Bacterial Resistance to Antimicrobials

SECOND EDITION
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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 incurable 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.

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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.

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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.
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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.
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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
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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 cytomorphologically 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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