Perspectives

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Vicious Circles: Looking Back on Resistance Plasmids

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DURING the past few years, the popular press has often made play with lurid articles describing the rise in appearance of "superbugs" and emphasizing the menace posed by multiply antibiotic-resistant bacterial pathogens, such as the Gram-negative *Pseudomonas aeruginosa* or Gram-positive *Staphylococcus aureus* and *Enterococcus faecalis*. At medical conventions, infectious-diseases experts regale audiences with descriptions of their pet "Andromeda strain," the drug-resistant bacterial species that causes the most serious and difficult-to-treat infections. *Trends in Microbiology* prepared a special issue entitled "Drug Resistance: The New Apocalypse?" TV and radio shows have echoed these grim stories that dramatize the end of the antibiotic era. What is not realized by the lay public is that these supposed exposés are reporting nothing new; they are simply describing the continuing manifestations of a phenomenon that was first evident in the 1950s and has increased ever since. The sad commentary is that so little has been done to try to avoid the problem.

The rapid development of antibiotic resistance in bacteria, now well recognized, first came as a series of events that evoked both surprise and disbelief. At the beginning of the antibiotic era in the early 1950s, microbial geneticists were more involved in discussions of the controversial topic of the mechanism of acquired antibiotic resistance in bacteria than in considerations of the clinical significance of this resistance. Many symposia and publications were concerned with hot debate on the subject of mutation *vs.* adaptation in the generation of antibiotic-resistant phenotypes in bacteria. Resistance to antibiotics in bacterial pathogens during the course of treatment of infectious diseases was considered by bacterial geneticists to be an event of low probability, and the notion of multiple drug resistance was heretical. Although this period was the heyday of studies on the nature of bacterial conjugation and the role of the F factor in this process, it was considered unlikely, at the time, that sexual mechanisms would contribute to any extent in the development of antibiotic resistance in bacteria. Coincidentally, however, in war-torn Japan, serious outbreaks of dysentery due to *Shigella dysenteriae* were responding with decreasing effectiveness to sulfonamides, the principal antibacterial treatment available at the time. By 1952, more than 80% of Shigella isolates in Japan were sulfonamide-resistant; it is of interest to note that the sulfonamide drugs are not, strictly speaking, antibiotics, since they do not occur naturally. When antibiotics such as streptomycin, tetracycline, and chloramphenicol became available, it was found that their efficacy was temporary, resistant strains developing in the afflicted population as these drugs were used clinically. By 1955 there were reports of the isolation of *S. dysenteriae* strains resistant to as many as four different antibiotics; the first publication of this phenomenon was apparently that of KITAMOTO *et al.* (1956). Numerous other reports confirmed that multiple antibiotic resistance in bacteria was not a rare, unusual observation in Japan; it was epidemic. The papers describing these developments have been widely referenced but very rarely read in the West, since the majority were published in Japanese journals.

Not long after, in 1959–60, it was found that the multiple antibiotic resistance of *Shigella dysenteriae* strains could be transferred to other Enterobacteriaceae, simply by mixing liquid cultures of resistant and sensitive bacteria and plating on solid medium containing the appropriate antibiotics as selective agents (OCHIAI *et al.* 1959; AKIBA *et al.* 1960). The mechanism by which the transfer occurred was revealed when the laboratories of S. MITSUHASHI (HARADA *et al.* 1960), R. NAKAYA (NAKAYA and NAKAMURA 1960a), and T. WATANABE (WATANABE and FUKUSAWA 1960a) all showed that cell-to-cell contact was required for resistance-gene transfer, indicating that bacterial conjugation was involved. This was subsequently confirmed by experiments that showed that blending (agitation) interfered with transfer and that acridine orange treatment of multiply resistant strains caused loss of the resistance determinants. Since HIROTA (1960) had shown previously that bacteria could be "cured" of the F plasmid by this treatment, a functional similarity between F plasmids and resistance factors was established; resistance factors were correctly deduced to be episomes. Most of this

work was published in Japanese journals, and it was only in late 1960 that two papers describing the phenomenon of transferable multiple antibiotic resistance appeared in *Biochemical and Biophysical Research Communications*. The publication of these studies was greeted with considerable skepticism; in fact, NAKAYA's paper was under review for nearly six months after submission, and it was (apparently) the receipt of WATANABE's manuscript that led the editors to the realization that the scientific basis for these observations was valid; NAKAYA *et al.* (1960b) and WATANABE and FUKUSAWA (1960b) were published back-to-back. WATANABE became the person most closely identified with the early work on R factors, publishing a long series of papers in *Medical Biology* (in Japanese) and subsequently the *Journal of Bacteriology* (in English) over many years; he wrote the first comprehensive summary of all the work done on R factors in Japan for *Bacteriological Reviews* (WATANABE 1963). A more detailed account of the early history of R factors is given by STANLEY FALKOW (1975).

In the United Kingdom in 1959, NAOMI DATTA initiated her studies of multiple antibiotic resistance by identifying clinical isolates of *Salmonella typhimurium* that were resistant to three antibiotics, only one of which had been used to treat the patients! On seeing the NAKAYA and WATANABE papers, DATTA tested her isolates for cell-to-cell transfer of resistance determinants and confirmed that gene transfer, dependent on cell contact, was involved in the spread of resistance among the bacterial species she was studying. This was the earliest demonstration of transmissible resistance outside Japan. Like the Japanese workers, DATTA encountered opposition to her findings; however, she had the advantage of working in Hammersmith Hospital next door to the bacterial genetics unit of BILL HAYES, who in NAOMI's words "looked at her plates" and gave her confidence in what she was doing. DATTA's results were published in 1962 in the *Journal of Hygiene*—hardly a high-impact journal by today's ratings. Other microbiologists working with multiple antibiotic resistance bacteria had similar experiences with skeptical colleagues, perhaps none so difficult as those of G. LEBEK, who obtained evidence for transferable, multiple antibiotic resistance in *Salmonella typhimurium* and *Escherichia coli* isolated from children in 1960. The presentation of his results was met with "harsh and unpleasant" criticism (his words) in Munich and LEBEK was dismissed. He was unemployed for several months and then accepted a position in Bern, Switzerland. A report of his work was eventually published (LEBEK 1963).

However, additional reports of multiple antibiotic resistance followed worldwide; with the paper of DAVID SMITH (1966) describing the isolation of transferable drug resistance in hospitals in Boston, the concept was generally recognized and the study of R-plasmid biology was on its way.

The number of different antibiotic-resistance determinants increased greatly as multiply antibiotic strains were studied in different countries; there were indications that the resistance determinants often reflected the geography of antibiotic usage. Subsequent studies led to characterization of a variety of types of R factors, many of which were detected outside the Enterobacteriaceae, in pseudomonads, staphylococci, streptococci, *etc.*; very few bacterial species do not possess endogenous plasmids. The magnitude of the clinical problems raised by antibiotic resistance was clearly identified by those working in the field, who often spoke out against the indiscriminate use of antibiotics in the treatment of common human diseases and the massive use of antibiotics in agriculture and as animal growth promotants. Into the 1980s, only about 50% of the antibiotics produced were employed for human use, and this is probably true today. Press coverage of the threat and its consequences was less evident thirty years ago, and even today the subject of antibiotic resistance is brought to the attention of the general public in daily newspapers only briefly, when news of other disasters is lacking.

The problem of antibiotic resistance is now an established fact of life in the clinical treatment of infectious diseases. Microbes have survived almost fifty years of onslaught by millions of tons of toxic agents by employing the processes of gene acquisition and gene transfer on a scale that is still not fully appreciated.

My interest in the subject of transmissible antibiotic resistance began in 1962 upon joining B. D. DAVIS' laboratory as a postdoctoral fellow to work on the mechanism of action of streptomycin. I was intrigued by antibiotic resistance and began to study the biochemical basis of streptomycin resistance in mutants of *E. coli* isolated in the laboratory. This work demonstrated that the structural components of ribosomes were altered by these mutations, which were the first genetic markers identified for these organelles. I met T. WATANABE in 1963 and read his review, but it was not until 1965 when I went to Paris to work with FRANÇOIS JACOB that transmissible antibiotic resistance caught my interest. YUKINORI HIROTA was in JACOB's laboratory, and he described in great detail (usually over a bottle of wine) the background to the R-factor problem in Japan. HIROTA gave me an *E. coli* strain carrying R plasmid NR-1 (variously known as R222 and R100), and JACOB agreed to let me study the resistant strains with HIROTA's help as a sideline to my primary research on genetic studies of *lac* regulation. We found that the ribosomes of the resistant strains remained sensitive to streptomycin, but that incubation of the antibiotic with cell-free extracts from the resistant strain led to inactivation of the antibiotics. Incidentally, although antibiotic-resistant clinical isolates had been isolated (in France) in 1954, it was not until 1968 that they were shown to carry transferable R plasmids. On my move to Wisconsin in 1967, more extensive biochemical studies with TAKESHI YAMADA and DONALD TIPPER (who knew how to do electrophoresis)

showed that streptomycin was inactivated by *O*-adenylylation in the strains carrying plasmid NR1 (YAMADA *et al.* 1968). This unusual modification prompted analysis of other R-factor-mediated antibiotic resistance mechanisms, and some eight biochemically distinct mechanisms have now been identified in bacteria.

The covalently closed circular form of isolated R-plasmid DNA was demonstrated in the late 1960s, which led to extensive physical mapping of the plasmids from a variety of sources. Although plasmid biology (as such) is little discussed in modern courses (many students, if asked to name a plasmid, being likely to mention one of the popular cloning vectors), the discovery of R plasmids revolutionized thinking about gene acquisition and gene exchange in nature, with important consequences for an understanding of the evolution of bacterial genomes. In addition, bacterial resistance plasmids have played a seminal role in the development of recombinant DNA technology and its applications. The study of plasmid-determined resistance in bacteria has been of enormous benefit to molecular science, both fundamental and applied. It is not possible to describe all of these studies, but some are worthy of mention. In 1972, STANLEY COHEN *et al.* successfully transformed competent *E. coli* with R-plasmid DNA. This demonstrated the ability of *E. coli* not only to take up DNA, but also to serve as a useful host strain for the expression of plasmid-encoded genes, converting a laboratory tool into an important industrial microorganism. This experiment also anticipated the development of plasmid replicons as cloning vectors and the use of resistance determinants as markers in the transfer of recombinant plasmids from one bacterial host to another. R plasmids and their bacterial components have played a seminal role in the development of genetic engineering and biotechnology in other ways; many of the restriction endonucleases currently used are plasmid-encoded.

Another highly significant characteristic of R-plasmid structure came to light in 1973. BOB HEDGES, working in NAOMI DATTA's laboratory, was carrying out compatibility tests with R plasmids. On introducing two different R plasmids into the same strain of *E. coli*, he recovered a "recombinant plasmid" from an event in which the ampicillin resistance gene of one plasmid had seemingly jumped onto the other plasmid; no other kind of recombinant was formed. HEDGES and ALAN JACOB carried out more extensive studies of this phenomenon and in 1974 proposed the existence of "transposons" in bacteria (HEDGES and JACOB 1974).

In the following year, several groups characterized a variety of transposable antibiotic resistance genes and demonstrated transposition between bacterial replicons; the molecular nature of "transposons" was established. Subsequent studies confirmed that most R-factor-encoded antibiotic resistance genes (in both Gram-negative and Gram-positive bacteria) are components of transposable elements. Parenthetically, in early experiments on the transduction of multiple antibiotic resistance carried out in Japan, it was found that the resistance genes were not subsequently transferable by conjugation. The stable transconjugants may well have been the products of transposition! Recent studies of the fine structure of transposable elements have provided clues to the mechanism by which resistance genes can be acquired by plasmids and so disseminated within the bacterial population. Resistance-gene cassettes (of unknown origin) are acquired by a mechanism of integrase-catalyzed, site-specific integration into an element named an integron. Integrons are natural, broad-host-range gene-expression cassettes in which transcription and translation of any inserted open reading frame(s) can be effected (STOKES and HALL 1989).

Thus, by use of a combination of different replicons, recombination systems, and gene transfer mechanisms, bacteria have access to an enormous pool of antibiotic resistance genes in the environment that are on tap when needed. Nonetheless, it is now clear that mutation also plays an important role in the development and evolution of antibiotic resistance in bacteria. The appearance of multiple antibiotic resistance in *Mycobacterium tuberculosis* is the result of point mutations, as is resistance to the newer fluoroquinolone antibiotics in pathogens such as *P. aeruginosa* and *S. aureus,* to give just two examples. The evolution of extended β-lactamases in Enterobacteriaceae is largely the result of a series of point mutations in plasmid-encoded resistance genes, showing that extensive natural protein engineering to change enzyme characteristics is yet another strategy that permits bacteria to evade the unwelcome appearance of novel antibiotic analogs.

Finally, after many years, methicillin-resistant *S. aureus* (MRSA), one of the earliest and most resilient of resistant bacterial lines, has been characterized biochemically. MRSA was discovered in 1960, one year after the introduction of penicillinase-resistant antibiotics into clinical practice, and has now become a pathogen of global importance with an incidence of more than 80% in some hospitals. These strains appear to have descended from a single clone of *S. aureus* which acquired the *mec*A gene, encoding a methicillin-insensitive enzyme involved in cell-wall biosynthesis, by chromosomal insertion (DE LENCASTRE *et al.* 1994). The source of the *mec*A gene remains a mystery and, in fact, the resistance genes of all R factors are due to acquisition of DNA encoding novel functions from unknown sources (DAVIES 1994).

In conclusion, while I do not support the "doom and gloom" approach of the popular press, one must recognize that a serious problem exists as a result of the inheritance of antibiotic resistance genes by bacterial pathogens. Bacteria are survivors, and no matter what novel antimicrobial substance is introduced into clinical practice, they resist and thrive. One has to marvel at the ability of microbes to thwart our best efforts to con-

trol or eliminate them, but after all, bacteria have inhabited the earth and resisted many hostile environments for close to four billion years, so we can hardly expect to get rid of them in a mere half century of effort.

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LITERATURE CITED

AKIBA, T., K. KOYAMA, Y. ISHIKI, S. KIMURA and T. FUKUSHIMA, 1960 On the mechanism of the development of multiple-drug-resistant clones of *Shigella*. Nihon Iji Shimpo **1866:** 45–50.

COHEN, S. N., A. C. Y. CHANG and L. HSU, 1972 Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *E. coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69:** 2110–2114.

DATTA, N., 1962 Transmissible drug resistance in an epidemic strain of *Salmonella typhimurium*. J. Hygiene **60:** 301–310.

DAVIES, J., 1994 Inactivation of antibiotics and the dissemination of resistance genes. Science **264:** 375–382.

DE LENCASTRE, H., B. L. M. DE JONGE, P. R. MATTHEWS and A. TOMASZ, 1994 Molecular aspects of methicillin resistance in *Staphylococcus aureus*. J. Antimicrob. Chemother. **33:** 7–24.

FALKOW, S., 1975 *Infectious Multiple Drug Resistance*. Pion Ltd., London.

HARADA, K., M. SUZUKI, M. KAMEDA and S. MITSUHASHI, 1960 On the drug resistance of entire bacteria. 2. Transmission of the drug resistance among *Enterobacteriaceae*. Jpn. J. Exp. Med. **30:** 289–299.

HEDGES, R. W., and A. JACOB, 1974 Transposition of ampicillin resistance from RP4 to other replicons. Mol. Gen. Genet. **132:** 31–40.

HIROTA, Y., 1960 The effect of acridine dyes on mating type factors in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **46:** 57–64.

KITAMOTO, O., N. KASAI, K. FUKAYA and A. KAWASHIMA, 1956 Drug sensitivity of the *Shigella* strains isolated in 1955. J. Jpn. Assoc. Infect. Dis. **30:** 403–404.

LEBEK, G., 1963 Über die Enstehung mehrfachresistanter Salmonellen. Ein experimenteller Beitrag. Zbl. Bakt., Abt. I, Orig. **188:** 494–499.

NAKAYA, R., and A. NAKAMURA, 1960a Mechanism of acquisition of drug resistance by prevalent strains of *Shigella*. Presented at the 13th Meeting of the Kanto Branch of the Society of Japanese Bacteriologists.

NAKAYA, R., A. NAKAMURA and Y. MURATA, 1960b Resistance transfer agents in *Shigella*. Biochem. Biophys. Res. Commun. **3:** 654–659.

OCHIAI, K., T. YAMANAKA, K. KIMURA and O. SAWADA, 1959 Studies on inheritance of drug resistance between *Shigella* strains and *Escherichia coli* strains. Nihon Iji Shimpo **1861:** 34–46.

SMITH, D. H., 1966 *Salmonella* with transferable drug resistance. N. Engl. J. Med. **275:** 626–630.

STOKES, H. W., and R. M. HALL, 1989 A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol. Microbiol. **3:** 1669–1683.

WATANABE, T., 1963 Infective heredity of multiple drug resistance in bacteria. Bacteriol. Rev. **27:** 87–115.

WATANABE, T., and T. FUKUSAWA, 1960a Episomic resistance factors in *Enterobacteriaceae*. 1. Transfer of resistance factors by conjugation among *Enterobacteriaceae*. Med. Biol. **56:** 56–59.

WATANABE, T., and T. FUKUSAWA, 1960b "Resistance transfer factor" an episome in *Enterobacteriaceae*. Biochem. Biophys. Res. Commun. **3:** 87–115.

YAMADA, T., D. TIPPER and J. DAVIES, 1968 Enzymatic inactivation of streptomycin by R factor-resistant *Escherichia coli*. Nature **219:** 288–291.