Mechanisms of Bacterial Antibiotic Resistance

Ying Zhang, MD, PhD
Department of Molecular Microbiology & Immunology
Bloomberg School of Public Health
Johns Hopkins University
Email: yzhang@jhsph.edu
History

- Paul Ehrlich: **Methylene blue** to fight malaria (1891), **trypan red** against trypanosomes (1904), **Compound 606** (Salvarsan) (**yellow**), the first antibiotic (1910) against syphilis. Coined terms "magic bullet”, "chemotherapy”
- Alexander Fleming: 1928, penicillin (**Penicillium notatum**)
- Gerhard Domagk: 1935, sulfa drugs, prontosil, sulfanilamide, isoniazid
- Rene Dubos: 1939, tyrothricin (gramicidin/tyrocidin) from **B. brevis** (topical use against G+ bacteria)
- Selman Waksman and Albert Schatz: 1943, streptomycin—first aminoglycoside (**Streptomyces**) against TB, coined the term “antibiotics”
- Chloramphenicol, 1947, from **Streptomyces venezuelae**
- Tetracycline: 1948, from **Streptomyces**
The famous Fleming photograph showing the action of the mould PENICILLIUM NOTATUM on colonies of staphylococci.
Antibiotics

Antibiotics are derived primarily from three major sources:
- molds or fungi
- bacteria: Streptomyces, Bacillus
- synthetic or semisynthetic

used internally or topically, inhibit or kill pathogens
work best on actively growing organisms, but not on non-growing persisters or spores
Bacteriostatic versus Bactericidal

- Static: inhibit growth
- Cidal: kill
- Cidal or static is not absolute, depending on drug concentration, bacterial species, phase of growth of the organism, and even the number of bacteria
- MIC (minimum inhibitory concentration): agar dilution; broth dilution, automated antibiotic susceptibility testing
- MBC (minimum bactericidal concentration)
Antibiotic Combination

- **Additive:** drug combination is more active than either drug alone and the response represents a sum of two drug effects.
- **Synergism:** combination has a greater effect than the sum of the two individual drug effects.
- **Antagonism:** combination has less activity than that of individual drug alone.
Broad versus Narrow Spectrum

- **Tetracycline**: typical broad spectrum antibiotic, active against G+ and G- bacteria, Mycobacterium, Rickettsia, protozoan
- **Penicillin**: primarily G+ bacteria,
- **Gentamycin**: G- bacteria
- **Pyrazinamide**: specific for M. tuberculosis
Mechanism of Action: Five major classes of antibiotics

- Inhibition of cell wall synthesis (beta-lactams, glycopeptides): most common
- Inhibition of protein synthesis (aminoglycoside, chloramphenicol, tetracycline, macrolides)
- Disruption of membrane permeability (polymyxin B for G- bacteria, gramicidin and daptomycin for G+ bacteria)
- Inhibition of nucleic acid synthesis (fluoroquinolones for DNA and rifampin for RNA synthesis)
- Anti-metabolite (sulfa drugs)
Antibiotic Resistance - a condition in which there is insensitivity to drugs that usually cause growth inhibition or cell death at a given concentration

- People cannot be effectively treated
- People are ill for longer
- People are at a greater risk of dying
- Epidemics are prolonged
- Others are at a greater risk of infection

(http://www.who.int/infectiousdiseasereport/2000(graphs/5_resistance.htm))
The History of Medicine

- 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.

— Anonymous

(From: http://www.who.int/infectious-disease-report/2000/)
The Big Guns of Resistance:
Bacterial Pathogens (WHO)

- Pneumonia: Strep pneumo, penicillin-R
- Diarrhoeal diseases: Shigella dysenteriae, Salmonella typhi, Vibrio cholerae
- Tuberculosis: MDR/XDR-TB, lengthy therapy
- Hospital-acquired infections: Salmonella, Pseudomonas and Klebsiella—most notably in developing nations; methicillin-resistant Staphylococcus aureus (MRSA), VISA (vancomycin-intermediate Staph aureus) and vancomycin-resistant Enterococcus (VRE)
- Gonorrhoea: antibiotic abuse has propelled a once-curable nuisance into a potentially life-threatening contagion—one of the major healthcare disasters of the 20th century
Causes of Resistance Problem

- Antibiotic overuse, abuse or misuse (misdiagnosis)
  - In Taiwan, 55% of patients arriving in ER had antimicrobials in urine.
  - Antibiotic resistance costs US $5-$24 billion/year
- Counterfeit Drugs
- Antibiotic use in animal husbandry and food: Avoparcin (vancomycin) use in livestock-> VRE jumping from animals to humans; chicken contaminated with MDR-campylobacter
- Globalization and resistance
- Resistance and hospitals: more than 70% of the bacteria that cause hospital-acquired infections are resistant to at least one of the antibiotics most commonly used to treat them
Antibiotic Resistance

- **Natural Resistance**: Bacteria may be inherently resistant to an antibiotic. Streptomyces has some genes responsible for resistance to its own antibiotic; or a Gram- bacteria have an outer membrane as a permeability barrier against antibiotic (e.g., penicillin); or an organism lacks a transport system for the antibiotic; or efflux pumps; or it lacks the target (e.g. INH-mycolic acid synthesis) of the antibiotic

- **Acquired Resistance**: Bacteria can develop resistance to antibiotics due to (1) mutations; (2) mobile genetic elements, e.g., plasmids or transposons or integrons carrying antibiotic resistance gene
Antibiotic Resistance Mechanisms

- Two Types of Antibiotic Resistance:
  - Genetic resistance: due to chromosomal mutations or acquisition of antibiotic resistance genes on plasmids or transposons
  - Phenotypic resistance: due to changes in bacterial physiological state as in stationary phase, antibiotic persisters, dormant state
How Do Bacteria Acquire Resistance?

- Resistance due to drug selection or drug induction?
  - 1950s, Joshua Lederberg devised replica plating-> demonstrating selection of pre-existing resistant mutant- growth dependent *Spontaneous mutations*
  - 1988, John Cairns showed mutations arise also in non-dividing or slowly dividing cells and have some relation to the selective pressure used. These mutations, named *adaptive mutations*, arise only in the presence of a non-lethal selective pressure that favors them.
  - Drug induction also plays a role, e.g., efflux

- Natural selection of spontaneous mutants in a large bacterial population: mutation frequency to rifampin=$10^{-8}$, INH=$10^{-6}$

- Drug combination to avoid resistance: mutants resistant to both RIF and INH occurs at $10^{-14}$
Mechanisms of Drug Resistance

(A) Chromosomal mutations:
1. Reduced permeability/uptake
2. Enhanced efflux
3. Enzymatic inactivation (beta-lactamase)
4. Alteration of drug target
5. Loss of enzymes involved in drug activation (as in isoniazid resistance-KatG, pyrazinamide resistance-PncA)

(B) Plasmid or transposon mediated:
Multidrug Resistance (MDR)

- Plasmid-mediated: 1959 Japanese found plasmid-mediated MDR (sulfonamides, streptomycin, chloramphenicol, tetracycline) in *Shigella* species

- Sequential accumulation of chromosomal mutations, one at a time, leading to MDR
A. CHROMOSOMAL MUTATIONS

1. Reduced Permeability/Uptake

- Outer membrane porin mutations (cross-resistance): Neisseria gonorrhoeae porin mutation cause resistance to penicillin and tetracycline; Enterobacter aerogenes porin mutation cause cephalosporin resistance
2. Increased Efflux Activity

(many examples)

- Membrane bound proteins involved in extrusion of antibiotics out of bacterial cell, energy-dependent (ATP, proton motive force)
- Tetracyclines (first efflux mechanism):
  - efflux proteins - TetA to G in G- bacteria; TetK and TetL in G+ bacteria
  - Macrolides (Staph), ATP-dependent fluoroquinolones (pseudomonas sp., Staph, enterococci), streptogramins (Staph)
- Cross-resistance by efflux pump:
3. Enzymatic Inactivation

- Beta-lactamases (penicillinase, Abraham and Chain, 1940) cleave beta-lactam antibiotics and cause resistance
  - Superbug: NDM-1, or New Delhi metallo-beta-lactamase degrades carbapenem (carbapenemase), on plasmid, in Klebsiella pneumoniae, Escherichia coli, horizontal gene transfer, first detected in North Carolina, in 1996
- Aminoglycoside-inactivating enzymes (adding groups acetyl, adenyl, phosphoryl to inactivate the antibiotic)
- Chloramphenicol acetyl transferase: add acetyl group to inactivate chloramphenicol
- Streptogramin acetyl transferase: found in Staph, Enterococci
4. Alteration of Drug Target (numerous examples)

- Penicillin-binding proteins (PBP/transpeptidase): alteration due to mutations cause resistance to beta-lactams commonly in G+ bacteria (e.g., methicillin-resistance in S. aureus, mecA encoding PBP2a)

- Vancomycin resistance: 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 vancomycin
A Susceptible bacteria

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>L-Ala</td>
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<tr>
<td>D-Glu</td>
<td>D-Glu</td>
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<tr>
<td>L-Lys</td>
<td>L-Lys</td>
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<tr>
<td>D-Ala</td>
<td>D-Ala</td>
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Normal cross-linking in peptidoglycan

B Resistant bacteria

Pyruvate $\rightarrow$ lactate and

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<tbody>
<tr>
<td>L-Ala</td>
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<tr>
<td>D-Glu</td>
<td>D-Glu</td>
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<tr>
<td>L-Lys</td>
<td>L-Lys</td>
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<tr>
<td>D-Ala</td>
<td>D-Ala</td>
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<tr>
<td>D-lactate</td>
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One enzyme (VanH) catalyzes conversion of pyruvate to lactate

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<tbody>
<tr>
<td>L-Ala</td>
<td>L-Ala</td>
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<tr>
<td>D-Glu</td>
<td>D-Glu</td>
</tr>
<tr>
<td>L-Lys</td>
<td>L-Lys</td>
</tr>
<tr>
<td>D-Ala</td>
<td>D-Ala</td>
</tr>
<tr>
<td>D-lactate</td>
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Second enzyme (VanA or VanB) leads to formation of D-Ala-D-lactate; vancomycin cannot bind

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<th>NAM</th>
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<tbody>
<tr>
<td>L-Ala</td>
<td>L-Ala</td>
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<tr>
<td>D-Glu</td>
<td>D-Glu</td>
</tr>
<tr>
<td>L-Lys</td>
<td>L-Lys</td>
</tr>
<tr>
<td>D-Ala</td>
<td>D-Ala</td>
</tr>
<tr>
<td>D-Ala + D-Ala</td>
<td></td>
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</tbody>
</table>

Third enzyme (VanX) cleaves one D-Ala from D-Ala-D-Ala synthesized by usual pathway; vancomycin cannot bind D-Ala

From: Bacterial Pathogenesis, Salyers and Whitt
4. Alteration of Drug Target-Cont

- Resistance to quinolones: mutations in DNA gyrase A, B subunits
- Resistance to rifampin: mutations in rpoB encoding beta-subunit of RNA polymerase cause rifampin resistance
- Resistance to macrolides, lincosamides and streptogramins, oxazolidinone: rRNA methylases (ermA, B, F, G) methylate an adenine on 23S rRNA (50S ribosome) and mediates resistance to these antibiotics, common in G+ cocci and Bacteroides
4. Alteration of Drug Target-Cont

- Resistance to trimethoprim and sulfonamides:
  Mutations in enzymes involved in folic acid synthesis, mutations causing resistance to either trimethoprim or sulfonamides occur frequently but resistance to both agents are rare—thus a combination of both trimethoprim and sulfonamides is used.
5. Resistance Caused by Loss of Enzymes Involved in Drug Activation

- The following drugs are prodrugs that need to be activated by bacterial enzymes for activity, and mutations in the enzymes cause inability to activate the drug, leading to resistance: e.g.
  - Isoniazid (INH): KatG (catalase-peroxidase) activate INH to produce active metabolites which then inhibit multiple targets including mycolic acid synthesis
  - Pyrazinamide (PZA): PncA (nicotinamidase/PZase) activate PZA to active form pyrazinoic acid (POA), which targets membrane and disrupts energy metabolism
  - Metronidazole (MTZ): RdxA (nitroreductase) activates MTZ to reactive form that damages DNA, and mutations in this enzyme cause resistance
Regulation of Resistance Genes

- Repressors: TetR, tet resistance
- Attenuation: erythromycin resistance genes (erm): without erythromycin, stem-loop structure form in mRNA which buries RBS and start codon; but with erythromycin cause RBS and start codon to expose, which results in expression of erm gene (methylase) and modifies ribosomes->growth
- Insertion sequence (IS) and promoter mutations: ampC of Enterobacter sp. poorly expressed, when IS is inserted before ampC gene-> overexpression of ampC
Figure 11–6  Repressor-mediated regulation of the efflux type of tetracycline resistance genes.
Figure 11–7  Regulation of resistance genes by attenuation. rbs, ribosome binding site.
B. TRANSFER OF RESISTANCE GENES

- Conjugation: Plasmids and Transposons:
- Plasmid-mediated: vancomycin resistance (vanA) in Enterococcus faecium (1988)
  - strA-strB streptomycin-resistance genes can be carried on plasmid in Shigella flexneri, on transposon (Tn5393) in pseudomonas sp
  - Plasmid-mediated sulfonamide and trimethoprim resistance in G- bacteria: plasmids carry drug-insensitive dihydropteroate synthase or dihydrofolate reductase
  - Plasmid-mediated quinolone resistance (qnr gene) in G- bacteria: qnr encodes pentapeptide repeats (DNA mimic) that bind to DNA gyrase and protect it, causing low level resistance (Jacoby, 1998)
Transposon-Mediated

- Transposons carrying drug resistance genes: Resistance genes flanked by insertion sequences in complex transposon
- Integrons: transposon that carry integrase gene and att site and a promoter P; integrase integrate circular DNA containing a promoter-less resistance gene cassette into the att site whose upstream contains a promoter for the expression of resistance genes
- Conjugative transposons: located in chromosome, but can excise and transfer from donor to recipient chromosome or plasmid, broader host range - among G+, G-, and between G+ and G-; e.g. Salmonella, Vibrio, Bacteroides
Phenotypic Resistance-changes in physiological state (not genetic mutations)

- Bacteria can become nonsusceptible to antibiotics when not growing as in stationary phase, biofilms, persisters, dormant state; but bacteria are still susceptible to antibiotics when growing again
Human Infections Involving Biofilms
(some examples)

Orthopedic devices: *S. aureus* and *S. epidermidis*
- Central venous catheters: *S. epidermidis* and others
- Sutures: *Staphylococcus epidermidis* and *S. aureus*
- Peritoneal dialysis (CAPD) peritonitis: A variety of bacteria and fungi
- Dental caries: Acidogenic Gram-positive cocci (e.g., *Streptococcus*)
- Periodontitis: Gram-negative anaerobic oral bacteria
- Otitis media: Nontypable strains of *Haemophilus influenzae*
- Necrotizing fasciitis: Group A streptococci
- Osteomyelitis: Various bacterial and fungal species--often mixed
- Native valve endocarditis: Viridans group streptococci
- Cystic fibrosis pneumonia: *P. aeruginosa* and *Burkholderia cepacia*
Biofilm Formation

Bacteria attach reversibly

Early biofilm

1st maturation phase

Mature biofilm

2nd maturation phase

Dispersion phase, single cell dislodge
### Susceptibility of planktonic and biofilm bacteria to selected antibiotics

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antibiotic</th>
<th>MIC planktonic (µg/ml)</th>
<th>Biofilm phenotype (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>Vancomycin</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>Imipenem</td>
<td>1</td>
<td>&gt;1024</td>
</tr>
<tr>
<td>E. coli</td>
<td>Ampicillin</td>
<td>2</td>
<td>512</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>Ampicillin</td>
<td>2</td>
<td>&gt;5000</td>
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<tr>
<td>S. sanguis</td>
<td>Doxycycline</td>
<td>0.063</td>
<td>3.15</td>
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Concentration required for 99% reduction
How bacteria in biofilm become resistant to antibiotics: slow penetration, slow growth/low metabolism, subpopulation of spore-like persisters

- **Slow penetration**
  Antibiotic (yellow) may fail to penetrate beyond the surface layers of the biofilm

- **Resistant phenotype**
  Some of the bacteria may differentiate into a protected phenotypic state (green)

- **Altered microenvironment**
  In zones of nutrient depletion or waste product accumulation (red), antibiotic action may be antagonised

*Substratum*
BacterialPersisters

- The phenomenon of bacterial persisters was first described by Joseph Bigger in 1944.
- Penicillin could not completely sterilize Staphylococcal culture in vitro. The residual persisters (about 1%) not killed by antibiotic were still susceptible to the same antibiotic upon subculture.
- The resistance (tolerance) in persisters is phenotypic and distinct from the genetic resistance.
Current Model of Persisters

- HipA (Moyed and colleagues in 1983, 1986)
- Lewis et al. performed microarray on ampicillin persisters and proposed toxin-antitoxin (TA) module persister model where inappropriate expression of toxin leads to persister formation (2004)
- Neyfakh et al. found overexpression of any unrelated proteins such as DnaJ etc can cause higher persister formation (2006), raising question about the specificity and validity of TA model
PhoU is a new persister switch in *E. coli*  
*(Li Y and Zhang Y, AAC, 2007, 51:2092-9)*

- E. coli transposon (mini-Tn10) screen with Ampicillin and identified PhoU mutant that failed to produce persisters
- PhoU mutant has a very dramatic phenotype characterized by reduced persister formation, 1000 fold less persister frequency (5x10^{-8}) compared with wild type strain W3110 (5x10^{-5})
- Increased sensitivity to a diverse range of antibiotics (norfloxacin, gentamicin, tetracycline) in MIC/MBC tests (2 fold more susceptible)
- Increased sensitivity to various stresses (starvation, acid pH, weak acids, heat)
- The PhoU mutant phenotypes can be complemented by wild type *phoU* gene
PhoU mutant is more susceptible to various antibiotics

**MIC and MBC determination (µg/ml)**

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<tr>
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<tbody>
<tr>
<td>Ampicillin</td>
<td>3.1/12.5</td>
<td>1.5/6.25</td>
<td>3.1/12.5</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>2.5/5</td>
<td>1.25/2.5</td>
<td>2.5/5</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>2/8</td>
<td>0.25/1</td>
<td>2/4</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0.5/1</td>
<td>0.125/0.5</td>
<td>0.5/1</td>
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PhoU mutant is more susceptible to antibiotics than wild type in stationary phase.
L-form Bacteria and Persistence


- L-form bacteria, or cell wall defective bacteria, were first described by Emmy Klieneberger (1892-1985) in 1935: L-form named after Lister Institute (London) where she worked
- L-form bacteria form “fried egg” structure, with a dense core or center and a less dense periphery
- Many bacterial species can form L-form under appropriate conditions (soft agar, osmotic protectant like sucrose or salt, and cell wall inhibitors) in vitro, but don’t grow in normal culture media
- L-forms can form in vivo (due to lysozyme or other host factors that act on cell wall)
- Similar to biofilms, resistant to some antibiotics and immune clearance - a special form of persistence, underlie chronic recurrent infections, e.g., UTI
- Survival strategy formed in response to stress
Morphologies of L-form Bacteria
No CWD growth (24)


Small colony size (18)


Reduced colony numbers (10)

- wcaL, vacJ, yrbD, yrbE, rffD, ompA, recA, fis, cpxA, fcl

Control CWD colonies

BW25113 parent strain

Measures to prevent the spread of drug-resistant bacteria

- Better treatment strategies, immunization programs, improved hygiene, nutrition, and initiatives targeting poor populations
- Antibiotic resistance surveillance
- Better education of healthcare professionals
- Critical investment of time, effort, money, cooperation, philanthropy and personal commitment on the part of individuals, governments, large pharmaceutical companies and private and public organizations
Limiting Drug Resistance

(i) Antibiotics should be used only when necessary

(ii) Antibiotics can be employed such that high concentrations of drug is maintained over long periods (i.e., taking all of one's pills over the prescribed duration of a treatment)

(iii) Antibiotics may be used in combination to prevent resistance and improve the efficacy of treatment
Combating Drug-Resistant Bacteria

- New antibiotic development: target screens versus whole organism screens; target selection; combinatorial chemistry; rational drug design (based on structure of target); efflux inhibitors; genomics/microarray/proteomics

-Irony: Drug companies are getting out of antibiotic development (99% candidates fail, not as profitable as other more commonly used drugs)

- Phage therapy: Russian origin

- Mobilizing host defense mechanism: defensins, Vaccine development: prevent disease->minimize the need to use antibiotics

- Use of normal bacterial flora: use of engineered drug-resistant E. coli (a commercial product) to restore normal flora
"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."

— Joshua Lederberg, Nobel Laureate