KATHLEEN F. KEYES
Evolution and Ecology of Florfenicol Antibiotic Resistance
(Under the direction of MARGIE D. LEE)

The evolution of antibiotic resistance has become a ubiquitous problem in both human and veterinary medicine. Florfenicol, a veterinary fluorinated analogue of thiamphenicol and chloramphenicol, was approved in early 1996 for use in cattle for the treatment of infectious respiratory diseases that formerly responded to chloramphenicol. There is currently much interest in the potential use of florfenicol in industrial poultry farming. However, florfenicol resistance has already emerged in a number of veterinary bacterial isolates, including *Escherichia coli* and *Salmonella enterica* serovar Typhimurium DT104 in cattle. The gene *flo*, which confers resistance to florfenicol and chloramphenicol, has previously been identified in *Photobacterium piscicida* and *Salmonella* DT104.

Resistance to florfenicol was detected in avian *Escherichia coli* isolates from clinical samples of sick chickens, although this antibiotic has as yet never been used in poultry. All florfenicol-resistant *E. coli* isolates were also positive for the florfenicol-resistance gene *flo* by polymerase chain reaction (PCR) screening. Molecular typing demonstrated that *flo* was independently acquired and is encoded on high-molecular-weight plasmids. Two of the florfenicol-resistant isolates also contained *intI*1, the DNA integrase gene that is characteristic of Class 1 integrons, which are mobile transmissible elements deemed important in horizontal transfer of antibiotic resistance genes.

INDEX WORDS: Florfenicol, Antibiotic, Antibiotic resistance, Antibiotic resistance genes, *flo*, *Escherichia coli*, Plasmids, Integrons, Poultry diseases

EVOLUTION AND ECOLOGY OF FLORFENICOL ANTIBIOTIC RESISTANCE

by

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DEDICATION

To the Past, Present, and Future…

I dedicate this thesis to my mother and father, my husband, and my daughter… They have each in their own way taught me that with hard work and perseverance, almost anything is possible.

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The University is a Paradise. Rivers of Knowledge are there. Arts and Sciences flow from thence. Counsell Tables are Horti conclusi, *(as it is said in the Canticles)* Gardens that are walled in, *and they are* Fontes signati. Wells that are sealed up; *bottomless depths of unsearchable Counsels there.*

John Donne

…Desire is a tricky thing. It can change a quick outing to the store for milk into a lifelong, shoeless quest through the Himalayas in search of enlightenment. It can put you on the road to Canterbury without your realizing it at first. And some version of that is what happened.

Michael Paternati in *Driving Mr. Albert*

Going back to school is one thing, a simple thing, but *finishing* (or trying to finish, *again*) is, as the Wizard said, "a horse of another color." Three years ago, I wanted to go back to graduate school and get (*finish* this time) a master's degree…I mean I *really* wanted it…but as Mr. Paternati declares, "desire is a tricky thing." We all need second chances at sometime during our lives, and actually doing something with that chance is one of the greatest gifts one can receive. I would like to thank all my family, friends, and the scholars who helped (or forced!) me to use my second (academic) chance…

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TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

"…the late twentieth century will be witness to the virtual elimination of infectious disease as a significant factor in social life…to write about infectious disease is almost to write of something that has passed into history".

Sir Macfarlane Burnet, 1962
Australian virologist and Nobel laureate

"The war against infectious diseases has been won".

William H. Stewart, 1969
U.S. Surgeon General

"For the fourth time in three years, health officials have identified a U.S. hospital patient infected by a strain of bacteria that has developed partial resistance to a treatment of last resort…The Center for Disease Control and Prevention said a 63-year-old women who was in an Illinois nursing home in the last stages of kidney disease and died in the hospital in April of a heart infection was infected with *Staphylococcus aureus* that had become resistant to the potent antibiotic vancomycin. Vancomycin is one of the few antibiotics effective against bacteria resistant to other drugs…Experts have warned for years that antibiotics are used too generously, fostering resistant "superbugs"."

Atlanta Journal-Constitution, January 9, 2000

"They have the genes, we have the brains, but I do not know who will end up victorious."

Joshua Lederberg, 1999
Academy of Medicine and Nobel laureate

The philosopher and critic George Santayana stated in 1905 that those who cannot remember the past are condemned to repeat it. He was commenting on the notion of historical, as well as scientific "progress" and human nature, but could well have been foretelling the future of disease treatment at the beginning of the twenty first century. Many aspects of history are unanticipated and unforeseeable, but man's optimism with his own achievements can sometimes overshadow what might seem obvious in retrospect…

Thinking about how scientific history concerning antibiotics and bacterial resistance has played out over the last 60 years, reminded me of stories my Dad used to tell about medical practice back in what sounded like (to me anyway) the “Dark Ages”… My father had just completed his medical studies and residency program in internal medicine at the University of Illinois when World War II broke out in 1939. Bacterial infections such as scarlet fever, pneumococcal pneumonia, TB, and diphtheria were both common and very serious before 1944, when the first antibiotic drugs became available. Physicians had to know how to work quickly because these infections could escalate rapidly out of control. Dr. John W. Keyes became a well-trained, observant diagnostician with an excellent “bedside manner”. A good thing, too, since state-of-the-art therapy at that time involved for the most part, good diagnosis, tender-loving-care, and sometimes oxygen. In 1942, my father was drafted into the Army Medical Corps and was stationed at an army hospital based on Staten Island, New York. He treated soldiers returning from many “exotic” battlefields, with many exotic diseases to match. Two years into the war, the army issued its first meager supplies of penicillin. Physicians were instructed to use this drug sparingly, in doses of about 5,000 units, less than a third of what is considered the minimum dose for a “minor” (and non-resistant) infection in the year 2001. In those days (before bacteria became widely resistant to antibiotics), such doses were capable of performing “miracles”.

But not all miracles last forever and even Nobel laureates and Surgeon Generals cannot always predict the future. Last year, scientists meeting at the International Conference on Emerging and Infectious Diseases in Atlanta tackled a wide array of topics, from “exotic” diseases, such as the West Nile virus that showed up initially in

New York City (coincidentally on Staten Island, of all places, and that has now spread along the entire length of the Eastern Seaboard), to food-borne bugs, to common household germs. A common thread that ran through many of these presentations was the difficulty, to near impossibility, in treating many of these pathogens, as well as their increasing resistance to the currently available antibiotics. The rapid global expansion of bacteria resistant to antibiotics is one of the most significant developments over the past decade in emerging bacterial diseases.

Antibiotics, the "wonder drugs" of the 1940s, and touted in the 1960s and 1970s as the so-called "magic bullet" that would put an end to infectious disease, have in many cases fallen short of their mark and become more of a boomerang at the beginning of the new millennium. Bacteria that have evolved resistance to these drugs are now one of human and veterinary medicine's biggest headaches. Like superpowers in an ever-escalating arms race, the bacteria are under constant selective pressure to develop new defenses and the pharmaceutical industry is faced with the challenge of developing new antimicrobial agents against these populations of constantly evolving bacteria. Not only are these resistant bacteria a significant medical and veterinary problem, but also their economic impact can be enormous in terms of prolonged, and sometimes unsuccessful treatment. A number of the so-called "emerging pathogens", such as *Salmonella typhimurium* DT104 and vancomycin resistant enterococci (VRE) and *Staphylococcus aureus* (VRSA), which have grabbed headlines lately, exhibit multiple antibiotic resistance and are extremely difficult to treat.

In reality, antibiotic resistance is not a new thing…resistance was documented almost as soon as the first antibiotics were used. In 1940, a penicillin resistant

Escherichia coli was reported, and close on its heels, in 1944, a similar penicillin resistance was reported in *Staphylococcus aureus*. Over the past two decades, antibiotic resistance has increased in virtually every species of bacteria examined. The last decade of the twentieth century heralded in the era of multiple drug resistant bacteria.

The last decade has also witnessed an increasing body of scientific research on the mechanisms by which bacteria render antibiotics ineffectual and the genes responsible for such resistance. We now know that antibiotic resistance occurs not only by mutation of microbial genes which code for antibiotic uptake into cells or the binding sites for antibiotics, but in fact mostly by the acquisition of heterologous resistance genes from external sources. Antibiotics may then act as the selective pressure to enhance the development of resistance and promote the transfer of resistance genes between bacteria. This gene movement, or horizontal gene transfer, produces extremely dynamic genomes and these lateral transfers of resistance genes have effectively changed the ecological and pathogenic character of many bacterial species. There is increasing evidence that extensive horizontal transfer of antibiotic resistance genes is occurring in nature, between clinical and nonclinical bacteria, between animal and human intestinal bacteria, and between intestinal and soil bacteria. The thought is that commensal and environmental bacteria, although not pathogens themselves, can function as “reservoirs” or storage chambers for resistance genes that can potentially be transferred to human pathogens.

Given the dramatic increase in multiple drug resistant bacteria, and the now mounting evidence of horizontal transfer of resistance from one organism to another, we may indeed witness an increase in disease pathogens for which there are no antibiotic solutions…what some have dubbed the “post-antibiotic era”. The molecular “tools” now

exist to decipher the genes responsible for antibiotic resistance, the mechanisms that they encode, and how these genes move through and between different bacterial populations. Successful treatment, surveillance, and control measures will depend upon knowing which antibiotic resistance genes already exist in the population, the reservoirs of those genes, and the mechanisms by which such genes are exchanged among microorganisms.

My father died seven years ago, in 1994. His medical practice spanned many years, from those before the advent of the miracle drug penicillin, to those where some tough, resistant "superbugs" were untreatable by virtually all antibiotics. He lived long enough to see many of the new microbial threats such as HIV develop, as well as old enemies, such as pneumococcal pneumonia, rear their ugly heads. He might be justified in asking whether doctors in the year 2001 are better or worse equipped to treat such "common-place" diseases as bacterial pneumonia, than were physicians in his "pre-antibiotic" days. The answer to that question may depend on whether researchers, as well as physicians and veterinarians, heed knowledge about how bacteria evolve and the way we think about disease. Rather than viewing the relationship between disease and bacteria in a one-way, linear fashion, with the only goal to decrease the risk to animals and humans, we need to seek ways to better understand the molecular and evolutionary ecology between microbes, man, and his environment.

This thesis will review, in general, the molecular basis and transfer of antibiotic resistance, and in particular, the evolution and ecology of bacterial resistance to florfenicol, a relatively new veterinary antibiotic.

CHAPTER 2

LITERATURE REVIEW

The Antibiotics: An Overview

Definition and Description of Action:

The term “antibiotic” from the Greek derivation that literally means “against life” first appeared in the scientific literature in 1942 (Waksman and Woodruff 1942; Waksman et al. 1942). Coined by Selman Waksman to embrace those newly discovered antimicrobial substances, such as pyocyanin, penicillin, actimycin and others, he used this term to describe and define those substances of microbial origin, which specifically inhibit the growth of other microorganisms. The usage for this term has now been extended to include any low-molecular-weight compound, whether it be a microbial or other living organism’s metabolite or synthetic compound, that at low concentrations will kill or inhibit the growth of other microorganisms (O’Grady et al. 1997).

Products obtained by chemical modification of natural antibiotics or of other products of microbial metabolism are called semisynthetic antibiotics (Lancini et al. 1995). A number of the antimicrobial agents which are inhibitors of bacterial nucleic acid synthesis, membrane-active compounds, or those used solely for the treatment of mycobacterial disease are purely synthetic chemicals, rather than antibiotics in the strict sense (Greenwood 1995b). There is also an increasing appearance in the scientific literature of the search for and description of antibiotic products from various herbaceous and woody plants (Desta 1993; Cosentino et al. 1999; Hammer et al. 1999; Dorman and Dean 2000), as well as insects and mammals (Gabay 1994).

Antibiotic agents affect the growth of bacteria in three general ways. Those that kill bacterial cells outright, but where lysis or cell rupture does not occur, are known as *bacteriocidal*. Bacteriocidal antibiotics generally bind tightly to their cellular targets and are not removed by dilution (Lancini et al. 1995; Madigan et al. 1999). *Bacteriolytic* agents induce killing by cell lysis and include those antibiotics which inhibit cell wall synthesis and those that damage the cytoplasmic membrane (Lancini et al. 1995; Madigan et al. 1999). Antibiotics that do not kill, but inhibit growth are known as *bacteriostatic*. Bacteriostatic antibiotics are frequently inhibitors of protein synthesis and act by binding to ribosomes. The binding is not tight, and when the concentration of the antibiotic is lowered, it becomes free from the ribosome and growth is resumed (Lancini et al. 1995).

The sensitivity of microorganisms to antibiotics varies. Gram-positive bacteria are usually more sensitive to antibiotics than gram-negative bacteria, although some antibiotics act only on gram-negative species (Greenwood 1997). An antibiotic that kills or inhibits the growth of many types of bacteria is called a *broad-spectrum* antibiotic (Madigan et al. 1999). A broad-spectrum antibiotic will generally find wider medical usage than a *narrow-spectrum* antibiotic, which is one that acts only on a single group of organisms. Narrow-spectrum antibiotics may be quite useful, however, for control of bacteria that fail to respond to other antibiotics. Some antibiotics have an extremely narrow range of action, being effective for only one or two bacterial species (O'Grady et al. 1997; Madigan et al. 1999).

Sources of Antibiotics

The majority of antibiotics in human and veterinary use as antibacterial agents are "natural products", elaborated as secondary metabolites by living organisms, primarily bacteria and fungi (Bennet and Bentley 1989; Davies 1990; Vining 1990). The production of antibiotics is not rigorously species-specific: organisms belonging to different species, genera, or even orders can produce the same antibiotic. And the reverse is also true, that strains classified taxonomically as members of the same species can produce different antibiotics. As a general rule, however, the more "distantly" related the organisms are on the taxonomic scale, the less probable it is that they will produce the same antibiotic (Lancini et al.1995).

Most antibiotics are the products of the secondary metabolism of three main groups of microorganisms: actinomycetes, eubacteria, and filamentous fungi (Greenwood 1995b). The actinomycetes produce the largest number and greatest variety of known antibiotics, with more than 6000 active substances isolated from them. More than 50% of the antibiotics described are produced by this bacterial order, and in particular by the genus, *Streptomyces* (Lancini et al. 1995). The spore-forming bacilli and pseudomonads of the Eubacteria, produce a fair number of antibiotics, with approximately 1000 substances described to date (Lancini et al.1995). The myxobacteria, although little studied, have also revealed a high frequency of production of antibiotics (Lancini et al. 1995). Two genera of the lower fungi, *Aspergillus* and *Penicillium*, produce several kinds of secondary metabolites with a relatively high antibiotic activity (Lancini et al 1995; Madigan et al. 1999). Only a few antibiotics described (so far) are produced by the higher fungi, algae, and plants, and they generally show low antimicrobial activity and

little specificity (Lancini et al. 1995). This may change, however, with the race to find new "classes" of antibiotics, with new modes of action.

Currently products obtained by chemical modification of natural antibiotics or of other products of microbial metabolism are called semisynthetic antibiotics (O'Grady et al. 1997). The requirement that the term "antibiotics" be applied only to those compounds that are "microbial metabolites" is no longer strictly applied in the literature, with the burgeoning search and research in the last decade for plants containing substances with antibiotic properties.

Targets and Mechanisms of Action of Antibiotics

Most antibiotics are directed against some target that is peculiar to bacteria, interfering with some structure or process that is essential to bacterial growth and/or survival, while causing little or no harm to the eukaryotic host harboring the infecting bacteria (Betina 1983). Antibiotics block the growth of sensitive bacteria by inhibiting the action of a molecule, usually a macromolecule, such as an enzyme or a nucleic acid, essential for cell multiplication. At the molecular level, this means that the antibiotic molecule is able to bind to a specific site on the target macromolecule, forming a molecular complex which is no longer able to accomplish its original function (Lancini et al. 1995).

To determine the mechanism of action of an antibiotic requires identification of the target molecule and its function. It is usually easier to identify the *function* that is blocked rather than the particular macromolecule involved. There are four proven principle targets for the main classes of antibiotics: (1.) bacterial cell-wall biosynthesis (peptidoglycan); (2.) bacterial protein synthesis (bacterial ribosomes); and (3.) bacterial

DNA replication and repair (bacterial enzymes involved in DNA supercoiling) and (4.) cytoplasmic membrane function (Walsh 2000). A few antibiotics are antimetabolites, acting as competitive inhibitors and mimicking important growth factors needed in cell metabolism (O'Grady et al. 1997).

Principle Classes of Antibiotics

Various schemes for classification of antibiotics have been proposed, although none have been universally adapted. In general, antibiotics and othe chemotherapeutic agents are grouped together based either on mechanism of action or more usually chemical structure (Lancini et al 1995; Madigan et al. 1999). Currently, those natural or semisynthetic antibiotics that have a similar basic chemical structure are grouped together into one "class" and named after the member first discovered or after a principle chemical property. This classification is useful in practice, as the components of one class usually share many biological properties. The major classes of antibiotics include: (1.) β-lactam antibiotics (penicillins and cephalosporins), which inhibit cell wall biosynthesis, (2.) glycopeptides, which inhibit cell wall biosynthesis (3.) tetracyclines, which inhibit protein synthesis by binding to the 30S ribosomal subunit, (4.) aminoglycosides, which inhibit protein synthesis by binding to the 30S ribosomal subunit, (4.) macrolides and lincosamides, which inhibit protein synthesis by binding to the 50S ribosomal subunit, (5.) quinolones, which inhibit DNA replication, and (6.) miscellaneous drugs, such as the anti-tubercular drug rifampin, which inhibits DNA-directed RNA polymerase, and chloramphenicol and analogues, which inhibit protein synthesis by binding to the 50S ribosomal subunit (Greenwood 1995b; Lancini et al. 1995; O'Grady et al. 1997).

Clinical Uses of Antibiotics and Necessary Criteria for Effective Therapy

The drug treatment of infectious diseases is based on the ability of antibiotics to inhibit the multiplication of the infecting bacteria without an intolerable toxic effect on the cells or the metabolic functions of the human or animal host. This inhibition makes it easier for the host's defenses to overcome infection. For an antibiotic to be useful in the clinical treatment of an animal or human infection, it must satisfy a number of criteria:

(1.) Activity against one or more pathogenic bacteria (Lancini et al. 1995). In general, the antibiotic should have a wide-spectrum of activity, that is, it should kill or inhibit the growth of many different kinds of bacteria. Broad-spectrum antibiotics are useful in human and veterinary medical practice because the same symptoms can be caused by different bacteria and it is often not practical to wait for the isolation and identification of the causative organism before therapy is started. Unfortunately, this can lead to reduced numbers of resident normal flora and can allow other pathogens (which are normally outcompeted by the normal flora) to "overgrow" and cause infection.

(2.) Good absorption and distribution: To be effective, an antibiotic must be absorbed, reach the site of infection and remain there at inhibitory concentrations for a sufficient time (Greenwood 1995b). It must be eliminated from the body within a reasonable length of time to avoid accumulation and potential toxic consequences (Lancini et al. 1995).

(3.) Lack of toxicity: The antibiotic should be without any intolerable toxicity to the host at therapeutic doses (O'Grady 1997). Most antibiotics are directed against targets that are peculiar to bacteria. This minimizes the risk of toxicity to the host animal or

human. Serious adverse reactions are tolerable only if the antibiotic is to be used in diseases that are extremely severe or potentially lethal..

Antibiotics are not only used clinically for the treatment of bacterial disease and infection, but also prophylactically to decrease the infectious disease risk of surgery, cancer chemotherapy, and organ transplants to a level low enough to make these procedures medically possible (http://www.healthsci.tufts.edu/apua).

Non-clinical Uses of Antibiotics

Not only are antibiotics used for the therapeutic treatment of disease in animals and humans, but they also play a role in the supposed "prophylactic" use in animal disease and as growth promoters in animal husbandry, are used in agriculture, and have become essential tools for research in genetics, microbiology, and molecular biology.

Then and Now…A Brief History of the Discovery and Usage of Antibiotics

Man's search for remedies against aches, pain, and illness is probably as old as mankind itself, but before the actual etiological agents of infectious disease were identified, treatment relied for the most part on the vagaries of chance and empirical observation.

Prehistoric findings indicate that humans may have used "natural" antibiotics more than 5,000 years ago. The 5,200-year old Tyrolean "Ice Man" discovered in 1991 in the Oztal Alps is the world's most ancient known human mummy (Hess et al. 1998). An analysis of the content of the Ice Man's rectum revealed eggs of the intestinal parasite *Trichuris trichiura* (Aspock et al. 1996), which causes a condition often resulting in severe abdominal pain and cyclic anemia. Also found along with the Ice Man were a number of his "personal effects," including two, odd cork-like lumps the size of walnuts,

each of which was pierced and tied to a leather thong (Capasso 1998). The lumps were identified as being the woody fruits of *Pitoporus betulinus,* a bracket fungus (Aspock et al. 1996) that contains toxic resins and agaric acid, which are powerful purgatives and result in bouts of diarrhea (Capasso 1998). The fungus also contains oils which have antibiotic activity against mycobacteria. This discovery suggests that the Ice Man may well have been "aware" of his intestinal parasites and was treating them with measured doses of *Piptoporus betulinus*.

Much of ancient "folk" medicine was also based on observations that "natural substances" could alleviate the symptoms of disease. Ancient Egyptian texts from the middle of the 16th Century BC, such as the Great Medical Papyrus, describe treatment of a pustular scalp ailment by rubbing the crumbs of moldy, wheaten loaf on it (Ebell 1937). Inflammations of the bladder and urinary tract were also treated with moldy, wheaten preparations, which enjoyed the reputation of "soothing the pipes" (Bottcher 1964). The Egyptian documents also describe dispensation of a "medicinal earth", which possessed curative properties and carried with it the recommendation, "prepared by Schow for Re" (Ebell 1937; Bottcher 1964)…and what more could one want from a remedy than one prepared by one god for another (!). The ancient Egyptians had worked out a system of antibiotically active concoctions: the moldy bread no doubt a goodly dose of penicillin and the "medicinal" dirt, with its accumulation of secondary metabolites or natural antibiotics from the soil organisms.

In 3500 BC, Sumerian physicians treated ailing patients with a beer soup laced with ground snakeskins and turtle shells (Kramer 1981; Magner 1992). About a thousand years later, ancient Babylonians used a medicine called "kuthacht", a combination of

whey, salt and bread crumbs, for healing infected eyes and “hizmi-cuscta”, a special microbrew beer for treatment of skin tuberculosis (Bottcher 1964). Presumably, the therapeutic action of these medicines was owed to the yeast and molds employed in their fermentation.

The existence of what we now call “scholarly medicine” dates from the time of Hippocrates, the father of modern medicine, 460-370 BC. The Greeks and Romans had a substantial herb and dirt “pharmacopoeia” to fight the diseases and infections they believed were caused by miasmas or “bad air” (Hoel et al. 1997). Soil, medicinal plants and herbs and other substances in this pharmacy may have contained the secondary metabolites and compounds wielding antibiotic activity. Miracle cures of the Middle Ages included a continuation of the methods known to the Greeks and Romans. A great number of pharmacopoeias and recipe books have survived from the 16th Century and they tell of the types of ingredients that the “pharmacists” used then in the preparation of their medicines. Christian Franz Paullini, a very learned Renaissance man of his time writes in his *Miraculous Dirt-Pharmacopoeia* of the following popular “medical” knowledge: “The water that collects on the stumps of oak trees, if filtered through a cloth and applied lukewarm usually affords relief, and I have known it to be used internally with beneficial results” (Bottcher 1964). This “folk” medicine again reflects antibiotic activity at work, in that the bark of oak trees contains tannins and other organic acids known to be active against several types of bacteria (Glombitza et al. 1978; Abo et al. 1999) and old stumps of oak trees definitely harbor many types of bacteria, actinomycetes, fungi, and lichens, all of which may contribute antibiotic substances to the water.

It is in the 16th Century, as well, where the first chemotherapeutic approaches were used against syphilis, by the Veronese physician Girolamo Fracastoro (Fracastoro [trans.] 1984). He recommended mercury compounds as a specific antibacterial treatment for the treatment of this disease…giving rise to the saying, "One night with Venus—A lifetime with Mercury" (Bottcher 1964; Goldwater 1972).

It wasn't until the second half of the 19th Century that Robert Koch observed and first reported in 1877 (Koch 1876-1877) that some microorganisms could destroy others, a phenomenon confirmed by Louis Pasteur (Pasteur 1880) and believed might be utilized in medicine. By the end of that century, the German bacteriologist Paul Ehrlich, often called the "father" of scientific antibacterial chemotherapy, had begun his quest to systematically seek out new, antimicrobial compounds (Himmelweit 1960). When he found a compound that showed at least limited antimicrobial activity, he would set about trying to modify the molecule to produce derivatives or closely related compounds in order to find more effective ones, an approach still used by drug chemists today. In 1909, Paul Ehrlich discovered the first chemical "cure" for a disease, the arsenical compound, arsphenimine (marketed as Salvarsan), that was selectively toxic for *Treponema pallidum* (Ehrlich and Hata 1910). The medical profession dubbed this compound "the magic bullet" because it killed the specific "germs" that caused syphilis.

Over the next few, short decades, from the late 1920s to the 1960s, came the true era of "wonder drugs" known as antibiotics. In 1935 the German bacteriologist and pharmacologist Gerhard Domagk discovered that the dye protonsil red used to tint cloth was effective in the treatment of streptococcal infections in mice (Domagk 1935). When his own young daughter was close to dying with a streptococcal infection, he in

desperation injected this dye into the girl (Schadewaldt 1985). Her fever dropped almost immediately, with a recovery which was termed at that time nothing short of miraculous. A Swiss-borne chemist, Daniel Bovert, then showed that the antibacterial activity of protonsil red was due to the sulfanilamide component of the dye. This compound already lay in the public domain when its antimicrobial effects were discovered and by 1940 sulfanilamide was available under at least thirty-three different trade names and a start had been made on producing the numerous sulfanilamide derivatives that subsequently became available (Magner 1992).

It was in early September of 1928 that Alexander Fleming, returning from holiday to his laboratory at St. Mary's Hospital in London, made his famous observation on an old uncovered culture plate of bacteria. Along with the "expected" staphylococci on the petri plate, he noticed a blue-green mold and "something" in the mold was attacking the bacteria. He identified the mold as *Penicillium notatum*, cultured it in nutrient broth, filtered it, and discovered in the filtrate a substance that ravaged bacteria. The discovery he named penicillin (Fleming 1929). Fleming was unable to purify the penicillin himself and was also unable to arouse much interest in his discovery. It took the economic and political (as well as medical) pressures of disease-ridden soldiers and civilians of World War II to awaken pharmaceutical company's interest in penicillin. The British pathologist/bacteriologist Howard Florey and German chemist Ernst Chain extracted the first "real" sample of penicillin in the spring of 1942, which was a million times more powerful than Fleming's original filtrate. Penicillin became the starting point for modern antibiotic therapy. Mass production of penicillin began in 1941 in the United States, shortly after our country became involved in the war, for use by the military to treat sick

and wounded soldiers. In 1945, Fleming, Florey, and Chain were awarded the Nobel Prize for Medicine (Hoel et al. 1997).

Close on the heels of Alexander Fleming's discovery, and inspired by it, came the investigations of Selman Waksman into the antimicrobial substances produced by the soil-borne fungi and microorganisms that destroy or slow the growth of other microbes. In 1940, he initiated a systematic search for non-toxic antibiotics produced by soil microorganisms, particularly the actinomycetes (Waksman et al. 1942). Waksman and his students screened more than 10,000 soil cultures; only 1,000 of these inhibited bacteria in their preliminary tests and then just 100 of these showed promise in later tests (Welsch 1942). Only ten could be actually isolated, but amazingly they struck gold with one. In 1943, a clump of dirt taken from the neck of a sick chicken and containing a peculiar mold was found to have "killer" activity against *Mycobacterium tuberculosis* (Schatz and Waksman 1944). This new antibiotic, which they called streptomycin after the organism *Streptomyces* from which it was isolated, was found to have a spectrum of activity which complemented penicillin, by inhibiting many gram-negative bacilli (Schatz et al. 1944). The discovery of streptomycin, the first aminoglycoside antibiotic, triggered a treasure hunt for more naturally occurring antibiotics, with pharmaceutical companies joining the fray. Soil samples from all over the world were screened and thousands of antimicrobial substances were discovered by this means, although most were found to be too toxic for clinical use. Soon, more broad-spectrum penicillins, including the first of the cephalosporins isolated from the microbial flora of a sewage outflow, and other aminoglycosides, were found (Botzu 1948; Burton and Abraham 1951). By the middle of the 1950s, representatives of most of the major families of antibiotics, including

chloramphenicol, tetracyclines, and macrolides, had been discovered (Glasby 1992). Since 1960, only a few novel antimicrobial substances have been discovered. Chemists and microbiologists have primarily been successful in chemically modifying existing agents in an attempt to derive new compounds with enhanced properties, such as the numerous semi-synthetic derivatives of penicillin and cephalosporins (O'Grady et al. 1997). The antibacterial potential of "non-antibiotic" substances has been exploited as well, and in fact all the synthetic classes of antibiotics that are in current therapeutic use, such as the nitrofurans, imidazoles, and quinolones, have emerged through a lucky mixture of biochemical knowledge, inspired hunch, and serendipity. Today, there are over 5,000 known antibiotics, although only about 100 of them are in are in clinical use (O'Grady et al. 1997).

The Scope of Antibiotic Therapy and the Development of Resistance

Sometime during 1999, as the second millennium drew to a close, a number of "surveys" were conducted as to what the greatest discoveries and inventions of the twentieth century were. The discovery of antibiotics for therapeutic use against infectious disease was in the "top five" of all such lists. Without question, the appearance in the late 1930s and 1940s, and the continuing discoveries of the next three decades, of potent, non-toxic antibiotic agents that were selectively active against bacteria revolutionized the treatment of bacterial infections and disease. Indeed, the discovery of these first "wonder drugs" was declared by some in the medical establishment to herald the end of bacterial infection and disease as a threat to public health. No longer would tuberculosis, dysentery, pneumonia, and other killers take their enormous toll on mankind and its social systems (or so it was thought). The initial

success of antibiotics during the 1960s and 1970s in “conquering” many bacterial infections led to the opinion by many that infectious diseases had lost their threat. However, with the benefit of more than half a century’s worth of hindsight, we are now able to take a more dispassionate, and perhaps objective, view of the benefits and limitations of antibiotic therapy.

What the past fifty years has shown us and what scientific research in fields such as bacterial genetics and microbial ecology continue to reveal are two main things. First, that microbes display a truly amazing versatility in terms of their ability to avoid, withstand or repel the antibiotic onslaught (Jacoby and Archer 1991; Neu 1992; Courvalin 1996). Secondly, it is often the use of antibiotics that disturbs the delicate bacterial ecology within the body of both humans as well as animals, allowing the proliferation of resistant species, and sometimes initiating new infections that are worse that the one originally treated (Levy 1997). The historical cycle that we have witnessed over and over in the last fifty years is that drugs were discovered, diseases (supposedly) were conquered, and more drugs were discovered in an inexhaustible go-around. Old disease “scourges” of both humans and animals, re-emerged as significant problems of the 1980’s. Antibiotic drug resistance allowed diseases such as cholera, bacterial meningitis, tuberculosis, pneumonia, and even plague to spring back with a renewed vengeance (Neu 1992; Caputo et al. 1993; Fraimow and Abrutyn 1995; Doern et al. 1996). By the 1990’s, many scientists as well as physicians and veterinarians, were sounding the warning bells about the alarming increase and rate of spread of antibiotic resistance, with concomitant dire predictions concerning the lack of remaining effective antibacterial drugs. Among hospital and community acquired infections from organisms

including *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, and vancomycin-resistant enterococci (VRE), there are now multi-drug resistant isolates, which means that some individuals may contract infections for which there is only one drug with which to treat, or in some cases, none (Levy 1998b). As the new century opens, we now know that bacteria resistant to antibiotics are one of the greatest problems facing clinicians in both human and veterinary medicine.

Why *do* antibiotics stop being effective? How do we define antibiotic resistance? How do bacteria become resistant and why are antibiotic "superbugs" the new medical and veterinary problem of the twenty first century?

What is Resistance?

Antibiotic resistance is probably as old as the "antibiotics" themselves, presumably having developed within antibiotic-producing microorganisms as a means of protecting themselves from these substances (see section: **Origins of Antibiotic Resistance Genes**). Antibiotic resistance in clinical bacterial isolates has been recorded since these agents were first brought into use. The first antibiotic resistance mechanism was identified in 1940 when Abraham and Chain described the presence of a penicillinase, an enzyme that inactivates penicillin, in resistant *Escherichia coli* (Abraham and Chain 1940). Close on its heels, a similar penicillin resistance was reported in 1944 in an isolate of *Staphylococcus aureus* (Kirby 1944). Therefore, even before penicillin came into widespread use, resistance to this drug was already recognized in both Gram-positive and Gram-negative bacteria. Although the enormity and complexity of the current problem of antibiotic resistance could hardly have been foretold in the early days of antibiotic use, even Alexander Fleming was able to recognize

the threat and factors that would promote its appearance (Fleming 1929). Multiple antibiotic resistant pathogenic bacteria have now been recognized by the World Health Organization as one of the most serious problems that complicates medical treatment of bacterial infections (WHO 1997).

Bacterial species vary tremendously in their susceptibility to an antibiotic. The term *antibiotic resistance* is often used in a general sense to signify the lack of effect of an antibiotic agent on a bacterial cell. A commonly accepted definition states that a bacterial strain derived from a species that is susceptible to an antibiotic is said to be *resistant* when it is inhibited by the minimal concentration of the antibiotic that inhibits the growth of typical strains of that species (Greenwood 1995). The resulting resistance is dependent on the relationship between (1.) a given bacterial strain, (2.) the particular antibiotic, and (3.) the concentration of that antibiotic (Lancini et al. 1995). However, the "real" decision as to whether a given bacterial isolate is labeled *sensitive* or *resistant* depends ultimately on the likelihood that an infection will respond to treatment with a given antibiotic. The clinical literature sometimes mistakenly refers to an organism as "resistant" when it is inhibited *in vitro* by an antibiotic concentration which is greater than that achievable *in vivo*. In fact, however, therapeutic success depends not only on the activity of an antibiotic against a particular bacterial isolate but also on the pharmacokinetic behavior of the drug, that is, its reaching the site of infection in sufficient concentration. A bacterial strain is considered truly resistant to a given antibiotic only if it can grow in the presence of a concentration equal to or greater than that which the antibiotic can reach in the serum or tissues (Towner 1995).

The Genetic Basis of Antibiotic Resistance

Bacterial resistance to antibiotics is essentially of two main types, *intrinsic resistance*, a natural property of the bacteria, or *acquired resistance*, which results from mutation or via the acquisition of new genetic material.

Intrinsic resistance refers to bacteria that are insensitive, in their natural state, to an antibiotic without the acquisition of resistance factors. Not all bacterial species are intrinsically sensitive to all antibiotics. The most obvious determinant of intrinsic resistance is the absence or inaccessibility of the target for the drug's action. For example, polyene antibiotics, such as amphotericin B, kill fungi by tightly binding to the sterols in the fungal cell membrane and altering permeability of the fungal cell. Since bacterial membranes do not contain sterols, bacteria are instrinsically resistant to this class of antibiotics (O'Grady et al. 1997). Similarly, the lipopolysaccharide (LPS) outer envelope of Gram-negative bacteria is important in determining resistance patterns, since numerous antibiotics cannot penetrate this barrier to reach their intracellular target (Brody 1994). Some other examples of intrinsic resistance include that of *Haemophilus influenzae* and its ability to move between interstitial cells of the host where it cannot be reached by large, hydrophilic antibiotics such as gentamycin (van Schilfgaarde et al. 1999) and the *Streptomycetes* sp. that produce antibiotics as a means of self-protection, necessitating an intrinsic resistance to those antibiotics (Benveniste and Davies 1973; Fouces et al. 1999). Intrinsic resistance is often predictable in a clinical situation and should not pose a problem if care is used choosing the appropriate antibiotic. Of greater concern is the acquisition or emergence of resistance in previously sensitive bacterial isolates, sometimes during the course of therapy itself.

Acquired resistance in bacteria occurs when a bacterium that has been sensitive to antibiotics develops resistance. This may happen by mutation or by acquisition of new DNA. Mutation is a spontaneous event that occurs regardless of whether an antibiotic is present. Changes in only a few base pairs, causing substitution of one or a few amino acids in a crucial target (e.g. enzyme, cell structure, or cell wall) may lead to new resistant strains of bacteria (Sanders and Wiedemann 1988). In any large population of bacterial cells a few individual cells may spontaneously become resistant. Such "resistant" cells have no particular advantage in the absence of an antibiotic, but following the introduction of treatment with an antimicrobial agent, all sensitive bacterial cells will be killed, so that the (initially) very few resistant cells can proliferate until they form a completely resistant population. Many antibiotics have been shown to select, both *in vitro* and *in vivo*, for this type of acquired resistance in many different bacterial species (Towner 1995). This problem has been recognized as being particularly troublesome in the long-term treatment of tuberculosis with antitubercular drugs (van Soolingen et al. 2000; Lee et al. 2001). Hospitalized patients treated with antimicrobials prone to select altered resistance traits can quickly become colonized or infected with these resistant strains (Medeiros 1993).

By far, the much more prevalent type of acquired resistance occurs when genes conferring antibiotic resistance transfer from a resistant bacterial cell to a sensitive one. Bacteria can acquire these foreign genes by three main mechanisms: transformation, transduction, and conjugation (Mazodier and Davies 1991; see section **Mechanisms of Gene Transfer Between Bacteria** as follows in this thesis for a detailed review of this subject). These foreign genes can provide a resistant version of the normal cellular

target, an additional drug efflux pump, or enzymes that modify the antibiotic, rendering it inactive (Salyers and Amabile-Cuevas 1997). Many of the antibiotic resistance genes are carried on plasmids, transposons, or integrons that can act as vectors that transfer these genes to other members of the species, as well as to another genus or species of bacteria (Ochman et al. 2000; see section **Vectors of Antibiotic Resistance** in this thesis for a detailed review of this subject).

Suffice it to say that however resistance appears in a previously sensitive bacterial isolate or population, it will probably become widespread only under the selective pressures produced by the presence of appropriate antibiotics. The development of individual resistant bacterial cells does not have to happen often or on a large scale. A single mutation or transfer event can, if the appropriate selective pressures are operating, lead to the replacement of a sensitive population by a resistant one (Towner 1995).

Mechanisms of Antibiotic Resistance

Bacteria exploit a variety of mechanisms to combat antibiotics. These strategies include limiting the intracellular concentrations of the antibiotic by decreased influx or increased efflux of the drug, modification or neutralization of the antibiotic by enzymes that reversibly or irreversibly inactivate the drug, alteration of the "target" of the antibiotic so that the drug no longer interferes with it, and elimination of the target altogether by the utilization of different metabolic pathways. Bacteria may use or combine multiple mechanisms against a single agent or class of agents, or a single change may result in development of resistance to several different agents.

Diminished Intracellular Drug Concentration: Decreased Permeability and Efflux

For antibiotics to be effective they must reach their specific bacterial targets and accumulate at concentrations that can act within some reasonable time frame. Many gram-negative bacteria can face the presence of toxic compounds, including antibiotics, by excluding them from the cell. The mechanisms by which intracellular concentrations of drugs are limited include decreased permeability through the outer membrane, decreased uptake through the cytoplasmic membrane, and active efflux back out across the cytoplasmic and outer membrane.

Decreased Uptake/Reduced Permeability of the Cell

Permeability barriers are an important component of the intrinsic resistance of many organisms. The outer membrane contains porin proteins, which form channels that allow the exchange of nutrients and other substances, including antibiotics, between the extracellular environment and the periplasmic space (Nikaido et al. 1983; Yoshimura et al. 1983; Nikaido 1988). Changes in the cell envelope, including loss of outer membrane porins or alterations of the lipopolysaccharide (LPS) layer, can be partially responsible for decreased susceptibility to a wide range of antibiotics (Cohen et al. 1988b, Rajyaguru and Muszynski 1997).

Several well-documented examples are recorded in the literature. For example, in order for β-lactam antibiotics to bind penicillin-binding proteins (PBPs), they must penetrate porin channels in the outer membrane and traverse through the periplasmic space (Sanders 1983). Diffusion of some β-lactam drugs through porin channels is limited by the presence of bulky subunits on the acyl side chain of the β-lactam nucleus; diffusion of others is limited by electrostatic charge (Yoshimura and Nikaido 1985).

Changes in outer membrane permeability serve to limit drug influx and decrease drug availability to PBPs located on the inner membrane (Nakaido 1988).

The high intrinsic resistance of *Pseudomonas aeruginosa* results from the complex interactions of several mechanisms, among which the rather impermeable porin pathway plays a key role (Yoshimura and Nikaido 1982). The outer membrane porin OprD facilitates the uptake of basic amino acids, small peptides, and carbapenem antibiotics such as imipenem and meropenem (Trias and Nikaido 1990). All of these molecules share a common binding site inside the OprD channel (Fukuoka et al.1991). Resistance of clinical strains to both imipenem and meropenem is increasingly observed as the result of the loss of protein OprD (Lynch et al. 1987; Quinn et al. 1986).

Another example of reduced permeability concerns two outer membrane proteins (OMPs) of *E. coli*, OmpF and OmpC, that have been identified and characterized (Nikaido et al. 1983; Yoshimura and Nikaido 1985; Nikaido and Rosenberg 1983). An additional (mutant) channel, PhoE, is produced in mutants of *E.coli* deficient in OmpF and OmpC under conditions of phosphate depletion (Nikaido et al. 1983). OmpF has the widest pore diameter and the highest permeability to β-lactam drugs (Nikaido and Rosenberg 1983). Zwitterionic compounds, such as ampicillin, cefaclor, cephalexin and imipenem, have much higher penetration rates through OmpF than do anionic compounds, such as carbenicillin, cefamandole, cefuroxime, and piperacillin (Yoshimura and Nikaido 1985). Permeability of zwitterionic compounds through PhoE channels is poor; however, anionic compounds have higher penetration rates through PhoE channels than through OmpF channels (Nikaido and Rosenberg 1983). *E. coli* mutants deficient in OmpF channels are resistant to β-lactam antibiotics as a

consequence of reduced penetration through OmpC channels produced by those strains (Harder and Nikaido 1981; Nikaido and Rosenberg 1983; Yoshimura and Nikaido 1985). Additionally, enzymatic degradation of β-lactam antibiotics is more efficient in OmpF-deficient mutants because of the slower drug penetration that occurs through OmpC channels (Sanders 1983).

Resistance mediated by decreased uptake across the metabolically active cytoplasmic membrane is best demonstrated by small colony aminoglycoside-resistant mutants of staphylococci, but this mechanism is less common than other mechanisms of aminoglycoside resistance (Nikaido 1994).

Active Efflux

Besides preventing an antibiotic from entering the cell by the elimination or modification of entry ports, some bacteria are able to protect sensitive targets and become resistant to antibiotics by manufacturing "pumps" that export the drug out as fast as it enters. This mechanism has been characterized in both bacterial strains that produce antibiotics and in clinical isolates, as well. Efflux is due to the presence of specialized membrane proteins, which fall into one of two basic "mechanistic" classes. One group resembles the multiple drug resistance (*mdr*) determinants found in tumor cell lines resistant to methotrexate and similar anticancer agents (Guilfoile and Hutchinson 1991). Like the Mdr proteins, the antibiotic resistance proteins are characterized by the presence of several membrane spanning domains and mediate resistance to more than one type of "inhibitor". The role of *mdr*-like resistance determinants in antibiotic-producing organisms is likely an immunity or "self-protection" mechanism for bacteria to prevent being killed by their own chemical

weapons and the mechanism may well have originated in these bacteria (Davies 1992, 1997).

In addition to the large family of Mdr-like membrane-associated active export systems, there is another group of resistance determinants that acts to limit the accumulation of specific antibiotics within the cell cytoplasm. The archetype of this mechanism is the active efflux "pump" found in tetracycline-resistant bacteria. Bacterial protein synthesis machinery, including the ribosomes, is located within the cytoplasm, so that antibiotics that are inhibitors of protein synthesis, such as tetracyclines, must pass through the cell membranes and then accumulate to a high enough concentration to block the particular susceptibility step in protein assembly. Plasmid-encoded tetracycline resistance based on energy-dependent efflux was first described in *E. coli* (McMurry et al. 1980). It is now recognized that both gram-positive and gram-negative bacteria that become resistant to tetracyclines commonly overproduce related cytoplasmic membrane proteins that act as an export or efflux pump for the drug (Levy 1992a; Paulsen et al. 1996). The drug is pumped out faster than it can diffuse in, so intrabacterial concentrations are kept low and ineffectual; bacterial protein synthesis proceeds at relatively unimpeded rates. A similar type of resistance has been recognized for chloramphenicol (Bissonette et al. 1991) and the erythromycin class of macrolide antibiotics (Ross et al. 1990; Paulsen et al. 1996).

It is increasingly recognized that both drug-specific, as well as multidrug efflux pumps are emerging as a major mechanism of antibiotic resistance and a major problem in clinical medicine (Levy 1992a; Saier et al. 1998). Over 100 different transport proteins that play an important physiological role in the extrusion of toxic substances

have been described and similar numbers of chromosomally encoded multidrug-efflux systems are found in pathogens and non-pathogens (Saier et al. 1998). Bacteria have found that these pumps confer such an advantage that the genes have been conserved (Nikaido and Saier 1992) and their dissemination has been extensive (Saier et al. 1998). Active antimicrobial efflux systems have been documented as playing a role in resistance to many different antibiotics, including the macrolides, tetracyclines, quinolones, chloramphenicol, and β-lactams. Multidrug efflux systems may also work in concert with outer membrane permeability changes to enhance the level of expressed resistance (Nikaido 1994).

Modification/Inactivation of the Antibiotic

Although the above two mechanisms prevent the antibiotic from accumulating in the desired compartment, they leave the antibiotic unchanged. Another strategy of resistance is the destruction or inactivation of the chemical "warhead" of the antibiotic. The resistant bacteria produce an enzyme that is capable of either degrading the antibiotic or chemically modifying it into an inactive form. Antibiotics can be inactivated either by enzymatic cleavage or by chemical modification such that they no longer interact with the target site or are no longer taken up by the bacteria (Pratt 1990). Inactivating enzymes remain the predominant mechanism of resistance to several major classes of antibiotics, with chemical modification conferring clinical resistance to the aminoglycoside antibiotics, chloramphenicol, penicillins, cephalosporins, and other β-lactam antibiotics (Pratt 1990; Brody 1994).

The mechanism of drug inactivation/modification was first characterized when Abraham and Chain (1940) described an enzyme produced by *E. coli* which they

showed destroyed penicillin. They called the enzyme penicillinase and their description was contemporaneous with the first clinical use of penicillin in December 1940. This classic example of chemical modification is resistance mediated through the hydrolytic deactivation of the β-lactam ring in the penicillins by elaboration of the hydrolytic enzyme β-lactamase in resistant bacteria (Philippon et al. 1985). β-lactamase enzymatically cleaves the four-membered β-lactam ring, rendering the antibiotic inactive. Over 200 types of β-lactamase enzymes have been described (Bush et al. 1995). These enzymes can be either plasmid- or chromosomally-encoded and their expression can be constitutive or induced. Most β-lactamases act to some degree against both penicillins and cephalosporins; others are more specific, namely cephalosporinases (such as the AmpC enzyme found in *Enterobacter*) or the penicillinases (such as the penicillinase of *S. aureus*). β-lactamases are widespread among both gram-positive and gram-negative species and exhibit varying degrees of inhibition by β-lactamase inhibitors, such as clavulanic acid (Livermore 1995).

Aminoglycoside modifying enzymes are the primary mechanism of acquired aminoglycoside resistance in both gram-positive and gram-negative bacteria and are usually encoded by resistance genes on plasmids (Shaw et al. 1993). The three major classes of these enzymes are acetyltransferases, phosphotransferases, and adenyltransferases. Each class modifies the aminoglycoside by transfer of the "indicated" chemical group (i.e. an acetyl, phosphoryl, or adenyl group) to a specified side chain. The nomenclature is based on the chemical group that is transferred and the site to which it is transferred. For example, an enzyme that phosphorylates the 3'-hydroxyl group of an aminoglycoside, would be designated a 3'-phosphotransferase

(APH[3']). There are numerous subgroups of enzymes based on which aminoglycosides serve as substrates; these are designated by a Roman numeral. Modification of the aminoglycosides does not appear to result in extracellular drug inactivation, but rather in decreased drug transport or modification during transport with decreased ribosomal binding (Bryan and Kwan 1983).

Resistance to chloramphenicol and macrolides can also be mediated by modifying or inactivating enzymes (Davies 1994). Numerous different gram-positive and gram-negative chloramphenicol acetyltransferases have been described. They all seem to act in a three-step process: acetylation of the 3'-hydroxyl group of chloramphenicol, intramolecular rearrangement to form chloramphenicol-1-acetate, and reacetylation of the 3'-hydroxyl group. Gram-positive chloramphenicol acetyltransferases are usually inducible, whereas gram-negative enzymes are most frequently constitutively produced. This reflects a similarity to the β-lactamase situation in which gram-positive organisms must excrete the enzyme into the environment, whereas gram-negative bacteria can contain the enzymatic activity within the periplasmic space.

Alteration of the target

Another resistance strategy focuses not on removal or destruction of the antibiotic, but on a "reprogramming" or camouflaging of the normal target to the antibiotic in the bacteria. Many antibiotics act by inactivating a target protein that can generically be called a "receptor". Resistant bacteria, sometimes by as little as a single mutational event in the primary target, develop a target protein that will not bind the antibiotic, or less often, a target that retains its function even after formation of the target-antibiotic complex.

There is a wide array of different types of target modifications used by bacteria to develop resistance to a variety of antibiotics. For example, the ribosome of *Staphylococcus aureus* can become insensitive to the antibiotic erythromycin following specific enzymatic modifications in the rRNA (Davies 1992). Resistant bacteria have emerged that are able to mono- or dimethylate a specific adenine residue, A2058, in the peptidyl transferase loop of the 23S RNA component of the ribosome. This modification is carried out by the methyl transferase enzyme Erm that does not impair protein biosynthesis in the bacteria, but does lower the affinity of all the members of the erythromycin class of drugs for the RNA (Bussiere et al. 1998). The Erm mechanism is the main resistance route in antibiotic resistant clinical isolates of *S. aureus* and is present in erythromycin producing organisms as a self-immunity mechanism (Walsh 2000).

Enterococci are regarded as being inherently resistant to cephalosporins because the enzymes responsible for the production of the polymer peptidoglycan for cell wall synthesis, the penicillin binding proteins (PBPs), have a low affinity for them and are therefore not inhibited. Infections caused by *Enterococcus* spp. fail to respond to cephalosporins because the enzymes, penicillin binding proteins (PBPs), responsible for the synthesis of the major cell wall component peptidoglycan have a low affinity for them and are not inhibited. Penicillins and cephalosporins are effective against most strains of *Streptococcus pneumoniae*, but by acquiring DNA sequences from other species of *Streptococci* encoding PBPs that do not bind these antibiotics, the strains of *S. pneumoniae* become resistant (Higashi et al. 1999). The novel PBP is called PBPX and because the gene consists of foreign DNA interspersed with novel DNA it is termed a

"mosaic gene". The emergence of high-level cephalosporin resistance among penicillin-resistant pneumococci by this mechanism also illustrates the rapid and flexible way bacterial resistance to antibiotics can emerge (Reichmann et al. 1997). In both these cases, DNA from the foreign streptococcal species enters the *S. pneumoniae* led by a process called transformation. Naked DNA strands are actively taken up across the bacterial cell wall and become incorporated by the process of homologous recombinations.

Modification of the gene encoding DNA gyrase is the major mechanism of resistance to quinolones, and target modification is also important in resistance to macrolides, tetracyclines, rifampin, and mupirocin (Spratt 1994).

Elimination of the target/Bypass Pathways

Some bacteria have gone beyond simple target modification and have acquired novel systems in which the need for the target has been eliminated by creation of new metabolic pathways to bypass the primary target. Resistant bacteria protect themselves from the antibiotic by the production of an alternative target (usually an enzyme) that is resistant to inhibition by the antibiotic, while continuing to produce the original sensitive target; the alternative enzyme "bypasses" the effect of the antibiotic. The most well documented examples of this mechanism are the alternative penicillin binding protein (PBP2a) in methicillin resistant *Staphylococcus aureus* (MRSA), resistance to the glycopeptides in enterococci, and the major means of acquired resistance to the folate antagonists.

The alternative penicillin binding protein (PBP2a) is produced in addition to the "normal" penicillin binding proteins by methicillin resistant *Staphylococcus aureus*

(MRSA). The protein is encoded by the *mecA* gene and because PBP2A is not inhibited by antibiotics such as flucoxacillin, the bacterial cell continues to synthesize peptidoglycan and hence has a structurally sound cell wall (Michel and Gutmann 1997; Higashi et al. 1999).

The appearance in 1987 of vancomycin resistant enterococci has aroused much interest because the genes involved can be transferred to *S. aureus*, and this can thus theoretically result in a vancomycin resistant MRSA. The mechanism also represents a variant of the alternative target mechanism of resistance (Leclercq and Courvalin 1997). In vancomycin-resistant enterococci (VRE), the *van* gene clusters mediate resistance to the glycopeptide antibiotics. The *vanHAX* genes encode a new pathway of enzymes that reduces pyruvate to D-lactate (*vanH),* add D-alanine and D-lactate together to produce D-Ala-D-lac (*vanA*), and then hydrolyze the normal metabolite D-Ala-D-Ala while sparing D-Ala-D-Lac (*vanX*) (Walsh et al. 1996). In this cell, only the D-Ala-D-Lac accumulates and serves as a substrate to be elongated and presented at the termini of the peptidoglycan strands. The “reprogramming” of peptidoglycan to end in D-Ala-D-Lac rather than the normal D-Ala-D-Ala has no effect on the cross-linking efficiency carried out by the transpeptidating PBPs, but the switch from the D,D-dipeptide terminus to D,D-desipeptide terminus lowers the binding affinity of vancomycin by about 1,000-fold (Bugg et al. 1991) and enables the VRE to grow at 1,000-fold-higher levels of the antibiotic.

Ecology and Evolution of Antibiotic Resistance

Origins of Antibiotic Resistance Genes

The “true” origin(s) of antibiotic resistance genes currently found in clinical bacterial isolates from human and animals will probably never be known, as the biochemical and molecular basis of resistance was yet to be discovered at the time antibiotics were first introduced into clinical use and the initial cases of resistant bacteria first reported. Several hypotheses have been put forward, however, as to what the original source of some of these resistance determinants might be.

A number of reports suggest that “housekeeping genes”, such as the sugar kinases and acyltransferases, may have evolved in function to modify aminoglycoside antibiotics (Udou et al. 1989; Shaw et al. 1993; Rather et al. 1993). Some earlier studies have also suggested that the genes for protein kinases and perhaps protein acetyltransferases were the “ancestral” sources of some classes of aminoglycoside-modifying enzyme genes (Heinzel et al. 1988; Martin et al. 1988). Payie et al. (1995) demonstrated that an aminoglycoside acetyltranferase (AAC2’), encoded as a chromosomal gene in the bacteria *Providencia stuartii*, may play an accessory role in cell wall peptidoglycan formation.

Another hypothesis, first reported in the early 1970s, suggests that the resistance determinants exist(ed) somewhere “in nature” as chromosomal genes and they were “picked up” to become the antibiotic resistance genes found on plasmids in resistant clinical isolates (Watanabe 1971). Several other early studies speculate that the source of these genes in nature may be the antibiotic-producing organisms (Walker and Walker 1970; Benveniste and Davies 1973), which must also encode the resistance mechanisms

to protect themselves against self-destruction (Cundliffe 1989). There are several lines of evidence which support the notion that the antibiotic resistance genes now present in human and animal bacterial isolates are recently acquired from an "outside source", and that many probably originated in organisms that produce these antibiotics (Davies 1994, Davies 1997).

A retrospective study that examined bacteria from the "Murray collection", a large group of clinical isolates preserved from the "pre-antibiotic era", found almost every isolate to be sensitive to antibiotics (and lacking resistance genes), although they did harbor a range of plasmids capable of conjugative transfer (Hughes and Datta 1983). This data suggests that the appearance of resistance genes in human and animal clinical isolates is a relatively recent event, with the multiresistant plasmids found in pathogens created during the past five decades.

Further evidence that the antibiotic resistance genes were not derived from the current bacterial "host" in which the resistance plasmid resides comes from DNA sequencing studies of the β-lactamases (Bush et al. 1995) and the aminoglycoside enzymes (Shaw et al. 1993). Despite similarities within the protein studies of these two families, there are very substantial sequence differences. Being that the evolutionary time frame is less than fifty years, it is not possible to derive a model in which evolution of these genes could have occurred by mutation from common ancestral genes. Davies (1997) suggests they must have been derived from independent sources, perhaps with the help of "gene shuffling" mechanisms.

Resistance genes on R plasmids also differ in base composition and codon usage from the flanking genes in the host (Davies 1997). This is very similar to the situation

found for genes determining microbial pathogenicity, that can be identified as “islands” of alien nucleic acid sequence inherited as an independent gene cluster from an external source (Blum et al. 1994).

Finally, many bacteria and fungi that produce antibiotics possess antibiotic resistance determinants that are “biochemical homologues” to those found in clinical bacteria (Davies 1997). For example, many of the species of *Streptomyces* both produce an antibiotic and have the concomitant resistance gene to it, a gene also found in an wide array of resistant clinical bacteria, e.g. *Streptomyces erythreus* produces the antibiotic erythromycin and also harbors the *erm* gene, *S. kanamyceticus* produces kanamycin and possesses the *aac* gene (Cundliffe 1989; Davies 1994). The first confirmation of “natural” resistance gene transfer in a clinical setting comes from a study of human infections in which mixed cultures of tetracycline-producing *Streptomyces* spp. and nontuberculous *Mycobacterium* spp. were found to possess identical genes for resistance to tetracycline (Pang et al. 1994). Both microbial species contained the resistance genes *tet*K and *tet*L, known to be the basis of resistance to tetracycline in gram-positive bacteria. What was surprising, however, was that both also contained the tetracycline resistance genes *otr*A and *otr*B, previously identified in the tetracycline-producing species *Streptomyces rimosus*. The tetK and tetL genes are considered “foreign” genes to both species (with a G+C content different from both) and must have been acquired as the result of a relatively recent gene transfer (Pang et al. 1994). This evidence is consistent with a gene transfer event between the streptomycetes and the mycobacteria, although it is not known which is the donor and which is the recipient.

Mechanisms of Gene Transfer Between Bacteria

Bacteria will often gain a defense against an antibiotic by acquiring a resistance gene from other bacteria in their vicinity. Bacteria can pick up and swap these resistance genes from other bacterial cells by one of three main mechanisms: transformation, conjugation, and transduction.

Transformation is the process in which bacteria can take up free DNA from the surrounding environment. The discovery of bacterial transformation dates from the late 1920's by the British scientist Frederick Griffith. The famed "dead mouse experiment" showed that bacterial cells that express one phenotype can be transformed into cells that express a different phenotype (Griffith 1928). Subsequent research by Avery, McLeod, and McCarty determined that the "transforming principle" was DNA (Avery et al. 1944). The importance of transformation in the spread of antibiotic resistance genes is not clear. Natural transformation in both gram-negative and gram-positive bacteria requires that the freed DNA remain stable and that potential recipient cells become competent to take it up. A gene is said to be successfully exchanged through transformation if it is taken in as part of a full plasmid, or if a fragment of DNA, containing the gene, becomes integrated into a recipient's chromosome. Unless a plasmid is transferred intact, transformation requires partial homology for DNA integration, as it depends on *recA* for controlled transformation (Olsen 1999). This would considerably narrow the range of bacteria that can successfully "transform" each other. Until recently, it was assumed that transformation would not occur in most places, because free DNA would not be stable in soil or water. However several studies have now demonstrated that free DNA can become stable by associating with soil components and that this DNA can be taken up by

competent cells (Lorenz and Wackeragel 1994; Miller 1998). Another investigation indicates that plasmid DNA has at times been transferred in river water and in the epilithon ("slimy layer") on river stones (Williams et al. 1996). There are no studies at this time indicating definitively that chromosomal genes have been transferred by transformation in aquatic or terrestrial environments.

Resistance genes can also be transferred by transduction, first described in studies relating to the *Salmonella* bacteriophage P22 (Zinder and Lederberg, 1952). In transduction, bacteriophages (viruses that infect bacteria) "extract" genetic material from one bacterial cell and inject it into another. Bacteriophages are capable of transferring whole plasmids and pieces of chromosomes between hosts, although full chromosomes are too big to fit into bacteriophages (Lewin 1997). Unlike transformation, transduction can occur in a wide range of bacteria, although again its importance in the spread of antibiotic resistance genes is not well defined. Laboratory experiments indicate that some bacteriophages, such as 71A-6 from *Vibrio vulnificus* (Khan et al. 2001) can apparently infect several species and even genera of bacteria, suggesting they might capable of "broad-casting" bacterial genes, such as those for resistance, well beyond the locale where they first took up the genes (Miller 1998). Transfer of antibiotic resistance genes by transduction has actually been demonstrated in *Staphylococcus* (el-Sohl et al. 1986) and in *Pseudomonas* (Hupkova et al. 1994).

Probably the most common way resistance genes are picked up is by conjugation, where there is formation of a "bridge" between two bacteria, through which genetic material can be transferred from one to another by cell-to-cell contact. There are a number of conjugative elements and types of mobile DNA, such as plasmids,

transposons, and integrons, that can be transferred in this fashion (see next section: **Vectors of Antibiotic Resistance**). Conjugation was first described in 1946 by Lederberg and Tatum, based on studies that the intestinal bacteria *E. coli* uses a process resembling sex to exchange the circular, extrachromosomal elements, now known as plasmids (Lederberg and Tatum 1946). Plasmids were not linked to transferable drug resistance ("R-factors") until the early 1960s, in a retrospective study describing the emergence of multiple antibiotic resistance in clinical isolates of *Shigella* from 1952-1955 (Wantanbe and Fukasawa 1961). The same resistance patterns were observed in many strains of different serotypes of *Shigella*, as well as in *E. coli* and *Citrobacter freundii* strains isolated from the same patients, ruling out the possibility of clonal selection. The resistance could also be transferred to sensitive strains by cell-to-cell contact, but not when a filter (permeable to DNA and bacteriophages) separated the bacteria. Conjugative plasmids were present in pathogenic clinical isolates of bacteria, such as *Shigella*, *E. coli*, *Klebsiella*, and *Proteus* (from the Murray collection) prior to the use of antibiotics, but they did not carry resistance genes (Hughes and Datta 1983). It has been suggested that the evolution of R-plasmids, some of which carry as many as seven different resistance determinants, is a recent evolutionary event, selected for by the intensive use of antibiotics in clinical therapy (Davies 1997).

Vectors of Antibiotic Resistance: Plasmids, Transposons, and Integrons

While the spread of antibiotic resistance is mediated through the mechanisms of transformation, conjugation, and transduction, the actual transfer of resistance genes from one DNA molecule to another is mediated by "vectors" in the form of specialized mobile genetic elements. Mobile genetic elements are integral parts of most bacterial genomes

and bacteria employ a diverse array of these mobile elements, including most commonly plasmids, transposons, and integrons, to disseminate and stockpile antibiotic resistance genes. They often act as "molecular genetic toolboxes" (Mahillon 1998) at a cell's disposal, in case of necessary (in terms of a bacteria's changing environment) genetic and genomic re-arrangement or transfers. These vectors are central to the integration of resistant genes into the bacterial chromosome, as well as the development of multiresistant strains of bacteria due to plasmid carriage.

Plasmids

Plasmids are one of the key players in the team of mobile genetic elements that fuel bacterial adaptability and diversity, and are capable of promoting the rapid spread of antibiotic resistance genes. Most bacteria contain plasmids in addition to their chromosomes (Stanisich 1988). By definition, a plasmid is a unit of extra-chromosomal inheritance (Thomas 2000). Most plasmids are self-replicating, circular molecules of DNA that are maintained in a bacterial cell in a stable manner (Lewin 1997). Some plasmids also have alternative lifestyles. They can exist either in the autonomous extra-chromosomal state or they can be inserted into the bacterial chromosome and are then carried as part of it like any other genetic sequence (Lewin 1997). Such units are properly called "episomes", although the terms "plasmid" and "episome" are sometimes used loosely as though interchangeable.

Natural plasmids vary considerably in size (from a few hundreds of base pairs to hundreds of kilobases), in copy number (from 1 to about 30), in host range (from one to several species), and in the various genetic traits conferred to their hosts (resistance to antibiotics and heavy metals, pathogenicity factors, degradation of aromatic compounds,

UV resistance, etc.) (Top et al. 2000). Whatever the size of a plasmid, however, their genes and sequences required for plasmid replication and its control are usually clustered in a small region called the basic replicon (Espinosa et al. 2000). Generally, basic replicons do not exceed 3 kb and consist of: (1.) an origin of replication (*ori*); (2.) *cop/inc* genes(s) involved in the control of the initiation of replication and, in most cases, (3.) *rep* gene(s) encoding Rep proteins required for replication, and often participating in its control (Espinosa et al. 2000).

Plasmids, as self-replicating and mobile genetic elements that are separate from the chromosome, generally provide accessory, but not essential functions to their hosts. In particular, traits that confer adaptations to locally restrictive conditions tend to cluster on plasmids (Eberhard 1989). As such, and in spite of any energetic drain imposed upon host cell metabolism, plasmids can be viewed as desirable elements for host cells. They provide a mechanism for carriage of functions that can be required for survival under conditions of environmental stress, but that are dispensable at other times. From the plasmid's selfish point of view, it makes sense to provide selectable functions to potential host cells, as this can enhance host competitiveness and thus host and plasmid survivability in cases of selective pressure in the environment (van Elsas et al. 2000).

Plasmids can be classified in a variety of ways depending on the purpose of classification, but the most commonly used system is based on replicon type, leading to the generation of incompatibility groups. Often it has been convenient to divide plasmids into two main groups, narrow and broad-host range or promiscuous plasmids. Broad host range plasmids are defined as those for which replication is not restricted to one particular species of bacteria, or more typically, a plasmid that can replicate in many of a

selected group of host species (Valla 1998; Szpirer et al. 1999). Broad host range plasmids may further be subdivided into two sub-groups, those which are conjugative or self-transmissible and non-conjugative plasmids. However, many of the non-conjugative plasmids may be conjugatively transferred via mobilization mediated by other plasmids in the same cell.

Bacteria isolated more than 50 years ago had the plasmids, but not the resistance genes that are found on those plasmids today (Hughes and Datta 1983). The discovery in the early 1960's of infectious multiple resistance plasmids in pathogenic bacteria (Akiba et al. 1960) provided the first unveiling of a "vector" of antibiotic resistance and the first notion of how antibiotic resistance genes move around. This discovery led directly to the finding that these resistance plasmids moved between different bacterial species (Mitsuhashi et al. 1977), thereby demonstrating that horizontal gene transfer was a natural process among wild-type bacteria and that plasmids could serve as natural vectors for antibiotic resistance genes. The Akiba and Mitsuhasi results changed the way we think about the epidemiology of antibiotic resistant bacteria. Plasmids can play an important role in the acquisition and dissemination of antibiotic resistance genes in bacteria. We now know that plasmids can encode a basic repertoire of "survival" functions that ensure that they can replicate and spread efficiently and that many plasmids carry genes that specify resistance to antibiotics.

Plasmids can act as vectors of these antibiotic resistance genes and promote the rapid spread of antibiotic resistance in a number of ways (see review in van Elsas et al. 2000). First, in many cases, when a gene, such one encoding antibiotic resistance, moves onto a plasmid, its copy number per bacterium rises, so that there is a greater chance for

mutations to show up in one of the copies. Secondly, plasmids that are self-transmissible or mobilizable increase the chance of antibiotic genes moving between bacteria. Thirdly, plasmids remove the need for an antibiotic gene to integrate into the bacterial chromosome in order to become established in a new bacterium. Furthermore, most plasmids can replicate within the species of at least one genus, so that they can easily spread between the species of that genus. A significant number of plasmids can also replicate within the species of many genera, what are known as "broad-host-range" plasmids. It is these self-transmissible, broad-host-range plasmids that are possibly the most active vehicles for a potential "horizontal gene pool" (Thomas 2000) of antibiotic resistance genes that are available to many bacteria, of many species, and even families.

Transposons

Antibiotic resistance genes on plasmids or in the bacterial chromosome are often located on transposons, genetic elements capable of moving from one DNA molecule to another, independent from the normal *recA*-dependent type of recombination. Following the discovery of R-factors, it was found that many antibiotic resistance genes were located on small pieces of DNA that can independently move around. The original concept of a DNA segment capable of physical movement inside a genome dates back to the early 1970s, when Hedges and Jacob (1971) proposed that the gene associated with resistance to ampicillin was part of a mobile genetic entity they named a "transposon". This was, in fact, the end of a long story that started with the obscure, then ridiculed, work of Barbara McClintock on the variegation of maize kernels (McClintock 1951). In the early 1950s, she postulated, based purely on non-molecular genetic experiments, the existence of "controlling" elements that were not following Mendel's rules of

segregation, but rather behaved as if they had some physical independence inside the plant genome. These same genetic elements or "jumping genes" are now known as transposons.

Transposons are linear pieces of DNA that range in size from 2.5 to 23 kilobase pairs (kb) and contain two identical insertion sequences (IS elements), one at each end of the molecule (Lewin 1997). Transposons are capable of moving from one DNA molecule to another, independent from the normal, *recA*-dependent type of recombination. The insertion sequences act as a point of insertion into a new molecule, as well as contain the transposase enzyme required for transposition (Kleckner 1981). Different genes, such as toxin genes and antibiotic resistance genes, can be located between the IS elements. The IS sequences of some transposons (e.g. Tn*5*) are themselves capable of independent movement, not carrying with them the antibiotic resistance genes (Berg and Berg 1983). These IS elements, are therefore sometimes found, not only flanking resistance genes, but also inserted into the bacterial chromosome at random sites. This finding suggests that transposons such as Tn*5* may have evolved by the movement of two IS elements to flank an antibiotic resistance gene, thus forming a composite unit (Berg et al. 1984). A "composite transposon" is a modular unit consisting of an antibiotic resistance gene(s) flanked by two IS elements.

Transposons appear to be ubiquitous in nature and have been identified in many types of organisms, including plants and animals, as well as bacteria (Berg and Howe 1989; Sherrat 1989). There are several "classes" of transposons, grouped according to their general structural and functional organization, using features such as size, conserved DNA regions, number of open reading frames (ORFs), presence of host genes, and

particularly the transpositional pathway (Mahillon 1998). Some transposons, such as Tn*10*, which encodes resistance to tetracycline, move in a "conservative" manner, without replication (Kleckner 1989). Other transposons, however, copy themselves to a new location, as well as maintaining the original location; the transposition includes replication of the transposon (review in Lupski 1987). Conjugative transposons, found in many gram-positive and a few gram-negative bacteria, are integrated DNA segments, that excise from the chromosome to form a circular intermediate. The circular intermediate then transfers itself to a recipient where it integrates once again into the chromosome (Salyers and Shoemaker 1997). Conjugative transposons, resemble conventional transposons, in that they integrate into DNA, but the mechanism of integration is completely different from that used by "regular" transposons. Unlike regular transposons, conjugative transposons regenerate the DNA segment from which they are excising and form a circular intermediate that is self-transmissible by conjugation. Conjugative transposons resemble plasmids in that they are transferred by conjugation and have a circular intermediate, but the circular intermediate does not replicate (Salyers and Shoemaker 1997).

Transposons enable antibiotic resistance genes to spread rapidly by both vertical evolution (successive generations) within a particular strain, as well as horizontal spread to other bacteria. Horizontal spread may occur by the movement of transposons to transmissible plasmids or to transducing bacteriophages that have a broad host range. Conjugative transposons are probably responsible for at least as much resistance gene transfer as plasmids, especially among gram-positive bacteria and they also have a very broad host range. Conjugative transposons, for example, Tn*916*, can transfer not only

among species within the gram-positive or within the gram-negative group, but also between gram-positive and gram-negative bacteria (Bettram et al. 1991; Scott et al. 1988; Scott and Churchward 1995). Like self-transmissible plasmids, conjugative transposons can mediate the transfer of other DNA. They can mobilize co-resident plasmids and some can mediate the transfer of linked segments of chromosomal DNA (Salyers et al 1999). Unlike phages and some plasmids, most conjugative transposons do not exclude closely related elements unless they have a single site that is no longer available once it is filled. This allows a bacterial strain to acquire more than one conjugative transposon. There is also some evidence that related conjugative transposons increase each other's transposition frequencies when they are present in the same strain (Salyers and Shoemaker 1997).

Integrons and Gene Cassettes

Although plasmids and transposons play a significant role as vectors in the acquisition and dissemination of antibiotic resistance genes, we now know that there are other routes for the incorporation of resistance genes into the bacterial genome, particularly into the plasmids and the transposons themselves. Detailed genetic analysis revealed that the regions that flank antibiotic resistance genes in many transposons and several broad host range plasmids of gram-negative bacteria often show a high degree of similarity (Ouellete and Roy 1987; Sundstrom et al. 1988; Cameron et al. 1986; Wiedermann et al. 1987; Hall and Vockler 1987). This realization led to the discovery of a new mobile genetic element, dubbed the "integron" (Stokes and Hall 1989). The term integron was originally coined to describe the group of apparently mobile elements which contain one or more antibiotic resistance genes located at a specific site, and also contain

the determinants of the site-specific recombination system responsible for insertion of the resistance genes (Stokes and Hall 1989).

Integrons are “natural” vectors, gene capture and expression systems (Ploy et al. 2000), for exogenous DNA that incorporate open reading frames and convert them into functional genes. The integron vector codes for an integrase gene (*intI*) that mediates recombination between a proximal primary recombination site (*attI*) and a secondary target called an *attC* site (or 59 base pair element). The *attC* site is normally found associated with a single open reading frame (ORF), and the *attC*-ORF structure is termed a gene cassette (Hall and Collis 1995). Insertion of the cassette at the *attI* site, which is located downstream of a resident promoter internal to the *intI* gene, drives expression of the encoded proteins (Levesque et al. 1994). Most of the *attC* sites of the integrons cassettes identified to date are unique. Their length and sequence vary considerably (from 57 to 141 bp), with similarities primarily restricted to their boundaries. Two consensus sequences of 7 bp frames the boundaries of each *attC* site and are designated the “core site” and “inverse core site”. These are the actual targets of the recombination process (Hall et al. 1991).

More than 60 different antibiotic resistance genes, covering many of the antimicrobial drugs presently in use, have been characterized in cassette structures (Mazel and Davies 1998; Mazel et al. 2000; Nield et al. 2001), as well as genes conferring resistance to disinfectants and heavy metals, such as mercury. Open reading frames encoding unknown function, but associated with *attC* sites in the form of cassettes, have also been described among several integrons (Recchia and Hall 1995).

At least eight distinct classes of chromosomal and plasmid-borne integrons have been identified, based on sequence differences between the integrases they encode. Classes 1, 2, and 3 are the best studied and all are implicated in antibiotic resistance (Recchia and Hall 1995).

Class 1 includes the majority of integrons found in clinical isolates to date and members of this group were those originally classified as integrons (Stokes and Hall 1989). These elements constitute the most intensively studied integrons and are the only group for which gene cassette movement has been demonstrated experimentally (Hall and Collis 1995). The integrase of class 1 integrons, *intI*1, is a 337 amino acid protein (Ploy et al. 2000a, 2000c). The structure of the class 1 integrons includes a 5' and 3' conserved segment and a variable region (Levesque and Roy 1993; Liebert et al. 1999; Paulsen et al. 1993; Stokes and Hall 1989). The 5' conserved segment consists of the integrase gene, *IntI*1, and a promoter region for expression of the inserted gene cassette(s) (Stoke and Hall 1989). Most class 1 integrons have a 3' end region containing three open reading frames. The first, *qac*EΔ1, is a truncated derivative of the *qac*E gene, conferring resistance to quaternary ammonium compounds (Paulsen et al. 1993). The second reading frame is the *sul*1 gene, which encodes resistance to sulfonamides (Stokes and Hall 1989). The third open reading frame, ORF5, does not code for any known function. The variable region, located between the two conserved segments, is the site for the insertion of the antibiotic gene cassettes (Gravel et al. 1998; Hansson et al. 1997; Stokes et al. 1997).

Class 2 integrons are present on transposon Tn*7* and related elements. The *intI*2 integrase gene encodes a protein exhibiting 46% homology with *intI*1, but truncated by

12 amino acids (Ploy et al. 2000b). The 3' end region of class 2 integrons is composed of genes involved in the transposition mechanism of Tn*7* (Recchia and Hall 1995). Class 2 integrons contain a dihydrofolate reductase gene cassette (Fling and Richards 1983).

A third class of integrons, whose integrase *IntI*3 exhibits 61% identity with *IntI*1, was described in 1995 (Arakawa et al. 1995) in *Serratia marcescens*. Only one integron of class 3 has been described (Arakawa et al. 1995) in a transposon-like element with several gene re-arrangements (Shibata et al. 1999). This integron carried the bla_{imp} gene, which encodes broad-spectrum β–lactam antibiotic resistance.

A fourth class of integron, dubbed the "super-integron", has been identified in several *Vibrio* species, including *V. cholerae* (Mazel et al. 1998) and *V. metschnikovii* (Rowe-Magnus et al. 1999). This chromosomal super-structure harbors hundreds of gene cassettes, and the encoded functions, when identifiable, are linked to adaptations extending beyond antibiotic resistance and pathogenicity (Mazel et al. 1998; Heidelberg et al. 2000). The cassette-associated *attC* sites of Class 4 integrons, termed VCRs, for *Vibrio cholerae* repeats, display a high degree of sequence relatedness (Rowe-Magnus et al. 1999) and the activity of the associated integrase is identical to that of the class 1 integrase, *intI*1. The integrase enzymes found in both these species of *Vibrio*, *IntI*4 and *IntI*6 respectively, apparently insert repeated sequences of genes in clusters that mirror the gene cassette arrays typically found in other integrons (Rowe-Magnus et al. 1999; Mazel et al. 1998). Equivalent integron superstructures have now been identified in nine distinct bacterial genera (Rowe-Magnus et al. 2001). The partial sequence for a fifth *intI* gene, *intI*5, associated with *Vibrio mimicus*, has been reported as having 75%

identity with *intI*4 (Clark et al. 2000). It is unclear whether this represents a new integron class or whether it represents another example of a super-integron.

Lastly, a very recent study recovered integron sequences from environmental DNA samples (Nield et al. 2001). The sequence diversity of nearly complete integron sequences in these samples was sufficient to classify them as belonging to three previously undescribed classes of integrons (Nield et al. 2001).

The integron/gene cassette system provides a simple mechanism for the acquisition and dissemination of "new" antibiotic resistance genes by existing bacterial genomes. Although integrons themselves are defective for self-transposition (Brown et al. 1996; Rowe-Magnus and Mazel 1999), they are often found associated with insertion sequences, transposons and/or conjugative plasmids which can serve as vehicles for their intra- and interspecies transmission (Rowe-Magnus et al. 1999; Liebert et al. 1999). The potency of such a highly efficient gene capture and expression system, in combination with broad-host range mobility, is obvious. Indeed, the strength of this partnership is confirmed by the wide spread occurrence of integrons in nature, in human, animal, and environmental isolates (Nield et al. 2001; Goldstein et al. 2001; Bass et al. 1999; Chang et al. 1997, Schnabel and Jones 1999; Daly et al. 2000; Lucey et al. 2000). That horizontal and vertical transfer readily occurs is demonstrated by the presence of many "common" gene cassettes among the Enterobacteriaceae and *Pseudomonas*, as well as the marked differences in codon usage among cassettes in the same integron, indicating that the antibiotic resistance determinants are of diverse origin (Rowe-Magnus and Mazel 1999).

The integron/gene cassette system also allows bacteria to stockpile exogenous DNA, such as antibiotic resistance genes and is believed to have played an important role in the development of multiple drug resistance. The finding of super-integrons with gene cassettes coding for other determinants, such as biochemical functions and virulence factors (Rowe-Magnus et al. 1999) implies a role for these structures in bacterial genome evolution before the antibiotic era. Integrons and super-integrons may represent a means for rapid adaptation to unpredictable changes in the environment by allowing bacteria to scavenge foreign genes that may ultimately endow them with increased fitness.

Selection for Antibiotic Resistance: the Role of Antibiotics

Bacteria become resistant to antibiotics either by mutations or by acquisition of a "pre-existing" resistance gene (or genes) by horizontal transfer from another organism. Prior to 1950, scientists were divided into two camps: those that believed resistance to be the result of post-treatment adaptation by the microbes, that is, that bacteria were "trained" to withstand the "poisonous" antibiotics (Hinshelwood 1946) and those that believed that random mutations were responsible for the development of all types of microbial diversity. The matter was solved in 1952 by a publication that introduced the technique of "replica-plating", which allowed the study of mutations independent from selection pressures (Lederberg and Lederberg 1952). The experiment in this study proved that streptomycin-resistant *E. coli* could be isolated and propagated without the selection pressure from streptomycin. This led to the important realization that mutations, as well as gene transfer of resistance determinants, for that matter, can happen independent of the use of antibiotics.

Once a mutation exists in, or an antibiotic resistance gene is acquired by a bacterium in the population, and antibiotics are then introduced, there exists the conditions whereby that "resistant bacterium" may become predominant in the population. Emerging clones of resistant bacteria actually are the result of selection by antibiotics (Levy 1992b; Levy 1998a). Antibiotic use selects, and promotes the evolution and growth of, bacteria that are resistant to that drug. The selection process is fairly straightforward and the scenario unfolds as follows. When bacteria are exposed to an antibiotic, bacterial cells that are susceptible to the drug will die. But cells that have some resistance from the start, or that acquire it later (through mutation or gene exchange) may survive and grow, especially if too little drug is given to overwhelm every last cell that is present. Those cells, facing reduced competition from the susceptible bacteria, will then go on to proliferate. When confronted with the antibiotic, the most resistant cells in a group will inevitably outcompete all others. Over time, such "antibiotic-resistant populations" can become predominant in a localized environment, be it the gut of an animal or human, an ICU, a whole hospital, a nursing home, or the broad community. Recognizing that antibiotics select for the growth and dissemination of otherwise rare, resistant microorganisms, this ability of antibiotics has been termed "the antibiotic paradox" (Levy 1992b): that antibiotics meant to overwhelm bacteria in a clinical infection, can actually select for the survivors which then thwart their efficacy.

It has also been argued that resistance cannot be fully explained by antibiotics selecting antibiotic resistant organisms, as they are in part (the bacteria, that is) only the "side-effects" of the evolution of subcellular entities that infect microorganisms and spread resistance genes (Heinemann 1998). This is akin to the "selfish gene" theory

(Dawkins 1976): i.e. the selection is not necessarily for resistant bacteria, but for the "vectors" that carry the resistance genes. Most resistance genes are mobile, moving from organism to organism as part of horizontally mobile elements, such as viruses, conjugative plasmids, transposons, and integrons, or flow between organisms through processes such as natural transformation (Lorenz and Wackernagel 1994; Baquero et al. 1998; Souza and Eguiarte 1997). The evolution of these horizontally mobile elements, because of their autonomous reproduction, can be very different from the "cellular life" carrying such elements (Souza and Eguiarte 1997).

There are also a number of ways in which antibiotics have been shown to specifically effect the development and evolution of resistance in bacteria. Some antibiotics are known to enhance the antibiotic resistance gene transfer between bacteria in humans and animals (Salyers and Shoemaker 1996) and influence the frequency of such events *in vitro* (Davies and Wright 1997). Antibiotics have been shown to increase gene transfer frequency in the laboratory by reducing the effectiveness of the cell surface as a barrier to the release and uptake of genetic material or making the bacteria susceptible to fusion with other bacteria and vesicles. For example, antibiotics have been shown to influence DNA uptake by weakening the bacterial cell wall and making cells more permeable to DNA, and/or decreasing the concentration of periplasmic nucleases (Vaara 1992; Dreiseikelmann 1994). β-lactam antibiotics have been shown to increase the frequency of interspecies DNA transmission between combinations of *E. coli*, *Staphylococcus aureus*, *Listeria moncytogenes*, *Streptococcus faecalis* and *Bacillus anthracis* (Ivins et al. 1988; Trieu-Cuot 1993).

Some antibiotics induce the genes controlling horizontal transfer by acting on the "vectors" themselves, and consequently, on their antibiotic resistance genes. For example, transmission of some conjugative transposons is regulated by levels of the antibiotic. Tetracycline stimulates transmission of a transposon that carries tetracycline resistance and is native to the gram-negative *Bacterioides*, by 100-1000 fold (Salyers et al. 1995). The transmission of *Tn*925, a conjugative transposon native to many gram-positive bacteria and like the *Bacteriodes* transposon encodes tetracycline resistance, is also responsive to the drug. The transmission of this transposon to antibiotic-sensitive bacteria was enhanced 5-100 fold following culture of the resistant host in tetracycline (Torres et al. 1991).

Although antibiotics may effectively halt bacterial reproduction, they rarely inhibit the metabolism necessary for accepting and distributing the genes on plasmids Such cells, called "dead vectors" because the antibiotic prevents the cells from dividing, none-the-less may sometimes remain active in the process of conjugation via plasmid-mediated transfer of genes (Heinemann 1998). For example, bacteria harboring plasmids that contain antibiotic resistance genes may continue to transfer the plasmid long after the "donor" bacterium has been killed. Mitomycin-C at some doses irreversibly damages DNA, but bacteria killed by these agents can still receive plasmids and then redistribute them (Heinemann 2000). Thus, it is possible that an antibiotic may convert a bacterial pathogen into a vector that is undetectable by conventional culture assays, but may persist in the patient or any other local "environment". If such "sneaky" vectors persist long enough, they may transfer antibiotic resistance genes to other microorganisms in the patient or environment (Heinemann 1998).

Some antibiotics are able to induce resistance to other drugs. For example, induction or constitutive expression in multiple-antibiotic resistance operator (*marO*) or repressor (*marR*) mutants confers cross-resistance upon cells to dissimilar agents by decreasing intracellular concentrations (Miller and Sulavik 1996). The *marO* and *marR* mutants selected on tetracycline or chloramphenicol were 1000 times more likely to also acquire resistance to the structurally unrelated fluoroquinolones, compared with populations exposed first to the fluoroquinolone itself (Cohen et al. 1989).

Some bacteria can adapt their physiology to new environments following the reception of a signal (usually from the new environment) to switch physiological states (Heinemann 1999). By mimicking signals that induce alternative physiological states, some antibiotics appear to induce phenotypic resistance to themselves and other drugs. For example, it was found that exposing *Pseudomonas aeruginosa* to the aminoglycoside antibiotic gentamycin for brief periods of time, induces resistance to the drug among those few that survive the initial exposures (Karlowsky et al. 1997). The resistance phenotype can also be induced by depriving the bacteria of Mg^{2+}. Gentamycin-resistant cells concomitantly display resistance to other toxic agents, such as netilmicin, tobramycin, neomycin, kanamycin, streptomycin, polymyxin, and EDTA (Karlowsky et al. 1997). That this is a “physiological” rather than “mutational” adaptation is inferred from the uniform appearance of the phenotype, after induction by either Mg^{2++} starvation or gentamycin, and then its uniform reversal in descendent generations. Although the mechanism of physiological adaptation is not known, it may be associated with a secondary effect that gentamycin has on the pathway for drug uptake.

Antibiotics can also act to increase recombination and mutation rates. Drugs that cause DNA damage, such mitomycin-C, can directly elevate the frequency of mutation (Lorenz and Wackernagel 1994). Antibiotics that affect "translational fidelity" can also boost the mutation rate in bacteria. Mutants arose more frequently during culture of wild-type strains of *E. coli* in the presence of streptomycin, which decreases translational fidelity, than during culture in the absence of streptomycin (Boe 1992). The antibiotic was not a mutagen *per se*, because the mutation rate of strains resistant to streptomycin was independent of culture conditions. Theoretically, by stimulating the basal mutation rate, antibiotics increase the probability that a new resistance determinant will arise in a population. If the mutant gene is on some type of horizontally transmissible vector, the gene has the potential to be disseminated even independently of the survival of the host cell.

As a final footnote, antibiotics may be (or may have been) involved in the development of antibiotic resistance by virtue of actually being the source of antibiotic resistance genes. A study by Webb and Davies (1993) found that a number of commercial antibiotic preparations were contaminated with DNA sequences containing antibiotic resistance genes from the organism used in their production. These researchers proposed that genes encoding resistance to antibiotics have inadvertently been co-administered with antibiotics for years and that under the simultaneous selection pressure of the antibiotic, uptake of one or more resistance genes by a bacterium in the host could lead to antibiotic resistant organisms being constructed by natural "genetic engineering". Subsequent inter- and intraspecific genetic transfers would permit other bacteria to become resistant to the antibiotics also. They also propose that, as some antibiotic-

producing organisms possess resistance mechanisms for several antibiotics, including some for antibiotics not produced by the organism, resistance to an antibiotic may be acquired from a preparation of a different antibiotic that is contaminated with DNA.

The Cost of Resistance

Conventional wisdom in the older evolutionary and microbiological literature assumed that bacteria (and other microorganisms) must pay a metabolic or physiologic "price" for the acquisition of antibiotic resistance, be it by mutation or carriage of an accessory element, such as a plasmid. In an environment that contains an antibiotic, possession of a corresponding resistance gene is clearly beneficial to a bacterium and worth "the cost". It was thought, however, that in the absence of an antibiotic, resistant genotypes may have reduced growth rates and be at a competitive disadvantage compared to their sensitive counterparts. It was believed that the frequency and rates of ascent and dissemination of antibiotic resistance in bacterial populations was directly related to the volume of antibiotic use and inversely related to the cost that resistance imposes on the fitness of the bacteria (Andersson and Levin 1999).

A number of older studies have indeed shown that resistant genotypes are less fit than their sensitive progenitors in an "antibiotic-free" medium, indicating a cost to the mechanisms of resistance. The principle element of these studies involved competition between sensitive and resistant genotypes that are otherwise isogenic (Lenski 1997). Some of these studies demonstrated costs associated with the carriage of plasmids and expression of plasmid-encoded resistance functions (Zund and Lebek 1980), whereas other studies have demonstrated the side-effects of mutations that impair growth (Jin and Gross 1989).

These older studies were performed, however, by putting an antibiotic resistance gene (either a plasmid-encoded function or a chromosomal mutation) into "naïve" bacteria, which have had no evolutionary history of association with the resistance genes (Lenski 1997). Evolutionary theory suggests that bacteria might overcome the cost of resistance by evolving adaptations that counteract the harmful "side-effects" of resistance genes. Several recent laboratory studies have now shown, that although most resistance genes do engender some fitness cost, at least initially, that cost of resistance may be substantially diminished, even eliminated, by evolutionary changes in bacteria over rather short periods of time.

Bouma and Lenski (1988) found that growth of *E. coli* containing the drug resistance plasmid pACYC184 for 500 generations, with selection for maintenance of the plasmid, resulted in adaptation of the host to eliminate the cost of plasmid carriage. The "adapted" *E. coli* host containing pACYC184 had a competitive advantage over the original plasmid-free *E. coli* host, even when grown in the absence of the antibiotic. Selection for increased fitness during long-term growth resulted in a strain that actually grows better with pACYC184 than without it. A similar study examined the co-evolution of *E. coli* and a derivative plasmid pBR322 that encodes resistance to ampicillin and tetracycline (Modi et al. 1991). After approximately 800 generations, they found that the cost of plasmid carriage to the bacterial host had been significantly reduced.

The finding that the cost of antibiotic resistance can be reduced is not restricted to plasmid-encoded resistance. Schrag and Perrot (1996) examined mutations in the *rpsL* gene of *E. coli* that confers resistance to streptomycin. These resistant mutants have altered ribosomes and a lower rate of peptide-chain elongation (which may account for

the cost of resistance). In the absence of the antibiotic, cells that carried the mutations had a substantial growth disadvantage compared to the streptomycin-sensitive parent strain. However, allowing the streptomycin-resistant *rpsL* mutants to evolve for 180 generations, in the absence of streptomycin, resulted in faster growing mutants that had almost no growth disadvantage compared to their streptomycin-susceptible parents. Contrary to expectations, this "cost-reduction" was achieved not by back mutations at the *rpsL* gene, but by secondary site mutation(s) outside of the *rpsL* gene, that increased their growth rate, without loss of streptomycin resistance (Schrag and Perrot 1996). In fact, it was found that having adapted to the *rpsL* mutations, there was now a fitness cost to losing streptomycin resistance (Schrag et al. 1997). The implication is that resistance to streptomycin (by ribosomal alterations) may initially slow growth, but secondary mutations quickly arise that eliminate this cost, producing "fitter" variants in which reversion to antibiotic susceptibility may now be costly.

The conclusions of these more recent *in vitro* studies are that bacteria do appear to adapt to the cost of antibiotic resistance, through secondary mutations that compensate for the loss of fitness, but do not reduce the level of resistance. However, these experiments were all performed in the laboratory under highly simplified conditions. It is very difficult in a natural setting to perform the rigorous manipulative experiments that are necessary to quantify subtle differences in competitive fitness. It is known from some experimental research that interactions between bacteria and the complex environment of their hosts cannot be gleaned from *in vitro* studies alone. The genes expressed by bacteria inhabiting a mammalian host are very different from those expressed by bacteria living in a petri dish (Waldor and Mekalanos 1996). There are two studies, however, that

suggest that bacteria inhabiting an animal host also evolve to compensate for the cost of resistance. An unpublished random survey of *E. coli* collected from the CDC day-care center in Atlanta, GA (Levin 2001) found that more than 25% of the bacteria sampled from the diapers of 25 infants were still resistant to streptomycin, an antibiotic physicians have rarely used in this country during the last 30 years. A study by Bjorkman et al. (2000) also showed that not only do bacteria in animal hosts adapt to the cost of resistance through secondary mutations that compensate for the loss of fitness, but that in *Salmonella*, the process of adaptation to the costs of resistance are different, depending on whether the bacteria "grew" in mice or in culture broth. The investigators again looked at streptomycin resistance conferred by mutations in the *rpsL* gene. Although neither culture broth nor mice were treated with streptomycin, the adaptation to the costs of streptomycin resistance was through compensatory mutations rather than reversion to a drug-sensitive phenotype. Interestingly, however, the mutations were different, depending on whether the bacteria had grown up in mice or in culture broth. In broth-grown bacteria, all compensatory changes were extragenic, that is, located in the *rpsD* and *rpsE* genes, not the *rpsL* gene. In contrast, in all ten mice studied the compensatory mutations were located in *rpsL*, within the same codon. All the streptomycin resistance compensatory mutations were accompanied by relatively high bacterial fitness, regardless of whether the bacteria were grown in mice or broth. One immediate implication of this study is that the nature of evolved, compensatory changes is environment-dependent, which suggests that evolution to compensate for fitness losses caused by resistance or other genetic alterations, might occur by different mechanisms depending on whether within a host or outside a host. The inference is that making predictions about the

evolution of antibiotic resistant bacteria *in vivo* requires that at least some of the experiments be performed *in vivo*.

From a clinical and public health perspective, the current studies have clear, and rather frightening implications. The biological cost incurred by antibiotic resistance has often been touted as a route to combating the ever-increasing number of drug resistant pathogenic bacteria. The rationale is that because resistance exacts a cost, its incidence will wane if we administer antibiotics "more prudently" (Levy 1994; 1997). These new studies on the "costs of resistance" challenge what has currently become the public health policy view (WHO 1997), that the frequency of antibiotic resistant bacteria will decline if antibiotic usage is reduced. These new studies demonstrate that even when bacterial resistance is associated with fitness costs, subsequent bacterial evolution reduces these burdens without reducing the level of resistance and that the rapid adaptation to the cost of resistance can occur in the absence of drugs selecting for resistance. These studies imply that since bacteria adapt to the cost of antibiotic resistance through secondary mutations that compensate for the loss of fitness, but do not reduce the level of resistance, it may not be enough to stop using the antibiotic…the bacteria are not going to revert to what they were before. Our current policies on antibiotic usage and restrictions may be a case of "locking the barn door after the horse is already out" and antibiotics that have lost their effectiveness may never again become the powerful weapons they once were.

Florfenicol Antibiotic Resistance

The Antibiotic Florfenicol: Description and Characteristics

One relatively new drug in the veterinary antimicrobial arsenal is florfenicol, or Nuflor (Schering-Plough, Kenilworth, New Jersey). Florfenicol [D-*d*-threo-3-fluoro-2-

dichromoacetamide-1-(4-methylsulfonylphenyl)-1-propanol] is a synthetic, broad-spectrum derivative of chloramphenicol, in which the nitro group is replaced by a methyl-sulphonyl group in the *para* position and the hydroxyl group by a fluorine (Neu and Fu 1980; Syriopoulou et al. 1981; Food and Drug Administration, FOI Summary NADA 141-063 1996).

This antibiotic was developed as a replacement drug for chloramphenicol, whose clinical use in both humans and animals was curtailed due to the serious side effects in humans, including bone marrow toxicity and irreversible aplastic anemia, reportedly linked to the nitro group in the benzene nucleus or its metabolites, in the bone marrow cells of genetically disposed individuals (Yunis et al. 1974, 1980; Yunis 1988). The search for a replacement for chloramphenicol was also necessitated due to significant increases in antibiotic resistance in members of the Enterobacteriaceae (Finland 1970; Anderson and Smith 1972; Kayser and Wurst 1974) and *Haemophilus influenzae* (Philpott-Howard and Williams 1982; Fraise et al. 1986), bacteria once successfully treated by this antibiotic. Florfenicol is not inactivated by the chloramphenicol acetyl tranferase (CAT) enzymes, which are responsible for most of the plasmid-mediated resistance to chloramphenicol (Dorman and Foster 1982), since the 3'-hydroxyl group, which is the site of acetylation in chloramphenicol, is replaced by a fluorine atom in florfenicol. It was speculated early on that florfenicol would be an effective substitute against many of the chloramphenicol-resistant strains of bacteria. An injectable formulation of this antibiotic is currently licensed for use in the treatment of bovine respiratory disease.

Florfenicol has been shown to have a broad-spectrum of activity, similar to that of chloramphenicol, except that it is active at lower levels than chloramphenicol against a wide range of clinical bacterial isolates (Neu and Fu 1980; Syriopoulou et al. 1981; Graham et al. 1988). Florfenicol is primarily bacteriostatic and its mechanism of action, like that of chloramphenicol, is inhibition of protein synthesis by binding the 50S subunit of the bacterial ribosome (Syriopoulou et al. 1981; Cannon 1990).

In vitro and Pharmacokinetic Studies

In vitro and *in vivo* antibacterial activity of florfenicol have been reported in the literature against a number of common bacterial pathogens. The initial *in vitro* activity studies conducted on florfenicol showed it to have much greater antimicrobial activity against a number of pathogenic bacteria than chloramphenicol (Neu and Fu 1980; Syriopoulou et al. 1981). Florfenicol was also shown in these studies to have antibacterial activity against some bacteria that are resistant to chloramphenicol (Neu and Fu 1980; Syriopoulou et al. 1981). In their studies using human clinical isolates, Neu and Fu (1980) found that florfenicol gave activity as good as, or better than chloramphenicol in most of the isolates tested, including many that were resistant to chloramphenicol. Florfenicol was active against the enteric bacteria *Citrobacter*, *Proteus mirabilis*, *Shigella*, *Salmonella*, *Providencia*, and *Bacteroides*. Isolates of *Staphylococcus aureus*, *Enterococcus*, and *Haemophilus influenzae* were also sensitive to florfenicol. Of a group of 15 specific chloramphenicol-resistant organisms, only *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Acinetobacter* exhibited resistance to both chloramphenicol and florfenicol. A second early *in vitro* study also found florfenicol to be more active than chloramphenicol against strains of *Shigella*

dysenteriae, *Salmonella typhi*, *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* (Syriopoulou et al. 1981).

Florfenicol was also shown to have excellent activity against a number of bacteria affecting cattle, including the primary bacterial pathogens involved in bovine pneumonias and "shipping fever," *Mannheimia haemolytica*, *Pasteurella multocida*, and *Haemophilus somnus*, making the antibiotic an ideal candidate for bovine respiratory disease (Varma et al. 1986a, 1986b; Varma et al. 1991). Indeed, this is currently the only approved usage of the antibiotic. A recent study also shows that these particular bacteria remain completely sensitive to florfenicol, with no evidence of developing resistance (DeRosa et al. 2000). In one other study determining the antimicrobial susceptibility of *Arcanobacterium pyogenes* isolated from cattle and pigs (Yoshimura et al. 2000) and another examining the *in vitro* susceptibility of *E. coli* isolated from diarrhoeic dairy calves (Orden et al. 2000), all isolates tested were also susceptible to florfenicol. However, in a study of the *in vitro* activity of florfenicol against field isolates of *Mycoplasma bovis*, there was a broad range in the MIC values, with more than 20% of the isolates being resistant to the drug (Ayling et al. 2000).

Several studies have examined the *in vitro* antibacterial activity of florfenicol against various fish pathogens. Fukui et al. (1987) found that florfenicol showed strong antibacterial activity against five common gram-negative pathogens encountered in aquaculture, *Photobacterium damselae* (formerly known as *Pasteurella piscicida*), *Vibrio anguillarium*, *Edwardsiella tarda*, *Aeromonas hydrophila*, and *Aeromonas salmonicida*, with little evidence of development of resistance. Subsequent *in vitro* studies also documented that *Vibrio anguillarium* isolated from the cultured ayu fish and

Photobacterium damselae (formerly classified as *Pasteurella piscicida*) from cultured yellowtail fish were highly sensitive to florfenicol (Yasunaga and Tsukahara 1988; Yasunaga and Yasumuto 1988; Kusuda et al. 1990; Zhao et al. 1992; Kim and Aoki 1993a). However, by 1992, florfenicol resistant strains of *P. piscicida* started to appear in yellowtail fish farms (Kim and Aoki 1993b). A more recent study examined the susceptibility of *Flavobacterium psychrophilum*, the causal agent of rainbow trout fry syndrome (RTFS), to florfenicol and found that all bacterial isolates tested were considered susceptible or moderately susceptible to the drug (Rangdale et al. 1997). Another recent study that investigated the *in vitro* antibacterial effects of florfenicol, chloramphenicol, and thiamphenicol against bacterial isolates from fish, soft-shell turtles, and shellfish, found that although florfenicol had the greatest antibacterial effect of the three drugs, there was a very wide range of MICs (from 0.20-100 μg/ml, where>25 μg/ml was considered resistant) and 17% of the aquatic isolates were resistant (Ho et al. 2000).

Finally, two recent reports included the *in vitro* activity of florfenicol against pathogenic bacteria isolated from poultry. In a study that included the *in vitro* activity of florfenicol against bacteria isolated from tissues of diseased turkey poults, examples of florfenicol resistant *E. coli*, *Citrobacter*, *Enterobacter*, *Klebsiella*, and *Pseudomonas* were found (Salmon and Watts 2000). Florfenicol resistant *E. coli* isolates were also detected in litter and clinical samples cultured from sick chickens (Keyes et al. 2000).

The initial pharmacokinetic studies of florfenicol were conducted in veal calves (Varma et al. 1986a; Adams et al. 1987), non-lactating dairy cows (Bretzlaff et al. 1987), and feed lot cattle (Varma et al. 1991) and provided information about the disposition of

the drug and effectiveness of routes of administration. Further studies to determine the absolute systemic availability of florfenicol after intramuscular (IM) administration showed that florfenicol attains a maximum of 3 μg/ml in the serum of feeder calves (Lobell et al. 1994; Food and Drug Administration NADA #141-063 1996) following IM administration of 20 mg/kg body weight to calf. Florfenicol was detectable in the serum of most of these animals through 60 hours, with a mean concentration of 0.19 μg/ml (Food and Drug Administration NADA #141-063 1996). Relatively low serum protein binding of florfenicol, 12.7%, 13.2%, and 18.3% at serum concentrations of 0.5, 3.0, and 16.0 μg/ml, respectively, was also reported (Lobell et al. 1994; Food and Drug Administration NADA #141-063 1996). Tissue florfenicol concentrations were about equal or higher than the corresponding serum concentrations (Adams et al. 1987; Lobell et al. 1994). Because of its low serum protein binding and extensive tissue distribution, florfenicol would be expected to reach clinically effective concentrations at sites of infection. The manufacturer's recommendations for use of florfenicol in the treatment of bovine respiratory disease (BRD) are in part based on these pharmacokinetic parameters.

Pharmacokinetic studies of florfenicol have also been conducted in horses (McKellar and Varma 1996), goats (Atef et al. 2000), pigs (Ueda et al. 1995), fish (Fukui et al. 1987), and poultry (Afifi and Abo El-Sooud 1997; Rios et al. 1997; El-Banna 1998), although there is no approval or label recommendations of the drug for these species. The peak plasma florfenicol concentration in ducks and chickens was found to be similar to that for cattle, approximately 3 μg/ml, with similar levels in the liver, kidney, and lung tissues (Afifi and Abo El-Sooud 1997; Rios et al. 1997; El-Banna 1998).

The Genetic Basis and Mechanism(s) of Florfenicol Resistance

In 1996, Aoki and Kim identified a gene sequence, pp-*flo*, on a plasmid from the fish pathogen *Photobacterium damselae* subspecies *piscicida* (formerly known as *Pasteurella piscicida*) isolated in Japan, that conferred resistance to both chloramphenicol and florfenicol (Kim and Aoki 1996). This was the first published report of a gene sequence associated with antibiotic resistance to florfenicol. Several recent studies have also revealed similar sequences in other bacteria that also confer dual resistance to both florfenicol and chloramphenicol. Two reports published almost simultaneously in May of 1999, described a gene, flo_{ST}/*floR*, with nearly complete homology to the pp-*flo* gene among *Salmonella typhimurium* DT104 strains isolated from cattle (Bolton et al. 1999; Arcangioli et al. 1999). These studies found that *Salmonella* DT104 harbors a chromosomal locus in which the *flo* gene and a tetracycline operon *tetR-tetA* are bracketed by two integron-like structures (Arcangioli et al. 1999; Bolton et al. 1999; Briggs and Fratamico 1999). This same antibiotic resistance gene cluster, including the *flo* gene, has also been found in *Salmonella enterica* serovar Agona isolates recovered from poultry in Belgium (Cloeckaert et al. 2000b). The *flo* gene has also been recently identified in florfenicol-resistant *Escherichia coli* from poultry in the United States (Keyes et al. 1999) and florfenicol-resistant *E. coli* from cattle in Europe (Cloeckaert et al. 2000a) and the United States (White et al. 2000). Molecular typing demonstrated that *flo* was located on high-molecular-weight plasmids in both the *E. coli* isolates from poultry (Keyes et al. 2000) and the *E. coli* cattle isolates from Europe (Cloeckaert et al. 2000a). The *flo* gene was also detected on high-molecular-weight plasmids in the majority, though not all, of the American florfenicol-resistant *E. coli*

isolates from cattle, suggesting that several of the resistant isolates may possess a chromosomally-encoded *flo* gene (White et al. 2000).

The mechanism of dual resistance to florfenicol and chloramphenicol conferred by the *flo* gene is not completely understood, but is currently thought to occur by a non-enzymatic efflux-type pump. It was suggested in one of the first studies examining *in vitro* activity of florfenicol that the mechanism of resistance to chloramphenicol analogues lacking a 3'-hydroxyl group is not by chloramphenicol acetyl transferase (CAT) enzymes, but may involve changes in cell membrane permeability or a mutation in a ribosome binding site (Syriopoulou et al. 1981). Kim and Aoki (1996) found that the predicted amino acid sequence encoded by pp-*flo* consisted of about 70% hydrophobic amino acid residues and showed 47.4% homology to the hydrophobic CmlA polypeptide that is responsible for non-enzymatic chloramphenicol resistance (Bissonette et al. 1991). The CmlA protein has significant similarity to the family of efflux and bacterial transport proteins. It is known that florfenicol is active against chloramphenicol resistant isolates coding for chloramphenicol acetyl transferases (Cannon et al. 1990) and chloramphenicol exporters, such as CmlA (Bissonette et al. 1990). Kim and Aoki (1996) also suggested that the predicted hydrophobic polypeptide product of the pp-*flo* gene may be a similar type of membrane protein having some role in florfenicol (and chloramphenicol) resistance. Subsequent studies on the resistance gene *flo* showed that the predicted amino acid sequence for the Flo protein to be virtually identical to that proposed for pp-*flo* (Arcangioli et al. 1999; Bolton et al. 1999). The Flo protein presented about 48% (Arcangioli et al. 1999) to 57% (Bolton et al. 1999) identity with CmlA protein of *Pseudomonas aeruginosa*.

A hydropathy analysis of both Flo and CmlA showed 12 hydrophobic putative transmembrane domains (Arcangioli et al. 1999). The occurrence of the same conserved motifs between Flo and CmlA suggest that the Flo protein belongs to the same 12-transmembrane segment (TMS) family of efflux pumps as CmlA (Paulsen et al. 1996). A similar, but more detailed hydropathy study examined the location of the basic and acidic residues on the transmembrane segments of CmlA and Flo (Supakorndej et al. 2000), predicting that differences between the two might determine which drug, florfenicol or chloramphenicol, is pumped out. Continued research is necessary to further characterize the putative efflux mechanism attributed to the expression of the *flo* gene in florfenicol-resistant bacterial isolates.

A second distinct gene that also mediates combined resistance to both florfenicol and chloramphenicol has reportedly been isolated from a 16.5 kb plasmid in *Staphylococcus sciuri* (Schwarz et al. 2000). This gene, designated *cfr*, represents a novel type of transferable florfenicol/chloramphenicol resistance determinant, with no homology to known genes encoding either chloramphenicol or florfenicol resistance. The mechanism of resistance mediated by *cfr* is unknown**.** Comparison of the Cfr amino acid sequence as deduced from the nucleotide sequence also revealed no homology to any known chloramphenicol acetyl-transferases (Murray and Shaw 1997) or putative efflux proteins (Dorman and Foster 1982; Kim and Aoki, 1996; Arcangioli et al. 1999; White et al. 2000) known to be associated with resistance to florfenicol and/or chloramphenicol resistance**.**

Evolution and Ecology of Florfenicol Resistance

Because florfenicol has been in clinical use for such a short period of time, there is very little information in the published literature concerning the ecology and evolution of resistance to this drug. However, this very fact, the brief time that florfenicol has been in use, allows an examination of resistance that is not always available. The development of bacterial antibiotic resistance is currently studied by analysis of the so-called "end-product"…the organism causing the therapeutic problem. Most information gained, therefore, is by the study of "already resistant" bacteria, with big, missing pieces concerning the earliest stages of the development of resistance itself. This is, of course, the case for florfenicol resistant organisms as well, but because florfenicol is such a new antibiotic and we know exactly when it first came into approved use and for what purpose, it may be possible to trace back some of the missing pieces more easily. It may be possible to attempt to "reconstruct" where and how resistance to this drug first arose and it is certainly possible to get a handle on the dissemination of this resistance and how it is possibly being transferred between bacteria.

Resistance to florfenicol has now been documented in many species of bacteria isolated from a wide variety of host animals. This includes resistant isolates of significant bacterial pathogens such as *Salmonella enterica* serovar Typhimurium DT104 from cattle (Arcangioli et al. 1999; Bolton et al. 1999), *E. coli* in cattle (Cloeckaert et al. 2000a; White et al. 2000) and poultry (Keyes et al. 2000; Salmon and Watts 2000), and *Photobacterium damselae* in cultured fish (Kim et al. 1993). Emergence of this resistance, a dual resistance to both florfenicol and chloramphenicol, has been associated with two new antibiotic resistance genes, *flo* and *cfr*. First identified

on a plasmid in the fish pathogen *Photobacterium damselae*, formerly known as *Pasteurella piscicida* (Kim and Aoki 1996), the *flo* gene has now been detected in *Salmonella enterica* serovar Typhimurium DT104 from cattle in Europe (Arcangioli et al. 1999) and the United States (Bolton et al. 1999), *Salmonella enterica* serovar Agona from poultry in Belgium (Cloeckaert et al. 2000b), *E. coli* from cattle (Cloeckaert et al. 2000a), chickens (Keyes et al. 2000), and horses, swine, and dogs (Lee and Maurer 2000), and in clinical samples of *Citrobacter*, *Klebsiella*, and *Pseudomonas* (Lee and Maurer 2000). The *cfr* gene has been detected only in *Staphylococcus sciuri*, so far (Schwarz et al. 2000). This isolate was obtained from the nasal swab of a calf with a respiratory infection (Schwarz et al. 2000).

Although it is not possible to know the exact origin of either the *flo* or *cfr* gene, it is possible to pinpoint when resistance arose, at least in two of the species of resistant bacteria. The laboratories of Aoki and Kitao in Japan had been monitoring antibiotic resistance and the distribution of R-plasmids in *Pasteurella piscicida*, the causative agent of pseudotuberculosis in cultured yellowtail, since 1975 (Aoki and Kitao 1985). Florfenicol resistant strains of *P. piscicida* started to appear in yellowtail farms during 1992 and a transferable R-plasmid encoded with cross-resistance to both florfenicol and chloramphenicol was identified in these resistant strains (Kim et al. 1993). A previous study of 175 strains of *P. piscicida* collected from cultured yellowtail in different areas of Japan from 1989-1991, documented complete sensitivity of all the strains to florfenicol (Kim and Aoki 1993). Florfenicol had been used as an antimicrobial agent in fish farms since 1990 (Kim et al. 1993). In this case, florfenicol resistance appeared to emerge within two years of the first use of the drug.

A ten-year retrospective study of chloramphenicol-resistant *Salmonella enterica* serovar Typhimurium isolates from the French bovine culture collection documents a somewhat different profile of the emergence of florfenicol resistance in this bacterial species. Chloramphenicol was banned in Europe in August 1994 for veterinary use in farm animals. In January 1995 florfenicol was licensed in France for therapeutic use with a specific indication for bovine respiratory disease. Soon after its introduction, the French National Network of Surveillance and Bovine Antibioresistance (RESABO) detected strains of *S. typhimurium* which appeared to be cross-resistant to both chloramphenicol and florfenicol (Martel et al. 1995). However, a study which looked at 86 strains *S. typhimurium* isolated between 1985 and 1995 from clinical cases of bovine salmonellosis and chosen for their resistance to chloramphenicol (Arcangioli et al. 2000) pinpoints the emergence of resistance a bit more precisely. The first strain resistant to florfenicol was isolated in 1989. By 1992-1993, resistance to florfenicol was present in almost 50% of the strains. In 1994, the phenotype expressing cross-resistance to chloramphenicol and florfenicol was predominant (Arcangioli et al. 2000). Thus, the first resistant strain was detected in 1989, six years before florfenicol was approved for veterinary use and by 1994, the year before introduction of the drug, resistant strains of *S. typhimurium* outnumbered florfenicol-sensitive strains. The antibiotic gene *flo* was detected in all florfenicol-resistant isolates from this study (Arcangioli et al. 2000) and all the florfenicol resistant strains were grouped into a single ribotype, the pattern described for *S. typhimurium* DT104 (Guerra et al. 1997; Arcangioli et al. 2000).

Clues to the emergence and spread of florfenicol resistance in the wide variety of bacteria that it now appears in can be obtained by examining the genetic environment in

which the resistance gene is located. The *flo* gene itself appears to be highly conserved in all the bacteria in which it has been detected (Kim and Aoki 1996; Arcangioli et al. 1999, 2000; Bolton et al. 1999; Cloeckaert et al. 2000a, 2000b; Keyes et al. 2000; and White et al. 2000). In *Salmonella enterica* serovar Typhimurium DT104, the *flo* gene is located in a chromosomal locus and flanked on one side by a Class 1 integron containing the streptomycin/spectinomycin resistance gene *aadA2* and on the other by a second integron carrying the bla_{PSE-1} gene (Arcangioli et al. 1999; Briggs and Fratamico 1999). *Salmonella enterica* serovar Agona isolates harbor the *flo* gene in an identical chromosomal location (Cloeckaert et al. 2000b). Integrons, a major agent in dissemination of antibiotic genes, might explain how the *flo* gene moved into (and/or out of?) its chromosomal location in these bacteria.

The pp-*flo* gene from *Photobacterium damselae* is located near the oriV replicon of the large molecular weight plasmid RS1010 (Kim and Aoki 1996). This plasmid is promiscuous and can easily be transferred within and between bacterial species. The *flo* gene was also identified on several structurally different high molecular weight plasmids in florfenicol-resistant *E. coli* strains associated with sick chickens (Keyes et al. 2000) and diseased cattle in France, Germany (Cloeckaert et al. 2000a), and the United States (White et al. 2000). The flanking regions of *flo* in most of the bovine isolates were similar to that of pp-*flo* of *Photobacterium damselae* (Cloeckaert et al. 2000a; White et al. 2000), suggesting the possibility that florfenicol resistance in these isolates arose from intraspecies transfer of broad-host range plasmids. Cloeckaert et al. (2000a) found that the *flo* gene was bracketed by two closely related open reading frames, whose predicted amino acid sequence revealed homology to putative transposases, suggesting

that it might be part of a transposable element. These researchers suggest that this might explain its mobility and location on different plasmids, as well as perhaps playing a role in the formation of the *Salmonella* DT104 antibiotic resistance gene cluster. White et al. (2000) also found that not all of the *flo*-positive bovine *E. coli* contained the *flo* gene on plasmids, suggesting a possible chromosomal location of the gene in these isolates.

As an interesting addendum, a study just published this month (Cloeckaert et al. 2001) showed that the nonenzymatic chloramphenicol resistance described in the late 1970s (Gaffney and Foster 1978) and mediated by the IncC plasmid R55, initially isolated from *Klebsiella pneumoniae* (Gaffney et al. 1981), is conferred by a *flo* gene variant. Comparative sequence analysis showed that this R55 *flo* gene variant is 95 to 97% identical to the previously reported *flo* genes of *Salmonella enterica* serovar Typhimurium and the *E. coli* animal isolates, and confers florfenicol cross-resistance to the same extent as the previously described *flo* genes. From a "historical" point of view, this is actually the first report of florfenicol resistance, long before clinical use of the antibiotic itself or reports of resistance in *Photobacterium damselae* (Kim and Aoki 1996), *Salmonella enterica* serovars Typhimurium DT104 (Arcangioli et al. 1999; Bolton et al. 1999; Briggs and Fratamico 1999) or Agona (Cloeckaert et al. 2000), or *E. coli* (Cloeckaert et al. 2000; Keyes et al. 2000; White et al. 2000). This study provides further evidence for the possibility that the presence of florfenicol resistance in many gram-negative bacteria arose and is spread through the transfer of the *flo* gene by broad host range plasmids.

The second known gene conferring florfenicol (and chloramphenicol) resistance, *cfr,* is from a *Staphylococcus sciuri* isolate obtained from the nasal swab of a calf

suffering from a respiratory tract infection (Schwarz et al. 2000). Antibiotic resistance is common among *S. sciuri* isolates and a number of plasmids carrying one or more resistance genes have been identified (Schwarz et al. 1990; Schwarz and Grolz-Krug 1991; Schwarz and Noble, 1994). The florfenicol resistance gene *cfr* was isolated from a large, 16.5 kb plasmid that also carried the inducible erythromycin *ermC* gene (Schwarz et al. 2000). The *cfr* gene seems to have no relationship that we can discern to the gram-negative *flo* genes and so little of the flanking region of *cfr* as it appears in *S. sciuri* is known, that it is difficult to make any predictions of how this gene is, or has been transferred, other than it is on a plasmid.

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CHAPTER 3

DETECTION OF FLORFENICOL RESISTANCE GENES IN *ESCHERICHIA COLI* ISOLATED FROM SICK CHICKENS [1]

[1] Keyes, Kathleen, Charlene Hudson, John J. Maurer, Stephan Thayer, David G. White, and Margie D. Lee. 2000. Antimicrobial Agents and Chemotherapy. 44:421-424.
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Abstract

Florfenicol is an antibiotic approved for veterinary use in cattle in the United States in 1996. Although this drug is not used in poultry, we have detected resistance to florfenicol in clinical isolates of avian *Escherichia coli*. Molecular typing demonstrated that the florfenicol resistance gene, *flo*, was independently acquired and is plasmid encoded.

Florfenicol is a synthetic, fluorinated analogue of chloramphenicol, which lacks chloramphenicol's associated human health risk (11). It has been used in Asia for aquaculture since the 1980s (12). In early 1996, an injectable formulation of florfenicol was approved for the treatment of bovine respiratory disease in the United States. It has not yet been approved for poultry, and, in fact, an animal feed formulation is not available.

Florfenicol is bacteriostatic, and its mechanism of action is similar to that of chloramphenicol (7,22). The mechanism of resistance to florfenicol is unknown but is associated with the *flo* determinant, a highly conserved gene sequence detected in *Salmonella enterica* serovar Typhimurium DT104 (4,6) and in the fish pathogen *Pasteurella piscicida* (*Photobacterium damsela*) (15). The *flo* gene confers resistance to both chloramphenicol and florfenicol (4,14).

Resistance to chloramphenicol is most commonly mediated by mono- and diacetylation via chloramphenicol acetyltransferase (CAT) enzymes, which prevents the binding of chloramphenicol to the 50S ribosomal subunit (21). None of the genes encoding CAT has been shown to confer resistance to florfenicol, and there is no homology between the CATs and Flo (9). Another mediator of chloramphenicol resistance, the *cmlA* gene of *Pseudomonas aeruginosa*, is believed to be a non-enzymatic efflux pump (3). CmlA is approximately 50% similar in amino acid sequence to Flo (4), but it is not known whether *cmlA* confers resistance to florfenicol.

Our study examined the prevalence of florfenicol resistance in clinical avian *Escherichia coli* isolates. We hypothesized that there were pre-existing genes in these bacterial isolates that conferred resistance to florfenicol and that this resistance might

limit the future usefulness of the drug in other veterinary species. We report here the presence and incidence of the florfenicol resistance gene, *flo*, in avian *E. coli*.

The characteristics of the avian *E. coli* isolates are presented in Table 1. Of the 100 isolates cultured from litter and from clinical and postmortem material, 11 were found to be resistant to chloramphenicol by disc diffusion (30 μg). All 11 chloramphenicol-resistant isolates were multidrug resistant, and four of these isolates, avian *E. coli* isolates 5334, 5790, 5840, and 6468, were also resistant to florfenicol by disc diffusion (< 21-mm-diameter zone of inhibition with a 30-μg disc). *S. enterica* serovar Typhimurium DT104, for which the MIC of florfenicol was 64 μg/ml, was used as the positive control (4). *E. coli* K-12 containing *cmlA* (3) exhibited a florfenicol MIC of 2 μg/ml, as did *E. coli* DH5α, the negative control. All of the *flo*-containing isolates exhibited florfenicol MICs of at least 32 μg/ml. The florfenicol MIC for two isolates which did not contain *flo* was 8 μg/ml. The breakpoints for florfenicol resistance recently adopted for bovine respiratory pathogens are 2 (sensitive), 4 (intermediate), and 8 (resistant) μg/ml (T. Shyrock, Chairholder, Veterinary Antimicrobial Susceptibility Testing Subcommittee, National Committee for Clinical Laboratory Standards, personal communication, 1999).

The florfenicol-resistant *E. coli* isolates came from clinical samples sent from different poultry farms in Georgia and North Carolina. To determine whether they represent dissemination of a clonal strain, random amplification of polymorphic DNA (RAPD) by the method of Maurer et al. was employed (17). RAPD analysis showed four distinct patterns, or RAPD types (data not shown), suggesting that florfenicol resistance is not limited to a particular strain of avian *E. coli.*

DNA-DNA colony hybridizations with probes specific for *cmlA*, *flo*, and *int* were done to correlate the presence of these genes with florfenicol resistance. PCR was used to generate the DNA probes; Table 2 lists the primers employed and the expected sizes of the PCR products. The identities of the PCR products were confirmed by DNA sequencing. The PCR mixture consisted of 2 mM $MgCl_2$, 0.2mM deoxyribonucleoside triphosphates (digoxigenin labeled), 50 pmol of each oligonucleotide primer, and 0.5 U of *Taq* polymerase (Boehringer Mannheim, Indianapolis, Ind.). The program parameters for the hot-air thermocycler were 30 cycles of (i) 94°C for 1 s, (ii) 40°C for 1 s, and (iii) 72°C for 15 s. The PCR products were purified by using WIZARD DNA Clean-Up System kits (Promega) and combined with hybridization buffer, containing 0.75M sodium chloride, 1% nonfat dry milk, 0.1% *N*-laurylsarcosine, and 0.2% sodium dodecyl sulfate; they were kept frozen at -20°C until use. Bacterial cells were patched onto nylon membranes with toothpicks, and DNA-DNA hybridizations was performed as described by Sambrook et al. (20), with hybridizations and washes being done at 68°C. Hybridizing DNA fragments were detected by using an anti-digoxigenin antibody-alkaline phosphatase conjugate with a color substrate solution of 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (XP).

Results of the DNA colony hybridizations are shown in Table 1. Only one isolate, *E. coli* 6468, contained *cmlA*, whereas all the four florfenicol-resistant isolates contained *flo*. The *int* gene probe revealed that 9 of 11 isolates were positive for the *int* sequence, indicating that integron-related genes were commonly present in multidrug-resistant clinical isolates. DNA-DNA hybridization was also used to assess whether the *flo* gene was plasmid associated. The *flo* gene was used to probe plasmids isolated from

the *flo*-positive avian *E. coli* isolates (Fig. 1). Plasmid DNA was isolated by the S1 nuclease method of Barton et al. (2) and separated by pulsed-field gel electrophoresis (pulse time, 2 to 40 s; voltage, 6V/cm; 25h). The DNA was transferred from the agarose gel to a nylon membrane with a vacuum blotter (Bio-Rad, Hercules, Calif.) according to the manufacturer's recommendations. The procedure for DNA-DNA hybridizations was performed as described above. Three of the four isolates contained *flo* on high-molecular-weight plasmids of 186 and 204 kb. The florfenicol resistance determinant appears to be present in a variety of large-molecular-weight *Xba*I DNA fragments in avian *E. coli*, in contrast to the mapping of the *flo* resistance gene to a 10-kb *Xba*I fragment of *S. enterica* serovar Typhimurium DT104 (Fig. 1). Therefore, its location in avian *E. coli* may be similar to its placement in large-molecular-weight R plasmids in *Pasteurella piscicida* (14); however, the differences in sizes of the plasmids and fragments suggest that the gene was independently acquired.

Two of the four florfenicol-resistant isolates also contained *int*, the DNA integrase gene that is characteristic of integrons, which are transmissible elements deemed important in the horizontal transfer of antibiotic resistance genes. In *Salmonella* strain DT104, *flo* is chromosomally located between two integrons (6). Many of the antibiotic resistance genes found in gram-negative bacteria are located within integrons, which are mobile genetic elements (18). The integrase acts as a site-specific DNA recombinase in the insertion of antibiotic resistance genes into these elements (8, 18). For example, *cmlA* is present within the integron of Tn*1696*, which makes up part of the *Pseudomonas aeruginosa* IncP plasmid R1033 (3). The *cmlA* drug resistance gene does not appear to be responsible for high-level florfenicol resistance, since we found that only one

florfenicol-resistant *E. coli* isolate possessed the gene and since *E. coli* containing *cmlA* was sensitive to florfenicol.

Our study demonstrates the persistence of chloramphenicol resistance in avian *E. coli*, although this drug has not been used therapeutically in food animals since its use was officially banned in 1988 (13). We also demonstrated that a low percentage (4%) of clinical avian *E. coli* isolates already display resistance to florfenicol, although the drug is not used therapeutically in chickens. In fact, a feed formulation is not currently available in the United States. Poultry production is rather unique in the United States since the processing company owns the birds and the feed; farmers are contracted to house the animals. The processing company employs veterinarians who are responsible for vaccination and medication of the birds in the face of illness. Therefore, the attending veterinarian prescribes the antibiotics used for treatment of disease, and it is improbable that the birds from which we isolated florfenicol-resistant *E. coli* had ever been exposed to florfenicol.

Antimicrobials are useful therapeutic agents only if the drug concentrations achieved in the serum and tissue are greater than the MIC of the drug. Florfenicol attains a maximum concentration of 3 μg/ml in the serum of feeder calves (16). The manufacturer of florfenicol reports an MIC of 1 μg/ml or less against 90% of the bacterial isolates from natural infections in cattle (5, 11, 23,). Pharmacokinetic studies have shown that the peak plasma florfenicol concentration in ducks and chickens is approximately 3 μg/ml, with similar levels in the liver, kidney, and lung tissues (1, 10, 19). Our study showed that all 11 chloramphenicol-resistant avian *E. coli* isolates had

florfenicol MICs greater than 3 μg/ml, suggesting that this antimicrobial agent may not be therapeutically successful in some cases.

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TABLE 1. Characteristics of chloramphenicol-resistant avian *E. coli* [A] isolates.

Isolate	Source	Site of isolation	Clinical signs	Antibiotic resistance [B]	Genes [C] detected by DNA hybridization		MIC (μg/ml) of:	
					flo	*int*	Chloramphenicol	Florfenicol
A35	VA	Litter sample	None	TE S SU NA SR EN	-	+	200	4
A410	VA	Litter sample	None	TE S SU NA SR EN	-	+	400	4
3518	GA	Yolk sac	Colibacillosis	AU TE S NA	-	+	100	8
5334	NC	Trachea	None	TE NE S SU	+	-	100	64
5361	GA	Peritoneum	Colibacillosis	AU TE S SU NA SR EN	-	+	200	8
5790	GA	Trachea	Respiratory disease	AU TE S SU	+	+	100	32
5965	GA	Trachea	Airsacculitis	AU TE S SU	-	+	400	4
5967	GA	Trachea	Airsacculitis	AU TE S SU	-	+	200	4
6005	GA	Trachea	Airsacculitis	AU TE GE S SU	-	+	100	4
6468	GA	Trachea	Respiratory disease	AU TE GE S SU	+	+	100	32
5840	GA	Trachea	Respiratory disease	TE S SU	+	-	100	64
Salmonella DT104	____ [D]			TE S SU	+	+	100	64
DH5α				NA	-	-	0.78	2

[A] None of the *E. coli* isolates were resistant to ceftiofur, an antibiotic that is routinely included in antibiotic susceptibility tests of poultry isolates.

[B] Antibiotic resistance was determined by disc diffusion. Abbreviations for tetracyclines: AU, chlortetracycline (15μg) and TE, tetracycline (30μg). Abbreviations for quinolones/fluoroquinolones: NA, nalidixic acid (30μg); SR, sarafloxacin (5μg); EN, enrofloxacin (5μg). Abbreviation for sulfonamides: SU, sulfisoxazole (250μg). Abbreviations for aminoglycosides: GE, gentamicin (10μg); NE, neomycin (30μg); and S, streptomycin (10μg).

[C] Isolates were not probed for the presence of *cat* genes.

[D] _____ data from reference 6.

TABLE 2. PCR-generated DNA probes for detecting presence of integrons, *cmlA* and *flo*.

Probe		Oligonucleotide primers[a]	Sequence accession no.	Product size (bp)	Template source	Reference for primers
flo	F:	5'-TATCTCCCTGTCGTTCCAG-3'	D37826	399	*S. enterica* serovar	This study.
	R:	5'-AGAACTCGCCGATCAATG-3'			Typhimurium (DT104)	
cmlA	F:	5'-CCGCCACGGTGTTGTTGTTATC-3'	M64556	698	Plasmid R1033	This study.
	R:	5'-CACCTTGCCTGCCCATCATTAG-3'				
int	F:	5'-CCTCCCGCACGATGATC-3'		280	Plasmid pDU202	25
	R:	5'-TCCACGCATCGTCAGGC-3'				

[a]F, forward primer; R, reverse primer.

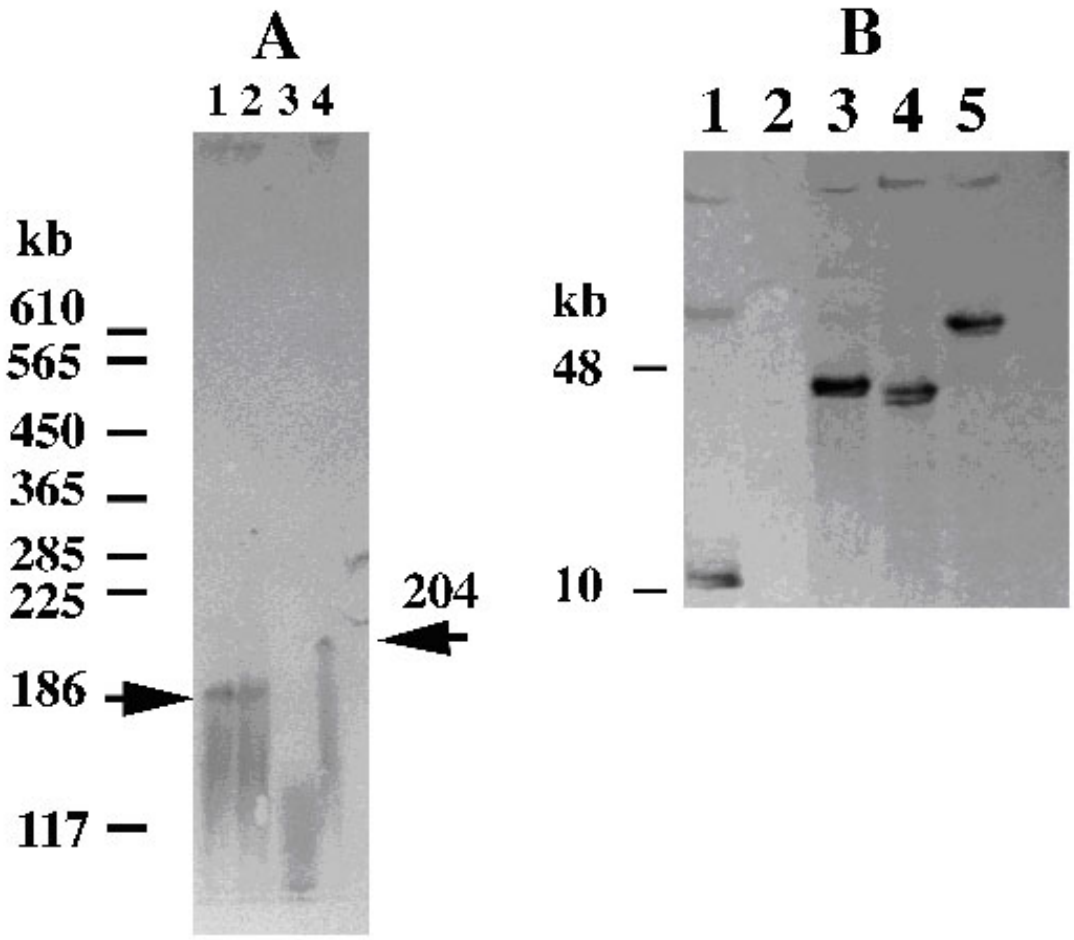

Figure 1. Localization of the florfenicol resistance gene *flo* to large molecular-weight plasmids in avian *E. coli*. (A) Large-sized plasmids (>100 kb) were separated by pulsed-field gel electrophoresis and probed with labeled *flo*. Lanes: 1, avian *E. coli* isolate 5334; 2, isolate 5790; 3, isolate 5840; 4, isolate 6468. Arrows identify plasmid bands recognized by the probe. (B) The location of *flo* in the avian *E. coli* chromosome was mapped by pulsed-field gel electrophoresis. *E. coli* genomic DNA was cut with *Xba*I, separated by pulsed-field gel electrophoresis and probed with labeled *flo*. Lanes 1, *S. enterica* serovar Typhimurium DT104; 2, *E. coli* K12 LE392; 3 to 5, avian *E. coli* isolates 5334 (lane 3), 5790 (lane 4), and 6468 (lane 5).

CHAPTER 4

CONCLUDING REMARKS: LOOKING BACKWARDS, LOOKING AHEAD

"Suddenly a flash of understanding, a spark that leaps across to the soul."
Plato

"The present contains nothing more than the past, and what is found in the effect was already in the cause.
Henri Bergson

Lessons from the Past

This year my daughter learned, both in her world history class and her Latin II class, about how Rome won control of Italy (an event deemed significant in the development of Western Civilization). The story, as she learned it, goes something like this. In 390 B.C., Rome's walls were successfully stormed by marauding Gauls, a people from the Po River Valley, north of the Apennines. The Gauls sacked Rome, leaving it to ruins. The Romans were forced to pay a humiliating bribe to persuade the Gauls to leave. The Romans recovered rapidly, though. They built a stronger, larger wall around their city and the reconstructed Rome spanned 1,000 acres. Foreign troops would not sack the city again for 800 years. Eventually, Romans controlled all of the Italian boot, except its heel and toe. For centuries, those southern regions of Italy had been colonized by the Greeks. The Greek cities watched the rise of Roman power with alarm. In 282 B.C., Greek colonists sought aid from Pyrrhus, a king in western Greece. A brilliant general, Pyrrhus brought 20,000 soldiers to fight the Romans. Twice, Pyrrhus's army slammed into the Roman legions and drove them from the fields. In each battle, however, the Greek army suffered terrible losses. Pyrrhus learned a bitter lesson of warfare (and of

life): You can win every battle and still lose the war. In 275 B.C., the Romans finally drove Pyrrhus and his tired, decimated troops back to Greece. Ever since, a victory gained at too high a price has been known as a "Pyrrhic victory".

My daughter, who likes to study history rather than the sciences like the rest of her family, often despairs, "But what is the *use* of history?" I tell her that her study of history, her explorations and explanations, have taught me much (since I am quite the dolt about such matters)…in fact, it was her discussion of how the term "a Pyrrhic Victory" originated that struck such a note with me when I was thinking about the "antibiotic paradox" and thinking about what to say in these concluding remarks. And I tell her, that to me, *everything* about biology is really "history" and that everything we study in biology in contingent on change, or what we biologist-scientists call evolution. Every living creature (even bacteria, I tell her) is a product of its past. When a biologist asks "Why?, he is really asking, "How did this come about?" He (like herself) is a historian.

The point of this rather long-winded (historical) recitation is that the Romans and Greeks put me in the mind of bacteria, antibiotics, and the development of resistance…although it initially appeared that man had "won the battle" against a number of bacterial diseases with the introduction of specific antibiotics, we may have ultimately "lost the war". It appears from a historical examination of the development of resistance to new antibiotics, that it takes bacteria almost no time at all to develop some level of resistance to virtually all of them. The bacteria, like the Romans, will be around when the "human" supplied antibiotics (like the Greeks) have long been vanquished from their surroundings…But perhaps it is not too late for *us* to learn the value of paying attention

to the lessons of the past, as it concerns the development of antibiotic resistance. What does hindsight tell us about the present, that is, how does what we already know help us to understand something "new"?

The history of antibiotic resistance is as old as the antibiotics themselves. Antibiotic-producing soil bacteria have probably been elaborating "self-protection" mechanisms (resistance mechanisms) to prevent annihilation by their own chemical weapons and other toxic substances encountered in their surroundings since very near their beginnings. It has certainly been the case that once an antibiotic enters widespread human and animal therapeutic use, its days are numbered; clinically significant resistance appears in a period of months to years (Davies 1996). For penicillin, the first antibiotic to be used in human clinical practice, widespread resistance began to be noted within two years of its introduction in the mid-1940s. This appears to be typical for a resistance mechanism involving the action of one gene product and its spread through bacterial populations. Vancomycin resistance, the recent scourge of hospital corridors with its source in life-threatening vancomycin-resistant enterococci (VRE), became "noticeable" in 1987 and spread dramatically in the ensuing 4-6 years (Murry 1997). VRE have collected five genes that are necessary and sufficient for high-level resistance (Arthur and Courvalin 1993; Walsh et al. 1996) and this may explain the 29 years delay between the introduction of vancomycin (in 1958) and the development of clinically important resistance. The historical record tells us, then, that the development of resistance is not a matter of "if", but only a matter of "when" (Davies 1994; Levy 1998b).

In the last 50 years bacteria have demonstrated a remarkable ability to develop and share resistance to almost every antibiotic that has been developed, often by quite

unexpected mechanisms and much more readily than was originally predicted. They can be resistant in their natural state or they can acquire resistance genes by mutation or from other bacteria. There is a large reservoir of antibiotic resistance genes, in bacterial chromosomes and in extra-chromosomal pieces of DNA, that encode different mechanisms of drug resistance, such as enzymes that inactivate antibiotics, altered antibiotic targets, and efflux pumps. During times of stress, commensal and pathogenic bacteria demonstrate the ability to become hypermutable and they duplicate and share "survival information", such as resistance genes, that can be encoded on plasmids, transposons, and integrons. Bacteria collect and exchange genetic information with extraordinary facility and often lack of species specificity, permitting antibiotic resistance already present in the "environment" to be picked up and passed on from one bacteria to another. And bacteria have also demonstrated an amazing capacity to retain genetic information that provides them with an evolutionary advantage, conserving advantageous mutations (even in the presence of DNA repair mechanisms that can correct them), as well as plasmids, transposons, integrons and other pieces of foreign DNA.

We know, then, that the development of antibiotic resistance in any particular bacteria to any specific antibiotic is contingent on many factors…mutation rates of existing genes, what antibiotic resistance genes reside in the surrounding environmental pool of bacteria, the vectors in which those resistance genes are carried, how easily those genes can be transferred to another bacterial individual, genus, or species, the selective pressures (such as the presence of the particular antibiotic in the environment) at work in the environment, and others (of which some we are probably not even aware).

In the past, detection of the development of antibiotic resistance was mostly an "after the fact" matter…relying on traditional microbiological culture methods and antimicrobial sensitivity testing of bacterial isolates after use of and exposure to a particular antibiotic. It is now possible, however, to utilize molecular methods such as PCR and Southern blotting, to actually determine the presence of specific antibiotic resistance genes and the resistance mechanisms that they encode. Molecular tools such as these allow not only the determination of antibiotic resistance, but the "tracking" of resistance genes in any bacterial population, and may even aid in predicting the trajectory of the path of resistance in human and veterinary pathogens before a particular antibiotic is used.

A Problem in the Present

The problem of antibiotic resistance extends not only into the realm of human clinical medicine, but into that of veterinary medicine, as well. In animal husbandry, the use of antibiotics in areas outside normal therapy, such as prophylaxis, has also played an important role in controlling losses due to infectious disease, thus helping to meet the growing demand for animal protein. There has been an escalating development of antibiotic resistance in bacteria responsible for animal disease, some think due, in part, to the selective pressure of antibiotics that many animals (and their resident bacteria) are bombarded with.

Antibiotic resistance among avian bacterial isolates is common and of great concern to the poultry industry. In particular, diseases resulting from *E. coli* infections, such as colibacillosis, air sacculitis, and cellulitis, which cause high mortality and morbidity in chickens (Gross 1991), are now commonly resistant to two or more

antibiotics (Irwin et al. 1989). Currently the only antibiotics proven effective at combating infection in poultry are two fluoroquinolone antibiotics, sarafloxacin and enrofloxacin. The utility of even these drugs is probably short-lived, not only because *E. coli* is commonly becoming resistant to them (Bazile-Pham-Khac et al. 1996; Blanco et al. 1997; Turtura et al. 1990; White et al. 1998), but also due to the impending ban by the FDA of fluoroquinolone use in chickens and turkeys…thus the race is on to develop new antimicrobial agents for poultry diseases.

The laboratory work for this thesis examined the prevalence of florfenicol resistance in clinical avian *E. coli* isolates. Florfenicol, a relatively new veterinary antibiotic, is currently approved for use in the United States only in the treatment of bovine respiratory disease. This thesis study demonstrated that a low percentage of clinical isolates of avian *E. coli* already display resistance to florfenicol, although the drug has never been used therapeutically in chickens. Antimicrobials are useful therapeutic agents only if the drug concentrations achieved in the serum and tissue are greater than the minimum inhibitory concentration (MIC) of the drug. Florfenicol attains a maximum concentration of 3 μg/ml in the serum of feeder calves (Lobell 1994). The manufacturer of florfenicol reports an MIC of 1 μg/ml or less against 90% of the bacterial isolates from natural infections in cattle (Booker 1997; Food and Drug Administration 1996; Wilson 1996). Pharmacokinetic studies have also shown that the peak plasma florfenicol concentration in ducks and chickens is approximately 3 μg/ml, with similar levels in the liver, kidney, and lung tissues (Affifi and Abo El-Sooud 1997; El-Banna 1998; Rios et al. 1997). This study showed that all florfenicol resistant avian *E. coli* isolates had florfenicol MICs of greater than 3 μg/ml, suggesting that this antimicrobial

agent may not be a successful new candidate for the treatment of *E. coli* infections in poultry.

This study also demonstrated the presence of the gene *flo* in all of the florfenicol resistant avian *E. coli* isolates. This gene is known to confer resistance to both florfenicol and chloramphenicol and has been reported in a number of other florfenicol resistant bacterial isolates from a number of animal hosts (Kim and Aoki 1996; Bolton et al. 1999; Arcangioli et al. 1999; Cloeckaert et al. 2000a, 2000b; White et al. 2000). Three out of four florfenicol resistant avian *E. coli* isolates contained the *flo* gene on high molecular-weight plasmids of 186 and 204 kb, respectively. The location of the florfenicol resistance determinant *flo* in avian *E. coli* may be similar to its placement in the large molecular-weight R-plasmid reported in *Photobacterium damselae* (Kim et al. 1993); however the differences in the sizes of the plasmids suggest dissemination of not a common plasmid, but perhaps a closely related one. The same resistance gene *flo* has been documented as being located on the chromosome of the multi-drug resistant *Salmonella* DT104 in between two integron-like structures (Briggs and Fratamico 1999). Several recent papers have also described the presence of the *flo* gene in both another serovar of *Salmonella enterica* in poultry, as well as in European *E. coli* isolates in cattle. The presence of the highly conserved *flo* gene sequence in all of these bacterial isolates from different animal hosts strongly suggests mobility of this gene. The occurrence of the *flo* gene on plasmids could explain this distribution in several bacterial species from different animal hosts. It may also be significant in terms of how rapidly florfenicol resistance can spread between isolates of one species, across genus and species lines, as well as into the environmental pool of bacteria. The presence of the *flo* gene on plasmids,

which often carry other resistance genes, might also implicate that the spread of this gene may be the result of co-selection in the presence of antibiotics other than florfenicol. It may be that *flo* itself is mobile, located within some sort of transposon or integron-like element, and has been independently acquired. The detection of the known florfenicol resistance gene in the avian *E. coli* from this study, as well as those bacterial isolates from other animal hosts never treated therapeutically with florfenicol itself, also suggests that this drug may not be very effective for the treatment of poultry diseases…the resistance gene may already be fairly widespread and easily transferable, even in the absence of any selective pressure by the antibiotic itself.

The riddle remains as to how this particular gene and the resistance it encodes moves around and what influences its transfer. Investigations are currently underway in the laboratory of Dr. Margie Lee to identify the genetic environment of the *flo* gene in a number of clinical bacterial isolates, including *E. coli* isolated from chickens, dogs, horses and cattle, *Salmonella* sp. isolates from pigs, cows, and exotic pet birds, *Citrobacter* sp. from horses, *Klebsiella* sp. from dogs and cows, and *Pseudomonas* isolates from dogs, cows, and horses. Examination of the sequences flanking the *flo* gene may aid in determining the origin of this gene, as well as providing insight into how *flo* is transferred between different bacterial genera and species. An assessment of whether *flo* is present and in what proportions in any particular bacterial population, as well knowledge concerning how the gene actually moves around in the population, is essential to determine whether the antibiotic florfenicol should be approved for use against other veterinary pathogens in new host animals.

Lessons for the Future: Rethinking the Past

On October 31 of last year, the Food and Drug Administration, in a rare (and very controversial) reversal of policy, announced plans to ban two antibiotics in the fluoroquinolone family that are used by poultry farmers to treat respiratory problems in chickens and turkeys. The two fluoroquinolones in question, sarafloxacin and enrofloxacin, are not approved for use in humans. However, others within that drug family, such as ciprofloxacin, are considered to be of vital importance for treating a broad range of human clinical infections. The FDA's concern is that once you have resistance in any members of that class of antibiotics, that it may confer cross-resistance to all members within the class. Agency officials also contend that the use of these drugs increases the likelihood that humans will become infected with fluoroquinolone-resistant strains of *Campylobacter*, a pathogen contracted primarily from eating chickens and the nation's most common bacterial food borne illness. The proposed ban would be the first by the agency specifically aimed at reducing the use of antibiotics in agriculture and animal husbandry, a practice frequently criticized as contributing to the overall level of antibiotic resistance among pathogens and also raising the risk of animal-to-human transmission of resistant organisms.

As they say, hindsight is always 20/20, but perhaps the above controversy might have been avoided if more consideration had been paid to what is already well known about the "how and wherefores" concerning the development of antibiotic resistance. Sarafloxacin was approved in late 1995 for use in poultry drinking water to control illnesses caused by *E. coli* bacteria and was the first fluoroquinolone approved for use in food animals. Ciprofloxacin was already in use to treat human infections and problems

concerning cross-resistance between related antibiotics were well established and documented in the scientific literature. There was already a growing amount of evidence concerning the transfer of resistant bacteria and their genes from animals to humans, as well.

The Lessons

Sometimes being cognizant of the "lessons of the past", that is, how the past has molded the present, can provide clues as to how the present can influence and help us avoid problems (such as those exemplified by fluoroquinolone use in poultry) in the future. Although extensive worldwide use of antibiotics in medicine, animal care, and agriculture may have accelerated the evolution and spread of antibiotic resistance in bacteria, it has not changed the nature of it. What do we now know about the basis of antibiotic resistance and about how it develops and spreads that can provide "lessons for the future"? And what can and should we do with this knowledge to impede the further emergence and spread of resistant bacterial strains?

Perhaps the first most important lesson to be appreciated is that bacteria are remarkably "adaptable". They have extraordinary genetic flexibility, in terms of both mechanisms and functions, and will continue to evolve and acquire new mechanisms of resistance to antibiotics. Bacterial genomes represent a large natural pool of diverse genetic information, including antibiotic resistance genes, that can be "accessed" under appropriate selective pressures, using a variety of gene acquisition and dissemination mechanisms. Human application of antibiotics on a massive scale can be a major selective pressure that activates these genetic systems, selecting for those resistance genes that promote the survival of a particular (resistant) bacterial population.

From this first lesson follows the second: the development of antibiotic resistance is inevitable. Antibiotic resistance is an undeniable fact and will continue to be a problem as long as we use the currently available antibiotics in the way we do now. The advances of the "antimicrobial era" are being dissipated by the emergence and spread of resistant bacteria, accelerated by the intensive use of antibiotics in humans and animals over the past 50-60 years. There is now a resurgence of "old" diseases in both humans and animals which we thought were conquered through the development of modern antibiotics. As we are cautioned to learn from past human wars to avoid the dangers of a future war, so should we take heed of what we have already learned about the arms race between antibiotics and the development by bacterial resistance to them.

The third lesson to be noted is actually a conclusion based on the second. Past strategies and policies for dealing with resistance have, at best, been only partially effective. New approaches to the problem of antibiotic resistance are badly needed. What can we do, based on knowledge we already have, to solve the problem of continued emergence and spread of antibiotic resistance?

Prudent Use of Antibiotics

First and foremost would be what at once seems the easiest and most obvious: "smart" use of antibiotics. There are numerous anecdotal examples of the relationship between antibiotic consumption and the emergence of resistance (Levy et al. 1987; Moellering 1990). Given these reports, it seems clear that inappropriate use of antibiotics should be discouraged at all costs. It is incumbent upon the medical establishment and individual practitioners to avoid the overuse, "imprudent" use, and outright abuse of antibiotics (Levy 1992) and utilize them only in situations in which the likelihood of

benefit is sufficient to justify the use. Thus, using antibiotics for prophylactic purposes in settings where the likelihood of infection is low should be discouraged. Likewise, routine use of antibiotics for the treatment of such things as “the common cold” and other infections unlikely to respond to antibiotic therapy, represents another potential source of selective pressure for resistant organisms.

Antibiotics are available without prescription in many parts of the world (Levy et al. 1987). This fact is thought to account for some of the increased resistance seen in developing countries. However there is also increasing pressure from pharmaceutical companies in this country to release certain antibiotics for over-the-counter use in the treatment of ailments such urinary tract infections and other “simple” bacterial infections. Given the potential for inappropriate use of antibiotics in this setting, as well as the increased likelihood that this type of use would increase the selective pressure that leads to resistance, such a change in approval policies does not make sense.

There is also heavy use of antibiotics in agriculture, aquaculture, and a variety of veterinary settings. Appropriate use and regulation of antibiotics under these circumstances is critical, as well. For example, there is some evidence that the veterinary use of a glycopeptide antibiotic, avoparcin, has added to the pressure that has lead to the development of vancomycin-resistant enterococci in certain countries in Europe (Perez-Trallero and Zigorraga 1995). There also appears to be some evidence that the use of antibiotics in aquaculture is associated with the emergence or resistance in a variety of gram-negative bacteria, including *E. coli* and *Aeromonas salmonacida*. The widespread use of subtherapeutic concentrations of antibiotics as growth promoters in many farm animals has also been reported to lead to the emergence of resistant organisms.

Subtherapeutic levels of tetracycline have actually been shown to increase the frequency of transfer of resistance plasmids in the guts of animals (Doucet-Populaire et al. 1991). Farmers and those involved in any sort of animal husbandry should be educated and helped to find inexpensive alternatives for encouraging animal growth. Improved hygiene and the use of probiotics, for instance, could go a long way to enhancing livestock development.

Under the heading of "prudent use" should also come federal policies concerning the approval process of new antibiotics. The problem of cross-resistance between antibiotics must be evaluated for each and every new drug used in humans and animals. Approval of any new antibiotic for veterinary use, especially in food animals, should be contingent on the fact that no "relative" of the drug is already in human use. The current problem concerning fluoroquinolone use in poultry is a case in point of what can happen when this is not done.

Lastly, individual consumers themselves should think twice before jumping on the "antibacterial bandwagon" which seems to be flourishing in the media and on grocery shelves, waiting to be taken home. A proliferation of everything from hand lotions to dishwashing detergent is now imbued with antibacterial agents, including triclocarbon, triclosan, and such quaternary ammonium compounds such as benzalkonium chloride. While there is little evidence that the addition of antibacterials to household products really wards off infection, there are now laboratory findings that indicate that these chemicals can select for bacteria resistant both to the antibacterial preparations and to antibiotics, as well (McMurry et al. 1998). These antibacterial agents, like antibiotics themselves, can alter the "ecological mix" of bacteria…they kill susceptible bacteria

while promoting the growth of resistant strains. These resistant bacteria may include those present from the start. But they can also include those unable to gain a foothold previously, but that can now thrive, thanks to the destruction of the "competitors". Theoretically, the "interlopers", once they have a chance to proliferate, can become the agents of new disease. This scenario may not be as far-fetched as it sounds…it is exactly what happens in the case of iatrogenic colitis and yeast infections seen in hospitalized, immunosuppressed patients who have been "dosed" with prophylactic antibiotics and whose normal flora has been wiped out.

Control and Prevention of Dissemination of Existing Antibiotic Resistance

The popular conception of antibiotic resistance has, until recently, been perceived as greatest in health care facilities. Although this is no longer true (if it ever was), control and prevention of the dissemination of antibiotic resistant organisms in the community, with special reference to hospitals and nursing homes, represents another widely recommended solution to the problem of antibiotic resistance. However, the fact that resistant bacteria continue to plague our hospitals and special care facilities, despite sophisticated attempts at infection control, suggests that this is not an effective strategy. Hospitals may actually serve as "test-tubes" for resistant organisms. As the severity of illness among patients in modern hospitals continues to rise and as we use therapeutic regimens that result in immunosuppression, as well as antibiotics that wipe out the normal flora, it may well be virtually impossible to prevent the emergence of resistant bacteria in the hospital setting. Some suggestions that have been made to prevent the continued emergence of resistant organisms in health care settings include increased aspects of hygiene, such as good hand washing technique, keeping patients affected with

multiresistant organisms in separate rooms, where they are seen by gloved and gowned health practitioners, and rotation of antibiotics used in the intensive care unit (Moellering 1990). Although all of these practices are good ones, which should continue, there is, to date, no concrete evidence that such policies have been at all effective in preventing the continued spread of resistant bacteria.

Discovery and Development of New Antibiotics

Having new antibiotics could provide more options for treatment. During the 1980s, drug manufacturers, concurring with the opinion that infectious diseases were essentially conquered, cut back severely on the search for additional antibiotics. At that time, if one drug failed, another in the arsenal would usually work. With this state of affairs coming to an end, researchers are again searching for novel antibiotics.

One new strategy is to give new life, so to speak, to existing antibiotics. For example, many bacteria evade penicillin and its relatives by switching on the enzyme penicillinase that degrades these compounds. A series of β-lactamase inhibitors, including clavulanic acid, sulbactam, and tazobactam, have been developed for combination with the β-lactam drugs, such as amoxicillin, ticarcillin, piperacillin, and certain cephalosporins. The resulting β-lactamase inhibitor-β-lactam combinations have a broad spectrum of activity against β-lactamase-producing bacteria, which would otherwise be resistant to the parent drug alone (Moellering 1991).

Another new approach in the development of antibiotics is the search for antibacterial activity due to a novel mechanism, for example defining new targets, such as genes or enzymatic functions, responsible for a crucial cellular activity. Once this has been done, inhibitors or blockers of the function or gene product can be developed. The

recent explosion in bacterial genomics offers a great opportunity for drug discovery pharmaceutical scientists to identify such novel essential genes that can serve as drug targets. With the proliferation of genomic information that is becoming available, it is only a matter of time and patience before a number of novel targets can be identified. The resistance issues are perceived to be less, as there would be no drugs in use to "pre-select" a resistant population. Novel mechanisms that have not been effectively exploited as drug targets include transport mechanisms, such as the multi-drug transport and metalloid transport systems. The newer tetracyclines that inhibit or evade efflux pumps are a variation of this sort of strategy (Levy and Nelson 1998). With these antibiotics, the drug jams the microbial pump that ejects tetracycline from bacteria; with the pump inactivated, tetracycline can penetrate bacterial cells effectively. The diphenolic methanes, or bis-phenols, that have been described as antimicrobial agents working through inhibition of a two-component transduction system represent another novel type of agent (Domagala et al. 1998). The bis-phenols are thought to effect bacterial processes that are not associated with the mechanism of action of other antimicrobial agents, and therefore its has been postulated that cross-resistance with other classes of antibiotics should be rare.

Use of Molecular Tools in Detection and Surveillance of Resistance

As we now know, while some antibiotic resistance is due to random mutations in the bacterial genome, most resistance is mediated by acquired genes whose presence in a cell is usually synonymous with a resistant phenotype. Most resistance genes harbored by bacteria are expressed, although exceptions to this rule have been documented (Flamm et al. 1993). Genetic tests aimed at the detection of resistance genes in bacterial isolates

by using DNA probes or PCR are based on the supposition that gene carriage equals resistance. Since genetic methods, including DNA probes and PCR-based assays, are now used in many clinical laboratories for other applications, the extension of these methods to the detection of antibiotic resistance genes is a natural next step. The use of these molecular tools in clinical laboratories to detect antibiotic resistance genes will allow us not only to identify resistance in clinical isolates, but also to keep a very careful eye on the emergence and further spread of resistance. In particular, there are several good reasons to pursue the identification of antibiotic resistance genes by genetic methods and the time has come to strongly encourage the development of molecular diagnostics in clinical laboratories and to utilize methods that are capable of more than just "identification" of resistance. First, DNA probes and/or nucleic acid amplification techniques are helpful for arbitrating MIC results that are at or near the breakpoint for resistance. For example, oxacillin-resistant isolates of *Staphylococcus aureus* with MICs between 2 and 8 μg/ml may either contain the methicillin resistance gene *mec* or may produce high levels of β-lactamase that slowly hydrolyze oxacillin (Chambers 1988). While vancomycin would be the drug of choice for the former cases, β-lactamase "hyperproducers" can be more effectively treated with penicillinase-stable β-lactams or β-lactam/β-lactamase inhibitor compounds (Massanari et al. 1988). A test showing the absence of the *mec* gene suggests that a physician could use an antibiotic other than vancomycin to treat the infection.

Second, genetic methods can be used to directly detect antibiotic resistance genes or mutations that result in resistance in organisms from clinical specimens in order to guide therapy early in the course of a patient's disease, long before cultures are positive.

This would aid in avoiding inappropriate antibiotics, thus preventing the selective pressure from such drugs in the development of resistance, as well as resulting in more successful therapy. For example, there are PCR assays that can detect mutations in the *rpoB* locus associated with rifampin resistance in *Mycobacterium tuberculosis* (Telenti et al. 1993). Such mutations indicate that the strain is at least resistant to rifampin and may be resistant to several drugs. A positive PCR result for mutations in the *rpoB* locus would direct the physician to avoid rifampin and use alternative antibiotics.

Third, molecular-based tests are more accurate than antibiograms for monitoring the epidemiological spread of a particular resistance gene in a hospital or a patient population. For instance, using PCR assays to track the spread of the *vanA* vancomycin resistance gene in enterococci has helped document the spread of multiresistant enterococci along the eastern seaboard of the United States (Clark et al. 1993). Antibiograms cannot differentiate between bacteria containing the *vanA* gene and those with derepressed *vanB* genes (Arthur and Courvalin 1993).

Lastly, genetic methods can be used as the so-called “gold standard” for the determination of antibiotic resistance when the accuracy of new susceptibility testing methods that use clinical isolates or stock cultures with border-line MICs is being evaluated (Huang 1993).

Genetic tests, such as PCR-based assays and the use of DNA probes for antibiotic resistance genes, need to become easier to use and more cost-effective. Hopefully, once the demand for such tests is high enough, the development and use will follow.

The use of molecular techniques currently used in research studies should also become mandatory in any and all epidemiology and surveillance work concerning the

development of antibiotic resistance, in order to detect the presence of novel and emerging resistance patterns, particularly now that the public health concern has arisen in the potential role played by animal populations in the spreading of antibiotic resistant strains and resistance genes to humans. Recent studies document the prevalence of plasmids, transposons, and integrons found carrying antibiotic resistance genes in environmental bacteria from material such as farm soil, sewage and pig manure, and their role in dissemination of antibiotic resistance genes. There is also the question concerning the potential for transfer of antibiotic resistance genes from genetically modified organisms, such as plants and bacteria in the environment, as well as the potential of transfer from genetically engineered plant material or contaminated food animals to bacteria in the human or animal gut. And we know that surveillance of antibiotic resistance should not only be confined to bacteria isolated from sick animals, be it in human or veterinary medicine, but that we need to assess the presence of resistance genes in bacteria from the normal flora of animals, as well as from environmental bacterial isolates…this will be what really tells us about the extent of the spread of antibiotic resistance.

Continued Basic Research in Molecular Evolution and Ecology of Resistance

In spite of significant increases over the last few decades in knowledge of the molecular mechanisms of antibiotic resistance and their gene determinants, our understanding about much of the ecological and evolutionary aspects in the development of resistance is still rudimentary. It goes without saying, that it remains imperative to continue basic scientific research in all areas on the development of resistance and how each area affects another. In order to have more control over the development of

resistance, it is essential that an understanding of the earliest stages of the process be obtained. We need to know, for instance, what happens ("microbiologically") when a new antibiotic is first introduced for therapeutic use on a large scale? Where and how does resistance first arise and how soon can the consequences be identified? Why is resistance apparently so stable and how do bacteria tolerate their increased genetic load or growth-retarding mutations, in the competition for survival in highly competitive environments? This information is necessary not only for a more complete understanding of the development of antibiotic resistance, but in order to develop more rational public health policies.

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The end of the twentieth century has left us with a clear message: It will not be enough to just find a drug to use as a lethal weapon against the resistant pathogens we have now and will have in our future. We need to remember our history lessons and learn theirs…we must understand why they are the way they are, and use this understanding to manage their evolution

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