Appendix 1- 2

Appendix 1

Material and Methods

Organisms

H. volcanii and *E. coli*
E. coli K12 is available from Carolina (https://www.carolina.com/bacteria/escherichia-coli-living-k-12-strain-nutrient-broth-tube/155068.pr?s_cid=ppc_gl_dsa&gclid=CjwKCAjwj8eJBhA5EiwAg3z0m_SF49rLF63PdGwTRNenHS23fXMQFEhMHGwSXoiyA-Yt1DkSyGfRixoC_qEQAvD_BwE).
H. volcanii is available upon request from the Pohlschroder laboratory.
A kit using another halophilic archaeon, *Halobacterium* sp. NRC-1, for the Kirby-Bauer disk diffusion susceptibility test is commercially available (https://www.carolina.com/catalog/detail.jsp?prodId=154776) and could be adapted to the focus on cell biological differences between archaea and bacteria. While use of this kit would avoid having to make the media, the instructors must have the financial means to purchase the kit. The doubling time of *H. salinarum* is also close to twice that of *H. volcanii*, and it requires significantly higher salt concentrations.

Materials

1. *E. coli* strain
2. *H. volcanii* strain
3. *E. coli* agar plates
4. *H. volcanii* agar plates
5. Q-tips
6. Antibiotic sensitivity disks. For example:
    1. Ampicillin (10 µg)
    2. Novobiocin (5 µg)
    3. Gentamicin (10 µg)
    4. Kanamycin (30 µg)
    5. Streptomycin (10 µg)
7. Tweezers
8. 37 °C incubator (optional: 45 °C incubator)

Other antibiotics can be utilized besides the ones listed here to highlight different antibiotic susceptibilities due to the cell biology of *H. volcanii* and *E. coli*. We recommend using five disks per plate to allow for optimal spacing given the zones of inhibition that will result, and we have provided a template for students to use (Supplemental Figure 1).
H. volcanii can be grown at 37 °C, which then means only one incubator is needed, but they will require a few days longer to grow than if they are grown at their optimal temperature of 45 °C (four to six days rather than three to five).

Media preparation

H. volcanii media

The laboratory-grade, semi-defined (Hv-Cab) media recipe used in this study, a less defined but easier to prepare medium (MGM) recipe, and the recipes for preparing 30% saltwater and 100x trace elements concentrated stock solutions are listed below. The recipe for preparing *E. coli* plates with either LB or NZCYM is also included.

It is recommended that the flask used is able to hold at least two times the amount of volume of medium being prepared.

Solid Hv-Cab agar plate (1.5% wt/vol) medium composition (per 1 L medium) (1):

1. Add 400 mL of ddH_2O, 5 g of Casamino acids, 2.35 mL of 1 M KOH, and 15 g of agar to the flask with an aluminum foil cap and stir bar and heat until it boils and all agar dissolves. It is recommended to boil the medium twice to ensure all agar has dissolved.
2. Add 600 mL of 30% saltwater stock (see below for instructions on preparing the 30% saltwater stock solution) and then autoclave for 20-30 min. Alternatively, *H. volcanii* medium can be boiled three times (until the mixture is clear and all salts are dissolved) or can be placed in a pressure cooker and kept in for about 20 minutes after steam start at middle power.
3. Add the remainder of the reagents to the flask: 3 mL of 1 M $CaCl_2$, 800 µL of thiamine, 100 µL of biotin and 10 mL of 100x trace elements (see below for instructions on preparing 100x trace element concentrated stock solution). Depending on the *H. volcanii* strain used, other nutrients may need to be added. For example, *H. volcanii* strain H53, used here, requires the addition of 1 mL of uracil (50 mg/mL in DMSO) and 5 mL of tryptophan (10 mg/mL in ddH_2O) stock solutions.
4. Allow the medium to continue stirring, and pour the medium into Petri dishes once it has cooled to about 60 °C.
5. Store plates upside down in a plastic bag at 4 °C for long-term storage or at room temperature for short-term (two weeks) storage.

30% saltwater stock solution composition (3):

1. 240 g/L NaCl
2. 35 g/L $MgSO_4 \cdot 7H_2O$
3. 30 g/L $MgCl_2 \cdot 6H_2O$
4. 7 g/L KCl
5. 5 mL 1 M $CaCl_2$

Add ddH_2O to bring the final volume to 1 L.

Adjust the pH of the solution to 7.5 with HCl or Tris Base after all salts are dissolved.

100x trace elements concentrated stock solution composition (1):

1. 5 g/L $Na_2EDTA \bullet 2H_2O$
2. 0.8 g/L $FeCl_3$
3. 0.05 g/L $ZnCl_2$
4. 0.01 g/L $CuCl_2$
5. 0.01 g/L $CoCl_2$
6. 0.01 g/L H_3BO_3
7. 1.6 g/L $MnCl_2$
8. 0.01 g/L $NiSO_4$
9. 0.01 g/L $Na_2MoO_4 \bullet 2H_2O$

Add ddH_2O to bring the final volume to 1 L.

Filter the brown precipitate by 0.2 µm sterile filtration.
Adjust the pH of the solution to 7.0 with NaOH (AR grade, Sigma).
Stock solutions can be stored at room temperature in aliquots.

As an alternative to Hv-Cab, MGM (modified growth medium) can be used to grow *H. volcanii.*

Solid MGM agar plate (1.5% wt/vol) composition (per 1 L medium) (2):

1. Add 400 mL of ddH_2O, 5 g Tryptone (Bacto TM Tryptone, Pancreatic Digest of Casein by Becton, Dickinson and Company), 3 g Yeast Extract (Bacto TM Yeast Extract, Dickinson and Company), and 15 g of agar to the flask with an aluminum foil cap and stir bar and heat until it boils and all agar dissolves. It is recommended to boil the medium twice to ensure all agar has dissolved.
2. Add 600 mL of 30% saltwater stock (see below for instructions on preparing the 30% saltwater stock solution).
3. Autoclave for 20-30 min. Alternatively, *H. volcanii* medium can be boiled three times (until the mixture is clear and all salts are dissolved) or can be placed in a pressure cooker and kept in for about 20 minutes after steam start at middle power.
4. Allow the medium to continue stirring after sterilization, and pour the medium into Petri dishes once it has cooled to about 60 °C.
5. Store plates upside down in a plastic bag at 4 °C for long-term storage or at room temperature for short-term (two weeks) storage.

<u>*E. coli* media</u>

Solid LB *E. coli* agar plate (1.5% wt/vol) medium composition (per 1 L medium):

1. Add 1 L of ddH_2O, 22 g of LB, and 15 g of agar to the flask with an aluminum foil cap and stir bar and heat until it boils and all agar dissolves. Double boiling is recommended to ensure all agar has dissolved.
2. Autoclave for 20-30 min. Alternatively, *E. coli* medium can be placed in a pressure cooker and kept in for about 20 minutes after steam start at middle power.
3. Allow the medium to continue stirring after sterilization, and pour the medium into Petri dishes once it cools to about 60 °C.
4. Store plates upside down in a plastic bag at 4 °C for long-term storage or at room temperature for short-term (two weeks) storage.

As an alternative to LB, NZCYM medium can be used to grow *E. coli*. Add 20 g of NZCYM, instead of 22 g of LB, to the recipe listed above.
If instructors do not wish to make their own plates, *E. coli* agar plates can be purchased from a wide variety of vendors.

<u>Plate storage</u>
Plates containing *H. volcanii* streaks can be stored at room temperature for at least a year in a closed container or ziplock bag to prevent dehydration.
Plates containing *E. coli* can be stored in the refrigerator for up to a month.

Procedure

Instructor pre-lab

1. At least one week before the student lab, prepare fresh bacterial and archaeal streaks on agar plates.
    1. *H. volcanii:* from a frozen stock, filter disk, or an older plate of *H. volcanii*, streak the archaea on agar plates and incubate for 4-6 days at 37 °C or 3-5 days at 45 °C.
    2. *E. coli:* from a frozen stock, vial containing a liquid culture, or an older plate of *E. coli,* streak the bacteria on *E. coli* agar plates and incubate overnight at 37 °C. These plates can be kept in the fridge for up to a week but can also be streaked the night before the experiment.

First laboratory period

1. Distribute one *E. coli* agar plate and one *H. volcanii* agar plate to each group.
2. Using a sterile Q-tip, allow students to pick a few colonies from the *E. coli* and *H. volcanii* plates previously prepared by the instructor and have them streak the cells onto the corresponding agar plate, being sure to gently streak along the entire surface of the agar plate with the Q-tip. Upon rotating the plate 45 degrees, students will repeat the streaking process to ensure complete coverage of the plate with the cells, as the aim is to obtain a lawn. Once the plates have been streaked by the students, the plates can be placed on the printed-out template marking optimal distancing of the disks (Supplemental Figure 1). Students place antibiotic disks on the plate with tweezers.
3. Plates are then incubated to allow a lawn to grow. For *E. coli*, we recommend removing the plates 12-24 hours after incubation at 37 °C and storing them in the fridge until the next lab session. *H. volcanii* should remain in a 37 °C incubator for at least 4-6 days. If a 45 °C incubator is available, lawns will have formed after 3-5 days. *H. volcanii* plates can be stored at room temperature until the next lab session.
4. Either at the end of the first lab session or during a lesson prior to the second lab session, students discuss relevant topics (antibiotic sensitivity, antibiotic resistance, antibiotic action, etc.) via guiding questions (see Appendix 2).

Second laboratory period (at least four days after the first period)

1. Students should collect their plates, analyze them, and record their results.
2. Discussion can be prompted using guiding questions such as those provided in Appendix 2.

High School Modification

Organisms

H. volcanii is available upon request from the Pohlschroder laboratory. High school teachers may opt to not use *E. coli* and instead just use *H. volcanii* and then show an image of an *E. coli* Kirby-Bauer plate.

Materials

The materials for a high school experiment are the same as listed in the above section with the exception of *E. coli* and *E. coli* agar plates. Additionally, if an incubator is not available, a homemade incubator can be built to use instead by using a Styrofoam cooler box and a heating pad that lines the bottom of the box. Details can be found in the supplemental material of Kouassi et al. 2017 (3).

Media

If the reagents listed above for preparing *H. volcanii* media are not available, we have also included the Kouassi et al. 2017 (1) *H. volcanii* agar plate recipe that mostly uses ingredients available at a grocery store.
It is recommended that the flask/pot being used is able to hold at least two times the amount of volume of medium being prepared.

Solid *H. volcanii* agar plate (1.5% wt/vol) medium composition using mostly grocery store ingredients (per 1 L medium) (2):

1. For 1 L of medium, add 1 L of water, 5 g of tryptone, and 5 g of agar to a flask or bottle and place on a hot plate to boil. Double boiling is recommended to ensure all agar has dissolved.
2. Add all salts (150 g Morton Salt, 50 g Epsom Salt, 10 g Nu-Salt, and 5 Antacid) to the flask or bottle and boil three times. Alternatively, *H. volcanii* medium can be placed in a pressure cooker and kept in for about 20 minutes after steam start at middle power.
3. Continuously stir the flask or bottle until the medium has cooled to about 60 °C before pouring into Petri dishes.
4. Store plates upside down in a plastic bag at 4 °C for long-term storage or at room temperature for short-term (two weeks) storage.

Procedure

The instructor pre-lab is the same for the high school modification with the exception of only preparing *H. volcanii* plates rather than also preparing plates for *E. coli*.

The first and second laboratory periods for students are the same for the high school modification, only the students will just prepare plates with antibiotic disks for *H. volcanii*. During the second laboratory period, students will receive an image of an *E. coli* Kirby-Bauer plate (Fig. 2A) for comparison with *H. volcanii*.

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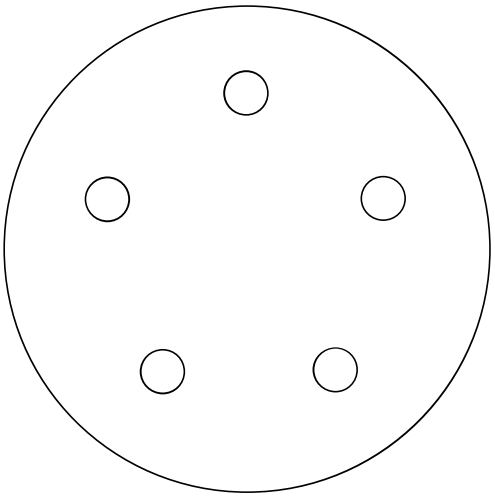

Supplemental File 1: Template for antibiotic disk placement
Copies of this template can be printed, and students can place the template underneath their agar plate streaks to aid in placing antibiotic disks at optimal distances.

Appendix 2

Antibiotic targets

Supplemental Table 1
Lists of suitable antibiotics, their targets, and the affected organisms. Reading resources on the topic are cited.

Antibiotic	Antibiotic target	Organism(s) affected	Reference(s)
Cell wall synthesis			
Ampicillin	Attachment to penicillin-binding proteins	*E. coli*	1, 2, 3, 4
Protein synthesis inhibitor			
Tetracyclines	30S ribosome (inhibits aminoacyl tRNA binding to ribosome)	*E. coli*	1, 2, 3, 4
Streptomycin	30S ribosome (mistranslation by tRNA mismatching)	*E. coli*	1, 3, 4
Gentamicin	30S ribosome (mistranslation by tRNA mismatching)	*E. coli*	1, 3, 4
Kanamycin	30S ribosome (mistranslation by tRNA mismatching)	*E. coli*	5
DNA synthesis inhibitors			
Novobiocin	DNA gyrase	*H. volcanii* and *E. coli*	2, 6, 7

Discussion questions

Pre-lab discussion questions

1. Based on your knowledge on the cell biology of archaea and bacteria and specific antibiotic targets, which antibiotics do you anticipate will inhibit the growth of both prokaryotes? Which ones will only inhibit *E. coli* or *H. volcanii* growth? Explain your answers.

2. Is an antimicrobial agent that effectively kills bacteria always a good antibiotic? Why or why not?
3. What criteria need to be considered in searching for an ideal antibiotic?
4. Antibiotics in the medical field are used to combat bacterial infections. However, many antibiotics are isolated from bacteria in the soil. What roles do antibiotics play in natural environments?
5. Why have only a handful of antibiotics been identified that specifically inhibit the growth of archaea?
6. Have antibiotic resistance mechanisms only evolved since the use of commercially produced antibiotics?
7. How can antibiotic resistance genes be transferred from one organism to another?

Post-lab result analysis

1. Based on the presence or absence of zones of inhibition, to which antibiotic(s) was *E. coli* resistant? Sensitive? Are your results consistent with your predictions? If not, what are some reasons for the discrepancies?
2. Based on the presence or absence of zones of inhibition, to which antibiotic(s) was *H. volcanii* resistant? Sensitive? Are your results consistent with your predictions? If not, what are some reasons for the discrepancies?
3. Measure the zones of inhibition for the antibiotics that prevented growth. Do certain antibiotics seem to be more or less effective at prohibiting growth?

Post-lab discussion questions

1. If you were a doctor and there was a shortage of gentamicin to treat an *E. coli* infection, which antibiotic would you use? Why?
2. If you were to design a novel drug that inhibits *H. volcanii* growth, which cellular components would you select as potential targets? Why?
3. Imagine you did this experiment again, but when you were examining your plates, you noticed that a single colony formed within a zone of inhibition. What may have happened?
4. The mega plate video (8) shows the evolution of antibiotic resistance in a bacterium over just two weeks. If you left your plate with antibiotic disks in the incubator for 14 days, why is it unlikely that you would observe the gradual increase of resistance to an antibiotic, eventually allowing *E. coli* growth close to the antibiotic disk?

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