Springer Protocols

Methods in Molecular Biology 646

Clostridium difficile

Methods and Protocols

Edited by
Peter Mullany
Adam P. Roberts

Humana Press

METHODS IN MOLECULAR BIOLOGY™

Series Editor
John M. Walker
School of Life Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

For other titles published in this series, go to
www.springer.com/series/7651

Clostridium difficile

Methods and Protocols

Edited by

Peter Mullany and Adam P. Roberts

UCL Eastman Dental Institute, London, UK

Editors
Peter Mullany
Eastman Dental Institute
Division of Microbial Diseases
University College, London
256 Gray's Inn Road
London
United Kingdom WC1X 8LD
p.mullany@eastman.ucl.ac.uk

Adam P. Roberts
Eastman Dental Institute
Division of Microbial Diseases
University College, London
256 Gray's Inn Road
London
United Kingdom WC1X 8LD
a.roberts@eastman.ucl.ac.uk

ISSN 1064-3745 e-ISSN 1940-6029
ISBN 978-1-60327-364-0 e-ISBN 978-1-60327-365-7
DOI 10.1007/978-1-60327-365-7
Springer New York Dordrecht Heidelberg London

Library of Congress Control Number: 2010923237

© Springer Science+Business Media, LLC 2010
All rights reserved. This work may not be translated or copied in whole or in part without the written permission of the publisher (Humana Press, c/o Springer Science+Business Media, LLC, 233 Spring Street, New York, NY 10013, USA), except for brief excerpts in connection with reviews or scholarly analysis. Use in connection with any form of information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed is forbidden.
The use in this publication of trade names, trademarks, service marks, and similar terms, even if they are not identified as such, is not to be taken as an expression of opinion as to whether or not they are subject to proprietary rights.

Cover illustration: *C.difficile* makes intimate contact through numerous flagella with microvilli on the mucosal lining of hamster cecum following a 48 hour oral infection with spores. We compare this 630 fully sequenced strain (originating from a patient with pseudomembranous colitis) with that of B1 (isolated from a patient with diarrhea) to reveal differences in behaviour during infection of the host. Scanning electron micrograph: bacterium length 4.5μm.

Printed on acid-free paper

Humana Press is part of Springer Science+Business Media (www.springer.com)

Preface

Clostridium difficile is a major nosocomial pathogen and has been shown to be a primary cause of antibiotic-associated disease. Recently, there has been an emergence of highly transmissible and frequently antibiotic-resistant strains, and the organism has become a considerable burden on health-care systems worldwide. At the same time, there has been a dramatic increase in our ability to study the organism. This book brings together the key workers in *C. difficile* research to describe the recently developed methods for studying the organism. These range from methods for isolation of the organism, molecular typing, genomics, genetic manipulation, and the use of animal models. We are now therefore in a position to gain an in-depth understanding of how this organism is transmitted and how it causes disease.

Peter Mullany
Adam P. Roberts

Contents

Preface . . . . *v*
Contributors . . . . *ix*

PART I INTRODUCTION TO *CLOSTRIDIUM DIFFICILE* AND THE DISEASE IT CAUSES

1. *Clostridium difficile*: No Longer an Enigmatic Pathogen? 3
   Adam P. Roberts and Peter Mullany
2. *Clostridium difficile* and the Disease It Causes 9
   Torbjörn Norén

PART II ISOLATION AND CULTURE TECHNIQUES

3. *Clostridium difficile* Isolation and Culture Techniques 39
   Mike Wren

PART III METHODS FOR TYPING AND EPIDEMIOLOGICAL STUDIES

4. Molecular Typing Methods for *Clostridium difficile*: Pulsed-Field Gel Electrophoresis and PCR Ribotyping 55
   Sandra Janezic and Maja Rupnik
5. *Clostridium difficile* Toxinotyping 67
   Maja Rupnik
6. Multilocus Sequence Typing for *Clostridium difficile* 77
   Ludovic Lemée and Jean-Louis Pons

PART IV BIOCHEMISTRY OF THE ORGANISM

7. Molecular Methods to Study Transcriptional Regulation of *Clostridium difficile* Toxin Genes 93
   Ana Antunes and Bruno Dupuy
8. Dissecting the Cell Surface 117
   Robert Fagan and Neil Fairweather
9. Human Intestinal Epithelial Response(s) to *Clostridium difficile* 135
   Nazila V. Jafari, Elaine Allan, and Mona Bajaj-Elliott

PART V GENOMICS

10. Comparative Genome Analysis of *Clostridium difficile* Using DNA Microarrays 149
    Richard Stabler, Lisa Dawson, and Brendan Wren

PART VI DEVELOPMENT OF SYSTEMS FOR GENETIC ANALYSIS OF THE ORGANISM

11. ClosTron-Targeted Mutagenesis . . . 165
John T. Heap, Stephen T. Cartman, Sarah A. Kuehne, Clare Cooksley, and Nigel P. Minton

12. Methods for Gene Cloning and Targeted Mutagenesis . . . 183
Glen P. Carter, Dena Lyras, Rachael Poon, Pauline M. Howarth, and Julian I. Rood

13. Transposon Mutagenesis in *Clostridium difficile* . . . 203
Haitham A. Hussain, Adam P. Roberts, Rachael Whalan, and Peter Mullany

PART VII ANIMAL MODELS OF DISEASE

14. Refinement of the Hamster Model of *Clostridium difficile* Disease . . . 215
Gillian Douce and David Goulding

15. Methods for Working with the Mouse Model . . . 229
Anne Collignon

Subject Index . . . 239

Contributors

ELAINE ALLAN • *Division of Microbial Diseases, UCL Eastman Dental Institute, London, UK*

ANA ANTUNES • *Unité de Toxines et Pathogénie Bactérienne, Institut Pasteur, Paris, France*

MONA BAJAJ-ELLIOTT • *Infectious Disease and Microbiology Unit, Institute of Child Health, London, UK*

GLEN P. CARTER • *Department of Microbiology, Monash University, Clayton, Australia*

STEPHEN T. CARTMAN • *Centre for Biomolecular Sciences, Institute of Infection Immunity and Inflammation, BBSRC Sustainable BioEnergy Centre, School of Molecular Medical Sciences, University of Nottingham, Nottingham, UK*

ANNE COLLIGNON • *Faculté de Pharmacie, Université Paris Sud, Châtenay Malabry, France*

CLARE COOKSLEY • *Centre for Biomolecular Sciences, Institute of Infection Immunity and Inflammation, BBSRC Sustainable BioEnergy Centre, School of Molecular Medical Sciences, University of Nottingham, Nottingham, UK*

LISA DAWSON • *Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK*

GILLIAN DOUCE • *Division of Infection and Immunity, IBLS, Glasgow Biomedical Research Centre, University of Glasgow, Glasgow, UK*

BRUNO DUPUY • *Unité de Toxines et Pathogénie Bactérienne, Institut Pasteur, Paris, France*

ROBERT FAGAN • *Centre for Molecular Microbiology and Infection, Imperial College London, London, UK*

NEIL FAIRWEATHER • *Centre for Molecular Microbiology and Infection, Imperial College London, London, UK*

DAVID GOULDING • *Wellcome Trust Genome Campus, Wellcome Trust Sanger Institute, Cambridge, UK*

JOHN T. HEAP • *Centre for Biomolecular Sciences, Institute of Infection Immunity and Inflammation, BBSRC Sustainable BioEnergy Centre, School of Molecular Medical Sciences, University of Nottingham, Nottingham, UK*

PAULINE M. HOWARTH • *Department of Microbiology, Monash University, Clayton, Australia*

HAITHAM A. HUSSAIN • *Division of Microbial Diseases, UCL Eastman Dental Institute, London, UK*

NAZILA V. JAFARI • *Infectious Disease and Microbiology Unit, Institute of Child Health, London, UK*

SANDRA JANEZIC • *Institute of Public Health Maribor, Centre for Microbiology, Maribor, Slovenia; Faculty of Medicine, University of Maribor, Maribor, Slovenia*

SARAH A. KUEHNE • *Centre for Biomolecular Sciences, Institute of Infection Immunity and Inflammation, BBSRC Sustainable BioEnergy Centre, School of Molecular Medical Sciences, University of Nottingham, Nottingham, UK*

LUDOVIC LEMÉE • *Groupe de Recherche sur les Antimicrobiens et les Micro-organismes (GRAM EA 2656, IFR 23), Faculté de Médecine-Pharmacie, Université de Rouen, Rouen Cedex, France*

DENA LYRAS • *Department of Microbiology, Monash University, Clayton, Australia*

NIGEL P. MINTON • *Centre for Biomolecular Sciences, Institute of Infection Immunity and Inflammation, BBSRC Sustainable BioEnergy Centre, School of Molecular Medical Sciences, University of Nottingham, Nottingham, UK*

PETER MULLANY • *Division of Microbial Diseases, UCL Eastman Dental Institute, London, UK*

TORBJÖRN NORÉN • *Department of Infectious Diseases, Orebro University Hospital and Orebro University, Orebro, Sweden*

ADAM P. ROBERTS • *Division of Microbial Diseases, UCL Eastman Dental Institute, London, UK*

JEAN-LOUIS PONS • *Groupe de Recherche sur les Antimicrobiens et les Micro-organismes (GRAM EA 2656, IFR 23), Faculté de Médecine-Pharmacie, Université de Rouen, Rouen Cedex, France*

RACHAEL POON • *Department of Microbiology, Monash University, Clayton, Australia*

JULIAN I. ROOD • *Department of Microbiology, Monash University, Clayton, Australia*

MAJA RUPNIK • *Institute of Public Health Maribor, Centre for Microbiology, Maribor, Slovenia; Faculty of Medicine, University of Maribor, Maribor, Slovenia*

RICHARD STABLER • *Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK*

RACHAEL WHALAN • *Division of Microbial Diseases, UCL Eastman Dental Institute, London, UK*

BRENDAN WREN • *Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK*

MIKE WREN • *Clinical Microbiology Laboratory, University College Hospital, London, UK*

Part I

Introduction to *Clostridium difficile* and the Disease It Causes

Chapter 1

Clostridium difficile: No Longer an Enigmatic Pathogen?

Adam P. Roberts and Peter Mullany

Abstract

Never before has there been a more timely opportunity to investigate the molecular genetics of *Clostridium difficile*. Over the last few years the perception of *C. difficile* has changed from an obscure, and often under-researched, bacterium to one of major clinical importance, at least in industrialized nations. Coupled with the increased interest in this organism researchers now have a greater understanding of its genetic content and molecular epidemiology; a direct consequence of the multiple *C. difficile* genomes which have been, and currently are being, sequenced. Concurrent with the sequencing efforts have been the development of tools to genetically manipulate the organism. We are now in a position to answer fundamental questions about the biology and pathogenicity of the organism. The techniques detailed in this volume should allow researchers to enter a new era of *C. difficile* research where it is possible, finally, to fulfil Koch's molecular postulates and determine empirically how this once enigmatic organism is able to cause disease.

Key words: *Clostridium difficile*, molecular biology, genetics, experimental techniques.

1. An Historical Perspective

Clostridium difficile is an anaerobic spore forming organism that was initially discovered in 1935 in the stools of newborns (1). However, it was not until 1978 that it was identified as the main causative agent of antibiotic-associated diarrhoea and pseudomembranous colitis, a severe necrotizing and often fatal disease of the colon (2). In the last few years there has been an increase in *C. difficile*-associated disease (CDAD) which has become more serious and refractory to standard treatment with the emergence of epidemic strains (3). The increase in the reporting of these epidemic strains was first observed in Canada (4) and

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_1, © Springer Science+Business Media, LLC 2010

was subsequently found in the USA (5). Analysis of these outbreaks showed that they were caused by a single strain, designated NAP1 ribotype 027 (6). Related epidemic strains of *C. difficile* have now been identified as the cause of hospital outbreaks in the UK (7) and 16 other European countries including Austria (8), Belgium (9), France (10) and the Netherlands (11). More recently, the strain has been identified in Japan (12) and Australia (13).

At the same time as this emerging strain was being reported a number of milestones in the development of *C. difficile* genetics were reached including the completion of the first genome sequence (14, 15) and major developments in the genetic tools available for the manipulation of this organism (16–18). This has provided researchers an unprecedented opportunity to increase our understanding of the molecular biology and virulence mechanisms of the organism.

2. Overview of the Book

In this book we have brought together experts in the molecular analysis of *C. difficile* from around the world to explain the techniques they use in detail, providing a host of methods for the study of DNA, RNA and proteins, typing methodologies, animal studies and immunological studies.

Following a comprehensive review of the diseases caused by *C. difficile* (**Chapter 2**) an in-depth explanation of isolation and culture techniques is presented (**Chapter 3**). Following this there is a detailed explanation of some of the various typing methodologies (**Part III**). Recent comparisons of the typing methods have shown that PCR-ribotyping and the library of PCR ribotypes held at the Anaerobe Reference Laboratory in Cardiff, UK, are the benchmarks to which most typing studies are compared (19).

Following this section we explore methods used to investigate the transcription of the toxin genes (**Chapter 7**), dissection of cell-surface proteins (**Chapter 8**) and immunological responses of human cells to *C. difficile* (**Chapter 9**). These sections provide most of the techniques needed to study transcription in the organism, protein biochemistry and interactions with human cells and host response.

Next there are detailed explanations of how to carry out comparative genomic analysis using microarrays (**Chapter 10**). The previous work from this group has shown an extraordinary small number of common genes within *C. difficile* species (20) with only 19.7% of genes shared by all strains analysed. These were

from diverse origins a possible explanation for this observation is that *C. difficile* undergoes extensive horizontal gene transfer.

The next section introduces various ways of generating mutations in *C. difficile*. **Chapter 11** explains the methodology associated with the Clostron gene knockout system (17) which allows the generation of defined mutants by using a derivative of a mutagenesis system based on the *Lactococcal* group II intron Ll.LtrB (21). **Chapter 12** describes in detail the methodology involved in generating defined recombinatorial mutants using an *Escherichia coli–C. difficile* shuttle vector based on pIP404 which is unstable in *C. difficile* (22). Finally in this section (**Chapter 13**) we describe the methodology used to generate relatively random insertion libraries using the broad host range conjugative transposon Tn*916* (23). The use of these random insertion libraries enables the identification of novel regulatory genes.

Finally the last section describes the two widely used animal models, both of which have their advantages (**Chapters 14** and **15**). The use of the animal model is fundamentally important in understanding the infection process, as is highlighted by a recent paper describing the effect of toxin B on virulence (24).

3. Questions, Controversies and Future Research

One of the most pressing questions currently being asked by many *C. difficile* researchers is "Are the epidemic PCR ribotype 027 strains hypervirulent?" While there was a great deal of hype over the alleged hypervirulence in the media and the popular press there seems to be very little scientific evidence to corroborate this. It was hypothesized early on in the emergence of ribotype 027 strains that mutations in the *tcdC* gene, the negative regulator of toxin production, may be responsible for the increase severity of disease (25); however, a recent analysis of ribotype 027 strains found no evidence that this deletion could predict hypertoxin production (26). Additionally a retrospective analysis of patient data found no evidence of hypervirulence in PCR ribotype 027 strains compared to other ribotypes (27). This brings into question our understanding of the fundamental pathogenicity of this organism and shows that there is an urgent need for a deeper understanding of the virulence and colonization mechanisms. The short fall in this understanding is illustrated if we compare what is known about *C. difficile* to what is known about other "model" enteric pathogens such as *E. coli* and *Salmonella typhimurium*. One of the major differences between the researches of these pathogens is the tools available. In order to understand *C. difficile* better there is still a need for tailor-made molecular tools. While

there have been recent advances which have pushed *C. difficile* research into the molecular age there are still gaps in the available tool kits, e.g. there is still need for a stable, integrative, complementation vector which will insert into a specific, transcriptionally defined, chromosomal site.

In addition to new tools the annotation of the 630 genome has identified seven genes whose predicted proteins share homology to proteins involved in competence (CD0133, CD1272, CD1273, CD1409, CD1899, CD2475 and CD2497). With the amount of DNA that is putatively foreign in the *C. difficile* (15) genome and the presence of these competence related genes, it seems likely that at some time in the life cycle of *C. difficile* it will become competent, presumably when it becomes stressed or detects a suitable environmental signal such as cell density. Work should focus on trying to determine the conditions in which this is likely to occur and what contribution, if any, these proteins make to this process. A repeatable, efficient transformation procedure for *C. difficile* has long been seen as one of the most beneficial developments for research into this organism.

References

1. Hall IC and O'Toole E. (1935) Intestinal flora in new born infants: with a description of a new pathogenic anaerobe, *Bacillus difficilis*. *Am J Child Dis* **49**, 390–402.
2. Bartlett JG, Chang TW, Gurwith M, Gorbach SL and Onderdonk AB. (1978) Antibiotic-associated pseudomembranous colitis due to toxin producing clostridia. *N Engl J Med* **298**, 531–537.
3. Bartlett JG. (2006) Narrative review: the new epidemic of *Clostridium difficile*-associated enteric disease. *Ann Intern Med* **145**, 758–764.
4. Pepin J, Valiquette L, Alary ME, Villemure P, Pelletier A, Forget K, Pépin K and Chouinard D. (2004) *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. *CMAJ* **171**, 466–472.
5. McDonald LC, Kilgore GE, Thompson A, Owens RC, Kazakova SV, Sambol SP, Johnson S and Gerding DN. (2005) An epidemic, toxin-gene variant strain of *Clostridium difficile*. *N Engl J Med* **353**, 2433–2441.
6. Loo VG, Poirer I, Miller MA, Oughton M, Libman MD, Michaud S, Bourgault AM, Nguyen T, Frenette C, Kelly M, Vibien A, Brassard P, Fenn S, Dewar K, Hudson TJ, Horn R, René P, Monczak Y and Dascal A. (2005) A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. *N Engl J Med* **353**, 2442–2449.
7. Smith A. (2005) Outbreak of *Clostridium difficile* infection in an English hospital linked to hypertoxin-producing strains in Canada and the US. *Euro Surveill* **10**, 050630.
8. Indra A, Huhulescu S, Hasenberger P, Schmid D, Alfery C, Wuerzner R, Fille M, Gattringer K, Kuijper E and Allerberger F. (2006) First isolation of *Clostridium difficile* PCR ribotype 027 in Austria. *Euro Surveill* **11**, E060914.3.
9. Joseph R, Demeyer D, Vanrenterghem D, van den Berg R, Kuijper E and Delmee M. (2005) First isolation of *Clostridium difficile* PCR ribotype 027, toxinotype III in Belgium. *Euro Surveill* **10**, E051020.4.
10. Tachon M, Cattoen C, Blanckaert K, Poujol I, Carbonne A, Barbut F, Petit JC and Coignard B. (2006) First cluster of *C. difficile* toxinotype III, PCR-ribotype 027 associated disease in France: preliminary report. *Euro Surveill* **11**, E060504.1.
11. Kuijper EJ, Coignard B, Tull P and ESCMID Study Group for *Clostridium difficile*; EU Member States; European Centre for Disease Prevention and Control. (2006) Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin Microbiol Infect* **12**(Suppl 6), 2–18.

12. Kato H, Ito Y, van den Berg RJ, Kuijper EJ and Arakawa Y. (2007) First isolation of *Clostridium difficile* 027 in Japan. *Euro Surveill* **12**, E070111.3.
13. Riley TV, Thean S, Hool G and Golledge CL. (2009) First Australian isolation of epidemic *Clostridium difficile* PCR ribotype 027. *Med J Aust* **190**, 706–708.
14. Wust J, Sullivan NM, Hardegger U and Wilkins TD. (1982) Investigation of an outbreak of antibiotic-associated colitis by various typing methods. *J Clin Microbiol* **16**, 1096–1101.
15. Sebaihia M, Wren BW, Mullany P, Fairweather NF, Minton N, Stabler R, Thomson NR, Roberts AP, Cerdeño-Tárraga AM, Wang H, et al. (2006) The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. *Nat Genet* **38**, 779–786.
16. O'Connor JR, Lyras D, Farrow KA, Adams V, Powell DR, Hinds J, Cheung JK and Rood JI. (2006) Construction and analysis of chromosomal *Clostridium difficile* mutants. *Mol Microbiol* **61**, 1335–1351.
17. Heap JT, Pennington OJ, Cartman ST, Carter GP and Minton NP. (2007) The ClosTron: a universal gene knock-out system for the genus *Clostridium. J Microbiol Methods* **70**, 452–464.
18. Heap JT, Pennington OJ, Cartman ST and Minton NP. (2009) A modular system for *Clostridium shuttle* plasmids. *J Microbiol Methods* **78**, 79–85.
19. Kuijper EJ, van den Berg RJ and Brazier JS. (2009) Comparison of molecular typing methods applied to *Clostridium difficile. Methods Mol Biol* **551**, 159–171.
20. Stabler RA, Gerding DN, Songer JG, Drudy D, Brazier JS, Trinh HT, Witney AA, Hinds J and Wren BW. (2006) Comparative phylogenomics of *Clostridium difficile* reveals clade specificity and microevolution of hypervirulent strains. *J Bacteriol* **188**, 7297–7305.
21. Karberg M, Guo H, Zhong J, Coon R, Perutka J and Lambowitz AM. (2001) Group II introns as controllable gene targeting vectors for genetic manipulation of bacteria. *Nat Biotechnol* **19**, 1162–1167.
22. O'Connor JR, Lyras D, Farrow KA, Adams V, Powell DR, Hinds J, Cheung JK and Rood JI. (2006) Construction and analysis of chromosomal *Clostridium difficile* mutants. *Mol Microbiol* **61**, 1335–1351.
23. Hussain HA, Roberts AP and Mullany P. (2005) Generation of an erythromycin-sensitive derivative of *Clostridium difficile* strain 630 (630 Δ*erm*) and demonstration that the conjugative transposon Tn*916*ΔE enters the genome of this strain at multiple sites. *J Med Microbiol* **54**, 137–141.
24. Lyras D, O'Connor JR, Howarth PM, Sambol SP, Carter GP, Phumoonna T, Poon R, Adams V, Vedantam G, Johnson S, Gerding DN and Rood JI. (2009) Toxin B is essential for virulence of *Clostridium difficile. Nature* **458**, 1176–1179.
25. Spigaglia P and Mastrantonio P:. (2002) Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. *J Clin Microbiol* **40**, 3470–3475.
26. Murray R, Boyd D, Levett PN, Mulvey MR and Alfa MJ. (2009) Truncation in the *tcdC* region of the *Clostridium difficile* PathLoc of clinical isolates does not predict increased biological activity of Toxin B or Toxin A. *BMC Infect Dis* **9**, 103.
27. Morgan OW, Rodrigues B, Elston T, Verlander NQ, Brown DFJ, Brazier J and Reacher M. (2008) Clinical severity of *Clostridium difficile* PCR ribotype 027: a case-case study. *PLoS ONE* **3**(3), e1812.

Chapter 2

Clostridium difficile and the Disease It Causes

Torbjörn Norén

Abstract

Clostridium difficile is a spore-forming, toxin-producing, anaerobic bacterium abundant in soils and water. Frequent and early colonization of the human intestinal flora is common and often asymptomatic. Antimicrobials given commonly disrupt the intestinal microflora and through proliferation in colon and production of toxin A and B it precipitates *C. difficile* infection (CDI). The enterocytic detachment and bowel inflammation provoke *C. difficile*-associated diarrhoea (CDAD) sometimes developing into severe pseudomembranous colitis (PMC) and paralytic ileus. Infection is acquired from an endogenous source or from spores in the environment, most easily facilitated during hospital stay. In the elderly, comorbidity, hospitalization and antimicrobial treatment present as major risk factors and the slow recolonization of the normal flora likely responsible for single or multiple recurrences of CDI (25–50%) post therapy. The key procedure for diagnosis is toxin detection from stool specimens and sometimes in combination with culture to increase sensitivity. In mild cases stopping the offending antimicrobial will lead to resolution (25%) but standard therapy still consist of either oral metronidazole or vancomycin. Alternative agents are presently being developed and fidaxomicin, as well as nitrothiazolide are promising. Furthermore, host factors like low antitoxin A levels in serum relates to increased risk of recurrence and small numbers of patients have received immunoglobulin with good results. An immunogenic toxoid vaccine has been developed and human colostrum rich in specific secretory Ig A also support the future use of immunotherapy. Today we experience a tenfold increase of CDI incidence in the western world and both epidemics and therapeutic failure of metronidazole is contributing to morbidity and mortality. The current epidemic of the *C. difficile* strain NAP1/027 emerging in 2002 in Canada and the USA has now spread to most parts of Europe and virulence factors like high toxin production and sporulation challenge the therapeutic situation and cause great concern among infection control workers. Excessive use of modern fluoroquinolones is thought to play an important role in facilitating this epidemic since NAP1/027 was shown to have acquired moxifloxacin resistance compared to historical strains of the same genotype. Both the current epidemic like this and other local outbreaks from resistant or virulent strains warrant culture to be routinely performed enabling susceptibility testing and typing of the pathogen. Genotyping is most commonly done today by pulse-field gel electrophoresis (PFGE) or PCR ribotyping but multilocus variable-number tandem-repeat analysis (MLVA) seems promising. Epidemiological surveillance using all these tools will help us to better understand the global spread of *C. difficile*.

Key words: *Clostridium difficile*, CDI, CDAD, NAP1/027.

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_2, © Springer Science+Business Media, LLC 2010

1. Introduction

From the moment of birth we are continuously colonized throughout our gastrointestinal tract by a complex flow of microorganisms. The equilibrium at any stage of life is crucial to health and involves the commensal relation between gut epithelium and the bacterial, fungal and parasitic luminal microbiota. Oral contamination of food particles supplemented by the respiratory mucociliary transport is processed and sterilized through the acidic environment of the stomach. Successful invasion past this point depends on gastric acidity and the ability of microbes to pass as resistant spores or remain hidden in the mucus barriers. Furthermore, gastroduodenal and nasogastric tubes used in artificial nutrition as well as modern proton pump inhibitors will successfully disrupt this functional barrier. Passing through the small intestine results in a luminal proliferation of approximately 10^6 bacteria per millilitre (1) entering colon where resorbtion of water increases concentration to 10^{12} bacteria per gram of faeces (2). Due to the luminal distance from oxygenic vessels of the mucosa the environment favour a 1,000- to 10,000-fold majority of anaerobic bacteria (1). Among some 40 anaerobic species, the *Clostridium* and *Bacteroides–Prevotella* group make up approximately 99% of all anaerobes in the colon (3). Abundant Bifidobacterium and *Lactobacillus* are also very prominent members of the colonic flora (4) but their proportion decline with age. Hence, this fact gives a rationale for such protective supplementation of commensal bacteria (probiotica) in the aging individual. Through all ages the gut equilibrium depends on the sessile and enterocytic bacterial interaction including cell adhesion and immunological control. This protective state of the normal intestinal ecology is often defined as colonization resistance (5).

The *Clostridium* is a heterogeneous group of gram-positive usually obligate anaerobic rod-shaped bacteria capable of endospore formation thereby possessing formidable survival qualities. Abundant in most soils and open freshwater on earth (6), oral colonization through contaminated food sources make *Clostridium* spp. early settlers and members of the gastrointestinal microbiota in animals. In addition to the formation of spores, an additional important feature of the *Clostridium* species is the frequent and diverse production of exotoxins unique to this bacterial genus (7). Approximately 30 out of the 80 known clostridial species are recognized as animal pathogens and 30% of these produce disease in man (8). Pathogens like *C. tetani, C. perfringens* and *C. botulinum* exhibit potent toxins causing deadly disease in humans such as tetanus, gas gangrene and food poisoning, respectively. *Clostridium difficile* is also an important enteropathogen,

provoking toxin-mediated diarrhoea and colitis, referred to as *C. difficile*-associated diarrhoea (CDAD) or *C. difficile* infection (CDI).

The premature tolerance of our gut-associated lymphoid tissue (GALT) to *C. difficile* toxins enables the colonization of this toxin-producing bacteria without secretory diarrhoea in early childhood. Throughout the lining of the gastrointestinal mucosa numerous mucus producing enterocytes are crowded with M cells for transcellular antigen uptake. In the subepithelial GALT dendritic cells and macrophages recognize luminal antigens like clostridium toxins. These are presented to T- and B lymphocytes located in subepithelial follicles of the Peyer's patches and initiate production of neutralizing antibodies (9). Healthy neonates and infants not yet with a stable microflora exhibit asymptomatic early *C. difficile* colonization rates of 25–80% without diarrhoea (10) attributed to immaturity of GALT or absence of a putative toxin receptor (11). Early colonization is influenced by breast-feeding and reduces carriage rates by half and features mainly non-toxin-producing strains up to 6 month of age (12). The natural tolerance is progressively lost and CDAD as being the most common cause of nosocomial diarrhoea (32%) was recently recorded among children with a median age of 1.3 years (13).

In healthy adults >60% have detectable serum IgG and IgA to both toxins A and B (14) but low colonization rates (15). This is not related to prior experience of CDAD and might be a result of childhood immunity or the frequent contact with these soilborne bacteria in the environment (16). Thus a colonized status do not predispose to CDAD when entering hospital (17) but the immunological response when contracting diarrhoea is crucial to the risk of recurrent disease (18, 19). For example, the antibody response in the asymptomatic colonized patients was 48 times more protective against diarrhoea compared to nonresponders (20). This was also supported by the protection observed in gnotobiotic mice given monoclonal IgG prior to successful *C. difficile* challenge (21). Similarly, specific mucosal IgA has been found to be low in faeces from patients with recurrent CDAD (22) and secretory immune activity is believed to reflect the serum responses to *C. difficile* toxins.

Since the introduction of antimicrobial therapy antibiotic-associated diarrhoea (AAD) has emerged as an increasing problem, presumably due to disruption of the indigenous intestinal microflora. Severe cases of pseudomembranous colitis (PMC) (**Fig. 2.1**) were frequently observed and findings of *Staphylococcus aureus* in faeces led to successful treatment of these conditions with vancomycin in the 1950s (23). In the 1970s PMC was blamed on exposure to clindamycin (24) and the fatal effect of this antibiotic in hamsters developing this pathology was highlighted. Pathogenesis was linked to cytotoxicity (25) and this

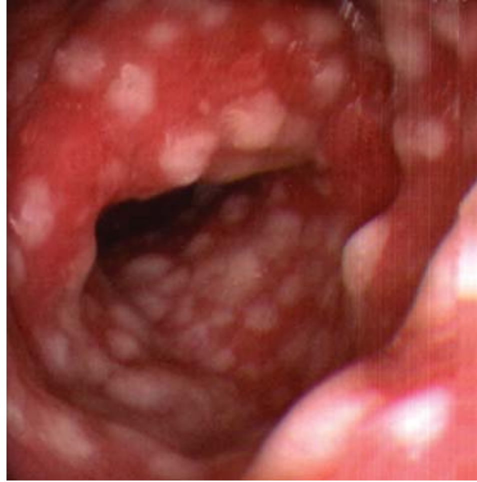


Fig. 2.1. Moderate pseudomembranous colitis (PMC) visualized by the typical inflammatory mucosa interspaced with light protruding knobs of ulcerative necrotic debris covered by fibrin representing pseudomembranes.

effect could be neutralized by antitoxin from *C. sordellii* (26), still without any corresponding pathogen in culture. Later we have learned that the neutralization of *C. sordellii* antitoxin was indeed an effect of cross-reaction with *C. difficile* toxins belonging to the same protein family. Bartlett and colleagues finally discovered the connection between *C. difficile* and PMC in 1978 (27) following treatment with clindamycin. PMC is diagnosed by endoscope or during surgery and is a highly specific (95–99%) test for toxin-producing *C. difficile* (28).

Approximately 10–25% of all AAD is related to *C. difficile* (29) and this fraction relates very well to laboratory reports on 10–20% of stool specimens submitted for *C. difficile* toxin testing being found positive (29–31). Other pathogens, besides *S. aureus* (32), proposed as contributing to AAD are *C. perfringens* type A (33), enterotoxin-producing *Bacteroides fragilis* (ETBF) (34), *Candida albicans* (35) and possibly salmonella (36) but most AAD is unexplained.

1.1. *C. difficile* and the Effect of Toxins

The pathogenicity of *C. difficile* is mediated by two toxins, A and B, and explained by a disruption of the enterocytic actin-skeleton via the Rho-metabolism (37, 38). Toxin A (308 kDa) and B (270 kDa) are prototype members of the protein family large clostridial cytotoxins (LCTs) (39, 40) and attach by the carboxy-terminal to an unknown receptor on the mucosal enterocyte. This important adhesion could depend on capsule and cell-wall components such as S-layer adhesins P36 and P47 or other proposed immunostimulative flagellar or fibronectin-binding proteins (41). Via translocation of the central part the enzymatic

(toxic) amino-terminal it is exposed to interior glucosylation of Rho proteins vital for enterocyte cytoskeleton formation (42). This leads to depolymerization of actin filaments with loss of internal architecture, rounding of cells and disruption of tight-junctions holding cells together. Destruction of villus and brush border membranes is followed by mucosal loss and inflammatory response, which accelerate the malfunction of the epithelium (43). Focal layers of fibrin organize to cover the ulcerative lesions, which stand out on inflammatory necrotic debris forming knob-like confluent pseudomembranes visualized by endoscope examination (**Fig. 2.2**) (44).

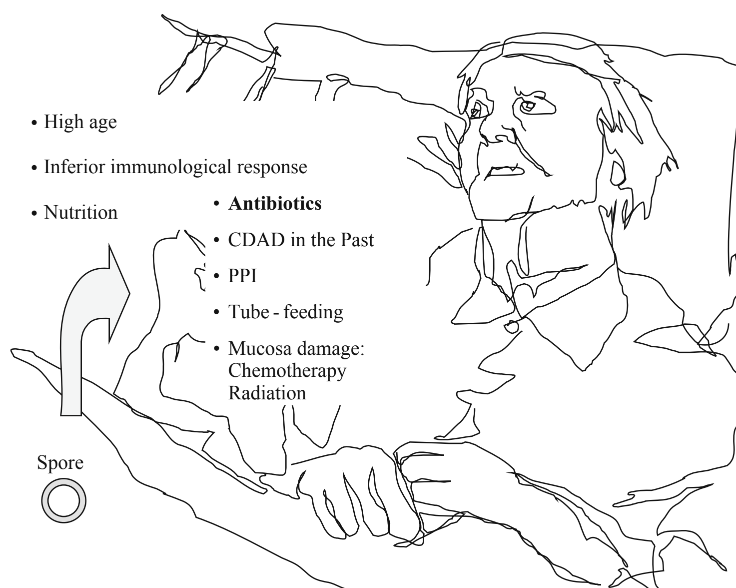


Fig. 2.2. Risk factors for *Clostridium difficile*-associated diarrhoea (CDAD).

Toxin-mediated diarrhoea is sometimes caused by strains only producing toxin B, lacking functional toxin A gene (45), but most clinical isolates have both toxin A and B resulting in synergistic pathological qualities (46). Some strains of *C. difficile* also produce a third chromosomaly encoded toxin called binary toxin (CDT) (47). This is an actin-specific ADP-ribosyltransferase with high homology in structure and function to the *C. perfringens* ι-toxin (47). Six to thirty percentage of toxin A- and B-positive strains also produce CDT (48). In addition, 2% of toxin-negative strains (A^- B^-), not believed to cause CDAD, produce CDT but a majority of those were from asymptomatic patients. Hence, the role of this toxin is so far considered to be of minor importance.

1.2. Colonization and Diarrhoea

A disruption of the indigenous colonic microflora is usually caused by antibiotic therapy and is the main factor that predisposes for infection with *C. difficile* and CDAD (49). The role of

antibiotics in destruction of colonization resistance and CDAD has been proven in several animal models where subsequent inoculation of normal faecal flora reversibly makes *C. difficile* and symptoms disappear (50, 51). Both in vitro inhibition of *C. difficile* growth when exposed to faecal emulsions from healthy adults (52) and in vivo curative nasogastric supplementation of normal enteric flora in human CDAD (53, 54) support this importance of colonization resistance restoration. Asymptomatic carriage of *C. difficile* in healthy adults in community is reported uncommon (1–15%) (55–58) and hence endogenous origin of *C. difficile* in CDAD has been questioned. Recent studies have, however, found evidence of the presence of specific toxin B from over 50% of healthy subjects (59). This indicates that the endogenous *C. difficile* population might rather be minute than absent in normal gut flora. On the other hand, the environment could very well act as a continuous daily source of infection enhanced by, for example, from strongly contaminated hospital wards. Nevertheless, whether transient or permanent, *C. difficile* carriage (60) predisposes for CDAD. This is often seen when patients are admitted to hospitals as 15–21% of patients are colonized with *C. difficile* and antibiotic treatment subsequently triggers onset of CDAD (20, 61). Infected patients, environmental surfaces and hands of health-care workers aid in transmission of *C. difficile* (61). Although asymptomatic carriers will have no diarrhoea (17, 62) they will contribute to the distribution of long-lived spores to the environment (63). While colonization of *C. difficile* is the basic risk condition, mainly acquired in hospital, there are several other important risk factors and these are illustrated in **Fig. 2.2**. Antibiotic treatment is the most important risk factor (10) and almost all existing antimicrobials have been reported to cause CDAD. The most cited agents are clindamycin (24), ampicillins and cephalosporins (64, 65) constituting around 90% of all precipitating antimicrobials in CDAD (66). Not surprising, repeated courses or multiple use of antimicrobials increases the risk of CDAD (67). Low-risk antibiotics like trimethoprim, rifampicin, tetracyclines, aminoglycosides and former generations of quinolones (68) have a limited impact on endogenous anaerobes and are seldom the cause of CDAD. Following the introduction of new generations of fluoroquinolones, exerting a more profound anaerobic activity, the risk of CDAD increases after gatifloxacin (69), levofloxacin (70) or moxifloxacin use (71). This is also exemplified by the extensive epidemics in the USA and Canada (72, 73) attributed to excessive fluoroquinolone prescription (71). Successful interventions by banning cephalosporin use clearly reduce the frequency of CDAD (74) The risk of CDAD increases with age by tenfold for those between 60 and 90 years of age (15, 75) and is partly confounded by other recognized risk factors of the host including weaning immunity and

low levels of antitoxin A and B antibodies (18). In addition, concomitant disease requiring frequent hospitalization or prolonged hospital stay (71) involving several precipitating treatments, i.e. antibiotic/antineoplastic treatment (76) and enteral tube feeding (77), will establish recurrent CDAD. Any use of proton pump inhibitors (78) and enemas (79) is reported to increase the risk of CDAD to some extent. Starvation conditions following surgical procedures or elemental diet (59) deprived of the nonessential amino acids cysteine or proline (80) are proposed as examples of nutritional risk conditions for CDAD but need further evaluation in clinical practice.

1.3. Clinical Recognition and Manifestation of CDAD

CDAD depends on altered colonic flora, significant amount of toxin-producing *C. difficile*, nutritional status or other factors regulating toxin expression, as well as defective host immunity (81) (**Fig. 2.1**). The interplay between these conditions decides the onset of diarrhoea generally defined as ≥3 loose stools a day presenting a mild self-limiting disease. The bacterium itself is not invasive and the pathological manifestations relate to the production and adhesion of toxins causing mucosal inflammation. For mild disease the endoscopic view presents an unspecific reddish and edematous picture. If the course of diarrhoea is not resolved by the interruption of the precipitating factor, i.e. antibiotic, progressive abdominal cramps, bloody stools and fever will worsen the condition and in 1–3% of CDAD patients fulminate colitis may develop (82). A defined histopathological and endoscopic entity of this severe condition is pseudomembranous colitis (PMC) where whitish yellow visible plaques (pseudomembranes) representing fibrin-covered inflammatory protrusions cover the inner colonic wall (**Fig. 2.2**). Severely ill patients may mimic clinical improvement by resolving diarrhoea, but often this is instead a sign of paralytic ileus with a picture of toxic megacolon. In this stage of disease the benefit of oral therapy is reduced and the mortality escalate up to 25–40% in spite of life-saving surgery (83). The overall mortality in CDAD is often hard to estimate due to serious concomitant diseases confounding the evaluations and has mostly been found around 4–5% (84).

The key procedure for CDAD diagnosis is toxin detection from a faecal specimen and sometimes, to increase sensitivity, culture is also done (85). Culturing the organism supplies additional support on diagnosis where no other cause is found and gives opportunity for further epidemiological information on outbreaks. Bearing in mind the high frequency of asymptomatic carriers (86) as well as the existence of nontoxigenic *C. difficile* (87) the diagnostic evaluation based on culture alone must be cautious. Detection of cytotoxin B in tissue cell culture, together with the isolation of *C. difficile* on selective medium, has for long been considered the gold standard (88). Though highly

sensitive (detects down to 10 pg of toxin B), disadvantages of its slow (24–48 h to result) and costly procedure leave CDAD diagnosis today often performed by using a variety of enzyme immunoassays (EIA) test kits for toxin (88–92). These more rapid tests have a good specificity (75–100%) but a lower sensitivity (75–85%) (89) detecting between 100 and 1,000 pg of toxin (93) and with up to 25% false negatives. Both test kits for toxin A alone and toxin A and B combined are commercially available and the latter should be preferred since 1–2% of CDAD involve toxin A-negative strains (94). A recently reported novel toxin A^{+}/toxin B^{-} CDT^{+} strain prevalent in 3.5% in the Calgary area in Canada may in part explain the 97% detection rate of CDAD by the cytotoxin B assay (95). Thus, combination of tests could be advocated though limited by costs. Multiple testing of stools increases the sensitivity (5–10%) but is not cost-effective (96). Different techniques for direct detection of toxin by polymerase chain reaction (PCR) has been tried (97, 98), but lack of reproducibility and frequent protease degradation are still problems to be solved.

Radiology gives information in severe cases where ileus or colon dilatation is suspected and endoscopy of the colon gives diagnosis by observing typical pseudomembranes in 41% of CDAD (99). However, if restricted only to sigmoidoscopy 10% will be missed and will be false negative (100).

1.4. Severe CDAD (S-CDAD) or Fulminant Colitis

Most clinicians agree on crude observations like megacolon, peritonitis, ileus or need of ICU and surgery to qualify as S-CDAD. More predictive attempts have recently been done to evaluate clinical and/or laboratory markers as risk factors for S-CDAD. Several different sets of markers such as diarrhoeal frequency, leucocytosis (101), fever, abdominal pain (102), ileus, hypotension, renal insufficiency (82), hypoalbuminemia, mental confusion and pseudomembranes (103–105) have been proposed, but a common accepted prediction score is not yet developed. In fact, this illustrates one of the most difficult challenges of CDAD, i.e. how to timely intercept the initiation of progressive paralysis, megacolon and subsequent septic peritonitis by colectomy. Various clinical pictures confuse optimal treatment since both toxic symptoms, including vomiting, lethargy and tachycardia or paralytic absence of diarrhoea due to postoperative drugs, sometimes rapidly evolve. High leucocyte counts (>15–$25{,}000/mm^3$) or even leukemoid reactions are thought to be the most convincing marker of such serious complication (84, 106), while elevated arterial lactate in the ICU setting may be even a better predictor (107, 108).

1.5. Change of CDAD Global Epidemiology and Clinical Severity

A recent outbreak of CDAD with increased severity, recurrence rate and mortality was initially observed in 2003 in Canada and the USA (73, 84, 109). From 1991 to 2003 a dramatic tenfold

incidence was noted in the risk group of >65 years of age and rates of toxic megacolon and refractory disease was reported. The mortality within 30 days increased from 4.7 to 13.8% and the 12-month attributable CDAD mortality of 16.7% compared to controls (110). Furthermore, in connection with relatively poor outcome from metronidazole treatment Musher et al. stated recurrence rates as high as 58% and a mortality in the range of 21–33% depending on response to therapy, not necessarily involving PMC (111).

The explanation for this epidemic was a highly virulent strain of *C. difficile*, referred to as the pulse-field type 1 (NAP1), PCR ribotype 027 and toxinotype III, diagnosed as the infective agent in over 80% of CDAD the cases. Soon after finding this epidemic strain in Canada and in the USA it was also isolated in various outbreaks in England, Scotland, Ireland, Belgium, France, Poland and the Netherlands (112, 113) on all sites in connection with increased severity. The virulence was thought to be caused by an in vitro 16-fold (toxin A) to 23-fold (toxin B) increase of toxin production compared to controls and its excellent sporulation capacity (114). The observation of an 18 bp deletion within the regulatory *tcdC* gene has been a theoretical explanation to this, although identical deletions have been found in less virulent strains and the magnitude of toxin levels have been hard to reproduce (115). Several studies have instead suggested the parallel increased use of modern fluoroquinolones, i.e. gatifloxacin and moxifloxacin, as the major reason for selection of CDAD caused by epidemic type 027 strains (71). In support of this, historic pre-epidemic isolates of type 027 are mostly susceptible to moxifloxacin (73, 115) and not related to severe disease or outbreaks, while emergent epidemic isolates are highly resistant. This current epidemic is still expanding by cases in Austria, Denmark and Finland and in most places under control by increased diagnostic efforts, hygiene interventions and antimicrobial restriction, including of the fluoroquinolones (116–118).

2. Treatment of CDAD

The initial step in treatment is to stop the offending antimicrobial and this leads to spontaneous cure in up to 25% of patients with mild disease (99, 119). In the remaining cases with established diarrhoea and more profound symptoms of fever and abdominal pain, specific antimicrobial therapy is necessary. The use of antimotility agents will theoretically retain active toxin in colon and should be avoided. As for probiotics these are mainly used in recurrent CDAD to facilitate the restoration of intestinal flora.

2.1. Standard Antibiotics

The most common used agents are metronidazole and vancomycin with similar cure rates of 93–98% and recurrence rates between 6 and 16% (99, 120–122). Vancomycin given orally has minimal absorption and impressive concentrations in stools (1,000 μg/ml) exceeding the MIC of *C. difficile* by 1,000-fold (86, 123). The recommended dose is 250 mg four times daily (124). Emerging resistance of vancomycin-resistant enterococci (VRE) in the gut is blamed on the use of oral vancomycin (125), and together with comparable high cost, metronidazole is now first choice in CDAD therapy. Vancomycin is preferred in metronidazole failures (persistent diarrhoea on day 3–5), pregnant and lactating women as well as in those with intolerable side effects from metronidazole or drug interactions (mainly the warfarins) (126). Oral metronidazole is well absorbed leaving a mean faecal concentration of only 9.3 μg/ml, still exceeding MIC_{90} (0.25–1.0 μg/ml) of circulating strains by almost a tenfold (127). Faecal metronidazole levels rest on a biliary secretion of 14% (128) compensating for the metabolism in the liver. Most importantly luminal concentration is increased by colonic inflammatory exudation, however, undetectable upon recovery (127). Similar reports on the relation between inflammatory activity in Mb Crohn and faecal concentration of metronidazole (1–23 μg/ml vs. not detectible in healthy controls) support this view (129). Obviously, this questions the optimal length of metronidazole therapy with maintained effective gut concentrations after the improvement of diarrhoea. The recommended doses of metronidazole are either 250 mg four times daily or 400 mg three times daily for 10–14 days based on comparative studies (120, 121). In spite of the resistance in *C. difficile* isolates reported up to 6.3% for metronidazole (130–135) and 3% intermediate resistance for vancomycin (130), no relation to clinical outcome has yet been found. Frequent therapeutic failures, increased morbidity and risk of recurrence have lately been found in metronidazole treatment (111, 136–138) and might suggest the use of vancomycin for S-CDAD (105).

2.2. Alternative Antibiotics

The narrow spectrum antibiotic fusidic acid derived from *Fusidium coccineum* has for years been used to treat staphylococcal bone and soft tissue infections. It resembles the pharmacological behaviour of metronidazole with good resorbtion and exudation in inflammatory parts of colon but without any effect on important gram-negative anaerobes like the *Bacteroides–Prevotella* group. *C. difficile* isolates are generally highly susceptible to this agent (139) and fusidic acid has emerged as a promising alternative therapy for CDAD. Several studies have proven efficacy similar to both vancomycin and metronidazole (121, 140, 141) in treatment of CDAD, achieving cure rates between 75 and 96%. In these trials daily doses of 0.5–1.5 g have been used with

variable length of treatment (7–21 days) and from clinical practice a lower regime of 250 mg tid, for 7 days seems sufficient. However, fusidic acid-resistant *C. difficile* develop in 55% of follow-up isolates from fusidic acid-treated CDAD patients (142). In analogy with the resistance in *S. aureus* after topical treatment with fusidic acid (143), this was due to resistance mutations in *fusA* (144) and complicates its use in nosocomial settings. Again this phenomena, like for metronidazole resistance, did not affect clinical outcome.

Rifampicin with excellent tissue penetration has been tried in CDAD sporadically. One study with seven patients had no relapses using rifampicin in combination with vancomycin (145). Recently a prospective randomized study of metronidazole vs. metronidazole and rifampicin (39 patients) found a disappointing recurrence rate (46%) and a high mortality (6/19) with the combination of rifampicin and metronidazole (146).

Teicoplanin has also been tested effective in CDAD (147) and as effective as metronidazole, vancomycin and fusidic acid (121) and a favourably low frequency of recurrences (7%). Unfortunately the glycopeptide similarity to vancomycin disqualifies its use by also selecting VRE and is not recommended in CDAD.

Ramoplanin, a lipoglycodepsipeptide antimicrobial derived from *Actinoplanes* strain (ATCC 33076), is an oral nonabsorbable agent used for treating VRE (148). Using a different mode of peptidoglycan action compared to the similar glycopeptide no cross-resistance has yet been observed (149, 150) and *C. difficile* is highly susceptible (151) this agent is currently in phase III studies and could be of future use in CDAD.

Fidaxomicin (OPT-80) (Optimer) is a nonabsorbable macrocycle with no gram-negative activity and preventing CDAD lethality and recurrence in the hamster model (152). In humans a low recurrence rate of 4.8% in phase II studies is promising and currently a phase III study is being performed.

Nitrothiazolide is marketed in the USA as an antiparasitic agent but is highly effective against *C. difficile* in vitro (153). A randomized double-blind trial showed this agent was comparable to metronidazole in response and recurrence rate (111). Although not yet available on CDAD indication, this agent could be of use in metronidazole failures or intolerant patients (154).

2.3. Immunotherapy

There is substantial evidence that the immune response to *C. difficile* toxins plays a major role in determining host susceptibility to CDAD (20, 155, 156). The significance of low antibody levels related to increased risk of recurrent CDAD (22, 157, 158) is well known. Hence, treatment with normal pooled human intravenous immunoglobulin (IVIG) containing specific toxin A antibodies has both resolved severe CDAD and prevented recurrences in man (18, 159–161). Toxoid vaccine immunization in cows and poultry have produced protective antibodies in

bovine colostrum and egg-yolk, respectively, and supplemented orally protected challenged hamsters (162, 163). Similar oral experiments in humans have failed, presumably due to acidic stomach degradation (164). Human colostrum rich in specific secretory IgA have been shown to effectively neutralize *C. difficile* toxins (165). This has led to further development of a presumptive toxoid (toxin A) vaccine given intramuscularly with antibody response well over the threshold (IgG for toxin A) seen in asymptomatic *C. difficile* carriers or CDAD patients (166). Preliminary results in three CDAD patients indicate protection against recurrence in 2 months follow-up (167). Poor IgA response, data mostly from young volunteers in a 2 month vaccine course, question the individual benefit in the aged nonresponding CDAD patient of natural disease (16).

2.4. Toxin binders

Toxin-binding strategies have included unselective cholestyramine (168) but constipation and findings of interactive binding of vancomycin have discouraged use in CDAD (169). However, this approach is being reconsidered by using a nonresorbable high molecular (>400 kDa) toxin-binding anionic polymer (Tolavamer), proven alone as effective as vancomycin clearing diarrhoea and preventing recurrences in phase II trials (170). Clearly, inactivating liberated toxins without interfering with the normal microflora is a tempting strategy. Preliminary results from the largest comparative multicenter phase III study of CDAD treatment (PACT) show, however, inferiority of Tolavamer to both metronidazole and vancomycin in both clinical response and time to resolution. Still the role as adjunctive therapy in S-CDAD or high-risk patients could be of value, considering the alternative mode of action.

2.5. Treatment of Recurrent CDAD

Recurrent CDAD will occur in 10–65% of initially cured patients and the higher estimates in multiple recurrent CDAD (171–174). This follows from sustained impact on colonization resistance where new courses on antibiotics or hospitalization provide conditions for CDAD to return frequently for years despite repeated standard therapies (173). Hence, recurrence is not regarded as therapeutic failure and recurrent CDAD responds well to repeated courses of metronidazole or vancomycin (99). For multiple recurrences a theoretically adopted tapered 6-week vancomycin regimen: 125 mg × 4 for the first week and withdrawal of 125 mg each week for 4 weeks and finishing by 125 mg every other day in week 5 and every third day the last week is sometimes used (175). Still, this gave a 31% recurrence rate and instead a primary 21-day pulsed treatment has been more successful (174). This would allow most of the spores to germinate and to be eliminated in their vegetative form. Culture of persistent toxin-producing *C. difficile* post-treatment is a common observation and increases the risk of recurrence (176). Recurrent strains of

C. difficile can be of identical genotype as the preceding strain (relapse) or a new strain may appear from environment (reinfection). The proportion of this is of epidemiological interest and is often reported on a 1:1 ratio (177) but is strongly influenced by hygiene measures in hospital environment as well as local dominance of epidemic strains. Surely both the density of spores in the environment and the intrinsic susceptibility of the host will play in favour of recurrent CDAD (178).

2.6. Probiotics

Probiotics are regarded as supplemental living cultures of nonpathogenic bacteria or fungi acting as a refill or as beneficial substitutes of the commensal intestinal flora disrupted in diarrhoeal disease (179). Their role in therapy has been protective during treatment and preventive while awaiting restoration of colonization resistance, and thus mainly effective in recurrent CDAD. Most commonly used is the nonpathogenic yeast *Saccharomyces boulardii* (180, 181), *Lactobacillus GG* (182, 183), *Lactobacillus acidophilus* and *Bifidobacteria* (184). Several studies have indicated the beneficial role of probiotics in AAD and CDAD but open design and small study populations often confuse the picture (185). Curative therapy in combination with *S. boulardii* is best documented and reduced the risk of relapse significantly (p=0.04) (28, 180, 186, 187) by specific protease activity on proposed receptor sites (188). *Lactobacillus GG* has also proven beneficial preventing recurrent CDAD (189).

Faecal flora substitution is an effective strategy involving instant replacement of a functional luminal faecal tissue. Most commonly a diluted faecal sample from a healthy donor is transferred through rectal enema and breaks the cycle of recurrent CDAD (190, 191). A more tasteful approach giving selected commensal donor bacteria as rectal infusion and this has also been effective (54). Even administration of non-toxin-producing *C. difficile* apparently gives protection against CDAD (192, 193) probably by competing for suitable ecological niches among toxin-producing members of the species. In fact, following CDAD patients posttreatment we found 10% (twice the proportion of total CDAD strains circulating) carrying nontoxigenic *C. difficile* in follow-up and none of these patients experienced relapse (unpublished data).

3. Antimicrobial Susceptibility Testing of *C. difficile* and Implications in CDAD

A high antimicrobial activity or a low minimal inhibiting concentration (MIC, µg/ml) of *C. difficile* isolates is desired when evaluating both low precipitating risk of CDAD and potential use in therapy. Susceptibility testing of *C. difficile* is not generally

done because it is a labour-intensive procedure and lack of useful breakpoints. A susceptible therapeutic drug concentration, in serum often used for systemic infection, is not, however, relevant to that of an intraluminal gut infection. Notably this could be crucial to highly absorbed antimicrobials like metronidazole mounting a 9.3 μg/ml concentration in faeces (127) during standard treatment, when compared to the pharmacological breakpoint MIC for susceptible isolate set at 4 μg/ml by the Swedish Reference Group for Antibiotics (SRGA) (http://www.srga.org) or at 8 μg/ml by others (151, 194).

The recommended method for susceptibility testing of anaerobes is agar dilution (194) together with the broth microdilution approved for the *Bacteroides fragilis* group (195). Both methods have been used to determine MIC in *C. difficile*. Etest (Biodisc, Solna) is easier to perform and is used routinely for a wide variety of bacteria in many laboratories. Measurements of MIC are comparable for all antimicrobials except for metronidazole, where somewhat lower values (1–2 steps of dilution) are detected (131, 196). Although the Etest is not approved by the latest CLSI standard (formerly NCCLS) 2007 (194), this test is increasingly used for *C. difficile*.

The importance of drug resistance in *C. difficile* for the outcome of CDAD treatment is limited as most clinical isolates usually are highly susceptible to both metronidazole and vancomycin (131, 139). Resistance to certain precipitating antimicrobials may be of importance when reviewing antibiotic guidelines as illustrated by clindamycin or moxifloxacin resistance (117, 197) and referring to frequent outbreaks of *ermB* positive clindamycin-resistant strains (198–200) or the fluoroquinolone-resistant NAP1/027 strain. The success of the epidemic NAP1/027 probably involves a nosocomial selection mechanism due to fluoroquinolone resistance in isolates and overuse of modern fluoroquinolones like levofloxacin, moxifloxacin or gatifloxacin (69, 71, 201), although not confirmed in all places (202). Treatment failure has not yet been related to resistance in offending isolates (134) furthermore, resistance to metronidazole or vancomycin in *C. difficile* isolates observed by Pelaez et al. (130) relating to increased risk of recurrence has not yet been verified by others. Currently in the NAP1/027 epidemic there are reports on failure of metronidazole therapy (111, 136, 203), but again, not related to metronidazole resistance. So far, a majority of recovered resistant isolates are nontoxigenic and not relevant to clinical outcome (131). Instead, maybe the extended damage of high toxin exposure of NAP1/027 in combination with suboptimal concentrations of metronidazole is enough to affect treatment efficacy (103). Offending *C. difficile* isolates in CDAD are often resistant to precipitating antimicrobials, but the overall anaerobicity in the colon will be as important in preparing the

environment for spore germination of *C. difficile*. All clinical isolates are always highly resistant to third-generation cephalosporins and ciprofloxacin (204). Moxifloxacin resistance (>32 μg/ml), the marker of the epidemic NAP1/027, is currently used for screening purposes but moxifloxacin also exist in other multiresistant *C. difficile* (205). Clindamycin resistance is common among strains causing outbreaks (199) but in a non-epidemic setting only 10% will be highly resistant (MIC >256 μg/ml) and 34% of moderate to low resistance (MIC 4–8 μg/ml) (204). Most frequently this resistance is due to the presence of the *ermB* gene and which encode resistance to methylase–lincosamide–streptogramin (MLS) which includes erythromycin (206). As for rifampicin 88% of isolates are highly susceptible (MIC <0.003 μg/ml) and the remaining fully resistant (131, 196). *C. difficile* has a low MIC to the newly introduced antimicrobials like linezolid, daptomycin and tigecycline which should not select for *C. difficile* during therapy. Penicillin derivatives like pc V and piperacillin have a diverse impact on the anaerobic flora including *C. difficile* with MIC_{90} ranging between 4 and >256 μg/ml (207).

4. Typing of *C. difficile*

To detect epidemic spread or clusters of CDAD, due to virulent genotypes, culture and typing are essential in order to respond by hygiene measures and antimicrobial restriction.

Several phenotypic and genotypic techniques have been used over the years but problems dealing with typeability, reproducibility and discrimination have left only a few for common application in epidemiology.

Early phenotypic classifications were antimicrobial or bacteriophage susceptibility patterns (208), immunoblotting (209) and SDS-polyacrylamide gel electrophoresis protein fingerprinting (SDS-PAGE) (210). All these techniques suffered from lack of reproducibility. Agglutinations with antisera differentiating four serovars were done early (211) but systematic serogrouping introduced by Delmée et al. (212) was the first method evaluated in international epidemiology and this main phenotypic procedure is still referred in modern genotyping of today. Genotyping gradually replaced the phenotypic procedures mentioned above. Early work on *C. difficile* plasmids (210, 213) yielded low typeability (30–60%). Turning to chromosomal DNA, the restriction enzyme analysis (REA) using different enzymes (*Hind*III, *Eco*RI, *Cfo*I or *Bam*HI) identified 22–55 distinct electrophoretic patterns (214, 215) and was highly discriminatory and reproducible. However,

the complex banding image (>50 products) required costly computer software for discrimination.

Arbitrarily primed PCR (AP-PCR) permits the detection of polymorphisms without prior knowledge of the target nucleotide sequence (216) and primers (up to 53 bp) are mostly used singly and are nonspecific (217–220). Sometimes a multilevel AP-PCR also referred to as random amplified polymorphic DNA (RAPD) with two or three shorter primers (10–19 bp) have been applied for higher discrimination between types (209, 221). Depending on the primer used the same strain can present different banding patterns (219) and often sensitivity to variable amounts of DNA presenting faint bands (209, 222). This makes reproducibility a major inter-laboratory problem (219). Still in local settings tracing outbreak strains, AP-PCR is an easy technique with satisfactory typeability and discriminative power (222, 223).

PCR ribotyping is based on the genetic stability of the 16S rRNA and 23S rRNA genes scattered as multiple copies of operons on the genome of *C. difficile* and the variable intergenic regions separating the two genes (224). Both the number of operons and length of the spacer regions are different between strains and this condition is used for discrimination (217). Specific oligonucleotide primers target the 3′ end of 16S rRNA and the 5′ end of the 23S rRNA and PCR amplification result in up to 24 different bands detected on gel electrophoresis. Sensitivity to the DNA quantity (problem of AP-PCR and RAPD) was shown to be minimized (225) and simplified DNA extraction as well as primer modification gave shorter (250–600 bp) and fewer bands (3–10) detected on more amenable agarose gels. Comparative studies proved good correlation between PCR ribotyping and both serotypes and PyMS (226).

Pulse-field gel electrophoresis (PFGE) is able to separate large DNA fragments of the whole genome after digestion of a set of restriction enzymes (*sma*I, *Ksp*I, *Sac*II or *Nru*I) and when used for *C. difficile* it yielded up to 10 fragment length polymorphisms per strain (227).

Several studies have compared AP-PCR/RAPD, PFGE and PCR ribotyping (223, 228–232) mostly in favour of the latter two, whose typing results are as discriminatory and with good correlation. Drawbacks for PFGE are the cost of equipment, its complexity and time-consuming nature (twice that of PCR ribotyping). Unfortunately, some strains are also untypeable (sero group G), presumably due to DNA degrading endonucleases (223). While PCR ribotyping is found inferior to PFGE in discrimination occasionally (233) this method has still propagated as the most widely used typing method. Applied by the International *Clostridium difficile* Study Group they demonstrated common geographical distribution of independent strain collections from the UK, Belgium and the USA (234). Similar findings were

done by combining both PCR ribotyping and PFGE (232) and in toxin A defective strains (235, 236). Presently, these methods enable us to recognize nationally dominant strains like the PCR ribotype UK1 of British isolates (237) and global epidemiological shifts like for the PCR ribotype 027 in North America and Europe (73, 113, 114). Toxinotyping was lately introduced on basis of the sequenced gene locus PaLoc including the toxin A (*TcdA*) and B (*TcdB*) gene (42). From variant morphology in the amplified RFLP's 10 distinct toxinotypes (I-X) compared to the reference strain VPI 10463 (toxinotype 0) could be identified (238). The strains were epidemiologically independent and existing in several countries and were consistent with serogrouping, PFGE and PCR ribotyping (236) offering another independent typing technique often used today to characterize international strains. For example, the present epidemic strain of North America and Europe is labelled according to all three typing techniques used today: PCR ribotype 027/NAP1(PFGE)/toxinotype III (114). Further development of genotyping tools today give us more opportunities to subtype and discriminate between outbreak strains. Modern techniques like multi locus sequence typing (MLST), surface-layer protein A sequence typing (*slpA*ST) and especially multilocus variable-number tandem-repeat analysis (MLVA) seem promising when compared to other modes of typing and might help us to better understand global spread of *C. difficile* (239).

References

1. Novogrudsky A and Plaut AG. (2003) The bacterial flora of the healthy gastrointestinal tract: colonization of the human colon. In: Yamada T, ed., *Gastroenterology*, 4th ed., Lippincott Williams & Wilkins, Philadelphia, 584–585.
2. Savage DC. (1981) The microbial flora in the gastrointestinal tract. *Prog Clin Biol Res* **77**, 893–908.
3. Mai V and Morris JG, Jr. (2004) Colonic bacterial flora: changing understandings in the molecular age. *J Nutr* **134**, 459–464.
4. Evaldson G, Heimdahl A, Kager L and Nord CE. (1982) The normal human anaerobic microflora. *Scand J Infect Dis Suppl* **35**, 9–15.
5. Vollaard EJ and Clasener HA. (1994) Colonization resistance. *Antimicrob Agents Chemother* **38**, 409–414.
6. Al Saif N and Brazier JS. (1996) The distribution of *Clostridium difficile* in the environment of South Wales. *J Med Microbiol* **45**, 133–137.
7. Hatheway CL. (1990) Toxigenic clostridia. *Clin Microbiol Rev* **3**, 66–98.
8. Murray PR. (2005) *Clostridium*. In: Murray PR, Rosenthal KS and Pfaller MA, eds., *Medical Microbiology*, 5th ed., Mosby, St. Louis.
9. Debard N, Sierro F and Kraehenbuhl JP. (1999) Development of Peyer's patches, follicle-associated epithelium and M cell: lessons from immunodeficient and knockout mice. *Semin Immunol* **11**, 183–191.
10. Kelly CP, Pothoulakis C and LaMont JT. (1994) *Clostridium difficile* colitis. *N Engl J Med* **330**, 257–262.
11. Eglow R, Pothoulakis C, Itzkowitz S, et al. (1992) Diminished *Clostridium difficile* toxin A sensitivity in newborn rabbit ileum is associated with decreased toxin A receptor. *J Clin Invest* **90**, 822–829.
12. Tullus K, Aronsson B, Marcus S and Mollby R. (1989) Intestinal colonization with *Clostridium difficile* in infants up to 18

months of age. *Eur J Clin Microbiol Infect Dis* **8**, 390–393.
13. Langley JM, LeBlanc JC, Hanakowski M and Goloubeva O. (2002) The role of *Clostridium difficile* and viruses as causes of nosocomial diarrhea in children. *Infect Control Hosp Epidemiol* **23**, 660–664.
14. Viscidi R, Laughon BE, Yolken R, et al. (1983) Serum antibody response to toxins A and B of *Clostridium difficile. J Infect Dis* **148**, 93–100.
15. Kelly CP and LaMont JT. (1998) *Clostridium difficile* infection. *Annu Rev Med* **49**, 375–390.
16. Wilcox M and Minton J. (2001) Role of antibody response in outcome of antibiotic-associated diarrhoea. *Lancet* **357**, 158–159.
17. Shim JK, Johnson S, Samore MH, Bliss DZ and Gerding DN. (1998) Primary symptomless colonisation by *Clostridium difficile* and decreased risk of subsequent diarrhoea. *Lancet* **351**, 633–636.
18. Leung DY, Kelly CP, Boguniewicz M, Pothoulakis C, LaMont JT and Flores A. (1991) Treatment with intravenously administered gamma globulin of chronic relapsing colitis induced by *Clostridium difficile* toxin. *J Pediatr* **118**, 633–637.
19. Johnson S, Sypura WD, Gerding DN, Ewing SL and Janoff EN. (1995) Selective neutralization of a bacterial enterotoxin by serum immunoglobulin A in response to mucosal disease. *Infect Immun* **63**, 3166–3173.
20. Kyne L, Warny M, Qamar A and Kelly CP. (2000) Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. *N Engl J Med* **342**, 390–397.
21. Corthier G, Muller MC, Wilkins TD, Lyerly D and L'Haridon R. (1991) Protection against experimental pseudomembranous colitis in gnotobiotic mice by use of monoclonal antibodies against *Clostridium difficile* toxin A. *Infect Immun* **59**, 1192–1195.
22. Warny M, Vaerman JP, Avesani V and Delmee M. (1994) Human antibody response to *Clostridium difficile* toxin A in relation to clinical course of infection. *Infect Immun* **62**, 384–389.
23. Khan MY and Hall WH. (1966) Staphylococcal enterocolitis – treatment with oral vancomycin. *Ann Intern Med* **65**, 1–8.
24. Tedesco FJ, Barton RW and Alpers DH. (1974) Clindamycin-associated colitis. A prospective study. *Ann Intern Med* **81**, 429–433.
25. Larson HE, Parry JV, Price AB, Davies DR, Dolby J and Tyrrell DA. (1977) Undescribed toxin in pseudomembranous colitis. *Br Med J* **1**, 1246–1248.
26. Rifkin GD, Fekety R and Silva J. (1978) Neutralization by *Clostridium sordellii* antitoxin of toxins implicated in clindamycin-induced cecitis in the hamster. *Gastroenterology* **75**, 422–424.
27. Bartlett JG, Chang TW, Gurwith M, Gorbach SL and Onderdonk AB. (1978) Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. *N Engl J Med* **298**, 531–534.
28. McFarland LV, Surawicz CM, Greenberg RN, et al. (1995) Prevention of beta-lactam-associated diarrhea by *Saccharomyces boulardii* compared with placebo. *Am J Gastroenterol* **90**, 439–448.
29. Gerding DN. (1989) Disease associated with *Clostridium difficile* infection. *Ann Intern Med* **110**, 255–257.
30. Norén T, Åkerlund T, Bäck E, et al. (2004) Molecular epidemiology of hospital-associated and community-acquired *Clostridium difficile* infection in a Swedish county. *J Clin Microbiol* **42**, 3635–3643.
31. Bartlett JG. (1992) Antibiotic-associated diarrhea. *Clin Infect Dis* **15**, 573–581.
32. Boyce JM and Havill NL. (2005) Nosocomial antibiotic-associated diarrhea associated with enterotoxin-producing strains of methicillin-resistant *Staphylococcus aureus. Am J Gastroenterol* **100**, 1828–1834.
33. Pituch H, van den Braak N, van Belkum A, et al. (2002) Characterization of *Clostridium perfringens* strains isolated from Polish patients with suspected antibiotic-associated diarrhea. *Med Sci Monit* **8**, BR85–BR88.
34. Cohen SH, Shetab R, Tang-Feldman YJ, Sarma P, Silva J, Jr. and Prindiville TP. (2006) Prevalence of enterotoxigenic *Bacteroides fragilis* in hospital-acquired diarrhea. *Diagn Microbiol Infect Dis* **55**, 251–254.
35. Danna PL, Urban C, Bellin E and Rahal JJ. (1991) Role of candida in pathogenesis of antibiotic-associated diarrhoea in elderly inpatients. *Lancet* **337**, 511–514.
36. Hovius SE and Rietra PJ. (1982) Salmonella colitis clinically presenting as a pseudomembranous colitis. *Neth J Surg* **34**, 81–82.
37. Sullivan NM, Pellett S and Wilkins TD. (1982) Purification and characterization of toxins A and B of *Clostridium difficile. Infect Immun* **35**, 1032–1040.
38. Just I, Fritz G, Aktories K, et al. (1994) *Clostridium difficile* toxin B acts on the

GTP-binding protein Rho. *J Biol Chem* **269**, 10706–10712.

39. Just I, Selzer J, Wilm M, von Eichel-Streiber C, Mann M and Aktories K. (1995) Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature* **375**, 500–503.
40. Just I, Wilm M, Selzer J, et al. (1995) The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. *J Biol Chem* **270**, 13932–13936.
41. Pechine S, Janoir C, Boureau H, et al. (2007) Diminished intestinal colonization by *Clostridium difficile* and immune response in mice after mucosal immunization with surface proteins of *Clostridium difficile*. *Vaccine* **25**, 3946–3954.
42. von Eichel-Streiber C, Boquet P, Sauerborn M and Thelestam M. (1996) Large clostridial cytotoxins – a family of glycosyltransferases modifying small GTP-binding proteins. *Trends Microbiol* **4**, 375–382.
43. Triadafilopoulos G, Pothoulakis C, O'Brien MJ and LaMont JT. (1987) Differential effects of *Clostridium difficile* toxins A and B on rabbit ileum. *Gastroenterology* **93**, 273–279.
44. Price AB and Davies DR. (1977) Pseudomembranous colitis. *J Clin Pathol* **30**, 1–12.
45. Lyerly DM, Barroso LA, Wilkins TD, Depitre C and Corthier G. (1992) Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile*. *Infect Immun* **60**, 4633–4639.
46. Libby JM, Jortner BS and Wilkins TD. (1982) Effects of the two toxins of *Clostridium difficile* in antibiotic-associated cecitis in hamsters. *Infect Immun* **36**, 822–829.
47. Popoff MR, Rubin EJ, Gill DM and Boquet P. (1988) Actin-specific ADP-ribosyltransferase produced by a *Clostridium difficile* strain. *Infect Immun* **56**, 2299–2306.
48. Goncalves C, Decre D, Barbut F, Burghoffer B and Petit JC. (2004) Prevalence and characterization of a binary toxin (actin-specific ADP-ribosyltransferase) from *Clostridium difficile*. *J Clin Microbiol* **42**, 1933–1939.
49. McFarland LV. (1998) Epidemiology, risk factors and treatments for antibiotic-associated diarrhea. *Dig Dis* **16**, 292–307.
50. Wilson KH, Silva J and Fekety FR. (1981) Suppression of *Clostridium difficile* by normal hamster cecal flora and prevention of antibiotic-associated cecitis. *Infect Immun* **34**, 626–628.
51. Onderdonk AB, Cisneros RL and Bartlett JG. (1980) *Clostridium difficile* in gnotobiotic mice. *Infect Immun* **28**, 277–282.
52. Borriello SP and Barclay FE. (1986) An in-vitro model of colonisation resistance to *Clostridium difficile* infection. *J Med Microbiol* **21**, 299–309.
53. Aas J, Gessert CE and Bakken JS. (2003) Recurrent *Clostridium difficile* colitis: case series involving 18 patients treated with donor stool administered via a nasogastric tube. *Clin Infect Dis* **36**, 580–585.
54. Tvede M and Rask-Madsen J. (1989) Bacteriotherapy for chronic relapsing *Clostridium difficile* diarrhoea in six patients. *Lancet* **1**, 1156–1160.
55. Aronsson B, Mollby R and Nord CE. (1985) Antimicrobial agents and *Clostridium difficile* in acute enteric disease: epidemiological data from Sweden, 1980–1982. *J Infect Dis* **151**, 476–481.
56. Larson HE, Price AB, Honour P and Borriello SP. (1978) *Clostridium difficile* and the aetiology of pseudomembranous colitis. *Lancet* **1**, 1063–1066.
57. Nakamura S, Mikawa M, Nakashio S, et al. (1981) Isolation of *Clostridium difficile* from the feces and the antibody in sera of young and elderly adults. *Microbiol Immunol* **25**, 345–351.
58. Viscidi R, Willey S and Bartlett JG. (1981) Isolation rates and toxigenic potential of *Clostridium difficile* isolates from various patient populations. *Gastroenterology* **81**, 5–9.
59. Iizuka M, Itou H, Konno S, et al. (2004) Elemental diet modulates the growth of *Clostridium difficile* in the gut flora. *Aliment Pharmacol Ther* **20**(Suppl 1), 151–157.
60. Brazier JS. (1998) The epidemiology and typing of *Clostridium difficile*. *J Antimicrob Chemother* **41**(Suppl C), 47–57.
61. McFarland LV, Mulligan ME, Kwok RY and Stamm WE. (1989) Nosocomial acquisition of *Clostridium difficile* infection. *N Engl J Med* **320**, 204–210.
62. Bobulsky GS, Al-Nassir WN, Riggs MM, Sethi AK and Donskey CJ. (2008) *Clostridium difficile* skin contamination in patients with *C. difficile*-associated disease. *Clin Infect Dis* **46**, 447–450.
63. Riggs MM, Sethi AK, Zabarsky TF, Eckstein EC, Jump RL and Donskey CJ. (2007) Asymptomatic carriers are a potential source for transmission of epidemic and nonepidemic *Clostridium difficile* strains among long-term care facility residents. *Clin Infect Dis* **45**, 992–998.

64. de Lalla F, Privitera G, Ortisi G, et al. (1989) Third generation cephalosporins as a risk factor for *Clostridium difficile*-associated disease: a four-year survey in a general hospital. *J Antimicrob Chemother* **23**, 623–631.
65. Riley TV. (1996) Antibiotic-associated diarrhoea. A costly problem. *PharmacoEconomics* **10**, 1–3.
66. Spencer RC. (1998) The role of antimicrobial agents in the aetiology of *Clostridium difficile*-associated disease. *J Antimicrob Chemother* **41**(Suppl C), 21–27.
67. Gerding DN, Olson MM, Peterson LR, et al. (1986) *Clostridium difficile*-associated diarrhea and colitis in adults. A prospective case-controlled epidemiologic study. *Arch Intern Med* **146**, 95–100.
68. Golledge CL, Carson CF, O'Neill GL, Bowman RA and Riley TV. (1992) Ciprofloxacin and *Clostridium difficile*-associated diarrhoea. *J Antimicrob Chemother* **30**, 141–147.
69. Gaynes R, Rimland D, Killum E, et al. (2004) Outbreak of *Clostridium difficile* infection in a long-term care facility: association with gatifloxacin use. *Clin Infect Dis* **38**, 640–645.
70. Khurana A, Vinayek N, Recco RA, Go ES and Zaman MM. (2004) The incidence of *Clostridium difficile*-associated and non-*C. difficile*-associated diarrhea after use of gatifloxacin and levofloxacin in an acute-care facility. *Clin Infect Dis* **39**, 602–603.
71. Pepin J, Saheb N, Coulombe MA, et al. (2005) Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*-associated diarrhea: a cohort study during an epidemic in Quebec. *Clin Infect Dis* **41**, 1254–1260.
72. Loo VG, Poirier L, Miller MA, et al. (2005) A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. *N Engl J Med* **353**, 2442–2449.
73. McDonald LC, Killgore GE, Thompson A, et al. (2005) An epidemic, toxin gene-variant strain of *Clostridium difficile*. *N Engl J Med* **353**, 2433–2441.
74. Thomas C, Stevenson M, Williamson DJ and Riley TV. (2002) *Clostridium difficile*-associated diarrhea: epidemiological data from Western Australia associated with a modified antibiotic policy. *Clin Infect Dis* **35**, 1457–1462.
75. Karlstrom O, Fryklund B, Tullus K and Burman LG. (1998) A prospective nationwide study of *Clostridium difficile*-associated diarrhea in Sweden. The Swedish *Clostridium difficile* Study Group. *Clin Infect Dis* **26**, 141–145.
76. Anand A and Glatt AE. (1993) *Clostridium difficile* infection associated with antineoplastic chemotherapy: a review. *Clin Infect Dis* **17**, 109–113.
77. Bliss DZ, Johnson S, Savik K, Clabots CR, Willard K and Gerding DN. (1998) Acquisition of *Clostridium difficile* and *Clostridium difficile*-associated diarrhea in hospitalized patients receiving tube feeding. *Ann Intern Med* **129**, 1012–1019.
78. Dial S, Alrasadi K, Manoukian C, Huang A and Menzies D. (2004) Risk of *Clostridium difficile* diarrhea among hospital inpatients prescribed proton pump inhibitors: cohort and case-control studies. *CMAJ* **171**, 33–38.
79. McFarland LV, Surawicz CM and Stamm WE. (1990) Risk factors for *Clostridium difficile* carriage and *C. difficile*-associated diarrhea in a cohort of hospitalized patients. *J Infect Dis* **162**, 678–684.
80. Karlsson S, Lindberg A, Norin E, Burman LG and Åkerlund T. (2000) Toxins, butyric acid, and other short-chain fatty acids are coordinately expressed and down-regulated by cysteine in *Clostridium difficile*. *Infect Immun* **68**, 5881–5888.
81. Wilson KH. (1993) The microecology of *Clostridium difficile*. *Clin Infect Dis* **16**(Suppl 4), S214–S218.
82. Dallal RM, Harbrecht BG, Boujoukas AJ, et al. (2002) Fulminant *Clostridium difficile*: an underappreciated and increasing cause of death and complications. *Ann Surg* **235**, 363–372.
83. Kuijper EJ, van Dissel JT and Wilcox MH. (2007) *Clostridium difficile*: changing epidemiology and new treatment options. *Curr Opin Infect Dis* **20**, 376–383.
84. Pepin J, Valiquette L, Alary ME, et al. (2004) *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. *CMAJ* **171**, 466–472.
85. Delmee M. (2001) Laboratory diagnosis of *Clostridium difficile* disease. *Clin Microbiol Infect* **7**, 411–416.
86. Johnson S, Homann SR, Bettin KM, et al. (1992) Treatment of asymptomatic *Clostridium difficile* carriers (fecal excretors) with vancomycin or metronidazole. A randomized, placebo-controlled trial. *Ann Intern Med* **117**, 297–302.
87. Djuretic T, Wall PG and Brazier JS. (1999) *Clostridium difficile*: an update on its

epidemiology and role in hospital outbreaks in England and Wales. *J Hosp Infect* **41**, 213–218.
88. Mylonakis E, Ryan ET and Calderwood SB. (2001) *Clostridium difficile* – associated diarrhea: a review. *Arch Intern Med* **161**, 525–533.
89. Barbut F, Kajzer C, Planas N and Petit JC. (1993) Comparison of three enzyme immunoassays, a cytotoxicity assay, and toxigenic culture for diagnosis of *Clostridium difficile*-associated diarrhea. *J Clin Microbiol* **31**, 963–967.
90. Fekety R and Shah AB. (1993) Diagnosis and treatment of *Clostridium difficile* colitis. *J Am Med Assoc* **269**, 71–75.
91. Gerding DN and Brazier JS. (1993) Optimal methods for identifying *Clostridium difficile* infections. *Clin Infect Dis* **16**(Suppl 4), S439–S442.
92. Peterson LR, Kelly PJ and Nordbrock HA. (1996) Role of culture and toxin detection in laboratory testing for diagnosis of *Clostridium difficile*-associated diarrhea. *Eur J Clin Microbiol Infect Dis* **15**, 330–336.
93. Bartlett JG. (2002) Clinical practice. Antibiotic-associated diarrhea. *N Engl J Med* **346**, 334–339.
94. Johnson S, Kent SA, O'Leary KJ, et al. (2001) Fatal pseudomembranous colitis associated with a variant *clostridium difficile* strain not detected by toxin A immunoassay. *Ann Intern Med* **135**, 434–438.
95. MacCannell DR, Louie TJ, Gregson DB, et al. (2006) Molecular analysis of *Clostridium difficile* PCR ribotype 027 isolates from Eastern and Western Canada. *J Clin Microbiol* **44**, 2147–2152.
96. Renshaw AA, Stelling JM and Doolittle MH. (1996) The lack of value of repeated *Clostridium difficile* cytotoxicity assays. *Arch Pathol Lab Med* **120**, 49–52.
97. Gumerlock PH, Tang YJ, Weiss JB and Silva J, Jr. (1993) Specific detection of toxigenic strains of *Clostridium difficile* in stool specimens. *J Clin Microbiol* **31**, 507–511.
98. Wolfhagen MJ, Fluit AC, Torensma R, Poppelier MJ and Verhoef J. (1994) Rapid detection of toxigenic *Clostridium difficile* in fecal samples by magnetic immuno PCR assay. *J Clin Microbiol* **32**, 1629–1633.
99. Olson MM, Shanholtzer CJ, Lee JT, Jr. and Gerding DN. (1994) Ten years of prospective *Clostridium difficile*-associated disease surveillance and treatment at the Minneapolis VA Medical Center, 1982–1991. *Infect Control Hosp Epidemiol* **15**, 371–381.
100. Tedesco FJ, Corless JK and Brownstein RE. (1982) Rectal sparing in antibiotic-associated pseudomembranous colitis: a prospective study. *Gastroenterology* **83**, 1259–1260.
101. Bulusu M, Narayan S, Shetler K and Triadafilopoulos G. (2000) Leukocytosis as a harbinger and surrogate marker of *Clostridium difficile* infection in hospitalized patients with diarrhea. *Am J Gastroenterol* **95**, 3137–3141.
102. Dubberke ER, Sadhu J, Gatti R, et al. (2007) Severity of *Clostridium difficile*-associated disease (CDAD) in allogeneic stem cell transplant recipients: evaluation of a CDAD severity grading system. *Infect Control Hosp Epidemiol* **28**, 208–211.
103. Belmares J, Gerding DN, Parada JP, Miskevics S, Weaver F and Johnson S. (2007) Outcome of metronidazole therapy for *Clostridium difficile* disease and correlation with a scoring system. *J Infect* **55**, 495–501.
104. Rubin MS, Bodenstein LE and Kent KC. (1995) Severe *Clostridium difficile* colitis. *Dis Colon Rectum* **38**, 350–354.
105. Zar FA, Bakkanagari SR, Moorthi KM and Davis MB. (2007) A comparison of vancomycin and metronidazole for the treatment of *Clostridium difficile*-associated diarrhea, stratified by disease severity. *Clin Infect Dis* **45**, 302–307.
106. Marinella MA, Burdette SD, Bedimo R and Markert RJ. (2004) Leukemoid reactions complicating colitis due to *Clostridium difficile*. *South Med J* **97**, 959–963.
107. Byrn JC, Maun DC, Gingold DS, Baril DT, Ozao JJ and Divino CM. (2008) Predictors of mortality after colectomy for fulminant *Clostridium difficile* colitis. *Arch Surg* **143**, 150–154, discussion 5.
108. Lamontagne F, Labbe AC, Haeck O, et al. (2007) Impact of emergency colectomy on survival of patients with fulminant *Clostridium difficile* colitis during an epidemic caused by a hypervirulent strain. *Ann Surg* **245**, 267–272.
109. Muto CA, Pokrywka M, Shutt K, et al. (2005) A large outbreak of *Clostridium difficile*-associated disease with an unexpected proportion of deaths and colectomies at a teaching hospital following increased fluoroquinolone use. *Infect Control Hosp Epidemiol* **26**, 273–280.
110. Pepin J, Valiquette L and Cossette B. (2005) Mortality attributable to nosocomial *Clostridium difficile*-associated disease during an epidemic caused by a hypervirulent strain in Quebec. *CMAJ* **173**, 1037–1042.

111. Musher DM, Aslam S, Logan N, et al. (2005) Relatively poor outcome after treatment of *Clostridium difficile* colitis with metronidazole. *Clin Infect Dis* **40**, 1586–1590.
112. Kuijper EJ, Coignard B and Tull P. (2006) Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin Microbiol Infect* **12**(Suppl 6), 2–18.
113. Kuijper EJ, van den Berg RJ, Debast S, et al. (2006) *Clostridium difficile* ribotype 027, toxinotype III, the Netherlands. *Emerg Infect Dis* **12**, 827–830.
114. Warny M, Pepin J, Fang A, et al. (2005) Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet* **366**, 1079–1084.
115. Åkerlund T, Persson I, Unemo M, et al. (2008) Increased sporulation rate of epidemic *Clostridium difficile* Type 027/NAP1. *J Clin Microbiol* **46**, 1530–1533.
116. Biller P, Shank B, Lind L, et al. (2007) Moxifloxacin therapy as a risk factor for *Clostridium difficile*-associated disease during an outbreak: attempts to control a new epidemic strain. *Infect Control Hosp Epidemiol* **28**, 198–201.
117. Valiquette L, Cossette B, Garant MP, Diab H and Pepin J. (2007) Impact of a reduction in the use of high-risk antibiotics on the course of an epidemic of *Clostridium difficile*-associated disease caused by the hypervirulent NAP1/027 strain. *Clin Infect Dis* **45**(Suppl 2), S112–S121.
118. Fowler S, Webber A, Cooper BS, et al. (2007) Successful use of feedback to improve antibiotic prescribing and reduce *Clostridium difficile* infection: a controlled interrupted time series. *J Antimicrob Chemother* **59**, 990–995.
119. Bartlett JG. (1984) Treatment of antibiotic-associated pseudomembranous colitis. *Rev Infect Dis* **6**(Suppl 1), S235–S241.
120. Teasley DG, Gerding DN, Olson MM, et al. (1983) Prospective randomised trial of metronidazole versus vancomycin for *Clostridium difficile*-associated diarrhoea and colitis. *Lancet* **2**, 1043–1046.
121. Wenisch C, Parschalk B, Hasenhundl M, Hirschl AM and Graninger W. (1996) Comparison of vancomycin, teicoplanin, metronidazole, and fusidic acid for the treatment of *Clostridium difficile*-associated diarrhea. *Clin Infect Dis* **22**, 813–818.
122. Wilcox MH and Spencer RC. (1992) *Clostridium difficile* infection: responses, relapses and re-infections. *J Hosp Infect* **22**, 85–92.
123. Tedesco F, Markham R, Gurwith M, Christie D and Bartlett JG. (1978) Oral vancomycin for antibiotic-associated pseudomembranous colitis. *Lancet* **2**, 226–228.
124. Fekety R, Silva J, Kauffman C, Buggy B and Deery HG. (1989) Treatment of antibiotic-associated *Clostridium difficile* colitis with oral vancomycin: comparison of two dosage regimens. *Am J Med* **86**, 15–19.
125. Dever LL, Smith SM, Handwerger S and Eng RH. (1995) Vancomycin-dependent Enterococcus faecium isolated from stool following oral vancomycin therapy. *J Clin Microbiol* **33**, 2770–2773.
126. Gerding DN. (2000) Treatment of *Clostridium difficile*-associated diarrhea and colitis. *Curr Top Microbiol Immunol* **250**, 127–139.
127. Bolton RP and Culshaw MA. (1986) Faecal metronidazole concentrations during oral and intravenous therapy for antibiotic associated colitis due to *Clostridium difficile*. *Gut* **27**, 1169–1172.
128. Molavi A, LeFrock JL and Prince RA. (1982) Metronidazole. *Med Clin North Am* **66**, 121–133.
129. Krook A, Lindstrom B, Kjellander J, Jarnerot G and Bodin L. (1981) Relation between concentrations of metronidazole and *Bacteroides spp* in faeces of patients with Crohn's disease and healthy individuals. *J Clin Pathol* **34**, 645–650.
130. Pelaez T, Alcala L, Alonso R, Rodriguez-Creixems M, Garcia-Lechuz JM and Bouza E. (2002) Reassessment of *Clostridium difficile* susceptibility to metronidazole and vancomycin. *Antimicrob Agents Chemother* **46**, 1647–1650.
131. Barbut F, Decre D, Burghoffer B, et al. (1999) Antimicrobial susceptibilities and serogroups of clinical strains of *Clostridium difficile* isolated in France in 1991 and 1997. *Antimicrob Agents Chemother* **43**, 2607–2611.
132. Brazier JS, Fawley W, Freeman J and Wilcox MH. (2001) Reduced susceptibility of *Clostridium difficile* to metronidazole. *J Antimicrob Chemother* **48**, 741–742.
133. Bishara J, Bloch Y, Garty M, Behor J and Samra Z. (2006) Antimicrobial resistance of *Clostridium difficile* isolates in a tertiary medical center, Israel. *Diagn Microbiol Infect Dis* **54**, 141–144.
134. Johnson S, Sanchez JL and Gerding DN. (2000) Metronidazole resistance in *Clostridium difficile*. *Clin Infect Dis* **31**, 625–626.

135. Wong SS, Woo PC, Luk WK and Yuen KY. (1999) Susceptibility testing of *Clostridium difficile* against metronidazole and vancomycin by disk diffusion and Etest. *Diagn Microbiol Infect Dis* **34**, 1–6.
136. Fernandez A, Anand G and Friedenberg F. (2004) Factors associated with failure of metronidazole in *Clostridium difficile*-associated disease. *J Clin Gastroenterol* **38**, 414–418.
137. Gerding DN. (2005) Metronidazole for *Clostridium difficile*-associated disease: is it okay for mom? *Clin Infect Dis* **40**, 1598–1600.
138. Modena S, Gollamudi S and Friedenberg F. (2006) Continuation of antibiotics is associated with failure of metronidazole for *Clostridium difficile*-associated diarrhea. *J Clin Gastroenterol* **40**, 49–54.
139. Leroi MJ, Siarakas S and Gottlieb T. (2002) E test susceptibility testing of nosocomial *Clostridium difficile* isolates against metronidazole, vancomycin, fusidic acid and the novel agents moxifloxacin, gatifloxacin, and linezolid. *Eur J Clin Microbiol Infect Dis* **21**, 72–74.
140. Cronberg S, Castor B and Thoren A. (1984) Fusidic acid for the treatment of antibiotic-associated colitis induced by *Clostridium difficile*. *Infection* **12**, 276–279.
141. Wullt M and Odenholt I. (2004) A double-blind randomized controlled trial of fusidic acid and metronidazole for treatment of an initial episode of *Clostridium difficile*-associated diarrhoea. *J Antimicrob Chemother* **54**, 211–216.
142. Norén T, Wullt M, Åkerlund T, Bäck E, Odenholt I and Burman LG. (2006) Frequent emergence of resistance in *Clostridium difficile* during treatment of *C. difficile*-associated diarrhea with fusidic acid. *Antimicrob Agents Chemother* **50**, 3028–3032.
143. Besier S, Ludwig A, Brade V and Wichelhaus TA. (2003) Molecular analysis of fusidic acid resistance in *Staphylococcus aureus*. *Mol Microbiol* **47**, 463–469.
144. Noren T, Åkerlund T, Wullt M, Burman LG and Unemo M. (2007) Mutations in fusA associated with posttherapy fusidic acid resistance in *Clostridium difficile*. *Antimicrob Agents Chemother* **51**, 1840–1843.
145. Buggy BP, Fekety R and Silva J, Jr. (1987) Therapy of relapsing *Clostridium difficile*-associated diarrhea and colitis with the combination of vancomycin and rifampin. *J Clin Gastroenterol* **9**, 155–159.
146. Lagrotteria D, Holmes S, Smieja M, Smaill F and Lee C. (2006) Prospective, randomized inpatient study of oral metronidazole versus oral metronidazole and rifampin for treatment of primary episode of *Clostridium difficile*-associated diarrhea. *Clin Infect Dis* **43**, 547–552.
147. de Lalla F, Privitera G, Rinaldi E, Ortisi G, Santoro D and Rizzardini G. (1989) Treatment of *Clostridium difficile*-associated disease with teicoplanin. *Antimicrob Agents Chemother* **33**, 1125–1127.
148. Montecalvo MA. (2003) Ramoplanin: a novel antimicrobial agent with the potential to prevent vancomycin-resistant enterococcal infection in high-risk patients. *J Antimicrob Chemother* **51**(Suppl 3), iii31–iii35.
149. Somner EA and Reynolds PE. (1990) Inhibition of peptidoglycan biosynthesis by ramoplanin. *Antimicrob Agents Chemother* **34**, 413–419.
150. Cudic P, Kranz JK, Behenna DC, et al. (2002) Complexation of peptidoglycan intermediates by the lipoglycodepsipeptide antibiotic ramoplanin: minimal structural requirements for intermolecular complexation and fibril formation. *Proc Natl Acad Sci USA* **99**, 7384–7389.
151. Pelaez T, Alcala L, Alonso R, et al. (2005) In vitro activity of ramoplanin against *Clostridium difficile*, including strains with reduced susceptibility to vancomycin or with resistance to metronidazole. *Antimicrob Agents Chemother* **49**, 1157–1159.
152. Ackermann G, Loffler B, Adler D and Rodloff AC. (2004) In vitro activity of OPT-80 against *Clostridium difficile*. *Antimicrob Agents Chemother* **48**, 2280–2282.
153. McVay CS and Rolfe RD. (2000) In vitro and in vivo activities of nitazoxanide against *Clostridium difficile*. *Antimicrob Agents Chemother* **44**, 2254–2258.
154. Bartlett JG. (2006) New drugs for *Clostridium difficile* infection. *Clin Infect Dis* **43**, 428–431.
155. Kelly CP. (1996) Immune response to *Clostridium difficile* infection. *Eur J Gastroenterol Hepatol* **8**, 1048–1053.
156. Kyne L, Warny M, Qamar A and Kelly CP. (2001) Association between antibody response to toxin A and protection against recurrent *Clostridium difficile* diarrhoea. *Lancet* **357**, 189–193.
157. Aronsson B, Granstrom M, Mollby R and Nord CE. (1985) Serum antibody response to *Clostridium difficile* toxins in patients with *Clostridium difficile* diarrhoea. *Infection* **13**, 97–101.
158. Bacon AE, 3rd and Fekety R. (1994) Immunoglobulin G directed against toxins

A and B of *Clostridium difficile* in the general population and patients with antibiotic-associated diarrhea. *Diagn Microbiol Infect Dis* **18**, 205–209.
159. Beales IL. (2002) Intravenous immunoglobulin for recurrent *Clostridium difficile* diarrhoea. *Gut* **51**, 456.
160. Salcedo J, Keates S, Pothoulakis C, et al. (1997) Intravenous immunoglobulin therapy for severe *Clostridium difficile* colitis. *Gut* **41**, 366–370.
161. Warny M, Denie C, Delmee M and Lefebvre C. (1995) Gamma globulin administration in relapsing *Clostridium difficile*-induced pseudomembranous colitis with a defective antibody response to toxin A. *Acta Clin Belg* **50**, 36–39.
162. Kelly CP, Pothoulakis C, Vavva F, et al. (1996) Anti-*Clostridium difficile* bovine immunoglobulin concentrate inhibits cytotoxicity and enterotoxicity of *C. difficile* toxins. *Antimicrob Agents Chemother* **40**, 373–379.
163. Lyerly DM, Bostwick EF, Binion SB and Wilkins TD. (1991) Passive immunization of hamsters against disease caused by *Clostridium difficile* by use of bovine immunoglobulin G concentrate. *Infect Immun* **59**, 2215–2218.
164. Kelly CP, Chetham S, Keates S, et al. (1997) Survival of anti-*Clostridium difficile* bovine immunoglobulin concentrate in the human gastrointestinal tract. *Antimicrob Agents Chemother* **41**, 236–241.
165. Kim K, Pickering LK, DuPont HL, Sullivan N and Wilkins T. (1984) In vitro and in vivo neutralizing activity of human colostrum and milk against purified toxins A and B of *Clostridium difficile. J Infect Dis* **150**, 57–62.
166. Aboudola S, Kotloff KL, Kyne L, et al. (2003) *Clostridium difficile* vaccine and serum immunoglobulin G antibody response to toxin A. *Infect Immun* **71**, 1608–1610.
167. Sougioultzis S, Kyne L, Drudy D, et al. (2005) *Clostridium difficile* toxoid vaccine in recurrent *C. difficile*-associated diarrhea. *Gastroenterology* **128**, 764–770.
168. Kreutzer EW and Milligan FD. (1978) Treatment of antibiotic-associated pseudomembranous colitis with cholestyramine resin. *Johns Hopkins Med J* **143**, 67–72.
169. Taylor NS, Thorne GM and Bartlett JG. (1981) Comparison of two toxins produced by *Clostridium difficile. Infect Immun* **34**, 1036–1043.
170. Louie TJ, Peppe J, Watt CK, et al. (2006) Tolevamer, a novel nonantibiotic polymer, compared with vancomycin in the treatment of mild to moderately severe *Clostridium difficile*-associated diarrhea. *Clin Infect Dis* **43**, 411–420.
171. Joyce AM and Burns DL. (2002) Recurrent *Clostridium difficile* colitis. Tackling a tenacious nosocomial infection. *Postgrad Med* **112**, 53–4, 7–8, 65 passim.
172. Kyne L and Kelly CP. (2001) Recurrent *Clostridium difficile* diarrhoea. *Gut* **49**, 152–153.
173. McFarland LV, Surawicz CM, Rubin M, Fekety R, Elmer GW and Greenberg RN. (1999) Recurrent *Clostridium difficile* disease: epidemiology and clinical characteristics. *Infect Control Hosp Epidemiol* **20**, 43–50.
174. McFarland LV, Elmer GW and Surawicz CM. (2002) Breaking the cycle: treatment strategies for 163 cases of recurrent *Clostridium difficile* disease. *Am J Gastroenterol* **97**, 1769–1775.
175. Tedesco FJ, Gordon D and Fortson WC. (1985) Approach to patients with multiple relapses of antibiotic-associated pseudomembranous colitis. *Am J Gastroenterol* **80**, 867–868.
176. Issack MI and Elliott TS. (1990) *Clostridium difficile* carriage after infection. *Lancet* **335**, 610–611.
177. Wilcox MH, Fawley WN, Settle CD and Davidson A. (1998) Recurrence of symptoms in *Clostridium difficile* infection – relapse or reinfection? *J Hosp Infect* **38**, 93–100.
178. Barbut F, Richard A, Hamadi K, Chomette V, Burghoffer B and Petit JC. (2000) Epidemiology of recurrences or reinfections of *Clostridium difficile*-associated diarrhea. *J Clin Microbiol* **38**, 2386–2388.
179. Hamilton-Miller JM. (1997) Living in the 'post-antibiotic era': could the use of probiotics be an effective strategy? *Clin Microbiol Infect* **3**, 2–3.
180. McFarland LV, Surawicz CM, Greenberg RN, et al. (1994) A randomized placebo-controlled trial of *Saccharomyces boulardii* in combination with standard antibiotics for *Clostridium difficile* disease. *J Am Med Assoc* **271**, 1913–1918.
181. Surawicz CM, McFarland LV, Greenberg RN, et al. (2000) The search for a better treatment for recurrent *Clostridium difficile* disease: use of high-dose vancomycin combined with *Saccharomyces boulardii. Clin Infect Dis* **31**, 1012–1017.
182. Gorbach SL, Chang TW and Goldin B. (1987) Successful treatment of relapsing

Clostridium difficile colitis with *Lactobacillus GG. Lancet* **2**, 1519.
183. Thomas MR, Litin SC, Osmon DR, Corr AP, Weaver AL and Lohse CM. (2001) Lack of effect of *Lactobacillus GG* on antibiotic-associated diarrhea: a randomized, placebo-controlled trial. *Mayo Clin Proc* **76**, 883–889.
184. Orrhage K and Nord CE. (2000) Bifidobacteria and lactobacilli in human health. *Drugs Exp Clin Res* **26**, 95–111.
185. McFarland LV. (2006) Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of *Clostridium difficile* disease. *Am J Gastroenterol* **101**, 812–822.
186. Kimmey MB, Elmer GW, Surawicz CM and McFarland LV. (1990) Prevention of further recurrences of *Clostridium difficile* colitis with *Saccharomyces boulardii. Dig Dis Sci* **35**, 897–901.
187. Surawicz CM, McFarland LV, Elmer G and Chinn J. (1989) Treatment of recurrent *Clostridium difficile* colitis with vancomycin and *Saccharomyces boulardii. Am J Gastroenterol* **84**, 1285–1287.
188. Czerucka D and Rampal P. (2002) Experimental effects of *Saccharomyces boulardii* on diarrheal pathogens. *Microbes Infect* **4**, 733–739.
189. Biller JA, Katz AJ, Flores AF, Buie TM and Gorbach SL. (1995) Treatment of recurrent *Clostridium difficile* colitis with Lactobacillus GG. *J Pediatr Gastroenterol Nutr* **21**, 224–226.
190. Bowden TA, Jr., Mansberger AR, Jr. and Lykins LE. (1981) Pseudomembranous enterocolitis: mechanism for restoring floral homeostasis. *Am Surg* **47**, 178–183.
191. Schwan A, Sjolin S, Trottestam U and Aronsson B. (1983) Relapsing *Clostridium difficile* enterocolitis cured by rectal infusion of homologous faeces. *Lancet* **2**, 845.
192. Merrigan MM, Sambol SP, Johnson S and Gerding DN. (2003) Prevention of fatal *Clostridium difficile*-associated disease during continuous administration of clindamycin in hamsters. *J Infect Dis* **188**, 1922–1927.
193. Seal D, Borriello SP, Barclay F, Welch A, Piper M and Bonnycastle M. (1987) Treatment of relapsing *Clostridium difficile* diarrhoea by administration of a non-toxigenic strain. *Eur J Clin Microbiol* **6**, 51–53.
194. NCCLS. (2007) *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard.* In: Hecht DW, Citron DM, Cox M, et al., eds., 7th ed., NCCLS, Pennsylvania, 1–47.
195. Baverud V, Gunnarsson A, Karlsson M and Franklin A. (2004) Antimicrobial susceptibility of equine and environmental isolates of *Clostridium difficile. Microb Drug Resist* **10**, 57–63.
196. Poilane I, Cruaud P, Torlotin JC and Collignon A. (2000) Comparison of the E test to the reference agar dilution method for antibiotic susceptibility testing of *Clostridium difficile. Clin Microbiol Infect* **6**, 155–156.
197. Climo MW, Israel DS, Wong ES, Williams D, Coudron P and Markowitz SM. (1998) Hospital-wide restriction of clindamycin: effect on the incidence of *Clostridium difficile*-associated diarrhea and cost. *Ann Intern Med* **128**, 989–995.
198. Tang-Feldman YJ, Henderson JP, Ackermann G, et al. (2005) Prevalence of the *ermB* gene in *Clostridium difficile* strains isolated at a university teaching hospital from 1987 through 1998. *Clin Infect Dis* **40**, 1537–1540.
199. Johnson S, Samore MH, Farrow KA, et al. (1999) Epidemics of diarrhea caused by a clindamycin-resistant strain of *Clostridium difficile* in four hospitals. *N Engl J Med* **341**, 1645–1651.
200. Pituch H, Obuch-Woszczatynski P, Meisel-Mikolajczyk F and Luczak M. (2003) Detection of *ermB* gene responsible for high level resistance to clindamycin (MLS type resistance) among *Clostridium difficile* strains isolated from antibiotic associated diarrhea (AAD). *Med Dosw Mikrobiol* **55**, 61–66.
201. Linder JA, Huang ES, Steinman MA, Gonzales R and Stafford RS. (2005) Fluoroquinolone prescribing in the United States: 1995 to 2002. *Am J Med* **118**, 259–268.
202. McFarland LV, Clarridge JE, Beneda HW and Raugi GJ. (2007) Fluoroquinolone use and risk factors for *Clostridium difficile*-associated disease within a Veterans Administration Health Care System. *Clin Infect Dis* **45**, 1141–1151.
203. Pepin J, Alary ME, Valiquette L, et al. (2005) Increasing risk of relapse after treatment of *Clostridium difficile* colitis in Quebec, Canada. *Clin Infect Dis* **40**, 1591–1597.
204. Aspevall O, Lundberg A, Burman LG, Åkerlund T and Svenungsson B. (2006) Antimicrobial susceptibility pattern of *Clostridium difficile* and its relation to PCR ribotypes in a Swedish University Hospital. *Antimicrob Agents Chemother* **50**, 1890–1892.
205. Schmidt C, Loffler B and Ackermann G. (2007) Antimicrobial phenotypes and

molecular basis in clinical strains of *Clostridium difficile*. *Diagn Microbiol Infect Dis* **59**, 1–5.
206. Leclercq R. (2002) Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. *Clin Infect Dis* **34**, 482–492.
207. Norén T, Bäck E, Alriksson I and Unemo M. (2006) New antimicrobials and resistance in *C. difficile* isolates and the potential risk of *Clostridium difficile*-associated diarrhea. *46th Interscience Conference on Antimicrobial Agents and Chemotherapy*, abstract E-742.
208. Tabaqchali S. (1990) Epidemiologic markers of *Clostridium difficile*. *Rev Infect Dis* **12**(Suppl 2), S192–S199.
209. Tang YJ, Houston ST, Gumerlock PH, et al. (1995) Comparison of arbitrarily primed PCR with restriction endonuclease and immunoblot analyses for typing *Clostridium difficile* isolates. *J Clin Microbiol* **33**, 3169–3173.
210. Wust J, Sullivan NM, Hardegger U and Wilkins TD. (1982) Investigation of an outbreak of antibiotic-associated colitis by various typing methods. *J Clin Microbiol* **16**, 1096–1101.
211. Nakamura S, Serikawa T, Mikawa M, Nakashio S, Yamakawa K and Nishida S. (1981) Agglutination, toxigenicity and sorbitol fermentation of *Clostridium difficile*. *Microbiol Immunol* **25**, 863–870.
212. Delmee M, Homel M and Wauters G. (1985) Serogrouping of *Clostridium difficile* strains by slide agglutination. *J Clin Microbiol* **21**, 323–327.
213. Mulligan ME, Peterson LR, Kwok RY, Clabots CR and Gerding DN. (1988) Immunoblots and plasmid fingerprints compared with serotyping and polyacrylamide gel electrophoresis for typing *Clostridium difficile*. *J Clin Microbiol* **26**, 41–46.
214. Peerbooms PG, Kuijt P and Maclaren DM. (1987) Application of chromosomal restriction endonuclease digest analysis for use as typing method for *Clostridium difficile*. *J Clin Pathol* **40**, 771–776.
215. Wren BW and Tabaqchali S. (1987) Restriction endonuclease DNA analysis of *Clostridium difficile*. *J Clin Microbiol* **25**, 2402–2404.
216. Welsh J and McClelland M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* **18**, 7213–7218.
217. Brazier JS. (2001) Typing of *Clostridium difficile*. *Clin Microbiol Infect* **7**, 428–431.
218. Killgore GE and Kato H. (1994) Use of arbitrary primer PCR to type *Clostridium difficile* and comparison of results with those by immunoblot typing. *J Clin Microbiol* **32**, 1591–1593.
219. Martirosian G, Kuipers S, Verbrugh H, van Belkum A and Meisel-Mikolajczyk F. (1995) PCR ribotyping and arbitrarily primed PCR for typing strains of *Clostridium difficile* from a Polish maternity hospital. *J Clin Microbiol* **33**, 2016–2021.
220. Wilks M and Tabaqchali S. (1994) Typing of *Clostridium difficile* by polymerase chain reaction with an arbitrary primer. *J Hosp Infect* **28**, 231–234.
221. Barbut F, Mario N, Delmee M, Gozian J and Petit JC. (1993) Genomic fingerprinting of *Clostridium difficile* isolates by using a random amplified polymorphic DNA (RAPD) assay. *FEMS Microbiol Lett* **114**, 161–166.
222. Lemann F, Chambon C, Barbut F, et al. (1997) Arbitrary primed PCR rules out *Clostridium difficile* cross-infection among patients in a haematology unit. *J Hosp Infect* **35**, 107–115.
223. Collier MC, Stock F, DeGirolami PC, Samore MH and Cartwright CP. (1996) Comparison of PCR-based approaches to molecular epidemiologic analysis of *Clostridium difficile*. *J Clin Microbiol* **34**, 1153–1157.
224. Gurtler V. (1993) Typing of *Clostridium difficile* strains by PCR-amplification of variable length 16S-23S rDNA spacer regions. *J Gen Microbiol* **139**, 3089–3097.
225. Cartwright CP, Stock F, Beekmann SE, Williams EC and Gill VJ. (1995) PCR amplification of rRNA intergenic spacer regions as a method for epidemiologic typing of *Clostridium difficile*. *J Clin Microbiol* **33**, 184–187.
226. Al Saif NM, O'Neill GL, Magee JT, Brazier JS and Duerden BI. (1998) PCR-ribotyping and pyrolysis mass spectrometry fingerprinting of environmental and hospital isolates of *Clostridium difficile*. *J Med Microbiol* **47**, 117–121.
227. Talon D, Bailly P, Delmee M, et al. (1995) Use of pulsed-field gel electrophoresis for investigation of an outbreak of *Clostridium difficile* infection among geriatric patients. *Eur J Clin Microbiol Infect Dis* **14**, 987–993.
228. Bidet P, Lalande V, Salauze B, et al. (2000) Comparison of PCR-ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for typing *Clostridium difficile*. *J Clin Microbiol* **38**, 2484–2487.

229. Kato H, Kita H, Karasawa T, et al. (2001) Colonisation and transmission of *Clostridium difficile* in healthy individuals examined by PCR ribotyping and pulsed-field gel electrophoresis. *J Med Microbiol* **50**, 720–727.
230. Klaassen CH, van Haren HA and Horrevorts AM. (2002) Molecular fingerprinting of *Clostridium difficile* isolates: pulsed-field gel electrophoresis versus amplified fragment length polymorphism. *J Clin Microbiol* **40**, 101–104.
231. Kristjansson M, Samore MH, Gerding DN, et al. (1994) Comparison of restriction endonuclease analysis, ribotyping, and pulsed-field gel electrophoresis for molecular differentiation of *Clostridium difficile* strains. *J Clin Microbiol* **32**, 1963–1969.
232. Spigaglia P, Cardines R, Rossi S, Menozzi MG and Mastrantonio P. (2001) Molecular typing and long-term comparison of *Clostridium difficile* strains by pulsed-field gel electrophoresis and PCR-ribotyping. *J Med Microbiol* **50**, 407–414.
233. van Dijck P, Avesani V and Delmee M. (1996) Genotyping of outbreak-related and sporadic isolates of *Clostridium difficile* belonging to serogroup C. *J Clin Microbiol* **34**, 3049–3055.
234. Brazier JS, Mulligan ME, Delmee M and Tabaqchali S. (1997) Preliminary findings of the international typing study on *Clostridium difficile*. International *Clostridium difficile* Study Group. *Clin Infect Dis* **25** (Suppl 2), S199–S201.
235. Barbut F, Lalande V, Burghoffer B, Thien HV, Grimprel E and Petit JC. (2002) Prevalence and genetic characterization of toxin A variant strains of *Clostridium difficile* among adults and children with diarrhea in France. *J Clin Microbiol* **40**, 2079–2083.
236. Rupnik M, Brazier JS, Duerden BI, Grabnar M and Stubbs SL. (2001) Comparison of toxinotyping and PCR ribotyping of *Clostridium difficile* strains and description of novel toxinotypes. *Microbiology* **147**, 439–447.
237. Stubbs SL, Brazier JS, O'Neill GL and Duerden BI. (1999) PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. *J Clin Microbiol* **37**, 461–463.
238. Rupnik M, Avesani V, Janc M, von Eichel-Streiber C and Delmee M. (1998) A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J Clin Microbiol* **36**, 2240–2247.
239. Killgore G, Thompson A, Johnson S, et al. (2008) Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. *J Clin Microbiol* **46**, 431–437.

Part II

Isolation and Culture Techniques

Chapter 3

Clostridium difficile Isolation and Culture Techniques

Mike Wren

Abstract

Clostridium difficile infection (CDI) occurs as a disease with a spectrum of severity ranging from mild, self-limiting diarrhoea to a severe colitis, pseudomembraneous colitis or toxic megacolon. The disease arises as a major complication of antibiotic therapy and is most commonly acquired in hospital. The laboratory investigation of faecal samples is supportive of a clinical suspicion that a patient has the disease. Currently the mainstay of diagnosis is the demonstration of *C. difficile* toxins in a diarrhoeal sample; only a few laboratories set up cultures for the organism. However, toxin tests should not be used as stand alone tests since some patients with disease do not have detectable levels of toxin in their faeces. Furthermore, other patients may have large amounts of toxin in the faeces and yet remain well. A combination of tests, therefore, should be used to help the physician to establish a diagnosis of CDI. This combination of tests should include culture (with toxin testing of the isolate), demonstration of toxin direct from the faeces and the detection of *C. difficile* antigen. This chapter outlines the methods used to establish the laboratory diagnosis of CDI and also includes the investigation of environmental samples when it is required to monitor them for the presence of *C. difficile*.

Key words: *Clostridium difficile*, isolation, Cefoxitin Cycloserine Egg Yolk agar, alcohol shock, glutamate dehydrogenase, enterotoxin, cytotoxin.

1. Introduction

Traditionally tests used for the diagnosis of *Clostridium difficile* infection (CDI) have been based on the detection of the organism by culture and demonstration of the toxins and antigen in the faeces of the infected patient.

When *C. difficile* was first described to be a cause of colitis and pseudomembraneous colitis the emphasis was placed on the laboratory's ability to isolate the organism. Selective media were

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_3, © Springer Science+Business Media, LLC 2010

developed to separate *C. difficile* from the normal colonic flora. Culture by itself does not differentiate toxigenic from nontoxigenic strains which can both colonise the colon. It soon became apparent that the detection of toxin in the faeces was required to establish that a patient had active CDI. Cytotoxin (toxin B) detection originally used mammalian or primate cell lines (such as HeP2, fibroblasts or Vero cells) to detect the toxin. The toxic effect could then be neutralised with specific antitoxin thereby establishing the specificity of the test.

Enzyme Immunoassays (ELISA) were subsequently developed to give laboratories without technical expertise in tissue culture the ability to detect faecal toxin in a technically easy and rapid manner. The incidence and clinical importance (in terms of morbidity and mortality) of CDI make it imperative that on-site local testing is available in all hospital laboratories.

Many of the commercially available ELISA tests have been clinically evaluated (1, 2). The significance of toxin A negative and toxin B positive (A–B+) strains means that any ELISA kits used must be able to detect both toxins (3). These kits also have a lower sensitivity such that some cases of disease may be missed if only a single faecal toxin test is used. Hence the additional use of so-called toxigenic culture (culture followed by toxin testing of the isolate) has been advocated by some workers (4).

More recently the polymerase chain reaction (PCR) based on the detection of the toxin B gene (*tcdB*) directly from faeces (making it a real-time test) has been stated to enhance the diagnosis of CDI (5, 6). This is likely to become available in a commercial format in the near future.

2. Materials

2.1. Culture and Isolation

1. CCFA (cefoxitin cycloserine fructose agar):
   Dehydrated powder and antibiotic supplement (Oxoid Ltd., Wade Road, Basingstoke, Hants, UK).
2. CCEY (cefoxitin cycloserine egg yolk agar: Brazier's medium):
   Dehydrated powder and the antibiotic supplement (Bioconnections, Thorpe Arch Estate, Wetherby, Leeds, UK).
   CCEY made as pre-poured plates may be purchased commercially (Oxoid Ltd.).
3. CCEYL (cefoxitin cycloserine egg yolk agar with lysozyme):
   Make up as the CCEY formulation and the antibiotic supplement and add a filter sterilised solution of lysozyme to

give a final concentration of 5 mg/l of lysozyme before pouring into Petri-dishes.

4. Egg yolk agar is made by adding egg yolk emulsion (Oxoid Ltd.) to a good anaerobic agar to give a final concentration of 7%.
5. Methylated spirit Industrial 74 OP (Fisher Scientific UK Ltd, Bishop Meadow Rd., Loughborough, Leics, UK).
6. Physiological saline (Oxoid Ltd., UK).
7. Roberston's cooked meat broth (Oxoid Ltd., UK).
8. Phosphate buffered saline tablets (Oxoid Ltd., UK).
9. Ringer's solution tablets (Oxoid Ltd., UK).
10. Spot indole reagent (Bioconnections, UK).
11. *C. difficile* latex kits (Oxoid Ltd., UK).
12. UV light source (360 nm) (Model UVL 21 Black Ray Lamp: UVP Inc., San Gabriel, California, USA).
13. Rodac (contact) plates (Sterilin Ltd., UK).

2.2. Cell Lines and Media

1. HeP2 and other cell lines (e.g. Vero cells, fibroblasts) may be obtained from European Collection of Cell Cultures, CAMR, Salisbury, Wiltshire, SP4 0JG, UK
2. Hanks salt solution; Eagle's MEM plus Earle's salts and glutamine; Foetal Calf Serum, (Sigma-Aldrich Ltd., Fancy Rd., Poole, Dorset, BH12 4QH, UK)
3. Trypsin, penicillin and streptomycin (Sigma-Aldrich Ltd., UK)

3. Methods

3.1. Culture from Faeces

Patients with *C. difficile* infection generally have an inflammatory diarrhoea. Isolation of the organism from the faeces is necessary for epidemiological investigations (including typing) and may be necessary to help for establishing a diagnosis in patients with disease when other tests give equivocal results (*see* **Note 1**) (7).

1. For liquid faeces add an equal volume of faeces and industrial alcohol (*see* **Note 2**) in a sterile container. Mix for 20 s using a vortex mixer to ensure thorough mixing.

   For semisolid faeces emulsify a pea-sized portion of the faeces in physiological saline in a sterile container and mix well. Add an equal volume of industrial alcohol. Mix on a vortex mixer for 20 s to ensure thorough mixing.

2. Leave to stand at room temperature for 30–60 min. Culture approximately 100 μl (three drops from a Pasteur pipette) of the settled faecal layer onto *C. difficile* selective medium (Brazier's CCEY is recommended).
3. Incubate the plates anaerobically at 37°C for 48 h in an anaerobic chamber or anaerobic jar (*see* **Note 3**).

Culture of *C. difficile* from the faeces of patients with diarrhoea followed by toxin testing of the isolate increased the detection rate of diseased patients since some samples from these patients had a negative faecal toxin test (4).

3.2. Culture from Environmental Samples

Because spores of *C. difficile* may suffer prolonged stress whilst in the environment CCEY supplemented with lysozyme (CCEYL) (8, 9) may give better isolation rates from environmental samples (*see* **Note 4**). Environmental sampling may yield useful information when investigating clusters of cases within a unit or ward. It should not be used, however, as a tool for monitoring cleaning efficiency. Routine environmental sampling serves no good purpose.

3.2.1. Water

1. Collect 500 ml samples of water in sterile containers.
2. Filter 100 ml aliquots through 0.45 μm membrane filters.
3. Place the filters into Robertson's cooked meat broth for enrichment and incubate at incubate for 48 h at 37°C.
4. After the 48 h of incubation have elapsed culture the broth onto CCEY or CCEYL and incubate these plates anaerobically at 37°C for 48–72 h.
5. Membranes may be placed directly onto CCEY or CCEYL plates and incubated anaerobically at 37°C for 48–96 h and the colonies counted to give a viable count of cells per 100 ml.

3.2.2. Soil Samples

1. Place 5 g samples of the soil into 5 ml of physiological saline, mix well and add 5 ml of industrial alcohol.
2. Mix on a vortex mixer for 20 s to ensure thorough mixing.
3. Leave to stand at room temperature for 30–60 min.
4. Culture the sediment onto CCEY or CCEYL plates and incubate anaerobically at 37°C for 48 h. Negative cultures may be incubated for a further 48 h before discarding.

3.2.3. Detection of C. difficile from Environmental Surfaces

Surfaces may be sampled by the use of Rodac contact plates or by swabbing surfaces and enriching the organism from these swabs before selective plating (9).

1. Rodac contact plates made using CCEYL medium are pressed firmly against the surface under investigation and held in place for 5–10 s.

2. The plates are incubated anaerobically at 37°C for 48–96 h. Initial examination is made at 48 h and a second further examination after 96 h before discarding. Counts may then be performed of colony forming units (CFU) per unit area if desired.
3. For surfaces that cannot be accessed by contact plates swabbing of surfaces may be performed. Sterile cotton swabs premoistened with nutrient broth are used for swabbing and then placed into a tube of pre-reduced Robertson's cooked meat medium (*see* **Note 5**) incubated anaerobically at 37°C for 48 h.
4. The broth is then subcultured to CCEY and the plates incubated anaerobically at 37°C for 48 h. Negative cultures at this time may be further incubated for another 48 h before discarding as negative.

3.2.4. Detection of C. difficile *in Air Samples*

The recovery of *C. difficile* spores from air occurs in areas where patients with diarrhoea due to *C. difficile* are being nursed. The examination of air samples is not performed routinely for this organism but may be required when complete analysis of ward air is needed.

1. At 30 min intervals (how many samplings are determined by the amount of activity in the identified area) draw 250 l of air through a slit sampler.
2. Concentrate the particular matter into 1 ml of sterile Ringer's solution.
3. Add an equal volume of Industrial alcohol, mix on a vortex mixer for 20 s to ensure thorough mixing and stand at room temperature for 30–60 min.
4. Culture 100 μl aliquots onto CCEYL media and incubate anaerobically at 37°C for 48 h.

3.2.5. Detection of C. difficile *in Meat Samples*

1. Add 5 g of meat sample to 20 ml of enrichment broth (10).
2. Incubate the broth at 37°C for 10 days.
3. After incubation add 2 ml of enrichment broth to 2 ml of industrial alcohol, mix on a vortex mixer for 20 s and stand at room temperature for 30–60 min.
4. Centrifuge this mixture at 3,000×*g* for 10 min.
5. Culture the sediment onto CCEYL agar and incubate anaerobically at 37°C for 48 h. Examine and reincubate the plates for a further 48 h if negative before discarding.

3.3. Differentiation of C. difficile *from Other Similar Clostridia*

On CCEY and CCEYL single well isolated colonies appear as yellow circular colonies with a filamentous edge. One edge of the colony is often pointed toward the direction of the spread. They

are flat in profile with a typical "ground glass" appearance measuring 3–5 mm in diameter. On blood agar incubated for 48 h colonies typically are greyish with a slightly whiter centre, irregular edged and measuring approximately 5 mm in diameter (*see* **Figs. 3.1** and **3.2**).

As CCEY and CCEYL are not completely selective for *C. difficile* a few other clostridia (especially from faecal samples) may grow on these media showing similar colonial morphology

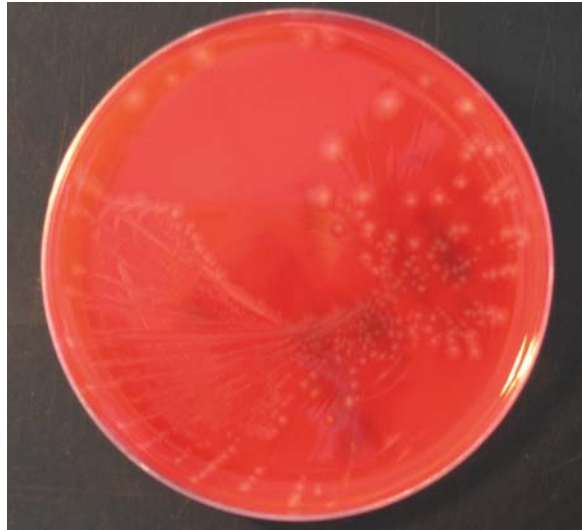


Fig. 3.1. Plate 1 showing *C. difficile* colonies on blood agar; 48 h anaerobic incubation.

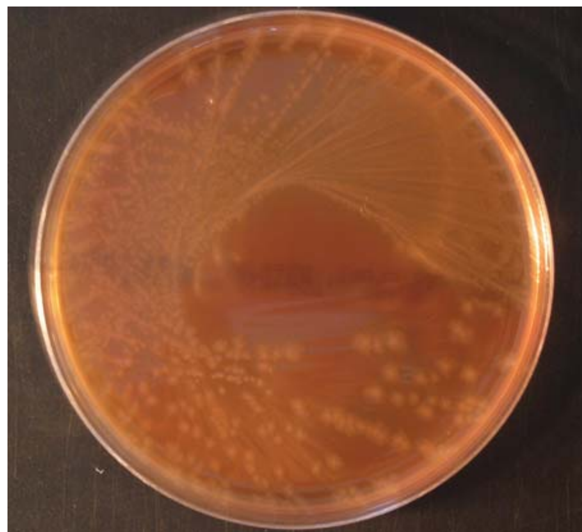


Fig. 3.2. Plate 2 showing *C. difficile* colonies on Brazier's CCEY agar; 48 h anaerobic incubation.

Table 3.1
Biochemical differentiation of organisms capable of growth on CCEY agar

	C. difficile	C. glycolicum	C. innocuum	C. bifermentans *and* C. sordellii
C. difficile latex	+	+	–	+
UV fluorescence[a]	+	–	+	–
Lecithinase on CCEY medium	–	–	–	+
Spot indole	–	–	–	+

[a]Yellow-green.

and may lead to confusion. *Clostridium glycolicum*, *C. innocuum*, *C. bifermentans* and *C. sordellii* may grow on CCEY/CCEYL and therefore, colonies of these species must be differentiated from those of *C. difficile* (**Table 3.1**).

1. Subculture the colonies under investigation onto blood agar to obtain a pure culture.
2. Incubate the culture anaerobically for 48 h at 37°C.
3. Colonies of *C. difficile* possess a typical "elephant dung" or "horse manure" odour.
4. Test the colonies for agglutination with a *C. difficile* latex kit (Oxoid Ltd.).
5. Examine the plate under long-wave UV light (360 nm) to check for yellow-green fluorescence. Wear UV protective goggles or face shield when performing this test.
6. Test the colonies for indole production using the spot indole reagent.
7. Subculture to egg yolk agar and incubate anaerobically at 37°C for 24 h and check for lecithinase production.

These tests should be performed as a minimal panel for the identification of *C. difficile*. Once the isolate is proven to be *C. difficile* the testing the isolate for toxin production may be performed as part of the "toxigenic culture" determination (*see* **Notes 6** and **7** concerning testing isolates for toxin and identification).

3.4. Detection of C. difficile *Glutamate Dehydrogenase (GDH) in Faeces*

Actively growing vegetative cells of *C. difficile* constitutively produce glutamate dehydrogenase and in patients who are either colonised or infected GDH accumulates in the faeces. This may be detected using a commercial kit which utilises a mouse monoclonal antibody directed against the *C. difficile* specific GDH. Culture and GDH results closely parallel each other (*see* **Note 8**).

The negative predictive value of GDH is very high (NPV = 99.3%) (11) and the absence of GDH from the faeces indicates a very high likelihood that *C. difficile* is not present at the time of testing. All cells of *C. difficile*, both toxigenic and nontoxigenic, produce GDH, therefore, it is not an indicator of potential pathogenicity but may be used as a rapid screening test for the presence/absence of the organism particularly when large numbers of samples are being tested.

1. Make a 1:20 dilution of the liquid faeces in the diluent (to which has been added 1 drop of conjugate). Mix well.
2. Add 400 μl of this mixture to the sample well.
3. Stand at room temperature for 15 min.
4. Add 300 μl of wash buffer to the reaction well. Allow to soak in completely.
5. Add two drops of substrate to the reaction window.
6. Stand at room temperature for a further 10 min and read after the 10 min have elapsed.
7. A positive GDH test is indicated by the appearance of a blue line in the test window.

3.5. Detection of C. difficile Toxin in Faeces

Production of toxin by isolates of *C. difficile* occurs in the intestine and is responsible for the inflammatory diarrhoea in susceptible patients. The toxins of *C. difficile* can be detected in the faeces of diseased patients by either using a tissue culture cell line to detect the cytotoxin (toxinB) or by an ELISA for both enterotoxin (toxin A) and cytotoxin (toxin B).

3.5.1. Demonstration of Cytotoxin by Tissue Culture Cell Line (After Edelstein)

3.5.1.1. Preparation of Cell Line

1. Pour off and discard the fluid medium from the master culture in the cell line flask (12).
2. Wash the cells in the flask with Hank's balanced salt solution.
3. Repeat stage 2.
4. Add 5 ml of a 10% solution of trypsin to the washed cells and gently tap the flask making sure the trypsin covers the cell sheet. Stand at room temperature until the cells have detached from the side of the flask.
5. Resuspend the detached cells in 10 ml Earle's MEM medium containing 10% foetal calf serum, 20 units/ml penicillin and 20 μg/ml streptomycin.
6. Perform a cell count using a counting chamber and adjust the cell density to 5×10^4 cells/ml using additional growth medium.
7. Transfer an aliquot to another flask to maintain the continuity of the cell line (if an immortal cell line is being used).

8. Transfer 180 μl of the cell suspension to the number of required wells of a sterile flat-bottomed microtitre tray and cover with the sterile lid.
9. Incubate the tray in 5% carbon dioxide in air at 37°C for 24 h. A monolayer of cells should develop at the bottom of each well.

3.5.1.2. Faecal Preparation

1. Add an equal volume of liquid faeces and phosphate buffered saline (pH 7.0) (PBS) and mix using the vortex mixer for 20 s.
2. Centrifuge this suspension at 3,000×*g* for 15–50 min to obtain a clear supernatant.
3. Filter this clear supernatant through a 0.45 μm membrane filter.
4. Transfer 50 μl of the supernatant into each of two sterile tubes.
5. Add 50 μl of *C. difficile* antitoxin to one tube and 50 μl of PBS to the second tube.

3.5.1.3. Test Proper

Using three wells per analysis add 20 μl of faecal filtrate to well 1, 20 μl of filtrate-antitoxin mixture to well 2 and 20 μl of PBS (negative control) to well 3. If cells in tubes are used add enough stool filtrate to give a 1:10 dilution of the filtrate in the medium in the tube.

1. Inoculate one well in the plate with a filtered (stage 3 above) broth culture supernatant of a known toxigenic strain of *C. difficile* as a positive control to check the performance of the cell line.
2. Incubate the tray in 5–10% carbon dioxide in air at 37°C for 18–24 h and read. Examine using an inverted microscope for any cytopathic effect denoted by rounding of the cells and an increase in their refractility (*see* **Note 9**).
3. Reincubate any negatives for a further 18 h before discarding.

3.5.1.4. Interpretation (Note Re Positives in Wells 1 and 2)

Well 1	Well 2	Well 3	Reported result
Test faecal supernatant well	Test faecal supernatant + *C. difficile* antitoxin	Negative control (PBS only)	
>90% rounding of cells	No change	No change	*C. difficile* toxin detected
No change	No change	No change	No *C. difficile* toxin detected

3.5.1.5. Limitations of the Cytotoxin Assay

1. Tests should be performed on fresh samples. If testing cannot be performed at this time samples may be stored at 4°C until testing can be performed with little reduction in cytotoxin titre (13).
2. Good potency antitoxin should be used. A commercial source of this antitoxin should be sourced by the user (*see* **Note 10**).

3.5.2. Demonstration of Toxins A and B by Commercial ELISA Systems

Laboratories using an ELISA test for the detection of *C. difficile* toxins in faeces almost exclusively use a commercial ELISA kit. These kits are simple to use, convenient and are standardised and controlled by the manufacturer. Meticulous attention to detail is required to ensure optimum performance of the kit (*see* **Note 11**).

It must be remembered that ELISA toxin test results must not be taken as stand alone tests for the diagnosis of *C. difficile* infection as commercial kits do have sensitivity and specificity issues (*see* **Note 12**).

3.5.2.1. Test Principles

1. The ELISA tests are based on the use of antibodies against toxins A and B of *C. difficile*. These antibodies are immobilised on the base of a microtitre tray well or in a line fixed on a pad or membrane.
2. Faecal samples are mixed with a diluent containing a conjugate. The conjugates consist of antibodies to toxin A and toxin B both linked to horseradish peroxidase. These bind any toxin present in the faecal sample.
3. The faecal-conjugate suspension is added to the fixed antibodies (well or membrane) and allowed to react at room temperature for a determined time period during which time any free toxins in the faeces bind to the antibody-conjugate.
4. A washing step or series of washing steps follow to remove any unbound conjugate. A chromogenic substrate (usually tetramethylbenzidine) is then added.
5. After the substrate addition a short room temperature incubation follows to allow the horseradish peroxidase to act on the substrate.
6. When using a plate ELISA a stop solution is then added to develop the colour for reading in a spectrophotometer at 450/620–650 nm. Membrane and pad devices do not require this step as the fixed antibodies appear as a blue line or spot in the device and are read by eye.

3.5.2.2. Limitations to ELISA and Membrane Devices

1. All devices must operate within the manufacturers recommendations giving the desired results with the internal kit controls and the users own external controls.

2. Proper collection of the sample must be ensured (for example, urine must not be present in the faecal sample).
3. A negative test may not exclude the possibility of disease. Some patients with severe disease may demonstrate a negative ELISA test for a number of days (14). The amount of toxin present in the faecal sample may be in a concentration below the limit of detection of the kit being used.
4. Very weak or equivocal reactions should be repeated on a *fresh* specimen submitted from the patient. *Repeating the test on the same sample serves no good purpose.*
5. Some strains of *C. sordellii* may produce toxins that immunologically cross react with the toxins of *C. difficile* (15). However, *C. sordellii* is not thought to cause antibiotic-associated diarrhoea.

3.6. The Future of Detection of C. difficile *in Faecal Samples*

The detection of *C. difficile* in faecal samples using the currently established tests has sensitivity and specificity issues particularly in patients with severe disease. This has led workers in the field to develop real-time PCR methods for the accurate and rapid detection of *C. difficile*. In one prospective multicentre study real-time PCR was compared to an immunocard method and to the cell cytotoxin assay method (used as the gold standard). Based on sensitivity and negative predictive values both the immunocard and the PCR methods were both considered to be useful diagnostic methods (6).

In another study van den Berg et al. concluded that real-time PCR had the highest concordance with toxigenic culture and was the preferred method for the diagnosis of CDI in patients with diarrhoea (16). The use of fluorescence resonance transfer probes in a real-time PCR against toxigenic culture showed the PCR to be sensitive and specific (17). Detection of *C. difficile* in the faeces of patients with CDI using PCR methods will undoubtedly become available commercially in the near future although expense may prohibit the immediate introduction into the diagnostic laboratory.

4. Notes

1. Performing toxigenic culture has been shown to be useful in patients who develop severe CDI but initially have negative tests for toxins both by cell culture assay and by ELISA. Performance of toxigenic culture is given in **Note 6**.
2. Industrial alcohol is methylated spirit (74OP) and can be used in place of absolute ethanol. The methylated spirit is

much cheaper and there is no difference in the ability to select *C. difficile* spores.

3. Cultures may be examined daily without removal from the anaerobic atmosphere when using an anaerobic chamber. When anaerobic jars are used a full 48 h of incubation must elapse before jars are opened. Young colonies of *C. difficile* are particularly sensitive to oxygen on selective agar.
4. The addition of 5 mg/l of lysozyme to CCEY increases the ability to recover spores from environmental samples where smaller numbers of *C. difficile* spores may be present (*see* reference 9). This has been postulated to be due to the ability of lysozyme to overcome the detrimental effect on spore germination that is caused by stress induced damage to cell envelope proteins.
5. Premoistening of swabs with broth increases the ability to pick up organisms and spores from the environmental surface. Robertson's cooked meat medium is a very rich broth medium and gives good growth of organisms from small innocula. Prereduction of the broth is achieved by slightly loosening the cap of the bottle of broth and placing the bottle into a boiling water bath for 20–30 min to drive off any air. After removal from the water bath, tighten the cap of the bottle and allow to cool at room temperature. Use on the same day.
6. Toxin testing of the isolate is done by subculturing a colony into broth and incubating the broth anaerobically at 37°C for 48 h. The broth is centrifuged and the supernatant tested for toxin by ELISA or by cell culture assay (sterilising by filtration through a 0.45 μm filter will be required prior to using tissue culture assay). If this information is urgently required five colonies may be emulsified in the ELISA kit diluent and tested directly. Those that are negative, however, must be confirmed using the broth method.
7. CCEY gives rise to very similar colonies when either *C. glycolicum* or *C. innocuum* is cultured. Definitive identification that the isolate as *C. difficile* is essential to prevent the reporting of a false positive culture and thereby subjecting the patient to unnecessary therapy. The latex reagent reacts with the common antigen which is present in *C. difficile* and strains of *C. glycolicum*, *C. innocuum*, *C. bifermentans* and *C. sordellii*. The other tests are required to prevent false positive reports that may occur if the latex reagent is used as the only test.
8. The author has experience using the GDH commercial kit manufactured by Techlab Inc., Blacksburg, Virginia, USA. It is rare to obtain a positive culture in the presence of a negative GDH test.

9. The appearance of the cytopathic effect of *C. difficile* toxin B may differ depending upon the cell line used. It is recommended that *C. difficile* toxin is used as a control when first setting up the cell line to become familiar with the demonstrated effect.
10. Good potency antitoxin is obtainable from Techlab Inc., USA.
11. Faecal samples should be tested on the day they are taken. *C. difficile* toxins will deteriorate on storage at room temperature. If same day testing cannot be performed the specimen must be kept at 4°C until it can be performed. This must not be more than 72 h. Freezing the specimen may result in loss of toxin titre, especially when samples are repeatedly frozen and thawed (13).
12. This is especially important in patients with severe CDI. The reporting of a negative faecal toxin test in such a patient may result in the denial of effective therapy to a very ill patient allowing the disease to progress.

References

1. Barbut F, Delmee M, Brazier JS, Petit JC, Poxton IR, Rupnik M, Lalande V, Schneider C, Mastrantonio P, Alonso R, Kuiper E and Tvede M. (2003) ESCMID Study Group on *Costridium difficile* (ESGD). A European survey of diagnostic methods and testing protocols for *Clostridium difficile. Clin Microbiol Infect* **9**, 989–996.
2. Turgeon DK, Novicki TJ, Quick J, Carlsson L, Miller P, Ulness B, Cent A, Ashley R, Coyle M, Limaye AP, Cookson BT and Fritsche TR. (2003) Six rapid tests for direct detection of *Clostridium difficile* and its toxins in fecal samples compared with the fibroblast cytotoxicity assay. *J Clin Microbiol* **41**, 667–670.
3. Massey V, Gregson DB, Chagla AH, Storey M, John MA and Hussain Z. (2003) Clinical usefulness of components of the Triage immunoassay, enzyme immunoassay for toxins A and B, and cytotoxin B tissue culture assay for the diagnosis of *Clostridium difficile* diarrhoea. *Am J Clin Pathol* **119**, 45–49.
4. Delmee M, Van Broeck J, Simon A, Janssens M and Avensi V. (2005) Laboratory diagnosis of *Clostridium difficile*-associated diarrhoea: a plea for culture. *J Med Microbiol* **54**, 187–191.
5. Morelli MS, Rouster SD, Giannella RA and Sherman KE. (2004) Clinical application of polymerase chain reaction to diagnose *Clostridium difficile* in hospitalised patients with diarrhoea. *Clin Gastroenterol Hepatol* **2**, 669–674.
6. van den berg RJ, Bruijnesteijn van Coppenraet LS, Gerritsen HJ, Endtz HP, van der Vorm ER and Kuijper EJ. (2005) Prospective multicentre evaluation of a new immunoassay and real-time PCR for rapid diagnosis of *Clostridium difficile*-associated diarrhoea in hospitalised patients. *J Clin Microbiol* **43**, 5338–5340.
7. Borriello SP and Honour P. (1981) Simplified procedure for routine isolation of *Clostridium difficile* from faeces. *J Clin Microbiol* **18**, 1124–1127.
8. Al-Saif NM, Brazier JS and Duerden BI. (1997) Environmental studies of *Clostridium difficile.* In: Eley AR and Bennett KW eds., *Anaerobic Pathogens*, Sheffield Academic Press, London, 207–213.
9. Wilcox MH, Fawley WN and Parnell P. (2000) Value of lysozyme agar incorporation and alkaline thioglycollate exposure for the environmental recovery of *Clostridium difficile. J Hosp Infect* **44**, 65–69.
10. Rodriguez-Palacios A, Staemfli HR, Duffield T and Weese JS. (2007) *Clostridium difficile* in retail ground meat, Canada. *Emerging Infect Dis* **13**, 485–487.
11. Fenner L, Widmer AF, Goy G, Rudin S and Frei R. (2008) Reliable diagnostic algorithm for detection of *Clostridium difficile. J Clin Microbiol* **46**, 328–330.

12. Edelstein MAC. (1988) Isolation and identification of *Clostridium difficile*; tissue culturecytotoxicity assay. In: Rolfe RD and Finegold SM eds., Clostridium difficile*: Its Role in Intestinal Disease*, Academic Press, London, 288–307.
13. Freeman J and Wilcox MH. (2003) The effects of storage conditions on viability of *Clostridium difficile* vegetative cells and spores and toxin activity in human faeces. *J Clin Pathol* **56**, 126–128.
14. Wren MWD, Coen PG and Shetty NP. (2007) What is the true burden of *Clostridium difficile* disease? *J Hosp Infect* **67**, 196–197.
15. Baldacini O, Girardot R, Green GA, Rihn B and Montiel H. (1992) Comparative study of immunological properties and cytotoxic effects of *Clostridium difficile* toxin B and *Clostridium sordellii* toxin L. *Toxicon* **30**, 129–140.
16. van den Berg R, Vaessen N, Endtz HP, Schulin T, van der Norm ER and Kuijper E. (2007) Evaluation of real-time PCR and conventional diagnostic methods for the detection of *Clostridium difficile*-associated diarrhoea in a prospective multicentre study. *J Med Microbiol* **56**, 36–42.
17. Sloan LM, Duresko BJ, Gustafson DR and Rosenblatt JE. (2008) Comparison of real-time PCR for detection of the tcdC gene with four immunoassays and culture in the diagnosis of *Clostridium difficile* infection. *J Clin Microbiol* **46**, 1996–2001.

Part III

Methods for Typing and Epidemiological Studies

Chapter 4

Molecular Typing Methods for *Clostridium difficile*: Pulsed-Field Gel Electrophoresis and PCR Ribotyping

Sandra Janezic and Maja Rupnik

Abstract

Molecular typing methods for *Clostridium difficile* are based on gel electrophoresis of restriction fragments (endonuclease restriction analysis, REA; pulsed field gel electrophoresis PFGE; toxinotyping), PCR amplification (PCR ribotyping, arbitrarily primed PCR, multilocus variable-number tandem-repeat analysis MLVA), and sequence analysis (multilocus sequence typing MLST; slpA typing, tandem repeat sequence typing). We will describe two standard methods (PCR ribotyping predominantly used throughout Europe and PFGE which is predominantly used in North America) and will discuss the difficulties of inter-laboratory comparability and unification of typing nomenclature.

Key words: *Clostridium difficile*, typing, PCR ribotype, pulsed field gel electrophoresis, typing nomenclature.

1. Introduction

Since 1978 when *Clostridium difficile* was first recognized as a human pathogen it was often associated with hospital outbreaks (1). For this reason typing techniques were developed very early. As in other genera, initial phenotypic methods, mostly serogrouping (2), were replaced by molecular methods.

Typing is used to follow and investigate outbreaks (3–6), to identify the emergence of new strains with increased virulence (7), to track transmission of *C. difficile* not only locally but also globally (8), and to clarify possible animal–human transmission (9–11). Current typing methods are summarized in **Table 4.1**. Three of those (restriction endonuclease analysis, REA; pulsed

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_4, © Springer Science+Business Media, LLC 2010

Table 4.1
Overview of molecular typing methods described for *C. difficile*

Type of method	Method designation	References[a]
Restriction-based methods	Restriction endonuclease analysis (REA)	(12, 13)
	Pulsed-field gel electrophoresis (PFGE)[b]	(15, 16)
	Toxinotyping	(31, www. mf.uni-mb.si/mikro/tox)
Amplification-based methods	PCR ribotyping (agarose gels)	(22, 23)
	PCR ribotyping (sequencer based)	(24, webribo. ages.at)
	Multilocus variable-number tandem-repeat analysis (MLVA)	(32, 33)
	Other amplification-based methods (AP PCR, AFLP)	(5, 7, 25, 26, 34)
Sequence-based methods	Multilocus sequence typing, MLST	(35, 36)
	slpA typing	(37, 38)
	Tandem repeat sequence typing	(39)

[a]Where applicable only first description of the method is cited.
[b]Publications on PFGE and *C. difficile* are numerous and only two examples are cited here.

field gel electrophoresis, PFGE; PCR ribotyping) are currently considered as standard methods and are schematically presented in **Fig. 4.1**. New and more discriminatory methods such as MLVA (multilocus variable-number tandem-repeat analysis) are likely to be increasingly used in routine outbreak investigations (6).

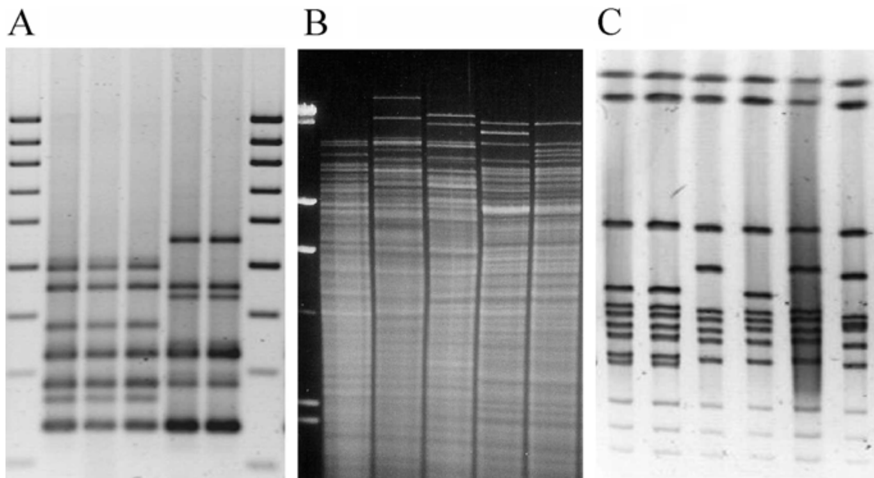


Fig. 4.1. Presentation of three standard molecular typing methods used for *C. difficile*. (**a**) PCR ribotyping, (**b**) restriction endonuclease analysis (REA), and (**c**) pulsed filed gel electrophoresis PFGE.

1.1. Restriction Endonuclease Analysis (REA)

For REA the whole bacterial DNA is cut with *Hin*dIII and resulting bands are visualized on agarose gels. As *Hin*dIII is a 6 bp cutter with numerous restriction sites in the genome the band pattern is not as clear as in PFGE (**Fig. 4.1**). Automated interpretation is still not possible and probably for this reason the method is not widely used. It was first described by Kuijper et al. (12) but later implemented by Gerding and colleagues (13). The Gerding laboratory maintains a collection of mostly clinical *C. difficile* isolates obtained from multiple US and other sources over a 20-year period. Isolates showing six or fewer visible restriction band differences (a similarity index of 90%) are placed within the same REA group and designated by letter. Isolates with identical restriction patterns are assigned a specific REA type designated by number (e.g., CF1, CF2).

This collection was important for the comparison of modern and historical strains during the emergence of the type BI/NAP1/027 and it demonstrated that such strains were present already in the past but were rare and that their increased virulence is correlated with the emergence of fluoroquinolone resistance (14).

1.2. Pulsed Field Gel Electrophoresis – PFGE

PFGE was one of the first molecular typing methods described for *C. difficile* and is still considered the standard in North America (Canada and USA). Initially, some types were untypable because of DNA degradation; however, the new improved protocols have increased typability to almost 100% (15).

Most groups use *Sma*I restriction (3, 15–17), while in some cases *Sac*II is more discriminatory (18; Janezic, unpublished data).

*Sma*I whole genome restriction gives 7–15 restriction fragments ranging from 10 to 1,100 kbp, while *Sac*II gives 10–20 fragments in the same size range. Band profiles can be analyzed visually or with appropriate software (e.g., BioNumerics, Applied Maths) (*see* **Note 1**). Strains with $\geq$ 80% similarity in band pattern are usually regarded as a single pulsotype. North America uses NAP and type number for designation of pulsotypes (North American Pulsotype; NAP1, NAP2, etc.). To date there is no standard protocol available for easy inter-laboratory comparison of pulsotypes.

1.3. PCR Ribotyping

In *C. difficile* PCR ribotyping is based on amplification of intergenic spacer region (ITS) between 16S and 23S rDNA (**Fig. 4.1**). Because this operon is present in several copies in *C. difficile* genome and copies also differ in the length of ITS a single primer pair can result in a pattern of bands ranging from 200 to 700 bp. The bands are usually visualized on an agarose gel. Resulting band

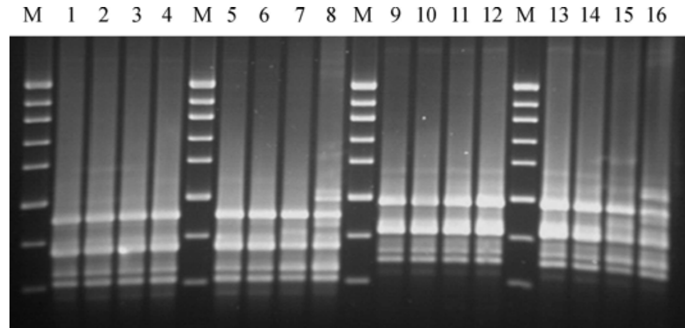


Fig. 4.2. Comparison of two PCR ribotyping methods using slightly different primers and thermocycling conditions. Lanes 1–8 primers described by Bidet et al.; lanes 9–15 primers described by Stubbs et al. M: 100 bp ladder.

patterns can be analyzed either visually or with suitable software (usually BioNumerics, Applied Maths) (*see* **Note 1**).

PCR ribotyping was described by several groups (19–23). Currently, most laboratories will use primers and conditions described by Stubbs et al. (23) or Bidet et al. (22) and both methods will give comparable band patterns (**Fig. 4.2**).

PCR ribotype is defined as a group of strains with identical band pattern. A single band difference represents a new ribotype. A large collection of strains from multiple sources is maintained at Anaerobe Reference Unit, Cardiff, UK. It contains more than 200 ribotypes designated by numbers (e.g., 001, 027, 106, …) (23).

PCR ribotyping is the standard typing method in Europe. However, the agarose gel analysis provides an obstacle in standardization and hence PCR ribotype can only be correctly assigned if the laboratory has reference strain(s). If the reference ribotype strains are not available a local nomenclature is used and types can be only compared within the local collection. Recently, a new method of capillary gel electrophoresis-based PCR ribotyping, supported by a web-based database has been developed which might be a solution to the problems associated with comparison of typing results between laboratories (24).

1.4. Comparative Studies and Unification of Typing Nomenclatures

Molecular typing methods differ in their discriminatory power and in the time needed to obtain the results. Many studies have compared two or more typing methods within the local setting (3, 7, 25, 26) and some large comparative international studies have typed larger and well-characterized strain collections (27, 28).

PFGE has good discriminatory power but it is labor-intensive, taking 4–5 days from pure culture to the result. REA has also very good discriminatory power but because of difficulty and subjectivity in interpretation of the banding patterns it is only performed in a single laboratory. MLVA is a method with great discriminatory power and the results are easily exchangeable

between laboratories (28). PCR ribotyping is quick and easy but inter-laboratory data exchange is difficult due to the lack of standardization.

Two difficulties are currently associated with *C. difficile* typing. While there are good methods available to type strains in the local environment, the global, inter-laboratory comparison is impossible without exchange of reference strains. Secondly, North America and Europe use two different typing systems (PFGE and PCR ribotyping, respectively) and this obviously affects the international comparability. There were some early attempts to unify typing nomenclature, e.g., to assign a correlation between specific pulsotype and PCR ribotype (29) and this was used for the first time during the recent emergence of NAP1/BI/027 (14, 30). Development of easy interchangeable methods like MLVA, sequencer-based PCR ribotyping or single locus-based sequence typing methods should improve this situation.

2. Materials

2.1. Pulsed Field Gel Electrophoresis (PFGE)

1. Blood agar plates
2. Brain heart infusion broth (BHI)
3. Cell suspension buffer (CSB): 0.18 M NaCl, 10 mM Tris (pH 8.0) (*see* **Note 2**).
4. TE2 buffer: 10 mM Tris (pH 8.0), 2 mM EDTA (pH 8.0) (*see* **Note 2**).
5. Cell lysis buffer: 10 mM Tris (pH 8.0), 0.5 M EDTA, 1% (w/v) sodium dodecyl sulfate (SDS) (*see* **Note 3**).
6. Proteinase K (Sigma) (*see* **Note 4**).
7. Restriction endonuclease *Sma*I, *Sac*II, and *Xba*I with appropriate 10X NEBuffer (New England Biolabs).
8. Pre-restriction incubation mixture: 10X NEBuffer diluted 1:10 in nuclease-free water.
9. Restriction mixture: 1X NEBuffer and 15 U of *Sma*I, *Sac*II, or *Xba*I.
10. TBE buffer: Prepare 5X stock with 0.445 M Tris, 0.445 M boric acid, and 10 mM EDTA. Store at room temperature. Working solution (0.3X) is prepared by diluting 60 ml of 5X TBE with 940 ml of distilled water.
11. Pulsed Field Certified™ agarose (Bio-Rad, California).
12. DNA staining solution: 0.2 μg/ml of ethidium bromide (EtBr) in distilled water.
13. *Salmonella* ser. Braenderup is used as a reference standard (*see* **Note 5**).

2.2. PCR Ribotyping

1. TAE buffer: Prepare 50X stock with 2.0 M Tris, 2.0 M acetic acid, and 50 mM EDTA. Adjust pH to 7.5–8.0. Working solution (1X) is prepared by diluting 20 ml of 50X TAE with 980 ml of distilled water. Cool the buffer to 4–8°C before use.
2. DNA staining solution: 0.2 μg/ml of ethidium bromide (EtBr) in distilled water.

3. Method

3.1. PFGE

Inoculate 3–5 *C. difficile* colonies from blood agar plate (28–48 h culture) into 5 ml of brain heart infusion broth (BHI) and incubate in an anaerobic atmosphere at 37°C.

Inoculate 0.1 ml of overnight culture into 5 ml of fresh pre-reduced BHI and incubate for 5 h anaerobically at 37°C.

Prepare 1.5% gel by mixing 0.3 g of Pulsed Field Certified™ agarose and 20 ml of TE2 buffer. Dissolve agarose by heating in a microwave oven. Cool the agarose to 60°C and maintain the temperature until use.

Remove 3 ml of culture, centrifuge, and wash it in 500 μl of CSB buffer. Pellet suspension by centrifugation at 10,000×*g* for 10 min, resuspend the pellet in CSB buffer, and adjust the concentration of suspension to 1.5×10^9 bacteria/ml.

To prepare agarose plugs, mix an equal volume of cell suspension and 1.5% agarose. Mix gently by pipetting. Immediately dispense the mixture into appropriate well of the plug mold (avoid bubbles). Leave plugs to solidify at 4°C for 15–30 min.

In 2 ml tubes prepare 990 μl of cell lysis buffer and 10 μl of proteinase K in final concentration of 0.1 mg/ml. Transfer the plugs from mold to the tube and incubate overnight at 37°C.

Carefully pour off the lysis buffer and add 1 ml of TE2 buffer. Incubate at 37°C for 30 min. Pour off TE2 buffer and repeat the washing step five times. If plugs are not used immediately, store them at 4°C in TE2 buffer.

Remove the plug from TE2 buffer with a spatula and place it on parafilm or glass slide. Cut off the small slice of a plug (approximately 5 × 3 mm) with a scalpel and transfer it to the tube containing 100 μl of pre-restriction incubation mixture. The shape and size of the plug slice will depend on the size of comb teeth used for casting the gel. Incubate for 30 min at room temperature.

Pour off the pre-restriction incubation mixture and add 100 μl of restriction mixture. Incubate at 37°C (for *Sac*II and *Xba*I) or at room temperature (for *Sma*I) for at least 4 h or overnight.

The following instructions are meant for the Biometra PFGE system.

Prepare a 1.0% gel by mixing 3.0 g of Pulsed Field Certified™ agarose (Bio-Rad, California) and 300 ml of 0.3X TBE buffer. Dissolve agarose by heating in a microwave oven. Mix the agarose with a magnetic stirrer during heating (*see* **Note 6**). Save a small volume (approximately 5 ml) of melted and cooled agarose to fix plugs on comb teeth and to seal wells after plugs are loaded. Agarose can be kept at room temperature, melted and reused when needed.

Remove plug slices from tubes (excess buffer should be removed) and load them on the bottom of comb teeth. Load *S.* ser Braenderup standard on first, and then every fifth lane. Seal the plugs with 1% agarose (50–60°C).

Level the gel form and position the comb teeth. Carefully pour the agarose (cooled to 50–60°C) and let the gel to solidify for 30–45 min. Remove the comb and seal the holes with 1% agarose.

Pour 2.4 l of 0.3X TBE buffer into electrophoresis chamber and let the buffer to cool to 13°C. Place the gel casting tray with the gel in the electrophoresis chamber. Assemble the PFGE system following the manufacturer's instructions. Select the following conditions for electrophoresis: initial switch time of 2 s, final switch time of 60 s, voltage 200 V, included angle 120°, temperature 13°C, and run time 21 h.

When electrophoresis is over stain the gel with ethidium bromide for 15–30 min and then destain the gel in distilled water for 20–60 min. Capture the image with gel documentation system.

3.2. PCR Ribotyping

Primers described by Bidet et al. are used to amplify intergenic regions between 16S and 23S rDNA. Sequence of primers (5′–3′):

Primer annealing on 3′ end of 16S rRNA gene: GTGCGGCTGGATCACCTCCT

Primer annealing on 5′ end of 23S rRNA gene: CCCTGCACCCTTAATAACTTGACC.

Reaction mixture:

H_2O	37.0 µl
10X buffer with $MgCl_2$[a]	5.0 µl
20 mM dNTPs	2.0 µl
Primer 1 (50 pmol/µl)	1.0 µl
Primer 2 (50 pmol/µl)	1.0 µl
Taq DNA polymerase (5 U/µl)	0.25 µl

[a]Final concentration of $MgCl_2$ in reaction mixture should be 1.5 mM.

Distribute PCR mastermix in PCR tubes and add 3 μl of crude template DNA or 2 μl of pure DNA.

Amplification conditions:

- Initial denaturation at 95°C for 5 min
- 35 cycles of
  1 min at 95°C for denaturation
  1 min at 57°C for annealing
  1 min at 72°C for elongation
- Final elongation at 72°C for 10 min

After amplification, concentrate the products by heating at 75°C for 45 min (leave the lid of thermal cycler and caps on the tubes open so that the water can evaporate). For electrophoresis 20 μl of PCR product is used.

Agarose gel electrophoresis:

Prepare 3% agarose gel (Certified™ Low Range Ultra Agarose; Bio-Rad, California, USA) in 1X TAE buffer. Dissolve the agarose by heating in a microwave oven. Gently mix the agarose with a magnetic stirrer during heating (avoiding bubbles) (*see* **Note 6**). Be careful not to overboil the agarose. If it starts to overboil, pause the microwave and allow to calm down. Continue until all the agarose has dissolved. Carefully pour the agarose (cooled to 50–60°C) and let it solidify for 30–45 min before running the electrophoresis at 2.5 V/cm for 5 h. Keep the buffer cold during electrophoresis (*see* **Note 7**).

After electrophoresis stain the gel with ethidium bromide for 10–20 min and destain in distilled water for 10–30 min. Capture the image with gel documentation system. PCR ribotypes for which the reference strains are available are designated by standard Cardiff nomenclature, while others are designated by internal nomenclature.

4. Notes

1. BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) is often used for analysis of banding patterns. It offers general platform for data analysis, databasing, and exchanging data in uniform way. Other gel analysis softwares (e.g., provided with hardware for gel imaging) can be used as well.
2. CSB buffer is stored at room temperature.
3. Cell lysis buffer can be stored at room temperature. Because SDS in buffer will precipitate at room temperature, store the bottle for 2–4 h (depending of the buffer volume) at about 37°C prior to use.

4. Prepare 10 mg/ml stock solution, aliquot, and store at –20°C.
5. Salmonella DNA must be digested with *Xba*I to give the appropriate band pattern. Follow instructions for *C. difficile* for making plugs and preparing restriction digest. Agarose plugs can be stored in TE2 buffer at 4°C for at least 3 months.
6. If using microwave oven for melting the agarose use only stirrers that are coated in plastic. Do not put metal stirrers in microwave oven.
7. To prevent excessive heating of the buffer and consecutive DNA degradation during electrophoresis you can either change the buffer every 1.5–2 h or you can surround the electrophoresis chamber with ice bags.

References

1. Bartlet JG. (1994) *Clostridium difficile*: history of its role as an enteric pathogen and the current state of knowledge about the organism. *Clin Infect Dis* **18**(4), 265–272.
2. Delmée M, Homel M and Wauters G. (1985) Serogrouping of *Clostridium difficile* strains by slide aglutination. *J Clin Microbiol* **21**, 323–327.
3. Bidet P, Lalande V, Salauze B, Burghoffer B, Avesani V, Delmee M, Rossier A, Barbut F and Petitt JC. (2000) Comparison of PCR-ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for typing *Clostridium difficile*. *J Clin Microbiol* **38**(7), 2484–2487.
4. Kato H, Kato N, Watanabe K, Ueno K, Ushijima H, Hashira S and Abe T. (1994) Application of typing by pulsed-field gel electrophoresis to the study of *Clostridium difficile* in a neonatal intensive care unit. *J Clin Microbiol* **32**(9), 2067–2070.
5. van den Berg RJ, Claas EC, Oyib DH, Klaassen CH, Dijkshoorn L, Brazier JS and Kuijper EJ. (2004) Characterization of toxin A-negative, toxin B-positive *Clostridium difficile* isolates from outbreaks in different countries by amplified fragment length polymorphism and PCR ribotyping. *J Clin Microbiol* **42**(3), 1035–1041.
6. Fawley M, Freeman J, Smith C, Hermanus C, van den Berg RJ, Kuijper EJ and Wilcox MH. (2008) Use of highly discriminatory fingerprinting to analyze clusters of *Clostridium difficile* infection cases due to epidemic ribotype 027 strains. *J Clin Microbiol* **46**, 954–960.
7. Fawley NW, Parnell P, Verity P, Freeman J and Wilcox MH. (2005) Molecular epidemiology of endemic *Clostridium difficile* infection and the significance of the United Kingdom epidemic strain (PCR ribotype 1). *J Clin Microbiol* **43**, 2685–2696.
8. Barbut F, Mastrantonio P, Delmée M, Brazier J, Kuijper E and Poxton I. (2007) Prospective study of *Clostridium difficile* infections in Europe with phenotypic and genotypic characterisation of the isolates. *Clin Microbiol Infect* **13**, 1048–1057.
9. Arroyo LG, Kruth SA, Willey BM, Staempfli HR, Low DE and Weese JS. (2005) PCR ribotyping of *Clostridium difficile* isolates originating from human and animal sources. *J Med Microbiol* **54**, 163–166.
10. Keel K, Brazier JS, Post KW, Weese SJ and Songer JG. (2007) Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. *J Clin Microbiol* **45**, 1963–1964.
11. Jhung MA, Thompson AD, Killgore GE, Zukovski WE, Songer G, Warny M, Johnson S, Gerding DN, McDonald LC and Limbago BM. (2008) Toxinotype V *Clostridium difficile* in humans and food animals. *Emerg Infect Dis* **14**, 1039–1045.
12. Kuijper EJ, Oudbier JH, Stuifbergen WNHM, Jansz A and Zanen HC. (1987) Application of whole-cell DNA restriction endonuclease profiles to the epidemiology of *Clostridium difficile*-induced diarrhea. *J Clin Microbiol* **25**, 751–753.
13. Clabots CR, Johnson S, Bettin K, Mathie PA, Mulligan ME, Schaberg DR, Peterson

LR and Gerding DN. (1993) Development of a rapid and efficient restriction endonuclease analysis typing system for *Clostridium difficile* and correlation with other typing systems. *J Clin Microbiol* **31**(7), 1870–1875.

14. McDonald LC, Killgore GE, Thompson A, Owens RC, Jr., Kazakova SV, Sambol SP, Johnson S and Gerding DN. (2005) An epidemic, toxin gene-variant strain of *Clostridium difficile N Engl J Med* **353**(23), 2433–2441.
15. Gal M, Northey G and Brazier JS. (2005) A modified pulsed-field gel electrophoresis (PFGE) protocol for subtyping previously non-PFGE typeable isolates of *Clostridium difficile* polymerase chain reaction ribotype 001. *J Hosp Infect* **61**(3), 231–236.
16. van Dijck P, Avesani V and Delmee M. (1996) Genotyping of outbreak-related and sporadic isolates of *Clostridium difficile* belonging to serogroup C. *J Clin Microbiol* **34**(12), 3049–3055.
17. Spigaglia P, Cardines R, Rossi S, Menozzi MG and Mastrantonio P. (2001) Molecular typing and long-term comparison of *Clostridium difficile* strains by pulsed-field gel electrophoresis and PCR-ribotyping. *J Med Microbiol* **50**(5), 407–414.
18. Kato H, Kato N, Watanabe K, Iwai N, Nakamura H, Yamamoto T, Suzuki K, Kim S-M, Chong Y and Wasito EB. (1998) Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. *J Clin Microbiol* **36**, 2178–2182.
19. Gurtler V. (1993) Typing of *Clostridium difficile* strains by PCR-amplification of variable length 16S-23S rDNA spacer regions. *J Gen Microbiol* **139**, 3089–3097.
20. Cartwright P, Stock F, Beekmann SE, Williams EC and Gill VE. (1995) PCR amplification of rRNA intergenic spacer regions as a method for epidemiologic typing of *Clostridium difficile*. *J Clin Microbiol* **33**, 184–187.
21. O'Neill GL, Ogunsola FT, Brazier JS and Duerden BI. (1996) Modification of a PCR ribotyping method for application as a routine typing scheme for *Clostridium difficile*. *Anaerobe* **2**, 205–209.
22. Bidet P, Barbut F, Lalande V, Burghoffer B and Petit JC. (1999) Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. *FEMS Microbiol Lett* **175**, 261–266.
23. Stubbs SL, Brazier JS, O'Neill GL and Duerden BI. (1999) PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. *J Clin Microbiol* **37**, 461–463.
24. Indra A, Huhulescu S, Scheeweis P, Hasenberger P, Kernbichler S, Fiedler A, Wewalka G, Allerberger F and Kuijper E. (2008) Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. *J Med Microbiol* **57**(11), 1377–1382.
25. Killgore G and Kato H. (1994) Use of arbitrary primer PCR to type *Clostridium difficile* and comparison of results with those by immunoblot typing. *J Clin Microbiol* **32**, 1591–1593.
26. Spigaglia P and Mastrantonio P. (2003) Evaluation of repetitive element sequence-based PCR as a molecular typing method for *Clostridium difficile*. *J Clin Microbiol* **41**, 2454–2457.
27. Johnson S, Sambol SP, Brazier JS, Delmee M, Avesani V, Merrigan MM and Gerding DN. (2003) International typing study of toxin A-negative, toxin B-positive *Clostridium difficile* variants. *J Clin Microbiol* **41**, 1543–1547.
28. Killgore G, Thompson A, Johnson S, Brazier J, Kuijper E, Pepin J, Frost EH, Savelkoul P, Nicholson B, van den Berg RJ, Kato H, Sambol SP, Zukowski W, Woods C, Limbago B, Gerding DN and McDonald LC. (2008) Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. *J Clin Microbiol* **46**(2), 431–437.
29. Brazier JS, Mulligan ME, Tabaqchali S and the international *C. difficile* Study Group. (1997) Preliminary findings of the international typing study on *Clostridium difficile*. *Clin Infect Dis* **25**(Suppl 2), S199–S201.
30. Kuijper EJ, Coignard B and Tull P. (2006) Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin Microbiol Infect* **12**(Suppl 6), 2–18.
31. Rupnik M, Avesani V, Janc M, von Eichel–Streiber C and Delmée M. (1998) A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J Clin Microbiol* **36**, 2240–2247.
32. Marsh JW, O'Leary MM, Shutt KA, Pasculle W, Johnson S, Gerding DN, Muto CA and Harrison LH. (2006) Multilocus

variable-number tandem repeat analysis for investigation of *Clostridium difficile* transmission in hospitals. *J Clin Microbiol* **44**(7), 2558–2566.

33. van den Berg RJ, Schaap I, Templeton KE, Klaassen CHW and Kuijper EJ. (2007) Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable-number tandem-repeat analysis. *J Clin Microbiol* **45**, 1024–1028.
34. Klaassen CH, van Haren HA and Horrevorts AM. (2002) Molecular fingerprinting of *Clostridium difficile* isolates: pulsed-field gel electrophoresis versus amplified fragment length polymorphism. *J Clin Microbiol* **40**(1), 101–104.
35. Lemee L, Dhalluin A, Pestel-Caron M, Lemeland J and Pons J. (2004) Multilocus sequence typing analysis of human and animal *Clostridium difficile* isolates of various toxigenic types. *J Clin Microbiol* **42**, 2609–2617.
36. Lemee L, Bourgeois I, Ruffin E, Collignon A, Lemeland JF and Pons JL. (2005) Multilocus sequence analysis and comparative evolution of virulence-associated genes and housekeeping genes of *Clostridium difficile*. *Microbiology* **151**, 3171–3180.
37. Kato H, Yokoyama T and Arakawa Y. (2005) Typing by sequencing the slpA gene of *Clostridium difficile* strains causing multiple outbreaks in Japan. *J Med Microbiol* **54**, 167–171.
38. Karjalainen T, Saumier N, Barc MC, Delmee M and Collignon A. (2002) *Clostridium difficile* genotyping based on slpA variable region in S-layer gene sequence: an alternative to serotyping. *J Clin Microbiol* **40**(7), 2452–2458.
39. Zeiss H, Rupnik M, Kuijper EJ, Hermanus C, Michielsen D, Janssens K and Nubel U. (2009) Typing *Clostridium difficile* strains based on tandem repeat sequences. *BMC Microbiol* **9**, 6.

Chapter 5

Clostridium difficile Toxinotyping

Maja Rupnik

Abstract

Clostridium difficile shows considerable variability in the PaLoc region encoding two main virulence factors, toxins TcdA and TcdB. Strains with changes in PaLoc are defined as variant toxinotypes and currently 27 such groups are recognized (I to XXVII). Toxinotype 0 includes strains with PaLoc identical to the reference laboratory strain VPI 10463. Toxinotyping is a RFLP-PCR-based method using a combination of restriction patterns of part of *tcdB* and *tcdA* genes for determination of toxinotype. Variations in PaLoc can affect the toxin production or could result in production of toxins with altered properties.

Key words: *Clostridium difficile*, toxinotype, PaLoc, variability, variant toxins, toxin genes.

1. Introduction

Clostridium difficile toxins A (TcdA) and B (TcdB) are encoded on a large, well-defined 19.6 kb chromosomal region called pathogenicity locus (PaLoc) (1, 2). In strains not producing TcdA and TcdB the PaLoc is replaced by short 115 bp nucleotide sequence (**Fig. 5.1**).

Initially, two groups of strains with different variant forms of toxin genes *tcdA* and *tcdB* were described, namely strain 8864 (3, 4) and strains from serogroup F (5). Subsequently it was shown that several other natural variations of *C. difficile* PaLoc exist and groups of strains with identical changes in their PaLoc were defined as toxinotypes (6, 7). Currently there are XXIV *C. difficile* toxinotypes published (7). However, the number is changing over time and a regularly updated overview (up to toxinotype XXVII) can be found at www.mf.uni-mb.si/mikro/tox.

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_5,

© Springer Science+Business Media, LLC 2010

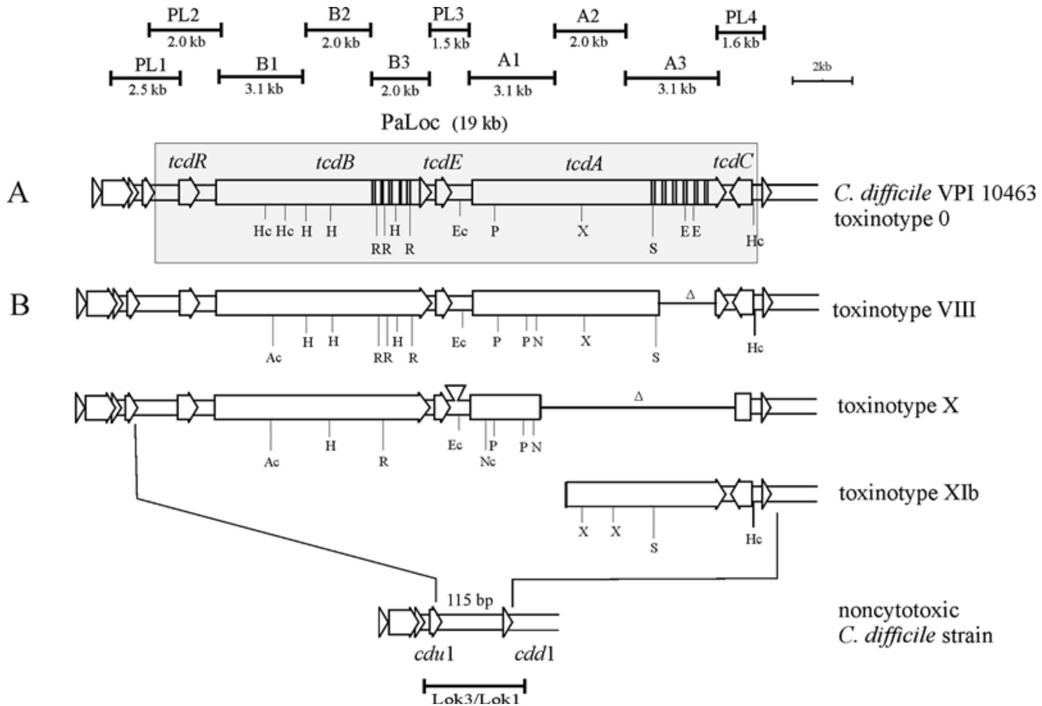


Fig. 5.1. Toxin coding PaLoc region of four different representative toxinotypes. (**a**) Strain VPI 10463 is a reference strain to which all changes in PaLoc are compared. PCRs used for amplification are indicated and B1 and A3 fragments are used for toxinotyping. (**b**) In variant toxinotypes (here only three examples are shown) changes can be deletions, insertions, or changes in restriction sites. In nontoxinogenic strains PaLoc is replaced by short sequence of 115 bp. Restriction enzymes (presence or absence and not exact positions of restriction site are shown): Ac, *Acc*I; E, *Eco*RI; Ec, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; N, *Nsi*I; P, *Pst*I; R, *Rsa*I; S, *Spe*I; X, *Xba*I.

Toxinotyping is a RFLP-PCR-based method for detection of toxinotypes. PaLoc region is covered by 10 overlapping PCR products (**Fig. 5.1**). Six of them amplify both toxin genes and four amplify accessory genes and intergenic regions. For determination of toxinotype only two PCR fragments are used, namely B1 covering first third of the *tcdB* gene and A3 covering repetitive regions of *tcdA* gene. Only in a few cases the RFLPs in B1 and A3 are identical for two toxinotypes and additional PCR fragments have to be amplified to differentiate among them (**Fig. 5.2**).

Changes in PaLoc can be insertions, deletions, or point mutations. Sequencing of the entire *tcdB* gene from toxinotype representatives has confirmed that RFLPs used in toxinotyping are good markers for substantial changes over the entire genes. Minimal identity between two different variant forms of *tcdB* genes on nucleotide level is between 99 and 87%. Gene *tcdC* is a very variable part of PaLoc (8, 9) but its amplification is not a part of the toxinotyping.

Variant toxinotypes also correlate well with other molecular typing methods, e.g., PCR ribotype always contains strains from

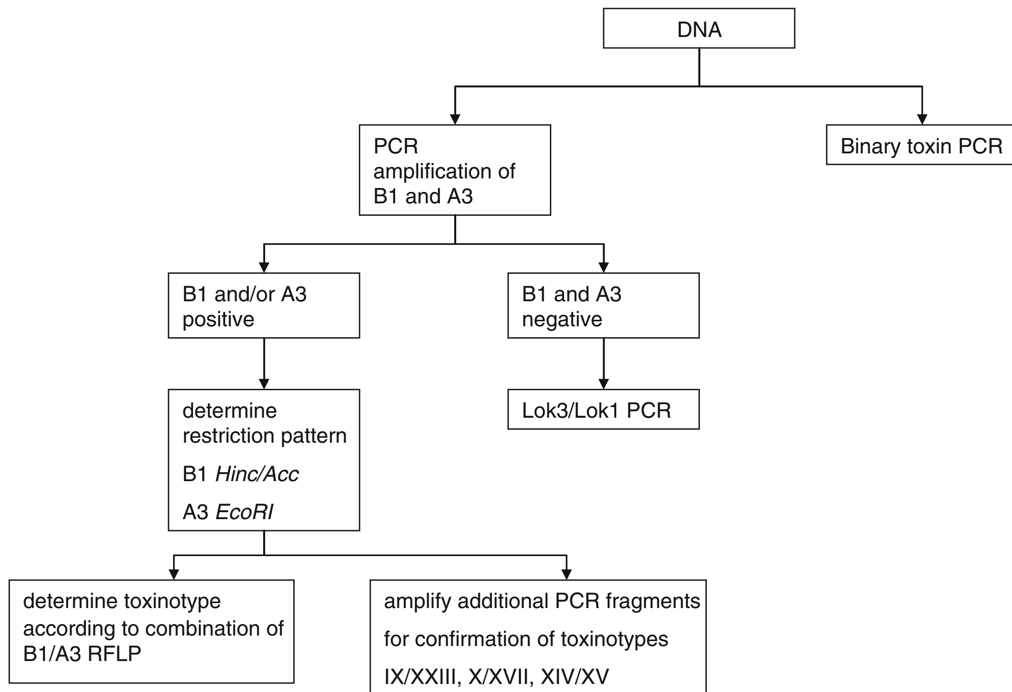


Fig. 5.2. Workflow for toxinotyping of *C. difficile* strains. Amplification of binary toxin gene is usually performed in parallel as many of toxinotypes are binary toxin positive.

a given toxinotype, while a given toxinotype can include several PCR ribotypes (7).

Variant toxinotypes can have toxin production other than TcdA-positive, TcdB-positive (7) or could produce toxins with altered properties (10). Many variant toxinotypes produce also a third toxin, binary toxin CDT (11, 12).

The proportion of variant strains within *C. difficile* isolates is changing and is also dependant on hospital, country, and host. In animals *C. difficile* variants can represent from 40 to 100% of all isolated strains and in humans from 2 to 25% of all isolated strains.

2. Materials

1. Chelex 100 resin (BioRad, Sigma)
2. Taq polimerase with additionally supplied $MgCl_2$ (*see* **Note 1**).
3. dNTPs. Usually supplied in concentration of 100 mM of each dNTP. Mix equal volumes of all four dNTPs (mix concentration 100 mM, each dNTP in concentration of 25 mM). Dilute with H_2O 1:4 to obtain final working solution with concentration 20 mM (5 mM for each dNTP).

4. Tetramethylammonium chloride (TMA, Sigma or Fluka). Usually supplied as 5 M solution. Dilute with dest H_2O to final solution of 10^{-3} M.
5. Restriction enzymes *Hinc*II, *Acc*I, *Eco*RI (different suppliers)
6. Agarose and 0.5X TBE buffer for electrophoresis (5X TBE: for 1 l dissolve in H_2O 54 g Tris base, 27.5 g boric acid, and 20 ml 0.5 M EDTA pH 8.0).
7. Primers are described in **Table 5.1**.
8. Control strains. A3 fragments can show length differences prior to restriction. To correctly determine some of such deleted forms use of control strains of toxinotype VIII and VI is recommended. The reference *C. difficile* strain VPI 10463 (toxinotype 0) is used as a control in every PCR reaction.

3. Methods

Toxinotyping is performed in three steps: (1) PCR amplification of B1 and A3 fragments, (2) restriction of amplified fragments, and (3) determination of toxinotypes according to the combination of B1 and A3 restriction types (**Fig. 5.2**). We include in every toxinotyping confirmation of the presence of the CDTb component of binary toxin because variant strains are usually CDT positive (12).

3.1. Isolation of DNA with Chelex-100

1. Resuspend 0.3 g Chelex-100 in 5 ml of sterile water and vortex.
2. Aliquot 0.1 ml of Chelex suspension in 1.5 ml tubes.
3. Take 1 μl loop-full of overnight culture from blood agar plate, resuspend it in the Chelex suspension and vortex.
4. Boil for 10 min.
5. Centrifuge at maximum speed for 10 min in tabletop centrifuge.
6. Transfer 70 μl of supernatant (crude DNA) into a fresh tube.
7. Store at 4°C for up to 1 week. If frozen the sample should not be repeatedly frozen and thawed.

3.2. Amplification of B1 and A3 PCR Products

Prepare mastermix according to **Table 5.2**.

Distribute PCR mastermix in PCR tubes in 46 μl aliquots. Add 3 μl of crude DNA (prepared by Chelex) or 3 μl of 1:100 diluted pure genomic DNA (*see* **Note 2**).

Table 5.1
Primers used for toxinotyping

PCR fragment	Expected fragment length	Primer	Sequence	Purpose
B1	3.1 kb	B1C	5′-AGAAAATTTTATGAGTTTAGTTAATAGAAA-3′	Toxinotyping
		B2N	5′-CAGATAATGTAGGAAGTAAGTCTATAG-3′	
A3	3.1 kb	A3C	5′-TATTGATAGCACCTGATTTATATACAAG-3′	Toxinotyping
		A4N	5′-TTATCAAACATATATTTTAGCCATATATC-3′	
A1	3.1 kb	A1C	5′-GGA GGT TTT TAT GTC TTT AAT ATC TAA AGA-3′	Confirmation of certain toxinotypes
		A2N	5′-CCC TCT GTT ATT GTA GGT AGT ACA TTT A-3′	
PL3	1.5 kb	Lok6	5′-GTT GTT TAG ATT TAG ATG AAA AGA-3′	Confirmation of certain toxinotypes
		PrimA	5′-CTT GGT CTA ATG CTA TAT GCG AG-3′	
Tox-	115 bp (in PaLoc neg strains)	Lok3	5′-TTT ACC AGA AAA AGT AGC TTT AA-3′	Confirmation of PaLoc absence
		Lok1	5′-AAA ATA TAC TGC ACA TCT GTA TAC-3′	

Table 5.2
PCR mastermix compositions for different PCR fragments

	B1 (μl)	A3 (μl)	A1 (μl)	PL3 (μl)	Tox-(μl)
H_2O	77	67	75	77	75
10X buffer (1.5 mM $MgCl_2$)	10	10	10	10	10
10^{-3} M TMA	–	10	–	–	–
25 mM $MgCl_2$	2	2	4	2	4
20 mM dNTPs[a]	4	4	4	4	4
Primer 1 (10 pmol/μl)	1	1	1	1	1
Primer 2 (10 pmol/μl)	1	1	1	1	1
Taq polymerase (5 U/μl)	0.25	0.25	0.25	0.25	0.25

[a]20 mM refers to entire nucleotide concentration in the solution; each single nucleotide is in 5 mM concentration.

Table 5.3
Amplification conditions used for toxinotyping

B1 PCR[a]	A3 PCR	PL3 PCR	Tox-A1 PCR
93°C 3 min	93°C 3 min	93°C 3 min	93°C 3 min
35 cycles	35 cycles	30 cycles	30 cycles
57°C 8 min	47°C 8 min	47°C 5 min	52°C 1 min
93°C 3 s	93°C 3 s	93°C 3 s	72°C 1 min 93°C 45 s
47°C 10 min	47°C 10 min	47°C 10 min	72°C 10 min

[a]Annealing temperature for B1 PCR can be from 55 to 57°C to avoid nonspecific fragments.

3.3. PCR Program

PCR programs used are summarized in **Table 5.3**. Because of the length of the fragments two-step PCR program is used for some reactions.

3.4. Restrictions and Analysis

1. Check PCR products on 1% agarose gels. Some large deletions in A3-PCR fragment can already be detected (types of A3 fragment 5, 6, 7, 10 and 11). A3 fragments 5 and 6 are very close on the gels, therefore, it is necessary to run such gels for longer time or to include a control strain of toxinotype VI which will show the A3 fragment type 5.
2. B1 amplified products are cut with two restriction enzymes, *Acc*I and *Hin*cII. Digests are visualized on 1% agarose gels and restriction pattern 1–7 is determined for each strain (**Fig. 5.3**).
3. A3 amplified products are cut with only one restriction enzyme, *Eco*RI. Digests are visualized on 1% agarose gels and restriction pattern 1–14 is determined for each strain

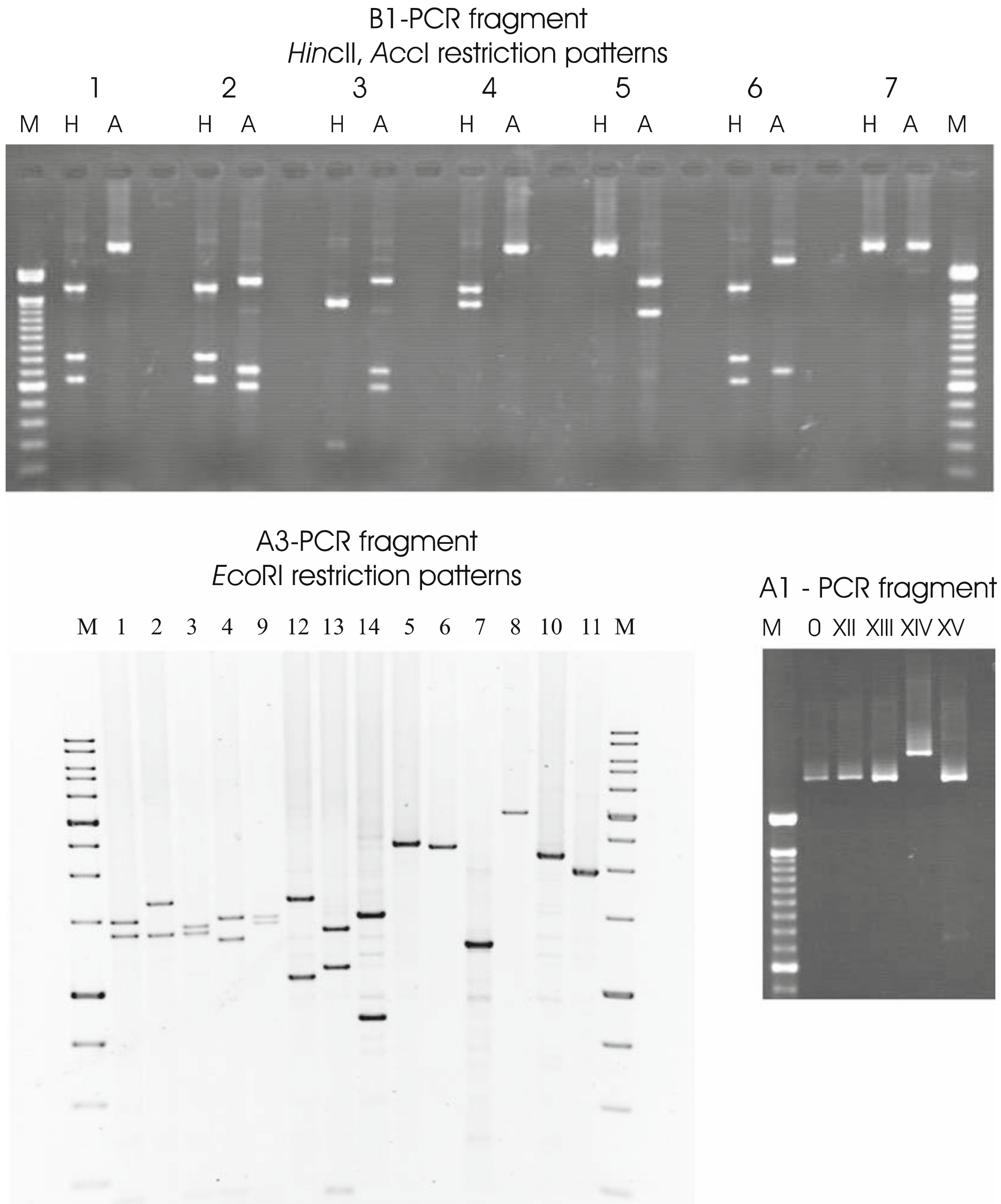


Fig. 5.3. Restriction fragment length polymorphisms found in amplified fragments B1 and A3. Note that A3 restriction type 8 represents whole length form without EcoRI restriction site and types 5–7 and 10–11 represent deleted forms that do not have EcoRI restriction sites. Fragment A1 is used only for confirmation of some toxinotypes. In this case restriction is not performed but the amplified fragment lengths differ in some types due to the presence of an ISTron.

Table 5.4
Types of RFLP patterns of B1 and A3 PCR fragments in different toxinotypes

Toxinotype	B1 fragment types of *Hinc/Acc* restrictions	A3 fragment types of *Eco*RI restrictions	Remarks
0	1	1	
XIa	Neg	5	
XIb	Neg	8	
XXIV	1	1	• Variant *tcdC* gene • Presence of binary toxin genes, which is unusual for strain with VPI like *tcd* genes
II	1	3	
I	1	4	
XIX	1	5	
XX	1	6	
XIII	1	9	
XVIII	1	11	
XXVI	1	13	
XXVII	1	14	
IV	2	2	
VI VII V XVI	3 3 3 3	5 6 8 10	Types V, VI, and VII very similar to each other in all *tcd* fragments except in A3
XXII	4	1	• ISTron in A1 (hence larger as in the VPI 10463 strain)
III	4	2	
XXV	4	12	
XXI	5	1	
IX XXIII	5 5	2 2	Differentiation of types IX and XXIII: • In XXIII longer A1 due to the presence of ISTron
VIII	5	7	
X XVII	5 5	Neg Neg	Differentiation of types X and XVII: • In XVII longer A1 due to the presence of ISTron • No insertion in PL3 in XVII
XII	6	1	
XIV XV	7 7	2 2	Differentiation of types XIV and XV: • In XIV longer A1 due to the presence of ISTron

Neg, not amplified.

(**Fig. 5.3**). Note that deleted versions of A3 fragment usually do not have *Eco*RI restriction sites.

4. With known types of restriction patterns for B1 and A3 fragments toxinotype can be determined from **Table 5.4** (*see* **Note 3**).

4. Notes

1. PCR fragments are large and Taq polymerases of high quality should be used.
2. A3 PCR fragment (covering repetitive regions) is sometimes difficult to amplify. Therefore, TMA should be added as described in protocols above. Also, increasing the amount of DNA will decrease the amplification.
3. With amplification conditions described sometimes nonspecific bands in B1 fragments are observed, depending on thermocycler used. In this case we suggest trying different annealing/elongation temperature (55–57°C).

Acknowledgments

This work was supported by grant J3-0194-0377-08.

References

1. Braun V, Hundesberger T, Leukel P, Sauerborn M and von Eichel–Streiber C. (1996) Definition of the single integration site of the pathogenicity locus in *Clostridium difficile. Gene* **181**, 29–38.
2. Rupnik M, Dupuy B, Fairweather NF, Gerding DN, Johnson S, Just I, Lyerly DM, Popoff MR, Rood JI, Sonenshein AL, Thelestam M, Wren BW, Wilkins TD and Eichel-Streiber CV. (2005) Revised nomenclature of *Clostridium difficile* toxins and associated genes. *J Med Microbiol* **54**, 113–117.
3. Borriello SP, Wren BW, Hyde S, Seddon SV, Sibbons P, Krishna MM, Tabaqchali S, Manek S and Price AB. (1992) Molecular, immunological, and biological characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile. Infect Immun* **60**, 4192–4199.
4. Lyerly DM, Barroso LA, Wilkins TD, Depitre C and Corthier G. (1992) Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile. Infect Immun* **60**, 4633–4639.
5. Depitre C, Delmée M, Avesani V, L'Haridon R, Roels A, Popoff M and Corthier G. (1993) Serogroup F strains of *Clostridium difficile* produce toxin B but not toxin A. *J Med Microbiol* **38**, 434–441.
6. Rupnik M, Avesani V, Janc M, von Eichel–Streiber C and Delmée M. (1998) A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J Clin Microbiol* **36**, 2240–2247.
7. Rupnik M. (2008) Heterogeneity of large clostridial toxins: importance of *Clostridium difficile* toxinotypes. *FEMS Microbiol Rev* **32**, 541–555.

8. Spigaglia P and Mastrantonio P. (2002) Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. *J Clin Microbiol* **40**, 3470–3475.
9. Curry SR, Marsh JW, Muto CA, O'Leary MM, Pasculle AW and Harrison LH. (2007) *tcdC* genotypes associated with severe TcdC truncation in an epidemic clone and other strains of *Clostridium difficile*. *J Clin Microbiol* **45**, 215–221.
10. Chaves-Olarte E, Freer E, Parra A, Guzman-Verri C, Moreno E and Thelestam M. (2003) R-Ras glucosylation and transient RhoA activation determine the cytopathic effect produced by toxin B variants from toxin A-negative strains of *Clostridium difficile*. *J Biol Chem* **278**, 7956–7963.
11. Perelle S, Gibert M, Bourlioux P, Corthier G and Popoff MR. (1997) Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. *Infect Immun* **65**, 1402–1407.
12. Stubbs S, Rupnik M, Gibert M, Brazier J, Duerden B and Popoff M. (2000) Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. *FEMS Microbiol Lett* **186**, 307–312.

Chapter 6

Multilocus Sequence Typing for *Clostridium difficile*

Ludovic Lemée and Jean-Louis Pons

Abstract

Multilocus sequence typing (MLST), a nucleotide sequence-based characterization of allelic polymorphism of housekeeping genes, has been proposed as a new approach for population and evolutionary genetics and global epidemiology of bacterial pathogens. MLST provides unambiguous sequence data that can be generated from various laboratories and should be shared in a common web database. Here are presented most of materials, methods, and programs or software necessary to perform MLST on *Clostridium difficile*.

We also describe an example of an MLST scheme for *C. difficile* based on sequence analysis of six housekeeping gene loci and use a set of 74 *C. difficile* isolates from various hosts, geographic sources, and PCR-toxigenic types (A+B+, A–B+, and A–B–). Thirty-two "sequence types" (ST) are defined from the combination of allelic data, which correlate well with toxigenic types. The estimation of linkage disequilibrium between loci reveals a clonal population structure. Mutational evolution of *C. difficile* is characterized, with point mutation generating new alleles at a frequency eightfold higher than recombinational exchange. Phylogenetic analysis shows that human and animal isolates do not cluster in distinct lineages, and that no hypervirulent lineage can be characterized within the population of toxigenic human isolates studied (strains from pseudomembranous colitis and antibiotic-associated diarrhea do not cluster in distinct lineages). However, all A–B+ variant isolates belong to a divergent but very homogeneous lineage in the population studied.

An MLST database specific for this species is now hosted at the web site of the Institut Pasteur Paris. Since MLST data reflect evolutionary genetics of the species, they could be used as typing markers, possibly in combination with virulence genes data, for long-term global epidemiology of *C. difficile*.

Key words: *Clostridium difficile*, multilocus sequence typing (MLST), phylogeny, population genetics, molecular typing.

1. Introduction

Clostridium difficile is recognized as a major enteric pathogen involved in antibiotic-associated diarrhea or pseudomembranous colitis from nosocomial or community origin. Because of its

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_6, © Springer Science+Business Media, LLC 2010

epidemic potential, many molecular typing methods have been proposed to investigate epidemic outbreaks: pulsed-field gel electrophoresis (PFGE), PCR-ribotyping, random amplified polymorphic DNA (RAPD) analysis, amplified fragment length polymorphism (AFLP), or multilocus variable-number tandem-repeat analysis (MLVA) (1–4). Clearly, PCR-ribotyping emerged as a simple and universal typing method for the investigation of the epidemiology of *C. difficile*, especially in the context of intercontinental spread of an hypervirulent strain belonging to PCR-ribotype 027 from North America to Europe in 2004–2006 (5–7). However, the need for a molecular typing method allowing long-term epidemiology and phylogeny of *C. difficile* led us to develop a multilocus sequence typing (MLST) scheme for this species (8). MLST, which characterizes multilocus genotypes of bacterial isolates by using 400- to 500-bp intragenic sequences of a set (generally five to seven) of housekeeping genes, has been developed as a strategy to characterize clonal relationships and filiation within bacterial populations. It was initially proposed for population genetics analysis of *Neisseria meningitidis* (9), *Streptococcus pneumoniae* (10), and *Staphylococcus aureus* (11) and allowed the characterization of recombinant population structure for the former and of clonal population structure for the latter. MLST combines the advantages of (i) a sequence-based typing method, which makes the data unambiguous, readily comparable between different laboratories, and allows the elaboration of a shared central database that can be enriched from various laboratories in the world, and of (ii) a phylogenetic approach to genetic diversity, since it is based on sequence polymorphism of housekeeping genes apart from selective pressure. In addition, it may be further developed for the multilocus analysis of virulence-associated genes, as already reported for *C. difficile* (12) or *Listeria monocytogenes* (13), opening the field of the study of phylogeny of virulence in bacterial pathogens.

2. Materials

2.1. DNA Extraction from Bacterial Isolates

2.1.1. Manual DNA Extraction

1. Blood agar culture of bacterial isolates
2. Microcentrifuge tubes
3. Boiling water bath
4. Microcentrifuge
5. Optionally, commercial kit for bacterial DNA isolation, such as InstaGene Matrix (Biorad ®)

2.1.2. Automated DNA Extraction

1. Blood agar culture of bacterial isolates
2. Microcentrifuge tubes
3. Automated extractor, such as MagNA Pure (Roche diagnostics®) with its specific reagents
4. Boiling water bath
5. Microcentrifuge

2.2. PCR Amplification and DNA Sequencing of Internal Fragments of Housekeeping Genes

1. PCR mixture
2. Primers (0.5 μM each) designed to allow the amplification of 400- to 500-bp fragments of the selected housekeeping genes (**Table 6.1**)
3. Deoxynucleoside triphosphate (200 μM each)
4. Taq DNA polymerase (Applied Biosystems®) (1.25 U in a final volume of 50 μl)
5. Amplification buffer: provided with Taq DNA polymerase
6. 2.5 mM $MgCl_2$
7. DNA: 10 μl
8. DNA thermal cycler
9. Qiaquick gel extraction kit (Qiagen®) for the purification of PCR products
10. ABI-PRISM BigDye terminator sequencing kit v3.1 (Applied Biosystems®) for the sequencing reaction of PCR products
11. Automated DNA sequencing analyzer

Table 6.1
PCR and sequencing primers used for six housekeeping loci in *C. difficile*

Gene	PCR and sequencing primers (5′ → 3′)	Size (bp) of analyzed fragments
aroE	Forward: CTAGTAGGTGAAAAACTCTCTCA Reverse: ACTGGTGTAGCATTTAATATTATATC	410
dutA	Forward: CCTAATTTTGCTCACAAAGGT Reverse: AAATCCAGTTGAGCCAAACC	325
gmk	Forward: TCA GGT GCA GGA AAA GGT AC Reverse: TCT GTT TCT GTA CCT CTT CCA AC	292
recA	Forward: CCA GAT ACA GGT GAA CAG GC Reverse: TTT AAC ATT TTC TCT TCC TTG TCC	379
sodA	Forward: TATSCWTATGATGCWYTWGARCC Reverse: TARTAAGCATGYTCCCAAACATC	416
tpi	Forward: GCAGGAAACTGGAAAATGCATAA Reverse: CAGATTGGCTCATATGCAACAAC	395

2.3. Computer Analysis of Sequence Data (*see* Note 1)

Many of the programs for analysis of MLST data are available from the MLST web site hosted at Oxford University (http://pubmlst.org/):

1. Allele assignment: NRDB program (http://pubmlst.org/software/analysis/)
2. Clustering from the matrix of allelic profiles: START program (http://pubmlst.org/software/analysis/)
3. Nucleotide sequences alignment: BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html)
4. Gene trees elaboration: PHYLIP package (http://pubmlst.org/perl/mlstanalyse/mlstanalyse.pl?site=pubmlst) or MEGA software (http://www.megasoftware.net/)
5. Index of association (*Ia*) between alleles: http://pubmlst.org/software/analysis/
6. Analysis of clonal complexes: BURST program (http://pubmlst.org/perl/mlstanalyse/mlstanalyse.pl?site=pubmlst)

3. Methods

3.1. Bacterial Isolates

The choice of bacterial isolates to include in the study is critical to allow phylogenetic analysis. Isolates from various geographic origins, hosts, and clinical sources should be collected. Isolates must be epidemiologically unrelated; this can be checked by a simple and rapid typing method such as PCR-ribotyping (14, 15).

3.2. DNA Extraction from Bacterial Isolates

If using simple boiling, a bacterial colony from a 24 h anaerobic blood agar culture is resuspended in 1 mL of distilled water in a microcentrifuge tube. The sample is boiled for 20 min prior to being centrifuged to pellet bacterial debris. Ten microliters of the supernatant, containing the genomic DNA, is used for PCR amplification. If using a commercial kit or automated DNA extractor, follow the manufacturer's specifications (*see* **Note 2**).

3.3. Selection of Housekeeping Target Genes

A set of five to seven housekeeping genes is selected over the *C. difficile* chromosome (**Fig. 6.1**). Loci should be distributed in various regions of the whole genome to avoid genetic linkage. The loci must be amplified in 100% of isolates to avoid the introduction of null alleles that interfere in the multilocus analysis; this can be checked by amplification of each target gene in a representative set (10%) of the whole population of isolates to be studied.

In our experience, the following loci are suitable for an MLST analysis in *C. difficile*: *aroE* (shikimate dehydrogenase),

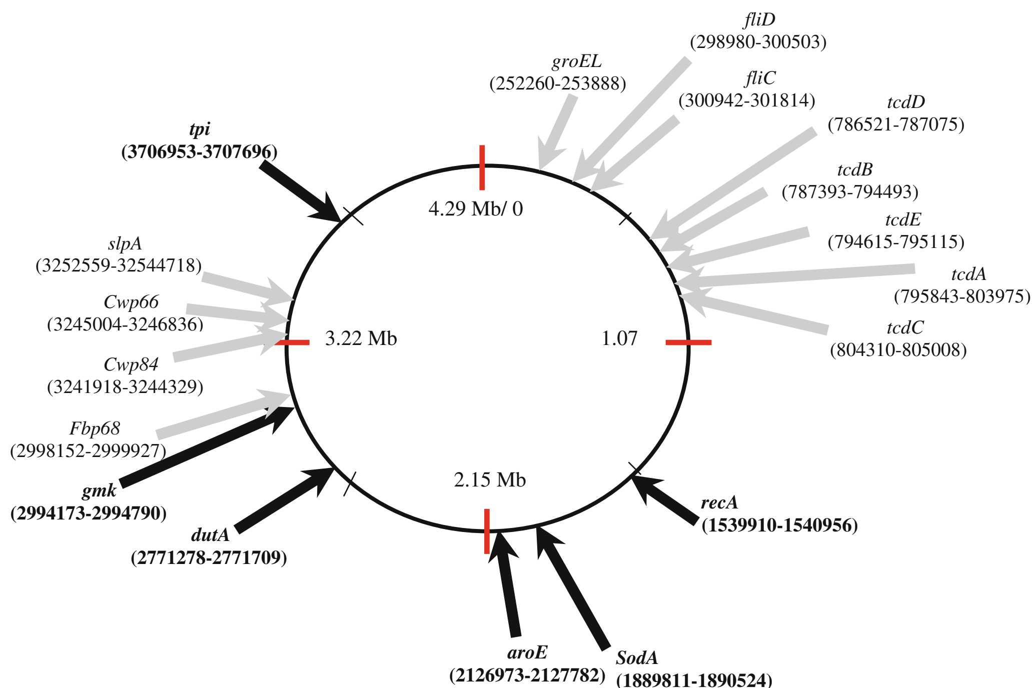


Fig. 6.1. Genomic locations of the loci analyzed in the *C. difficile* 630 genome. The six housekeeping loci are shown in *bold* with *black arrows* (8); virulence-associated genes, also investigated by multilocus analysis (12), are shown with *gray arrows*.

dutA (dUTP pyrophosphatase), *gmk* (guanylate kinase), *recA* (recombinase), *sodA* (superoxide dismutase), *tpi* (triosephosphate isomerase).

3.4. PCR Amplification and DNA Sequencing of Internal Fragments (400–500 bp) of Housekeeping Genes

PCR is performed according to the following procedure: the PCR mixtures are heated for 3 min at 95°C and then a touchdown procedure follows, consisting of 30 s at 95°C, annealing for 30 s at temperatures decreasing from 60 to 50°C during the first 11 cycles (with 1°C decremental steps in cycles 1–11) and ending with an extension step at 72°C for 30 s. Forty cycles are performed. PCR products are purified with the Qiaquick gel extraction kit (Qiagen®) according to the manufacturer's recommendations and sequenced (200–500 ng of DNA) on both strands with PCR forward and reverse primers (*see* **Note 3**).

3.5. Computer Analysis of Sequence Data

3.5.1. Definition of Sequence Types

Definition of sequence types: for a given locus the different sequences described in the collection of isolates studied are assigned allele numbers; multilocus combination of alleles are then obtained for the various isolates, and each unique multilocus allelic profile is assigned a sequence type (ST). Single point polymorphisms must be confirmed by sequencing both DNA strands from two separate PCR experiments.

3.5.2. Polymorphism Analysis

The following data should be collected for polymorphism analysis:

- Number of individual alleles for each locus, number of polymorphic sites on a given locus, number of nucleotide differences between alleles of a given locus
- Ratios of nonsynonymous to synonymous substitutions (dN/dS): the START program may be used to calculate these ratios for each housekeeping gene, which reflect the degree of selection operating on a given locus (a low ratio indicates a limited contribution of environmental selection to the sequence variation of the housekeeping gene)
- Number of different STs in the population studied

3.5.3. Clustering of Allelic Profiles

Clustering of allelic profiles by UPGMA (unweighted pair-group method with arithmetic averages). The START program is used to elaborate a matrix of pairwise similarities between the allelic profiles. The examination of the dendrogram (*see* **Fig. 6.2** for an example in *C. difficile*) will allow:

- To highlight the main phylogenetic lineages in the bacterial population studied
- To check for putative correlation between ST or lineage and geographic origin, host, clinical course, toxigenic type, etc.

3.5.4. Composite Sequence-Based Analysis

Since a single nucleotide polymorphism as well as multiple polymorphic sites are sufficient to generate a new allele, there may be a bias in the estimation of genetic distance between isolates, which considers alleles differing by only one point mutation or by multiple polymorphic sites in the same manner. To avoid this bias, the sequenced gene fragments of the loci analyzed can be spliced together to obtain a concatenated composite sequence for each of the isolates, in order to determine the overall divergence of the sequences of the loci analyzed (*see* **Fig. 6.3** for an example in *C. difficile*).

3.5.5. Estimation of Relative Contributions of Recombination and Mutation to Genomic Evolution in the Population Studied

- Visual examination of sequences: visual examination of sequences can reveal recombinational events as a portion of sequence exhibiting a high proportion of polymorphic sites
- The index of association (Ia) (16) gives a quantitative evaluation of the linkage between alleles from the different loci. The observed variance in the distribution of allelic mismatches in all pairwise comparisons of the allelic profiles is compared to that expected in a freely recombining population (linkage equilibrium)

  Ia is calculated using the formula

$$Ia = (V_{\text{obs}}/V_{\text{exp}}) - 1$$

where V_{obs} is the observed variance of multilocus profiles analyzed and V_{exp} is the expected variance of multilocus profiles in the case of random association between alleles.

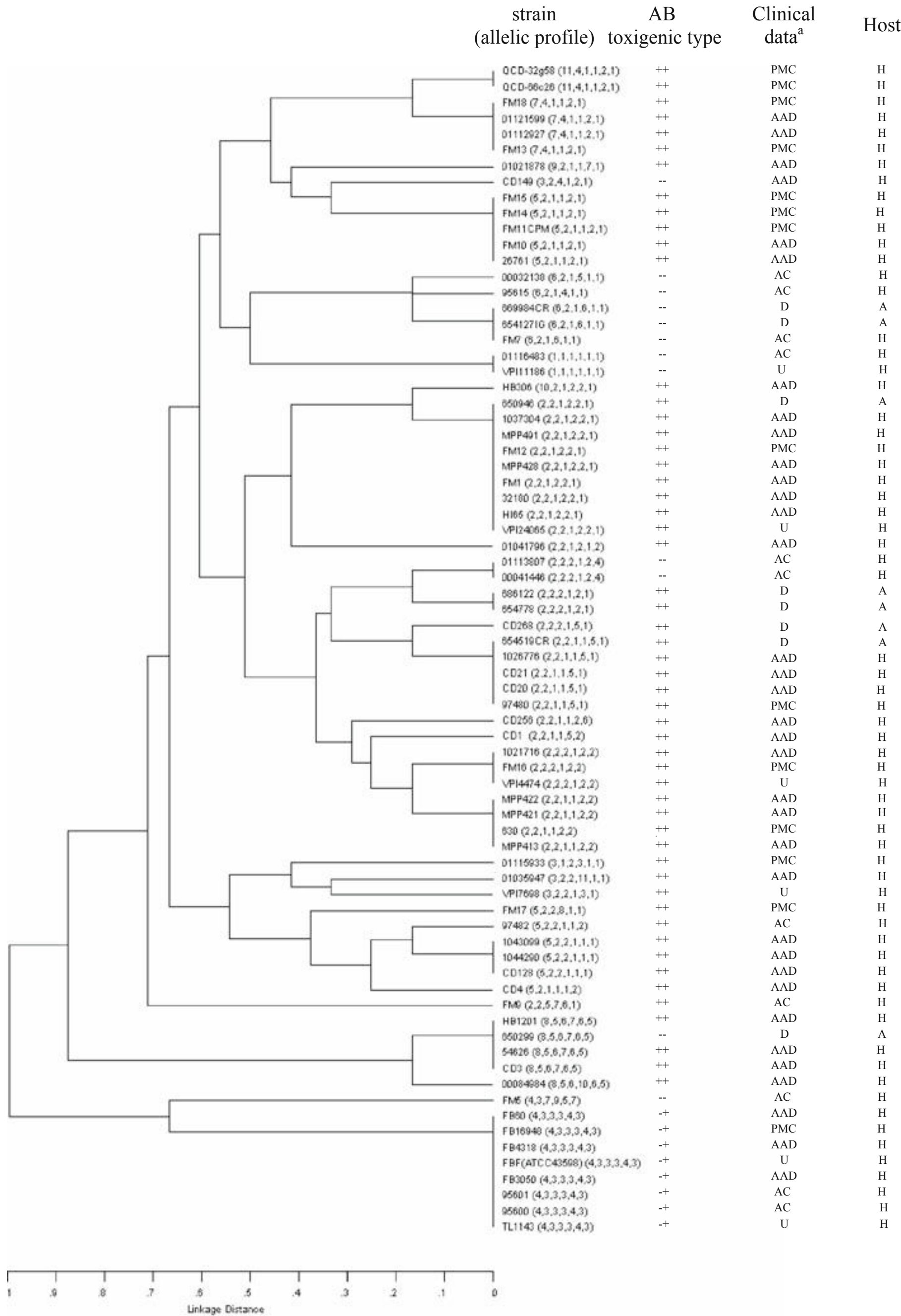


Fig. 6.2. Dendrogram showing clustering analysis (UPGMA) of 74 *C. difficile* isolates after data compilation from six housekeeping genes. AAD, antibiotic-associated diarrhea; D, diarrhea; PMC, pseudomembranous colitis; AC, asymptomatic carriage; U, unknown. H, human; A, animal.

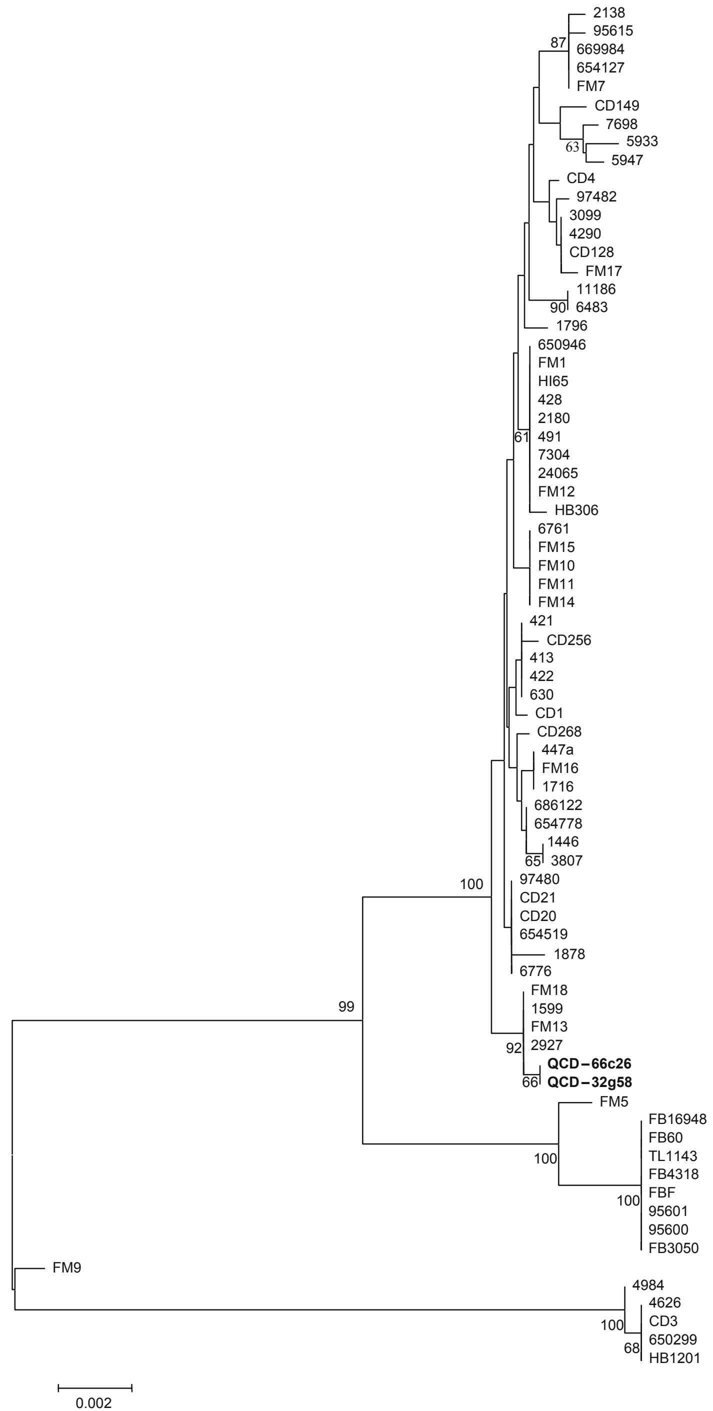


Fig. 6.3. Dendrogram showing genetic relationships of the same 74 *C. difficile* strains as those displayed in **Fig. 6.2** but based on composite sequence of the six housekeeping genes. The topologies of the two dendrograms (**Figs. 6.2** and **6.3**) are comparable, but composite sequence analysis gives a better quantification of the genetic relationships within strains or STs than allelic profiles analysis.

Values near 0 reflect a recombinant population, as described for example in *N. meningitidis* (9), values higher than 0.5 will reflect significant linkage disequilibrium, and thus a clonal evolution mode, as described in *S. aureus* (11) or in *C. difficile* (8).

Of note, a limitation of this approach is that it does not consider the nature of differences between allele sequences (point mutations or recombination of larger sequences).

– Dendrograms based on allelic variation of each gene: dendrograms may be elaborated for each separate locus to check for congruence between loci. The congruence of topologies of trees of different loci suggests a probable coevolution. Conversely, loci exhibiting trees with noncongruent topologies most probably reflect recombinational events and/or environmental selective pressure (*see* **Fig. 6.4** for an example).

– Estimation of relative contributions of recombinational exchange and point mutation to clonal divergence (17). Multilocus combination of allele numbers defines the MLST genotype, also called “sequence type” (ST), as explained before. The eBURST program can cluster STs that share high genetic similarity into clonal complexes (CCs). These STs share 100% genetic identity at all but one housekeeping loci with at least one other member of the CC. The founding genotype for each CC is then defined parsimoniously as the ST that differs from the highest number of other STs in the CC at only one locus out of the loci analyzed. In a CC, the STs that differ from the founding ST in only one out of the loci analyzed are named “single-locus variants” (SLVs). Then it is possible to evaluate the respective roles of mutation and recombination in the microgenomic evolution of the bacterial genome, using comparisons between sequences of the founding ST and its SLVs at the divergent locus (**Fig. 6.5**): (i) if the difference between the founding ST and the SLV consists only in one nucleotide polymorphism, and if this new allele is not present elsewhere in the database, the most probable origin of the divergence is point mutation; (ii) if the difference between the founding ST and its SLV consists in two or more substitutions, the most probable origin of the divergence is recombination. The eBURST program provides also a very useful graphic representation of the filiation of the isolates within a species, but only when a sufficient number of isolates (probably at least 200–300 isolates) have been analyzed. For *C. difficile*, an additional set of isolates is currently under MLST analysis and should allow to obtain such a representation of clonal complexes and their relationships from eBURST analysis.

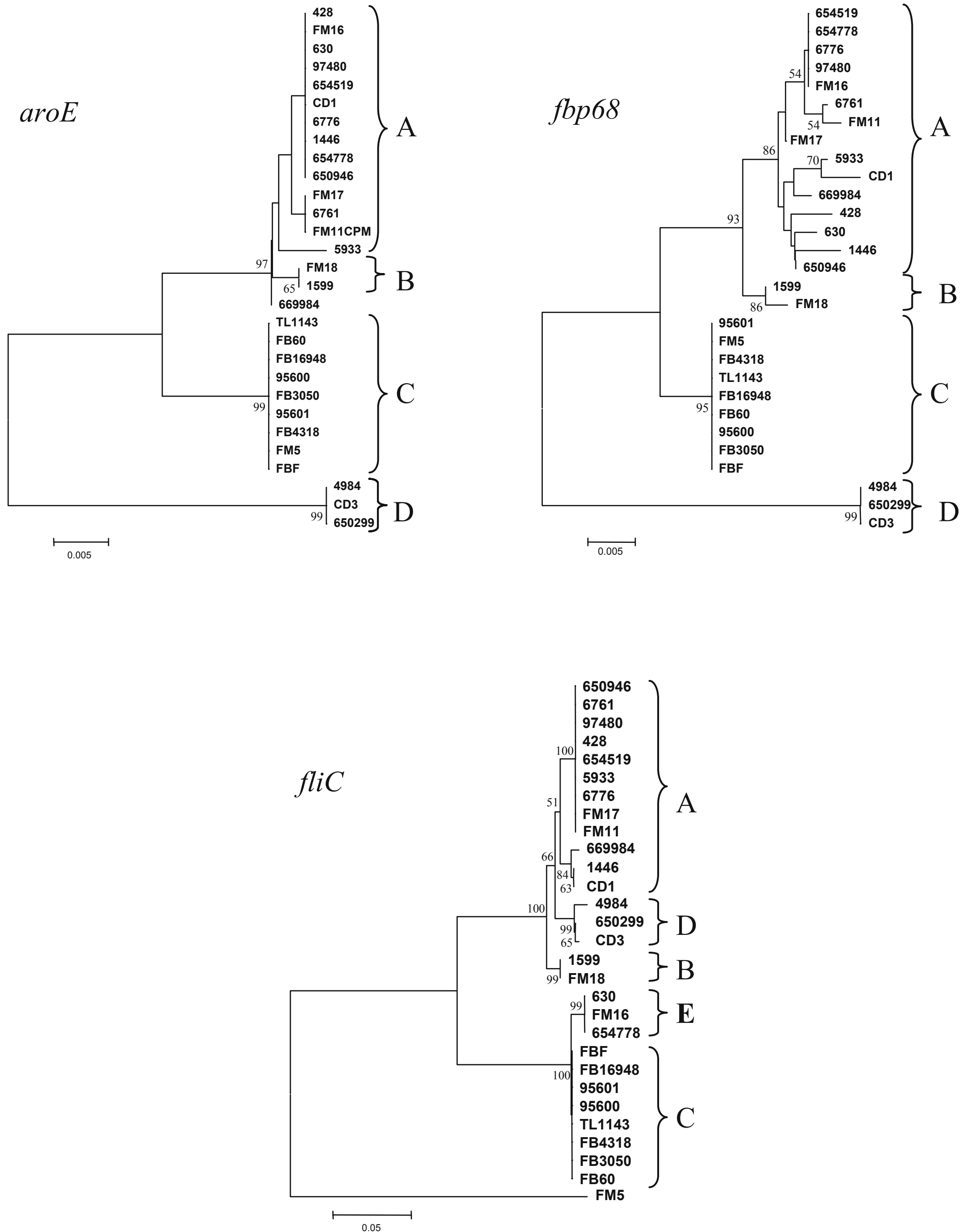


Fig. 6.4. Examples of congruent and noncongruent monolocus trees. Dendrograms show genetic relationships between *C. difficile* isolates based on allele sequences of three individual loci. For this species, the dendrograms from *aroE* (a housekeeping gene) and *fbp68* (a virulence-associated gene) are strongly congruent; conversely, the dendrogram from *fliC* (a virulence-associated gene) is noncongruent with *aroE* and *fbp68* (*see* cluster E in the *fliC* tree).

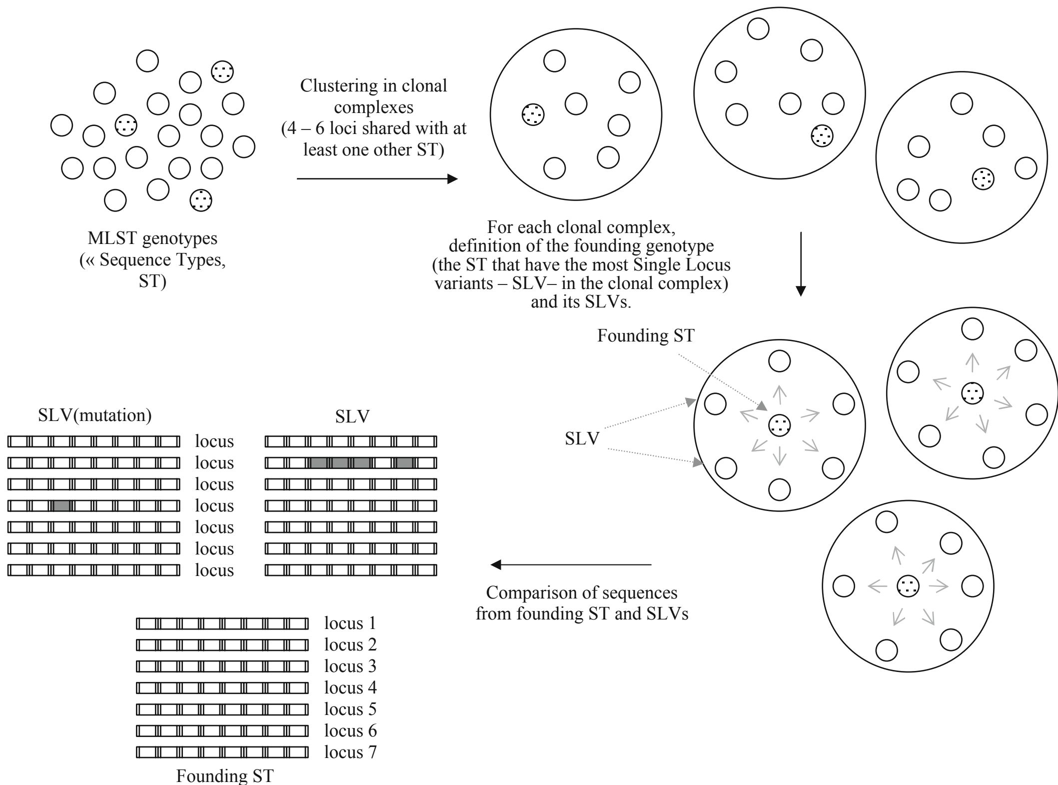


Fig. 6.5. Analysis of clonal complexes for the estimation of rates of mutation/recombination in the genome microevolution of a bacteria using MLST data and the eBURST program.

3.6. Applications of MLST in C. difficile

MLST provides a sequence-based molecular approach that is suitable for population genetics, global epidemiology, and phylogeny of *C. difficile*, since it records neutral variations accumulating within the sequences of housekeeping genes. Compared to molecular typing methods, MLST may be less discriminating than methods exploring genome regions exhibiting rapid variability, but reflects more ancestral genetic relationships and is thus suitable for phylogenetic analysis.

Our experience in MLST of *C. difficile* led us to raise the following questions (8): what is the correlation between human and animal isolates, is there any correlation between ST or lineage and toxigenic type (based on toxins A and B encoding genes), is there any correlation between ST or lineage and clinical severity of digestive tract infection (diarrhea or pseudomembranous colitis), what is the extent of clonality (linkage disequilibrium between alleles) in *C. difficile*, what are the relative impact of recombination and mutation on clonal divergence in *C. difficile*?

From the 74 strains initially analyzed, allelic profiles allow the definition of 32 different sequence types (STs) (8). These STs do not correlate with geographic source, but do correlate to toxigenic type. The dendrogram generated from a matrix of

pairwise genetic distances shows that animal strains analyzed do not constitute a distinct lineage from human strains and that no hypervirulent lineage can be characterized within the population of toxigenic human strains studied (strains recovered from pseudomembranous colitis and antibiotic-associated diarrhea do not cluster in distinct lineages). However, A–B+ variant strains share the same ST which appears as a divergent lineage in the population studied, indicating a single evolutionary origin. The population structure was further examined by analysis of allelic polymorphism. The dendrogram generated from composite sequence-based analysis reveals a homogeneous population associated with three divergent lineages, one of which is restricted to A–B+ variant strains. *C. difficile* exhibits a clonal population structure, as revealed by the estimation of linkage disequilibrium (*Ia*) between loci. The analysis of alleles within clonal complexes estimates that point mutation generates new alleles at a frequency eightfold higher than recombinational exchange, and the congruence of the dendrograms generated from separate housekeeping loci confirms the mutational evolution of this species. However, many of MLST analysis and information on population genetics of *C. difficile* will be clarified when more strains are analyzed; it would be, for example, very interesting to explore the phylogenetic origin of the PCR-ribotype 027 hypervirulent lineage. We are currently increasing and developing the MLST database on *C. difficile* (http://www.pasteur.fr/recherche/genopole/PF8/mlst/index.html), focusing on representative European strains and, among others, on 027 strains.

When applied to virulence-associated genes, MLST (that should be named MvLST in this case) may compare their evolution to that of housekeeping genes and provide information on phylogeny of virulence-associated genes (toxins genes, colonization-associated genes), and on the impact of recombination and/or environmental (digestive tract) selective pressure on their evolution (12). In addition, the selection of two or three highly polymorphic virulence-associated genes allows molecular typing schemes based on unambiguous sequence data that are well suited to short epidemiology (investigation of nosocomial or community spread) and that can be shared by various laboratories. Virulence-associated genes such as *slpA*, *cwp66*, or *fliC* are putatively interesting targets in this approach.

4. Notes

1. *MLST analysis.* Although the majority of parameters of MLST analysis can be performed using free programs and software as mentioned before, commercially available

software suites, such as Bionumerics (Applied Maths NV®), can also be used and are easier to use, especially when using MLST on large scale.

2. *DNA extraction from bacterial isolates.* In our experience, although simple boiling or automated extraction can be used, the best way is the use of a commercial kit, such as InstaGene Matrix (Biorad®): It is faster and cheaper than automated extraction and produces more stable DNA than simple boiling, if PCRs are not immediately performed.
3. *PCR amplification of housekeeping genes.* Although the PCR mixture described before (**Section 2.2**) gives good results, it is easier to use a ready-to-use preMIX for PCR amplification, such as ReddyMix, ABGene®: steps of PCR and gel electrophoresis are greatly simplified, and in our experience, no hampering in the purification of PCR products or in the sequencing reaction was observed. In addition, for some strains and for some loci, especially *dutA*, a better rate of amplification can be achieved with a 55–45°C touchdown PCR protocol (instead of 60–50°C protocol).

References

1. van den Berg RJ, Schaap I, Templeton KE, Klaassen CH and Kuijper EJ. (2007) Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable-number tandem-repeat analysis. *J Clin Microbiol* **45**, 1024–1028.
2. Barbut F, Mario N, Delmee M, Gozian J and Petit JC. (1993) Genomic fingerprinting of *Clostridium difficile* isolates by using a random amplified polymorphic DNA (RAPD) assay. *FEMS Microbiol Lett* **114**, 161–166.
3. Cartwright CP, Stock F, Beekmann SE, Williams EC and Gill VJ. (1995) PCR amplification of rRNA intergenic spacer regions as a method for epidemiologic typing of *Clostridium difficile. J Clin Microbiol* **33**, 184–187.
4. Klaassen CH, van Haren HA and Horrevorts AM. (2002) Molecular fingerprinting of *Clostridium difficile* isolates: pulsed-field gel electrophoresis versus amplified fragment length polymorphism. *J Clin Microbiol* **40**, 101–104.
5. Goorhuis A, Van der Kooi T, Vaessen N, Dekker FW, Van den Berg R, Harmanus C, van den Hof S, Notermans DW and Kuijper EJ. (2007) Spread and epidemiology of *Clostridium difficile* polymerase chain reaction ribotype 027/toxinotype III in The Netherlands. *Clin Infect Dis* **45**, 695–703.
6. Killgore G, Thompson A, Johnson S, Brazier J, Kuijper E, Pepin J, Frost EH, Savelkoul P, Nicholson B, van den Berg RJ, et al. (2008) Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein a gene sequence typing. *J Clin Microbiol* **46**, 431–437.
7. Curry SR, Marsh JW, Muto CA, O'Leary MM, Pasculle AW and Harrison LH. (2007) *tcdC* genotypes associated with severe TcdC truncation in an epidemic clone and other strains of *Clostridium difficile. J Clin Microbiol* **45**, 215–221.
8. Lemee L, Dhalluin A, Pestel-Caron M, Lemeland JF and Pons JL. (2004) Multilocus sequence typing analysis of human and animal *Clostridium difficile* isolates of various toxigenic types. *J Clin Microbiol* **42**, 2609–2617.
9. Feil EJ, Maiden MC, Achtman M and Spratt BG. (1999) The relative contributions of recombination and mutation to the

divergence of clones of *Neisseria meningitidis. Mol Biol Evol* **16**, 1496–1502.
10. Enright MC and Spratt BG. (1998) A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. *Microbiology* **144**(Pt 11), 3049–3060.
11. Feil EJ, Cooper JE, Grundmann H, Robinson DA, Enright MC, Berendt T, Peacock SJ, Smith JM, Murphy M, Spratt BG, Moore CE and Day NP. (2003) How clonal is *Staphylococcus aureus*? *J Bacteriol* **185**, 3307–3316.
12. Lemee L, Bourgeois I, Ruffin E, Collignon A, Lemeland JF and Pons JL. (2005) Multilocus sequence analysis and comparative evolution of virulence-associated genes and housekeeping genes of *Clostridium difficile*. *Microbiology* **151**, 3171–3180.
13. Zhang W, Jayarao BM and Knabel SJ. (2004) Multi-virulence-locus sequence typing of *Listeria monocytogenes. Appl Environ Microbiol* **70**, 913–920.
14. Stubbs SL, Brazier JS, O'Neill GL and Duerden BI. (1999) PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. *J Clin Microbiol* **37**, 461–463.
15. Bidet P, Barbut F, Lalande V, Burghoffer B and Petit JC. (1999) Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. *FEMS Microbiol Lett* **175**, 261–266.
16. Smith JM, Smith NH, O'Rourke M and Spratt BG. (1993) How clonal are bacteria? *Proc Natl Acad Sci USA* **90**, 4384–4388.
17. Feil EJ, Holmes EC, Bessen DE, Chan MS, Day NP, Enright MC, Goldstein R, Hood DW, Kalia A, Moore CE, Zhou J and Spratt BG. (2001) Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences. *Proc Natl Acad Sci USA* **98**, 182–187.

Part IV

Biochemistry of the Organism

Chapter 7

Molecular Methods to Study Transcriptional Regulation of *Clostridium difficile* Toxin Genes

Ana Antunes and Bruno Dupuy

Abstract

Toxin A (TcdA) and Toxin B (TcdB) are the major virulence factors that contribute to the pathogenesis of *Clostridium difficile*-associated diarrhoea (CDAD). These enterotoxins act by glucosylation of members of the Rho protein family of small GTP-binding proteins. This leads to the disorganization of the host cell actin cytoskeleton (cytopathic effect) and apoptosis (cytotoxic effect). Due to their glucosyltransferase activity, they are referred as "clostridial glucosylating toxins". The severe form of CDAD has been recently correlated to the levels of toxin production. This reinforces the idea that regulation of toxin production is an important part of the *C. difficile* infection. Genes encoding TcdA (*tcdA*) and TcdB (*tcdB*) are present in a pathogenicity locus (PaLoc) that also includes three accessory genes: *tcdR*, *tcdE* and *tcdC*. TcdR is an alternative RNA polymerase sigma factor that positively regulates toxin gene transcription as well as its own. TcdE has high homologies with bacteriophage holin proteins. TcdC negatively regulates toxin synthesis by interfering with the RNA polymerase formed with TcdR. Therefore, TcdR and TcdC constitute specific regulators of toxin gene transcription thereby tightly regulating toxin synthesis. In addition a variety of environmental signals, such as the presence of carbon sources or amino acids in the growth medium, and temperature also regulate toxin synthesis.

Key words: TcdA, TcdB, TcdR, TcdC, *Clostridium difficile*, regulation, toxin synthesis.

1. Introduction

Clostridium difficile is one of the major pathogenic clostridia. *C. difficile*-associated diarrhoea is the most frequently occurring nosocomial diarrhoea in many hospitals of industrialized countries. Most of the virulent strains of *C. difficile* produce two toxins: toxin A (TcdA) and toxin B (TcdB) that are single-chain proteins with high molecular weight of 308 and 270 kDa,

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_7, © Springer Science+Business Media, LLC 2010

respectively, and are regarded as the primary virulence factors. The toxins are endocytosed by the intestinal epithelial cells and when they are inside the cell, both toxins monoglucosylate and inactivate members of the Rho protein family of small GTP-binding proteins leading to the modification of the actin cytoskeleton, loss of cell shape (cytopathic effect) and to the induction of apoptosis (cytotoxic effect) (1, 2). TcdA and TcdB are classified as typical AB toxins harbouring a catalytic domain and a binding/translocation domain. The N-terminal possesses the glucosyltransferase activity and the receptor-binding domain is located at the C-terminus. Both toxins are part of the "clostridial glucosylating toxins" family, defined by their inherent glucosyltransferase (GT) activity (1). Some toxinogenic strains also produce a binary toxin with actin-specific ADP-ribosyltransferase activity named "*Clostridium difficile* toxin" (CDT). This toxin is the product of two genes (*cdtA* and *cdtB*) encoding, respectively, the enzymatic and the binding components. CDT causes enterotoxic effects independently of TcdA and TcdB but is not required for the virulence of *C. difficile* (3) and its role in CDAD pathogenesis remains to be identified. The severest forms of CDAD have recently been correlated to the toxin levels during host infection (4), reinforcing the idea that regulation of toxin production is central to *C. difficile* pathogenesis. The genes encoding TcdA (*tcdA*) and TcdB (*tcdB*) are present in a pathogenicity locus (PaLoc) of 19.6 kb which is only found in *C. difficile* toxinogenic strains (5). This locus possesses in addition three accessory genes, *tcdR*, *tcdE* and *tcdC*. We have shown through in vitro run-off transcription and gel retardation experiments that *tcdR* encodes a specific RNA polymerase sigma factor that activates toxin genes transcription as well as its own promoter (6, 7). In fact, TcdR is part of a new group of the σ^{70} family (group V) that also includes UviA, a sigma factor that directs the bacteriocin gene transcription of *Clostridium perfringens*; BotR and TetR that activate transcription of the botulinum and tetanus toxin genes, respectively (8). The gene at the right end of the PaLoc named *tcdC* encodes a negative regulator of toxin genes expression. In fact, *tcdC* is expressed at high levels during exponential growth and is shut off at the onset of stationary phase, coincident with the start of transcription of all other *tcd* genes. We have recently shown using different in vitro approaches such as gel retardation, run-off transcription assays and surface plasmon resonance binding assays that TcdC is capable of interfering with the ability of TcdR-containing holoenzyme to recognize the *tcd* promoters (9). Finally, the last accessory gene of the PaLoc is *tcdE* that encodes a holin-like protein that could play a role in toxins secretion, as both toxins lack a signal peptide (10). Toxins as well as their regulatory proteins are regulated in response to a variety of environmental signals. We demonstrated through RNAse

protection assays that glucose or any other sugar transported by the phosphotransferase system (PTS) inhibits transcription of toxin genes (11). Similarly, it has been shown that transition from an ambient temperature to mammalian body temperature modifies toxin production to a significant extent (12). Moreover, cysteine and its derivates also strongly inhibit toxin production when present in excess in the growth medium (13). Other physiological conditions, such as the presence of bicarbonate or biotin, variation of the oxidation–reduction potential, and subinhibitory concentrations of antibiotics (14–17) also contribute to the regulation of toxin synthesis, demonstrating the complexity of the regulation of *C. difficile* pathogenicity in response to environmental stresses.

2. Materials

All solutions are prepared with DEPC-treated water.

2.1. Primer Extension to Define the 5′-Ends of Toxin Gene mRNAs

2.1.1. Total RNA Extraction (see **Note 1**)

1. Trizol (Gibco-BRL) (storage at 4°C and avoid light contact).
2. Lysis solution: 10 mM Tris–HCl pH 7.0, 10 mM EDTA, 20% sucrose.
3. Lysozyme (make a 100 mg/ml solution and prepare single use aliquots that are kept at –20°C).
4. Ammonium acetate 10 M solution.

2.1.2. Radioactive Labelling of Oligonucleotides

1. T4 polynucleotide kinase (New England Biolabs).
2. 150 μCi [γ-^{32}P]ATP (Amersham).
3. Ammonium acetate 10 M solution.

2.1.3. Primer Extension Reaction

1. Ammonium acetate 10 M solution.
2. Glycogen (20 mg/ml).
3. Hybridization buffer: 80% formamide, 0.5 M NaCl, 1 mM EDTA, 40 mM PIPES pH 6.8.
4. RT buffer: 50 mM Tris pH 8.0, 50 mM KCl, 8 mM $MgCl_2$ and prior to use 4 mM DTT.
5. ATP, CTP, GTP 10 mM (Roche).
6. RNasin, AMV reverse transcriptase enzyme (Promega).
7. RNase A solution (1 mg/ml).

8. EDTA 0.5 M solution.
9. Phenol–chloroform solution (v/v).
10. Loading dye: 98% formamide, 10 mM EDTA, 0.2% bromophenol blue, 0.2% xylene cyanol.

2.1.4. DNA Sequencing

1. Sequenase^{TM} Version 2.0 DNA sequencing kit (USB).
2. [α-^{35}S] dATP (Amersham).
3. TE 1X buffer: 10 mM Tris, 1 mM EDTA, pH 7.6.

2.1.5. Sequencing DNA Gel

1. 40% acrylamide/bis solution (19:1) (Bio-Rad) (*see* **Note 2**).
2. Urea (Bio-Rad).
3. Ammonium persulfate (APS) 10% solution (prepare in water and *see* **Note 3**).
4. *N,N,N,N′*-tetramethylethylenediamine (TEMED, Bio-Rad).
5. TBE 1X solution: 90 mM Tris–HCl, 64.6 mM boric acid, 2.5 mM EDTA, pH 8.3.
6. PlusOne^{TM} Repel-Silane ES (Amersham).

2.2. RNase Protection Assay to Define the Relevant Transcription Units and Confirm the 5′-Ends of Toxin Gene mRNAs

2.2.1. Probes Synthesis

1. Riboprobe® in vitro transcription system (Promega).
2. GENECLEAN® kit (Q-BIOgene).
3. Transcription buffer (5X): 200 mM Tris (pH 7.9), 30 mM $MgCl_2$, 10 mM spermidine and 50 mM NaCl.
4. Dithiotheritol (DTT) 0.75 M solution.
5. ATP, CTP, GTP 10 mM and UTP 1 mM (Roche).
6. 50 μCi [α-^{32}P]UTP (Amersham).
7. RNAsin (2 U/μl) and RQI DNAse (Promega).
8. Phenol–chloroform solution (v/v).
9. Ammonium acetate 10 M solution.
10. Glycogen (20 mg/ml).

2.2.2. Hybridization of Labelled Probe to Total RNA

1. Sodium acetate 3 M solution.
2. Hybridization buffer: 80% formamide, 40 mM PIPES, pH 6.4, 0.4 M NaCl, 1 mM EDTA.

2.2.3. Digestion of Unhybridized RNA Regions

1. T2 digestion buffer: 50 mM NaOAc pH 5.4, 2 mM EDTA, 0.1 M NaCl, 10 U RNAse T2 (Ambion) and kept at 4°C (*see* **Note 4**).
2. 20 mg/ml proteinase K solution.
3. Sodium acetate 0.3 M solution.

4. Glycogen (20 mg/ml).
5. Loading dye buffer: 98% formamide, 10 mM EDTA, 0.2% bromophenol blue, 0.2% xylene cyanol.
6. Preparation of a denaturing gel of 5% polyacrylamide-7 M urea (*see* **Section 2.1.5**).

2.3. Construction of Transcriptional Fusions and Measurement of Reporter Gene Activity (with β-Glucuronidase) (see Note 13)

1. Z buffer: 60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, pH 7.0 and add prior to use 50 mM β-mercaptoethanol). Do not autoclave.
2. Toluene (caution, very toxic).
3. PNPG substrate solution [PNPG (*para*-nitrophenyl-β-D-glucuronide, N-627, Sigma) 2 mg/ml].
4. Na_2CO_3 1 M solution.

2.4. Overexpression and Purification of Regulator Proteins (TcdR and TcdC)

2.4.1. Overexpression and Purification of His-Tagged Proteins

1. Luria–Bertani (LB) medium: 1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5, H_2O up to 1 l.
2. Lysis buffer: 20 mM Tris–HCl pH 7.9, 0.3 M NaCl, 10% (v/v) glycerol, 10 mM imidazole.
3. Complete Mini free EDTA Protease inhibitor cocktail (Roche).
4. Ni^+-NTA resin (Qiagen).
5. Imidazole solution at 500 mM (fresh solution).
6. Elution buffer: 20 mM Tris–HCl pH 7.9, 0.3 M NaCl, 10% (v/v) glycerol.

2.4.2. Preparation of 12 % Sodium Dodecyl Sulfate Polyacrylamide Gel (SDS-PAGE)

1. 0.25 M Tris–HCl pH 6.8, and 0.75 M Tris–HCl pH 8.8.
2. SDS 10%.
3. 40% acrylamide/bis solution (37.5:1, Bio-Rad) (*see* **Note 2**).
4. APS 10% (*see* **Note 3**) and TEMED.
5. Isobutanol solution. Store at room temperature away from the light.
6. Laemli buffer (10X): 190 mM Tris, 1.9 M glycine, 1% SDS. Store at room temperature.
7. Prestained molecular weight marker (Invitrogen).

2.4.3. Identification of Fractions with His-Tagged Proteins and Storage

1. Protein loading buffer (2X): 0.1 M Tris pH 6.8, 0.2 M DTT, 4% SDS, 0.2% bromophenol blue, 20% (v/v) glycerol.
2. Dialysis buffer: 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 50% (v/v) glycerol.
3. Protein assay dye reagent concentrate (Bio-Rad).

2.5. Gel Retardation Assays to Show Interactions of Regulator Proteins (TcdR or TcdC) with Toxin Gene Promoters

2.5.1. Radioactive Labelling of DNA Probes

1. [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham).
2. T4 polynucleotide kinase (New England Biolabs).

2.5.2. Preparation of a 4.5 % Polyacrylamide TBE 1X Gel

1. TBE 10X solution: 900 mM Tris–HCl, 646 mM Boric acid, 25 mM EDTA, pH 8.3.
2. 40% acrylamide/bis solution (37.5:1, Bio-Rad) (*see* **Note 2**).
3. APS 10% (*see* **Note 3**) and TEMED.

2.5.3. Protein–DNA Interaction

1. Glutamate buffer: 40 mM HEPES, pH 8.0, 100 mM $MgCl_2$, 100 mM potassium glutamate, 500 μg/ml BSA.
2. Holo or core enzyme of the *Escherichia coli* RNA polymerase (Epicentre).
3. Heparin–dye solution: 150 μg/ml heparin, 0.1% bromophenol blue, 50% (v/v) sucrose.

2.6. In Vitro Run-Off Transcription Assay

1. RNAP 5X buffer: 200 mM Tris–HCl pH 8.0, 50 mM $MgCl_2$, 0.5 mM EDTA pH 8.0, 0.5 M KCl, 0.5 mg/ml BSA, 25% (v/v) glycerol.
2. NTP mix: 2 mM ATP, GTP, CTP and 0.5 mM UTP (Roche).
3. RNAsin (2 U/μl) (Promega).
4. 20 mM DTT solution (fresh solution).
5. Holo or core enzyme of the *E. coli* RNA polymerase (Epicentre).
6. DNA sequencing loading buffer: 98% formamide, 10 mM EDTA, 0.2% bromophenol blue, 0.2% xylene cyanol.

2.7. Dot-Blot/Affinity Chromatography Assays

2.7.1. Immunoblot Detection of TcdR or TcdC – Core Enzyme Interaction

1. Nitrocellulose membrane (Amersham).
2. Phosphate buffered saline 10X (PBS): 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4, H_2O up to a litre, pH 7.4. Autoclave 120°C for 20 min.
3. Blocking buffer: PBS 1X with 5% powdered milk.
4. TBS buffer: 30 ml NaCl 5 M, 50 ml Tris–HCl 1 M pH 7.5 and H_2O up to 1 l.

5. TBS milk buffer: TBS buffer with 50 g of powdered milk.
6. Rabbit anti-TcdR antibody (6).
7. Rabbit antibodies (Amersham).
8. Dithiobis(succinimidyl)propionate (DSP, Sigma) 50 mM (prepare a fresh solution in chloroform or acetone and store at 4°C protected from light, *see* **Note 5**).
9. Chemiluminescence kit (Amersham, Pharmacia).

2.7.2. Affinity Chromatography Detection of TcdC–TcdR Interaction

1. Dialysis buffer: 300 mM NaCl, 50 mM sodium phosphate pH 8.0, 50% (v/v) glycerol.
2. Cross-linker buffer: 50 mM HEPES pH 7.5, 2 mM EDTA, 7.5% (v/v) glycerol, Complete Mini free EDTA Protease inhibitor cocktail (Roche).
3. Cross-linker buffer with 50 mM imidazole.
4. 0.1 M glycine.
5. Ni^{+}-NTA resin (Qiagen).
6. Mouse anti-TcdR antibodies (7).

3. Methods

C. difficile toxin production and regulation is a very complex mechanism that allows efficient synthesis of toxins according to specific environmental conditions (11, 12). In order to understand toxin gene expression, the first step is to identify and to characterize *C. difficile* toxin promoters. This can be accomplished through primer extension that measures the 5′-ends of toxin transcripts including the transcription start point (+1). To verify that toxin gene mRNA 5′-ends deduced from primer extension are not the result of an abortive transcription, we can perform RNAse T2 protection assays. This technique also defines the relevant transcriptional unit(s) more precisely and it showed that *tcdA* and *tcdB* are transcribed mainly from their own promoters in late exponential phase although monocistronic transcripts are also detected but much less abundant (11, 18, 19). The identification of toxin promoters is a necessary step before undertaking in vivo studies of toxin gene expression. Accordingly, transcriptional fusion vectors are constructed using *E. coli gusA* as a reporter gene and transformed in *C. perfringens* used as a surrogate host (6, 11) (*see* **Note 13**). Until recently *C. perfringens* was chosen because no procedure was available to transform

and manipulate *C. difficile* (*see* **Chapter 6**). Toxin promoters alone are not sufficient to induce toxin transcription. However, when we introduced the *tcd–gusA fusions* into the surrogate host carrying a plasmid expressing *tcdR*, expression of the GusA activity is detected (example **Fig. 7.3**) indicating that TcdR acts as a positive regulator of toxin expression (6, 20). The same approach was used to show that TcdC negatively regulates toxin gene transcription (9, 19). To further characterize the molecular mechanism by which TcdR and TcdC regulate toxin gene transcription, overexpression and purification of both proteins can be carried out and interactions with toxin promoters or with the RNA polymerase can be demonstrated by gel retardation assays, in vitro run-off transcription and dot-blot analysis. The results of these techniques demonstrated that TcdR does not interact with toxin promoters by itself but it acts as an alternative sigma factor. TcdR interacts directly with RNA polymerase core enzyme and confers to the holoenzyme complex formed with TcdR the ability to recognize toxin promoters (6). TcdC does not interact directly with toxin promoters, however, it interacts with the RNA core enzyme and TcdR, as shown by affinity chromatography experiments, thereby destabilizing the TcdR-containing holoenzyme (9). Finally, all these molecular methods can be applied to study the mode of action of the regulators and their target genes in *C. difficile*.

3.1. Primer Extension

3.1.1. Total RNA Extraction (see **Note 1**)

1. Pick 5 ml of culture at the time-phase chosen and centrifuge at 12,000×*g* for 20 min at 4°C.
2. Resuspend pellet in 200 μl of lysis solution containing 1 mg of lysosyme. Incubate 30 min at 37°C. Wash cell protoplasts once in the same buffer.
3. Add 1 ml TRIzol and incubate 5 min at room temperature.
4. Add 200 μl chloroform and vortex.
5. Centrifuge at 12,000×*g* for 15 min at 4°C.
6. Transfer the aqueous phase to a new tube.
7. Add 0.5 ml isopropanol and leave 30 min at –20°C to precipitate RNA.
8. Centrifuge at 12,000×*g* for 20 min at 4°C.
9. Wash pellet with 1 ml of 70% (v/v) ethanol. Centrifuge at 12,000×*g* for 5 min at 4°C. Briefly dry (*see* **Note 6**).
10. Resuspend pellet in 50–100 μl of H_2O-DEPC (*see* **Note 7**).

3.1.2. Radioactive Labelling of Oligonucleotides

1. Radioactively labelled oligonucleotides are generated with T4 polynucleotide kinase by mixing 50 pmol of the oligonucleotide with 1 μl of polynucleotide kinase buffer 10X,

3 µl [γ-^{32}P]ATP (150 µCi final), 10 U of T4 polynucleotide kinase and H_2O-DEPC up to 10 µl final. Incubate at 37°C for 30 min. Inactivate enzyme by heating at 70°C for 10 min (*see* **Note 8A**).

2. Precipitate the DNA to remove all other components, by adding 120 µl of ammonium acetate, 40 µl of H_2O and 550 µl of ice-cold ethanol 100%. Incubate 30 min at –20°C.
3. Centrifuge at 12,000×*g* for 20 min at 4°C. Wash the pellet with 70% (v/v) ethanol. Centrifuge at 12,000×*g* for 10 min at 4°C and briefly dry the pellet.
4. Resuspend in 100 µl of H_2O-DEPC.

*3.1.3. Primer Extension Reaction (*see **Note 8B**)

1. Mix 20 µg of total RNA with 10 µl of the labelled oligonucleotide (2.5 × 10^6 cpm) and H_2O-DEPC up to 50 µl.
2. Precipitate with 120 µl of ammonium acetate, 550 µl of ice-cold ethanol 100% and 1 µl of glycogen. Incubate 30 min on ice. Centrifuge at 12,000×*g* for 20 min at 4°C. Wash the pellet with 70% (v/v) ethanol. Centrifuge at 12,000×*g* for 10 min at 4°C and briefly dry pellet.
3. Resuspend the pellet in 20 µl of hybridization buffer and heat the mix at 80°C for 5 min and cooling to 30°C.
4. Precipitate again with 1 µl of glycogen, 30 µl of H_2O-DEPC, 120 µl of ammonium acetate and 550 µl of ice-cold ethanol 100%. Incubate 30 min on ice. Centrifuge at 12,000×*g* for 20 min at 4°C. Wash the pellet with 70% (v/v) ethanol. Centrifuge at 12,000×*g* for 10 min at 4°C and briefly dry pellet. Resuspend in 25 µl of RT buffer.
5. Add 1 mM of each dNTP, plus 1 µl of RNAsine and 1 µl of AMV reverse transcriptase. Incubate at 42°C for 60 min.
6. Add 1 µl of RNAse A and 2 µl of EDTA. Incubate at 37°C for 30 min.
7. Add H_2O-DEPC up to 100 µl.
8. Extract the nucleic acids by adding equal volume of a phenol/chloroform solution (v/v). Vortex and centrifuge at 12,000×*g* for 5 min at room temperature.
9. Transfer to a new tube the upper phase and precipitate (*see* **Step 2** above).
10. Resuspend the pellet in 5 µl loading dye and load on sequencing DNA gel.

3.1.4. DNA Sequencing

The DNA sequencing reactions of the extension products are performed using the Sequenase Version 2.0 kit according to the manufacture instructions.

3.1.5. Extension Products Analysis by Sequencing DNA Gel

Prepare a 5% polyacrylamide-7 M urea gel to migrate samples.

1. Mix 31.5 g of urea with 7.5 ml of TBE 1X, 9.4 ml of polyacrylamide solution and H_2O-DEPC up to 60 ml. Heat at 50°C and dissolve with agitation.
2. When the mix solution is dissolved, complete with H_2O-DEPC till 75 ml and cooling to room temperature.
3. Meanwhile after cleaning with H_2O (*see* **Note 9**) assemble plates with spacers and clamps.
4. Add 600 μl APS and 60 μl TEMED to the mix. Agitate gently to minimize bubble formation.
5. Using a 50 ml syringe, pour the gel carefully without introducing bubbles.
6. Insert the top spacer. The gel will take 30–45 min to polymerize.
7. Remove the spacer out and clean the wells with TBE 1X buffer running buffer.
8. Pre-run the gel at 50 W for 60 min.
9. Place the reactions in a heat block at 90°C for 5 min and cool them quickly on ice.
10. Load your samples and run the gel at 50 W until the first blue is in the bottom of the gel.
11. Transfer the gel on Whatman paper (3 M) and dry under vacuum.
12. Place the dried gel in an X-ray film cassette with a film and expose it for a suitable time (from 1 h to an overnight). An example of the results is shown in **Fig. 7.1**.

3.2. RNase Protection Assay

3.2.1. Probes Synthesis

Radioactively labelled antisense RNA probes are generated using the Riboprobe® in vitro transcription system.

1. Digest 2 μg of the template plasmid DNA with the appropriate restriction enzyme in order to linearize it.
2. Migrate your digested plasmid in an electrophoresis TAE 1X gel and purify the DNA fragment using the GeneClean kit and resuspend it in H_2O-DEPC to 1 μg/μl DNA concentration.
3. In a sterile microcentrifuge tube add 5 μl transcription buffer, 1 μl of each ATP, CTP and GTP, 1 μl of cold UTP, 1 μl DTT, 1 U RNAsin, 1 μl of linearized plasmid DNA (1 μg), 5 μl [α-^{32}P]UTP (50 μCi total), 10 U of T7 or Sp6 DNA polymerase and H_2O-DEPC to a final volume of 25 μl. Incubate at 37°C for 1 h.

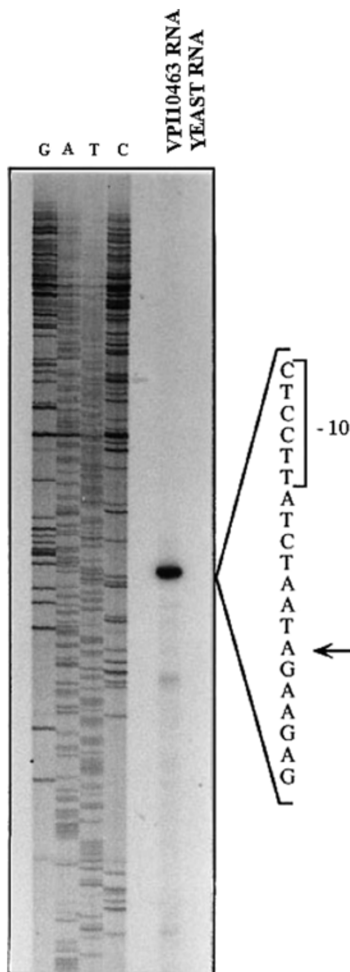


Fig. 7.1. Estimation of the 5′-termini of *tcdA* transcript by primer extension. RNA extracted from *C. difficile* strain VPI10463 was annealed to the specific primer and extended using avian myeloblastosis virus reverse transcriptase. In control experiments, *Saccharomyces cerevisiae* (yeast) RNA was used instead of bacteria RNA. The sequencing reactions (lanes GATC) produced with the same oligonucleotides on a DNA template are aligned to the left of the primer extensions (adapted from Dupuy and Sonenshein (11)).

4. Add 1 μl of RQ1 DNAse and incubate at 37°C for 15 min.
5. Adjust the volume to up 100 μl with H_2O-DEPC and extract with an equal volume of phenol–chloroform solution (v/v).
6. Precipitate nucleic acids with 35 μl of sodium acetate and 325 μl of cold ethanol 100%. Incubate at –20°C for 30 min. Centrifuge at 12,000×*g* for 5 min at 4°C (*see* **Note 11**).
7. Dry the pellet and resuspend in 100 μl H_2O-DEPC.

3.2.2. Hybridization of Labelled Probe total to RNA

1. Mix 2.5×10^5 cpm of radioactively antisense RNA probe with 20 μg of total RNA and complete the volume up to 100 μl with H_2O-DEPC (*see* **Note 10**).
2. Precipitate nucleic acids as in **Section 3.2.1**, Step 6.
3. Dry pellet and resuspend in 20 μl of hybridization buffer (*see* **Note** 7).
4. Heat samples at 70°C and then reset water bath to 42°C and leave it incubate for at least 3 h (*see* **Note 12**).

3.2.3. Digestion of Unhybridized RNA Regions

1. To each reaction add 300 μl of cold T2 digestion buffer. Incubate at 30°C for 60 min.
2. Incubate at 37°C for 30 min with 10 μl proteinase K to stop the reaction.
3. Precipitate RNA hybrid samples with 120 μl of sodium acetate, 1 μl glycogen and 900 μl volume of ethanol 100%. Incubate at –20°C for 30 min.
4. Centrifuge at 12,000×*g* for 15 min at 4°C and wash the pellet in 300 μl of 70% (v/v) ethanol. Centrifuge at 12,000×*g* for 5 min at 4°C and dry the pellet.
5. Resuspend in 5 μl of loading dye buffer.
6. Prepare a denaturing gel of 5% polyacrylamide-7 M urea (*see* **Section 3.1.5**).
7. Pre-run the gel at 50 W for 60 min.
8. Heat samples at 80°C for 3 min and chill them on ice.
9. Load your samples and run the gel at 50 W.
10. Transfer the gel to a Whatman paper 3 M and dry under vacuum.
11. Quantify the radioactivity in the dried gels may be carried out using the Storm PhosphorImager (Molecular Dynamics). An example of the results is shown in **Fig. 7.2**.

3.3. Measurement of β-Glucuronidase Activity of Transcriptional Fusions

Construct of transcriptional fusions such as *tcd* promoter–*gusA* used in *C. perfringens* has been described in Mani and Dupuy (6). In these examples, the *tcd* promoters and their N-terminal coding sequences are fused in-frame to the *E. coli gusA* coding sequence inside an *E. coli–C. perfringens* shuttle vector which is further transformed in *C. perfringens* strain SM101 that do not have β-glucuronidase activity (11). Then we measured the β-glucuronidase activity. For such in vivo transcription studies, other reporter genes have been used as well as other surrogate hosts (*see* **Note 13**).

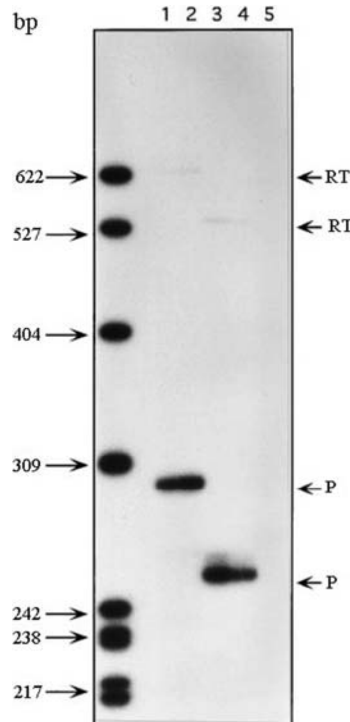


Fig. 7.2. RNase T2 mapping of the 5′-termini of *tcdA* mRNA using two antisense RNA probes (lanes 1–4) or sense RNA probe (lane 5) (adapted from Dupuy and Sonenshein (11)).

3.3.1. β-Glucuronidase Assay

1. Collect 1 ml samples taken at different time growth and record OD_{600}. Centrifuge the cell samples at 12,000×*g* for 5 min at 4°C, remove the supernatant and store pellets at –80°C (*see* **Note 14**).
2. Resuspend pellets in 0.8 ml of Z buffer (with β-mercaptoethanol) and then add 8 μl of toluene (*see* **Note 15**). Tubes are capped and vortex for 1 min.
3. Incubate on ice for 10 min and then at 37°C for 30 min with caps open to evaporate toluene.
4. Add 160 μl of PNPG solution to start the enzyme reaction (T0). Mix and incubate at 37°C until slightly yellow colour develops.
5. Stop reaction by adding 0.4 ml Na_2CO_3 solution (T1) and record incubation time (T1–T0) (*see* **Note 16**).
6. Tubes are centrifuged for 10 min at 12,000×*g* to remove cell debris. Measure the colour reaction in a spectrophotometer at 405 nm and calculate the specific activity in Miller units (*see* **Note 17**A). An example of the results is shown in **Fig. 7.3**.

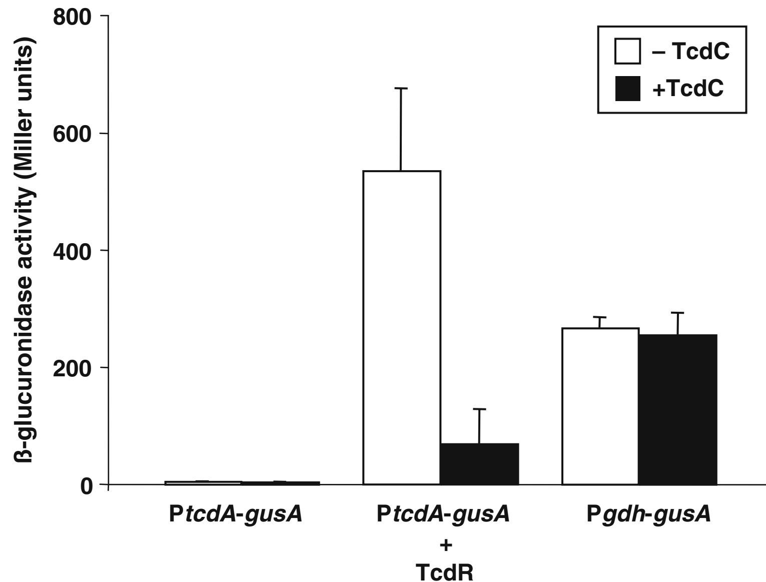


Fig. 7.3. Expression of P*tcdA–gusA* and P*gdh–gusA* fusions in stationary phase cells of *C. perfringens*. Transcriptional fusion vectors were introduced into *C. perfringens* carrying either the empty vector (*left bars*) or the vector-borne *tcdR* gene (*middle bars*). β-Glucuronidase activity was determined in cell containing (*dark bars*) or not (*white bars*) *tcdC* (adapted from Matamouros et al. (9)).

3.4. Overexpression and Purification of Transcriptional Regulators such as TcdR and TcdC

3.4.1. Overexpression and Purification of His-Tagged Proteins

1. *TcdR* and *tcdC* genes are amplified by PCR from *C. difficile* genomic DNA and cloned into an expression vector like pET28b or pET16b (Novagen). These constructs create a translational fusion adding a histidine tag at the C- or N-terminal of the coding sequences, respectively, and place it under the control of the T7 promoter. Then, the plasmids are introduced by electroporation into *E. coli* strain BL21λDE3 (pLysS), carrying the isoproprуl β-D-thiogalactoside (IPTG)-inducible T7 RNA polymerase.
2. Prepare an overnight preculture of *E. coli* strain BL21λDE3 (carrying *tcdR* or *tcdC* overexpression plasmids) in LB broth.
3. Next day inoculate 2 l of LB broth (1/100 dilution) and incubate at 22°C with agitation (*see* **Note 17B**). For cells expressing TcdR, add IPTG at 1 mM final when cells reach OD_{600} = 3 and continue incubation for 3 h (*see* **Note 17B**).
4. Harvest cells by centrifugation at 12,000×*g* for 20 min at 4°C.
5. Resuspend in 20 ml of lysis buffer containing Complete Mini free EDTA Protease inhibitor cocktail and disrupted by sonication.

6. Remove cell debris by centrifugation at 12,000×*g* for 30 min at 4°C.
7. Transfer the supernatant to a new tube and add 0.5 ml of Ni^{+}-NTA resin. Allow proteins to bind to the resin for 1–3 h at 4°C with soft rotation.
8. Wash in batch three times with 5 ml of lysis buffer for 10 min at 4°C.
9. Transfer the resin solution to a chromatography column already equilibrated with lysis buffer.
10. Wash with 5 ml of lysis buffer.
11. The His-tagged proteins are eluted with 5 ml of elution buffer supplemented with a gradient of 40, 60, 100, 200 and 250 mM imidazole.

3.4.2. Prepare a 12 % Sodium Dodecyl Sulfate Polyacrylamide Gel (SDS-PAGE)

1. Wash the glass plates with ethanol and then with H_2O. Assemble the plates with spacers and clamps.
2. Prepare the resolving gel by mixing 3 ml of acrylamide/bis solution with 4.8 ml of Tris–HCl 0.75 M, 2.07 ml of H_2O, 100 μl SDS, 20 μl APS and 10 μl TEMED. Pour the gel leaving space for the stacking gel and overlay with isobutanol solution. Leave it polymerize (30–45 min).
3. Remove the isobutanol and rinse the top of the gel with water and remove traces with Whatman paper.
4. Prepare the stacking gel by mixing 0.4 ml of acrylamide/bis solution with 1.5 ml Tris 0.25 M, 1.1 ml H_2O, 30 μl SDS, 75 μl APS and 10 μl TEMED. Pour the gel on the top of the resolving gel and insert the comb. Leave it polymerize (30 min). Prepare 1 l of Laemli buffer 1X.
5. Remove comb and wash wells with Laemli buffer 1X.

3.4.3. Identification of Fractions with His-Tagged Proteins and Storage

1. Mix 25 μl of each fraction with protein loading buffer and heat at 90°C for 5 min. Load samples on the gel and the pre-stained molecular weight marker.
2. Add the Laemli buffer 1X to the gel chamber and run at 50 mA.
3. Stain the gels with Coomassie brilliant blue.
4. Fractions containing highly purified His-tagged protein are pooled and dialyzed overnight at 4°C against 1 l of dialysis buffer.
5. Protein concentration is determined spectrophotometrically by the Bradford protein method using the Protein Assay Dye Reagent Concentrate.
6. Make aliquots of the protein purified and store them at –80°C.

3.5. Gel Retardation Assays

3.5.1. Radioactive Labelling of DNA Probes

1. PCR amplification of DNA fragments corresponding to the toxin promoters.
2. End-label DNA fragments with T4 polynucleotide kinase and [γ-^{32}P]ATP (*see* **Section 3.1.2**).

3.5.2. Preparation of 4.5 % Polyacrylamide TBE 1X Gel

1. Clean plates with ethanol and distilled H_2O. Assemble plates with spacers and clamps.
2. Mix 3 ml of TBE with 3.4 ml acrylamide/bis solution, 23.4 ml H_2O, 150 μl APS and 50 μl TEMED. Pour the gel and insert the comb. Leave it to polymerize (30 min).
3. Pre-run the gel with TBE 1X buffer for 30 min at 150 V at room temperature.

3.5.3. Protein–DNA Interaction

1. Mix 50–200 nM of *E. coli* RNA polymerase core enzyme with fourfold molar excess of TcdR and add glutamate buffer up to 10 μl final. Incubate for 30 min at 37°C (*see* **Note 18**).
2. Add to the mix 0.2 nM of labelled fragment. Incubate for 60 min at room temperature.
3. Add 3 μl of heparin–dye to the mix and load during electrophoresis (100 V).
4. Run the gel at 200 V for around 2 h.
5. Transfer the gel to a Whatman paper and dry under vacuum.
6. Place the dried gel in an X-ray film cassette with a film and expose it for a suitable time (from 1 h to one overnight). An example of the results is shown in **Fig. 7.4**.

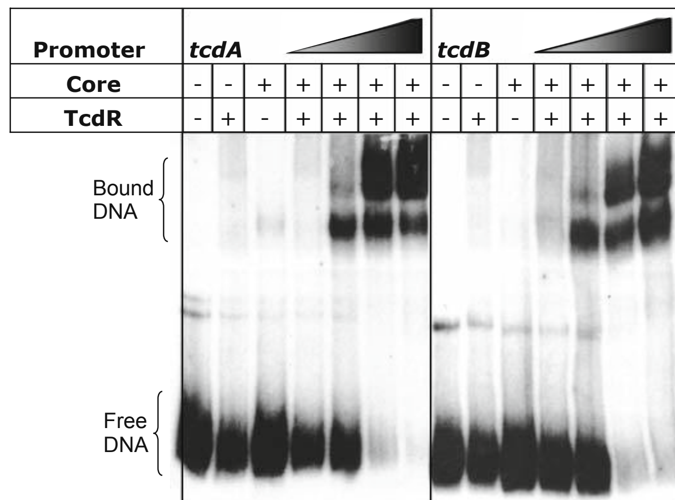


Fig. 7.4. Gel mobility retardation of *tcd* promoters with *E. coli* RNA polymerase core enzyme and increasing amounts of TcdR (*triangles*) (adapted from Mani and Dupuy (6)).

3.6. In Vitro Run-Off Transcription Assay

3.6.1. Labelling of the pBR322–MspI Marker

1. Digest 5 μg of pBR322 with MspI restriction enzyme. Inactivate enzyme by heating at 65°C for 20 min.
2. Mix 5 μl of pBR322 MspI digested with 2.5 μl of One-Phor-All buffer, 2 μl of [α-^{32}P]dCTP, 1 μl of Klenow and H_2O up to 25 μl. Incubate 30 min at room temperature.
3. Add 25 μl of H_2O. Purify DNA with QIAquick columns and elute in 40 μl of H_2O.

3.6.2. In Vitro Transcription Reactions

To create DNA template, linearize a plasmid containing the promoter of interest with a unique downstream restriction enzyme that should generate a run-off transcript around 100–200 nucleotides.

1. Extract DNA plasmid two times with 1 volume of phenol–chloroform solution. Vortex. Centrifuge at 12,000×*g* for 5 min.
2. Transfer as much as possible of the upper phase and precipitate with 2.5 volume of 100% ethanol (30 min at –20°C). Centrifuge 12,000×*g* for 20 min and wash with 70% (v/v) ethanol. Centrifuge 12,000×*g* for 5 min and dry pellet.
3. Resuspend the DNA fragment in appropriate volume of TE to give a final concentration of 1 μg/μl.
4. Preincubate 0.5 mM of *E. coli* RNA polymerase core enzyme with a fourfold excess of the regulator protein for 30 min at 37°C (for TcdR and TcdC *see* **Note 18**).
5. Add to the mix 2 μl RNAP buffer, 1 μl $MnCl_2$, 1 μl NTP mix, 0.25 μl [γ-^{32}P]UTP (2.5 μCi final); 1 μl RNAsin, 0.5 μl DTT and 1.25 μl H_2O-DEPC. Mix gently.
6. Add 1 μl of linearized DNA plasmid. Incubate at 37°C for 10–15 min.
7. Add 5 μl of DNA sequencing loading buffer to samples and heat them at 80°C for 10 min.
8. Prepare a denaturing gel of 5% polyacrylamide-8 M urea, as in **Section 3.1.5** with 36 g of urea instead of 31.5 g.
9. Pre-run the gel at 50 W for 60 min.
10. Load the samples and the pBR322–MspI marker (3 μl) and run at 50 W (*see* **Note 19**).
11. Transfer the gel to a Whatman paper 3 M and dry under vacuum.
12. Expose overnight to 24 h to an X-ray film. An example of the results is shown in **Fig. 7.5**.

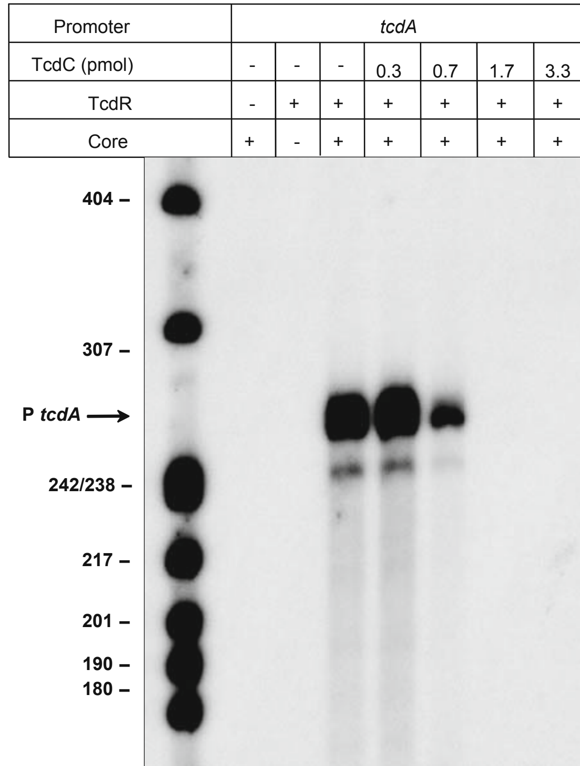


Fig. 7.5. Inhibition of TcdR transcriptional activity by purified TcdC. Run-off transcription reactions were performed using *E. coli* RNAP core enzyme and a DNA fragment containing the *tcdA* promoter, incubate in the absence or presence of TcdR (adapted from Matamouros et al. (9)).

3.7. Dot-Blot/Affinity Chromatography Assays (see Note 20)

3.7.1. Immunoblot Detection of TcdR – Core Enzyme Interaction

1. Immobilize 1 μg of *E. coli* RNA polymerase core enzyme on nitrocellulose membranes.
2. Incubate membranes with 20 ml of blocking buffer at 4°C during an overnight.
3. Remove the blocking buffer.
4. Add 200 μg of whole-cell extract of *E. coli* BL21λDE3 carrying TcdR-expressing plasmid (*see* **Note 21**) to the membranes. Incubate 2 h at 37°C.
5. Wash the membranes with TBS milk 1 h at room temperature.
6. Incubate with rabbit anti-TcdR antibody 1 h at 37°C.
7. Wash the membrane with TBS milk 1 h at room temperature.
8. Incubate with anti-rabbit antibody 1 h at 37°C.
9. Wash three times with TBS milk for 15 min and then with TBS for 15 min.

10. Develop the membrane using an enhanced chemiluminescence kit.

3.7.2. Affinity Chromatography Detection of TcdC–TcdR Interaction

1. Mix 250 μg soluble extract of *E. coli* BL21λDE3 overexpressing TcdR and 1 μg of purified TcdC in cross-linker buffer to a final volume of 500 μl. Incubate 10 min at room temperature.
2. Add DSP to 1 mM final concentration of the reaction mix. Incubate for 1 h at 4°C.
3. Add glycine to 0.1 M final concentration to stop the reaction.
4. Add 50 μl of Ni^{+}-NTA resin to samples. Incubate for an additional 1 h at 4°C with gently agitation, followed by 30 min at 37°C.
5. Wash samples three times with 200 μl of cross-link buffer containing 50 mM imidazole.
6. Add 20 μl of protein loading buffer and heat at 100°C for 5 min.
7. Load samples and run on a 12% SDS-PAGE gel.
8. Execute a Western blot to detect TcdR.

4. Notes

1. There are alternative protocols to extract *C. difficile* RNA, like extracting with small beads and purifying using the RNeasy kit (Qiagen) (21).
2. Avoid contact with unpolymerized acrylamide. It is a neurotoxin that can be absorbed through the skin. Wear gloves and safety glasses when handling solutions containing acrylamide.
3. Make a 10% solution and prepare single use aliquots of ~200 μl that are kept at –20°C.
4. There is a variety of RNases that can be used in the RNase protection assay, with different base cleavage preference. For instance, the RNase T2 cleaves after all four residues, but preferentially after A residues. The RNase T1 cleaves after G residues, RNase A cleaves after C and U residues and in general a mixture of the two is used. Recently *E. coli* RNase I that like RNase T2 cleaves after all ribonucleotides without base preference became commercially available (22).

5. DSP is a homobifunctional, thiol-cleavable and membrane permeable cross-linker. It is used in chemical cross-linking of intracellular protein prior to cell lysis, immunoprecipitation and for the permanent bond of transient weak proteins interactions (23, 24).
6. Leave it dry at room temperature and do not vacuum dry (it may damage the RNA).
7. To determine the purity of the RNA, the ratio A260/A280 should be between 1.9 and 2.1. If not, repeat a phenol–chloroform extraction and ethanol precipitation. To check the RNA integrity run the RNA on a denaturing agarose gel and stain with ethidium bromide.

8A. Polynucleotide kinase is inhibited by ammonium ions, therefore, DNA should not be precipitated in the presence of ammonium ions prior to phosphorylation.

8B. The primer extension experiment can be made by using avian myeloblastosis virus reverse transcriptase (Invitrogen Life Science) and desoxynucleoside triphosphates, one of which is radioactively labelled [α-^{32}P] dNTP.

9. Optionally, before assembling the plates rinse them with PlusOne^{TM} Repel-Silane ES (Amersham) and let it dry. This product inhibits sticking of polyacrylamide gels to glass surfaces, making it easier to remove the gel from the plates afterwards.
10. The probe should be stored at –80°C and used within 3 days of preparation to minimize background.
11. The co-precipitation of the RNA probe with the RNA sample ensures that the subsequent annealing is reproducible. Nevertheless, the probe and sample may be added without precipitation to the hybridization buffer, but the additional volume will alter the final hybridization conditions. Moreover, the ethanol precipitation step allows removing inhibitors and contaminants that may still be present in RNA sample or probe.
12. Most samples anneal efficiently at 37–45°C in the hybridization buffer.
13. Other transcriptional fusions of toxin promoters (P*tcd*) have been described using different reporter genes and hosts. For instance, toxin promoter fused to the toxin A repeating units (ARU) reporter gene and assayed in *E. coli* BL21(DE3) strain (20), or Ptcd-*lacZ* fusions measuring the β-galactosidase activity in *Bacillus subtilis* SMY strain (25) and P*tcd–gusA* fusions quantifying the β-glucuronidase activity in *C. difficile* CD37 strain (7).
14. You can freeze the pellets at –80°C indefinitely.

15. Toluene opens holes in the cell membrane. Use this product with caution as it is highly flammable.

16. Optimal time of incubation is between 20 and 60 min. Up to 6 h may be required for reliable detection of very low activities.

17A. The β-glucuronidase specific activity can be calculated as follows: the absorbance at OD_{405} (**Section 3.3.1**) × 1,000/[OD_{600} of cells (**Section 3.3**) × Incubation time T1–T0 (min) × 1.25 × volume of sample (ml)] (11).

17B. Incubation temperature of expression cells of 37°C is generally used when protein stays soluble. However, if overexpressed protein forms inclusion bodies we have to change some culture growth conditions such as temperature. The OD_{600} of cells may be also different according to the solubility of the protein overexpressed (generally at the mid-exponential growth phase).

18. During the incubation the RNA polymerase holoenzyme is formed, having TcdR as sigma factor, which will allow toxin promoter recognition by the RNA polymerase. For the reactions in presence of TcdC, incubate 2 pmol of TcdR with different amounts of TcdC at 37°C for 15 min. Add 0.5 mM *E. coli* RNA polymerase core enzyme and incubate for further 15 min at 37°C.

19. Besides running a ^{32}P-labelled pBR322/MspI marker to quantify the size of the transcript produced, DNA sequencing of the transcript (*see* **Section 3.1.4**) can be done to localize the transcript product.

20. Other techniques permitted to show TcdR or TcdC interactions with the RNAP core enzyme like using surface plasmon resonance analysis (Biocore, 9) (**Fig. 7.6**).

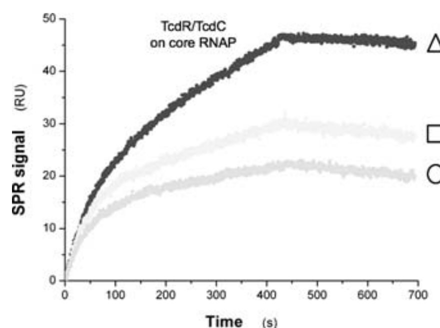


Fig. 7.6. Surface plasmon resonance analysis of the binding of TcdC and TcdR to RNAP. TcdR (18 nM) overimmobilized core RNAP in the presence of different concentrations of TcdC [0 nM (in *triangle*), 10 nM (in *square*) and 20 nM (in *circle*)] (adapted from Matamouros et al. (9)).

However, this technique demands the use of very specific and expensive technology equipment that may not be present in all laboratories.

21. Make in parallel a negative control by mixing whole-cell extract of *E. coli* BL21λDE3 carrying the expressing vector without TcdR, therefore, removing the background that may exist.

Acknowledgement

This work was supported by funds from Pasteur Institute and the research grant (AI057637) from the US public health service to Bruno Dupuy and from a predoctoral fellowship (SFRH/BD/16399/2004) from the Fundação para a Ciência e a Tecnologia (Portugal) to Ana Antunes.

References

1. Genth H, Dreger SC, Huelsenbeck J and Just I. (2008) *Clostridium difficile* toxins: more than mere inhibitors of Rho proteins. *Int J Biochem Cell Biol* **40**, 592–597.
2. Just I, Selzer J, Wilm M, von Eichel-Streiber C, Mann M and Aktories K. (1995) Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature* **375**, 500–503.
3. Perelle S, Gibert M, Bourlioux P, Corthier G and Popoff MR. (1997) Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. *Infect Immun* **65**, 1402–1407.
4. Akerlund T, Persson I, Unemo M, et al. (2008) Increased sporulation rate of epidemic *Clostridium difficile* Type 027/NAP1. *J Clin Microbiol* **46**, 1530–1533.
5. Braun V, Hundsberger T, Leukel P, Sauerborn M and von Eichel-Streiber C. (1996) Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. *Gene* **181**, 29–38.
6. Mani N and Dupuy B. (2001) Regulation of toxin synthesis in *Clostridium difficile* by an alternative RNA polymerase sigma factor. *Proc Natl Acad Sci USA* **98**, 5844–5849.
7. Mani N, Lyras D, Barroso L, et al. (2002) Environmental response and autoregulation of *Clostridium difficile* TxeR, a sigma factor for toxin gene expression. *J Bacteriol* **184**, 5971–5978.
8. Dupuy B and Matamouros S. (2006) Regulation of toxin and bacteriocin synthesis in Clostridium species by a new subgroup of RNA polymerase sigma-factors. *Res Microbiol* **157**, 201–205.
9. Matamouros S, England P and Dupuy B. (2007) *Clostridium difficile* toxin expression is inhibited by the novel regulator TcdC. *Mol Microbiol* **64**, 1274–1288.
10. Tan KS, Wee BY and Song KP. (2001) Evidence for holin function of tcdE gene in the pathogenicity of *Clostridium difficile*. *J Med Microbiol* **50**, 613–619.
11. Dupuy B and Sonenshein AL. (1998) Regulated transcription of *Clostridium difficile* toxin genes. *Mol Microbiol* **27**, 107–120.
12. Karlsson S, Dupuy B, Mukherjee K, Norin E, Burman LG and Akerlund T. (2003) Expression of *Clostridium difficile* toxins A and B and their sigma factor TcdD is controlled by temperature. *Infect Immun* **71**, 1784–1793.
13. Karlsson S, Lindberg A, Norin E, Burman LG and Akerlund T. (2000) Toxins, Butyric acid, and other short-chain fatty acids are coordinately expressed and down-regulated by cysteine in *Clostridium difficile*. *Infect Immun* **68**, 5881–5888.
14. Karlsson S, Burman L and Akerlund T. (1999) Suppression of toxin production in *Clostridium difficile* VPI 10463 by amino acids. *Microbiology* **145**, 1683–1693.
15. Ikeda D, Karasawa T, Yamakawa K, Tanaka R, Namiki M and Nakamura S. (1998) Effect of isoleucine on toxin production by

Clostridium difficile in a defined medium. *Zentralbl Bakteriol* **287**, 375–386.

16. Yamakawa K, Karasawa T, Ikoma S and Nakamura S. (1996) Enhancement of *Clostridium difficile* toxin production in biotin-limited conditions. *J Med Microbiol* **44**, 111–114.
17. Karasawa T, Maegawa T, Nojiri T, Yamakawa K and Nakamura S. (1997) Effect of arginine on toxin production by *Clostridium difficile* in defined medium. *Microbiol Immunol* **41**, 581–585.
18. Hammond GA, Lyerly DM and Johnson JL. (1997) Transcriptional analysis of the toxigenic element of *Clostridium difficile*. *Microb Pathog* **22**, 143–154.
19. Hundsberger T, Braun V, Weidmann M, Leukel P, Sauerborn M and von Eichel-Streiber C. (1997) Transcription analysis of the genes *tcdA-E* of the pathogenicity locus of *Clostridium difficile*. *Eur J Biochem* **244**, 735–742.
20. Moncrief JS, Barroso LA and Wilkins TD. (1997) Positive regulation of *Clostridium difficile* toxins. *Infect Immun* **65**, 1105–1108.
21. Dineen SS, Villapakkam AC, Nordman JT and Sonenshein AL. (2007) Repression of *Clostridium difficile* toxin gene expression by CodY. *Mol Microbiol* **66**(1), 206–219.
22. Prediger EA. (2001) Detection and quantitation of mRNAs using ribonuclease protection assays. *Methods Mol Biol* **160**, 495–505.
23. Darder M, Takada K, Pariente F, Lorenzo E and Abruna HD. (1999) Dithiobissuccinimidyl propionate as an anchor for assembling peroxidases at electrodes surfaces and its application in a H_2O_2 biosensor. *Anal Chem* **71**, 5530–5537.
24. Lomant AJ and Fairbanks G. (1976) Chemical probes of extended biological structures: synthesis and properties of the cleavable protein cross-linking reagent [35S]dithiobis(succinimidyl propionate). *J Mol Biol* **104**, 243–261.
25. Dupuy B, Raffestin S, Matamouros S, Mani N, Popoff MR and Sonenshein AL. (2006) Regulation of toxin and bacteriocin gene expression in Clostridium by interchangeable RNA polymerase sigma factors. *Mol Microbiol* **60**, 1044–1057.

Chapter 8

Dissecting the Cell Surface

Robert Fagan and Neil Fairweather

Abstract

The bacterial cell surface is an important structure as it mediates interactions with the external environment. In the case of pathogens like *Clostridium difficile*, the cell wall and its components also have to mediate interactions with the host cells and their products. In this chapter we discuss the various methods used for dissecting the cell surface and the biochemical and immunological procedures that are commonly used to analyse the properties of the proteins within the cell wall. A major consideration is the S-layer which in *C. difficile* shows considerable variation in sequence and between strains, a property which is also reflected in its antigenic properties.

Key words: Cell wall, S-layer, protein purification, ELISA, immunofluorescence.

1. Introduction

Interactions between bacteria and their host invariably involve surface structures on the bacteria. These are usually proteinaceous, but can be modified by, for example glycosylation. In Gram-positive bacteria, most surface proteins are by necessity anchored to the cell wall, either covalently as in the case of the well-described LPXTG proteins of *Staphylococcus aureus* and other species (1) or non-covalently as is the case in the family of cell wall proteins of *Clostridium difficile* and perhaps some other clostridia, e.g. *Clostridium botulinum* and *Clostridium tetani* (2, 3). The functions of the cell wall proteins of *C. difficile* are far from understood, although the two major wall proteins which are produced from the precursor SlpA assemble to form the S-layer of the bacterium and are involved in adhesion to host cells (2, 4). In *C. difficile*, *C. tetani* and *C. botulinum*, families of putative

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_8, © Springer Science+Business Media, LLC 2010

cell wall proteins have been identified, and members are characterised by possessing one or more Pf04122 cell wall-binding motifs (5–8). The nature of the interaction between this motif and the underlying cell wall is not entirely understood but is thought to involve interactions between the motifs, with homology to Bacillus cell wall-binding proteins, and either peptidoglycan or a secondary cell wall polymer (9–11). As these interactions are non-covalent it is possible to break these bonds by a number of different methods releasing these surface proteins into the extracellular fraction.

2. Materials

2.1. Growth and Storage of C. difficile

1. Liquid growth medium: Prepare BHI broth (Oxoid) according to the manufacturers instructions and sterilise by autoclaving. Sterilised media can be stored for several months at room temperature.
2. Solid growth media: *C. difficile* can be grown successfully on either BHI agar or blood agar base supplemented with 7.5% defibrinated horse blood. BHI agar and blood agar base should be prepared according to the manufacturers instructions and sterilised by autoclaving. Cooled solidified agar can be stored in bottles at room temperature for several months. To prepare agar plates re-melt the agar using a commercial microwave, steamer or re-autoclave. *Ensure the lid of the bottle is loosened prior to heating*. For blood agar, cool the molten agar base to approx. 55°C prior to addition of blood to avoid lysis of blood cells. As plates may be incubated for several days at 37°C to allow growth of *C. difficile* it is advisable to pour agar plates considerably thicker than is normal for other organisms to avoid drying of the agar during growth.
3. Glycerol (70%), filter sterilised through a 0.2-μm syringe filter.
4. Robertson's double cooked meat broth in 25 ml aliquots in glass universals.

2.2. Preparation of S-Layer Extracts Using Low-pH Glycine

1. Phosphate-buffered saline (PBS): PBS may be purchased in tablet form (Sigma) for convenient preparation of working solutions or as a 10X concentrated liquid stock solution which must be diluted 1:10 prior to use (*see* **Notes 1** and **2**).
2. Low-pH glycine solution: 0.2 M glycine–HCl, pH 2.2.

3. Neutralisation solution: 2 M Tris base.
4. pH indicator strips with a range of 0–14.

2.3. Alternative Methods of S-Layer Protein Extraction

1. The reagents for these techniques are specified in **Section 3.8**.

2.4. Extraction of Cell Wall Proteins

1. Tris–sucrose (TS) buffer: 10 mM Tris–HCl pH 6.9, 10 mM $MgCl_2$, 0.5 M sucrose.
2. Digestion buffer: TS with 60 μg/ml mutanolysin, 1 mg/ml lysozyme, 50 μg/ml lysostaphin, 250 μg/ml RNase A, 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF).

2.5. SDS-PAGE

1. Resolving gel buffer (4X): 1.5 M Tris–HCl, pH 8.8.
2. Stacking gel buffer (4X): 0.5 M Tris–HCl, pH 6.8.
3. 10% SDS (w/v).
4. 30% acrylamide/bis-acrylamide (37.5:1) solution (Sigma) (*see* **Note 3**).
5. N,N,N',N'-tetramethylethylenediamine (TEMED).
6. Ammonium persulphate (APS) (10%). Store frozen in small aliquots (<200 μl). The number of freeze-thaw cycles should be minimised.
7. Water-saturated butanol or 96% ethanol.
8. Running buffer (10X): 0.25 M Tris base, 1.92 M glycine, 1% SDS. Dilute to 1X with H_2O before use.
9. Laemmli sample buffer (2X): 150 mM Tris–HCl, pH 6.8, 30% (v/v) glycerol, 1.5% (w/v) SDS, 15% (v/v) β-mercaptoethanol, 2 μg/ml bromophenol blue.
10. Protein marker broad range (New England Biolabs) or equivalent.
11. Coomassie stain: 0.25% (w/v) Coomassie brilliant blue R-250, 45% (v/v) methanol, 10% (v/v) acetic acid.
12. Coomassie destain: 45% (v/v) methanol, 10% (v/v) acetic acid.
13. Acetic acid (10%).

2.6. Native PAGE

1. Bis-Tris buffer (3.5X): 1.25 M bis-Tris, pH 6.5.
2. Acrylamide, APS, TEMED, Coomassie stain and destain as above.
3. Native sample buffer (2X): 150 mM Tris–HCl, pH 6.8, 30% (v/v) glycerol, 2 μg/ml bromophenol blue.
4. Sodium bisulphite: prepare a 1 M stock solution and store at 4°C.

5. Native running buffer (5X): 250 mM MOPS, 250 mM Tris base, 5 mM EDTA (from 0.5 M, pH 8.0 stock).

2.7. Western Immunoblotting from SDS and Native Gels

1. Immobilon-P PVDF membrane (Millipore).
2. Methanol.
3. Cathode buffer: 25 mM Tris–HCl, pH 9.4, 40 mM glycine, 10% (v/v) methanol.
4. Anode I buffer: 0.3 M Tris–HCl, pH 10.4, 10% (v/v) methanol.
5. Anode II buffer: 25 mM Tris–HCl, pH 10.4, 10% (v/v) methanol.
6. Ponceau S stain: 0.5% (w/v) Ponceau S, 1% (v/v) acetic acid.
7. Antibody dilution solution: 3% (w/v) non-fat milk in PBS, 0.01% Tween-20.
8. Antibodies raised against your protein of interest.
9. HRP-conjugated secondary antibodies recognising the species used to raise antibodies above.
10. PBS.
11. SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).
12. Clingfilm.
13. Stripping solution: 25 mM glycine–HCl, pH 2.0, 1% (w/v) SDS.

2.8. Immunofluorescence

1. PBS.
2. Formaldehyde (8%).
3. NH_4Cl_2 (20 mM).
4. Clean glass microscope slides and cover slips.
5. Blocking solution: 2% (w/v) bovine serum albumin (BSA) in PBS.
6. Primary antibodies to proteins of interest. For detection of more than one protein the antibodies must be raised in different species to allow detection with species-specific secondary antibodies.
7. Fluorescently labelled secondary antibodies recognising the species used to raise antibodies above. For detection of more than one protein use secondary antibodies with different colour fluorescent labels.
8. ProLong Gold anti-fade reagent (Invitrogen).
9. Epifluorescent or confocal microscope.

2.9. ELISA Analysis of Protein–Protein Interactions

1. Coating dilution buffer: PBS
2. Wash buffer: PBS supplemented with 0.05% Tween-20 (PBS-T).
3. Blocking/dilution buffer: 3% non-fat milk in PBS-T.
4. Developing solution: Prepare 20 ml of developing solution as follows: 25 mM citric acid, 50 mM Na_2HPO_4, 1X *o*-phenylenediamine dihydrochloride (OPD) tablet (Sigma). Prepare 0.1 M citric acid and 0.2 M Na_2HPO_4 stocks. Immediately before use add 0.04% (v/v) H_2O_2.
5. Stop solution: 3 M H_2SO_4

3. Methods

***3.1. Growth and Storage of* C. difficile**

1. Warm and pre-reduce media (both liquid and solid) by incubation at 37°C under anaerobic conditions for at least 1 h prior to inoculation. The length of this incubation step should be increased for large volumes of media, e.g. >100 ml BHI broth. Agar plates should be streaked using standard microbiological techniques. When resuscitating frozen cultures of *C. difficile* (*see* below) it is possible to streak plates on the laboratory bench prior to incubation in an anaerobic environment. This will slow the growth of the bacteria (by up to 24 h) but does not greatly reduce the recovery efficiency. Broth should be inoculated with a single colony of the desired *C. difficile* strain under anaerobic conditions. Classical aseptic technique is impossible in an anaerobic environment so care should be taken to avoid contamination, e.g. use disposable loops and work quickly. The growth of broth cultures inoculated on the laboratory bench is highly variable and unreliable and as a result inoculation in this manner is not advised for most applications.
2. Stocks of *C. difficile* strains can be stored successfully in several ways. Traditionally strains were stored as cultures in glass universals of Robertson's double cooked broth. This is still a reliable method for long-term storage of *C. difficile* but the stored cultures are prone to contamination. To prepare a stock culture inoculate a Robertson's broth with a single colony of *C. difficile* and incubate at 37°C under anaerobic conditions for at least 4 days. The culture can then be stored sealed in the laboratory at room temperature. To resuscitate the strain mix the Robertson's broth well and transfer a loopful to a fresh blood agar plate, streak and incubate as normal. Alternatively strains can be stored frozen

at –80°C with 20% glycerol as a cryoprotectant. Prepare a liquid overnight culture of the strain to be frozen as described above. Following overnight growth transfer 1 ml of culture to a cryovial (1.8 ml, Nunc) containing 400 μl pre-reduced sterile 70% glycerol, mix well and freeze immediately. To resuscitate, scrape a small amount of ice from the top of the frozen culture with a disposable loop and streak on blood agar. Incubate as normal. Avoid thawing of the frozen culture as repeated freeze-thaw cycles reduces viability of the stock.

3.2. Preparation of S-Layer Extracts Using Low-pH Glycine

1. Prepare an overnight liquid *C. difficile* culture in BHI broth. The culture should be prepared such that the subsequent steps can be carried out not longer than 18-h post-inoculation (*see* **Note 4**). A typical 50 ml culture will yield approximately 0.5–1 mg of total surface protein with the two major S-layer proteins representing approximately 90–95% of this total. Culture volume can be readily scaled-up to increase total protein yield.
2. Harvest the *C. difficile* 16–18-h post-inoculation by centrifugation at 3,500×*g* for 10 min at room temperature (*see* **Note 5**).
3. Discard the supernatant into a strong disinfectant such as 2% Virkon and gently resuspend the bacterial pellet in 1/10 volume PBS. Repeat centrifugation.
4. Discard the supernatant again into disinfectant and invert tubes briefly over absorbent paper. Residual PBS will affect the pH in subsequent steps and reduce protein yield so it is important to remove as much as possible at this point. A single wash step is sufficient for routine isolation of S-layers and associated proteins but if required this wash step may be repeated several times to increase stringency and remove loosely attached proteins.
5. Resuspend the bacterial pellet in 1/100 volume of low-pH glycine and incubate with gentle agitation at room temperature for 20 min (*see* **Note 6**).
6. Transfer the bacterial suspension to microfuge tubes and centrifuge at maximum speed in a bench top microfuge at 4°C for 10 min.
7. Carefully remove the supernatant containing the surface proteins to a clean tube and neutralise the pH by addition of 2 M Tris base. The pH can be estimated by pipetting 20 μl of protein solution onto a broad range pH indicator strip. Add a small volume of 2 M Tris (e.g. 20 μl per 500 μl low-pH glycine), mix gently and measure the pH again. Repeat until the pH is in the 7–8 range. The total volume of 2 M

Tris required must be determined empirically and will vary somewhat depending on several factors including any possible carryover of PBS from the wash step(s) but will be in the region of 20–40 μl per 500 μl of low-pH glycine.

3.3. Alternative Methods of S-Layer Protein Extraction

The S-layer proteins and other cell surface-associated proteins may be removed from *C. difficile* using a variety of chemical treatments, including guanidinium hydrochloride, urea, EDTA or LiCl (12–14). These methods yield preparations containing the two S-layer proteins together with varying quantities of other cell wall proteins. In our hands use of EDTA or LiCl does not yield equimolar quantities of the HMW and LMW SLPs, but tends to produce preparations rich in the LMW SLP. Equimolar amounts of the HMW and LMW SLPs are produced using GuHCl and urea, but the latter method in our hands yields increased amounts of other cell wall proteins. For some purposes, these methods may therefore be preferable.

1. Prepare a 50 ml overnight culture of *C. difficile* as described in **Section 3.2**, Step 1 above.
2. Follow the protocol for extraction with low-pH glycine, but after the washing steps, replace the glycine with 1/100 volume of one of the following solutions:
   - 8 M urea, 50 mM Tris–HCl pH 8.0, 1 mM phenyl-methyl-sulphonide-fluoride
   - 1 or 5 M LiCl.
   - 10 or 70 mM Na_2EDTA in PBS pH 7.4 (incubate with EDTA at 45°C for 30 min instead of 20 min at room temperature).
3. Incubate with gentle agitation at room temperature for 20 min and follow the procedures described above for low-pH glycine extraction (*see* **Note 8**).

3.4. Extraction of Cell Wall Proteins

In order to isolate the S-layer proteins together with other proteins that are present within the cell wall, the following procedure can be used. This procedure was developed from methods described previously (15, 16).

1. Prepare a 20 ml overnight liquid *C. difficile* culture in BHI broth and centrifuge the culture to obtain a bacterial pellet as described in **Section 3.2**, Steps 1 and 2 above.
2. Wash the bacterial pellet twice by centrifugation and resuspension, once in PBS and once in Tris–sucrose buffer.
3. Resuspend the bacterial pellet in 2 ml digestion buffer in a 15-ml Falcon tube and incubate the tube horizontally for 2 h at 37°C with gentle rotating agitation. This can be performed in a 37°C constant temperature room, or alternatively using an incubator with gentle rotation. In our

laboratory we find a Hybaid hybridisation oven ideal for this purpose.

4. Observe cells for lysis under a light microscope using x 40 objective lens and phase contrast. The cells should exhibit signs of protoplast formation; however, formation of protoplasts is rarely complete.
5. Pellet the cells by centrifugation (16,700×*g*, 5 min). The supernatant fluids contain the cell wall-associated proteins which can be analysed by gel electrophoresis.

3.5. SDS-PAGE

The following methods for denaturing acrylamide gel electrophoresis of proteins are based on the method of Laemmli (17) using the Bio-Rad Mini-Protean 3 gel system but are readily adaptable for use with other apparatus. Using this method, proteins are denatured and reduced in SDS and β-mercaptoethanol and separated on the basis of their size as they travel through a polyacrylamide gel towards the anode. SDS binds to most proteins in a constant weight ratio, masking their natural charge with its own negative charge and giving each protein a similar mass:charge ratio allowing separation on the basis of size rather than charge. The discontinuous gel is formed using buffers of differing composition and pH to first focus the separating proteins into narrow well-defined bands and then separate these focused proteins on the basis of their size.

1. Using clean, dry glass plates assemble the gel casting apparatus according to the manufacturer's instructions.
2. Prepare the acrylamide gel solutions as outlined in **Tables 8.1** and **8.2** *without APS and TEMED.* Both resolving gel and stacking gel solutions can be prepared at the same time.

Table 8.1
Recipe for SDS-PAGE resolving gel. Sufficient for two 1.0mm mini-gels

	6%	8%	10%	12%	15%
30% Acrylamide/ bis-acrylamide (ml)	2	2.7	3.3	4	5
1.5 M Tris–HCl, pH 8.8 (ml)	2.5	2.5	2.5	2.5	2.5
H_2O (ml)	5.4	4.7	4.1	3.4	2.4
10% SDS (μl)	100	100	100	100	100
10% APS (μl)	50	50	50	50	50
TEMED (μl)	10	10	10	10	10

Table 8.2
Recipe for SDS-PAGE stacking gel. Sufficient for two 1.0mm mini-gels

	5%
30% Acrylamide/bis-acrylamide	833 μl
0.5 M Tris–HCl, pH 6.8	1.25 ml
H_2O	2.87 ml
10% SDS	50 μl
10% APS	25 μl
TEMED	5 μl

Add APS and TEMED to the resolving gel solution, mix gently and pipette approximately 4 ml of the solution between the glass plates. Overlay 0.5 ml of ethanol or water-saturated butanol on top of the acrylamide solution to exclude oxygen (which inhibits polymerisation) and give an even straight edge to the top of the resolving gel. Allow to polymerise for approximately 30 min. The remaining unused resolving gel solution can be used as a guide for the polymerisation state of the gel.

3. Once the resolving gel has polymerised, Pour off the ethanol or butanol and allow to dry briefly.
4. Add APS and TEMED to the stacking gel solution and mix gently. Pipette the solution on top of the resolving gel, filling all of the remaining space between the glass plates, and gently insert the desired comb. Allow to polymerise for approximately 30 min.
5. Prepare the running buffer by dilution of the 10X stock. 450 ml is sufficient for two gels using the Bio-Rad Mini-Protean 3 system.
6. Prepare all samples to be analysed using the 2X Laemmli sample buffer such that the final concentration of sample buffer is 1X. Some samples (e.g. whole bacterial samples) require boiling (5 min, 100°C) prior to electrophoresis to ensure cell lysis and complete denaturing of proteins, however, this is generally not required for purified protein samples.
7. When the stacking gel is polymerised gently remove the comb and assemble the gel running apparatus. Fill the upper gel compartment with running buffer and pour the remaining buffer into the lower gel compartment.
8. Load 5–20 μl of sample per well including one well reserved for the protein size marker. Add an equal volume

of 1X Laemmli sample buffer to any empty wells to prevent samples spreading horizontally into the empty lanes during electrophoresis.

9. Connect the gel running apparatus to a power supply and run at 200 V until the bromophenol blue dye front has reached the bottom of the gel (approximately 40 min for a 12% gel).
10. Disassemble the apparatus and the glass plates and remove the gel for staining (for Western immunoblotting *see* **Section 3.7** below). Place the gel in a suitably sized glass or plastic dish and completely cover with Coomassie stain solution. Incubate at room temperature with gentle agitation for at least 2 h. Pour off the stain and cover the stained gel with Coomassie destain solution. Incubate with gentle agitation at least 1 h. Repeat with fresh destain solution until the background staining has reached an acceptable level. A further incubation step with 10% acetic acid is useful to completely remove any remaining background staining and rehydrate the gel.

3.6. Native PAGE

Native or non-denaturing polyacrylamide gel electrophoresis has many applications in studying protein sample dispersity, oligomerisation state and protein–protein interactions. Several different protocols are available for the separation of proteins under non-denaturing conditions; the following protocol is applicable for the separation of proteins with pIs below 6.5. Under non-denaturing conditions the migration of proteins through a polyacrylamide gel will be influenced by their size, shape and charge. As a result, accurate size determination is impossible using these methods. However, this protocol can be modified to produce Blue-Native gels (18) which will allow a degree of size determination (*see* **Note 7**). The following methods are optimised for use with the Bio-Rad Mini-Protean 3 gel system but are readily adaptable for use with other apparatus.

1. Using clean, dry glass plates assemble the gel casting apparatus according to the manufacturer's instructions.
2. Prepare the acrylamide gel solutions as outlined in **Table 8.3** *without APS and TEMED.*
3. Prepare and pour resolving and stacking gels as for SDS-PAGE gels (*see* **Section 3.3** above).
4. Prepare all protein samples to be analysed using the 2X Native gel sample buffer such that the final concentration of sample buffer is 1X. Unlike samples for SDS-PAGE Native gel samples should never be heated.
5. Prepare 1X running buffer by dilution of the 5X stock and if required addition of sodium bisulphite to a final concentration of 1 mM. Prepare 1X running buffer fresh immediately

Table 8.3
Recipe for native bis-tris polyacrylamide gels. Sufficient for two 1.0-mm mini-gels

	12% Resolving gel	4.5% Stacking gel
3.5X Bis-tris buffer (ml)	3.8	1
30% Acrylamide/ bis-acrylamide (ml)	5.4	0.5
H_2O (ml)	4.2	2
10% APS (μl)	50	24
TEMED (μl)	14	12

before use. Sodium bisulphite should be added if removal of inter- or intramolecular disulphide bonds is desired. 450 ml of 1X buffer is sufficient for two gels using the Mini-Protean 3 system.

6. When the Stacking gel is polymerised gently remove the comb and assemble the gel running apparatus. Fill the upper gel compartment with running buffer and pour the remaining buffer into the lower gel compartment.
7. Load 5–20 μl of sample per well. If using an adapted Blue-Native protocol (*see* **Note 7**), reserve a lane for a suitable native protein molecular weight marker. In contrast to SDS-PAGE, loading empty lanes with 1X sample buffer is generally not necessary for Native gels.
8. Connect the gel running apparatus to a power supply and run at 120 V. The electrophoresis time should be determined empirically for individual applications and sample types. As a guide, 3 h is sufficient for clear resolution of *C. difficile* S-layer heterodimeric complexes from monomers of the two S-layer proteins.
9. Disassemble the apparatus and the glass plates and remove the gel for staining (for Western immunoblotting *see* **Section 3.7** below). Stain and destain gels as described above for SDS-PAGE (*see* **Section 3.3**). Note, polyacrylamide gels without SDS are quite sticky and are easy to tear when removing from the glass plates. If required, the gel can be floated off one of the glass plates by submersion in Coomassie stain solution.

3.7. Western Immunoblotting from SDS and Native Gels

Most standard protocols are applicable for the electrotransfer of proteins from both denaturing and non-denaturing polyacrylamide gels. However, the following protocol is optimised for the transfer of a wide size range of proteins, allows faster detection

than most standard methods and gives generally lower background and higher sensitivity. This protocol has been optimised for use with a Bio-Rad Trans-blot SD semi-dry transfer system but should be readily applicable for use with any semi-dry apparatus. It is also possible to perform a wet electrotransfer using standard methods and then continue with the rapid detection protocol.

1. Separate protein samples on a native or denaturing gel as described above (*see* **Sections 3.4 and 3.5**).
2. Carefully remove the gel from the glass plates and equilibrate in Cathode buffer at room temperature for 10 min.
3. While the gel is equilibrating cut a piece of Immobilon-P PVDF membrane to the size of the gel to be transferred. Wet briefly in methanol, incubate in Milli-Q water for 2 min and finally equilibrate in Anode II buffer for 5 min.
4. Cut six pieces of Whatman 3-mm paper 1 cm a side longer than the gel and membrane. Wet three of the pieces in Cathode buffer, two in Anode I buffer and one in Anode II buffer.
5. Assemble the transfer stack as follows: Place two pieces of filter paper wet in Anode I on the anode followed by the piece wet in Anode II, the equilibrated membrane, the equilibrated gel and finally the three pieces of filter paper wet in cathode buffer. Gently roll a pipette over the transfer stack to remove any air bubbles.
6. Finish assembling the semi-dry apparatus, connect to a power pack and run at 15 V for 15 min (suitable for the transfer of a single mini-gel).
7. Remove the membrane from the transfer stack to a clean plastic dish (10 cm × 10 cm petri dishes are useful for this and later steps). To evaluate the efficiency of transfer, pour over enough Ponceau S stain to cover the membrane and incubate at room temperature for 2–5 min. Pour off the Ponceau S stain and rinse the membrane with Milli-Q water to decrease background staining. The transferred proteins should be clearly visible. Repeated washes will eventually remove all of the Ponceau S stain, however, residual stain will not affect any of the subsequent steps.
8. Wet the membrane in methanol to dehydrate and dry by incubation at room temperature for 15–30 min. The surface of the dried PVDF membrane is hydrophobic and as such will not interact with antibody. As a result the blocking step typical of other Western immunoblot protocols can be omitted shortening the detection procedure.

9. Prepare the primary antibody by dilution of the antibody stock in PBS with 3% non-fat powdered milk and 0.01% Tween-20 and add to the dried membrane. The dilution must be determined for each individual antibody. About 10 ml of diluted primary antibody is sufficient to cover a single membrane in a small plastic dish. For precious antibodies it is possible to use significantly less than 10 ml by adding the diluted antibody to the membrane in a heat-sealed plastic pouch. Incubate at room temperature with gentle agitation for 1 h.
10. Pour the primary antibody away and wash the membrane four times with PBS. To wash, completely cover the membrane with PBS (approx. 50 ml), agitate gently for a few seconds and pour away the PBS. No incubation steps are necessary.
11. Prepare the secondary HRP-conjugated antibody as described above at the dilution suggested by the manufacturer. Add to the membrane and incubate with gentle agitation for 30 min.
12. Wash the membrane as before with PBS. Leave the membrane in PBS following the final wash.
13. Prepare sufficient enhanced chemiluminescent (ECL) substrate to cover the membrane (approx. 0.1 ml/cm^2). Remove the membrane from the PBS, drain briefly on absorbent paper, place on a clean dry surface and pipette the substrate carefully onto the membrane, ensuring the entire surface of the membrane is covered. Incubate for 2–5 min at room temperature, drain briefly and completely wrap in clingfilm to prevent the membrane drying out.
14. Expose the membrane using either an X-ray film or a luminescence imager such as the Fuji LAS-3000.

3.8. Immunofluorescence

1. Prepare a 5 ml overnight culture of *C. difficile* in BHI as described in **Section 3.1** above.
2. Dilute the overnight culture 1:50 in fresh BHI and grow to mid-logarithmic phase ($OD_{600\ nm} \sim 0.6$).
3. Harvest 400 µl of cells by centrifugation at 6,800×*g* for 1 min.
4. Discard the supernatant, wash the pellet by resuspension in 1 ml PBS and centrifuge as above. Repeat twice.
5. Following the final wash resuspend the pellets in 400 µl 8% formaldehyde and incubate at room temperature for 15 min to fix the bacteria.

6. Harvest and wash the cells three times with PBS as described above.
7. Resuspend the pellet in 400 μl 20 mM NH_4Cl_2 and incubate at room temperature for 15 min to quench any remaining formaldehyde.
8. Harvest and wash twice with PBS as before.
9. Resuspend the pellet in 40 μl PBS. Spot 10 μl of the resuspended bacteria onto a clean microscope slide and allow to dry.
10. Once dry, rinse the slides gently with PBS, drain briefly on absorbant paper and cover with 400 μl blocking solution. Incubate at 4°C overnight.
11. Rinse the blocked slides once with PBS and drain on absorbant paper.
12. Dilute the primary antibody(ies) in blocking solution and pipette onto the slide completely covering the spot of dried, fixed bacterial cells. About 15 μl is generally sufficient to cover a single spot. The optimum dilution for each antibody must be determined empirically but will be of the order of 100-fold more concentrated than would be used in Western immunoblots. Lay the slides on a base of wet tissue paper (to minimise evaporation of the antibody solution), cover with an opaque lid (e.g. an inverted tip box) and incubate at room temperature for 45 min.
13. Rinse the slides briefly with PBS and then immerse in a bath of PBS for 1 min.
14. Drain the slides on absorbant paper and add the appropriate secondary antibody(ies) diluted in blocking solution. Use the dilution suggested by the manufacturer for immunofluorescence. Incubate the slides at room temperature for 45 min as before.
15. Wash the slides with PBS as before.
16. Drain and allow to dry in air at room temperature.
17. Once dry, pipette 10 μl of ProLong Gold anti-fade reagent onto the dried bacterial cell spot and carefully lower a coverslip into place. Lower the coverslip one side at a time to exclude air bubbles. Cover the slide with an opaque lid and leave to dry overnight at room temperature.
18. Use a suitable epifluorescence or confocal microscope to visualise the fluorescence. The microscope must have filters appropriate to the emission spectra the fluorescent labels used. We use a Ziess Imager M1 fluorescence microscope with filters for GFP and Cy3 and a Zeiss AxioCam MRM camera.

3.9. ELISA Analysis of Protein–Protein Interactions

Enzyme-linked immunosorbent assays (ELISAs) have many applications including the analysis of protein–protein interactions. However, for detailed analysis of the kinetics of such interactions, more quantitative techniques such as surface plasmon resonance (Biacore, GE Healthcare) should be employed. To study protein–protein interactions using ELISA, one protein is bound to the surface of a specially treated microtitre plate and a dilution series of a second protein is overlaid in solution. Interaction of the proteins is then detected using a specific primary antibody to the second protein and an appropriate HRP-conjugated secondary antibody.

1. Dilute the coating protein to 10 μg/ml in PBS. Add 50 μl to each well of a Nunc Maxisorp microtitre plate. It is also important to coat one or more wells on the same microtitre plate with the second protein of interest to act as a positive control for antibody detection. If desired, a dilution series of the second protein will also give an indication of the detection sensitivity. Wrap the plate in cling film to minimise evaporation and incubate overnight at 4°C.
2. Prepare 2 l of wash buffer in a large open container. Remove the coated plate from the fridge and discard excess coating solution into a suitable waste receptacle (*see* **Note 9**).
3. Completely submerge the plate in wash buffer, ensuring all wells fill. Discard the wash buffer in the wells as described above. Repeat twice more. After the final wash step invert the plate and tap vigorously onto a pile of paper towels to remove any remaining wash buffer.
4. Add 100 μl of blocking buffer to each well, wrap the plate in cling film and incubate at 37°C for 2 h. This blocks any remaining protein-binding sites on the plate surface.
5. During this incubation prepare a dilution series of the second protein in blocking/dilution buffer. The highest concentration of proteins used must be determined empirically (*see* **Note 10**).
6. Discard the blocking/dilution buffer and tap away excess on paper towels.
7. Add 50 μl of diluted secondary overlay protein per well, wrap in cling film and incubate at 37°C for 1 h. It is important to incubate some wells with blocking/dilution buffer alone. This will control for any cross-reaction between the primary antibody (*see* below) and the protein used to coat the plate.
8. During this incubation step prepare the primary antibody in blocking/dilution buffer. The primary antibody should be specific for the second overlay protein. The dilution of

primary antibody must be determined empirically for any given antibody but as a rule will be the order of 5- to 10-fold more concentrated than would be used in a Western blot.

9. Discard excess overlay solution and wash as before.
10. Add 50 μl of diluted primary antibody per well and incubate at 37°C for 1 h.
11. During this incubation step prepare the secondary HRP-conjugated antibody in blocking/dilution buffer at the concentration suggested by the manufacturer.
12. Discard excess primary antibody solution and wash as before.
13. Add 50 μl of diluted secondary antibody per well and incubate at 37°C for 1 h.
14. Prepare the developing solution but do not add the H_2O_2 until immediately before use.
15. Discard excess secondary antibody solution and wash as before.
16. Add 50 μl of developing solution (with H_2O_2) per well and incubate at room temperature. A yellow colour, indicative of detected bound secondary protein, will gradually develop. Colour development will be strongest in the wells containing the highest concentration of overlay protein. When these wells exhibit a distinct yellow colour, add 20 μl H_2SO_4 to all wells to stop the reaction.
17. Measure the colour development using a microtitre plate reader set to measure absorbance at 492 nm.

4. Notes

1. All chemical solutions should be prepared with Milli-Q grade water or similar with a resistivity of 18.2 $M\Omega cm^{-1}$ unless otherwise stated in the text.
2. All solutions should be stored at room temperature unless otherwise stated in the text.
3. Acrylamide is a neurotoxin in its unpolymerised form and is also a carcinogen. Care should be taken when handling these solutions, handle all solutions and any laboratory apparatus that may be contaminated with gloves.
4. Under normal conditions, growth of *C. difficile* for longer than 18 h results in clumping of the bacteria upon

resuspension in low-pH glycine. This drastically reduces the yield of surface proteins. The growth rate of a particular strain will affect this timing somewhat. If clumping is observed in low-pH glycine reduce growth by at least 1 h.

5. All centrifugation and wash steps involving *C. difficile* should be carried out at room temperature. Incubation at lower temperatures results in bacterial pellets that are hard to resuspend and can reduce protein yield.
6. Incubation for 20 min will remove approximately 95% of all surface proteins. Lengthening this incubation has negligible effect on the protein yield, however, if removal of more protein is required this low-pH stripping step can be repeated with fresh low-pH glycine.
7. Blue-Native gel electrophoresis: The native charge on proteins can be masked by inclusion of 0.02% Coomassie Brilliant Blue stain in the upper gel running buffer – analogous to the use of SDS in SDS-PAGE but without denaturing the proteins. If the gel is to be silver stained following electrophoresis, reduce the Coomassie concentration in the upper gel buffer to 0.002%. This allows migration of proteins through the gel based solely on their size and shape. This will allow accurate size determination of globular proteins using commercially available molecular size markers. However, size determination of non-globular proteins is still highly unreliable.
8. An optional step of ultracentrifugation (100,000×*g* for 30 min at 4°C) can be employed to pellet unlysed cells and bacterial debris in any of these alternative extraction procedures.
9. For this and later steps in the ELISA protocol the best way to discard liquid from the wells of a microtitre plate is to simply invert the plate over a sink or waste receptacle and rapidly flick the plate from side to side. This is sufficient for removing most liquid, e.g. between multiple wash steps. The plate should then be inverted and patted onto a pile of dry paper towels to remove trace liquid before adding a different solution to the wells.
10. To obtain a complete curve with saturation at the maximum and minimum it is necessary to include sufficient protein dilutions. As a result it is often advisable to try a first experiment with a broad range of protein concentrations, e.g. fivefold dilutions from a concentrated stock (0.1–1 mg/ml). Once the first broad range binding curve has been determined you can then repeat with a tighter range of protein concentrations, e.g. twofold dilutions.

Acknowledgements

We thank Anne Wright, Omar Qazi and Natalie Welsh for developing and optimising some of the procedures described in this chapter.

References

1. Navarre WW and Schneewind O. (1999) Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol Mol Biol Rev* **63**, 174–229.
2. Calabi E, Ward S, Wren B, et al. (2001) Molecular characterization of the surface layer proteins from *Clostridium difficile*. *Mol Microbiol* **40**, 1187–1199.
3. Qazi O, Brailsford A, Wright A, Faraar J, Campbell J and Fairweather N. (2007) Identification and characterization of the surface-layer protein of *Clostridium tetani*. *FEMS Microbiol Lett* **274**, 126–131.
4. Calabi E, Calabi F, Phillips AD and Fairweather N. (2002) Binding of *Clostridium difficile* surface layer proteins to gastrointestinal tissues. *Infect Immun* **70**, 5770–5778.
5. Sebaihia M, Peck MW, Minton NP, et al. (2007) Genome sequence of a proteolytic (Group I) *Clostridium botulinum* strain Hall A and comparative analysis of the clostridial genomes. *Genome Res* **17**, 1082–1092.
6. Sebaihia M, Wren BW, Mullany P, et al. (2006) The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. *Nat Genet* **38**, 779–786.
7. Bruggemann H, Baumer S, Fricke WF, et al. (2003) The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. *PNAS* **100**, 1316–1321.
8. Bruggemann H and Gottschalk G. (2008) Comparative genomics of Clostridia: link between the ecological niche and cell surface properties. *Ann NY Acad Sci* **1125**, 73–81.
9. Karjalainen T, Waligora-Dupriet A-J, Cerquetti M, et al. (2001) Molecular and genomic analysis of genes encoding surface-anchored proteins from *Clostridium difficile*. *Infect Immun* **69**, 3442–3446.
10. Emerson J and Fairweather NF. (2009) Surface structures of *C. difficile* and other clostridia. In: Bruggemann H, Gottschalk G, eds., *Clostridia – Molecular Biology in the Post-genomic Era*, Caister Academic Press, Norfolk.
11. Herbold DR and Glaser L. (1975) Interaction of N-acetylmuramic acid L-alanine amidase with cell wall polymers. *J Biol Chem* **250**, 7231–7238.
12. McCoubrey J and Poxton IR. (2001) Variation in the surface layer proteins of *Clostridium difficile*. *FEMS Immunol Med Microbiol* **31**, 131–135.
13. Masuda K, Itoh M and Kawata T. (1989) Characterization and reassembly of a regular array in the cell wall of *Clostridium difficile* GA I4131. *Microbiol Immunol* **33**, 287–298.
14. Wright A. Proteomic and bioinformatic analysis of surface proteins from *Clostridium difficile*, PhD thesis. University of London, 2006.
15. Schneewind O, Model P and Fischetti VA. (1992) Sorting of protein A to the staphylococcal cell wall. *Cell* **70**, 267–281.
16. Jonquieres R, Bierne H, Fiedler F, Gounon P and Cossart P. (1999) Interaction between the protein InlB of *Listeria monocytogenes* and lipoteichoic acid: a novel mechanism of protein association at the surface of gram-positive bacteria. *Mol Microbiol* **34**, 902–914.
17. Laemmli UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
18. Wittig I, Braun HP and Schagger H. (2006) Blue native PAGE. *Nat Prot* **1**, 418–428.

Chapter 9

Human Intestinal Epithelial Response(s) to *Clostridium difficile*

Nazila V. Jafari, Elaine Allan, and Mona Bajaj-Elliott

Abstract

Clostridium difficile is a gram-positive, spore-forming, toxin-producing anaerobic bacillus that is being increasingly implicated as the leading cause of diarrhea and colitis, particularly in hospitalized, elderly patients. Studies to date suggest that *C. difficile* toxins A and B play a major role in the observed colonic inflammation and associated disease pathogenesis; however, the role of other potential bacterial factors at present remains unknown. Early effects of *C. difficile* on host intestinal epithelia include modest induction of innate immune responses with progressive loss of intestinal epithelial cell barrier function and cell death.

Key words: Intestinal barrier, innate immunity, inflammation.

1. Introduction

The intestinal epithelium is the first host cell that *Clostridium difficile* comes in contact with prior to triggering infection and disease (1, 2). The intestinal epithelium is a single-cell layer that maintains homeostasis in health by performing a multitude of tasks. Its primary role is to conduct electrolyte and nutrient absorption and yet at the same time it provides a barrier against potentially harmful antigenic, toxic, or infectious agents present in the intestinal lumen (3). These cells are held together by tight junction-associated proteins that control epithelial permeability and polarity (4). *C. difficile* toxins A and B cause disruption of epithelial barrier function and initiate apoptosis in a variety of cell lines, suggesting these cellular events may also occur in in vivo (5, 6).

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_9, © Springer Science+Business Media, LLC 2010

The anaerobic nature of the bacterium makes infection by live, viable *C. difficile* refractory to analyze in routine in vitro mammalian coculture conditions, thus limiting investigation of host–pathogen interactions to those modulated by dead bacteria. These studies remain informative as bacterial signature motifs (flagellin, peptidoglycan) do not require viability to interact with host pattern-recognition receptors (PRRs). Our current understanding of the role of epithelial PRRs in recognizing *C. difficile* and triggering downstream cellular events is limited. Published data suggest that the epithelia are able to express a range of cytokines and chemokines in response to infection (7). Intestinal epithelial cells are also responsible for production of antimicrobial peptides/proteins, which are components of innate immunity against microorganisms. The induction of innate immune genes is generally followed both at the mRNA (semi-quantitative and quantitative RT-PCR) and protein (ELISA, Luminex) level. In recent years, identification of proteins involved in formation of tight junction which control cell–cell adhesion and movement of solutes across the paracellular space has allowed biologists to study modulation of these processes during infection (4). Claudin, occuldin, and JAMA-1 proteins are components of the tight junctions and their cellular appearance can be detected by immunofluorescence. The transepithelial electrical resistance which gives a measure of the extent of disruption of cell–cell adhesion can be followed simultaneously with the influx of labeled small molecules (e.g., inulin) to the basolateral surface. Collectively, these techniques indicate how bacterial components affect epithelial cell biology during the acute phase of infection.

2. Materials

2.1. Cell Culture

1. Cell cultures; Caco-2 (ATCC, HTB-37), HT-29 (ATCC, HTB-38), and T-84 (ATCC, CCL-248) human colon carcinoma cells.
2. Dulbecco's Modified Eagle Medium with GlutaMAX-I (DMEM) (Invitrogen/Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (Invitrogen/Gibco), 1% penicillin–streptomycin solution (Invitrogen/Gibco), 1% L-glutamine 200 mM (Invitrogen/Gibco), and 1% MEM nonessential amino acid solution (Invitrogen/Gibco).
3. Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (Sigma-Aldrich, Poole, UK) supplemented with 10% fetal bovine serum (Invitrogen/Gibco), 1% penicillin–streptomycin solution (Invitrogen/Gibco), 1% L-glutamine

200 mM (Invitrogen/Gibco), and 1% MEM nonessential amino acid solution (Invitrogen/Gibco).

4. Trypsin–EDTA (0.05% Trypsin, 0.53 mM EDTA·4Na) (Invitrogen/Gibco).
5. D-PBS, Dulbecco's phosphate buffered saline (Invitrogen/Gibco).

2.2. Preparation of Bacterial Strains

1. Brain heart infusion (BHI) (Oxoid, Basingstoke, England).
2. Brain heart infusion (BHI) agar (Oxoid).
3. Defibrinated horse blood (5%) (E & O Laboratories Limited, Burnhouse, Scotland).
4. *Clostridium difficile* selective supplement (Oxoid).

2.3. RNA Extraction from Cell Cultures

1. D-PBS, Dulbecco's phosphate buffered saline (Invitrogen/Gibco).
2. TRIzol reagent (Invitrogen, Paisley, UK).
3. Chloroform, minimum 99% (Sigma-Aldrich, Poole, UK).
4. 2-Propanol, for molecular biology, minimum 99% (Sigma-Aldrich).
5. Ethanol, 190 proof, for molecular biology (Sigma-Aldrich).
6. Nuclease-free water (Severn Biotech Ltd, Kidderminster, UK).
7. RNA storage solution (Ambion, Warrington, UK).

2.4. cDNA Synthesis

1. Oligo(dT)$_{12-18}$ primer (Invitrogen).
2. 10 mM dNTP mix (Invitrogen).
3. 5X first-strand buffer (Invitrogen).
4. 0.1 M DTT (Invitrogen).
5. SuperScript™ II reverse transcriptase (Invitrogen).

2.5. RT-PCR

1. BioMix™ Red (Bioline, London, UK).
2. Water (ddH_2O) (Severn Biotech Ltd).
3. Primers (Sigma-Genosys, Pampisford, UK).

2.6. Gel Electrophoresis

1. Agarose electrophoresis grade (Invitrogen).
2. Ethidium bromide solution, 10 mg/ml (Sigma-Aldrich).
3. Tris–Borate–EDTA buffer (TBE), 10X concentrate (Sigma-Aldrich).

2.7. Real-Time PCR

The contents of TURBO DNA-*free*™ kit are as follows:

- 10X TURBO DNase buffer (Ambion).
- TURBO DNase (Ambion).
- DNase inactivation reagent (Ambion).

1. SYBR green PCR master mix (Qiagen, Crawley, UK).
2. Nuclease-free water (Severn Biotech Ltd).
3. Primers (Sigma-Genosys).

2.8. Enzyme-Linked Immunosorbent Assay (ELISA)

The content of ELISA sets (eBioscience, Insight Biotechnology Limited, Wembley, UK):

1. Capture antibody: pretitrated, purified antibody.
2. Detection antibody: pretitrated, biotin-conjugated antibody.
3. Standard: recombinant cytokine for generating standard curve and calibrating samples.
4. ELISA coating buffer powder (*see* **Note 1**).
5. Assay diluent: 5X concentrated (*see* **Note 2**).
6. Detection enzyme: pretitrated avidin-horseradish peroxidase (Av-HRP).
7. Substrate solution: tetramethylbenzidine (TMB) substrate solution.
8. Certificate of analysis: lot-specific instructions for dilution of antibodies and standards.
9. 96-Well plate (*see* **Note 3**).

Other materials needed are as follows:

1. Wash buffer 1X PBS with 0.05% Tween-20 (Sigma-Aldrich).
2. Stop solution: 1 M H_3PO_4 or 2 N H_2SO_4 solution.

2.9. Luminex Fluorokine MAP Cytokine

Materials provided by Fluorokine® MAP cytokine multiplex kits are (R & D System Europe Ltd, Abingdon, UK) are follows:

1. Standard cocktail 1 and 2: recombinant human cytokines in a buffered protein base.
2. Standard value card 1 and 2: card listing the standard cocktail 1 and 2 reconstitution volume and working standard concentrations.
3. Microparticle diluent: a buffered protein base.
4. Calibrator diluent RD5K concentrate: a buffered protein base for cell culture supernatant samples.
5. Calibrator diluent RD6-40: a buffered protein base for serum/plasma samples.
6. Wash buffer concentrate: a solution of buffered surfactant.
7. Biotin antibody diluent: a buffered protein base.
8. Streptavidin–PE: a streptavidin–phycoerythrin conjugate.

2.10. Occludin Immunofluorescence

1. D-PBS, Dulbecco's phosphate buffered saline (Invitrogen/Gibco).

2. Paraformaldehyde (8) (Sigma-Aldrich).
3. Triton X-100 (8) (Sigma-Aldrich).
4. Bovine serum albumin (8) (Invitrogen/Gibco).
5. Occludin antibody (8) (Invitrogen).
6. Fluorescein isothiocyanate-conjugated secondary antibody (8) (Sigma-Aldrich).

2.11. Measurement of Transepithelial Electrical Resistance (TEER)

1. Transwell permeable support tissue culture treated (5) (Corning Incorporated, Corning, USA)
2. Epithelial volt-ohmmeter (World Precision Instrument, Stevenage, England)

3. Methods

3.1. Cell Culture Preparation/ Maintenance

1. Caco-2 (ATCC, HTB-37), HT-29 (ATCC, HTB-38), and T-84 (ATCC, CCL-248) human colon carcinoma cells are grown respectively in Dulbecco's Modified Eagle Medium (DMEM) and DMEM F-12 Ham medium supplemented with 10% fetal calf serum, 1% L-glutamine, 1% penicillin–streptomycin, and 1% nonessential amino acid. Cultures are maintained at 37°C in 5% CO_2. When cell lines are confluent they are briefly rinsed with PBS and detached using 3 ml of trypsin–EDTA for 2–3 min (*see* **Note 4**).
2. 10 ml of complete growth medium is added and centrifuged (*see* **Note 5**).
3. Caco-2 and HT-29 pellets are resuspended in 10 ml of DMEM (*see* **Note 6**) and appropriate aliquots of the cell suspensions are added to new 75 cm^2 culture flasks with complete growth medium. Cells are incubated at 37°C.
4. The T-84 cell pellet is resuspended in 10 ml of DMEM F-12 Ham and 1:2 to 1:4 ratios of the cell suspensions are added to new 75 cm^2 culture flasks with complete growth medium. Cells are incubated at 37°C.

3.2. Preparation of Bacterial Strains and Cell Line Infection

1. *C. difficile* strains are cultured either on BHI agar supplemented with 5% defibrinated horse blood and *C. difficile* selective supplement or in BHI broth.
2. Cultures on BHI agar are grown in an anaerobic chamber for 48 h at 37°C.
3. 10 ml of BHI broth is inoculated and incubated in anaerobic chamber until stationary phase is achieved (8–9 h).

4. Confluent Caco-2 and HT-29 monolayers are infected with *C. difficile* strains at the multiplicity of infection (MOI) of (100–300) and assessed at specific times (8–24 h) postinfection (**Figs**. **9.1**, **9.2**, **9.3**, and **9.4**) (9).

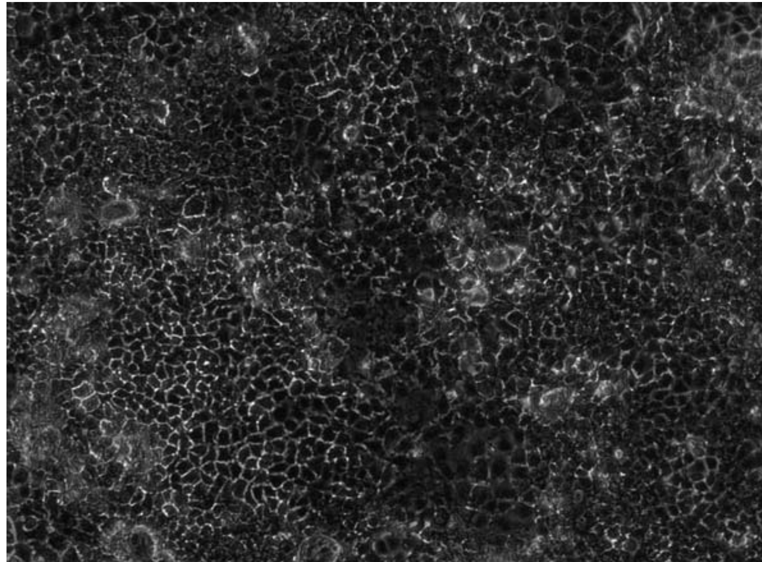


Fig. 9.1. Uninfected Caco-2 monolayer.

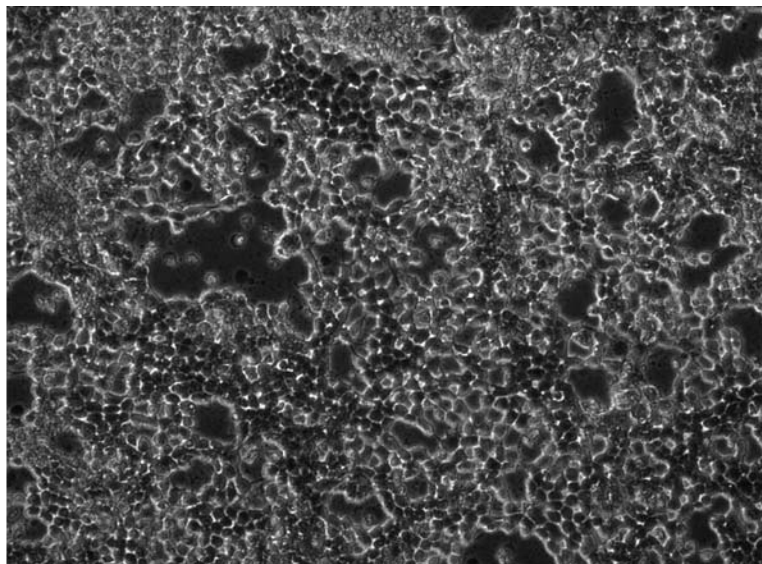


Fig. 9.2. Caco-2 monolayer disruption 24 h postinfection with *C. difficile* R20291 strain.

3.3. RNA Extraction

1. Aspirate medium off the cells and wash with PBS. Add 1 ml of TRIzol and transfer homogenized cells (*see* **Note 7**) to sterile 1.5 ml microcentrifuge tubes and allow to stand at room temperature for 5 min.

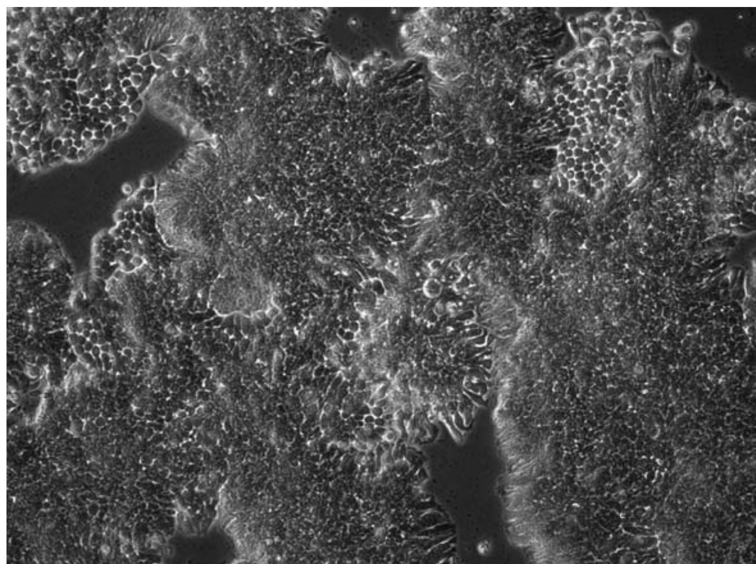


Fig. 9.3. Uninfected HT-29 cells.

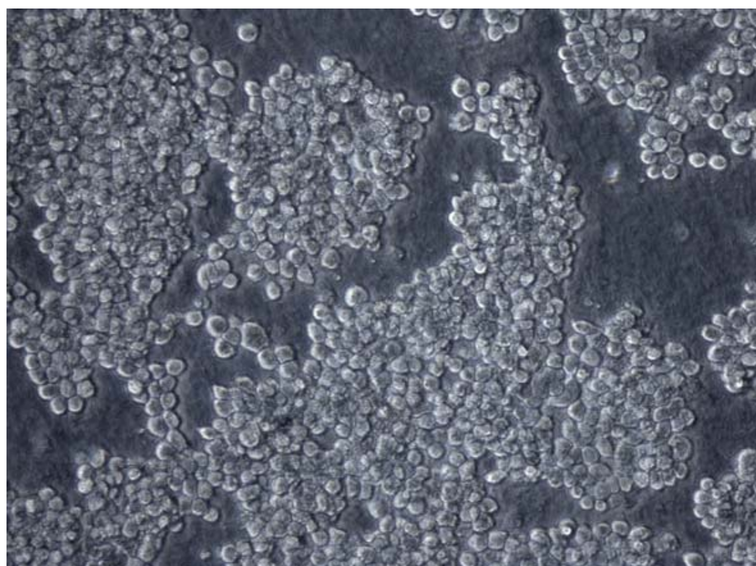


Fig. 9.4. HT-29 cell rounding in response to toxin A *C. difficile* R20291 strain at 8 h postinfection.

2. Add 200 μl (5:1 ratio; TRIzol: chloroform) of chloroform and vortex for 15 s. Incubate at room temperature for 3 min, then centrifuge at 12,000*g* at 4°C (*see* **Note 8**) for 15–20 min.

3. Transfer aqueous layer to a new sterile tube (*see* **Note 9**) containing 500 μl isopropanol and vortex. Incubate at room temperature for 10 min (or overnight at –20°C), then centrifuge at 12,000*g* at 4°C for 15 min.
4. Remove supernatant and wash RNA pellet with 1 ml of 75% nuclease-free ethanol and centrifuge at 7,500×*g* at 4°C for 5 min.
5. Remove supernatant and pipette off any remaining ethanol (*see* **Note 10**). Resuspend pellet in 25–50 μl of RNA storage solution and store the aliquots at –70°C.
6. Measure total RNA by NanoDrop ND-1000 spectrophotometer at 260 and 280 nm. Use the ratio of 260/280 to assess the purity of RNA sample.

3.4. cDNA Synthesis

1. Add the following components to a nuclease-free microcentrifuge tube:
   - Oligo $(dT)_{12-18}$ (500 μg/ml) 1 μl (or random hexamers)
   - 5 μg total RNA × μl
   - dNTP Mix 1 μl
   - Sterile, nuclease-free water to give final volume = 13 μl
2. Heat mixture to 65°C for 5 min and rapidly transfer on ice for at least 2 min (*see* **Note 11**). Add the followings to the contents of the tube:
   - 5X first-strand buffer 4 μl
   - 0.1 M DTT 2 μl
3. Mix contents of the tubes gently and incubate at 25°C for 2 min.
4. Add 1 μl of SuperScript II RT and mix by pipetting.
5. Incubate at 42°C for 50 min.
6. Inactivate the reaction by heating at 70°C for 15 min.

3.5. RT-PCR

The prepared cDNA is used as a template for amplification in PCR.

1. Add the following to a PCR tube and mix them gently:
   - BioMix™ Red 12.5 μl
   - Nuclease-free water 9.5–10.5 μl
   - Forward primer (10 μM) 1 μl
   - Reverse primer (10 μM) 1 μl
   - cDNA 1–2 μl
2. Heat reaction to 94°C for 2 min to denature.
3. Perform 15–40 cycles of PCR. Use the recommended annealing and extension temperatures for the required primers (*see* **Note 12**).

3.6. Real-Time PCR

Real-time PCR is a quantitative PCR. Synthesize cDNA using DNase-treated RNA samples with TURBO DNA-*free*™ kit according to manufacturer's protocol.

Add the following reagents to a microcentrifuge tube and mix gently:

- SYBR green PCR master mix 10 μl
- Required primers (*see* **Note 13**) 1 μl
- Nuclease-free water 7 μl
- cDNA 2 μl

Analyze samples using a real-time PCR machine (Rotor-Gene, Corbett Life Science).

3.7. Gel Electrophoresis

1. Make up 1.5–2% agarose gel.
2. Submerse the gel in a tank filled with 1X TBE buffer.
3. Using a pipette, load RNA markers and RT-PCR reaction samples into slots made in the agarose gel. The molecular weight of samples can be estimated by comparison of the molecular weight of standards.
4. Acquire the image of samples using a UV imager.

3.8. Enzyme-Linked Immunosorbent Assay (ELISA)

1. Coat 96-well ELISA plate with 100 μl/well of capture antibody diluted in coating buffer as manufacturer's instructions. Seal the plate and incubate overnight at 4°C.
2. Aspirate wells and wash five times with 250 μl/well wash buffer (*see* **Note 14**).
3. Add 100 μl/well 1X assay diluent and incubate at room temperature for 1 h, then wash as in Step 2.
4. Using 1X assay diluent, dilute standards, add 100 μl/well of standard to the appropriate wells. Perform twofold serial dilutions of the standards to make the standard curve. Add 100 μl of samples to the appropriate wells and cover the plate and incubate at room temperature for 2 h.
5. Wash as in Step 2.
6. Add 100 μl of detection antibody diluted in 1X assay diluent. Seal the plate and incubate at room temperature for 1 h.
7. Wash as in Step 2.
8. Add 100 μl/well of avidin-HRP diluted in 1X assay diluent. Seal the plate and incubate at room temperature in dark for 30 min.
9. Aspirate and wash wells (*see* Step 2) in wash buffer repeating for a total of seven washes.

10. Add 100 μl of substrate solution to each well. Incubate plate at room temperature for 15 min.
11. Add 50 μl of stop solution to each well.
12. Read plate at 450 nm using a microplate spectrophotometer.

3.9. Luminex

1. Prepare all reagents, working standards, and samples as manufacturer's instruction.
2. Pre-wet the filter-bottomed microplate by filling each well with 100 μl of wash buffer. Remove the liquid through the filter at the bottom of the plate.
3. Resuspend the diluted microparticle mixture by vortexing. Add 50 μl of the microparticle mixture to each well of the microplate.
4. Add 50 μl of standard or sample per well. Mix and seal the plate with a foil and incubate for 3 h at room temperature on a shaker at 500 rpm.
5. Remove the liquid and wash each well with 100 μl of wash buffer. Perform the wash procedure three times.
6. Add 50 μl of diluted biotin antibody cocktail to each well. Cover with foil and incubate for 1 h at room temperature on the shaker at 500 rpm.
7. Repeat the wash as in Step 5.
8. Add 50 μl of diluted Streptavidin–PE to each well. Cover with foil and incubate for 1 h at room temperature on the shaker at 500 rpm.
9. Repeat the wash as in Step 5.
10. Resuspend the microparticles by adding 100 μl of wash buffer to each well. Incubate for 2 min on the shaker at 500 rpm.
11. Read within 90 min using a Luminex analyzer machine.

3.10. Occludin Immunofluorescence

1. Infect confluent T-84 monolayers with *C. difficile* strains for specific time (10).
2. Wash with cold phosphate-buffered saline.
3. Fix with 4% paraformaldehyde at room temperature for 10 min (8), then permeabilize in 0.1% Triton X-100/PBS for 30 min at room temperature.
4. Block cells with 5% bovine serum albumin/PBS for 1 h and incubate with 4 μg/ml occludin antibody overnight at 4°C (8).
5. Use (1:100 dilution) fluorescein isothiocyanate-conjugated secondary antibody for 1 h and cover with foil (8).

6. Visualize using a fluorescence microscope (Leica DMLB, CoolSNAP-Pro$_{cf}$ camera).

3.11. Measurement of Transepithelial Electrical Resistance (TEER)

1. Measure resistance of confluent T84 monolayer on Transwell inserts (CoStar) prior infection (resistance readings between 1,200 and 1,500 Ωcm^2) (11).
2. Infect T84 cells with bacterial culture (8).
3. Measure transepithelial electrical resistance (TEER) at specific times using an epithelial volt-ohmmeter voltmeter (*see* **Note 15**). Use Ohm's law ($V = IR$) to calculate monolayer resistance (10).

4. Notes

1. Reconstitute ELISA coating buffer powder to 1 l with dH_2O and filter the solution with 0.22 μM filter.
2. Make up 1X working solution by adding 10 ml of 5X assay diluent to 40 ml of distilled water.
3. Use only provided 96-well plates or suggested (Corning Costar 9018 or NUNC MaxiSorp flat-bottom) plates.
4. Longer incubation in trypsin–EDTA will lead to increased proteolytic cleavage, consequently cells will take longer to recover; therefore, avoid long incubation time in trypsin–EDTA, just enough time for cells to detach.
5. The growth media contains FCS which is a protein inhibitor of trypsin. Rapid addition of media is recommended.
6. Cell pellets should be washed twice to ensure complete removal of trypsin.
7. Complete homogenization (passing solution through syringe and needle) is recommended to ensure RNA isolation from cellular contents.
8. Centrifugation at 4°C gives a tighter phase separation than that obtained at room temperature.
9. Ensure no phenolic content gets transferred as it will denature the enzymes in later stages.
10. Leave pellet to air-dry to remove all ethanol.
11. Rapid transfer to ice aids in specificity of primer binding.
12. If nonspecific bands are obtained, optimize conditions for PCR by performing gradient temperature PCR and/or varying Mg^{2+} concentration between 0.5 and 2.5 mM.

Do not exceed 15–20 pmol/μl primer concentration and ensure there is minimal propensity for primer dimer formation.

13. Use only primers designed for real-time PCR (for example, that generate a product no larger than 150 bp). Before use, check the concentration and annealing temperature of the primers.
14. Allow time for soaking approximately 1–2 min during each wash step. This helps to increase the effectiveness of the washes.
15. Ensure that probe does not touch the cell monolayer as it may lead to microscopic damage.

References

1. Cloud J and Kelly CP. (2007) Update on *Clostridium difficile* associated disease. *Curr Opin Gastroenterol* **23**(1), 4–9.
2. Issa M, Ananthakrishnan AN and Binion DG. (2008) *Clostridium difficile* and inflammatory bowel disease. *Inflamm Bowel Dis* **14**(10), 1432–1442.
3. Bajaj-Elliott M and Sanderson IR 2008. Nutrition and gene expression, pp. 209–222 in *Nutrition in Pediatrics*, 4th ed., BC Decker Inc, Hamilton, Ontario.
4. Pastorelli L, Vecchi M, Cominelli F and Pizarro T. (2008) Genetic regulation of epithelial barrier function: recent insights into the pathogenesis of inflammatory bowel disease. *Inflamm Bowel Dis Monit* **8**(4), 124–133.
5. Sutton PA, et al. (2008) Essential role of toxin A in *C. difficile* 027 and reference strain supernatant-mediated disruption of Caco-2 intestinal epithelial barrier function. *Clin Exp Immunol* **153**(3), 439–447.
6. Johal SS, et al. (2004) Differential effects of varying concentrations of *Clostridium difficile* toxin A on epithelial barrier function and expression of cytokines. *J Infect Dis* **189**(11), 2110–2119.
7. Mahida YR, et al. (1996) Effect of *Clostridium difficile* toxin A on human intestinal epithelial cells: induction of interleukin 8 production and apoptosis after cell detachment. *Gut* **38**(3), 337–347.
8. Viswanathan VK, et al. (2004) Comparative analysis of EspF from enteropathogenic and enterohemorrhagic Escherichia coli in alteration of epithelial barrier function. *Infect Immun* **72**(6), 3218–3227.
9. Canny G, et al. (2006) Toxigenic *C. difficile* induced inflammatory marker expression by human intestinal epithelial cells is asymmetrical. *Life Sci* **78**(9), 920–925.
10. Viswanathan VK, et al. (2008) Enteropathogenic *E. coli*-induced barrier function alteration is not a consequence of host cell apoptosis. *Am J Physiol Gastrointest Liver Physiol* **294**(5), G1165–G1170.
11. Tirumalasetty PP and Eley JG. (2006) Permeability enhancing effects of the alkylglycoside, octylglucoside, on insulin permeation across epithelial membrane in vitro. *J Pharm Sci* **9**(1), 32–39.

Part V

Genomics

Chapter 10

Comparative Genome Analysis of *Clostridium difficile* Using DNA Microarrays

Richard Stabler, Lisa Dawson, and Brendan Wren

Abstract

Clostridium difficile is a pathogen on the move, as evidenced by the rapid transcontinental spread of the so-called hypervirulent 027 strains, followed by the emergence of further PCR ribotypes such as 017, 078 and 106. This provides a rare opportunity to study the evolution of virulence in action. However, to fully exploit this opportunity, robust phylogenetic methods on a diverse set of characterised strains are required to provide a reference evolutionary framework to study *C. difficile* epidemiology, ecology and virulence. Traditional phylogenetic classification of bacteria to study evolutionary relatedness is based on the characterisation of a limited number of genes, rRNA or signature sequences. However, due to the acquisition of DNA through lateral gene transfer, the differences between closely related bacterial strains can be vast. By contrast, whole genome sequencing comparisons allow all genes to be compared. Nevertheless, whole-scale genome sequencing remains an expensive endeavour and such comparisons are limited to only a handful of strains. DNA microarrays represent an alternative technology for whole genome comparisons enabling a "birds eye view" of all the genes absent or present in a given genome as compared to the reference genome on the microarray.

Key words: *Clostridium difficile*, microarray, comparative phylogenomics, Bayesian data analysis.

1. Introduction

Harnessing DNA microarray information through interrogative and robust algorithms has enabled a true "comparative phylogenomics" approach to be developed. Recent comparative genomics studies have been undertaken on increasingly large collections of strains from defined origins. A common feature from many of these studies has been the unexpectedly large genetic diversity between strains within the same species, blurring our definition of

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_10, © Springer Science+Business Media, LLC 2010

species boundaries. Whole genome comparisons typically identify sets of "core genes" shared by all strains in a species and "accessory genes" present in one or more strains in a species that often result from gene acquisition. It is these differences that can often be used to identify genes/genetic islands related to "gain-of-function traits" in pathogenic strains. Uncovering the mechanisms behind this variability is fundamental in understanding and ultimately counteracting infection. Microarray technology, allied to complex mathematical analysis to determine phylogeny, has provided a sensitive and robust method to examine the genetic relatedness of bacterial populations. The genetic relationships described by Bayesian phylogeny of a DNA–DNA microarray data set can then be correlated against the known phenotypes and ecological behaviour of each bacterial strain in the analysis; this is particularly useful when studying the epidemiology and host association of pathogens. We have termed this technique comparative phylogenomics and have applied it to a number of bacterial pathogens including *C. difficile* (1–4).

Comparative phylogenomic experimental design is based on competitive hybridisations of a collection of test strains against a common reference (**Fig. 10.1**). The selection of strains to be tested is central to any comparative phylogenetic analysis. There are two major parameters which should be considered, first, are strains from well-documented sources and second are strains from diverse ecological, geographical and host sources. A typical common reference would be the strain which has been sequenced and the microarray was designed from in the case of single strain microarrays. Alternatively on multi-strain or pan-species microarrays the strain that most of the reporters were originally designed from would be used as a common reference. Compound or mixed

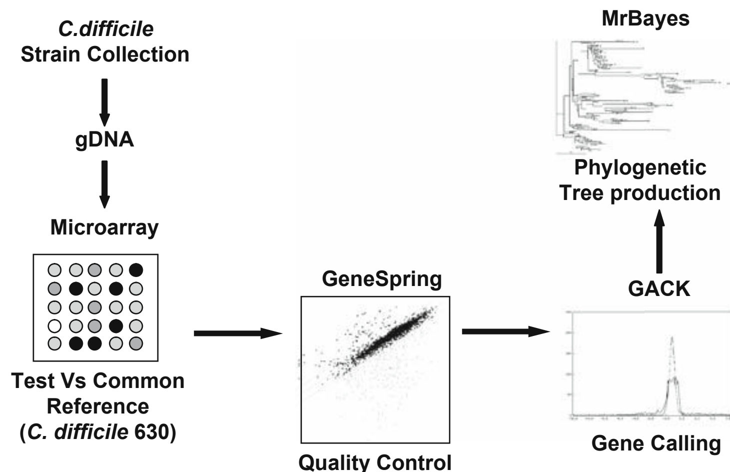


Fig. 10.1. Pipeline diagram for comparative phylogenomics.

genomic DNA from many strains is not recommended as a common reference for comparative phylogenomics. Genomic DNAs from test strains are labelled with one fluorescent dye (e.g. Cy5) and reference with an alternative dye (e.g. Cy3). This results in all test samples being labelled with same dye. Replicate hybridisations can be used to improve data quality; however, for large strain collections this maybe impractical. Additionally dye swaps are not required as all test samples will have the same dye-associated bias and will not effect results. In this chapter we describe the steps involved in generating a comparative phylogenomic tree using *C. difficile* as an example.

2. Materials

2.1. Growth Media

1. Cefoxitin–cycloserine egg yolk (CCEY) agar (BioConnections, Leeds, UK)
2. CCEY supplement (BioConnections)
3. Defibrinated horse blood (TCS Biosciences Ltd, Botolph Claydon, UK)
4. Egg yolk suspension (BioConnections)
5. Cooked meat media (Oxoid, Basingstoke, UK)
6. BHI broth (Oxoid)
7. Blood agar base No. 2 (Oxoid)
8. *C. difficile* supplement (Sigma, St. Louis, MO, USA)
9. Glycerol (Sigma)

2.2. DNA Extraction

1. Lysozyme (Sigma)
2. Mutanolysin (Sigma)
3. Lysostaphin (Sigma)
4. RNase A (Qiagen, Crawley, UK)
5. Proteinase K (Sigma)
6. 20% SDS (Sigma)
7. Phenol–chloroform isoamyl alcohol 25:24:1 (Sigma)
8. Chloroform (Sigma)
9. Phase lock tubes 15 ml (Eppendorf, Hamburg, Germany)
10. Tris–EDTA (TE) (Sigma)

2.3. Microarray Hybridisations

1. 1.5- and 0.5-ml laboratory plus amber microtubes (Alpha Laboratories, Eastleigh, UK)
2. Random primers (Invitrogen, Paisley, UK)

3. REact buffer (Invitrogen)
4. dNTP's (Promega, Madison, WI, USA)
5. dCTP-Cy3 and dCTP-Cy5 (GE Healthcare, Fairfield, USA)
6. Klenow large-fragment DNA polymerase (Invitrogen)
7. Bovine Serum Albumin (BSA) (Sigma)
8. MinElute PCR purification kit (Promega, Southampton, UK)
9. Molecular biology grade dH_2O (Sigma)
10. 20X SSC (Sigma)
11. 20% SDS (Sigma)
12. Lifterslip 22 × 25 mm (Eerie Scientific, Portsmouth, NH, USA)
13. Coplin jar (Fisher, Loughborough, UK)
14. Slide staining trough (Fisher)
15. Staining rack (Fisher)
16. Hybridisation chamber (Fisher)

2.4. Hardware

1. Anaerobic cabinet (Don Whitley Scientific Ltd, Shipley, UK)
2. Microarray laser scanner (Affymetrix GMS418, Santa Clara, CA, USA)

2.5. Software

1. Microarray laser scanner software (Affymetrix GMS418 ArrayReader)
2. Data acquisition software (ImaGene, BioDiscovery, El Segundo, CA, USA)
3. Data analysis software (GeneSpring v7.3.1, Agilent, Santa Clara, CA, USA)
4. Excel (Microsoft)
5. GACK (morphbank.ebc.uu.se/mrbayes/info.php)
6. MrBayes (mrbayes.csit.fsu.edu)
7. Notepad (Microsoft)
8. TreeView (taxonomy.zoology.gla.ac.uk/rod/treeview.html)

3. Methods

3.1. Growth of *C. difficile* Strain Collection

1. *Clostridium difficile* strains are grown from cooked meat media stocks on CCEY agar plates (according to manufacturer's instructions). Briefly 24 g CCEY agar in 450 ml

dH_2O is autoclaved and allowed to cool to 42°C. To the molten agar 1 vial of CCEY supplement rehydrated in 5 ml sterile dH_2O is added plus 5 ml defibrinated horse blood and 20 ml egg yolk suspension. Twenty-five millilitre aliquots of molten CCEY agar are then poured into Petri dishes.

2. Cooked meat media stocks of *C. difficile* are agitated briefly. Cooked meat media supernatant (50 μl) is pipetted on to CCEY plate surface. The supernatant is spread across the CCEY agar plate with a sterile loop or hockey stick. Plates are incubated in anaerobic conditions for 24–48 h at 37°C.
3. BHI broth (500 ml) containing one vial *C. difficile* supplement rehydrated in 5 ml sterile dH_2O and 0.05% cysteine is pre-reduced and divided into 10 ml aliquots in disposable 28-ml universals. Three colonies of *C. difficile* are used to inoculate a BHI broth aliquot and incubated for 20–24 h at 37°C in an anaerobic chamber.
4. Cultures of *C. difficile* are checked for contamination by using sterility plates. Sterility plates consist of 20 g blood agar base No. 2 in 465 ml dH_2O, autoclaved and allow to cool to 42°C. About 35 ml (7%) defibrinated horse blood is added and the agar is poured into Petri dishes and allow to set. Using a sterile loop, spread *C. difficile* culture onto one sterility plate and incubate anaerobically overnight to check for growth of pure *C. difficile* colonies. Using a sterile loop, spread onto a second sterility plate and incubate in the aerobically overnight to check for no growth.

3.2. DNA Extractions (see Note 1)

1. Spin down overnight cultures (3,000×*g* for 5–10 min, 4°C) from **Section 3.1**, Step 3. Remove the supernatant and resuspend bacterial pellet in 3 ml of 50 mM EDTA. Once the pellet had been thoroughly resuspended add 750 μl (20 mg/ml) lysozyme, 100 μl (10 U/ml) mutanolysin and 100 μl (5 mg/ml) lysostaphin. Mix thoroughly but do not vortex. Add 20 μl RNase and mix but do not vortex. Incubate at 37°C for ≥1 h.
2. To the bacterial suspension add 90 μl of (25 mg/ml) Proteinase K. Add 90 μl of 20% SDS and mix thoroughly but do not vortex. Incubate at 50°C for 1 h.
3. Centrifuge a 15-ml phase lock tube at 1,500×*g* for 2 min to prepare gel. In a fume hood add 3 ml phenol chloroform isoamyl alcohol to phase lock tubes (taken from under protective buffer). Add bacterial suspension from Step 2 and vortex for 10 s, ensuring that the cap is closed before vortexing. Centrifuge at 1,500×*g* for 5 min.
4. In the fume hood, transfer upper phase into a sterile 15-ml centrifuge tube. Add 3 ml of chloroform and vortex for 10 s. Centrifuge at 3,000×*g* for 5 min.

5. Repeat Step 4.
6. Add 8 ml of 100% ethanol to a sterile 15-ml centrifuge tube and add the supernatant from Step 5. Incubate overnight at –20°C.
7. Centrifuge samples at 3,000×*g* for 30 min at 4°C, then remove and discard supernatant. Wash pellet with 1 ml of 70% ethanol (made with nuclease-free water). Centrifuge at 3,000×*g* for 15 min at 4°C and carefully discard supernatant. Pulse in centrifuge and carefully remove any residual ethanol. Air dry for 5 min and resuspend in 50–100 µl 1X TE. Incubate at 4°C to fully resuspend gDNA.

3.3. Microarray Hybridisation (see Note 2)

1. Label test and control in separate amber microfuge tubes. To 2–5 µg gDNA add 1 µl random primers and dH_2O to a final volume of 41.5 µl. Incubate at 95°C for 5 min and snap cool on ice for 2 min (5).
2. Briefly centrifuge and add 5 µl REact buffer, 1 µl dNTP's (5 mM dA/G/TTP, 2 mM dCTP), 0.5 µl dCTP-Cy3 (test) or dCTP-Cy5 (control) and 1 µl Klenow large-fragment polymerase. Mix thoroughly.
3. Incubate at 37°C for 90 min in the dark to label the gDNA (*see* **Note 3**).
4. Unincorporated Cy-dyes and reagents are removed from the fluorescently labelled products using a MinElute clean-up kit. The Cy3- and Cy5-labelled samples are combined. Add 500 µl PBI to the Cy3/Cy5 mix and pipette on to MinElute column. Centrifuge at 11,000×*g* for 1 min and dispose of elute.
5. Pipette 500 µl PE wash on to column and centrifuge at 11,000×*g* for 1 min and dispose of elute.
6. Pipette 250 µl PE wash on to column and centrifuge at 11,000×*g* for 1 min and dispose of elute. Centrifuge at 11,000×*g* for a further 1 min to remove final traces of ethanol.
7. Place MinElute column in a clean 1.5-ml centrifuge tube. Carefully pipette 15.9 µl dH_2O onto the column membrane. Allow to stand for 1 min and centrifuge at 11,000×*g*. About 14.9 µl of the elute is then transferred to a clean 0.5-ml amber tube.
8. To Cy3-/Cy5-labelled gDNA from Step 7 add 4.6 µl filtered 20X SSC and 3.5 µl filtered 2% SDS. Store this hybridisation solution in the dark until required.
9. To prehybridise the microarrays add 8.75 ml 20 x SSC to a Coplin jar. Add 5 ml BSA at 100 mg/ml (filter sterilised) (*see* **Note 4**)**,** 36 ml dH_2O and 250 µl 20% SDS. Preheat

to 65°C for at least 30 min. Place up to five microarrays (as required) in the Coplin jar and incubate at 65°C for 20 min. Wash vigorously for 1 min in dH_2O using a dedicated staining trough and rack. Transfer rack immediately and wash vigorously for 1 min in 100% iso-propanol using a dedicated staining trough. Spin dry in a 50-ml Falcon tube at 500×*g* for 2 min and store in a dark, dry, dust-free environment until use.

10. To set up the hybridisations heat the hybridisation solution from Step 8 to 95°C for 2 min and allow to cool slowly to room temperature (*see* **Note 5**). Place a LiftSlip over microarray (*see* **Note 6**). Pipette Cy3-/Cy5-labelled gDNA under LifterSlip (*see* **Note 7**). Place microarray in a hybridisation chamber and seal (*see* **Note 8**). Place chamber in 65°C water bath overnight in the dark.
11. Wash microarrays vigorously for 2 min in 400 ml wash A preheated to 65°C (wash A: 1X SSC, 0.05% SDS) using a dedicated staining trough and rack (*see* **Note 9**).
12. Transfer the staining rack quickly into a dedicated staining trough containing 400 ml wash B (wash B: 0.06 x SSC) and wash microarrays for vigorously 2 min.
13. Repeat Step 12 in fresh wash B for 2 min using a dedicated staining trough.
14. Centrifuge dry in a 50-ml Falcon tube at 500×*g* for 2 min. Transfer microarrays to a clean, dry and dark microarray container.

3.4. Microarray Laser Scanning (see Note 10)

1. Load microarray into the microarray scanner following the manufacturers' instructions.
2. Set scan area (*see* **Note 11**)
3. Set photomultiplier amplification gain and scan slide.
4. Adjust gain to optimise fluorescent dynamic range (*see* **Note 12**). Save image of optimal gain scan for both Cy3 and Cy5.

3.5. Data Extraction (ImaGene) (see Note 13)

1. Open data extraction software and load settings file (*see* **Note 14**).
2. Import optimal Cy3 and Cy5.tif images into data acquisition software, ensuring images overlap exactly (*see* **Note 15**).
3. Load template file and ensure correctly overlay of images (*see* **Note 16**).
4. Adjust template to match fluorescent spots on microarray (*see* **Note 17**).
5. Mark any artefacts (*see* **Note 18**) and calculate fluorescent intensity and quality measurements.

3.6. Quality Control (GeneSpring) (See Note 19)

1. Load (paired) text files of fluorescent data measurements into software and set up a new experiment.
2. Input strain information into parameters.
3. Adjust interpretation to replicate duplicate hybridisations if required.
4. Adjust interpretation to display "Present and marginal" data only to remove poor quality data as identified during data acquisition (**Section 3.5**).
5. Copy annotated gene lists of (average) raw and control fluorescent data from all genes present on the microarray.
6. Pass exported data into Excel. For each strain set up columns to calculate raw intensity divided by control intensity (ratio) and the $\log_2$ of the ratio value for every gene. (*see* **Note 20**).

3.7. GACK Calling of Present and Absent (see Note 21)

1. GACK requires the data to be in a tab-delimited format. To construct the input file set up columns in Excel, as shown in **Fig. 10.2**, and save worksheet as tab-delimited text (.txt) (6).

UNIQID	NAME	GWEIGHT	Exp1	Exp2	Exp3
EWEIGHT			1	1	1
uniqid1	gene1	1	–1.945	–1.334	–2.038
uniqid2	gene2	1	0.536	0.705	0.454
uniqid3	gene3	1		1.952	–0.904
uniqid4	gene4	1	0.267	0.674	1.068

GID	UNIQID	NAME	GWEIGHT	Exp1	Exp2	Exp3
AID						
EWEIGHT				1	1	1
gene12x	uniqid1	gene1	1	–1.945	–1.334	–2.038
gene24x	uniqid2	gene2	1	0.536	0.705	0.454
gene26x	uniqid3	gene3	1		1.952	–0.904
gene31x	uniqid4	gene4	1	0.267	0.674	1.068

Fig. 10.2. GACK input files. Input files must be in a tab-delimited format. EWEIGHT (experiment weight) and GWEIGHT (gene weight) should be set to 1 for all experiments and genes. UNIQID is typically a systematic numbering to ensure no duplication (e.g. 1, 2, 3, 4 or CDS1, CDS2, CDS3, CDS4), which may occur with gene names. A blank data point indicates where poor quality data have been removed and will be ignored by GACK.

2. Open the GACK programme and browse to locate input file.
3. Select to “Generate graphs”, “No Smoothing” and “Normal curve” for peak modelling.
4. Select to generate “Binary output” and ensure binary %EPP is set to 0% (*see* **Note 22**).
5. Select “Create logfile” and input desired log file name.
6. Select “Run GACK” and exit on completion. The generated graphs can be viewed to see the EPP model compared to the input data.

3.8. Generate Phylogeny Using Bayesian Cluster Algorithm (MrBayes) (see Note 23)

1. MrBayes requires that the input file is in Nexus (FASTA) format (*see* **Fig. 10.3**). This can be achieved by transposing the data from columns to rows (*see* **Note 24**).
2. Add MrBayes header (*see* **Fig. 10.3**).
3. Add MrBayes footer (*see* **Fig. 10.3**) (*see* **Note 25**).
4. MrBayes does not have a windows GUI so has to be run using MS-DOS. Do this Run “cmd” from “start” menu to get MS-DOS C prompt.
5. Navigate to the directory where the Nexus file is stored and then run MrBayes (*see* **Note 26**).

```
#NEXUS
Begin data;
      Dimensions ntax =5 nchar =70;
      Format datatype=standard missing=? matchchar =.;
      Matrix
Strain1
11111111100?0111100011111111100000110100000010 0?0?00000?01110000???0?0
Strain2
101100111?0??01???0?1111111?111000101??0??001011001000000??00?10?010??
Strain3
111?000000??0??0000?0111011110000000???000000?000?00000101100????00?00
Strain4
?0100011100??000000?0000001??00110011?11111011000111100?00?100?0?0?0?0
Strain5
00100011100?0?00000001000111100000?000?000000?0?0?0000010??0000?00000?0;
End;
begin mrbayes;
      log start filename=Demo.txt;
      lset coding = noabsence rate = gamma ngammacat = 16;
mcmc    ngen = 1000000    nruns = 2    nchains = 8    temp = 0.5    printfreq = 100
samplefreq = 100 Startingtree = Random burnin = 10000 savebrlens = yes;
end;
```

Fig. 10.3. Example of a MrBayes input NEXUS file. ntax sets the number of strains in the matrix, nchar sets number of genes, datatype = standard sets the input data to binary, log start ensures all screen output is recorded to “Demo.txt”. ngen sets the number of generations, nruns=2 enables two simultaneous MrBayes runs; however, to minimise RAM bottlenecks these can be performed separately using nruns=1, nchains=8 sets the number of parallel chains (e.g. one cold chain plus seven heated chains), printfreq and samplefreq set the interval between which the cold chain is recorded.

6. Execute input file either directly from C prompt command or from MrBayes interface.
7. At the end of the run, check that the average standard deviation of split frequencies is less than 0.1 to indicate convergence of the two runs. This indicates that the two separate models have converged on a similar phylogeny. If convergence has not been reached allow MrBayes to continue until convergence has been achieved. MrBayes records all sampled trees to the [input_file_name].t file. The sample files can be viewed in many analysis programmes (e.g. Treeview, PAUP, MacClade).

3.9. Viewing Trees

1. Start the Treeview programme and open.t file from MrBayes output from either run1 or run2.
2. Select last tree generated to view the modelled phylogeny from the converged runs.

4. Notes

1. The quality of the nucleic acid samples used is paramount to obtaining quality microarray data. Once prepared, it is essential that the concentration is accurately measured (e.g. using a Nanodrop instrument) and is assessed for degradation (e.g. agarose gel electrophoresis).
2. Comparative phylogenetics is based on the assigning of "present" or "absent" calls to each gene/reporter present on a microarray. Therefore, it is imperative that the microarray design is based on a minimum-cross hybridisation style design and/or take into account some data are not gene specific. Any microarray platform based on competitive (two colour) hybridisations can be used; from spotted PCR products, spotted oligonucleotides to in situ manufactured microarrays, e.g. Agilent IJISS microarrays.
3. Cy-dyes are photosensitive and both dye stocks and labelling reactions should be kept in the dark to minimise photo-degradation.
4. BSA can be prepared in large batches and stored as 5 ml aliquots at –20°C until required.
5. Do not snap cool as this will result in precipitation of the SDS solution.
6. Place LifterSlip carefully onto microarray using tweezers ensuring that the raised edge is face down. Minimise movement of the LifterSlip as this may lead to scratches on the microarray surface.

7. Pipette the hybridisation solution quickly along one edge of the LifterSlip to ensure that the solution wicks under the LifterSlip without forming bubbles, which can lead to patches of non-hybridisation.
8. Add dH_2O into the hybridisation chamber to maintain humidity as per the manufactures' instructions.
9. Wash A, straining trough and staining rack are placed in an 65°C overnight to achieve correct temperature. Staining troughs will break if not preheated. Transfer microarrays from hybridisation chamber to staining rack in wash A as quickly as possible. Holding the microarray in wash A first will allow the LifterSlip to float free.
10. Microarrays should be scanned as soon as possible after washing.
11. Not all microarray scanners allow the scan area to be pre-determined.
12. It is essential that laser scanning of the hybridised microarray maximises the data from the microarray. For example, scanning using low PMT gain will result in low signal fluorescence and poor quality data resulting in unreliable hybridisation ratios. Too high gain setting will result in saturation of the PMT detection levels again resulting in inaccurate ratios. To optimise the dynamic range of fluorescence an optimal scan gain is used such that the gain is sufficiently low to ensure that no pixels are saturated but sufficiently high that signal strength is at maximum. To maximise the quality of the data multiple images can be combined (e.g. using MAVI).
13. There are a number of different software packages (e.g. ImaGene, BlueFuse) used to convert scanned microarray images (usually 16-bit.tiff) but all perform the same function of identifying where each of the microarray "spots" are located, perform measurements on both spot fluorescence and surrounding "background" fluorescence.
14. It is essential that user modifiable settings are always the same between microarray analyses. This can be ensured by using setting files or SOPs prior to any analysis. It is also useful to employ any available measurements on spot quality within the acquisitions software. This will allow the removal of poor quality data later in the analysis as even "empty" spots will have some very low level of fluorescence, which will give false or inaccurate data in subsequent analysis. However, it is essential that these settings and quality measurements do not remove valid data. For example, poor quality can be considered if spot fluorescence is less than twice the background for *both* "test" and

"reference". This ensures that spots that are present in the "reference" but not in the "test" or visa versa are not marked as poor quality.

15. With some microarray scanners the Cy3 and Cy5 images will not align correctly and have to be manually aligned; this is vital to ensure that the spots identified by the software are identical for both Cy3 and Cy5.
16. Templates include the number and location of spots on the microarray as well as gene ID information. The format of this data will depend on the data acquisition software used, ImaGene template file uses a.txt file for gene ID's and a user-defined .grd grid map combined.
17. This step adjusts where the software looks for microarray spots to match the physical size of the spots on the microarray. Some data acquisition software performs this step automatically without any user input.
18. Artefacts are hairs, scratches, fluorescent marks, etc., that mask the true signal of the reporter and need to be excluded. Identification of these problem areas here allows for removal of this poor quality data during quality control (**Section 3.6**).
19. This step involves the averaging of replicate spots on the microarray and removal of data marked as unreliable during the data acquisition step (**Section 3.5**). This quality control can be done manually or through other data analysis software packages. The suggested method using GeneSpring is described here.
20. The raw fluorescent data must be converted to ratio data and then log'd to convert into a normal distributed data set. The data is usually formatted using log base 2 ($\log_2$), however, other log bases can be used but this must be taken into account in the following steps.
21. Genome analysis by Charlie Kim (GACK) uses the ratio data of DNA–DNA hybridisations to identify genes that are conserved (present), contain some sequence divergence (typically less than 5%) or are absent or highly divergent (approximately <95% identity). GACK calculates an EPP (estimated probability of presence) value for each gene; this is then converted into either graded binary or trinary data. GACK requires that data are in a tab-delimited format (**Fig. 10.2**).
22. For analysis by MrBayes a binary output is required, this is usually set at the conservative 0% EPP. GACK binary output identifies a gene as present (1) or absent (0) based on the EPP cut-off. Setting the EPP to 0% only identifies genes with a 0% probability of being present as "absent".

Trinary can be used to designate present (1), divergent (0) or absent (–1). Divergent category is typically genes with an EPP between 0 and 100%. Where data have been removed "empty" data points will be used. The binary output will be in file called filename.bgk and trinary in filename.tgk. Both files can be examined in Excel if required.

23. MrBayes utilises the binary data from GACK to calculate the phylogeny, however, the data have to be converted into Nexus format (**Fig. 10.3**) to be accessible to MrBayes. First empty data points have to be replaced with "?". This can be achieved by opening the GACK output file into Excel and using "find and replace" to find " " and replace "?", but ensuring that the "match entire cell contents" option is selected.
24. The data can be transposed from column to row in Excel, however, due to a limit to the number of columns and alternative maybe required, for example using a Perl script. This output is then saved in tab-delimited text format for further editing.
25. The Bayesian model used four-chain Markov chain Monte Carlo (MCMC), 16-category gamma distribution (ngammacat=16) with 1 million iterations (ngen=1,000,000) with a heat of 0.5 (temp=0.5). Phylogenetic trees were sampled every 100th iteration (printfreq=100 samplefreq=100) and tree structure convergence was statistically assessed across all potential phylogenies [except an initial tree burnin, usually set at the first 25% of sampled trees (burnin=2,500)].
26. For example >mrbayes.exe [input_file_name].nex or > mrbayes.exe -i [input_file_name].nex.

Acknowledgments

The work described in this chapter was funded by the Biotechnology and Biological Sciences Research Council and the Wellcome Trust.

References

1. Champion OL, Gaunt MW, Gundogdu O, Elmi A, Witney AA, Hinds J, Dorrell N and Wren BW. (2005) Comparative phylogenomics of the food-borne pathogen *Campylobacter jejuni* reveals genetic markers predictive of infection source. *Proc Natl Acad Sci USA* **102**(44), 16043–16048.
2. Hinchliffe SJ, Isherwood KE, Stabler RA, Prentice MB, Rakin A, Nichols RA, Oyston PC, Hinds J, Titball RW and Wren BW.

(2003) Application of DNA microarrays to study the evolutionary genomics of *Yersinia pestis* and *Yersinia pseudotuberculosis*. *Genome Res* **13**(9), 2018–2029.

3. Howard SL, Gaunt MW, Hinds J, Witney AA, Stabler R and Wren BW. (2006) Application of comparative phylogenomics to study the evolution of *Yersinia enterocolitica* and to identify genetic differences relating to pathogenicity. *J Bacteriol* **188**(10), 3645–3653.
4. Stabler RA, Gerding DN, Songer JG, Drudy D, Brazier JS, Trinh HT, Witney AA, Hinds J and Wren BW. (2006) Comparative phylogenomics of *Clostridium difficile* reveals clade specificity and microevolution of hypervirulent strains. *J Bacteriol* **188**(20), 7297–7305.
5. Dorrell N, Mangan JA, Laing KG, Hinds J, Linton D, Al-Ghusein H, Barrell BG, Parkhill J, Stoker NG, Karlyshev AV, Butcher PD and Wren BW. (2001 Oct) Whole genome comparison of *Campylobacter jejuni* human isolates using a low-cost microarray reveals extensive genetic diversity. *Genome Res* **11**(10), 1706–1715.
6. Kim CC, Joyce EA, Chan K and Falkow S. (2002) Improved analytical methods for microarray-based genome-composition analysis. *Genome Biol* **3**, 11.

Part VI

Development of Systems for Genetic Analysis of the Organism

Chapter 11

ClosTron-Targeted Mutagenesis

John T. Heap, Stephen T. Cartman, Sarah A. Kuehne, Clare Cooksley, and Nigel P. Minton

Abstract

Members of the genus *Clostridium* have long been recognised as important to humankind and its animals, both in terms of the diseases they cause and the useful biological processes they undertake. This has led to increasing efforts directed at deriving greater information on their basic biology, most notably through genome sequence. Accordingly, annotated sequences of all of the most important species are now available. However, full exploitation of the data generated has been hindered by the lack of mutational tools that may be used in functional genomic studies. Thus, the number of clostridial mutants generated has until recently been disappointingly small. In particular, the construction of directed mutants using classical homologous recombination-based methods has met with only limited success. Moreover, most of these few mutants were constructed by the unstable integration of a plasmid into the chromosome via a single crossover event. As an alternative, recombination-independent strategies have been devised that are reliant upon a re-targeted group II intron. One element in particular, the ClosTron, provides the facility for the positive selection of insertional mutants. The generation of mutants using the ClosTron is extremely rapid (as little as 10 days) and is highly efficient and reproducible. Furthermore, the insertions made are extremely stable. Its deployment has considerably expanded available options for clostridial functional genomic studies.

Key words: Clostridia, ClosTron, Mutagenesis, Group II Intron, Gene Knock-out, Shuttle Vector, *Clostridium difficile*, *Clostridium acetobutylicum*, *Clostridium botulinum*, *Clostridium sporogenes.*

1. Introduction

Group II introns are widespread among both eukaryotic organelle and prokaryotic genomes, where they are found in protein coding and RNA genes. Through the action of a multifunctional intron-encoded protein (IEP), these introns are able to self-catalytically splice out of the RNA of the host gene. The paradigm is the 'Ll.LtrB intron' from the *Lactococcus lactis* gene *ltrB*, which can

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_11, © Springer Science+Business Media, LLC 2010

be found in the chromosome and on certain conjugative plasmids. In a series of very elegant studies, the Lambowitz laboratory have characterised the mechanistic details of its replication and insertion (retro-homing) into intron-free copies of *ltrB*. Through the identification of those factors important in specificity, crucially including base-pairing between intron RNA and target site DNA, they were able to devise procedures whereby defined changes to the intron sequence could be employed to target Ll.LtrB-derived introns to almost any gene of interest (1, 2). The IEP-encoding gene *ltrA* was moved from within the Ll.LtrB intron to a distal plasmid location, allowing the *ltrA* gene to be lost along with the plasmid after the mutagenesis procedure. LtrA is required for mobility of the intron, so its absence prevents the possibility of undesirable secondary mutations in constructed strains. The retargeted intron elements created have been termed "Targetrons".

Whilst incredibly useful, basic Targetron technology is disadvantaged by an inability to select cells in which the intron element has inserted into the desired location. Intron integration frequencies vary widely between target sites and can make the screening effort required to isolate a mutant prohibitively laborious, unless a simple phenotypic screen is available to detect the desired mutation. To circumvent this deficiency, the Lambowitz laboratory constructed an artificial "twintron", whereby the Ll.LtrB intron contains an antibiotic resistance gene which has been inactivated through the insertion of a region of DNA encoding a group I intron. These three elements are orientated relative to one another such that the group I intron is lost (through self-catalytic splicing) during group II intron retro-homing. Consequently the inserted group II intron carries a functional antibiotic resistance gene, allowing positive selection of the desired insertional event (3, 4). This new marker was termed a retrotransposition-activated selectable marker (RAM), and a plasmid incorporating this feature based on the *kan* gene, pACD4K-C, is available from Sigma-Aldrich.

Basic Targetron technology was first used to generate a *plc* mutant of *Clostridium perfringens* (5) using a simple phenotypic plate assay to identify the desired mutant. The available RAM elements could not be used as the encoded antibiotic resistance genes cannot easily be used in *Clostridium* spp. To improve the utility of the method, we constructed a new element incorporating a clostridial RAM based on the *ermB* gene of the *Enterococcus faecalis* plasmid pAMβ1. This new element, the ClosTron (6), allows for the positive selection (using erythromycin) of integration of group II intron elements into any gene, without recourse to a phenotypic screen, and in clostridial species in which, unlike *C. perfringens*, mutants have seldom or never previously been obtained. The plasmid carrying the ClosTron has been designated pMTL007 (**Fig. 11.1**)

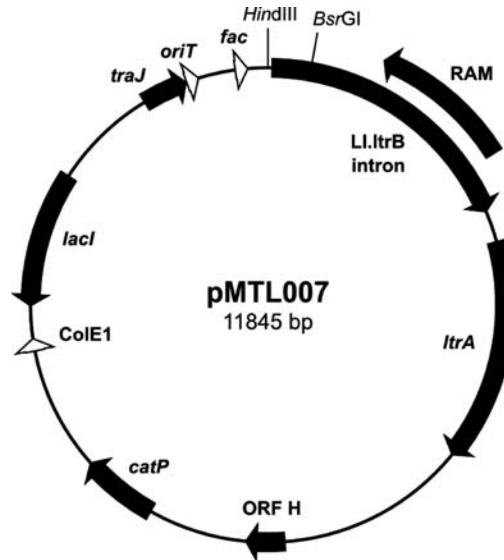


Fig. 11.1. Plasmid pMTL007. For full details of its construction *see* reference (6).

2. Materials

2.1. Strains

1. For cloning, a typical strain such as *Escherichia coli* TOP10 (Invitrogen) is suitable. *E. coli* CA434 is an effective conjugation donor for pMTL007 (7). *Escherichia coli* TOP10 containing pAN2 can be used to methylate pMTL007 (6) prior to electroporation into *Clostridium acetobutylicum* ATCC 824.
2. In our laboratory the ClosTron mutagenesis method has been used successfully with *Clostridium difficile* 630ΔErm (8), *C. difficile* R20291, *Clostridium sporogenes* NCTC 10696, *C. sporogenes* DSM 795, *Clostridium botulinum* ATCC 3502, *C. acetobutylicum* ATCC 824 and *Clostridium beijerinckii* NCIMB 8052.
3. The method has additionally been successfully employed in collaborator laboratories in *Clostridium sordelli* and group II *C. botulinum* strains.

2.2. Media and Buffers

Rich complex media such as those listed below are appropriate for all stages of the gene knock-out protocol using pMTL007. In all these instances, solidified media is made by adding 1% (w/v) bacteriological agar.

1. LB medium: 10 g/l tryptone extract, 5 g/l yeast extract, 5 g/l NaCl; or 2xYT medium: 16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl is routinely used for *E. coli*.

2. BHI medium: Oxoid, 37 g/l brain heart infusion is used for *C. difficile*.
3. TYG medium: 30 g/l tryptone, 20 g/l yeast extract, 1 g sodium thioglycollate is routinely used for *C. sporogenes* and group I *C. botulinum*.
4. 2xYTG medium: 16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, 5 g/l glucose is routinely used for *C. beijerinckii*.
5. CGM medium: 5 g/l yeast extract, 0.75 g/l KH_2PO_4, 0.75 g/l K_2HPO_4, 0.4 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $MnSO_4 \cdot H_2O$, 0.01 g/l $FeSO_4 \cdot 7H_2O$, 2 g/l $(NH_4)_2SO_4$, 1 g/l NaCl, 2 g/l asparagine, 50 g/l glucose, 0.5 g/l cysteine and 1 mg/l rezazurin is routinely used for *C. acetobutylicum*.
6. Electroporation buffer for *C. acetobutylicum* ATCC 824 is 5 mM sodium phosphate buffer at pH 7.4 containing 270 mM sucrose.

2.3. Antibiotic Supplements

1. *Escherichia coli* transformant cells containing pMTL007 are selected and maintained using chloramphenicol at 25 μg/ml in solid medium and 12.5 μg/ml in liquid medium.
2. *Escherichia coli* transformant cells containing pAN2 are selected and maintained using tetracycline at 10 μg/ml.
3. *Clostridium difficile* transconjugant cells containing pMTL007 are selected and maintained using D-cycloserine at 250 μg/ml, 8 μg/ml cefoxitin (commercially available from Oxoid in a mixture called *C. difficile* selective supplement) and thiamphenicol at 15 μg/ml in solid medium and 7.5 μg/ml in liquid medium.
4. Transconjugant cells of other *Clostridium* spp containing pMTL007 are selected and maintained using D-cycloserine at 250 μg/ml and thiamphenicol at 15 μg/ml in solid medium and 7.5 μg/ml in liquid medium.
5. Transformant cells of other *Clostridium* spp containing pMTL007 are selected and maintained using thiamphenicol at 15 μg/ml in solid medium and 7.5 μg/ml in liquid medium.

3. Methods

The following protocol represents a day-by-day account of the individual steps required to generate a clostridial clone in which the group II intron has been inserted into the desired region (gene) of DNA. In overview:

Day 1

- Perform re-targeting PCR.
- Digest PCR product and pMTL007 vector with *Hin*dIII and *Bsr*GI.
- Ligate digested PCR product into digested pMTL007 vector.
- Transform ligation reactions into *E. coli* cloning strain.

Day 2

- Inspect ligation and control plates.
- Pick colonies and inoculate overnight cultures.

Day 3

- Determine sequence of new targeting region.
- Miniprep plasmid DNA from overnight cultures.
- Screen clones by restriction analysis.
- Send plasmid DNA for sequencing.

Day 4

- Transform the re-targeted pMTL007 plasmid into a suitable *E. coli* strain as required by the plasmid transfer method to be employed on Day 5.
- Inoculate an overnight culture with the target clostridial strain.

Day 5

- Mix conjugative donors and recipients and incubate on a plate for 8 h to allow transfer of re-targeted pMTL007 by conjugation or
- Electroporate competent cells of *E. coli* carrying an appropriate methylase gene with purified re-targeted pMTL007.
- Plate transconjugants or electrotransformants onto selective growth medium and incubate overnight.

[Transformants/transconjugants may require 1–3 days incubation dependent on organism]

Day 6

- Inspect transformation/conjugation plates.
- Re-streak a single colony onto a fresh selective plate.
- Use the same colony to inoculate an overnight culture if appropriate for the host strain.

Day 7

- If required for the host strain, induce intron expression in liquid culture using IPTG.
- Plate integrants onto selective growth medium and incubate overnight.

[Integrants may require 1–3 days incubation, dependent on organism]

Day 8

- Inspect integrant (knock-out) plates.
- Pick colonies and re-streak onto fresh selective plates.

Day 9

- Re-streak isolated clones onto fresh selective plates.
- Use the same colonies to inoculate overnight cultures.

Day 10

- Prepare genomic DNA from overnight cultures.
- Use PCR to screen colonies for the desired integration event.
- Send PCR products for nucleotide sequencing
- Re-streak integrants to screen for plasmid loss.

3.1. Intron Re-targeting Primer Design

The initial step in the procedure is to use a computer algorithm to identify sites in the sequence of interest to which the Ll.LtrB intron could likely be targeted, as described by Perutka et al. (2). Next, a derivative of the Ll.LtrB intron is designed to target such a site using modified EBS1/δ and EBS2 sequences, which are responsible for recognition of the target site by base-pairing. Corresponding IBS sequences required for splicing are also designed. These two steps may most simply be achieved through the purchase of a Targetron Gene Knock-out System kit (from Sigma-Aldrich) which includes a unique access code allowing the Targetron Design Site (http://www.sigma-genosys.com/targetron/) to be used several times. This service provides a straightforward, automatic tool for both the identification of target sites and the design of PCR primers (*see* **Note 1**). Before using the Targetron Design Site, the Targetron Test Site (http://www.sigma-genosys.com/targetron/checkSequence.aspx) can be used to determine the number of possible intron insertion sites within each gene (or portion of gene) free of charge.

3.2. Mutant Generation

3.2.1. Day 1

SOE PCR (splicing by overlap extension PCR) is used to mutate the region of the Ll.LtrB intron responsible for target specificity. The PCR primers required to make the appropriate mutations were designed previously using the computer algorithm. The resulting PCR product contains the targeting region (EBS1/δ, EBS2 and IBS sequences) of an intron that will insert into your gene of interest. By using an appropriate mixture of four primers and a special template, the SOE PCR can be performed in a single tube without the intermediate purification steps and second round of PCR usually required for SOE PCR.

1. Assemble a four-primer mixture by mixing 12 μl dH_20 with 2 μl IBS (100 μM), EBS1d (100 μM), EBS2 (20 μM) and EBS Universal (20 μM) primers. The IBS, EBS1d and EBS2 primers are specific to the selected target site, whereas the EBS Universal primer (5′-CGAAATTAGAAACTTGCGTTCAGTAAAC-3′) is common to all re-targeting PCRs.
2. Assemble PCR reaction using your preferred PCR enzyme/buffer system, including as primers and template 1 μl of the above 4-primer mixture and 1 μl of Intron PCR Template (supplied in the Sigma Targetron kit). The Intron PCR Template can be diluted at least 10-fold without adversely affecting the PCR reaction. Making a dilute stock of the Intron PCR Template is therefore a useful way to conserve limited quantities of the template.
3. Perform PCR using the following cycling conditions: Denature 94°C for 30 s followed by 30 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 30 s with a final extension of 72°C for 2 min.
4. Although the PCR product is only ~350 bp in length, the presence of extensive secondary structure in the template increases the rate of mis-incorporation (the PCR error rate). The risk of an early PCR error predominating among the products present at the end of a single reaction can be ameliorated by performing the PCR reaction in triplicate, and after thermocycling is complete, pooling the three reactions together.
5. Visualise the PCR product(s) using agarose gel electrophoresis. A 1% (w/v) agarose gel should provide adequate resolution. The desired ~350 bp PCR product should be bright (*see* **Note 2**)
6. The desired ~350 bp PCR product DNA must be purified to remove the other PCR reaction components. Normally purification by a standard PCR clean-up method should suffice, especially if the desired ~350 bp PCR product was the only visible band. To be especially rigorous, or if other bands are clearly visible, the desired PCR product can be purified by a standard agarose gel purification method.
7. The purified PCR product is digested with *Hin*dIII and *Bsr*GI prior to ligation into pMTL007. The plasmid is linearised with the same pair of restriction endonucleases to generate compatible cohesive ends. Ensure sufficient incubation time is allowed for the DNA to be digested to completion. Partial digestion reactions cannot be read-

ily identified by gel electrophoresis. If desired, an alkaline phosphatase treatment can be performed on the linearised pMTL007 at this stage.

8. Analyse the pMTL007 plasmid restriction digestion by electrophoresis on a 1% agarose gel. Load the entire reaction mixture. Two bands should be visible, one at ~350 bp (which corresponds to the excised targeting region) and one at ~11.5 kbp (which corresponds to the pMTL007 plasmid backbone). Excise and gel-purify the ~11.5 kbp pMTL007 backbone band.
9. Purify the new targeting region from the digestion of the PCR product using a standard PCR clean-up method. Agarose gel purification is not necessary.
10. The digested PCR product is ligated into pMTL007 to retarget the intron to the gene of interest. A vector-only control reaction is also performed to verify complete digestion of the pMTL007 plasmid. Sufficient ligation has usually occurred after incubation at room temperature for 30 min.
11. The ligation reaction mixtures are transformed by electroporation or heat shock into a standard *E. coli* cloning strain, such as DH5α or TOP10. *Escherichia coli* cells which have been transformed with pMTL007 can be selected by plating the transformation mixture onto media supplemented with 25 μg/ml chloramphenicol and incubating the plates at 37°C overnight.

3.2.2. Day 2

1. After incubation at 37°C for 24–48 h chloramphenicol-resistant colonies should be large enough to pick. If the restriction digestion and ligation reactions were successful, there should be numerous colonies on the ligation plate and very few or no colonies on the ligation control plate.
2. If there are numerous colonies on the vector-only control plate, consider repeating the restriction digestions and ligations. Treating the linearised pMTL007 vector with alkaline phosphatase after the restriction digestion should reduce the number of colonies on the vector-only control plate.
3. If few or no colonies are obtained, longer incubation of the ligation reaction, a larger-scale ligation or alternative incubation conditions may increase efficiency and yield more colonies.
4. Pick several colonies and use each to inoculate 5 ml of fresh LB broth supplemented with 12.5 μg/ml chloramphenicol. Incubate the cultures overnight at 37°C and 200 rpm shaking. To keep the clones, re-streak the same colonies onto fresh selective plates.

```
      HindIII            IBS primer
AAAA AAGCTT ATAATTATCCTTACGTGACGGTTAAGTGCGCCCAGATAGGGTGTTAA

GTCAAGTAGTTTAAGGTACTACTCTGTAAGATAACACAGAAAACAGCCAACCTAACC

GAAAAGCGAAAGCTGATACGGGAACAGAGCACGGTTGGAAAGCGATGAGTTACCTAA

AGACAATCGGGTACGACTGAGTCGCAATGTTAATCAGATATAAGGTATAAGTTGTGT
                           EBS2 primer
TTACTGAACGCAAGTTTCTAATTTCGGTTTCACGTCGATAGAGGAAAGTGTCTGAAA

CCTCTAGTACAAAGAAAGGTAAGTTACGTTAACCGACTTATCTGTTATCACCACATT
                              Reverse complement of EBS1d primer
TGTACA ATCTG
BsrGI
```

Fig. 11.2. The targeting region from pMTL007. The unique *Hin*dIII and *Bsr*GI restriction sites are shown in *white*.

3.2.3. Day 3

1. The expected sequence of the new targeting region can be easily derived from the sequence of the existing pMTL007 targeting region, which is shown in **Fig. 11.2**. Replace the sequence within the three boxed regions with the sequence of your target-specific IBS primer, EBS2 primer or the reverse complement of the EBS1d primer as indicated. The resulting sequence will contain the base changes required to target the intron to your gene of interest.
2. Using a standard miniprep method, purify plasmid DNA from each of the overnight cultures.
3. If possible, the purified plasmid miniprep DNA could be screened by restriction analysis prior to sequencing. However, the new targeting region generated by PCR differs by only a few base pairs from the old targeting region excised from pMTL007, and these few differences may or may not result in any restriction site differences between the old and new targeting regions.
4. Send plasmid DNA from one or more clones for sequencing. The primers 5402F-F1 (5′-TTAAGGAGGTGTA TTTCATATGACCATGATTACG-3′) and pMTL007-R1 (5′-AGGGTATCCCCAGTTAGTGTTAAGTCTTGG-3′) are suitable for use in sequencing reactions using an annealing temperature of 50°C.

3.2.4. Day 4

The procedures on the fourth day largely depend upon the method by which the re-targeted pMTL007 plasmid is to be transferred from *E. coli* into the clostridial host.

In any case, a successfully re-targeted pMTL007 plasmid, verified by sequencing, is first identified. Ten millilitre of an appropriate anaerobic broth should also be inoculated with your

target clostridial host and incubated at 37°C under anaerobic conditions overnight.

Some clostridia can be electrotransformed without taking measures to overcome their native restriction system(s), including strains of *C. beijerinckii*.

1. Use the *E. coli* clone containing the re-targeted pMTL007 plasmid to inoculate 5 ml of LB broth supplemented with 12.5 μg/ml chloramphenicol.
2. Incubate the culture at 37°C and 200 rpm shaking overnight.

For species which can be electrotransformed, but in which a restriction barrier prevents DNA transfer, the re-targeted pMTL007 plasmid must first be protectively methylated. A suitable method is described for the example of *C. acetobutylicum* ATCC 824:

1. The re-targeted pMTL007 plasmid is re-transformed into *E. coli* cells containing the plasmid pAN2 (6). pAN2 expresses a DNA methylase from *Bacillus* phage Φ3T with identical specificity to the DNA methylase from *C. acetobutylicum* ATCC824. After transformation, allow the cells a typical 1-h recovery period in rich medium.
2. Use the transformed cells to inoculate 5 ml of LB broth supplemented with 12.5 μg/ml chloramphenicol (to select for pMTL007) and 10 μg/ml tetracycline (to select for pAN2).
3. Incubate the culture at 37°C and 200 rpm shaking overnight. The presence of the pAN2 plasmid in these cells will cause the pMTL007 plasmid to be methylated and protected against the *C. acetobutylicum* ATCC824 DNA restriction endonuclease Cac824I.

Plasmids can only be introduced into some clostridia, such as *C. difficile* and *C. sporogenes* (7, 9), by conjugative transfer from *E. coli* donors:

1. Re-transform the re-targeted pMTL007 plasmid into *E. coli* CA434 cells (7). This host contains plasmid R702, which is capable of mobilising shuttle plasmids that include the RK2 origin of transfer (*oriT*), such as pMTL007. After transformation, allow the cells a typical 1-h recovery in LB broth
2. Use the transformed cells to inoculate 5 ml of LB broth supplemented with 12.5 μg/ml chloramphenicol to select for pMTL007.
3. Incubate the culture at 37°C and 200 rpm shaking overnight.

3.2.5. Day 5

For clostridial hosts in which the re-targeted plasmid is introduced by conjugative transfer, mix conjugal donors and recipients and incubate on a plate for 8 h to allow transfer of pMTL007 by conjugation:

1. Pellet 1 ml of the stationary overnight culture of *E. coli* CA434 cells harbouring your re-targeted pMTL007 plasmid by centrifugation at 8,000 rpm for 1 min. Discard the supernatant, then wash the cells by re-suspending them in 0.5 ml of sterile PBS. Centrifuge as before and discard the supernatant.
2. Re-suspend the conjugal donor pellet in 200 μl of the stationary overnight culture of conjugal recipient cells (your target organism).
3. Pipette the entire conjugation mixture onto a single non-selective plate containing an appropriate anaerobic solid growth medium in discrete drops or "spots". Do not invert the plate. Incubate the plate at 37°C for 8 h under anaerobic conditions to allow conjugal transfer of the re-targeted pMTL007 plasmid from the *E. coli* donor to the clostridial recipient.
4. Pipette 1 ml of anaerobic sterile PBS onto the conjugation plate. Using a sterile spreader, scrape the layer of cells off the plate and re-suspend them in the PBS.
5. Using a pipette, aspirate as much of the conjugation slurry as possible into a fresh microtube. Spread all of the slurry onto several fresh plates of an appropriate anaerobic solid growth medium, supplemented with a counterselection agent to select against the *E. coli* conjugal donor (*see* **Section 2.3**) and 15 μg/ml thiamphenicol to select for the re-targeted pMTL007 plasmid.
6. Incubate the plates at 37°C under anaerobic conditions for 1–3 days.

For clostridial strains which can be electrotransformed; purify plasmid DNA from *E. coli*, prepare electrocompetent clostridial cells, and transfer the re-targeted pMTL007 plasmid by electroporation:

1. Use a standard mini plasmid preparation method to isolate plasmid DNA from the overnight culture. In the case of *E. coli* cells containing both pAN2 and a re-targeted pMTL007 plasmid, the purified DNA will be protected against the *C. acetobutylicum* ATCC824 DNA restriction endonuclease *Cac*824I. Protection of the plasmid DNA can be demonstrated by restriction digestion with *Fnu*4HI, an isoschizomer of *Cac*824I (i.e. it recognises the same sequence GCNGC) which is also blocked by methylation.
2. Prepare competent cells of the target organism and electroporate with pMTL007. Methods for the preparation and electroporation of electrocompetent *C. acetobutylicum*, *C. beijerinckii* cells and *C. botulinum* are described in the literature (10–13) (*see* **Note 3**).

- Use a loop of *C. acetobutylicum* ATCC824 cells from a fresh plate to inoculate 10 ml 2xYTG broth, and mix by vortexing. Serially dilute this suspension into further 10 ml volumes to a 10^{-3} dilution.
- Incubate all cultures anaerobically at 37°C overnight.
- Inoculate 60 ml 2xYTG medium with the whole 10 ml of the most dilute overnight culture showing growth, which is likely to correspond to cells in exponential phase.
- Incubate anaerobically at 37°C until the OD_{600} reaches approximately 1.1.
- Split the culture among centrifuge tubes, place on ice and transfer out of the anaerobic cabinet.
- Pellet cells by centrifugation at 5,000×*g* for 10 min at 4°C. Place the tubes on ice, transfer back into the anaerobic cabinet and carefully discard the supernatants.
- Re-suspend the pellets in a total of 10 ml of ice-cold electroporation buffer (*see* 2.2 Media and Buffers) and pool the cells into a single centrifuge tube. Place the tube on ice and transfer out of the anaerobic cabinet.
- Pellet cells as before. Place the tube on ice, transfer back into the anaerobic cabinet and carefully discard the supernatant.
- Re-suspend the pellet in 2.3 ml of cold electroporation buffer.
- Add 20 μl of methylated plasmid DNA solution containing 2–10 μg DNA to each chilled 0.4-cm gap electroporation cuvette. Transfer cuvettes into the anaerobic cabinet on ice.
- Add 570 μl of cell suspension to each cuvette.
- Electroporate the cells (2.0 kV, 25 μF, ∞Ω) and immediately add 1 ml 2xYTG medium. Mix gently and transfer the contents of the cuvette to a tube containing 9 ml 2xYTG medium.
- Incubate anaerobically at 37°C for 1–3 h to allow recovery.
- Pellet cells by centrifugation at 5,000×*g* for 10 min at room temperature. Transfer back into the anaerobic cabinet, discard supernatants and re-suspend each pellet in 1 ml of 2xYTG before plating out onto suitable selective CGM agar plates (supplemented with 15 μg/ml thiamphenicol in the case of pMTL007).

3. After the recovery period, plate the transformation mixture onto several fresh plates containing an appropriate anaerobic solid growth medium supplemented with

7.5 μg/ml thiamphenicol to select for the re-targeted pMTL007 plasmid.

4. Incubate the plates at 37°C under anaerobic conditions for 1–3 days.

3.2.6. Day 6

1. Inspect transformation/conjugation plates. After incubation at 37°C for 24–72 h thiamphenicol-resistant colonies should be large enough to pick.
2. Re-streak a single colony onto a fresh plate of the same selective medium for safe keeping.
3. For organisms in which IPTG induction of pMTL007 is necessary to induce intron expression and in which pMTL007 can be maintained in liquid culture (such as *C. sporogenes* and *C. botulinum*) use the same colony to inoculate 1 ml of an appropriate anaerobic liquid growth medium supplemented with 7.5 μg/ml thiamphenicol. Incubate the culture at 37°C under anaerobic conditions overnight.

3.2.7. Day 7

For organisms in which IPTG induction of pMTL007 is necessary:

1. Inoculate 1 ml of fresh selective broth with 100 μl of the overnight culture of pMTL007 transformants/transconjugants.
2. Incubate the culture at 37°C under anaerobic conditions until there is visible growth, indicating the culture is in exponential phase (typically after incubation for 1 h).
3. Induce intron expression with IPTG. Add IPTG to the culture to a final concentration of 1 mM. Incubate at 37°C under anaerobic conditions for 1–3 h.
4. Wash cells and allow recovery period. Pellet the cells by centrifugation at 5,000×*g* for 1 min. Discard the supernatant, then wash the cells by re-suspending them in 0.5 ml of sterile PBS. Centrifuge as before and discard the supernatant. Re-suspend the pellet in 1 ml of an appropriate anaerobic liquid growth medium, unsupplemented with antibiotics and incubate at 37°C under anaerobic conditions for 1–3 h.
5. Plate the integration mixture onto fresh plates containing an appropriate anaerobic solid growth medium supplemented with 2.5 μg/ml erythromycin to select for presence of the spliced ErmRAM, which indicates intron integration. The integration frequency may vary widely depending upon the organism, duration of induction and recovery steps, targeting region and potentially the phenotype of the mutant. Therefore, spread three plates with 100 μl of neat, 100 μl of 100-fold diluted and 100 μl of 5-fold concentrated integration mixture.

6. If you wish to determine the frequency of the integration event, you should also plate serial dilutions of the integration mixture onto fresh plate(s) containing an appropriate anaerobic solid growth medium unsupplemented with antibiotics.
7. Incubate the plates at 37°C under anaerobic conditions for 1–3 days.
8. Generally, 2.5 μg/ml erythromycin provides clean selection. Higher concentrations of erythromycin can be used if background growth is observed, although reduced integration frequencies will be observed and longer incubations may be necessary.

For organisms in which IPTG induction of pMTL007 is not necessary, such as *C. acetobutylicum* and *C. difficile*, integrant clones may be selected by simply re-streaking transconjugants/transformants directly onto suitable selective media (i.e. containing erythromycin).

3.2.8. Day 8

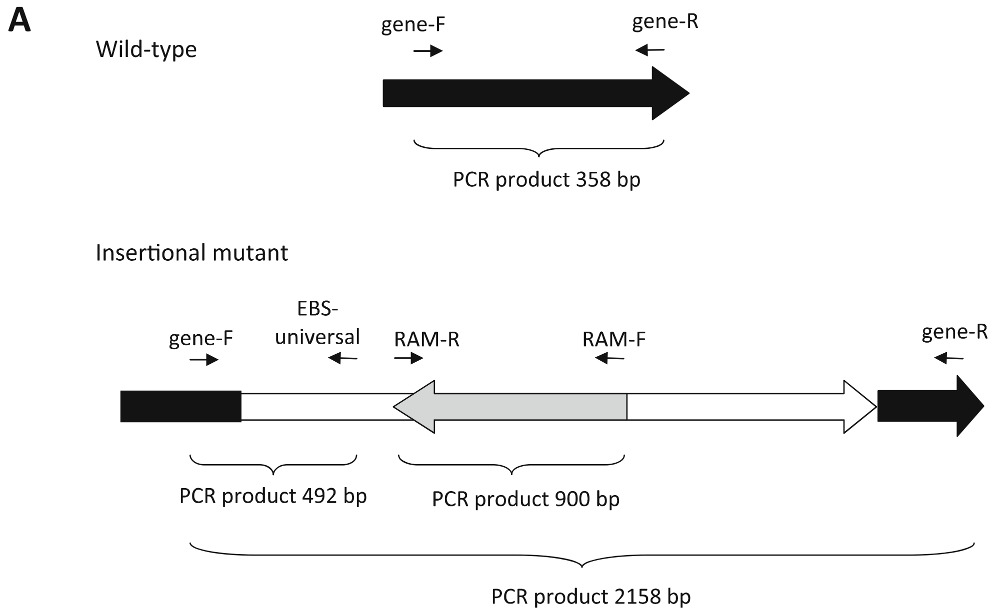
1. Inspect integrant (mutant) plates. After incubation at 37°C for 24–72 h erythromycin-resistant colonies should be large enough to pick.
2. As soon as they are large enough, pick several erythromycin-resistant colonies and re-streak to single colonies on fresh selective (erythromycin-supplemented) plates to isolate clones.

3.2.9. Day 9

1. Re-streak isolated clones (single colonies from the re-streak plates) onto fresh selective (erythromycin-supplemented) plates for safe keeping.
2. Use the same colonies to inoculate 1 ml of an appropriate anaerobic liquid growth medium supplemented with 2.5 μg/ml erythromycin to select for the integrants.
3. Incubate the cultures at 37°C under anaerobic conditions overnight.

3.2.10. Day 10

1. Prepare genomic DNA from overnight cultures. Using a standard genomic DNA preparation method for the organism, purify genomic DNA from each of the overnight cultures. For example, the Qiagen DNeasy Tissue kit is suitable for preparing genomic DNA from many organisms.
2. Use PCR to screen colonies for the desired integration event. PCR using several different combinations of primers can be used to characterise the intron integrants, as shown in **Fig. 11.3**.
3. PCR using the ErmRAM forward primer (5′-ACGCGTT ATATTGATAAAAATAATAATAGTGGG-3′) and reverse primer (5′-ACGCGTGCGACTCATAGAATTATTTCCT



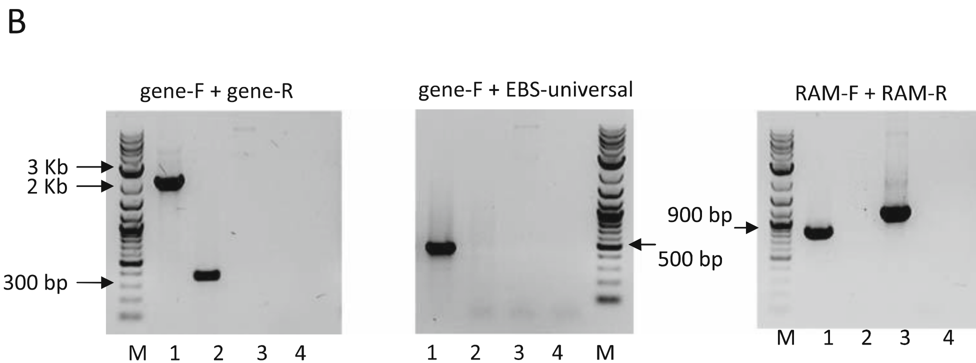


Fig. 11.3. Three PCR screens routinely carried out on putative ClosTron mutants. Primers gene-F and gene-R are used to screen the target gene either side of the insertion. The product obtained from the mutant will be 1,800 bp larger than the wild type. Primers gene-F and EBS Universal are used to screen one of the intron–exon junctions of the mutant. A product should not be visible in the wild type. Primers RAM-F and RAM-R are used to demonstrate the spliced form of the RAM in the mutant. The spliced RAM will give a product approximately 400 bp smaller than the unspliced RAM (observed in the plasmid control reaction). M, 2 log DNA ladder; 1, putative ClosTron mutant genomic DNA; 2, wild type genomic DNA; 3, pMTL007 plasmid DNA; 4, water negative control.

CCCG-3′) demonstrates RAM splicing, yielding a 900-bp product in the presence of the spliced (and therefore integrated) RAM, and a 1,300-bp product in the presence of the full-length RAM. The presence of both bands shows the presence of a spliced RAM, and also the presence of the full-length RAM on pMTL007, indicating that the plasmid had not yet been lost from this clone when the genomic

DNA was prepared, and some plasmid DNA was co-purified with the genomic DNA.

4. The most useful primer combinations for screening are those which amplify across the intron–exon junctions. These primer combinations should only give a PCR product if the intron has integrated into its intended target site, and this can be verified by checking the size of the PCR product and sequencing the PCR product if desired. Appropriate primer combinations for PCR across the intron–exon junctions will differ depending upon the orientation of the intron insertion. For example, *see* **Fig. 11.2**.
5. Re-streak integrants to screen for plasmid loss. Plasmid pMTL007 uses the replication region from *Clostridium butyricum* plasmid pCB102. Consequently, pMTL007 is readily lost from many clostridial strains once thiamphenicol selection is removed. Integrants verified by PCR screening should be replicated (after passaging under erythromycin selection if necessary) onto fresh plates containing an appropriate anaerobic solid growth medium supplemented with 15 μg/ml thiamphenicol and also onto control plates supplemented with 2.5 μg/ml erythromycin. A transformant/transconjugant colony (from Day 6) should also be replicated onto the thiamphenicol-supplemented plates as a positive control.
6. All integrants should grow on erythromycin-supplemented plates, but only those which still retain the pMTL007 plasmid will grow on thiamphenicol-supplemented plates. The transformant/transconjugant positive control should grow on thiamphenicol-supplemented plates.
7. In many strains, pMTL007 will be lost during the minimum required passaging on Day 8 and Day 9, and additional passaging will not be required. Other strains may require more extensive passaging.

3.3. Refinements to the Method

In light of data gathered during validation (6) and subsequent use of pMTL007, a series of plasmids featuring a number of refinements have been made (14) – *see* http://www.clostron.com for full details. Most notably:

1. Production of group II intron RNA is directed by the strong constitutive *fdx* promoter of *C. sporogenes.* We previously demonstrated that inducible control of intron expression was not necessary or advantageous. Furthermore, constitutive expression obviates the need to include an IPTG induction step in the protocol. Accordingly, integrant cells of all strains may be selected by re-streaking transconjugants/transformants directly onto selective media.

2. The targeting region of the intron has been replaced by a smaller *lacZα* "stuffer" sequence. Screening for re-targeting of the original plasmid pMTL007 is not generally easily achieved without sequencing. Re-targeted derivatives of all the new plasmids are easily identified by PCR, restriction analysis or blue/white screening.
3. In some plasmids the ErmRAM is flanked by Flippase Recognition Target (FRT) sites. Expression of FLP recombinase in strains constructed using these plasmids leads to excision and loss of the sequence between the two FRT sites, leading to an unmarked strain. The ErmRAM can therefore be "re-cycled" and used in further steps to construct strains containing multiple mutations.
4. The *Sal*I site in domain IV of the intron is now unique. It is known that the intron can tolerate the insertion or deletion of sequence in the non-structural domain IV and retain mobility, but in the original plasmid pMTL007 there is no unique restriction site in this region. The presence of a unique *Sal*I site allows the convenient, one-step cloning of "cargo" sequence to be delivered by the intron.

4. Notes

1. Each sequence submitted is limited to 3,500 bp in length, although this can include sequence from more than one gene, so researchers may wish to add several genes (or portions of several genes) together into a single sequence before submission.
2. Weak bands of ~100 and ~250 bp may also be visible.
3. Some labs also use their own methods or their own variations on published protocols. For example, the following protocol can be used to prepare and electroporate competent cells of *C. acetobutylicum* ATCC824 (essentially as described by Mermelstein and Papoutsakis (10)). Use pre-reduced buffer and media.

Acknowledgements

The authors wish to acknowledge the financial support of the BBSRC (grants BB/D522797/1, BD/D522289/1 BB/F003390/1), the MRC (G0601176) and SysMO (project number 2; http://www.sysmo.net).

References

1. Mohr G, Smith D, Belfort M and Lambowitz AM. (2000) Rules for DNA target-site recognition by a lactococcal group II intron enable retargeting of the intron to specific DNA sequences. *Genes Dev* **14**, 559–573.
2. Perutka J, Wang W, Goerlitz D and Lambowitz AM. (2004) Use of computer-designed group II introns to disrupt *Escherichia coli* DExH/D-box protein and DNA helicase genes. *J Mol Biol* **336**, 421–439.
3. Karberg M, Guo H, Zhong J, Coon R, Perutka J and Lambowitz AM. (2001) Group II introns as controllable gene targeting vectors for genetic manipulation of bacteria. *Nat Biotechnol* **19**, 1162–1167.
4. Zhong J, Karberg M and Lambowitz AM. (2003) Targeted and random bacterial gene disruption using a group II intron (targetron) vector containing a retrotransposition-activated selectable marker. *Nucleic Acids Res* **31**, 1656–1664.
5. Chen Y, McClane BA, Fisher DJ, Rood JI and Gupta P. (2005) Construction of an alpha toxin gene knockout mutant of *Clostridium perfringens* type A by use of a mobile group II intron. *Appl Environ Microbiol* **71**, 7542–7547.
6. Heap JT, Pennington OJ, Cartman ST, Carter GP and Minton NP. (2007) The ClosTron: a universal gene knock-out system for the genus *Clostridium*. *J Microbiol Methods* **70**, 452–464.
7. Purdy D, O'Keeffe TA, Elmore M, Herbert M, McLeod A, Bokori-Brown M, Ostrowski A and Minton NP. (2002) Conjugative transfer of clostridial shuttle vectors from *Escherichia coli* to *Clostridium difficile* through circumvention of the restriction barrier. *Mol Microbiol* **46**, 439–452.
8. Hussain HA, Roberts AP and Mullany P. (2005) Generation of an erythromycin-sensitive derivative of *Clostridium difficile* strain 630 (630Deltaerm) and demonstration that the conjugative transposon Tn916DeltaE enters the genome of this strain at multiple sites. *J Med Microbiol* **54**, 137–141.
9. Theys J, Pennington OP, Dubois L, Anlezark G, Vaughan T, Mengesha A, Landuyt W, Anné J, Burke PJ, Dûrre P, Wouters BG, Minton NP and Lambin P. (2006) Repeated cycles of *Clostridium*-directed enzyme prodrug therapy result in sustained antitumour effects in vivo. *British J Cancer* **95**, 1212–1219.
10. Mermelstein LD and Papoutsakis ET. (1993) In vivo methylation in *Escherichia coli* by the *Bacillus subtilis* phage_ 3T I methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. *Appl Environ Microbiol* **59**, 1077–1081.
11. Mauchline ML, Davis TO and Minton NP. (1999) Clostridia. In: Demain AL, Davies JE eds., *Manual of Industrial Microbiology and Biotechnology*, ASM Press, Herndon, VA, pp. 475–492.
12. Davis TO, Henderson I, Brehm JK and Minton NP. (2000) Development of a transformation and gene reporter system for group II, non-proteolytic *Clostridium botulinum* type B strains. *J Mol Microbiol Biotechnol* **2**, 59–69.
13. Zhou YT and Johnson EA. (1993) Genetic transformation of *Clostridium botulinum* Hall A by electroporation. *Biotechnol Lett* **15**, 121–126.
14. Heap JT, Kuehne SA, Ehsaan M, Cartman ST, Cooksley CM, Scott JC, Minton NP. (2010) The ClosTron: Mutagenesis in *Clostridium* refined and streamlined. *J Microbiol Methods* **80**, 49–55.

Chapter 12

Methods for Gene Cloning and Targeted Mutagenesis

Glen P. Carter, Dena Lyras, Rachael Poon, Pauline M. Howarth, and Julian I. Rood

Abstract

Clostridium difficile is the causative agent of a range of intestinal diseases, collectively referred to as *Clostridium difficile*-associated disease (CDAD). The recent emergence of "hypervirulent" strains associated with increased rates of mortality and severity of disease in humans has highlighted the need to study this organism at the molecular level. These studies will increase our knowledge of the mechanisms by which *C. difficile* causes disease and facilitate the rational design of new and improved therapeutics. The study of *C. difficile* has long been hampered by difficulties in genetically manipulating the organism. It has been only recently (within the last decade) that methods have been developed to introduce plasmid DNA into *C. difficile* and most importantly to enable the generation of isogenic mutants in this emerging human pathogen. These methods are essential prerequisites for the effective study of gene function in this important bacterium.

Key words: *Clostridium difficile*, recombination vector, conjugation, single cross-over, complementation.

1. Introduction

The study of *Clostridium difficile* at the molecular level has long been hampered by the difficulty in genetically manipulating this organism. It is only within the last 10 years that reliable and reproducible systems have been developed to enable us to introduce plasmid DNA into the organism (1, 2) and even more recently, within the last 2 years, to make directed mutants (3, 4). Both processes are prerequisites for the efficient study of a bacterial species at the molecular level. Several plasmids that can be transferred into different isolates of *C. difficile* by RP4-mediated conjugation

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_12, © Springer Science+Business Media, LLC 2010

from specific *Escherichia coli* conjugative donor strains have now been developed. The first of these plasmids is pMTL9301 and its derivatives (1). These plasmids are based on the native *C. difficile* pCD6 plasmid and as such are extremely stable in *C. difficile*, making them ideally suited to gene cloning and complementation experiments (1). The second set of plasmids, including pJIR1456 (5) and pJIR2816 (3), is based on pIP404 from *C. perfringens* and has been shown to be unstable in *C. difficile*. The inherent instability of these "recombination vectors" has facilitated the use of pJIR1456 and pJIR2816 for the generation of several directed chromosomal mutants of *C. difficile* by homologous recombination (3, 6).

The following sections describe the materials and methods used to generate directed chromosomal mutants in *C. difficile* through the use of pIP404-derived vectors, the subsequent analysis of these mutations and finally the genetic complementation of the mutations that are generated.

2. Materials

2.1. Standard Molecular Biology Procedures

2.1.1. PCR Amplification

(1) Gene-specific oligonucleotide primers: Make up to a final concentration of 100 μM in sterile distilled water. Store at –20°C.

(2) Taq DNA polymerase and 10X PCR buffer with magnesium (Roche). Store at –20°C.

(3) dNTPs: Make up to a final concentration of 2 mM with 1X TE and dispense in 50 μl aliquots. Store at –20°C.

(4) Genomic DNA template. Store at –20°C.

(5) Distilled water. Autoclave and store at room temperature.

2.1.2. Restriction Digestion of Plasmid DNA

(1) Purified plasmid DNA. Store at –20°C.

(2) Restriction enzymes and buffers. Store according to manufacturer's instructions.

(3) Distilled water. Autoclave and store at room temperature.

2.1.3. Ligation of DNA Fragments

(1) T4 DNA ligase and 10X ligase buffer (Promega): Store at –20°C. This enzyme is very heat labile. Dispense buffer into single use 10 μl aliquots to avoid repeated freeze/thawing.

(2) Distilled water. Autoclave and store at room temperature.

2.1.4. Electroporation

(1) Dialysis discs (Millipore). Store dry and at room temperature.

(2) Electrocompetent *E. coli* DH5α or Top10 (Invitrogen). Store 30 μl aliquots at –70°C.

(3) 0.1-mm gap electroporation cuvettes (Biorad).
(4) Phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4. Autoclave and store at room temperature.
(5) 2YT medium: 16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl (and 15 g/l agar for solid media). Autoclave and store liquid media at room temperature.

2.1.5. Gel Electrophoresis

(1) 6X DNA-loading dye: 30% (v/v) glycerol, 0.3% (w/v) bromophenol blue, 0.3% xylene cyanol. Store at room temperature.
(2) TAE buffer: 40 mM Tris–acetate, 2 mM EDTA, pH 8.5. Store at room temperature.
(3) Seakem Agarose: Dissolve in TAE buffer to a concentration of 0.8% (w/v) by heating to greater than 55°C. Molten agarose can be stored above 55°C.
(4) DNA molecular weight markers: 1 kb hyperladder (Bioline).

2.2. Construction of Recombination Vectors

2.2.1. PCR Amplification of Internal Gene Fragment

(1) PCR amplification materials as detailed above.
(2) Gel electrophoresis materials as detailed above.
(3) PCR purification kit (Qiagen).

2.2.2. Cloning

(1) Purified pJIR2816 plasmid DNA. Store at –20°C.
(2) Restriction digestion of plasmid DNA materials as detailed above.
(3) Gel electrophoresis materials as detailed above.
(4) Gel extraction kit (Qiagen).
(5) T4 DNA ligase and 10X ligase buffer (Promega) as detailed above.

2.2.3. Introduction of Insertional Inactivation Plasmids into E. coli

(1) *E. coli* HB101(pVS520) (7).
(2) Electroporation materials as detailed above.
(3) Chloramphenicol: Dissolve in absolute ethanol to a final concentration of 25 mg/ml. Store at –20°C.

2.2.4. Analysis of E. coli *Transformants Carrying Insertional Inactivation Plasmid*

(1) 2YT medium: As described above.
(2) Chloramphenicol: As described above.
(3) Tetracycline: Dissolve in absolute ethanol to a final concentration of 10 mg/ml. Wrap tube in foil to protect from light and store at –20°C.

(4) Qiaprep spin miniprep kit (Qiagen).
(5) Restriction digestion of plasmid DNA materials as detailed above.
(6) Gel electrophoresis materials as detailed above.

2.3. Isolation of Chromosomal *C. difficile* Mutants

(1) BHIS medium (8): Brain heart infusion 37 g/l, yeast extract 5 g/l (and agar 15 g/l for solid media). Autoclave and store liquid media at room temperature. Immediately prior to use add 0.1% (w/v) sterile L-cysteine, 0.5% (w/v) sterile glucose and 0.09% (w/v) $FeSO_4$.
(2) 2YT medium: As described above.
(3) Phosphate-buffered saline (PBS): As described above.
(4) Tetracycline: As described above.
(5) Thiamphenicol: Dissolve in methanol to a final concentration of 10 mg/ml. Store at –20°C. Note that thiamphenicol is a derivative of chloramphenicol; it is used in place of the latter for selection purposes in clostridial matings or transformations utilising vectors carrying the chloramphenicol resistance gene, *catP*.
(6) D-cycloserine: Dissolve in sterile distilled water to a final concentration of 20 mg/ml. Make up fresh and use immediately.
(7) Cefoxitin: Dissolve in sterile distilled water to a final concentration of 8 mg/ml. Store at –20°C.

2.4. Analysis of *C. difficile* Mutants by Southern Hybridisation

2.4.1. Probe Synthesis

(1) PCR DIG probe synthesis kit (Roche).
(2) Purified pJIR2816 plasmid DNA and purified *C. difficile* genomic DNA.
(3) *catP*-specific oligonucleotides (*see* **Note 1**) and gene-specific oligonucleotide primers: Dissolve in sterile distilled water to a final concentration of 100 μM. Store at –20°C.
(4) Gel electrophoresis materials as detailed above.

2.4.2. Transfer

(1) Genomic DNA extracted from putative *C. difficile* mutants.
(2) TAE buffer: 40 mM Tris–acetate, 2 mM EDTA, pH 8.5. Store at room temperature.
(3) Seakem Agarose: Dissolve in TAE buffer to a final concentration of 0.8% (w/v). Use immediately, but can be stored at 60°C.
(4) DNA-loading dye: As described above.
(5) DIG-labelled DNA molecular weight markers (Roche).
(6) Depurination solution: 250 mM HCl. Store at room temperature.

(7) Denaturation solution: 0.2 M NaOH, 0.5 M NaCl. Store at room temperature.
(8) Neutralisation solution: 165 mM tri-sodium citrate, 1.5 M NaCl, 0.25 M Tris–HCl (pH 7.5). Store at room temperature.
(9) Hybond-N+ membrane (Amersham). Store dry at room temperature.
(10) 7X pieces of Whatman 3 MM paper.
(11) 20X SSC: 3 M NaCl, 300 mM tri-sodium citrate, pH 7.0. Dilute to desired concentration with distilled water immediately prior to use.

2.4.3. Hybridisation

(1) Prehybridisation buffer: 83 mM tri-sodium citrate, 0.75 mM NaCl, 0.05% (w/v) blocking reagent, 1% (v/v) *N*-lauroylsarcosine, 0.02% (v/v) SDS. Make up fresh and use immediately.
(2) 2X Wash solution: 2X SSC, 0.1% (v/v) SDS. Make fresh and use immediately.
(3) 0.2X Wash solution: 0.2X SSC, 0.1% (v/v) SDS. Make fresh and use immediately.

2.4.4. Detection

(1) Maleic acid buffer: 0.1 M maleic acid, 0.15 M NaCl, adjust to pH 7.5 with solid NaOH. Store at room temperature.
(2) Washing buffer: Maleic acid, 0.3% (v/v) Tween-20. Store at room temperature.
(3) 10X Blocking solution: 10% (w/v) blocking reagent in maleic acid. Autoclave and store at 4°C. Immediately prior to use dilute 1 in 10 in maleic acid buffer.
(4) Anti-DIG-AP conjugate (Roche). Store at 4°C.
(5) Detection buffer: 0.1 M Tris–HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5. Store at room temperature.
(6) CDP-star: Store at 4°C and protect from light.
(7) X-ray film. Store at room temperature and protect from light.

2.5. Complementation

2.5.1. Construction of Complementation Vectors

(1) PCR amplification materials as detailed above.
(2) Restriction digestion of plasmid DNA materials as detailed above.
(3) *Fsp*I restriction enzyme and buffer 4 (New England Biolabs).
(4) Gel electrophoresis materials as detailed above.
(5) Gel extraction kit (Qiagen).

(6) Ligation of DNA fragments materials as detailed above.

(7) Electroporation materials as detailed above.

(8) *E. coli* DH5α or Top10 (invitrogen).

(9) Erythromycin: Dissolve in absolute ethanol to a final concentration of 50 mg/ml. Store at –20°C.

2.5.2. Transfer of Complementation Plasmids into C. difficile *by RP4-Mediated Conjugation from* E. coli

(1) Isolation of chromosomal *C. difficile* mutants as detailed above.

(2) Erythromycin: Dissolve in absolute ethanol to a final concentration of 10 mg/ml. Store at –20°C.

2.5.3. Analysis

(1) BHIS medium: Prepared and stored as above.

(2) Erythromycin: Dissolve in absolute ethanol to a final concentration of 50 mg/ml. Store at –20°C.

(3) Lysozyme: Dissolve in buffer P1 from Qiaprep spin miniprep kit (Qiagen) to a final concentration of 30 mg/ml. Use immediately, do not store.

(4) Qiaprep spin miniprep kit (Qiagen).

(5) Electroporation materials as described above.

(6) Restriction digestion of plasmid DNA materials as described above.

(7) Gel electrophoresis materials as described above.

3. Methods

Construction of directed chromosomal mutants in *C. difficile* through the use of pIP404-based recombination vectors can broadly be split into three parts. The first is the construction of the insertional inactivation plasmid, which consists of one of the recombination vectors in which has been cloned a PCR fragment that has a region of homology with the gene of interest. The second part involves the transfer of this plasmid into *C. difficile* by RP4-mediated conjugation from *E. coli* and selection of plasmid-bearing *C. difficile* transconjugants. The final stage involves analysis of the mutation and confirmation that the plasmid has inserted into the gene of interest by homologous recombination. We have also included a section on complementation, which is required when studying gene function at the molecular level in order to meet molecular Koch's postulates.

3.1. Construction of an Insertional Inactivation Vector for a Gene Knockout in C. difficile

3.1.1. Amplification of an Internal Gene Fragment from the Gene of Interest

(1) Oligonucleotide primers are designed to amplify an internal fragment of the gene of interest flanked by restriction sites that enable the synthesised gene fragment to be cloned into the *lacZα* polylinker region present in the recombination vector pJIR2816 (**Fig. 12.1**). Genomic DNA is used as the template for PCR (*see* **Note 2**).

(2) There are many different PCR systems currently available, and any one of them is appropriate for amplifying the internal gene fragment. The following procedure describes the use of *Taq* DNA polymerase and 10X PCR buffer with $MgCl_2$ supplied by Roche.

(3) The components used in a typical PCR reaction are shown in **Table 12.1**. The reagents are added to a sterile PCR tube, mixed thoroughly, placed in a thermal cycler and PCR amplification performed.

(4) Optimal PCR amplification conditions should be determined empirically for each combination of oligonucleotide

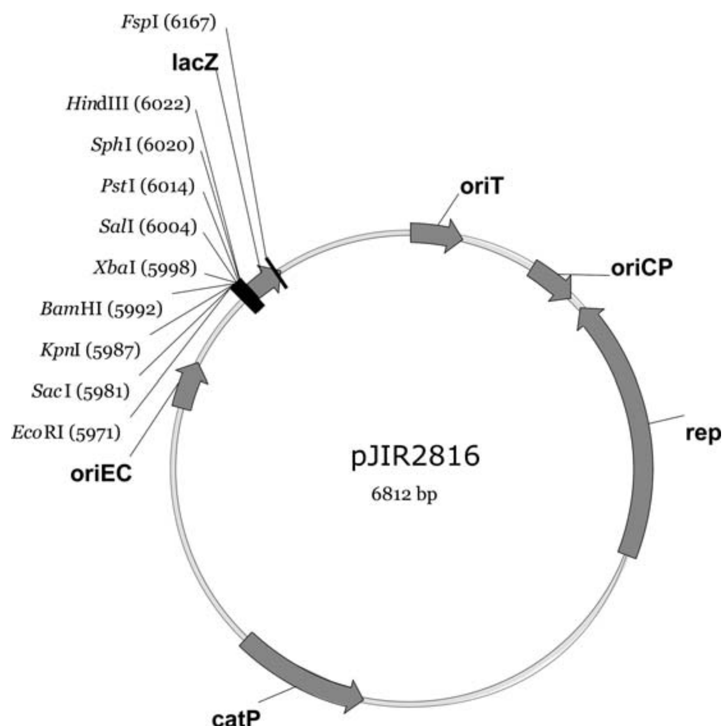


Fig. 12.1. Schematic representation of recombination plasmid pJIR2816. The position and orientation of the *C. perfringens* pIP404 plasmid replication region (*rep* and *oriCP*) and the *E. coli* ColE1 plasmid replication region (*oriEC*) are shown, as is the transfer origin (*oriT*), the chloramphenicol resistance marker (*catP*) and the *lacZα* multiple cloning site (*lacZ*). The restriction endonuclease recognition sites shown are unique and can be used to clone homologous gene fragments into plasmid pJIR2816.

Table 12.1
Reagents added to a standard PCR reaction

Reagent	Volume/concentration
10X PCR buffer with $MgCl_2$	5 μl
Taq DNA polymerase	0.5 μl
DNA template	10–100 ng genomic *C. difficile* DNA
Forward and reverse primers	200 nM each primer, final concentration
dNTPs	200 μM final concentration
Sterile distilled water	Variable volume
Total volume	50 μl

Table 12.2
Typical PCR cycling program

PCR step	Temperature (°C)	Time	Number of cycles
Initial denaturation	95	4 min	–
Denaturation	95	30 s	30
Annealing	50	30 s	30
Extension	72	1 min/kb amplified	30
Final extension	72	10 min	–

primers and DNA template. However, a standard PCR program is outlined in **Table 12.2**.

(5) After the PCR reactions are complete, 5 μl is removed from each tube and analysed by gel electrophoresis as follows.

(6) The 5 μl aliquot is mixed with DNA-loading dye and loaded into the wells of an agarose minigel prepared using TAE buffer, along with a DNA molecular weight ladder. The gel is then subjected to electrophoresis at 100 V for approximately 45 min.

(7) The gel is subsequently stained with 0.5 μg/ml ethidium bromide for approximately 1 min and destained in distilled water for at least 30 min (*see* **Note 3**).

(8) DNA is then visualised with UV light by placing the gel onto a UV transilluminator.

(9) If the correct-sized product has been amplified, then the remainder of the PCR product is purified using a Qiagen PCR purification kit.

3.1.2. Cloning the Amplified Gene Fragment into the Recombination Vector pJIR2816

(1) Restriction digests with the enzymes corresponding to the PCR-incorporated restriction sites is then performed using 10–20 μl of the purified PCR product and approximately 1–2 μg of purified pJIR2816 plasmid DNA. The procedure for the restriction digest is dependant on the enzyme used and should follow the manufacturer's instructions (*see* **Note 4**).

(2) Following restriction digestion, DNA-loading dye is added and the reactions are analysed by gel electrophoresis, as already described.

(3) The desired DNA fragments are then extracted from the agarose gel using a Qiagen gel extraction kit.

(4) The extracted fragments corresponding to the pJIR2816 vector backbone and the internal gene fragment are then ligated together with T4 DNA ligase overnight at room temperature using standard protocols (9).

(5) The following day, the ligation mixture is dialysed against distilled water by pipetting the reaction mixture onto the surface of a millipore dialysis disc that is floating in a petri dish full of distilled water and incubating for at least 30 min at room temperature.

3.1.3. Introduction of the Insertional Inactivation Plasmid into E. coli *by Electroporation*

(1) Whilst the ligation reactions are being dialysed aliquots of electrocompetent *E. coli* cells are thawed on ice for 10 min.

(2) Maximum volumes of 5 μl from each dialysed mixture are removed and added to separate 30 μl aliquots of the pre-thawed electrocompetent *E. coli* HB101(pVS520) cells.

(3) The cells and ligation reaction are mixed gently and then transferred to a pre-chilled 0.1-mm gap electroporation cuvette (Biorad).

(4) Any condensation or moisture is carefully removed from the electroporation cuvette. The individual cuvettes are placed in the chamber of an electroporator (BTX model 620, Harvard Apparatus) and an electric charge is passed through the cells. The settings used are: 1.8 kV, 200 Ω and 25 μF. A time constant of greater than 5 should be achieved.

(5) The cells are immediately resuspended in 1 ml of 2YT medium and incubated for 1 h at 37°C with shaking at 150–200 rpm.

(6) Following this incubation, dilutions are made in PBS and 100 μl aliquots of cell suspension are spread onto 2YT agar plates supplemented with 25 μg/ml chloramphenicol and 10 μg/ml tetracycline.

(7) The plates are inverted and incubated at 37°C overnight.

(8) Any colonies that grow are patched onto the same medium and analysed as follows.

(9) *See* **Note 5**.

3.1.4. Analysis of E. coli *Transformants by Restriction Digestion*

(1) Inoculate 5 ml of 2YT broth supplemented with 25 μg/ml chloramphenicol and 10 μg/ml tetracycline with a single colony of each putative clone and incubate overnight at 37°C with shaking at 150–200 rpm.

(2) The following morning 1.5 ml is transferred to a sterile tube and the cells pelleted by centrifugation at 3,000×*g* for 1 min at room temperature.

(3) The supernatants are discarded and plasmid is extracted using a Qiaprep spin miniprep kit, according to the manufacturer's instructions.

(4) Appropriate restriction digests are then performed on the purified plasmid DNA to confirm the successful cloning of the internal gene fragment into pJIR2816. Restriction digest conditions are dependant on the enzymes used and should be performed according to the manufacturer's conditions. Control digests of pJIR2816 are always included.

(5) The restriction digests are analysed by gel electrophoresis as described previously.

(6) If this analysis confirms that the plasmids are as predicted, then the insertional inactivation plasmids can be introduced into *C. difficile* by RP4-mediated conjugation.

3.2. Transfer of the Insertional Inactivation Plasmids into C. difficile *by RP4-Mediated Conjugation from* E. coli

(1) Inoculate 20 ml of BHIS broth with a single colony of the *C. difficile* strain to be mutated and incubate overnight in an anaerobic environment at 37°C (*see* **Note 6**).

(2) Inoculate 5 ml of 2YT broth supplemented with appropriate antibiotics with a single colony of *E. coli* HB101 (pVS520) carrying the insertional inactivation vector constructed above, and incubate overnight at 37°C with shaking at 150–200 rpm.

(3) The following morning 1 ml aliquots are removed from each culture and the cells pelleted by centrifugation at 3,000×*g* for 1 min at RT.

(4) The cell pellets are then washed with 1 ml sterile PBS, followed by centrifugation for 1 min at 3,000×*g*.

(5) The bacterial pellets are resuspended in 100 μl of sterile PBS, transferred into the same tube, mixed and spread onto a thick non-selective BHIS agar plate.

(6) The agar plate is then incubated for 7 h at 37°C under anaerobic conditions. Do not invert the plate.

(7) The conjugation mixture is washed aseptically from the plate with 1 ml of sterile PBS, a sterile spreader is used to scrape the layer of cells from the plate. The cell slurry is then aspirated from the plate using a pipette and transferred to a sterile tube.

(8) The cell slurry is diluted 10- and 100-fold with sterile PBS and a 100 μl volume of each dilution as well as the undiluted cell slurry are spread separately onto BHIS agar supplemented with 10 μg/ml thiamphenicol.

(9) After incubation overnight at 37°C under anaerobic conditions the plates are then washed using 1 ml of sterile PBS, using a sterile spreader as before. The cell slurry is transferred to a fresh sterile tube (*see* **Note 7**). Aliquots of 100 μl are spread onto BHIS agar plates supplemented with 250 μg/ml D-cycloserine, 8 μg/ml cefoxitin and 10 μg/ml thiamphenicol. The plates are then incubated for 48–72 h at 37°C under anaerobic conditions (*see* **Note 8**).

(10) Colonies that grow are then patched onto BHIS agar plates supplemented with 250 μg/ml D-cycloserine, 8 μg/ml cefoxitin and 10 μg/ml thiamphenicol, and incubated for 24–48 h at 37°C under anaerobic conditions.

(11) Patches that show growth are then subcultured on the same growth medium twice before analysis by Southern blotting.

3.3. Analysis of Chromosomal C. difficile Mutants by Southern Blotting

3.3.1. DNA Hybridisation Probe Synthesis

(1) This procedure assumes the use of a PCR DIG synthesis kit (Roche) and is adapted from the user's manual. However, there are several alternative procedures available.

(2) For synthesis of the *catP* probe, pJIR2816 plasmid DNA is used as the PCR template together with oligonucleotide primers JRP2142 and JRP2143 (3) (*see* **Note 1**). For synthesis of the gene-specific probe, genomic DNA is used as the PCR template. For both probes a labelled and unlabelled control probe is synthesised.

(3) For each probe, the reactions detailed in **Table 12.3** are assembled in sterile PCR tubes.

(4) The reagents are mixed thoroughly, the tubes are placed in a thermal cycler and PCR is performed.

(5) Optimal PCR conditions should be determined for each combination of primers and template. The standard PCR program described before can be used.

Table 12.3
Reactions used to generate a DIG-labelled DNA probe

Reagent	DIG-labelled probe	Unlabelled control probe
Sterile distilled water	Variable volume	Variable volume
10X PCR buffer with $MgCl_2$	5 μl	5 μl
10X PCR DIG mix	5 μl	–
dNTPs	200 μM final concentration	200 μM final concentration
Forward and reverse primers	1 μM each primer, final concentration	1 μM each primer, final concentration
Expand enzyme mix	0.75 μl	0.75 μl
Template DNA	–10 ng genomic DNA or –10 pg plasmid DNA	–10 ng genomic DNA or –10 pg plasmid DNA
Total reaction volume	50 μl	50 μl

(6) After the PCR reactions are complete, a 5 μl aliquot is analysed by gel electrophoresis as described previously (*see* **Note 9**).

(7) If the reactions have been successful, the labelled probes can be stored at 4°C until use. If no product or the wrong product is obtained then standard PCR troubleshooting should be performed to optimise the PCR cycling conditions.

3.3.2. Transfer of DNA

(1) Genomic DNA is isolated from the putative mutants to be tested (*see* **Note 10**).

(2) Approximately 5 μg of genomic DNA is digested with appropriate restriction enzymes overnight, according to the manufacturer's instructions.

(3) The digested DNA is mixed with DNA-loading dye and loaded into the wells of an 18.2 cm × 15 cm 0.8% agarose gel prepared using TAE buffer.

(4) The gel is then run overnight in TAE buffer at 30 V, or until the DNA bands are well separated.

(5) To depurinate the larger gDNA fragments the gel is submerged in 250 mM HCl with gentle shaking at room temperature for 10–15 min. The low molecular weight end of the gel is elevated out of the solution. Depurination improves the transfer of DNA fragments greater than 10 kb in size.

(6) The gel is rinsed briefly in distilled water and then submerged in denaturation solution for 30 min at room temperature with gentle shaking.

(7) The gel is again rinsed briefly in distilled water and submerged in neutralisation solution for 30 min at room temperature with gentle shaking.

(8) The gel is washed a second time in neutralisation solution as described in **Step 7**.

(9) The DNA is transferred from the gel onto a nylon or nitrocellulose membrane overnight using the following process (*see* **Note 11**):

- A piece of Whatman 3 MM is soaked with 2X SSC and placed on top of a bridge resting in a reservoir of 10X SSC
- The gel is inverted and placed on top of the soaked sheet of Whatman 3 MM paper and any air bubbles that may have formed are removed.
- The Hybond-N+ membrane (Amersham Pharmacia Biotech.) is cut to the size of the gel, soaked in 2X SSC and placed on top of the gel. Again, any air bubbles that may have formed are removed.
- Three pieces of Whatman 3 MM paper cut to the size of the gel are soaked in 2X SSC and placed on top of the membrane.
- Three pieces of dry Whatman 3 MM paper cut to the size of the gel, a stack of paper towels, a glass plate and a 400–500 g weight are then added to complete the assembly.

(10) After overnight transfer at RT, the membrane is rinsed briefly in 2X SSC and allowed to dry at room temperature for approximately 10 min.

(11) The DNA is then cross-linked to the membrane by exposure to UV light (254 nm) for 3 min (*see* **Note 12**).

3.3.3. Hybridisation

(1) This procedure requires the prior preparation of a labelled *catP* DNA probe and a labelled DNA probe specific for the mutated gene.

(2) The membrane is placed in a hybridisation bag containing prehybridisation buffer (20 ml/100 cm^2 filter surface area), the bag is heat sealed and incubated for at least 3 h at 65°C.

(3) The hybridisation solution, which consists of 75 ng of either the *catP* or gene-specific DNA probe in 5 ml of prehybridisation buffer, is boiled for 5 min and then placed on ice for 5 min.

(4) The prehybridisation buffer is removed from the hybridisation bag and the hybridisation buffer is added. The bag is then resealed and incubated overnight at 65°C.

(5) The membrane is removed from the hybridisation bag and washed twice in 2X wash solution for 5 min at room temperature with gentle shaking.

(6) The membrane is then washed a further two times in 0.2X wash solution for 15 min at room temperature with gentle shaking.

(7) The membrane is now ready for hybridisation detection.

3.3.4. Detection

(1) This procedure assumes the use of a DIG-labelled DNA probe for hybridisation and is based on the user's manual from the CDP-star detection kit (Roche). Alternative detection procedures are available.

(2) The membrane is submerged in washing buffer for 5 min at room temperature with gentle shaking.

(3) The membrane is then submerged in 100 ml of blocking solution for 30 min at room temperature with gentle shaking.

(4) During this incubation, antibody solution is prepared by diluting 2 μl of the anti-DIG-AP conjugate into 20 ml of blocking solution.

(5) The membrane is then removed from the blocking solution and incubated for 30 min at room temperature with gentle shaking in antibody solution.

(6) The membrane is then washed twice in 100 ml of washing buffer for 15 min at room temperature with gentle shaking.

(7) The membrane is equilibrated in 20 ml of detection buffer for 5 min at room temperature with gentle shaking, whilst 20 μl of CDP-star reagent is diluted in 20 ml of detection buffer.

(8) The membrane is placed in a hybridisation bag and 2 ml of the diluted CDP-star reagent is added, the bag is sealed and incubated for 5 min at room temperature with gentle shaking.

(9) The membrane is then sealed in a fresh hybridisation bag and is exposed to X-ray film for 15 s to 5 min at room temperature.

(10) The X-ray film is then developed using standard procedures.

(11) A typical blot is shown in **Fig. 12.2**.

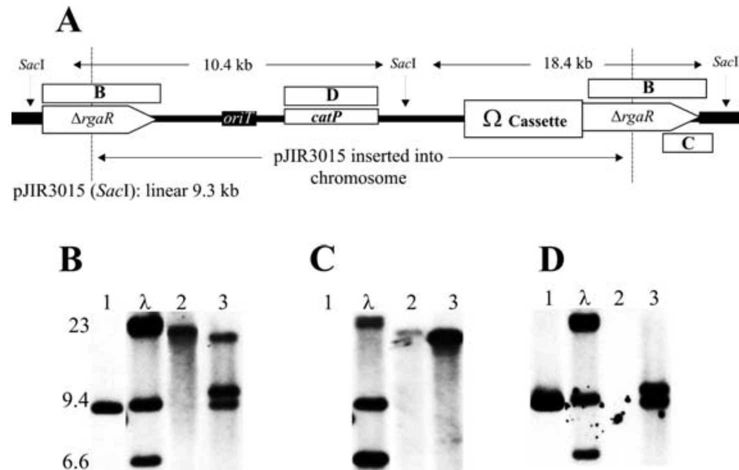


Fig. 12.2. Southern hybridisation of *rgaR* mutants. Genomic DNA was digested with *Sac*I prior to electrophoresis on 0.8% agarose gels. The Southern blots were probed as indicated. (**a**) Schematic indicating the arrangement of the *rgaR* region in the chromosome of the *rgaR* mutant. The expected sizes of restriction fragments are shown, and the regions to which the probes are expected to hybridise are marked. The predicted size of the intact *rgaR* band in the wild-type strain JIR8094 is 19.5 kb. (**b**) Southern blot probed with the *rgaR* gene. (**c**) Southern blot probed with the 3′ end of the *rgaR* coding region. (**d**). Southern blot probed with *catP*. Legend: lanes (1) JIR3015, *rgaR* recombination vector, (l) λ-*Hin*dIII molecular weight markers, sizes as indicated (kb), (2) JIR8094, wild-type strain, (3) JIR8223, *rgaR* mutant. This figure was originally published as Fig. 5 in (3) and is reproduced with permission of the publisher.

3.4. Complementation of Chromosomal Mutants

3.4.1. Construction of the Complementation Vectors

(1) The intact gene and its upstream promoter region (*see* **Note 13**) is PCR amplified using genomic DNA as the template and forward and reverse primers designed to insert flanking restriction sites that will enable cloning of the gene into the *Fsp*I site of the shuttle plasmid pMTL9301.

(2) Analysis of the PCR reaction by gel electrophoresis is then carried out as described before.

(3) The PCR reactions are purified and 10–20 μl of purified DNA is subjected to restriction digestion with the appropriate restriction enzymes for 2–3 h, according to the manufacturer's instructions. *See* **Note 4**.

(4) Approximately 1–2 μg of purified pMTL9301 plasmid DNA is subjected to restriction digestion with *Fsp*I for 2–3 h.

(5) The restriction digests are then analysed by gel electrophoresis as before.

(6) The desired DNA fragments are then extracted from the gel and the fragments are ligated together, as already described.

(7) The ligation reactions are then introduced into *E. coli* HB101(pVS520) cells by electroporation as before and 100 μl aliquots of electroporation mixture spread onto 2YT agar plates supplemented with 400 μg/ml erythromycin and 10 μg/ml tetracycline.

(8) Any colonies that grow after incubation at 37°C overnight are then analysed by restriction digestion to determine whether the gene of interest has been successfully cloned into pMTL9301.

3.4.2. Transfer of the Complementation Vectors into C. difficile *by Conjugation from* E. coli

(1) If the desired clones are obtained, then complementation experiments can be performed by transfer of the complementation plasmid into the previously generated *C. difficile* mutants, via RP4-mediated conjugation from *E. coli*, as already described.

(2) After incubation of the conjugation mixture on non-selective BHIS agar plates for 7 h at 37°C under anaerobic conditions, the cell slurry is removed as before with PBS and a sterile scraper and transferred to a sterile tube and diluted with PBS.

(3) Approximately 100 μl of various dilutions of cell slurry is spread directly onto BHIS agar supplemented with 250 μg/ml D-cycloserine, 8 μg/ml cefoxitin and 10 μg/ml erythromycin.

(4) The plates are then inverted and incubated at 37°C under anaerobic conditions for 24–48 h.

(5) Any colonies that grow are patched twice onto BHIS agar plates containing 250 μg/ml D-cycloserine, 8 μg/ml cefoxitin, 10 μg/ml erythromycin and 10 μg/ml thiamphenicol.

3.4.3. Analysis of Complemented C. difficile *Strains*

(1) Inoculate 10 ml of BHIS broth supplemented with 10 μg/ml erythromycin with a single colony of each putative complemented *C. difficile* isolate and grow at 37°C under anaerobic conditions overnight (*see* **Note 6**).

(2) A 5 ml aliquot is removed and the cells are pelleted by centrifugation at 5,000×*g* for 10 min.

(3) The supernatant is discarded and the pellet is resuspended in 250 μl buffer P1 from the Qiaprep spin miniprep kit to which 30 μg/ml lysozyme has been added.

(4) The lysis reaction is then incubated at 37°C for 30 min.

(5) Following this incubation, the standard procedure described in the manufacturer's instructions for the Qiaprep spin miniprep kit is followed.

(6) Approximately 10 μl of purified plasmid is then introduced into *E. coli* Top10 or DH5α cells by electroporation, as described previously. Aliquots of the electroporation mixture are plated onto 2YT agar supplemented with 400 μg/ml erythromycin and incubated at 37°C overnight.

(7) Any colonies that grow are analysed as described before by extraction of plasmid DNA followed by restriction digest analysis.

(8) *Clostridium difficile* isolates containing the correct complementation plasmids are then subjected to appropriate phenotypic or microarray analysis.

4. Notes

(1) JRP2142 nucleotide sequence: 5′ CTCAGTACTGAGAGGGAACTTAGATGGTAT 3′; JRP2143 nucleotide sequence: 5′ CCGGGATCCTTAGGGTAACAAAAACACC 3′ (3).

(2) The size of the internal gene fragment should be at least 300 bp to facilitate efficient homologous recombination in *C. difficile*.

(3) Ethidium bromide is a highly toxic carcinogen (10). Nontoxic alternatives such as SYBR safe (Invitrogen) are now available for staining DNA gels.

(4) If the enzymes used generate ligatable ends, then an additional incubation with alkaline phosphase is carried out according to the manufacturer's instructions. The objective is to prevent re-ligation of the recombination vector backbone.

(5) If an *E. coli* strain such as DH5α or Top10 (Invitrogen) is used, then blue/white selection can be used to identify plasmids containing the desired insert. This is achieved by addition of 50 μg/ml X-gal to the 2YT agar plates. Plasmids must then be introduced into the conjugationally proficient donor HB101(pVS520).

(6) All *C. difficile* growth media must be pre-reduced before use by incubation for at least 4 h in an anaerobic atmosphere or by boiling for 10 min.

(7) To increase the cell yield the wash step can be repeated up to three times. The cell slurry is collected in the same tube.

(8) Due to the instability of these plasmids in *C. difficile*, it may be necessary to repeat the conjugation experiments several times before any transconjugants are obtained.

(9) If the reactions have been successful, the unlabelled reaction should yield a product of the expected size, whilst the labelled reaction should appear slightly larger due to the presence of the incorporated DIG.

(10) Different procedures or commercial kits are available for extraction of genomic DNA.

(11) A schematic representation of a typical Southern hybridisation transfer set up can be found at https://www.roche-applied-science.com/sis/lad/lad_docs/dig91-119.pdf, along with an in-depth transfer protocol.

(12) The UV exposure time can have a significant impact on the success of the Southern blot and should be optimised empirically. Exposure time can be anywhere between 30 s and 10 min. Cross-linkage of DNA to the membrane can also be achieved by baking the filter at 80°C for 2 h.

(13) A minimum of 300 bp of additional upstream sequence should be included to ensure amplification of the native promoter region.

Acknowledgements

Research in this laboratory was supported by grants from the Australian National Health and Medical Research Council and grant AI057637 from the United States National Institute of Allergy and Infectious Diseases.

References

1. Purdy D, O'Keeffe TA, Elmore M, Herbert M, McLeod A, Bokori-Brown M, Ostrowski A and Minton NP. (2002) Conjugative transfer of clostridial shuttle vectors from *Escherichia coli* to *Clostridium difficile* through circumvention of the restriction barrier. *Mol Microbiol* **46**, 439–452.
2. Mani N, Lyras D, Barroso L, Howarth P, Wilkins T, Rood JI, Sonenshein AL and Dupuy B. (2002) Environmental response and autoregulation of *Clostridium difficile* TxeR, a sigma factor for toxin gene expression. *J Bacteriol* **184**, 5971–5978.
3. O'Connor JR, Lyras D, Farrow KA, Adams V, Powell DR, Hinds J, Cheung JK and Rood JI. (2006) Construction and analysis of chromosomal *Clostridium difficile* mutants. *Mol Microbiol* **61**, 1335–1351.
4. Heap JT, Pennington OJ, Cartman ST, Carter GP and Minton NP. (2007) The ClosTron: a universal gene knock-out system for the genus *Clostridium*. *J Microbiol Methods* **70**, 452–464.
5. Lyras D and Rood JI. (1998) Conjugative transfer of RP4-*oriT* shuttle vectors from *Escherichia coli* to *Clostridium perfringens*. *Plasmid* **39**, 160–164.
6. Dineen SS, Villapakkam AC, Nordman JT and Sonenshein AL. (2007) Repression of *Clostridium difficile* toxin gene

expression by CodY. *Mol Microbiol* **66**, 206–219.
7. Palombo EA, Yusoff K, Stanisich VA, Krishnapillai V and Willetts NS. (1989) Cloning and genetic analysis of *tra* cistrons of the Tra 2/Tra 3 region of plasmid RP1. *Plasmid* **22**, 59–69.
8. Smith CJ, Markowitz SM and Macrina FL. (1981) Transferable tetracycline resistance in *Clostridium difficile. Antimicrob Agents Chemother* **19**, 997–1003.
9. Sambrook J and Russell DW. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbour, NY.
10. Ames BN, Lee FD and Durston WE. (1973) An improved bacterial test system for the detection and classification of mutagens and carcinogens. *Proc Natl Acad Sci USA* **70**, 782–786.

Chapter 13

Transposon Mutagenesis in *Clostridium difficile*

Haitham A. Hussain, Adam P. Roberts, Rachael Whalan, and Peter Mullany

Abstract

Genetic manipulation of *Clostridium difficile* is notoriously difficult, currently there is only one reliable method for generating random mutations in the organism and that is to use the conjugative transposon Tn*916*. Tn*916* enters the genome of most strains of *C. difficile* with no obvious target site preference. In order to use the genome strain *C. difficile* 630 for transposon mutagenesis a erythromycin-sensitive derivative *C. difficile* 630Δ*erm* was constructed and the Tn*916* derivative, Tn*916*ΔE, was shown to enter the genome at multiple sites enabling the construction of a Tn*916* insertion library.

Key words: Tn*916*, *Clostridium difficile*, 630Δ*erm*, transposon mutagenesis, mutant library.

1. Introduction

Conjugative transposons are mobile genetic elements capable of both intracellular transposition and intercellular conjugation. The largest family of conjugative transposons discovered to date is typified by the 18 kb, tetracycline resistance conferring, Tn*916* which was originally discovered in *Enterococcus faecalis* (1, 2). Tn*916* has an extremely broad host range and has been found in, or introduced into, over 30 different genera of bacteria (2, 3) where it integrates predominantly at A+T rich regions within the genome (4). Tn*916* has previously been used as an insertional mutagen in various species of bacteria (5, 6). In order to increase the general utility of Tn*916* as a tool for genetic manipulation an erythromycin-resistant derivative of the element, Tn*916*ΔE, was constructed (7). *Clostridium difficile* strain 630,

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_13, © Springer Science+Business Media, LLC 2010

whose genome has recently been published (8), is a tetracycline and erythromycin-resistant clinical isolate (9). Tetracycline resistance is conferred by a Tn*916*-like conjugative transposon designated Tn*5397* (10). Our previous analysis has demonstrated that the tetracycline resistance is stable in this strain (11). However, erythromycin resistance is not stable and an erythromycin-sensitive derivative of this strain designated *C. difficile* 630Δ*erm* has been isolated (11). This strain has undergone a chromosomal deletion event removing one copy of the two *erm*(B) genes present on a mobilisable transposon Tn*5398* (11, 12). The remaining copy of *erm*(B) does not confer erythromycin resistance, however, it should be remembered that reversion to erythromycin resistance does occur in 1×10^9 cells (11). This strain has been used as a host for Tn*916*ΔE and this transposon enters the *C. difficile* 630Δ*erm* genome at multiple sites providing the opportunity to construct a transposon insertion library in this strain. The ability to construct random transposon insertion libraries is an essential part in discovering new gene function. This chapter details the experimental procedures involved in both constructing and characterising a transposon insertion library in *C. difficile* strain 630Δ*erm*.

2. Materials

2.1. Bacterial Strains and Growth Media

1. Recipient strain, *C. difficile* 630Δ*erm* (11) (*see* **Note 1**).
2. Donor strain, *Bacillus subtilis*:Tn*916*ΔE (BS59A) (unpublished) (*see* **Note 2**).
3. Brain heart infusion (BHI) agar (Oxoid, Basingstoke, UK).
4. BHI broth (Oxoid).
5. Cooked meat medium (Oxoid).

2.2. Filter-Mating

1. Nitrocellulose 0.45-μm pore size filters (Sartorius Ltd, Epsom, UK) (*see* **Note 3**).
2. Anaerobic cabinet (Don Whitley Scientific Ltd, Shipley, UK) supplied with anaerobic gas (10% CO_2, 10% H_2, 80% N_2).
3. Erythromycin (Sigma, Pool, UK).
4. Tetracycline (Sigma).
5. *Clostridium difficile* selective supplement (Oxoid).
6. Sterile disposable spreaders (VWR International, Lutterworth, UK).
7. Sterile forceps.
8. Sterile universal bottle 28 ml (Sarstedt, Leicester, UK).

2.3. Determining the Quality of the Library

1. Gram-positive and yeast DNA isolation kit (Gentra Systems, supplied by Flowgen, Nottingham, UK).
2. QIAEXII gel extraction kit (Qiagen).
3. Primers (Sigma Genosys, Haverhill, UK) for probe amplification (intxis1; 5′-CGCCAAAGGATCCTGTATATG-3′and intxis2; 5′-GCTGTAGGTTTTATCAGCTTTTGC-3′).
4. Taq DNA polymerase (Promega, Southampton, UK).
5. Restriction enzyme, *Hinc*II (Promega).
6. Agarose and TAE buffer for gel electrophoresis.
7. ECL hybridisation kit (Amersham, Little Chalfont, UK).

2.4. Determination of the Insertions Sites in Mutants

1. Primers specific for the ends of Tn*916* (LEO 5′-GGTTTTGACCTTGATAAAGTGTGATAAGTCC-3′and REO 5′-CGAAAGCACATAGAATAAGGCTTTACGAGC-3′) (13): the Sp6 primer (5′-GATTTAGGTGACACTATAG-3′) and T7 primers (5′-TAATACGACTCACTATAGGG-3′) for binding to pUC18.
2. Taq DNA polymerase (Promega).
3. Deoxynucleotide triphosphates (Promega).
4. T4 DNA ligase (Promega).
5. Various restriction enzymes (six cutters) (Promega).
6. Calf intestine alkaline phosphatase (Promega).

2.5. Storage of Library

1. 2.2-ml 96-well storage plates (ABgene, Epsom, UK)
2. 96 cap sealing mat (ABgene)
3. Glycerol (Sigma)
4. Cooked meat medium (Oxoid)
5. Glass universal tubes (VWR International)

3. Methods

3.1. Growth of Donor and Recipient Strains

1. The *B. subtilis* donor and *C. difficile* recipient strains are grown from stocks. *Bacillus subtilis* BS59A is grown up overnight aerobically at 37°C on BHI agar containing 10 μg/ml of freshly prepared erythromycin.
2. *Clostridium difficile* 630Δ*erm* is grown anaerobically for 3 days at 37°C in an anaerobic chamber on BHI agar containing 10 μg/ml tetracycline and *C. difficile* selective supplement (according to manufacturer's instructions).

3. A single colony of *C. difficile* 630Δ*erm* is used to inoculate 20 ml pre-reduced (*see* **Note 4**) BHI broth containing 5 μg/ml tetracycline and incubated anaerobically for 20–24 h at 37°C in the anaerobic chamber.
4. A single colony of *B. subtilis* BS59A is used to inoculate 5 ml BHI broth containing 10 μg/ml erythromycin and incubated for 18 h at 37°C. The 5 ml culture is used to inoculate a 100 ml BHI broth containing 10 μg/ml erythromycin and grown at 37°C until mid-exponential phase (OD_{600} of 0.5–0.6) (*see* **Note 5**).

3.2. Filter-Mating

1. Using sterile forceps place the desired number of filters onto freshly prepared, antibiotic and supplement-free BHI agar plates. Place these in the anaerobic chamber to pre-warm and pre-reduce.
2. Place 1 ml fresh, antibiotic-free BHI broth into a 20-ml universal tube (one tube per filter) and place in anaerobic chamber to pre-reduce and pre-warm.
3. *Bacillus subtilis* and *C. difficile* cells are centrifuged and washed twice with pre-warmed (37°C) or pre-warmed and pre-reduced BHI broth, respectively. Both *B. subtilis* and *C. difficile* are resuspended in 1 ml aliquots of BHI broth.
4. Cultures of donor and recipient are mixed in an anaerobic environment and 100 μl is spread, using sterile spreaders, on nitrocellulose 0.45-μm pore size filters previously placed on BHI agar plates. These are incubated for 18–24 h at 37°C anaerobically (*see* **Note 6**).
5. The filters are removed from the agar plates using sterile forceps and placed in the 20-ml universal bottles containing pre-reduced and pre-warmed 1 ml BHI broth. These are vortexed vigorously to resuspend the cells in the broth (*see* **Note** 7).
6. Next 100 μl aliquots are spread on BHI agar supplemented with *C. difficile* selective supplement and 10 μg/ml erythromycin for *C. difficile* 630ΔE incubated anaerobically for 3–4 days checking for growth every day.
7. Putative transconjugants are sub-cultured on fresh selective plates and incubated for a further 3–4 days.

3.3. Determination of the Quality of the Library

1. In order to determine the quality of the library choose 10 individual transconjugants (from different filters) to check by Southern blot hybridisation.
2. Inoculate a fresh 10 ml pre-warmed and pre-reduced BHI broth containing erythromycin at 10 μg/ml with each of the chosen transconjugants. Incubate 48 h anaerobically.

3. Carry out genomic DNA extractions for each of the transconjugant *C. difficile* cultures using the Gram-positive and yeast DNA isolation kit.
4. After checking the quality of the DNA by electrophoresis, digest it with *Hinc*II and carry out a Southern blot of this restricted DNA. Digesting with *Hinc*II will release a Tn*916*:genome junction fragment which contains the *int*Tn integrase gene (13). Therefore, when probed with *int*Tn the number of hybridising fragments should represent the number of copies of Tn*916*ΔE in the genome with the length of the fragment being determined by the position of the nearest *Hinc*II site to the occupied target site.
5. Amplify the *int*Tn probe using pAM120 DNA (14) as a template and the primers intxis1 and intxis2. The typical PCR program is as follows: 94°C for 4 min followed by 25–30 cycles of 94°C for 30 sec, 50–60°C for 1.5 min and 72°C for 1–3 min, followed by a final incubation at 72°C for 10 min and a rapid thermal ramp and hold at 4°C until analysis.
6. Probe the Southern blot with the probe according to the manufacturer's instructions (Amersham). A typical blot of 9 independent Tn*916*ΔE containing *C. difficile* 630Δ*erm* transconjugants is shown in **Fig. 13.1**.
7. If all of the putative transconjugants have different insertions the library can either be stored (*see* **Section 3.5** below) or used directly in the phenotypic assay of your choice.

3.4. Determination of the Target Site in Mutants

1. Carry out a Southern blot (as described in **Section 3.3** above) on any putative mutant selected during your phenotypic assays to determine the number of copies of Tn*916*ΔE.
2. If there is a single copy of Tn*916*ΔE direct genomic sequencing can be carried out and the resulting sequence analysed by BLASTing it against the genome sequence (the *C. difficile* 630 chromosome and plasmid have been deposited in the EMBL database under accession numbers AM180355 and AM180356, respectively).
3. If there are two or more copies of Tn*916*ΔE present, i.e. there are more than one hybridising bands to the *int*Tn probe, the quickest way to determine the insertion site sequences is to use single specific primer (ssp) PCR.
4. Digest the DNA from the selected mutant with a (6 bp) restriction enzyme such as *Hin*dIII, *Hinc*II, *Eco*R1 or *Bam*HI.
5. Digest pUC18 with the same enzymes and dephosphorylate.

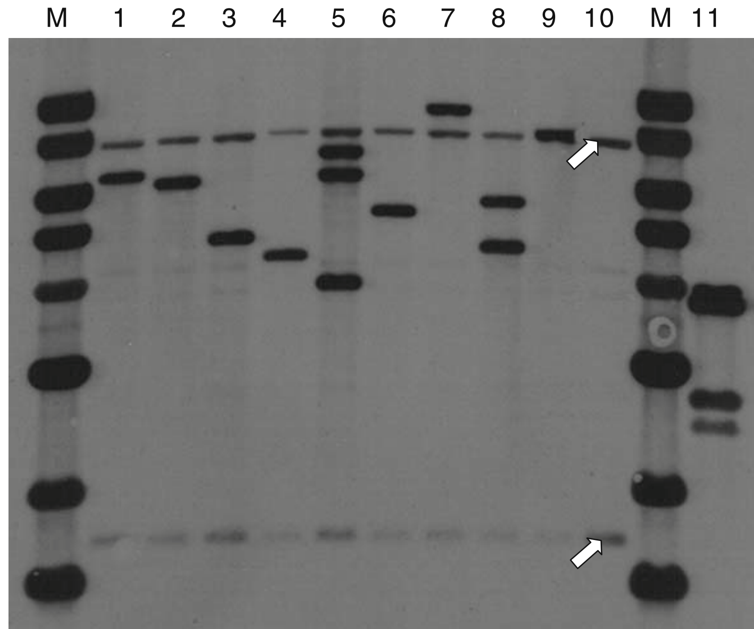


Fig. 13.1. Southern blot, probed with an *int*-derived PCR amplicon, revealing different insertion sites of nine independently generated (each from different filters) *C. difficile* 630Δ*erm* transconjugants. M, molecular mass marker; Lanes 1–9, *C. difficile* 630Δ*erm*::Tn*916*ΔE transconjugants; Lane 10, *C. difficile* 630Δ*erm*; Lane 11, pAM120 DNA (positive control). The two *white arrows* indicate background hybridisation to the *C. difficile* 630Δ*erm* genome (8). It can be clearly seen that all contain insertions are in different genomic locations and two transconjugants (in lanes 5 and 8) contain more than one copy of Tn*916*ΔE (three copies in lane 5, two copies in lane 8).

6. Ligate the restricted genomic DNA to the restricted and dephosphorylated pUC18 DNA using T4 DNA ligase.
7. Carry out PCR using one of the primers reading out of Tn*916* (15) and one of either SP6 or T7.
8. Run the reaction on a gel and excise all bands.
9. Sequence the bands with the Tn*916*-specific primer used in the reaction (*see* **Note 8**).
10. The bands that correspond to the correct amplicons will give you a sequence that corresponds to the end of Tn*916* (accession number; NC_006372) followed by *C. difficile* genomic DNA sequence (*see* **Section 3.4**, Step 2).

3.5. Storing *C. difficile* Library

1. Single putative transconjugants can either be inoculated into cooked meat broth medium and incubated anaerobically for 3 days and then stored at room temperature for further analysis.
2. Alternatively transconjugants can be frozen in 2.2-ml 96-well storage plates. A single colony of a putative

transconjugant is used to inoculate 1 ml pre-reduced BHI broth containing 10 μg/ml erythromycin in a single well of a 2.2-ml 96-well plate and incubated for 24 h anaerobically. Three hundred microlitres is then transferred to a fresh 96-well storage plate containing 300 μl BHI broth plus 60% glycerol and mixed by pipetting. The 2.2-ml 96-well storage plate is then sealed with a 96 cap sealing mat and stored at –80°C.

4. Notes

1. Alternative recipient *C. difficile* strains can be used. However, one needs to know if the recipient strain is resistant to tetracycline (encoded by Tn*916*) or contains an *erm* gene encoding the MLS phenotype (16) as currently only tetracycline and MLS encoding versions of Tn*916* are available. Also one needs to keep in mind that in at least one *C. difficile* strain (CD37) Tn*916* enters the genome at a highly preferred site (15). Additionally when choosing a *C. difficile* strain in which to create an insertion library, always consider if the strain has or is currently being sequenced as there is a great deal of variation among *C. difficile* strains (17).
2. Alternative *B. subtilis* donor strains, depending on the desired conjugative transposon, can be used, e.g. if the wild-type Tn*916* element is required *B. subtilis*::Tn*916* (strain BS34A) (18) should be used.
3. The choice of filter is important. The reason for using a 0.45-μm pore size filter as opposed to plating directly onto agar is that the filter is believed to bring the bacteria together and hold them in intimate contact (19). Additionally it has been shown that a filter with a pore size of 0.45 μm results in more transconjugants than a filter-mating carried out on a 0.22-μm pore size filter (19). The side of the filter on which the mating is carried out is also important as each side is different. It is recommended that filter-mating is carried out on the front side of the filter, which exhibits a more sponge-like appearance under the scanning electron microscope. The front side of the filters recommended here has a grid on the front, which is placed face upwards on the agar plate.
4. It is important to pre-reduce all broths used to grow *C. difficile*. We routinely incubate broths in the anaerobic chamber for at least 4 h prior to use (with the lids of the tubes slightly open to allow gaseous exchange). If, after autoclaving,

broths are transferred to the anaerobic chamber immediately they will be ready to use once cooled to the desired temperature.

5. The amount of donor and recipient cells should be optimised for each mating pair. It is recommended that a small number of filter-mating experiments (3–5 filters) are carried out prior to library construction for optimisation.
6. We routinely incubate the filter-mating experiment overnight; however, transfer is likely to occur within 4 h (20).
7. While it is ideal to carry out every step of the filter-mating in anaerobic conditions we routinely carry out this vortex step aerobically.
8. Some of the bands resulting from the ssp-PCR are likely to be from mispriming and from small fragment ligation to two copies of pUC18 which can result in an amplicon with just the forward or reverse primer. Therefore, by sequencing with the Tn*916*-specific primer it cuts down on the amount of analysis as most of the erroneous amplicons simply will not return usable data.

Acknowledgements

The work described in this chapter was funded by the BBSRC (grant number; 364/E13746) and the Medical Research Council (grant number; G0601176).

References

1. Franke AE and Clewell DB. (1981) Evidence for a chromosome-borne resistance transposon (Tn*916*) in Streptococcus faecalis that is capable of "conjugal" transfer in the absence of a conjugative plasmid. *J Bacteriol* **145**, 494–502.
2. Roberts AP and Mullany P. (2009) A modular master on the move; the Tn*916* family of mobile genetic elements. *Trends in Microbiol* **17**, 251–258.
3. Rice LB. (1998) Tn*916* family conjugative transposons and dissemination of antimicrobial resistance determinants. *Antimicrob Agents Chemother* **42**, 1871–1877.
4. Nelson KE, Richardson DL and Dougherty BA. (1997) Tn*916* transposition in *Haemophilus influenzae* Rd: preferential insertion into noncoding DNA. *Microb Comp Genomics* **2**, 313–321.
5. Stephens DS, Swartley JS, Kathariou S and Morse SA. (1991) Insertion of Tn*916* in *Neisseria meningitidis* resulting in loss of group B capsular polysaccharide. *Infect Immun* **59**, 4097–4102.
6. Clemans DL, Kolenbrander PE, Debabov DV, Zhang Q, Lunsford RD, Sakone H, Whittaker CJ, Heaton MP and Neuhaus FC. (1999) Insertional inactivation of genes responsible for the D-alanylation of lipoteichoic acid in *Streptococcus gordonii* DL1 (Challis) affects intrageneric coaggregations. *Infect Immun* **67**, 2464–2474.

7. Rubens CE and Heggen LM. (1988) Tn*916*ΔE: a Tn*916* transposon derivative expressing erythromycin resistance. *Plasmid* **20**, 137–142.
8. Sebaihia M, Wren BW, Mullany P, Fairweather NF, Minton N, Stabler R, Thomson NR, Roberts AP, Cerdeño-Tárraga AM, Wang H, Holden MT, Wright A, Churcher C, Quail MA, Baker S, Bason N, Brooks K, Chillingworth T, Cronin A, Davis P, Dowd L, Fraser A, Feltwell T, Hance Z, Holroyd S, Jagels K, Moule S, Mungall K, Price C, Rabbinowitsch E, Sharp S, Simmonds M, Stevens K, Unwin L, Whithead S, Dupuy B, Dougan G, Barrell B and Parkhill J. (1996) The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. *Nat Genet* **38**, 779–786.
9. Wust J, Sullivan NM, Hardegger U and Wilkins TD. (1982) Investigation of an outbreak of antibiotic-associated colitis by various typing methods. *J Clin Microbiol* **16**, 1096–1101.
10. Roberts AP, Johanesen PA, Lyras D, Mullany P and Rood JI. (2001) Comparison of Tn*5397* from *Clostridium difficile*, Tn*916* from *Enterococcus faecalis* and the CW459*tet*(M) element from *Clostridium perfringens* shows that they have similar conjugation regions but different insertion and excision modules. *Microbiology* **147**, 1243–1251.
11. Hussain HA, Roberts AP and Mullany P. (2005) Generation of an erythromycin-sensitive derivative of *Clostridium difficile* strain 630 (630Δ*erm*) and demonstration that the conjugative transposon Tn*916*ΔE enters the genome of this strain at multiple sites. *J Med Microbiol* **54**, 137–141.
12. Mullany P, Wilks M and Tabaqchali S. (1994) Transfer of macrolide-lincosamide-streptogramin B (MLS) resistance in *Clostridium difficile* is linked to a gene homologous with toxin A and is mediated by a conjugative transposon, Tn*5398*. *J Antimicrob Chemother* **35**, 305–315.
13. Flannagan SE, Zitzow LA, Su YA and Clewell DB. (1994) Nucleotide sequence of the 18-kb conjugative transposon Tn*916* from *Enterococcus faecalis*. *Plasmid* **32**, 350–354.
14. Gawron-Burke C and Clewell DB. (1884) Regeneration of insertionally inactivated streptococcal DNA fragments after excision of transposon Tn*916* in *Escherichia coli*: strategy for targeting and cloning of genes from Gram-positive bacteria. *J Bacteriol* **159**, 214–221.
15. Wang H, Roberts AP and Mullany P. (2000) DNA sequence of the insertional hot spot of Tn*916* in the *Clostridium difficile* genome and discovery of a Tn*916*-like element in an environmental isolate integrated in the same hot spot. *FEMS Microbiol Lett* **192**, 15–20.
16. Arthur M, Brisson-Noël A and Courvalin P. (1987) Origin and evolution of genes specifying resistance to macrolide, lincosamide and streptogramin antibiotics: data and hypotheses. *J Antimicrob Chemother* **20**, 783–802.
17. Stabler RA, Gerding DN, Songer JG, Drudy D, Brazier JS, Trinh HT, Witney AA, Hinds J and Wren BW. (2006) Comparative phylogenomics of *Clostridium difficile* reveals clade specificity and microevolution of hypervirulent strains. *J Bacteriol* **188**, 7297–7305.
18. Roberts AP, Hennequin C, Elmore M, Collignon A, Karjalainen T, Minton N and Mullany P. (2003) Development of an integrative vector for the expression of antisense RNA in *Clostridium difficile*. *J Microbiol Methods* **55**, 617–624.
19. Sasaki Y, Taketomo N and Sasaki T. (1988) Factors affecting transfer frequency of pAM beta 1 from *Streptococcus faecalis* to *Lactobacillus plantarum*. *J Bacteriol* **170**, 5939–5942.
20. Flannagan SE and Clewell DB. (1991) Conjugative transfer of Tn*916* in *Enterococcus faecalis*: trans activation of homologous transposons. *J Bacteriol* **173**, 7136–7141.

Part VII

Animal Models of Disease

Chapter 14

Refinement of the Hamster Model of *Clostridium difficile* Disease

Gillian Douce and David Goulding

Abstract

The Golden Syrian hamster is widely regarded as the most relevant small animal model of *Clostridium difficile* disease as oral infection of animals pre-treated with antibiotics reproduces many of the symptoms observed in man. These include diarrhoea, histological damage, colonisation of the large bowel and sporulation of the organism at the terminal stage of the disease. However, infection results in a fatal outcome, which in the past has been used as an experimental endpoint. More recently, attempts have been made to refine the model to maximise the scientific data generated whilst minimising animal suffering. This has been achieved using a combination of qualitative and quantitative measurements taken during the course of the infection and at post-mortem. This has allowed timing of experiments to be optimised to ensure appropriate monitoring of animals during the acute phase of infection and provides opportunities to establish appropriate humane endpoints to these experiments.

Key words: *Clostridium difficile*, Golden Syrian hamster, telemetry, colonisation, histological damage.

1. Introduction

Clostridium difficile is a spore-forming Gram-positive anaerobic bacteria that causes gastrointestinal infections in humans with symptoms that range from asymptomatic colonisation to severe diarrhoea, pseudomembraneous colitis and death (1). *Clostridium difficile*-associated diarrhoea (CDAD) characteristically occurs following treatment with broad-spectrum antibiotics. This treatment disrupts the normal gut microflora, encouraging *C. difficile* to establish. It has become a significant problem in both hospitals and care homes as the elderly are especially vulnerable (2).

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_14,

© Springer Science+Business Media, LLC 2010

Until recently, the pathogenesis of this organism was largely attributed to the production of two large toxins (A and B) by the bacteria. In fact, oral delivery of purified toxins to hamsters reproduces many of the symptoms associated with infection with the whole organism (3). However, the inability to fully protect animals vaccinated with the toxins (4, 5), together with the isolation of strains showing variation in toxin A (including deletion of large sections of the toxin) but which still cause clinical disease (6), suggests that other factors contribute toward disease severity.

To better understand the factors the dynamic interaction between the pathogen and the host in vivo studies either in animals or directly in the clinic are required. Whilst several in vitro systems have been used to characterise specific aspects of CDAD (7), overall these have not significantly extended our understanding of this pathogen. At present the best small animal model of CDAD is acknowledged as the hamster, as many of the clinical symptoms including diarrhoea, histological damage and relapse of the condition following removal of treatment can be reproduced (8, 9). In contrast, infection in the mouse results in a much milder disease in which the mice become transiently colonised but show limited pathology (10). Whilst use of the mouse is advantageous as it provides an opportunity to consider the contribution of specific host genes on the infection process (by the use of transgenics), its milder symptoms reduce the capacity to determine the significant impact of new treatments on the full range of symptoms observed in the hamster. This chapter describes the methodology used to both successfully infect and characterise the interaction of the *C. difficile* with the gut tissue in the hamster model.

2. Materials

2.1. Bacteria and Propagation

1. CCFA media containing 7% horse blood is used for routine culturing of *C. difficile*.
2. All bacteria are grown under anaerobic conditions within a miniMacs workstation (Don Whitley)
3. Amphotericin B (Sigma) used at 10 μg/ml.
4. Erythromycin (Sigma) used at 5 μg/ml.
5. Taurocolic acid used at 0.1% (w/v).

2.2. Animals

1. Female Syrian Golden hamsters approximately 100 g in weight (Harlan/Olac) caged individually (*see* **Note 1**).

2.3. Insertion and Monitoring Through Telemetric Chips

1. Telemetric chips: G2 E-mittor chips (MiniMiiter, Oregan, USA). These are surface sterilised prior to use with Cidex-activated glutaraldehyde solution (Johnson and Johnson) for 30 min and then rinsed in at least three changes of sterile water prior to insertion.
2. Detection system: MiniMitter Vital View 4000 Telemetry System (Linton Instruments, Norfolk).

2.4. Infection

1. Clindamycin 2 phosphate (Sigma) made up at 15 mg/ml in distilled water. Filtered aliquots stored in batches of 650 μl at –20°C (sufficient to treat three animals).
2. Filter-topped sterile cages or IVC units.
3. Autoclaved cages, bedding and water bottles (15 psi, 20 min cycle).
4. Sterile bedding, food (B&K, Hull), water (Baxters SA, Glasgow).
5. Sterile surgeons gloves (JustGloves, UK).
6. Sterile gavage needles: These should be surface sterilised as described for telemetry chips. Immediately prior to use, the needle should be flushed through with PBS. If more than one animal is to be treated the needle should be subject to cleaning between each animal. If possible, different needles should be used for treatment with antibiotics and infection with bacteria.

2.5. Confirmation of Strain Used Pre- and Post-infection

1. Genomic DNA, purified using Genomic DNA isolation kit (Promega, Madison, USA) from 24 h broth culture grown organisms.
2. Taq Polymerase (Invitrogen, UK) and appropriate supplied buffers.
3. Seven pairs of primers (as described in (11)) (400 nM).
4. dNTP's (deoxyribonucleoside 5′ triphosphates) (200 μM)
5. PCR conditions: 1 cycle, 94°C, 5 min; 35 cycles, 94°C, 1 min, 50°C, 1 min, 72°C, 1 min; 1 cycle, 72°C, 10 min.

2.6. Infected Tissue Microscopy

1. Formal saline (Sigma-Aldrich) 10% formalin of which 4% is formaldehyde.
2. Fine pastettes (Alpha lab, Hampshire, UK).
3. Haemotoxin and eosin (Sigma, UK).

2.7. Electron Microscopy

2.7.1. Transmission EM

1. Glutaraldehyde (2.5%) (TAAB) and 2% paraformaldehyde (TAAB) in 0.1 M sodium cacodylate buffer (Sigma). Calcium chloride and magnesium chloride (Sigma).
2. Osmium tetroxide solution (1%) (TAAB), 1% tannic acid (TAAB), 1% sodium sulphate (Sigma).

3. Ethanol (Sigma). Propylene oxide (TAAB). TAAB 812 embedding resin. Specimen rotator and 60°C oven (both TAAB).
4. Leica EMUC6 Ultramicrotome. Toluidine blue solution made with 1% sodium borate (Sigma) and 1% toluidine blue (TAAB). Saturated uranyl acetate (TAAB). Lead acetate and sodium citrate to make lead citrate (Sigma). FEI 120 kV Spirit Biotwin TEM with Tietz F415 digital Temcam.

2.7.2. Scanning EM

1. Thiocarbohydrazide (1%) (Sigma) Liquid carbon dioxide (BOC); Bal-tec CPD030 Critical point dryer; Bal-tec SCD050 Gold sputter coater; Hitachi S-4800 SEM.

2.7.3. Fine Structure Immunocytochemistry – Immuno EM

1. Leica FS freeze-substitution unit. PBS tablets (Sigma). 0.02 M glycine (Sigma) 10% foetal calf serum (Invitrogen) prepared by inactivating for 20 min at 56°C and spinning at 22,000 rpm for 1 h before aliquoting and freezing at –80°C. Protein A gold (University Medical Centre, Utrecht). Lowicryl HM20 low-temperature resin (Agar Scientific).

2.7.4. Formvar-Carbon-Coated Grids (TAAB). Anticapillary Forceps Type N5AC (TAAB)

3. Methods

One of the main challenges to this model is the susceptibility of animal's post-antibiotic treatment to unexpected and unwanted sources of *C. difficile* from the local environment of the animal facility. In addition, hamsters may arrive within the unit colonised with a naturally occurring animal *C. difficile* strains. In this context, ingestion of *C. difficile* spores or infection with other intestinal pathogens generates highly variable results that are often impossible to interpret. Other sources of infection include other animals within the unit, contaminated bedding material or exposure through contamination of equipment used during the course of handling and inoculation. To minimise this risk, animals should be housed individually and post-antibiotic treatment is provided with sterile cages, food, bedding and water. Animals should be handled in a contained and chemically cleaned environment by an individual gowned and gloved for sterile work. If possible, all procedures should be carried out in a class 2 cabinet.

3.1. Preparation of Animals for Infection: Insertion of Telemetric Chips

1. Each animal is anaesthetised (using inhaled fluorothane and oxygen) and a laprotomy is performed.
2. The small telemetric chip is inserted into the peritoneal cavity where it is secured to the peritoneal membrane using thread.
3. The wounds are closed and the animals monitored in recovery. Pain relief is administered immediately after surgery and 24 h later.
4. Wounds and animal weights are monitored for the next 7 days to ensure no post-operational complications occur.
5. Animals are then left for a minimum of 3 weeks before subject to further procedures.

3.2. Preparation of Spores for Infection

Infection of the animals can be achieved using both vegetative and spore preparations of the organism. The advantage of spore preparation is that oxygen exposure during procedure of infection has no detrimental effect upon the number of viable bacteria to which the animal is exposed. In contrast, spores can be made in advance and stored in batches at –80°C. This allows consistency of infection when comparing, for example the efficacy of drug treatments on the infection process (*see* **Note 2**).

1. The strain of *C. difficile* required is initially streaked out onto CCFA plates to provide single colonies.
2. Two or three typical colonies are then used to inoculate several fresh TCCFA plates, which are grown under anaerobic conditions at 37°C for 5–7 days. The plates are then removed from the anaerobic cabinet and left overnight at room temperature to maximise sporulation.
3. Growth from the plates is then removed by addition of 1 ml of 100% ethanol to the plates and loosening of bacterial growth using a sterile disposal spreader. The resultant spore suspension can then be removed to a sterile Eppendorf.
4. Any remaining material can then be collected by washing the plate surface with an additional 1 ml PBS.
5. The preparation should be washed by centrifugation and the pellet re-suspended in 1 ml of PBS before being split into 100 μl aliquots that can be stored at –80°C.
6. The approximate number of spores can be determined in these aliquots prior to use by recovery of a single aliquot and serial dilution of the material it contains.
7. One hundred microlitre volumes of each dilution are then spread over the surface of a TCCFA agar plate. These should then be incubated anaerobically at 37°C for at least 48 h before enumeration of the CFU/ml of the sample.

3.3. Pre-treatment of Animals with Antibiotics

To ensure infection with the *C. difficile* spores, animals are generally treated with parenteral or oral clindamycin, between 3 and 24 h prior to infection. However, it has recently been shown that the timing of clindamycin treatment can significantly affect the development of disease especially with strains of the organism that have an increased sensitivity to this antibiotic. This is especially relevant when considering the epidemic 027 strains or strains containing a disrupted *ermB* gene, which show no evidence of infection if given 1-day post-treatment with clindamycin but show the more typical profile of disease if given 5-day post-treatment.

1. Sterile filter-topped cages containing sterile food water and bedding should be prepared prior to the treatment of the animals.
2. Animals should be handled from this point only by those wearing sterile gloves.
3. Animals should be treated with Clindamycin (30 mg/kg) by oral gavage or s.c. by injection.
4. Animals should then be placed in the filtered sterile cages, which can be placed on telemetry receiver pads. Data of pre- and post-clindamycin treatment can then be collected to ensure that treatment does not influence the normal temperature and activity of the animal. If animals are subject to the 5-day antibiotic regimen, cages should be changed 24-h post-treatment.

3.4. Infection of Animals

1. Prior to infection, an aliquot of bacterial spores should be defrosted, centrifuged to pellet the spores and re-suspended in an appropriate volume of sterile PBS to allow delivery of approximately 100 spores in a volume of 200 μl.
2. To determine that the number of spores in the preparation does not vary during the infection procedure, a viable count is carried out on the sample prior to and post-challenge of the animals.
3. Animals are challenged orally with the spore preparation and placed into fresh sterile filter-topped cages which are returned to the transformer/receiver plates to allow collection of temperature and activity data.
4. Every 24 h of survival post-infection, the animals are transferred to fresh sterile cages. Each animal is monitored several times a day (especially 24–48 h) for the development of diarrhoea.

3.5. Symptoms of Infection

The first outward obvious sign of infection is diarrhoea. For some strains, infection manifests as a rapid production of several faecal pellets, whilst for other strains the diarrhoea is less profuse. Animals appear well, active and continue to take food for 1–2 h

post-onset of this symptom. After this stage the animals become increasingly less active and 2–5 h post-onset begin to lose several degrees of core body temperature (*see* **Fig. 14.1**).

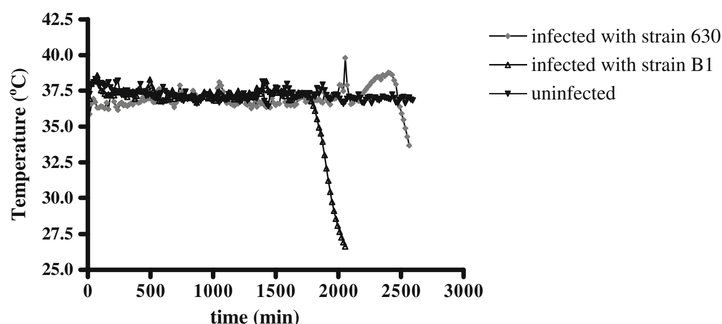


Fig. 14.1. Monitoring of core body temperature following infection with approximately 100 spores of *C. difficile*. This figure represents typical profiles observed with uninfected animals (◊), animals infected with strain B1(▼) and animals infected with strain 630 (▲).

When using the same strain of the organism (and particularly the same batch of spores), the time to death with some strains appears highly reproducible. Using the telemetry data as a guide, it is possible to use a small number of animals to determine the most appropriate time to infect the animals with spores, such that the onset of symptoms falls within the normal working day. This allows opportunities for maximal observation and appropriate culling of an infected animal before significant suffering has occurred.

3.6. Bacteriological Assessment of Infection – Vegetative Cells and Spores

This can be determined using faecal material or post-mortem by analysis of bacterial loads in specific sections of the gut. This can be further divided into organisms found in the lumen of the tissue and those more intimately associated with the mucosal tissue.

1. Tissues should be dissected as described above.
2. Each should then be opened longitudinally and washed with at least 2 × 10 ml of PBS. These washes should contain the majority of lumen-associated organisms (LA).
3. Once clear of significant material, the tissue can be removed to a fresh container. If significant faecal material remains apparent, further washing stages should be included.
4. The tissue should then be weighed and then placed within a bag for homogenisation (weighing the tissue allows bacteriology to be expressed CFU/g of tissue). A further 5 ml of PBS is added to aid maceration.
5. The tissue is then homogenised for 2 min (Stomacher 80) and then the suspension containing tissue-associated (TA) organisms recovered.

6. The number of viable bacteria in the lumen or associated with tissue can then be assessed by serial dilution of these suspensions in PBS and plating of the bacteria on TCCFA plates.
7. Plates should be incubated for a minimum of 48 h before assessment for growth.
8. To establish the relative number of spores to vegetative cells in the preparation, the samples should be analysed as above and then placed in a water bath at 56°C for a minimum of 10 min. This is sufficient to kill the vegetative forms of the organisms. The analysis can then be repeated to determine the percentage of the population that are spores.

3.7. Strain Identity Confirmation

To clarify the strain recovered at the end of the infection is identical to that given at the start, a modified version of the MVLA typing is used. This requires the amplification of seven repeat regions of the genomic DNA of the infecting and recovered strains.

1. Genomic DNA from the strains to be tested is prepared and subjected to PCR using the seven pairs of primers (11).
2. Once amplified, these fragments are separated by gel electrophoresis on a 0.8% agarose gel.
3. The resultant banding pattern is suitably discriminating to confirm, when compared alongside the original strain, the identity of the infecting strain (*see* **Fig. 14.2**).
4. For additional confirmation this fragments can be subjected to sequencing and the resultant sequences compared to the original strain used in the infection.

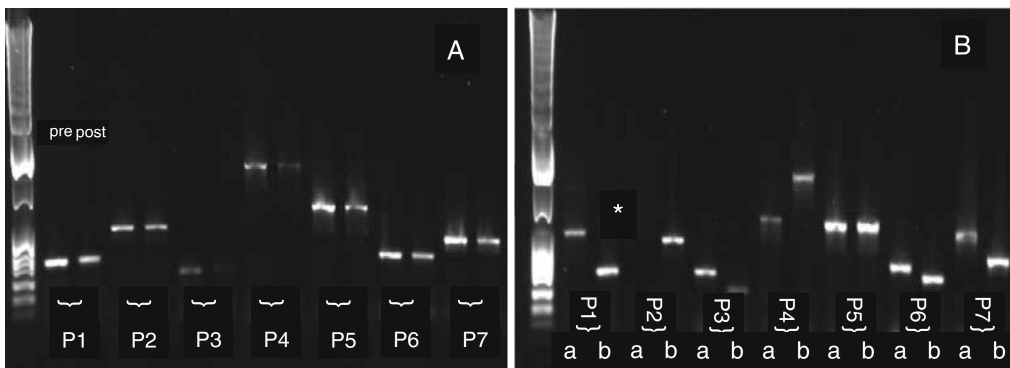


Fig. 14.2. The use of MVLA PCR patterns to confirm the identity of strains of *C. difficile* pre- and post-infection of the hamster. (**a**) Shows the amplified bands using all seven pairs of primers pre- and post-infection with *C. difficile* BI-6. Note the band sizes are identical. (**b**) Shows the same seven amplified bands from either *C. difficile* 630 (a) or *C. difficile* BI-6. Using these primers only one of the amplified fragments (P5) generates a band of equal size in each strain.

3.8. Gross Histological Analysis of Infected Gut Tissue

One of the difficulties associated with histological assessment of the tissue is the friable nature of the infected gut when compared to normal gut tissue. To ensure minimal damage as a consequence of handling, relatively large sections (2–3 cm) are taken and fixed in formal saline.

1. Animals are dissected and the viscera exposed.
2. The gut from the bottom of the stomach to the rectum is removed and separated into separate sections, which includes the small and large bowel and the caecum.
3. For the large and small bowel, sections of tissue are cut and to ensure rapid fixing, formal saline is introduced into the lumen of the section using a fine tipped pastette. The section can then be lowered into a bath of formal saline and tissue is allowed to fix for a minimum of 24 h at 22°C before sectioning and staining with haematoxylin and eosin. Sections taken from the caecum should be at least 1 cm^2 and are fixed directly without cleaning in a bath of formal saline.
4. Sections can be viewed by conventional microscopy.

3.9. Fine Structure Analysis of Infected Gut Tissue by Electron Microscopy

3.9.1. TEM

1. Rings of colon and caecum approximately 5 mm in thickness are dissected, opened and placed in a primary fixative (containing 2% paraformaldehyde with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.42 with added 0.1 and 0.05% magnesium and calcium chloride) for 15 min at room temperature followed by 45 min on ice immediately after dissection.
2. The specimens are then washed in ice-cold sodium cacodylate buffer with added chlorides three times over 15 min, before being placed in 1% osmium tetroxide in sodium cacodylate buffer for 1 h at room temperature.
3. The samples are then rinsed a further three times in buffer and then treated with mordant (1% tannic acid) followed by 10 min in 1% sodium sulphate before dehydrating in an ethanol series for 30 min each in 20, 30, 50, 70, 90 and 95% of ethanol, staining with 2% uranyl acetate at the 30% ethanol stage, finally being submerged for 3 × 20 min in 100% ethanol.
4. The samples are treated for 2 × 15 min in propylene oxide (PO), which are then exchanged to a 1:1 PO to TAAB 812 resin for at least 1 h and finally for neat resin (with a few drops of PO) over night.
5. The specimens are then embedded in a flat moulded tray and cured in an oven at 60°C.

One-micron thick sections are cut on a Leica EMUC6 ultramicrotome and stained with toluidine blue on a hotplate at 100°C

for observation on the light microscope. Areas of interest are then ultrathin sectioned at 70 nm, contrasted with saturated uranyl acetate and lead citrate and imaged on an FEI 120 kV Spirit Biotwin transmission electron microscope with a Tietz F415 digital Temcam.

The resultant pictures show the organisms interacting with the immune cells at the surface of and within the mucosal surface (**Fig. 14.3**). However, the extent to which organisms gain access to deeper tissues appears dependent on the strain of *C. difficile* used in the infection.

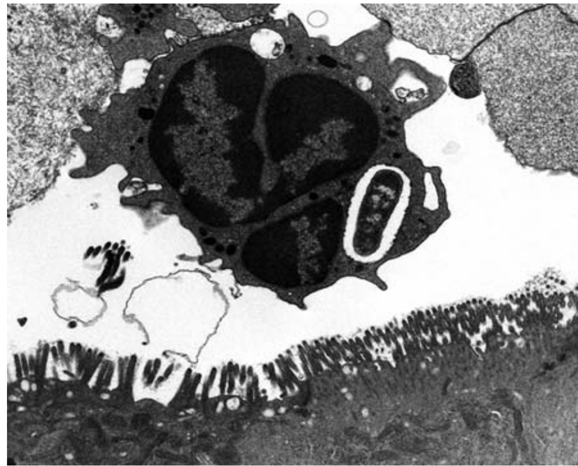


Fig. 14.3. Polymorphonucleocytes engulfing *C. difficile* B1 at the mucosal membrane.

3.9.2. SEM

1. Rings of colon and caecum are processed as above except that after initial osmification tissues are further impregnated with 1% aqueous thiocarbohydrazide and osmium tetroxide layers following the protocol for OTOTO (Malick and Wilson 1975).
2. Dehydration is followed by critical point drying in a Bal-tec CPD030.
3. Tissues are mounted on aluminium stubs with silver dag, sputter coated with 2 nm of gold in a Bal-tec SCD050 and examined on a Hitachi S-4800 scanning electron microscope.

Captured images indicate the production of a number of surface structures including long filaments that resemble flagellar (**Fig. 14.4**).

3.9.3. Immuno EM

1. Rings of colon and caecum are fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M PBS at pH 7.4 at 37°C

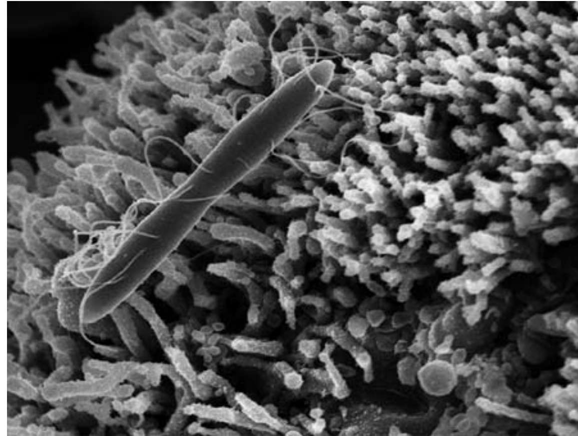


Fig. 14.4. *Clostridium difficile* 630 at the surface of the mucosal membrane. This SEM image shows the bacteria expressing long thin filaments that look like flagellar 24-h post-infection.

for 10 min and then at room temperature for a combined total of 2 h.

2. Tissues are then rinsed in PBS and processed to Lowicryl HM20 by progressive lowering of temperature in a Leica FS unit. Samples are dehydrated in an ethanol series, 30% at +4°C, 50% at +1°C, 70 and 90% at –20°C and 100% ethanol at –30 and –50°C for 30 min each, impregnated with Lowicryl/ethanol 1:3, 1:1, 3:1 for an hour each followed by neat Lowicryl overnight before embedding and UV polymerisation at –50°C.
3. Fifty-nanometre ultrathin sections are cut and collected on Formar/carbon-coated grids for labelling by lifting and transferring each grid with anticapillary forceps across a series of 50 μl reagent droplets (listed below) and 200 μl wash/blocking droplets on a Parafilm strip spread over and adhered to the workbench on a puddle of water to keep it flat.
4. Sections are blocked with 0.02 M glycine in PBS for 10 min followed by 10% foetal calf serum in PBS (blocking diluent for all following steps) for 1 h, labelled with serum raised to heat-killed *C. difficile* 630, diluted 1:200 for 30 min to 1 h and rinsed in PBS three times over 15 min.
5. Sections are then incubated on 10 nm protein A gold for 20 min, washed sequentially on 10 distilled water drops over 10 min, contrasted with uranyl acetate and lead citrate and imaged on the TEM.

Organisms or particular structures on the *C. difficile* bacteria can be confirmed using specific serum (**Fig. 14.5**).

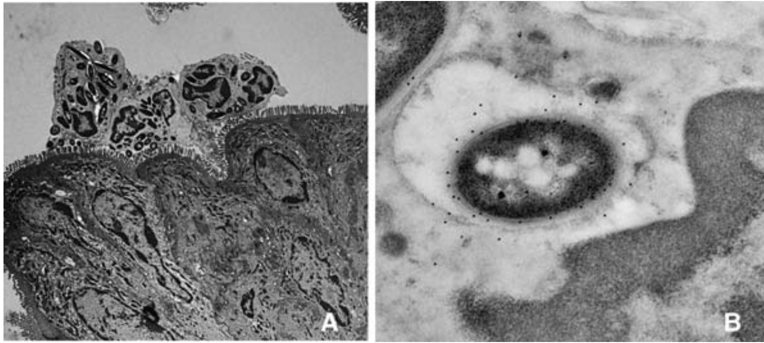


Fig. 14.5. Confirmation of the identity of the bacteria interacting with immune cells using immunohistochemistry. The bacteria observed interacting with neutrophils and eosinophils at the mucosal surface can be identified as *C. difficile* using gold-labelled-specific anti-serum.

4. Notes

1. As indicated, one of the difficulties that arise when using the model is the potential for cross-contamination. This should be minimised where possible by separating infected animals from stock animals, minimising the number of different strains used within a single experiment and regularly deep cleaning the local environment. All experiments should contain an environmental control animal which has been treated with antibiotics. This animal should remain well throughout the length of the study if cross infection has been eliminated.
2. If a single inoculation needle is used to infect several animals with the same strain, it is useful to decontaminate this between each animal using a combination of antimicrobial agents such as Virkon and 70% ethanol. Whilst this is unlikely to impact on the potential for cross-contamination with *C. difficile* spores, it will reduce the spread of other contaminating flora between the animals.
3 As the profile of infection appears to vary between strains of *C. difficile* (time to acute phase of the disease, damage caused as a consequence of infection, dose of spores required for reproducible infection) small numbers of animals should be used in initial experiments to establish the appropriate parameters for each strain. In addition, it is advisable to begin with a strain of known behaviour in the animal model.
4. For recovery of organisms from tissues and faecal material, the use of more selective media such as Brazier's can aid in the distinction of *C. difficile* from other gut flora.
5. Infection doses. The dose (no of spore-forming units) required to initiate a reproducible infection can vary

between strains, particularly with 027 strains which require at least a 10-fold increase in the dose required. In addition, we have had limited infections when heat shock rather than ethanol is used to create the spores used in infection.

Acknowledgements

Work in Glasgow was sponsored by a Wellcome Trust grant 080860 to GRD. DG is funded by the Wellcome Trust.

References

1. Barbut F, et al. (2007) Clinical features of *Clostridium difficile* – associated infections and molecular characterisation of strains: results of a restrospective study, 2000–2004. *Infect Control Hosp Epidemiol* **28**, 131–139.
2. Spencer RC. (1998) Clinical impact and associated costs of *Clostridium difficile* associated disease. *J Antimicrob Chemother* **41**, 5–12.
3. Bartlett JG, et al. (1977) Clindamycin associated colitis due to a toxin producing species of Clostridium in hamsters. *J Infect Dis* **136**, 701–705.
4. Torres JF, et al. (1995) Evaluation of formalin inactivated *Clostridium difficile* vaccines administered by parenteral and mucosal routes of immunisation in hamsters. *Infect Immun* **63**, 4619–4627.
5. Kim PH and Rolfe RD. (1989) Immunisation of adult hamsters against *Clostridium difficile* associated ileocecitis and transfer of protection to infant hamsters. *Infect Immun* **5**(5), 2984–2992.
6. Drudy D, et al. (2007) Emergence and control of fluoroquinolone-resistant, toxin A-negative, toxin B-positive *Clostridium difficile. Infect Control Hosp Epidemiol* **28**, 932–940.
7. Cerquetti M, et al. (2002) Binding of *Clostridium difficile* to Caco-2 epithelial cell line and extracellular matrix proteins. *FEMS Immunol Med Microbiol* **32**, 211–218.
8. Razaq N, et al. (2007) Infection of hamsters with historic and epidemic BI types of *Clostridium difficile. J Infect Dis* **196**, 1813–1817.
9. Sambol SP, et al. (2001) Infection of hamsters with epidemiologically important strains of *Clostridium difficile. J Infect Dis* **183**, 1760–1766.
10. Naaber P, et al. (1998) Bacterial translocation, intestinal microflora and morphological changes in experimental models of *Clostridium difficile* infection. *J Med Micro* **47**, 591–598.
11. Marsh JW, et al. (2006) Multilocus variable-number tandem-repeat analysis for investigation of *Clostridium difficile* transmission in hospitals. *J Clin Microbiol* **44**, 2558–2566.
12. Malik LE and Wilson RB. (1975) Modified thiocarbohydrazide procedure for scanning electron microscopy: routine use for normal, pathological, or experimental tissue. *Stain Technology* **50**, 265–269.

Chapter 15

Methods for Working with the Mouse Model

Anne Collignon

Abstract

Mouse models have been developed to study the pathogenic process of *Clostridium difficile* infections, first the intestinal colonization and second the toxin production. These models have also been used to test the role of environmental conditions that modulate infection. Different mouse models have been used successfully to study *C. difficile* infections such as conventional mice, gnotobiotic mouse models including the monoxenic *C. difficile* mouse model, and the human microbiota-associated mouse model. The advantages and disadvantages of these models are discussed.

Key words: *Clostridium difficile*, pathogenesis, conventional mice, gnotobiotic mouse models, human microbiota-associated mouse model.

1. Introduction

Various animal models have been used for studying *Clostridium difficile* pathogenesis, each of which has intrinsic advantages and disadvantages of complexity, convenience, and suitability (1–4).

In this chapter, we describe the mouse models, which have been developed to study intestinal colonization and toxin production by *C. difficile*. In addition, these models have also been used to test the role of environmental conditions that can modulate the pathogenic process.

1.1. Conventional Mice

Conventional mice have many limitations, but they have been used to follow *C. difficile* colonization. They are relatively resistant to *C. difficile* infection and do not develop a fatal infection (3, 4).

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7_15, © Springer Science+Business Media, LLC 2010

A number of studies have been undertaken in mice to test the hypothesis that the intestinal microbiota components that normally suppress *C. difficile* are eliminated by antibiotic administration, allowing the pathogen to attain unusually high population levels (2, 5, 6). They demonstrated the importance of antibiotics in predisposing to gastrointestinal colonization with this pathogen. Consequently in this model, the challenge with *C. difficile* must be performed after disruption of the intestinal microbiota barrier by antibiotics.

1.2. Gnotobiotic Mice

Gnotoxenic mice are axenic (germ-free) animals which are inoculated with pure bacterial strains. The animals are kept in a sterile environment to prevent any other bacterial contamination. The advantage of gnotobiotic animals lies in the ability to control the composition of the environment in which a multicellular organism develops and functions (7).

All bacteria are able to colonize the intestinal tracts of germ-free animals. When introduced into germ-free mice, *C. difficile* rapidly establishes a stable level (4, 7–9). In contrast, mice with a conventional microbiota are resistant to *C. difficile* intestinal colonization. Experiments in gnotobiotic animals support the importance of the intestinal microbiota in protecting the host against *C. difficile*-associated disease.

1.2.1. Monoxenic C. difficile (CD) Mouse Model

Monoxenic mice are inoculated with a single species. The monoxenic CD mouse model is a simplified model, which can be used to study direct effects of the bacteria on the host without the interference of the intestinal barrier microbiota. The monoxenic CD mouse model has been developed to study *C. difficile* intestinal colonization and in vivo toxin production. It has allowed comparisons of strain virulence. The morbidity and mortality due to *C. difficile* in this monoxenic model depend on the toxin production of the strain. With the highly toxigenic strain VPI 10463, Wilson et al. (4) and Corthier et al. (9) have found that most mice died from severe ileocaecitis with caecal epithelial ulceration and submucosal inflammation.

Protective effects mediated by probiotics have been studied in gnotoxenic mouse models. Corthier et al. assessed the role of the yeast *Saccharomyces boulardii* against *C. difficile* (10). The yeast was given to axenic mice in drinking water. *Clostridium difficile* challenge with the lethal VPI 10463 strain was made 4 days later. The results showed that living yeast can prevent death. *Clostridium difficile* colonization was not inhibited but the toxin level was highly reduced in surviving animals. This protective effect can be explained by the proteolytic activity of the yeast. Similarly, these authors demonstrated the preventing role of low-protein diet. Protection was always related to a low level of toxin

production. This work suggested that protein composition of the diet may affect the pathogenic process (11).

We have used this model to determine whether flagella play a role in intestinal implantation of *C. difficile* (12).

1.2.2. Gnotoxenic Mouse Model of Colonization Resistance to C. difficile

The gnotobiotic mouse model has been used extensively to study environmental factors involved in the outcome of *C. difficile* infections.

In conventional animals, the gut microbiota exerts an antagonistic effect against the multiplication of *C. difficile*. When defined cultures are tested in gnotobiotic mice, the resistance produced is less than that obtained in the conventional animal (6). However, Ducluzeau et al. (13) demonstrated antagonistic effects of simplified fractions of intestinal microbiota obtained from conventional animals comparable to that of the total microbiota.

We have previously described a simplified in vivo model of microbiota barrier against *C. difficile*. It has been obtained in trixenic mice. Three species are involved in this barrier effect: *Clostridium indolis*, *Clostridium cocleatum*, and *Clostridium fusiformis*. In this simplified model, the implantation of the strains in the digestive tract is sequential: the first strain capable of colonization is *C. indolis*, then *C. cocleatum*, and last *C. fusiformis* (14–16). The colonization resistance could be due to a competition between *C. fusiformis* and *C. difficile* for nutrients or receptor sites (17).

1.2.3. Human Microbiota-Associated (HMA) Mouse Model

In an attempt to circumvent some of the problems associated with the use of conventional animals in experimental studies, and yet retain their advantages of complex microbiota, the colonization of germ-free mice with human fecal organisms has been explored (7). This method maintains the microbiota in an in vivo environment by inoculating germ-free mice with a suspension of freshly collected human feces.

Several studies have been undertaken with traditional culture methods and reported that the composition of the fecal microbiota in the animal model was stable for a prolonged period of time and exerted a barrier effect (18–21). Thus, the results of these studies indicate that the human fecal microbiota retains its bacteriological and enzymatic characteristics when associated with gnotobiotic rodents. Such a system provides a model for studying human gut microbial ecology. In particular, it facilitates studies of the interaction between pathogens and the human gut microbiota.

We have studied the fecal microbiota of HMA mice with molecular methods combining FISH with flow cytometry before, during, and after antibiotic treatment by amoxicillin and clavulanic acid (22). The most important groups determined by FISH were the *Clostridium coccoides–Eubacterium rectale* and

Bacteroides–Porphyromonas–Prevotella groups which represent the dominant microbiota. The *Bifidobacterium* and *Lactobacillus* groups and *Enterobacteriaceae* were not detected in our mouse model since they represent a subdominant population and their levels were not high enough to be detectable by FISH.

During amoxicillin and clavulanic acid treatment, the *C. coccoides–E. rectale* group decreased dramatically, whereas the *Bacteroides–Porphyromonas–Prevotella* group and the *Enterobacteriaceae* group increased even though the total microbiota was not quantitatively modified. We also used this model to follow *C. difficile* intestinal colonization after mucosal immunization of the mice with various combination of *C. difficile* surface proteins (23).

2. Materials

2.1. Conventional Mice

1. Animals: BALB/c or C3H mice (Janvier breeding, Charles River breeding laboratories, France)
2. Animal care facilities: classical
3. Diet: classical rodent diet A03 (SAFE, France)

2.2. Gnotobiotic Mice

1. Animals. 6–12 weeks germ-free mice (C3H/HeN or BALB/c), from Charles River Breeding Laboratories (France) (*see* **Note 1**).
2. Germ-free and gnotobiotic rodent facilities. Sterile isolators and transfer devices (plastic isolator, DPTE transfer device, La Calhène, France) (**Fig. 15.1**).
3. Diet: sterilized by irradiation, vacuum packed, RO3-40 (SAFE, France)
4. Autoclave and sterilization apparatus (Sterilizator FPS92A, JCE Biotechnology, France).
5. Sterilizing liquid agent: Soproper (Peracetic acid 3.5% and hydrogen peroxyde) (Air Liquide Seppic, France).

2.3. Dilution and Culture Media

1. LCY buffer 0.2% (w/v) acid casein hydrolysate, 0.5% (w/v) NaCl, 0.1% (w/v) KH_2PO_4, 0.2% (w/v) yeast extract, pH 7.0.
2. Stool diluent 0.5% (w/v) NaCl, 0.2% (w/v) glucose, 0.03% (w/v) cysteine–HCl.
3. Tryptone–Glucose–Yeast (TGY) infusion broth (Difco).
4. Brain heart infusion broth (Difco).

Fig. 15.1. Sterile isolator with its cylindrical transfer device.

5. GAPTT medium 1% (w/v) yeast hydrolysate, 1.5% (w/v) Bacto-peptone, 1% (w/v) Bacto-tryptone, 0.1% (v/v) Tween 80, Bacto agar 1%, pH 6.5.
6. *Clostridium difficile* selective medium: Columbia agar, 5% horse blood, 25 mg/l D-cycloserine, and 8 mg/l cefoxitin (supplement SR96 Oxoid) (*see* **Note 2**).
7. Ultra-Turrax apparatus (T25-Janke&Kunkel, IKA-Labortechnik, Germany).

3. Methods

3.1. Conventional Mice

1. Breeding conditions. They are housed in cages under normal conditions. They are fed standard rodent diet. Food, water, bedding, and cages are autoclaved.
2. *Clostridium difficile* challenge. The mice are administered by intra gastric (i.g.) gavage 100 mg/kg cefoxitin sodium salt (0.5 ml/mouse) during five consecutive days to disrupt the normal intestinal microbiota to facilitate *C. difficile* infection. Twenty-four hours after the last administration of the antibiotic, the mice are challenged by i.g. administration of 0.5 ml of *C. difficile* 24-h culture (10^8 CFU/ml) (*see* **Notes 3** and **4**).
3. *Clostridium difficile* colonization process. Fecal samples are collected and processed anaerobically in an anaerobic chamber. The feces are homogenized and diluted in LCY buffer and enumerated using serial dilutions. Tenfold serial

dilutions (0.1 ml) are inoculated on *C. difficile* selective medium. After 48 h of incubation in anaerobiosis, colonies characteristic of *C. difficile* are counted.

3.2. Gnotobiotic Mouse Models: Sterilization Procedures

1. Sterilization procedure by autoclaving. Classical procedure (120°C, 60 min) for all the materials which support this process (metal transfer devices, feeding tubes, cages, litter, and all stainless material).
2. Sterilization procedure by evaporation. Procedure used for the plastic isolators, plastic cylinders, plastic transfer tubes, etc. The FPS92A sterilization apparatus with Soproper is used according to recommendations of the supplier and strict security rules. The Soproper is vaporized and releases peracetic acid and hydrogen peroxide. The sterilization process must last at least 2 h followed by rinsing at least 2 h (*see* **Note 5**).

3.3. Gnotobiotic Mouse Models: Breeding Conditions

1. Germ-free mice (C3H or BALB/c) are maintained in sterile isolators with sterilized bedding. They received standard nutrition sterilized by irradiation and water sterilized by autoclaving ad libitum (*see* **Note 6**).

3.4. Monoxenic C. difficile *(CD) Mouse Model*

1. *Clostridium difficile challenge*. Axenic mice are challenged by i.g. administration of 0.5 ml of a 24-h *C. difficile* culture in TGY broth (10^8 CFU/ml) (*see* **Notes 7** and **8**).
2. *Clostridium difficile colonization*. Population levels in feces were measured by viable counts. At 1, 2, 6, and 7 days, a fecal sample of each mouse was collected at the anus and weighed. The feces were homogenized in an anaerobic chamber and diluted in LCY buffer and enumerated using serial dilutions. Dilutions were seeded in duplicate and cultured in 1.5% agar GAPTT tubes (8 × 400 mm) or *C. difficile* selective medium in petri dishes. After 48 h of incubation in anaerobiosis, characteristic colonies are counted.
3. *Clostridium difficile association to the mouse caecum*. Seven days after *C. difficile* challenge, the mice are sacrificed and introduced into an anaerobic chamber. The entire caecum of each mouse is removed, rinsed by gentle shaking eight times in a phosphate buffer pH 7.2 and weighed. Each caecum is crushed with an Ultra-Turrax apparatus (T25-Janke&Kunkel, IKA-Labortechnik, Germany) for 1 min at 13,500 rpm and diluted in LCY buffer in order to obtain a concentration of 10 mg/ml. Serial dilutions are seeded in duplicate and cultured in 1% agar GAPTT tubes (*see* **Note 9**).

3.5. Gnotoxenic Mouse Model of Colonization Resistance Against C. difficile

1. *Anaerobic culture of the strains*. The three strains of *C. indolis*, *C. cocleatum*, and *C. fusiformis* are cultured in BHI broth in strict anaerobiosis for 72 h.
2. *Obtention of the trixenic mouse model*. Axenic mice are first challenged by i.g. administration with a culture of *C. indolis* (0.5 ml). After 1 week, the *C. indolis* monoxenic mice are i.g. challenged with a culture of *C. cocleatum* (0.5 ml). One week later, the dixenic mice are i.g. challenged with 0.5 ml of a culture of *C. fusiformis*.
3. *Clostridium difficile challenge*. Mice are challenged by i.g. administration of 0.5 ml of a 24-h *C. difficile culture* (10^8 CFU/ml).

3.6. Human Microbiota-Associated (HMA) Mouse Model

Fresh human stool from a healthy donor is collected in anaerobic boxes (Anaerocult, Merck, Germany). Dilutions of stool (1:10) are anaerobically prepared in NaCl (5 g/l), glucose (2 g/l), and cysteine–HCl (0.3 g/l).

Administration to axenic mice. The fecal suspension is immediately administered to germ-free mice through a single gastric gavage (0.5 ml) (*see* **Note 10**).

Clostridium difficile challenge. The mice are administered by i.g. gavage 150 mg/kg amoxicillin and clavulanic acid, 0.3 ml/mouse during 7 consecutive days to disrupt the barrier microbiota. Twenty-four hours after to the last administration of the antibiotic, the mice are challenged by i.g. administration of 10^8 CFU/ml of *C. difficile* (*see* **Note 11**).

Clostridium difficile colonization. Fecal samples are obtained after administration of *C. difficile* and processed anaerobically. Ten milligrams of feces were suspended in 1 ml of LCY, and 100 μl of tenfold serial dilutions are cultured on GAPPT or CCFA. Typical colonies are counted after 48-h incubation in anaerobiosis.

4. Notes

1. We obtained gnotobiotic mice from Institut National de Recherche Agronomique, Jouy-en-Josas, France (http://www.jouy.inra.fr).
2. Taurocholate 0.1% (Sigma) can be added to allow the recovery of spores. Taurocholate solution is sterilized by filtration.
3. Animals are kept for at least 1 week to allow an acclimatization period after arrival in the animal care facilities before experiments.

4. The disadvantages of conventional animals are quantitative and qualitative fluctuations in the bacterial populations of the normal intestinal microbiota that can occur depending on the breeding conditions. This variability makes interpretation of results difficult.
5. All the handlings with Soproper must be done with all the safety procedures as recommended by the supplier (with gloves and goggles).
6. Sterility of animals can be confirmed by testing representative animals from each cage by fecal culture.
7. Intra gastric gavage is done with adapted tube feeding without food and drink restriction and without anesthesia.
8. Maintain several animals per cage so that cross-infection occurs. Allow natural coprophagy to take place in order to increase cross-infection.
9. The numbers of bacteria present in the suspension are enumerated using serial dilutions in LCY. Dilutions are seeded in duplicate and cultured in tubes (8 × 400 mm) containing 1% GAPTT agar at 37°C for 24–48 h. Viable *C. difficile* are enumerated according to Raibaud (7).
10. These first passage HMA mice can serve as future donors. Germ-free mice can be gastrically intubated twice a week for 2 weeks with 0.5 ml of a suspension of feces from a gnotobiotic mouse harboring a human fecal microbiota. It is also possible after mating to obtain HMA offspring.
11. Augmentin®, Amoxicillin 1 g/clavulanic acid 200 mg.

Acknowledgments

The author would like to thank Séverine Péchiné, Alban Le Monnier, and Sandra Hoys for their advice. The author also thanks her colleagues from the U.E.P.S.D., INRA, Jouy-en-Josas, for their help in gnotoxenic experiments and particularly Sylvie Rabot who is responsible for the gnotobiotic animals.

References

1. Wilson KH, Sheagren JV and Freter R. (1985) Population dynamics of ingested *Clostridium difficile* in the gastrointestinal tract of the Syrian hamster. *J Infect Dis* **151**, 355–361.
2. Larson HE and Borriello SP. (1990) Quantitative study of antibiotic-induced susceptibility to *Clostridium difficile* enterocecitis in hamsters. *Antimicrob Agents Chemother* **34**, 1348–1353.

3. Lucas F, Elmer GW, Brot-Laroche E and Corthier G. (1989) Fixation of *Clostridium difficile* toxin A and cholera toxin to intestinal brush border membranes from axenic and conventional mice. *Infect Immun* **57**, 1680–1683.
4. Wilson KH, Sheagren JN, Freter R, Weatherbee L and Lyerly L. (1986) Gnotobiotic models for study of the microbial ecology of *Clostridium difficile* and *Escherichia coli. J Infect Dis* **153**, 547–551.
5. Borriello SP, Barclay FE and Welch AR. (1988) Evaluation of the predictive capability of an in vitro model of colonization resistance to *Clostridium difficile* infection. *Microb Ecol Health Dis* **1**, 61–64.
6. Wilson KH and Perini F. (1988) Role of competition for nutrients in suppression of *Clostridium difficile* by the colonic microflora. *Infect Immun* **56**, 2610–2614.
7. Raibaud P, Ducluzeau R, Dubos R, Hudault S, Bewa H and Muller MC. (1980) Implantation of bacteria from the digestive tract of man and various animals in gnotobiotic mice. *Am J Clin Nutr* **33**, 2440–2447.
8. Onderdonk AB, Cisneros RL and Bartlett JG. (1980) *Clostridium difficile* in gnotobiotic mice. *Infect Immun* **28**, 277–282.
9. Corthier G, Dubos F and Raibaud P. (1985) Modulation of cytotoxin production by *Clostridium difficile* in the intestinal tract of gnotobiotic mice inoculated with various human intestinal bacteria. *Appl Environm Microbiol* **49**, 250–252.
10. Corthier G, Dubos. F and Ducluzeau R. (1986) Prevention of *Clostridium difficile* induced mortality in gnotobiotic mice by *Saccharomyces boulardii. Can J Microbiol* **32**, 894–896.
11. Corthier G and Muller MC. (1988) Emergence in gnotobiotic mice of nontoxinogenic clones of *Clostridium difficile* from a toxinogenic one. *Infect Immun* **56**, 1500–1504.
12. Tasteyre A, Barc MC, Collignon A and Karjalainen T. (2001) Role of flagellar proteins of *Clostridium difficile* in adherence and gut colonization. *Infect Immun* **69**, 7937–7940.
13. Ducluzeau R, Ladire M, Callut C, Raibaud P and Abrams GD. (1977) Antagonistic effect of extremely oxygen-sensitive clostridia from the microflora of conventional mice and of *Escherichia coli* against *Shigella flexneri* in the digestive tract of gnotobiotic mice. *Infect Immun* **17**, 415–424.
14. Boureau H, Decré D, Carlier JP, Guichet C and Bourlioux P. (1993) Identification of a *Clostridium cocleatum* strain involved in an anti-*Clostridium difficile* barrier effect and determination of its mucin-degrading enzymes. *Res Microbiol* **44**, 405–410.
15. Boureau H, Salanon C, Decaens C and Bourlioux P. (1994) Caecal localisation of the specific microbiota resistant to *Clostridium difficile* colonisation in gnotobiotic mice. *Microb Ecol Health Dis* **7**, 111–117.
16. Karjalainen T, Ramaldes M, Leblond F, Boureau H and Bourlioux P. (1997) The cloning of sialidase genes from *Clostridium indolis* and *Clostridium cocleatum. Clin Infect Dis* **25**, S156–S157.
17. Thomas V, Rochet V, Boureau H, Ekstrand C, Bulteau S, Doré J, Collignon A and Bourlioux P. (2002) Molecular characterization and spatial analysis of a simplified gut microbiota displaying colonization resistance against *Clostridium difficile. Microb Ecol Health Dis* **14**, 203–210.
18. Hirayama K, Kawamura S and Mitsuoka T. (1991) Development and stability of human fecal flora in the intestine of ex-germ-free mice. *Microb Ecol Health Dis* **4**, 95–99.
19. Hazenberg MP, Bakker M and Verschoor-Burggraaf A. (1981) Effects of human intestinal microflora on germ-free mice. *J Appl Bacteriol* **50**, 95.
20. Wong WC, Hentges DJ and Dougherty SH. (1996) Adequacy of the human fecal microbiota associated mouse as a model for studying the ecology of the human intestinal tract. *Microbial Ecol Health Dis* **9**, 187–198.
21. Imaoka A, Setoyama H, Takagi A, Matsumoto S and Umesaki Y. (2004) Improvement of human faecal flora-associated mouse model for evaluation of the functional foods. *J Appl Microbiol* **96**, 656–663.
22. Barc MC, Bourlioux F, Rigottier-Gois L, Charrin-Sarnel C, Janoir C, Boureau H, Doré J and Collignon A. (2004) Effect of amoxicillin clavulanic acid on human faecal flora in a gnotobiotic mouse model assessed with fluorescence hybridization using group specific 16S rRNA probes in combination with flow cytometry. *Antimicrob Agents Chemother* **48**, 1365–1368.
23. Péchiné S, Janoir C, Boureau H, Gleizes A, Tsapis N, Hoys S, Fattal E and Collignon A. (2007) Diminished intestinal colonization by *Clostridium difficile* and immune response in mice after mucosal immunization with surface proteins of *Clostridium difficile. Vaccine* **25**, 3946–3954.

Subject Index

A

Actin filaments 13
Age 6, 10–11, 13–14, 17
Alcohol shock 39
Aminoglycosides 14
Amphotericin B 216
Ampicillin 14
Anaerobe reference unit (Cardiff UK) 58
Animal strains of *C. difficile* 88
Antibiotic associated diarrhoea 3, 49
AroE 79–81, 86

B

Bacillus subtilis 112, 204–206
Bacillus subtilis::Tn*916*ΔE 204
Bacteroides fragilis 12, 22
Bayesian data analysis 150
Bicarbonate 95
Bifidobacterium 10, 232
Binary toxin 13, 69–70, 74
Biotin 95, 138, 144
BlueFuse 159
Brain heart infusion 59–60, 137, 168, 186, 204, 232
Braziers medium 40
Breast feeding 11

C

Caecum 223–224, 234
Candida albicans 12
CatP 186, 189, 193, 195, 197
CDNA synthesis 137, 142
Cefoxatin cycloserine egg yolk agar 40
Cefoxatin cycloserine fructose agar 40
Cell surface 4, 117–133
Cell wall binding protein 118
Cell wall protein 117–119, 123–124
Cephalosporin 14, 23
Chloramphenicol . . 168, 172, 174, 185–186, 189, 191–192
Claudin 136
Clindamycin 11–12, 14, 22–23, 217, 220
Clostridium acetobutylicum 167
Clostridium beijerinckii 167
Clostridium bifermentans 45, 50
Clostridium botulinum 117, 167
Clostridium cocleatum 231
Clostridium difficile 3–114, 117, 135–161, 167–168, 203–226, 233–235
  630 . . . . . 167, 205, 225
  associated diarrhoea 13, 215
  Δerm 167, 204–208
  fusiformis 231
  glycolcium 45, 50
  indolis 231, 235
  innocuum 45, 50
  perfringens . . 10, 12–13, 94, 99, 104, 106, 166, 184, 189
  R20291 . . . . . 140–141, 167
  sordellii 12, 45, 49–50
  sporogenes 167–168, 174, 177, 180
  tetani 10, 117
  VPI 10463 . . . . . 68, 70, 230
Clostron 5, 165–181
Colon 3, 10, 16–18, 22, 40, 136, 139, 223–224
Colonisation 215
Colonisation resistance 231
Colostrum 20
Comparative phylogenomics 149–151
Complementation 6, 184, 187–188, 197–199
Complementation vectors 187–188, 197–198
Conjugation 167, 169, 174–175, 183, 188, 192–193, 198–200
Conjugative transposon 5, 203–204, 209
Conventional mice 229–230, 232–234
Coomassie brilliant blue 107, 119, 133
Core genes 150
Cysteine 15, 95, 153, 168, 186, 232, 235
Cytokine 136, 138
Cytopathic effect 47, 51, 94

D

Daptomycin 23
Diarrhoea 3, 11, 13–18, 20–21, 41–43, 46, 49, 93, 215–216, 220–221
Difimicin 19
Directed mutants 183
Dot blot 98–100, 110–111
DutA 79, 81, 89

E

E. coli CA434 . . . . . 167, 174–175
E. coli DH5 alpha 184, 188
E. coli HB101 . . . . . 185, 191–192, 198
E. coli Top 10 . . . . . 167, 172, 184, 188, 199
Electron microscopy 217–218, 223–226
Electroporation 106, 167–168, 172, 175–176, 184–185, 188, 191–192, 198–199
Elephant dung odour 45
ELISA . . . 40, 46, 48–50, 121, 131–133, 136, 138, 143–145
Endoscope 12–13
Enterotoxin 12, 46
Epidemic 3–5, 14, 17, 21–23, 78, 220
ErmB 22–23, 166, 220

P. Mullany, A.P. Roberts (eds.), *Clostridium difficile*, Methods in Molecular Biology 646,
DOI 10.1007/978-1-60327-365-7, © Springer Science+Business Media, LLC 2010

Erm RAM ... 166
European collection of cell cultures ... 41
Exon binding sites (EBS) ... 171, 179

F

Faecal emulsion ... 14
Fibroblasts ... 40–41
Fibronectin binding proteins ... 12
Filter-mating ... 204, 206, 209–210
FISH ... 231–232
Flagella ... 231
Flow cytometry ... 231
Fluoroquinolones ... 14, 17, 22
Food poisoning ... 10
Fusidic acid ... 18–19

G

GACK ... 150, 152, 156–157, 160–161
Gas gangrene ... 10
Gatifloxacin ... 14, 17, 22
Gel retardation assays ... 98, 100, 108
GeneSpring ... 150, 152, 156, 160
Genome sequence ... 4, 207
Genomic DNA ... 70, 80, 106, 150–151, 170, 178–180, 184, 186, 189, 193–194, 197, 200, 207–208, 217, 222
Genotyping ... 23, 25
Glucosyltransferase activity ... 94
Glutamate dehydrogenase ... 45–46
Glycine ... 97, 99, 111, 118–120, 122–123, 133, 218, 225
Gmk ... 79, 81
Gnotobiotic mice ... 11, 230–232, 235
Gnotoxenic mice ... 230
Golden Syrian hamster ... 215
Group II intron ... 5, 165–166, 168, 180
Guanidinium hydrochloride ... 123
Gut associated lymphoid tissue ... 11
Gut epithelium ... 10

H

Hamsters ... 11, 19–20, 215–227
Hanks balance salt solution ... 41, 46
Histological damage ... 216
Holin ... 94
Holoenzyme ... 94, 100, 113
Homeostasis ... 135
Hospital ward ... 14
Human microbiota associated mouse model ... 231–232, 235
Human stool ... 235
Hybridisation ... 124, 150–152, 154–156, 158–160, 186–188, 193–197, 200, 205, 208

I

IgA ... 11, 20
IgG ... 11, 20
ImaGene ... 152, 155, 159–160
Immunocytochemistry ... 218
Immunotherapy ... 19–20
Index of association (IA) ... 80, 82
Indole ... 21, 45
Infection of animals ... 220–221
Inflammation ... 15, 230
Innate immunity ... 136
Insertional mutagen ... 203
Insertional mutagenesis ... 203
Intergenic spacer region ... 57
Intestinal barriers ... 230
Intestinal epithelial ... 94, 135–146
Intron encoded protein ... 165

J

JAMA-1 ... 136

K

Kan gene ... 166
Klenow ... 109, 152, 154
Koch's postulates ... 188

L

Lactobacillus ... 10, 21, 232
Lactococcus lactis ... 165
LacZ ... 112, 181, 189
Large clostridial cytotoxins ... 12
Levofloxacin ... 14, 22
Lincosamide ... 23
Linezolid ... 23
Listeria monocytogeneses ... 78
Ll.LtrB intron ... 165–166, 170
Lymphocytes ... 11
Lysostaphin ... 119, 151, 153

M

Membrane filters ... 42, 47
Methylase ... 23, 169, 174
Metronidazole ... 17–20, 22
Mice ... 11, 216, 229–236
Microarray ... 4, 149–161, 199
MLST data base ... 80, 87–88
Molecular typing ... 55–63, 68, 78, 87–88
Monoxenic mice ... 230, 235
Mouse ... 45, 99, 216, 229–236
Moxifloxacin ... 14, 17, 22–23
Mr Bayes ... 150, 152, 157–158, 160–161
Mucus ... 10–11
Multi locus sequence typing ... 25
Multi locus variable number tandem repeat analysis ... 25, 56, 78
Multiresistant ... 23
Mutanolysin ... 119, 151, 153
MvLST ... 88

N

Nanodrop ... 142, 158
Neisseria meningitides ... 78
NEXUS ... 157, 161
Nitrothiazolide ... 19
Non toxigenic *C. difficile* ... 15, 21–22, 40, 45–46
North American pulsed field type ... 1, 57

O

Occuldin ... 136
Oral colonisation ... 215
Oxygen ... 50, 125, 219

P

PACD4K-C . . . 166
PaLoc . . . 25, 67–68, 71, 94
PAM120 . . . 207–208
PAMβ1 . . . 166
PAN2 . . . 167–168, 174–175
Paralytic ileus . . . 15
PCD6 . . . 184
PCR-ribotyping . . . 4, 78, 80
Peptidoglycan . . . 19, 118, 136
Peyer's patches . . . 11
PHYLIP . . . 80
Phylogenetic classification . . . 149
PIP404 . . . 5, 184, 188–189
Piperacillin . . . 23
PJIR1546 . . . 184
PJIR2816 . . . 184–186, 189, 191–193
Plasmid . . . 100, 102, 109–110, 166–167, 169–177, 179–181, 184–189, 191–194, 199, 207
PMTL007 . . . 166–169, 171–181
PMTL9301 . . . 184, 197–198
Ponceau S stain . . . 120, 128
Primary antibody . . . 129–132
Primer extension . . . 95–96, 99–103, 112
Probiotics . . . 17, 21, 230
Promoters . . . 94, 98–100, 104, 108, 112
Proteinase K . . . 59–60, 96, 104, 151, 153
Protein assays . . . 97, 107
Proton pump inhibitor . . . 10, 15
Protoplast . . . 100, 124
Pseudomembranous colitis . . . 3, 11–12, 15, 83, 87–88
Pulsed field gel electrophoresis . . . 55–63, 78

Q

Quinolones . . . 14

R

Ramoplanin . . . 19
Real time PCR . . . 49, 137–138, 143, 146
RecA . . . 79, 81
Recombination vector . . . 184–186, 188–189, 191, 197, 199
Reinfection . . . 21
Reporter genes . . . 104, 112
Restriction endonuclease analysis . . . 55–57
Restriction fragment length polymorphism . . . 73
Retargeted intron . . . 166
Retro-homing . . . 166
Retro transposition activated selectable marker (RAM) . . . 157, 166, 179
Reverse transcriptase PCR . . . 95, 101, 103, 112, 137
Rho protein . . . 13, 94
Rifampicin . . . 14, 19, 23
RNA extraction . . . 95, 100, 137, 140–142
RNA polymerase . . . 94, 98, 100, 106, 108–110, 113
RNAse . . . 94–97, 99, 101–105, 111, 119, 151, 153
RNase protection assays . . . 94–95, 99
Robertson's Cooked meat medium . . . 43, 50
Rodac contact plates . . . 42
RP4 mediated conjugation . . . 183, 188, 192–193, 198
Run off transcription assay . . . 94, 98, 109–110

S

Saccharomyces boulardii . . . 21, 230
Secondary antibody . . . 130–132, 139, 144
Selective medium . . . 15, 42, 177, 233–234
Self catalytic splicing . . . 166
Sequence type . . . 81–82, 85, 87
Sigma factor . . . 94, 100, 113
Sigmoidoscopy . . . 16
Single cross over . . . 183
Single locus variant . . . 85, 87
S-layer . . . 12, 117–119, 122–123, 127
Splicing by over lap extension PCR . . . 170
Spore . . 3, 10, 13–14, 20–21, 23, 42–43, 50, 215, 218–222, 226–227, 235
Spore preparation . . . 219–220
Sporulation . . . 17, 219
Staphylococcus aureus . . . 11, 78, 117
Stomach . . . 10, 20, 221, 223
Stools . . . 3, 15–16, 18
Streptogramin . . . 23
Streptomycin . . . 41, 46, 136, 139
Surface layer protein A sequence typing . . . 25

T

Targetron . . . 166, 170–171
TccD . . . 81
TcdA . . . 25, 67–69, 81, 93–94, 99, 105–106, 108, 110
TcdB . . . 25, 40, 67–69, 81, 94, 99, 108
TcdC . . . 5, 17, 68, 74, 81, 94, 97–100, 106–107, 109–111, 113
TcdE . . . 68, 81, 94
TcdR . . . 68, 94, 97–100, 106–111, 113–114
Teicoplanin . . . 19
Telemetry . . . 217, 220–221
Tetanus . . . 10, 94
Tetracycline . . . 14, 168, 174, 185–186, 191–192, 198, 203–206, 209
Thiamphenicol . . . 168, 175–177, 180, 186, 193, 198
Tigecycline . . . 23
Tight junctions . . . 136
Tn5397 . . . 204
Tn5398 . . . 204
Tn916 . . . 5, 203–205, 208–210
Tn916ΔE . . . 203–204, 207–208
Tolevamer . . . 9
Toxic megacolon . . . 15, 17
Toxin A . . 12–13, 15–17, 19–20, 25, 40, 45–46, 48–49, 93, 112, 141, 216–217
Toxin B . . . 5, 13–17, 20, 40, 46–48, 50–51, 69–70, 93, 216
Toxinotyping . . . 25, 56, 67–75
Tpi . . . 79, 81
Transcriptional fusions . . . 97, 99, 104–106, 112
Transepithelial electrical resistance . . . 136, 139, 145
Treeview . . . 152, 158
Trimethoprim . . . 14
Twintron . . . 166
Typing . . . 4, 23–25, 41, 55–63, 67–75, 77–89

V

Vaccine . . . 19–20
Vancomycin . . . 11, 18–20, 22
Vancomycin resistant enterococci . . . 18
Vero cells . . . 40–41
Virkon . . . 122, 226
Virulence . . . 4–5, 17, 55, 57, 78, 81, 86, 88, 94, 230
Virulence factors . . . 94

W

Western blotting . . . 111, 132