



- The hardest part of this lab is flaming the slides. Too little flaming and the microbes get washed off of the slide. Too much flaming and they are burned to a cinder. You may wish to modify this lab so that each group passes the slide through the flame at a measured speed or number of passes until they learn this skill.
- Always have students dispose of their Petri dishes in the biohazard bag.
- If you wish to make LB agar, use the following recipe for 1 liter of agar (makes enough for about 90 60x15mm Petri dishes:

5g yeast extract (Fisher, #DF0127-15-1, 100g, \$32.05)

10g Bacto-Tryptone (Fisher, #DF0123-15-5, \$22.55)

5g NaCl

15g agar (Fisher, #DF0140-15-4, 100g, 61.95)

H<sub>2</sub>O to 1L

Mix the ingredients. To ensure complete dissolution, place the flask containing LB agar in a pan of water and boil the water, swirling the contents of the flask occasionally, until there are no solid particles seen in the solution. Pour the LB agar into two 500ml flasks and stopper the flasks with nonabsorbent cotton. Autoclave the agar at 121°C, 20 psi for 30 minutes. If you do not have an autoclave, you can pressure-cook it for 1 hour. Allow the media (LB agar) to cool to 55°C, then pour the plates using the sterile technique described in this lab.

Allow the plates to cool on the bench-top for as long as possible (at least overnight, but being out for 2-3 days will help to eliminate condensation forming on the lids). To check for contamination, you may wish to place an uninoculated dish in an incubator at 37°C overnight. No growth of bacteria means no contamination. Store the dishes in the sleeves they came in, upside down in the refrigerator. Do not store plates longer than several weeks.

- To make LB broth, use the above recipe, but omit the agar. Broth can be sterilized in half-filled and stoppered test tubes.
- Inoculate tubes of broth containing *Alcaligenes faecalis*, *Bacillus cereus* and *Serratia marcescens*
- several days prior to this activity for student use. Incubate the tubes overnight at 37°C. The tubes should be gently swirled prior to use to mix

### Correlations to State and National Standards:

- **Colorado State Standard 3:** Life Science-- Students know and understand the characteristics and structure of living things, the processes of life, and how living things interact with each other and their environment.
- **Colorado State Standard 5:** Life Science-- Students know and understand interrelationships among science, technology, and human activity and how they can affect the world.
- **Colorado State Standard 6:** Life Science--Students understand that science involves a particular way of knowing and understand common connections among scientific disciplines.

- **National Content Standard C (Life Science):** As a result of their activities in grades 9-12, all students should develop understanding of the cell; the molecular basis of heredity; biological evolution; interdependence of organisms, matter, energy, and organization in living systems; and behavior of organisms.

**Correlation to Confronting the Microbe Menace:**

Cross reference information given on Bacteria, and Antibiotics found on the DVD 2000 and beyond confronting the microbe menace with lab 5.

General Information on Bacteria	T5C5	07:45
Size: Analogy one, ping pong ball	T5C5	07:51
Analogy two, ruler	T5C5	08:11
Gram Stain Identification	T5C6	08:55
Chart: Bacteria Are Everywhere and Numerous	T5C7	09:45
Slide: Normal Flora of the Mouth Bacteria Gram Strained	T5C9	12:11
Video: Bacteria E.Coli, show actual reproduction	T5C10	13:25
Chart: Some Bacterial cause Disease (Sometimes)	T5C14	16:52
Chart: Examples of Bacterial Diseases	T5C16	19:30
Chart: Infectious Agents Are Easily Spread	T5C18	21:50
Combat Infectious Diseases		
Chart: How to combat infectious Disease	T5C20	24:21
Chart: Antibiotics	T5C21	25:18
Chart: Antibiotic Mechanisms	T5C23	27:02
Picture E. Coli on a plate Disk Diffusion Kirby-Bauer	T5C24	27:25
Video E. Coli being lysis	T5C25	27:54
Chart: Antibiotics	T5C26	28:39
Picture: E. Coli on plate that is antibiotic resistant.	T5C27	29:30
Chart: Antibiotic Resistance	T5C38	29:38
Demonstration of Super Bug (Antibiotic Resistant	T5C29	31:45
Chart: Antibiotic Resistance (Super Bug)	T5C29	32:09
Video: Conjugation	T5C31	34:52
Chart: Shelf Life of New Antibiotic	T5C32	35:29

**Supplementary Materials:**

### URL'S and titles of useful web sites

1. Antibiotics  
[WWW.ultranet.com/~jkimball/BiologyPages/A/Antibiotics.html](http://WWW.ultranet.com/~jkimball/BiologyPages/A/Antibiotics.html)
2. Penicillin and other Antibiotics  
[helios.bto.ed.ac.uk/bto/microbes/penicill.htm](http://helios.bto.ed.ac.uk/bto/microbes/penicill.htm)
3. Antibiotics Factory Farm Project  
[www.factoryfarm.org/antibiotics.html](http://www.factoryfarm.org/antibiotics.html)
4. Antibiotics  
[www.bact.wisc.edu/MicrotexBook/ControlG.antibiotic.html](http://www.bact.wisc.edu/MicrotexBook/ControlG.antibiotic.html)
5. Antibiotic Politics  
[helium.vancouver.wsu.edu/~kendall/politics.htm](http://helium.vancouver.wsu.edu/~kendall/politics.htm)
6. Antibiotics  
[helium.vancouver.wsu.edu/~kendall/index.htm](http://helium.vancouver.wsu.edu/~kendall/index.htm)
7. What are Antibiotics  
[helium.vancouver.wsu.edu/~kendall/whatareantibiotics.htm](http://helium.vancouver.wsu.edu/~kendall/whatareantibiotics.htm)
8. Chapter#18 Food Borne Diseases  
[www.slic2.wsu.edu:82/hurbert/micro101/pages/Chap18.html](http://www.slic2.wsu.edu:82/hurbert/micro101/pages/Chap18.html)
9. Guardian? Unlimited Special Reports/Antibiotics in food  
[www.guardian.co.uk/antibiotics/](http://www.guardian.co.uk/antibiotics/)
10. Antibiotic Attack  
[www.asklive.org.grants/lecturesbiointer...Attack/a 2.html](http://www.asklive.org.grants/lecturesbiointer...Attack/a%20.html)
11. Evolution: "Microbes: What They Do and How Antibiotics Change Them" by Maura J. eade-“ Callahan, Ph. D  
[www.actionbioscience.org/evolution/meade callahan.html](http://www.actionbioscience.org/evolution/meade%20callahan.html)
12. APUA: Ecology of Antibiotics  
[www.healthsci.tufts.edu/apua/Ecology/ecology.html](http://www.healthsci.tufts.edu/apua/Ecology/ecology.html)
13. Antibiotics--- Penicillins &Its Derivatives, Vancomycin derivatives  
[www.infoallglobe.com/wriers/Antibotics...term paper.htm](http://www.infoallglobe.com/wriers/Antibotics...term%20paper.htm)
14. The Rise (and Fall) of Antibiotics  
[www.naturalrearing.com/J In Learning/Misc/Antibiotics.html](http://www.naturalrearing.com/J%20In%20Learning/Misc/Antibiotics.html)

15. Time.com: The Antibiotics Crisis  
 time.com/time/health/article/0,8599,93929,00.html

Bibliography if print resources

**1. Antimicrobial Use and Antimicrobial Resistance: A Population Perspective**

Emerging Infectious Diseases  
 April 2002  
 Page(s) : 347-354  
 Health and Human Services Department (HHS)  
 National Center for Infectious Diseases (NCID)  
 SuDoc Number : HE 20.7817/8/4

2. Natural Microbial Compounds May Control Strep and Staph Infections  
 Agricultural Research Service News, Jan. 3, 2000, 3K, SIRS Government Reporter
3. Miracle Drug Vs Superbug  
 FDA Consumer Nov./Dec. 1998, 15K SIRS Researcher
4. Antibiotics  
 World Health, Gale Group 2000
5. Antibiotics  
 U\*X\*L Science U\*X\*L 1998

**Materials Price List/ordering Information**

Carolina -1-800-334-5551, [www.carolina.com](http://www.carolina.com)

Item	Ordering Number	Price
Alcaligenes faecalis	Ww15-4835	\$ 9.75 per vial
Bacillus cereus	Ww 15-4780A	\$ 9.75 per vial
Serratia marcescens	15-5450A	\$ 9.75 per vial
TSA Media tubes	BA-82-7322	\$ 13.73 pack of 10
TSA Dehydrated media	BA-78-8420	\$ 17.95 /100grams

**Life Science Products**

Call for current prices 1-800-245-5774 [www.lifesciprod.com](http://www.lifesciprod.com) [lspi@lifesciprod.com](mailto:lspi@lifesciprod.com)

Item	Ordering Number	Price
60x15mm Petri dish (500/case)	LS-6606	\$48.70
Red 12"x24" Biohazard Bags (200/pack)	LS-4812-R3	\$32.50
Sterile Cotton-Tipped		

Applicator Swabs (100/pack)	AP-4304	\$6.90
--------------------------------	---------	--------

**Fisher Scientific** Call for current prices 1-800-766-7000

<i>Item</i>	<b>Ordering Number</b>	<b>Price</b>
Dehydrated LB agar	DF0140-15-4	\$61.99

### **Lab #3: Unstained Preparations and Simple Stains**

#### **Introduction:**

Because bacteria are extremely small and usually transparent, special techniques have been developed to see them. A special type of microscope, called an oil immersion microscope, is required to see such a small creature. When light passes from one medium to another, it changes direction. You have probably seen a spoon in a glass of water that appears to have a broken handle. This happens because water has a different “refractive index” than air and so light changes direction as it passes from air through water. The handle of the spoon in the air appears to be in one place and the rest of the spoon in the water appears to be some place else, unattached to the handle in the air. This is because the light, which is “carrying the image” of the spoon, has been bent relative to the light in the air. The oil immersion microscope uses a drop of oil between the microscope slide and the objective lens to decrease the amount of refraction, or bending, of light because the oil and the glass has very similar refractive indices. This enables smaller images to be visualized than you would have without the oil.

To make it easier to see the almost transparent bacteria, stains are often used. There are generally two types of stains: simple stains and differential stains. All stains must have at least two properties. Stains must be colored and they must react with some cellular component. Simple stains are general, all-purpose stains while differential stains are usually for staining certain structures within the cell. Simple stains are often basic dyes (a salt with the color in the positive ion). Because the cell membrane is a negatively charged structure, the positively charged dye will stick to it. Usually the cells are exposed to the stain briefly, then the excess stain is washed away and the cell can be visualized. We will find out about differential stains in the next lab. Preparation of

bacteria involves two steps, making a “smear” to cause the organisms to stick to the glass slide and then staining the smear.

**Purpose:** After reading the Introduction and the Procedure, explain the purpose in doing this lab in the space below.

**Hypothesis:**

Because this is another skills lab and not an inquiry, no hypothesis is needed.

**Materials:**

3 Microscope Slides	3 Cover Slips	Oil Immersion Microscope
95% Ethanol (EtOH)	Test Tube Holder	Latex Gloves
Bunsen Burner	Matches	Concave Slide
Soap	Clothes Pin	Kim wipes

24 hr. agar and broth cultures of *Alcaligenes faecalis*, *Bacillus cereus* and *Serratia marcescens*

Stains: Crystal Violet, Methylene Blue and Safranin

**Safety:**

1. A microbiology lab is potentially a very dangerous place. For this reason it is extremely important that you follow all safety guidelines and always practice sterile technique when handling microbes, unless instructed otherwise.
2. There should be no books or papers at your workstation except this lab packet.
3. Never have any food or drink at your workstation.
4. Always thoroughly wash your hands with disinfectant soap or alcohol before leaving your workstation.
5. Never open a Petri dish after you have inoculated it and allowed it to incubate overnight.
6. Always dispose of used material in the biohazard bag, unless instructed otherwise.

**Procedure:**

Preparation of the Smear

1. It is best if you wear gloves when you are staining slides. This is so your fingers aren't stained. More importantly (for the slide), oil from your fingers will prevent the bacteria or the stain from sticking to the slide during the smear preparation. For this

reason, you should wear gloves and touch only the edges of the slide with your fingers.

2. Lay several sheets of paper towel on your work surface. Always lay the slides on the paper towels if you need to set them somewhere.
3. Thoroughly wash and rinse three glass slides with soap and water.
4. Dry the slides with a paper towel. Place several drops of 95% ethanol (EtOH) on the slides and wipe the ethanol on both sides of the slide with a clean paper towel.
5. Place the slides on the towels you have laid on the work area. Add several drops of 95% ethanol to the area of the slide where the smear will be placed and allow the ethanol to air dry or use Kimwipes to dry them.
6. Hold the slide with the clothespin. Briefly pass the slide of the slide to which the smear will be attached through the flame of the Bunsen burner. Caution: Too much heating will break the slide.
7. Being careful not to touch the middle of the slide where the smear will go, write the names of the organisms to be smeared over to one side of the slide. On the following page are the three organisms that you will be smearing.

*A. faecalis*      *B. cereus*      *S. marcescens*

Steps 1 through 7 are standard for cleaning slides in preparation of smearing and staining.

Always follow these steps, unless instructed other wise.

8. Using the fine tipped marking pen, make two circles about 1 cm in diameter as shown in the diagram below. Mark one circle A (for agar) and the other B (for broth).

9. Swirl the test tube of broth until it is equally murky throughout. Use your inoculating loop to place a small drop of water in the A circle.

10. Flame the loop and allow it to cool for 10-15 seconds. Transfer a **small** amount of bacteria from an agar culture to the A circle of the appropriate slide. **Caution:** It is easy to get too many cells from agar. Less than a pinhead is all that's needed. Mix

the bacteria on the loop in the water. The water should be turbid (cloudy), not opaque. Flame the loop. If you think you have too many bacteria in the circle, wash them off, blot the slide dry and try again.

11. Gently swirl the contents of the test tube (broth culture) that contains the same type of bacteria as the agar culture you just smeared on the slide.
12. Pick up the loop and the test tube containing the broth culture. Remove the cap of the test tube with your pinky, flame the mouth of the test tube and the loop.
13. Transfer 2-3 loopfuls of broth culture to the B circle of the appropriate slide.
14. Flame the test tube and replace the cap. Flame the loop. Allow the slide to air dry while your partners try their hand at making smears with the other cultures.
15. After the slide has air dried, pick up the slide with the clothespin and fix the bacteria to the slide by passing it through the flame of the Bunsen burner several times.  
**Caution:** Too much heating will burn the bacteria; too little heating and they may wash off while staining!

#### Staining the Smear

1. After the slide has completely cooled, hold the slide with the test tube holder or a clothespin over the sink.
2. Flood the slide with stain. Make sure that each smear is covered completely. Do not allow the stain to dry on the smear. Add more stain, if necessary. Use the following table for smear and staining times.

<u>Bacteria</u>	<u>Stain</u>	<u>Time</u>
<i>A. faecalis</i>	Crystal Violet	30 seconds
<i>B. cereus</i>	Methylene Blue	1 minute
<i>S. marcescens</i>	Safranin	1 minute

3. After the appropriate amount of time, rinse the excess stain from the slide by holding under a stream of gently flowing cold water. Do not let the stream of water to fall directly on the smear, but let it flow over the smear.
4. Remove the excess water by tapping the long edge of the slide on the paper toweling. Allow the slide to air dry.

### Using the Oil Immersion Microscope

1. Place a single drop of immersion oil on the portion of the slide that you wish to view.
2. Select the oil immersion objective lens (usually it is the highest, or 100X, power).
3. While watching from one side, carefully lower the objective lens until it is just touching the drop of oil.
4. Look into the ocular (eyepiece) lens and very slowly and carefully; continue to lower the objective lens until the bacteria come into focus. Record the necessary information in the appropriate data table.
5. When all of the group members have viewed the slide, rotate the nosepiece so that the lowest power objective lens is over the slide. Move the coarse adjustment knob to raise the objective lenses and then remove the slide. Place another slide on the stage and repeat steps 1-5 until everybody has seen all of the slides.
6. After all of your group members have viewed all of the slides, clean the slides and the tabletop with EtOH.
7. While wearing gloves, place a drop or two of xylene on a piece of lens paper. *Gently* dab the xylene on the high power objective lens. *Gently* use a dry piece of lens paper to finish cleaning the lens. Caution: Do not skip this step because the oil can dry on the lens, making nasty goobers that are difficult to remove and impossible to see through. Also, do NOT use anything except lens paper on the lenses.

#### **Results:**

Observations of Stained Bacteria			
<u>Bacteria</u>	<u>Color</u>	<u>Shape</u>	<u>Drawing</u>

#### **“Analysis/Conclusion:”**

1. Use the microscope to compare the stains made from the broth with those from agar. How easily you can find or see individual bacteria from either source? Which source gave you better results and why?
2. What are some of the benefits to getting bacteria for staining from agar? What are some of the disadvantages?
3. What are some of the benefits to getting bacteria for staining from broth? What are some of the disadvantages?
4. Which step was the most difficult to perform in the preparation of the stained slides? How can you make this step more successful in the future?
5. Describe the differences in shapes of the three different types of bacteria.

## Teachers Answers

### Analysis/Conclusion:

1. Use the microscope to compare the stains made from the broth with those from agar. How easily you can find or see individual bacteria from either source?  
Which source gave you better results and why?

*The lab allows for students not only to see individual bacteria but also the stain of the particular bacteria.*

*A faecalis – Crystal Violet*

*B. cereus- Methylene Blue*

*S. marcescens- Safranin*

*Depending on the students' lab techniques they should get the following information:*

*Best- Broth- this should give the student the best viewing of individual bacteria because the concentration is not as great.*

*Agar- this allows students to see colonies but their smears will most likely have to many bacteria to be able to individual bacteria.*

2. What are some of the benefits of getting bacteria for staining from agar? What are some of the disadvantages?

*Benefits:*

- *Students will be able to identify colony type, so that they can get a pure bacteria strain.*
- *Students will be able to see contamination if it has taken place.*

*Disadvantage*

- *The concentration of the bacteria on each smear makes it hard to see the individual bacteria.*

3. What are some of the benefits to getting bacteria for staining from broth? What are some of the disadvantages?

*Benefits*

- *The concentration of the bacteria is not as great so the student will be able to see individual bacteria easier.*

*Disadvantages*

- *Contamination is not as easy see.*
- *Students may not get enough bacteria on the plate.*

4. Which step was the most difficult to perform in the preparation of the stained slides? How can you make this step more successful in the future?

*This will depend on the lab group. It may be the fixation section, or the time that the bacteria are exposed to the stain. They even may wash the bacteria from the slide.*

5. Describe the differences in shapes of the three different types of bacteria

*The three shapes of the bacteria are:*

- *Bacillis is rod shaped.*
- *Coccus is round.*
- *Spirillum is spiral.*