

Experiments in Molecular Genetics

Jeffrey H. Miller

Experiments in Molecular Genetics

Jeffrey H. Miller

Society of Fellows, Harvard University
and
Département de Biologie Moléculaire
Université de Genève

EXPERIMENTS IN MOLECULAR GENETICS

© 1972 by Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724
All rights reserved.

International Standard Book Number: 0-87969-106-9

Library of Congress Catalog Card Number: 72-78914

Printed in the United States of America

Cover photos: Mutator colonies stained for both constitutive β -galactosidase (blue) and constitutive alkaline phosphatase (yellow). For description of methods see Experiment 3. Photograph courtesy of G. Hombrecher from the laboratory of W. Vielmetter, Institut Genetik, Universität Köln.

Frontispiece: Mutator colony stained with Giemsa stain (see Experiment 3). Photograph courtesy of G. Hombrecher from the laboratory of W. Vielmetter, Institut Genetik, Universität Köln.



Foreword

One of the more obvious conclusions from the biology of the past twenty-five years is that biochemistry moves faster when it can utilize genetic methods. Likewise, much of what we now call genetics would not have been discovered had not biochemical methods been introduced. Though today many of the practitioners who merge the biochemical and genetic approaches call themselves molecular biologists, there are many other scientists who see no reason to change their names, seeing nothing dishonorable with the terms biochemist and geneticist. But no matter what we call ourselves today, almost no one argues for a purest approach which sees virtue in solving a problem solely by genetic crosses or by massive numbers of postdoctoral biochemists. Of course, there still exist many important problems where now only one of these approaches can be applied. Increasingly, however, we find we can remove this limitation by choosing a more appropriate organism to work with.

Now there is hardly any discussion as to the organism of first choice for probing a fundamental biological problem. *E. coli* stands so far in the forefront that it frequently provokes boredom on the part of the uninitiated, who may have started reading about it in high school and who in college found it hard to take a biology or biochemistry course where it did not sneak in. Thus, it is easy to say that the days of *E. coli*'s domination must soon pass, and so on to embryology and those organisms which have nuclei, mitochondria, and perhaps a number of chloroplasts.

But sighting a new frontier does not always mean that the time is ripe for everyone to open it up. Over and over the past decade has produced examples of key problems where the use of mutants was indispensable to their solution. This situation is still true today. Virtually each new issue of the *Proceedings of the National Academy of Science* contains one or more incisive articles whose conclusions are based on the use of specific *E. coli* mutants.

So knowledge of how to work with *E. coli* as a genetic system is likely to remain

a key ingredient in the biology curriculum for many years to come. Teaching bacterial genetics, however, as a purely formal subject without integration into the mainstream of modern biochemistry and molecular biology would be a very dull job. It would fail to convey the excitement of current genetic research and leave the impression that it is an esoteric topic best suited for those who only live genetics. Instead we believe the *E. coli* genetics is best taught in the context of contemporary research where mutants are vital for solving fundamental biological dilemmas.

This is the way the bacterial genetics course at Cold Spring Harbor has been taught since its inception the summer of 1950. First taught by M. Demerec, E. Witkin, and V. Bryson, it has been given here each succeeding summer to ten to twenty students of highly diverse backgrounds, ranging from the pure physical scientist to the applied microbiologist. By now some 250 people have come here for this specific purpose. Many have been strongly influenced by this experience and quickly settled on subsequent careers in molecular genetics.

Now we have to face the fact, however, that the three-week interval in which our summer courses must be given is only sufficient for a small fraction of the experiments that make up bacterial genetics. Inevitably, the experiments which are chosen for a given summer must reflect the specific research interests of the instructors of that year. Furthermore, the number of people who need to be familiar with current tricks for doing bacterial genetics greatly exceeds those that can come here to take our course or go for a learning period to a lab that specializes on this topic. Yet the intelligent novice should have at his disposal a way to become familiar with all the new procedures, if not the "lore" that he might someday need.

The moment thus seems propitious to bring forth an all-inclusive manual where "everything you need to know" about bacterial genetics can be found. In getting Jeffrey Miller to do this job, we have been most fortunate. He is old enough already to be a master in this field, yet, when he started writing he was too young to know how much work is necessary to turn out a good book. The final result, I believe, is a superb job; one, I hope, that will find widescale use for many years to come.

Cold Spring Harbor
March, 1972

J. D. Watson

Preface

Why do we still study *E. coli*? One attraction of working with such cells is that they represent a simplified system. The possibility of harvesting a large number of cells in a short time, and the advantage offered by a haploid organism containing only one chromosome and which can double every 20 minutes, have prompted many investigators to use *E. coli* for genetics research. Their work has considerably increased our knowledge of this organism and has resulted in the development of numerous specialized techniques, many of which we use in this manual.

Most importantly, there remain a vast number of basic problems in the field of cell biology which are as yet poorly understood in even a relatively simple system like *E. coli*. DNA replication, recombination, and repair are examples. Much about the mechanism of transcriptional (mRNA) control is unclear, for instance, positive control. The question of how the synthesis of ribosomal and transfer RNA is controlled is still largely unanswered. Many details of protein synthesis have not yet been elucidated, and the DNA and RNA sequences coding for the initiation and termination of transcription and translation are just now being deduced. The problem of controlled degradation of mRNA and of proteins still is mostly unsolved, and the study of membranes and transport may also be dependent on systems such as *E. coli*. Finally, there remain many unresolved aspects of intermediary metabolism.

How can bacterial genetics help solve these problems? The answer to this question is the subject of this manual. The basic approach that this field offers is the isolation of mutants. The discovery of new control systems, the tailoring of enzymes by genetic manipulation, and the definition of genes involved in biochemical pathways and processes are all direct results of this approach. Therefore, much of the text describes methods for the induction, isolation, characterization, and mapping of different types of mutations.

To facilitate the teaching of experimental molecular biology, we have compiled a series of experiments which can be done on a class basis and which cover many of the areas of modern bacterial genetics. Many of these experiments have been performed by student groups, such as the summer Bacterial Genetics Courses at Cold Spring Harbor. We use the *lac* operon for illustration often, and a review text, *The Lactose Operon* (Cold Spring Harbor Laboratory, 1970) has recently been published which provides a valuable summary of work on this basic system. We were fortunate to be able to draw on the excellent experimental manual by Clowes and Hayes (John Wiley and Sons, Inc., 1968) which served as a model for much of this book.

We have also tried to bring together into one volume as many recipes and methods as possible to enable investigators to use this text as a research handbook. Although the *lac* system is used for demonstrative purposes throughout part of the manual, almost all of the methods and techniques described are general. Thus, the description of indicator plates, mutagenesis, Hfr crosses, strain construction, hybridization, and enzyme assays are applicable to a wide variety of systems. Also, we hope that compiling these techniques will enable investigators not thoroughly acquainted with genetic manipulations in *E. coli* to form strategies for building strains and isolating mutants. One of the recent advances in bacterial genetics has been the development of techniques for incorporating bacterial genes into the DNA of certain phages. These specialized transducing phage are then used to provide DNA greatly enriched for the specific gene of interest. Experiments utilizing current methods for isolating these phage are presented in detail in this manual.

We have attempted to arrange these experiments in order of increasing difficulty and have tried to introduce the concepts of some of the later experiments in earlier sections. For instance, Experiment 42 (The Isolation of *trp-lac* Fusion Strains) is a series of steps, each of which has been covered previously. The object of the experiment is to isolate strains in which the *lac* genes are under the control of the *trp* operon. First, phage-resistant mutants are isolated and examined on lactose indicator plates. Recombination and complementation tests are then used to determine the end points of the deletions. Finally, Hfr crosses and F' transfers are employed to prepare and test *trpR* derivatives of the fusion strains. (This experiment was successfully performed by different groups of 20 students at Cold Spring Harbor in 1969 and 1970.) We would like to emphasize, however, that there is no mandatory order to these experiments. We have included many more experiments in this manual than could possibly be accomplished in a one-semester course. This gives the students and instructors a large freedom of choice in selecting experiments and planning courses. For instance, Unit VI is certainly optional since this requires special equipment which may be unavailable in some laboratories.

In order to facilitate the use of this manual, we have made available strain kits containing the 79 strains described here and 5 lysates in small, 1-dram, agar-filled stab bottles. We also include in each kit a precision-made device, described in the Appendix, which is used for interrupted matings. Kits can be obtained from Cold Spring Harbor Laboratory for \$100 to cover costs of handling, packaging, and mailing.

Acknowledgments

It is a pleasure to acknowledge the help of many people without whom this manual could never have been compiled. First of all I am indebted to Jim Watson, who conceived of the idea to produce this manual, who encouraged and advised me throughout the preparation of the book, and who spent many hours reading the final manuscript and the proofs.

This book is a collection of experiments written by several authors. I wrote the introductory material, Units I–V, Unit IX through the end of Experiment 59, and part of Unit VII. Terry Platt wrote Unit VIII, Appendix VI, and parts of Unit VII; Bill Haseltine contributed Experiments 44–47 in Unit VI; Jack Greenblatt prepared Experiments 60–62 in Unit IX; and Benno Müller-Hill wrote Experiment 43 in Unit VI and parts of Unit VII. Also, Larry Taylor and Brooks Low contributed Appendices IV and II, respectively, and Ernesto Bade wrote part of Experiment 47. I have reviewed and edited all of this material to put together the final manual, and I am solely responsible for any errors which are present.

Significant parts of this manuscript were read by Charles Yanofsky, Bob Weisberg, Frank Stahl, Joel Kirschbaum, Don Ganem, John Scaife, Larry Taylor, Ray Gesteland, Nancy Hopkins, Geoffrey Zubay, and Terry Platt. I am grateful for their comments. I am particularly indebted to David Botstein, Brooks Low, and Bill Reznikoff who read most of the first draft in detail and suggested many revisions, and also to David Zipser, Ahmad Bukhari, and Ernesto Bade for reading parts of the proofs.

During the initial preparation of the experimental protocols I benefited from the advice of many people. In particular I would like to thank Ed Lin, Bob Weisberg, Joel Kirschbaum, Don Ganem, Larry Taylor, Brooks Low, Max Gottesman, Jon Beckwith, Ethan Signer, Walter Gilbert, Dan Morse, Julian and Marilyn Gross, Lucien Caro, Klaus Weber, David Zipser, Ahmad Bukhari, Jürgen Schrenk, Mike Malamy, Ekke and Linde Bautz, Marc Van Montagu, and David Dressler. Several of these experiments were retested specifically for this manual by Joel Kirschbaum and Don Ganem. I am grateful for their help.

I would also like to thank Madeline Szadkowski for typing the final manuscript, Judy Gordon for doing the copy editing and proof reading, and Glen Lyle and Judy Gordon for preparing the design of the book. Hanna Neubauer and F. W. Taylor, Co., Northport, N.Y., contributed the art work.

I wrote this manual while a junior fellow of the Society of Fellows of Harvard University, and I am most grateful for their support. I also used the facilities of the Harvard Biological Laboratories (in the Watson-Gilbert group), the Genetics Institute of the University of Cologne (in the laboratory of B. Müller-Hill), and the Cold Spring Harbor Laboratories during this period, and am indebted to these institutions for their support.

Finally, I would like to thank all of the people in the Cold Spring Harbor community who made my past two summers at Cold Spring Harbor (where most of this manual was prepared) enjoyable. In particular I am indebted to Elfie, John, and all the Cairns; Liz and Jim Watson; Frauka, Henry, and the Westphals; and Marianne, Walter, and the Kellers.

*Cold Spring Harbor
January, 1972*

Jeffrey H. Miller

Table of Contents

	Foreword	v
	Preface	vii
	Acknowledgments	ix
Introduction	Introduction to the <i>lac</i> System	3
	Format; Use of Materials	6
	Nomenclature	7
	Strain List	13
Unit I	Introductory Experiments	
Experiment 1	Determination of Viable Cell Counts: Bacterial Growth Curves	31
2	Preparation and Plaque Assay of a Phage Stock	37
3	Behavior of Mutants on Indicator Plates	47
4	Replica Plating	56
		xi

Unit II Matings between Male and Female Cells

Experiment 5	Episome Transfers: Direct Selection	82
6	Interrupted Matings and the Time of Marker Entry	86
7	Location of Markers on the <i>E. coli</i> Chromosome by the Gradient of Transmission	93
8	Determination of the Point of Origin of Hfr Strains	96
9	Test for the Sex Factor by Phage Sensitivity	98
10	Production of F ⁻ Phenocopies	101
11	Curing of Episomes from <i>E. coli</i> Strains with Acridine Orange	104
12	Recombination Deficient Strains: Influence on Episome Transfer and Recombination	107

Unit III Mutagenesis and the Isolation of Mutants

Experiment 13	Ultraviolet Light Mutagenesis	121
14	Nitrosoguanidine Mutagenesis	125
15	Mutagenesis with a Frameshift Mutagen: ICR 191	130
16	2-Aminopurine and Nitrous Acid Mutagenesis Other Methods: EMS, Hydroxylamine, 5-Bromouracil, Sodium Bisulfite, Mu-1 Phage	135
17	Reversion Tests	140
18	Isolation of Temperature-sensitive Mutants in the <i>lac</i> Operon	144
19	Isolation of Spontaneous Lac ⁻ and i ⁻ Mutants	146
20	Complementation Tests of Lac Mutants—Intracistronic Complementation	153
21	Fine Structure Mapping of Lac ⁻ Mutants	159
22	Isolation of Nonsense Mutants	167
23	Isolation of Nonsense Suppressor Strains	173
24	Detecting Polar Mutants in the <i>lac</i> Operon	180

Unit IV	Strain Construction	
	Episome Transfers	
Experiment 25	F' Factor Transfers—Inability to Select for Recipient	190
26	F' Factor Transfers—Inability to Select against Donor	193
27	Construction of a Temperature-sensitive Episome	196
28	Generalized Transduction; Use of P1 in Strain Construction	201
29	F' Factor-mediated Chromosome Transfer	206
	Manipulation of Genetic Markers	
30	Use of P2 Phage to Isolate His ⁻ Strains of <i>E. coli</i>	215
31	Selection of Thy ⁻ Strains with Trimethoprim	218
32	Isolation of Valine-resistant and Antibiotic-resistant Mutants	221
	A. Valine Resistance	
	B. Nalidixic Acid Resistance	
	C. Rifampicin Resistance	
	D. Ampicillin Resistance	
	Additional Markers Used for Selection	
	Isolation of Auxotrophic Mutants	
33	Penicillin and Ampicillin Treatment for the Isolation of Auxotrophic Mutants	230
	Other Enrichment Techniques	
34	Isolation and Mapping of Temperature-sensitive Lethal Mutants	235
	Construction of Hfr Strains	
35	Selection for Integration of F Factor	249
36	Conversion of an F ⁻ Strain to an Hfr Strain	253
	A. Conversion of an F ⁻ Strain to HfrH	
	B. Conversion of an F ⁻ Strain to Hfr Cavalli	
37	F' Factor Formation; Use of Rec ⁻ Strains to Isolate F' Factors Carrying Different Regions of the <i>E. coli</i> Chromosome	259
38	Use of Homogenotization in Strain Construction	265

Unit V	Transduction and the Isolation of Specialized Transducing Phage Lines	
Experiment 39	Construction of Specialized Transducing Phage Lines	274
	Positioning Genes Near Prophage Attachment Sites	
40	Transpositions of the <i>lac</i> Region	284
	A. Selection of F' <i>lac</i> Integrations	287
	B. Transpositions at the <i>tonB</i> Locus	290
41A	Insertion of λ DNA into New Sites on the Chromosome	293
41B	Bringing Two Genes into Close Proximity by Isolation of a Fused Episome	301
	Operon Fusion	
42A	Isolation of Fusion Strains for the <i>lac</i> and <i>trp</i> Operons	304
42B	Construction of <i>trpR</i> ⁻ Derivatives of <i>trp-lac</i> Fusion Strains	312
Unit VI	Transformation with λh80d<i>lac</i> DNA and Measurement of <i>lac</i> Messenger RNA	
	<i>Bill Haseltine and Benno Müller-Hill</i>	
Experiment 43	Preparation of λ h80d <i>lac</i> Phage and Transformation with λ h80d <i>lac</i> DNA	319
44	Isolation of RNA Labeled <i>in vivo</i> after IPTG Induction	328
45	Separation of the Strands of λ plac DNA	331
46	Hybridization with <i>lac</i> mRNA	338
47	Determination of the Sedimentation Constant of <i>lac</i> mRNA	344
Unit VII	Assays of the <i>lac</i> Operon Enzymes	
	<i>Terry Platt, Benno Müller-Hill, and Jeffrey H. Miller</i>	
Experiment 48	Assay of β -Galactosidase	352
49	Time Course of β -Galactosidase Induction	356
50	Assay of <i>lac</i> Permease	360

Table of Contents**xv**

51	Assay of <i>lac</i> Repressor by Binding to Inducer	363
52	Assay of <i>lac</i> Repressor by Binding to Operator	367
53	Assay of Transacetylase	371
54	Assay for α Complementation	374

Unit VIII **Protein Purifications**
Terry Platt

Experiment 55	Purification of the <i>lac</i> Repressor	385
56	Determination of the Binding Constant of <i>lac</i> Repressor for IPTG	394
57	Purification of β -Galactosidase	398

Unit IX **Cyclic AMP, Catabolite Repression, and
Cell-free Enzyme Synthesis**
Jack Greenblatt and Jeffrey H. Miller

Experiment 58	Cyclic AMP and Catabolite Repression	412
59	Isolation of Mutants Missing a Factor Necessary for the Expression of Catabolite-sensitive Operons	415
60	Cell-free Synthesis of β -Galactosidase	419
61	Catabolite Repression in a Cell-free System	425
62	Repression of β -Galactosidase Synthesis in a Cell-free System	427

Appendices

I	Formulas and Recipes	431
II	Operation of Mating Interrupter <i>Brooks Low</i>	436
III	Linkage Map of <i>E. coli</i>	439

IV	General Mapping Strategy: Mapping Strategy for Two Loci Concerned with Pyridoxine Biosynthesis <i>A. L. Taylor</i>	451
V	Lambda Genetic Elements	455
VI	Assay for <i>lac</i> Repressor by Millipore Filter <i>Terry Platt</i>	458
VII	Selected F' Factors from <i>E. coli</i> K12	460
VIII	Addresses for Laboratory Supplies	461

INTRODUCTION