

MICROBIAL PHYSIOLOGY

LAB MANUAL

MBOI 3440

2007

**MBIO 3440 PHYSIOLOGY SCHEDULE****2007****Lab Location: 201 and 204 Buller**

TITLE	WEEK #	DATE		
		Lab Experiment	Data Due <sup>a</sup>	Report Due <sup>b</sup>
Lab 1: Review of Bacterial dilution and plating technique	1	Jan 9		Jan 16
Lab 2: Kinetics of Threonine Deaminase from <i>Echerichia coli</i> Part II: Colorimetric determination of degradative threonine deaminase	2	Jan 16		Jan 23
Lab 2: Kinetics of Threonine Deaminase from <i>Echerichia coli</i> Part III Enzyme coupled reaction determination of biosynthetic threonine deaminase.	3	Jan 23		Jan 30
Class data analysis report				Feb 6
NO LAB	4	Jan 30		
Lab 3: Control of Cell Division and D-phase in <i>E. coli</i>	5	Feb 6	Feb 9	Feb 20
MID-TERM BREAK	6	Feb 13		
Lab 4: Bacterial Motility and Chemotaxis	7	Feb 20	Feb 23	Mar 6
Lab 5: Environmental Stress Shock in Bacteria	8	Feb 27	Mar 2	Mar 13
Lab 6: Instrumental Tutorial and Demonstration	9	Mar 6		
<b>Lab exam</b>	12	<b>March 27</b>		

<sup>a</sup> All lab data is due by 2:30 pm on due date. Honesty Declaration does not need to be attached to data handed-in.

<sup>b</sup>All lab reports are due by 4:30 pm on due date.

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## GENERAL INSTRUCTIONS

Lab Instructor:	Dr. L. Cameron	Office: 414B
Demonstrators:	Ben Wiseman	Lab: 410
	Vikash Rha	Lab: 410
Lab Location:	204B	

**WEBSITE:** [www.umanitoba.ca/faculties/science/microbiology/staff/cameron/](http://www.umanitoba.ca/faculties/science/microbiology/staff/cameron/)

OR via University of Manitoba Microbiology Homepage:

<http://www.umanitoba.ca/faculties/science/microbiology/labinformation.htm>

Information available at the website: changes/corrections, additional information, data, lab exam outline, marks

## REGULATIONS

1. Lab attendance is compulsory.
2. All students must wear a lab coat. Read the lab procedure thoroughly before coming to lab. You need a fine point permanent marker. Food or beverage is not permitted in the lab.
3. Read lab standard operations procedure before attending lab.
4. Students work individually for lab 1, remainder of labs in pairs.
5. Each student's bench area must be thoroughly cleaned and wiped with cleaner before leaving the lab. Student bench area will be checked by the demonstrator. Marks will be subtracted from final lab mark (technique) if this is not performed each lab day.

## EVALUATION

1. The lab is worth 20% of the final course mark.
  - Lab reports..... 8%
  - Lab exam.....12%
  - Lab technique mark..... up to 2% of final lab mark may be subtracted if lab conduct and performance is shown to be poor.
2. Students must pass the lab to complete the course (50%).
3. The lab exam will be held in the lab during normal lab time, starting at 2:30 pm (refer to schedule for date). The lab exam is 1.5 h.
4. Lab reports are to be handed in as stated in schedule by 4:30 pm of that day. Hand in lab reports and lab data through slotted drawer in filing cabinet in room 204 Buller ONLY. Instructor and demonstrators do not accept lab reports. If handing in lab late, 1 mark will be subtracted for each class day late. Marked lab reports will be returned to students the next week. A late report will not be accepted after that report has been returned to the class.
5. Lab report marks are final unless an obvious error in addition of marks has been made. However, if a student feels they have a legitimate complaint, please direct attention to lab instructor.
6. Approximately two weeks prior to the lab exam, a brief outline of lab exam format and information content will be available on the website. You are responsible for fundamental information presented during the tutorial lab.
7. You must notify the lab instructor no later than two school days after the missed lab. A Doctor's certificate is required for a missed lab exam. All deferrals will write the lab exam at a scheduled time set by the instructor. Failure to comply will result in a zero on your lab exam.

8. **Plagiarism (copying another student’s lab report (present or previous year) or copying published literature without citing is a violation of University regulations. Refer to the STUDENT DISCIPLINE BY-LAW in your student handbook (rule book) for action taken for plagiarism.**

### LAB REPORT PRESENTATION INSTRUCTIONS

[Before handing in your report review report to ensure that all information is included. When printing Excel spreadsheets make sure you have selected all information before printing. If you are using text boxes, they must be completely within the selected area or they do not print.]

1. All reports must have an Honesty Declaration attached at end of report. Available as a pdf file on lab website.
2. A reference file is available in the science library (2 hour reserve). You will need to use the reference material to write the lab reports.
3. Lab reports must be **typed**. Up to 10% of the mark subtracted for reports not typed. Must use Word lab report format or data sheets and/or Excel spreadsheet(s) if available on lab website.
4. Number pages.
5. On the front page of the report state:
  - Course name and number
  - Experiment number and Title
  - Group # and section #
  - Individual or Group name(s). If handing in an individual report, also include lab partners name.
  - GROUP report or INDIVIDUAL report
  - Date
6. Lab report information is to be presented exactly as requested in lab manual. Number sections the same as the lab manual.
7. Lab report 1 is done as an individual effort. Each students hands in a lab 1 report. The remainder of the lab report may be done as an individual effort or a group effort by the two students that carried out the experiment. One report or two reports may be handed in per group. The decision on the number of reports per group is totally dependent on members of the group. This decision may be changed any time during the term. Therefore for each lab report the group has the option to hand in one or two reports exclusive of what has been done before or after that particular report. Indicate on the cover page of the report if the report is a group report or an individual report. If handing in an individual report also include lab partner’s name.
8. Always include a sample of each type of calculation in your lab report.
9. If a group’s data is not workable, borrow data from another group and reference. Non workable refers to data that cannot be plotted, used for calculations or required analysis. It does not necessarily mean the expected data.
10. Cite reference in text of lab report and record full reference at end of lab report. When should you cite and reference. The following is a good definition of plagiarism that explains when you should cite a reference. **“The unacknowledged use of another person’s work, in the form of original ideas, strategies, and research, as well as another person’s writing, in the form of sentences, phases and innovative**

**terminology.**" (Spatt<sup>1</sup>, 1983, p.438) This is done by using bracketed reference number that you used when listing references at end of lab report or by bracketing first authors name and date. Quote text unless you paraphrase completely in your own words. But remember, quotes should only be a small part of your work. If you are using the name year system, list the references alphabetically. Some examples are as follows (McMillan<sup>2</sup> 1997):

Binder V, Hendriksen C, Kreiner S. 1985. Prognosis in Crohn's disease - - based on results from regional patient group from county of Copenhagen. *Gut* 26:146-50.

Danforth DN, editor. 1982. *Obstetrics and gynecology*. 4<sup>th</sup> ed. Philadelphia: Harper and Row. 1316 p.

Petter JJ. 1965. The lemurs of Madagascar. In: DeVore I, editor. *Primate behavior: field studies of monkeys and apes*. New York: Holt, Rinehart and Winston. p 2920319.

If journal article assessed on the internet, site as journal. However, if available only on the web, reference as follows:

Kingsolver JC, Srygley RB. Experimental analyses of body size, flight and survival in pierid butterflies. *Evol. Ecol. Res.* [serial online] 2000;2:593-612. Available from: Colgate University online catalog. Accessed 2000 Oct 3.

11. Personal or Professional Electronic sources<sup>2</sup>:

**Cite in-text** by putting the following in parentheses, author's last name or file name (if no author's name is available) and publication date or the date of access (if no publication date is available).

**At the end of report list:** author or organization, publication date or date last revised, title of Web site, URL site in angle brackets, and the date accessed.

Cameron, L. 60.344 Microbial Physiology Lab Information

<[http://www.umanitoba.ca/faculties/science/microbiology/staff/cameron/60\\_344.htm](http://www.umanitoba.ca/faculties/science/microbiology/staff/cameron/60_344.htm)>. Accessed 2004 April 12.

**Table presentation** (if format not available on the website)

- Table number and title (legend) presented above the table body.
- Number tables using arabic numbers, even if only one table in a report.
- Include enough information in title to completely describe table, eliminating the necessity to search elsewhere in the lab report to understand information presented in table. Table title starts with an incomplete sentence. Additional complete sentences may be included to adequately describe the table (this also applies to figures).
- If abbreviations are used in table, indicate what abbreviations mean as a footnote. Other footnotes may be required to clarify material in the table.
- Like information should be in columns making it easier to view the table.
- Data in columns should be listed under the centre of each heading. Align decimal points and dashes. If a number value is less than 1 always include zero before the decimal.
- Column or Row headings should be complete and self explanatory. A heading is a separate entity from the title. It cannot be assumed information given in the title is adequate for a heading. The unit of measurement should only be included in the heading, not in column data.

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<sup>1</sup>Spatt, B. (1983). *Writing from Sources*. New York: St. Martin's Press.

<sup>2</sup>McMillan V.E. 1997. *Writing Papers in the Biological Sciences*. 2<sup>nd</sup> ed. Boston: Bedford Books: 1997. 197 p. and McMillan, V.E. 2001. *Writing Papers in the Biological Sciences*. 3<sup>rd</sup> ed. Boston: Bedford Books. 123 p.

- Group related column headings under larger headings.
- If information is the same for each column or row do not include but treat as a footnote.
- Make the table as concise as possible but include all necessary information. For example, any constant experimental conditions that would change the data presented.
- Tables should be properly set up with a straight edge. Horizontal lines must be included but it not necessary to always include vertical lines.

### **Figure presentation (graphs, diagrams, photographs, films)**

(all graphs must be computer generated - where applicable, required Excel presentation procedure is given in the lab appendix)

- Figures are to be numbered separate from tables, using arabic numbers. Include figure number even if only one figure.
- Figure number and figure legend should be presented below the graph. The figure legend, like the table, starts with an incomplete sentence describing the graph. Do not repeat just the labels of the x- and y-axis but present in a descriptive manner. Additional sentences should be included if additional information is required to completely describe figure, for example, any constant experimental conditions that affect the data presented.
- All diagrams, photographs, and films are figures and should be completely labelled.
- For figures of graphs, there is one dependent variable plotted and one or more independent variables plotted. The dependent variable is a function of the independent variable. It is accepted practise to plot the independent variable on the x-axis and the dependent variable on the y-axis. For example the measurement of absorbance (dependent) with increasing concentration of protein (independent). The size of the graph should fit the plot(s). The axis should not necessarily start at zero. Place graph completely within graph grid, this includes axis labels and legend. The overall size of graph should not be too large but should not be so small that information is obscured. The graph must be completely labelled (always include units). Use different symbols for each plot (not different coloured pens) on a graph. If more than one plot, explain symbols in legend or in a key included in the body of the graph. Graph plots can be drawn in a number of ways (this depends on the plot): (a) best fit straight line, (b) join each point with a straight line, and (c) use a flexible curve ruler or french curve. Do not drawn a free hand line.

Note: When writing your lab reports you are frequently requested to present both a table and a figure for a given set of data, similar to keeping a research journal. This is not the accepted practice for papers published in journals or books. Usually either a table or a figure is presented for a given set of data and depending on nature of data, it may only be summarized in the text. How do you make a choice of data presentation? The aim is to effectively and efficiently demonstrate what you want to show, for example, correlations, comparisons, pattern, trends, etc (

McMillan V.E. 1997. Writing Papers in the Biological Sciences. 2<sup>nd</sup> ed. Boston: Bedford Books: 1997. 197 p.)

**MICROBIAL PHYSIOLOGY 60.344**

The majority of references available on-line, see lab website.

For references not available on-line there is a lab reference binder (2 h reserve) in Science and Technology Library.

**NUMBER    REFERENCE**

**Lab 2 Kinetics of Theonine Deaminase from *Escherichia coli***

- 1     Hirata, M., M. Tokushige, A. Inagaki, & O. Hayaishi. 1965. Nucleotide Activation of Threonine Deaminase from *Escherichia coli*. J. Biol. Chem. 240:1711-1716. Not available on-line.
- 2     Nelson, D.L. & M.M. Cox. 2000. In Principles of Biochemistry, 4th edition. New York: WH Freeman and Company p.202-212, 225-227,846-848. (not available on-line)

**Lab 3 Control of Cell Division and D-phase in *E. coli***

- 3     Donachie, W. D. 1969 Control of Cell Division in *Escherichia coli*: Experiments with Thymine Starvation. J. Bact. 100:260-268. (ASM journal)
- 4     Kubitschek, H. E. 1974 Estimation of the D Period from Residual Division after Exposure of Exponential Phase Bacteria to Chloramphenicol. Molec. Gen. Genet. 135:123-130. (Springer Link)

**Lab 4 Bacterial Motility and Chemotaxis**

- 5     Tso, Wung-Wai & J. Adler. 1974. Negative Chemotaxis in *Escherichia coli*. J. Bact. 118: 560-564, 566-569. (ASM journal)
- 6     Mao, H, Cremer, P. S, & Manson, M. D. 2003. A sensitive, versatile microfluidic assay for bacterial chemotaxis. PNAS 100: 5449-5454.
- 7     Zhulin, IB, Rowsell, EH, Johnson, MS, Taylor, BL. 1997. Glycerol elicits energy taxis of *Escherichia coli* and *Salmonella typhimurium*. J. Bact. 179: 3196-3201. (ASM journal)
- 8     Alexandre, G, Zhulin, IB. 2001. More than one way to sense chemicals. J. Bact. 183:4681-4686. (ASM journal)

**Lab 5 Environmental Stress Shock in Bacteria**

- 9     Michel, G.P.F., & J. Starka. 1986. Effect of Ethanol and Heat Stresses on the Protein Pattern of *Zymomonas mobilis* Journal of Bacteriology. 165, 1040-1042. (ASM journal)
- 10    Neidhardt, F.C., VanBogelen, R.A. & V. Vaughn. 1984. The Genetics and Regulation of Heat-Shock Proteins. Ann. Rev. Genet. 18: 295-329 (UM NETDOC, search via GOGGLE SCHOLAR - under Science or search Science under UM NetDoc)
- 11    VanBogelen, R. A., P. M. Kelley, and F. C. Neidhardt. 1987. Differential Induction of Heat Shock, SOS, and Oxidative Stress Regulons and Accumulation of Nucleotides in *Escherichia coli*. J. Bacteriology, 169:26-32. (ASM journal)

## LAB STANDARD OPERATIONS PROCEDURE (SOP)

**Bench area:** Wash bench area before and after use with bench top cleaner.

**Personal safety:** You must wear a lab coat. Wear coat only in the lab, transport separately outside of the lab (in a plastic bag). Long hair must be tied back. Shoes should be closed toed. Wash hands with antibacterial soap before leaving the lab. No eating or drinking in the lab. Use aseptic technique for transfer of bacteria. This is to protect yourself as much as to ensure the purity of your culture. Protect hands with gloves and eyes with glasses when needed. The gloves provided in the lab are to be disposed of in the Petri plate container. If you forget your lab coat, dark blue lab coats are available in the lab, sign IN/OUT sheet. Long hair must be tied back. Closed toed shoes must be worn.

**Biohazards:** Know biosafety risk groups. Handle all cultures as potential pathogens. Never mouth pipette. Always use a pro-pipette. If you spill a culture, cover the spill with paper towels. Pour bench top cleaner over the towels to saturate. Gather up soaked towels and discard. Wipe area to dryness with fresh paper towels. Wash hands with soap and water. Place cultures on discard trolley. All cultures are autoclaved before disposing. Dispose of eppendorf tubes<sup>a</sup> in petri plate containers. Dispose of pipetman tips<sup>a</sup> and disposable pipettes in clear plastic lined basins along with glass or plastic Pasteur pipets, broken glassware, glass slides, brittle plastic objects, plastic 1 ml cuvetts, metal objects<sup>a</sup> (not needles or blades). Discard disposable pipettes (1 ml and 10 ml) in yellow plastic pails. Bacteria dilutions may to be poured down the sink and the tubes rinsed before placing on the discard trolley. Rinse sink with lots of water.

<sup>a</sup> due to the multi-use nature of the teaching lab, all eppendorf tubes, pipetman tips, Pasteur pipets, brittle plastic or metal objects will be treated the same as similar items contaminated with microorganisms.

When handling level 2 microorganisms you must wear disposable gloves, make sure any cuts on your hands are covered with a bandage, and be aware of the possibility of bacteria aerosol when you flame your loop.

**Glassware (unbroken):** Remove tape and pen markings (use alcohol) from glassware before placing on discard trolley. Used glassware should be rinsed and placed on the discard trolley. Rinsed test tubes should be placed in tray provided on the discard trolley.

**Petri plate culture and non-sharps solid culture material disposal:** use covered plastic containers lined with clear plastic bags for contaminated petri dishes or any bacteria contaminated solid non-sharps material (eppendorf tubes, API strips, antibiotic strips, microtitration plates, etc)

**Hazardous material disposal:** Examples: radioactive material, ethidium bromide, sovents, etc. The lab demonstrator will instruct proper disposal methods for labs that contain hazardous materials. These materials must be disposed of in appropriately labelled containers and disposed via the safety office. Use fumehood when recommended. A MSDS binder available in lab gives information on all hazardous materials used in the lab. Use extreme care with flammable solvents. Alcohol used to flame spread rod should never be positioned within 40 cm of flame. Never put a very hot spread rod into a beaker of alcohol. The alcohol may catch fire. Many of the immunochemicals are preserved in 0.1% Na azide...handle with gloved hands. Handle caustic (acids and bases) solutions with care. Never discard an acid or base greater than one molar down the sink. Discard in labelled glass containers provided. Use lots of water when discard caustic solutions (< 1M). These materials are disposed of through the university safety office. Never pour solvents down the sink (eg. phenol, ether, chloroform, etc). Discard in labelled containers provided.

**Sharps disposal:** Dispose of all sharps (needles, syringes, razors, scalpel blades) in specified biohazard container. Dispose of syringe with needle attached - do not take apart. Do not replace the needle cap before disposing (high frequency of accidents occur when replacing cap). Sharp's containers are autoclaved before disposing. All syringe tops must be disposed of in the biohazard sharp's container even if never used for transfer of biological material, it is a perceived (by general public) biohazard.

**General garbage disposal:** Nothing 'pointy' should be disposed in the general waste basket, eg. disposal pipets, tips, can top, toothpicks, sticks, etc. Nothing that has come into contact with biological material should be disposed in general waste container. No liquids, the caretaker does not know what the liquid is!

**Broken glass disposal:** Dispose of broken glass in labelled plastic containers lined with clear plastic. Transferred to boxes before discarding.

**Know location:** Exits, fire extinguisher, eye wash, sink shower, and first aid kit. This information is given in the first pre-lab.

**Equipment operation:** Know how to operate equipment before use. DO NOT use equipment unless you know exactly how to operate the equipment. The demonstrator is always available to assist. Please follow instructions in appendix for proper clean up of Spectronic 20D. Ensure the spec tubes are thoroughly washed and rinsed with distilled water before replacing in rack upside down as you (hopefully) found the tubes.

**Leave your bench area clean** All equipment and supplies should be returned to original location.

## LABORATORY BIOSAFETY GUIDE

Although you only use level 1 bacteria in this lab, it is still important that you follow standard operation procedures, SOP (see above) and work with care as level 2 bacteria are used in this location by other labs..

The University of Manitoba Biosafety Guide (Feb 2000) and Health Canada Laboratory Biosafety Guidelines booklets are available in your lab. Biosafety information is also available at the Health Canada websites:

Guidelines: <http://www.hc-sc.gc.ca/pphb-dgsp/ols-bsl/lbg-ldmbl/index.html> Health Canada [http://www.umanitoba.ca/campus/health\\_and\\_safety/](http://www.umanitoba.ca/campus/health_and_safety/)  
MSDS (infectious agents): <http://www.hc-sc.gc.ca/pphb-dgsp/msds-ftss/index.html>

There is no listing of level 1 agents in the guidelines or MSDS pamphlets

**Risk group 1 bacteria** are low individual and community risk and are unlikely to cause disease in healthy workers.

**Risk group 2 bacteria** are moderate individual risk and limited community risk. Bacteria in this group can cause human or animal disease but are unlikely to infect healthy laboratory workers. Effective treatment is available. Risk of spreading is limited.

CONTAINMENT LEVEL 1 (UM biosafety guide p. 11)

- microbiology lab with washable walls, countertops and hand wash sink
- established safe laboratory practices (hand washing and disinfection of countertops)
- general WHMIS safety training
- UM lab registration

CONTAINMENT LEVEL 2 (UM biosafety guide p.11)

- all of level 1 specifications
- biosafety permit
- biological safety cabinet (not required)
- biohazard signage
- a written standard operations procedure
- MSDS for the infectious agent

## WHMIS

The Workplace Hazardous Materials Information System (WHMIS) is a system for safe management of hazardous materials. WHMIS is legislated by both the federal and provincial governments.

Under WHMIS legislation, laboratories are considered to be a workplace, and students are workers. By law, all workers must be familiar with the basic elements of the WHMIS system. The WHMIS program includes:

1. Cautionary labels on containers of controlled products. Consumer products, explosives, cosmetics, drugs and foods, radioactive materials, and pest control products are regulated separately, under different legislation.
2. Provision of a Material Safety Data Sheet (MSDS) for each controlled product.
3. A worker education program

### 1. A. SUPPLIER LABELS

Controlled products must have a label of prescribed design which includes the following information:

**PRODUCT IDENTIFIER** - trade name or chemical name

**SUPPLIER IDENTIFIER** - supplier's name and address

**MSDS REFERENCE** - usually, "See MSDS supplied"

**HAZARD SYMBOL** - (see illustration on next page)

**RISK PHRASES** - describes nature of hazards

**PRECAUTIONARY MEASURES**

**FIRST AID MEASURES**

### B. WORKPLACE LABELS

All material dispensed in a workplace container must be labelled with the **Product Name**, **Precautionary Measures** (simplified) and **Reference to Availability of MSDS**.

## 2. MSDS

Material Safety Data Sheets (MSDS) are available for each lab. Refer to binder located in each lab (Room 201 binder is located in Room 204). Also main binders are located in the Microbiology preparation room, 307/309 Buller. MSDS are also available on the internet. See introductory page in MSDS binder for best website.

The MSDS will provide: relevant technical information on the substance, chemical hazard data, control measures, accident prevention information, handling, storage and disposal procedures, and emergency procedures to follow in the event of an accident.

## 3. SAFETY

The Laboratory Supervisor will provide information on the location and use of safety equipment, and emergency procedures.



## INTRODUCTION

The microbial physiology experiments, Control of Cell Division and D-phase in *E. coli*, Bacterial Motility and Chemotaxis, Environmental Stress Shock in Bacteria and Kinetics of Threonine Deaminase from *Escherichia coli*, have been chosen for several reasons.

(1) The repetition of basic microbiology lab skills. This gives each student the chance to repeat the basic skills until they obtain a high level of competence in bacteria manipulation. (2) Many of the experiments require a high degree of preparation, organization and coordination with a lab partner, these are all-important lab skills. (3) The different physiological experiments augment the lecture notes allowing the students exposure to practical application of theoretical concepts. (4) Critical reading of reference material and application to practical experiment when writing the lab report. A tutorial is also included to give the students exposure to other lab equipment and techniques that (a) were used in preparation of an experiment, (b) demonstrate alternative way of performing an experiment, and (c) present additional information related to an experiment.

## **LAB 1          REVIEW OF BACTERIAL DILUTION AND PLATING TECHNIQUE**

### **OBJECT**

The object of this experiment is two fold, (1) introduction of students to lab environment and (2) review of basic microbiology techniques.

Many of the experiments in this lab require accurate bacteria titration and good plating technique. Without these skills the experimental data is useless. Today's lab is short, but subsequent labs may be lengthy. You have a short period of time to complete a complex experiment. Come to lab well prepared.

### **INTRODUCTION**

Refer to appendix for additional information on bacterial pure culture technique.

#### **Aseptic technique**

This technique involves avoiding any contact of the pure culture, sterile medium, and sterile surfaces with contaminating microorganisms. This is accomplished by the work area cleaned with AIRx109, the transfer loop sterilized by heating with a Bunsen burner before and after transferring, and the work done quickly and efficiently to minimize the time of exposure during which contamination of the culture or laboratory worker can occur. The normal steps for transferring a culture from one vessel to another are (1) flame the transfer loop and allow to air cool, (2) open and flame the mouth of tubes/flasks, (3) pick up some of the culture growth and transfer to fresh medium, (4) flame the mouth of the culture vessels, and reseal them, and (5) re-flame the inoculation loop. Similar technique is used to transfer culture from a petri plate (only the petri plate is not flamed!) and to transfer cultures using sterile pipettes (the pipette canister is flamed after removing top and then flamed again after removal of pipette before replacing top). It is essential after removal of sterile caps, plugs, or pipette canister tops that they are kept in your hand sterile side down before replacing. **DO NOT** place on bench surface. If using pipetmen, do not flame pipetmen or tip box but do flame the dilution tube. All pipet tips are sterile and the pipetmen do not come into contact with sterile culture. When removing a tip from a tip container treat container as you would a agar petri plate. Tilt the lid open enough to remove tip using the pipetman. Replace lid as soon as possible. Never leave the lid off the box of pipetmen tips. You may temporarily discard tips in a tissue before discarding the lot in the petri plate container - do not use glass beakers.

## PROCEDURE

**For this lab students work individually. For all subsequent labs the students work in pairs.**

### Week 1

Each student is to work with one log phase *E. coli* culture (Students work individually for this lab). The *E. coli* log phase culture was grown at 37°C with shaking for 4 hours in LB medium.

1. Prepare 10-fold serial dilutions  $10^{-1}$  to  $10^{-8}$  of bacterial culture in saline. Mix or vortex culture before starting serial dilution. Mix tube after each transfer. Mix by vortexing, drawing solution up and down in pipette, or shaking or tapping the bottom of the tube. Prepare dilutions in a total volume of 1 ml using 5 inch metal capped test tubes.

P200 pipetmen should be used when transferring 0.1 ml and P1000 pipetman should be used to measure 0.9 ml or 1 ml. **It is extremely important that you do not turn the dial of the p1000 above 1 ml or the P200 above 200  $\mu$ l. This will permanently damage the pipetman. Make sure you get assistance from the demonstrator if you are not sure of operation. Refer to appendix for details on pipetmen operation.** What is the volume range of the P1000 and the P200? Check visually what each volume you measure looks like in the tip. This will allow you to correlate expected volume with volume set, which in turn allows you to spot pipetman inaccuracy. This also allows you to spot bubbles in tip.

Use separate tips for each dilution and dilution plating.

Preparation of 10 fold- serial dilutions: transfer 100  $\mu$ l of vortexed culture to 0.9 ml saline ( $10^{-1}$  dilution), vortex, then transfer 100  $\mu$ l of  $10^{-1}$  dilution to 0.9 ml saline ( $10^{-2}$  dilution), vortex - repeat this process until you have prepared to  $10^{-8}$  dilution.

2. Plate 0.1 ml  $10^{-4}$  to  $10^{-8}$  dilution **in duplicate** on T-soy plates. Total of 10 plates.
3. Use spread plate technique to distribute the bacteria evenly over the surface of the T-soy plate.
  - 1) Aseptically transfer 0.1 ml of culture or dilution to centre surface of agar plate. Never lift the lid of agar plate completely off the plate or place on bench surface. Best method is with the lid tilted above the plate.
  - 2) Dip hockey stick spreader in bottle of alcohol.
  - 3) Flame the spreader until alcohol ignites. Immediately remove spreader from flame and wait until alcohol completely burns off. Cool slightly.
  - 4) Open lid keeping it tilted over the plate and touch spreader to surface of plate that does not contain culture drop. If the spreader is still hot, it will kill bacteria. Still holding the lid tilted over plate, move the plate around spreading bacteria evenly over agar surface. (Use turntable to rotate plate if available.)

Note: 0.1 ml culture or dilution is the standard plating volume used for this lab and all subsequent physiology labs.
4. Allow plates to dry right side up at room temperature for 5 min before incubating upside down at 37°C overnight.

5. **Next day\* count colonies on plates** (all plates containing separated colonies) and record plate count data. **DO NOT DISCARD PLATES. Place ALL your plates on side bench area where indicated. Just stack upside down - do not use Petri plate containers.** NO MASKING TAPE whatsoever. Plates will be marked for technique which includes correct plate labelling and all aspects of dilution and spread plate technique. Label plates with your complete name, no initials. No data is handed in for this lab.  
\*If you cannot count your plates the next day. Put in student cold box located in room 203 off room 201. Plates must be counted by Thursday as that is the last day that plates are marked. Do not leave you plates in the cold box for marking but put on the designated bench location (information obtained in pre-lab).

An automatic colony counter (see lab manual appendix) may be used even though the colonies are marked. If you need assistance, ask a TA to demonstrate.

**LAB 1 REPORT** ( Lab format available as a Word document on lab website just save file, open in Word and enter requested information)

1. a) Record requested information in the following table.

Table 1. Determination of titre (bacteria/ml) for <i>E. coli</i> growing on LB medium.			
Dilution	<i>E. coli</i> plate counts		<i>E. coli</i> Titre (bacteria/ml)
	plate 1	plate 2	
$10^{-4}$			
$10^{-5}$			
$10^{-6}$			
$10^{-7}$			
$10^{-8}$			

TNTC = too numerous to count

0.1 ml of each dilution added per plate

\*Put an asterisk by plate counts used to calculate titre.

2. b) Calculate *E. coli* titre (bacteria/ml).

Notes:

- (1) For this lab and all subsequent labs, significant *E. coli* plate count numbers are between 30 and as high as bacteria can be counted accurately (colonies not overlapping), this includes >300 if colonies not overlapping. If no *E. coli* plate counts (for all dilutions plated) are above 30, use numbers below 30 to calculate the number of bacteria per ml - state below significance but only data available.
- (2) For each sample (eg. time point) there should only be one calculated bacteria/ml value per culture, time point, etc.
- (3) Make sure you footnote the numbers used to calculate the titre in your table.

4. 2. Practical: For this part of the lab report, the culture plates handed in are evaluated on plating technique, plate labelling and accurate bacterial titration.

## Questions

- 2 1. The following plate count results have been obtained in the MBIO 3440 lab in recent years. Give one possible reason why.

Student plate results.	One possible explanation for the student's results. Be specific to experiment protocol.
None of the dilution plates have any bacteria colonies after 24 h. Assume the dilution tubes were properly prepared and contain bacteria. Also assume the student incubated the plates at 37°C.	
Although there is apparent good plate results many of the colonies have run together.	
More than one size type of colony on the plate. Assume the initial culture was pure.	
The bacteria colonies on the 10-fold dilution plates do not decrease by 10-fold.	

- 1 3. a) What volume is measured for the following readout on (i) P20, (ii) P200 and (iii) P1000 Pipetman?

Pipetman readout	Volume measured by Pipetman Type ( $\mu\text{l}$ )		
	P20	P200	P1000
0			
9			
2			

- b) What Pipetman would use to measure? P20, P200 or P1000?

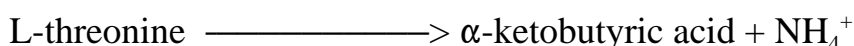
Volume measured ( $\mu\text{l}$ )	Pipetmen used.
120	
12	
1200	

## OBJECT

The object of this experiment is three-fold; first to appreciate the differences of threonine deaminase two iso-enzymes, one enzyme participates in the biosynthesis of L-isoleucine from threonine and the other enzyme is involved in the catabolism of L-threonine under anaerobic conditions, second to use kinetic analysis to determine  $K_m$  and  $V_{max}$ , and third to observe the effect of activator and inhibitor on enzyme kinetics.

## INTRODUCTION

Threonine deaminase catalyses the following lyase reaction:

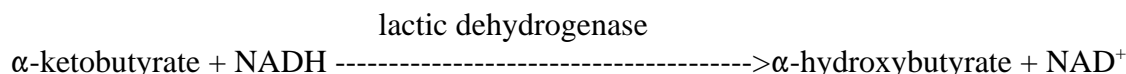
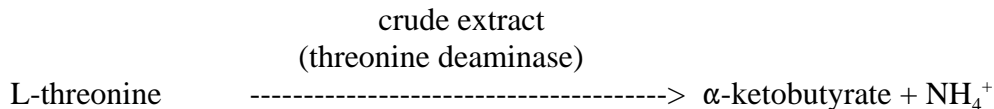


The biodegradative threonine deaminase differs from the biosynthetic threonine deaminase in that the biodegradative enzyme is an inducible enzyme that is synthesized in the presence of mixed amino acids and the absence of glucose and oxygen. The degradative threonine deaminase is activated by AMP, adenosine 5'-monophosphate. The biosynthetic threonine deaminase is a constitutive enzyme that is subject to end product repression and is inhibited by L-isoleucine.

The degradative threonine deaminase enzyme is determined using a colorimetric assay. 2,4-dinitrophenylhydrazine in the presence of  $\alpha$ -ketobutyrate forms 2,4-dinitrophenylhydrazone, a coloured product. The coloured product is directly proportional to the concentration of  $\alpha$ -ketobutyrate.

The biosynthetic threonine deaminase enzyme is determined using a NADH coupled enzymatic reaction to expose you to an alternate method of measuring threonine deaminase activity. NADH coupled enzymatic reaction is a commonly used method to measure enzyme activity as long as the enzyme of interest can be linked to a dehydrogenase enzyme.

The NADH coupled reactions:



An excess of lactic dehydrogenase and NADH is added to drive the reaction forward and to completion. The reaction is followed at 10 second intervals on the spectrophotometer which allows us to observe the decrease in absorbance at 340 nm due to the conversion of NADH to  $\text{NAD}^+$ . NADH absorbs at 340 nm while  $\text{NAD}^+$  does not absorb. The rate of decrease (slope) is directly proportional to the velocity of threonine deaminase. The coupled lactic dehydrogenase enzyme reaction is not rate limiting, therefore the velocity of threonine deaminase is dependent on threonine concentration. Even though the slope is negative the rate (velocity) is the absolute value of the slope.

Enzyme kinetics is used to determine the mechanism of action of threonine deaminase using

both classical Lineweaver-Burk plot (report) and Michaelis-Menten<sup>3</sup> computer analysis (tutorial). Although both methods are acceptable for the calculation of  $K_m$  and  $V_{max}$ , the Michaelis-Menton method is more accurate as there is no manipulation of data before calculating values. Also the  $K_m$  calculation is based equally on all substrate concentrations. The Lineweaver-Burk analysis is weighed heavily towards the low substrate concentration when calculating the  $K_m$  due to the nature of the double reciprocal plot.

Using Lineweaver-Burk analysis the mode of action of activator (AMP) and mixed inhibitor (isoleucine) are determined.

During the lab tutorial, class Threonine Deaminase kinetic data is used to calculate  $V_{max}$  and  $K_m$  using the Michaelis-Menton plot ( $V$  vs  $S$  plot) to obtain the hyperbola regression equation  $y = (ax)/(b + x)$ . This is possible since this is the Michaelis-Menton equation,  $V_o = V_{max} [S]/K_m + [S]$  where  $V_o$  is the velocity ( $V$ ),  $y$  is the y-intercept,  $x$  is substrate concentration  $[S]$ ,  $a$  is  $V_{max}$  (maximum velocity) and  $b$  is the Michaelis Menton constant,  $K_m$ .

## PROCEDURE

### Part I: Crude Cell Extract Preparation

#### Crude Degradative Enzyme Preparation

The *E. coli* cultures were grown overnight at 37°C without aeration in mixed amino acids without glucose (LB medium). The cells were harvested by centrifugation. Washed with buffer and resuspended in buffer at 1 gram wet weight of cells per 10 ml of buffer (20 mM potassium phosphate buffer, pH 7.4 (containing 5 mM  $\beta$ -mercaptoethanol). The *E. coli* cells were broken by French Press. The cell debris was removed by centrifugation at 20,000 x g (15000 rpm) for 20 min. The supernatant is dialyzed overnight against 20 mM potassium phosphate buffer, pH 7.4 to remove salts and small molecules.

The dialyzed supernatant is the degradative crude extract.

#### Crude Biosynthetic Enzyme Preparation

The *E. coli* cultures were grown overnight at 37°C with aeration in minimal medium containing glucose. The cells were harvested by centrifugation. Washed with 20 mM potassium phosphate buffer, pH 7.4 and resuspended in buffer at 1 gram wet weight per 10 ml 20 mM potassium phosphate buffer, pH 7.4 containing 5 mM  $\beta$ -mercaptoethanol. The *E. coli* cells were broken by French Press. The cell debris was removed by centrifugation at 20,000 x g (15000 rpm) for 20 min. The supernatant is dialyzed overnight against 20 mM potassium phosphate buffer, pH 7.4 to remove salts and small molecules. The dialyzed supernatant is the biosynthetic crude extract.

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<sup>3</sup>Nelson, D.L. & M.M. Cox. 2000. In Principles of Biochemistry, 4th edition. New York: WH Freeman and Company p.202-212, 225-227, 846-848.

**Week 2**

STUDENT LAB STARTS HERE.

**Part II: Colorimetric determination of DEGRADATIVE Threonine Deaminase**

1. Record protein concentration of degradative crude enzyme extract (found on board in lab).
2. Each group prepares one set of 4 reactions (varying concentration of threonine). Use 4 eppendorf tubes - label each tube with threonine concentration.

Degradative threonine deaminase isozyme	
Group Number	Effector added
1,4,7,10,13,16,19,22	CONTROL (none)
2,5,8,11,14,17,20,23	0.1 mM 5'-AMP
3,6,9,12,15,18,21,24	1.0 mM 5'-AMP

**Do not add enzyme until ready to start taking samples. Keep all components on ice.**

Assay components for DEGRADATIVE THREONINE DEAMINASE (AMP as the effector)						
Threonine final concentration	Components added ( $\mu$ l)				Enzyme extract	Distilled water <sup>b</sup>
	1 M potassium phosphate buffer, pH 7.4	0.2 M L-Threonine	10 mM 5'-AMP <sup>a</sup> (designated groups add only one of the following concentrations)			
			0.1 mM f.c.	1.0 mM f.c.		
Tube 1 0.005 M	100	25	10	100	100 <sup>a</sup>	to 1000 $\mu$ l
Tube 2 0.01 M	100	50	10	100	100 <sup>a</sup>	to 1000 $\mu$ l
Tube 3 0.05 M	100	250	10	100	100 <sup>a</sup>	to 1000 $\mu$ l
Tube 4 0.1 M	100	500	10	100	100 <sup>a</sup>	to 1000 $\mu$ l

f.c. = final concentration

<sup>a</sup> if your effector is 0.1 mM AMP add only 25  $\mu$ l enzyme extract per assay; if your effector is 1.0 mM AMP add only 10  $\mu$ l enzyme extract per assay

<sup>b</sup>Each group needs to calculate the exact amount of distilled water to add such that the total volume of each reaction mixture is 1000  $\mu$ l.

Take care not to create bubbles as bubbles really mess up your absorbance values.

- Step up microtitration sample tray. Add 4  $\mu\text{l}$  50% TCA **to bottom** of each well A1 to A7, B1 to B7, C1 to C7 & D1 to D7 inclusive. These four rows are for your experiment samples. Each group must also set up a standard curve using wells E1 to E5 and F1 to F5 (duplicate). **Standard Curve.** Add respectively 4, 6, 8, 10, 12  $\mu\text{l}$  0.5 mg/ml  $\alpha$ -ketobutyrate to wells E1 to E5, repeat for wells F1 to F5. Add distilled water to 40  $\mu\text{l}$  in each well, i.e. respectively 36, 34, 32, 30, 28  $\mu\text{l}$  for rows E and F.

Get everything ready to start sample experiment, tips, pipetmen set, etc.

- Start each sample reaction by adding enzyme last (start timing - zero time). Several large timers are available in the lab or use lab clock. Quickly mix by shaking tube. Then **immediately** remove a 40  $\mu\text{l}$  sample and put in the bottom of well A1 of microtitration plate (zero-time). The sample must come into contact with the 50% TCA (sample will turn slightly cloudy). **Use a separate tip for each sample.** Put reaction tube in 37°C waterbath and hold in water for remainder of experiment. Do not remove tube from waterbath when removing timed samples.
- At 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 min after start of reaction remove 40  $\mu\text{l}$  sample and put in the bottom of the appropriate microtitration well (A2 to A7).
- Repeat steps 4 and 5 for remaining three reaction mixtures (varying concentrations of threonine) using microtitration wells respectively; B1 to B7, C1 to C7 and D1 to D7.
- When all samples (including standard curve samples) are complete, add 120  $\mu\text{l}$  0.025% 2,4-dinitrophenylhydrazine in 0.5 M HCl to each microtitration directly into the sample. Use a separate tip for each sample. Incubate microtitration plate at room temperature for 15 min.
- Add 40  $\mu\text{l}$  40% KOH to bottom of each well using a separate tip for each sample. Let stand for 15 min or more to allow colour to stabilize. Wipe bottom of plate with a small amount of ethanol and a tissue just before reading absorbances. Do not touch the bottom of plate.
- Read sample absorbance in computer linked microtitration plate spectrophotometer (Dr. Londry's lab). A teaching assistant will help you.
  - The reader should be turned on 30 min prior to assay.
  - Select **SOFT Max** icon. Software used to analyze microtitration plate results.
  - If the program is not already set up, go to **Set Up, Instrument**, put in the following parameters: endpoint L1, nm =490, automix, select OK.
  - Go to **Control** in the pull down menu, select **read plate**. Asks for file name, just cancel.
  - The spectrophotometer moves the plate into the reader, reads absorbance, and returns plate.
  - Select **File, print Window**. The printer prints out data as a table of optical density (absorbance) identical to plate layout.

### Week 3

#### Part III Enzyme coupled reaction determination of biosynthetic threonine deaminase.

1. Record protein concentration of biosynthetic crude enzyme extract (found on board in lab).
2. Each group prepares one set of 4 reaction cuvetts (varying concentration of threonine). Use 4 disposable plastic cuvetts. Label the top non-transmission side with threonine concentration. The cuvetts are made of plastic that permit transmission of short wavelength light.

Biosynthetic threonine deaminase isozyme	
Group Number	Effector added
1,4,7,10,13,16,19,22	0.03 mM isoleucine
2,5,8,11,14,17,20,23	0.01 mM isoleucine
3,6,9,12,15,18,21,24	CONTROL (none)

You may set up all tubes but **do not add enzyme until ready to start experiment**. Do not add NADH to your first reaction mixture to allow you to blank the spec20. Keep all components on ice.

Assay Components for BIOSYNTHETIC THREONINE DEAMINASE (isoleucine as effector)										
Threonine f.c.	Components added ( $\mu$ l)									Distilled water
	1 M potassium phosphate buffer, pH 8.0	1 M $\text{NH}_4\text{Cl}$	1 mM pyridoxal phosphate	3 mM NADH	lactic dehydrogenase (9090 units/ml)	0.2 M L-Threonine	1 mM Isoleucine <sup>b</sup> (designated groups add only one of the following concentrations)		Enzyme extract	
							0.01 mM f.c.	0.03 mM f.c.		
Tube 1 0.005 M	100	20	20	100	40	25	10	30	200	to 1 ml
Tube 2 0.01 M	100	20	20	100	40	50	10	30	200	to 1 ml
Tube 3 0.05 M	100	20	20	100	40	250	10	30	200	to 1 ml
Tube 4 0.1 M	100	20	20	100	40	500	10	30	200	to 1 ml <sup>c</sup>

f.c. = final concentration

<sup>b</sup>do not add if you are the control group

<sup>c</sup> if you are adding 0.15 mM isoleucine the final volume will be 1.01 ml (this is acceptable)

1. Turn on the spec20D 15 minutes prior to start of experiment. Set at 350 nm. Ideally the wavelength should be 340 nm but that is the lower limit of the spec 20D and for some specs it just doesn't work. Since the reaction volume is only 1 ml use a spec 20D adaptor to hold the 1 ml cuvetts. See diagram for instructions. The cuvetts adaptor only fits in the spec 20 sideways, open side facing right. The lid must close.



2. Use your first reaction mixture tube to blank spec20D. Add NADH after blanking spec20D. Take absorbance reading, zero time.
3. Add biosynthetic crude extract at zero time.
5. Start recording absorbance values at 20 seconds and every 10 seconds thereafter until 120 seconds. Use table available in lab manual or on website to collect data.
6. Repeat steps 4 and 5 for remaining three reaction mixtures (varying concentrations of threonine).

60.344 Lab 2: Biosynthetic Threonine Deaminase DATA SHEET Effector:				
Time (sec)	Absorbance at 350 nm for varying Threonine concentrations			
	0.005 M	0.01 M	0.05 M	0.1 M
0				
20				
30				
40				
50				
60				
70				
80				
90				
100				
110				
120				

Note: available on website as a word document for your own use only - must use excel format for lab report.

**LAB 2 REPORT** - handed in over a period of three weeks

Part	Date	Experiment Section Handed In - Excel format available on lab website. Record all requested information. See lab 2 appendix for details on figure presentation. (The spreadsheet consists of 4 worksheets, see tab at bottom of worksheet to select the required worksheet.)
1	Jan 24	Completed MS Excel spreadsheet of group DEGRADATIVE threonine deaminase standard curve and kinetic analysis*
2	Jan 31	Completed MS Excel spreadsheet of group BIOSYNTHETIC threonine deaminase kinetic analysis* . Reminder - the velocity is the absolute value of the slope, ie. it doesn't matter if the slope is negative or positive.
3	Feb 7	Completed MS Excel spreadsheet of class <sup>b</sup> Threonine deaminase kinetic analysis and questions

\*if your data is not workable, borrow data ONLY to complete report, not another group's completed report

<sup>b</sup>class data will be posted on the website (entered into page 4 of spreadsheet - lower tab of spreadsheet) as soon as possible after the 2<sup>nd</sup> part of report is handed in.

Mark distribution: Part 1, (2 marks standard curve, 6 marks degradative; Part 2, 6 marks, Part 3, 6 marks.

**Question** - include with class data report (due Feb 7)

Question format available in Report Excel spreadsheet, worksheet - Report Question (see Tab at bottom of sheet).

1. Hirata et al ( 1965) presented the effect of various nucleotides on threonine deaminase activity in table format listing Km and Vmax values (Table 2 and Table 4). Include x-axis and y-axis for each nucleotide in the Excel spreadsheet table provide. Using this excerpted information draw a completely labelled Lineweaver-Burke graph showing the effect of each nucleotide on threonine deaminase activity. Do not include sample calculations, marked on values presented.

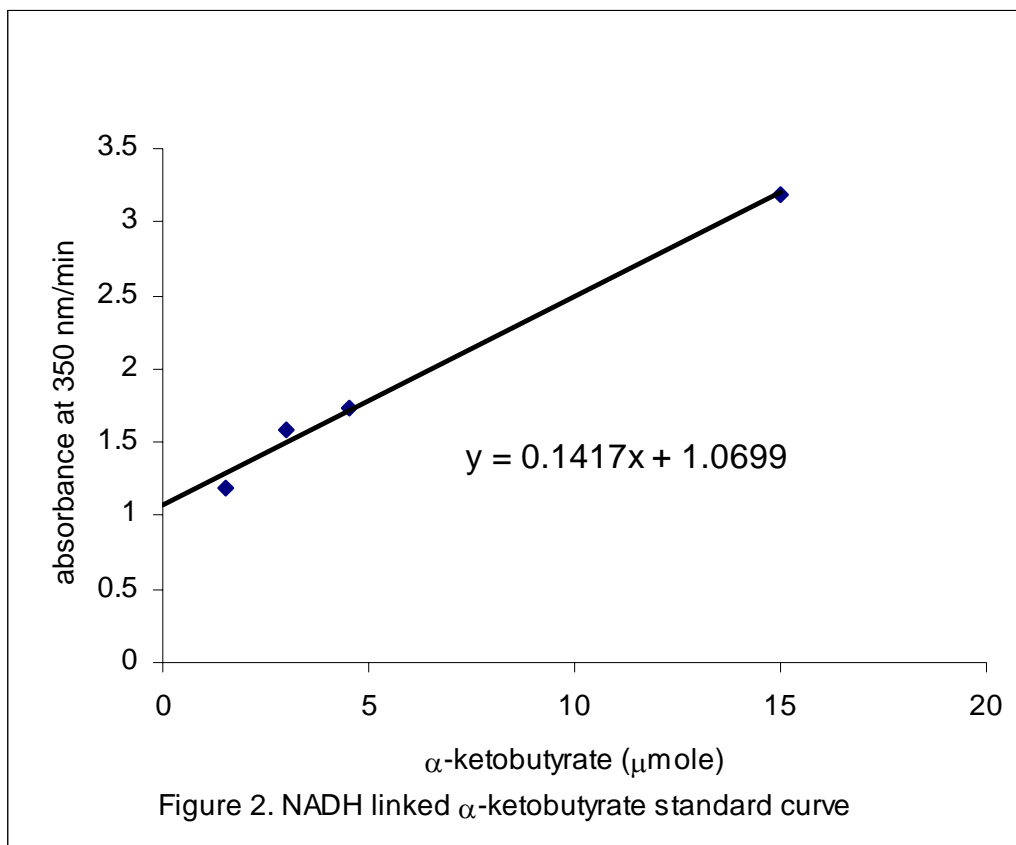
**REFERENCES** - References used to prepare lab, may or may not be required.

Hatfield, G. W. & H. E. Unbarger. L-Threonine Deaminase, Biosynthetic. Methods in Enzymology. Vol. 8, 212, 215,576

Hirata, M., M. Tokushige, A. Inagaki, & O. Hayaishi. 1965. Nucleotide Activation of Threonine Deaminase from *Escherichia coli*. J. Biol. Chem. 240:1711-1716.

Manual of Methods for General Microbiology. p.376-379

Nelson, D.L. & M.M. Cox. 2000. In Principles of Biochemistry, 4th edition. New York: WH Freeman and Company p.202-212, 225-227,846-848.



## **EXCEL OPERATIONS AND STANDARD CURVE PLOT METHODS FOR 60.344 Lab.**

Procedures may vary depending on excel version. There are numerous ways to use Excel, only one described below. HINT: Right click on whatever you want to change and select appropriate item from the pull down menu.

### **Formula and Function calculations**

Put the cursor in the cell where you want to enter formula or function. Type in formula or select function button. In the spreadsheet just set up formula for one cell then copy and paste for similar calculations.

### **Cell Formulae Notations:**

Remember to use correct mathematical brackets in your formulae.

start equation with an = sign, do not leave spaces between entries

\* multiply

/ divide

+ add

- minus

^power or use the POWER function

### **Insertion of Text boxes into Excel spreadsheet**

The majority of spreadsheets require numerical sample calculations. Often it is tedious to work in Excel.

One solution is to carry out the sample calculation in Word then copy paste to Text box on the excel spreadsheet, easily moved resized. The text box button is located in the drawing

## **MS EXCEL CHARTS**

### **$\alpha$ -ketobutyrate Standard Curve**

Select Insert pull down menu.

Select chart - (standard types), select (XY) Scatter chart type. Defaults to only markers present. Click next. Select Column.

Put cursor in data range box, click. Hold down CTRL key, then use the mouse to click and hold down to select x-axis data ( $\mu$ mole  $\alpha$ -ketobutyrate), then y-axis data (absorbance at 490 nm).

Click next.

Chart Options menu appears. Under default titles tab, enter title and axes labels. Unfortunately the title defaults to the top of the graph. After you are finished setting up graph, just select and move below graph.

Mostly likely you will need to move the graph up. May require resizing, font size change, 8 is good, etc.

Click next. Keep the default setting (graph in data page).

Click finish.

Right click any empty area on graph area. Select format plot area. Change the background color to white. OR CLEAR. Click OK.

Put the cursor on any one of the plot markers. Right click. Select Add Trendline. (Linear Regression Line). Under the default Type tab the linear regression box should be highlighted. This is what you want.

Select the Options tab. DO NOT force linear regression through zero as reagent is only linear over a defined sample concentration dependent on assay components. Most likely does not extend to zero. Change the Forecast backward from zero to whatever value is the difference between zero and your first x-axis value (draws the linear regression line to the y-axis). Select display equation on chart. Select display R-squared value on chart. Press OK. Move the display to close to the trendline. Select legend - delete as only one plot.

Move the graph to specified area on data sheet.

Select page setup, select print to fit portrait page. Select area you want to print, includes table, calculations and graph - all on one page. Print.

R-squared value: The value is between 0 and 1. The R squared value is an indicator of how closely the estimated values used to draw the linear regression trendline correspond to the actual data. The linear regression trendline is most reliable when R value is close to 1.

**Absorbance vs time plots:**

Select Insert pull down menu.

Select chart - (standard types), select (XY) Scatter chart type. Defaults to only markers present. Click next. Put cursor in data range box, click. Make sure there is no default information in box - delete if present as Excel will often automatically select data. Use the mouse to click and hold down to select the first y-axis data (0.005 M Threonine). Column should be selected - just check.

Select series tab. Series 1 plot is highlighted. Put the cursor in  $x$  values box, click. Again make sure there is no information in this box - delete if present. Use the mouse to click and hold down to select x-axis data (time in min or sec). Put the cursor in Name box. Select appropriate concentration of threonine. Click ADD under series box. Repeat above for remaining three concentrations of threonine.

Click next.

Chart Options menu appears. Under default titles tab, enter figure title and axes labels. Unfortunately the title defaults to the top of the graph. After you are finished setting up graph, just select and move below graph. Mostly likely you will need to move the graph up. May require resizing, font size change (8 is best), etc. Legend should contain each of the threonine concentrations. Make sure to leave the values as numbers as need for preparation of Lineweaver-Burk plot. Just add a title above legend after set up graph (using menu below spreadsheet) to label legend Threonine (M).

Click next. Keep the default setting (graph in data page).

Click finish.

Right click any empty area on graph area. Select format plot area. Change the background color to white. Click OK.

Right click on any horizontal grid line. Select clear.

Put the cursor on any one of the plot markers. Right click. Select Add Trendline - Select the default linear regression box.

Select the Options tab. Select display equation on chart. Press OK. If required, move the equation so completely visible. Add a linear regression line and equation for three remaining plots.

Move the graph to specified area on data sheet.

Print to fit portrait page (all information on one page..)

**Lineweaver-Burk plots:**

Select Insert pull down menu.

Select chart - (standard types), select (XY) Scatter chart type. Defaults to only markers present. Click next. Put cursor in data range box, click. Make sure there is no default information in box - delete if present as Excel will often automatically select data. Use the mouse to click and hold down to select 1/V (specific velocity) for different concentrations of threonine. Row should be selected - just check.

Select series tab. Series 1 plot is highlighted. Put the cursor in  $x$  values box, click. Again make sure there is no information in this box - delete if present. Use the mouse to click and hold down to select x-axis data 1/S data. No name is required for group data but select a name for class data by typing in effector or control or selecting box with label. For class data add remaining plots as above.

Click next.

Chart Options menu appears. Under default titles tab, enter figure title and axes labels. Unfortunately the title defaults to the top of the graph. After you are finished setting up graph, just select and move below graph. Mostly likely you will need to move the graph up. May require resizing, font size change (8 is best), etc. Click the legend tab. Remove check mark from legend - not required as only one plot. For class data keep the legend.

Click next. Keep the default setting (graph in data page).

Click finish.

Right click any empty area on graph area. Select format plot area. Change the background color to white. Click OK.

Right click on any horizontal grid line. Select clear.

Right click on x-axis scale. Select Format Axis. Select scale tab. Change minimum scale value from zero to less than plot x-axis intercept, eg. -300.

Normally a Lineweaver-Burk plot does not have a border. Right click on border. Select Clear or Format Plot Area. Select None under Border.

Put the cursor on any one of the plot markers. Right click. Select Add Trendline - Select the default linear regression box.

Select the Options tab. You need to change the Forecast from zero default to approximate value of plot x-axis intercept. This extends the regression line to the x-axis. Select display equation on chart. Press OK. If required, move the equation so completely visible. For class data, add a linear regression line and equation for remaining plots. The regression line notation will appear in the legend, just delete as not required.

Move the graph to specified area on data sheet.

Print to fit portrait page.

### **LAB 3 CONTROL OF CELL DIVISION AND DETERMINATION OF D-PHASE IN *E. COLI***

#### **OBJECT**

The object of this experiment is to first show that the inhibition of DNA replication prevents cell division but does not interfere with cell growth and to secondly demonstrate that *E. coli* cells, that have completed a round of DNA replication, proceed to divide despite protein starvation allowing the calculation of D-phase.

#### **INTRODUCTION**

In *E. coli* there are two events of fixed duration that make up the division cycle; the C-phase which is the time required to replicate the chromosome and the D-phase which is the time that lapses after completion of DNA synthesis and cell division. The only variable in the division cycle is the initiation of DNA replication which is reflected in the generation time. The initiation of DNA replication depends on cell growth conditions and requires protein synthesis. Consequently the generation time is dependent upon cell growth conditions. If protein synthesis is blocked the initiation of DNA replication is blocked, however cell division still continues for *E. coli* cells that have completed a round of DNA replication. Refer to reference papers for additional information.

In this lab cell growth as measured by absorbance and cell division as measured by plate count will be followed in an *E. coli* culture. Firstly, inhibition of DNA replication prevents cell division but does not interfere with cell growth will be demonstrated by removal of thymine from *E. coli* thymine auxotroph. After this thymine will be added to the growth medium to follow recovery of cell division and continuation of cell growth. Secondly, the addition of chloramphenicol to *E. coli* culture will demonstrate protein starvation does not prevent cell division of cells that have completed a round of DNA replication. The data obtained from chloramphenicol treated cells allows determination of the D-phase.

## D-PHASE THEORY

The D-phase calculation method presented here is based on a paper by H.E. Kubitschek (1974). The following is an excerpt. Refer to reference paper for more details. The D-phase is estimated from residual cell division that occurs after exposing exponentially growing *E. coli* cells to chloramphenicol. Chloramphenicol prevents protein synthesis which prevents initiation of DNA replication. Only those cells that have completed a round of DNA replication are capable of cell division. The fraction of cell capable of performing cell division (R) is related to the initial number of cells ( $N_0$ ) at the time of addition of inhibitor (chloramphenicol), to the final number of cells (N) when all possible cell divisions are complete, and to the time period between DNA replication completion and cell division (D) by the following equation. T is the generation time of *E. coli* culture.

$$N/N_0 = 1 + R = 2^{D/T}$$

The cell division rate (r) is equal  $1/T$ . The term  $\ln(1 + R)$  increases with the cell division rate (r) as follows:

$$\ln(1 + R) = Dr \ln 2$$

D is constant, independent of the cell division rate.

T = generation time calculation = refer to basic microbiology book.

## PROCEDURE

### Week 5

#### READ COMPLETE EXPERIMENT BEFORE STARTING

1. An overnight culture of *E. coli* UM242 (Pro<sup>-</sup>, Thy<sup>-</sup>) was subcultured in M9Ca medium (minimal medium containing casaminoacids + proline + thymidine) with shaking at 37°C until an exponential culture was obtained (~3 hours).
2. The cultures (a) and (b) were centrifuged and resuspended in (a) 2 x 6 ml M9Ca complete medium (plus thymidine), (b) 2 x 6 ml M9Ca medium (minus thymidine) respectively. Medium was prewarmed to 37°C before resuspension to ensure maintenance of exponential growing phase.  
*E. coli* cultures (c) and (d) are dispensed in 2 x 6 ml M9Ca complete medium. Your cultures are located on the 37°C culture rotor labelled as M9Ca complete or M9Ca minus thymidine. Each group selects two tubes of the appropriate medium depending on the experiment your group performs. Label top of caps with your group number, and side with experiment (a), (b), (c) or (d). One tube should be labelled bacterial/ml and the other absorbance.  
Note: Thymidine (Thy) is substituted for thymine for practical reasons as it is more soluble in aqueous solution. Thymidine and thymine are equivalent.

STUDENT LAB STARTS HERE.

**Remember to record absorbance value at all time points.**

**This is a long lab, be sure you come to lab well prepared. The lab should be open before the lab allowing you (if your schedule allows) to label plates and dilution tubes before 2:30 pm.**

**Supplies are usually in the lab by lunch time. Actual experiment cannot be started before 2:30 pm.**

Cultures are assigned as follows:

- E. coli* UM242 culture (a) for groups - 1, 5, 9, 13, 17, 21
- E. coli* UM242 culture (b) for groups - 2, 6, 10, 14, 18, 22
- E. coli* UM242 culture (c) for groups - 3, 7, 11, 15, 19, 23
- E. coli* UM242 culture (d) for groups - 4, 8, 12, 16, 20, 24

Each group works with two 6 ml *E. coli* culture a, b, c or d. Label one tube bacteria/ml and other absorbance sampling for 0.1 ml and 0.2 ml at time intervals from respective tubes. Label top of tube with your group # for quick identification. Casamino acids has been added to the defined medium to shorten replication time. The tubes are incubated on the culture rotor. Remove and replace as quickly as possible to ensure the rotor is stopped only briefly. Turn on rotor immediately upon removing or replacing cultures. Make sure you tubes are balance opposite each other.

BACTERIA MANIPULATION, DILUTION AND PLATING for cultures (a) Glucose M9Ca Minimal Medium (plus Thymidine) and (b) Glucose M9Ca Minimal Medium (minus Thymidine).

3. The time of thymidine removal is 2:15 pm for cultures (a) and (b), This is zero time of experiments (a) and (b).
4. **Culture (b) only: At 40 min from time zero add thymidine** at a final concentration of 40  $\mu\text{g/ml}$  to culture lacking thymidine. Stock thymidine concentration is 10 mg/ml. Calculate volume of 10 mg/ml thymidine to add **to each 6 ml culture** yourself then check with demonstrator to ensure calculated correctly.
5. **Cultures (a) and (b):** At 30, 40, 60, 80, 100, 130, and 160 min after start of experiment, transfer 0.1 ml of culture to 0.9 ml saline ( $10^{-1}$  dilution). Prepare 10-fold serial dilutions ( $10^{-2}$  to  $10^{-7}$ ) for each time point. Plate in duplicate on T-soy plates 0.1 ml of  $10^{-4}$  to  $10^{-7}$  dilutions.

Put on ice only if unable to perform dilutions and plating immediately.

BACTERIA MANIPULATION, DILUTION AND PLATING for cultures (c) MINUS CHLORAMPHENICOL and (d) PLUS CHLORAMPHENICOL

1. **Culture (d) only:** Add chloramphenicol at a final concentration of 50 µg/ml. The time of addition of chloramphenicol is zero time, ie., when your group starts the experiment. Stock chloramphenicol concentration is 10 mg/ml. Calculate volume of 10mg/ml chloramphenicol to add to your **two 6 ml *E. coli* cultures** yourself, then check with demonstrator to ensure calculation is correct.
2. **Cultures (c) and (d):** At zero time immediately transfer 0.1 ml culture to 0.9 ml saline ( $10^{-1}$  dilution). Then prepare 10-fold serial dilutions ( $10^{-2}$  to  $10^{-7}$ ). Plate 0.1 ml of  $10^{-4}$  to  $10^{-7}$  dilutions in duplicate on T-soy plates.  
Put on ice only if unable to perform dilutions and plating immediately.
3. Repeat step 2 for cultures (c) and (d) at 15, 30, 45, 60, 90, and 120 minutes.

SPEC20D ABSORBANCE READING for all cultures, (a) (b) (c) & (d)

Refer to appendix for spec20D operation procedure.

1. Using the spectronic 20D at 540 nm, measure the absorbance of the culture **at all time points** specified for each experiment.
2. Measurement of absorbance (use plastic cuvet holder) is performed by transferring 0.2 ml culture to a Spectronic 20D plastic cuvet containing 0.8 ml saline (1 in 5 dilution - be sure to correct absorbance reading to that of the culture before plotting). The spectronic 20D blank is 1 ml saline.  
Note: If unable to read absorbance shortly after sampling, put sample on ice until you have time to read the absorbance.

INCUBATION AND BACTERIA PLATE COUNT DATA COLLECTION for all cultures, (a) (b) (c) & (d)

1. Incubate plates at 37°C overnight.
2. Next day record plate count data. After counting DISCARD PLATES in petri plate waste container for this lab and subsequent labs. If you are unable to record you data the next day, transfer your plates to the student cold box (room 203 off room 201). Read your plate counts as soon as possible. Must be recorded by Friday. Do not store plates in the cold box after counting.
3. **Each group submits ONE COPY of plate count data SHEET and optical density data by 2:30 pm Friday (see schedule for date). Place data through slotted drawer in filing cabinet located in room 204 or email data [le\\_cameron@umanitoba.ca](mailto:le_cameron@umanitoba.ca) by 2:30 pm Friday.** Keep the original copy of the data for lab report write up.
  - (a) Include all requested data. Plate count data must be complete for all dilutions plated, ie. include data above (TNTC<sup>a</sup>) and below significant plate counts.
  - <sup>a</sup>too numerous to count
  - (b) Class data will available on website as soon as possible.
  - (c) Remember you must **keep the original copy of your data** for lab report write up.

(available on website as a WORD and Excel document)

Date: \_\_\_\_\_

Group Number: \_\_\_\_\_ Group names: \_\_\_\_\_

Complete **ONE** of the following tables.

Circle your group's experiment:

(a) Glucose M9Ca Minimal Medium (plus Thymidine) OR (b) Glucose M9Ca Minimal Medium (minus Thymidine)

Time (min)	absorbance <sup>a</sup> (540 nm) x5	Duplicate <i>E. coli</i> UM242 plate count data for 0.1 ml of dilution plated							
		10 <sup>-4</sup>		10 <sup>-5</sup>		10 <sup>-6</sup>		10 <sup>-7</sup>	
		plate 1	plate 2	plate 1	plate 2	plate 1	plate 2	plate 1	plate 2
30									
40									
60									
80									
100									
130									
160									

<sup>a</sup>before recording absorbance multiple absorbance by 5 to take into consideration the 5-fold dilution in saline

OR (c) minus chloramphenicol OR (d) plus chloramphenicol

Time (min)	absorbance <sup>a</sup> (540 nm) x5	Duplicate <i>E. coli</i> UM242 plate count data for 0.1 ml of dilution plated							
		10 <sup>-4</sup>		10 <sup>-5</sup>		10 <sup>-6</sup>		10 <sup>-7</sup>	
		plate 1	plate 2	plate 1	plate 2	plate 1	plate 2	plate 1	plate 2
0									
15									
30									
45									
60									
90									
120									

<sup>a</sup>before recording absorbance multiple absorbance by 5 to take into consideration the 5-fold dilution in saline

**LAB 3 REPORT**

**Data Presentation and Analysis**

Excel spreadsheet format available on lab website. First worksheet is for group data while the second and third worksheets are for class data. Select by clicking tabs below worksheet. When available class

data will be entered into worksheets.

Group Data:

- 1 1. Append data sheet of *E. coli* UM242 plate count and absorbance data. On sheet indicate plate count data used to calculate bacteria/ml.
- 3 2. Included completed Excel spreadsheet (group data) of bacteria/ml for each time point for your group data. Record all requested information including sample titre calculation. Insert figure of bacteria/ml (left y-axis log format) and absorbance at 540 nm (right y-axis) vs time. See appendix for Excel figure presentation. Include all important information in figure title.

Class Data:

- 1 1. Included completed Excel worksheets of class data.
- 2 2. For worksheet **class data (a) and (b)**, insert one figure (one graph) of bacteria/ml (left y-axis log format) and absorbance at 540 nm (right y-axis) vs time. Include all important information in figure title. See lab appendix for figure presentation details.
- 5 3. For worksheet **class data (c) and (d)**, insert one figure (one graph) of bacteria/ml (left y-axis log format) and absorbance at 540 nm (right y-axis) vs time. Include all important information in figure title. Include exponential regression plot and equation for culture (c). See lab appendix for figure presentation details. In worksheet include requested growth rate constant and related information. Also include requested D-phase value and related information. Show all requested calculations.

Required Formulae (must be used in Excel spreadsheet):

$r = (\log N_t - \log N_o) / (\log 2 * t)$  The values for  $N_t$  and  $N_o$  must be taken from the exponential regression line for culture (c) not just the first and last values for bacteria/ml entered in table.

$t$  is the time interval between  $N_o$  and  $N_t$ .

Use the regression formula ( $y = ce^{bx}$ ) on your graph to calculate these values and enter in required cells on worksheet. Also include R-squared value on graph.

$e = 2.718$  (not required as you use the EXP function in Excel)

$D = (\ln N - \ln N_o) / r * \ln 2$  The value for  $N_o$  is zero time for culture (d). The value for  $N_t$  is calculated using the Excel function INTERCEPT. When calculating the y-intercept (N) consider only culture (d) data after the titre has levelled off indicated by titre value only increasing very slightly or titre going slightly up and down due to standard deviation.

**Questions**

Include references used to answer questions. (Cite in text and list at end of report.)

- 0.5 1. a) Kubitschek<sup>4</sup> (1974) showed that when  $\ln(1 + R)$  is plotted again divisions/hr a linear line occurs (figure 2). State why there is a linear relationship.
- 0.5 b) Explain why Kubitschek (1974) add chloramphenicol to the culture.
- 1 2. In your lab you measured cell division by measuring cell number while Donachie<sup>5</sup> (1969) observed cell division using both C<sup>14</sup>-thymine and cell number. How do the experimental results differ and explain why.

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**Lab 3 EXCEL OPERATIONS AND STANDARD CURVE PLOT METHODS.**

Procedures may vary depending on excel version. There are numerous ways to use Excel, only one described below. HINT: Right click on whatever you want to change and select appropriate item from the pull down menu.

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<sup>4</sup>Kubitschek, H. E. 1974 Estimation of the D Period from Residual Division after Exposure of Exponential Phase Bacteria to Chloramphenicol. Molec. Gen. Genet. 135:123-130.

<sup>5</sup>Donachie, W. D. 1969 Control of Cell Division in *Escherichia coli*: Experiments with Thymine Starvation. J. Bact. 100:260-268.

## Formula and Function calculations

Put the cursor in the cell where you want to enter formula or function. Type in formula or select function button. In the spreadsheet just set up formula for one cell then copy and paste for similar calculations.

### Cell Formulae Notations:

Remember to use correct **mathematical brackets** in your formulae.

start equation with an = sign, do not leave spaces between entries

\* multiply

/ divide

+ add

- minus

^power or use the POWER function

## FUNCTIONS

### INTERCEPT

Calculates the point at which a line will intercept the y-axis using a best fit regression through known x and y values. Ideally the best fit regression should be horizontal to determine the y-axis intercept.

Use the INTERCEPT FUNCTION to determine N (culture (d)) required for calculation of D-phase.

Put the cursor in the cell where you want to record standard deviation.

Select paste function button ( $f_x$ ), then Statistical under function category. Select INTERCEPT under function name. Click OK. A pop-up menu appears. If it covers the data you want to use, just move. Put the cursor in known y's box.

Remember to select only data after the line has levelled out.

Using your mouse click the first or last cell of data set.

Hold down button and scroll down to the last or first cell in the data set. Release button.

Put the cursor in the known x's box and insert data.

Click OK on pop-up menu.

The y-axis intercept value appears in your selected cell (should be formatted to scientific number category).

### EXP (e raised to the power of a given number)

You do not actually use the function alone but incorporate it into the exponential equation required to calculate  $N_0$  and  $N_t$  for culture (c).

Example:

for  $y = ce^{bx}$  the exponential regression equation

$$y = 5E+08e^{0.0202x}$$

$$\text{for time (x) 0 min (N}_0\text{)} : =(5E+08)*(EXP(0.0202*0)) = 5E+08 \text{ bacteria/ml}$$

$$\text{for time (x) 120 min (N}_t\text{)} : =(5E+08)*(EXP(0.0202*120)) = 5.65E+09 \text{ bacteria/ml}$$

## MS EXCEL CHARTS - two y-axes

Notes:

(i) Bacteria/ml values are scientific number category which permits growth rate constant calculation and keeps all data consistent.

(ii) Excel sometimes is not 'Science Friendly', this is one of those cases where two y-axes are required on a scatter plot. Sigma Plot is an excellent Excel based graph program but is not available on the University computers (expensive) so we need to make do.

Select Insert pull down menu.

Select chart - (standard types), select (XY) Scatter chart type. Select chart with data points connected by lines. Click next.

Put cursor in data range box, click. Make sure there is no default information in box - delete if present as Excel will often automatically select data. Use the mouse to click and hold down to select the first y-

axis data (either bacteria/ml or absorbance at 540 nm). Column should be selected - just check. Select series tab. Series 1 plot is highlighted. Put the cursor in x values box, click. Again make sure there is no information in this box - delete if present. Use the mouse to click and hold down to select x-axis data (time (min)). Put the cursor in Name box. Select appropriate heading (eg + or - thymidine or chloramphenicol). Click ADD under series box. Repeat above for remaining y-axes data. All data will default to one axis - okay for now. Click next.

Chart Options menu appears. Under default titles tab, enter figure title and axes labels (enter right y-axis name). Unfortunately the title defaults to the top of the graph. After you are finished setting up graph, just select and move below graph. Mostly likely you will need to move the graph up. May require resizing, font size change (8 is best), etc. When changing font select the entire graph box. May need to redo after adding second y-axis. Click next. Keep the default setting (graph in data page). Click finish.

Right click any empty area on graph area. Select format plot area. Change the background color to white. Click OK.

Right click on any horizontal grid line. Select clear.

Right click on absorbance plot. Select Format Data series. Select Axis tab. Change plot series on - from primary to secondary axis. Click OK. Repeat for all other plots you want to put on second y-axis. Now you need to add a right y-axis name. Right click anywhere in the plot area. Select Chart Options. Select Title tab. Enter second value (y) axis. Just leave second value (x) axis blank.

Right click left axis (bacteria/ml) to change to log scale. Select Format Axis. Select scale tab. Check Logarithmic scale. Make sure the value range fits the size of your graph. For log bacteria/ml most likely need only  $10^2$  to  $10^3$  range. The object is to have your plot fit the size of graph. Click OK.

Add Trendline for culture (c) class data only: Put the cursor on any one of the plot markers. Right click. Select Add Trendline - Select the default exponential regression box.

Select the Options tab. Select display equation and  $R^2$  value on chart. Press OK. If required, move the equation so completely visible. You require this regression equation to calculate  $N_0$  and  $N_t$  for growth rate constant calculation.

Move the legend to empty space on graph. Resize graph if required.

Move the graph to specified area on data sheet.

Make sure you select print all information on one page under page setup. Exception is worksheet 3 (cultures (c) and (d)) as too much information. Print graph separately by selecting just the graph before printing. Then print spread sheet and calculations together.

**LAB 4****MOTILITY AND CHEMOTAXIS****OBJECT**

The object of this experiment is to distinguish true motility from Brownian movement and to visualize the functions of motility and the bacteria's ability to respond to the environment.

**INTRODUCTION**

Motile organisms are attracted or repelled by chemicals, respectively positive and negative chemotaxis. In *E. coli* motility is permitted by the presence of six randomly located flagella, organelles that consist of helical filaments that are driven by a rotary motor at the base of the flagella. Run motion (straight line motion) results from counterclockwise filament rotation where the flagella work together. Tumbling motion is clockwise flagella motion where the flagella pull in opposite directions resulting in chaotic angular motion. The following diagram illustrates bacterial motility in the presence and absence of an unidirectional attractant. Although the pattern of runs and tumbles is similar, the presence of attractant favors runs and suppresses tumbling.

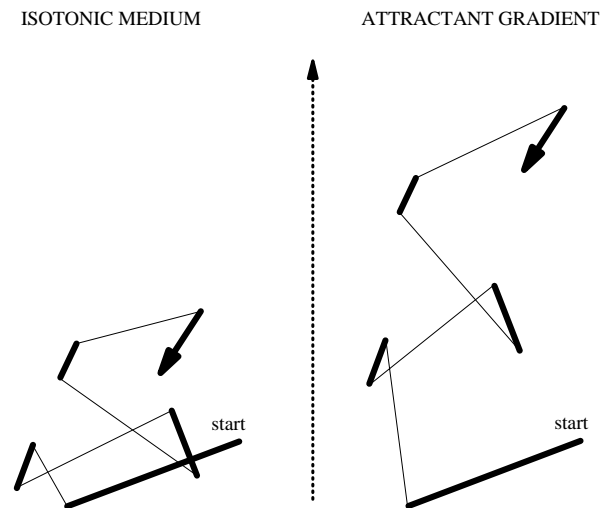


Figure 2: Bacterial chemotaxis motility patterns.

Note: Centre arrow indicates unidirectional gradient of attractant.

## PROCEDURE

### Week 7

#### Part I: Preparation of Bacteria (prepared prior to start of lab)

To allow for maximum motility, the *E. coli* strain was first grown on semi-soft tryptone agar plate and the cells at edge of swarm were collected for further study. The plates were incubate overnight at 28°C. The cells were gently suspended in a small amount chemotaxis buffer or chemotaxis buffer plus leucine. The cells were washed (centrifuged, supernatant discarded and pellet resuspended in buffer) twice with chemotaxis buffer and resuspended gently using chemotaxis buffer or chemotaxis buffer plus leucine at the same bacteria cell concentration. The resuspended cells were incubated without shaking at 37°C for a minimum of 2 hours before the lab. The *E. coli* tubes are labelled with group number, *E. coli* growth medium, *E. coli* suspension medium.

#### Part II: Chemotaxis Assay by Slide Technique

Refer to the following table for assigned experiment by group number.

Group Number	<i>E. coli</i> suspension medium	Chemical added to capillary tube
1,7,13,19	chemotaxis buffer	10 mM glycerol
2,8,14,20	chemotaxis buffer	10 mM galactose
3,9,15,21	chemotaxis buffer	10 mM aspartate
4,10,16,22	chemotaxis buffer	chemotaxis buffer
5,11,17,23	chemotaxis buffer plus 10 mM leucine	chemotaxis buffer
6,12,18,24	chemotaxis buffer plus 10 mM leucine	chemotaxis buffer plus 10 mM leucine

1. Flame seal one end of the capillary tube. After cooling use the capillary tube, flame sealed at one end, to take up attractant or repellent. This is performed by passing the open end of the capillary over flame and plunging into an eppendorf tube containing 1 ml liquid assigned in your group's experiment. As the capillary cools, the liquid is drawn up to the first graduated line (1  $\mu$ l) on the capillary - this may take several trials to get the correct timing. An approximate 1  $\mu$ l volume is acceptable. Return capillary to assigned liquid. It is essential that the capillary contents do not start to dry out as this may form an air bubble at the end of the capillary preventing bacteria from moving into the capillary.
2. A U-shaped capillary tube has been glued to a slide as shown in diagram. Pipet 220  $\mu$ l of your group's assigned *E. coli* cell suspension onto the microscope slide in the horseshoe formed by the U-shaped capillary tube on the slide (refer to diagram).

- Remove capillary tube containing 1  $\mu\text{l}$  liquid from assigned liquid. Make sure there is no liquid on the outside of the capillary. Insert open end into U-shaped chamber containing *E. coli* suspension as shown in diagram. Place cover slip on top. Make sure the bacterial suspension completely covers the end of the capillary tube and is in contact with the cover slip. Support sealed end of capillary tube with a glass slide when placing in incubator.

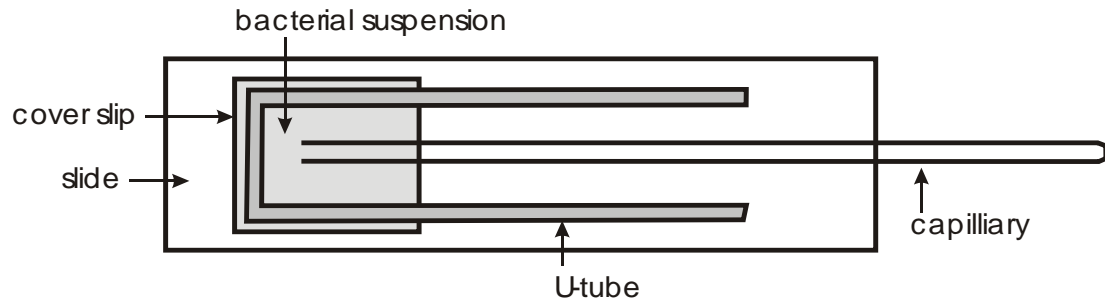


Figure 3. Chemotaxis assay apparatus

- Incubate on heating plate for 1.5 hours (30°C). The heating plate is a metal tray over a waterbath. The tray surface is covered with a damp cloth to maintain humidity. Place slides directly on damp cloth. Cover metal tray with foil to maintain a high humidity. This procedure should prevent evaporation of your sample, however it is important to monitor the chemotaxis slide while incubating to ensure that the tip of the capillary tube is in contact with the bacterial suspension throughout the incubation time. The bacterial suspensions should also remain in contact with the cover slip throughout the incubation time. **This is an important step that requires attention by each group.**
- Transfer 200  $\mu\text{l}$  tryptone broth from a test tube of broth provided to an eppendorf tube. After one and a half hours remove capillary, rinse with a thin stream of water from a squirt bottle. Break\* off sealed end of the capillary while holding the open end over the tryptone broth tube. Use a syringe\* with a tubing attachment for the capillary tube to squirt contents of capillary into the eppendorf containing 200  $\mu\text{l}$  tryptone. While doing this, touch the tip of the capillary to the side of the tube just above broth. Cap tube and vortex sample to ensure homogeneous suspension of bacteria before titrating.  
\*pull up plunger part way before attaching syringe to capillary tube
- Prepare serial dilutions (in saline) using sterile 5" metal capped test tubes in a total volume of 1 ml:  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$ .
- Spread plate 0.1 ml of each dilution in duplicate on tryptone agar.
- Also prepare dilutions of original cell suspension in saline;  $10^{-1}$  to  $10^{-8}$ . Spread plate in duplicate 0.1 ml of dilutions  $10^{-4}$  to  $10^{-8}$  on tryptone agar.
- Allow plates to dry upright, invert, and incubate at 37°C overnight.

10. **Each group submits ONE COPY of plate count data sheet by 2:30 pm Friday (see schedule for date). Place data through slotted drawer in filing cabinet located in room 204 or email data [le\\_cameron@umanitoba.ca](mailto:le_cameron@umanitoba.ca) by 2:30 pm Friday.**
- (a) Include all requested data. Plate count data must be complete for all dilutions plated, ie. include data above (TNTC<sup>a</sup>) and below significant plate counts.  
<sup>a</sup>too numerous to count
  - (b) Class data will available on website as soon as possible.
  - (c) Remember you must **keep the original copy of your data** for lab report write up.

**LAB 4 MOTILITY AND CHEMOTAXIS DATA SHEET**

(available on website as a WORD and Excel document)

Date: \_\_\_\_\_

Group Number: \_\_\_\_\_

Group names: \_\_\_\_\_

*E. coli* suspension medium: \_\_\_\_\_

Chemical added to capillary tube: \_\_\_\_\_

Dilution	<i>E. coli</i> plate count data for original culture		<i>E. coli</i> plate count data for tryptone broth (contains capillary contents in 200 $\mu$ l)	
	plate 1	plate 2	plate 1	plate 2
$10^{-1}$	N/A	N/A		
$10^{-2}$	N/A	N/A		
$10^{-3}$	N/A	N/A		
$10^{-4}$				
$10^{-5}$				
$10^{-6}$			N/A	N/A
$10^{-7}$			N/A	N/A
$10^{-8}$			N/A	N/A

TNTC = too numerous to count

N/A = not plated

## LAB 4 REPORT

### Data Presentation and Analysis

#### Group data:

- 3 1. Include completed group data Excel spreadsheet. Include all requested information, data and sample calculations. This includes determination of bacteria/ml of the original *E. coli* culture and tryptone broth culture. It also includes determination of the total number of bacteria in capillary. First determine the total number of bacteria on the microscope slide and in the capillary. Using these values, determine percentage of the total population that migrated into the capillary.

#### Class data:

- 3 1. Include completed class data Excel spreadsheet. Include all requested information.(percentage of the total population that migrated into the capillary). No sample calculation required. Also include chemotaxis interpretation, ie., control (random motility), attractant, repellent. Wherever relevant footnote chemoreceptor involved and cite reference.

**continued on the next page**

## Question

- 2 1. In 1969 Adler<sup>6</sup> found glycerol to be a non-attractant. Then Oosawa and Imae<sup>7</sup> (1983) showed that glycerol could be a repellent under selected experimental conditions. Zhulin et al<sup>8</sup> (1997) confirmed the negative chemotaxis response and also showed that glycerol could be an attractant. In the following table state the experimental conditions that promote each type of glycerol chemotaxis. Explain why for each.  
Table is available as a Word document on lab website.

Table 3. Glycerol chemotaxis in <i>Escherichia coli</i> .		
Type of chemotaxis	Experimental Conditions	Explanation Why
non-attractant		
repellent		
attractant		

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<sup>6</sup>Adler, J. 1969. Chemoreceptors in bacteria. *Science*. 166: 1588-1597.

<sup>7</sup>Oosawa, K, Imae, Y. 1983. Glycerol and ethylene glycol: members of a new class of repellents of *Escherichia coli* chemotaxis. *J. Bact.* 154: 104-112.

<sup>8</sup>Zhulin, IB, Rowsell, EH, Johnson, MS, Taylor, BL. 1997. Glycerol elicits energy taxis of *Escherichia coli* and *Salmonella typhimurium*. *J. Bact.* 179: 3196-3201.

## LAB 5 ENVIRONMENTAL STRESS SHOCK IN BACTERIA

### OBJECT

The object of this experiment is to appreciate the methods by which vegetative bacterial cells are capable of resisting hostile environments.

### INTRODUCTION

In *E. coli* at least four stress regulons are involved in the protection against environmental stress, nutrient limitation, and damage caused by toxic or physical agents. The four stress regulons are the heat shock regulon, the oxidation stress regulon, the SOS regulon, and the stringent regulon. Each regulon produces enzymes that protect the bacteria against stress damage. *E. coli* may induce only one regulon or a combination of regulons in an attempt to survive environmental stress agents. Refer to reference papers for additional information.

### PROCEDURE

#### Week 8

Refer to the following table for assigned experiment by group number.

Group Number	Pretreatment	Shock Treatment
1,14	untreated cells	10 mM H <sub>2</sub> O <sub>2</sub>
2,15	60 μM H <sub>2</sub> O <sub>2</sub>	10 mM H <sub>2</sub> O <sub>2</sub>
3,16	60 μM H <sub>2</sub> O <sub>2</sub> + 50 μg/ml chloramphenicol	10 mM H <sub>2</sub> O <sub>2</sub>
4,17	60 μM H <sub>2</sub> O <sub>2</sub>	50°C
5,18	untreated cells	50°C
6,19	42°C	50°C
7,20	42°C	10 mM H <sub>2</sub> O <sub>2</sub>
8,21	42°C	10% EtOH
9,22	42°C + 50 μg/ml chloramphenicol	50°C
10,23	untreated cells	10% EtOH
11,24	4% EtOH	10% EtOH
12,25	4% EtOH	50°C
13,26	4% EtOH + 50 μg/ml chloramphenicol	10% EtOH

H<sub>2</sub>O<sub>2</sub> supplied at 10 mM and 1 M stock. Use appropriate stock solution.

Chloramphenicol supplied at 10 mg/ml stock solution.

EtOH supplied at 95% stock solution.

Note: All treatments are at 30°C unless otherwise stated.

Come to lab prepared. It is a good idea to organize and label all necessary tubes and plates prior to start of lab. Throughout the term the lab is open before start of lab for experiment set up and labelling of tubes. Supplies are usually in the lab by lunch time.

**Cultures should remain shaking in appropriate waterbath whenever possible.** Quickly remove and return culture to waterbath for additions and sampling.

1. *E. coli* cells were grown overnight with shaking at 30°C in M9 Glucose broth.
2. Day of lab, the *E. coli* cells were subcultured into 5 ml fresh M9 Glucose (large screw capped culture tube) and incubated at 30°C with shaking for 3 to 4 hours (approximately 0.8 absorbance at 600 nm).
3. **Student lab starts.** Take one 5 ml *E. coli* culture and clearly label with your group number and student name(s).
4. **Pretreatment:** Each group should add prescribed pretreatment from stock solution to tube of *E. coli* culture, unless you are a control group. It is important to add the correct concentration. **After calculating, check with demonstrator to ensure that you are adding the correct volume.** All cultures except 42°C pretreatment should be incubated in a 30°C shaking waterbath for 30 min. Incubate 42°C pretreatment cultures in the 42°C shaking waterbath. CONTROL cultures (no pretreatment) should also be incubated at 30°C for 30 min before shock treatment. This ensures the same number of cells in the control culture as pretreated cells.
5. **Shock Treatment:** After pretreatment, add prescribed shock treatment chemical to cell suspension or shift to 50°C waterbath. **Again add correct volume for concentration required from stock solution.**
6. At 0, 20, 40 and 60 min after addition of shock treatment chemical, or temperature upshift, transfer 0.1 ml culture to a labelled sterile 5" metal capped test tube containing 0.9 ml saline ( $10^{-1}$  dilution for each time point) and place on ice. Continue to prepare 10-fold serial dilutions ( $10^{-2}$  to  $10^{-7}$ ) in saline (total volume of 1 ml) for each time point. If you prepare dilutions immediately, you do not need to place on ice. Spread plate 0.1 ml of dilutions  $10^{-2}$  to  $10^{-7}$  in duplicate on nutrient agar plates. There is a wide range of dilutions plated for each time point to ensure significant plate counts are obtained. Class data plate counts should vary considerably depending on treatment.
7. Incubate plates at 37°C overnight.
8. Next day record plate count data.

9. **Each group submits ONE COPY of plate count data by 2:30 pm Friday (see schedule for date). Place data through slotted drawer in filing cabinet located in room 204 or email data [le\\_cameron@umanitoba.ca](mailto:le_cameron@umanitoba.ca) by 2:30 pm Friday.**
- (a) Include all requested data. Plate count data must be complete for all dilutions plated, ie. include data above (TNTC<sup>a</sup>) and below significant plate counts.
- <sup>a</sup>too numerous to count
- (b) Class data will available on website as soon as possible.
- (c) Remember you must **keep the original copy of your data** for lab report write up.

#### REFERENCES

- Michel, G.P.F., & J. Starka. 1986. Effect of Ethanol and Heat Stresses on the Protein Pattern of *Zymomonas mobilis*. J. Bacteriology. 165, 1040-1042
- \*Neidhardt, F.C., VanBogelen, R.A. & V. Vaughn. 1984. The Genetics and Regulation of Heat-Shock Proteins. Ann. Rev. Genet. 18: 295-329
- VanBogelen, R. A., P. M. Kelley, and F. C. Neidhardt. 1987. Differential Induction of Heat Shock, SOS, and Oxidative Stress Regulons and Accumulation of Nucleotides in *Escherichia coli*. J. Bacteriology, 169: 26-32.

**Lab 5: Environmental Stress Shock in Bacteria DATA SHEET**

(available on website as a WORD document)

Date: \_\_\_\_\_

Group number: \_\_\_\_\_

Group names: \_\_\_\_\_

*E. coli* pretreatment: \_\_\_\_\_*E. coli* shock treatment: \_\_\_\_\_

dilution	<i>E. coli</i> plate count data at time intervals			
	0 min	20 min	40 min	60 min
10 <sup>-2</sup>				
10 <sup>-2</sup>				
10 <sup>-3</sup>				
10 <sup>-3</sup>				
10 <sup>-4</sup>				
10 <sup>-4</sup>				
10 <sup>-5</sup>				
10 <sup>-5</sup>				
10 <sup>-6</sup>				
10 <sup>-6</sup>				
10 <sup>-7</sup>				
10 <sup>-7</sup>				

0.1ml of each dilution plated in duplicate

TNTC = too numerous to count

## LAB 5 REPORT

### Data Presentation and Analysis

See website for Lab 5 Excel spreadsheets (consists of two worksheets group data and class data).

#### Group Data

- 3 1. Include group data Excel worksheet. Include all requested information and sample calculations - bacteria/ ml and percentage survival\*.  
 \* Percentage survival =  $\frac{\text{Bacteria/ml at time interval}}{\text{Bacterial/ml at 0 time}} \times 100$

#### Class Data

- 6 2. Include class data Excel worksheet. Record all requested information (% survival, interpretation, and relative amounts of type(s) of stress proteins). Include three figures plotting % survival (y-axis log format) vs time. There should be ONE figure for each shock treatment (figure 1. hydrogen peroxide shock treatment, figure 2. heat shock treatment and figure 3. ethanol shock treatment). Each graph should include a control and all pretreatments used for that particular shock treatment. Figure should be complete, similar to previous lab report figures including both a legend and figure title. Join markers with straight lines.

### Question

#### Question

- 1 1. Michel & Starka<sup>9</sup> (1986) investigated the effect of ethanol and heat stresses on the protein pattern of *Zymomonas mobilis*.  
 a) In figure 1 each figure has two axes one labeled pH and the other MW. Explain what this means with respect to practical experiment performed.  
 b) Explain why the cells must be labelled for a short time, “a 6 min labeling was carried out with <sup>35</sup>S-methionine”.  
 c) Figure 1 results are fluorographs. Explain why they must be fluorographs not just stained gels.

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<sup>9</sup>Michel, G.P.F., & J. Starka. 1986. Effect of Ethanol and Heat Stresses on the Protein Pattern of *Zymomonas mobilis* Journal of Bacteriology. 165, 1040-1042.

## APPENDIX

### MEDIA AND SOLUTIONS

#### Minimal Media (M9 Media)

$K_2HPO_4$  ..... 10.5 g  
 $KH_2PO_4$  ..... 4.5 g  
 $(NH_4)_2SO_4$  ..... 1.0 g  
 Sodium Citrate  $\cdot 2H_2O$  ..... 0.5 g  
 Distilled Water ..... to final volume of 1 liter (including additions)  
 Autoclave. Cool.  
 Add  $MgSO_4 \cdot 7H_2O$ , thiamine, required amino acids or nucleotides and carbon source as required. (general glucose unless otherwise required) as specified below.

#### Notes:

- (i) M9 media - defined medium that contains minimum requirements for wild type *E. coli* growth = salts, magnesium and a carbon source which is usually glucose.  
 (ii) M9Ca: Minimal media plus 4 g/liter casamino acids (acid hydrolyzed casein)

#### STOCK SOLUTIONS FOR Minimal Media (M9 or M9Ca minimal media)

##### $MgSO_4 \cdot 7H_2O$ [final concentration 25gms/liter]

$MgSO_4$ .....2.5 g  
 Dist.  $H_2O$ ..... to final volume of 100 ml  
 Filter sterilize and add 1 ml stock per 100 ml medium.

##### 20% Carbon Source

Carbon Source.....20 g  
 Dist.  $H_2O$ ..... to final volume of 100 ml  
 Autoclave and add 1 ml stock per 99 ml medium.

##### THIAMINE [final concentration 5 $\mu$ g/ml] - B<sub>1</sub>

Thiamine.....1.0 g  
 Dist.  $H_2O$ ..... to final volume of 100 ml  
 Filter sterilize and add 0.5 ml per 100 ml of medium.

##### Thymidine [ final concentration 40 $\mu$ g/ml] (thymine and thymidine are interchangeable - thymidine is used because it is easier to dissolve in water).

Thymidine.....0.4 g  
 Dist.  $H_2O$ .....100 ml  
 (NaOH may be added to help dissolve)  
 Filter sterilize and add 1 ml of stock solution to 99 ml of medium.  
 NOTE: this solution must be kept warm when adding to the broth (ie. in a beaker of warm water).

##### L PROLINE [final concentration 20 $\mu$ g/ml]

L Proline.....0.2 g  
 Dist.  $H_2O$ .....to final volume of 100 ml  
 Filter sterilize and add 1 ml stock solution per 99 ml of medium.

TRYPTONE BROTH - complex medium 10 g Tryptone, 5 g NaCl per liter (final volume) distilled water. pH~7.0. Autoclave.

TRYPTONE AGAR PLATES - 10 g Tryptone, 5 g NaCl, 15 g agar ~pH 7.0 per liter (final volume) distilled water. Autoclave.

SEMI SOFT TRYPTONE AGAR - 5 g tryptone, 2.5 g NaCl, 7 g agar ~ pH 7.2 per liter (final volume) distilled water. Autoclave.

1 M Potassium Phosphate Buffer (pH 7.4 and / pH 8.2)

A)  $K_2HPO_4$  ..... 87.1 g

Distilled Water ..... to 500 ml

B)  $KH_2PO_4$  ..... 68.05 g

Distilled Water ..... to 500 ml

Add B to A until desired pH is reached.

(approximately 36 ml of "B" to 100 ml of "A" for pH 7.4 - 7.5)

(approximately 8 ml of "B" to 100 ml of "A" for pH 8.2 )

Chemotaxis Buffer (10 mM potassium phosphate buffer, pH 7.0 + 0.1 mM EDTA.

$K_2HPO_4$  ..... 3.5 g

Distilled Water ..... to final volume of 2000 ml

$KH_2PO_4$  ..... 2.7 g

Distilled Water ..... to a final volume of 2000 ml

Add the monopotassium to a volume of the dipotassium phosphate until the pH is 7.0. Add 2 ml of 0.5 M EDTA (pH 8.0) per liter of buffer.

## EXAMPLE OF MEDIA PREPARATION PROTOCOL

### 500 ml M9 Medium with glucose + proline + thiamine

1. **Prepare salts solution.** Add ~400 ml double distilled water to a 500 or 1000 ml beaker. Place on a stirrer and put a stirring bar in the water. Start stirring. Slowly add 5.3 g  $K_2HPO_4$ . Stir until dissolved. Slowly add 2.3 g  $KH_2PO_4$ . Stir until dissolved. Slowly add 0.5 g  $(NH_4)_2SO_4$ . Stir until dissolved. Slowly add 0.25 g sodium citrate.  $2H_2O$ . Stir until dissolved. pH should be ~7.0. Check and adjust with acid or base if necessary. Bring volume to 490 ml with distilled water.
2. Prepare **20% glucose stock solution.** The glucose solution must be prepared separately. Put ~60 ml double distilled water in a beaker on a stirrer. Add stirrer and start stirring. Slowly add 20 g sucrose - make sure no clumps form. Stir until dissolved. Bring volume to 100 ml. Pour into a milk dilution bottle. If any carbohydrate is added to salt solution (phosphates present) and subsequently autoclaved, the carbohydrate will caramelize. The carbohydrate that has caramelized (phosphates interact with the carbohydrate upon heating) is no longer available as a carbon source for the microorganism. Prepare **1% thiamine stock solution** and **0.2% proline stock** solution in a similar manner.
3. Autoclave all media solutions.
5. When solutions have cooled to below 50°C, add 5 ml glucose solution, 5 ml proline solution and 250  $\mu$ l thiamine stock solution to salt solution.

## SAMPLE CALCULATION

What volume of 95% ethanol should be added to 5 ml culture to obtain a final concentration of 4% (v/v)?

v = volume

You must consider the volume you are adding in addition to the volume of the culture.

$$C_1(V_1 + V_2) = C_2V_2$$

$$\text{eg. } 4\%(5 \text{ ml} + V_2) = 95\%V_2$$

$$V_2 = .22 \text{ ml}$$

$C_1$  is the final concentration of the solution

$V_1$  is the solution initial volume

$C_2$  is the concentration of the solution you are adding

$V_2$  is the volume of the solution added

## SOLUTION FUNCTIONS

### Lab 2 Kinetics of Threonine Deaminase from *Escherichia coli*

Crude cell extract preparation - enzyme extracts suspended in potassium phosphate buffer, pH 7.4 and  $\beta$ -mercaptoethanol.

Potassium phosphate buffer, pH 7.4 Buffers at pH where the enzyme is stable, plus optimum ionic strength of stability of enzyme.  $\beta$ -mercaptoethanol - reducing agent.

$\beta$ -mercaptoethanol. Whenever working with cell components (in vitro) must have a reducing agent in low concentration to prevent degradation of the enzyme by oxygen present in the air. At low concentration the reducing agent does not break disulfide bonds.

pyridoxal phosphate - cofactor for the biosynthetic threonine deaminase

TCA (trichloroacetic acid) - used to stop threonine deaminase enzyme reaction. Precipitates protein (enzyme), therefore stops reaction.

Ammonium chloride - prevents the dissociation of biosynthetic threonine deaminase at pH 8.0. (could also use KCl).

**SAMPLE CALCULATION of the number of bacteria per ml**

Data for example calculations using the following sample data

Dilution plated	Number of colonies	
	Plate 1	Plate 2
10 <sup>-2</sup>	TNTC	TNTC
10 <sup>-3</sup>	320	316
10 <sup>-4</sup>	34	27
10 <sup>-5</sup>	2	3

TNTC = too numerous to count

Terms

Plating factor = reciprocal of volume plated

Dilution factor = reciprocal of dilution for significant counts

Significant plate counts = the sum of the plate counts at significant dilution divided by number of plates. Often more than one dilution has significant plate counts. It is important to use all significant plate count data. There are several ways to deal with data that has more than one significant plate count dilution.

Number of plates = number of significant plates

Calculation

Do not average an average value as it incorporates error in your calculation (not statistically accurate).

Use one of the following methods to calculate bacteria titre.

Bring all significant counts to the same dilution:

$$\text{Bacteria/ml} = \frac{\text{significant plate counts}}{\text{number of plates}} \times \text{dilution factor} \times \text{plating factor}$$

$(320 + 316 + 340)/3 \times 1/10^{-3} \times 1/10^{-1} = 3.25 \times 10^6$  bacteria/ml, since the smallest number of significant figures for plate counts is two, the answer is  $3.3 \times 10^6$  bacteria/ml

Or calculate the titre for each significant plate count and average.

$$\text{Bacteria/ml} = \text{significant plate count} \times \text{dilution factor} \times \text{plating factor}$$

$$320 \times 1/10^{-3} \times 1/10^{-1} = 3.20 \times 10^6 \text{ bacteria/ml}$$

$$316 \times 1/10^{-3} \times 1/10^{-1} = 3.16 \times 10^6 \text{ bacteria/ml}$$

$$34 \times 1/10^{-4} \times 1/10^{-1} = 3.4 \times 10^6 \text{ bacteria/ml}$$

Average all values:  $(3.20 \times 10^6 + 3.16 \times 10^6 + 3.4 \times 10^6)/3 = 3.25 \times 10^6$  bacteria/ml, since the smallest number of significant figures for plate counts is two, the answer is  $3.3 \times 10^6$  bacteria/ml

If the titre is used for further calculations keep the decimal value until the final value is obtained.

Then round to the appropriate decimal place.

## **AUTOMATIC COLONY COUNTER**

There are several makes of automatic colony counters in this department. All automatic colony counters work on the same principle. The counter registers a count every time you touch the colony with the counter probe as long as the L-shaped probe is inserted into the agar at the edge of the plate. This completes the electrical circuit through the agar from the L-shaped probe to the counter probe (needle shaped probe) touching the colony.

### **Operation**

1. Push or flip the power switch to turn on counter.
2. Press the button on the counter that resets the counter to zero.
3. Place agar culture plate on counter and remove cover.
4. Insert L-shaped probe into the agar at the edge of the plate.
5. Count colonies by touching each colony with the counter probe tip (needle shaped probe).
6. Remove plate, replace lid.
7. Remember to turn off power switch when you are finished counting.

### Notes:

- (i) Use a marker to divide the plate into sections or use the grid on the automatic colony counter to facilitate counting.
- (ii) The counter also comes with a magnifying glass but it is not required unless you are counting very small closely spaced colonies.

## SPECTRONIC 20D OPERATION

The spectronic 20D is a single beam spectrophotometer. The wavelength range is 340 nm to 600 nm with a nominal spectral slit width of 20 nm that is constant over the wavelength range. The wavelength accuracy is 2.5 nm. The absorbance range is 0 to 1.999 A. The spectronic 20D is supplied with ½ inch test tubes.

### SAMPLE MEASUREMENT: Absorbance

1. Remove dust cover. Turn on **Power Switch** clockwise. Allow the spectrophotometer to warm up for 15 min.
2. Set the required wavelength with the **Wavelength Control Knob**.
3. Set the display mode to TRANSMISSION by pressing the **MODE CONTROL KEY** until the LED beside TRANSMISSION is lit.
4. The sample compartment should be empty and closed. Adjust the display to 0.0%T with the **Zero Control Knob** (same as power switch).
5. Fill a spec 20D ½ inch test tube with blank solution. The tube should be at least ½ full. Wipe the test tube with tissue to ensure no liquid drops, dust or fingerprints. Place the test tube in **Sample Compartment** and align the guide mark on the test tube with the guide mark at the front of the sample compartment. Press test tube firmly into sample compartment and close lid.
6. Press the **MODE CONTROL KEY** until the LED beside ABSORBANCE is lit. Adjust the display to 0.0% A with the **Transmission/Absorbance Control Knob**. Remove the test tube from the sample compartment.
7. Put test tube containing sample(s) in **Sample Compartment** and close lid. Read absorbance directly from display.
8. When all measurements are complete, turn off the spec 20D and replace dust cover. Thoroughly rinse all spec 20D test tubes with distilled water, and place test tubes in spec 20D rack upside down.

### COMMENTS

1. Keep all solutions free of bubbles.
2. The display must be reset to 100%T or 0.0A every time the wavelength is changed.

## PIPETMAN OPERATION

(Excerpted from Gilson pipetman operation manual.)

In your lab, you have available three different pipetmen depending on the lab. If you look at the top of the plunger it states the size of the pipetman.

P20 measures accurately from 2  $\mu\text{l}$  to 20  $\mu\text{l}$ .

P200 measures accurately from 20  $\mu\text{l}$  to 200  $\mu\text{l}$ .

P1000 measures accurately from 100  $\mu\text{l}$  to 1000  $\mu\text{l}$ .

Never turn the pipetman above the maximum volume; 20  $\mu\text{l}$  for P20, 200  $\mu\text{l}$  for P200, and 1000  $\mu\text{l}$  for P1000 as this breaks the pipetman. The scale on the pipettor is read different for each type - refer to Figure 5 for an example of how to read the scale.

1. Setting the volume: The required volume is set on the digital volumeter by turning the knurled adjustment ring (Figure 5, 2-A). The volumeter display is read from top to bottom in  $\mu\text{l}$  for P20 and P200 and ml for P1000 (Figure 5, 2).
2. Place a disposable tip on the shaft of the Pipetman. Press on firmly with a slight twisting motion to ensure an airtight seal. Depress the push-button to the first positive stop (Fig. 5, 3-A). While holding the Pipetman vertical, immerse the tip 2-4 mm into the sample liquid. Release the push-button slowly to draw up the sample (Fig. 5, 3-B). Wait 1 to 2 seconds, then withdraw the tip from the sample.
3. To dispense the sample, place the tip end at a 10-45° angle against the inside wall of the vessel and depress the push-button SMOOTHLY to the first stop (Fig 5, 3-C). Wait 1 to 2 seconds and then depress the push-button completely to expel any residual liquid (Fig. 5, 3-D). With the push-button fully depressed, carefully withdraw the Pipetman, sliding the tip along the inside wall of the tube. Release the push-button. Remove the used tip by depressing the tip ejector button (Figure 5, 1-F).

**pipetman diagram**

## PURE CULTURE METHODS

In a microbial physiology lab it is essential that pure culture methods be practised. The growth of a microorganism in pure culture means that all other microorganisms be eliminated. As microorganisms are ubiquitous in the environment, obtaining and maintaining a pure culture requires:

- sterilization - elimination of other microorganisms
- aseptic transfer - movement of microorganisms from one place to another without contamination
- isolation - separation of microorganisms being cultured from other microorganisms
- preservation - maintenance of the pure culture

**Sterilization:** All materials that come into contact with the pure culture must be sterilized. A variety of ways are used to sterilize liquids, containers, and instruments: autoclaving (steam at 15 lb/sq. in.) for 15 min, exposure to radiation, and filtration.

**Isolation:** Pure cultures of microorganisms can be isolated by several different methods; streak plate, spread plate, and pour plate. All the methods involve separation of single bacterium on solid (agar) media where it grows into a colony (clone) which is a pure culture of a single microorganism type.

Streak Plate - In the streak plate technique a loopful of bacterial cells is streaked across the surface of nutrient agar plate. This method of streaking established a dilution gradient so that single colonies develop.

Spread Plate - In the spread plate method a drop of microbial suspension (usually 0.1 ml) is placed on the centre of an agar plate and spread evenly over the surface of the agar using a sterile glass rod. Usually appropriate culture dilutions are made to obtain separated single colonies when plated. Refer to spread plate technique section for additional information.

**Preservation:** For short term storage (2-4 weeks) colonies of most bacterial strains can be maintained at 4°C on the surface of agar media if the plates are tightly wrapped. Also liquid cultures can be maintained at 4°C for short term storage.

For medium term storage (1-2 years) most strains of bacteria can be maintained as stab cultures. Such cultures are usually prepared in small screw capped bottles containing 2 to 3 ml of agar medium. First dip a sterile straight wire into a dense liquid bacterial culture, then stab deep into agar medium, and incubate overnight with a loose fitting cap. The cap is then tightened, wrapped with parafilm, and stored in the dark at room temperature or 4°C.

For long term storage, bacteria can be maintained in 15% glycerol (1 ml in a small screw capped vial) at low temperature without significant loss of bacteria. The bacterial culture can be stored at -20°C for a few years or at -70°C for many years.

**FINAL LAB EXAM: Microbiology 60.344 MICROBIAL PHYSIOLOGY****DATE: Sample exam      PAGE: 1 of 2      TIME: 1.5 h****INSTRUCTOR: Dr. L. Cameron****Student Name: \_\_\_\_\_ Student Number: \_\_\_\_\_****WRITE IN PEN ONLY****CONCISELY ANSWER ALL QUESTIONS in spaces provided (spaces removed from sample exam). ANSWERS ACCEPTABLE IN POINT FORM.**

8

1. Briefly answer each of the following questions.

a) Threonine deaminase is present in *E. coli* as two iso-enzymes. State experimental growth conditions required for production of biosynthetic iso-enzyme of threonine deaminase.

b) In the chemotaxis assay, *E. coli* was grown in glycerol or galactose media, suspended in chemotaxis buffer, and 1 mM glycerol added to the capillary served as a control. Explain why glycerol was added to the capillary instead of chemotaxis buffer.

c) What is the mechanism of breaking *E. coli* cells in the French Press?

d) Extremely high voltage (75,000 volts) is required to run the electron microscope. Explain why.

e) Chemotaxis assay using the slide method requires constant attendance by the student while the slide is incubating on the 30°C heating tray. What experimental conditions must be maintained? Explanation must include diagram.

f) What volume of 10 mM H<sub>2</sub>O<sub>2</sub> would you add to a 5 ml *E. coli* culture to give a final concentration of 40 μM H<sub>2</sub>O<sub>2</sub> in the *E. coli* culture?

g) Calculate bacteria titre (bacteria/ml) for the following bacteria plate count data.

dilution	bacteria counts (0.1 ml bacteria plated on each plate)	
	plate 1	plate 2

10 <sup>-3</sup>	457	432
10 <sup>-4</sup>	43	45
10 <sup>-5</sup>	1	4
10 <sup>-6</sup>	0	1

h) How do you prepare 50 ml of 1 mM pyridoxal phosphate? Formula weight of pyridoxal phosphate = 247.

CONTINUED ON PAGE 2...

**FINAL LAB EXAM: Microbiology 60.344 MICROBIAL PHYSIOLOGY****DATE: Sample exam      PAGE: 2 of 2      TIME: 1.5 h****INSTRUCTOR: Dr. L. Cameron**

- 6      2. The following reagents are used in experiments or demonstrations in your physiology lab. What is the function of each reagent?
- a) 5 mM leucine in *E. coli* motility and chemotaxis
  - b) pyridoxal phosphate in threonine deaminase assay
  - c) 50% TCA in threonine deaminase determination
  - d) 5 mM  $\beta$ -mercaptoethanol added during preparation of crude threonine deaminase extract
  - e) NADH in threonine deaminase determination
  - f) 0.025% 2,4-dinitrophenylhydrazine in 0.5 M HCl in threonine deaminase determination
- 4      3. *E. coli* strain, UM242 Thy<sup>-</sup>, is the bacterial strain that is used in your physiology lab, control of cell division. Explain why with reference to the object and data analysis of the experiment.
- 3      4. State all experimental data that is required to calculate the D-phase of *E. coli*. Relate data to D-phase formula calculation.
- 1      5. a) Schematically graph data to demonstrate heat shock stress in *E. coli* for the following conditions; untreated cells, cells pretreated with H<sub>2</sub>O<sub>2</sub>, ethanol, and 42°C, and cells pretreated with 42°C plus 100  $\mu$ g/ml chloramphenicol. Graph must be completely labelled and figure legend included.
- 2      b) Explain graph results at the physiological level.
- 2      6. a) Schematically present expected experimental data on one Lineweaver-Burk plot for biosynthetic threonine deaminase kinetics. Assume experiment was similar to that stated in lab manual. The effector is present in varying concentrations; 10 mM, 1 mM, 0.5 mM, and 0.1 mM effector.
- 2      b) For the colorimetric threonine deaminase enzyme assay, state data analysis required prior to preparing a Lineweaver-Burke graph.

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 28 total

- END -