

Investigating the Mechanisms of Antimicrobial Resistance

Kali Iyer^{1*} & Nicole Yokubynas^{2*}

*Authors contributed to equally to this work.

¹Honours Integrated Science - Biology Concentration Student, McMaster University

²Honours Integrated Science - Biochemistry Concentration Student, McMaster University

Direct Correspondence To: iyerk2@mcmaster.ca, yokubyna@mcmaster.ca

Abstract

Since their discovery, antimicrobial agents have revolutionized the treatment of infectious disease. Increased use of these agents, however, contributed to the concomitant development of microbial resistance, rendering these treatments less effective, or often useless¹. Despite considerable pressures on the scientific community, few new classes of antimicrobial agents have been discovered since the antibiotic era (1950-1970). Microbes' astonishing ability to adapt to antibiotics poses a serious threat to the modern health care system². In order to reduce the prevalence of resistance and develop new antimicrobial agents, it is crucial to understand the origins of antibiotics, the development of resistance through evolutionary mechanisms, and the biochemical mode of action of antibiotics along with their associated resistance pathways. This review investigates the various mechanisms of antibiotic resistance, from both an evolutionary and biochemical standpoint. Microbes are able to adapt and mutate at unparalleled rates through mechanisms such as horizontal gene transfer and high reproduction rates³. Acquired resistance mechanisms include modifying enzymes, point mutations in the target site of antibiotics, and reduced uptake of antibiotics⁴. This paper concludes by considering responses to the current crisis in microbial resistance, such as preventative measures and the development of new antibiotics.

Introduction

The introduction of antibiotics to treat infectious disease was one of the most significant medical advances of the 20th century. Initially, it was easy for society to disregard the few early cases of antibiotic failure in favour of their astronomical cure rates. As a result, many consider antibiotic resistance a recent problem². However, human use of antibiotics, together with microbial resistance, predates their modern use. For example, ancient remains of Nubian people from 350-550 CE have been found to contain traces of antibiotic compounds, and traditional Chinese medicine has incorporated the use of plant-based antibiotics for thousands of years to treat an array of illnesses². Resistance to major antibiotics such as penicillin and streptomycin was also reported almost immediately after their discovery². Despite this, persistent and widespread ignorance of resistance and the misuse of antibiotics have created a contemporary medical crisis.

The antibiotic revolution started in the lab of Paul Ehrlich (1854-1915). With the observation that common dyes affected certain strains of bacteria differently due to their chemical composition, he developed a novel screening program to search for compounds that targeted syphilis, a prominent disease of that time⁵. In 1909, Ehrlich successfully identified the antibiotic arsphenamine (Salvarsan). It was considered the "magic bullet" for syphilis and was the first recorded compound with antimicrobial activity⁶. Coincidentally, Ehrlich was also studying resistance in several organisms. He noticed that the practice of delivering drugs by increasing the dosage until it was therapeutically effective was leading to an

increase in resistance; he therefore recommended maximal dosages for treatment. By 1913, although the exact mechanisms for resistance were unknown, the idea of drug resistant bacteria was common knowledge of the scientific community⁷.

Alexander Fleming (1881-1955) is famous for his serendipitous discovery of penicillin; however it was not until Ernst Chain (1906-1979) and Howard Florey (1898-1968) published a paper on the antimicrobial value of penicillin and optimized purification techniques⁸ that the antibiotic era dawned². For decades, many new classes of antibiotics were synthesized or purified, resulting in a dramatic drop in infectious fatalities and the belief that the battle against microbes had been won⁶. One of the most shocking aspects about widespread use of antibiotics in this era was the scientific community's negligence, as the ability of bacteria to rapidly adapt was known, but not acknowledged⁹. The antibiotic era was followed by a dry spell of three decades during which resistance continued to grow and the rate of antibiotic discovery plummeted as pharmaceutical companies switched to more lucrative fields. This left a void in treatment options for patients with microbes resistant to drugs available at the time (Wright, G. (2014). In-person Interview.). Finally, in 2000, the dry spell was broken by the development of linezolid, the first of the class of oxazolidinones, followed by daptomycin in 2003. These antibiotics could kill bacteria resistant to a variety of older antibiotics, making them extremely attractive to the medical community¹⁰. Society needs to use these historical lessons about misuses of these drugs to protect the effectiveness of antibiotic treatment.

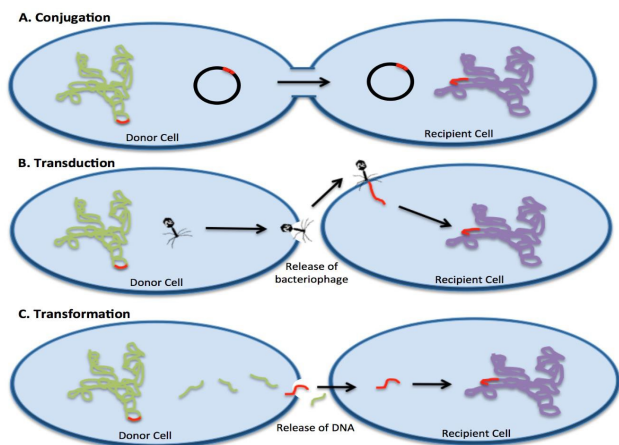


Figure 1. Horizontal Gene Transfer Mechanisms. (A) Bacterial Conjugation occurs through direct contact of the cells allowing the formation of a bridge in which replicated plasmids containing the *r* genes move. (B) Transduction is facilitated by the infection of bacteriophages that integrate the *r* genes into the hosts DNA along with bacteriophage DNA. (C) Bacterial transformation occurs when DNA containing *r* genes from one cell is released into the environment and is taken up by the other cell. *R* genes are indicated in red, donor DNA in green, and recipient DNA in purple¹¹.

Antibiotic resistance is now a common term, and society is starting to experience its significant implications. Researchers are feeling the pressure to learn about mechanisms of resistance in order to develop novel treatments and stay ahead of the microbes. However, to date, microbes have demonstrated an uncanny ability to adapt to antibiotics due to their high reproducibility, ability to acquire foreign DNA, and their high spontaneous mutation rates¹. Consideration of key processes involved in the bacterial transfer of genes and the associated biochemical mechanisms of antibiotic resistance is vital to understanding resistance now and for future generations.

Evolutionary Mechanisms of Antibiotic Resistance

Resistant genes (*r* genes) have been discovered in soil samples that are over 300 000 years old, indicating that they were present in microbes that could not have come in contact with modern antibiotics (Wright, G. (2014). In-person Interview.). *R* genes make up the antibiotic resistome (a collection of antibiotic resistant genes) and can exist in any microbe as either cryptic or expressed (Brown, E. (2014). In-person Interview.). Since bacteria reproduce at unparalleled rates, *r* genes spread rapidly to produce entire colonies of resistant organisms. Microbes have developed a plethora of mechanisms, not only for developing resistance, but also for sharing it amongst themselves⁹.

Table 1. Summary of target function of antibiotics and associated classes of antibiotics that participate in each type of inhibition^{4,14,15}

Process	Target	Reason for Selective	Common Examples
Interference with Cell Wall Biosynthesis	Enzymes that synthesize and cross-link peptidoglycan or prevent the peptide substrate from reacting with the transpeptidases or transglycosylases	Mammalian cells lack peptidoglycan	β -lactams (penicillin, cephalosporins) Glycopeptides (vancomycin, teicoplanin)
Bacterial Protein Synthesis Inhibition	Bacterial ribosomal subunits (50s, 70s and 30s)	Protein synthesis in eukaryotic cells occurs via the 80s ribosome	Macrolides, Tetracyclines, Aminoglycosides, Chloramphenicol, Linezolid, and Clindamycin
Interferes with Nucleic Acid Synthesis	DNA gyrase and RNA polymerase that prevent DNA replication	Not as selective as mechanisms are shared between eukaryotic and prokaryotic cells	Quinolones (Fluoroquinolones), Rifampicin, and Metronidazole
Inhibition of Metabolic Pathways	Folate Biosynthesis	Folic acid synthesis in bacteria differs radically	Sulfonamides, Trimethoprim
Disruption of Membrane Function	Membrane, Efflux Pumps	Not as selective as mechanisms are shared between eukaryotic and prokaryotic cells	Polymyxins and Amphotericin B

Mutation

The simplest mechanism by which microbes develop resistance is through spontaneous mutation. All cells have mechanisms to protect against mutations, such as proofreading enzymes that reduce the average mutation rate from 10^{-5} to 10^{-7} errors for every nucleotide synthesized. For bacteria, because of their high replication rate, this results in approximately three mutations every hour⁹. Rarely will a single mutation allow a cell to evade death from an antibiotic; however, given ample time and the occurrence of stepwise mutations, microbes have an increased chance of success⁹.

Mutation can also result from the selective pressures initiated with the use of antibiotics, as they encourage the spread of *r* genes through favourable selection, and induce mutations through SOS mechanisms. When bacteria are under stress they begin to synthesize an SOS gene sequence, producing more proteins involved in DNA synthesis and repair. These proteins are not as precise and result in a higher mutation rate⁹. Occasionally, mutator mutations can occur. These are errors in proteins that synthesize or repair DNA, and result in an increased mutation rate of up to 100 fold⁹.

Bacterial Promiscuity

Horizontal gene transfer (HGT) is the ability for bacteria to share DNA between organisms through methods other than reproduction as shown in Figure 1. In order for HGT to have an effect, bacteria must be able to uptake and incorporate foreign DNA³. Conjugation is the most common HGT mechanism and is dependent on the presence of plasmids. In conjugation, plasmids from the donor microbe are replicated and one copy is transferred into the recipient cell, which can then be amalgamated into the chromosomes of the recipient (Figure 1A)³. Transduction, another mechanism of HGT, is facilitated by bacteriophages (viruses that use bacteria as hosts to reproduce). Occasionally, bacterial DNA is integrated into the DNA of the bacteriophage progeny. When these progeny infect other bacterial cells, the initial bacterial DNA is also transferred into the new host. The bacteria can live with these new genes for some time if the virus enters a lysogenic state (Figure 1B). Finally, transformation occurs when DNA is released from one cell and taken up by another through the cell membrane to be incorporated into the host cell (Figure 1C)⁹. Awareness of gene transfer through the microbe community allows us to better understand how *r* genes are transmitted and can aid in anticipating future resistance. In order to avoid returning to the pre-antibiotic era, we must anticipate and prepare for resistance, as the bacterial evolutionary mechanisms in place will continue to operate.

Biochemical Mechanisms of Antibiotic Resistance

How Antibiotics Work

The mode of action (MOA) of all antibiotics is to interfere with vital bacterial function thereby inhibiting their growth, while maintaining minimal toxicity to the patient¹². Antimicrobial agents can be either bacteriostatic (agents that inhibit multiplication) or bactericidal (agents that directly kill the bacteria)⁴. Antibiotics achieve their low toxicity to eukaryotic cells by targeting cell components or processes specifically unique to bacterial cells (e.g. peptidoglycan, bacterial ribosomal subunits, folate biosynthesis, etc). The processes that the major classes of antibiotics disrupt are summarized in Table 1¹³.

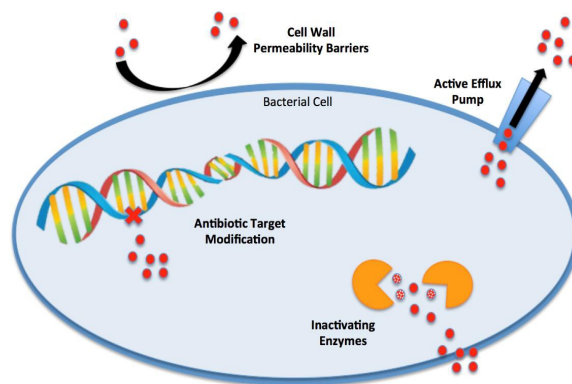


Figure 2. Biochemical Mechanisms of Antibiotic Resistance. Diagram of common mechanisms employed in bacteria associated with antibiotic resistance. Mechanisms include target modification, inactivating enzymes, active efflux, and modification of cell wall permeability. Mechanisms are not standalone within bacteria and within various strains of bacteria, thus adding complexity such as multiple drug resistance and cross-resistance. Antibiotics are indicated as red circles.

Development of Resistance

The major mechanisms of resistance correspond to the main MOA of antibiotics and include enzymes that inactivate the microbial agent, mutations in the target site that reduce the binding ability of the antibiotic, and devices that decrease the amount of antibiotic through reduced uptake or increased efflux¹⁶. These major classes of mechanisms and corresponding examples are outlined in Figure 2 and Table 2. Acquired resistance mechanisms are amplified by the selective pressures resulting from the misuse of antibiotics and present a major threat to the future of antibiotic therapy¹³.

Target Modification

One major method by which bacteria acquire resistance is through point mutations in select genes¹⁸. These mutations can arise spontaneously, or through the mechanisms of HGT as discussed previously. Mutations commonly occur at the binding site of the antibiotic target protein and result in an altered structure or conformation to reduce binding affinity of the antibiotic^{7,14}. For example, target modification used by the bacterium vancomycin-resistant enterococci (VRE) reduces the binding affinity of vancomycin by 1000 fold, rendering it nearly ineffective¹³.

Enzyme Inactivation

Certain enzymes within bacteria have the ability to alter the structure of antibiotics so as to render them ineffective^{4,19}. The precise mechanism varies with both the bacterial strain and the antibiotic, however most mechanisms are similar amongst similar classes of antibiotics¹⁹. A characteristic example is enzymes that inactivate β -lactams, such as penicillin. β -lactamases hydrolyze the β -lactam ring, which prevents the inhibition of the final cross-linking step in peptidoglycan formation by the antibiotic and renders the antibiotic ineffective. Other

enzymes have the ability to add chemical substituents to an antibiotic, reducing target binding affinity⁴. For example, certain enzymes act on the amino and hydroxyl groups of aminoglycosides to add substituents such as a phosphate groups. With the added substituents, the drug can no longer bind to its ribosomal target to inhibit protein synthesis¹⁹.

Table 2. Summary of the common types of resistant mechanisms and examples of known antibiotics affected by each form of resistance^{4, 12, 17, 18}

Types of Resistance	Examples
Target Modification	An amino acid substitution by VRE alters the D-Alanyl-D-Alanine structure in vancomycin, which prevents cell wall crosslinking. Vancomycin binds to the D-Ala-D-Ala terminus via hydrogen bonding at its active site, inhibiting the formation of peptidoglycan. The mutation alters an amide linkage to an ester linkage, which reduces the number of hydrogen bonds and introduces electronic repulsion, restricting antibiotic binding.
Enzyme Inactivation (Inactivation of β -lactams)	β -lactamases break and open the β -lactam ring (essential to inactivate transpeptidases in the cross-linking formation process), which renders the antibiotic ineffective.
Enzyme Inactivation (Add Chemical Substituents)	Enzymes add phosphate groups on the amino and hydroxyl group of aminoglycosides, which inhibit the antibiotic from binding to ribosomes to inhibit protein synthesis.
Enzyme Inactivation (Via Point Mutations)	Mutations in the gene that encodes for the enzyme dihydrofolate reductase eliminates the binding affinity of trimethoprim and its enzyme inhibition action.
Active Efflux	A resistance mechanism seen in an array of antibiotics, such as tetracycline, as it is not a standalone mechanism.
Cell Wall Permeability Barriers	Mutations in the antibiotic binding proteins in bacterial cell wall can reduce affinity to antibiotics such as β -lactams and glycopeptides to reduce the overall permeability of the cell wall.

Lastly, enzyme inactivation can occur via point mutations. Mutations result in amino acid substitutions that alter the structure and chemical properties of the antibiotic binding site, which

produce drug resistant forms of key enzymes that regulate vital metabolic activity¹³. An example of this is resistance to trimethoprim, whereby mutations in the gene encoding the enzyme dihydrofolate reductase eliminate the binding affinity of the drug and thus its enzyme inhibition action¹⁴.

Active Efflux and Reduced Permeability

Bacterial cells can develop resistance through active efflux and reduced permeability, which lower the intracellular concentrations of antibiotics. Active efflux occurs as a result of the overexpression of genes that code for efflux pumps when the antibiotic is detected in the cell⁴. The antibiotic is then removed from the cell at a faster rate than it enters, resulting in ineffective intracellular concentrations¹⁴. Cell wall permeability can be reduced through mutation of the antibiotic binding proteins in the cell wall, leaving it less susceptible to lysis, or through the reduced formation of crosslinks⁴. Both of these mechanisms often act as an enhancement that accompanies other types of resistance mechanisms. In addition, bacteria with alterations in permeability and efflux mechanisms must be able to withstand the associated reduction in nutritional intake¹³.

Resistance is Complex

In addition to these complex individual mechanisms, researchers are also faced with phenomena such as cross resistance (developed resistance to antibiotics that are chemically related) and multiple drug resistance (acquired resistance to unrelated chemical compounds due to the synergistic effects of the outlined mechanisms)¹³. As well, individual classes of antibiotics can be resisted simultaneously through multiple mechanisms²⁰. Understanding the biochemical mechanisms behind resistance is crucial to improving existing antimicrobial reagents and for designing new classes of antibiotics that are not hindered by currently known mechanisms of resistance.

Conclusion and Future Directions

Antimicrobial resistance is an ancient complication. R genes have been circulating the resistome for nearly 3.8 billion years, and will continue to accumulate with time¹². The development of antibacterial resistance was inevitable because of the sheer number of microbes, their short replication time, and the gene mutation frequency of bacteria. Bacteria have complex mechanisms allowing them to share selective resistant genes that code for mechanisms such as efflux pumps, target modification, reduced permeability, and modifying enzymes that allow the bacteria to avoid the harmful effects of antibiotics¹⁶.

Humans play an integral role in the development of resistance. The misuse of antibiotics dramatically increases the rate of resistance through selective pressures⁹. Society must adhere to strict use protocols to limit the spread of antibiotic resistance. These include dose concentration and duration guidelines⁹, ensuring antibiotics are prescribed only

for bacterial infections, and eliminating the use of sub-threshold doses of antibiotics for agricultural practice¹⁵.

The latter is especially important because transmission of resistant genes from animals to humans quickly results in the antibiotic becoming ineffective in human treatment¹. While agricultural use of antibiotics may have many economic benefits in terms of yield and product quality, the negative impact of this practice on the future of health care may be monumental.

In order to develop new antibiotics, we must look beyond the scope of what is known. One new avenue under investigation is the use of analog structures of natural molecules that have co-evolved with bacterial resistance to produce semi-synthetic derivatives¹. Another approach is to synthesize molecules that are more closely related to their natural counterparts. This may result in chemical libraries of molecules that are able to penetrate cells and avoid efflux. It is predicted that antibiotics will become very specific towards the target site and the type of infection. This type of treatment goes alongside the development of highly technical diagnostic assays to determine the characteristics of a particular infection (Brown, E. (2014) In-person Interview).

A full appreciation of both the history and emergence of antibiotic resistance, in addition to the evolutionary and biochemical mechanisms of the development of resistance will enable researchers to better understand areas of success and failure for future improvement of the treatment of infectious disease. It is only through this understanding that we can avoid an era in which humans are once again at the mercy of microbes.

References

1. Walsh, C. & Wright, G. Introduction: antibiotic resistance. *Chemical reviews* 105, 391-394 (2005).
2. Aminov, R. I. A brief history of the antibiotic era: lessons learned and challenges for the future. *Frontiers in microbiology* 1, 134 (2010).
3. Davies, J. & Davies, D. Origins and evolution of antibiotic resistance. *Microbiology and molecular biology reviews : MMBR* 74, 417-433 (2010).
4. Walsh, C. Molecular mechanisms that confer antibacterial drug resistance. *Nature* 406, 775-781 (2000).
5. Zaffiri, L., Gardner, J. & Toledo-Pereyra, L. H. History of antibiotics. From salvarsan to cephalosporins. *Journal of investigative surgery : the official journal of the Academy of Surgical Research* 25, 67-77 (2012).
6. Clardy, J., Fischbach, M. A. & Currie, C. R. The natural history of antibiotics. *Current biology : CB* 19, R437-441 (2009).
7. Wax, R. G., Lewis, K., Salyers, A. A. & Taber, H. *Bacterial resistance to antimicrobials*. (CRC Press, 2007).
8. Chain, E. *et al.* Penicillin as a chemotherapeutic agent. *The lancet* 236, 226-228 (1940).
9. Drlica, K. S. & Perlin, D. S. *Antibiotic resistance: Understanding and responding to an emerging crisis*. (FT Press, 2010).
10. Perry, C. M. & Jarvis, B. Linezolid. *Drugs* 61, 525-551 (2001).
11. Furuya, E. Y. & Lowy, F. D. Antimicrobial-resistant bacteria in the community setting. *Nature Reviews Microbiology* 4, 36-45 (2006).
12. Sosa, A. d. J. *et al.* *Antimicrobial resistance in developing countries*. (Springer, 2010).
13. Sefton, A. M. Mechanisms of antimicrobial resistance. *Drugs* 62, 557-566 (2002).
14. Wright, G. D. Molecular mechanisms of antibiotic resistance. *Chemical Communications* 47, 4055-4061 (2011).
15. Wright, G. D. & Poinar, H. Antibiotic resistance is ancient: implications for drug discovery. *Trends in microbiology* 20, 157-159 (2012).
16. Jack, D. B. Biochemistry of antimicrobial action, 4th ed, by T. J. Franklin and G. A. Snow, Chapman and Hall, 1989, 216 pages. ISBN 0-412-30260-8, paperback. *Biopharmaceutics & Drug Disposition* 11, 553-553 (1990).
17. Fischbach, M. A. & Walsh, C. T. Antibiotics for emerging pathogens. *Science* 325, 1089-1093 (2009).
18. Tenover, F. C. Mechanisms of antimicrobial resistance in bacteria. *The American journal of medicine* 119, S3-S10 (2006).
19. Todar, K. Online Textbook of Bacteriology. 2011. *Bacterial Endotoxin*. [textbookofbacteriology.net/endotoxin.html].
20. Cox, G. & Wright, G. D. Intrinsic antibiotic resistance: mechanisms, origins, challenges and solutions. *International Journal of Medical Microbiology* 303, 287-292 (2013).