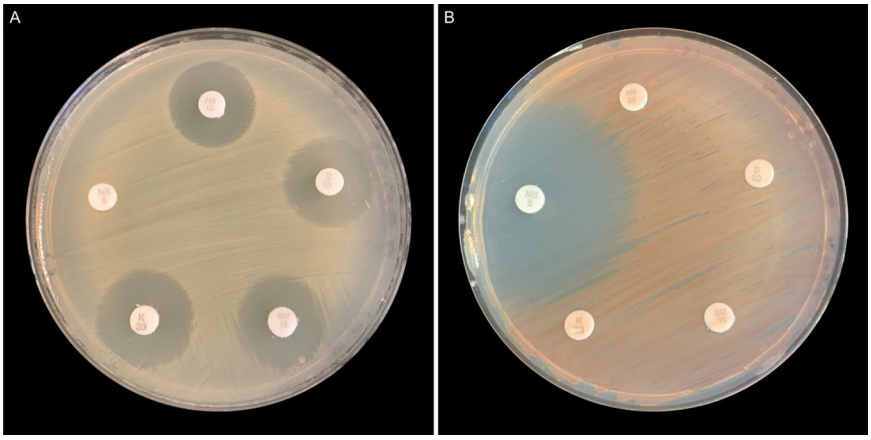

FIG 2. *H. volcanii* and *E. coli* are susceptible to distinct antibiotics. Cells of each organism were spread on their respective agar plates, and antibiotic disks were placed on the plates to test for antibiotic susceptibility. Ampicillin (AM) (top), streptomycin (S), gentamicin (GM), kanamycin (K), and novobiocin (NB) antibiotics (clockwise) were used. (A) The *E. coli* plate was imaged after overnight incubation. (B) The *H. volcanii* plate was incubated for 5 days before imaging. The *E. coli* strain used here is DH5α.

are then incubated at 37°C (the optimal temperature of *H. volcanii* is 45°C, but it can grow at 37°C, eliminating the need for two incubators) and will be analyzed during the second lab meeting (Fig. 2).

Materials

The materials needed are listed in Appendix 1. *E. coli* strain K-12 can be purchased from Carolina, while *H. volcanii* is available upon request from Pohlschroder's lab. Note that other *E. coli* strains besides K-12 can be used; when performing this experiment in our lab, we used the DH5α strain. The protocols for preparing both *H. volcanii* and *E. coli* media are outlined in Appendix 1. An alternative to the standard *H. volcanii* laboratory medium has been published by Kouassi et al. and uses ingredients available at grocery stores (12), which may be more suitable for high school classrooms; this protocol is also included in Appendix 1.

Intended audience

This laboratory exercise is intended for undergraduate students taking a microbiology course. It can be introduced into the curriculum for biology majors or nonbiology majors since the level of the postlab analysis is at the discretion of the instructor.

We also provide an affordable version of this experiment that can be used in a high school setting (see Appendix 1). This version uses only *H. volcanii* and provides a photo of a plate with *E. coli* (Fig. 2A). Using only *H. volcanii* allows high school students to have hands-on experience while reducing the cost, as high-salt plates can be prepared by the teachers without an autoclave and do not have any risk of contamination (see "Safety issues").

Safety issues

The nonpathogenic nature of *H. volcanii* eliminates any risk associated with younger, less experienced scientists handling prokaryotes. Furthermore, its high-salt-growth requirement eliminates the need for sterile conditions in preparing and handling the *H. volcanii* agar plates. The *E. coli* strain K-12 is also nonpathogenic and classified as a biosafety level 1 (BSL1) organism. Students only handle *E. coli* plates containing lawns of this organism, reducing the risk of contaminating these plates with other bacteria. Undergraduate students receive safety training at the beginning of the semester, and all experiments follow ASM Guidelines for Biosafety in Teaching Laboratories (https://asm.org/Guideline/ASM-Guidelines-for-Biosafety-in-Teaching-Laborator).

CONCLUSION

Recent publications have shown that the haloarchaeon *H. volcanii* is ideal for incorporation of hands-on experiments that might otherwise require sterile techniques and be cost-prohibitive to some undergraduate institutions and high schools (12, 13). This laboratory activity is a twist to the standard Kirby-Bauer disk diffusion susceptibility test to teach students about archaea, a domain of life that is commonly understudied in all levels of academia, and provide an excellent hands-on, equitable, and accessible microbiology experiment.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.13 MB.

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