Regulation of Bacterial Virulence

EDITED BY
Michael L. Vasil
Andrew J. Darwin

Regulation of Bacterial Virulence

Regulation of Bacterial Virulence

EDITED BY

Michael L. Vasil

University of Colorado School of Medicine
Aurora, Colorado

Andrew J. Darwin

New York University School of Medicine
New York, New York

ASM PRESS WASHINGTON, DC

Copyright © 2013 by ASM Press. ASM Press is a registered trademark of the American Society for Microbiology. All rights reserved. No part of this publication may be reproduced or transmitted in whole or in part or reused in any form or by any means, electronic or mechanical, including photocopying and recording, or by any information storage and retrieval system, without permission in writing from the publisher.

Disclaimer: To the best of the publisher's knowledge, this publication provides information concerning the subject matter covered that is accurate as of the date of publication. The publisher is not providing legal, medical, or other professional services. Any reference herein to any specific commercial products, procedures, or services by trade name, trademark, manufacturer, or otherwise does not constitute or imply endorsement, recommendation, or favored status by the American Society for Microbiology (ASM). The views and opinions of the author(s) expressed in this publication do not necessarily state or reflect those of ASM, and they shall not be used to advertise or endorse any product.

Library of Congress Cataloging-in-Publication Data

Regulation of bacterial virulence / edited by Michael L. Vasil and Andrew J. Darwin.
p. ; cm.
Includes bibliographical references and index.
ISBN 978-1-55581-676-6 (hardcover : alk. paper) — ISBN 1-55581-676-2 (hardcover : alk. paper)
I. Vasil, Michael Lawrence, 1945– II. Darwin, Andrew J.
[DNLM: 1. Bacteria—pathogenicity. 2. Bacteria—genetics. 3. Bacterial Toxins. 4. Bacteriophages—physiology. 5. Virulence—physiology. 6. Virulence Factors—physiology. QW 50]
572.8´293—dc23
2012016281
eISBN: 978-1-55581-852-4

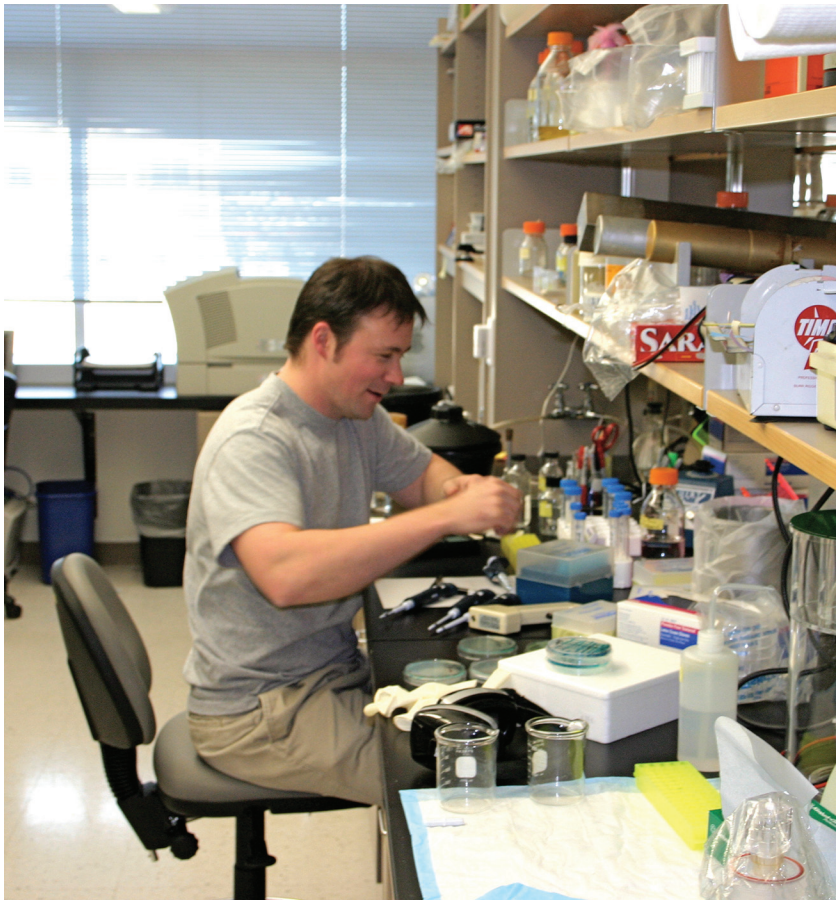
10 9 8 7 6 5 4 3 2 1

All Rights Reserved
Printed in the United States of America

Address editorial correspondence to ASM Press, 1752 N St., N.W., Washington, DC 20036-2904, USA

Send orders to ASM Press, P.O. Box 605, Herndon, VA 20172, USA
Phone: 800-546-2416; 703-661-1593
Fax: 703-661-1501
E-mail: books@asmusa.org
Online: http://estore.asm.org

Cover image: Perle Fine, *Roaring Wind*, 1958. Oil collage on canvas with aluminum foil, 42 × 52 inches. Collection of Drs. Thomas and Marika Herskovic. From the book *American Abstract and Figurative Expressionism* (New York School Press, Franklin Lakes, NJ, 2009).
Cover design: Ed Atkeson, Berg Design.



Michael Vasil dedicates this book to the memory of Martin Stonehouse, Ph.D.,
who relished science and loved life to the fullest.
He left his loving wife, Carly, his sons, Ronan and Morgan, his family,
and all of us much too soon, 29 October 2011.

Andrew Darwin dedicates this book to his parents, Frank and Pauline.
They have never pushed but always supported.

CONTENTS

CONTRIBUTORS

Meredith A. Benson
Department of Microbiology
New York University School of Medicine
New York, NY 10016

Sandra Billig
Department of Microbiology
University of Osnabrück
D-49076 Osnabrück, Germany

Alex Böhm
Institute for Molecular Infection Biology
University of Würzburg
Würzburg, Germany

Evan Bradley
Department of Molecular Biology & Microbiology
Tufts University School of Medicine
136 Harrison Avenue
Boston, MA 02111

Daniel J. Bretl
Department of Microbiology and Molecular Genetics
Center for Infectious Disease Research
Medical College of Wisconsin
Milwaukee, WI 53226

Andrew Camilli
Department of Molecular Biology & Microbiology
Tufts University School of Medicine
136 Harrison Avenue
Boston, MA 02111

Paul E. Carlson, Jr.
Department of Microbiology and Immunology
University of Michigan Medical School, Box 0620
Ann Arbor, MI 48108

Glen P. Carter
Department of Microbiology
Monash University
Clayton, Victoria 3800, Australia

Josephine R. Chandler
Department of Microbiology
University of Washington School of Medicine
1705 NE Pacific Street
Seattle, WA 98195

Yanjie Chao
Institute for Molecular Infection Biology
University of Würzburg
Würzburg, Germany

Jackie K. Cheung
Department of Microbiology
Monash University
Clayton, Victoria 3800, Australia

Peter J. Christie
Department of Microbiology and Molecular Genetics
University of Texas Medical School at Houston
Houston, TX 77030

Colin P. Corcoran
Institute for Molecular Infection Biology
University of Würzburg
Würzburg, Germany

Shipan Dai
Center for Microbial Interface Biology
Department of Microbial Infection and Immunity
The Ohio State University
Columbus, OH 43210

Jennifer L. Dale
Department of Microbiology and
Molecular Genetics
University of Texas—Houston Medical School
Houston, TX 77030

Shandee D. Dixon
Department of Microbiology and Immunology
University of Michigan Medical School, Box 0620
Ann Arbor, MI 48108

Robert K. Ernst
Department of Microbial Pathogenesis
University of Maryland, Baltimore
Baltimore, MD 21201

Allison J. Farrand
Department of Pathology
Microbiology and Immunology
Vanderbilt University Medical Center
Nashville, TN 37232

Alfonso Felipe-López
Department of Microbiology
University of Osnabrück
D-49076 Osnabrück, Germany

Nancy E. Freitag
Department of Microbiology and Immunology
University of Illinois at Chicago College
of Medicine
Chicago, IL 60612

Kathrin S. Fröhlich
Institute for Molecular Infection Biology
University of Würzburg
Würzburg, Germany

Joanna B. Goldberg
Department of Microbiology, Immunology,
and Cancer Biology
University of Virginia
Charlottesville, VA 22908

E. Peter Greenberg
Department of Microbiology
University of Washington, School of Medicine
1705 NE Pacific Street
Seattle, WA 98195-7242

Charley Gruber
Department of Microbiology
UT Southwestern Medical Center
Dallas, TX 75390

John S. Gunn
Center for Microbial Interface Biology
Department of Microbial Infection and Immunity
The Ohio State University
Columbus, OH 43210

Sanjay K. Gupta
Institute for Molecular Infection Biology
University of Würzburg
Würzburg, Germany

Philip C. Hanna
Department of Microbiology and Immunology
University of Michigan Medical School, Box 0620
Ann Arbor, MI 48108

Nadja Heidrich
Institute for Molecular Infection Biology
University of Würzburg
Würzburg, Germany

Calvin A. Henard
Department of Microbiology
University of Colorado Denver
School of Medicine
Aurora, CO 80045

Michael Hensel
Department of Microbiology
University of Osnabrück
D-49076 Osnabrück, Germany

Thomas J. Hiscox
Department of Microbiology
Monash University
Clayton, Victoria 3800, Australia

Lauren E. Hittle
Department of Microbial Pathogenesis
University of Maryland, Baltimore
Baltimore, MD 21201

Ansel Hsiao
Center for Genome Sciences & Systems Biology
Washington University
School of Medicine
St. Louis, MO 63110

Scott J. Hultgren
Department of Molecular Microbiology
Washington University School of Medicine
St. Louis, MO 63110

Michael P. Jennings
The Institute for Glycomics
Griffith University
Gold Coast Campus
Parklands Drive
Southport, QLD 4222, Australia

Barbara I. Kazmierczak
Department of Medicine
Yale University School of Medicine
333 Cedar Street, Box 208022
New Haven, CT 06520-8022

Erica N. Kintz
Department of Microbiology, Immunology, and Cancer Biology
University of Virginia
Charlottesville, VA 22908

Theresa M. Koehler
Department of Microbiology and Molecular Genetics
University of Texas—Houston Medical School
Houston, TX 77030

Jenny A. Laverde-Gomez
Department of Microbiology and Molecular Genetics
University of Texas Medical School at Houston
Houston, TX 77030

Audrey Le Gouellec
TheREx, TIMC-IMAG Laboratory
UMR 5525 CNRS
Université Joseph Fourier
Grenoble, France

Lee-Yean Low
Department of Microbiology
Monash University
Clayton, Victoria 3800, Australia

Dena Lyras
Department of Microbiology
Monash University
Clayton, Victoria 3800, Australia

Kate E. Mackin
Department of Microbiology
Monash University
Clayton, Victoria 3800, Australia

Charlotte D. Majerczyk
Department of Microbiology
University of Washington School of Medicine
1705 NE Pacific Street
Seattle, WA 98195

EmilyKate McDonough
Department of Molecular Biology & Microbiology
Tufts University School of Medicine
136 Harrison Avenue
Boston, MA 02111

Kathleen A. McDonough
Wadsworth Center
New York State Department of Health
Albany, NY 12201-2002

Masatoshi Miyakoshi
Institute for Molecular Infection Biology
University of Würzburg
Würzburg, Germany

Nrusingh P. Mohapatra
Center for Microbial Interface Biology
Department of Microbial Infection and Immunity
The Ohio State University
Columbus, OH 43210

Thomas S. Murray
Department of Basic Medical Sciences
Quinnipiac University School of Medicine
275 Mt. Carmel Avenue, N1-HSC
Hamden, CT 06518-1908

Abiodun D. Ogunniyi
Research Centre for Infectious Diseases
School of Molecular and Biomedical Science
University of Adelaide
Adelaide, SA 5005, Australia

Yuta Okkotsu
Department of Microbiology
University of Colorado School of Medicine
Aurora, CO 80045

Gregory C. Palmer
Institute for Cellular and Molecular Biology
The University of Texas at Austin
Austin, TX 78712

Kai Papenfort
Department of Molecular Biology
Princeton University
Princeton, NJ 08544

Matthew R. Parsek
Department of Microbiology
University of Washington
Seattle, WA 98195

James C. Paton
Research Centre for Infectious Diseases
School of Molecular and Biomedical Science,
University of Adelaide
Adelaide, SA 5005, Australia

Ian R. Peak
The Institute for Glycomics
Griffith University
Gold Coast Campus
Parklands Drive
Southport, QLD 4222, Australia

Robert D. Perry
Department of Microbiology, Immunology, and Molecular Genetics
University of Kentucky
Lexington, KY 40536-0298

Benoit Polack
TheREx, TIMC-IMAG Laboratory
UMR 5525 CNRS
Université Joseph Fourier
Grenoble, France

Daniel A. Powell
Department of Microbial Pathogenesis
University of Maryland, Baltimore
Baltimore, MD 21201

Christopher L. Pritchett
East Tennessee State University
Department of Health Sciences
Johnson City, TN 37614

Julian I. Rood
Department of Microbiology
Monash University
Clayton, Victoria 3800, Australia

Mayukh Sarkar
Department of Microbiology and Molecular Genetics
University of Texas Medical School at Houston
Houston, TX 77030

Michael J. Schurr
Department of Microbiology
University of Colorado School of Medicine
Aurora, CO 80045

Drew J. Schwartz
Department of Molecular Microbiology
Washington University School of Medicine
St. Louis, MO 63110

Cynthia M. Sharma
Research Centre of Infectious Diseases
University of Würzburg
Würzburg, Germany

Dakang Shen
School of Cellular and Molecular Medicine
University of Bristol
University Walk
Bristol BS8 1TD, United Kingdom

Eric P. Skaar
Department of Pathology, Microbiology and Immunology
Vanderbilt University Medical Center
Nashville, TN 37232

Karen Skorupski
Department of Microbiology and Immunology
Dartmouth Medical School
Hanover, NH 03755

Vanessa Sperandio
Department of Microbiology
UT Southwestern Medical Center
Dallas, TX 75390

Yogitha N. Srikhanta
Department of Microbiology and Immunology
The University of Melbourne
Royal Parade, Parkville
Melbourne, VIC 3010, Australia

Andrew M. Stern
Department of Microbiology
Perelman School of Medicine
University of Pennsylvania
Philadelphia, PA 19104

Ronald K. Taylor
Department of Microbiology and Immunology
Dartmouth Medical School
Hanover, NH 03755

Victor J. Torres
Department of Microbiology
New York University School of Medicine
New York, NY 10016

Bertrand Toussaint
TheREx, TIMC-IMAG Laboratory
UMR 5525 CNRS
Université Joseph Fourier
Grenoble, France

Boo Shan Tseng
Department of Microbiology
University of Washington
Seattle, WA 98195

Andrés Vázquez-Torres
Department of Microbiology
University of Colorado Denver School of Medicine
Aurora, CO 80045

Jörg Vogel
Institute for Molecular Infection Biology
University of Würzburg
Würzburg, Germany

Jovanka M. Voyich
Department of Immunology and
Infectious Diseases
Montana State University
Bozeman, MT 59718

Aimee K. Wessel
Section of Molecular Genetics and Microbiology
The University of Texas at Austin
Austin, TX 78712

Marvin Whiteley
Institute for Cellular and Molecular Biology and
Section of Molecular Genetics and Microbiology
The University of Texas at Austin
Austin, TX 78712

Bobbi Xayarath
Department of Microbiology and Immunology
University of Illinois at Chicago College of Medicine
Chicago, IL 60612

Thomas C. Zahrt
Department of Microbiology and Molecular Genetics
Center for Infectious Disease Research
Medical College of Wisconsin
Milwaukee, WI 53226

Jun Zhu
Department of Microbiology
Perelman School of Medicine
University of Pennsylvania
Philadelphia, PA 19104

Wilma Ziebuhr
Institute for Molecular Infection Biology
University of Würzburg
Würzburg, Germany

PREFACE

Arguably, the theme of virulence regulation within the field of bacterial pathogenesis began as early as the 1930s, with a relatively straightforward observation about the inhibitory effect of iron on the in vitro production of diphtheria toxin by *Corynebacterium diphtheriae*. Three independent laboratories reported this important discovery (those of Pappenheimer and Johnson, Locke and Main, and Pope). It was then nearly two decades later before the next major leap of insight into the regulation of diphtheria toxin came about. In 1951, Freeman reported in the *Journal of Bacteriology* that the conversion of a nontoxigenic (i.e., avirulent) strain of *C. diphtheriae* to one that expresses diphtheria toxin required exposure of the avirulent strain to lysates containing phage B (β) but not phage A. Ultimately, these two discoveries provided an extraordinary amount of stimulating fodder to generations of other investigators. First, they established a solid foundation for the further understanding of the mechanisms of *C. diphtheriae* toxin regulation. Second, they offered novel and fascinating paradigms that were clearly worthy of further investigation in the context of the regulation of virulence in a plethora of other animal, as well as plant, bacterial pathogens.

In the time following those key discoveries, there have been thousands of publications directly relating to the topic of this book (>8,000 references found in a PubMed search from 1980, with the query "Regulation of Bacterial Virulence"). Clearly, this field is advancing at a remarkable pace. As a consequence, we felt that it would be worthwhile at this time to assemble a compendium of many of the more fascinating and contemporary insights relating to this topic, from outstanding authorities in the field, with the wish to stimulate further research efforts.

Therefore, in this book we have attempted to provide a wide range of topics that represent a balance between the newest information along more established lines of investigation (e.g., iron, chapters 5, 6, and 16), as well as information describing refreshing new paradigms that have been investigated within only the past few years (e.g., vesicle formation and host signaling, chapters 23 and 27). It is true that the book devotes significant focus toward some areas, such as the effects of iron on bacterial virulence. Most likely this is a consequence of both its early discovery in relation to the regulation of bacterial virulence (see above) and the increasing realization that the role of environmental iron levels in virulence is magnificently complex, from the standpoint of both the pathogen and the host. That is, iron has an impact that reaches far beyond simply regulating the expression of virulence determinants. Although iron was subsequently discovered to affect the expression of other major bacterial toxins (e.g., Shiga toxin and *Pseudomonas aeruginosa* exotoxin A), environmental iron levels have also been shown to have an extraordinary impact on increasingly intricate processes relating to bacterial virulence, including biofilm formation, basic physiological processes, resistance to oxidative stress, and basic intermediary metabolism (see chapters 1, 5, 6, 9, 16, and 22).

Another example of how early observations can establish an important paradigm is provided by the requirement of a bacteriophage in the regulation of bacterial virulence, as described above with the β phage of *C. diphtheriae*. Decades later came the observations about the requirement of a different type of bacteriophage in the production of cholera toxin. In fact, cholera toxin provides an amazingly complex story about virulence gene regulation, as well as the intricate overlapping control mechanisms of different virulence factors (see chapter 12). For this reason, *Vibrio cholerae* features prominently in more than one chapter. Even so, it is clear that much still needs to be explored about the regulation of cholera toxin expression and how phage-associated genes affect the virulence of *V. cholerae*.

We have also provided chapters (see chapters 2, 27, and 28) from outstanding authors who are investigating the regulation of extremely complex behaviors of bacterial pathogens. These include descriptions of how some bacteria (e.g., *P. aeruginosa*) control gene regulation before, during, and after their transition from an acute infection to a more chronic one. Along similar lines, also included is a chapter (chapter 28) that provides new insights about the regulatory transition of *V. cholerae* from inside a human host to its more natural environments, such as estuaries, where

it exists in planktonic form as well as in biofilms, and then back into a human host.

Last, but not least, we gratefully acknowledge all the other outstanding chapters we were not able to mention above, due to space constraints of this preface. The omission of any chapter in this book would most certainly diminish its value. As the editors, we offer our sincere thanks to all of the authors for their dedication and hard work toward the production of this book.

It is hoped that the exciting discoveries described by all of the wonderful authors of this book will be as inspirational to both young and more seasoned investigators, as the early observations about the regulation of diphtheria toxin were to scores of scientists for decades. We can only hope that this will most certainly be so.

Michael L. Vasil
Andrew J. Darwin

I. GLOBAL CHANGES DURING AND BETWEEN DIFFERENT STATES OF INFECTIONS

Regulation of Bacterial Virulence
Edited by Michael L. Vasil and Andrew J. Darwin
© 2013 ASM Press, Washington, DC doi:10.1128/9781555818524.ch1

Chapter 1

Factors That Impact *Pseudomonas aeruginosa* Biofilm Structure and Function

Boo Shan Tseng and Matthew R. Parsek

INTRODUCTION

Biofilm microbiology has been an area of recent intense research. The importance of biofilms both in the environment and in human disease has researchers trying to understand the molecular determinants involved in their formation. The hope is that by identifying such determinants, we might be able to control biofilms.

Approximately a decade ago, the hypothesis that biofilm formation is a developmental process was put forth (O'Toole et al., 2000a; Stoodley et al., 2002). The thought is that like long-studied developmental processes, such as spore formation in *Bacillus subtilis*, biofilm development is a sophisticated, genetically orchestrated series of events. Subsequently, several researchers have attempted to identify genetic elements that play a role in biofilm development. We have since learned that different regulatory and signaling systems in different species are important. We have also learned that environmental conditions are critical, with sudden changes capable of both promoting and dissolving biofilms.

Chronic infections are linked to the biofilm mode of growth. Since bacteria within a biofilm are less susceptible to antimicrobial treatment than planktonic cells, the relative antimicrobial tolerance of these communities has also been a focus of biofilm-related research. Understanding the mechanisms underpinning tolerance may be key in the therapeutic targeting of chronic infections. Currently, the antimicrobial tolerance of biofilms is thought to be multifactorial. Slow-growing subpopulations within the community, biofilm-related patterns of gene expression, and reduced penetration of antimicrobials have all been linked to tolerance. Not surprisingly, the development of biofilms has also been linked to tolerance. Different biofilm development patterns have been shown to result in different mature biofilm structures. These different structures have been shown to differ in their susceptibilities towards antimicrobial treatment.

This review focuses on the relationships among biofilm development, the environment, and antimicrobial tolerance for the paradigm organism *Pseudomonas aeruginosa*.

WHAT IS A BIOFILM?

Historically, the seemingly simple question of what a biofilm is has generated heated debate within the biofilm research community. Is a pellicle at the air-liquid interface of a standing liquid culture or a colony on a plate a biofilm? Do single cells attached to a surface constitute a biofilm? If not, how many cells are required? At the root of this controversy are the qualitative definitions traditionally used to designate a "biofilm," usually involving some type of microscopic assessment.

As researchers learn more about the molecular mechanisms that control the switch from planktonic to biofilm-associated lifestyles, some patterns are emerging that aid in defining the term biofilm. While no universal molecular definition exists to diagnose the biofilm mode of growth, elevated levels of the intracellular signal cyclic-di-GMP (c-di-GMP) appear to be a key focal point for defining biofilms among many gram-negative species (Dow et al., 2007). A high level of this signal is crucial in shutting off flagellar production and function while promoting expression of key biofilm matrix polysaccharides and extracellular proteins. There is also evidence in gram-positive species, such as *Bacillus subtilis*, that the regulation of motility and matrix production are carefully coordinated in an inverse fashion (Blair et al., 2008).

Although a universal molecular determinant indicative of the biofilm lifestyle may not exist, diagnosing the biofilm state should be achievable for

Boo Shan Tseng and Matthew R. Parsek • Department of Microbiology, University of Washington, Seattle, WA 98195.

some species. The ability to define cells as being in the biofilm lifestyle may be beneficial in studying complex environmental or clinical samples. It may also be useful in analyzing cells that have features of both biofilm and planktonic behavior, such as cells that are swarming on a surface. As researchers continue to explore the basis of biofilm formation, a more universal molecular definition of a biofilm may emerge.

BIOFILM DEVELOMENT AND STRUCTURE IN *PSEUDOMONAS AERUGINOSA*

The old, conventional view of a biofilm was of a structurally homogeneous collection of cells enclosed by a slimy matrix. The work of Lawrence and others in the 1990s revolutionized this view of biofilm structure (Lawrence et al., 1991; Stoodley et al., 1994; Massol-Deya et al., 1995). Using confocal scanning laser microscopy, they demonstrated that biofilms are morphologically complex communities. These studies have led to the suggestion that biofilm production is a highly regulated developmental process (O'Toole et al., 2000a; Stoodley et al., 2002). Investigators have discovered that biofilms grown in vitro reproducibly form specific structures that are affected by a plethora of conditions. For *P. aeruginosa*, two general biofilm shapes have been observed using the flow cell system: structured biofilms and flat biofilms. The biological significance of the different three-dimensional biofilm structures produced by the same species is still unclear. Are different cellular functions or processes used to produce these different structures? Are these structures regulated by different pathways? Is the antimicrobial tolerance of the biofilm affected by its structure? What is the relevance of the structure in the modeling of environmental or clinical biofilms? While these questions remain unresolved, many researchers have made headway in our understanding of biofilm morphology.

Structured Biofilms

A structured biofilm consists of a thin layer of cells on a substratum that is punctuated by large cellular clusters. Some of these biofilms resemble mushrooms, with a large cap-like population of cells atop a "stalk" of cells. Others of this class have more mound-like or pillar-like cellular clusters, which are composed of large cellular aggregates. These types of biofilms are all characterized by their "rough" and fairly heterogeneous nature (Costerton et al., 1995). There are at least two routes by which *P. aeruginosa* produces a structured biofilm. The first we call "structured biofilm I" (Fig. 1). Several developmentally discrete steps have been linked to their formation. First, bacterial cells interact with a surface in a reversible manner, in which they can still detach from the surface. Some of these cells become irreversibly attached to the surface. Irreversible attachment is thought to be linked to the production of exopolysaccharide, which aids in the adherence to the surface. Cells proceed to expand clonally, producing an aggregate that is primarily derived from the initial attached cell. Ultimately, cells in the interior of the aggregates disperse, leaving a doughnut-like structure behind (Stoodley et al., 2002).

The development of the second type of structured biofilm ("structured biofilm II" [Fig. 1]) is more complex. This involves the interaction of at least two subpopulations of cells: "stalk" and "cap" cells. By growing two differentially labeled isogenic populations of *P. aeruginosa* in a flow cell system with glucose minimal medium, Klausen and colleagues showed that in an individual mushroom, the stalk cells consisted primarily of one population of labeled cells, while the caps were mixtures of the two labeled populations (Klausen et al., 2003a). This result suggests that the stalk is created through the clonal expansion of a surface-adhered, nonmotile cell and the cap is created through the migration of motile cells on top of the stalk. In agreement with this model, after photobleaching a section of a green fluorescent protein (GFP)-labeled mushroom, the cap recovered fluorescence, while the stalk did not (Haagensen et al., 2007). This result suggests that new cells can move into the cap region of the biofilm but not into the stalk region. This pattern of development, however, has only been shown under specific culturing conditions, namely, a defined medium with glucose as the primary carbon source.

Flat Biofilms

In contrast to structured biofilms, flat biofilms are more homogeneous in appearance. These biofilms are suggested to form initially in a manner similar to that of structured biofilms. By mixing two differentially labeled isogenic populations of *P. aeruginosa* in a flow cell system with a citrate-based defined medium, Klausen and colleagues showed that in early stages of biofilm development, flat biofilms start as microcolonies of one or the other labeled population, suggesting that these microcolonies form from clonal expansion, similar to the structured biofilms. Unlike the structured biofilms, however, cells move, divide, and intermix as the biofilm matures. The substratum between the microcolonies become filled with motile cells of either population, and eventually a flat biofilm

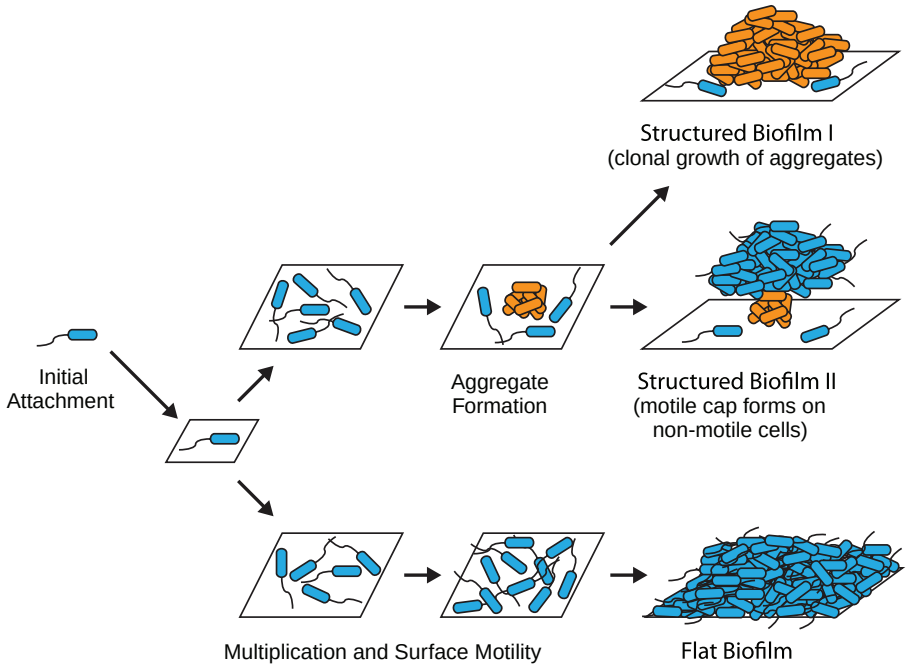


Figure 1. Models of flat versus structured biofilm formation in *P. aeruginosa*. Initial steps of biofilm formation are suggested to be the same for flat and structured biofilms. After initial steps, however, maturation of the different structures is suggested to follow different developmental pathways. There are two forms of structured biofilms (structured biofilms I and II). Aggregates of cells clonally expand to produce the structured biofilm I phenotype, while motile cells move to top nonmotile aggregates of cells to form mushroom-shaped structures in the structured biofilm II phenotype. Flat biofilms are formed through the clonal expansion of motile cells. Blue cylinders represent motile cells, and orange cylinders represent nonmotile cells. Adapted from Kirisits and Parsek, 2006, with permission from John Wiley and Sons.
doi:10.1128/9781555818524.ch1f1

is produced (Klausen et al., 2003b). Data from Shrout and colleagues indicate a role for swarming motility in this process (Shrout et al., 2006).

FACTORS THAT INFLUENCE BIOFILM STRUCTURE

While a specific strain of *P. aeruginosa* consistently produces the same biofilm structure under one laboratory culturing condition, the same strain produces a very different biofilm structure under different culturing conditions. While the environmental sensing mechanisms and the regulatory pathways leading to the formation of specific biofilm structures have not been fully elucidated, it is clear that many factors are important for this process.

Motility

One of the major differences between flat and structured biofilm development is related to the motility of the cells (Shrout et al., 2006). The two biofilms are suggested to start similarly, with individual attached cells growing clonally to create very small cellular aggregates. Cells forming flat biofilms move on the surface, grow, and fill in the space between microcolonies. On the other hand, cells that are destined to produce structured biofilms have two possible developmental paths. To create the structured biofilm I phenotype, cells statically grow, producing larger aggregates, while structured biofilm II is created by a motile population of cells migrating to the top of existing aggregates of nonmotile cells. Monte Carlo simulations suggest that modulating cellular motility affects the biofilm structure (Shrout et al., 2006). In agreement with the mathematical modeling, mutations in the biosynthesis of motility appendages (i.e., type IV pili and flagella) affect biofilm morphology (O'Toole and Kolter, 1998a; Heydorn et al., 2002; Klausen et al., 2003a; Klausen et al., 2003b; Landry et al., 2006; Barken et al., 2008; Yang et al., 2009). Furthermore, the production of rhamnolipids, which is suggested to be important for a type of surface motility called swarming, has been shown to be important for biofilm formation (Davey et al., 2003; Lequette and Greenberg, 2005; Pamp and Tolker-Nielsen, 2007; Glick et al., 2010).

Type IV pili

Twitching motility is a type IV pilus-dependent translocation of bacteria on a surface. Type IV pilus-dependent surface motility was traditionally thought to be based on a grappling-hook-type model, in which the polar type IV pilus extends, binds to the surface, and retracts, dragging the cell body behind it (Mattick, 2002). Recent data, however, suggest that type IV pilus-dependent motility is more complex (Gibiansky et al., 2010; Jin et al., 2011). Through the inactivation of the genes involved in pilus biogenesis, many studies have shown that type IV pili and twitching are important in multiple steps of biofilm development (O'Toole and Kolter, 1998a; Heydorn et al., 2002; Klausen et al., 2003a; Klausen et al., 2003b; Barken et al., 2008; Yang et al., 2009).

Attachment. Using the PA14 strain of *P. aeruginosa*, O'Toole and Kolter showed that under static conditions with a glucose-casamino acids-based minimal medium, various mutants in type IV pilus biogenesis were defective in adhering to a plastic surface (O'Toole and Kolter, 1998b). However, a PAO1 mutant with a deletion in *pilA*, which encodes the major structural subunit of the pilus, was not defective in adhering to glass under flowing conditions with a citrate minimal medium (Klausen et al., 2003b). While strain and/or surface chemistry differences could possibly explain this discrepancy (O'Toole and Kolter, 1998a; Heydorn et al., 2000), Klausen and colleagues suggested that the difference between the two results was based on the nutritional conditions (see more below on how nutrition affects biofilm morphology). Consistent with the O'Toole and Kolter study, under static conditions, the PAO1 Δ*pilA* mutant was defective for attachment in a glucose-casamino acids minimal medium but not a citrate minimal medium (Klausen et al., 2003b). Landry et al. further supported this result, showing that a PAO1 Δ*pilA* mutant was defective in attaching to a glass surface under flowing conditions with a glucose-rich medium (Landry et al., 2006). Therefore, the role of the type IV pili in the attachment of bacteria to a surface is nutrient dependent.

Flat biofilm formation. Under conditions in which *P. aeruginosa* forms flat biofilms, type IV pilus mutants form irregular rough, structured biofilms (Fig. 2). These biofilms appear as isolated microcolonies and fail to spread on the substratum (Heydorn et al., 2002; Klausen et al., 2003a; Klausen et al., 2003b). In addition, on the basis of findings obtained by mixing two differentially labeled populations of Δ*pilA* mutants, Klausen et al. suggest that these microcolonies arose from clonal growth (Fig. 2A) (Klausen et al., 2003a). Since wild-type flat biofilms start as isolated microcolonies, pilus mutants are defective in progressing to the next developmental stage where cells spread to occupy the areas between the microcolonies. This observation has led to the hypothesis that twitching-based motility is in part responsible for the flat biofilm structure (Klausen et al., 2003b). As further evidence for this hypothesis, cells that have increased twitch rates are correlated with the formation of flat biofilms (Singh et al., 2002; Landry et al., 2006).

Structured biofilm formation. Under conditions that lead to wild-type cells forming structured biofilms, Δ*pilA* mutants form biofilms with isolated microcolonies (Klausen et al., 2003a). The Δ*pilA* mutant strain was unable to form mushroom caps, as biofilms formed from a mixture of cyan fluorescent protein (CFP)-labeled Δ*pilA* mutants and YFP-labeled wild-type cells have clonal populations of CFP-labeled stalks and yellow fluorescent protein (YFP)-labeled caps. Oddly, most of the stalks are CFP labeled in these experiments, suggesting that cells defective in twitching are better at forming the stalks than those that can twitch (Klausen et al., 2003a). While this defect in cap formation of Δ*pilA* mutants may be attributed to the inability to twitch, it could also be due to the lack of pilus structures on the surface of the cells. Suggesting that the latter may be the case, Barken et al. showed that Δ*pilH* mutants, which have increased surface piliation but decreased twitching motility, can form caps and irregular mushroom-shaped biofilms (Barken et al., 2008).

Flagella

The flagellum is a highly regulated, complex structure, composed essentially of a filament and a motor that drives filament rotation (Berg, 2003). *P. aeruginosa* has a single polar flagellum. Similar to that of type IV pili, the role of the flagellum in biofilm development is nutrient dependent (O'Toole and Kolter, 1998a; Klausen et al., 2003b; Shrout et al., 2006; Barken et al., 2008). O'Toole and Kolter showed that under static conditions with a glucose-casamino acids minimal medium, a mutant in flagellar biosynthesis was defective in attachment to a plastic surface (O'Toole and Kolter, 1998a). This phenotype, however, can be rescued by using a different growth medium (O'Toole and Kolter, 1998a; Klausen et al., 2003b). In addition to attachment, mutants in flagellar biosynthesis form mature biofilms that are different from that of wild-type cells. Under conditions where wild-type cells form flat biofilms, a Δ*fliM*

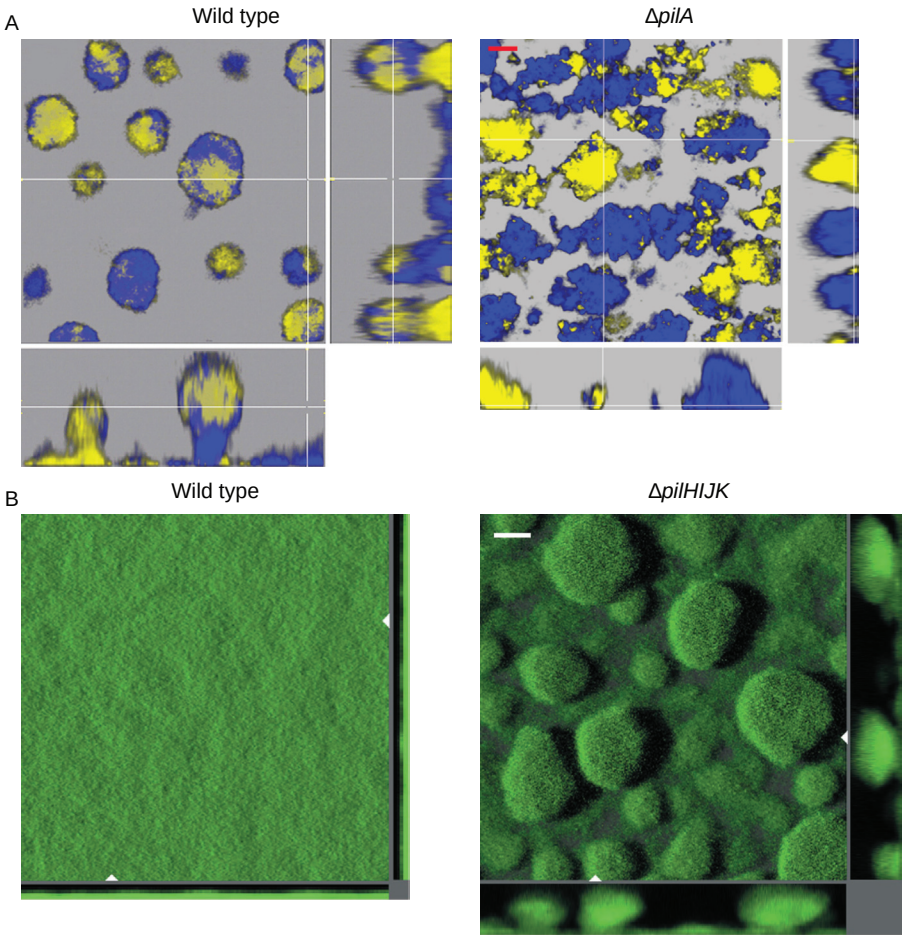


Figure 2. Type IV pilus mutants form biofilms that are morphologically distinct from those of the wild-type strain. (A) Four-day-old biofilms of wild-type and Δ*pilA* strains of *P. aeruginosa* grown in glucose-based minimal medium. A 1:1 mixture of CFP- and YFP-expressing cells of the same strain was used to initiate the biofilm. (B) Ninety-eight-hour-old biofilms of GFP-expressing wild-type and Δ*pilHIJK* strains of *P. aeruginosa* grown in citrate-based minimal medium. Crosshairs (A) and white triangles (B) indicate the positions of vertical cross sections shown on the right and bottom of each image. Scale bar, 20 μm. Modified from Klausen et al., 2003a (A), and Heydorn et al., 2000 (B), with permission from John Wiley and Sons and the Society for General Microbiology, respectively.
doi:10.1128/9781555818524.ch1f2

mutant strain produced structured biofilms that contain more biomass than wild-type biofilms, suggesting that flagellum-dependent motility is important for producing flat biofilms (Klausen et al., 2003b). In support of this hypothesis, multiple studies have shown that flagellar mutants are defective in surface-associated motility (Kohler et al., 2000; Landry et al., 2006; Shrout et al., 2006; Barken et al., 2008).

Chemotaxis

Chemotaxis is traditionally defined as the directed motion of swimming bacteria in planktonic culture. Chemotaxis, however, has also been observed on a surface and is suggested to play a role in biofilm formation (Kearns et al., 2001). A mutant in CheR1, a methyltransferase that regulates chemotaxis, samples less of the surface than a wild-type cell and is defective in changing direction (Schmidt et al., 2011). Furthermore, mutants in chemotaxis produce mound-like biofilms, similar to flagellar mutants, under conditions where wild-type cells form flat biofilms (Li et al., 2007; Barken et al., 2008). While it is unknown what the chemotactic machinery is sensing during biofilm formation, these results provide further evidence that flagellum-mediated motility and chemotaxis are important for producing the mature biofilm structure.

Rhamnolipids

Rhamnolipids are surfactants that aid in swarming motility, and their production is quorum sensing

(QS) regulated (Kohler et al., 2000; Lequette and Greenberg, 2005). While rhamnolipids were originally suggested to be necessary for swarming (Kohler et al., 2000), the requirement for rhamnolipids in swarming on agar surfaces is nutrient dependent (Shrout et al., 2006). However, since there are no genetic approaches for manipulating swarming without directly interfering with flagellum- or pilus-dependent motility, most studies have used mutants that eliminate rhamnolipid production to investigate the role of swarming in biofilm formation. Therefore, these studies make a statement about the role of rhamnolipids in biofilm formation, while the role of swarming is less clear.

Biofilms formed by a strain containing a mutation in *rhlA*, a gene encoding a rhamnolipid biosynthetic enzyme (Soberon-Chavez et al., 2005), are structurally distinct from those of wild-type cells. Under conditions where wild-type biofilms are structured with large aggregates, a Δ*rhlA* mutant forms flat biofilms (Davey et al., 2003; Boles et al., 2005; Pamp and Tolker-Nielsen, 2007). However, if a Δ*rhlA* mutant is mixed with cells that can produce rhamnolipids, Δ*rhlA* cells can participate in structured biofilm formation, suggesting that rhamnolipids serve as a community good (Pamp and Tolker-Nielsen, 2007). Interestingly, in these mixed-population studies, the cells that produce rhamnolipids form the stalks, while the cells deficient for rhamnolipid production form the caps of the aggregates, with a developmental pattern similar to that of structured biofilm II (Pamp and Tolker-Nielsen, 2007). In agreement with this observation, Lequette and Greenberg observed that in structured wild-type biofilms, *rhlA* was expressed mainly in the stalk, with little expression in the cap portion of the mushroom (Lequette and Greenberg, 2005). Together, these results suggest that rhamnolipid production in the stalk is important for cap formation under conditions where *P. aeruginosa* forms biofilms of the structured biofilm II class.

Completely contrary to the above-described flat biofilm morphology for Δ*rhlA* mutants, under iron-limiting conditions where wild-type cells form flat biofilms, Δ*rhlA* cells formed structured biofilms (Glick et al., 2010). This phenotype is not completely surprising, as iron levels influence rhamnolipid production. Under iron-limiting conditions, wild-type *P. aeruginosa* increases expression of rhamnolipid biosynthetic genes (Glick et al., 2010). Furthermore, Glick and colleagues showed that in iron-limiting media, Δ*rhlA* cells are defective for twitching, but this deficiency can be rescued by using iron-replete media (Glick et al., 2010). Interestingly, the Δ*rhlA* cells can also form structured biofilms, like wild-type cells, under iron-replete conditions. Glick and colleagues suggest that the timing of *rhlA* expression under iron-limiting versus replete conditions in wild-type biofilms may explain these results (Glick et al., 2010). Ultimately, these results with those described above show that the role of rhamnolipid production, and possibly swarming, in biofilm development is complicated and confounded by multiple environmental factors.

In addition to their role in biofilm morphology, rhamnolipids affect both biofilm initiation and dispersal. Exogenous addition of rhamnolipids can block biofilm initiation (Davey et al., 2003) and disperse preformed biofilms (Boles et al., 2005). This effect of excess rhamnolipids on biofilm initiation and dispersal is suggested to be due to their surfactant property, disrupting cell-cell and cell-surface interactions. Supporting this interpretation, Boles and colleagues show that another surfactant, sodium dodecyl sulfate (SDS), can also induce dispersal of a preformed biofilm in a manner similar to that of rhamnolipids (Boles et al., 2005). Since these studies are based on addition of excess rhamnolipids exogenously, they suggest that rhamnolipid production, which starts in planktonic cells at the transition into stationary phase (Lequette and Greenberg, 2005), is tightly regulated throughout biofilm development.

Nutrition

P. aeruginosa is renowned for its ability to grow in almost any environment and can use a wide variety of sources to meet its nutritional requirements. The nutritional conditions under which biofilms develop greatly affect their morphology. For instance, changing the carbon source in the media leads to drastically different biofilm structures (Wolfaardt et al., 1994; Klausen et al., 2003a; Klausen et al., 2003b; Shrout et al., 2006). Based on mathematical modeling, nutrient limitation is a major driving force in determining biofilm structure (Wimpenny and Colasanti, 1997; Picioreanu et al., 1998). In these models, rougher structured biofilms form under limiting nutrient conditions and growth was limited by nutrient transfer. In comparison, denser, flat biofilms form under nutrient-rich conditions and were limited by the growth rate of the microorganisms. These simplistic models, however, do not fully describe the complexity with which nutrients, such as carbon source and iron levels, affect biofilm morphology.

Carbon sources

From halogenated aromatics to intermediates of the tricarboxylic acid (TCA) cycle, *P. aeruginosa* can use a diverse group of organic compounds as a sole carbon source. Most defined media for laboratory culturing of *P. aeruginosa* provide either TCA