Mechanisms of Antibiotic Resistance in the Microbial World

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I. An Historical Overview of Antibiotics

Allow me to begin with an historical overview of antibiotics. The antibiotics field was initiated when Paul EHRLICH first coined the term 'magic bullet', or chemotherapy, to designate the use of antimicrobial compounds to treat microbial infections. In 1910, EHRLICH discovered the first antibiotic drug, Salvarsan, which was used against syphilis.

EHRLICH was followed by Alexander FLEMING, who discovered penicillin by accident in 1928. Then, in the 1935, Gerhard DOMAGK discovered the sulfa drugs, thereby paving the way to the discovery of the anti-TB drug Isoniazid. Then, in 1939, René DUBOS became the first scientist to discover an antibiotic after purposely looking for it in soil microbes. DUBOS discovered Gramicidin, which is still used today to treat skin infections.

Finally, in 1943, the first TB drug, Streptomycin, was discovered by Selman WAKSMAN and Albert SCHATZ. WAKSMAN was also the one who coined the term 'antibiotics'. Thus, antibiotics have been used to treat bacterial infections since the 1940s.

II. The Basic Characteristics of Antibiotics

Today, there are about 4 000 compounds with antibiotic properties. Antibiotics are used to treat and prevent infections, and to promote growth in animals.

Antibiotics are derived from three sources: moulds or fungi; bacteria; or synthetic or semi-synthetic compounds. They can be used either internally or topically, and their function is to either inhibit the growth of pathogens or to kill them. Antibiotics can thus be divided into Bacteriostatic drugs, which merely inhibit the growth of the pathogen, and Bacteriocidal drugs, which actually kill the bacteria. However, the distinction is not absolute, and depends on the drug concentration, the bacterial species, and the phase of growth.

Antibiotics are more effective against actively growing bacteria, than against non-growing persisters or spores. When two antibiotics are used in combination, the effect could be additive, synergistic, or antagonistic.

Antibiotics can also be divided into broad-spectrum and narrow-spectrum antibiotics. For example, Tetracycline, a broad spectrum antibiotic, is active against G+ bacteria, G- bacteria, and even against mycobacteria; whereas penicillin, which has a relatively narrow spectrum, can be used mainly against G+ bacteria. Other antibiotics, such as Pyrazinamide, have an even narrower spectrum, and can be used merely against *Mycobacterium tuberculosis*.

Antibiotics fight against bacteria by inhibiting certain vital processes of bacterial cells or metabolism. Based on these processes, we can divide antibiotics into five major classes:

1. Cell wall inhibitors, such as Penicillin and Vancomycin.

2. Inhibitors of nucleic acid synthesis, such as Fluoroquinolones, which inhibits DNA synthesis, and Rifampin, which inhibits RNA synthesis.

3. Protein synthesis inhibitors, such as Aminoglycoside.

4. Anti-metabolites, such as the sulfa drugs.

5. Antibiotics that can damage the membrane of the cell, such as Polymyxin B, Gramicidin and Daptomycin.

III. Drug Resistance

1. The Scope of the Drug Resistance Problem

Drug resistant bacteria have been posing a major challenge to the effective control of bacterial infections for quite some time. The WHO has compiled a list of all the major antibiotic resistant bacteria. For pneumonia, they include Streptococcus pneumoniae (penicillin-R). For diarrhoeal diseases, they include Shigella dysenteriae, Salmonella typhi, and Vibrio cholerae. In the case of *M. tuberculosis*, MDR-TB and XDR-TB are becoming an increasingly significant problem. Finally, Hospital-acquired infections and Gonorrhoea infections are also increasingly drug resistant. The problem is so severe that it prompted an anonymous writer to write a short poem, which reflects our ongoing fight with bacteria.

- 2000 B.C. – Here, eat this root
- 1000 A.D. – That root is heathen. Here, say this prayer.
- 1850 A.D. – That prayer is superstition. Here, drink this potion.
- 1920 A.D. – That potion is snake oil. Here, swallow this pill.
- 1945 A.D. – That pill is ineffective. Here, take this penicillin.
- 1955 A.D. – Oops....bugs mutated. Here, take this tetracycline.
- 1960-1999 – 39 more "oops"...Here, take this more powerful antibiotic.
- 2000 A.D. – The bugs have won! Here, eat this root.

Drug resistance refers to a situation in which the drugs that usually destroy the bacteria no longer do so. It implies that people can no longer be effectively treated against the bacteria. Consequently, they are ill for longer periods of time; and they face a greater risk of dying. Furthermore, epidemics are prolonged, putting more people at a risk of becoming infected. Antibiotic resistance is an extremely expensive problem. Its costs in the US alone are estimated at US $5-$24 billion per year.

2. Causes for Drug Resistance

One of the main causes of antibiotics drug resistance is antibiotic overuse, abuse, and in some cases, misuse, due to incorrect diagnosis. A second cause is counterfeit drugs. Antibiotic use

in animal husbandry is also creating some drug resistant bacteria, which can be transmitted to humans. Increased globalisation could also cause the spread of drug resistance. Finally, hospital settings often give rise to antibiotic resistant bacteria.

3. Natural and Acquired Resistance

Antibiotic resistance can be divided into natural resistance and acquired resistance. Natural resistance means that the bacteria are 'intrinsically' resistant. For example, Streptomyces has some genes responsible for resistance to its own antibiotic. Other examples include organisms that lack a transport system or a target for the antibiotics. In other cases, the resistance can be due to increased efflux activity.

Acquired resistance refers to bacteria that are usually sensitive to antibiotics, but are liable to develop resistance. Acquired resistance is often caused by mutations in chromosomal genes, or by the acquisition of mobile genetic elements, such as plasmids or transposons, which carry the antibiotic resistance genes.

4. Genetic and Phenotypic Resistance

Broadly speaking, antibiotic resistance could also be divided into genetic drug resistance, which is the one most commonly discussed, and phenotypic drug resistance, which is a more subtle type. Genetic resistance is due to chromosomal mutations or acquisition of antibiotic resistance genes on plasmids or transposons. Phenotypic resistance is due to changes in the bacterial physiological state, such as the stationary phase, antibiotic persisters, and the dormant state.

IV. Genetic Drug Resistance Mechanisms

Until the 1950s, it was not clear how the bacteria acquire drug resistance. Then, Joshua LEDERBERG devised replica plating, and demonstrated that the antibiotic resistant mutants are pre-existing. Thus, the antibiotics merely selected these mutants.

Then, in 1988, John CAIRNS showed that when the bacteria are not growing, they are nevertheless able to acquire new mutations, due to some genetic alteration process. Those mutations are called adaptive mutations. It was never formally proven that adaptive mutations cause antibiotic resistance; however, it is possible, particularly in non-growing forms of bacteria.

There are five major mechanisms of antibiotic drug resistance, which are due to chromosomal mutations:

1. Reduced permeability or uptake.
2. Enhanced efflux.
3. Enzymatic inactivation.
4. Alteration or over-expression of the drug target.
5. Loss of enzymes involved in drug activation. This mechanism is relatively new.

1. MDR Resistance

The Multi-drug Resistance (MDR) mechanism can be caused by different mechanisms in different organisms. For example, in 1959, the Japanese found Shigella species that were resistant to Sulfonamides, Streptomycin, Chloramphenicol, and Tetracycline. The resistance was due to plasmid, which carried different antibiotic resistance genes. The other MDR mechanism is due to sequential accumulation of chromosomal mutations in different drug resistant genes, as in the case of MDR-TB and XDR-TB.

2. Examples of Chromosomal Mutations

Let us now examine some examples of chromosomal mutations.

a. Reduced Permeability or Uptake

The first mechanism is reduced permeability or uptake of the bacteria. For example, Neisseria gonorrhoea porin can acquire mutations that can cause resistance to penicillin and tetracycline. Another example is Enterobacter aerogenes porin, which can acquire mutations that cause cephalosporin resistance.

b. Increased Efflux Activity

There are many examples of the second mechanism, increased efflux activity. The first one, Tetracycline efflux was discovered in the early 1980s. TetK serves as an example for an efflux-mediated Tetracycline resistance. Under normal conditions, the efflux gene, TetK, is not expressed, due to a suppressor that is bound to the promoter region. However, in the presence of Tetracycline, it binds to the repressor, relieves the suppression, and causes transcription and translation of the efflux pump, thereby leading to Tetracycline resistance.

c. Enzymatic Inactivation

A famous example of the third mechanism, enzymatic inactivation, is Beta-lactamases, which can cleave beta-lactam antibiotics and cause resistance. A second example is the Aminoglycoside-inactivating enzymes, which can add Acetyl, Adenyl, and Phosphoryl groups to inactivate the antibiotic. Finally, both Chloramphenicol Acetyl Transferase and Streptogramin Acetyl Transferase can add an acetyl group to inactivate the two antibiotics, respectively.

d. Alteration of Drug Target

An example close to home to the alteration or over-expression of the drug target is inhA, which has a -15C to T promoter mutation. This mutation causes over-expression of the drug target InhA, and lead to a low-level isoniazid (INH) resistance in *M. tuberculosis*. A second noteworthy example is penicillin resistance, which is due to alterations in penicillin binding proteins.

A third example is vancomycin resistance. Under susceptible conditions, vancomycin prevents cross-linking of peptidoglycan by binding to D-Ala-D-Ala dipeptide of the muramyl peptide. Most G+ bacteria acquire vancomycin resistance by changing D-Ala-D-Ala to D-Ala-D-lactate, which does not bind to vancomycin.

A fourth example is mutations in DNA gyrase A and B subunits in quinolone resistance is another example of an alteration of the drug target. Finally, in Rifampicin resistance, there are mutations in *rpoB* gene encoding beta-subunit of RNA polymerase.

e. Loss of Enzymes in Drug Activation

As I have noted earlier, loss of enzymes involved in drug activation is a relatively new mechanism of drug resistance. In this case, the antibiotic itself is a prodrug, which has no direct activity against the bacteria. Rather, it relies on the activation by a bacterial enzyme.

INH can serve as a useful example. KatG (catalase-peroxidase) is an enzyme involved in the activation of INH, which produces a range of reactive metabolites including reactive oxygen species and then reactive organic radicals, which then inhibit multiple targets, including mycolic acid synthesis.

Another example is the Metronidazole (MTZ) prodrug. MTZ is activated through RdxA (nitroreductase), and then forms reactive species that damage the DNA. Thus, mutations in this enzyme cause resistance to Metronidazole.

3. Regulation of Resistance Genes

Bacteria are extremely versatile in becoming resistant to antibiotics, and are actually able to regulate their drug resistance genes. One example is due to repressors, as in the case of Tetracycline, which I have shown earlier as an example of efflux mediated drug resistance.

A second example, which relates to erythromycin resistance genes (erm) is due to attenuation. In the absence of erythromycin, a stem-loop structure forms in the mRNA, which buries the Ribosome Binding Site (RBS) and the start codon. Thus, in the absence of the antibiotics, the drug resistance gene is not expressed. However, low concentrations of erythromycin cause the RBS and start codon to be exposed, causing a translation of the drug resistance gene, erm, resulting in the expression the gene.

4. Transfer of Resistance Genes

In addition to chromosomal mutations, a second broad category of drug resistance is due to mobile genetic elements, such as plasmids or transposons, which carry drug resistant genes. Allow me to offer several examples:

- Streptomycin-resistance genes, strA- and strB, which can be carried on plasmid, and cause Streptomycin resistant.

- Sulfa drug resistance, caused by plasmids that carry the drug insensitive form of the enzyme.

- A relatively new mechanism is the plasmid-mediated qnr (quinolone resistance). The qnr gene encodes a device called pentapeptide, which is a DNA mimic. Pentapeptide binds to the DNA gyrase and thus helps prevent the quinolone drug from binding to the gyrase, thereby causing low-level resistance.

Transposons can also carry drug resistant genes. It is noteworthy that plasmids and transposons are not involved in drug resistance mechanisms in TB.

V. Phenotypic Drug Resistance

Phenotypic drug resistance refers to the fact that when the bacteria are not growing, they can become unsusceptible to antibiotics. Then, when the bacteria are sub-cultured into a fresh media, and they begin to grow again, they regain their antibiotic susceptibility. This complex mechanism has been posing significant problems as in biofilm infections and particularly for TB chemotherapy.

1. Biofilm Infections

Drug resistance is also becoming a major problem in human infections involving biofilm. For example, some orthopaedic devices can have *Staphylococcus aureus* and *Staphylococcus epidermidis* infections. Once these devices are infected with the biofilm, it is extremely difficult to eliminate the biofilm completely merely by using antibiotics. Often, the orthopaedic device must be replaced.

2. Biofilm Formation

Let us review how biofilms are formed. Initially, the bacteria simply attaches to surfaces irreversibly, and then irreversibly. Then, early biofilms are formed, and turn into mature biofilms. They are then able to release new organisms off the structure. Biofilm bacteria are extremely resistant to antibiotics. When we compare the susceptibility of the planktonic form and biofilm, we observe that antibiotic imipenem can destroy planktonic organisms of *P. aeruginosa* effectively at 1 (μg/ml), but require at least 1024 (μg/ml) to fight against biofilm.

The Biofilm structure is extremely complex. The bacteria are divided into different sub-populations, ranging from an almost spore-like sub-population, to a more actively metabolising population at the colony surface.

3. Salicylate -Induced Antibiotic Resistance

Another form of phenotypic drug resistance is mediated by salicylic acid, which is the active component in aspirin. Different organisms have been found to have Salicylate-mediated drug resistance. E. coli is the best example, and additional ones include Klebsiella, Pseudomonas, Burkholderia, and also *M. tuberculosis*. In has been demonstrated that in the presence of Salicylate, TB bacteria is less susceptible to INH, Rifampicin, EMB, and PAS. Preliminary experiments in the mouse model of TB demonstrate that aspirin can antagonise the activity of INH, indicating that it might also have some effect in-vivo.

Let us examine the mechanism of Salicylate-induced antibiotic resistance in E. coli. There is Multiple Antibiotics Resistance (MAR) operon in the E.coli where the MarR is the repressor. Salicylate binds to MarR in order to release the suppression of the MarAB operon. MarA encodes a transcription factor, which in turn, activates the transcription of the efflux pump acrAB, as well as the membrane channel tolC, which is required for the functioning of the pump. Thus, the first drug resistance mechanism is conducted through increased efflux. In a second mechanism, MarA enhances the transcription of micF, an antisense RNA for ompF, a membrane porin required for entry of antibiotics. Thus, micF shuts down the expression of ompF through antisense. When the porin expression is reduced, the drug intake is reduced as well.

VI. Bacterial Persisters

The bacterial persisters are an important example of the phenotypic resistance. Persistence was first discovered with penicillin in 1944. Joseph BIGGER demonstrated that penicillin can kill merely 99% of the bacteria. The remaining 1% of the bacteria were persisters. When these persisters were cultured to fresh media, they regained susceptibility to antibiotics.

1. The Toxin-Antitoxin (TA) Model

For many years, the mechanism of persisted resistance to antibiotics remained unknown. Then, in the 1980s, Harris MOYED found the HipA gene being involved in persistence in E. coli. Later, a group headed by Kim LEWIS discovered that HipA and HipB form a toxin-antitoxin (TA) module, in which an inappropriate expression of toxin leads to persister formation. The TA model was initially discovered on plasmids, but was later observed in chromosomes of many bacterial species. When a toxin is expressed, it shuts down transcription and translation. Thus, the activity of toxins and anti-toxins must be carefully regulated, to prevent cells from dying.

The model has difficulties explaining persistence in organisms that do not have TA modules. More recently, a Chicago group has demonstrated that if any toxic proteins are expressed, they can induce persister formation, regardless of the toxin-antitoxin module. These findings raises some questions as to the validity of the toxin-antitoxin theory.

2. PhoU

Recently, we have identified a new persisted gene, PhoU, through a transposon-based screen in E. coli. The PhoU mutant displays high susceptibility to a range of different antibiotics, such as Ampicillin, Streptomycin, Sulfa drugs, and Quinolone drugs. It is also more susceptible to different conditions, such as heat, starvation, acid pH, and weak acids. The PhoU mutant phenotypes can be complemented by a wild type PhoU gene.

An interesting feature of the PhoU mutant is that it is highly susceptible to Ampicillin in the stationary phase. Many other antibiotics, especially penicillin, are not active against stationary phase bacteria, but merely against growing bacteria. We have also demonstrated, through microarray experiments, that the PhoU mutant has a hyper-active metabolism.

Thus, PhoU appears to be a suppressor mechanism for cellular metabolism. When it is expressed, it shuts down cellular metabolism. Although the detailed mechanism is not clear yet, we believe that PhoU could be an interesting drug target for killing persister bacteria. We are therefore currently trying to raise the interest of some pharmaceutical companies in designing drugs targetting PhoU.

I. Managing the Drug Resistance Problem

1. Limiting the Spread of Drug Resistant Bacteria

Several measures could be used to prevent the spread of drug resistant bacteria. First, we could use better treatment strategies; better immunization programmes; improved hygiene and nutrition; and initiatives targeting the poor populations. Second, it might be useful to establish antibiotic resistance surveillance programmes. Third, better education of health care professionals is required to prevent the prescription of unnecessary antibiotics. It is

noteworthy that significant investment of time, effort, and money is necessary in order to control antibiotic resistant bacteria.

Of course, as long as antibiotics are used, antibiotics resistance is bound to occur. However, we might be able to reduce the drug resistance problem. One strategy is to ensure that antibiotics are used only when necessary. A second strategy is to ensure that they are used for the appropriate amount of time; that is, that the treatment is not stopped before it is completed. Patient compliance is a key problem in that respect. A third strategy for limiting drug resistance is to use antibiotics combinations. Unfortunately, while all these strategies seem sound in theory, in reality, the problem persists.

2. Development of New Antibiotics

Another possibility is to develop new antibiotics. However, that is not an easy task. The sad irony is that many pharmaceutical companies have decided to abandon their antibiotic development programmes when new antibiotics are needed most, since 99% of the drug candidates fail, and antibiotics are not as profitable as other, more commonly used, drugs.

The traditional approach of screening microbes for antibiotics is not efficient. A second approach, which utilises target-based screening, became popular when genomics tools became available. However, although the idea is appealing, in reality, it is extremely difficult. Many companies have tried this approach, and so far they have all failed. The whole organism-based approach is more feasible but the conditions of screen need careful consideration.

3. Phage Therapy

Phage therapy can also be used to deal with antibiotics resistance. This approach had already been used by the Russians during the Second World War, and has been gaining popularity again in recent years. Phage can be applied on the wounds of a patient to kill the bacteria, and has proven to be quite effective. Of course, it cannot be used for internal infections, and the bacteria might also develop phage resistance.

4. Mobilisation of Host Defence Mechanisms

Yet another approach is to mobilise host defence mechanisms. This can be achieved through the mobilisation of innate immunity such as defensins, or through the development of vaccines, which make antibiotics less necessary. The idea is to boast the immune response capability to control the bacterial infection. Of course, that approach is not always successful.

5. The Use of Normal Bacterial Flora

Finally, one could also potentially use normal bacterial flora to suppress some pathogens.

II. Conclusion

I would like to end my talk with a quote by the Nobel Prize laureate Joshua LEDERBERG: "*Antibiotic resistance as a phenomenon is, in itself, not surprising. Nor is it new. It is, however, newly worrying, because it is accumulating and accelerating, while the world's tools for combating it decrease in power and number.*"

This description may sound gloomy, but unfortunately, it is rather precise. We must remember that the bugs have been on this planet much longer than we have and can develop

resistance to any antibiotics used to treat them. We have to use a combination of approaches as discussed above to minimize the resistance problem, and hopefully can live in peace with the microbes.

III. Question and Answer Session

Kathryn DERIEMER

Do you believe that *M. tuberculosis* forms biofilms *in vivo*?

Ying ZHANG

Absolutely, I do not doubt it. There is an extensive controversy on the definition of biofilm, and different models are used. Basically, biofilms merely demonstrate the remarkable plasticity of bacteria, and their ability to adapt to various stress environments, including antibiotics. For example, in the case of tuberculosis, the bacteria form clumps; they aggregate together.

However, I am not sure whether the fact that *M. tuberculosis* develops biofilms assists us in the development of new approaches for control, since we must still find a way to cope with the biofilm persisters.

Participant

What are the functions of PhoU?

Ying ZHANG

The function is not clear. In E.coli, it is the last gene of the PST operon, which is involved in phosphate uptake. Thus, when it expresses, it suppresses the PST operon. It is thus a repressor of phosphate metabolism. Recently, PhoU has been shown to be expressed in biofilms. Thus, we believe it is involved in bacterial survival and persister formation.

Participant

Can antibiotics kill the persistent bacteria?

Ying ZHANG

Antibiotics are able to kill merely growing forms of bacteria. They cannot kill the persisters, which are a non-growing form of bacteria. The persisters are thought to be taken care of by the host immune system. That system of the human body is usually greatly underestimated.

Participant

Do you believe the efflux pumps play an important role in *M. tuberculosis* resistance?

Ying ZHANG

To my knowledge, it has not been shown that the efflux pumps found in *M. tuberculosis* play any role in clinical drug resistance.

Qian GAO

Can phenotypic resistance explain the problems in drug susceptibility testing?

Ying ZHANG

It is true that phenotypic resistance is defined by the non-growth of the bacteria, while drug susceptibility testing, such as MIC, rely on the growth of the tested organism. The drug susceptibility tests can detect merely the more stable genetic drug resistance, mediated by gene mutations not phenotypic drug resistance.

Participant

What is the difference between persistence and latency?

Ying ZHANG

There is indeed some confusion in this area. I use the term persistence to refer to a bacterial property, and reserve the term latency to describe the host. However, host latency refers to a combination of the bacterial and host factors.

It is possible that the bacteria in a latent tuberculosis infection would still continue to grow; thus, the bacteria are not necessarily latent themselves. Rather, latency indicates that the host is infected by the bacteria, but has not shown any obvious symptoms of the disease.

Participant

Are there more powerful tools for identifying the drug resistance mechanism in tuberculosis?

Ying ZHANG

The genetic tools are indeed more powerful today, and it is possible to knock-out and overexpress genes in *M. tuberculosis*. However, some drug resistance mechanisms remain to be identified. Even with INH, the mechanism is not completely known. While some procedures exist, a tremendous amount of luck is still necessary to identify new mechanism of drug resistance.

Participant

Could you clarify the difference between the dormant and persistent status?

Ying ZHANG

There is a lot of confusion here , since the terms ‘persisters’ and ‘dormant bacteria are both used. We proposed a new Yin-Yang model model (Y. Zhang, Clin Pharmacol Ther. 2007, 82:595-600). I believe that in theory, there are numerous sub-populations of bacteria, even among persisters. Among the persister population, I would refer to the bacteria that requires resuscitation in order to form CFU as ‘dormant’. Persisters, on the other hand, are defined by their non-susceptibility to stress and antibiotics. I believe the problem is that people use the term ‘dormant bacteria’ and ‘persister’ rather loosely, without rigorously defining the condition.