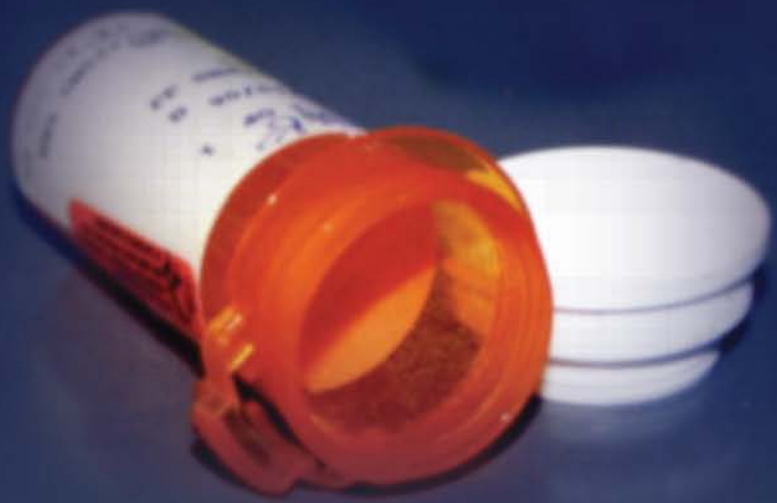
Bacterial Resistance to Antimicrobials

SECOND EDITION



Edited by
Richard G. Wax • Kim Lewis
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CRC Press
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Contents

Preface

On June 9, 1999, the *New York Times* published a lengthy obituary for Anne Miller. Ms. Miller, who was 90 when she died, was not a celebrity or a high-profile politician. Her claim to fame was that, at the age of 33, she had been one of the first people to be given the new and largely untested antibiotic penicillin. The transformation in her condition, which occurred within days, from a young woman slipping into death to a woman who could sit up in bed, eat meals, and chat with visitors was a stunning demonstration of what was to become commonplace in a new era of medicine. Such seemingly miraculous cures soon led physicians and the public to call antibiotics "miracle drugs."

Since then, antibiotics have not only saved people with pneumonia and other dreaded diseases, such as tuberculosis, but also have become the foundation on which much of modern medicine rests. Antibiotics make routine surgery feasible. They protect cancer patients whose chemotherapy had rendered them temporarily susceptible to a variety of infections. They even cure diseases like ulcers that had been considered uncurable chronic conditions. In recent years, antibiotic use has been extended to agriculture, where it plays an important role in preventing infections and in promoting animal growth.

The success of antibiotics in so many areas has, ironically, led antibiotics to become an endangered category of drugs. Bacteria have once again demonstrated their enormous genetic flexibility by becoming resistant to one antibiotic after another. At first, bacterial resistance to antibiotics, such as penicillin, did not seem very alarming because new antibiotics were regularly being discovered and introduced into clinical use. In the 1970s, however, a scant two decades after the introduction of the first antibiotics, the number of new antibiotics entering the pipeline from laboratory to clinic began to decrease. Antibiotic discovery and development are expensive, especially considering the speed with which bacterial resistance can arise. And they are becoming more and more difficult to discover and develop. These factors have led pharmaceutical companies to be less and less interested in antibiotic production. One company after another has shut down or cut back on its antibiotic discovery program.

Finally, the medical community has begun to take antibiotic-resistant bacteria seriously. The public has also become alarmed. This alarm is reflected in the number of articles in the popular press anguishing about the new "superbugs." Agricultural use of antibiotics has been called into question as a possible threat to human health. There is also the potential fallout if antibiotics were to be "lost." Medical researchers have failed to cure many diseases, and the public accepts these failures with grumbling stoicism. But what if overuse of antibiotics caused physicians to lose a cure, an event that would be a first in history? How would this affect public confidence in the medical community?

This book explores many of the aspects of the growing problem posed by antibiotic-resistant bacteria. What is unique about this book is that it is a blend of the purely scientific and the practical, an approach that is essential because antibiotic resistance is a social and economic problem as well as a scientific problem. Chapter 1 explores the history of antibiotics and how bacteria became resistant to them. Understanding the forces leading to the overuse and abuse of antibiotics that have sped the appearance of ever more resistant bacteria is important because it impresses on people the need for rapid and effective future action. The speed with which resistance has arisen is something that everyone needs to appreciate.

Chapter 2 discusses the ecology of antibiotic resistance genes. In recent years, scientists have realized that there is more to the epidemiology of resistance than the transmission of resistant strains of bacteria. Resistance genes are also moving from one bacterium to another, across species and genus lines. Bacteria do not have to spend years mutating their way to resistance; they can become resistant within hours by obtaining genes from other bacteria. Also clear from this chapter, however, is how primitive and inadequate our understanding of resistance ecology still is.

Chapters 3 through 14 describe the means by which bacteria become resistant to antibiotics, methods of detecting resistance genes, and the latest findings on resistance or susceptibility specific to particular groups of bacteria. The bacteria that cause human and animal disease exhibit a staggering diversity. There is no one answer to the question of how bacteria become resistant to antibiotics. Understanding resistance mechanisms is the foundation for more rational design of new antibiotics that are themselves resistant to resistance mechanisms.

A complementary approach, exemplified by combination of a compound that inhibits bacterial β-lactamases with a β-lactam antibiotic, offers great promise. More such successes are needed. To take such an approach, however, is necessary to understand the mechanisms of resistance at a very basic level. Even in the case of the β-lactamase inhibitors, variations in the mechanisms of resistance have foiled this approach in some bacteria that do not use β-lactamases as a resistance mechanism. These chapters pull together all of the information on resistance mechanisms in different groups of bacteria in a way that should help future efforts to develop such combination therapies.

Chapters 15 and 16 examine the public health aspects of the resistance problem. Science alone is not going to solve the resistance problem. Communicating scientific advances and new understandings of forces that promote the rapid development of resistance is essential if the public is to join in the effort to slow the increase in bacterial resistance to antibiotics. Taking antibiotics is a personal matter for most people, a decision made by them and their physicians. As long as antibiotic use remains a personal matter and is not put in the context of public welfare, it is unlikely that progress will be made toward saving antibiotics.

Chapter 17 addresses the problem of finding and developing new antibiotics. This chapter is written by an "insider," a scientist who runs an antibiotic discovery program and thus knows the industry side of the problem. Since the resistance genie is out of the bottle and it will not be easy to put him back in, the continued discovery of new antibiotics is going to be a critical part of the effort to combat resistant bacterial strains. This effort is a critical legacy that we owe our children, who are the ones most likely to bear the consequences of the crisis we have precipitated.

This book is one-stop shopping for anyone interested in all of the facets of bacterial resistance to antibiotics. The breadth of the topics covered reflects the input of a diversity of editors, some of whom have spent their careers in the ivory tower of academic research, some who have had an interest in the public health issues involving the resistance problem, and some who have had direct experience with antibiotic discovery and development. The book represents a unique contribution to the continuing discussion of the best ways to respond to the challenge posed by resistant bacteria. Victory in this battle is not going to be easy. After all, our bacterial adversaries have had a 3-billion-year evolutionary head start. Their diversity and ability to respond to adversity are amazing and frightening. Disseminating information and thus stimulating more scientists to become part of the solution to the problem of resistant bacteria is our best strategy for victory.

Richard G. Wax
Kim Lewis
Abigail A. Salyers
Harry Taber

About the Editors

Richard G. Wax, Ph.D., was an Associate Research Fellow at Pfizer Global Research until his retirement in 2005. He received his B.S. in Chemical Engineering from the Polytechnic University of New York and his M.S. in Biophysics from Yale University. He followed his mentor, Professor Ernest Pollard, to The Pennsylvania State University, University Park, where he received his Ph.D. in Biophysics. He was a Staff Fellow at the U.S. National Institutes of Health (NIH), Bethesda, Maryland, and an NIH Special Fellow at the Weizmann Institute, Rehovot, Israel.

Dr. Wax's career has focused on secondary metabolism. In early research he developed a medium, AGFK, which is now the primary means used for germinating *Bacillus subtilis* spores. At the Merck Research Laboratories his laboratory created high-yielding mutants that allowed commercially feasible antibiotic production. He was co-discoverer of efrotomycin, an antibiotic that acts specifically on bacterial elongation factor Tu. Prior to joining Pfizer he served as Section Head of the Fermentation Group at the Frederick Cancer Research Facility, Frederick, Maryland.

Dr. Wax's avocation is a study of the roles of microbes in altering human history, and he has published and lectured on this subject.

Kim Lewis, Ph.D., is Professor of Biology and Director of the Antimicrobial Discovery Center at Northeastern University in Boston. He is also Director of NovoBiotic Pharmaceuticals, a biotechnology company focused on discovery of antibiotics from previously uncultured bacteria. Dr. Lewis received his B.S. in Biochemistry and his Ph.D. in Microbiology from Moscow University. After moving to the United States, he was a faculty member at the Massachusetts Institute of Technology, the University of Maryland, and Tufts University.

Dr. Lewis has worked in the field of multidrug pumps and established a program for studying antimicrobial tolerance of biofilms and persister cells.

Abigail A. Salyers, Ph.D., is Arends Professor for Molecular and Cellular Biology in the Department of Microbiology at the University of Illinois (Urbana-Champaign). She received her B.A. and Ph.D. from The George Washington University. She spent several years at the Virginia Polytechnic Institute Anaerobe Laboratory, where she began to work on human colonic *Bacteroides* spp.

From 1995 to 1999 Dr. Salyers was a Co-Director of the Microbial Diversity summer course at the Marine Biological Laboratory, Woods Hole, Massachusetts. She was President of the American Society for Microbiology from 2001 to 2002. Her current research focuses on the mechanisms and ecology of antibiotic resistance gene transfer in the human colon, with particular emphasis on *Bacteroides* species.

She is the author of *Revenge of the Microbes*, a book on antibiotics and antibiotic-resistant bacteria that is directed at the general public.

Harry Taber, Ph.D., is Director of the Division of Laboratory Quality Certification at the Wadsworth Center of the New York State Department of Health, Albany, New York. He received his B.A. in Chemistry from Reed College in Portland, Oregon and his Ph.D. in Biochemistry from the University of Rochester School of Medicine and Dentistry in Rochester, New York. He received postdoctoral training at Rochester, the National Institutes of Health in Bethesda, Maryland, and the Centre National de la Recherche Scientifique in Gif-sur-Yvette, France. He was on the Microbiology faculties of the University of Rochester and Albany Medical College before joining the Wadsworth Center as a Research Scientist. He is Past Director of the Division of Infectious Disease at Wadsworth.

Dr. Taber's research has been in the area of genetic regulation of bacterial respiratory systems, particularly as this regulation affects sporulation of *Bacillus subtilis* and the uptake of aminoglycoside antibiotics. He has broad interests in the public health aspects of bacterial antibiotic resistance and in the use of genotyping technologies for tuberculosis control.

Contributors

Keith A. Bostian
Mpex Pharmaceuticals Inc.
San Diego, Connecticut

Jooyoung Cha
Department of Chemistry and Biochemistry
University of Notre Dame
Notre Dame, Indiana

Stewart T. Cole
Unité de Génétique Moléculaire Bactérienne
Institut Pasteur
Paris, France

Christopher Gerard Dowson
Department of Biological Sciences
University of Warwick
Coventry, United Kingdom

George M. Eliopoulos
Division of Infectious Diseases
Beth Israel Deaconess Medical Center
Boston, Massachusetts

Ad C. Fluit
Eijkman-Winkler Institute
University Medical Center Utrecht
Utrecht, the Netherlands

Cindy R. Friedman
Centers for Disease Control and Prevention
Atlanta, Georgia

Nafsika H. Georgopapadakou
MethylGene Inc.
Montreal, Quebec, Canada

Keeta S. Gilmore
Schepens Eye Research Institute
Boston, Massachusetts

Michael S. Gilmore
Schepens Eye Research Institute
Boston, Massachusetts

David C. Hooper
Division of Infectious Diseases
Massachusetts General Hospital
Boston, Massachusetts

Lakshmi P. Kotra
Departments of Pharmaceutical Sciences and Chemistry
University of Toronto
and
Division of Cell and Molecular Biology
Toronto General Research Institute
University Health Network
Toronto, Ontario, Canada

Olga Lomovskaya
Mpex Pharmaceuticals Inc.
San Diego, Connecticut

Paul F. Miller
Infectious Diseases Therapeutic Area
Pfizer Global Research and Development
Groton, Connecticut

Shahriar Mobashery
Departments of Chemistry and Biochemistry
University of Notre Dame
Notre Dame, Indiana

Steven J. Projan
Wyeth Research
Cambridge, Massachusetts

Alex S. Pym
Unit for Clinical and Biomedical Tuberculosis Research
South African Medical Research Council
Durban, South Africa

Philip N. Rather
Departments of Microbiology and Immunology
Emory University School of Medicine
Atlanta, Georgia

Daniel F. Sahm
Eurofins Medinet
Herndon, Virginia

David Schlesinger
Department of Microbiology
University of Illinois
Urbana, Illinois

Paul Shears
Sheffield Teaching Hospitals
NHS Trust
Sheffield, United Kingdom

Nadja Shoemaker
Department of Microbiology
University of Illinois
Urbana, Illinois

Arjun Srinivasan
Centers for Disease Control and Prevention
Atlanta, Georgia

William C. Summers
Yale University School of Medicine
New Haven, Connecticut

Krzysztof Trzcinski
Departments of Epidemiology, Immunology, and Infectious Diseases
Harvard School of Public Health
Boston, Massachusetts

Gerard D. Wright
Department of Biochemistry and Biomedical Sciences
McMaster University
Hamilton, Ontario, Canada

Helen I. Zgurskaya
Department of Chemistry and Biochemistry
University of Oklahoma
Norman, Oklahoma

1 Microbial Drug Resistance: A Historical Perspective

William C. Summers

CONTENTS

Almost as soon as it was known that microorganisms could be killed by certain substances, it was recognized that some microbes could survive normally lethal doses and were described as "drug-fast" (German: *-fest* = -proof, as in *feuerfest* = fire-proof; hence "drug-proof," in common usage by at least 1913). These early studies [1–3] conceived of microbial resistance in terms of "adaptation" to the toxic agents. By 1907, Ehrlich [4] more clearly focused on the concept of resistant organisms in his discussion of the development of resistance of *Trypanosoma brucei* to *p*-roseaniline, and in 1911 Morgenroth and Kaufmann [5] reported that pneumococci could develop resistance to ethylhydrocupreine. For every new agent that killed or inhibited microorganisms, resistance became an interest as well.

While we think of antibiotic resistance as a phenomenon of recent concern, the basic conceptions of the problems, the controversies, and even the fundamental mechanisms were well developed in the early decades of the twentieth century. These principles were, of course, elaborated in terms of resistance to anti-microbial toxins, such as the arsenicals, dyes, such as trypan red, and disinfectants, such as acid, phenols, and the like. However, by the time the first antibiotics were employed in the 1940s and resistance was first observed, the framework for understanding this phenomenon was already in place.

DRUG-FASTNESS

Drug-fastness became a topic of importance as microbiologists sought understanding of the growth, metabolism, and pathogenicity of bacteria, protozoa, and fungi. In 1913, Paul Ehrlich clearly described the basic mechanisms of drug action on microbes [6]: "parasites are only killed by those materials to which they have a certain relationship, by means of which they are fixed by them." He went on to describe specific drug binding (fixation) to specific organisms and elaborated "The principle of fixation in chemotherapy."

Once this principle was accepted, one could investigate how drugs are fixed by microbes, what kinds of cross-sensitivities existed, and what happened when organisms became resistant to chemotherapeutic agents. Ehrlich noted that both trypanosomes and spirochaetes, his favorite experimental organisms, exhibited different chemoreceptors that were specific for drugs of a given chemical class. Thus, there seemed to be a chemoreceptor for arsenic compounds (arsenious acid, arsanilic acid, and arsenophenylglycine) that differed from the receptor for azo-dyes (trypan red and trypan blue) as well as from the receptor for certain basic triphenylmethane dyes, such as fuchsin and methyl violet.

Drug-fastness, therefore, was readily explained as "a reduction of their (the chemoreceptors) affinity for certain chemical groupings connected with the remedy (the drug), which can only be regarded as purely chemical" [6]. Clearly, Ehrlich's approach was an outgrowth of his earlier work on histological staining and dye chemistry and reflected his strong chemical thinking.

Already in 1913, the problem of clinical drug resistance was confronting the physician and microbiologist. Ehrlich discussed the problem of "relapsing crops" of parasites as a result of the parasites' biological properties. His views were mildly selectionist, but he also held the common view that microbes had great adaptive power and that the few that managed to escape destruction by drugs (or immune serum) could subsequently change into new varieties that were drug-fast or serum-proof.

One corollary of the specific chemoreceptor hypothesis was that combined chemotherapy was best carried out with agents that attack entirely different chemoreceptors of the microbes. Ehrlich, who frequently resorted to military metaphors, wrote: "It is clear that in this manner a simultaneous and varied attack is directed at the parasites, in accordance with the military maxim: 'March apart but fight combined' " [6]. He also allowed for the possibility of drug synergism so that in favorable cases the effects of the drugs may be multiplied rather than simply additive. From the earliest days of chemotherapy, it appears that multiple drug therapy with agents with different mechanisms was seen as a way to circumvent the problem of "relapsing crops" or emergence of resistant organisms.

Ehrlich, too, realized the relationship between evolution of resistant variants and the dose of the agent used to treat the infection. Clinical practice often used remedies in increasing dosages, perhaps a therapeutic principle derived from empirical treatment practice of long tradition. He noted that these were precisely the conditions likely to lead to emergence of drug-fast organisms and developed the idea of "*therapia sterilisans magna*" (total sterilization) in which he advocated the maximum microbicidal dose that was non-toxic to the host [7]. Indeed, by 1916, there was

experimental confirmation in controlled *in vitro* laboratory studies that gradual increases in drug concentration would lead to outgrowth of resistant spirochetes, while exposure to initial high concentrations of antitreponemal agents (arsenicals, mercuric, and iodide compounds) would not [7].

DISINFECTION

Often early research on antimicrobial agents was directed to problems of "disinfection" and related matters of public health, and the origins and properties of resistant organisms became of concern in the "fight against germs" [8]. Protocols for inducing drug-resistance *in vivo* were elaborated, and the relevance of *in vitro* resistance to "natural" *in vivo* resistance was debated in the literature of the 1930s and 1940s. One interesting aspect, now forgotten, was the widespread belief in bacterial life cycles as an explanation for the changing properties of bacterial cultures under what we would now call "selection." This theory of bacterial life cycles [9–11], called "cyclogeny," held that bacteria had definite phases of growth, and that properties of bacteria, such as shape, nutritional requirements, pathogenicity, antigenic reactivities, and chemical resistances, were variable properties of the organism that simply reflected the growth phase of the culture. This cyclogenic variation revived an old nineteenth century controversy in bacteriology, namely that of Koch's monomorphism versus Cohn's polymorphism. Ferdinand Cohn believed that bacterial forms were highly variable so that one "species" of bacteria could exist in many shapes and with many different properties, while Robert Koch held that specific bacterial "species" had unique morphologies and properties that were unchanging. This debate, of course, had far-reaching implications both for problems of bacterial classification and for understanding variation and mutation of bacterial characteristics.

MICROBIAL METABOLISM AND ADAPTATION

The basic issue, as we would see it today, that faced microbiologists in the early days of antimicrobial research is one of "adaptation versus mutation." It was passionately debated and contested by leading microbiologists from the mid-1930s until the early 1960s. Even those who viewed most microbial resistance as some sort of heritable change, or mutation, were divided on the basic problem of whether the mutations arose in response to the agent, or occurred spontaneously and were simply observed after selection against the sensitive organisms. This problem was unresolved until the 1940s and 1950s, but has returned in a new form recently, as will be discussed subsequently.

As early as the 1920s, the ability of bacterial cells to undergo infrequent abrupt and permanent changes in characteristics was interpreted as a manifestation of the phenomenon of mutation as had been described in higher organisms [12]. The relation of these mutations to the growth conditions where they could be observed, was, however, unclear. In the 1930s, this question was confronted directly by I.M. Lewis [13], who studied the mutation of a lactose-negative strain of "*Bacillus coli mutabile*" (*Escherichia coli*) to lactose-utilizing proficiency. Lewis laboriously isolated colonies and found that even in the absence of growth in lactose, the ability to ferment this

sugar arose spontaneously in about one cell in 10^5. This work was the beginning of a long line of investigations that quite conclusively showed that mutation is (almost always) independent of selection.

The second kind of adaptation, that "due to chemical environment," is of special historical interest. As early as 1900, Frédéric Dienert [14] found that yeast that were grown for some time in galactose-containing medium became adapted to this medium and would grow rapidly without a lag when subcultured into fresh galactose medium, but that this "adaptation" was lost after a period of growth in glucose-containing medium. By 1930, Hennig Karström in Helsinki had found several instances of such adaptation [15]. For example, he found that a strain of *Bacillus aerogenes* could grow on ("ferment" to use the older term) xylose if "adapted" to do so, but that this strain could ferment glucose "constitutively" without the need for adaptation. When he examined the enzyme content of these adapted and unadapted cells, he found that there were some enzymes that were "constitutive" and some that were "adaptive." Thus, the metabolic properties of the culture mirrored the intracellular chemistry. By experiments in which the medium was changed in various ways, Karström and others showed that metabolic adaptation could sometimes take place even without measurable increase in cell numbers in the culture.

Marjory Stephenson, a leading mid-twentieth century bacterial physiologist, described these variations in her influential book, *Bacterial Metabolism* [16], as "Adaptation by Natural Selection" and "Adaptation due to Chemical Environment." The former included the phenomenon that is now termed mutation.

Between 1931 and the start of World War II, Stephenson and her students, John Yudkin and Ernest Gale, investigated bacterial metabolic variation in detail, often exploiting the lactose-fermenting system in enteric bacteria to study it. The mechanism of chemical adaptation, however, eluded them. The final paragraph of her monograph expressed her belief in the importance of the study of bacterial metabolism: "It (the bacterial cell) is immensely tolerant of experimental meddling and offers material for the study of processes of growth, variation and development of enzymes without parallel in any other biological material" [16].

In 1934, another research group on "bacterial chemistry" consisting of Paul Fildes and B.C.J.G. Knight was established at Middlesex Hospital in London [17]. Fildes and Knight investigated bacterial nutrition and established vitamin B1 (thiamine) as a growth factor for *Staphylococcus aureus*. Their work on bacterial growth factors suggested a unity of metabolic biochemistry at the cellular level, and they investigated the variations in growth factor requirements. One recurrent theme in their early work was the finding that they could "train" bacteria to grow on media deficient in some essential metabolite. For example, they could train *Bact. typhosum* (modern name *Salmonella typhi*) to grow on medium without tryptophan or without indole. Fildes noted that "during this time little attention was given to the mechanism of the training process, but it was certainly supposed that the enzyme make-up of the bacteria became altered as a result of a stimulus produced by the deficiency of the metabolite" [18].

By the mid-1940s, however, Fildes and his colleagues undertook a study of the mechanism of this ubiquitous "training." Was it another example of enzyme adaptation or was it something else? Using only simple growth curves, viable colony counts

on agar plates, and ingenious experimental designs, they concluded "that 'training' bacteria to dispense with certain nutritive substances normally essential may be looked upon as a cumbersome method for selecting genetic mutants" [18]. Little by little, the underlying mechanisms of the different kinds of biochemical variations seen in bacteria were becoming clear, and little by little, genetics was joining biochemistry as a powerful approach to study bacterial physiology. This understanding, of course, was central to discovering the underlying mechanisms involved in the variation of microbial behavior related to drug resistance.

This approach, however, was not uncontested and matters were not so easily settled as Arthur Koch pointed out in an important review of the field in 1981 [19]. A more extreme view of cellular metabolism was proposed by Cyril Hinshelwood, a Nobel Prize winner, no less, who argued that all variations in cellular functions, such as enzyme inductions, changes in nutritional requirements, and drug resistances, were but readjustments of complex multiple equilibria of chemical reactions already active in the cell [20].

ADAPTATION OR MUTATION?

With the discovery and development of antibiotics and their medical applications, drug resistance took on new relevance and new approaches became possible. No sooner were new antibiotics announced than reports of drug resistance appeared: sulfonamide resistance in 1939 [21], penicillin resistance in 1941 [22], and streptomycin resistance in 1946 [23], to cite a few early reports in the widely read literature. Research on resistance focused on three major problems: (*i*) cross-resistance to other agents, that is, was resistance to one agent accompanied by resistance to another agent? (*ii*) distribution of resistance in nature, that is, what was the prevalence of resistance in naturally occurring strains of the same organism from different sources? (*iii*) induction of resistance, that is, what regimens of drug exposure led to the induction or selection of resistant organisms?

While many practically useful results came from such research, two lines of investigation emerged that were later to prove scientifically interesting. Rare nutritional markers were somewhat limited and such mutations often resulted in loss of function, usually recessive traits that were difficult to manipulate experimentally. Drug resistance, on the other hand, provided a potent experimental tool to microbiologists who were studying bacterial genes and mutations because it allowed the analysis of events that took place at extremely low frequencies. For example, in 1936, Lewis [13] tested for preexisting, spontaneous mutations to lactose utilization in a previously lactose-negative strain of *E. coli*, but his results gave only indirect evidence for the random, spontaneous nature of bacterial mutation (as did the statistical approach of Luria and Delbrück in 1943 [24]). However, Lederberg and Lederberg [25] were able to use both streptomycin resistance and their newly devised replica plating technique to provide direct and convincing evidence to support the belief that mutations to drug resistance occurred even in the absence of the selective agent. Not only did such work on drug resistance clarify the nature of microbe–drug interactions, but it provided a much-needed tool to the nascent field of microbial genetics [26].

Just as Paul Ehrlich's 1913 summary of the principles of chemotherapy provided a window on early understanding of drug resistance, we can find a similar succinct presentation of the mid-twentieth century state of the field in a review by Bernard Davis in 1952 [27]. By this time, genetics of microbes had replaced microbial biochemistry as the fashionable mode of explanation for bacterial drug resistance. Although bacteria did not have a cytologically visible nucleus with stainable chromosomes, it was recognized that they had "nucleoid bodies" and that the material in this structure appeared to behave in a way similar to the chromosomes of higher organisms. Davis boldly (for the time) asserted that bacteria have nuclei, and that "within these nuclei are chromosomes that appear to undergo mitosis." He went even further to note that "some bacterial strains can inherit features (including acquired drug resistance) from two different parents, as in the sexual process of higher organisms." Thus, by the mid-twentieth century, bacteria had become "real" cells, with conventional genetic properties. If bacteria were like higher organisms, and since "almost all the inherited properties of animals or plants are transmitted by their genes," it was only logical, Davis argued, to consider genetic mutations as the basis for inherited drug resistance.

Davis, however, gave a fair consideration to the possible neo-Lamarckian hypothesis that single-cell organisms, where there is no separation between somatic and germ cells, might behave differently from higher sexually dimorphic organisms. To his mind, however, the recent work in microbial genetics by Luria and Delbrück [24], by Lederberg and Lederberg [25], and by Newcombe [28], settled the matter: the mutations to drug resistance were already present, having originated by some "spontaneous" process, and were simply selected by the application of the drug.

A very important clinical correlate of this new understanding of the nature of bacterial drug resistance was its application to combination chemotherapy. Since it became clear that mutations to resistance to different agents were independent events, the concept of multiple drug therapy, initially envisioned by Ehrlich [6], was refined and made precise. It was realized that adequate dosages and lengths of treatment were necessary if the emergence of resistant organisms was to be avoided [27,29].

DRUG DEPENDENCE

The second observation of basic significance was the odd phenomenon of drug dependence, which was first noted for streptomycin in 1947 by Miller and Bohnhoff [30]. This finding seemed to be restricted to streptomycin, but was extensively investigated at the time, and was thought to offer clues to the problems of antibiotic resistance in general. Later, however, this puzzling finding would be fundamental to understanding the functioning of the ribosome, and rather specific to the mode of action of streptomycin. The history of this aspect of drug resistance emphasizes our inability to predict the future course of research and our failure to identify, beforehand, just where the likely advances will take us.

MULTIPLE DRUG RESISTANCE AND CROSS RESISTANCE

In the 1950s, in the era of many new antibiotics and the emphasis on surveys of both cross resistance and distributions of resistance in natural microbial populations,

especially in Japan, it was recognized that many strains with multiple drug resistances were emerging. The appearance of such multiple drug resistance could not be adequately explained on the basis of random, independent mutational events. Also, the patterns of resistance were complex and did not fit a simple mutational model. For example, resistance to chloramphenicol was rarely, if ever, observed alone, but it was common in multiply-resistant strains. Careful epidemiological and bacteriological studies of drug-resistant strains in Japan led Akiba et al. [31] and Ochiai et al. [32] to suggest that multiple drug resistance may be transmissible both *in vivo* and *in vitro* between bacterial strains by so-called resistance transfer factors (RTFs) [33].

Genetic analysis of this phenomenon showed that the genes for these antibiotic resistance properties resided on the bacterial genome, yet were transmissible between strains albeit at low frequency. Further study showed that the transfer of these genes was mediated by a conjugal plasmid and that the resistance genes could associate with the conjugal plasmid; it was suggested that the resistance gene could be horizontally transmitted to other strains in a fashion similar to that for the integrative recombination for the temperate phage lambda [34]. It soon became clear, however, that the F-episome/F-lac system in *E. coli* was a better analogous genetic system. In some cases, the resistance genes and the transfer genes could be separated both genetically and physically [35]. Because of the promiscuous nature of the RTF, once a gene for drug resistance evolves, it can rapidly spread to other organisms. Additionally, because the R-factor plasmids replicate to high copy number, probably as a way to provide high levels of the drug-resistant protein, these plasmids have become the molecule of choice for molecular cloning technology.

With the better understanding of the genetics of drug resistance and the classification of the types of resistance, the biochemical bases for resistance were elucidated. Knowledge of the mechanism of action of an agent led to understanding of possible mechanisms of resistance. The specific role of penicillin in blocking cell wall biosynthesis, coupled with the knowledge of the structures of bacterial cell walls, could explain the sensitivity of Gram-positive organisms and the resistance of Gram-negative organisms to this antibiotic. Likewise, understanding of its metabolic fate led to the finding that penicillin was often inactivated by degradation by β-lactamase, which provides one mechanism of bacterial drug resistance. Detailed biochemical studies of the actions of antimicrobials have led to the understanding of the many ways in which microbes evolve to become resistant to such agents.

NEWLY FOUND MODES OF RESISTANCE

Not all voices for the adaptation hypothesis of drug resistance were drowned by the din of the genetic and conjugal mechanists. In the 1970s, mainly through the work of Samson and Cairns [36] and their colleagues, a variant of the adaptative model was revived and new mechanisms for bacterial drug resistance were discovered. Cairns and his colleagues observed that in accord with some of the older work, indeed, bacteria could be "trained" to resist certain agents by prior exposure to small, sublethal concentrations of the agent. They found that alkylating agents could induce the expression of specific genes whose products react with the alkylators, thus acting as a sink for further alkylating damage and rendering the cell hyper-resistant. While this

phenomenon seems to represent a specialized pathway for dealing with alkylation damages, it suggests that a century after its first observation, microbial drug resistance is still a fruitful and surprising area of research.

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2 Ecology of Antibiotic Resistance Genes

Abigail A. Salyers, Nadja Shoemaker, and David Schlesinger

CONTENTS

The movement of antibiotic resistance genes, as opposed to the movement of resistant bacterial strains, has become an issue of interest in connection with clinical and agricultural antibiotic use patterns. Evidence to date suggests that extensive DNA transfer is occurring in natural settings, such as the human intestine. This transfer activity, especially transfers that cross genus lines, is probably being mediated mainly by conjugative transfer of plasmids and conjugative transposons. Natural transformation and phage transduction probably contribute mainly to transfers within species or groups of closely related species, but the extent of this contribution is not clear. A considerable amount of information is available about the mechanisms of resistance gene transfer. The goal of future work on resistance ecology will focus on new approaches to detecting gene transfer events in nature and incorporating this information into a framework that explains and predicts the effects of human antibiotic use patterns on resistance development.

INTRODUCTION

For many years, surveillance systems designed to monitor patterns of bacterial resistance to antibiotics focused exclusively on antibiotic-resistant strains of bacteria. Moreover, of necessity, these surveillance efforts had to focus on a limited number of clinically important bacterial species such as *Staphylococcus aureus* [1–4] and *Salmonella* spp. [5]. A limitation of this approach is not just that it can monitor only a limited number of species but also that it does not take into account the dynamic nature of the bacterial genome. In theory, DNA is constantly flowing into and out of

bacterial cells located in a natural setting. Thus, the pattern of resistance gene distribution could be as important, if not more so, than the distribution of resistant strains of a particular species. This is especially true if resistance genes from one species can move to another species. Even if a newly acquired resistance gene is not expressed initially in a bacterial host, selective pressures imposed by the widespread clinical and agricultural use of antibiotics could select for promoter or codon usage mutations that allow the resistance gene to be expressed [6,7].

The importance of understanding the flow of resistance genes became particularly evident in discussions of possible impacts of agricultural use of antibiotics. In this case, initial attention focused on *Salmonella* and *Campylobacter* spp., types of bacteria that could cause human disease. Attention soon expanded, however, to include a broader question. Was it possible that even non-pathogenic bacteria, moving through the food supply from farm to the consumer, could transfer resistance genes to human intestinal bacteria [8–10]? Since human intestinal bacteria are a common cause of post-surgical infections [11,12], increased resistance due to acquisition of genes from swallowed bacteria passing through the intestinal tract could indeed have a direct impact on human health [13,14].

Assertions such as this prompted an old idea, called the "reservoir hypothesis" to resurface [15–17]. The reservoir hypothesis as it applies to human colonic bacteria is illustrated in Figure 2.1, but similar sorts of gene flows could occur almost anywhere in nature. According to the reservoir hypothesis, commensal bacteria in the colon, including those that could act as opportunistic pathogens and those that were truly non-pathogenic, exchange DNA with one another. They can also acquire DNA from or donate DNA to swallowed bacteria that cannot colonize the human colon, but spend enough time in the colon for DNA transfer to occur [18,19].

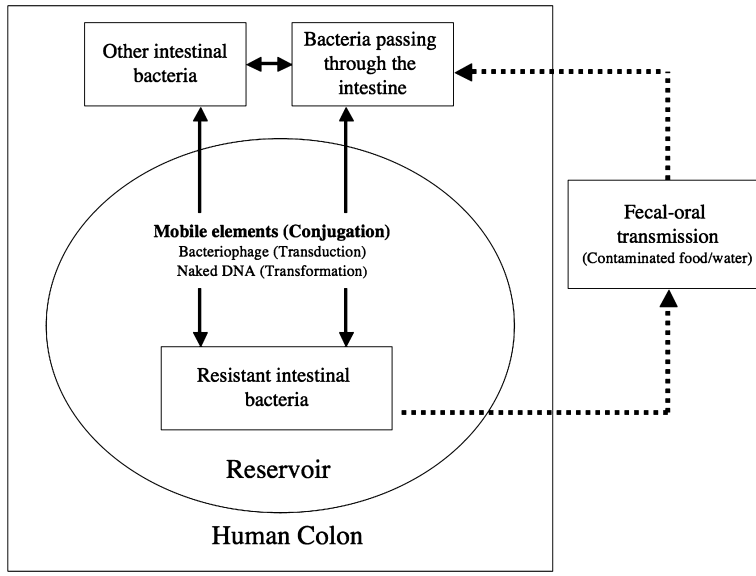

FIGURE 2.1 The reservoir hypothesis. Bacteria in the human colon serve as "reservoirs" for resistance genes that can be acquired from ingested bacteria.

But how likely are such exchanges to occur, especially broad host range transfers between members of different species and genera? This is the type of transfer that could be most problematic because it would allow resistance genes to move into bacteria capable of causing human disease. In trying to answer this question, attention has focused on conjugative gene transfer because this is the type of transfer known to be capable of crossing genus and phylum lines [20]. Initially, however, the focus was somewhat larger because early studies sought examples in which the same gene, with "same" defined as DNA sequence identity of more than 95%, was found in two very distantly related species of bacteria. That is, the only criterion was evidence that some sort of DNA transfer had occurred, without specifying the mechanism. The 95% cutoff was arbitrary but was motivated by the need to eliminate the possibility of convergent evolution. In convergent evolution, the same amino acid sequence might arise by selection from two different genes. Since two genes can differ by as much as 20% at the DNA sequence level and still have the same amino acid sequence, the requirement for 95% or higher DNA sequence identity seemed to be a good way to restrict attention to recent horizontal transfers of resistance genes.

In fact, the cutoff could have been 98%, because it proved all too easy to find resistance genes in different genera and species that were 98% to 100% identical at the DNA sequence level. Some examples are shown in Figure 2.2, where the resistance gene designation is shown inside the oval at the center and the names of Gram-positive and Gram-negative bacterial species found to have that gene are shown on either side of the oval. What is striking about this figure is that not only has the same gene been found in widely divergent species, but also in species commonly found in different locations. That is, the same genes were found not only in human colonic bacteria, but also in bacteria from other sites, such as soil, the intestinal tracts of

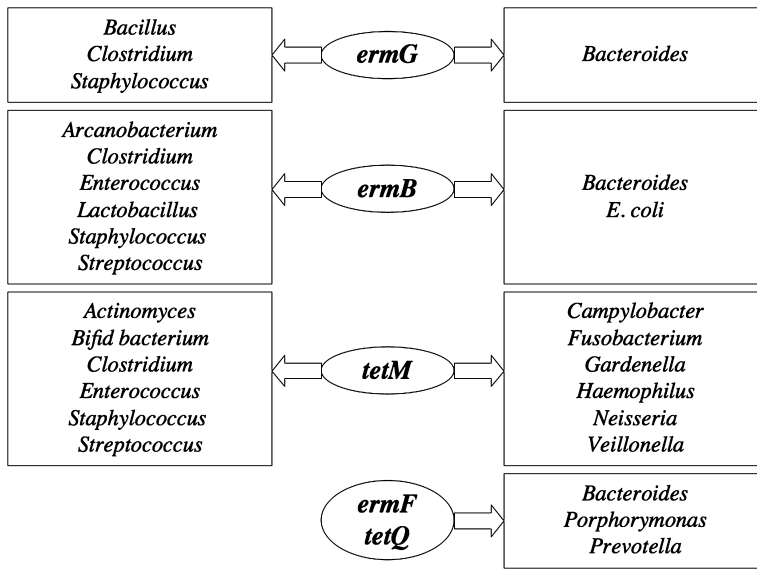

FIGURE 2.2 Example of genes with more than 95% sequence identity that have been found in distantly related bacteria from different sites.

non-human animals, and the human mouth. Most of these genes are genes that confer resistance to tetracycline (*tetM, tetQ*) or to macrolides (*ermB, ermF, ermG*). The tetracycline resistance genes are not the ones that encode efflux pumps, but encode a cytoplasmic protein that protects the bacterial ribosome from tetracycline. Why these two types of genes seem to be the ones most commonly found in different species and genera is not clear but could have something to do with the fact that they have a cytoplasmic location and thus do not need to be coupled with the proton motive force in membranes or to be secreted through the cytoplasmic membrane, requiring localization functions that could be species-specific.

A striking feature of all of the genes shown in Figure 2.2 is that they have been found almost exclusively on a type of integrated conjugative element called a conjugative transposon (CTn). CTns normally reside in the chromosome, but can excise to form a non-replicating circular intermediate, which transfers similarly to a plasmid. That is, there is a single stranded nick in the circular form, followed by transfer of a single strand of the DNA through a multi-protein complex that joins the cytoplasms of the donor and recipient. Once in the recipient, the circular copy of the CTn becomes double stranded and integrates into the recipient chromosome. Presumably the copy of the CTn in the donor has the same fate. Even if the copy of the CTn in the donor is sometimes lost, this affects only a small fraction of donor cells and the outcome of the process is a net increase in the number of bacteria carrying the CTn, especially if there is antibiotic selection for resistant cells.

CTns were first discovered in the Gram-positive bacteria and in the *Bacteroides* group of Gram-negative bacteria, but now that their existence is known, scientists are discovering CTns in other types of Gram-negative bacteria, such as *Vibrio cholerae, Salmonella* spp., and *Rhizobium* spp. [21–23]. There is no consistent nomenclature for this type of integrated transmissible element. They have also been called integrating, conjugative elements (ICE) elements and constins as well as CTns [24,25]. These alternative terms have the advantage that they avoid the word "transposon." Calling the CTns "transposons" is misleading, because their excision and integration is quite different from that of transposons, such as Tn*5* and Tn*10.* In fact, the enzyme that catalyzes the integration reaction, the CTn integrase, has most often proved to be a member of the tyrosine recombinase family, a family associated with many lambdoid phages. In some ways, the CTns resemble "phage" that travel from cell to cell through a multi-protein "capsid," similarly to the fusigenic viruses of mammalian cells. We will use the nomenclature CTn, because for better or worse, this nomenclature has been the one most commonly used in the literature.

Just as there are mobilizable plasmids that are transferred with the help of self-transmissible plasmids, there are also mobilizable transposons (MTns). The first of these to be discovered was NBU1, an MTn that is mobilized by a *Bacteroides* CTn, CTnDOT [26]. CTns can also mobilize plasmids [27,28].

MOVEMENT OF CTNS BETWEEN SPECIES OF HUMAN COLONIC *BACTEROIDES* SPP.

Figure 2.1 posits that gene transfer events occur between different species of colonic bacteria. What is the evidence that such transfers can occur and that if they do occur,

they are common? A first attempt to answer this question was made in a 2001 publication by Shoemaker et al. [29]. In this study, two sets of human colonic *Bacteroides* strains were screened. One set had been isolated prior to 1970 and was obtained from the culture collection of the now defunct Virginia Polytechnic Institute Anaerobe Laboratory (Blacksburg, Virginia, U.S.A.). The second set included isolates obtained after 1990. The two sets of strains were further divided into clinical and community isolates. The community isolates were derived from healthy people. The clinical isolates were obtained from patients with *Bacteroides* infections. The reason for looking at these two groups separately was that if the reservoir hypothesis is correct, both sets should follow the same pattern of gene acquisition, rather than clinical isolates exhibiting a different ecology as might be expected if events happened primarily in a clinical setting.

The patterns of antibiotic resistance genes seen in the clinical and community isolates were indeed similar. A striking difference was apparent, however, when the pre-1970 and post-1990 strains were compared. The older strains had a much lower rate of carriage of *tetQ* and the *erm* genes than the strains isolated after 1990. So, something had happened in the two-decade period that separated the two sets of strains, a period characterized by extensive use of antibiotics, such as tetracycline and the macrolides [30]. It is also surprising how high the carriage rate was in the isolates obtained prior to 1970, before the onset of intensive use of antibiotics in the treatment of human disease. This type of anomaly has been seen in other cases, such as detection of antibiotic-resistant bacteria in "pristine" environments [31,32]. This raises the question of whether antibiotics are the only force selecting for antibiotic-resistant bacteria, a still-unanswered question to which we will return at the end of this chapter.

The high number of strains in the post-1990 period that carry *tetQ*, even in the community isolates obtained from people who were not taking antibiotics, indicates that once acquired, *tetQ* is maintained very stably. Since, as already indicated, *tetQ* is found almost exclusively on a type of CTn exemplified by CTnDOT, a human *Bacteroides* CTn, this indicates that the CTn itself is also maintained very stably. It is interesting to note another characteristic of CTnDOT: its excision and transfer are stimulated 100- to 1000-fold by exposure of the bacteria to tetracycline [33–35]. Tetracycline is used not only to treat acute human infections, but also in dermatology and agriculture. In the treatment of acne, tetracycline is administered orally in relatively low doses over a period that can extend from months to years [36,37]. In agriculture, tetracycline has been used to stimulate growth of some animals [38]. Thus, long dosage regimens for tetracycline have been widespread and could have been responsible for the increased carriage of *tetQ* between 1970 and 1990.

The *tetQ* gene is not the only gene whose carriage has increased over the past few decades. Carriage of some of the *erm* genes, principally *ermB*, *ermF*, and *ermG*, increased dramatically between the pre-1970 and post-1990 period. A particularly interesting aspect of this increase in carriage by human colonic *Bacteroides* strains is that *ermB* and *ermG* were previously thought to be "Gram-positive" resistance genes, because they were found primarily in Gram-positive bacteria. These genes seem to have entered *Bacteroides* spp. only very recently [29]. Could they be coming in from Gram-positive bacteria? The largest population of bacteria in the human

colon is that of the Gram-positive anaerobes, a little studied and poorly understood group of bacteria [39,40]. Similarly, Gram-positive bacteria are the predominant population of bacteria in the human mouth and in the intestines of farm animals [41,42].

CHARTING THE MOVEMENT OF RESISTANCE GENES INTO *BACTEROIDES* SPP.

Given that the *ermB* and *ermG* genes had been found previously exclusively in the Gram-positive bacteria, is it possible that these genes were obtained from Gram-positive bacteria? Recently, it became possible to ask this question, because a CTn that carries *ermB,* CTnBST, was found in *Bacteroides* spp: It has been sequenced. The results of this analysis are both revealing and confusing [43]. We had hoped that the answer would be a simple one, that is, that a single CTn of Gram-positive origin would be revealed as having moved into *Bacteroides.* What we found was that the *ermB* gene was carried on a segment of DNA that is at least 7 kbp in size, and has integrated into a CTn that has been found previously in *Bacteroides fragilis*. The CTn is now clearly a chimera of Gram-positive and *Bacteroides*-like DNA. The *Bacteroides*-like DNA may not be from *Bacteroides* after all, however, because the percentage G+C content of the CTn outside the *ermB* region is higher than the percentage G+C content of *Bacteroides* spp. The chimeric nature of CTns and plasmids is becoming an old story. Recently, *tetM,* a Gram-positive tetracycline resistance gene, has been found in *Escherichia coli* [44]. Whether this gene is on a transmissible element remains to be seen. In the Gram-positive bacteria, *tetM* is usually found on CTns.

Some of the same resistance genes seen in human oral and colonic bacteria are also found in animal feces. The *ermB* and *tetQ* genes are examples of this. The *tetQ* gene was reported in a bacterium isolated from the rumen of cattle in the 1990s. This gene was not on a CTn but on a plasmid. Nonetheless, its DNA sequence was more than 95% identical to the sequences of the *tetQ* genes we were finding in human colonic bacteria [10]. More recently, the *ermB* gene has been found in isolates, mostly Gram positive, from a below-barn pig manure collection tank.

The overwhelming majority of reports of antibiotic resistance genes in bacteria isolated from animals and humans have focused on such foodborne pathogens as *Salmonella* and *Campylobacter.* Since these pathogens can colonize humans as well as animals, it is perhaps not surprising that they would move as resistant strains between human and animal reservoirs. More surprising is the apparent movement of genes, such as *tetQ* and *ermB* between members of the normal microflora of humans and animals, populations of bacteria that differ in species composition [10,29]. In these cases, it is almost certainly the genes that are moving rather than just the bacterial strains.

THE TRICLOSAN QUANDRY

A cause for concern is the widespread use of antimicrobial agents in products ranging from soaps to cutting boards. The story of triclosan is a good example of marketing gone wild. Triclosan is an antibacterial compound that has been added for years to plastic products to maintain the integrity of the products. One day, some marketing genius realized that by adding the label “antibacterial” to the product, the product

suddenly gained added value in the public eye. Soon, triclosan was being added to soaps, toothpaste, and mouthwash, among other products.

Initially, triclosan was thought to be a disinfectant, but it has since been found to have a specific mode of action. It inhibits fatty acid biosynthesis. In 1998, Stuart Levy and co-workers first showed that *E. coli* strains resistant to triclosan could be isolated and that these strains had a specific defect in fatty acid synthesis [45]. Since then, many studies of the mechanism of triclosan action have been published, but the question that is still hanging fire is the question of how widespread triclosan use might affect the distribution of antibiotic-resistant strains. Fortunately, obtaining approval to use other antibacterial compounds in personal products is not easy, so there may be time to evaluate the impact of triclosan before decisions on newer antibacterials are made. How best to evaluate the impact of triclosan? The most obvious approach is to assess the ease with which triclosan-resistant mutants are selected, but this is not the critical question. The critical question is whether triclosan use could cross-select for strains resistant to other antibiotics. This question remains to be answered.

Whatever the impact of triclosan use on antibiotic resistance patterns, the sudden popularity of "antibacterial" products is a cautionary tale. Public health officials were unprepared for the sudden advent of such products, and it remains unclear what the appropriate response to such changes in public consumption patterns is and how best the implications of such usage changes can be evaluated for safety.

THE ECOLOGY OF THE FUTURE

Although the ecology of antibiotic resistance genes is still a relatively new area, some problems and challenges are evident. First, very few systematic studies of the distribution and movement of resistance genes in nature have been done. Comparisons of the incidence of resistant strains in farms that do or do not use antibiotics are misleading if variables such as the proximity of water supplies that might be contaminated with antibiotics or the movement of wild birds and rodents between the farms are not taken into account. The finding of significant concentrations of antibiotics in some water sources has demonstrated what should have been obvious all along: antibiotics do not necessarily stay in the location where they are used. Antibiotics used in the hospital or in agriculture can appear later in water released from sewage treatment plants [46,47]. Or water recovered from animal manure and used to irrigate vegetable crops can spread antibiotics to locations where antibiotics are not being used intentionally [48–50].

An unanswered question is how widely distributed antibiotic resistance genes are in nature outside the human body. Our finding that even in strains isolated from humans prior to 1970, the *tetQ* gene was already present in nearly one-third of *Bacteroides* strains is perplexing since tetracycline use only became widespread in the 1960s and 1970s. Is it possible that there are non-antibiotic selections for antibiotic-resistant strains? Production of antibiotics by antibiotic-producing bacteria is very low in natural settings, but plant compounds that mimic antibiotics may be more abundant. Also, it is important to keep in mind that resistance genes are often linked on the same element. Integrons are an excellent example of this phenomenon. If a set of genes in an operon includes, for example, a cadmium resistance gene as

well as several antibiotic resistance genes, cadmium may select for maintenance of the antibiotic resistance genes. In the case of the *Bacteroides* CTns in our studies of human colonic bacteria, the CTnDOT type element contained both a tetracycline resistance gene, *tetQ*, and a macrolide resistance gene, *ermF*, so that selection for either resistance gene tends to select for maintenance of the other type [51,52].

Relatively few studies have been done to evaluate the distribution of antibiotic resistance patterns in environmental bacteria, especially bacteria in sites outside of farms or areas of human settlement. It would be informative to conduct a study similar to those often done by marine microbiologists, in which sites around the perimeter of an island that differ in the amount of human pollution are sampled and evaluated. Unfortunately, none of the major funding agencies regards this type of survey as part of its mission. Thus, the question of whether there is such a thing as a truly pristine site, free of antibiotic-resistant bacteria, remains unanswered. Also, surprisingly, the question of the extent to which animal or human pollution affects the incidence of antibiotic resistance genes is also unanswered.

Clearly, systematic surveys of antibiotic resistance gene distribution are needed, and ideally surveys should be guided by the principles developed by environmental microbiologists who have had long experience in ecology. An interesting approach to this type of analysis has been taken by Randall Singer and his associates. The approach is called landscape ecology [53]. It is a form of mathematical modeling that assesses correlations between antibiotic use patterns and the incidence of resistant strains. Proving association is not the same as proving cause and effect, but the fact that scientists are beginning to explore mathematical modeling of resistance patterns as a means of seeking possible cause-and-effect connections is encouraging.

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3 Global Response Systems That Confer Resistance

Paul F. Miller and Philip N. Rather

CONTENTS

The majority of attention on antibiotic resistance mechanisms has been justifiably focused on those factors that are highly transmissible among species and that lead to high levels of resistance to a specific class of antibiotics. Less is known about the ability of bacteria to alter their susceptibility to noxious agents by modulating their own intrinsic physiological systems. In this chapter, we describe two of the better-studied examples of this latter situation, both of which occur in Gram-negative species.

In the first example, the *mar*/*sox* regulatory network found in *Escherichia coli* is described. This system acts to modulate factors that limit the accumulation of a wide range of noxious agents, including several clinically important antibiotics. As such, we discuss a network of sensory and regulatory factors that operate to control the expression of genes whose products either actively extrude antibiotics or enhance the effectiveness of external permeability barriers. Because Chapter 4 specifically addresses efflux pumps, our discussion focuses on the structure and function of the *marRAB* and *soxRS* regulatory loci. We review evidence describing the high degree of molecular redundancy shared by these two regulatory systems, leading toward the concept that these are two semi-independent sensory systems that control a nearly identical set of target genes, although in quantitatively different ways. These differences may reflect the distinct types of signals that are sensed by the two systems, such that a protective response to inducers of one (e.g., superoxide generating compounds for *soxRS*) may require a slightly different gene expression pattern than would the response to inducers of the second (phenolic agents and antibiotics for *marRAB*).

In the second example, we describe the regulatory mechanisms controlling the *aac(2′)-Ia* gene in *Providencia stuartii*. The *aac(2′)-Ia* gene is a member of a growing family of chromosomally encoded aminoglycoside acetyltransferases that are intrinsic to certain bacterial species. Although the role of these acetyltransferases is largely unknown, the AAC(2′)-Ia enzyme in *P. stuartii* functions as a peptidoglycan *O*-acetyltransferase. Given the possibility of diverse functions for these enzymes, we anticipate that the regulation of these genes will involve distinct mechanisms. However, the information on *aac(2′)-Ia* expression that has been compiled to date may serve as a useful preliminary model for other systems.

INTRODUCTION

Microorganisms live in intimate proximity to their environment. For free-living species, this situation equates to the constant threat of exposure to a wide variety of potentially toxic agents produced either deliberately (e.g., by other organisms for defense against microbial encroachment) or as a consequence of normal organic turnover. Similarly, commensal and pathogenic organisms must protect themselves from both specific and non-specific agents elicited by the host. Not surprisingly, then, unicellular species have evolved an elaborate array of defenses designed to reduce or prevent the accumulation of unwanted toxic substances. There is, for example, a remarkable inventory of efflux systems that can be identified in the genomes of almost all bacteria. The mechanisms by which efflux pumps operate are discussed in Chapter 4 of this volume.

With such a genetic investment in defense systems, it also makes sense that these organisms would possess similarly intricate regulatory mechanisms, which allow them to control the deployment of these systems. In this chapter, we highlight our understanding of a few of the better-characterized regulatory systems, including global resistance systems and intrinsic modifying enzymes. Although the systems described in this chapter have been studied primarily in *E. coli* and *P. stuartii*, it is reasonable to expect that these systems will serve as formal paradigms for as yet undiscovered control networks in other bacterial species.

GLOBAL REGULATORS OF ANTIBIOTIC RESISTANCE IN *ESCHERICHIA COLI*

The *mar* Regulatory Locus

Undoubtedly, the best-characterized global antibiotic resistance regulatory system is the *mar* (multiple antibiotic resistance) system in *E. coli*. An excellent review of the molecular genetics of this system has been published [1]. Much of the detailed work described in that review is only summarized here, and the reader is encouraged to look to that source for additional detailed information. The *mar* locus was first described in 1983 in the pioneering studies of George and Levy. As a component of an ongoing effort to understand the mechanisms contributing to tetracycline resistance, these investigators identified a locus on the *E. coli* chromosome that was associated with the frequent emergence of low-level resistant strains [2]. Moreover, it was shown that these tetracycline-resistant (tet^r) strains had also acquired a concomitant resistance to other structurally unrelated antibiotics including chloramphenicol, rifampicin, and fluoroquinolones [2]; mechanistically this phenotype was associated with reduced accumulation and efflux of the affected agents [2–4]. The substrate spectrum for this system was later expanded to include certain organic solvents and disinfectants [5,6]. A Tn*5* insertion at the 34 min region of the chromosome reversed the resistance phenotype for all of these agents, and identified the genetic locus, which was designated as *mar* [7]. DNA sequence analysis of cloned genetic segments that could complement the Mar phenotype associated with either the Tn*5* insertion or a larger chromosomal deletion encompassing this region revealed a three-gene regulatory operon, designated *marRAB* [8–11]. The Tn*5* insertion originally isolated by George and Levy was located in the second gene, *marA*. Overexpression of this gene by itself was shown to be sufficient to confer the Mar phenotype in all cell types, including strains deleted for this region of the chromosome [12]. The deduced protein product of this gene, MarA, is related by amino acid sequence similarity to a family of transcriptional activators, the prototype for which is the AraC regulator that controls genes involved in the metabolism of arabinose [13]. This observation suggested that the Mar phenotype resulting from a mutation at the *mar* locus was likely due to an indirect mechanism, with MarA serving to control the expression of genes located elsewhere on the chromosome. It is presumably these target genes that are the more direct effectors of antibiotic resistance.

If overexpression of *marA* is sufficient to confer a Mar phenotype, then the *mar* locus must be capable of controlling the expression of *marA*. This proved to be the

case, and the first gene in the operon, *marR*, was determined to play a critical role in this process [9]. Unlike MarA, the MarR protein, at the time of its sequencing, bore little similarity to any known genes. However, analysis of selected Mar isolates showed that the majority of these bore mutations in *marR*, and concomitantly exhibited elevated levels of the *marRAB* transcript [9,11,14]. Introduction of a wild type copy of *marR* in *trans* on a plasmid reversed the Mar phenotype, indicating that the *marR* mutations were recessive, and that this gene encoded a repressor of *marRAB* operon expression. Results of genetic experiments suggested that the target for MarR repression is the operator/promoter region of the *marRAB* operon, *marOP*, as one could titrate the repressing activity of MarR simply by introducing additional copies of *marOP* on a plasmid [9,14]. This finding was confirmed biochemically by showing that purified MarR protein bound specifically to *marOP* DNA sequences [15].

At roughly the same time as the original George and Levy experiments, it was noted that exposing *E. coli* cells to the weak aromatic acid salicylate (SAL) induced a condition of phenotypic antibiotic resistance subsequently referred to as Par [16]. Notably, SAL treatment conferred resistance to the same diverse group of antibiotics as was observed for the *mar* mutants. These findings converged mechanistically when it was found, through the use of a *mar-lacZ* fusion, that SAL treatment led to an induction of *marRAB* expression [17]. Importantly, this was the first observation that connected the *mar* regulatory locus with extracellular stimuli. Deletion of the *marRAB* operon led to a greatly reduced responsiveness to SAL as an inducer of antibiotic resistance, and to a hypersensitivity to many of the same agents that were affected by the original *mar* mutants [11,12,17]. The extent to which this hypersensitivity was observed depended on the specific *E. coli* strain background in use [8,10,11].

The crystal structure of the MarR repressor has been determined at 2.3 Å of resolution by Alekshun and co-workers [18]. The structure reveals MarR as a dimer, with each subunit composed of six helical regions that mediate a protein–protein interface in each monomer. The DNA binding domain consisting of amino acids 61 to 121 adopts a winged helix fold from amino acids 55 to 100. The formation of the MarR crystal required the presence of SAL, a strong inducer that relieves MarR-mediated repression of the Mar regulon. Based on electron density, there appear to be two SAL binding sites, both of which are positioned near the DNA binding helix. The location of these sites is consistent with the ability of SAL to alter the DNA binding properties of MarR by directly interacting with the repressor.

These studies suggested that the following hierarchy could explain inducible antibiotic resistance mediated by the *marRAB* system. The *mar* locus is normally maintained in a quiescent state due to the autorepressor activity of the *marR* gene product. Exposure to a specific inducer such as SAL leads to the binding of the inducer by MarR, antagonizing its ability to mediate transcriptional repression of the *marRAB* operon. This results in an increase in transcription of the *marRAB* genes, leading to an increase in the abundance of the products of these genes in the cell. MarA, the proximal activator of target genes involved in the antibiotic resistance response, thus becomes available in sufficient quantities to diffuse to other sites on the chromosome and activate its target genes. A more detailed discussion of the targets and inducers in the *mar* regulatory network is provided below.

The *soxRS* System

Exposure of *E. coli* cells to various redox cycling agents, such as paraquat, leads to the induction of a number of genes that collectively constitute the superoxide stress response [19]. Constitutive mutants have been selected in which the expression of these target genes is elevated in the absence of any inducing agent. Such regulatory mutants typically map to the *soxR* locus, located at 92 min on the *E. coli* chromosome [20]. Notably, these constitutive regulatory mutants also exhibit a concomitant antibiotic resistance phenotype, which is remarkably similar to that observed with *mar* strains. In addition, one such regulatory mutant with a very similar phenotype, known as *soxQ1*, mapped to the *marA* locus [21].

Molecular dissection of the *soxR* locus revealed two divergently transcribed regulatory genes, *soxR* and *soxS*. The constitutive *sox* mutants mapped to *soxR* and have been referred to as *soxR*(Con) alleles, to distinguish them from non-functional mutants. Gene expression studies showed that the expression of *soxR* is unaffected by either superoxide generating agents or the constitutively activating mutations [22,23]. In contrast, expression of *soxS* is induced by redox cycling agents, such as menadione or paraquat, as well as by *soxR*(Con) mutants, and an intact *soxR* gene is required for induction of *soxS* expression as well as that of superoxide stress response target genes [22,23]. Similar to findings described above for *marA*, overexpression of *soxS* was shown to be sufficient to activate the expression of superoxide stress response target genes as well as confer the antibiotic resistance phenotype [22,23]. These findings, combined with the recognition that the SoxR protein contains iron–sulfur clusters in its C-terminal region that are characteristic of those involved with redox reactions, suggested that SoxR activity (and not expression) may be modulated in response to superoxide radicals, and led to a better molecular understanding of the two-stage model for control of this regulon [24,25]. In this model, exposure to agents or conditions leading to an accumulation of superoxide radicals results in the conversion of inactive SoxR to an activated form. Activated SoxR then induces the transcription of the adjacent *soxS* gene, whose product stimulates the expression of the unlinked regulon genes, the products of which presumably engender resistance to superoxide radical-generating agents and Mar-type antibiotics. Constitutive *soxR* mutants appear to be permanently in an activated conformation, which may explain why in these strains regulon genes are expressed even in the absence of a small molecule activator.

Additional observations tied the *soxRS* regulon to the *mar* system. Along with the observations that *soxR*(Con) mutants have a Mar phenotype, and that the *soxQ1* mutant mapped near *marA*, another mutant that was initially selected based on its strong Mar phenotype was found to map to the *soxR* locus [26]. Reconciliation of these genetic observations began when it was recognized that MarA and SoxS, the proximal activators in these regulatory systems, are closely related members of the AraC family of transcription factors [13]. Thus, overexpression of either *soxS* or *marA* leads to a Mar phenotype as well as induction of the superoxide stress response target genes. However, these regulators do not behave in completely redundant ways, as there appear to be quantitative differences in the effects of these activators on the different target genes that have been studied to date. For example, *marA* overexpression

tends to produce a greater level of antibiotic resistance and a smaller induction of superoxide stress response target genes, such as *nfo* (encodes endonuclease IV), than does *soxS* [21,26].

Studies of clinical isolates have verified the role of *soxRS* in resistance. In *E. coli,* fluoroquinolone-resistant clinical isolates exhibited mutations in *soxR* and *soxS* that resulted in higher levels of *soxS* expression and activation of downstream genes required for resistance [27–29]. In *Salmonella enterica* (serovar typhimurium), a quinolone-resistant isolate arose during treatment that contained a single point mutation in *soxR*. This substitution rendered SoxR constitutively active and increased expression of SoxS-dependent genes [30].

Rob—A Third Regulator?

E. coli contains another gene whose product exhibits significant amino acid sequence similarity to MarA and SoxS. This protein, known as Rob, was first identified as a factor that binds to the chromosomal origin of replication [31]. It is larger than either MarA or SoxS, and appears to contain an additional domain not found in the other two proteins. It is also different in that it is constitutively expressed at high levels, increasing in concentration as cells transition from logarithmic to stationary phase. Although higher-level induction of recombinant Rob accumulation has been shown to confer a Mar phenotype, and purified Rob protein has been shown to bind to MarA/SoxS target promoters *in vitro* [32,33], a physiological role for this protein in antibiotic resistance has yet to be demonstrated. In addition, mutants affecting intrinsic antibiotic resistance have yet to be linked to the *rob* gene. For these reasons, this interesting and mysterious protein will not be described further here.

A Single Regulon with Two Activators

As has been proposed recently, it now seems reasonable to consider the existence of a single stress response regulon that is controlled by multiple related regulators [34]. This could be called the *mar* regulon, as has been proposed, or be referred to by a more general descriptor to reflect the distinct stresses that led to its activation. Regardless, the important consequence from the perspective of this review is that intrinsic antibiotic resistance is affected. We shall now consider more distal and proximal components of this pathway.

Regulon Targets and Antibiotic Resistance

Recent work has led to a greater understanding of the target binding site in MarA and SoxS responsive promoters [34–36]. Work with MarA has suggested that this activator interacts with target promoters as a monomeric protein, and that it can bind in either of two orientations to effect transcription. However, the orientation of the binding site in a given promoter must be as it originally exists in that element; inverting it leads to a loss of MarA responsiveness. In addition, distinct spacing rules appear to exist regarding the distance between the "marbox" and the binding sites for RNA polymerase (RNAP), depending on whether the marbox is present in the + or − orientation. Marboxes that are located on the opposite strand from that of the

RNAP binding sites (−35 and −10 sequences) are positioned further upstream than are those that are found on the same strand as the RNP binding site [34,37,38]. It has been proposed that these positions and orientations allow MarA to interact productively with RNAP in either orientation.

Marboxes that have been found upstream from a number of target promoters in *E. coli* have been aligned to generate a consensus binding site [34]. Despite significant experimental work, this consensus remains quite degenerate. From the crystal structure studies of MarA, it has been proposed that MarA interacts with specific promoter elements by way of an interaction of complementary shapes that are held together by Van der Waals forces [39]. Whether the interaction of MarA with a marbox results in activation or repression of transcription appears to be related to the relative position and orientation of the marbox within a promoter element [40]. By inference, it seems reasonable to expect that many of the mechanistic observations made for MarA will also be applicable to SoxS. This is supported by biochemical studies conducted with this latter protein, and its interaction with known target genes [35,36,38,41]. Thus, several of the genes containing marbox elements in their promoters have been implicated by both genetic and biochemical methods as specific targets for MarA and/or SoxS control. Because of the focus of this volume, those key target genes implicated in antibiotic resistance are discussed in further detail here.

micF

One of the earliest physiological observations associated with the Mar phenotype was a down regulation of the major outer membrane porin OmpF [42]; this effect has also been observed following SAL treatment [43]. This porin forms a large outer membrane channel through which low-molecular-weight, water-soluble compounds can diffuse. Thus, a reduction in the abundance of this channel in *mar* mutants fits well with the reduced antibiotic accumulation phenotype observed with these strains. Studies of OmpF regulation revealed that one form of negative control involved a post-transcriptional repression mechanism mediated by the anti-sense RNA *micF* [44,45]. Experiments with *micF-lacZ* fusions as well as *micF* deletions demonstrated that *mar* mutants have elevated levels of *micF* expression, and that *mar*-mediated down regulation of OmpF requires an intact *micF* gene [12,46]. However, using strains deleted for the *ompF* gene, it was also shown that a simple loss of OmpF from the outer membrane was not sufficient to confer a Mar phenotype [12]. Thus, additional *marA* targets appeared to be required for a full Mar phenotype.

acrAB and tolC

Accumulating experimental evidence on the structure and function of efflux pumps in Gram-negative organisms [47] suggested that one of these export systems might play a role in *mar*-mediated antibiotic resistance. Subsequent genetic studies then showed that the multidrug efflux pump encoded by the *acrAB* genes is required for the Mar phenotype, as a deletion of *acrAB* completely eliminated the Mar phenotype associated with *mar* mutants [48]. Subsequently, it was noted that the promoter for the *acrAB* operon, as well as that of the *tolC* gene, whose product forms the outer

membrane channel component of the AcrAB pump, contains a marbox element [34,49], which is bound by both MarA and SoxS *in vitro*. This strongly suggests that the products of *acrAB* and *tolC,* which act in concert to increase antibiotic efflux, are both controlled by MarA.

marRAB

The promoter for the *marRAB* operon also contains a marbox element and is subject to autoactivation [50]. This observation helped rationalize earlier studies, which showed that high-level expression of either *soxS* or *marA* led to increased *marRAB* operon expression. The marbox in the *marRAB* promoter region is one of the most MarA-responsive elements studied to date [34]. Moreover, *marRAB* operon expression is subject to both transcriptional and translational regulation [51].

As mentioned above, the SoxS protein is expected to bind to virtually the same set of target gene promoters as MarA. This has been largely substantiated experimentally, and in many cases a SoxS interaction was demonstrated first [41]. If this is true, then the explanation for the different effects of *marA* versus *soxS* induction on multiple antibiotic resistance, or the superoxide stress response, must lie in the quantitative ways in which these two regulators interact with their target promoters. This hypothesis is supported by recent evidence [52]. The marbox elements in different regulon promoters respond differently to MarA or SoxS induction. This difference was shown to be due to specific nucleotide sequence differences among the various marbox elements, and it was possible to vary the responsiveness of a promoter to MarA compared with SoxS by changing the sequence of a specific marbox [52]. These findings may also provide an explanation for a perplexing observation associated with certain bases in the proposed consensus sequences. Some of the invariant positions in the consensus have nonetheless been shown to be dispensable for MarA responsiveness. While one can consider it reasonable to propose that MarA and SoxS control an almost identical set of target genes (although in quantitatively different ways), it seems possible that these positions may be more important for SoxS binding than they are for MarA.

Mechanisms of Regulon Induction and Physiological Roles

While much work has focused on the mechanisms by which MarA and SoxS interact with regulon target promoters, early studies were actually driven by physiological observations that gave insights into regulon induction. For the *mar* system, this work centered on the phenolic compound salicylate and its ability to stimulate *marRAB* expression [17]. As mentioned earlier, *marRAB* induction involves antagonism of the MarR repressor, apparently by a direct interaction with SAL [15,18]. The poor solubility of MarR in a purified form along with the relatively weak affinity of SAL for MarR has made biochemical characterization of this interaction difficult. In contrast, *soxRS* induction by superoxide inducing agents is somewhat better understood. Genetic and biochemical experiments demonstrated that superoxide radicals activate *soxS* transcription via their effects on SoxR [24,25]. As stated earlier, SoxR activation involves a cluster of iron–sulfur centers near the 3′ end of the protein, suggesting that a direct activation mechanism may be involved.