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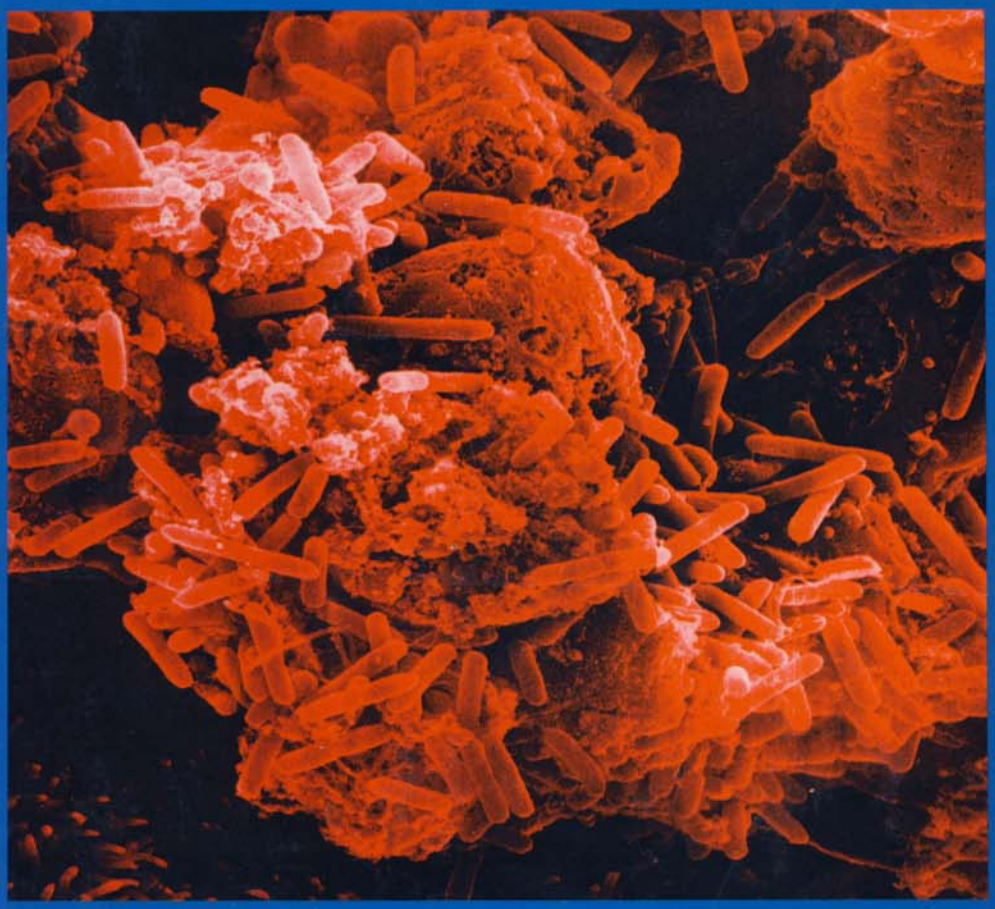
Bacterial Pathogenesis

Edited by
Peter Williams, Julian Ketley & George Salmond



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Edited by

Peter Williams

Department of Microbiology and Immunology
University of Leicester School of Medicine
Leicester

Julian Ketley

Department of Genetics
University of Leicester
Leicester

George Salmond

Department of Biochemistry
University of Cambridge
Cambridge

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Contributors

Carlos F. Amábile-Cuevas Department of Pharmacology, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 México DF, Mexico

John Bannantine Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Disease, Hamilton, Montana 59840, USA

F. Barras Laboratoire de Chimie Bactérienne, CNRS, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex, France

Steven V. Beer Department of Plant Pathology, Cornell University, Ithaca, New York 14853-4203, USA

Dagmar Beier Department of Molecular Biology, IRIS, Chiron Vaccines, Italy

Carol L. Bender 110 Noble Research Center, Department of Plant Pathology, Oklahoma State University, Stillwater, Oklahoma 74078-3032, USA

Charles S. Bestwick Department of Biological Sciences, Wye College, University of London, Ashford, Kent TN25 5AH, UK

Bernard Boher Laboratoire de Phytopathologie, ORSTOM, BP 5045, 34032 Montpellier, France

Ulla Bonas Institut des Sciences Végétales, Centre National de la Recherche Scientifiques, Avenue de la Terasse, 91198 Gif-sur-Yvette, France

J.C. Boucher Department of Microbiology and Immunology, 5641 Medical Science Building II, University of Michigan Medical School, Ann Arbor, Michigan 48109-0620, USA

Ian R. Brown Department of Biological Sciences, Wye College, University of London, Ashford, Kent TN25 5AH, UK

Bernard Burke Division of Infection and Immunity, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, UK

Roberto Cabrera Department of Public Health, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 México DF, Mexico

Miguel Càmara School of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

Trinad Chakraborty Institut für Medizinische Mikrobiologie, Frankfurter Strasse 107, D-35392 Giessen, Germany

Darlene Charboneau Pulmonary Diseases (111N), VA Medical Center, One Veterans Drive, Minneapolis, Minnesota 55417, USA

Siri Ram Chhabra School of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

Alan Collmer Department of Plant Pathology, Cornell University, Ithaca, New York 14853-4203, USA

Alejandro Cravioto Dean, Facultad de Medicina, Universidad Nacional Autónoma de México, Apartado Postal 70-443, 04510 México DF, Mexico

Michael J. Daniels The Sainsbury Laboratory, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK

Mavis Daykin School of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

V. Deretic Department of Microbiology and Immunology, 5641 Medical Science Building II, University of Michigan Medical School, Ann Arbor, Michigan 48109-0620, USA

Eugen Domann Institut für Medizinische Mikrobiologie, Frankfurter Strasse 107, D-35392 Giessen, Germany

Charles J. Dorman Department of Microbiology, Moyne Institute of Preventive Medicine, University of Dublin, Trinity College, Dublin 2, Ireland

Gordon Dougan Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AZ, UK

Ruth B. Dowling Host Defence Unit, Imperial College of Science, Technology and Medicine, National Heart and Lung Institute, London SW3 6LR, UK

Christoph von Eichel-Streiber Verfügungsgebäude für Forschung und Entwicklung, Institut für Medizinische Mikrobiologie und Hygiene, Obere Zahlbacherstrasse 63, D-55101 Mainz, Germany

Carlos Eslava Department of Public Health, Facultad de Medicina, Universidad Nacional Autónoma de México, Apartado Postal 70–443, 04510 México DF, Mexico

Margaret Essenberg Department of Biochemistry and Molecular Biology, 246B Noble Research Center, Stillwater, Oklahoma 744078-3055, USA

Paul Everest Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AZ, UK

Stanley Falkow Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305, USA

Michele Farris Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK

Alain Filloux Laboratoire d'Ingenierie des Systemes Macromoleculaires (LISM), CNRS/CBBM, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France

Timothy J. Foster Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College, Dublin 2, Ireland

L. Yolanda Fuchs Center for Research on Infectious Diseases, Instituto Nacional de Salud Pública, Cuernavaca Mor, Mexico

Murielle Giry Verfügungsgebäude für Forschung und Entwicklung, Institut für Medizinische Mikrobiologie und Hygiene, Obere Zahlbacherstrasse 63, D-55101 Mainz, Germany

Andrew Gorringe Microbial Antigen Department, Centre for Applied Microbiology and Research (CAMR), Porton Down, Salisbury SP4 0JG, UK

Ted Hackstadt Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Disease, Hamilton, Montana 59840, USA

Kim R. Hardie School of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG2 2RD, UK

S. Harris Department of Biological Sciences, Warwick University, Coventry CV4 7AL, UK

Henrik Hasman Department of Microbiology, Technical University of Denmark, DK-2800 Lyngby, Denmark

David L. Hasty Department of Anatomy and Neurobiology, University of Tennessee, Memphis, Tennessee 38163, USA

John Henderson Department of Microbiology, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU

Michael Hensel Lehrstuhl für Bakteriologie, Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, Pettenkoferstrasses 9a, D-80336, Munich, Germany

Jay C.D. Hinton Nuffield Department of Clinical Biochemistry, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK

David W. Holden Department of Infectious Diseases and Bacteriology, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 0NN, UK

Julie Holland School of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

Lawrence G. Hunt Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK

Margareta Ieven Associate Professor of Medical Microbiology, Laboratorium voor Microbiologie, Universitaire Ziekenhuis Antwerpen, Wilrijkstraat 10, B-2650, Edegem, Belgium

Alan D. Jackson Host Defence Unit, Imperial College of Science, Technology and Medicine, National Heart and Lung Institute, London SW3 6LR, UK

Julian M. Ketley Department of Genetics, University of Leicester, Leicester LE1 7RH, UK

Per Klemm Department of Microbiology, Technical University of Denmark, DK-2800 Lyngby, Denmark

Steven E. Lindow Department of Plant and Microbial Biology, University of California, 111 Koshland Hall, Berkeley, California 94720-3102, USA

Shu-Lin Liu Salmonella Genetic Stock Centre, Department of Biological Sciences, Calgary, Alberta, Canada T2N 1N4

Camille Locht Laboratoire de Microbiologie Génétique et Moléculaire, Institut National de la Santé et de la Recherche Médicale U447, Institut Pasteur de Lille, F59019 Lille Cedex, France

Yolanda Lopez-Vidal Department of Microbiology and Parasitology, Facultad de Medicina, Universidad Nacional Autónoma de México, Apartado Postal 70-443, 04510 México DF, Mexico

John W. Mansfield Department of Biological Sciences, Wye College, University of London, Ashford, Kent TN25 5AH, UK

Franco D. Menozzi Laboratoire de Microbiologie Génétique et Moléculaire, Institut National de la Santé et de la Recherche Médicale U447, Institut Pasteur de Lille, F-59019 Lille Cedex, France

Timothy J. Mitchell Division of Infection and Immunity, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, UK

Mahtab Moayeri Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA

Michael Moos Verfügungsgebäude für Forschung und Entwicklung, Institut für Medizinische Mikrobiologie und Hygiene, Obere Zahlbacherstrasse 63, D-55101 Mainz, Germany

Michel Nicole Laboratoire de Phytopathologie, ORSTOM, BP 5045, 34032 Montpellier, France

C. David O'Connor Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK

Mark Pallen Microbial Pathogenicity Research Group, Department of Medical Microbiology, St Bartholomew's and the Royal London School of Medicine and Dentistry, London EC1A 7BE, UK

Malcolm B. Perry Institute for Biological Sciences, National Research Council, Ottawa, Ontario, Canada K1A 0R6

Nicholas B.L. Powell Institute of Infections and Immunity, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, UK

B. Py Laboratoire de Chimie Bactérienne, CNRS, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex, France

Rino Rappuoli Department of Molecular Biology, IRIS, Chiron Vaccines, Italy

Mark Roberts Department of Veterinary Pathology, Glasgow University Veterinary School, Bearsden Road, Glasgow G61 1QH, UK

Ian Roberts Department of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, UK

N. Robson Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK

Ilan Rosenshine Department of Molecular Genetics and Biotechnology, The Hebrew University Faculty of Medicine, POB 12272, Jerusalem 9112, Israel

Jeffrey B. Rubins Pulmonary Diseases (111N), VA Medical Center, One Veterans Drive, Minneapolis, Minnesota 55417, USA

George P.C. Salmond Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK

Kenneth E. Sanderson Salmonella Genetic Stock Centre, Department of Biological Sciences, Calgary, Alberta, Canada T2N 1N4

Vincenzo Scarlato Department of Molecular Biology, IRIS, Chiron Vaccines, Italy

Silke Schäferkordt Institut für Medizinische Mikrobiologie, Frankfurter Strasse 107, D-35392 Giessen, Germany

Mark Schembri Department of Microbiology, Technical University of Denmark, DK-2800 Lyngby, Denmark

Marci A. Scidmore Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Disease, Hamilton, Montana 59840, USA

Anthony W. Smith School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK

R. Elizabeth Sockett School of Biological Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

Bodil Stentebjerg-Olesen Department of Microbiology, Technical University of Denmark, DK-2800 Lyngby, Denmark

Paul V. Tearle National Safety Officer, Public Health Laboratory Service, 61 Colindale Avenue, London NW9 5DF, UK

Raphael H. Valdivia Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305, USA

Fermin Valenzuela Department of Pharmacology, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudada Universitaria, 04510 México DF, Mexico

Arnoud H.M. Van Vliet Department of Genetics, University of Leicester, Leicester LE1 7RH, UK

Peter Vandamme Laboratorium voor Microbiologie, Universiteit Gent, Ledeganckstraat 35, B-9000 Gent, Belgium; and Laboratorium voor Medische Microbiologie, Universitaire Instelling Antwerpen, Universiteitsplein 1, B-2610 Wilrijk, Belgium

Timothy S. Wallis Institute for Animal Health, Compton, Berkshire RG20 7NN, UK

Manfred Weidmann Verfügungsgebäude für Forschung und Entwicklung, Institut für Medizinische Mikrobiologie und Hygiene, Obere Zahlbacherstrasse 63, D-55101 Mainz, Germany

Rodney A. Welch Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA

Chris Whitfield Department of Microbiology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Paul Williams School of Pharmaceutical Sciences and Institute of Infections and Immunity, University of Nottingham, Nottingham NG2 2RD, UK

Robert Wilson Host Defence Unit, Imperial College of Science, Technology and Medicine, National Heart and Lung Institute, London SW3 6LR, UK

Anne C. Wood Department of Genetics, University of Leicester, Leicester LE1 7RH, UK

Karl Wooldridge Department of Genetics, University of Leicester, Leicester LE1 7RH, UK

J. Neville Wright Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK

Kevin D. Young Department of Microbiology and Immunology, University of North Dakota School of Medicine, Grand Forks, North Dakota 58202-9037, USA

H. Yu Department of Microbiology and Immunology, 5641 Medical Science Building II, University of Michigan Medical School, Ann Arbor, Michigan 48109-0620, USA

Preface

'Il n'existe pas de sciences appliquées, mais seulement des applications de la science.'

'There are no such things as applied sciences, only the applications of science.'

Louis Pasteur

Not since the days of Koch and Pasteur has there been a more exciting or important time to study bacterial pathogenesis. Those of us who already work in the field would undoubtedly give our own particular and personal reasons for doing so, but most would probably mention three attractions: the obvious practical value, the challenges of new and fast-moving technologies, and the intellectual fascination of trying to unravel the complex dynamism of host–pathogen interactions.

◆◆◆◆◆◆ GLOBAL PROBLEMS

Bacterial diseases are on the increase, threatening the most vulnerable in our society. From the parochial British point of view, the recent emergence in Lanarkshire of *Escherichia coli* O157:H7 as a lethal pathogen, particularly among the elderly, is perhaps most deeply etched in the public consciousness, forcing us to rethink our eating habits and fuelling demands for a tighter legislative framework for the food industry. But all over the developed world, cases of food poisoning by *Salmonella*, *Listeria* and *Campylobacter* continue to pose major public health problems; the incidence of tuberculosis is increasing at a greater rate than at any time since the advent of antibiotics, especially among immunocompromised patients; multiply resistant 'superbugs' appear in hospitals with frightening regularity; and the control of infection in crop plants continues to impose indiscriminate economic burdens. Western society often seems to be surprised at having to come to terms once again with re-emerging infectious diseases that were commonplace little more than a century ago. We have good sanitation, antimicrobial agents, effective diagnostic techniques, multimillion dollar pharmaceutical and agrochemical industries. Yet the fact is that many of the microorganisms that were the great scourges of history are still with us, ready to re-emerge wherever standards of sanitation, hygiene and disease control break down, or whenever changes in lifestyle or medical and agricultural practices provide novel selective pressures for the evolution of 'new' pathogens. In developing countries, by contrast, infectious diseases remain a fact of everyday life for a large part of the human population, inflicting a continuing and continuous burden of mortality and morbidity on those least able to cope. Thus, first and foremost there are unassailable pragmatic reasons, medical, veterinary, agricultural, even humanitarian, for studying bacterial pathogenicity.

The second reason relates to the development in recent years of exciting new experimental approaches for studying bacterial pathogenicity. In particular, bacterial molecular genetics has reached such a level of methodological sophistication that it is now possible to manipulate genes, make defined mutants and analyze gene products in organisms that only recently seemed intractable to genetic analysis. It is axiomatic that bacterial pathogenicity is multifactorial, that it takes several characteristics of a particular organism to collaborate, simultaneously or sequentially, and in the face of host defense mechanisms, to effect the progress of an infection. From one perspective, therefore, host–pathogen relationships can be seen as highly evolved dynamic interactions in which the outcome (development of disease symptoms on the one hand, recovery of the host organism on the other) may depend ultimately upon seemingly insignificant factors that just tip the balance one way or the other. Another viewpoint, however, sees the victim of infection simply as one particular (albeit highly specialized) ecological niche that a bacterial pathogen may occupy during its normal life cycle. Such a habitat undoubtedly imposes powerful selective pressures for the acquisition of virulence factor genes, often in groups on plasmids or in so-called pathogenicity islands. Moreover, complex interacting regulatory circuits have evolved to allow coordinated expression of important genes at critical phases in the infection process. Indeed, it is interesting in this context to consider that transitions between the 'free-living' and infectious states of a pathogen require major adaptation of cellular physiology over and above those aspects that we generally consider to be associated with virulence. Strategies for the survival of pathogens outside their hosts are likely to be equally important areas of study.

At the heart of modern molecular genetic approaches to the study of bacterial pathogenicity is the application of Robert Koch's timeless principles to the analysis of individual components of an organism's repertoire of virulence determinants. 'Molecular Koch's postulates' allow for formal proof that a particular phenotype of a microorganism does indeed contribute to virulence; it should normally be found among isolates from disease, the cognate genes should be capable of isolation by molecular cloning, and the expression of the cloned genes in a non-pathogenic host should reproduce relevant features of disease in an appropriate infection model. Mutagenesis and the molecular and biochemical analysis of mutant proteins add further refinement. But perhaps most significant in this respect, a veritable quantum leap in the study of bacterial pathogenesis, is the availability of total genome sequences for a growing number of microorganisms.

Genomics complements traditional genetic approaches based on analysis of phenotypic changes by allowing us to infer functions of genes or their products from direct comparisons of nucleotide or amino acid sequences, and to design experimental approaches accordingly. In this context, it is interesting that the recent sequencing of the *E. coli* K-12 genome has uncovered an unexpectedly high percentage of genes of

unknown (or unpredictable) function. This clearly suggests that there are areas of *E. coli* physiology and general biology of which we are currently completely ignorant. Given the phenomenal effort that has gone into the study of *E. coli* over the past half century or more, this is a salutary reminder that only a limited repertoire of the total physiological traits of a bacterium can be uncovered by studying its behavior in the artefactual environment of a laboratory culture flask! By analogy, then, genomics should enable us to undertake the directed analysis of multiple genes in pathogens which might not have been uncovered by more conventional mutagenesis and screening strategies. Undoubtedly some of these 'new' genes will play roles in virulence, but, perhaps equally important, some may be crucial for survival and proliferation of pathogens away from their human, animal or plant hosts. In short, whole new areas of the biology of bacteria are still waiting to be discovered!

◆◆◆◆◆◆ INTELLECTUAL CHALLENGES

The third reason (if two are inadequate) to study bacterial pathogenicity is for the thrill of discovering how two very different organisms interact in the fluid, ever-changing environments that constitute the sites of bacterial infections. In the last decade there has been an explosion in our understanding of the complexities of the cell biology of eukaryotic cells in health and disease, especially the elucidation of intra- and intercellular signal transduction mechanisms. Much of our current knowledge has come from studies using cell, tissue and organ cultures, but observations made *in vitro* are now being used to inform the design of *in vivo* infection models. This, coupled with the development of techniques for the generation of transgenic plants and animals, means that it is now possible to begin to predict and test the effects of particular host cell functions on the progress of an infection in a way that has not been possible before. The study of bacterial pathogenicity has thus become truly multidisciplinary, and the challenge for the future will be to harness the power of various experimental approaches, microbiological, biochemical, genetical, epidemiological, clinical, veterinary and agricultural, to understanding the capacity of microorganisms to amaze us with their complexity, elegance and plasticity.

◆◆◆◆◆◆ CONTENTS AND OBJECTIVES

This is the context in which this book was conceived. Sections cover topics ranging from basic laboratory safety and diagnostics, through molecular genetic and cellular analysis of pathogens and their hosts, to the study of pathogens in populations. Each section has a lead author who is not only an acknowledged expert, but an enthusiast with the ability to communicate the excitement and challenges of a particular area of expertise.

Where appropriate, lead authors have recruited active bench scientists intimately involved in the development and application of relevant methodology to contribute short chapters explaining experimental strategies, describing tried and tested techniques (with recipes if necessary), and generally giving us the benefit of their experiences often gained through years of painstaking research. We have restricted ourselves to bacterial pathogens simply because of the similarity of basic cultural, biochemical and genetic techniques for prokaryotes. It is of course true that many of the general principles of infection by viruses and fungi are essentially the same as those for bacterial pathogens. Nevertheless, approaches to the study of the three classes of pathogen are fundamentally different in methodological terms, and we therefore felt that the inclusion of details for the analysis of viruses and fungi would have significantly increased the size, complexity and cost of the book. However, we felt that it was very important to include techniques for the study of both plant and animal (human) bacterial pathogens in order to stress the similarity of approaches used and to encourage dialog between workers in both fields.

We open (Section 1) with an essay from a laboratory that has been influential in the field of bacterial pathogenesis for more than a quarter of a century, but which elegantly and eloquently illustrates the excitement and challenges of the modern era of research on microbial pathogens. We then step back momentarily from the active, productive research group to consider readers who are newcomers to the field. Section 2 stresses the crucial importance of developing safe working practices. The intention is not to provide a manual of safety methods, which may very well vary according to the particular organism and techniques to be used, and even to the country in which the work is to be done. Rather, Section 2 describes the background against which national and international legislative frameworks have developed, with the aim of providing a philosophical basis for understanding the problems and assessing the risks of working with pathogenic microorganisms at a time when the field is becoming ever more reliant on interdisciplinary approaches. If safety is the watchword for everyday life in an active research laboratory, then surely confidence in the provenance and characteristics of the microorganisms we choose to study must be at the heart of developing consistent and meaningful research programmes. Section 3, therefore, highlights the importance of robust methods for detecting, speciating, and identifying bacteria to ensure reproducibility of experimental data and continuity in experimental design.

Assured of our ability to identify and recognize pathogenic microbes, and to work safely with them, we move next to consider the ways in which these organisms interact with their hosts during the progress of an infection. Animal and plant pathogens are covered in Sections 4 and 5, respectively, a separation dictated more by semantic and methodological differences in the two specialisms, than by fundamental phenomenological or philosophical schisms between two groups of research workers. Indeed, Sections 6, 7, and 8 then go on to present biochemical, molecular genetic and cell biological approaches that are applicable to the study of bacterial virulence determinants regardless of the target organism. This

reflects a growing realization in recent years that there are strongly converging themes in several aspects of bacterial pathogenesis of plants and animals. The production of similar bacterial pheromones (in quorum sensing regulation of virulence factor elaboration) and the multiple, common pathways for targeting of virulence factors are just two areas that highlight how plant and animal pathogens employ similar strategies in interacting with their respective hosts. We return to separate treatments of animal and plant pathogens again in Sections 9 and 10, when we go on to consider the reactions of host organisms to infection. This is simply a pragmatic recognition of the fact that animals and plants are different, but we hope that it will nevertheless encourage the dissemination of ideas from one branch of the discipline to the other. Note that, despite its topicality, we have deliberately shied away from including the theory and practice of large scale whole genome sequence analysis, preferring instead to concentrate on approaches that we feel will be relevant to the work of individual laboratories and research groups.

For our final two sections we go global! Section 11 forces us to evaluate laboratory studies in the context of their applicability to real human problems. It gives us a graphic illustration of the vastness of the problem of controlling infectious diseases worldwide, especially in the developing world where the monitoring of the use of anti-infective agents may be less than adequate. The global nature of research on bacterial pathogenesis is further highlighted by reference in Section 12 to the massive resources available on the World Wide Web for the analysis and interpretation of molecular data. Relevant databases (including, of course, whole genome sequences for an ever-increasing number of pathogens) are continually growing and analytical software is constantly improving, and so detailed descriptions would inevitably date too rapidly to be of lasting use. Instead, Section 12 (itself available on the Internet) concentrates on summarizing the range of materials available and, most importantly, indicating how it can be accessed.

◆◆◆◆◆◆ AND FINALLY ...

The ideal way to learn any particular technique is to go to a laboratory where they do it routinely; of course, this is often not possible for a variety of reasons, and in any case, given the increasingly multidisciplinary nature of the field, it is not always obvious what techniques are applicable to answering a particular question. Our aim in producing this book is to provide an invaluable source-book that will both advance the research of current workers in the field and provide the impetus for new recruits to contribute to it. We wish our readers well in their endeavors.

Peter Williams, Julian Ketley and
George Salmond

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SECTION I

Detection of Virulence Genes Expressed within Infected Cells

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1.1 Detection of Virulence Genes Expressed within Infected Cells

Raphael H. Valdivia and Stanley Falkow
Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305, USA

CONTENTS

INTRODUCTION

We can confidently predict that the complete nucleotide sequence of the chromosomes of most common bacterial pathogens will be known by the end of the next decade. The amount of comparative information will be staggering, and undoubtedly we will discover exciting new facets concerning the biology of microbial pathogenicity. However, nucleotide or amino acid homology does not necessarily define biological function. Already, sequencing has permitted us, for example, to understand that many effector genes of *Shigella flexneri* and *Salmonella typhimurium* employed during entry into host cells are highly homologous at the molecular level (Gijsegem *et al.*, 1993), yet these two species have distinctly different mechanisms of entry and intracellular trafficking. Moreover, the functions of a myriad of gene products remains unknown or the subjects of speculation at best. Consequently, while the wide availability of complete chromosomal sequences undoubtedly represents a biological revolution, it does not relieve us from seeking ways to define the biological functions of sequenced genes.

Microbial pathogenicity cannot be fully understood without an understanding of how the pathogen responds to different environments and challenges within its host. Presumably, the coordinate expression of a subset of genes during residence *in vivo* is necessary for the organism to colonize, survive and replicate within its host. Therefore, the identification of bacterial genes expressed preferentially within infected cells and infected animals is central to understanding how bacterial pathogens

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circumvent the immune system and cause disease. DNA sequence information will not reveal which bacterial genes will be expressed at a particular time during an infectious process nor whether a particular gene product will be transiently expressed or even essential for virulence. In this article, we briefly review some of the genetic methods that have been developed recently to detect genes that are exclusively expressed within invaded cells or infected animals. We will not review promising new biochemical methodologies that have been developed to examine differential messenger RNA (mRNA) expression of bacteria to identify genes that are expressed preferentially during *in vivo* growth (Chuang *et al.*, 1993; Plum and Clark–Curtiss, 1994; Kwaik and Pederson, 1996).

◆◆◆◆◆◆ METHODS USED TO IDENTIFY GENES IMPORTANT IN BACTERIAL VIRULENCE

Several years ago, our laboratory tried to adopt a molecular version of Koch's postulates to prove cause and effect relationships for suspected virulence factors (Falkow, 1988). The fundamental idea was that the relationship between a gene and a functional phenotype, such as virulence, might be established by investigating the pathogenesis of isogenic strains differing only in a defined genotype alteration. If reintroduction of the genetic sequence encoding for the putative virulence factor reconstitutes the pathogenic characteristics of the strain, the molecular Koch's postulates are fulfilled. One of the challenges of this approach is to identify candidate genes that lead to decreased virulence when disrupted.

Transposon mutagenesis has been the most commonly used tool for the identification of virulence genes. The use of transposon mutagenesis was strengthened by the parallel development of tissue culture infection systems that permitted straightforward screening of large numbers of mutants. Some of these approaches exploited the properties of particular cell lines (e.g. polarized cells) or extended the classic penicillin selection method to isolate mutants that were incapable of intracellular replication and therefore were spared the killing action of β-lactam antibiotics (Finlay *et al.*, 1988; Leung and Finlay, 1991). In early experiments, thousands of transposon mutants of *S. typhimurium* were screened individually in a macrophage infection model to identify genes essential for intracellular survival (Fields *et al.*, 1986). This approach revealed a number of mutants with altered envelope components, auxotrophic requirements, and susceptibility to host cell antibacterial compounds, which subsequently led to the identification of the two-component PhoP/PhoQ regulatory system that is required for *Salmonella* intracellular survival (Groisman *et al.*, 1989; Miller *et al.*, 1989).

Signature-Tagged Mutagenesis (STM)

In recent years, an exciting new methodology, STM, has furthered the use of transposon mutagenesis to identify essential virulence genes by

permitting the simultaneous screening of large pools of mutants in a single animal. STM was developed by David Holden and co-workers to identify *S. typhimurium* genes essential for growth in the spleen of infected mice (Hensel *et al.*, 1995). This system uses a 'negative-selection' strategy to identify avirulent strains created by transposon mutagenesis. Each transposon is tagged with a unique oligonucleotide sequence that allows for individual clones to be identified from a large pool of mutant strains. Thus, the protocol allows for parallel screening of large numbers of independent mutants in a minimal number of experimental animals.

The basic steps of this methodology include:

(1) Constructing a large pool of transposons, each individually tagged with a randomly generated, unique sequence.
(2) Generating a collection of tagged transposon mutant *S. typhimurium* strains, each of which is distributed in a separate well in a standard microtiter dish.
(3) Passing pools of mutants through a mouse model of infection to provide negative selection against strains with attenuated virulence (i.e. disruptions in essential genes involved in reaching or surviving within the spleen).
(4) Recovering the surviving virulent bacteria.
(5) Amplifying and labeling the tagged sequences within each transposon insert using the polymerase chain reaction (PCR).
(6) Identifying avirulent strains missing from the recovered pool of mutants.

The last step is accomplished by comparing the hybridization patterns produced by radiolabeled tags amplified from the input library and the mouse survivors to DNA dot blots derived from the input pool. Thus, the tags present in mutants deficient in pathogenic genes are absent from the final pool.

The initial application of this technology was highly successful. It permitted detection of a previously unexpected large pathogenicity island necessary for *in vivo Salmonella* growth (Shea *et al.*, 1996). Our laboratory has worked in collaboration with Dr Holden and his colleagues to examine the overlap between mutations that affect survival in the mouse spleen and mutations that affect other aspects of *Salmonella* pathogenesis, such as the ability to enter cultured epithelial cells or survive within macrophages. It is of considerable interest that the selection for genes essential for epithelial cell entry exclusively identifies genes associated with a previously described pathogenicity island. There is no overlap between genes associated with survival in the spleen and genes necessary for entry into cultured epithelial cells. However, genes involved in survival and persistence in macrophages *in vitro* sometimes overlap those found to be essential for *in vivo* survival (B. Rapauch, unpublished observations).

One limitation of the STM method (and probably of all such gene selection methods) is the difficulty in identifying avirulent mutants following oral challenge. A phenomenon described as the rule of independent action by Guy Meynell some 40 years ago comes into play (Meynell and

Stocker, 1957): in essence, oral challenge (at least with *Salmonella* and *Yersinia*) reveals that a limited subset of virulent bacterial clones progress beyond the mucosal barrier. Thus, if a mouse is challenged with a pool of 96 independent *S. typhimurium* clones, only one-third will be recovered from the mesenteric lymph nodes no matter how high the oral inoculum (J. Mecsas and B. Raupach, unpublished observations). Furthermore, in each mouse a different subset of virulent clones will be found in infected nodes. This does not mean that the methodology cannot be applied to oral infection, but only that more animals need to be infected to determine whether a particular clone is inherently restricted from reaching its cellular target within an animal. Indeed, the application of STM to animals infected orally with *Yersinia* in our laboratory (J. Mecsas, personal communication) has permitted identification of several genes, on both the virulence plasmid and the chromosome, that are essential for pathogenicity by the oral route.

The same fundamental idea of signature tags could be used to mark the genome of a microbe of interest without restricting oneself to transposon mutagenesis. This can be achieved with tagged lysogenic phages or by inserting tags directly into a region of the chromosome that is known not to be required for virulence. Provided that individual organisms can be labeled with a unique molecular 'tattoo', the basic STM approach can be used regardless of the method of mutagenesis used. In this way, the signature tags can be optimized and the relative virulence of each of the tagged strains can be predetermined before mutagenesis and negative selection (B. P. Cormack, personal communication).

STM is a promising new approach for identifying the genetic sequences that are necessary during different stages of infection (e.g. bowel, lymph nodes, and spleen). It takes into account the competitive aspects of virulent and non-virulent clones of the same species and has broad applicability. The only limitation is in the availability of suitable infection models.

◆◆◆◆◆◆ THE SEARCH FOR HOST-INDUCED VIRULENCE GENES

In vivo Expression Technology (IVET)

IVET was the first practical strategy described for selecting bacterial genes expressed preferentially during infection of an animal host (Mahan *et al.*, 1993). Random *S. typhimurium* DNA inserts were cloned upstream of a promoterless tandem *pur*A–*lac*Z gene fusion and introduced into the bacterial chromosome of an avirulent *purA*$^-$ strain by homologous recombination. Since *purA*$^-$ strains cannot grow in the host, bacteria can replicate only if they contain a suitable promoter expressed *in vivo*. The bacteria surviving growth in the animal were then screened on agar plates in search of *purA–lacZ* fusions that were silent under laboratory conditions (as judged by *lac*Z expression). Several variations of the IVET method based on antibiotic resistance and genetic recombina-

tion have been used successfully to detect genes that are expressed preferentially during infection (Camilli *et al.*, 1994; Camilli and Mekalanos, 1995; Mahan *et al.*, 1995). Some of the genes identified by IVET are involved in general biosynthetic processes or transcriptional regulation (e.g. integrated host factor (IHF)). While mutations in selected *in vivo* induced (*ivi*) genes led to a decrease in virulence, the role of many of these genes remains unclear. In some cases mutations within the genes were not significantly affected in their overall virulence for animals (Camilli and Mekalanos, 1995). This important methodology is still being refined. Clearly, using the initial experimental approach, the identification of *ivi* genes was dependent upon an arbitrary criterion for the absence of gene activity in laboratory-grown bacteria. The stringency of such criteria and the strength of any particular promoter fused to the selectable marker (*purA*, *cat* or *tnpR*) can heavily bias the type of genes identified. Nevertheless, further 'incarnations' of this important gene detection method will undoubtedly continue to be developed and be applied to the investigation of many pathogenic microbial species.

Identification of Host-induced Genes using Fluorescence-based Techniques: Differential Fluorescence Induction (DFI)

The adherence, internalization, and intracellular trafficking of bacterial pathogens in their host cells have been studied to single cell resolution with a variety of fluorescence-based technologies such as epifluorescence microscopy, laser scanning confocal microscopy, and flow cytometry. We have extended the single-cell resolution of fluorescence-based technologies to devise a flow cytometry-based selection method to detect genetic sequences that are expressed exclusively within cells or infected animals.

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* is a unique experimental tool that permits monitoring of gene expression and protein localization in living cells. GFP is stable and, unlike reporter molecules such as *lacZ* or luciferase, it does not require cofactors for its activity (Cubitt *et al.*, 1995). GFP had limitations as a reporter gene in bacteria because of its tendency to precipitate into non-fluorescent inclusion bodies, as well as a long lag observed from the time of the synthesis of the GFP protein to the post-translational chromophore formation. We were able to overcome some of these shortcomings by isolating, with the aid of a fluorescence-activated cell sorter (FACS), mutants of GFP that have enhanced fluorescence emission, increased cytoplasmic solubility, and an increased rate of chromophore formation (Cormack *et al.*, 1996).

GFP can be expressed in a variety of both Gram-positive and Gram-negative bacteria. The base composition of the DNA does not necessarily pose an obstacle since we have expressed *gfp* in microorganisms as diverse as *Bartonella henselae*, *Legionella pneumophila*, *Mycobacteria* spp., and a number of enteric Gram-negative species including *S. typhimurium* and *Yersinia pseudotuberculosis*. GFP-labeled bacteria, either alone or in association with mammalian cells, can be detected and sorted routinely by standard flow cytometry (Valdivia *et al.*, 1998).

The ease with which GFP can be detected in a number of pathogenic microbes suggested to us that the ability to separate microorganisms physically on the basis of their relative fluorescence intensity could provide the means for identifying genes induced in complex and poorly defined environments, including infected cells and animals. The flow cytometric separation of individual bacteria on the basis of fluorescence is analogous in conventional bacterial genetics to the manual screening of colonies on agar plates. However, the sorting speed of contemporary FACS machines (2–3000 bacteria per second) makes this screening process similar in efficiency to genetic selection. In principle, individual organisms with any degree of absolute fluorescence over the noise level can be specifically isolated. Therefore, unlike conventional selection methods, a GFP-based selection separates cells on the basis of small differences in fluorescence intensity with little or no bias towards strong gene expression. To that end, we have devised a gene selection strategy, termed DFI, to isolate genes induced in complex environments, including that experienced by *S. typhimurium* after entry into murine macrophages.

DFI is a FACS-enrichment cycle in which bacteria bearing random transcriptional fusions to *gfp* are sorted on the basis of the stimulus-dependent synthesis of GFP. We have recently used DFI to isolate bacterial genes that are induced by a transient exposure to a pH of 4.5 (Valdivia and Falkow, 1996). This selective environment was chosen because of evidence that *S. typhimurium* is exposed to and actually requires an acidic phagosome for it to complete a successful cellular infection (Rathman *et al.*, 1996). Briefly, a library of random promoters fused to *gfp* was subjected to pH 4.5 and all fluorescent bacteria were collected. Since bona-fide acid-inducible genes will not be expressed during growth at neutral pH, the collected population was exposed to media at pH 7, and the non-fluorescent or only weakly fluorescent population was collected. A final exposure of this non-fluorescent population to pH 4.5 yielded a large proportion of fluorescent bacteria. This population was highly enriched (30–50%) for bacteria bearing acid-induced gene fusions. DNA sequence analysis of eight of these genes – by no means an exhaustive analysis of all possible acid-inducible clones – showed that they were mostly related to genes known to possess pH-regulated activity (Valdivia and Falkow, 1996). Two of these acid-inducible genes were also found to be highly induced after entry into macrophages. One of the genes was *pagA*, a PhoP/PhoQ-regulated gene previously reported to be induced within macrophages (Alpuche–Aranda *et al.*, 1992). Another gene, *aas*, is a homologue of an *Escherichia coli* gene involved in phospholipid recycling and potentially is involved in cell membrane repair (Jackowski *et al.*, 1994).

Flow cytometry can also be exploited to identify loci that regulate a gene of interest. Thus, *Salmonella* bearing the *aas–gfp* fusion described above was subjected to transposon mutagenesis and FACS was used to isolate mutants that could no longer induce *aas–gfp* at pH 4.5. Non-fluorescent mutants were detected at a frequency of approximately 0.01% and several of these were found to map to the *ompR/envZ* locus. This two-component regulatory system is necessary for the acid- and macrophage-dependent expression of *aas-gfp*. Interestingly, *Salmonella ompR* mutants

are impaired in their ability to survive within murine macrophages (M. Rathmann, unpublished observation).

Most recently, we have applied DFI to isolate genes that are preferentially expressed within macrophages (Fig. 1.1). Briefly, we infected cultured macrophages with *S. typhimurium* bearing random *gfp* gene fusions and sorted intact cells on the basis of fluorescence from associated bacteria. Lysis of the macrophages and growth of the bacterial population on ordinary laboratory media yielded a population of both fluorescent and non-fluorescent microorganisms. The latter population contains *gfp* gene fusions that are silent under laboratory conditions and was used to infect macrophages at a ratio such that each cell was infected with at most one bacterium. Sorting macrophages that emit a fluorescent signal after bacterial infection provided a bacterial population that contained *gfp* fusions specifically activated in the host cell's intracellular environment. Thus far, we have identified 14 macrophage-inducible loci. A subset of these has previously been reported to comprise essential plasmid or chromosomal genes for *in vivo* survival including components of a type III secretion system necessary for intracellular survival (Shea *et al.*, 1996; Ochman *et al.*,

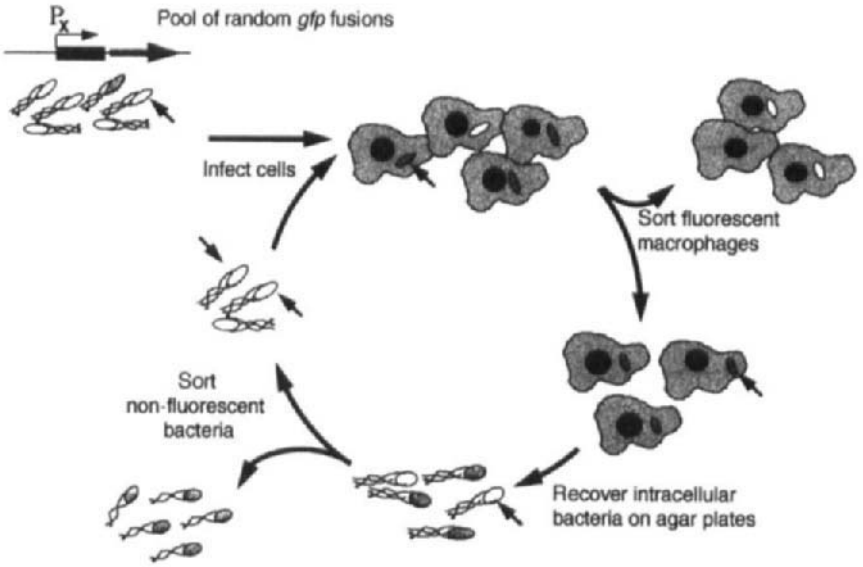

Figure 1.1. Identification of macrophage-inducible promoters by DFI. A library of *S. typhimurium* bearing random *gfp* gene fusions is used to infect murine macrophages. Macrophages showing any fluorescence because of their association with bacteria bearing a transcriptionally active *gfp* gene fusion are then collected with a FACS. Bacteria associated with the sorted macrophages are released by gentle detergent lysis and grown in tissue culture media. This population of cells is then analyzed by FACS and non-fluorescent bacteria are collected. This population is used to reinfect macrophages and fluorescent cells are once again sorted. This FACS-based cycle rapidly enriches for bacteria (arrow) bearing *gfp* fusions that are highly expressed within macrophages but remain silent under laboratory conditions. Px stands for random *S. typhimurium* DNA fragment with promoter activity.

1996; Valdivia and Falkow, 1998). We have also made some progress in understanding the regulation of these genes after bacterial entry into the macrophage. We find that there are at least two general classes of macrophage-inducible genes: one induced within the first hour of infection and the other induced about four hours after cell entry. This latter group is under the control of the two component regulatory system PhoP/PhoQ. PhoP/PhoQ-regulated genes (*pag*) have been shown to be induced late (4–5 h) after entry into the macrophage (Alpuche–Aranda *et al.*, 1992).

Our experience with DFI has not been restricted to studies with *S. typhimurium*. It has been possible to apply DFI to the isolation of iron-inducible and macrophage-inducible genes in *L. pneumophila* (D. Martin and S. Michaux–Charachon, unpublished observations). We expect this technique to be widely applicable to the isolation of *ivi* genes from a variety of bacterial pathogens.

◆◆◆◆◆ CONCLUDING REMARKS

The renewed enthusiasm for the study of the genetic and molecular basis of microbial pathogenicity has spawned a new family of experimental approaches to identify genes expressed exclusively during infection. In particular, we believe that an understanding of the invading microorganism's response to the innate elements of the immune system will be a key to understanding the pathogenesis of infection, as well as the means for designing a new generation of anti-infective agents and vaccines. IVET, STM and DFI represent the first forays for detecting and following specific virulence factors at discrete stages of interaction between the host and the invading parasite. Our experience using STM and DFI has shown that these two methods are complementary rather than redundant in the information they provide about the biological basis of pathogenicity. It is also possible to 'marry' both STM and DFI elements within a single transposon, which provides the means to mark a mutation specifically and assign a potential function to a specific gene fusion. The study of bacterial pathogenicity has never been more amenable to investigation. The development of cell culture methods, the explosion of microbial genomics, and the experimental approaches described here, places us at the threshold of understanding the precise nature of the interplay of microbial life and our own.

Our interaction with the microbial world is not just between the relatively few pathogenic microbes that harm us, but also includes the uneasy relationship with our 'normal' microbial flora. It is extraordinary that we know so little about the complex communities of microorganisms that inhabit our bodies and how they establish themselves in unique niches within us. How are they efficiently transferred to new susceptible hosts? How do they compete with other microbes for nutrients and achieve a suitable rate of cell division consistent with their survival? What do we know about the genes that *Escherichia coli* uses to establish itself as the

most numerous facultative microbe of the human bowel? The methods designed to detect the genes of pathogenicity will play an equally important role in the future for studying the many diverse microbes inhabiting complex communities and extreme environments. IVET, STM, and DFI are only the first approaches to what will become a focus in the coming years: the analysis of microbes outside the confines of the laboratory flask.

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