Prokaryotic Genomics

Prokaryotic Genomics

Edited by
Michel Blot

Birkhäuser Verlag
Basel · Boston · Berlin

Editor
Prof. Dr. Michel Blot
Université Joseph Fourier
460 rue de la piscine
38400 St Martin d'Hères
France

Library of Congress Cataloging-in-Publication Data
Prokaryotic genomics / edited by Michel Blot.
p. cm. – (Methods and tools in biosciences and medicine)
Includes bibliographical references and index.
ISBN 3764365978 (alk. paper) – ISBN 376436596X (soft cover : alk. paper)
1. Bacterial genetics–Laboratory manuals. 2. Genomics–Laboratory manuals.
3. Proteomics–Laboratory manuals. I. Blot, Michel. II. Series.

Deutsche Bibliothek Cataloging-in-Publication Data
Prokaryotic genomics / ed. by Michel Blot. – Basel;
Boston; Berlin: Birkhäuser, 2002
(Methods and tools in biosciences and medicine)
ISBN 3-7643-6597-8
ISBN 3-7643-6596-X

ISBN 3-7643-6597-8 Birkhäuser Verlag, Basel – Boston – Berlin
ISBN 3-7643-6596-X Birkhäuser Verlag, Basel – Boston – Berlin

Member of the BertelsmannSpringer Publishing Group
Printed on acid-free paper produced from chlorine-free pulp. TCF∞
Cover illustration: CRIM plasmids with IPTG-inducible promoters (see p. 82)
Printed in Germany
ISBN 3-7643-6597-8
ISBN 3-7643-6596-X
9 8 7 6 5 4 3 2 1

www.birkhauser.ch

Methods and Tools in Biosciences and Medicine

Microinjection,
edited by Juan Carlos Lacal, Rosario Perona and James Feramisco, 1999
DNA Profiling and DNA Fingerprinting,
edited by Jörg Epplen and Thomas Lubjuhn, 1999
Animal Toxins – Facts and Protocols,
edited by Hervé Rochat and Marie-France Martin-Eauclaire, 2000
Methods in Non-Aqueous Enzymology,
edited by Munishwar Nath Gupta, 2000
Techniques in Molecular Systematics and Evolution,
edited by Rob DeSalle, Gonzalo Giribet and Ward Wheeler, 2002
Methods for Affinity-Based Separations of Enzymes and Proteins,
edited by Munishwar Nath Gupta, 2002
Analytical Biotechnology,
edited by Thomas G.M. Schalkhammer, 2002
Prokaryotic Genomics,
edited by Michel Blot, 2003

In memory of Michel Blot

Michel Blot died at the age of 42 years in a tragic mountain accident on the 8th of September 2002. During his unusual and prematurely terminated career as a biologist he has conducted research in fields ranging from population biology to molecular genetics. Michel Blot's initial area of interest was classical population biology. After his theoretical training at the Pierre and Marie Curie university in Paris, he carried out part of his thesis work on the Kerguelen islands, in the extreme southern part of the Indian ocean, studying the population genetics of an indigenous mollusk. He received his Ph.D. from the university Paris VII in 1989. During his post-doctoral work in the laboratory of Werner Arber (laureate of the Nobel price of medicine in 1978) in Basel (Switzerland), he switched subject and became interested in bacterial genetics.

Michel Blot was appointed full professor at the University Joseph Fourier in Grenoble, France, in 1995 and set out to elucidate the molecular mechanisms of the evolution of bacterial cultures. Inspired by his background in population biology, he did not consider a bacterial culture as a population of millions of identical individuals but as cells that mutate, thereby producing a heterogeneous population that provides the substrate upon which evolutionary selection can act. During the past few years Michel and his group, with an ever-growing array of international collaborators, have established the role of insertion sequences as a motor of bacterial evolution. He cleverly exploited bacterial cultures that had been stored or grown for many years, making double usage of insertion sequences as molecular markers as well as causal elements of evolution. His research demonstrated how these elements provide genetic diversity and promote the evolutionary adaptation of bacteria to the environment in which they live. He has thus explored the entire spectrum from population biology to the study of molecular mechanisms of the regulation of the expression of genes involved in evolutionary adaptation.

The scientific merits of Michel Blot were recognized and honored by national and international science agencies and he was appointed junior member of the very prestigious Institut Universitaire de France in 1998. His success certainly rested partly on his extraordinary ability to convey to an audience, first-year students and established scientists alike, his enthusiasm and excitement for scientific research.

Michel Blot the scientist was well recognized, but Michel Blot the science politician has also left very distinctive marks in the national scientific community. Upon his arrival in Grenoble, he started off an initially very small microbiology laboratory and he invested a large amount of energy to make the laboratory grow and to provide an environment conducive to scientific re-

search. He did succeed in creating a laboratory where the scientific imagination can thrive. His combative style made Michel some enemies as well as friends, who will retain a lasting respect, affection and gratitude.

Michel Blot will be most missed by his wife and his two sons, but his absence will also be felt by all those working in the fields of bacterial and molecular evolution. We have lost, far too soon, an exceptional scientist and colleague who had still much to give professionally and personally.

List of Contributors

Avramova, Larisa, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA; e-mail: la@bilbo.bio.purdue.edu

Bouloc, Philippe, Laboratoire des Réseaux de Régulations, Institut de Génétique et Microbiologie, Université Paris-Sud, CNRS UMR8621, 91405 Orsay cedex, France; e-mail bouloc@infobiogen.fr

Camacho, Eva M., Departamento de Genetica, Universidad de Sevilla, Apartado 1095, 41080 Sevilla, Spain; e-mail: ecamacho@us.es

Casadesus, Josep, Departamento de Genetica, Universidad de Sevilla, Apartado 1095, 41080 Sevilla, Spain; e-mail: casadesus@us.es

Chong, Shaorong, New England Biolabs, Inc., 32 Tozer Road, Beverly, MA 01915, USA; e-mail: chong@neb.com

Dale, Colin, Department of Biochemistry, University of Arizona, Tucson, AZ 85721, USA; e-mail: cdale@email.arizona.edu

Datsenko, Kirill A., Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA; e-mail: datsenko@bilbo.bio.purdue.edu

Denizot, François, Laboratoire de Chimie Bactérienne, IBSM-CNRS, 31 Chemin Joseph Aiguier, 13402 Marseille cedex 20, France; e-mail: denizot@ibsm.cnrs-mrs.fr

Di Lallo, Gustavo, Dipartimento di Biologia, Università di Roma "Tor Vergata", Via della Ricerca Scientifica, 00133 Roma, Italy; e-mail: dilallo@uniroma2.it

D'Ari, Richard, Institut Jacques Monod, CNRS Universités Paris 6,7, 2 place Jussieu, 75251 Paris cedex 05, France; e-mail: dari@ijm.jussieu.fr

Ghelardini, Patrizia, Centro Acidi Nucleici del CNR, c/o Dipartimento di Genetica e Biologia Molecolare, Università "La Sapienza", P.le Aldo Moro 5, 00185 Roma, Italy; e-mail: ghelardini@uniroma2.it

Houseweart, Chad, Genome Therapeutics Corporation, 100 Beaver Street, Waltham, MA 02453, USA

Jourlin-Castelli, Cécile, Laboratoire de Chimie Bactérienne, IBSM-CNRS, 31 Chemin Joseph Aiguier, 13402 Marseille cedex 20, France; e-mail: jourlin@ibsm.cnrs-mrs.fr

Kenney, Teresa J., Genome Therapeutics Corporation, 100 Beaver Street, Waltham, MA 02453, USA

Kim, Soo-Ki, Department of Animal Products and Environmental Science, Konkuk University, 1 Hwayang-dong, Gwangin-gu, Seoul, 143–701, Korea; e-mail: mebong7@hanmail.net

Laub, Michael T., Bauer Center for Genomics Research, Harvard University, Cambridge, MA 02138, USA; e-mail: laub@cgr.harvard.edu

Lelong, Cécile, Plasticité et Expression des Génomes Microbiens, CNRS FRE2383, Université Joseph Fourier, 38041 Grenoble cedex, France; e-mail: cecile.lelong@ujf-grenoble.fr

MALOY, STANLEY, Center for Microbial Sciences, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182–4614, USA;
e-mail: smaloy@sciences.sdsu.edu

METZGAR, DAVID, Mail Code BCC-379, The Scripps Research Institute, 10550 North Torrey Pines Rd, La Jolla, CA, 92037, USA;
e-mail: dmetzgar@hermes.scripps.edu

OCHMAN, HOWARD, Department of Biochemistry, 233 Life Sciences, Southern University of Arizona, Tucson, AZ 85721, USA;
e-mail: hochman@email.arizona.edu

PAOLOZZI, LUCIANO, Dipartimento di Biologia, Università di Roma "Tor Vergata", Via della Ricerca Scientifica, 00133 Roma, Italy;
e-mail: paolozzi@bio.uniroma2.it

PERLER, FRAN, New England Biolabs, 32 Tozer Road, Beverly, MA 01915, USA; e-mail: perler@neb.com

RABILLOUD, THIERRY, DRDC/BECP, CEA Grenoble, 17, Avenue des Martyrs, 38054 Grenoble cedex 9, France; e-mail: thierry@sanrafael.ceng.cea.fr

ROSENZWEIG, R. FRANK, Division of Biological Sciences, University of Montana, Missoula, MT 59812, USA; e-mail: rrose@selway.umt.edu

SCHNEIDER, DOMINIQUE, Plasticité et Expression des Génomes Microbiens, CNRS FRE2383, Université Joseph Fourier, 38041 Grenoble cedex, France; e-mail: dominique.schneider@ujf-grenoble.fr

SMITH, WENDY, Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA; e-mail: wasmith@email.arizona.edu

SWIFT, SIMON, Division of Molecular Medicine and Pathology, Faculty of Medical and Health Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand; e-mail: s.swift@auckland.ac.nz

THIERAUF, ANNE, Department of Microbiology, University of Illinois, 601 S. Goodwin Ave, Urbana, IL 61801, USA; e-mail: thierauf@students.uiuc.edu

TINLAND, BRUNO, Monsanto Europe Africa, Avenue de Tervuren 270–272, 1150 Brussels, Belgium; e-mail: bruno.tinland@monsanto.com

VINELLA, DANIEL, Institut Jacques Monod, CNRS-Universités Paris 6,7, 2 place Jussieu, 75251 Paris cedex 05, France; e-mail: vinella@ijm.jussieu.fr

WALKER, SCOTT, Schering-Plough Research Institute, 2015 Galloping Hill Road, 4700 Kenilworth, NJ 07033, USA; e-mail: scott.walker@spcorp.com

WANNER, BARRY L., Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA; e-mail: BLW@bilbo.bio.purdue.edu

ZHOU, LU, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA; e-mail: zhou@purdue.edu

Preface

This manual reflects practical approaches to handling bacteria in the laboratory. It is designed to recall historical methods of bacterial genetics that have had recent developments and to present new techniques that allow full genome analysis. It has been written for microbiologists who need to group their protocols at the state of the art of a new millennium and also for scientists in other fields of life sciences who need to use bacteria for their research. Teachers, graduate students, and postdocs also will benefit from having these protocols to help them understand modern bacterial genetics.

I learned so much from these contributions from my colleagues that I have no doubt about the daily usefulness of this book.

April 2002 Michel Blot

Abbreviations

Acyl-HSL *N*-acyl homoserine lactone
Amp or Ap ampicillin
C carboxy
C10-HSL *N*-decanoyl-L-homoserine lactone
C12-HSL *N*-dodecanoyl-L-homoserine lactone
C14-HSL *N*-tetradecanoyl-L-homoserine lactone
C4-HSL *N*-butanoyl-L-homoserine lactone
C6-HSL *N*-hexanoyl-L-homoserine lactone
C8-HSL *N*-octanoyl-L-homoserine lactone
Cam or Cm chloramphenicol
CBD chitin binding domain
CHEF contour clamped homogenous electric field
CI consistency index
CRIM conditional-replication, integration, and modular
dCTP deoxycytidine triphosphate
deg. C degrees Celcius
DKP diketopiperazine
DMF dimethylformamide
DMSO dimethylsulfoxide
DNA deoxyribonucleic acid
dNTP deoxynucleotide dATP, dCTP, dGTP, dTTP
DTT dithiothreitol
EBU Evans Blue-Uranine
ECL enhanced chemiluminescence
ECOR *Escherichia coli* collection of reference
EDTA ethylamine diamine tetraacetic acid
EGTA ethylene glycol-*bis*(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid
EPL expressed protein ligation
GC guanine/cytosine
GFP green fluorescent protein
HPLC high pressure liquid chromatography
HT high transducing
Int integrase
int integrase; mutation prevents formation of stable lysogens
IPL intein-mediated protein ligation
IPTG isopropyl β-D-thiogalactopyranoside
Kan Kanamycin
kb kilobase
kDa kiloDalton
LB Luria-Bertani broth
βME β-mercaptoethanol
MESNA 2-mercaptoethanesulfonic acid
min. minimum
moi multiplicity of infection
N amino
NMR nuclear magnetic resonance
3-OH-C14:1–HSL *N*-(3-hydroxy-7-cis-tetradecanoyl)homo-serine lactone
3-OH-C4-HSL *N*-3-hydroxybutanoyl-L-homoserine lactone
ONPG *o*-nitrophenyl β-D-galactopyranoside
ORF open reading frame
OTG 1-S-octyl-β-D-thioglucoside
3-oxo-C10-HSL *N*-3-oxodecanoyl-L-homoserine lactone
3-oxo-C12-HSL *N*-3-oxododecanoyl-L-homoserine lactone
3-oxo-C14- HSL *N*-3-oxotetradecanoyl-L-homoserine lactone
3-oxo-C4-HSL *N*-3-oxobutanoyl-L-homoserine lactone
3-oxo-C6-HSL *N*-3-oxohexanoyl-L-homoserine lactone
3-oxo-C8-HSL *N*-3-oxooctanoyl-L-homoserine lactone
pac packaging site; DNA site where phage packaging is initiated
PCR Polymerase chain reaction
PFGE pulsed field gel electrophoresis
pfu plaque forming units
PMSF phenylmethylsulfonyl fluoride
QS quorum sensing
QSB quorum sensing blocker
RFLP restriction fragment length polymorphism
T0 time zero, initial cell population
T15, 30, 45 15, 30, 45 doublings of the initial population
Tc tetracycline
TCCP tris-(2-cyanoethyl)phosphine
TCEP tris-(2-carboxyethyl)phosphine
TE tris-EDTA buffer
TLC thin layer chromatography
T_m melting temperature
Tris-HCl tris(hydroxymethyl)amino-methane-hydrochloride
TYE tryptone-yeast extract
v:v volume:volume
vir virulent
w:v weight:volume
X-gal 5-bromo-4-chloro-3-indolyl-(-D-galactoside)
Xis excisionase

Contents

List of Contributors IX

Preface XI

Abbreviations XII

1 **Physical Analysis of Chromosome Size Variation** 1
Colin Dale, Wendy Smith and Howard Ochman

2 **Genetic Mapping in *Salmonella enterica*** 10
Josep Casadesus and Eva M. Camacho

3 **Insertion Sequences as Genomic Markers** 22
Dominique Schneider and Michel Blot

4 **The Use of Noncoding Microsatellite Length Analysis for Bacterial Strain Typing.** 34
David Metzgar

5 **How to Amplify Easily, on the Bacterial Chromosome, a Desired DNA Sequence** 41
Richard D'Ari and Daniel Vinella

6 **Generalized Transduction** 50
Anne Thierauf and Stanley Maloy

7 **Use of Conditional-replication, Integration, and Modular CRIM Plasmids to Make Single-copy *lacZ* Fusions** 65
Lu Zhou, Soo-Ki Kim, Larisa Avramova, Kirill A. Datsenko and Barry L. Wanner

8 **Genetic Footprinting for Bacterial Functional Genomics** 90
Scott S. Walker, Chad Houseweart and Teresa J. Kenney

9 **Gene Transfer to Plants through Bacterial Vectors** 102
Bruno Tinland

10 **Quorum Sensing: Approaches to Identify Signals and Signalling Genes in Gram-negative Bacteria** 110
Simon Swift

11 **Transcriptional Profiling in Bacteria Using Microarrays** 131
Michael T. Laub and R. Frank Rosenzweig

12 **Transcriptome Analysis by Macroarrays** 145
Cécile Jourlin-Castelli, François Denizot and Philippe Bouloc

13 **Prokaryotic Proteomics** 157
Cécile Lelong and Thierry Rabilloud

14 Intein-mediated Protein Purification 172
Shaorong Chong and Francine B. Perler

15 Two-hybrid Assay in *Escherichia coli* K12 194
Gustavo Di Lallo, Patrizia Ghelardini and Luciano Paolozzi

Guide to Protocols 205

Index 207

1 Physical Analysis of Chromosome Size Variation

Colin Dale, Wendy Smith and Howard Ochman

Contents

1 Introduction 1
2 Methods 2
Protocol 1: Preparation of bacteria in agarose 2
Protocol 2: Preparation of bacterial DNA 3
Protocol 3: Restriction digestion of bacterial DNA 3
Protocol 4: Pulsed-field gel electrophoresis 3
Protocol 5: Resolving multiple fragments following electrophoresis 5
2.1 Other considerations 5
Selecting appropriate restriction enzymes 5
Use of the I-CeuI intron encoded endonuclease 6
3 Results and discussion 6
3.1 Accurate determination of bacterial genome-size by PFGE 6
References 9

1 Introduction

Early characterization of genetic material from a wide range of organisms involved the determination of base composition and genome size. Aside from the intrinsic value of such information, these properties were studied because they could be obtained for the large number of samples where cytogenetic and transmission genetic analysis was onerous or obscure. As it turned out, these general features divulged some of the most fundamental aspects of gene and genome organization and evolution. The base compositional differences among bacteria led to theories about mutational processes that foreshadowed the neutral theory of molecular evolution [1–3] and, among eukaryotes, to the discovery of the isochore structuring within chromosomes [4]. With respect to genome-size variation, the results were equally consequential. Across life forms, there seemed to be little relationship between the amount of genetic material and the degree of organismal complexity (the so-called "C-value paradox"), which has led to inquiries about the amounts, the accumulation, and the function of non-coding DNA in genomes [5–9]. Within bacteria, genome-size would appear to have direct consequences on the biology of an organism:

Methods and Tools in Biosciences and Medicine
Prokaryotic Genomics, ed. by M. Blot

because of the high coding content of bacterial DNA, variation in genome-size implies differences in the absolute number of genes.

The sizes of microbial genomes were assessed by thermal denaturation and/or reassociation kinetics [10–12], sedimentation and buoyant density [13, 14], and electron microscopic visualization [15]. However, the advent of pulsed-field gel electrophoresis (PFGE) [16, 17] clearly changed the way that chromosomes could be studied and the types of questions addressed. Moreover, the sizes of DNA fragments that could be readily resolved by PFGE were ideally suited to the known size range of bacterial chromosomes. Therefore, it was not surprising that the technique was rapidly adopted by geneticists, microbiologists, population biologists, epidemiologists, and taxonomists as a means to examine bacterial genomes. At last count, well over 200 bacterial species, and numerous samples within species, were characterized by PFGE [18]. The following sections describe the PFGE procedures used in our laboratory to examine genome-size variation and genetic polymorphism within enteric bacteria. These procedures can be readily adapted to investigate genome-size variation within any microbial group.

2 Methods

Variations in PFGE methodology have been developed to accommodate differences in bacterial growth rate, cellular composition, and genome size. The procedures detailed below are tailored to the preparation and analysis of genomic DNA from Escherichia coli using the contour clamped homogenous electric field (CHEF) electrophoresis method [19]. The CHEF method can be used to resolve DNA fragments of up to 10 Mbp in size and is currently the method of choice for most applications.

Protocol 1 Preparation of bacteria in agarose

1. Pellet cells from a 2 ml overnight bacterial culture (8000 × g, 1 min).
2. Wash bacterial pellet in TE (10 mM Tris·Cl [pH 8.0], 1 mM EDTA) and resuspend in 100 µl TES (50 mM Tris·Cl [pH 8.0], 100 mM EDTA, 25% [w/v] sucrose).
3. Add 20 µl 4 mg/ml lysozyme (in TES) and 180 µl 1% InCert agarose (FMC) in TES.
4. Transfer the bacteria/agarose suspension into 2-mm thick plastic molds and solidify at 4 °C.

Protocol 2 Preparation of bacterial DNA

1. Following solidification, transfer bacterial plugs into a minimum of 50 volumes of freshly prepared deproteinizing solution (0.5 M EDTA [pH 8.0], 1% [w/v] sarkosyl, 0.2 mg/ml proteinase K).
2. Allow digestion to proceed for 48 h at 37 °C, replacing the deproteinizing solution after 24 h.
3. Remove plugs from deproteinizing solution and transfer to 100 volumes of wash solution (50 mM EDTA, pH 8.0).
4. Wash plugs three times at room temperature with gentle agitation to remove proteinase and detergent prior to restriction digestion.
5. Optional: Wash plugs for 30 min in wash solution containing 10 μM PMSF (prepared fresh from a stock of 10 mM PMSF in ethanol, stored in a light-proof bottle at –20 °C). PMSF is a proteinase inhibitor and its use can improve subsequent digestion efficiency. This chemical is toxic and should be handled with great care. Eliminating the PMSF step still generates consistent and reliable results.
6. Plugs can be stored at 4 °C in 50 mM EDTA (pH 8.0) for several months.

Protocol 3 Restriction digestion of bacterial DNA

1. After washing and storage, plugs should be trimmed to a size convienient for restriction digestion in 1.5 ml Eppendorf tubes. To ensure adequate digestion, we recommend a size no greater than 2 mm × 5 mm × 5 mm.
2. The cut plugs should be equilibrated in 100 volumes of TE buffer at room temperature for 2 h and then equilibrated overnight (16 h) in 10 volumes of 1 × restriction buffer.
3. Prior to digestion, the restriction buffer should be removed and replaced. We conducted restriction digestions overnight in 1 × restriction buffer in a 250 μl reaction containing 0.1 units of restriction enzyme/microlitre. Many enzyme manufacturers provide specific recommendations for reaction volume, enzyme concentrations, and reaction conditions.
4. Following restriction digestion, electrophoresis should be carried out as soon as possible to avoid degradation of the plugs.

Protocol 4 Pulsed-field gel electrophoresis

Separation of DNA fragments during CHEF PFGE is governed by electric field strength, pulse time, temperature, buffer ionic strength, and gel concentration. In practice, electrophoresis can be optimized by the appropriate selection of gel concentration, run time, field strength, and pulse parameters. Despite several analytical methods designed to obtain optimal resolution, trial and error is sometimes needed to achieve an acceptable degree of separation. Occasionally, it is necessary to run the same plugs under different conditions in order to resolve all of the DNA fragments generated by restriction digestion. See Table 1 for some suggested conditions suitable for resolving DNA fragments in different size ranges.

Table 1 Recommended PFGE parameters

Size range	Initial switch time	Final switch time	Run time[1]
1–25 Kbp	0.1 s	2 s	10
5–50 Kbp	2 s	10 s	16
50–300 Kbp	7 s	25 s	30
100–450 Kbp	12 s	40 s	34
200–900 Kbp	25 s	75 s	40
400–1500 Kbp	50 s	120 s	60

[1] Utilizing a linear switching ramp, a 1% agarose gel at 14 °C

1. Prepare 4 l electrophoresis buffer (0.5 × TBE) and use some of this buffer to prepare a gel containing pulsed-field certified agarose.
2. Fill CHEF electrophoresis tank with remaining electrophoresis buffer and switch on buffer circulator and cooler (14 °C)
3. When the gel has completely solidified, insert digested plugs and marker plugs into available slots and seal with a drop of molten 1% agarose in 0.5 × TBE. We routinely use markers from NEB (Beverly, MA), including a yeast chromosome marker (225 Kbp to 1.9 Mbp) and a λ concatamer ladder (48.5 Kbp to 1 Mbp) when resolving bacterial genome fragment sizes.
4. When electrophoresis buffer has cooled to the appropriate temperature, transfer and secure gel in the PFGE apparatus, re-check all parameters, and start the run.
5. After the run, stain the gel with ethidium bromide and destain in water for at least 1 h. Prolonged destaining (up to 48 h) can improve visualization but may cause diffusion of smaller bands (< 100 Kbp).

Figure 1 shows the differences among strains of *E. coli* that have been identified by digestion with an 8-base cutter (*Not*I), followed by PFGE. As well as establishing and enabling comparisons of chromsome size of each of the strains, restriction fragment length polymorphisms (RFLPs) also are observed among strains. Bands that appear very brightly stained are apt to consist of co-

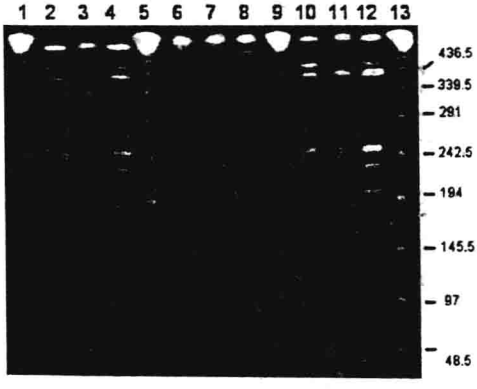

Figure 1 PFGE analysis of *E. coli* chromosomal DNA.
*Not*I-restricted genomic DNA from nine isolates of *E. coli* resolved by pulsed-field gel electrophoresis. Lanes 1, 5, 9, 13; Lambda concatamer PFGE ladder. All other lanes; *Not*I-restricted genomic DNA from isolates of *E.coli*. DNA fragments resolved by PFGE through a 1% gel (50 h, 170 V, 10–40 s switch time). Sizes of molecular weight standards shown.

migrating DNA fragments. To determine whether this is indeed the case, such bands are excised and redigested as described below.

Protocol 5 Resolving multiple fragments following electrophoresis

Occasionally, restriction digestion can generate DNA fragments that co-migrate during PFGE. For accurate determination of genome size, it is important to be able to resolve such multimers. This can be achieved by several different methods. First, it may be possible to separate co-migrating bands by optimizing electrophoretic conditions such that the resolution of fragments is enhanced over the desired size range (e. g. < 20 Kbp, 20–100 Kbp, and 100–700 Kbp). Also, conventional agarose gel electrophoresis should be applied for the separation of fragments < 20 Kbp. In the event that such optimization is insufficient, we have found that redigestion of the DNA fragment provides an accurate empirical means of analyzing mutiple DNA bands. In this method, the fragment of interest is physically excised from the gel and redigested with an alternate restriction enzyme. Resulting fragments generated by the second digestion reaction are resolved on a subsequent gel.

1. Excise the band of interest from gel in as small a piece of agarose as possible with a scalpel or razor blade.
2. Wash the gel band in 100 volumes of TE for 18 h to remove boric acid and excess EDTA. Change TE buffer at least twice during the washing period.
3. Equilibrate the gel band overnight in 10 volumes of 1 × restriction buffer and replace the restriction buffer prior to digestion.
4. Set up an overnight (16 h) restriction digest of the gel band in a 250 µl reaction containing 25–40 units of restriction enzyme. Be sure to check the manufacturer recommendations relating to reaction volume, enzyme concentrations, and reaction conditions.
5. Following restriction digestion, carry out PFGE as describe in protocol 4 to determine the complexity of the gel band.
6. Note that an apparently incomplete digestion may represent a case where one or more DNA species were not digested by a particular enzyme. In these cases, it is advantageous to digest bands with a number of different restriction enzymes.

2.1 Other considerations

Selecting appropriate restriction enzymes

For accurate resolution of genome sizes through PFGE, it is important to select restriction enzymes that cut a given DNA species at a low frequency (5–40 sites). For analysis of bacterial chromosomes, we favor the use of enzymes with 8-base recognition sites for DNA molecules > 3 Mbp and the use of non-degenerate 6-base cutters for molecules < 3 Mbp. If genome-size is unknown, we recommend the initial use of 8-base cutters.