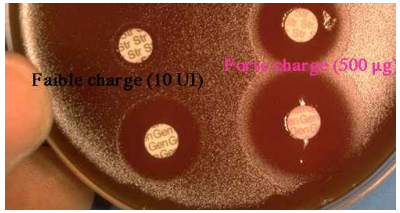
MECHANISMS OF RESISTANCE



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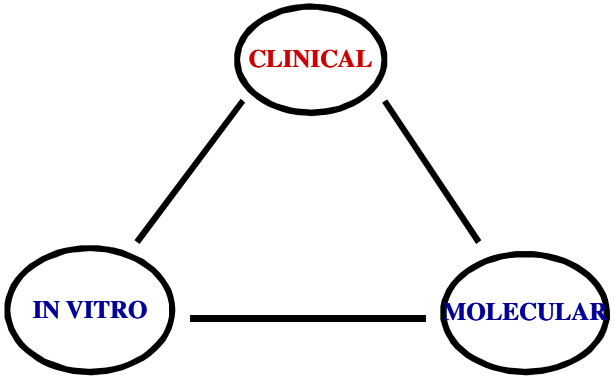
Evolutionary stages of resistance

- Tolerance: survive but not duplicate

precursor

- Resistance: Survive and duplicate

RESISTANCE is..



A resistant strain may be present due to:

- Intrinsic resistance
- Replacement with a more resistant species
- Replacement with a more resistant strain
- Transient gene expressions that cause temporary resistance (epigenetic resistance)
- Alterations in cell type (?)
- Genomic instability within a single strain (population bottleneck)

Clinical Resistance is a Multifactorial Issue

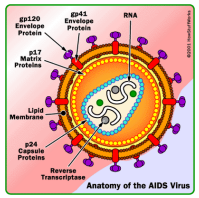
- **HOST**
  - Immune status
  - Site of infection
  - Severity of infection
  - Foreign devices
  - Noncompliance with drug regimen

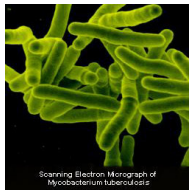
- **Organisms**
  - Initial MIC
  - Cell type: Bacteria/Yeast/hyphae..
  - Genomic stability
  - Biofilm production
  - Population bottlenecks

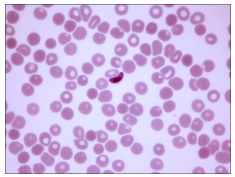
- **DRUG**
  - Efficacy nature
  - Dosing
  - Pharmacokinetics
  - Drug-drug interactions

Most Important Infectious Agents on the Planet

- *Mycobacterium tuberculosis*
- *Plasmodium falciparum*
- Human immunodeficiency virus



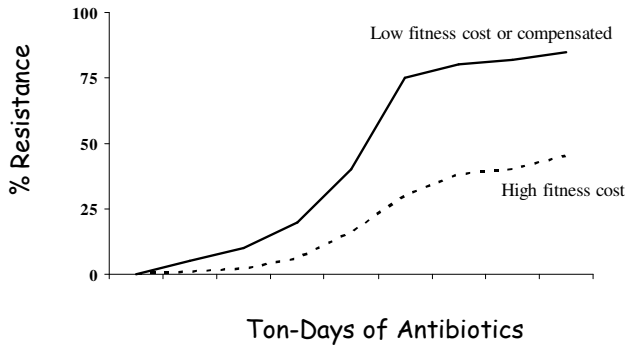




Most Common Bacterial Pathogens on the Planet

- *Mycobacterium tuberculosis*
- Enterics (*Salmonella enterica, Shigella sp., E. coli*)
- *Staphylococcus aureus*
- *Streptococcus* sp. (*S. pneumoniae, S. pyogenes*)
- *Neisseria* sp. (*N. gonorrhoeae, N. meningitidis*)
- *Hemophilus* sp.

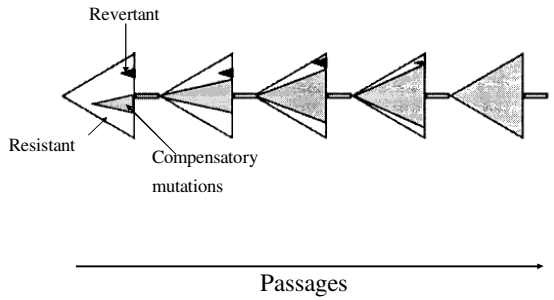
Selective Pressure



Population bottleneck
(genetic bottleneck)

- Evolutionary event in which a population or species is killed or otherwise prevented from reproducing, and the population is reduced by 50% or more.
- A graph of this change resembles the neck of a bottle, from wide to narrow; hence the name.
- Population bottlenecks increase genetic drift, as the rate of drift is inversely proportional to the population size.
- It also changes the relationship of natural selection.

Effect of Bottleneck on Emergence of Compensatory Mutations



Levin, Genetics 154:985, 2000

Compensatory Mutations

- Wild-type is best adapted, most fit
- Mutation (or acquisition of resistance genes) comes at a cost
- Cost can be compensated by
  - loss of resistance (by reversion or recombination)
  - by compensatory mutations
- Compensatory mutations are
  - environmentally determined
  - a consequence of a low recombination rate

Compensatory Mutations

- Favored when the rate of their generation exceeds reversion
- Bottlenecks play an important role
- Function to maintain resistance (implications for antibiotic control)
- Less likely to occur if gene exchange or recombination is common

Example of Compensatory Mutations

- *Salmonella typhimurium* LT2
  - SmR: resistant to streptomycin (ribosomal inhibitor)
- Cost: slow growth because of reduced rate of protein synthesis
- SmR passaged in broth (LB) and mice
- Mutants recovering fitness (fast growth) analyzed for acquisition of compensatory mutations

Bjorkman, Science 287:479 (2000)

Antibacterial resistance

Involve a stable genetic change.

- Alteration of bacterial genetic:
  - Mutation
    - Reduce target affinity
    - Allow production of drug-modifying enzyme
  - Transfer of genetic material
    - Transduction
    - Transformation
    - Conjugation

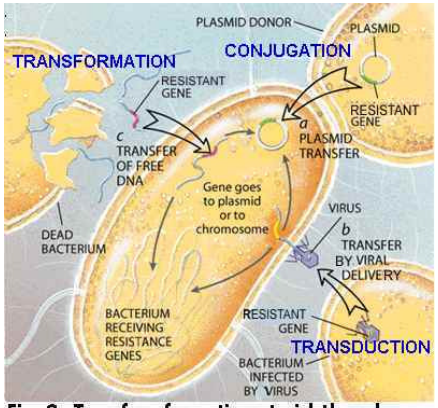


Fig. 2: Transfer of genetic material through transformation, transduction, and conjugation
image taken from Levy, 1998
Image: Tomo Narashima
http://www.sciam.com/1998/0398issue/0398levy.html

Genetics of Resistance

- Vertical genetic exchange: generation
  - Fluoroquinolone resistance in all bacterial species
  - All drug resistance in Mtb
  - Comparatively less advantageous
- Horizontal, transfer of mobile element and selection between bacteria:
  - Transducing phage (e.g., transfer of S. aureus *bla* plasmid)
  - Transformation, homologous recombination (e.g., penicillin-resistance in pneumococci)
  - Conjugation (e.g., vanA resistance of enterococci)
  - Comparatively more advantageous

Resistance Mediated by Horizontal Transfer

Toxins and Mobile Elements

Disease	Toxin	Organism	Element
Anthrax	Anthrax toxin	*Bacillus anthracis*	Plasmid
Cholera	Cholera toxin	*Vibrio cholerae*	Prophage
Food poisoning	Cpe toxin	*Clostridium perfringens*	Transposon
Toxic shock syndrome	TSST-1	*Staphylococcus aureus*	Pathogenicity island

Mobile Elements Involved in Resistance

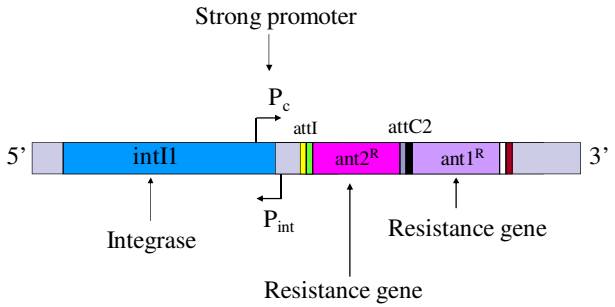
Element	Features
Insertion sequence	Inverted repeats + recombinases for site specific insertion, self copy
Transposon	Insertion sequence + other elements
Integron	Integrase + integrase recognition site, do not self copy, not themselves mobile
Chromosomal cassette	Pathogenicity island-like with *att* sites and recombinases, can be relatively large
Conjugative plasmid	DNA transfer knock-off of bacterial type IV secretion system

Resistance Integrons

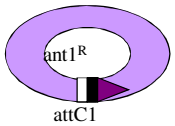
Integrons

- An integrase, gene cassette(s), and a cassette integration site (*att*), a strong promoter
- Not themselves mobile, but excise and integrate cassettes that are found on transposons, plasmids, chromosome
- Two major groups: resistance integrons and super-integrons (presumed ancestor, ~100 kb)

Integron Structural Features



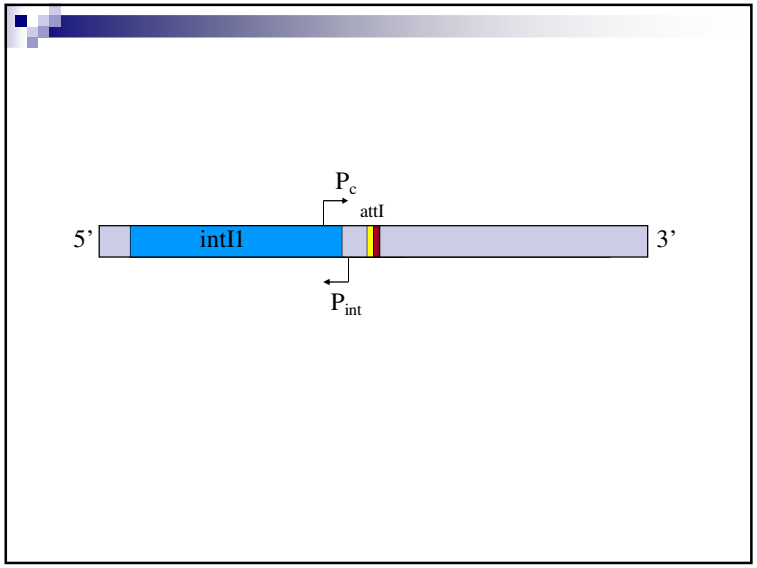
Gene Cassettes

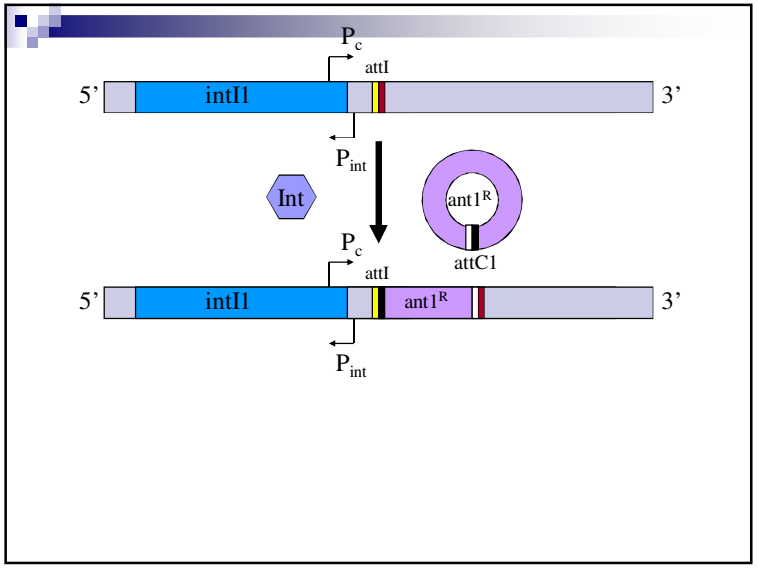


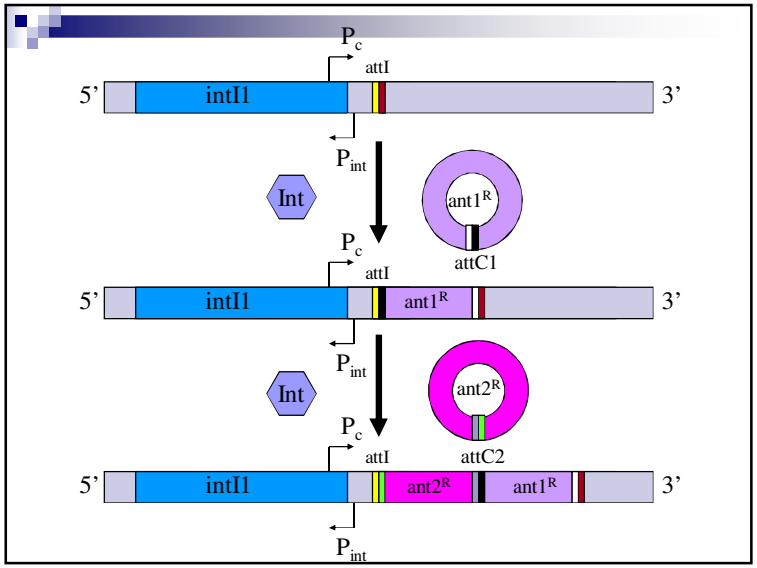
- Open reading frame, usually promoterless (provided by integron)
- A repeat sequence, attC, that is recognized by integrase
- Cassettes for β-lactamases, aminoglycoside resistance, chloramphenicol resistance, trimethoprim resistance, ciprofloxacin, etc

Integron, gene cassette, attC

- The backbone structure of an integron contains a conserved region encoding an integrase (*intI*) and a variable region with integrated gene cassettes.
- A gene cassette usually contains a single open reading frame and a recombination site, the *attC* site (59-base element).
- The *attC* sites consist of an inverse core site and a core site separated by an intervening palindrome of variable length.
- The inverse core site is defined as RYYYAAC and the core site as GTTRRRY (R = A or G, Y = C or T).


Pc
attI
5'
intI1
3'
Pint


Pc
attI
5'
intI1
3'
Pint
Int
ant1R
attC1
Pc
attI
5'
intI1
ant1R
3'
Pint


Pc
attI
5'
intI1
3'
Pint
Int
ant1R
attC1
Pc
attI
5'
intI1
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Pint
Int
ant2R
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attI
5'
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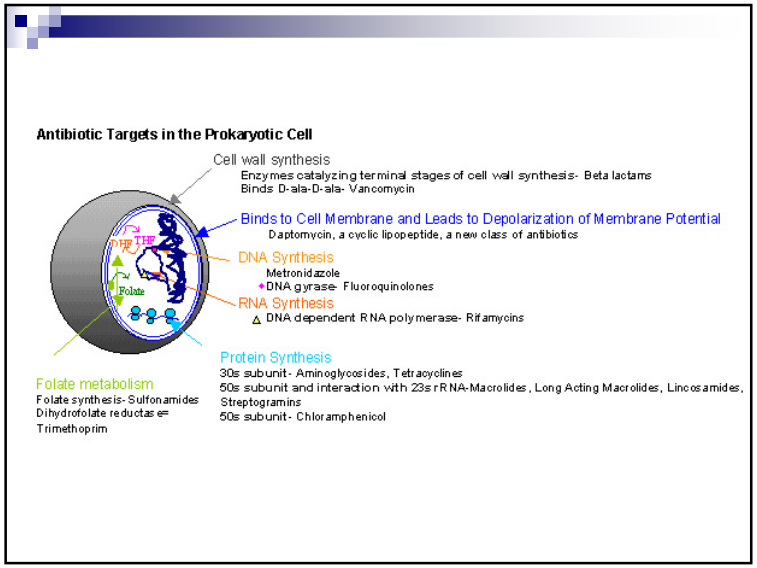
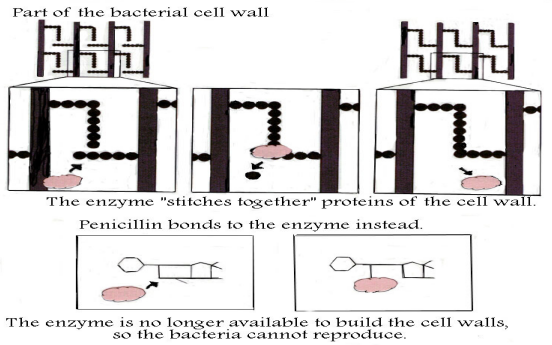
Pint


Antibiotic Targets in the Prokaryotic Cell
Cell wall synthesis
Enzymes catalyzing terminal stages of cell wall synthesis- Beta lactams
Binds D-ala-D-ala- Vancomycin
Binds to Cell Membrane and Leads to Depolarization of Membrane Potential
Daptomycin, a cyclic lipopeptide, a new class of antibiotics
DNA Synthesis
Metronidazole
DNA gyrase- Fluoroquinolones
RNA Synthesis
DNA dependent RNA polymerase- Rifamycins
Protein Synthesis
30s subunit- Aminoglycosides, Tetracyclines
50s subunit and interaction with 23s rRNA-Macrolides, Long Acting Macrolides, Lincosamides, Streptogramins
50s subunit- Chloramphenicol
Folate metabolism
Folate synthesis- Sulfonamides
Dihydrofolate reductase- Trimethoprim
DHF
THF
Folate

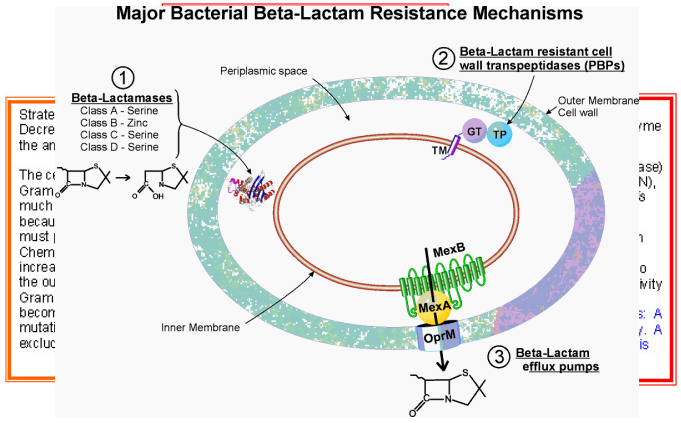
Resistance mechanisms

- Alternation target site
- Drug-destroying mechanisms
- Decreased uptake

Inhibitors of Cell Wall Synthesis: beta-lactam antibiotics



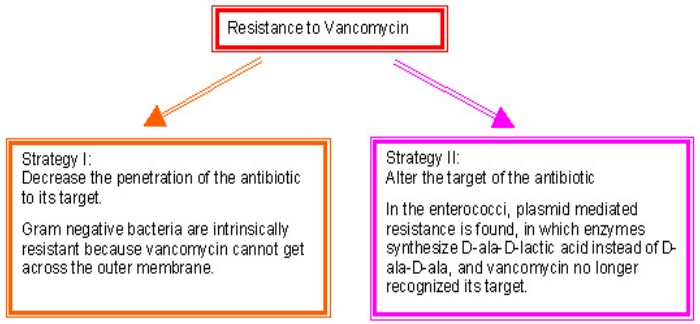
Gram positives are intrinsically susceptible to most β-lactam antibiotics.



Vancomycin

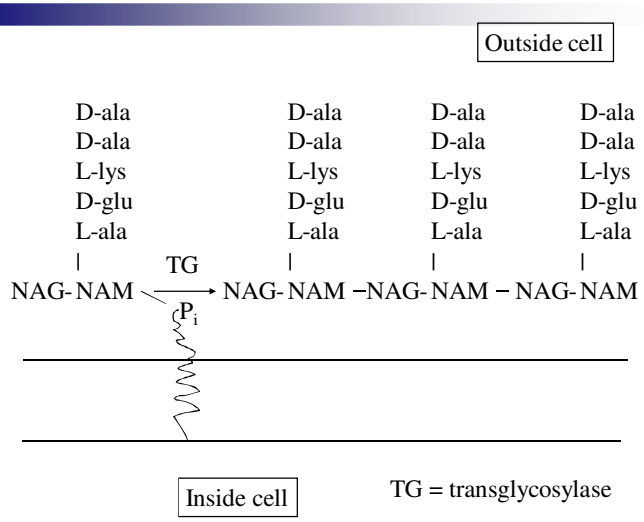
- A glycopeptide, ~1450 daltons
- Inhibitor of cell wall synthesis
- Target: D-alanyl-D-alanine
- Interferes with transglycosylation and transpeptidation (crosslinking) reactions
- Bactericidal

Vancomycin: blocks the polymerization of the N-acetyl muramic acid-N-acetyl glucosamine backbone of the peptidoglycan (cell wall) by binding to D-ala –D-ala.

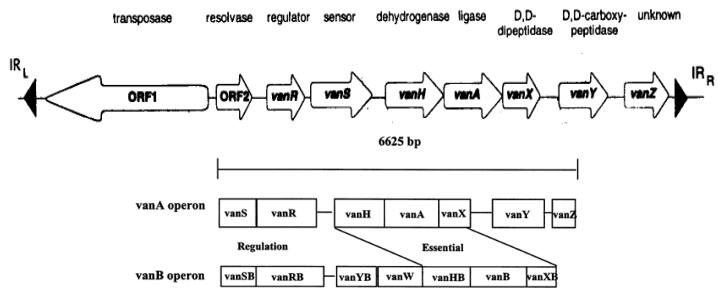


Players in vanA resistance

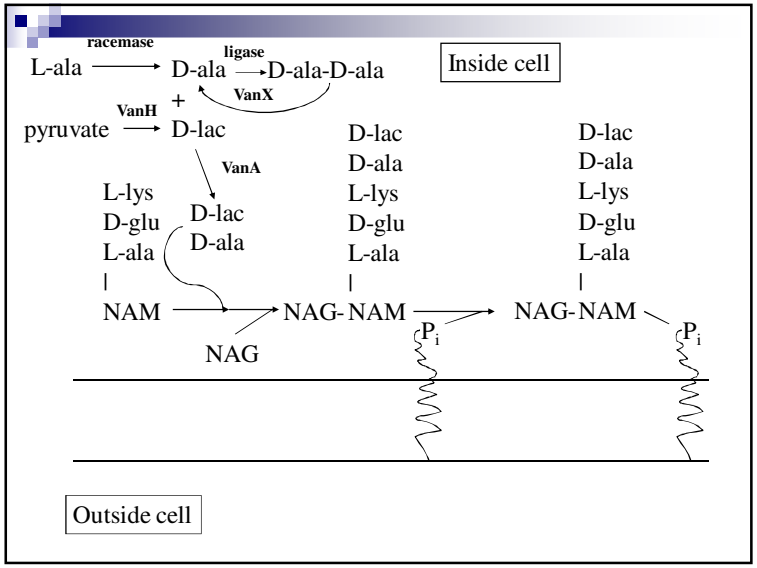
- *Enterococcus faecium* and *E. faecalis* donors
- A broad-host range conjugative plasmid
- Suitable recipient cell (pheromone producer)
- A transposon, Tn1546, resident on the plasmid
- The D-ala-D-ala target

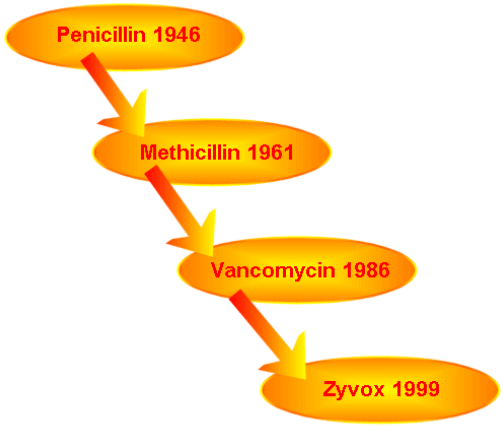


Map of Transposon Tn1546



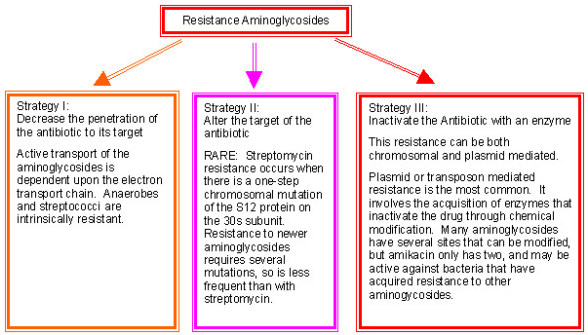
Comparison of arrangements of the VanA and VanB glycopeptide resistance operons. Essential genes and those involved in regulation of expression of the resistance determinant are marked.





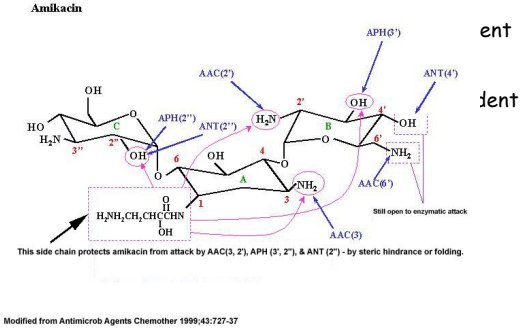
Inhibitors of Protein Synthesis: Aminoglycosides

- Streptomycin, the prototype aminoglycoside, works by binding to a specific protein, S12, on the 30s ribosomal subunit. (Gentamicin, Tobramycin, and Amikacin)



There are three types of aminoglycoside modifying enzymes:

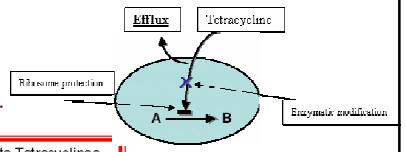
1. N-Acetyltransferases (AAC) - catalyzes acetyl CoA-deper
2. O-A ent adeny
3. O-P dent phosp



Modified from Antimicrob Agents Chemother 1999;43:727-37

Inhibitors of Protein Synthesis: Tetracyclines

- Tetracyclines block tRNA binding to the ribosome by binding the 30s ribosomal subunit. Tetracyclines are almost always bacteriostatic.



Resistance to Tetracyclines

Strategy I:
Decrease the penetration of the antibiotic to its target.

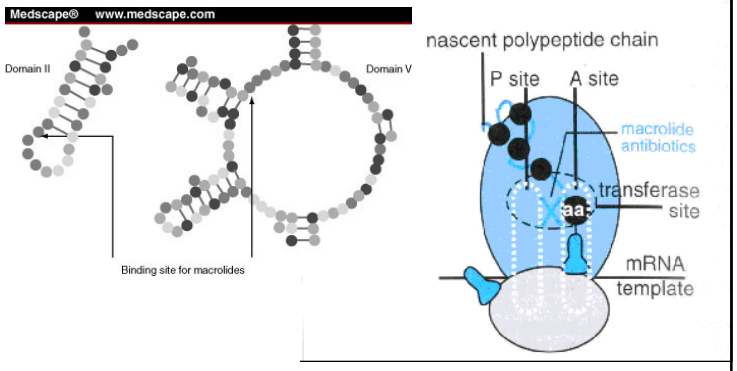
There is plasmid encoded resistance, which increases the efflux of the drugs.

Strategy II:
Alter the target of the antibiotic.

There is plasmid mediated resistance, which alters the ribosome so that it is less susceptible to the drugs.

Inhibitors of Protein Synthesis: The MSL Group: Macrolides (Erythromycin), Long Acting Macrolides (Azithromycin) and Lincosamides (Clindamycin) and Streptogramins (Syneroid)

- These antibiotics bind to the 50s ribosomal subunit and interact with the 23s ribosomal RNA. The overall effect is to block chain elongation. They can be bacteriocidal or bacteriostatic depending upon the organism.
- Streptogramins bind on the 50s ribosomal subunit near the macrolide binding site.
- The mechanism of action of telithromycin is similar to that of macrolides, however, appears to have greater affinity for the ribosomal binding site than macrolides



Resistance to The MSL Group

- Plasmid encoded resistance to one drug in this group means resistance to all.
- Resistance due to multidrug exporters affect macrolides but not clindamycin.
- Chromosomal resistance to streptogramins is seen.

Resistance to the Macrolide Group

Strategy II:
Alter the target of the antibiotic.

There is plasmid mediated resistance due to enzymes that methylate the 23s ribosomal RNA subunit so the drugs cannot recognize their targets.

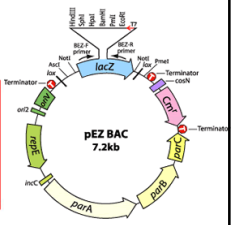
Inhibitors of Protein Synthesis: Chloramphenicol

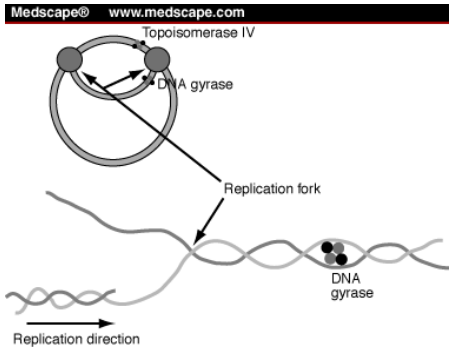
- Chloramphenicol binds the 50s ribosomal subunit and inhibits the translocation of the peptide chain from A site to P site. Chloramphenicol is bacteriostatic.

Resistance to Chloramphenicol

Strategy III:
Inactivate the Antibiotic with an enzyme.

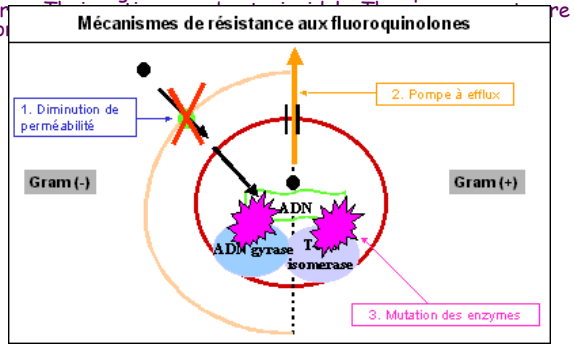
Plasmid encoded chloramphenicol acetyl-transferase inactivates the drug by acetylating it.





Inhibitors of DNA Synthesis: Quinolones (Ciprofloxacin, Levofloxacin, Trovafloxacin and others)

- Quinolones interfere with the activity of DNA gyrase. They prevent winding of the DNA helix into the supercoiled



Inhibitors of DNA Synthesis: Metronidazole

- Metronidazole is reduced to a substance that inhibits bacterial DNA synthesis. Its action is bacteriocidal, but its use is limited to anaerobic organisms.

Resistance to Metronidazole

- *Actinomyces* are intrinsically resistant.
- Acquired resistance to metronidazole is very unusual. However, very rare plasmid mediated resistance has been reported in *B. fragilis.*

Inhibitors of RNA Synthesis: Rifamycins (Rifampin)

- Rifamycins inhibit DNA dependent RNA polymerase by binding to the beta subunit and inhibiting initiation. This action is bacteriocidal. The drug in major use is rifampin, while rifabutin is used sporadically.

Resistance to Rifamycins

Strategy II:
Alter the target of the antibiotic

Resistance occurs when the polymerase is altered. This resistance is chromosomally mediated, and occurs in one step. It is important not to use Rifampin alone.

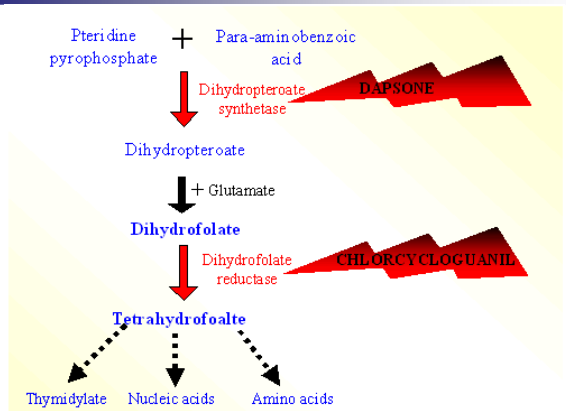
Cell Membrane Toxins:

The one drug in this class is Daptomycin.

- Daptomycin is a cyclic lipopeptide antibiotic with activity limited to Gram positive pathogens.
- Antibacterial effects are mediated by disruption of multiple aspects of bacterial plasma membrane function, including peptidoglycan synthesis, lipoteichoic acid synthesis, and bacterial membrane potential.
- These effects occur with no penetration of the cytoplasm. The mechanism of binding is not known.
- Daptomycin exhibits concentration-dependant bactericidal activity.

Resistance

- Daptomycin does not penetrate outer membrane of Gram negative bacteria, so these organisms are intrinsically resistant.
- Since this drug has not been widely used, transferable genes encoding resistance have not been described.



The pathway for folate biosynthesis in plasmodia. The figure shows the targets of dapsone and chlorcycloguanil, the active metabolite of chlorproguanil.

Inhibitors of Folate Metabolism:

Sulfonamides and Trimethoprim

- Sulfonamides competitively inhibit folate synthesis. (humans need an exogenous source of folate.)
- Trimethoprim competitively inhibits the bacterial dihydrofolate reductase, and is much less active toward the human enzyme.
- They are often given together because they act synergistically, and are bacteriocidal in combination.

Resistance to Inhibitors of Folate Metabolism

Strategy II:
Alter the target of the antibiotic.

Resistance to both sulfonamides and trimethoprim is due to the acquisition of genes encoding new enzymes that are not inhibited by the drugs. These enzymes are plasmid or transposon mediated.

Alternation of target site

- Aminoglycosides → Altered ribosomal protein
- Beta-lactam → Altered or new penicillin binding proteins
- Erythromycin → Ribosomal RNA methylation
- Quinolones → Altered DNA gyrase
- Sulphonamides → New drug-insensitive dihydropteroate synthase

Alternation of target site

- Tetracyclines → Ribosomal protection
- Trimethoprim → New drug-insensitive dihydrofolate reductase
- Vancomycin → Altered cell wall stem peptide

Drug-destroying mechanisms

- Aminoglycosides → Acetyltransferase, Nucleotidetransferase, Phosphotransferase.
- Beta-lactamase → Beta lactamase
- Chloramphenicol → Acetyltransferase

Decreased uptake (permeability)

Beta-lactam, chloramphenicol, quinolones, tetracyclines, trimethroprim.

Alternation in the permeability of the bacterial cell envelope

What drugs act as antifungal agents?

- present more difficult therapeutic problems than do bacterial infections.
- few agents that can be used to treat fungal infections. The fungal cell wall may be considered to be a prime target for selectively toxic antifungal agents because of its chitin structure, absent from human cells.
- No clinically available inhibitor of chitin synthesis analogous to the β-lactams exists at present, even though much effort is being directed towards developing such agents.

ANTIFUNGAL DRUGS by mode of action

- **Membrane disrupting agents**
  Amphotericin B, nystatin
- **Ergosterol synthesis inhibitors**
  Azoles, allylamines, morpholine
- **Nucleic acid inhibitor**
  Flucytosine
- **Anti-mitotic (spindle disruption)**
  Griseofulvin
- **Glucan synthesis inhibitors**
  Echinocandins
- **Chitin synthesis inhibitor**
  Nikkomycin
- **Protein synthesis inhibitors**
  Sordarins, azasordarins

Resistance to Amphotericin B

- Technical difficulties in detection of resistance *in vitro*
- *In vivo* resistance is rare

C. lusitaniae, C. krusei
C. neoformans
Trichosporon spp.
A. terreus
S. apiospermum
Fusarium spp.
...

Mechanisms of Amphotericin B Resistance

- Reduced ergosterol content (defective ERG2 or ERG3 genes)
- Alterations in sterol content (fecosterol, episterol: reduced affinity)
- Alterations in sterol to phospholipid ratio
- Reorientation or masking of ergosterol
- Stationary growth phase
- Previous exposure to azoles

Resistance to Azoles

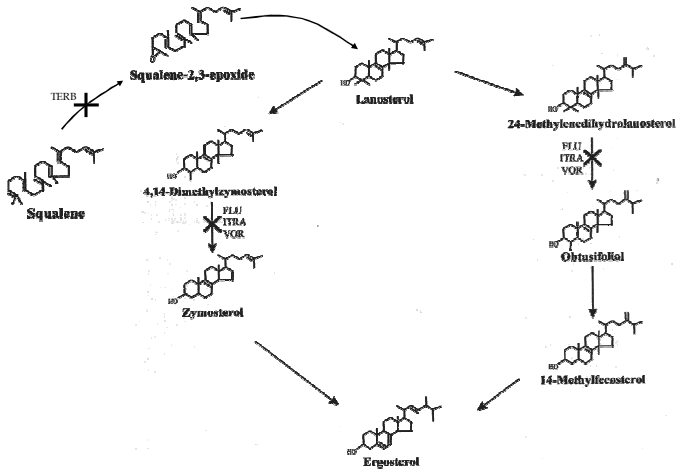
- Well-known particularly for fluconazole
- Data available also for other azoles
- A significant clinical problem

RESISTANCE TO FLUCONAZOLE

PRIMARY	*C. krusei*
	Aspergillus
	C. glabrata
	C. norvegensis...
SECONDARY	*C. albicans*
	C. dubliniensis...

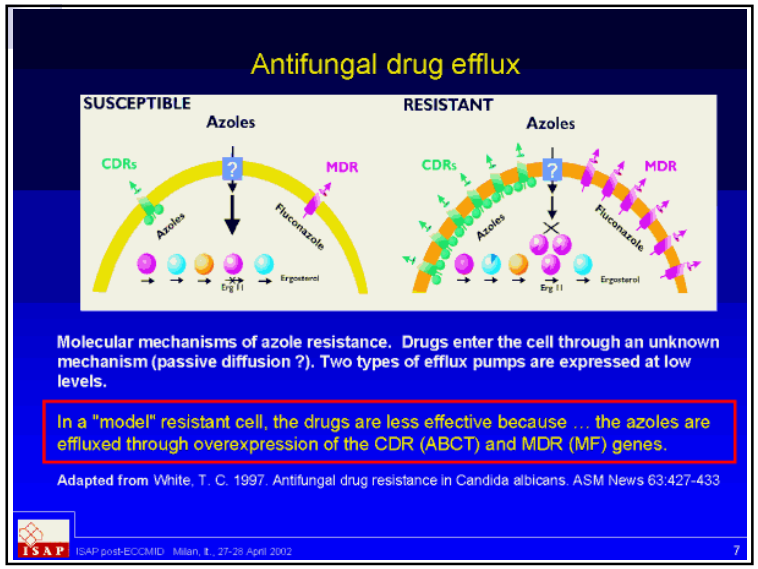
Mechanisms of Resistance to Azoles

- Alteration of lanosterol (14-alpha) demethylase
- Overexpression of lanosterol demethylase
- Energy-dependent efflux systems
  a. Major facilitator superfamily (MFS) proteins (BEN^r =MDR1 of *Candida*...)
  b. ATP-binding cassette (ABC) superfamily proteins (MDR, CDR of *Candida*)
- Changes in sterol and/or phospholipid composition of fungal cell membrane (decreased permeability)



Azole Resistance Molecular Aspects

- Single point mutation of ERG11 gene
  ⇨Altered lanosterol demethylase
- Overexpression of ERG11 gene
  ⇨Increased production of lanosterol demethylase
- Alterations in ERG3 or ERG5 genes
  ⇨Production of low affinity sterols
- Increase in mRNA levels of CDR1 or MDR1 genes
  ⇨Decreased accumulation of the azole in fungal cell



Resistance to Terbinafine

- Very rare
- Primary resistance to terbinafine in a *T. rubrum* strain (ICAAC 2001, abst. no. J-104)
- Mechanism: (?)
  CDR1-mediated efflux (possible)

Resistance to Flucytosine

- PRIMARY — non-*albicans Candida*
  C. neoformans
  Aspergillus (highest)
- SECONDARY — *C. albicans*
  C. neoformans

❁Secondary resistance develops following flucytosine MONOtherapy.

Mechanisms of Resistance to Flucytosine

- Loss of permease activity
- Loss of cytosine deaminase activity
- Decrease in the activity of UPRTase

Flucytosine Resistance Molecular Aspects

- FCY genes (FCY1, FCY2) encode for UPRTase
  - FCY/FCY homozygotes possess high UPRTase activity
  - FCY/fcy heterozygotes possess low UPRTase activity
  - fcy/fcy homozygotes possess barely detectable UPRTase activity

Resistance to Echinocandins

PRIMARY *C. neoformans*

Fusarium spp.

SECONDARY (?)

The only licensed member is caspofungin (Jan 2001, USA). Resistant mutants due to therapy are not available.

Echinocandin Resistance Molecular Aspects

- FKS1 encodes glucan synthase
- GNS1 encodes an enzyme involved in fatty acid elongation

  Resistance is observed following laboratory derived mutations in FKS1 or GNS1

- Other mechanisms (?)

Future Directions to Avoid Development of Resistance

- Proper dosing strategies
- Restricted and well-defined indications for prophylaxis with azoles

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- Fungi will continue to develop NEW resistance mechanisms!..

Final Word

- Antifungal resistance is a complex, gradual and multifactorial issue
- Several uncertainties remain
- Molecular assays to detect resistance are not simple
- The best way to improve the efficacy of antifungal therapy is to improve the immune status of the host

Closing Comments

- Given enough time and pressure, resistance will eventually occur when possible acquisition of resistance (and probably other traits as well) occurs by horizontal gene transfer
- Compensatory mutations are a way to mitigate the cost of resistance and maintain the trait in a fit pool (and there may be no way back)
- Hopefully, a better understanding of microbial pathogenesis can lead to reduce antibiotic pressure
- Have a happy day!