Molecular Microbiology Laboratory

A Writing-Intensive Course

Walt Ream
Bruce Geller
Janine Trempy
Katharine Field

MOLECULAR MICROBIOLOGY LABORATORY

A WRITING-INTENSIVE COURSE

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Introduction

I. WRITING-INTENSIVE COURSE

A. Goals

This 10-week course is designed to teach undergraduate students molecular biology techniques commonly used in the life sciences and to develop the students' scientific writing skills.

B. Means

The course contains four units that introduce procedures most life scientists will encounter during their careers. In the first unit, students prepare plasmid DNA, construct a restriction map of the plasmid, and transform it into *Escherichia coli*. The plasmid contains a luciferase reporter gene, which introduces the concept of reporter genes through firsthand experience. In the second unit, students express, purify, and analyze an affinity-tagged protein. The third unit requires intellectual input from students, who will isolate bacteria from environments that they choose. Each student will select one unknown bacterium to culture, examine by light microscopy, and identify by DNA sequence analysis. During this experiment students learn to isolate genomic DNA, perform a polymerase chain reaction (PCR), purify PCR products, and analyze DNA sequence data. The fourth unit teaches students to perform Southern blots and to prepare hybridization probes. The methods students use in this course are basic techniques that introduce the fundamental principles of molecular biology.

This is also a writing-intensive course. The manual contains a general discussion of scientific writing and critical reading, and it includes detailed instructions for preparation and peer review of lab reports. Additional writing exercises based upon journal articles accompany each experimental unit. The studies in these articles

employ the techniques used in the laboratory exercises. By evaluating these papers, students reinforce their understanding of the technology. Students see how diverse authors report their findings and how formats differ from one journal to another. They also discover that all scientific papers share several essential components. Lectures based on the book "How to Write and Publish a Scientific Paper," by Robert Day, discuss each section of a scientific paper in detail. To improve their copyediting skills, students read and discuss "Line by Line," an outstanding manual written by a copyeditor, Claire Kehrwald Cook. Thus, to build their writing skills and enhance their understanding of molecular microbiology, students compose and revise lab reports, edit their peers' reports, critique journal articles, and study writing manuals.

II. SCHEDULE

Day	Laboratory	Lecture	In-class writing	Hand in	Read
1		Introduction; how to write lab reports and proposals			Manual 1–43; Day Ch. 1–10
2	Purify plasmid; restriction	Restriction enzymes and mapping	Rewrite sentences	Flow Chart 1	Kragelund *et al.*, 1997
3	Agarose gel; transform	Transformation; reporter genes	Restriction mapping problems		Day Ch. 13–15 and 32–35; Day Append. 3–4
3+1	Examine plates				
4		Affinity-tagged protein purification	Peer review Report 1, critique Kragelund *et al.*, 1997	Report 1 draft	Bush *et al.*, 1991
5	Lyse cells; bind Ni resin	Lysozyme		Lab Report 1, Flow chart 2	Cook Ch. 1
6	SDS–PAGE	SDS–PAGE			Cook Ch. 2
7		How to read a journal article	Peer review Report 2, critique Bush *et al.*, 1991	Report 2 draft	Cook Ch. 3
8	Isolate bacteria		Peer review proposal	Proposal draft	Cook Ch. 4
8+1	Examine plates and streak				

9	Gram stain; microscopy; inoculate broth	PCR; rRNA-based phylogeny	Describe colonies	Lab Report 2	Cook Ch. 5
10	Prepare genomic DNA; freeze cultures	DNA purification		Flow Chart 3	Borneman and Triplett, 1997
11	PCR	Primer stock preparation	Write abstract for Borneman and Triplett, 1997		Rappé *et al.*, 1998
12	Purify PCR product	DNA sequencing; using GenBank		Proposal	*Nature* Editorial, 1996
13	Agarose gel and template preparation	Review	Sample problems		Review questions
14			Test		
15	Restriction; agarose gel	Southern blots; probes	Edit sequences	Flow Chart 4	Salyers, 1996
16	Blot gel; prepare probe		Peer review Report 3	Report 3 draft	Bengtsson, 1997
17	Hybridization		Peer review Editorial	Lab Report 3, Editorial draft	
18	Wash and develop blots		Discuss GMO (genetically modified organism) papers	Editorial	
19		Engineered crops	Peer review Report 4	Report 4 draft	
20		Summary		Lab report 4	

III. ATTENDANCE AND GRADING POLICIES

Attendance is **mandatory**. Each unexcused absence will result in a 5% deduction from your final grade. More than two absences will result in an Incomplete. Arrival more than 15 minutes late will count as half an absence.

Requests for an excused absence will be considered on a case-by-case basis, but exercises cannot be rescheduled. Students with an excused absence must complete all missed assignments.

A. Grading

Final Grade

A/A–	=	90–100% of top score
B+/B/B–	=	80–89%
C+/C/C–	=	65–79%
D	=	50–65%
F	=	below 50%

Lab reports	=	20% each × 4 = 80%
Test	=	20%

IV. LABORATORY RULES

You must prepare a flow chart prior to each experiment. You may not begin an experiment without a completed flow chart, which is due at the start of class. Feel free to ask questions when you do not understand the instructions or the principles involved.

You must have a rubber pipette bulb, a lab coat, and safety glasses. **Lab coats and protective eye wear are REQUIRED for the experiments that use phenol.** Please do not wear shorts or sandals because phenol causes severe chemical burns when it contacts skin; wash with water to remove phenol.

Assume that all bacteria you use may cause disease. Observe the following safety rules at all times:

1. **Do not pipette by mouth.**
2. **Wear a laboratory coat and safety glasses.**
3. **Do not eat, drink, or chew gum in the laboratory.**
4. **Disinfect your bench surface before and after you work.**
5. **Insert pipette into the rubber bulb gently** to avoid breaking the pipette, which could cut your hand.
6. Disinfect contaminated equipment and surfaces.
7. Place used liquid cultures, supernatants, and glassware in autoclave containers. Discard contaminated plates and plasticware (tips and tubes) in autoclave bags. Discard organic solvents (phenol and chloroform) in waste containers.
8. **Wash your hands after you finish working.**

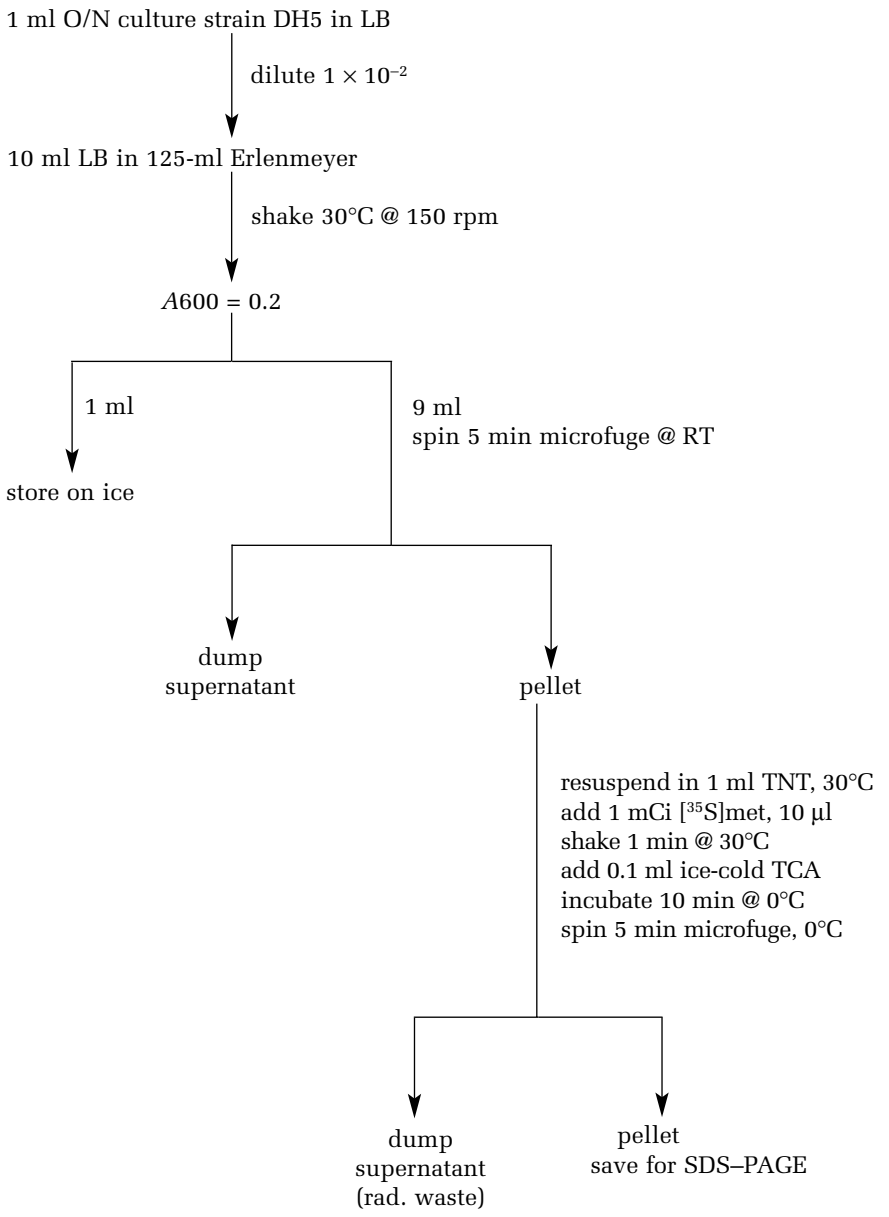
V. FLOW CHARTS

Prepare a flow chart in ink (not pencil) prior to each experiment and include it in your lab report. You may not participate in the laboratory exercise without a flow chart.

A flow chart outlines each procedure step by step and guides you through the experiment. If you modify a procedure during the course of an experiment, note these changes on the flow chart. Record observations on a separate page as you work.

Flow charts contain words, symbols, diagrams, and arrows. Begin your flow chart by listing the first step of the procedure. Use an arrow to connect the first step to the second, and so forth. The arrows indicate major procedural steps and direct your attention to the next task. The steps taken to proceed from one intermediate to the next are listed beside each arrow. A sample flow chart appears on the next page. Can you understand the experiment by reading the flow chart?

A. Sample Flow Chart



VI. PREPARING A LABORATORY REPORT

1. You must prepare a lab report for each experiment. You will need a pocket folder to turn in your lab reports. Due dates for rough drafts and finished lab reports are in the schedule.
2. In class, record data directly in a loose-leaf notebook using a **pen**, not a pencil. You will include these pages with your lab report.
3. Use a word processor to type your lab report. All material in the report must be typed, except for the flow chart and observations (data) recorded during class. **Word processors all have spell check tools.** There is no excuse for spelling errors that your word processor can detect. Some typographical errors, such as "form" in place of "from," can evade detection by your computer, so proofreading is also necessary.
4. Lab reports must include each of the following sections:
   a. **Name:** Your name.
   b. **Title:** Name the experiment. The title should be concise and specific. Tell the reader what you did.
   c. **Date:** List the dates the experiment was performed.
   d. **Purpose:** State the purpose of the experiment in one or two sentences. Be specific.
   e. **Methods:** List the procedures used, and reference the appropriate lab manual pages. Do not copy the methods from the lab manual; instead, note modifications to the procedure. Include complete mathematical calculations. Put the flow chart at the end of this section.
   f. **Results:** Introduce this section with one or two sentences that describe the experiment. Follow with a complete description of what you observed.

Present the observations you made during the experiment, not what you think should have happened. Do not repeat the methods section. Include raw data collected during the experiment at the end of this section. Never transcribe raw data.

Include graphs, tables, photographs, and DNA sequencing electropherograms in this section. Each figure and table must have a title, a number, and a legend that contains all information needed to interpret the data. Specify units on the abscissa and ordinate of graphs. Label columns and rows in tables. Number each lane on photographs used to document gel electrophoresis data, and indicate the contents of each lane in the figure legend. Place each figure or table immediately after the paragraph in which it is first cited or on the page following its first mention.

g. **Discussion:** Offer your interpretation of the data presented in the results section. Begin with a brief introduction that makes the purpose of the experiment clear. Next discuss the meaning of your observations. If you can interpret your data in more than one way, mention all the possibilities and indicate which alternative you think is correct. Discuss all your results, even if they are unexpected or negative. For example, you should explain the presence of unexpected bands on SDS–PAGE and agarose gels. If the experiment did not work, indicate what went wrong.

h. **Conclusions:** Summarize the meaning of *your* results in two or three sentences.

i. **References:** List references you used to prepare your lab report.

j. **Questions:** Include the answers at the end of the report. Show your calculations for mathematical questions.

VII. HOW TO EVALUATE LABORATORY REPORTS

Two of your classmates will review the first draft of your lab report before you submit it for a grade. This will give you an opportunity to improve it before you complete the final version. You will review two of your peers' draft reports for each experiment. The next pages show a sample of the peer review form that you will use. Copies of this form are included at the end of the manual. These guidelines will also help you write and revise your own lab reports.

Evaluate each section of the report separately beginning with the title. First consider overall issues: Does the section contain the appropriate material? Did the author omit information that belongs in this section, or does it contain material that belongs in another section? For example, did the author include discussion or methods in the results section? Is the writing organized logically? Do you understand what the author is trying to say? Do not correct spelling, punctuation, word choice, or sentence structure until you have addressed these global issues.

Each paragraph should focus on one idea and begin with a sentence that describes the content of the paragraph. As you read, suggest paragraph revisions by marking in the text or margins. Do not write the revision yourself; that is the author's responsibility. Indicate statements that are incorrect or unclear. Show the author where you found poor paragraph organization.

Next, edit for errors in spelling, punctuation, word choice, and sentence structure. If you are not sure whether a sentence is correct, ask an instructor or circle the questionable item and indicate the potential problem. Several of the books we recommend to students in this course can help you identify and correct poorly written sentences: "How to Write and Publish a Scientific Paper" and "Scientific English" by Robert A. Day, "Line by Line"

by Claire Kehrwald Cook, and "Elements of Style" by William Strunk and E. B. White.

Use the following checklist to evaluate a rough draft, including your own. Eight copies of the peer evaluation checklist (two for each lab report) are included in the Appendix. Indicate your suggestions on the checklist. Your evaluation matters, so please do it conscientiously. Give the completed checklist and edited draft to the author. **Submit the checklists and first draft with the final version of the lab report.**

A. Peer Review Checklist

Reviewer_____ Experiment no._____

Author_____ Date_____

1. Throughout the report, look for awkward sentence construction, poorly organized paragraphs, incorrect grammar, and misspelled words. Remember, even grammatically correct sentences can be awkward and difficult to read. Sentences should be simple and straightforward. Use active voice instead of passive voice, and eliminate redundant words. Remove vague, qualitative adjectives such as "large" or "small" and replace them with numbers. For example, "a 10-fold increase" is much more informative than "a large increase." Make certain new terms are explained clearly, and clarify statements that are ambiguous.
2. Check that the name, title, and date are present and legible.
3. Is the purpose stated clearly and concisely? Is the statement complete and accurate?
4. Are the methods described completely and references cited properly? Does the methods section clearly and concisely describe modifications made to the referenced procedure?
5. Check the flow chart, and ensure it was not retyped after the experiment was completed.
6. The results section should begin with a description of the work performed. After reading the results section, a scientist who has not read the methods section should understand what you did. However, it should not include all the details normally found in the methods section. Strike a balance between too little and too much information by describing the procedures well enough to understand the experiment, but do not include all the details required to repeat the experiment. Instead, detailed information belongs in

the methods section. For example, do not describe the composition of buffers or media in the results. However, the methods section should contain this information or reference a publication that does.

7. The order in which the results are presented should lead the reader through the experiment. Is the organization of the data logical? Is the presentation clear? Would changing the organization make the report easier to understand?
8. Are the figures and tables cited in the text, and do they have appropriate titles and legends? Is each lane of a gel photograph labeled, and does the figure legend list the contents of each lane?
9. Does the text accurately describe the figures? The data must support statements made in the text.
10. The discussion should begin with a brief introduction that makes the purpose of the experiment clear. This section should flow logically from an introduction through the results to a sound conclusion. The discussion may include background material that puts the need for the experiments in perspective. A good discussion does not merely reiterate information presented in the results section. After a brief recap of the question asked and the work done to answer it, the author should state the meaning of the data and indicate how the findings affect our current understanding of the field. In other words, what was the answer to the question, and what does that answer mean? Does the study establish new general principles? Is there more than one way to interpret the data? If so, the discussion should present both possibilities and suggest which the author believes is correct. Did the experiment produce unexpected results such as "extra" bands on a gel? The discussion must **not** ignore results that do not fit the author's expectations; instead, the discussion should contain a plausible explanation of such data. Make certain problems with the data are discussed.

11. The conclusion should summarize the experiment in two or three sentences. The data must support the conclusions.

12. What is best about this writing?

13. What needs to be improved the most?

B. Criteria for Grading Laboratory Reports

Section	Grade		
	A	**B**	**C**
Name	Legible		Illegible
Title	Descriptive Brief	Wordy	Vague
Date	Correct day/month/year		Incomplete
Purpose	Clear, concise statement of goal or hypothesis.	Wordy	Vague
Methods	Clear, succinct step by step. References correct. Changes noted.	Vague Wordy	Incorrect Incomplete
Results	Well organized. Describes experiments and results clearly. Includes primary data and careful analysis. Data labeled with correct units. Gel lanes labeled; legend lists contents. Prose clear and elegant. Active voice. Figures look neat.	Complete but difficult to understand. Organization confusing. Contains awkward sentences. Passive voice. Figures look sloppy.	Incorrect Incomplete Poor grammar and spelling. Unorganized. Figures not labeled. No legends.
Discussion	Concise and meaningful interpretation of results. Considers extant theories. Reasonable alternative explanations. Cites published papers.	Superficial. Theory not integrated with results. Importance unclear.	Incorrect Incomplete
Conclusion	Correct and concise.	Wordy	Vague or incorrect.
References	Did outside reading. Complete; correct format (J. Bact.).	Wrong format. Incomplete Irrelevant	None cited.
Questions	Correct and clearly written answers.	Answers vague or incomplete.	Incorrect

C. Checklist for Grading Laboratory Reports

This is the grading sheet that the instructors and teaching assistants will use to grade your lab reports. Appendix C contains four grading checklists; hand in one with each report.

Student's name:

Points awarded: A = 9–10, B = 8–9, C = 6.5–8, D = 5–6.5, F = 0–5.

Section/items scored	Letter grade	Points	Maximum score
Name, Title, Date			
In place, legible, correct, title informative and brief	——	——	10
Purpose			
Clear and concise	——	——	10
Correct	——	——	10
Grammar and spelling	——	——	10
Sentence structure	——	——	10
Methods			
Clear and concise	——	——	10
Correct	——	——	10
Complete, proper references	——	——	10
Grammar and spelling	——	——	10
Sentence structure	——	——	10
Flow chart	——	——	10
Results			
Overall organization	——	——	10
Paragraph structure	——	——	10
Sentence structure	——	——	10
Clear and concise	——	——	10
Grammar and spelling	——	——	10
Accurate	——	——	10
Thorough	——	——	10
Figures correctly labeled and neat	——	——	10
Results, not methods or discussion	——	——	10
Discussion			
Overall organization	——	——	10
Paragraph structure	——	——	10
Sentence structure	——	——	10
Clear and concise	——	——	10
Grammar and spelling	——	——	10
Logical, supported by data	——	——	10
Thorough	——	——	10
Integrates theory with results	——	——	10
Explains unexpected results	——	——	10
Conclusion			
Sentence structure	——	——	10
Clear and concise	——	——	10
Grammar and spelling	——	——	10
Logical, supported by data	——	——	10
Questions	——	——	70
Grand Total	——	——	400

VIII. HOW TO READ A JOURNAL ARTICLE

A. Organization

Title, Authors, Abstract, Introduction, Methods, Results, and Discussion

B. Order in Which to Read an Article

a. Title

b. Authors

c. Abstract

d. Introduction

e. Results

f. Discussion

g. Methods

The most important section of the paper is the results! In the abstract, introduction, and discussion, you are reading the authors' interpretations. In the results, you can assess what they found. *Your* interpretation of the data and its meaning may differ from the authors'. If the title catches your eye and the abstract piques your interest, read the introduction next. This section will provide sufficient background for you to understand the rationale behind the experiment. Skip the methods section and continue by reading the results. Make certain that the text accurately describes the data in the figures and tables. Sometimes the data do not support statements made in the text. You should never assume that the text is correct. As you read the results, decide for yourself what the results mean. In the discussion, the authors will try to convince you of the meaning and significance of their data. You must weigh their arguments and decide whether you agree.

C. Information Contained in Each Section

1. Title

The title describes the contents of the article using as few words as possible. The title is important for several reasons. It is the first thing that readers see, and it helps them decide whether to read the article. A good title will help readers find articles relevant to their interests because literature databases use key words from the title to list papers.

2. Authors

Over time you will recognize authors doing important work in your field. Literature searches based on their names will ensure that you see everything they publish.

3. Abstract

The abstract is a condensed version of the article. It presents the rationale for the study, reports the key results, and points out their significance. The abstract will let you decide whether you want to read the entire paper, and it provides an overview that will make the article easier to understand. Literature databases often supply abstracts on-line.

4. Introduction

The introduction supplies background information and puts the paper into perspective. It gives the rationale for the work and may include formal hypotheses. At the end of this section, the authors will *briefly* state what they did, what they observed, and what they concluded.

5. Results

This section is the meat of a paper. It describes the experiments and the observations the authors made. The

results section presents the data, often as figures, tables, and graphs. The authors do not discuss the meaning of their findings here. Use past tense.

6. Discussion

The authors summarize and interpret their data in the discussion section. They will show how their observations relate to each other to form a cohesive story. The authors will discuss how their data support (or contradict) current theories and extend previously published observations. The discussion will provide plausible explanations for unexpected results that contradict accepted theories. Often, discussion sections end by stating the significance of the work.

In the discussion section, the authors use the past tense to describe data from the results section and the present tense to cite facts established by previously published work.

7. Methods

The methods section describes in detail the procedures used. Read this section to learn about techniques that are unfamiliar to you. Study the methods section carefully if you intend to conduct similar experiments, or to better understand possible limitations of the experiments.

IX. REQUIRED AND SUGGESTED READINGS

A. Required Writing Manuals

Day, Robert A. (1998). "How to Write and Publish a Scientific Paper," 5th ed. Phoenix, AZ: Oryx Press. ISBN 1-57356-164-9.

This book is required reading for all students in this class. Day provides step-by-step instructions for writing a scientific paper, and he discusses common errors that occur in scientific writing. Day's style is lively, making this book a pleasure to read.

Cook, Claire Kehrwald (1985). "Line by Line: How to Improve Your Own Writing." Boston: Houghton Mifflin Co. ISBN 0-395-39391-4.

Strunk, William, Jr., and White, E. B. (1979). "The Elements of Style," 3rd ed. New York: Macmillan Publishing Co.

Although neither book addresses scientific writing in particular, we recommend these two books to students who want to improve their writing skills. Generations of writers have studied "Strunk and White," which has become a classic. It is short and packed with good advice. "Line by Line" is filled with examples of poorly written sentences together with improved versions. Cook illustrates a number of common mistakes and shows how a professional copy editor would fix them. Most students will benefit from reading this book.

B. Highly Recommended Resources for Writers

Day, Robert A. (1992). "Scientific English: A Guide for Scientists and Other Professionals." Phoenix, AZ: Oryx Press. ISBN 0-89774-722-4.

"Scientific English" is an excellent companion for "How to Write and Publish a Scientific Paper." In this book, Day discusses parts of speech, grammar, phrases, clauses, sentences, and paragraphs. It is not as tedious as it sounds due to Day's straightforward, readable prose. Most students will benefit from reading this book.

Kanare, Howard M. (1985). "Writing the Laboratory Notebook." Washington, DC: American Chemical Society. ISBN 0-8412-0906-5.

Kanare discusses how and why to write and organize lab notebooks. He includes a chapter on patent protection and a photograph of the notebook pages where Alexander Fleming recorded the discovery of penicillin.

Reif-Lehrer, Liane (1982). "Writing a Successful Grant Application." Boston: Science Books International, Inc. ISBN 0-86720-007-3.

This book is indispensable for those students who intend to embark on a career that will require them to write grant proposals. Grant writing differs from other types of scientific writing; this book will help improve your chances of success.

Three additional tools belong on the desk of any writer.

1. Webster's Dictionary.
2. Roget's Thesaurus. "A Thesaurus is the opposite of a dictionary. You turn to it when you have the meaning already but don't yet have the word." I. A. Richards
3. Burchfield, R. W., Ed. (1996). "The New Fowler's Modern English Usage," 3rd ed. Oxford, UK: Oxford University Press. ISBN 0-19-869126-2.

This comprehensive guide to the proper use of words is replete with examples. Do you want to know when you may end a sentence with a preposition? This book will tell you.

C. Required Journal Articles

1. Experiment 1

Kragelund, L., Hosbond, C., and Nybroe, O. (1997). Distribution of Metabolic Activity and Phosphate Starvation Response of *lux*-Tagged *Pseudomonas fluorescens* Reporter Bacteria in the Barley Rhizosphere. *Appl. Environ. Microbiol.* **63:**4920–4928 (www.journals.asm.org).

2. Experiment 2

Bush, G. L., Tassin, A.-M., Friden, H., and Meyer, D. I. (1991). Secretion in Yeast: Purification and *in vitro* Translocation of Chemical Amounts of Prepro-α-Factor. *J. Biol. Chem.* **266:**3811–3814.

3. Experiment 3

Borneman, J., and Triplett, E. W. (1997). Molecular Microbial Diversity in Soils from Eastern Amazonia: Evidence for Unusual Microorganisms and Microbial Population Shifts Associated with Deforestation. *Appl. Environ. Microbiol.* **63:**2647–2653 (www.journals.asm.org).

D. Suggested Background Reading

1. Experiment 1

Engebrecht, J., Nealson, K., and Silverman, M. (1983). Bacterial Bioluminescence: Isolation and Genetic Analysis of Functions from *Vibrio fischeri. Cell* **32:**773–781.

2. Experiment 3

Rappé, M. S., Suzuki, M. T., Vergin, K. L., and Giovannoni, S. J. (1998). Phylogenetic Diversity of Ultraplankton Plastid Small-Subunit rRNA Genes Recovered in Environmental Nucleic Acid Samples from the Pacific and Atlantic Coasts of the United States. *Appl. Environ. Microbiol.* **64:**294–303 (www.journals.asm.org).

E. Required Editorials

Editorial (1996). Distrust in Genetically Altered Foods. *Nature* **383:**559.

Salyers, A. (1996). The Real Threat from Antibiotics. *Nature* **384:**304.

Bengtsson, B. O. (1997). Pros and Cons of Foreign Genes in Crops. *Nature* **385:**290.

Borlaug, N. (2000). We Need Biotech to Feed the World (Editorial). *Wall Street Journal*, December 6, 2000.

X. PHRASES TO AVOID

Here are phrases that cause me to grind my teeth. Because students in previous classes used them frequently, I have listed them here so that you can avoid them in your writing.

Use "is" instead of "is known to be."
Use "were" instead of "were found to be," "were observed to be," or "were determined to be."
Use "was" instead of "was identified as being" or "it was observed that."
Use "may be" instead of "is thought to be."
"This data" is wrong; use "these data" instead.

You can eliminate "It was found that," "The results show that," "In this study," and "The purpose of this study was to examine." If you find these phrases in your writing, simply remove them and begin the sentence with the next word. For example, change "The results show that the *lux* operon was in orientation A" to "The *lux* operon was in orientation A."

Do not use lab jargon or slang. For example, scientists often use the word "spin" for "centrifugation" when they converse in the laboratory; do not use "spin" in your written reports. Similarly, many students use the slang "run on" or "run through" when they describe gel electrophoresis. The phrase "run through" might describe the outcome of a fencing match, but it has no place in a laboratory report. You should also realize that "electrophorese" is not a word; refer to Appendix 3 in Day's book if you do not believe me. I suggest that you "separate restriction fragments by electrophoresis through an agarose gel" rather than "run the cut DNA through an agarose gel."

Three additional mistakes deserve special mention. First, make certain to number (or otherwise label) each lane on the photograph of a gel, and write a figure legend

that includes the figure number, title, and the contents of each lane. Second, italicize genus, species, and gene names. Third, do not use "homology" when you mean "identity" or "similarity." The word "homology" means that two species share a common ancestor. If your data show that the 16S ribosomal RNA genes from two different species are 80% identical, say precisely that ("the genes are 80% identical"). Leave arguments about "homology" to the evolutionary biologists unless the purpose of your research is to establish evolutionary relationships.

XI. PIPETMAN'S CREED

This is my Pipetman. There are many like it, but this one is mine.
My Pipetman is my best friend. It is my life.
I must master it as I must master my life.
Without me, my Pipetman is useless.
Without my Pipetman, I am useless.
I must pipet my DNA true.
I must pipet faster than my competitor,
who is trying to publish before me.
I will.

Acknowledgments

We thank the teaching assistants and students who have suggested improvements to this course, and we are grateful to the Applied Biosystems Corporation for their support of this course through generous donations of PCR reagents. Special thanks to Trena Shanks of dawn design (www.bydawndesign.com), who prepared the figures in this book.

NOTES

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EXPERIMENT
1

Plasmid Purification and Restriction Mapping

I. INTRODUCTION

In this unit, you will isolate a plasmid from *Escherichia coli,* determine its structure by restriction mapping, and transform it into another *E. coli* strain.

II. BACKGROUND

A. Plasmid DNA Preparation

In this course, you will learn to work with plasmids because you will probably use them often in your career. Many methods have been developed to purify plasmid DNA from *E. coli* and other bacteria. These procedures employ a variety of methods to lyse cells and separate plasmid DNA from other cellular components. Detergents, organic solvents, alkali, or heat can lyse bacteria. Three factors dictate which method to use: the size of the plasmid, the bacterial strain, and the technique used to purify plasmid DNA from the lysate.

Large plasmids (>50 kb or kilobases) are easily damaged and must be released from cells by gentle lysis that minimizes osmotic shock. This reduces the shear forces that arise from disruption of the pressurized bacterial cells. More severe methods are used to lyse bacteria containing smaller plasmids, such as the plasmid you will purify in this unit. These procedures usually use ethylenediaminetetraacetic acid (EDTA) to chelate divalent cations and make the outer membrane permeable. Because nucleases require divalent cations (Mg^{2+}) for activity, EDTA in the lysate protects DNA from degradation. Many procedures use lysozyme to hydrolyze the peptidoglycan layer (Fig. 1.1). Addition of a detergent, sodium dodecyl sulfate (SDS), breaks the weakened cells

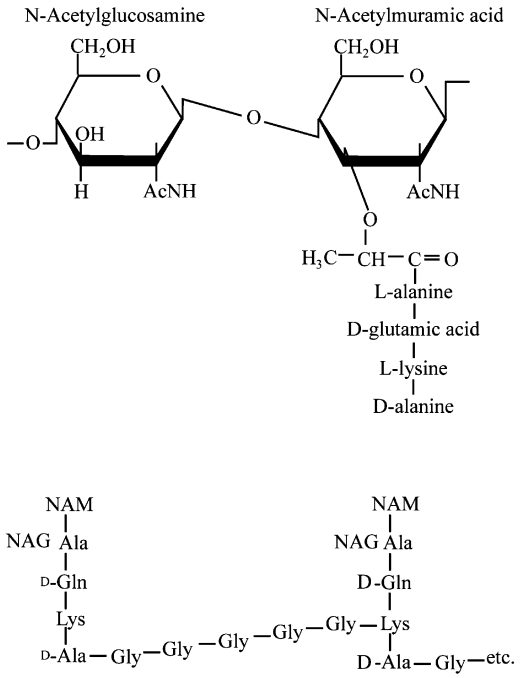


FIGURE 1.1

Peptidoglycan structure.

by dissolving the lipid bilayers. Both circular plasmid DNA and linear chromosomal DNA fragments are denatured by adding alkali (NaOH) or by boiling. When the pH of the lysate is rapidly returned to neutral, linear chromosomal DNA fragments remain denatured, whereas the intertwined strands of covalently closed, circular plasmid DNA "snap back" into duplex DNA molecules. Most of the denatured chromosomal DNA precipitates along with the cellular debris, leaving the plasmid DNA in solution.

In this unit, you will isolate a plasmid, either pKN800-A or pKN800-B, from *E. coli*. These plasmids carry a β-lactamase gene (*bla*) and the luciferase (*lux*) operon from *Vibrio fischeri*; see the article on the suggested reading list by Engebrecht *et al.* (Introduction, Section IX) for a

complete description of these plasmids. The *bla* gene confers ampicillin resistance to cells containing the plasmid. The *lux* operon consists of five structural genes and two regulatory genes, all of which are required for luminescence. Figure 1.2 shows how these plasmids were constructed; the A and B designations indicate different orientations of the *lux* operon relative to the vector plasmid (pBR322). You will create a restriction map of the plasmid you isolate to determine whether it carries *lux* in the A or B orientation.

To isolate one of these plasmids, you will use a standard "miniprep" method that uses EDTA, lysozyme, alkali, and SDS to lyse the cells. After lysis occurs, you will add potassium acetate to neutralize the lysate and precipitate the denatured chromosomal DNA and cellular debris, which you will remove by centrifugation. The supernatant will contain RNA from the cells in addition to plasmid DNA. Because these RNA molecules will obscure small restriction fragments (< 1 kb) on agarose gels, you will add ribonuclease A (RNase A) to eliminate the RNA. Extraction with a mixture of phenol and chloroform will remove RNase and cellular proteins from the DNA preparation. In the presence of salt (potassium acetate), ethanol causes DNA to precipitate from solution, allowing you to concentrate and desalt the DNA. The plasmid DNA will be pure enough to use for restriction mapping, cloning, DNA sequence analysis, or transformation into other strains of *E. coli*.

Restriction endonucleases cut both DNA strands at specific sequences leaving ends that are either staggered or blunt, depending on the enzyme used. Commonly used type II restriction endonucleases cut within palindromic target sequences 4–8 bp (base pairs) long. One strand of a palindromic DNA sequence is identical to its complement when each is read 5′ to 3′. The sequence 5′-GTCGAC-3′ is the palindrome recognized by *Sal*I, the restriction endonuclease used to construct the pKN800 plasmids. *Sal*I, isolated from *Streptomyces albus,* cuts

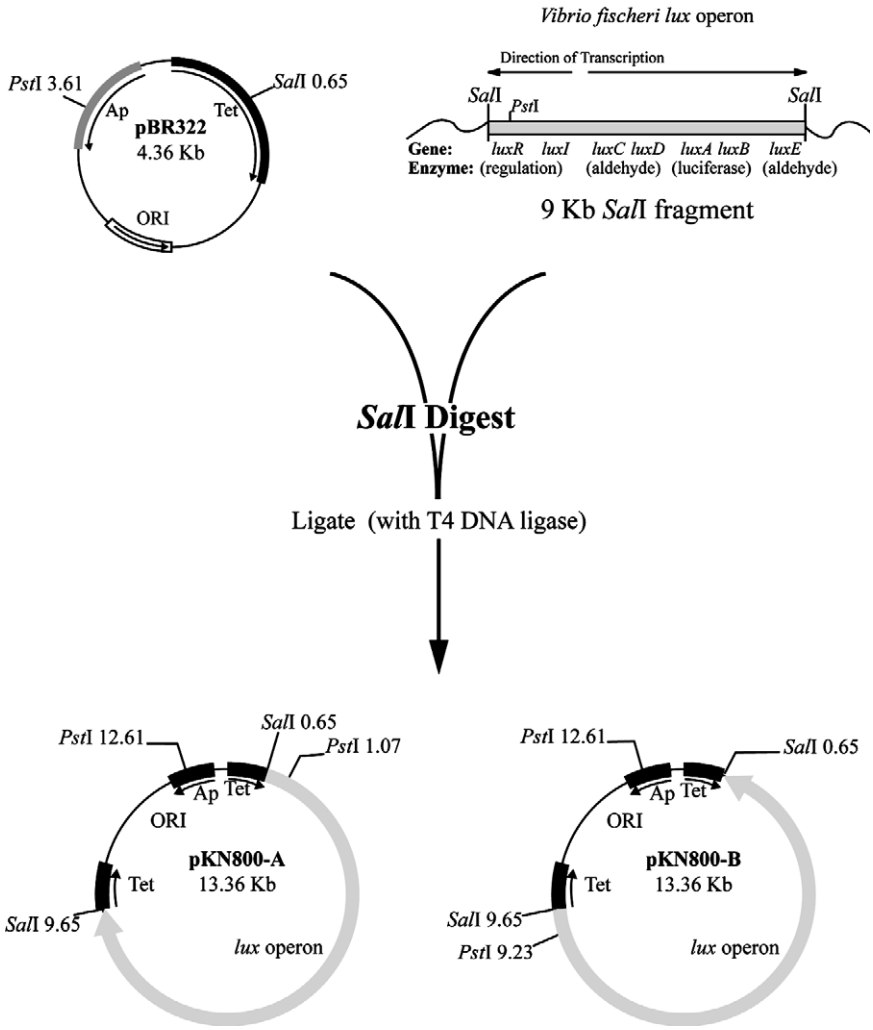


FIGURE 1.2

Construction and structure of pKN800-A and -B.

between the G and T bases on each strand (G^{v}TCGAC), leaving four unpaired bases (TCGA) at the 5′ end of each restriction fragment.

5′––––––GTCGAC––––––3′
3′––––––CAGCTG––––––5′

cut by *Sal*I to give:

5′––––––G TCGAC––––––3′
3′––––––CAGCT G––––––5′

The cohesive or "sticky" single-stranded ends of a *Sal*I restriction fragment can form base pairs with the identical ends of any other DNA fragment produced by *Sal*I cleavage. Once the ends of two restriction fragments form base pairs (*in vitro*), DNA ligase [plus adenosine triphosphate (ATP)] can create covalent phosphodiester bonds that join the fragments. A second ligation event (between the other ends of the two linked fragments) will form a circular DNA molecule, which will remain intact when transformed into *E. coli*. The RecBCD nuclease destroys linear DNAs introduced into *E. coli,* so circle formation is critical for successful cloning into plasmid vectors.

The entire *Vibrio fischeri lux* operon lies within a 9000-bp *Sal*I fragment, which was inserted into the single *Sal*I site of the plasmid cloning vector pBR322 to form the pKN800 plasmids (Fig. 1.2). Insertion of foreign DNA into the *Sal*I site of pBR322 disrupts the gene encoding tetracycline resistance. Thus, cells harboring recombinant plasmids will be resistant to ampicillin but sensitive to tetracycline. Cells transformed with recircularized pBR322 will exhibit resistance to both antibiotics. Recircularized vector lacking an insert is the most prevalent product of the simple ligation experiment depicted in Fig. 1.2. Screening for insertional inactivation of the *tet* gene simplifies the search for cells that contain recombinant plasmids. In our case, we can also detect cells that contain

recombinant plasmids by looking for cells that glow in the dark, which is the phenotype conferred by the *lux* operon.

The *lux* operon can insert into pBR322 in two possible orientations (Fig. 1.2). To distinguish orientation A from orientation B, you will cut the plasmid DNA with *Pst*I. The *lux* operon and pBR322 each contain a single *Pst*I site positioned asymmetrically relative to the *Sal*I sites. Although *Pst*I will cut each plasmid into two fragments, the sizes of the *Pst*I fragments produced will differ depending on the orientation of *lux* relative to pBR322.

The restriction endonuclease *Pst*I (isolated from *Providencia stuartii*) recognizes the base sequence:

5′––––––CTGCAG––––––3′
3′––––––GACGTC––––––5′

cut by *Pst*I to give:

5′––––––CTGCA G––––––3′
3′––––––G ACGTC––––––5′

Note that *Pst*I cleavage generates fragments with four unpaired bases at their 3′ ends. (Recall that *Sal*I fragments have single-stranded 5′ ends.)

Agarose gel electrophoresis separates DNA molecules based on their size and structure. DNA, which has a negative charge, will migrate from the cathode (negative, black lead) to the anode (positive, red lead) when an electrical field is applied across the gel. Agarose concentration and the ionic strength of the electrophoresis buffer affect the mobility of DNA through an agarose gel. The agarose gel acts as a molecular sieve that retards the migration of long DNA molecules more than short ones. Linear DNA molecules usually migrate through agarose gels faster than circular DNAs, which occur in two forms: covalently closed circular (ccc) and open circular (oc). Covalently closed circular DNAs form supercoils; picture a telephone cord twisted around itself. Although

small supercoiled DNAs migrate more rapidly than linear DNAs of the same length, most supercoiled circular DNAs migrate slower than the corresponding linear molecules. Most of the plasmid DNA in your preparation should be supercoiled. A break (nick) in one strand of a circular DNA molecule relieves the torsional strain that causes supercoiling and produces "relaxed" or "open" circular DNA, which migrates through agarose much more slowly than other topological forms (linear or supercoiled DNA) of the same size. Which would you rather try to pull through a chain-link fence, a piece of pipe (linear DNA), a hula hoop (relaxed circular DNA), or a hula hoop twisted into a straight (albeit fat) cylinder (supercoiled DNA)?

The location of DNA within an agarose gel can be determined directly by staining with ethidium bromide, a fluorescent dye. The planar structure of ethidium bromide allows it to intercalate (stack) between the nucleotide bases of DNA (Fig. 1.3). When the stained DNA is exposed to ultraviolet light, the ethidium emits visible (orange) light. Ethidium bromide staining can detect as little as 1 ng of DNA.

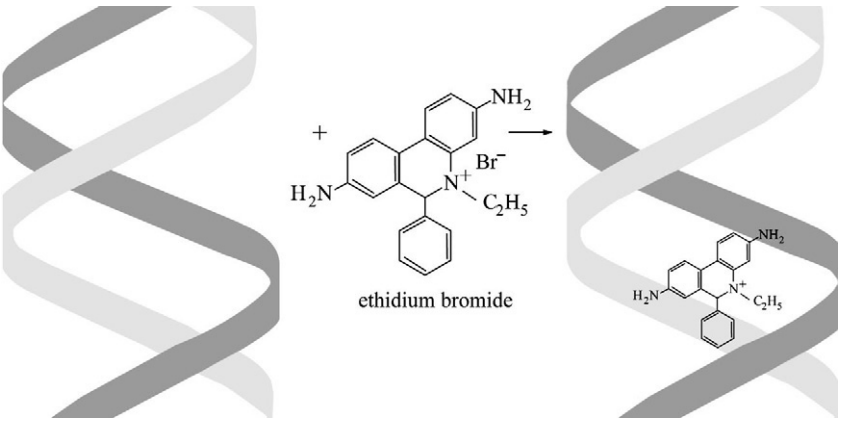


FIGURE 1.3

Chemical structure of ethidium bromide.

In this unit you will estimate the size of restriction fragments based on their electrophoretic mobility relative to molecular weight standards consisting of linear DNA fragments of known molecular weight. To make a standard curve, measure the migration distance of each band of the molecular weight standard (Fig. 1.4) and plot these data on semilogarithmic graph paper (with molecular weight on the log scale; Fig. 1.5). Next measure how far each restriction fragment has migrated and extrapolate from the standard curve to estimate their sizes.

Many commercial molecular weight standards contain known amounts of DNA in each band. You can estimate

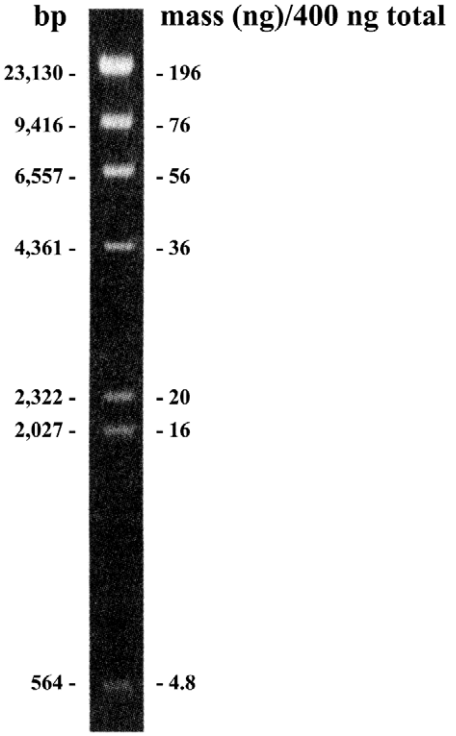


FIGURE 1.4

DNA molecular weight standards.

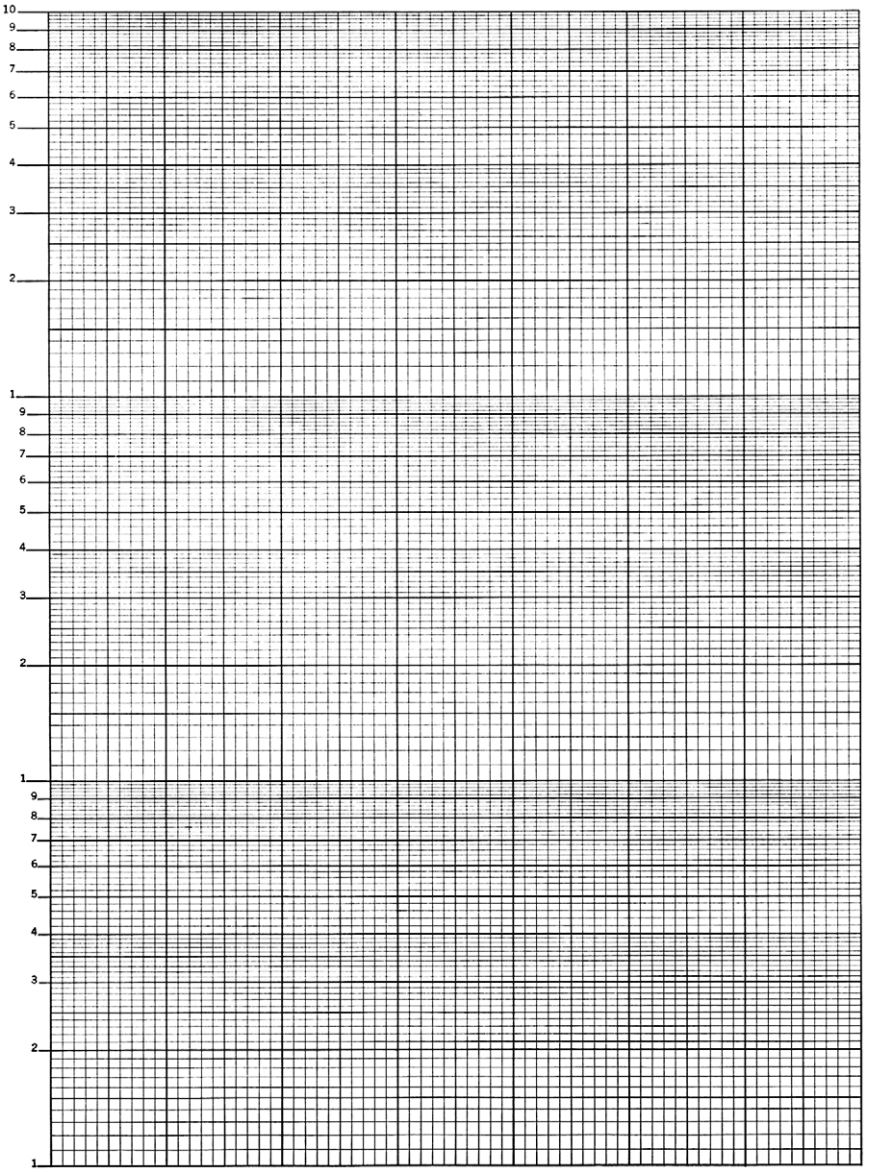


FIGURE 1.5

Semilogarithmic graph paper.

the amount of DNA in each band of your specimen by comparing their intensities to those of the known standards. Fluorescence is directly proportional to the quantity (nanograms) of DNA present, regardless of the molecular weight of the DNA molecules in the band.

Transformation is a simple, inexpensive, and effective way to introduce recombinant plasmids into *E. coli.* In this unit you will transform pKN800 DNA into a different strain of *E. coli,* select ampicillin-resistant transformants, and score colonies for luminescence. Some Gram-positive (*Streptococcus* and *Bacillus)* and Gram-negative (*Neisseria* and *Haemophilus*) bacteria normally become **competent** to take up extracellular DNA during specific stages of their growth cycle. Other bacteria, such as *E. coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*, only become competent under artificial conditions. Most transformation methods use ice-cold solutions of $CaCl_2$ or $RbCl_2$ followed by a brief heat shock to transform plasmid DNA into *E. coli*. **Electroporation**, which involves damaging the cell envelope with pulses of high voltage, is also used to introduce DNA into *E. coli* and a number of bacterial species that are difficult to transform with $CaCl_2$ treatment.

Micropipettor technique: You will use micropipettors (20 µl, 200 µl, and 1 ml capacity) with sterile tips (yellow for 20- and 200-µl pipettors; blue for 1-ml pipettor) to measure and transfer small volumes of liquid. Accurate measurements require proper technique. Sterile tips are supplied in racks held inside closed plastic boxes. To place a tip securely on the pipette barrel, press the barrel firmly inside a tip (while it is still in the rack) and withdraw the tip from the box. **Do not** use your hand, even with gloves, to put the tip on the pipettor. Use a clean tip each time you pipette from a reagent stock.

The pipettor has a two-stage plunger; use the stage with low resistance to measure and the high-resistance stage to expel all the liquid from the pipette tip. Press the plunger to the bottom of the low-resistance stage **before** you insert the tip in the liquid. Submerge the mouth of

the tip in the solution you wish to pipette, but do not go deeper than necessary. This will prevent drops from clinging to the outside of the pipette tip, which can occur when you measure viscous solutions such as restriction endonucleases. Storage buffers for most enzymes contain glycerol, which is especially viscous at –20°C, the temperature typically used to store enzymes.

Draw the liquid into the tip by releasing the plunger gradually; this should take 2 s. If you allow the spring to snap the plunger back too quickly, your measurements may be inaccurate. With your free hand, pick up the tube into which you will pipette the solution; this will allow you to see where the liquid goes. Move the pipette tip to the bottom of the tube and expel the solution as a single drop. Ensure that subsequent additions go into the same drop. After you pipette all the components into a single drop at the bottom of the tube, set the pipettor to approximately the volume of the drop. Insert the tip into the drop and mix the reagents by pipetting up and down several times. Do not create bubbles, which may cause enzymes in the mixture to oxidize.

III. PROCEDURE

A. Purify Plasmid pKN800 DNA (Class 2)

Work in pairs; wear gloves throughout the entire procedure to protect plasmid DNA from skin-borne nucleases and to protect your skin from phenol. Lab coats and eye protection are **required** when you (or others) are using phenol. Open containers of phenol only in the fume hoods. Do all steps aseptically.

1. **TAs do step 1:** Grow 5-ml cultures of *E. coli* strains NM522(pKN800-A) and NM522(pKN800-B) in Luria broth plus 50 μg/ml ampicillin; shake at 37°C overnight. Prepare a 5-ml culture for each pair of students. Centrifuge the cells at 4000 rpm for 10 min. Discard the supernatant (into a container that will be autoclaved after class) and resuspend the cell pellet in 150 μl of lysis buffer (50 m*M* glucose, 10 m*M* EDTA, 25 m*M* Tris·HCl, pH 8.0). Transfer the cells to a sterile 1.5-ml microcentrifuge tube and store on ice until class.
2. **Students start here:** Add 60 μl of a freshly prepared lysozyme solution (80 mg of lysozyme/ml in 10 m*M* Tris·HCl, pH 8) to the cell suspension and mix vigorously. Incubate at room temperature for 10 min. Mark the tube with your name and sample number.
3. Add 200 μl of 1% SDS–1 *M* NaOH solution to the cell suspension. Invert the tube GENTLY several times to mix the contents and then place on ice for 5 min. Note that the cells lyse at this point, increasing the viscosity of the solution. **Treat the cells gently because you do not want to shear the chromosomal DNA and release it from the membrane.** If the chromosomal DNA is sheared into small fragments, it will copurify with the plasmid DNA. The goal is to isolate only

plasmid DNA. The detergent (SDS) lyses the cells, and the NaOH increases the pH so that the DNA denatures.

4. Add 150 µl of 5 *M* potassium acetate to the lysate. Invert the tube GENTLY several times to mix the contents and then place on ice for 10 min. You should see a white flocculent precipitate. The potassium acetate returns the pH to neutral and helps precipitate the chromosomal DNA.

5. Centrifuge the tube at maximum speed (14,000 rpm) for 10 min. Be sure to place another sample on the opposite side of the rotor to balance it. The pellet contains chromosomal DNA and debris from the lysed cells. Use a micropipette to transfer the supernatant to a clean, sterile 1.5-ml microcentrifuge tube. Mark the tube with your name and sample number. The supernatant contains the plasmid DNA; you should have approximately 500 µl of this solution. Discard the pellet in a container that will be autoclaved after class.

6. Add 10 µl of RNase A (1 mg/ml in 25 m*M* Tris·HCl, pH 7.4) to the supernatant and invert the tube several times to mix; incubate at room temperature for 10 min. This step degrades the RNA.

7. Wear gloves, goggles, and lab coats. Add 500 µl of phenol–chloroform–isoamyl alcohol (25:24:1) to the supernatant. Vortex vigorously and centrifuge at full speed for 3 min to separate the aqueous (top) and organic (bottom) phases.

8. Transfer the **top** (aqueous) phase, which contains the plasmid DNA, into a clean, sterile 1.5-ml centrifuge tube. Mark the tube with your name and sample number. Avoid the interphase region, which is a white layer of protein that forms between the aqueous and organic phases. Cap the tubes containing the organic (bottom) phase; discard the phenol in the organic waste container.

9. Add 10 µl of 3.5 *M* sodium acetate to the aqueous phase containing the plasmid DNA. Mix well by inverting the tube several times. The sodium acetate (and potassium acetate added in step 4) provides the high salt concentration required for ethanol precipitation of the DNA.

10. Add 500 µl of ice-cold 95% ethanol to the DNA–salt solution. Mix well by inverting the tube several times; incubate on ice for 10 min.

11. Centrifuge at full speed for 10 min. Place the hinge of the tube lid to the outside of the rotor; this will help you locate the DNA pellet, which will adhere to the outer wall of the bottom of the tube. Pour off the alcohol gently but quickly; do not dislodge the DNA pellet from the tube. Centrifuge the tube for 10 s. Carefully remove the residual ethanol with a sterile pipette tip. Ask the teaching assistant if you are unsure how to do this. Label the tube with your name and sample number and write "pure pKN800" on the tube. Incubate the tube, with the cap open, in a vacuum desiccator or Speed Vac centrifuge until the ethanol has evaporated.

12. Dissolve the pellet in 50 µl of TE buffer (10 m*M* Tris, 1 m*M* EDTA, pH 8). Place in ice bucket. Store this sample in the freezer until next class.

B. Restriction of Plasmid pKN800 (Class 2)

1. Label two sterile 1.5-ml microcentrifuge tubes with your name and sample number. Write "pst" on one tube and "uncut" on the other.
2. Add 3 µl of 10× reaction buffer to both tubes (one labeled "pst" and the other labeled "uncut"). The reaction buffer is supplied by the manufacturer. Use fresh sterile tips.
3. Add 10 µl of sterile distilled water to the tube labeled "pst" and 12 µl to the tube labeled "uncut." Use fresh sterile tips for each addition.
4. Transfer 15 µl of pKN800 DNA into each tube. Use fresh sterile tips. Store these tubes and the rest of your plasmid DNA on ice.
5. Use a new sterile pipette tip to add 2 µl (20 units) of *Pst*I enzyme to the tube labeled "pst." Set the pipettor to 15 µl and mix the reagents by pipetting them up and down (inside the tube) several times. Mix thoroughly, but do not make bubbles. [Most companies sell restriction enzymes at a concentration of 10 units/µl. A unit of restriction endonuclease is usually defined as the amount of enzyme required to cut 1 µg of a particular DNA species in 1 h under specified reaction conditions.]
6. Centrifuge the tube for 10 s to bring all of the liquid to the bottom.
7. Incubate both samples in a 37°C waterbath for 45–60 min. Use a floating rack to hold the tubes.
8. Incubate the samples at 70°C for 10 min to inactivate the *Pst*I enzyme, then store the samples at 4°C until next class. **The heat treatment is optional** for this experiment. However, if you wanted to clone the restriction fragments, you would need to inactivate

the restriction enzyme before you attempted to ligate the DNA fragments to another vector. Heat will inactivate *Pst*I and many other restriction endonucleases, but some enzymes, such as *Bgl*II, are not destroyed by heating to 70 °C and must be extracted with phenol.

C. Agarose Gel Electrophoresis of *Pst*I-Digested pKN800 (Class 3)

1. **Cast an agarose gel.** Use an 0.8% agarose gel to separate the restriction fragments in your DNA samples. This percentage of agarose will separate linear DNA molecules that range from 0.6 to 10 kb.

   a. Seal the edges of a clean, dry, plastic gel-casting tray supplied with the electrophoresis apparatus. Our apparatus comes with a sealing clamp, but some models require that the ends be sealed with tape. Install the well former (comb) on the casting tray; set the comb depth so that a microscope slide will just fit between the tray and the tips of the comb's teeth. Make certain the comb is parallel to the edge of the casting tray.

   b. Add 0.8 g of agarose to a flask containing 100 ml of 1× TAE buffer (0.04 *M* Tris–acetate with 0.001 *M* EDTA). Mark the level of the liquid on the flask with a felt tip marker pen. Boil the liquid and swirl until the agarose dissolves completely. **Use heat-resistant gloves to hold the flask, and swirl the flask gently to prevent the molten agarose from boiling over.** Undissolved agarose appears as small, clear particles floating in the solution. Add distilled water to replace the liquid that evaporated. Use a microwave oven, hot plate, or steamer to melt the agarose.

   c. Cool the agarose solution to 55°C; the flask should be cool enough to touch. Ethidium bromide is a planar molecule that intercalates into DNA by stacking between the bases. This causes DNA polymerase to make frameshift mutations during DNA replication. Because ethidium bromide is mutagenic, **you should wear gloves and a lab coat.** Add 4 μl of ethidium bromide (10 mg/ml stock solution) per

100 ml of agarose solution; swirl to mix. Pour the cooled agarose solution into the casting tray. Pour to a height between 3 and 5 mm thick.

d. After the agarose has solidified, remove the casting tray from the clamp (or remove the tape from the ends of the tray) and place the tray in the electrophoresis apparatus. Add just enough TAE electrophoresis buffer to fill the buffer reservoirs and cover the gel to a depth of 1 mm. Lift the comb straight up to remove it from the gel. The buffer will fill the wells and prevent them from tearing as you remove the comb. The wells must be full of electrophoresis buffer before you load your samples into them.

2. Load the DNA samples onto the agarose gel.

a. Centrifuge the samples for 15 s.

b. Label two new tubes "pst" and "uncut."

c. Transfer 15 µl of each of your samples into the appropriately labeled tube. Store the remaining plasmid DNA on ice.

d. Add 2 µl of loading solution to each of the 15-µl samples. Mix well by pipetting up and down a few times. The loading solution contains bromophenol blue for tracking the progress of the electrophoresis and glycerol to increase the density of the sample so that it will sink to the bottom of the well in the gel. **Do not add loading solution to any of the other tubes.**

e. Load the samples into different wells of the submerged gel. Place the pipette tip just above the well and gently expel the sample, which will sink to the bottom of the well and displace the electrophoresis buffer. If you insert the pipette tip too deeply into the well, you may pierce the bottom of the well, allowing your sample to leak out.

f. Load 400 ng of phage λ DNA restricted with *Hin*dIII in one lane. Heat this DNA to 65°C immediately before loading; this will separate the two fragments (23,130 and 4361 bp) that contain the phage genome's cohesive ends (*cos* sites), which anneal to each other at room temperature. This DNA is already cut, so you do not need to digest it with *Hin*dIII. This molecular weight standard provides restriction fragments of known size that range from 23,130 to 564 bp (Fig. 1.4). You will use these standards to estimate the size of the restriction fragments in your samples.

g. Attach the electrical leads so that the red lead (anode) is at the bottom of the gel and the black lead (cathode) is at the top, nearest your samples. DNA has a negative charge and will migrate toward the anode. Apply 10 V/cm (75 V) to the gel. Turn the rheostat on the power supply gradually from 0 until the meter indicates the desired voltage. If you apply the full load all at once, you may blow the fuse. [**Begin the transformation (step D) while you wait for the electrophoresis to finish.**]

h. Turn off the current when the bromophenol blue dye is 1 cm from the bottom of the gel.

i. View the ethidium bromide-stained gel on an ultraviolet light transilluminator. **You must wear UV-blocking eyewear; a full face shield is best.** Do not look at the light source without proper eye protection, and protect your skin from the UV light.

j. Photograph the gel. Each student will receive a print to analyze and include in his or her lab report.

D. Transform *E. coli* Strain DH5α with pKN800 DNA (Class 3)

1. Competent Cell Preparation

The cells that you will use were purchased as frozen competent cells, but you may prepare competent cells yourself as follows. Grow *E. coli* DH5α*mcr* (or any other *E. coli* strain) to midlog phase (OD_{600} = 0.4) in 10 ml of LB broth and then chill on ice. Centrifuge cells at 3700 rpm for 10 min. at 4°C in a clinical centrifuge. Discard the supernatant (into a container that can be autoclaved) and place the cells on ice. Resuspend the cell pellet in 10 ml of ice-cold 100 m*M* $CaCl_2$ and incubate on ice for 30 min. Centrifuge the cells (3700 rpm, 10 min.), discard the supernatant, and place the cells on ice. Resuspend the cell pellet in 5 ml of ice-cold 100 m*M* $CaCl_2$ and incubate the cells on ice for 30 min. Centrifuge the cells, pour off the supernatant, and place the cells on ice. Resuspend the cell pellet in 1 ml of cold 100 m*M* $CaCl_2$ and incubate on ice for 15 min. The cells are now competent for transformation.

1. **Students start here.** Work in pairs; use aseptic technique throughout the procedure. Label one sterile 1.5-ml microcentrifuge tube "cut," a second tube "uncut," and a third tube "no DNA." Place the tubes on ice. Frozen competent cells will be thawed on ice immediately before use.
2. Stir the ice-cold competent cells with a sterile pipette tip and then transfer 50 µl to each chilled tube.
3. Add 5 µl of uncut or *Pst*I-cut plasmid DNA to the appropriate tube. Use a fresh sterile pipette tip for each transfer. Mix the DNA with the competent cells. Do not let the cells become warm. Incubate on ice for 40 min. Do the same incubation with the cells in the "no DNA" tube.

4. Place the tubes in a 37 °C water bath for **exactly** 20 s. Do not shake the tubes.

5. Place all tubes on ice for 2 min **immediately** after the heat shock.

6. Add 0.95 ml of room-temperature LB broth to each microcentrifuge tube and shake at 225 rpm for 60 min at 37 °C.

7. Spread cells from each culture on LB agar containing 50 μg/ml ampicillin. To ensure that you obtain single colonies, you will dilute some of the cultures before you spread them on the LB–ampicillin plates; see Table 1.1 for the appropriate dilutions. Use room-temperature LB broth to dilute the cells. Spread two plates for each dilution. The culture transformed with **uncut DNA** should contain the largest number of ampicillin-resistant transformants because supercoiled plasmid DNA transforms competent *E. coli* cells efficiently. Spread 0.1 ml of undiluted cells from this transformation on LB–ampicillin plates. Also dilute this culture 10-fold and spread 0.1 ml/plate on LB–ampicillin agar.

Table 1.1 Dilutions for Plating on LB–Ampicillin and LB Agar

Plates	**Culture transformed with**		
	Uncut DNA	**PstI-cut DNA**	**No DNA**
LB + Amp	0.1 ml of undiluted	0.1 ml of undiluted	0.1 ml of undiluted
LB + Amp	0.1 ml of 10^{-1} dilution		
LB			0.1 ml of 10^{-5} dilution
LB			0.1 ml of 10^{-6} dilution

8. The culture labeled "**no DNA**" is a negative control; this culture should not produce colonies on LB–ampicillin plates. Spread 0.1 ml of undiluted cells on LB–ampicillin plates. This control serves several purposes. It ensures that the competent cells were not resistant to ampicillin prior to transformation with pKN800 DNA, and it shows that the plates contained enough antibiotic to kill the untransformed cells. For these controls to be meaningful, you must prove that the culture contained viable (ampicillin-sensitive) cells. To do this, you will plate this culture on LB plates without ampicillin. Prepare 10^{-5}- and 10^{-6}-fold dilutions of the cells in the tube labeled "**no DNA,**" and plate 0.1 ml of each dilution on LB agar **without ampicillin**. The number of colonies on these LB plates will allow you to calculate the number of viable cells in the culture.

9. The culture transformed with ***Pst*I-cut DNA** is a control to test whether all of the plasmid DNA was cut with *Pst*I. Linear DNA does not transform *E. coli* because it is destroyed in the bacterial cell by exonuclease V (ExoV), which is also called the RecBCD nuclease. When *Pst*I-cut DNA enters *E. coli*, ExoV will destroy it, and no ampicillin-resistant colonies will grow. However, if *Pst*I did not cut all of the plasmid molecules, uncut plasmid DNA molecules can transform competent *E.* coli efficiently and lead to the growth of ampicillin-resistant transformants. To assess the extent of the *Pst*I digestion, spread 0.1 ml of undiluted cells transformed with ***Pst*I-cut DNA** on LB–ampicillin plates.

10. Incubate all plates at 30°C overnight. Examine the plates the next afternoon. Normally *E. coli* is grown at 37°C, but the luciferase enzyme works better at 30°C.

E. Observe Luciferase Reporter Gene Expression (Day after Class 3)

1. Take your plates from the 30°C incubator to a dark room.
2. Turn off the light and let your eyes acclimate to the dark for about 2 min.
3. Count the number of ampicillin-resistant transformants that glow in the dark.
4. Turn on the lights and count the colonies on each plate. Record the data.

IV. LABORATORY REPORT

Include the following in your laboratory report:

1. In the results section of your lab report, describe the pattern of restriction fragments you observed.
2. Include a photograph of your gel as a figure in your report. Label each lane on the photograph and indicate the contents of each lane in the figure legend.
3. Measure the distance that each molecular weight standard migrated during the electrophoresis. On semi-logarithmic graph paper, plot the size (in base pairs) of each fragment (log scale) versus the distance migrated (linear scale). If your gel did not work, copy one from another group or use the example shown in Fig. 1.6. The molecular weight standards used for this gel are shown in Figs. 1.4 and 1.7.
4. In a table, record the average number of ampicillin-resistant and luminescent transformants obtained in each transformation.
5. Calculate the efficiency of transformation, which is expressed as the number of transformants per microgram of plasmid DNA. Divide the total number of transformants by the amount of DNA used to transform. Take into account the dilutions that you made. To estimate the concentration of your plasmid DNA, compare the intensity of the uncut pKN800 band with that of the bands in the molecular weight standard. Figure 1.4 indicates the amount of DNA (in nanograms) contained in each band of the standard.

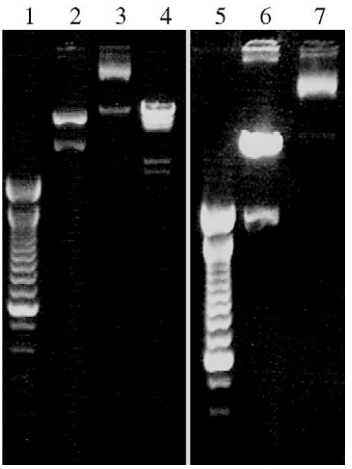


FIGURE 1.6

Agarose gel electrophoresis of uncut and *Pst*I-cut pKN800-A and -B plasmid DNA: lane 1, 100-bp ladder; lane 2, pKN800-A cut with *Pst*I; lane 3, uncut pKN800-A; lane 4, λ DNA cut with *Hin*dIII; lane 5, 100-bp ladder; lane 6, pKN800-B cut with *Pst*I; lane 7, uncut pKN800-B. The gel was cast with 2% 3:1 agarose; note the compression of the λ ladder (lane 4). The 2322- and 2027-bp *Hin*dIII fragments show clear separation. See Fig. 1.7 for a description of the 100-bp ladder.

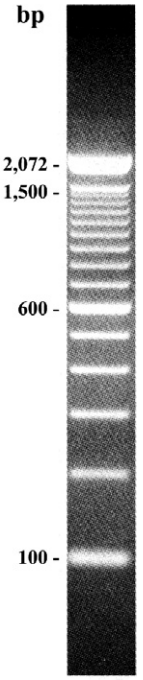


FIGURE 1.7

100-bp DNA molecular weight standards.

V. QUESTIONS

1. Did the uncut plasmid DNA form more than one band during agarose gel electrophoresis? What does each band represent?

2. Which orientation of the *lux* operon did your plasmid have, A or B (see Fig. 1.2)? How can you tell?

3. Did your *Pst*I digestion go to completion? How can you tell?

4. Did you get ampicillin-resistant transformants with restricted plasmid DNA? Explain.

5. Would you expect any of the transformations to yield ampicillin-resistant nonluminescent transformants? Justify your answer.

6. Why did you analyze the uncut plasmid on the gel?

7. Why did you plate bacteria that did not receive any plasmid DNA?

8. If there were no colonies on any of the LB–ampicillin plates, how could you distinguish between extremely poor transformation efficiency and incorrect medium? In other words, what control would indicate whether the plates were at fault?

VI. IN-CLASS WRITING EXERCISE (CLASS 2)

Rewrite these sentences, making them more succinct. Eliminate as many words as possible without sacrificing meaning.

1. From the results of this experiment, it was indicated that blue dextran (blue band) had a greater molecular weight than DNP–glycine (yellow band), as it was eluted first.

2. The procedures on pages 27–34 of the lab book were generally followed with the following three deviations as listed below:

3. Colonies that were yellow in color and white in color were seen.

4. A possible explanation for this result could be due to the fact that molecules larger then the largest pore size of the gel cannot diffuse into the gel pores.

VII. RESTRICTION MAPPING EXERCISES (CLASS 3)

1. This linear 10-kb DNA fragment contains both *Bam*HI and *Hin*dIII restriction sites. Draw the sites on the map; be specific about the location of the sites relative to each other and the ends of the fragment. There are two solutions.

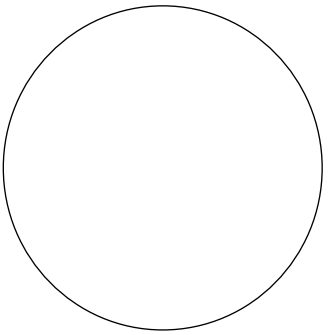
Digestion with	Fragment sizes (kb)
*Bam*HI	5.0, 3.0, 2.0
*Hin*dIII	5.5, 4.5
*Bam*HI and *Hin*dIII	5.0, 3.0, 1.5, 0.5

2. A covalently closed circular plasmid was digested with several restriction endonucleases and analyzed by agarose gel electrophoresis. The digestions produced restriction fragments of the following sizes:

Digestion with	Fragment sizes (kb)
*Eco*RI	6.0
*Pst*I	3.5, 2.0, 0.5
*Eco*RI and *Pst*I	2.5, 2.0, 1.0, 0.5
*Xba*I	3.8, 2.2
*Xba*I and *Pst*I	1.8, 1.7, 1.5, 0.5*

*Note that the 0.5-kb band produced by digestion with *Xba*I and *Pst*I appears twice as bright as expected on the agarose gel.

Draw a circular restriction map of this plasmid.



VIII. IN-CLASS WRITING EXERCISE (CLASS 4)

Critique of: Kragelund, L., Hosbond, C., and Nybroe, O. (1997). Distribution of Metabolic Activity and Phosphate Starvation Response of *lux*-Tagged *Pseudomonas fluorescens* Reporter Bacteria in the Barley Rhizosphere. *Appl. Environ. Microbiol.* **63:**4920–4928.

1. What is the rationale for this work, and where did you find it?

2. Did the abstract contain statements that you did not understand? Did the introduction clarify these statements?

3. Were any of the results unclear? If so, why?

4. What is the connection between this article and Experiment 1?

5. Did you notice an unusual finding in the second paragraph of the results section?

NOTES

EXPERIMENT
2

Affinity Purification of Histidine-Tagged FnbA Protein

I. INTRODUCTION

In this unit, you will express the *Staphylococcus aureus* fibronectin-binding protein gene (*fnbA*) in *Escherichia coli*. Next, you will purify FnbA protein by affinity chromatography and examine it by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The purified protein was originally used to immunize rabbits in order to obtain polyclonal antisera, which were used to detect FnbA. We are interested in FnbA because it is required for the infection of host tissue.

II. BACKGROUND

S. aureus causes bovine mastitis. The pathogen attaches to fibronectin, a secreted host protein that holds together the epithelial cells that line the mucosal surface of the udder. *S. aureus* fibronectin-binding protein contains a 115 amino-acid domain that allows the bacteria to bind host cells.

The 345-bp portion of the *fnbA* gene that encodes the fibronectin-binding domain was fused, in frame, to six histidine codons, which comprise the affinity tag. This gene fusion is expressed from a strong *E. coli* promoter called *tac*. The *tac* promoter is a hybrid of the *trp* and *lac* promoters; it is induced by a lactose analog, isopropyl thiogalactoside (IPTG). The entire construct is carried on a plasmid that encodes resistance to ampicillin. *E. coli* cells harboring this plasmid will produce the histidine-tagged, fibronectin-binding protein when they are cultured in the presence of IPTG.

Affinity tags, such as six histidine residues, are often attached to proteins to simplify their purification. Affinity tags may or may not affect the normal activity of the tagged protein; however, this is not a concern when the goal is to

purify a protein for use as an antigen. Histidine residues arranged properly within a protein bind nickel (Ni). Therefore, histidine-tagged proteins are purified by chromatography over a nickel-containing resin. Bacterial cells are disrupted, in this case by a proprietary detergent, and the extracts are passed over a nickel column. In theory, only the histidine-tagged protein binds, and the other proteins in the extract wash off the column. Addition of imidazole, which competes with the histidine-tagged protein for binding sites on the Ni resin, releases the tagged protein from the column. The purity and yield of the eluted protein can be assessed by SDS–PAGE. To learn more about this technology, see www.qiagen.com/. Under "Products & Services," select "QIAGEN Product Guide," then under "Protein Purification" select "The Ni-NTA System."

III. PROCEDURE

A. Lyse Bacteria (Class 5)

1. Each pair of students will receive 1.5 ml of IPTG-induced culture expressing histidine-tagged FnbA. Each group will also receive 1.5 ml of an uninduced culture as a negative control.
2. Centrifuge each culture for 2 min at maximum speed (14,000 rpm) in a microcentrifuge. Discard the supernatant into a flask that will be autoclaved after class.
3. Label a fresh microcentrifuge tube for each culture. Write "total" and either "I" (for induced) or "U" (for uninduced) on the tubes.
4. Suspend each cell pellet in 297 µl of Tris-buffered B-PER detergent plus 3 µl of phenyl methyl sulfonyl fluoride (PMSF) solution, which is a proteinase inhibitor. **Caution: PMSF is a poison; do not allow it to contact your skin. Use gloves.** Vortex vigorously until the cells are completely suspended.
5. Transfer 10 µl of each suspension to a fresh tube and hold on ice; use tubes from Step 3.
6. Centrifuge the remaining 290 µl for 5 min at maximum speed to separate the soluble and insoluble proteins. Technical note: Always place microcentrifuge tubes into a fixed-angle rotor in a specific orientation so that you know where the pellet is located, even if it is not visible. I always orient tubes so that the lid's hinge is on the outside of the rotor.
7. For each culture, label a fresh tube "sol" and either "I" or "U."
8. Transfer the supernatants (soluble fractions) to the tubes labeled "sol."

9. Suspend the pellet (insoluble material), which may be invisible, in 284 µl of Tris-buffered B-PER. Vortex vigorously for 1 min.
10. Add 6 µl of lysozyme solution to the resuspended pellet. **Do not add lysozyme to the supernatant**.
11. Vortex vigorously for 1 min.
12. Add 1 ml of diluted (1:10) B-PER reagent to the suspension and vortex for 1 min.
13. Centrifuge for 10 min at maximum speed in a microcentrifuge.
14. Discard the supernatant.
15. Repeat steps 12–14.
16. Dissolve each pellet, which may be invisible, in 290 µl of H_2O, and label the tubes "insol" and either "I" or "U."

B. Adsorb His-Tagged FnbA Protein to Nickel–Agarose (Class 5)

1. Combine the soluble and insoluble fractions from the induced culture. Do the same for the fractions from the uninduced culture.
2. Add 100 µl of the nickel-chelated agarose (50% suspension) to each of the combined fractions.
3. Close the tubes and agitate by rocking for 10 min at room temperature.
4. Centrifuge for 10 s.
5. Transfer each supernatant to tubes labeled "nonadsorb" and either "I" or "U."

C. Wash Resin and Elute Histidine-Tagged FnbA (Class 5)

1. Add 100 µl of wash buffer to each pellet and vortex until the resin is completely suspended.
2. Incubate 5 min at room temperature.
3. Centrifuge for 10 s.
4. Discard each supernatant.
5. Repeat steps 1–4.
6. Add 50 µl of elution buffer to each pellet, vortex vigorously, and incubate for 5 min at room temperature.
7. Centrifuge for 10 s.
8. Transfer the supernatants into tubes labeled "eluate 1-I" and "eluate 1-U." Store at –20°C.
9. Repeat steps 6–8. Place the second supernatants in tubes labeled "eluate 2-I" and "eluate 2-U." Store at –20°C.
10. Make sure all your tubes are labeled with your initials. The TAs will store your samples at –20°C until next class.

D. SDS–Polyacrylamide Gel Electrophoresis (Class 6)

1. You will use cast gels and a Mini-Protean II gel apparatus from BioRad. Unwrap the gel and remove the comb from the top of the gel.
2. Remove the tape from the bottom of the gel sandwich.
3. Insert the gel into the clamp; a TA will demonstrate how to do this. Two groups of students will use each gel, and each BioRad minigel apparatus holds two gels.
4. Align the clamp and gel such that their tops are flush.
5. Finger tighten the four screws on the clamp assembly. **Do not overtighten the screws!** Excessive pressure may distort the gel or crack the plates.
6. Install the gel and clamp into the electrophoresis apparatus. First insert the notches at the top of the gel assembly into the apparatus, then snap the lower ends of the assemblies together.
7. Add electrophoresis buffer to the top buffer chamber; note that both gels must be in place to seal the upper chamber. Check for leaks. If necessary, tighten the screws on the clamp to stop leaks. Fill the bottom buffer chamber.
8. Label three tubes for each culture: "nonadsorb," "eluate 1," and "eluate 2."
9. Transfer 10 µl from each of the samples listed in the previous step to the appropriate tube.
10. Add 5 µl of SDS sample buffer to each of the 10-µl samples: "total," "nonadsorb," "eluate 1," and "eluate 2." You should now have eight samples (four from each culture) for electrophoretic analysis. Note that the BioRad gels have 15 lanes. Reserve one lane for

molecular weight standards. If your team is sharing a gel with another group, each group is allotted only seven lanes and will need to omit one sample. Omit the "nonadsorb" sample from the uninduced culture. Eluates 1 and 2 from the induced culture contain similar amounts of His-tagged FnbA protein (Fig. 2.1), so it is not necessary to include both samples if space on the gel is limited.

11. Close the tubes and incubate at 95°C for 5 min, then cool to room temperature.

12. Centrifuge the boiled samples for 10 s.

13. Use a micropipette with a long, narrow tip (designed for loading thin gels) to transfer each sample to a different well of the SDS–polyacrylamide gel. Record which sample is in each lane of the gel.

14. Load 15 µl of protein standards in one lane.

15. Place the gels and electrode assembly in the electrophoresis tank.

16. Place the top on the tank to connect the electrodes to the power cables.

17. Connect the leads to the power supply and adjust the voltage to a constant 200 V. The current should start at about 100 mA.

18. Turn off the power supply when the blue dye nears the bottom of the gel.

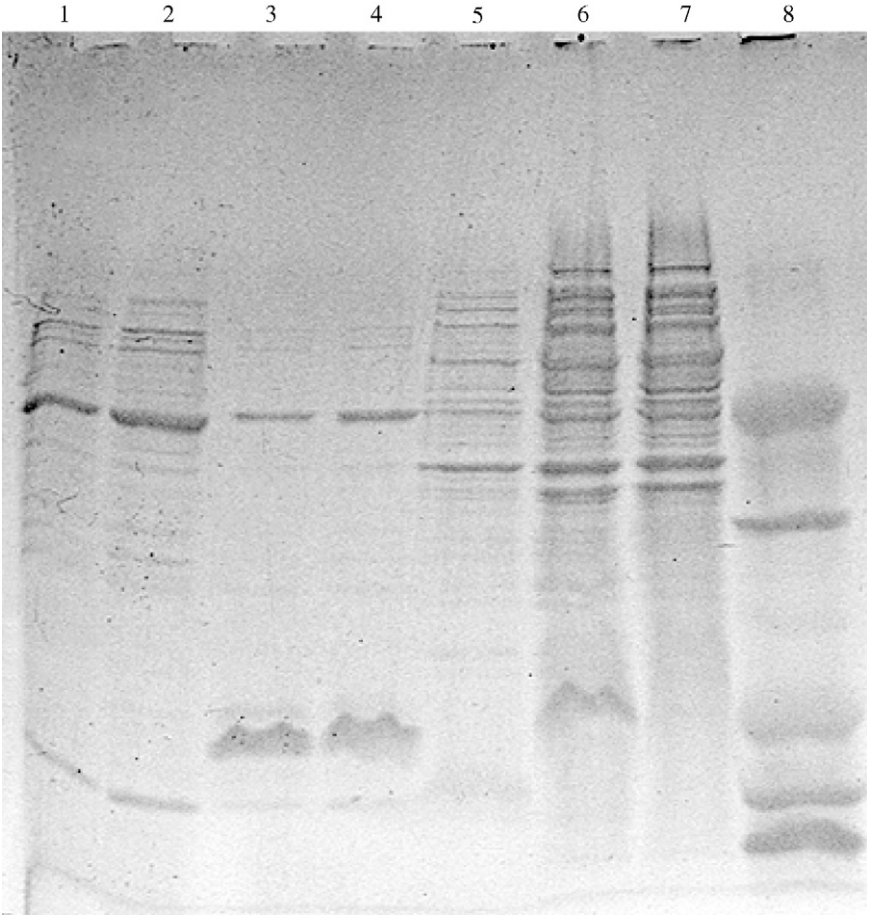


FIGURE 2.1

SDS–PAGE of His-tagged FnbA protein: eluate 2 (lane 1) and eluate 1 (lane 2) from uninduced cells; eluate 2 (lane 3) and eluate 1 (lane 4) from induced cells; flow through from induced cells (lane 5); total cells from induced (lane 6) and uninduced (lane 7) cultures; molecular weight standards (lane 8) (see Fig. 2.3).

E. Stain Gel (Class 6)

1. Remove the gel sandwich from the apparatus.
2. Separate the two glass plates of the gel sandwich. Cut the tape that holds the plates together, and use a spatula to pry the plates apart. As the glass plates separate, let the gel stick to the bottom plate.
3. After the top plate has been removed, submerge the gel and bottom plate in a tray that contains 100 ml of distilled water. Gently rock the tray until the gel floats off the bottom plate. Mechanical stress applied to the gel at this stage may cause it to crack later when it is dried.
4. Rock gently for 5 min.
5. Use a pipette or an aspirator to remove the water from the tray. Do not touch the gel with your hands or allow powder from your gloves to contact the gel.
6. Rinse the gel in distilled water two more times for 5 min each.
7. After the last rinse, add 20 ml of Coomassie G-250 stain.
8. Agitate gently and periodically check for protein band development. Staining intensity reaches a maximum within 1 hr. The background should remain clear, unless the SDS was not fully removed by the water rinses.
9. Photograph the gel for your report.

IV. LABORATORY REPORT

Include the following in your laboratory report:

1. Include the photograph of the SDS–polyacrylamide gel as a figure in your report.
2. Estimate the purity of your preparation of His–tagged FnbA protein. Did you detect contaminating proteins? How many contaminants did you see? What fraction of the protein in each eluate is FnbA?
3. Estimate the size of your purified protein. Measure the distance that each protein in the molecular weight standard migrated. On semilogarithmic graph paper (Fig. 2.2), plot a standard curve of molecular weight (log scale) versus migration distance (linear scale). Figure 2.3 shows the size of each protein in the molecular weight standard. Measure the migration distance of each major protein component in the eluate derived from the induced culture. Use the standard curve to estimate the molecular weights of His-tagged FnbA and any other proteins that constitute a significant fraction of the eluate.
4. Does the fraction you eluted from the Ni–agarose resin contain a contaminating protein that you did not observe in total cellular protein? If so, estimate its molecular weight and speculate about its source. Could this protein be lysozyme? Hint: Look at the legend for Fig. 2.3.

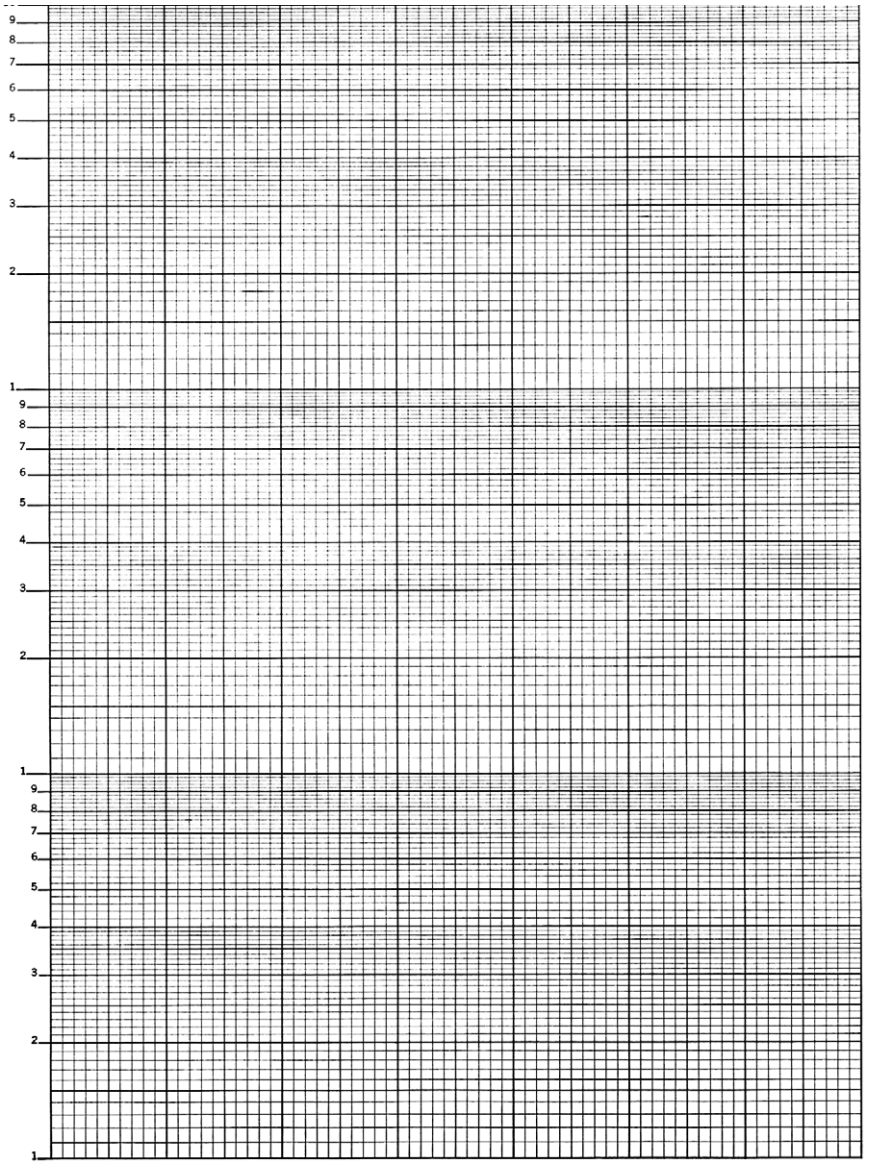


FIGURE 2.2

Semilogarithmic graph paper.

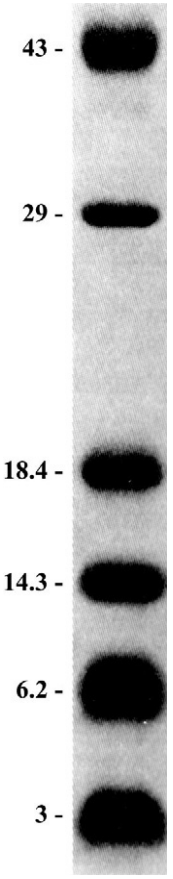


FIGURE 2.3

Protein molecular weight standards: ovalbumin (43,000), carbonic anhydrase (29,000), β-lactoglobulin (18,400), lysozyme (14,300), bovine trypsin inhibitor (6,200), and insulin (3,000).

V. QUESTIONS

1. What is the purpose of SDS in SDS–polyacrylamide gel electrophoresis?

2. Does the apparent molecular weight of your affinity-purified, His-tagged FnbA protein agree with the molecular weight predicted from the coding sequence of the gene fusion? Explain and show your calculations. Include the six histidine residues of the affinity tag in your calculations.

Note: You can either estimate the molecular weight of the His-tagged FnbA peptide, or you can predict the exact molecular weight based on the coding sequence. If you decide to estimate the molecular weight, use 110 as the molecular weight of an "average" amino acid. To predict the exact molecular weight, you will need the coding sequence for the "D repeat region," which is the portion of FnbA fused to the 6-His tag. You will find the *fnbA* gene sequence in GenBank accession J04151 or in *Proceedings of the National Academy of Sciences of the United States of America* **86**(2):699–703 (Jan. 15, 1989). The D repeat region begins at nucleotide 2350 and ends at nucleotide 2694.

3. An affinity tag may or may not affect the function of the tagged protein. Explain how you could determine whether His-tagged FnbA retained its function.

4. What are some advantages of the 6-His tag in protein purification compared to alternative methods? What are some disadvantages of affinity tags?

VI. IN-CLASS WRITING EXERCISE (CLASS 7)

Critique of: Bush, G. L., Tassin, A. M., Friden, H., and Meyer, D. I. (1991). Secretion in Yeast: Purification and *in vitro* Translocation of Chemical Amounts of Prepro-alpha-Factor *J. Biol. Chem.* **266**: 13811–13814.

1. What is the rationale for this work?

2. What is the connection between this article and Experiment 2?

3. The second paragraph of the introduction contains a number of unnecessary phrases and words. Simplify this paragraph without altering its meaning.

4. In the results section the authors state, "Prepro-alpha-factor (His)6 was translocated and glycosylated with approximately the same efficiency as the wild-type prepro-alpha-factor (Fig. 1B)." Do these data support this conclusion? Explain your answer.

5. Would you change the experiment depicted in Fig. 1? If so, how and why?

6. In the last paragraph of the discussion, the authors note that the glycosylation reaction (Fig. 3) did not go to completion. They proposed that the microsomes lacked sufficient substrate to glycosylate all the pre-protein added to the assays. What positive control would test whether the microsomal extracts remained active?

NOTES

EXPERIMENT
3

Polymerase Chain Reaction and DNA Sequence Analysis of Bacterial Ribosomal RNA Genes

I. INTRODUCTION

In this unit, you will culture a bacterium from a natural environment and purify genomic DNA from this organism. You will use this DNA as a template for polymerase chain reaction (PCR) amplification of 16S ribosomal RNA (rRNA) genes contained in the bacterial genome. Next, you will examine your PCR products by agarose gel electrophoresis. Finally, you will prepare the DNA for sequence analysis, which will enable you to identify the bacterium that you isolated.

II. BACKGROUND

The sequences of 16S rRNA genes provide an accurate means to identify bacterial groups. 16S rRNA is a component of ribosomes that is necessary for protein translation. This gene has several features that make it ideal for identification of bacterial groups and study of bacterial evolution. Several sites within the gene are nearly identical over a broad range of bacterial groups, and these sites flank highly variable regions shared by much smaller groups (Fig. 3.1). The highly conserved regions serve as priming sites for PCR amplification of more variable sequences that lie between two conserved sites. Experience shows that primers based on these highly conserved sites can prime PCR amplification of 16S rRNA genes from most bacteria. The sequence of the hypervariable regions of this PCR product will identify groups.

DNA amplification by PCR results in rapid *in vitro* synthesis of many copies of a specific DNA sequence from a large population of different DNA molecules (Fig. 3.2). The reaction contains template DNA (in this

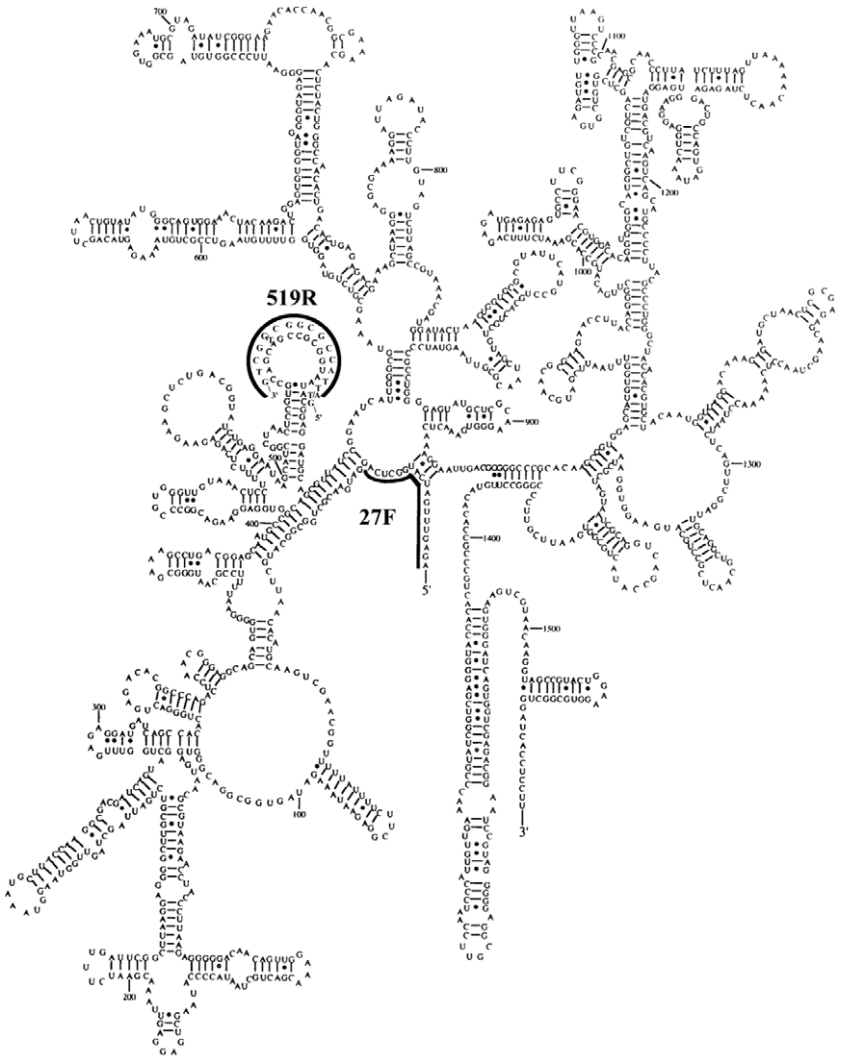


FIGURE 3.1

Proposed secondary structure for a 16S rRNA. Adapted with permission from: Rappé, M. S., Suzuki, M. T., Vergin, K. L., and Giovannoni, S. J. (1998). Phylogenetic Diversity of Ultraplankton Plastid Small-Subunit rRNA Genes Recovered in Environmental Nucleic Acid Samples from the Pacific and Atlantic Coasts of the United States. *Appl. Environ. Microbiol.* **64**: 294–330. Bars labeled 27F and 519R indicate the PCR primers used in Experiment 3.

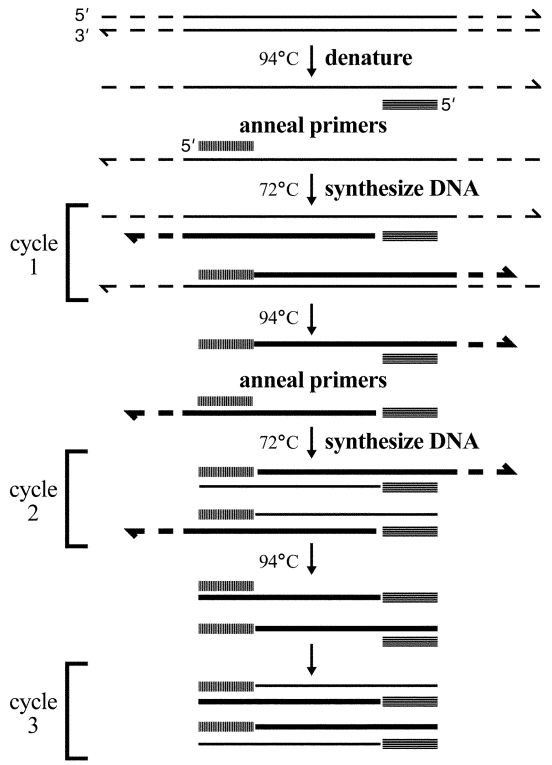


FIGURE 3.2

Polymerase chain reaction. The diagram shows the first three cycles of a standard PCR reaction.

case an entire bacterial genome), heat-stable *Taq* DNA polymerase (from *Thermus aquaticus*), deoxynucleoside triphosphates (dNTPs), magnesium, buffer, and two primer oligonucleotides. One primer complements a region upstream of the sequence being amplified; the other primer complements a region on the opposite strand downstream of this sequence (Fig. 3.2). The mixture is heated to 94°C to denature the template DNA, and then it is cooled enough to allow the primer oligonucleotides to anneal to their target sequences. Next the reaction

is raised to the optimum temperature for *Taq* DNA polymerase activity (72°C). We will repeat this cycle 35 times for our study.

During the first cycle, the primers anneal to the target sequence and are extended by DNA polymerase beyond the other primer-binding site (Fig. 3.2). In the second cycle, these new strands are used as templates, generating strands running from primer to primer. Thus, the final amplification product contains DNA molecules of a specific length. In theory, 35 cycles of amplification can produce 8,589,934,592 (2 to the 33rd power) copies from a single template molecule. Note that the first two cycles do not produce products of a specific length. Although some investigators have detected a single copy of template DNA, PCR reactions never yield the theoretical maximum of product DNA.

Because a single molecule of contaminating DNA can be amplified many times, you must take precautions to avoid introducing extraneous DNA. Use clean gloves, a clean work surface, and pipette tips with aerosol barriers. Always perform at least one negative control reaction with no added DNA for **each** reaction that contains template. The PCR kit we will use contains template and primers for a positive control reaction, which allows you to verify that the DNA polymerase is active and the dNTPs are not degraded.

DNA sequencing reactions require purified template DNAs free of PCR reagents and primers. You will use cartridges that contain a silica matrix (Fig. 3.3) to separate your PCR product from the rest of the reaction cocktail. DNA binds silica (glass) in the presence of high salt, whereas the other components of a PCR reaction do not. This will allow you to bind your PCR product to the silica matrix in the cartridge and wash away other components of the PCR reaction with a high-salt buffer. You will then elute your purified DNA with low-salt DNA buffer.

The "dideoxy" method of DNA sequencing is similar to PCR, but there are several important differences

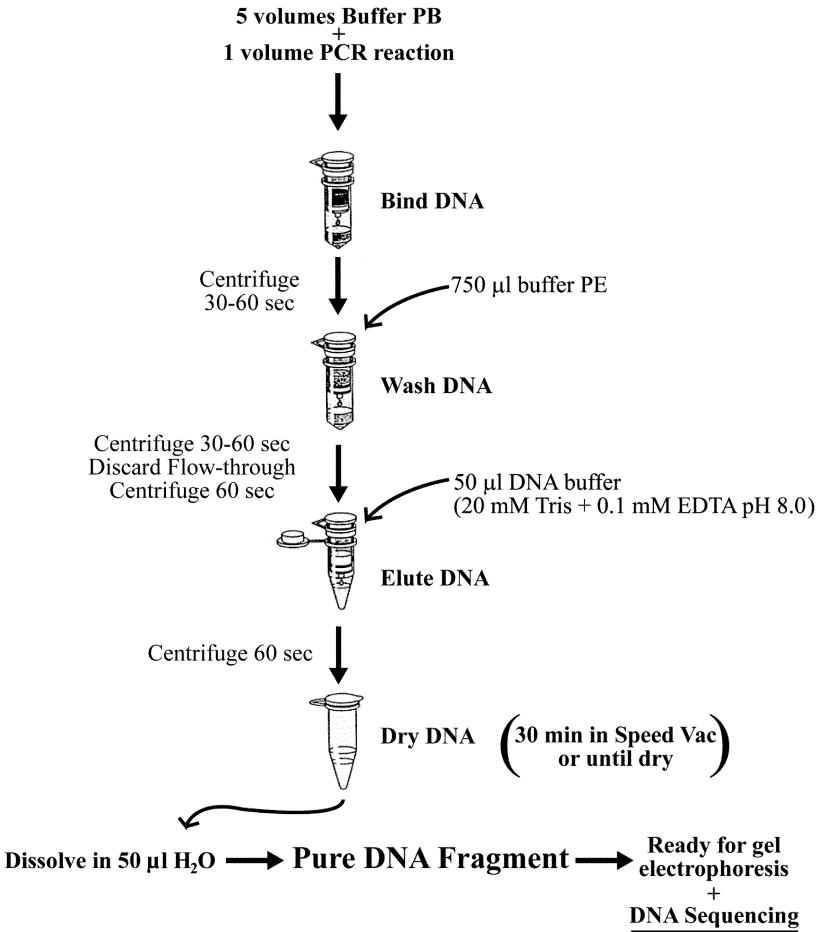


FIGURE 3.3

Purification of PCR products with a QiaQuick cartridge.

between these procedures. First, the template for DNA synthesis is a specific DNA molecule, not a complex mixture. Second, only one primer oligonucleotide is added to a sequencing reaction. Third, sequencing reactions contain both standard deoxynucleoside triphosphates (dNTPs) and dideoxynucleoside triphosphates

(ddNTPs; Fig. 3.4), which lack the 3′-hydroxyl group required for continued DNA synthesis (Fig. 3.5). Thus, incorporation of a ddNTP into a growing DNA chain prevents further DNA synthesis because there is no free 3′-OH group to form a phosphodiester bond with the 5′-phosphate of the next dNTP (Fig. 3.5). Finally, either the primer or ddNTPs ("terminators") are labeled with fluorescent molecules (Fig. 3.6).

In the "dye terminator" method that we will use, each ddNTP is labeled with a fluor that emits a different wavelength (color) of light (Fig. 3.6). The ratio of dNTPs to ddNTPs is adjusted so that adequate DNA synthesis occurs, but very few synthesis reactions will continue to the end of the template. The result is a population of DNA molecules that vary in length and extend from the primer at the 5′ end to a labeled ddNTP at the 3′ end. Ideally, the shortest molecule will contain the primer with a single ddNTP added to the 3′ end. The synthesis products should also contain molecules that terminated

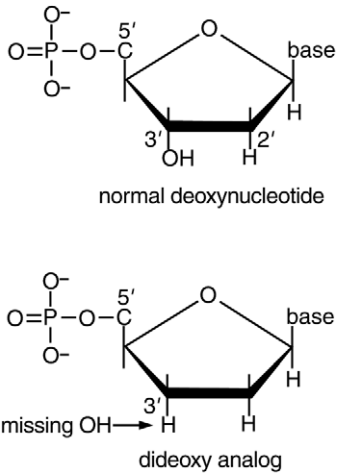


FIGURE 3.4

Chemical structures of normal and dideoxy nucleotides.

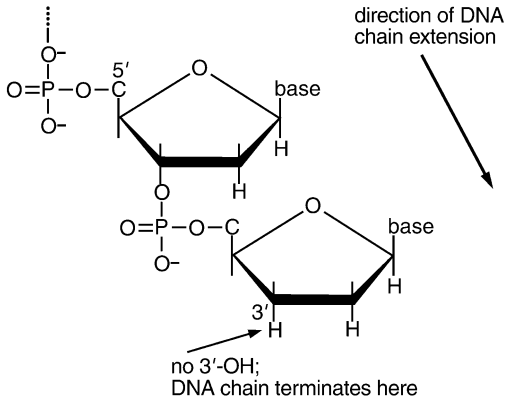


FIGURE 3.5

DNA chain termination by dideoxynucleotide incorporation.

at the second, third, fourth, fifth, and sixth incorporated bases and so on until the end of the template is reached (Fig. 3.6). The newly synthesized DNAs are denatured and separated according to their length by electrophoresis through long (~1 m) thin (~0.07 mm) polyacrylamide–urea gels, which are capable of resolving single-stranded DNA molecules that differ in length by a single nucleotide. As each DNA band passes a light source–detector at the bottom of the gel, the color and intensity of the band are recorded and plotted as a four-color electropherogram (Fig. 3.7).

In practice, the success of a sequencing reaction depends on the reagents, primer, reaction conditions, and the amount, length, sequence, and quality of the template. A sequencing reaction that yields 900 nucleotides of readable sequence is considered excellent; many sequencing facilities provide about 600 bases of sequence from a typical reaction.

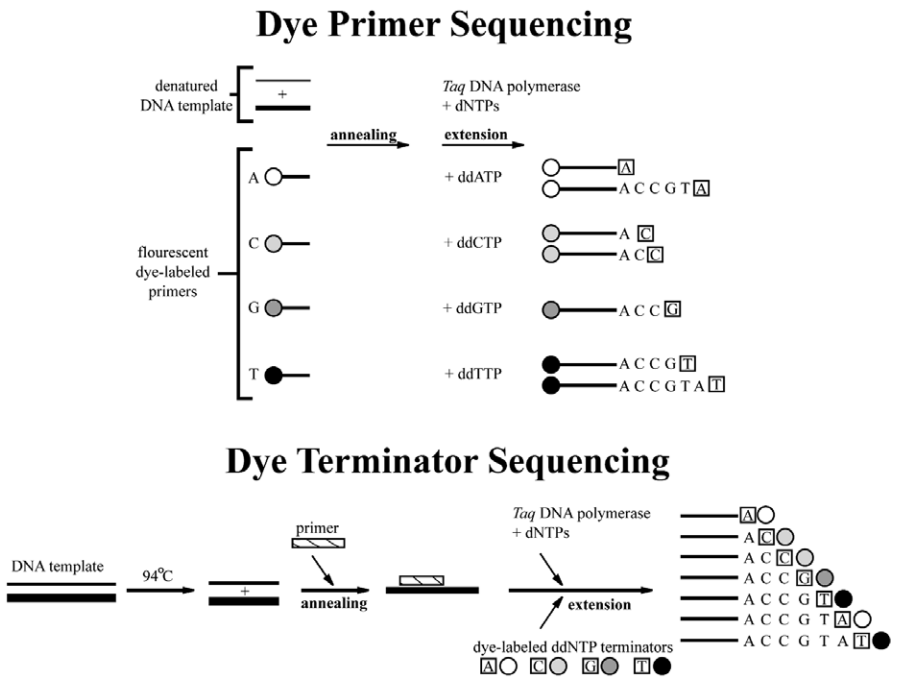


FIGURE 3.6

Dye primer and dye terminator DNA sequencing.

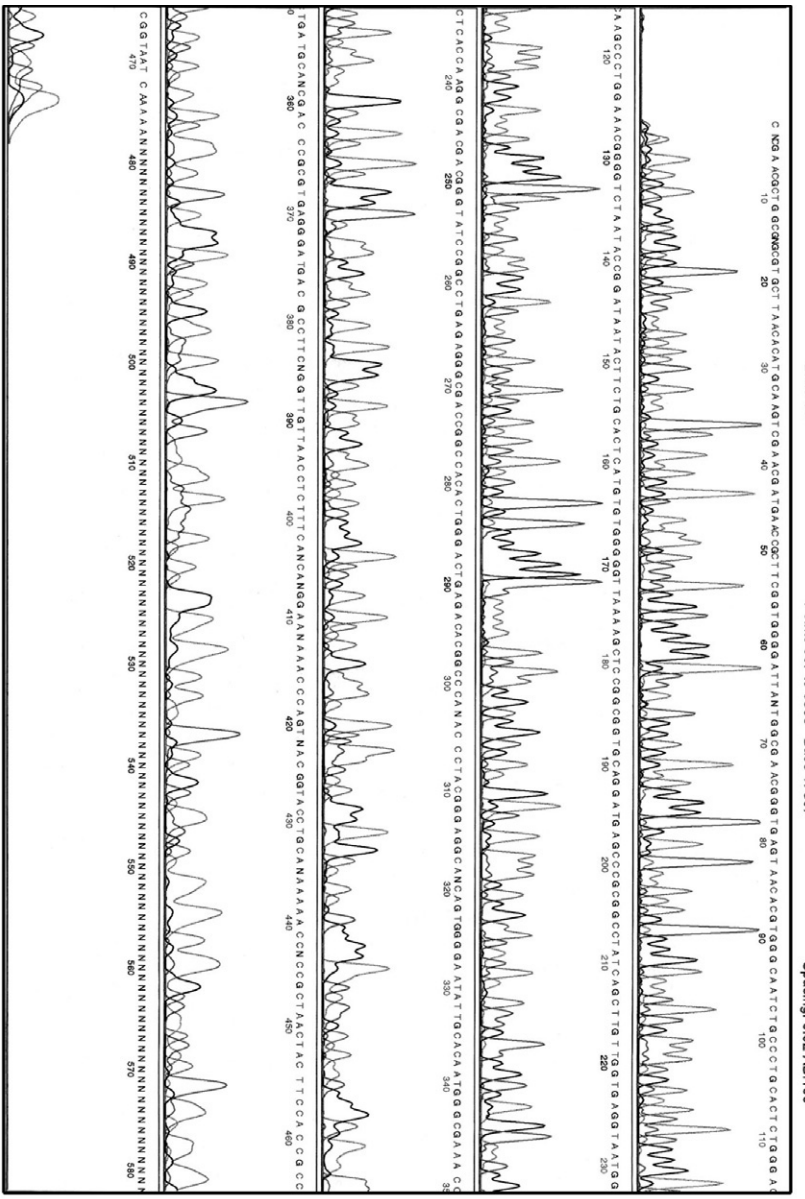


FIGURE 3.7

Partial 16S rRNA gene sequence from a *Streptomyces* species.

III. PROCEDURE

A. Isolate a Bacterium (Class 8)

Collect a sample from an environment that you believe will yield bacteria of interest to you and your colleagues. Select your collection sites carefully; in one of your writing assignments you must justify your choices. From the population of bacteria that you isolate, you will select one species to grow in pure culture. Next, you will purify genomic DNA from these cells and use the DNA as a template for PCR.

As you think about the experimental design, keep in mind that some of the bacterial species present in your sample will **not** grow on LB agar. Other species will grow poorly on this medium; you should avoid isolates that do not grow vigorously. Consider how the inability to culture all of the bacteria from a particular environment will affect interpretation of your data. Most environmental microbiologists studying bacterial populations in natural environments do not attempt to grow bacteria in culture. Instead, they extract DNA directly from the mixture of bacteria present in the sample. The extraction procedures used depend on the properties of the sample, so this approach is not feasible for this course. In addition, we want to teach you a reliable method to extract genomic DNA from a variety of bacterial species. We also want you to realize that this is not the approach an environmental microbiologist would use to learn about bacterial populations.

1. Label two LB agar plates with your name and two different sites where you plan to collect samples.

2. Use sterile cotton swabs to collect samples. If you swab a dry surface, first wet the swab with sterile water or LB broth. You may go outside the lab or

building to collect your sample. Remove the swab from its container, collect the sample, then streak it repeatedly across one edge of the LB agar. Return to the lab and complete streaking the plate with sterile toothpicks, wooden sticks, or an inoculating loop. Make three additional streaks, each with a fresh toothpick; this will create three "dilutions" of your original sample. Work from one edge of the original streak to make the second streak, make the third streak from the edge of the second, and so forth. (If you use a loop, touch it to an uninoculated portion of the agar to cool the loop before you allow it to contact the sample. After you finish, place the wire below the loop in a flame and allow transferred heat to kill bacteria in the loop. If you put the wet loop directly in the flame, you may create an aerosol of live bacteria if the sample "pops." Personally, I prefer toothpicks.)

3. Incubate the LB agar plates at an appropriate temperature. Bacteria from soil or water will probably grow best at room temperature or 25°C, whereas samples from the human body should be incubated at 37°C. Incubate your plates overnight and **examine them the next day** at your convenience. Think of step 4 as homework.

4. **One day after class 8:** Write a brief description of the bacterial (and fungal) colonies on your plates. Note the color, shape, size, and abundance of each colony type. Choose two different isolated bacterial colonies and use sterile toothpicks to streak each on fresh LB agar. Use at least three toothpicks so that you can spread the inoculum enough to produce single colonies. Incubate the streaked plates overnight at the proper temperature. **Two days after class 8** the TAs will move your streaked plates to the cold room.

   Handle these cultures (and all subcultures) as though they are human pathogens. They may be pathogenic, particularly if you sampled an environment conducive

to growth of human pathogens. Avoid all contact with the bacteria, and autoclave all cultures, supernatants, and contaminated tubes, pipettes, and tips.

5. **Class 9:** Use an isolated colony from each streak to inoculate LB broth.

   a. Label two sterile test tubes with the sample source and your name.

   b. Pipette 3 ml of LB broth into each tube.

   c. Use aseptic technique to inoculate each tube with an isolated colony from the streaked plates. You may use sterile inoculating sticks, toothpicks, or a loop. (I prefer sticks.) Note the locations of the two colonies you picked, and leave a portion of each colony on the plate so that you can transfer the cells to a microscope slide (see Section B).

   d. Incubate your cultures overnight at the appropriate temperature with vigorous aeration (shaking). The **next day** the TAs will move the cultures to the cold room. During class 10, you will select your favorite culture, freeze 1 ml of it, and prepare genomic DNA from 1.5 ml of culture.

B. Gram Stain and Light Microscopy (Class 9)

1. Prepare smears on clean microscope slides using a few cells from the isolated colonies that you inoculated into broth.
2. Air-dry and heat-fix the smears.
3. Place the slides in a staining rack, immerse them in crystal violet for 1 min and then rinse the slide with distilled water and drain.
4. Immerse the slides in iodine for 1 min, then rinse and blot dry.
5. Decolorize with 95% ethanol for 15 s, then rinse with distilled water and drain.
6. Counterstain with safranin for 20–30 s and then rinse, blot dry, and examine under a 100× oil immersion lens. Describe (in writing) the morphology of the bacteria; draw diagrams if necessary. Are they rods, cocci, or another shape? Do the cells form filaments or clusters? How large are the cells? Did the bacteria take the Gram stain? Leave this writing assignment on your bench at the end of class.

   Focusing a light microscope involves several steps. Place the slide on the stage and move it so that a promising area is over the light source. Move the low-power (10×) objective lens close to the slide. Raise the lens with the coarse adjustment knob until the specimen comes into focus. Put a drop of oil directly over the light path through the slide. Shift the oil immersion lens (100×) into place; the lens should barely touch the oil on the slide. Use the fine adjustment knob to focus on the specimen. Use the stage control knobs to find optimal viewing areas. Clean the oil immersion lens with lens paper when you finish.

7. If you isolated bacteria from soil, stain a sample with malachite green to look for spores. First, smear bacteria on a clean microscope slide, then air-dry and heat-fix the smears.
8. Place the slides over a beaker of boiling water and cover the specimen with malachite green. Steam for 5 min. Add additional stain if it boils off.
9. Wait until the slide cools, then rinse with distilled water for 30 s.
10. Counterstain with safranin for 20 s, then rinse with distilled water, blot dry, and observe under an oil immersion lens. Spores appear intense green, whereas cells stain red.

C. Freeze Cultures (Class 10)

You will complete experiment 3 with a single isolate. Choose your favorite culture. If possible, pick a culture that has grown to a high density, and avoid cultures that form clumps of cells. Transfer 1 ml of culture to a sterile, 1-dram screw-cap vial that contains 0.2 ml of dimethyl sulfoxide (DMSO). Shake the vial to mix the culture with the DMSO. Label the vial with your name and sample identification number. Lab tape will adhere to a glass vial in the freezer if you wrap it around the entire vial so that the ends overlap. Place the sample in the freezer box, which is stored at –80°C. Note the location of your specimen in the box. The cultures will remain viable indefinitely. Although it should not be necessary for this class, you can start a fresh culture by inoculating broth with a small chunk of frozen culture scraped from the frozen stock with a sterile stick. Do not allow the freezer stock to thaw. Place the remaining culture in a labeled, 1.5-ml microcentrifuge tube and purify genomic DNA from these cells (see Section III.D).

D. Purify Genomic DNA (Class 10)

1. Transfer 1.5 ml of your favorite culture to a 1.5-ml microcentrifuge tube. You will only characterize one of your isolates, so pick your favorite.
2. Centrifuge at maximum speed for 1 min at room temperature. Discard the supernatant safely in a container that will be autoclaved.
3. Suspend the cells in 450 µl of 25 m*M* Tris + 10 m*M* EDTA, pH 8; make certain the pellet is completely dispersed.
4. Add 20 µl of lysozyme (30 mg/ml); dissolve lysozyme in 25 m*M* Tris, pH 8, immediately before use. Incubate for 20 min at 37°C.
5. Add 10 µl of proteinase K (50 mg/ml; dissolve in distilled water immediately before use); incubate for 20 min at 50°C.
6. Add 20 µl of 25% SDS; incubate for 10 min at 68°C.
7. Add 57 µl of 5 *M* NaCl; vortex at full power for 1 min.
8. Incubate for 5 min at 68°C; vortex at full power for 1 min.
9. Add 0.5 ml of phenol–chloroform–isoamyl alcohol (25:24:1) equilibrated with 1 *M* Tris, pH 8. Mix well. Avoid all contact with these organic solvents and do not inhale vapors; work in a fume hood. Lab coats, gloves, eye protection, closed shoes (no sandals), and long pants (no shorts) are required. If phenol contacts your skin, wash thoroughly with water and seek medical attention.
10. Centrifuge at maximum speed in a microcentrifuge for 5 min at room temperature.
11. Transfer the aqueous (top) phase into a clean 1.5-ml microcentrifuge tube that contains 0.5 ml of chloroform–isoamyl alcohol (24:1). As you remove the top

phase, take care to leave proteins and other cell debris at the interphase. DNA attached to the membrane will tug at material in the interphase as you withdraw the aqueous phase. Discard organic solvents in an organic waste container.

12. Mix well. Centrifuge at maximum speed in a microcentrifuge for 2 min at room temperature.

13. Transfer the aqueous (top) phase into a clean 1.5-ml microcentrifuge tube. As you remove the top phase, take care to leave proteins and other cell debris at the interphase. Discard organic solvents in an organic waste container.

14. Add 1 ml of ethanol (ice cold), mix thoroughly, and hold at 0 or 4°C for 15 min.

15. Centrifuge for 5 min at maximum speed in a microcentrifuge; remember to orient the tube in the centrifuge rotor so that you know where the pellet will form. Room temperature is fine. Discard the supernatant.

16. Wash the DNA–RNA pellet with 0.5 ml of 70% ethanol. Let the tube stand for 1 min; do not disturb the pellet.

17. Centrifuge at maximum speed for 2 min at room temperature. Discard the supernatant carefully; the pellet will not stick tightly to the tube and may come loose.

18. Centrifuge the tube for 10 s to bring the residual ethanol to the bottom of the tube. Use a clean, sterile yellow pipette tip to remove all traces of ethanol, which inhibits PCR. Avoid the pellet; remember to orient the tube in the centrifuge rotor so that you know where the pellet will form.

Place tubes with lids open in a Speed Vac (a heated low-speed centrifuge connected to a vacuum system) for 5 min or you can air-dry the pellet.

19. Dissolve the DNA–RNA pellet in 50 µl of DNA buffer. Store frozen at –20°C.

E. PCR Amplification of 16S rRNA Genes (Class 11)

1. The teaching assistants will supply each student with a tube that contains 190 μl of PCR reaction mixture, which includes all components except template DNA. When you set up your PCR reaction, wear clean gloves and use pipette tips that have aerosol barrier filters. Note that P20 and P200 pipettors require different barrier tips. These precautions will help you avoid contaminating your PCR reactions with extraneous DNA.

2. Place 95 μl of reaction mixture in each of two 0.2-ml PCR reaction tubes. Handle these tubes gently; they have thin walls to permit rapid heat transfer, and this makes them fragile. Label these tubes only at the top of the conical portion, do not label the lid. Markings on the lid will burn off during the PCR.

3. Add 5 μl of template DNA to one PCR reaction mixture and mix well. Add 5 μl of sterile distilled water to the other PCR reaction and mix well; this is your no-template control, which will allow you to detect extraneous DNA in the PCR reagents. Store these tubes on ice until the entire class is ready to load the PCR machine.
   Each PCR reaction should contain:

10× buffer	10 μl
2 m*M* (each) dNTP mix	10 μl
25 m*M* $MgCl^2$	6 μl
50% acetamide	10 μl
27F primer, 10 μ*M*	2 μl
519R primer, 10 μ*M*	2 μl
Taq DNA polymerase	0.5 μl (2.5 units)
template DNA	100 ng (about 1–10 μl)
water	amount required to bring total volume to 100 μl

Primer sequences:

27F: 5′ AGA GTT TGA TC(C/A) TGG CTC AG 3′

519R: 5′ G(T/A)A TTA CCG CGG C(T/G)G CTG 3′

The *E. coli* 16S rRNA gene is about 1600 bp long. Most other organisms have 16S rRNA genes of similar length (±100 bp). The primers are numbered according to the *E. coli* gene. Therefore, this PCR will amplify a product approximately 529 bp long derived from the 5' end of the gene. This region is particularly informative.

Acetamide helps maintain single-stranded DNA and is useful for templates with high GC contents, which is typical for 16S rRNA genes.

4. Some PCR thermal cyclers have a heated lid, which prevents condensation of water on the lid of the PCR reaction tube. This obviates the need to overlay the sample with mineral oil, which is used to prevent sample evaporation in machines without a heated lid.

5. Cycle at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. Repeat for 35 cycles. Note where your tubes are in the heat block. The TA will remove the reactions upon completion and store them frozen until the next lab period.

F. Purify PCR Product (Class 12)

1. Transfer 10 µl of each completed PCR reaction into a clean microcentrifuge tube and store the samples at 4°C. Save samples from your PCR reaction and the no-template control. Later today you will examine these samples by agarose gel electrophoresis to estimate the yield of amplified DNA and to determine whether the no-template control indicates the presence of contaminating DNA in the reagents.
2. DNA sequence analysis of a PCR product requires removal of the other components of the PCR reaction. We will use cartridges (from Qiagen) that contain a silica membrane, which binds DNA in the presence of a high concentration of a chaotropic salt (a proprietary reagent, called buffer PB, supplied with the kit).
3. Mix the completed PCR reaction with 5 volumes (0.5 ml) of buffer PB. Pipette the mixture into the center of the cartridge. Make certain that you load the sample in the center. Centrifuge at full speed for 1 min, then discard the filtrate.
4. Add 750 µl of buffer PE (a proprietary wash buffer containing 70% ethanol). Centrifuge for 1 min, then discard the filtrate.
5. Centrifuge again for 1 min to remove all traces of ethanol.
6. Transfer the cartridge to a fresh microcentrifuge tube, then add 50 µl of DNA buffer (20 m*M* Tris, 0.1 m*M* EDTA, pH 8). Pipette the elution buffer onto the center of the membrane. Let stand for 5 min at room temperature. Centrifuge for 1 min.
7. Place samples in a Speed Vac for 30 min to remove all traces of ethanol. Bring the volume to 50 µl with distilled water.

G. Electrophoretic Analysis of PCR Product (Class 13)

1. Remove 5 μl of purified PCR product to estimate yield by agarose gel electrophoresis. Also examine 10 μl each of the unpurified PCR reaction and the negative control reaction. Include a known quantity (470 ng in 4 μl) of the low DNA mass ladder (Gibco/BRL) in separate lanes so that you can estimate the amount and size of your PCR product. Mix the DNA samples with 1 μl of loading solution (50% glycerol + 0.05% bromophenol blue); remember to include the two 10-μl samples (unpurified PCR product and negative control PCR reaction) you removed prior to purification.

2. Cast and load a 2% agarose gel (use 3:1 agarose from ISC Bio Express) for electrophoresis essentially as described in Experiment 1, Section III.C. The PCR products you want to analyze in this unit are smaller than the restriction fragments you studied previously. For optimum resolution of shorter DNA fragments, use a higher percentage of agarose (2% instead of 0.8%) and a different type of agarose (3:1 instead of standard).

H. DNA Sequence Analysis (Class 13)

The OSU Center for Gene Research Central Services Lab will perform the DNA sequence analysis. To sequence a 500-bp PCR product, they need 25 ng of template DNA mixed with 12 pmol of one primer in 12 μl of water. Normally you would sequence both strands of the DNA molecule to ensure that the sequence is correct. To sequence both strands of the PCR product, you would supply the sequencing facility with two template–primer stocks: one with 12 pmol of the 27F primer plus 25 ng of purified PCR product and another with the 519R primer plus 25 ng of PCR product. To save money, we will sequence only one strand of each template; use 12 pmol of the 27F primer oligonucleotide.

You will examine the DNA sequence results manually to look for ambiguities. If you had sequence data from both strands, you would use the sequence of one strand to resolve ambiguities in the other. After you have confirmed and corrected the sequence data, you will perform a computer search of the GenBank database to identify the bacterium you isolated. One program, called BLAST (Basic Local Alignment Search Tool), can search any database for either DNA or protein sequences from any source.

1. Use Netscape to reach BLAST at http://www.ncbi.nlm.nih.gov/BLAST/
2. Click on "Basic BLAST Search."
3. Leave the "Program" window set to "blastn" to search for a nucleotide sequence. Leave the "Database" window set to "nr" to search all nonredundant databases. (If you want an explanation of these options, click on the "Program" or "Database" button.)
4. Enter your sequence in the box under "Sequence in FASTA format." You can type the sequence by hand, or you can copy a file (from a word processor, for

example) and paste it into this box. Enter only nucleotide sequence in this box; numbers and other text are not permitted, although blank spaces within the sequence do not matter.

5. Click on "Submit Query."

Although you will not need this information here, you can perform literature database, GenBank, and BLAST searches from http://www.ncbi.nlm.nih.gov/

IV. LABORATORY REPORT

Include the following in your laboratory report:

1. Describe the source of your bacterium and the colony types seen on the initial streak plates, especially the one selected for DNA analysis. How rapidly did they grow under the conditions you used?
2. Report the changes that occurred during each step of the DNA isolation. When did the cells appear to lyse?
3. Was your PCR amplification successful? How many different PCR products did you detect by gel electrophoresis? How many did you expect? Did you see any products in the "no-template" negative control reaction? If your PCR reaction did not produce a detectable product, discuss possible explanations.
4. Use semilogarithmic graph paper (Fig. 3.8) to plot base pairs (log scale, *y* axis) versus distance migrated (linear scale, *x* axis) for the DNA mass ladder standards (Fig. 3.9) that you included on your gel. Include this graph in the results section. Measure the distances migrated from the bottom of the wells to the front edges of the bands. Estimate the size of your PCR product by comparing its migration distance to the standard curve. Indicate on your curve where the PCR product migrated. Include a photocopy of your gel. If your gel did not work, use one from another group or the example in Fig. 3.10.
5. Estimate the quantity of product in your PCR reaction before and after purification. Compare the intensity of the PCR product band with those of the DNA mass ladder markers on your gel. The amount of DNA in each band of the DNA mass ladder is shown in Fig. 3.9. The intensity (brightness) of each band is directly proportional to the amount of DNA the band contains.

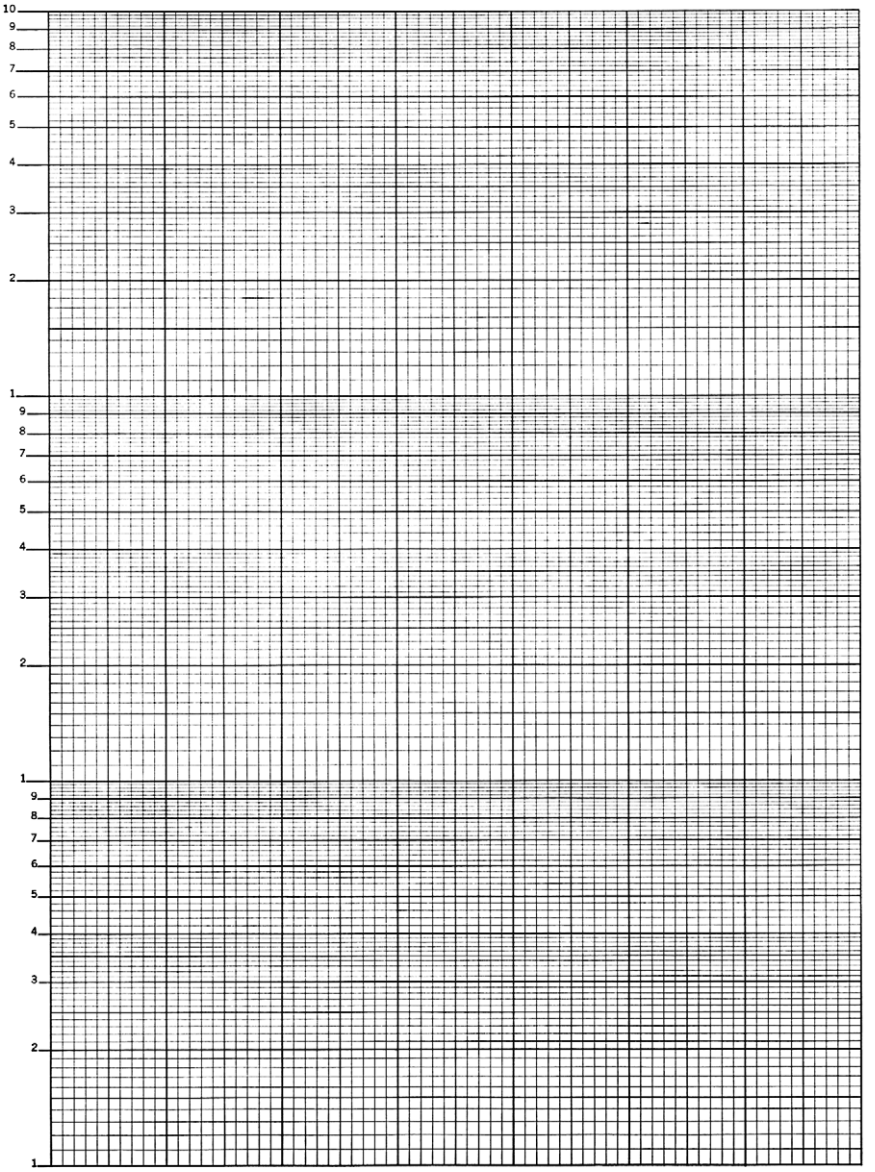


FIGURE 3.8

Semilogarithmic graph paper.

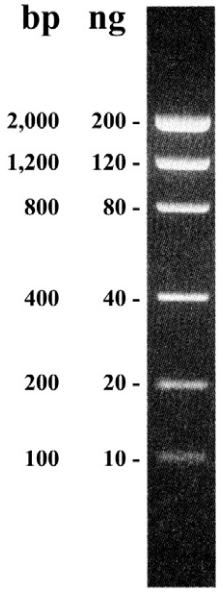


FIGURE 3.9

DNA mass ladder molecular weight standards.

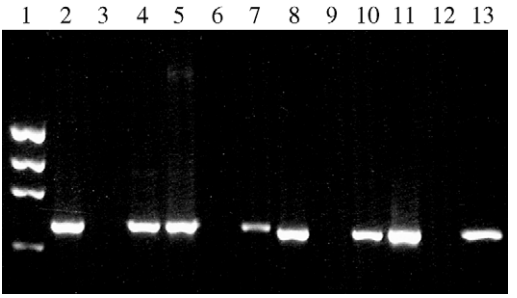


FIGURE 3.10

Agarose gel electrophoresis of PCR products. Lane 1 contains 4 μl (470 ng) of DNA mass ladder (Fig. 3.9). Lanes 2, 5, 8, and 11 contain unpurified PCR product from experiment 3. Lanes 4, 7, 10, and 13 contain purified PCR product recovered from the samples in lanes 5, 8, 11, and 2, respectively. Note the excellent recovery in lane 4 and the poor recovery in lane 7. Lanes 3, 6, 9, and 12 contain samples from no-template PCR reactions.

6. Estimate the yield (percent recovery) of purified PCR product. In step 5 you estimated the amount of PCR product present before and after purification. Simply divide the amount of purified product by the amount in the PCR reaction prior to purification. These numbers are directly comparable because you loaded 10% of each sample (10 µl of the original 100-µl PCR reaction and 5-µl of the 50-µl final volume of purified product). Show your calculation.

7. Based on your answers to the previous two questions, calculate the final concentration (in ng/µl) of your purified PCR product. Show your calculation.

8. Report the DNA sequence of the 16S rRNA gene from the bacterium you isolated. Were there ambiguities (Ns) in the sequence? When the DNA sequencing machine cannot identify the correct base at a particular position in a DNA sequence, it places the letter "N" at that position. If so, did you resolve them? If so, how? If not, what would you do next to resolve the ambiguities? Identify this organism based on the sequence.

V. QUESTIONS

1. What were the purposes of lysozyme, proteinase K, SDS, and EDTA in the DNA preparation?

2. Why did you perform a PCR reaction without added DNA? What do you conclude from your results?

3. Assume that the average molecular weight of a deoxynucleotide is 330 and that 1 absorbance (OD) unit at 260 nm indicates a concentration of 33 μg/ml for a short, single-stranded oligonucleotide (the figure is 50 μg /ml for double-stranded DNA). You receive a 20-nucleotide primer oligonucleotide from the DNA synthesis facility as a dry powder, which you dissolve in 1 ml of water. You pipette 5 μl of this into 295 μl of water and measure the absorbance at 260 nm; the reading is 2.000. What is the concentration of the primer stock? How much must you dilute it to make a 10 μ*M* stock for use in PCR? Show your calculations.

4. How many nanograms of each PCR primer constitutes 12 pmol? How many micrograms/milliliter of each primer does a 10 μ*M* stock solution contain? Show your calculations.

5. The following formula will allow you to estimate the melting temperature (T_m) of each primer that you used in your PCR.

$$T_m = 16.6 \log[\text{Na}] + 0.41(\%\ \text{G+C}) + 81.5 - 500/\text{bp}$$

where (%G+C) is the percentage expressed as a whole number (e.g., 50), not a fraction (e.g., 0.5), [Na] is the molar salt concentration, and bp indicates oligonucleotide length in base pairs. Assume the salt concentration in your PCR was 50 m*M* and calculate the T_m for each primer. Show your calculations.

6. PCR reactions can fail for several reasons. One or more of the reagents (*Taq* DNA polymerase, dNTPs, reaction buffer, or primers) may be faulty or present at the wrong concentration. Alternatively, the template DNA preparation may contain too little intact DNA, or the DNA may contain foreign substances (extracted from the specimen) that inhibit PCR. Finally, the sequences of the primer oligonucleotides may not complement the template sequences well enough to form stable duplexes under the reaction conditions used. How would you distinguish between these possibilities?

VI. IN-CLASS WRITING EXERCISE (CLASS 11)

Read the article by Borneman, J., and Triplett, E. W. (1997). Molecular Microbial Diversity in Soils from Eastern Amazonia: Evidence for Unusual Microorganisms and Microbial Population Shifts Associated with Deforestation. *Appl. Environ. Microbiol.* **63**: 2647–2653. Note that I have removed the abstract from your copy of this article. Of course you can find this paper in the library and copy the abstract, but that would defeat the purpose of this exercise. Other students have tried this, and I promise you that I **will** notice if you copy the original abstract. I will give you a copy of the abstract after you complete the following assignment.

1. Write an abstract for this paper.

2. What is the most significant difference between the procedure used by these authors to prepare template DNA and the method you used in Experiment 3? Explain why this difference is so important.

VII. WRITING ASSIGNMENT—PROPOSAL [CLASS 8 (DRAFT) AND CLASS 12]

We live in a world with finite resources. For every research project that we decide to pursue, there are others that we cannot perform because our resources are limited. Before you can do your research, you must convince your colleagues that it is worthwhile. Your proposal should begin with a general introduction so that your colleagues can understand the experimental system and its importance, even if they do not specialize in that area. Next, your proposal should ask a specific question that the research will answer, and it should indicate why we should care about the answer. Finally, the proposal should demonstrate how the study will answer the question. Most research is justified by direct benefits to people's lives. For example, can we find a species of *Streptomyces* that produces a novel antibiotic? Other research is justified only by curiosity. Can we find evidence of microbial life on Mars? Your research should be important, interesting, or both, and you must be able to explain this to others.

Write a brief (1- to 2-page) proposal to convince me that the unknown organism you isolated is interesting enough to warrant spending money to identify it by DNA sequence analysis.

VIII. PEER REVIEW OF PROPOSALS TO STUDY BACTERIAL ISOLATES

As a peer reviewer, comment on both presentation and content. Proposals must not contain typographical or grammatical errors. Sentences should express the author's thoughts clearly, and paragraphs should follow a logical train of thought. The document should have a neat, professional appearance.
Proposals need to address several issues:

1. What is the question?
2. Why should the reader care?
3. How will the work answer the question?

Reviewers must ask themselves whether the proposals they read answer these questions to their satisfaction. If they do not, suggest steps that the author can take to correct the deficiencies.

Note: Answers to the question, "Why should the reader care?" usually involve work of obvious importance to people's lives, work that addresses a fundamental issue in biology, or work of great general interest.

IX. SAMPLE PROPOSALS

A. An Outstanding Proposal

Streptomyces is a bacterial genus represented by over 500 species, a number of which are antibiotic producers. Some studies have reported that 50% of streptomycetes isolated are antibiotic producers. This genus is responsible for the manufacture of over 500 antibiotic substances (2), including chloramphenicol, erythromycin, neomycin, nystatin, streptomycin, and tetracycline (1, 3, 4), which play a major role in the treatment, control, and cure of human and animal diseases and which are used extensively for research (selectivity) in microbiological laboratories.

We have obtained a specimen isolated from pine soil that we believe is *Streptomyces* and have cross-streaked it with *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus* with excellent inhibition. This technique involves streaking one-third trypticase soy agar plate with the test organism and placing cross streaks of known bacterial species in close proximity to it. A clearing on the agar between the test and known bacteria indicates antibiotic production by the test organism.

The colony morphology, cell morphology, and earthy smell are all consistent with this organism. Also, a pathologist at Oregon State University examined the specimen under the microscope and stated that he believed it was *Streptomyces*.

Precise identification by DNA sequence analysis would tell us which strain we have isolated and, consequently, what antibiotics the strain generally produces. Chemical analysis is probably necessary to definitely identify these antibiotics. We request that funds be allotted for only the DNA sequence analysis at this time to help us narrow the range of possible antibiotics this organism is generating.

1. Madigan, M., Martinko, J., and Parker, J. (1997). "Biology of Microorganisms," 8th ed., p. 736. Upper Saddle River, NJ: Prentice-Hall Inc.

2. Mathews, C. K., and van Holde, K. E. (1996). "Biochemistry," 2nd ed., pp. 1026–1027. Menlo Park, CA: The Benjamin/Cummings Publishing Co.

3. Prescott, L. M., Harley, J. P., and Klein, D. A. (1996). "Microbiology," 4th ed., pp. 498–500. Dubuque, IA: Wm. C. Brown Publishers.

4. Stryer, L. (1995). "Biochemistry," 4th ed., pp. 902–903. New York: W.H. Freeman and Company.

B. A Poor Proposal

Polution: Man made? Natural?
A combination of both?

Who's t say that polution in todays world is a result of mankind. The possibility of naturally occuring polution I believe is out there. As can be seen with the human population, species population explossion is possible for any species. With the great increase of a population, be it humans, plants, or microorganisms, such as bacteria, the ecosystem in which they reside in is put to the limits of support. So, is todays polution problem strickly due to mankind? Or could it be a result of nature and mankind working together? If they are working together to create the polution, who's to say that they can not work together to clean it up.

The two environmental samples that were taken may in a way help to answer these questions, by way of a polymerase chain reaction (PCR) of the 16S rRNA of both samples. If any environmental influence, e it natural or man made, have caused chemical alterations, of any sort, it will be detecable at the variable region or V2 site. If the chemical alterations are found to have some degree of homology in bothe V2 sites then some factor that is the same for both samples has influance a mutation in the RNA sequance. The samples were taken from similar ecological nitches, aquatic, which contained very different ecosystems. The first sample was taken from a rock pit pool. The pool is located in solid rock and is stagnent unless it is raining, then naturally occring springs and rain water disterb it, as do animals that use it. With the spring water silt, clay is added along with organic matter from surrounding grass, trees, and brush. The pool was selected on the bases that humans were in no way involved in the ecosystem that it support or that the pool is apart of. The second sample was taken from a man made pond in an industrial park area. The only water source for the pond is rain water and rain

run off from surrounding areas. The pond is located on top of a mud, sand, and silt bottom and has various plant forms growing within along with fish. The ecosystem that the pond supports is in constent interaction with industrial by-products and humans as is the ecosystem that the pond is a apart of.

Perliminary tests, such as TNA plate cultivation and gram stain analysis has identified some unique characteristics of the microbes that were collected. Both plates cultured presented with very few and isolated colonies when incubated at 37 degrees celsius. Both sets of colonies showed small pin point colonies to about 2 mm in diameter. The colonies that developed from the rock pit pool were visibly a cloudy white in color with some clear spots contain within. The colonies from the pond showed a yellowish colored colonies with a slight pink tint to them. These colonies also presented a distinc odor when the plate lid was lifted. Microscopic analysis of the the gram stained cultures showd great similarity between to two cultures. Both cultures were gram positive bacillus with dipolar red spots at each end. The cells from the pond were in collections forming chains and also individual cells. The rock pit cells were only presented as individual cells. For pure culture isolation the two samples were inoculated to nutrient broth and incubated. The culture from the pond was placed at 37 degrees celsius and the rock pit culture was placed at 30 degrees celsius. The rock pit sample was initialy grown at 37 degree celsius and due to decreased growth and the cool environment that it was isolated from it was decided to place this culture at a lower temperature for further incubation in hopes of collecting a greater quantity of cells.

Because the samples were taken from two completely different ecosystems, with different human interaction levels, any homology in the V2 site on the 16S rRNA could be due to polution. With the source of any polution being different for both samples, the homology would suggest that yes polution could be man made, natural, or even a combination of both.

NOTES

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EXPERIMENT
4

Southern Blot Analysis of Bacterial rRNA Genes

I. INTRODUCTION

In this unit, you will use the Southern blot procedure to examine the rRNA genes in the bacterial genomic DNA that you prepared in Experiment 3. You will determine the number of restriction fragments that contain rRNA genes and estimate their sizes. You will also determine how much genomic DNA your preparation contained. If your PCR reaction failed (in Experiment 3), this experiment will indicate whether lack of template DNA was the problem. First, you will digest the genomic DNA that you prepared in Experiment 3 with a restriction endonuclease. Next, you will perform agarose gel electrophoresis to separate the restriction fragments according to their size. After you photograph the gel, you will denature the DNA *in situ* and then transfer (blot) it to a nylon membrane. Finally, you will prepare a labeled hybridization probe and use it to detect rRNA genes bound to the nylon filter.

II. BACKGROUND

A. Southern Blot Hybridization

The Southern blot procedure is one of the most important techniques used in molecular biology because it provides a simple means to examine a specific restriction fragment among a complex mixture of fragments that represents an entire genome. The procedure begins with techniques that you learned in Experiment 1: restriction endonuclease digestion and agarose gel electrophoresis. In this unit, you will digest genomic DNA rather than plasmid DNA. Due to the complexity of bacterial genomes, you will not see individual restriction fragments on the ethidium-stained agarose gel. Instead, you will detect particular

DNA fragments by hybridization with a labeled probe DNA that can form base pairs with (anneal to) the DNA sequence of interest.

Hybridization between probe and target DNAs can occur only if both are denatured. Double-stranded probe DNAs are boiled to separate the strands, and then the denatured DNA is chilled rapidly so that the complementary strands do not have an opportunity to reanneal. The DNA in the agarose gel cannot be denatured by boiling, which would melt the gel. Instead, the gel is soaked in NaOH to raise the pH high enough (pH ~ 13) to disrupt the hydrogen bonds that hold the strands together. After the target DNA is denatured, the gel is soaked in concentrated buffer to return the pH to neutral.

The denatured target DNA is transferred from the gel onto a nylon (or nitrocellulose) membrane. The capillary blot procedure is the simplest and least expensive means to transfer DNA from an agarose gel to a membrane. The gel is placed on a stack of filter paper (Whatman 3 mm) saturated with transfer solution, and the nylon membrane, also moistened with transfer solution, is laid on top of the gel. Another sheet of moistened Whatman filter paper protects the top of the nylon membrane, which must be kept scrupulously clean. Dry paper towels and a modest weight are added to the top of the stack. As the paper towels absorb the transfer solution, the DNA is deposited on the nylon membrane by capillary action. Electrophoretic or vacuum blotting devices increase the speed of transfer somewhat, but the final results are similar.

During capillary blotting, small DNA fragments transfer out of the agarose gel and onto the nylon membrane more readily than large fragments. For this reason, the procedure you will use includes a step that reduces the size of large restriction fragments in the gel just prior to blotting. The brief acid wash (Section III.C, step 1) removes a limited number of purine bases from the DNA. The next step, denaturation in NaOH, breaks DNA chains at depurinated sites, thereby reducing the length of the DNA fragments.

Blotting membranes are designed to trap DNA during capillary transfer and retain it throughout the subsequent hybridization and wash steps. Limited irradiation with ultraviolet (UV) light can improve DNA retention by covalently cross-linking the DNA to the membrane. This will increase the hybridization signal, provided that the cross-linking is not too extensive. Excessive cross-linking to the membrane will hinder access of the probe to complementary sequences in the target DNA.

B. Hybridization Probes

DNA (or RNA) probes usually contain either radioactive phosphorus (^{32}P) or a nonradioactive label such as digoxigenin (DIG), a steroid hapten (Fig. 4.1). Radioactive probes provide greater sensitivity and lower background than nonradioactive probes, but the logistics of handling radioisotopes safely preclude their use in a large laboratory class. To prepare a probe for bacterial 16S rRNA, you will include DIG-labeled dUTP in PCR reactions that amplify a portion of the *Escherichia coli* 16S rRNA gene. The PCR conditions will be similar to those used in Experiment 3, with two exceptions: (1) the template will be a plasmid that contains a rRNA gene, and (2) DIG-labeled dUTP will replace dTTP in the reaction cocktail.

A hybridization probe need not match the target sequence perfectly. The 16S rRNA genes present in the bacterial species you isolated from the environment will differ from the probe at some positions in the sequence, unless you isolated *E. coli* by coincidence. **Heteroduplex** DNA results when strands from similar DNA molecules anneal. If 1% of the bases in a heteroduplex are unpaired, the melting temperature (T_m) of the DNA molecule will be approximately 1.5°C lower than the T_m of the corresponding **homoduplex**. Hybridization incubations will be performed at approximately 25°C below T_m, which allows heteroduplex DNAs to form with up to 17% of

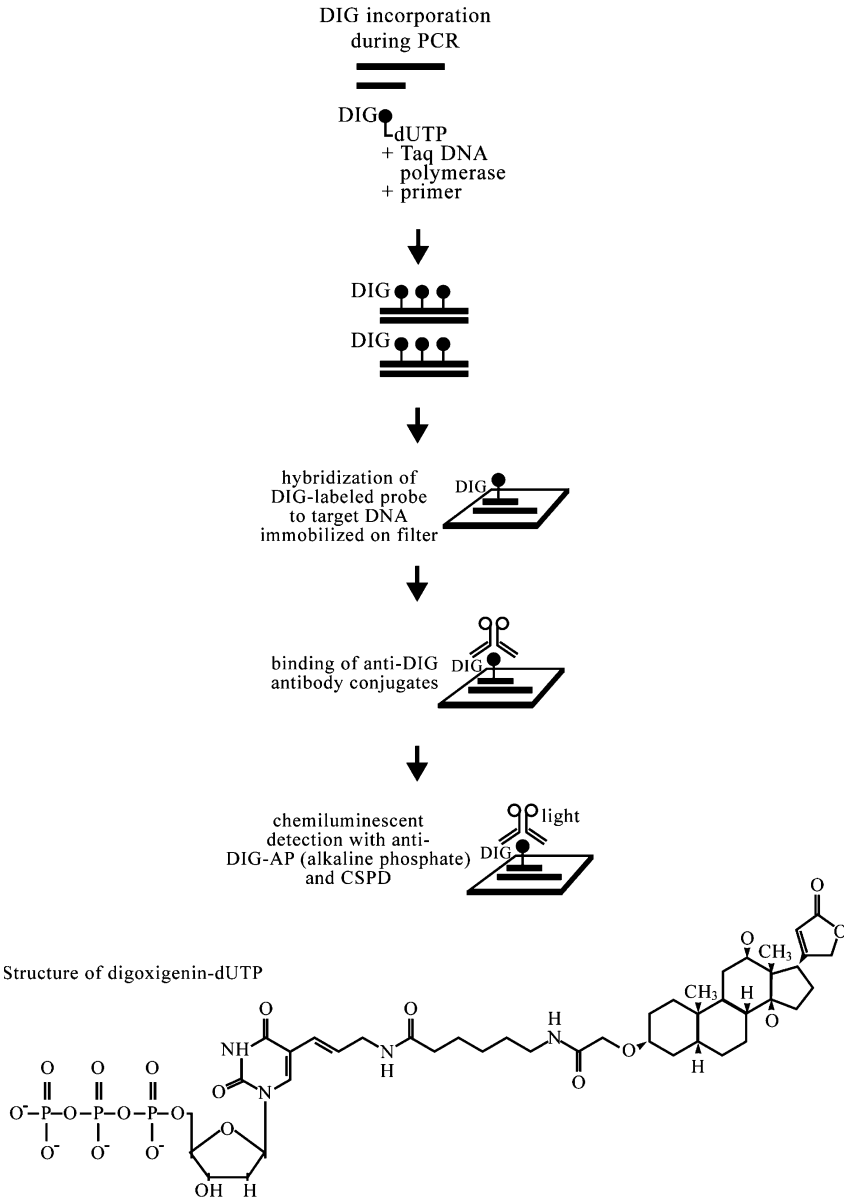

FIGURE 4.1

Chemical structure and use of digoxigenin.

the bases unpaired. In a Southern blot experiment, the **stringency** (the fraction of mismatched bases allowed between annealed probe and target DNA strands) is determined by the conditions under which the filter is washed after hybridization. Factors that affect T_m include salt concentration, the G+C content and length of the DNA sequence, and the fraction of unpaired bases. Use the following formula to estimate T_m:

$$T_m = 16.6 \log[\text{Na}] + 0.41\ (\%\ \text{G+C}) + 81.5 - 500/\text{bp},$$

where [Na] is the molar salt concentration, (%G+C) is the G/C content expressed as a whole number (i.e., for 50% G+C enter 50, not 0.5), and bp is base pairs. Washing stringency is adjusted by varying both salt concentration and temperature. Most protocols include both nonstringent and stringent washes. Which of the washes in Section III.F, steps 1–3, is the most stringent? (Remember that 20×SSC contains 3 *M* NaCl + 0.3 *M* Na citrate.) These washes will remove probe that is bound nonspecifically to genomic DNA on the filter. Only probe annealed to the target rRNA genes should remain bound.

Probe detection is the final step in our Southern blot experiment. You will use antibodies specific for digoxigenin to detect DIG-labeled probe that remains bound to rRNA genes. The antidigoxigenin antibodies are conjugated to alkaline phosphatase, which dephosphorylates a chemiluminescent substrate called CSPD (Fig. 4.2). (The chemical name is so cumbersome that I will not include it here.) Loss of the phosphate group destabilizes CSPD, which emits light at 477 nm as it breaks down. These light emissions occur at sites where anti-DIG antibodies have bound DIG-labeled probe annealed to target DNA sequences (rRNA genes in this case). You will use X-ray film to detect the chemiluminescence.

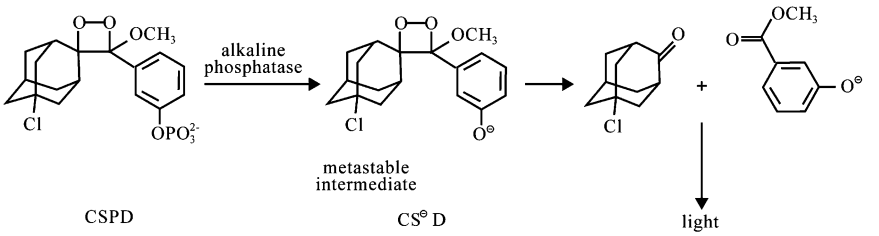

FIGURE 4.2

Chemiluminescence of CSPD.

III. PROCEDURE

A. Restriction of Genomic DNA (Class 15)

1. Place 5 µl of bacterial genomic DNA, which you saved from Experiment 3, into a microcentrifuge tube.
2. Add 6.5 µl of sterile distilled water, 1.5 µl of 10× *Eco*RI restriction buffer, and 1 µl of ribonuclease A + ribonuclease T1 mixture (5 mg/ml each). Mix.
3. Add 1 µl (10 units) of *Eco*RI restriction endonuclease. Mix, but do not introduce bubbles.
4. Incubate at 37 °C for 1 h.

B. Agarose Gel Electrophoresis (Class 15)

1. Pour an agarose gel, as described in Experiment 1, Section III.C.
2. Add 2 μl of loading solution to the restricted DNA and mix.
3. Load the entire sample into one lane of your agarose gel. Load phage λ DNA (500 ng) digested with *Hin*dIII into another lane on the agarose gel; this is the molecular weight standard.
4. Apply current until the bromophenol blue is near the bottom of the gel.
5. Place the gel on plastic wrap and photograph it under UV illumination. Place a ruler with fluorescent markings beside the gel so that you can measure the migration distance of each restriction fragment in the molecular weight standard. Keep the gel clean.
6. Wrap the gel with plastic wrap and store at 4°C until next class.

C. DNA Transfer by Blotting (Class 16)

1. Wash the gel (stored in plastic wrap at 4°C since last class) for 15 min in 0.25 *M* HCl; use 100 ml. Rinse the gel with distilled water after the acid wash.
2. Wash the gel twice for 15 min each in NaOH–NaCl solution (0.5 *M* NaOH + 1.5 *M* NaCl); use 100 ml per wash. Rinse the gel with distilled water after the second wash.
3. Wash the gel twice for 15 min in Tris–NaCl neutralization buffer (1 *M* Tris, pH 7.5, + 1.5 *M* NaCl); use 100 ml per wash.
4. Cut the nylon membrane to the same size as the gel. Use clean scissors or a new razor blade. Wear gloves

and use the liner sheet to keep the membrane clean. Mark one corner of your membrane with a soft pencil.

5. Float the membrane in a tray of distilled water to wet it by capillary action.

6. Soak the membrane in 20 ml of 10×SSC for 15 min. (10× SSC = saline–sodium citrate buffer: 1.5 *M* NaCl + 0.15 *M* sodium citrate, pH 7.0)

7. Cut eight sheets of Whatman 3-mm filter paper to the same size as the gel; saturate the filters with 10× SSC and place seven sheets on a large piece of plastic wrap.

8. Place the agarose gel on the SSC-saturated Whatman 3-mm paper. Invert the gel so the bottom face will be up (and in contact with the nylon membrane). Use finger pressure (wear clean gloves) to remove air bubbles trapped between the gel and filters.

9. Lay the nylon membrane on top of the gel with the pencil mark down; record which corner of the gel is aligned with the pencil mark on the filter. Once the membrane contacts the gel, do not move it, even if the gel and filter are not properly aligned. Use finger pressure to remove air bubbles.

10. Place one sheet of SSC-saturated Whatman 3-mm paper on top of the nylon membrane and remove air bubbles. Cover this with a 3-in. stack of dry paper towels (also cut to the same size as the gel). Wrap the entire stack in the plastic wrap, and set a modest weight on top of the paper towels.

11. Allow DNA transfer to continue until next class. Normal transfer time is 2–16 h; transfer is complete when the gel becomes 1 mm thick.

D. Probe Preparation (Class 16)

1. Mix the following components from the PCR DIG Probe Synthesis Kit (Roche catalog no. 1 636 090) in a sterile microcentrifuge tube. Keep the tube on ice, and use aerosol-resistant pipette tips.
   Add:

   5 µl of 10X PCR buffer (kit vial 3)
   5 µl of PCR DIG mix (kit vial 2)
   5 µl of 50% acetamide
   1 µl of 27F primer (10 µ*M*)
   1 µl of 519R primer (10 µ*M*)
   5 µl of template plasmid DNA (100 pg; the plasmid contains a 16S rRNA gene)
   27 µl of sterile distilled water
   0.75 µl of enzyme mix (kit vial 1)

2. Perform PCR as described in Section III.D of Experiment 3. Cycle at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. Repeat for 30 cycles. End with a 7-min incubation at 72°C for the final elongation step.
3. Analyze 10 µl of PCR product by agarose gel electrophoresis as described in Experiment 3, Section III.F.
4. Denature DIG-labeled probe by boiling for 5 min; chill in ice water and store frozen.

E. Hybridization (Class 17)

1. Wash the nylon filter for 20 min at room temperature in 50 ml of 0.2 *M* Tris, pH 7.5, + 2×SSC. Place filter, with pencil mark (DNA side) up, on dry Whatman 3-mm paper. Just as the filter begins to dry, irradiate it with 1200 μJ of UV light (using the Stratagene 1800 Stratalinker). This procedure links the DNA permanently to the membrane.
2. Incubate the filter with gentle agitation in 20 ml of DIG Easy Hyb solution (Roche catalog no. 1 603 558) at 42°C for 60 min. This blocking solution should be warmed to 42°C before use.
3. Boil 100 pg of DIG-labeled probe (approximately 5 μl of the PCR reaction) and add it to 2.5 ml of fresh DIG Easy Hyb solution (warmed to 42°C). Mix thoroughly, but do not create bubbles.
4. Remove the filter from the blocking solution and submerge it in 2.5 ml of probe–Easy Hyb mixture. Incubate at 42°C until next class.

F. Washing and Detection (Class 18)

1. Wash the filter twice in 50 ml of 2× SSC + 1% SDS; 5 min per wash. Agitate at 42°C.
2. Wash the filter twice in 50 ml of 2× SSC + 1% SDS; 15 min per wash. Agitate at 65°C.
3. Wash the filter twice in 50 ml of 0.1× SSC + 1% SDS; 15 min per wash. Agitate at 42°C.
4. Agitate the filter for 5 min at room temperature in 20 ml of washing buffer (0.1 *M* maleic acid + 0.15 *M* NaCl, pH 7.5, + 0.3% Tween 20).
5. Agitate the filter for 30 min at room temperature in 100 ml of Easy Hyb blocking solution.
6. Agitate the filter for 30 min at room temperature in 20 ml of antibody solution [anti-DIG–AP conjugate (kit vial 3) diluted 1:10,000 (to 75 mU/ml) in kit buffer 2].
7. Dilute CSPD (kit vial 5) 1:100 in detection buffer (0.1 *M* Tris + 0.1 *M* NaCl, pH 9.5).
8. Incubate the membrane in a sealed hybridization bag for 5 min at room temperature with 1–2 ml of diluted CSPD detection solution.
9. Remove the filter from detection solution and let the excess drip off the filter; blot the membrane briefly (DNA side up) on Whatman 3-mm paper. Do not allow the membrane to dry completely.
10. Seal the damp membrane in a hybridization bag and incubate for 5 min at 37°C.
11. Expose for 15 min at room temperature to X-ray film. Develop the film and examine the pattern of bands.

IV. LABORATORY REPORT

Include the following in your laboratory report:

1. Include the photograph of the ethidium-stained agarose gel in your report. Label each lane, and indicate their contents in the figure legend. Estimate the quantity of DNA present by comparing the total fluorescence of the genomic DNA with that of the molecular weight standard (500 ng of phage λ DNA). Assess whether the restriction endonuclease digested the DNA completely. Genomic DNA that has been digested with a restriction endonuclease will form a ladder of closely spaced bands that form a smear extending from the largest band to the bottom of the gel. The largest restriction fragments produced by a typical restriction endonuclease (with a 6-bp target sequence) usually range from 15,000 to 25,000 bp. In a lane that contains the proper amount of DNA, the "smear" will appear striated. This is due to the presence, at some positions, of multiple restriction fragments with the same electrophoretic mobility, which form brighter bands than surrounding single fragments. These striations disappear if the lane is overloaded. Because the largest restriction fragments are less abundant than smaller ones, the top of the smear should not appear brighter than the lower portions. If the top edge appears jagged rather than sharp, the restriction endonuclease did not cut all of its target sites in the genomic DNA. This is called a partial digest. Did each lane contain the proper amount (~2 μg) of genomic DNA, and was it digested to completion?
2. In the results section, include the developed blot as a figure. If your blot did not work, use the data in Fig. 4.3. Label each lane, and indicate their contents in the figure legend. How many different bands did you see? Measure the distance that each molecular weight

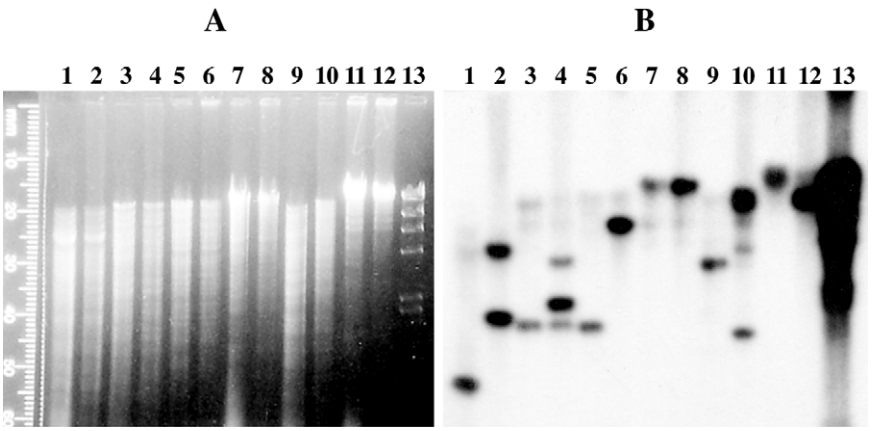

FIGURE 4.3

Southern blot analysis of 16S rRNA genes. Panel A shows a photograph of the ethidium-stained agarose gel. Panel B shows an X-ray film exposed to the blot, which was hybridized with a ^{32}P-labeled probe. Lanes 1–12 contain genomic DNA from *Agrobacterium tumefaciens*; these genomic DNAs were digested with *Eco*RI. Note that the DNA samples in lanes 7, 8, 11, and 12 were only partially cleaved by *Eco*RI, whereas DNA in the other lanes was digested completely. Lane 13 contains phage λ DNA digested with *Hin*dIII.

standard migrated, and do the same for each restriction fragment detected by the ribosomal DNA probe. On semilogarithmic graph paper (Fig. 4.4), plot the size (in base pairs) of each marker (log scale) versus the distance migrated (linear scale). Use this standard curve to estimate the size of each restriction fragment that contains an rRNA gene.

3. Discuss the copy number of rRNA genes in the bacterial species you analyzed.

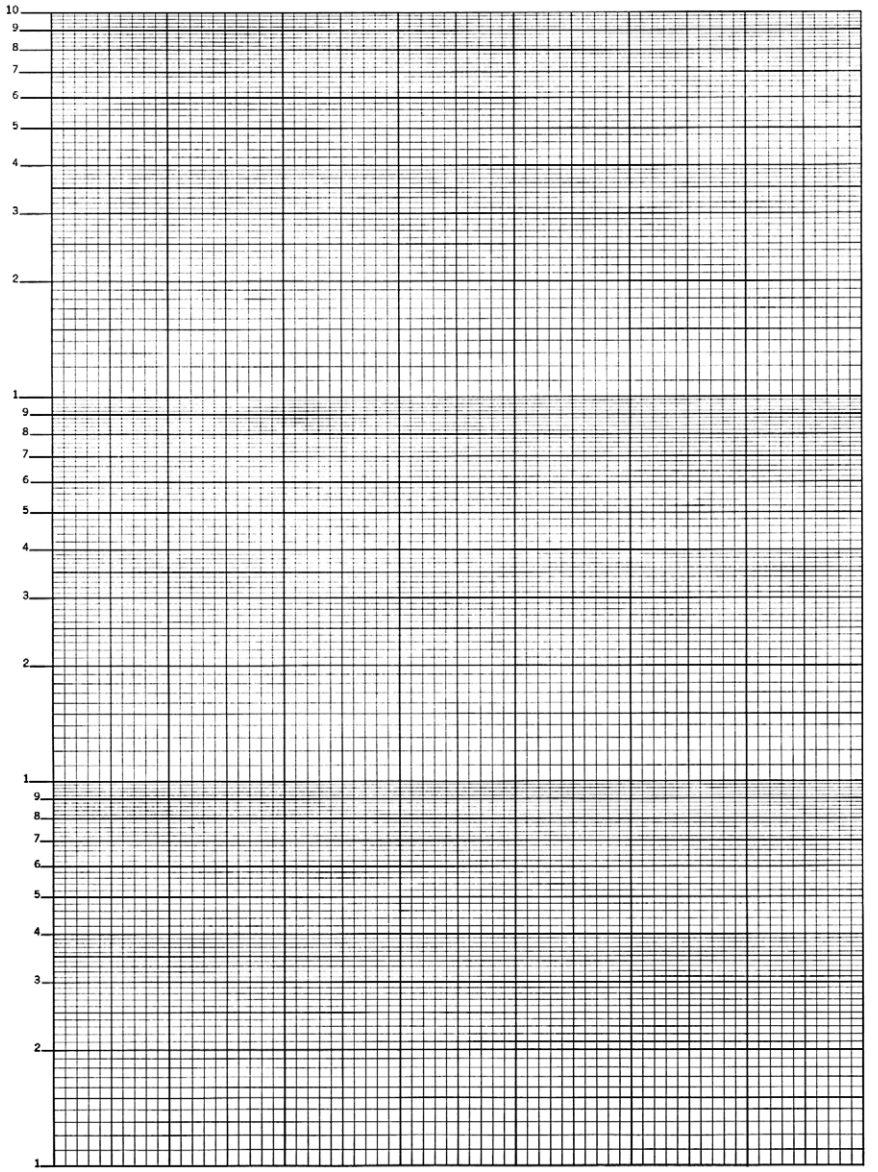

FIGURE 4.4

Semilogarithmic graph paper.

V. QUESTIONS

1. For each wash in Section III.F, steps 1–3, calculate the T_m of a 519-bp probe with a G+C content of 50% and perfect identity to the target sequence. Remember:

$$T_m = 16.6 \log[Na] + 0.41(\% \text{ G+C}) + 81.5 - 500/bp,$$

where % G+C is the percentage expressed as a whole number (e.g., 50), not a fraction (e.g., 0.5), [Na] is the molar salt concentration, and bp indicates probe length in base pairs. Show your calculations.

2. Which wash was the most stringent?

3. How many unpaired bases can a heteroduplex (formed with this probe) tolerate under the most stringent wash conditions?

4. Which was the least stringent wash?

5. Explain the purposes of the HCl, NaOH, and Tris washes that you used to prepare the agarose gel for blotting.

VI. WRITING EXERCISE—EDITORIAL ON GENETICALLY MODIFIED CROPS [CLASS 17 (DRAFT) AND CLASS 18]

Read the three editorials in *Nature*. These articles raise scientific and ethical issues, and they exemplify the critical role that scientific decisions play in everyday life. They also show that science does not operate in a vacuum devoid of politics or human considerations.

Write a brief (1- to 2-page) editorial on this issue. Support your opinion with facts from these articles and other sources. Begin with a brief summary of the problem. State your position on the controversy. Support your position by constructing arguments based on facts. Conclude the paper with a statement that summarizes your position.

Once you have written your article, read the newspaper editorial published by Norman Borlaug in the *Wall Street Journal*, December 6, 2000.

VII. IN-CLASS WRITING EXERCISE (CLASS 18)

The following questions refer to articles on genetically altered foods.

1. Do you think people from underdeveloped nations would have a different opinion on genetically modified foods than Europeans or Americans?

2. Do you think people are less likely to accept genetically engineered foods than genetically engineered medicines? Why?

3. Which values, ethical or scientific, are most important to you in this controversy? Rank them in terms of importance in your decision-making process.

NOTES

APPENDIX A: SAMPLE QUIZ

Name _____

Question 1 (50 points)

The DNA synthesis facility sends you a 20-nucleotide primer as a dry powder; this oligonucleotide has a molecular weight of 6600. You dissolve the oligonucleotide in 1 ml of sterile distilled water; this is your "concentrated primer stock." You pipette 5 µl of the concentrated primer stock into 495 µl of water and measure the absorbance at 260 nm; the reading is 0.61. A spectrophotometer reading of 1 absorbance (OD) unit at 260 nm indicates a concentration of 33 µg/ml for a short single-stranded oligonucleotide.

1. What is the concentration of the concentrated primer stock? Please show your calculations. You may express the answer as µg/ml, or you may give the micromolar (µ*M*) concentration.

2. How much must you dilute the concentrated primer stock to make a 10 μ*M* solution for use in PCR? Please show your calculations.

Name _____

Question 2 (30 points)

You have a PCR primer 25 nucleotides long that contains 50% G+C. This primer is 100% complementary to the template DNA, and your PCR reaction contains 100 m*M* NaCl. What is the melting temperature (T_m) of the duplex DNA formed between this primer oligonucleotide and the template DNA under these conditions?

$$T_m = 16.6 \log[Na] + 0.41(\%G+C) + 81.5 - 500/bp,$$

where (%G+C) is the percentage expressed as a whole number (for example, 50, not 0.5, indicates 50%), [Na] is the molar salt concentration, and bp indicates length of DNA–DNA hybrid in base pairs.

Name _____

Question 3 (70 points)

Restriction endonuclease digests of plasmid pMB311 produced these fragments:

*Eco*RI: 6.0 kb

*Pst*I: 3.5, 2.0, 0.5 kb

*Eco*RI and *Pst*I: 2.5, 2.0, 1.0, 0.5 kb

*Sal*I: 3.8, 2.2 kb

*Sal*I and *Pst*I: 1.8, 1.7, 1.5, 0.5, 0.5 kb (two 0.5–kb fragments)

Use this information to draw a circular restriction map of pMB311. Put the cleavage sites for *Eco*RI, *Pst*I, and *Sal*I on a single map.

Name _____

Question 4 (25 points)

Improve the following sentences.

1. Both the crude and purified PCR products were determined to be 560 bp in length.

2. It was estimated that the crude product was two times brighter than the ladder.

3. Identification of the unknown environmental organism was identified as being *Escherichia coli*.

4. DNA was extracted as described for *Agrobacterium* above.

5. Ends of restriction fragments produced by *Pst*I cleavage contain four unpaired bases that can hydrogen bond to the complimentary bases on the end of another *Pst*I fragment.

Name _____

Question 5 (10 points)

$$T_m = 16.6 \log[Na] + 0.41(\%G+C) + 81.5 - 500/bp,$$

where (%G+C) is the percentage expressed as a whole number (for example, 50, not 0.5, indicates 50%), [Na] is the molar salt concentration, and bp indicates length of DNA–DNA hybrid in base pairs. You have a radiolabeled, single-stranded DNA probe 250 nucleotides long that contains 50% G+C. You hybridize this probe to a Southern blot in a hybridization solution that contains 2×SSC; note that 20×SSC contains 3 *M* NaCl. Assume that the probe is 100% complementary to the target DNA on the Southern blot. What is the melting temperature (T_m) of the hybrid formed between this probe and the target DNA under these conditions?

Name _____

Question 6 (10 points)

In Experiment 3, you cultured unidentified bacteria, isolated genomic DNA from the cultured cells, and used this DNA as a template for PCR. The authors of the article on bacterial diversity in the Amazon isolated bacterial DNA directly from soil samples without growing the bacteria in culture. In one sentence, please explain why the authors chose this approach.

Name _____

Question 7 (15 points)

List three parameters that affect the melting temperature (T_m) of annealed primer–template duplex DNA in a PCR reaction.

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APPENDIX B: SAMPLE LABORATORY REPORTS

Date: January 18, 2001

Title: Affinity Purification and Molecular Weight Determination of 6His-*fnbA* Protein Domain

Purpose: The purpose of this experiment was to purify a fibronectin-binding protein domain from *Staphylococcus aureus* and determine its purity and molecular weight.

Procedure: *Escherichia coli* cells were induced to overexpress a 6-histidine-tagged fibronectin-binding protein domain sequence (6His-*fnbA*) cloned from *S. aureus*. The histidine-tagged protein domain was purified from lysed *E. coli* cells using affinity chromatography over a nickel-containing agar. The purified protein domain was subjected to SDS–polyacrylamide gel electrophoresis to determine the yield and purity of the protein domain. Also, the protein domain was compared to a standard protein ladder to approximate the molecular weight of the purified domain.

The procedures on pp. 23–27 of the lab manual were followed except for the following deviations (1). Step B-8: The first and second elution buffer supernatants were saved and analyzed via SDS–PAGE.

Results: A standard curve was generated by plotting log molecular weight (MW) versus distance migrated using MW standard proteins of known size (Figure 1).

The elution fractions (10 μl each of total uninduced, total induced, nonabsorb induced) were analyzed via SDS–PAGE.

After the gel was stained (Figure 2), the total induced and total uninduced fractions produced multiple light and dark bands. The nonabsorb induced fraction produced one dark band. The elution 1 induced and elution 2 induced fractions both produced the same two-band pattern. The elution 1 uninduced fraction produced one dark band. The elution 2 uninduced fraction did not produce any decipherable banding pattern. The bands corresponding to each elution fraction (except total induced and total uninduced) were measured to determine the distance migrated (mm) from the interface between the stacking gel and the resolving gel (Table 1). Figure 1 shows a comparison of the migration distance of the bands in lanes 5 and 6 (elution 1 induced and elution 2 induced) to the standard curve. The predicted molecular weight of the protein domains is ~13.3 kDa based on the coding sequence of the gene fusion:

fnbA sequence = 354 base pairs × 1 codon/3 base pairs
= 115 AA + 6 His = 121 AA; 121 AA × 110 Da
(average MW for AA) = 13.3 kDa

Discussion: Interpolation of Figure 1 estimates that the molecular weight of the protein corresponding to the lower bands in lanes 5 and 6 (elution 1 induced and elution 2 induced) is 15.8 kDa. This value is reasonably close to the predicted molecular weight of the protein domain (13.3 kDa). Furthermore, by comparison of lanes 2 and 3, there is clearly a dark band in the lane containing the total induced fraction (lane 3) that is not present in the lane containing the total uninduced fraction (lane 2). Additionally, this band has migrated the same distance as the lower dark bands in lanes 5 and 6, which contain the elution 1 induced and elution 2 induced fractions, respectively. These data suggest that these bands correspond to the protein domain from the fused 6His-*fnbA* coding sequence.

By comparing the upper bands in lanes 5–7 (elution 1 induced, elution 2 induced, elution 1 uninduced), Figure 2 shows that there is a protein of common molecular weight in these three elution fractions. Additionally, lane 7 (elution 1 uninduced) does not show the lower protein bands corresponding to the 6His-*fnbA* protein domain as seen in lanes 3, 5, and 6 (total induced, elution 1 induced, elution 2 induced). This suggests that both the induced and uninduced cells contain a common, high-molecular-weight protein that is either able to bind to the Ni-containing agar similarly to the *fnbA* protein domain or is unable to be eliminated in the purification process. Also, the appearance of the upper bands in lane 5 (elution 1 induced) and lane 6 (elution 2 induced) indicates that the purity of the 6His-*fnbA* protein domain is moderate due to contamination by this high-molecular-weight protein.

Conclusion: The 6His-*fnbA* protein domain has been moderately purified, and its estimated molecular weight is 15.8 kDa.

References

1. Geller, B., Ream, W., Field, K., and Trempy, J. (2001). "Molecular Microbiology Laboratory Manual," pp. 23–29. Oregon State University.

Title: Expression and Affinity Purification of a Histidine-Tagged Protein

Date: 01/17/01–01/19/01

Introduction

As referenced on p. 23 of the lab manual, *Staphylococcus aureus* causes bovine mastitis. Virulence is related to the production of a fibronectin-binding protein. The purpose of the experiment is to express the gene (or part of the gene) responsible for the production of the protein in *Escherichea coli* and subsequently to isolate the histidine-tagged protein produced by affinity chromatography. Once the purified protein has been identified using SDS–PAGE, it can be used for the production of antisera in rabbits.

Procedure

The procedure was carried out as shown on pp. 23–26 of the lab manual. A step-by-step method is given in flow chart form on the following pages. Exceptions: step 2, p. 24, need to vortex nickel-chelated agarose; step 8, p. 25, do not discard first supernatant; save as eluate 1; step 1, p. 25, need total sample tube for each culture.

Results

SDS–PAGE gel shown in Figure 1. Using the standard curve shown in Figure 2, the size of protein bands present within the uninduced eluate 1 and 2 samples are shown to be 9.4 and 56 kDa. A protein band present within the total induced sample, shown as 14 kDa, can be identified as the fibronectin-binding protein. The migration distances and protein sizes for bands other than these are shown in the table.

Calculation of fibronectin-binding protein size:

$$345/3 = 115$$

$$115 \times 110 \text{ (average amino acid MW)} = 12{,}650$$

$$156 \text{ (histidine MW)} \times 6 = 936$$

$$12{,}650 + 936 = 13{,}586 = 13.586 \text{ kDa}$$

Discussion

The clear problem with the interpretation of the results shown in gel A of Figure 1 lies with the lack of the protein ladder. In order to get around this, a second gel, run in the same tank, is being used in comparison. By looking at and comparing the distances traveled by distinct bands present on both gels, they are shown to be virtually identical, thus allowing the use of a standard curve constructed from the second gel for the assessment of the size of bands in the first, albeit with a margin of error.

A second problem comes with the fact that neither of the induced eluant samples appears on the gel. However, by using some of the results from another group it is possible to identify the fibronectin-binding protein. By using the standard curve, a distance traveled of 3.6 cm on the gel can be assigned to the calculated size. Theoretically speaking, the protein should be present only in the induced culture and would not be present within the nonadsorb sample. Thus, a band should exist at this point in the total I, E1 I, and E2 I samples. From the gel shown at the top of Figure 1, a fuzzy, indistinct band appears at this point in the total I sample. By then looking at the second gel this same band is found in all of the predicted samples.

The table on the previous page shows that many other proteins were identified as well as the fibronectin-binding protein. It is difficult to say what these proteins are, but some information can be gained from looking at common bands between the different samples. For example, a

protein of size 56 kDa is present in all of the samples, indicating that it has a his tag and yet is not pelleted with the bacterial matter.

As for a reason for the lack of the induced samples, there are a number of feasible explanations. At the section noted on the flow diagram on p. 1, the 284 µl of Tris buffer was added to the soluble fraction as well as the pellet. This would dilute the sample and may reduce the ability of centrifugation to collect the protein present. Another explanation is that the protein content was removed accidentally when the supernatant was discarded (step 4, p. 24, and step 8, p. 25 in lab book).

Conclusion

Although there were a number of errors during the experiment, using a degree of external data the his-tagged fibronectin-binding protein product was identified. In order to isolate the protein it would most likely be necessary to repeat the procedure, but nevertheless this run provides valuable information and would make a repeat easy.

APPENDIX C: GRADING CHECKLISTS

CHECKLIST FOR GRADING LABORATORY REPORT 1

Student's name:

Points awarded: A = 9–10, B = 8–9, C = 6.5–8, D = 5–6.5, F = 0–5.

Section/items scored	Letter grade	Points	Maximum score
Name, Title, Date			
In place, legible, correct, title informative and brief	——	——	10
Purpose			
Clear and concise	——	——	10
Correct	——	——	10
Grammar and spelling	——	——	10
Sentence structure	——	——	10
Methods	——	——	**10**
Clear and concise	——	——	10
Correct	——	——	10
Complete, proper references	——	——	10
Grammar and spelling	——	——	10
Sentence structure	——	——	10
Flow chart	——	——	10
Results			
Overall organization	——	——	10
Paragraph structure	——	——	10
Sentence structure	——	——	10
Clear and concise	——	——	10
Grammar and spelling	——	——	10
Accurate	——	——	10
Thorough	——	——	10
Figures correctly labeled and neat	——	——	10
Results, not methods or discussion	——	——	10

Continues

Checklist continued

Section/items scored	Letter grade	Points	Maximum score
Discussion			
Overall organization	——	——	10
Paragraph structure	——	——	10
Sentence structure	——	——	10
Clear and concise	——	——	10
Grammar and spelling	——	——	10
Logical, supported by data	——	——	10
Thorough			
Integrates theory with results	——	——	10
Explains unexpected results	——	——	10
Conclusion			
Sentence structure	——	——	10
Clear and concise	——	——	10
Grammar and spelling	——	——	10
Logical, supported by data	——	——	10
Questions	**——**	**——**	**70**
Grand Total	**——**	**——**	**400**

CHECKLIST FOR GRADING LABORATORY REPORT 2

Student's name:

Points Awarded: A = 9–10, B = 8–9, C = 6.5–8, D = 5–6.5, F = 0–5.

Section/items scored	Letter grade	Points	Maximum score
Name, Title, Date			
In place, legible, correct, title informative and brief	——	——	10
Purpose			
Clear and concise	——	——	10
Correct	——	——	10
Grammar and spelling	——	——	10
Sentence structure	——	——	10
Methods			
Clear and concise	——	——	10
Correct	——	——	10
Complete, proper references	——	——	10
Grammar and spelling	——	——	10
Sentence structure	——	——	10
Flow chart	——	——	10
Results			
Overall organization	——	——	10
Paragraph structure	——	——	10
Sentence structure	——	——	10
Clear and concise	——	——	10
Grammar and spelling	——	——	10
Accurate	——	——	10
Thorough	——	——	10
Figures correctly labeled and neat	——	——	10
Results, not methods or discussion	——	——	10

Continues

Checklist continued

Section/items scored	Letter grade	Points	Maximum score
Discussion			
Overall organization	___	___	10
Paragraph structure	___	___	10
Sentence structure	___	___	10
Clear and concise	___	___	10
Grammar and spelling	___	___	10
Logical, supported by data	___	___	10
Thorough	___	___	10
Integrates theory with results	___	___	10
Explains unexpected results	___	___	10
Conclusion			
Sentence structure	___	___	10
Clear and concise	___	___	10
Grammar and spelling	___	___	10
Logical, supported by data	___	___	10
Questions	**___**	**___**	**70**
Grand total	**___**	**___**	**400**

CHECKLIST FOR GRADING LABORATORY REPORT 3

Student's name:

Points Awarded: A = 9–10, B = 8–9, C = 6.5–8, D = 5–6.5, F = 0–5.

Section/items scored	Letter grade	Points	Maximum score
Name, Title, Date			
In place, legible, correct, title informative and brief	——	——	10
Purpose			
Clear and concise	——	——	10
Correct	——	——	10
Grammar and spelling	——	——	10
Sentence structure	——	——	10
Methods			
Clear and concise	——	——	10
Correct	——	——	10
Complete, proper references	——	——	10
Grammar and spelling	——	——	10
Sentence structure	——	——	10
Flow chart	——	——	10
Results			
Overall organization	——	——	10
Paragraph structure	——	——	10
Sentence structure	——	——	10
Clear and concise	——	——	10
Grammar and spelling	——	——	10
Accurate	——	——	10
Thorough	——	——	10
Figures correctly labeled and neat	——	——	10
Results, not methods or discussion	——	——	10

Continues

Checklist continued

Section/items scored	Letter grade	Points	Maximum score
Discussion			
Overall organization	___	___	10
Paragraph structure	___	___	10
Sentence structure	___	___	10
Clear and concise	___	___	10
Grammar and spelling	___	___	10
Logical, supported by data	___	___	10
Thorough	___	___	10
Integrates theory with results	___	___	10
Explains unexpected results	___	___	10
Conclusion			
Sentence structure	___	___	10
Clear and concise	___	___	10
Grammar and spelling	___	___	10
Logical, supported by data	___	___	10
Questions	**___**	**___**	**70**
Grand total	**___**	**___**	**400**

CHECKLIST FOR GRADING LABORATORY REPORT 4

Student's name:

Points Awarded: A = 9–10, B = 8–9, C = 6.5–8, D = 5–6.5, F = 0–5.

Section/items scored	Letter grade	Points	Maximum score
Name, Title, Date			
In place, legible, correct, title informative and brief	——	——	10
Purpose			
Clear and concise	——	——	10
Correct	——	——	10
Grammar and spelling	——	——	10
Sentence structure	——	——	10
Methods			
Clear and concise	——	——	10
Correct	——	——	10
Complete, proper references	——	——	10
Grammar and spelling	——	——	10
Sentence structure	——	——	10
Flow chart	——	——	10
Results			
Overall organization	——	——	10
Paragraph structure	——	——	10
Sentence structure	——	——	10
Clear and concise	——	——	10
Grammar and spelling	——	——	10
Accurate	——	——	10
Thorough	——	——	10
Figures correctly labeled and neat	——	——	10
Results, not methods or discussion	——	——	10

Continues

Checklist continued

Section/items scored	Letter grade	Points	Maximum score
Discussion			
Overall organization	___	___	10
Paragraph structure	___	___	10
Sentence structure	___	___	10
Clear and concise	___	___	10
Grammar and spelling	___	___	10
Logical, supported by data	___	___	10
Thorough	___	___	10
Integrates theory with results	___	___	10
Explains unexpected results	___	___	10
Conclusion			
Sentence structure	___	___	10
Clear and concise	___	___	10
Grammar and spelling	___	___	10
Logical, supported by data	___	___	10
Questions	**___**	**___**	**70**
Grand Total	**___**	**___**	**400**

APPENDIX D: PEER REVIEW CHECKLISTS

Reviewer_____ **Experiment no.**_____

Author_____ **Date**_____

1. Throughout the report, look for awkward sentence construction, poorly organized paragraphs, incorrect grammar, and misspelled words. Remember, even grammatically correct sentences can be awkward and difficult to read. Sentences should be simple and straightforward. Use active voice instead of passive voice, and eliminate redundant words. Remove vague, qualitative adjectives such as "large" or "small" and replace them with numbers. For example, "a 10-fold increase" is much more informative than "a large increase." Make certain new terms are explained clearly, and clarify statements that are ambiguous.

2. Check that the name, title, and date are present and legible.

3. Is the purpose stated clearly and concisely? Is the statement complete and accurate?

4. Are the methods described completely and references cited properly? Does the methods section clearly and concisely describe modifications made to the referenced procedure?

5. Check the flow chart, and ensure it was not retyped after the experiment was completed.

6. The results section should begin with a description of the work performed. After reading the results section, a scientist who has not read the methods section should understand what you did. However, it should not include all the details normally found in the methods section. Strike a balance between too little and too much information by describing the procedures well enough to understand the experiment, but do not include all the details required to repeat the experiment. Instead, detailed information belongs in the methods section. For example, do not describe the composition of buffers or media in the results. However, the methods section should contain this information or reference a publication that does.

7. The order in which the results are presented should lead the reader through the experiment. Is the organization of the data logical? Is the presentation clear? Would changing the organization make the report easier to understand?

8. Are the figures and tables cited in the text, and do they have appropriate titles and legends? Is each lane of a gel photograph labeled, and does the figure legend list the contents of each lane?

9. Does the text accurately describe the figures? The data must support statements made in the text.

10. The discussion should begin with a brief introduction that makes the purpose of the experiment clear. This section should flow logically from an introduction through the results to a sound conclusion. The discussion may include background material that puts the need for the experiments in perspective. A good discussion does not merely reiterate information presented in the results section. After a brief recap of the question asked and the work done to answer it, the author should state the meaning of the data and indicate how the findings affect our current understanding of the field. In other words, what was the answer to the question, and what does that answer mean? Does the study establish new general principles? Is there more than one way to interpret the data? If so, the discussion should present both possibilities and suggest which the author believes is correct. Did the experiment produce unexpected results such as "extra" bands on a gel? The discussion must **not** ignore results that do not fit the author's expectations; instead, the discussion should contain a plausible explanation of such data. Make certain problems with the data are discussed.

11. The conclusion should summarize the experiment in two or three sentences. The data must support the conclusions.

12. What is best about this writing?

13. What needs to be improved the most?

Reviewer_____ **Experiment no.**_____

Author_____ **Date**_____

1. Throughout the report, look for awkward sentence construction, poorly organized paragraphs, incorrect grammar, and misspelled words. Remember, even grammatically correct sentences can be awkward and difficult to read. Sentences should be simple and straightforward. Use active voice instead of passive voice, and eliminate redundant words. Remove vague, qualitative adjectives such as "large" or "small" and replace them with numbers. For example, "a 10-fold increase" is much more informative than "a large increase." Make certain new terms are explained clearly, and clarify statements that are ambiguous.

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8. Are the figures and tables cited in the text, and do they have appropriate titles and legends? Is each lane of a gel photograph labeled, and does the figure legend list the contents of each lane?

9. Does the text accurately describe the figures? The data must support statements made in the text.

10. The discussion should begin with a brief introduction that makes the purpose of the experiment clear. This section should flow logically from an introduction through the results to a sound conclusion. The discussion may include background material that puts the need for the experiments in perspective. A good discussion does not merely reiterate information presented in the results section. After a brief recap of the question asked and the work done to answer it, the author should state the meaning of the data and indicate how the findings affect our current understanding of the field. In other words, what was the answer to the question, and what does that answer mean? Does the study establish new general principles? Is there more than one way to interpret the data? If so, the discussion should present both possibilities and suggest which the author believes is correct. Did the experiment produce unexpected results such as "extra" bands on a gel? The discussion must **not** ignore results that do not fit the author's expectations; instead, the discussion should contain a plausible explanation of such data. Make certain problems with the data are discussed.

11. The conclusion should summarize the experiment in two or three sentences. The data must support the conclusions.

12. What is best about this writing?

13. What needs to be improved the most?

Reviewer_____ **Experiment no.**_____

Author_____ **Date**_____

1. Throughout the report, look for awkward sentence construction, poorly organized paragraphs, incorrect grammar, and misspelled words. Remember, even grammatically correct sentences can be awkward and difficult to read. Sentences should be simple and straightforward. Use active voice instead of passive voice, and eliminate redundant words. Remove vague, qualitative adjectives such as "large" or "small" and replace them with numbers. For example, "a 10-fold increase" is much more informative than "a large increase." Make certain new terms are explained clearly, and clarify statements that are ambiguous.

2. Check that the name, title, and date are present and legible.

3. Is the purpose stated clearly and concisely? Is the statement complete and accurate?

4. Are the methods described completely and references cited properly? Does the methods section clearly and concisely describe modifications made to the referenced procedure?

5. Check the flow chart, and ensure it was not retyped after the experiment was completed.

6. The results section should begin with a description of the work performed. After reading the results section, a scientist who has not read the methods section should understand what you did. However, it should not include all the details normally found in the methods section. Strike a balance between too little and too much information by describing the procedures well enough to understand the experiment, but do not include all the details required to repeat the experiment. Instead, detailed information belongs in the methods section. For example, do not describe the composition of buffers or media in the results. However, the methods section should contain this information or reference a publication that does.

7. The order in which the results are presented should lead the reader through the experiment. Is the organization of the data logical? Is the presentation clear? Would changing the organization make the report easier to understand?

8. Are the figures and tables cited in the text, and do they have appropriate titles and legends? Is each lane of a gel photograph labeled, and does the figure legend list the contents of each lane?

9. Does the text accurately describe the figures? The data must support statements made in the text.

10. The discussion should begin with a brief introduction that makes the purpose of the experiment clear. This section should flow logically from an introduction through the results to a sound conclusion. The discussion may include background material that puts the need for the experiments in perspective. A good discussion does not merely reiterate information presented in the results section. After a brief recap of the question asked and the work done to answer it, the author should state the meaning of the data and indicate how the findings affect our current understanding of the field. In other words, what was the answer to the question, and what does that answer mean? Does the study establish new general principles? Is there more than one way to interpret the data? If so, the discussion should present both possibilities and suggest which the author believes is correct. Did the experiment produce unexpected results such as "extra" bands on a gel? The discussion must **not** ignore results that do not fit the author's expectations; instead, the discussion should contain a plausible explanation of such data. Make certain problems with the data are discussed.

11. The conclusion should summarize the experiment in two or three sentences. The data must support the conclusions.

12. What is best about this writing?

13. What needs to be improved the most?

MOLECULAR MICROBIOLOGY LABORATORY PREPARATION MANUAL

This preparation manual is designed for a class of 48 students. In Experiments 1 and 2 students work in pairs, whereas during Experiments 3 and 4 they work individually. Accordingly, quantities of reagents required per pair are listed for the first two experiments, and materials needed by each student are indicated for the final two experiments. For each gel electrophoresis experiment, the number of lanes per pair (or per student) are listed; the number of electrophoresis rigs required will depend on how many lanes each gel contains. Each student (or pair) should have a set of pipettors as well as safety glasses, a lab coat, and gloves. Equipment required for the entire class is listed, but the precise number of microcentrifuges, vortexers, etc. is not. If possible, no more than six students (or three pairs) should share one piece of equipment. We have found that it is often wise to prepare 5–10% more reagents than are absolutely necessary.

EXPERIMENT 1: PLASMID PURIFICATION AND RESTRICTION MAPPING

Class 2: Purify and restrict plasmid DNA

Class 3: Agarose gel electrophoresis and transformation

Next Day: Examine plates

Teaching assistants: Verify with media kitchen and strain curator that all materials have been ordered and that equipment, strains, and materials are present by the date indicated. Set up laboratory at least 1 hr before class.

Media kitchen: Prepare media and solutions by date indicated. Order restriction enzymes and supplies.

Strain curator: Coordinate TAs' activities with media room. Prepare the two NM522 (pKN800) *Escherichia coli* strains (with different orientations of the *lux* operon relative to the vector) for the experiment. Have the media room prepare media, flasks, tubes, and solutions. **Prepare materials 1 week before class 2.**

Items for Strain Curator

2 LB–ampicillin (50 μg/ml) agar plates
2 sterile 250-ml flasks with 25 ml of LB–ampicillin broth
2 sterile 50-ml centrifuge tubes
32 sterile 1.5-ml microcentrifuge tubes
100 ml of lysis buffer: 50 m*M* glucose + 10 m*M* EDTA + 25 m*M* Tris, pH 8
Rinse all glassware with distilled water. Use sterile distilled water to prepare all reagents.

Schedule for Strain Curator

One week before class 2: Use aseptic technique. Streak both isolates (A and B orientations; Fig. 1.2) of *E. coli*

strain NM522 (pKN800) for single colonies on LB–ampicillin agar. Incubate overnight at 37°C.

Next day: Remove plates from 37°C incubator, seal with Parafilm, and store at 4°C.

Four days before class 2: At the end of the day inoculate both strains of the glow-in-the-dark NM522 (pKN800) from the LB–amp plates into 25 ml of LB–ampicillin broth. Incubate cultures in 250-ml flasks with aeration (200 rpm shaking) at 37°C overnight.

Next day:

1. Centrifuge each culture at 5000 × *g* for 10 min at 4°C.
2. Discard the supernatants.
3. Thoroughly resuspend each pellet in 2.5 ml of cold lysis buffer.
4. Label 32 sterile 1.5-ml microcentrifuge tubes with numbers from 1 to 32.
5. Dispense 0.15 ml of the resuspended pellets into the labeled 1.5-ml microcentrifuge tubes. Put NM522 (pKN800-A) in the odd-numbered tubes and NM522 (pKN800-B) in the even-numbered tubes.
6. Store the aliquots in a non-defrosting freezer until class 2.

Class 2: Purify and Restrict Plasmid DNA

Items per Pair

0.15 ml — 5 *M* potassium acetate. Dissolve 49.1 g of potassium acetate in 100 ml of sterile distilled water. Take 60 ml of this solution and add 11.5 ml of glacial acetic acid and 28.5 ml of sterile distilled water. The resulting solution is 3 *M* with respect to potassium and 5 *M* with respect to acetate. Store at room temperature.

0.01 ml — RNase A solution (1 mg/ml). Heat at 68°C for 15 min. Store at –20°C.

0.25 ml — phenol[1] equilibrated in 1 *M* Tris, pH 8.0. Store at 4°C. **Note**: Phenol must be **colorless**, not pink.

0.25 ml — isoamyl alcohol–chloroform.[1] Add 1 ml of isoamyl alcohol to 24 ml of chloroform.

0.01 ml — 3.5 *M* sodium acetate. If you have anhydrous sodium acetate, add 28.7 g to 50 ml of sterile distilled water, then adjust pH to 5.2 with glacial acetic acid and bring to a final volume of 100 ml with sterile distilled water. If you have sodium acetate trihydrate, add 47.6 g to 50 ml of sterile distilled water, then adjust the pH to 5.2 with glacial acetic acid and bring to a final volume of 100 ml with sterile distilled water. Store at room temperature.

1 ml — TE, pH 8: 10 m*M* Tris + 1 m*M* EDTA, pH 8. Mix 0.121 g of Tris base and 0.0372 g of disodium EDTA; add 50 ml of sterile distilled water, then bring pH to 8.0 with HCl. Bring final volume to 100 ml with sterile distilled water. Autoclave. Store at room temperature.

[1]Phenol, isoamyl alcohol, and chloroform may be combined (25:1:24) or ordered as a mixture from Gibco/BRL (catalog number 15593-031).

0.003 ml	10 × *Pst*I reaction buffer. Obtain from enzyme supplier (Fermentas or Gibco). Store frozen.
0.002 ml	*Pst*I enzyme (10–20 units/μl). Obtain from Fermentas or Gibco freezer. Store at –20°C in a non-defrosting freezer.
0.5 ml	95% ethanol (chill on ice on class day 2)
0.5 ml	sterile distilled water
18	sterile 1.5-ml microcentrifuge tubes
36	sterile yellow tips for P20 and P200 Pipetmen
21	sterile blue tips for P1000 Pipetman
0.15 ml	resuspended NM522(pKN800) pellet (stored in the freezer)
0.06 ml	lysozyme solution. 80 mg of lysozyme dissolved in 1 ml of sterile distilled water. Prepare 1 hr before class and store on ice.
0.2 ml	SDS–NaOH solution. 8.8 ml of sterile distilled water + 0.2 ml of 10 *N* NaOH + 1 ml of 10% SDS solution. Prepare 1 hr before class and store at room temperature. 10% SDS solution = 1 g of SDS in 9 ml of sterile distilled water.
1	microcentrifuge with tube holders
1	P20 Pipetman
1	P200 Pipetman
1	P1000 Pipetman
2	pairs of disposable gloves
2	goggles
1	ice bucket with ice
1	rack to hold microcentrifuge tubes
2	plastic beakers for discarded tips
1	forceps for handling sterile microcentrifuge tubes

Equipment Required for Class 2

1 37°C incubator

1 37°C waterbath

1 70°C waterbath or heating block

4 floating racks for microcentrifuge tubes in waterbaths

4 glass bottles for phenol waste

8 vortexers (or more)

1 Speedvac concentrator

Class 3: Agarose Gel Electrophoresis and Transformation

Materials for Entire Class

Place power supplies, agarose gel apparatus with combs and casting trays, UV transilluminator, microcentrifuges with racks, and four glass Pyrex dishes in the classroom.

250 ml 0.8% agarose

Dissolve 2.0 g of agarose in 250 ml of 1 × TAE electrophoresis buffer. Use a microwave or hotplate to melt the agarose.

1 liter 20 × TAE electrophoresis buffer

96.8 g of Tris base + 22.87 ml of glacial acetic acid + 0.75 g EDTA. Add distilled water to 1 liter. Mix well. Store at room temperature.

2.5 liters 1 × TAE

Prepare 250 ml of 1 × TAE to fill each electrophoresis apparatus x 8 gels. In addition, you will need 250 ml of 1 × TAE to prepare enough agarose for 8 gels (30 ml each).

0.24 ml loading solution

24 microcentrifuge tubes, each with 10 µl of 0.25% bromophenol blue + 30% glycerol dissolved in sterile distilled water. Store at room temperature.

5 ml ethidium bromide (10 mg/ml)

Dissolve 50 mg of ethidium bromide in 5 ml of sterile distilled water. Store in the refrigerator and protect from light. Do not aliquot.

Caution: Ethidium bromide is a carcinogen.

100 µl λ/*Hin*dIII molecular weight standards

Mix 4 µg of *Hin*dIII-cut λ DNA with 10 µl of loading solution and bring the total volume to 100 µl with DNA buffer (10 m*M* Tris + 0.1 m*M* EDTA). Use 10 µl/lane (400 ng/lane).

100 µl 100-bp ladder molecular weight standard
Mix 25 µl of stock (1 µg/µl) with 65 µl of DNA buffer + 10 µl of loading solution. Load 10 µl/lane (2.5 µg/lane).

5 sterile 1-ml pipettes

1 37°C waterbath with racks to hold microcentrifuge tubes

1 37°C shaking incubator with rack to hold microcentrifuge tubes

1 30°C dry incubator

1 UV light source, camera, film

Items per Pair

12	sterile 1.5-ml microcentrifuge tubes
20	sterile yellow tips
5	sterile blue tips
4	pairs of disposable gloves
1	ice bucket filled with ice
1	P20 Pipetman
1	P200 Pipetman
1	P1000 Pipetman
1	can 1-ml sterile pipets
12 ml	sterile LB broth
8	LB agar plates containing 50 µg/ml ampicillin
4	LB agar plates (no antibiotics)
1	alcohol bottle
2	safety glasses or goggles
1	ice bucket with ice
2	glass rods for spreading plates
175 µl	competent *E. coli* cells
10 µl	RNAse A (1 mg/ml; heat treated)

Strain curator: On **class day 3**, dilute a fresh overnight culture of *E. coli* strain NM522 (pKN800) 10^{-4}-,10^{-5}-, 10^{-6}-, and 10^{-7}-fold and plate 100 µl of each dilution on LB–amp plates. Incubate at 30°C overnight. These are control plates for the students to view fluorescent colonies the next day.

TA's role: Verify that all items are present by the start of class. The class (48 students) will pour eight 0.8% agarose minigels (30 ml each). Each gel will contain at least eight sample wells (2 lanes/pair × 3 pairs/gel + 1 marker = 7 lanes/gel).

Recipes

Lysis buffer: 4.5 ml of a 20% sterile glucose solution. Dissolve 20 g of glucose in 80 ml of sterile distilled water. Add 4 ml of sterile 0.25 *M* EDTA.Add 2.5 ml of sterile 1 *M* Tris, pH 8. Add 89 ml of sterile distilled water; adjust pH to 8. Filter sterilize. Store in refrigerator.

LB broth: 10 g/l tryptone (Difco) + 5 g/l yeast extract (Difco) + 10 g/l NaCl Adjust pH to 7.0 and autoclave.

LB agar: 10 g/l tryptone (Difco) + 5 g/l yeast extract (Difco) + 10 g/l NaCl + 15 g/l agar (Difco). Adjust pH to 7.0 and autoclave.

5% ampicillin: Dissolve 0.05 g of ampicillin in 1.0 ml of sterile distilled water. Filter sterilize. Store frozen.

LB–ampicillin broth: Same as LB medium, except add ampicillin to a final concentration of 50 µg/ml. After the LB broth has cooled to room temperature, add 0.10 ml of a fresh sterile 5% ampicillin solution to 100 ml of broth. Store at 4°C.

LB–ampicillin agar plates: Same as LB agar, but add ampicillin to a final concentration of 50 µg/ml. After the autoclaved LB agar has cooled to 55°C, add 0.10 ml of a fresh sterile 5% (50 mg/ml) ampicillin solution to 100 ml of medium. Let the plates dry overnight in a 37°C incubator, then store in plastic sleeves at 4°C.

Ordering Information

Item	Catalogue no.	Unit	Price
United States Biochemical Company (800-321-9322)			
Lysozyme	18645	1 g	$10.00
SDS	21651	25 g	$10.00
RNase A	21199	1 mg	$12.00
Phenol	20083	100 ml	$20.00
Sodium acetate	21608	500 g	$20.50
*Pst*I (order 2)	70595	10,000 units	$44.50
Agarose-LE	32802	25 g	$30.50
Ethidium bromide	32813	1 g	$5.50
Bromophenol blue	12370	5 g	$5.40
λDNA (*Hin*dIII-digested)	70061	200 mg	$50.00
Sigma Chemical Company (800-325-3010)			
Potassium acetate	P3542	100 gm	$8.60
Isoamyl alcohol	I1885	100 ml	$8.00
VWR (800-225-0440)			
Chloroform	IB05040	500 ml	$17.00
Gibco/BRL (800-828-6686)			
100-bp ladder	15628-019	50 μg	$75.00
λDNA (*Hin*dIII-digested)	15612-013	500 μg	$106.00
E. coli DH5α, competent, subcloning grade	18265-017	2 × 2 ml	$49 × 2

EXPERIMENT 2: AFFINITY PURIFICATION OF A HISTIDINE-TAGGED PROTEIN

Class 5: Lyse cells and bind His-tagged protein to Ni resin

Class 6: SDS–polyacrylamide gel electrophoresis

Prepare materials for strain curator 1 week before class 5.

1	LB kanamycin (25 μg/ml) + ampicillin (50 μg/ml) plate (fresh)
10 ml	LB kanamycin–ampicillin broth (fresh)
150 ml	LB kanamycin–ampicillin broth (fresh)
1 ml	0.1 *M* IPTG (isopropyl thiogalactoside; 28.2 mg/ml in water) Filter sterilize and store frozen.

Schedule for Strain Curator

Six days before class 5: Streak one culture of *E. coli* (pQE-FnbA) to a plate of LB kanamycin (25 μg/ml) plus ampicillin (50 μg/ml). Incubate at 37°C overnight.

Next day: Pick two single colonies and start two separate 5-ml cultures in LB kan–amp broth. Shake at 37°C overnight.

Next day: Store liquid culture at 4°C.

One day before class 5: Start two 5-ml cultures in LB kan–amp broth from single colonies as above. Shake 37°C overnight.

Class 5 (8:00 AM): Dilute one of the overnight cultures 1:50 [2 ml into 100 ml of warm (37°C) LB kan–amp]. Use a 1-liter flask to ensure proper aeration. Shake at 37°C until the OD = 0.5. Split culture in half. To one half add 1.0 ml of 0.1 *M* IPTG. Do not add anything to the other half.

Shake cultures at 37°C until noon. Aliquot 1.5 ml of each culture to separate microcentrifuge tubes. Each pair of students needs 1.5 ml of each culture. Label tubes ± IPTG; use differently colored tubes for induced and uninduced cultures.

Class 5: Lyse Cells and Bind His-Tagged Protein to Ni Resin

Items per Pair:

10 µl	PMSF solution (200 m*M* in isopropropyl alcohol, stored in freezer)
1.5 ml	IPTG-induced culture of *E. coli* (pQE-FnbA) in 1.5-ml tube
1.5 ml	uninduced culture of *E. coli* (pQE-FnbA) in 1.5-ml microcentrifuge tube
1	micropipettor, 2–20 µl (P20)
1	micropipettor, 20–200 µl (P200)
1	micropipettor, 200–1000 µl (P1000)
1 box	yellow tips for P20 and P200
1 box	blue tips for P1000
50	sterile microcentrifuge tubes
2 ml	B-PER buffered detergent reagent, Pierce Chemical Co.
5 ml	1:10 diluted B-PER buffered detergent reagent (Pierce)
20 µl	lysozyme (30 mg/ml) in water (made fresh; keep on ice)
250 µl	nickel-chelated agarose (50% suspension)
500 µl	wash buffer (Pierce)

250 µl	elution buffer (Pierce)
1	microcentrifuge tube rack
1	styrofoam container of ice

Equipment Needed for Class 5

8 (or more) microcentrifuges
12 (or more) vortexers

Class 6. SDS–PAGE

Items per Pair

50 µl	3 × sample buffer
25	sterile microcentrifuge tubes
1	micropipettor, 2–20 µl (P20)
1 box	yellow tips for P20
7	microcapillary tips for P20
1	15% polyacrylamide precast mini gel (BioRad) (need 1 gel/2 pairs = 12 gels/48 students)

Each pair of students will have eight samples: eluates 1 and 2, nonadsorbed proteins, and total cellular protein from both induced (IPTG-treated) and uninduced cultures. Each gel must accommodate two pairs of students. Because the BioRad gels (#161-0938) have fifteen wells, each pair must omit one sample (nonadsorbed proteins from uninduced cells). Thus, each pair will load seven samples; molecular weight standards will be loaded in the fifteenth lane.

1	small spatula
1	razor blade
400 ml	1 × Tris–glycine–SDS electrophoresis buffer (per 4 pairs)
5 µl	protein MW standards, low range (Gibco/BRL)
1	25-ml glass pipette
1	250- or 500-ml graduated cylinder
25 ml	gel code blue G-250 stain (BioRad)
1	gel staining tray
1	polyacrylamide gel electrophoresis apparatus (Need one BioRad mini-protean apparatus/

4 pairs = 6/48 students.) Each apparatus holds two gels.

1 microcentrifuge tube rack

1 styrofoam container of ice

Equipment Needed for Class 6

8 (or more) microcentrifuges
3 double-outlet power supplies for SDS–PAGE
1 95°C waterbath
1 light table (white light), camera, film

TA's role: Verify that all materials are present. Set out items for lab, including the students' samples from class 5. Adjust the waterbath to 95°C, and see that it is at the correct temperature by class time. Monitor use of the light source and camera.

Recipes

3 × Sample Buffer

reagent	*volume (µl)*
1 M Tris–HCl, pH 6.8	100
glycerol	400
10% SDS	320
1% bromophenol blue	20
β-mercaptoethanol	80

Ordering Information

Item	Catalog no.	Unit	Price
Pierce			
Nickel agarose kit (includes Pierce B-PER detergent, wash buffer, elution buffer, nickel agarose)	78300VE	1	$295
Gel code blue stain reagent	24590GV	500 ml	$29
PMSF (phenyl methyl sulfonyl fluoride)	36978	5 g	$62
IPTG	34060	1 g	$42
BioRad			
Precast 15% polyacrylamide gels, (15-well, 10/pkg)	161-0938	2 pkg	$82
10× Tris–glycine–SDS electrophoresis buffer	161-0732	1 liter	$20
Gibco/BRL			
Protein MW standards, low range	26000-018	500 µl	$69
Rainin			
Microcapillary pipette tips, round gel-well style	GT-250-6	2 × 200/rack	$29

EXPERIMENT 3: PCR AND DNA SEQUENCE ANALYSIS OF BACTERIAL rRNA GENES

Class 8: Isolate bacteria from environment

Next day: Examine plates and streak

Class 9: Gram stain, microscopy, inoculate broth

Class 10: Prepare genomic DNA, freeze cultures

Class 11: PCR

Class 12: Purify PCR product

Class 13: Agarose gel and template preparation

Prepare media, plates, swabs, toothpicks, inoculating sticks, pipettes, and freezer vials 1 week before class 8.

Note: Students work individually, NOT in pairs for Experiment 3.

Class 8: Isolate Bacteria from the Environment

Items per Student

4 LB agar plates

1 sterile test tube containing 1 ml of LB broth

6 sterile cotton swabs; **1 tube for each student**

20 sterile toothpicks; **1 bottle/student**

1 loop for streaking agar plates

1 Bunsen burner and striker

For Entire Class:

3 boxes to hold inoculated agar plates

TA's role: Verify that all items are present and in place when class 8 begins. Label the boxes 25, 30, and 37°C. Transfer boxes to warm rooms after class. The next day, **students** will retrieve their plates, pick and streak colonies, and place the streaked plates in the appropriate incubator. The following day, the **TAs** will examine the streaked plates for growth and move plates with adequate growth to the cold room. TAs will bring the streaked plates to class 9.

Class 9: Gram Stain, Microscopy, Inoculate Broth

Items per Student

2 sterile culture tubes containing 3 ml of LB broth (each)

1 rack for culture tubes

1 wire inoculating loop

2 sterile inoculating sticks

1 microscope

2 glass microscope slides and cover slips

1 Bunsen burner and striker

1 250-ml beaker

1 tripod with screen (to hold beaker over burner)

1 wash bottle filled with distilled water

1 reagent for Gram stain (crystal violet, Gram's iodine, 95% alcohol, safranin)

1 reagent for spore stain (5% malachite green, safranin)

Equipment Required for Class 9

3 racks for incubating culture tubes at 25, 30, and 37°C

3 shaker–incubators, one each at 25, 30, and 37°C

TA's role: Verify that all items are present and that incubators are at proper temperatures. Bring streaked plates to class 9. On the day after class 9, TAs will remove culture tubes from the incubators (provided they show adequate growth) and store them in the refrigerator until class 10. TAs will bring cultures to class 10.

Class 10: Prepare Genomic DNA, Freeze Cultures

Items per Student

2	3-ml LB broth cultures of unknown bacteria (isolated from the environment during class 8 and inoculated from streaks during class 9)
1	sterile 1-dram screw-cap freezer vials containing 0.2 ml of DMSO. Distribute DMSO to vials, replace caps, and autoclave.
1 ml	25 m*M* Tris + 10 m*M* EDTA, pH 8 (sterile). Mix 3 g of Tris base + 3.36 g of EDTA; add 950 ml of distilled water, adjust to pH 8 with HCl, and bring to 1 liter with distilled water. Autoclave. Store at room temperature.
40 µl	lysozyme (30 mg/ml). Dissolve in 25 m*M* Tris, pH 8, immediately before use. Keep on ice.
20 µl	proteinase K (50 mg/ml). Dissolve in sterile distilled water immediately before use. Keep on ice.
40 µl	25% SDS. Dissolve 25 g of SDS in sterile distilled water. Store at room temperature.

120 µl	5 *M* NaCl. Dissolve 29.2 g of NaCl in distilled water and bring to 100 ml. Autoclave. Store at room temperature.
1 ml	phenol–chloroform–isoamyl alcohol (25:24:1) Equilibrate with Tris, pH 8. Store at 4°C.
1 ml	chloroform–isoamyl alcohol (24:1)
4 ml	95% ethanol. Place on ice before class.
1 ml	70% ethanol. Place on ice before class.
1 ml	DNA buffer = 10 m*M* Tris + 0.1 m*M* EDTA, pH 8. Mix 1.21 g of Tris base with 1 ml of 100 m*M* EDTA, pH 8. Bring volume to 950 ml, adjust pH to 8 with HCl, and then bring to 1 liter with distilled water. Autoclave and store at room temperature. To make 100 m*M* EDTA, dissolve 3.36 g of EDTA in 100 ml of distilled water and adjust pH to 8 with NaOH.
4	sterile 1.5-ml microcentrifuge tubes
1	rack for microcentrifuge tubes
1	float for microcentrifuge tubes
1	box yellow pipette tips
1	box blue pipette tips
1	P20 Pipetman
1	P200 Pipetman
1	P1000 Pipetman
1	ice bucket
1	lab coat
1	safety goggles

Equipment Required for Class 10

8 (or more) microcentrifuges & 12 (or more) vortex mixers
water baths at 37°C, 50°C, and 68°C
floating racks for 1.5-ml microcentrifuge tubes
freezer box to hold 48 1-dram vials
Speed Vac centrifuge–concentrator
portable fume hoods
container for organic waste
autoclave bags for plates, microcentrifuge tubes, and tips
autoclave container for supernatants
autoclave rack for culture tubes

Class 11: PCR

Items per Student

190 µl PCR reaction mixture. **TA will prepare just before class**. Store on ice.

Mixture (190 µl/student) will contain[a]	*For 50 students*
20 µl of 10 × PCR buffer (Perkin Elmer)	1000 µl
20 µl of 2 m*M* (each) dNTP mix	1000 µl
12 µl of 25 m*M* $MgCl_2$	600 µl
20 µl of 50% acetamide	1000 µl
4 µl of 10 µ*M* 27F primer	200 µl
4 µl of 10 µ*M* 519R primer	200 µl
1 µl of *Taq* DNA polymerase (5 Units/µl)	50 µl
109 µl of distilled water	5450 µl
190 µl of total volume	**9500 µl**

[a]The 10 × PCR buffer, 2 m*M* dNTP mix, 25 m*M* $MgCl_2$, *Taq* DNA polymerase, distilled water, and 0.2-ml thin-wall reaction tubes are supplied by Perkin Elmer and are contained in the GeneAmp kit with AmpliTaq (N801-0055), the AmpliTaq 250 + Buffer II kit (N808-0161), the GeneAmp PCR core kit (N808-0009), the GeneAmp dNTPs kit (N808-0007), and the MicroAmp (0.2 ml) reaction tube with cap (N801-0540). One of each of these products is sufficient for 40 students. Order acetamide separately (Sigma).

Order PCR primers from Gibco/BRL.

Primer 27F:	5′-AGA GTT TGA TC(C/A) TGG CTC AG-3′
Primer 519R:	5′-G(T/A)A TTA CCG CGG C(T/G)G CTG-3′
10 µl	sterile distilled water (DNA free)
2	yellow BARRIER (aerosol-resistant) tips for P20
2	yellow BARRIER tips for P200
2	0.2-ml MicroAmp PCR reaction tubes, with cap (Perkin Elmer product no. N801-0540)
6	1.5-ml microcentrifuge tubes (sterile)
16	blue tips for Pipetman (sterile)
16	yellow tips for Pipetman (sterile)
1	P20 Pipetman
1	P200 Pipetman
1	P1000 Pipetman
2	pairs of disposable gloves
1	ice bucket and ice
1	rack for microcentrifuge tubes
1	plastic beaker for discarding used tips
1	forceps
1	thermal cycler with hot top

TA's role: Prepare PCR mix just before **class 11**. Check the concentrations of each primer before adding to the mix. Remove PCR reactions from thermal cycler and store them at –20°C until **class 12**. Help set up thermal cycler.

Class 12: Purify PCR Product

Items per Student

1 QiaQuick PCR purification cartridge (Qiagen)

0.5 ml QiaQuick buffer PB

0.75 ml QiaQuick buffer PE (contains 70% ethanol)

50 µl DNA buffer (20 m*M* Tris + 0.1 m*M* EDTA, pH 8), sterile

50 µl sterile distilled water

5 1.5-ml microcentrifuge tubes (sterile)

rack for microcentrifuge tubes

10 yellow tips (sterile)

4 blue tips (sterile)

P20, P200, and P1000 pipettors

1 pair disposable gloves

Equipment Required for Class 12

8 (or more) microcentrifuges

1 Speed Vac centrifuge–concentrator and vacuum connection

TA's role: Provide racks to hold tubes containing purified and unpurified PCR products and no-template control reactions. Collect these samples after class and store frozen until class 13.

Class 13: Electrophoresis of PCR Product and Template Preparation

Items per Student

12 pmol of primer oligonucleotide 27F in 1 µl of sterile distilled water

20 µl sterile distilled water

6 µl agarose gel loading solution (50% glycerol + 0.05% bromophenol blue)

4 1.5-ml microcentrifuge tubes (sterile)

1 rack for microcentrifuge tubes

10 yellow tips (sterile)

P20 pipettor

1 pair disposable gloves

Materials for Entire Class

6 µg Gibco/BRL low MW DNA mass ladder (500 ng/gel × 12 gels). Need 3 lanes/student × 4 students/gel + 1 marker = 13 wells/gel. Each student will have 3 samples: PCR product before and after purification and no-template control reaction. A total of 16 gels containing 10 wells will accommodate 48 students, 3 students/gel.

6 g NuSeive 3:1 agarose (0.5 g/25 ml of 2% gel × 12 gels)

6 liters 1 × TAE electrophoresis buffer. 40 m*M* Tris-acetate + 2 m*M* EDTA (25 ml/gel + 400 ml/tank × 12). 1 liter of 50 × TAE: 242 g of Tris base + 57.1 ml glacial acetic acid + 100 ml of 0.5 *M* EDTA, pH 8.0.

18 µl 10 mg/ml ethidium bromide/gel (1.5 µl/25 ml gel × 12 gels).

The class (48 students) will pour 12 2.0% 3:1 NuSeive agarose minigels (25 ml each). Each gel will contain 13 sample wells.

Equipment Required for Class 13

1 hotplate or microwave to melt agarose

12 125-ml flasks for agarose

12 agarose gel electrophoresis apparatus + casting trays and combs

6 dual-outlet power supplies and 12 sets of leads

1 UV transilluminator, camera, film, face shield

Speed Vac centrifuge–concentrator

TA's role: Place frozen PCR reactions at instructors' bench at front of lab. Set out gel casting trays with combs, electrophoresis apparatus, and power supplies. Supervise preparation of purified PCR products for sequencing; ensure that samples contain 25 ng of PCR product and 12 pmol of primer 27F in a total volume of 12 µl. Concentrate dilute samples in the Speed Vac, if necessary. Collect samples and submit to lab for sequencing.

EXPERIMENT 4: SOUTHERN BLOT ANALYSIS OF BACTERIAL rRNA GENES

Class 15: Restriction and agarose gel electrophoresis

Class 16: Blot gel, prepare probe

Class 17: Hybridization

Class 18: Wash and develop blots

Note: Students work individually, NOT in pairs for Experiment 4.

Class 15: Restriction and Agarose Gel Electrophoresis

Equipment Required for Class 15

8 gel rigs (with well formers and casting trays) (1 lane/student × 6 students/gel + 1 marker/gel = 7 lanes/gel)

4 dual-outlet power supplies and leads for 8 gels

water baths at 37 and 55°C

8 (or more) microcentrifuges

P20 Pipetmen

floating microcentrifuge tube holders

benchtop microcentrifuge tube racks

1.5-ml microcentrifuge tubes (at least 1 per student)

yellow tips (1 box per team)

plastic wrap

gloves

UV goggles or face shields

*Eco*RI (Fermentas; 10–20 units/µl; need 60 µl, 1 µl/student)

10 × *Eco*RI buffer (supplied with enzyme, 2 µl/student)

sterile distilled water (20 µl/student)

λ DNA cut with *Hin*dIII (8 µg; 1 µg/lane × 8 gels)

ethidium bromide (10 mg/ml, make 1 ml, use 1.5 µl/25 ml gel)

agarose (1.6 g total; 0.2 g/25 ml gel × 8 gels)

TAE electrophoresis buffer (5 liters). 40 m*M* Tris–acetate, 2 m*M* EDTA. 1 liter of 50 × buffer = 242 g of Tris base + 57.1 ml of glacial acetic acid + 100 ml of 0.5 *M* EDTA, pH 8.0.

loading solution: 0.05% bromophenol blue + 40% (w/v) glycerol in water; need 125 µl (2 µl/student × 48 + 2 µl/marker × 12).

Class 16: Blot Gel and Prepare Probes

Equipment Required for Class 16

8 paper cutters or scissors

8 clean razor blades or scalpels to cut nylon membranes

16 clean plastic trays to hold gels and nylon filters; 12 × 12 cm

shaker platforms to hold 8 trays (at room temperature)

PCR machine

P20 Pipetmen

ice buckets

Whatman 3-mm filter paper (at least 12 large sheets)

paper towels (6 bundles)

positively charged nylon membrane (enough for 8 gels; 9 × 9 cm)

gloves

plastic wrap

aerosol-resistant yellow tips (1 box/team)

0.2-ml thin-wall tubes for PCR machine (16)

0.25 *M* HCl; make 1 liter (100 ml/gel × 8) (12.5 ml of concentrated HCl + 487.5 ml of distilled water = 500 ml).

0.5 *M* NaOH + 1.5 *M* NaCl; make 2 liters (200 ml/gel × 8) (10 g of NaOH + 43.9 g of NaCl + 485 ml of distilled water = 500 ml).

1 *M* Tris, pH 7.5, + 1.5 *M* NaCl; make 2 liters (200 ml/gel × 8) (157.6 g of Tris base + 87.7 g of NaCl + 67.7 ml of concentrated HCl + 810 ml of distilled water).

20 × SSC: 3 *M* NaCl + 0.3 *M* sodium citrate, pH 7.0; make 2 liters (350.4 g of NaCl + 176.5 g of sodium citrate·$2H_2O$ + 7.2 ml of concentrated HCl + distilled water to 2 liters).

50% acetamide

10 µM 27F primer (BRL); 5′-AGA GTT TGA TC(C/A) TGG CTC AG-3′

10 µM 519R primer (BRL); 5′-G(T/A)A TTA CCG CGG C(T/G)G CTG-3′

PCR DIG Probe Synthesis Kit (Roche cat. no. 1 636 090)

mineral oil (PCR grade) (Sigma M5904)

plasmid template DNA containing 16S eubacterial rRNA gene

Class 17: Hybridize Blots

Equipment Required for Class 17

2 hybridization ovens

shakers (for 8 trays) at room temperature and 42°C

boiling water bath

ice bucket

P20 Pipetmen

racks for 50-ml tubes

graduated cylinders and pipettes (25 ml and 5 ml)

Stratalinker (UV light source with dosimeter)

scissors

8 plastic trays

yellow tips (1 box/team)

50-ml Falcon tubes (8; for hybridization oven)

Whatman 3-mm paper (3 sheets)

gloves

0.2 *M* Tris, pH 7.5, + 2 × SSC; make 500 ml (50 ml/filter × 8)

DIG Easy Hyb (Roche cat. no. 1 603 558); make 250 ml (30 ml/filter × 8) (heat to 42°C before class)

Class 18: Wash and Develop Blots

Equipment Required for Class 18

incubators and shakers (or hybridization ovens) at 42 and 65°C

shakers at room temperature

37°C incubator

darkroom, X-ray developer; X-ray film cassettes

P20 Pipetmen

graduated cylinders

5-ml pipettes (8)

scissors

plastic trays (8)

gloves

Whatman 3 mm (3 sheets)

yellow tips

X-ray film (Roche cat. no. 1666 657, 8 × 10 in., 2 sheets)

2 × SSC + 1% SDS; make 2 liters (2 × 100 ml/filter × 8 filters). Heat 1 liter to 42°C and 1 liter to 65°C before class.

0.1 × SSC + 1% SDS; make 1 liter (100 ml/filter × 8). Heat to 42°C.

maleic acid buffer; make 2 liters (0.1 *M* maleic acid + 0.15 *M* NaCl; adjust to pH 7.5 with NaOH).

maleic acid wash buffer + 0.3% (v/v) Tween 20; make 2.5 liters.

10 × blocking solution stock: Dissolve 10% (w/v) blocking reagent (DIG Wash & Block Buffer Set; Roche cat. no. 1585 762; kit vial 4) in maleic acid buffer. Dissolve

blocking reagent by constantly stirring on a heating block at 65°C or by heating in a microwave oven; autoclave and store at 4°C. The solution remains opaque. Make 10 ml.

blocking solution; make 1 liter (100 ml/filter × 8). Dilute 10 × stock 1:10 in maleic acid buffer.

Dilute anti-DIG-AP conjugate just before use; make 200 ml (20 ml/filter × 8) From DIG Wash & Block Buffer Set (Roche cat. no. 1585 762), dilute anti-DIG-AP conjugate (kit vial 3) 1:10,000 (to 75 mU/ml) in 1 × kit buffer 2.

detection buffer: 0.1 M Tris + 0.1 M NaCl, pH 9.5; make 250 ml (22 ml/filter × 8).

CSPD detection reagent; make 20 ml (2 ml/filter × 8). Dilute CSPD (kit vial 5) 1:100 in detection buffer.

Required and Suggested Readings

Secretion in Yeast

Purification and *in vitro* Translocation of Chemical Amounts of Prepro-α-factor*

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The *Saccharomyces cerevisiae* mating pheromone precursor, prepro-α-factor, can be translocated across yeast endoplasmic reticulum membranes post-translationally in an *in vitro* system. This characteristic makes prepro-α-factor potentially useful as a probe in the biochemical dissection of the mechanism of this basic cellular process. Efforts have been limited by the inability to isolate sufficient quantities of such secretory protein precursors in a translocation-competent form. We report here the one-step purification of chemical amounts of translocation-competent prepro-α-factor using nickel ion affinity chromatography on nitrilotriacetate resin. An oligonucleotide encoding 6 histidine residues was inserted into a genomic clone encoding prepro-α-factor 5′ of the naturally occurring translational stop codon by site-directed mutagenesis. The construct was expressed at high levels in a SecY⁻ strain of *Escherichia coli.* The produced preprotein was solubilized in 6 M guanidine hydrochloride and bound to nitrilotriacetate resin. Prepro-α-factor was recovered at a purity in excess of 95% by elution with 0.25 M imidazole, 8 M urea, which competitively displaced the histidine affinity tag from the nickel column. The chemical amounts of prepro-α-factor obtained in this way were determined to be competent for translocation across yeast microsomal membranes and for subsequent modifications such as signal sequence cleavage and *N*-linked glycosylation.

Protein translocation is the process whereby proteins are vectorially transported across a lipid bilayer. This process is encountered most frequently as proteins enter the secretory pathway by translocating across the membrane of the endoplasmic reticulum in eukaryotic cells. Understanding the molecular mechanisms of translocation is a major focus of research in many laboratories.

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The biochemical characterization of the elements involved in the translocation of proteins across endoplasmic reticulum membranes would be greatly facilitated by the ability to purify chemical amounts of those substrates which can be transferred across the membrane as full-length precursors, *i.e.* posttranslationally. Currently, study of the mechanism by which proteins are transported across the endoplasmic reticulum depends upon radiochemical amounts of proteins translated in a cell-free system (1). Amounts are so limiting that basic biochemical studies, such as an analysis of saturation of translocation sites, cannot be undertaken. Moreover, in eukaryotic systems the translocation of most proteins is coupled to their translation, *i.e.* transfer across the membrane is obligatorily co-translational. A major exception to this cotranslational requirement is found in the case of the *Saccharomyces cerevisiae* mating pheromone prepro-α-factor (2-4). It has been shown that prepro-α-factor even maintains its translocation competence when denatured in and diluted out of 8 M urea (5). This ability of prepro-α-factor to be posttranslationally translocated makes it an ideal substrate for study as translocation can effectively be uncoupled from translation. Were chemical quantities of this preprotein available, it would then represent an ideal probe in the biochemical dissection of the process of translocation.

The production of such large amounts of pure protein precursors has proven to be difficult, time-consuming, and inefficient. Although bacterial expression systems can be used to overproduce protein precursors, purification of the products has resulted in yields too low to be useful for *in vitro* studies. Typical purification schemes have employed multiple preparative electrophoretic separations or low efficiency chromatography steps such as antibody affinity columns (6), where often the denaturing conditions necessary to keep preproteins soluble have destroyed or severely limited antibody–antigen binding. A recent advance in the ability to isolate large amounts of recombinant proteins is the use of nickel affinity chromatography (7, 8). By cloning 4–6 contiguous histidine residues into the primary structure of a protein, a ligand is generated that can stably bind to the affinity column under even the harshest of denaturing conditions. Such conditions are often required to solubilize recombinant proteins located in bacterial inclusion bodies and preclude the use of antibodies as affinity adsorbents. The affinity of the histidine residues for binding to the nickel column is sufficiently high to enable extensive washing and removal of almost all contaminating proteins. The result is a nearly homogeneous population of recombinant proteins.

In this report, we describe the results of our successful efforts to use this technology to produce a translocation-competent yeast secretory protein precursor from extracts of *Escherichia coli.* Not only was the precursor chemically pure, but was as efficiently translocated, processed, and glycosylated as material that had been produced using the traditional cell-free system. Prepro-α-factor produced in this way can be used, in turn, as an affinity probe for the isolation and characterization of the cellular machinery which mediates protein targeting and transport.

MATERIALS AND METHODS

Plasmid Construction—An *Eco*RI-*Sal*I fragment from pGEM2-α36 (6) containing the entire coding sequence of prepro-α-factor was cloned into M13mp9. Single-stranded DNA from this construct was used

as a template for *in vitro* mutagenesis. Six histidine codons (CAT) were placed, in frame, just before the stop codon using a synthetic oligonucleotide and the Amersham *in vitro* mutagenesis kit (Amersham Corp.). The insertion of the histidine codons was verified by DNA sequencing. The *Eco*RI-*Sal*I fragment containing the mutagenized prepro-α-factor was then subcloned into pGEM2 (Promega Biotec, Madison, WI), under the control of the SP6 promoter to generate pGAH4 for subsequent use in a yeast *in vitro* transcription/translation/translocation system (9).

An *Nde*I site at the ATG (start of translation) codon was required for the in-frame cloning of prepro-α-factor into the bacterial expression vector, pJLA603 (10). The complete coding region of prepro-α-factor, containing the 6 histidine codons, was reconstructed by cloning the *Pst*I-*Sal*I fragment from pGAH4 into pBluescript II KS+ (Stratagene, La Jolla, CA). A *Pst*I fragment from prepro-α-factor in M13mp8 that had been mutagenized to contain an *Nde*I site at the start of translation was subcloned into the *Pst*I site of the α-factor fragment in pBluescript II KS+. The resulting *Nde*I-*Sal*I fragment contained the entire coding region of prepro-α-factor including an *Nde*I site and six codons for histidine. The integrity of the coding region was confirmed by sequence analysis. This fragment was subcloned into the *Nde*I-*Sal*I sites of the expression vector pJLA603 to generate pMAH1.

The oligonucleotide sequences used in the *in vitro* mutagenesis are as follows: for the insertion of the six histidine codons, 5′-ACCAATGTACCATCATCATCATCATCAT TAAGCCCGACTGATA-3′; for the creation of the *Nde*I site, 5′-CGATTAAACATATG AGATTT-3′.

Construction of Sec Y⁻ Strain—E. coli BL21(DE3) *secY*ts (F^-, *ompT*, r^-_B r^-_B, sec*Y*ts, Tc^R) was constructed from BL21(DE3) (F^-, *ompT*, r^-_B r^-_B,) (11) and CJ107 (F′, *lac, pro* [Δ*lac pro*], *secY*ts, with Tn10 closely linked to *sec Y*) (12) by transduction with $P1_{vir}$ Tetracycline-resistant transductants were selected at 30°C. These were further tested for temperature-sensitive growth at 42°C. The $SecY^-$ phenotype was analyzed by the accumulation of proOmpA at 42°C as described by Wolfe *et al*. (12) and by the accumulation at 42°C of unprocessed prepro-α-factor in cells transformed with pMAH1.

Expression of Prepro-α-factor in E. coli—BL21(DE3) *secY*ts cells were transformed by pMAH1 and maintained in Luria broth containing ampicillin (100 μg/ml) at 30°C. To induce expression of prepro-α-factor, cells were grown at 30°C in the same medium until an A_{600} nm of 0.6. The culture was transferred to 42°C to induce the $SecY^-$ phenotype and the expression of prepro-α-factor (10, 12). Cultures were maintained at the induction temperature for 4 h. The cell culture was centrifuged at 4,000×g for 10 min. The supernatant was discarded, and the cell pellet was stored at −20°C for subsequent purification.

Nickel Ion-NTA Affinity Chromatography—The purification of prepro-α-factor modified by the addition of the histidine hexamer (prepro-α-factor$(His)_6$) using Ni^{2+}-nitrilotriacetate (NTA)[1] resin (Diagen, Düsseldorf, Federal Republic of Germany) was accomplished following a modification of the procedure of Stüber *et al* (13). The stored bacterial cell pellet was thawed at room temperature for 15 min and then resuspended in 10 ml (per 100 ml of cell culture) buffer A (6 M guanidine-HCI, 0.1 M

[1]The abbreviations used are: NTA, nitrilotriacetate; SDS-PAGE, sodium dodecyl sulfate -polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

NaH_2PO_4, pH 8.0). The cells were lysed by continual mixing at room temperature for 1 h. The lysate was centrifuged at 10,000 × g for 10 min, and the supernatant was applied to a 13 × 1-cm column containing 2–3 ml of Ni^{2+}-charged NTA resin. The column was packed and equilibrated in buffer A. The column was washed sequentially with 7–10 ml of buffers B, C, and D (buffer B: 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0; buffer C: 8 M urea, 0.1 M NaH_2PO_4 0.01 M Tris-HCI, pH 6.3; buffer D: 8 M urea, 0.1 M NaH_2PO_4 0.01 M Tris-HCl, pH 5.9). The volume of each wash was varied to allow complete elution as measured by a UV column monitor Prepro-α-factor$(His)_6$ was eluted using buffer B containing 0.25 M imidazole pH 8.0. The column was washed in 3 ml of buffer B containing 0.5 M imidazole pH 8.0 and finally with buffer F (6 M guanidine-HCl, 0.2 M acetic acid) to detach any proteins remaining on the column.

Each column fraction was brought to pH 7 with 2 M Tris-HCl were pH 9.5, and stored at −20°C. Fractions containing guanidine-HCl were dialyzed against buffer B prior to analysis by SDS-PAGE. Prepro-α factor$(His)_6$-containing fractions were dialyzed against buffer B to remove the imidazole prior to use in translocation assays.

Assays for Translocation—In vitro transcription, translation, and translocation of pGEM2-α36 and pGAH4 were performed with essentially as described by Rothblatt and Meyer (9) with minor modifications. Ten μg of plasmid DNA was linearized using 20 units of *Pvull* (Boehringer Mannheim) for 1 h at 37°C. The linearized DNA was transcribed with 60 units of SP6 RNA polymerase (Promega) in a final reaction volume of 100 μl containing 40 mM Hepes, pH 7.5, 6 mM magnesium acetate, 2 mM spermadine, 5 mM dithiothreitol, 0.5 mM each of ATP, UTP, CTP, and GTP, 125 units of RNase inhibitor (Boehringer Mannheim) and 0.4 A_{260} units of $m^7G(5')ppp(5')G$. Transcription was allowed to proceed for 10 min at 40°C. One μl of 40 mM GTP was added to the reaction and it was left at 40°C for an additional 10 min. The transcription mixture was either put on ice for immediate use in cell-free translation or was quick-frozen in liquid nitrogen and stored at −80°C.

A 25-μl translation assay contained 0.5 μl of pGEM2-α36 or pGAH4 transcription mixture, 10 μl of yeast lysate (9) and was adjusted to the following concentrations: 42 mM Hepes, pH 7.4, 190 mM potassium acetate, 2.8 mM magnesium acetate, 20 mM creatine phosphate, 80 μg/ml creatine phosphokinase, 1.1 mM ATP, 30 μM of each of 19 amino acids (minus methionine), 0.2 mg/ml yeast tRNA, 1500 units/ml Rnase inhibitor, 25 μCi of [^{35}S]L-methionine and 1 μl of yeast membranes (50 A_{280} nm/ml). Translation/tanslocation reactions were incubated at 25°C for 1 h and then treated with proteinase K as described below.

Translocation of purified prepro-α-factor$(His)_6$ was carried out in a final reaction volume of 150 μl containing 88 mM potassium acetate, 3.5 mM magnesium acetate, 7.6% glycerol, 12 mM Hepes, pH 7.4, 1.6 mM creatine phosphate, 3.2 μg/ml creatine phosphokinase, 40 μM ATP, and 6 mM GTP. Approximately 6 μg of prepro-α-factor$(His)_6$ in a 20-μ1 volume was used in each reaction resulting in a final concentration of 1 M urea. Approximately 1 A_{280} nm unit of membranes, prepared according to the procedure of Rothblatt and Meyer (4), was used in each reaction. After the addition of all components, the reaction mixture was gently mixed and allowed to stand at 25°C for 1 h. The reaction was then divided into three equal aliquots, two of which were subjected to proteolysis at 0°C for 90 min at a final concentration of 0.2 mg/ml proteinase K in the presence and absence of 0.3% Triton X-100. Proteolytic digestion was stopped by the

addition of 6 mM phenylmethylsulfonyl fluoride, and the entire aliquot was analyzed using SDS-PAGE.

Electrophoresis and Immunoblotting—Electrophoresis of the *in vitro* translocation products and the column fractions was carried out using 13% polyacrylamide slab gels. SDS-PAGE and fluorography were carried out as described by Blobel and Dobberstein (14). Silver staining was according to the method of Ansorge (15). Proteins were transferred to 0.45-μm nitrocellulose membrane with electroblotting and stained with rabbit sera containing polyclonal antibodies raised against prepro-α-factor. The rabbit sera was blocked with whole cell bacterial lysate prior to staining the Western blot.

RESULTS AND DISCUSSION

Previous reports on the use of nickel ion affinity columns as a method of protein purification have made use of multiple histidine and/or tryptophan residues as the interacting ligand (7, 10, 16–21). The optimum situation for this type of study requires that the ligand bind the nickel ion efficiently, and be easily detachable under specific conditions. To date, no systematic studies have been carried out to define the optimum number and spacing of residues for efficient ligand-nickel interaction; it appears to be protein-specific. Smith *et al*. (18) made use of a histidine-tryptophan dipeptide for the purification of leuteinizing hormone-releasing hormone. Prepro-α-factor contains four such histidine-tryptophan dipeptides spaced along the protein, but our preliminary results showed that these were not sufficient for metal ion affinity purification under denaturing conditions. Thus, wild-type prepro-α-factor, expressed in a bacterial system, exhibited only weak binding to the nickel column and eluted over many fractions (data not shown). In order to provide prepro-α-factor with a strong nickel-binding site, an oligonucleotide encoding 6 histidines was spliced into the coding region, 5' to the naturally occurring stop codon.

Addition of C-terminal Histidine Residues to Prepro-α-factor Does Not Affect Its Translocation Competence—Prior to purifying large amounts of prepro-α-factor$(His)_6$ it was necessary to demonstrate that this version of the preprotein was as competent for translocation as wild-type prepro-α-factor. Previous studies, which have demonstrated that prepro-α-factor will translocate both co- and post-translationally, made use of a yeast cell-free translation system programmed with prepro-α-factor mRNA produced by *in vitro* transcription. Accordingly, the transcription of the prepro-α-factor$(His)_6$ gene was placed under the control of the same SP6 promoter in pGEM2 as the wild-type precursor. The transcription, translation, and translocation of radiochemical amounts of this construct was then compared with that of wild-type prepro-α-factor using the conventional yeast *in vitro* translocation system (2, 3, 9).

As shown in Fig. 1A, *lanes 1–3*, the presence of yeast microsomes resulted in cleavage of the signal peptide of prepro-α-factor and glycosylation of the propeptide (pro-α-factor has three sites for *N*-linked glycosylation). Translocation across the microsomal membrane was confirmed by the protease protection of the translocated products, pro-α-factor, and glycosylated pro-α-factor. In the absence of exogenous membranes, only a negligible quantity of prepro-α-factor was processed to pro-α-factor (*lane 4*); this results from the presence of a small amount of membranes which cannot be removed from the yeast lysate system during its preparation. Prepro-α-factor

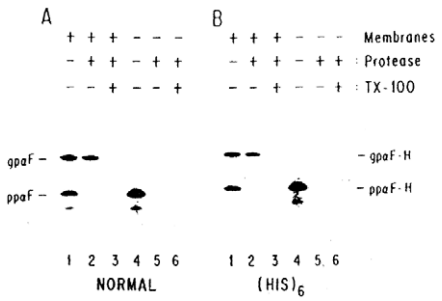

FIGURE 1 Translocation of radiolabeled prepro-α-factor and prepro-α-factor(His)$_6$. *In vitro* transcription and translation were carried out as described under "Materials and Methods." The presence of membranes, proteinase K, and Triton X-100 are indicated *above* the figure. *A*, wild-type prepro-α-factor translated from pGEM2-α36. *B,* prepro-α-factor(His)$_6$ translated from pGAH4. Translocation is demonstrated by the protection of glycosylated pro-α-factor (*gpαF*) from protease digestion in the presence of intact membranes (*lane 2*) and its digestion when the membranes were first disrupted by Triton X-100 (*lane 3*). Untranslocated prepro-α-factor (*ppαF*) was protease-sensitive even in the presence of membranes. No translocation occurred in the absence of membranes (*lanes 4–6*).

(His)6 was translocated and glycosylated with approximately the same efficiency as the wild-type prepro-α-factor (Fig. 1B).

Purification of Chemical Amounts of Prepro-α-factor Containing C-terminal Histidines—To facilitate the production of chemical quantities of prepro-α-factor the region encoding prepro-α-factor(His)$_6$ was subcloned into the bacterial expression vector pJLA603. It had previously been established that, in bacteria, prepro-α-factor is efficiently transported into the periplasmic spare and processed to pro-α-factor (22). Translocation shows a dependence upon a membrane potential and the *secY* gene product. Since obtaining large yields of prepro-α-factor from bacteria is a prerequisite to affinity purification, retention of the unprocessed form in the cytoplasm is paramount. Accordingly, a temperature-sensitive *sec Y* strain was constructed to eliminate the *in vivo* translocation and processing of prepro-α-factor to the pro form. This strain was transformed with a plasmid encoding prepro-α-factor(His)$_6$, and single colony isolates were tested for expression. Induction of both prepro-α-factor and the SecY$^-$ phenotype were accomplished by a shift in culture temperature from 30 to 42°C. This combination of host and plasmid resulted in the overexpression of full-length prepro-α-factor(His)$_6$.

Prepro-α-factor(His)$_6$ was purified from a bacterial cell extract, generated from cells lysed in 6 M guanidine-HCl, by passing the supernatant over a column of Ni^{2+}-NTA resin. Contaminating proteins were eluted using a pH gradient in 8 M urea consisting of steps at pH 8.0, 6.3, and 5.9. Prepro-α-factor(His)$_6$, retained during all aforementioned washes, was eluted from the column with an 8 M urea buffer, pH 8.0, containing 0.25 M imidazole. The elution profile from a typical column run is shown on a silver-stained polyacrylamide gel in Fig. 2*A*. An immunoblot of this elution profile demonstrates the binding efficiency of prepro-α-factor(His)$_6$ to the Ni^{2+}-NTA column (Fig. 2*B*). Prepro-α-factor(His)$_6$ was observed in the whole cell lysate *(lane 1*) and supernatant fraction (*lane 2*), but none was detected in the column flow-through (*lane 3*). Trace amounts of the protein were eluted in the pH 5.9 wash (*lane 7*), but the vast majority was eluted with the 0.25 *M* imidazole, pH 8.0 buffer (*lanes 8–10*). No detectable amount of prepro-α-factor(His)$_6$ appeared in the 0.5 *M* imidazole wash (*lane 11*) indicating that the 0.25 M imidazole elution was sufficient to remove all of the prepro-α-factor(His)$_6$ from the column. Prepro-α-factor(His)$_6$ represents 87% of the

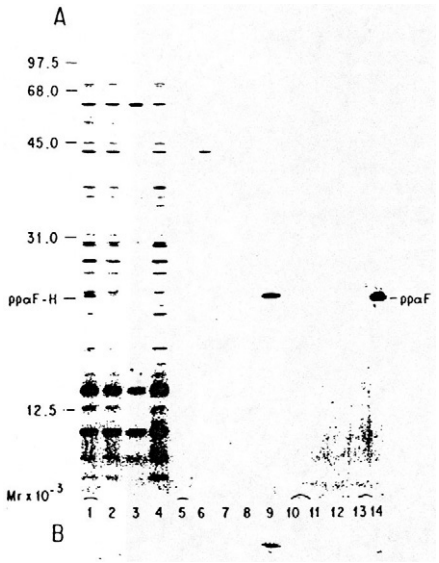

FIGURE 2 Purification of prepro-α-factor (His)6 from *E. coli* on NTA resin. A, silver-stained 13% SDS-PAGE gel of nickel-NTA column fractions. *B*, immunoblot of the same fractions decorated with rabbit anti-prepro-α-factor (ppαF) antibody. *Lane 1*, whole bacterial cell lysate; *lane 2*, supernatant from centrifugation at 10,000 × *g*; *lane 3*, column flow-through; *lanes 4–7*, washes with buffers A (6 M guanidine-HCl, pH 8.0), B (8 M urea, pH 8.0), C (8 M urea, pH 6.3), and D (8 M urea, pH 5.9), respectively; *lanes 8–10*, fractions from an elution with 0.25 M imidazole, pH 8.0; *lane 11*, wash with 0.5 M imidazole, pH 8.0; *lane 12*, wash with buffer F (6 M guanidine-HCl, 0.2 M acetic acid); *lane 13*, blank; *lane 14*, wild-type prepro-α-factor purified by preparative gel electrophoresis.

protein in the fraction shown in *lane 9*, and 98% of the protein in the fraction shown in *lane 10* as determined by densitometry of the silver-stained gel shown in Fig. 2. This corresponds to an 11–12-fold enrichment, as prepro-α-factor(His)$_6$ represented 7.6% of the total protein in the whole cell lysate (*lane 1*).

Overproduced, Purified Prepro-α-factor (His)$_6$ Is Translocation-competent—To show that the prepro-α-factor(His)$_6$ eluted from the Ni^{2+}-NTA column could be translocated, it was tested in an *in vitro* translocation reaction (Fig. 3). The column fractions containing purified prepro-α-factor(His)$_6$ were first dialyzed against three changes of the 8 M urea, pH 8.0 buffer (buffer B) to remove the imidazole. Prepro-α-factor(His)$_6$, diluted out of urea, was incubated in the presence and absence of yeast microsomal membranes. In the presence of membranes, prepro-α-factor(His)$_6$ was processed to pro-α-factor(His)$_6$ and was glycosylated (*lane 4*). The presence of unglycosylated pro-α-factor(His)$_6$ and the partially glycosylated forms indicated that the glycosylation reaction had not gone to

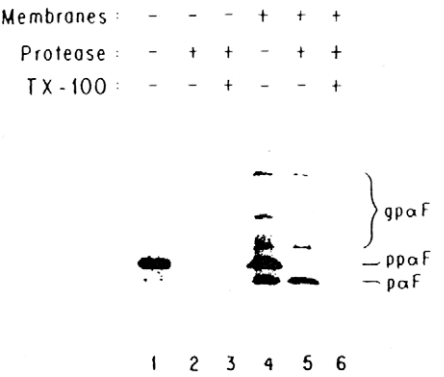

FIGURE 3 Translocation of chemical quantities of recombinant prepro-α-factor(His)$_6$. Translocation of prepro-α-factor(His)$_6$ (*ppαF*) was carried out as described under "Materials and Methods." *Lanes 1–3*, translocation reaction in the absence of membranes; *lanes 4–6*, translocation in the presence of membranes. The translocated products, glycosylated pro-α-factor (*gpαF*), and pro-α-factor (*pαF*) were protected from protease digestion by the membranes (*lane 5*), but not when the membranes were disrupted by detergent (*lane 6*).

completion. A plausible, and testable, explanation would be that the levels of dolichol-oligosaccharide substrate in the microsomes were insufficient to glycosylate the chemical quantities of preprotein that were translocated. As shown in a comparison of *lanes 4* and *5*, the (signal-cleaved) pro-α-factor(His)$_6$ and the glycosylated forms were protected from protease digestion by the membranes, whereas the untranslocated prepro-α-factor(His)$_6$ was not. The protected forms of pro-α-factor(His)$_6$ became protease-sensitive when the membranes were first disrupted by Triton X-100 (*lane 6*). Taken together, these data show that the prepro-α-factor(His)$_6$, produced and purified as described, represents an ideal substrate for probing the translocation reaction.

CONCLUSIONS

We have taken advantage of nickel ion affinity chromatography to produce large quantities of a pure preprotein that can serve as a substrate for translocation. The use of a histidine hexamer at the C terminus of prepro-α-factor allowed the quantitative purification of the protein from a whole cell bacterial lysate to 87–98% purity. The addition of histidines to the primary sequence had no apparent effect on the biological activity of the preprotein as measured by its ability to translocate and respond to antibodies. The material thus purified is currently being used to probe the yeast *in vitro* translocation system to identify and characterize other protein components.

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Bacterial Bioluminescence: Isolation and Genetic Analysis of Functions from Vibrio fischeri

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Recombinant E. coli that produce light were found in a clone library of hybrid plasmids containing DNA from the marine bacterium Vibrio fischeri. All luminescent clones had a 16 kb insert that encoded enzymatic activities for the light reaction as well as regulatory functions necessary for expression of the luminescence phenotype (Lux). Mutants generated by transposons Tn5 and mini-Mu were used to define Lux functions and to determine the genetic organization of the *lux* region. Regulatory and enzymatic functions were assigned to regions of two *lux* operons. With transcriptional fusions between the *lacZ* gene on transposon mini-Mu and the target gene, expression of *lux* operons could be measured in the absence of light production. The direction of transcription of *lux* operons was deduced from the orientation of mini-Mu insertions in the fusion plasmids. Induction of transcription of one *lux* operon required a function encoded by that operon (autoregulation). From these and other regulatory relationships, we propose a model for genetic control of light production.

INTRODUCTION

Luminescent bacteria are ubiquitous in marine environments, where they can exist planktonically, as gut symbionts, as saprophytes, as parasites, or in specialized light organs of certain fish and squid (Nealson and Hastings, 1979). The ecological significance for the host in these symbiotic associations has long been recognized (Morin et al., 1975). Luminescence can be used by these higher organisms for a variety of purposes, including attraction of prey, intraspecies communication and escape from predators. However, it is not certain what specific benefit these bacteria, either free living or in association with higher organisms, derive from this property. It is of interest to understand the adaptive advantage of light production for the bacteria and the genetic mechanisms that regulate expression of genes for luminescence (*lux*).

Light production by luminous bacteria is catalyzed by the enzyme luciferase, a mixed function oxidase consisting of two different subunits (alpha and beta), each approximately 40K MW (Ziegler and Baldwin, 1981). In the generation of light, luciferase catalyzes the oxidation of a reduced flavin and a long chain aldehyde, producing oxidized flavin and the corresponding long chain fatty acid

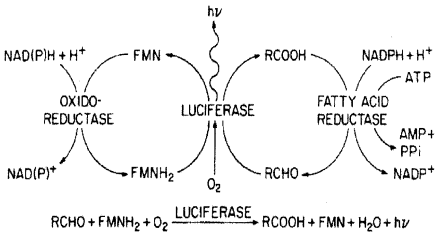

FIGURE 1 Substrates, products and pathways involved in the bacterial bioluminescence reaction.

(see Figure 1). A fatty acid reductase has been implicated in the recycling of the fatty acid to the aldehyde (Riendeau and Meighen, 1980). This enzyme was isolated from Photobacterium phosphoreum and shown to have two activities: an acyl protein synthetase (51K MW) which is ATP-dependent, and an acyl CoA reductase (58K MW) which is NADPH-dependent. The reducing power for the luminescence system is generated by an NAD(P)HFMN oxidoreductase (Jablonski and DeLuca, 1978). Other components necessary for light production may include enzymes involved in the de novo synthesis of a specific fatty acid or aldehyde.

Luciferase can constitute 5% or more of the cellular protein (Hastings et al., 1965), and 10% or more of cellular energy can be utilized in producing light (Karl and Nealson, 1980). With such a large energy commitment, it is not surprising that the system is highly regulated. Luminous bacteria synthesize a small sensory molecule, called autoinducer, which accumulates in the environment (Nealson, 1977). When this molecule reaches a critical concentration, induction of the luminescence system occurs, resulting in approximately a 1000-fold increase in light production. Luminescence appears to be a social phenomenon that occurs when cells are confined at high densities. Autoinducer has been isolated from Vibrio fischeri and was shown to be *N*-(β-ketocaproyl) homoserine lactone (Eberhard et al., 1981). This compound induces light production in all V. fischeri strains and in the closely related V. logei, but in no others (K. H. Nealson, unpublished results). Inhibitor studies suggested that autoinducer controls light production at the level of gene transcription (Nealson et al., 1970). As many as seven polypeptides have been reported to appear upon induction of the luminescence system in V. harveyi (Michaliszyn and Meighen, 1976).

Recently, the two genes for luciferase (*luxA* and *luxB*) from V. harveyi, a planktonic bacterium, were isolated by Belas et al. (1982). Expression of these *lux* genes in E. coli required the provision of promoters such as P_L and P_R of bacteriophage λ. Light production in these recombinant bacteria was dependent upon exogenous addition of aldehyde. Apparently the cloned fragment did not contain genes for accessory enzymes in the light reaction or the genetic elements necessary for expression of *luxA* and *luxB*. To understand the regulation of the luminescence system, we attempted to isolate the genes necessary for light production from the symbiont V. fischeri (MJ-1), which inhabits the light organ of the fish Monocentris japonicus (Tebo et al., 1979). This bacterium was particularly useful for analysis, since the autoinducer molecule for this species has been identified. We report the isolation and genetic characterization of a cloned fragment of DNA that encoded all the functions necessary for light production in E. coli, and which also contained the regulatory elements required for expression of the luminescence (Lux) phenotype.

RESULTS

Isolation of *lux* Genes

A library of recombinant plasmids was constructed by ligating Bam HI restriction

fragments of V. fischeri (MJ-1) DNA with vector pACYC184 (see Experimental Procedures). The resultant transformants in E. coli were examined visually in a dark room for the production of light. Of approximately 10,000 clones screened, three recombinants were luminescent. Addition of C_{14} aldehyde (tetradecanal) was not required for light production from these recombinant bacteria. Restriction digests of the hybrid plasmids from these clones were analyzed, and all three plasmids contained a 16 kb insert. The insert from one of these plasmids (pJE201) hybridized with a corresponding fragment in a Bam HI digest of MJ-1 DNA transferred to nitrocellulose paper by the method of Southern, 1975 (data not shown). A 9 kb Sal I fragment, internal to the Bam HI restriction sites, was subcloned into plasmid pBR322 (see Figure 3). This recombinant (pJE202) also produced light without addition of tetradecanal. A 5 kb Bgl II-Sal I fragment from the original insert was also cloned (pJE205). Cells with plasmid pJE205 were dependent on exogenous aldehyde for luminescence. Strains with other subclones (pJE204, pJE207) produced no light.

The regulation of expression of luminescence in recombinant clones was examined. Light production in V. fischeri depends upon the accumulation of an extracellular signal molecule, autoinducer, in the growth medium (Nealson, 1977). At low cell densities, little autoinducer is present, and no net synthesis of bioluminescence enzymes occurs. At higher cell densities, a critical concentration of autoinducer is reached, and synthesis of luminescence enzymes begins. Following induction, light production per cell increases exponentially. The effect of cell density on light production in V. fischeri is shown in Figure 2. Recombinant clones with pJE201 and pJE202 mirrored this behavior. Light production in strains with pJE201 was consistently lower than that observed with pJE202. This effect may result in part from differences in gene dosage (copy number), since pJE201 (~20 kb) was almost twice the size of pJE202 (~13 kb). Light production in cells with plasmid pJE205 was independent of cell density. Recombinant clones with pJE201 and pJE202 appeared to encode enzymatic functions necessary for light

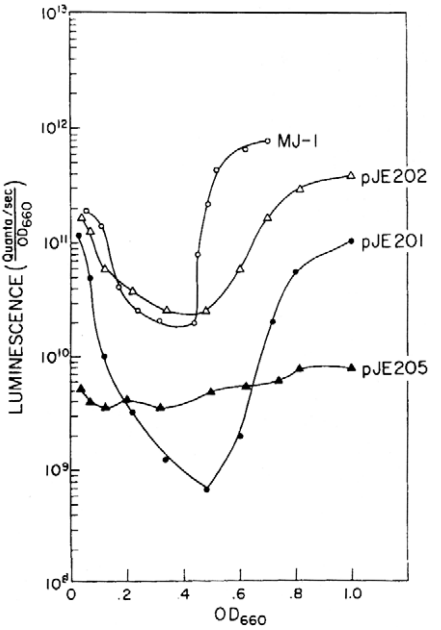

FIGURE 2 Expression of Luminescence in V. fischeri and E. coli Strains Containing Recombinant Plasmid Samples from growing cultures were removed every 30 min for measurement of light production and cell density (OD_{660}). Light production per unit of cell density is plotted as a function of the cell density. Induction occurred in V. fischeri at an OD_{660} of approximately 0.4 and in E. coli strains harboring plasmids pJE201 and pJE202 at an OD_{660} of approximately 0.55. C_{14} aldehyde was added to cells containing pJE205 prior to light measurements (see Experimental Procedures). Light emission was at 490 nm for V. fischeri (MJ-1) and for E. coil recombinants.

production in E. coli, as well as regulatory functions that controlled the expression of these luminescence activities.

Mutagenesis

Transposon-generated mutations were used to define the luminescence functions encoded by hybrid plasmids pJE201 and pJE202. Transposon Tn5 was used to mutagenize plasmid pJE201, and mini-Mu was used to mutagenize plasmid pJE202. Both Tn5 and mini-Mu mutate by insertional inactivation of the target gene, generally causing complete loss of function (null phenotype; Kleckner et al., 1977). If the target is in an operon, transposon insertion interferes with transcription of downstream genes. Thus these mutations can result in negation of several genetic functions. In addition, insertion of transposon mini-Mu can result in transcriptional fusions between the target gene and the *lacZ* gene carried on the mini-Mu (Casadaban and Cohen, 1979). More than 200 nonluminous (>100 fold reduction in light production) and dim (10 to 100 fold reduction in light production) mutants were isolated (see Experimental Procedures), and transposon insertions in plasmids pJE201 and pJE202 were located by restriction mapping. Figures 3A and 3B show the position of these elements in plasmids pJE201 and pJE202. All insertions that affected bioluminescence mapped within the Sal I sites and defined a coding region of about 9 kb. Mutant pJE325 produced normal levels of light, and this insertion mutation marked the leftward boundary of this coding region.

Complementation

Hybrid plasmids pJE201 (derivative of pACYC184) and pJE202 (derivative of pBR322) have compatible replicons and can coexist within a cell. Complementation studies were undertaken by introducing pJE201::Tn5 mutants (pJE300's) and pJE202::mini-Mu mutants (pJE400's) into the same E. coli strain (see Experimental Procedures). Because of the polar nature of Tn5 and mini-Mu insertions, mutations in trans in the same operon did not complement to give light production, while mutations in trans in different operons did complement. Thus complementation analysis defined operons and not individual genes within a transcriptional unit. Figure 4 shows the results of one complementation test. Light production occurred when the mutations on the plasmids complemented. Those cells that produced little or no light harbored plasmids with noncomplementing mutations. Since mutations in different operons complemented, and two complementation groups were observed, we concluded that *lux* genes were organized into two operons. These transcriptional units will be called operon L (left) and operon R (right). Operon L was approximately 1 kb, while operon R was about 7.5 kb in length. Results from all complementation tests are compiled in Table 1. Complementation tests with subclones pJE204, pJE207 and pJE205 were also performed, and the results were generally consistent with the presence of two operons. The properties of plasmid pJE205 will be discussed later in more detail.

Orientation

The direction of transcription of the *lux* operons was determined by measuring β-galactosidase activity resulting from transcriptional fusions with the *lacZ* gene of mini-Mu. Transposon mini-Mu is a sensitive tool for measuring transcription in vivo. The *IacZ* (and *lacY*) gene of E. coli is inserted

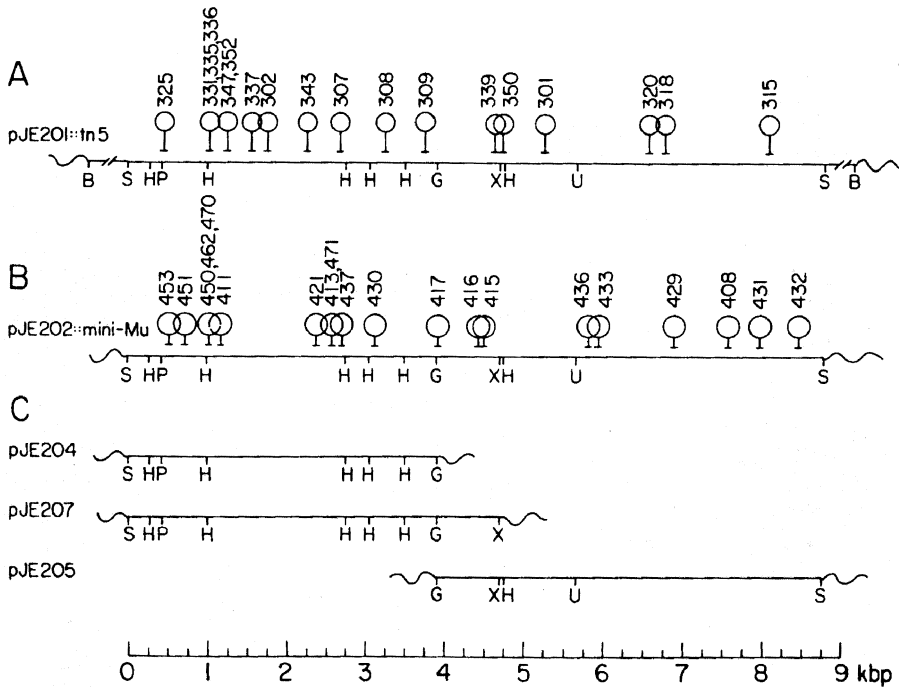

FIGURE 3 Restriction Maps of Cloned DNA in Hybrid Plasmids and Locations of Transposon Insertions (A) Plasmid pJE201 contains a Bam HI fragment from V. fischeri cloned into the Bam HI site of the plasmid vector pACYC184 (Chang and Cohen, 1978). B: Bam HI. S: Sal I. H: Hind III. P: Pst I. G: Bgl II. X: Xho I. U: Pvu II. Symbols above the map represent the location of Tn5 insertions positioned by analyzing Hind III and Bam HI plus Bgl II digests of the corresponding plasmids. Endonuclease sites in the Tn5 transposon came from the restriction map reported by Rothstein et al. (1980). (B) Plasmid pJE202 contains the Sal I fragment of pJE201 that was cloned into the plasmid vector pBR322 (Bolivar et al., 1977). Symbols represent mini-Mu insertions positioned by analyzing Hind Ill and Pst I plus Eco RI digests of the corresponding plasmids. A restriction map of mini-Mu used in positioning and orienting insertions was provided by M. Casadaban (personal communication). (C) Physical maps of subclones pJE204 and pJE207 in vehicle pACYC184 and pJE205 in vehicle pBR322. The position of transposon insertions was accurate to approximately ± 75 bp. Mutants with inserts pJE331, 335, 336, 450, 462, 470 were dim, 1%–10% of the light of the parent hybrid, while all other mutants were dark and produced less than 1% of the light of the parent hybrid plasmid.

adjacent to one end of Mu in such a way that transcription of this gene is dependent upon transcription of the target gene. Insertion of this transposon in one orientation in an operon usually causes transcriptional fusion between the target gene and the *lacZ* and results in the synthesis of β-galactosidase, while insertion of the transposon in the opposite orientation does not result in β-galactosidase synthesis (Casadaban and Cohen, 1979). The orientation of the mini-Mu was found by restriction analysis with

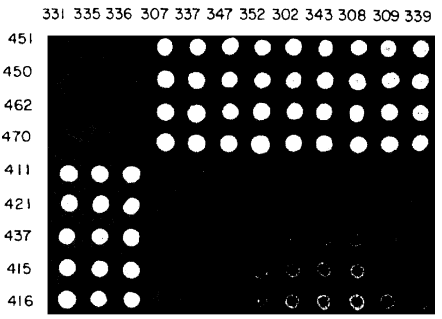

FIGURE 4 Complementation analysis. Light production in strains containing pairs of hybrid plasmids was measured. One pJE201::Tn5 hybrid plasmid (300's) and one pJE202::mini-Mu hybrid plasmid (400's) were transformed into E. coli strain HB101 Mu*cts*. Luminescent strains contain hybrid plasmids with complementing mutations. The figure shows a photographic double exposure taken by the strains' own light and also dark-field-illuminated to show the position of the strains. Bacteria (5 µl of liquid culture) were spotted on an L agar slab and incubated at 30˚C overnight before photography. Faint images result from dark-field illumination and not from bioluminescence. Table 1 summarizes the results of such complementation tests.

enzymes that cleaved DNA at asymmetric sites within the transposon (see Experimental Procedures). Mutants with mini-Mu insertions in operon R had high levels of β-galactosidase (approximately 2000 U in 1 ml culture at OD_{660} of 1.0) when the *lacZ* gene was oriented in the rightward direction, and had virtually no activity when the *lacZ* gene was oriented in the leftward direction. It was concluded that operon R was transcribed from left to right (see Figure 5). Measurement of β-galactosidase activity in mutants with mini-Mu in operon L (approximately 250 U at OD_{660} of 1.0) suggested leftward transcription of this operon. However, some activity was observed when the *lacZ* gene was oriented in the rightward direction, which could have been due to read-through from a plasmid promoter. Strains with *lacZ* fusions in operon R synthesized about tenfold more β-galactosidase than strains with fusions in operon L when dense (postinduction) cultures were assayed. Therefore, transcription of operon R exceeded that of operon L following induction of luminescence.

Bioluminescence Functions

Genetic functions necessary for the regulation of expression of bioluminescence and for the synthesis of enzymes involved in bioluminescence were assigned to regions of the cloned DNA fragment. Genes for the α and β subunits of the luciferase (*luxA* and *LuxB)* were located with hybridization probes. These included mixtures of oligonucleotides designed from the first five amino acids of the N-terminus of the α and β luciferase proteins of V. fischeri (Baldwin et al., 1979; Cohn et al., 1983) and fragments of DNA from the *luxA luxB* clone isolated from V. harveyi (Belas et al., 1982). Labeled probes were hybridized to Southern blots of restricted pJE201 plasmid DNA (data not shown). The locations of *luxA* and *luxB* are shown in Figure 5. In addition, cell extracts from strains with transposon mutations in the *luxA luxB* region (pJE339, 350, 301, 436, 433) had no in vitro luciferase activity. Transposon insertion in operon R downstream from *luxA* and *luxB* (pJE320, 315, 408) did not affect luciferase synthesis, while insertions upstream in operon R (pJE308, 421, 430) and in operon L (pJE331, 450, 451) greatly reduced but did not entirely eliminate the synthesis of luciferase.

Light production in recombinants containing plasmids pJE201 and pJE202 did not require the addition of the long chain aldehyde substrate, which suggested that the

TABLE 1 Complementation of *lux* Operon Mutations

pBR322 Replicon	pACYC184 Replicon																		
	331	**335**	**336**	**347**	**352**	**337**	**302**	**434**	**307**	**356**	**305**	**308**	**309**	**339**	**301**	**320**	**315**	**204**	**207**
451	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	ND
450	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
454	–	–	–	ND	ND	ND	+	+	ND	ND	ND	+	+	+	ND	ND	+	ND	ND
462	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
470	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
411	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
421	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
437	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
423	+	+	+	–	ND	ND	–	–	ND	–	–	ND	ND	–	–	–	–	ND	ND
414	+	+	+	–	ND	ND	–	–	ND	–	–	ND	ND	–	–	–	–	ND	ND
415	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
416	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
410	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	ND
435	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	ND
431	+	+	+	–	ND	ND	–	–	ND	–	–	ND	ND	–	–	–	–	ND	ND
432	+	+	+	–	ND	ND	–	–	ND	–	–	ND	ND	–	–	–	–	–	–
205	–	ND	ND	–	ND	ND	–	–	ND	ND	–	ND	ND	+	+	+	+	–	+

Complementation of *lux* operon defects was measured in Rec⁻ strains harboring pairs of hybrid plasmids (see Experimental Procedures). The transposon insertion mutations and subclones (pJE204, 205, 207) are described in Figure 3. The location of some transposon mutations in this table is not shown in Figure 3, but all mutations are arranged in the order of their location on the linear map. Plasmid numbers are used interchangeably with mutation numbers.

+: positive complementation. –: no complementation. ND: not determined.

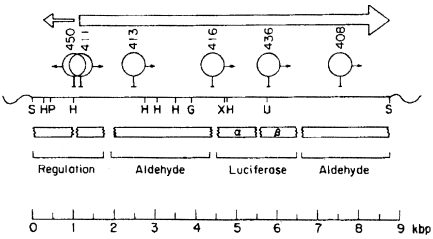

FIGURE 5 Genetic organization of lux functions. The physical map of the insert in plasmid pJE202 is shown with the locations of some of the mini-Mu insertions used to determine the direction of transcription of the two *lux* operons (L and R). Transposon insertions resulted in fusion between the *lacZ* gene and the target gene in the *lux* operon. Small arrows on transposon symbols: orientation of the *lacZ* gene. The direction of transcription of operon L (left) and operon R (right) was determined from the orientation of the *lacZ* gene. Large arrows: direction of transcription and location of the operons. β-galactosidase synthesis in fusion strains was taken as a measure of the level of expression of the operons, and the thickness of the large arrows represents the difference in levels of transcription (see text). Regions of function encoded by the V. fischeri DNA fragment are shown below the restriction map.

genetic functions necessary for aldehyde synthesis (see Figure 1) resided in the cloned fragment. Mutants with transposon insertions in operon R downstream from *luxA* and *luxB* (pJE320, 318, 315, 429, 408, 431, 432) were dark, but produced light if aldehyde was added to the cells. The 2 kb region for aldehyde function is shown in Figure 5. Results with plasmid pJE205 suggested that this region alone was not sufficient to encode functions for aldehyde production. Plasmid pJE205 contained the downstream portion of operon R, including the *luxA* and *luxB* genes and the region for aldehyde function discussed above. Expression of these genetic functions in pJE205 probably resulted from a transcriptional fusion to a plasmid promoter element (see Discussion). Light production with strains containing this plasmid required the addition of aldehyde. Requirement for aldehyde could be fulfilled by providing plasmid pJE207 but not plasmid pJE204 in trans. Plasmids pJE339, 301, 320 and 315 could also provide aldehyde function in trans (see Table I). It was concluded that another region in operon R upstream from *luxA* and *luxB* (see Figure 5) was necessary for aldehyde production.

Regions of DNA that were necessary for the synthesis of luciferase and the aldehyde substrate have been identified, but an additional function such as oxidoreductase (see Figure 1) could also be encoded by the recombinant plasmid. Studies of E. coli containing a small fragment with the luciferase (*luxA luxB*) genes of V. harveyi suggested that E. coli could provide reduced flavin for light production (Belas et al., 1982). If this were the case, an oxidoreductase function on the V. fischeri clone would be redundant and difficult to identify. Studies on oxidoreductase from V. harveyi indicated that synthesis of this enzyme was not inducible, and therefore was not coregulated with luciferase expression (Jablonski and DeLuca, 1978).

Expression of bioluminescence in the recombinant clones was very similar to that in V. fischeri (Figure 2), and the functions that controlled *lux* gene expression in V. fischeri could reside on the hybrid plasmid. E. coli strains containing plasmids pJE201 and pJE202 were capable of producing autoinducer identical in activity to that isolated from V. fischeri, and were also capable of responding to autoinducer isolated from V. fischeri or the recombinant E. coli. Extracellular autoinducer activity was detected by measuring stimulation of light production by V. fischeri or hybrid E. coli cells in the preinduction stage of growth, or

by using mutant strains with the dim phenotype (see below). Figure 6 shows a test for autoinducer production in which a receptive dim mutant was cocultivated with a variety of mutants on a nutrient agar plate. Analysis of strains with transposon mutations showed that a region in operon R was responsible for autoinducer activity. Mutations in plasmids pJE347, 352, 337, 411 abolished the ability to make autoinducer, while insertions in operon R downstream from this region did not affect the production of this activity. This function is located in the regulatory region shown in Figure 5 (5′ end of operon R).

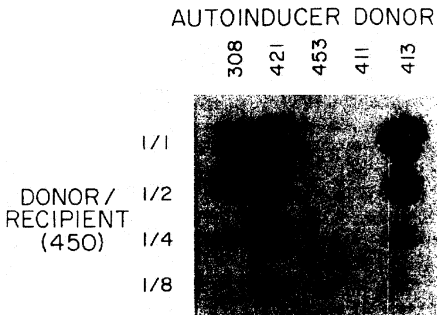

FIGURE 6 Assay for Autoinducer Production. Luminescence of dim mutants (see text) can be stimulated more than tenfold by exogenous autoinducer, and this receptive property was used to assay autoinducer production by dark mutants. The dim mutant recipient (HB101 with pJE450) was spotted (5 μl) in a 4 × 5 array on an L agar plate. Several serial dilutions of the donor strain to be tested were then spotted on the recipient strain. After overnight incubation, an autoradiograph was made by placing the culture plate in contact with x-ray film for 10 sec. Donor strains are arranged in vertical rows, and the donor:recipient ratio is shown at left. Strains containing plasmids pJE308, 421 and 413 stimulated the expression of luminescence, and therefore produced autoinducer, while strains with plasmids pJE411 and 453 did not.

Strains with transposon insertions in operon L had two phenotypes: strains harboring plasmids with insertions pJE451, 453 were dark (<1 % light production of wild-type hybrid), and strains with insertions pJE331, 335, 336, 450, 462, 470 were dim (1 %–10% light production of wild-type hybrid). Dark mutants produced no detectable autoinducer (see Figure 6), while dim mutants produced less than 10% of the amount found in strains with the parent plasmids. Exogenous addition of autoinducer (from V. fischeri or E. coli hybrids) did not stimulate light production with the dark mutants, but increased light production more than tenfold with the dim mutants. In vitro luciferase activity from these strains was greatly reduced (proportionate to the level of light production observed in the intact cells). Thus mutations in operon L affected the expression of the *luxA* and *luxB* genes in operon R, and this suggested that a product of operon L was required for expression of operon R. Defects in operon L could influence expression of operon R by preventing the synthesis of autoinducer or by affecting the response to autoinducer (or both). Transcription of operon R required the presence of autoinducer because, as shown below, transcription of operon R was prevented by mutation in the autoinducer region (pJE411), and transcription could be restored by provision of exogenous autoinducer. Addition of autoinducer to the dark mutants with transposon insertions in operon L did not stimulate expression of operon R (as measured by in vitro luciferase activity). Since the defect in these mutants could not be influenced by autoinducer addition, it was likely that a function necessary for response to this signal was missing in operon L mutants (see Figure 5). The transposon insertions in the dim mutants were clustered at the 5′ end of operon L, and it is possible that these mutations lowered

transcription of this operon by affecting the activity of a promoter element. The reduction of autoinducer synthesis in mutants with the transposons in operon L mentioned above could result from the trans effect of operon L mutations on operon R expression. Operon L appeared to encode a function which, in response to the presence of autoinducer, stimulated expression of operon R. Due to the polar effect of insertion mutations, we could not exclude the possibility that operon L contained functions for both response to autoinducer and production of this molecule.

Strains that contained plasmids with mini-Mu inserts synthesized β-galactosidase when the *lacZ* gene on the transposon had the same orientation as the target gene. The synthesis of β-galactosidase in strains containing fusions with operon R closely resembled the expression of light production in V. fischeri and in E. coli with pJE201 and pJE202. Little or no β-galactosidase synthesis took place in the preinduction stage of growth, but at cell densities at which induction of light production occurred β-galactosidase was induced and increased more than tenfold every cell division thereafter. All transposon insertions in operon R prevented light production, but with the fusion strains, transcription of operon R could be measured by assaying β-galactosidase. One plasmid (pJE411) containing a fusion of the *lacZ* gene with operon R was exceptional in that it directed the synthesis of a relatively small amount of β-galactosidase even at high cell densities (approximately 100 U in 1 ml of culture at OD_{660} of 1.0). This β-galactosidase activity was typical of that found in operon R fusion strains in the preinduction stage of growth. Plasmid pJE411 contained a transposon in the region necessary for autoinducer synthesis (see Figure 5). However, upon addition of exogenous autoinducer, induction of β-galactosidase synthesis in this fusion resulted and reached levels similar to those found with other operon R fusion strains in the postinduction stage of growth (approximately 2000 U at OD_{660} of 1.0). Thus expression of operon R was dependent on autoinducer. Furthermore, the transcription of at least one gene involved in the production of autoinducer was regulated by the presence of autoinducer. With similar fusions, transcription of operon L was not found to be influenced by the presence of autoinducer.

DISCUSSION

The genetic functions necessary for light production in E. coli were contained on a DNA fragment cloned from the marine symbiont V. fischeri. Regions of DNA that encoded functions for the luminescence reaction as well as functions required for regulatory control are shown in Figure 5. Two transcriptional units, operon L and operon R, were identified. Genes for the α and β subunits of the luciferase enzyme were located in operon R. This operon also contained functions for aldehyde production. We have not identified the specific enzymatic activities that correspond to the regions for aldehyde function. These activities could be involved in aldehyde cycling (see Figure 1), and possibly in the de novo synthesis of an aldehyde precursor (that is, fatty acid). The bioluminescence system probably interfaces with other cellular systems that furnish common organic intermediates. For example, the E. coli host might provide a fatty acid precursor. Furthermore, the oxidoreductase that generates reduced flavin might be common to many bacteria, and E. coli might have this activity. If E. coli were to duplicate a function required in V. fischeri, it would be difficult by genetic methods alone to recognize a redundant plasmid-encoded function or to determine whether that genetic

function had been cloned. We are presently attempting to identify oxidoreductase activity in the recombinant bacteria by biochemical methods.

In strains containing plasmid pJE205, constitutive light production was observed. This plasmid did not encode functions sufficient for aldehyde production, and consisted of an abbreviated fragment (see Figure 3) that contained the 3′ end of operon R. The plasmid tetracycline gene promoter, which was aligned with the *lux* genes on pJE205, could have supported transcription of these genes. This plasmid is similar in its properties to the *lux* gene clone from V. harveyi (Belas et al., 1982). In contrast with plasmid pJE205, plasmids pJE201 and pJE202 appeared to have the genetic elements for the regulation of luminescence in V. fischeri. Strains with these plasmids both synthesized autoinducer and were capable of response to this signal. The regions of DNA that encoded these regulatory functions are shown in Figure 5. Of particular interest was the finding that a region necessary for autoinducer synthesis was part of an operon that was controlled by the presence of autoinducer. Thus autoinducer synthesis was autoregulated. (Originally, "auto" referred to the self-directed species-specific activity of the inducer substance). Consequently, expression of operon R, which encoded the enzymatic activities for bioluminescence, would increase in an exponential manner after induction. Light production did in fact increase exponentially after induction. *Lux* gene expression appears to be controlled by a positive feedback circuit, and Figure 7 illustrates our model for regulation of light production. A low basal level of luminescence activity (1/100 to 1/1000 of induced activity) occurs under preinduction conditions, and a low level of autoinducer synthesis results. As a critical concentration of autoinducer is reached, transcription of operon R greatly increases as a result of the autoregulatory property of the *lux* gene control

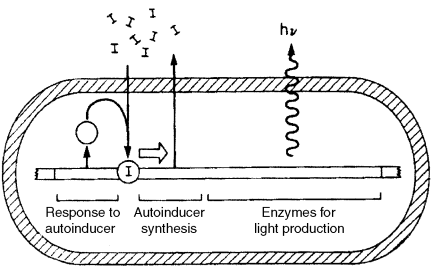

FIGURE 7 Model for genetic regulation of bioluminescence. Autoinducer and enzymes for light production are synthesized at low levels until a critical concentration of autoinducer (I) is reached (preinduction). At this time, sufficient autoinducer is present to interact with a "receptor" encoded by operon L, which then activates transcription of operon R. This in turn causes the production of more autoinducer, which further increases transcription of operon R, resulting in an exponential increase in light production.

circuit. The nature of the function necessary for response to autoinducer is not clear. This receptor-like function could actually reside in the membrane, which would eliminate the need for internalizing the signal molecule. Since expression of operon L did not appear to respond to autoinducer, the maximal level of bioluminescence might be limited by the amount of this "receptor."

It is apparent that the regulation of light production operates to limit expression to special circumstances. Luminescence would occur with dense populations in confined environments, such as in light organs or in gut tracts of fish and other marine animals. Such conditions may impose restraints on bacterial growth by oxygen (Nealson, 1979) or specific growth factor limitation, or by the presence of toxic substances. Light production may be a by-product of a process that deals with an environmental restraint such as those mentioned above. Hypotheses concerning the significance of bioluminescence can now be tested in an

organism such as E. coli, which is genetically and physiologically well characterized. Since this system can now be manipulated genetically, it should be possible to complete the identification of genes and gene products, and to understand better whether the bacteria derive a direct benefit from the luminescence functions.

EXPERIMENTAL PROCEDURES

Cloning Vibrio DNA

DNA was isolated from V. fischeri strain MJ-1 by the procedure of Ditta et al. (1981). Bam HI fragments of Vibrio DNA were ligated into the Bam HI site of vehicle pACYC184 (Chang and Cohen, 1978) at 15°C for 4–5 hr with T4 DNA ligase (Bethesda Research Labs). Hybrid molecules were recovered by transformation (Mandel and Higa, 1970) into E. coli strain ED8654 (*supE supF met hsdR*$^{-}$*hsdM*$^{+}$), and transformants were selected on L (Luria) agar containing 50 μg/ml of chloramphenicol. Hybrid plasmids, that is, pJE201, containing *lux* genes were recovered from luminescent clones. DNA fragments were subcloned into various sites in vehicle pBR322 (Bolivar et al., 1977) or pACYC184. Selection for transformants containing recombinants in pBR322 was on L agar plates containing 80 μg/ml ampicillin. Restriction endonucleases were purchased from New England BioLabs. Antibiotics were purchased from Calbiochem.

Light Measurements

Luminescence from 0.1 ml samples of well aerated cultures were measured with a photomultiplier apparatus (Rosson and Nealson, 1981) calibrated with the light standard of Hastings and Weber (1963). Light measurements were taken on MJ-1 grown at room temperature in L broth containing 2% NaCl. E. coli strains harboring recombinant plasmids were grown at 30°C in L broth. Ten microliters of a sonicated solution of tetradecanal (Aldrich; 10 μl in 10 ml distilled water) was added to cells that required this substrate (that is, strains with pJE205) approximately 1 min before light was measured. Optical density (660 nm) was measured prior to light measurements on a Bausch and Lomb Spectronic 20. Light produced by cells on agar surfaces was measured qualitatively either visually, by photography, or by plate contact printing with Kodak XAR-5 x-ray film. Light was also measured with an LKB 1211 Minibeta Scintillation Counter set to chemiluminescence mode.

Transposon Mutagenesis

Transposon Tn5 was used to mutate hybrid plasmid pJE201 by the procedure of Boyd et al. (1981). Strain HB101 (*hsdS recA ara proA lac galK rpsL*), harboring the target plasmid, was infected with λ::Tn5 at a multiplicity of infection of 10 and incubated at 30°C for 2 hr. The cells were harvested and spread onto L agar plates containing 50 μg/ml chloramphenicol (plasmid-encoded resistance) and 100 μg/ml kanamycin (Tn5 resistance). Resultant colonies that appeared after 24 hr were washed off the plate and inoculated into liquid medium for growth (under antibiotic selection) and for preparation of plasmid DNA (small-scale procedure of Birnboim and Doly, 1979). This pool of DNA was used to transform strain ED8654. Minimal amounts of DNA were used to avoid the isolation of multiply transformed bacteria. Isolation of sibling mutants was minimized by performing

20 independent mutant selections. More than 100 dark or dim mutants were picked. Plasmid DNA was isolated from mutants containing pJE201::Tn5 hybrids, and the locations of Tn5 inserts in the plasmid were determined by restriction analysis. Endonuclease sites useful for mapping were obtained from the restriction map reported by Rothstein et al. (1980). Only those mutants that were clearly different, as judged by phenotypes or the location of transposon insertion, or that originated from independent mutagenesis procedures were saved for further study.

Transposon mini-Mu was constructed by M. Casadaban, and consisted of an abbreviated derivative of the Mud phage (Chaconas et al., 1981). Except for the smaller size of mini-Mu (15.8 kb), and the substitution of kanamycin resistance for ampicillin resistance, this transposon was similar to Mud. Like Mud, mini-Mu could transpose and was capable of generating transcriptional fusions between the target gene and the *lacZ* (and *lacY*) gene on the transposon. This smaller version of the Mu was necessary to avoid the problems of instability and manipulation associated with very large plasmids. Plasmid pJE202 was moved by transformation into strain POI1681 (*araD araB::*Mu*cts* Δ*lac recA rpsL*), which contained mini-Mu (MudI1681 km^R ΔBam HI *cts*). This strain was heat-induced, and the resulting phage lysate was used to infect Rec^+ recipient strain MH3497 (*lac gal rpsL* Mu*cts*) obtained from M. Howe (University of Wisconsin). Mutated plasmid pJE202::mini-Mu were packaged by the Mu*cts* helper phage, and this phage preparation could be used for transduction ("Muduction" in Casadaban terminology). The recipient was Rec^+ to ensure recircularization of the transduced pJE202:: mini-Mu, and was also a Mu*cts* lysogen to ensure repression of the transposase functions of the incoming mini-Mu plasmid. The infected bacteria were plated on L agar containing 80 μg/ml ampicillin (plasmid resistance) and 100 μg /ml kanamycin (mini-Mu resistance). The resulting transformants were screened for light production and analyzed as with Tn5 mutants. More than 100 mutants were saved. Growth of cultures was always at 30°C to prevent induction of mini-Mu transposase functions, and to ensure expression of bioluminescence enzymes, which were subject to inactivation at temperatures of 37°C or higher. With a restriction map of mini-Mu provided by M. Casadaban, restriction sites useful for determining the location and orientation of the insert were found.

Complementation

Trans complementation of *lux* operon mutations was measured by cotransforming plasmids containing Tn5 (pJE300's) and mini-Mu (pJE400's) into a Rec^- E. coli strain, HB101 Mu*cts*. These plasmids have compatible replicons and can cohabit the same cell (Chang and Cohen, 1978). To maintain the hybrid plasmids, we propagated strains on L agar containing 80 μg/ml ampicillin (pBR322-encoded drug resistance), 50 μg/ml chloramphenicol (pACYC184-encoded resistance) and 100 μg/ml kanamycin (Tn5- and mini-Mu-encoded resistance). Light production was measured in these strains (see above), and complementation was scored as positive if light production was more than 10% of a control value and negative if less than 10%. Light production with a strain that contained both hybrids pJE201 and pJE202 was used as the control measurement. In practice, light production in strains scored as positive complementation was on average approximately 100-fold more intense than in those scored as negative.

Analysis of Functions

Luciferase assays were performed in vitro as previously described (Nealson, 1978). One milliliter of cells at OD_{660} of ~ 1.0 was harvested by centrifugation and lysed by suspension in a solution of 10 mM EDTA (pH 7.0), with 2 mg/ml lysozyme (Sigma) to a final volume of 100 μl. A 10 μl sample of the lysate was then mixed with 1.0 ml of a BSA solution (2 mg/ml containing 10 μl of a sonicated tetradecanal solution (10 μl in 10 ml H_2O). To this mixture, 0.5 ml of catalytically reduced $FMNH_2$ was introduced via syringe, and the enzyme activity was recorded as the peak of the luminescent flash which resulted. Autoinducer response was detected by placing 1 μl of a partially purified autoinducer preparation dissolved in ethyl acetate (Eberhard et al., 1981) obtained from M. Haygood (Scripps Institution of Oceanography) onto a 1/4 in Bacto Concentration Disc (Difco), which was placed on a lawn of cells to be assayed. Autoinducer production was assayed by growing cells overnight on an 0.2 μm Gelman filter in contact with an L agar plate, removing the filter, and plating a dim mutant (pJE331 or pJE450) on to this zone. Response to or excretion of autoinducer was also assayed by spotting combinations of donor and receptor mutants onto nutrient agar. With these methods, light production was measured after overnight growth by contact printing with x-ray film, or if done in liquid culture, light was measured with a photomultiplier apparatus. At low cell densities (preinduction period), V. fischeri or the E. coli hybrids expressed light in response to additions of cell-free liquid from growth cultures. Thus it was shown that autoinducer substance from E. coli hybrids could induce the expression of light in V. fischeri. β-galactosidase activity was measured with the SDS-$CHCl_3$ modification of Miller (1977), or detected qualitatively on MacConkey agar indicator plates (Difco) with 1% lactose. Bacterial hosts for the fusion plasmids were $LacZ^-$. To identify mutant strains that required aldehyde for luminescence, 1 μl tetradecanal was added to 100 μl culture samples prior to light measurement, or 5 μl tetradecanal was added to a filter pad attached to the lid of a petri plate containing bacterial colonies prior to contact printing with x-ray film. A variety of fatty acids was also tested, and was found to be ineffective in substituting for the presence of aldehyde.

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Distrust in Genetically Altered Foods

Attempts in Europe to resist imports of genetically modified maize from the United States risk a damaging trade battle. But opposition springs from science and public distrust, both of which need a considered response from industry and politicians alike.

PUBLIC attitudes to the safety of genetically engineered products in general, and foods in particular, are not 'rational' in the strictly scientific sense. Indeed, it would be surprising if they were. The rapid growth of interest in organic foods is only one consequence of the high environmental— and perhaps health — price that has been paid for past enthusiasm for chemical herbicides, insecticides and fertilizers. Yet while many consumers are voicing increasing demands for 'natural' products, farmers are seeking to cut costs and increase yields by moving in precisely the opposite direction through the use of crops that have been genetically tailored for greater efficiency of production.

Conflict has been inevitable. It should therefore come as little surprise that the critics of genetic engineering have seized a new opportunity—the first exports of genetically-engineered agricultural products from the United States to Europe at the end of the current growing season—to highlight their concerns (see page 564). Ironically their protests, timed to coincide with this week's World Food Day, partly reflect the success of the US agricultural industry in persuading regulators that their products are safe to grow. But they have also spotlighted both residual concerns about potential hazards, and the cultural challenge of handling low-level risk on both sides of the Atlantic.

Neither issue is straightforward, although the first is easier to address than the second. Critics of the new crops have raised a wide variety of concerns. Most are already being carefully watched for, such as the legitimate fear that genes for herbicide resistance might pass from a crop such as oil seed rape to its 'weedy' relatives (see *Nature* 380, 31; 1996). Others, such as the claim that stimulating the resistance of crops to chemical herbicides encourages the excessive use of such herbicides, concern broader questions of environmental policy that cannot be tackled through the regulation of genetically-engineered crops alone.

Separate from these is a more specific concern that has surfaced in a particularly acute form over one particular crop. The crop in question is a variety of herbicide-resistant corn (or maize) that has been developed by the Swiss company Ciba-Geigy to express an additional trait, namely toxicity to a major pest, the European corn-borer. The concern, highlighted earlier this year by Britain's Advisory Committee on Novel Foods and Processes (ACNFP), is that a gene resistant to the antibiotic ampicillin, used as a marker in an early stage of the development process, could—at least theoretically—be passed to man via bacteria lodged in the gut of animals which eat the maize unprocessed.

Ciba-Geigy's response to this concern, which has contributed directly to a regulatory

impasse in Brussels, is that it is exaggerated. Strictly speaking, the company may be right; certainly the series of events that would need to occur—including the transfer of the offending stretches of DNA to gut bacteria—have a low probability. But the risk, nevertheless, is there, and is a matter of genuine scientific debate. There is certainly wisdom is those members of the ACNPF who argue against attempts to shrug off any potential increase in antibiotic resistance in the population, even if small. European science advisers now addressing this issue should ensure that it receives full consideration in their recommendations.

Handling risks of this type has now become the most difficult task of regulators on both sides of the Atlantic. Enhancing scientific understanding is essential, but is not the whole solution. Just as challenging, but just as necessary, is the creation of trust. It is that which European consumers currently appear to lack, combining deep-rooted cultural fears of genetic manipulation with past experience of the aggressiveness of some agri-business companies (a tradition which Ciba-Geigy is perpetuating, by all accounts). If both technical and political monitoring procedures can be put in place that are sufficient to generate and maintain this trust, there is no reason why genetically-engineered foodstuffs should not enjoy success in the market. But without such procedures—or even recognition for their need—seed companies and their allies risk growing opposition.

The Real Threat from Antibiotics

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SIR—In your leading article "Distrust in genetically altered foods" (*Nature* **383**, 559; 1996) you side with the British Advisory Committee on Novel Foods and Processes (ACNFP) on the issue of an ampicillin resistance gene in some strains of transgenic corn. The ACNFP had decided that the ampicillin resistance gene in the transgenic corn posed an "unacceptable risk" because of the possibility that it might be transferred from the corn genome into the genomes of bacteria found in the intestinal tracts of animals and humans.

Following the ACNFP decision, a group of experts was convened in Talloires, France, in September to consider the issue. The meeting was convened by the Foundation for Nutritional Advancement, a private foundation associated with Tufts University in Massachusetts, and was attended by a small group of microbiologists and food safety experts from the United States, the United Kingdom and several other European countries. The unanimous conclusion of this group, of which I was a member, was that the ampicillin resistance gene in the transgenic corn posed no significant health hazard to humans or animals.

This conclusion was based not only on the fact that the probability that the gene would be transferred from corn to bacteria was negligible, but also on the fact that, even if such a transfer occurred, it would have no clinical impact. The ampicillin gene in the transgenic corn strain is the *bla* gene that is present on pUC19 and other plasmids used by molecular biologists. This gene, which encodes a β-lactamase, was originally cloned from a clinical strain isolated in the 1960s. This type of resistance gene poses no clinical problems today because there are many antibiotic formulations that easily control strains producing this type of β-lactamase.

By contrast, the β-lactamase genes that are currently causing problems in hospitals are modern genes that have evolved extensively during the past few decades to the point where they confer resistance not only to a wide variety of β-lactam antibiotics but also to β-lactamase inhibitors that have been used to 'recycle' antibiotics like ampicillin. Additionally, a new type of resistance to β-lactam antibiotics that is different from β-lactamases (mutant penicillin-binding proteins) is causing resistance problems in the Gram-positive bacteria.

Finding the old-style *bla* gene in a hospital isolate today would evoke yawns rather than cries of distress. Moreover, such a gene would most likely have been transferred from other hospital bacteria that carry the gene on transmissible genetic elements. The ACNFP failed to consider the clinical impact of a transfer of the *bla* gene from corn to bacteria in its analysis. Instead, it seemed to have assumed that all antibiotic resistance genes are equally dangerous, which is definitely not the case.

Unfortunately, *Nature* chose to focus attention on the extremely minor threat posed by transgenic corn while ignoring another European regulatory decision that is far more likely to have an impact on human health. In May, only a few months before the ACNFP decision, the European Scientific Committee for Animal Nutrition (SCAN) approved the continued use of the antibiotic avoparcin as a feed additive for farm animals. Avoparcin is an analogue of vancomycin and is known to select for resistance genes that confer resistance to vancomycin. Vancomycin resistance in Grampositive bacteria is one of the most serious resistance problems currently encountered in large US and European hospitals, where vancomycin is sometimes the only antibiotic left that is effective against multiply resistant strains of *Staphylococcus aureus*.

Certainly, there is room for argument about the extent to which feeding avoparcin to farm animals—and the concomitant exposure of farm workers and their bacteria to antibiotic selection—might contribute to an increased incidence of vancomycin-resistant clinical isolates, but this seems to me to be a far more serious issue than the remote possibility of transfer of an ampicillin resistance gene from corn to bacteria.

In the last paragraph of the leading article, you expressed concern about "deep rooted cultural fears of genetic manipulation" on the part of the public and stressed the importance of generating consumer trust and confidence in the new, genetically engineered foods. I do not know whether, as you contend, the seed companies are behaving in a way that increases consumer distrust of their product I do know, however, that we as scientists need to do a better job of communicating scientific issues to the public.

In my view your leading article is a case study in how misguided scientific emphasis can help to increase public confusion and anxiety about genetic engineering and its products. By choosing to give precious space to what is at best a very minor safety concern, while ignoring the real antibiotic resistance problems—such as the continued abuse and overuse of antibiotics by physicians, over-the-counter sale of antibiotics and use of antibiotics in animal feed—*Nature* is sending the wrong message to the public about the forces that are driving the increase in antibiotic resistance.

Pros and Cons of Foreign Genes in Crops

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Sir—In a leading article you expressed your scepticism about leaving antibiotics genes in agricultural transgenic plants (*Nature* **383**, 559; 1996). This view was criticized by Abigail Salyers (*Nature* **384**, 304; 1996), who believes that it may detract from the more important fight against "the continued abuse and overuse of antibiotics by physicians, over-the-counter sale of antibiotics, and use of antibiotics in animal feed". These positions should not, however, be pitted against each other.

It is easy to understand why there should be restrictions on the direct use of antibiotics in the human environment. It may be less obvious why regulating bodies should take a critical view of harmless antibiotics genes in agricultural plants. These genes are needed to introduce the transgenic characters into the plants, but are not vital for the growth of the plants in the field. Methods can therefore be designed to remove them selectively after they have finished their task.

There are two reasons why breeders should be asked not to leave such antibiotics genes in their products. The first is that all crop plants in the future will be transformed not only once but many times. If every transformation leaves another antibiotics gene in the plant, then it will soon become difficult for the plant breeders to find new 'harmless' antibiotics genes to use. The second reason has to do with what the public expects from the new gene technology. Commercial plants with transgenic properties will become generally accepted only if the arguments in favour of their use are strong and convincing. The best argument for them is, undoubtedly, the precision by which they have become altered, by comparison with, for example, classical plant breeding. But if gene technology is to be presented as a clean technology, then it must be clean. From personal experience in the Swedish Gene Technology Board, I know how difficult it is to argue for a new crop variety containing an interesting character if it also carries some antibiotics gene of no relevance to the needs of the farmer or the consumer.

So setting high standards for new transgenic plant varieties is not only a question about human health. It is also a way to protect a vital new technology against shortsighted uses that may later lead to severe setbacks.

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We Need Biotech to Feed the World

Norman Borlaug

Science is under attack in affluent nations, where antibiotech activists claim consumers are being poisoned by inorganic fertilizers and synthetic pesticides. They also claim that newer genetic engineering technologies decrease biodiversity and degrade the environment. Neither claim is true, but fear-mongering could be disastrous for less-developed nations.

Recently, in India, I confronted a move to outlaw inorganic, synthetic fertilizers. Government officials had been influenced by a cadre of international foes of technology. Officials told me that although Indian agriculture had greatly benefited from the use of such fertilizers in its Green Revolution—by which India achieved self-sufficiency in grain in the 1970s—they were now concerned that these products might have long-term negative effects. They wanted to revert to the exclusive use of so-called organic fertilizers.

They were correct about one thing—India *has* been the beneficiary of modern agricultural techniques. In the mid-1960s, both Pakistan and India saw widespread famine. I managed to persuade both governments to try the highly productive dwarf wheat and the improved integrated crop management practices that my colleagues and I developed at the International Maize and Wheat Center in Mexico.

The results speak for themselves: In 1965, wheat yields were 4.6 million tons in Pakistan and 12.3 million in India. By 1970, after the introduction of our new wheat, Pakistan produced nearly twice its amount, while India increased its yield to 20 million tons. The trend continues. This year Pakistan harvested 21 million tons, and India 73.5 million—all-time records.

This salutary trend will be reversed if misguided bureaucrats have their way. Such a law as India proposed would have seriously diminished the country's ability to feed its one billion people. Famine would again rear its ugly head.

The citizens of affluent nations may be able to pay more for food produced by "natural" or "organic" methods. The chronically undernourished people of impoverished nations cannot. They also cannot afford to have the promise of new agricultural technology nipped in the bud, as many antibiotechnology activists wish.

The latter have been agitating about the supposed threats to human health engendered by bioengineered foods. But such foods pose no greater threat to health than foods produced by conventional methods—probably even less. While activists inveigh against introducing a gene from one plant or one species into another, they fail to note that conventional breeders have been doing just that for many years.

Today we do it better. In the past, conventional plant breeders were forced to bring unwanted genes along with desirable ones when incorporating insect or disease resistance in a new crop variety. The extra genes often had negative effects, and it took years of crossbreeding and selection to oust them. Conventional plant breeding is crude in comparison to the methods being used in genetic engineering, where we move one or a few genes that we know are useful. We must do a better job of explaining such complexities to the general public, so people will not be vulnerable to antibiotech distortions.

Some environmental extremists bewail the use of genetic modification that allows crops to be herbicide resistant, or others that allow plants to produce their own insecticide. Among other charges, they suggest that herbicide resistance might be passed to wild relatives of the crops, and that insecticide-producing plants will decimate insect life and decrease biodiversity.

The truth is that resistance genes bred into crops by conventional means could also be spread to wild relatives by Mother Nature herself. Steps can be taken to minimize the possibility of that happening. Further, the suggestion that insecticide-producing plants will wipe out insects like Monarch butterflies is truly far-fetched. The most likely threat to the butterflies is a reduction of their winter habitat by encroaching development in Mexico.

What the activists don't want people to know is that one very good way to protect wildlife habitat is to ensure that marginal lands are not pressed into agricultural service in an attempt to feed burgeoning populations. In 1960 in the U.S., the production of the 17 most important food, feed, and fiber crops was 252 million tons. By 1999 it had increased to 700 million tons. It is important to note that the 1999 harvest was produced on 10 million fewer acres than were cultivated in 1960. If we had tried to produce the harvest of 1999 with the technology of 1960, we would have had to increase the cultivated area by about 460 million acres of land of the same quality—which we didn't have.

It is this type of arithmetic that is so important when considering how to feed the world's ever-increasing population. In 1914, when I was born, there were about 1.6 billion people in the world. Now it's about six billion, and we're adding about 85 million each year. We will not be able to feed the people of this millennium with the current agricultural techniques and practices. To insist that we can is a delusion that will condemn millions to hunger, malnutrition and starvation, as well as to social, economic and political chaos.

I visited Russia recently and spent some time at the newly renamed N.I. Vavilov Institute of Genetics and Crop Breeding in St. Petersburg. As I was leaving the conference room, a professor emeritus pulled me aside and pointed to the red chair at the head of the conference table, which was unoccupied during our meeting. "That's where Trofim Lysenko sat for 12 years when he destroyed our agricultural research programs and sent many of our top scientists to prison camps."

T.D. Lysenko, of course, was the pseudo-geneticist who insisted that Soviet agriculture must be run along politically correct party lines. Many who disagreed with Lysenko, including N.I. Vavilov, perished in prison camps. I fear that, like Lysenko, those ideologically opposed to technological advances will unduly influence our government and developing nations, as they have almost succeeded in doing in India. If they do, our prospects for feeding the world will be dim indeed.

I believe the world will be able to produce the food needed to feed the projected population of about 8.3 billion in the year

2025. I also believe that it can be done with little negative impact on the environment. But it cannot be attained without permitting the use of technologies now available, or without research to further improve and utilize new technologies, including biotechnology and recombinant DNA.

Mr. Borlaug, who was awarded the Nobel Peace Prize in 1970 for his accomplishments in agriculture, is a professor at Texas A&M University.

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