

Mechanisms of aminoglycoside resistance in mycobacteria

Advisor

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Reading

- Chapters in BBOM 10th: 20.6, 20.7, 20.9, 20.12, 24.3, 30.5, 12.24, pg. 202, pg. 339. BBOM: Madigan M.T., J.M. Martinko and J. Parker: "Brock - Biology of Microorganisms", 10th Edition 2003, Prentice Hall.
- Medizinische Mikrobiologie – F.H. Kayser, K.A. Bienz, J. Eckert, R.M. Zinkernagel (10. Auflage – Kapitel 3.6 Grundlagen der Antibiotikatherapie v.a. 3.6.7 Probleme der Resistenz; Kapitel 4.8 Mycobacterium
- Azucena, E., Mobashery, S. (2001) Aminoglycoside-modifying enzymes: mechanisms of catalytic processes and inhibition. Drug Resistance Updates 4: 106 – 117.

Objectives

Disinfection, antibiotic resistance, resistance mechanisms, cross resistance, ribosome as drug target

Background

a) Aminoglycosides

Aminoglycosides constitute a large family of water soluble, polycationic amino sugars of considerable structural diversity. They are often broad-spectrum antibacterial agents that are products of bacterial or fungal metabolism. The structures of several of these natural products have been altered chemically to generate semi-synthetic aminoglycosides with expanded activity and less side effects. Aminoglycosides bind to the small subunit of the bacterial ribosome (30S subunit) and induce misreading of the genetic code.

Aminoglycosides were applied for clinical use in the treatment of bacterial infections caused by a variety of both gram-negative and gram-positive bacterial pathogens. However, aminoglycosides are associated with considerable side effects for the host, i.e. ototoxicity and nephrotoxicity. These side effects at least partially are due to inhibition of human mitochondrial ribosomes.

Clinically acquired resistance towards aminoglycosides can be conferred by different mechanisms. These mechanisms are principle mechanisms of drug resistance:

- presence of antibiotic modifying enzymes (aminoglycoside nucleotidyl transferases (ANT), aminoglycoside acetyl transferases (AAC), aminoglycoside phosphotransferases (APH)
- altered structure of the target (ribosomal proteins, ribosomal RNA)
- altered membrane permeability
- extrusion of the antibiotics from the cell by efflux pumps

Some of these resistance mechanisms are specific for a particular aminoglycoside subclass, others confer broad range resistance towards a number of several subclasses, a phenomenon called cross resistance. As a consequence of cross resistance the entire class of aminoglycosides loses its therapeutic potential.

The first antibiotic used for the treatment of tuberculosis was streptomycin (an aminocyclitol aminoglycoside). However, resistant strains develop rapidly during therapy with a single antibiotic (mono-therapy). Nowadays, aminoglycosides are used for the therapy of multi-drug resistant *Mycobacterium tuberculosis* strains and for the treatment of infections with mycobacteria other than tuberculosis (MOTT).

b) Experimental system

Mycobacterium smegmatis is a non-pathogenic bacterium. It belongs to a genus comprising some of the world's most important pathogens, i.e. *Mycobacterium tuberculosis* and *Mycobacterium leprae*. These species are the causative agents of tuberculosis and leprosy, respectively. The genus *Mycobacterium* may be divided in two groups, the fast growing and the slow growing mycobacteria. *M. smegmatis* has a generation time of approximately 3 h and belongs to the subgroup of fast growing mycobacteria. *M. tuberculosis* has a generation time of approximately 24 h.

Ribosomal RNA genes are encoded as an operon (*rrn*) comprising the genes for 16S rRNA (*rrs*), 23S rRNA (*rrl*) and 5S rRNA (*rrf*). Most bacterial species contain a multitude of these operons; e.g. *Escherichia coli* 7; *Bacillus subtilis* 10. In contrast, mycobacteria are characterized by a surprisingly small number of rRNA operons. This limited number of rRNA operons makes mycobacteria particularly useful for the investigation of rRNA structure function relationships. As a model system a derivative of *M. smegmatis* (two functional rRNA operons: *rrnA* and *rrnB*) has been generated. This strain, *M. smegmatis rrnB*, is characterized by the presence of a single functional ribosomal RNA operon.

Additional literature (more specialized)

- Carter, A.P. et al., (2001) Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature*. **407**: 340 – 348.
- Böttger E.C. et al. (2001) Structural basis for selectivity and toxicity of ribosomal antibiotics. *EMBO-Rep*. **21**: 318 – 323.
- Glickman M.S., Jacobs, W.R. (2001) Microbial pathogenesis of *Mycobacterium tuberculosis*: dawn of a discipline. *Cell* **104**: 477 – 485.

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Theoretical work (30 min.)

Fill in the answers to the following questions:

1. Name 3 antibiotics and their targets. 3 P.
2. Define the term “selective toxicity”? 1 P.
3. Name 3 principle mechanisms of antibiotic resistance. 3 P
4. What is a broad-spectrum antibiotic? 1 P.
5. Why does antibiotic resistance occur and how can its occurrence be minimized? 2 P.
6. Explain the term “minimal inhibitory concentration” 1 P.
7. What is the meaning of “cross resistance”? 1 P.
8. Nominate 2 species of the genus *Mycobacterium*. 2 P.
9. What property, also used as a diagnostic feature, does mycolic acid confer on mycobacteria? 1 P.
10. What are the macromolecular components of a ribosome? 1 P.
11. What functional role does rRNA play in protein synthesis? 2 P.
12. Why do aminoglycosides have harmful effects on human beings (ototoxicity, nephrotoxicity)? 1 P.
13. Why are mycobacteria considered as model organisms for the investigation of rRNA structure function relationship? 1 P.

Score

20 – 19 Pts	6.0
18 – 17 Pts	5.5
16 – 15 Pts	5.0
14 – 13 Pts	4.5
12 – 10 Pts	4.0
9 – 8 Pts	3.5
7 – 6 Pts	3.0
5 – 4 Pts	2.5
3 – 2 Pts	2.0
1 Pt	1.5
< 1 Pt	1.0

Practical work

You will perform a disinfection experiment, dilution streak, determine antibiotic resistance phenotype and minimal inhibitory concentration of different *Mycobacterium smegmatis* strains.

Material and experimental protocols

Experiment 1

Disinfection - Bacteria residing on the skin

Material: Blood agar plate (1 per participant)

Investigate the number of bacteria residing on the skin.

- Make a thumb print on a blood agar plate
- Disinfect your hands with disinfectant
- Make a print with the disinfected thumb on the same agar plate

Seal the plate and incubate it at 37 °C for 2 days.

Evaluation: Determine the number of colonies before and after disinfection. Describe the colony morphology.

Experiment 2

Dilution Streak

Perform a dilution streak (Dreiösenausstrich; Vereinzelungsausstrich) for one of the strains on LB-agar.

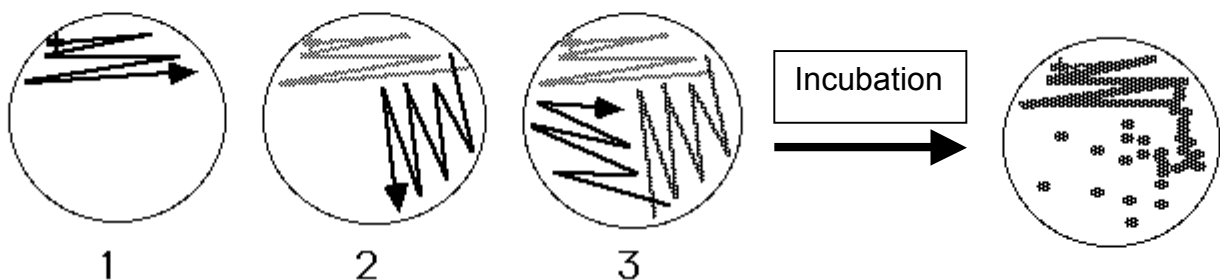
Material: LB-agar plate, rack for Eppendorf tubes, pen, sterile loops, parafilm, bacterial strains A, B, C, D, E **or** F

- take one loop of bacterial suspension and spread it with a single streak on one part (third) of the plate (see scheme)
- take a second sterile loop, streak once through the bacterial suspension on the plate and streak it on the second part of the plate
- take a third sterile loop and whip once through the second streak and streak it on the third part of the plate
- seal the plate with parafilm
- incubate at 37 °C (bottom up) for one week

Evaluation: Describe the colony morphology.

Streaking

Growth



Experiment 3**Determination of the antibiotic resistance phenotype of mycobacteria**

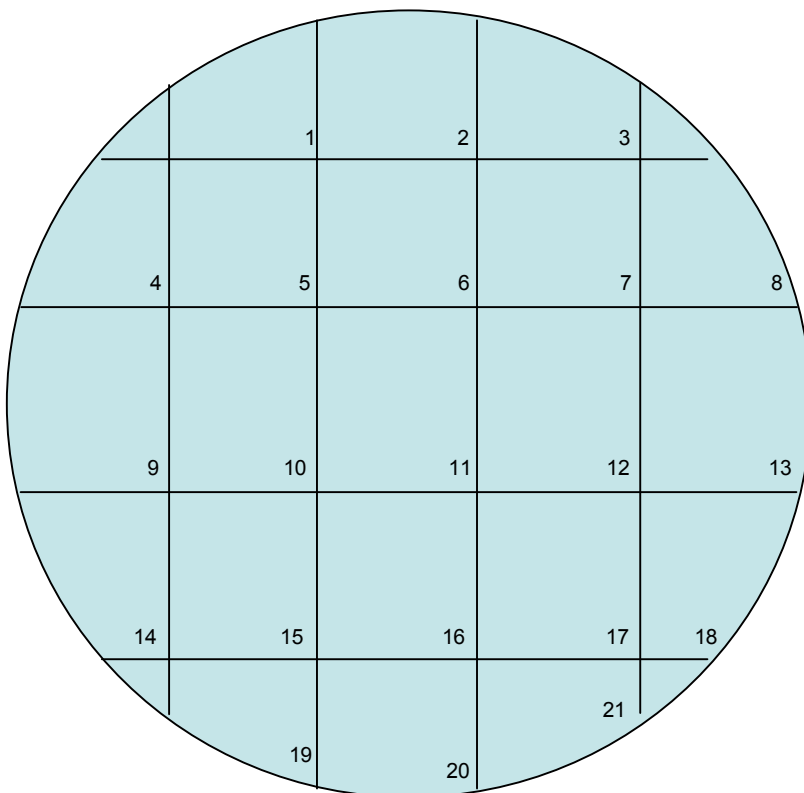
Material: Agar plates (1 each – LB, LB streptomycin (Sm), LB- kanamycin (Kan), LB- paromomycin (Par), LB-gentamicin (Gm), LB-amikacin (Am), LB-tobramycin (Tob)), LB-broth, sterile Eppendorf tubes, μ l-pipette, sterile tips, bacterial strains A, B, C, D, E, F

Preparation of serial dilutions

- prepare a 10-fold serial dilution of each of the 6 bacterial strains by mixing 100 μ l of the bacterial suspension with 900 μ l of LB-Tween broth in sterile Eppendorf-tubes (10^{-1} dilution).
- use a new tip for preparing each dilution
- transfer 100 μ l of the 10^{-1} dilution into another tube containing 900 μ l of broth. This results in the 10^{-2} dilution.
- transfer 100 μ l of the 10^{-2} dilution into a third tube containing 900 μ l of broth. This results in the 10^{-3} dilution.

Growth on agar plates

- draw a grid for 18 spots on the backside of each of the plates and enumerate each of the squares
- spot 2 μ l of the 10^{-1} , 10^{-2} and 10^{-3} dilution of each strain in one of the squares
- seal the plates with parafilm when plates are dry.
- incubate plates at 37 °C for 3 – 5 days (bottom up)



Evaluation

- judge growth of the bacteria and determine the resistance phenotype. Use the following symbols: + = growth = resistant; +/- = ?; 0 = no growth = susceptible.
- discuss putative resistance mechanisms

Strain	LB	Sm	Kan	Par	Gm	Am	Tob	resistance phenotype	genotype
A									
B									
C									
D									
E									
F									
	Control	aminocyclitol-aminoglycoside	2-deoxystreptamine-aminoglycoside						

Experiment 4: Determination of minimal inhibitory concentration by microbroth dilution

Material: 24 vials, LB-Tween broth, LB broth with kanamycin (1 ml 400 µg/ml); bacterial strains G, H

Microbroth dilution

- prepare 24 vials with one ml of LB broth
- enumerate 12 vials with G1 – G12 and the other 12 wells with H1 – H12
- add 1 ml of LB broth containing 400 µg/ml kanamycin to vial G1
- mix gently
- transfer 1 ml from vial G1 to vial G2
- mix gently
- continue this procedure to well G10
- after mixing G10 discard 1 ml
- add 1 ml of LB broth containing 400 µg/ml kanamycin to vial H1 and dilute according to the procedure performed for vials G
- vial 11 is the growth control without antibiotics
- vial 12 is the sterile control
- add 50 µl of a 1: 100 dilution of bacterial strain G to each of the vials G1 to G11
- add 50 µl of a 1: 100 dilution of bacterial strain H to each of the vials H1 to H11
- incubate at 37 °C (with constant shaking) for 3 – 5 days

Evaluation

- calculate the antibiotic concentration in each of the vials
- read growth in each of the vials
- determine the minimal inhibitory concentration, i.e. the lowest concentration of the antibiotic where no growth occurs.

Laboratory rules and Precautions

- you have to wear a lab coat
- no eating or drinking in the lab
- care must be taken not to contaminate the environment
- all material must be collected and be sterilized before disposal
- hands must be washed and disinfected
- surfaces must be wiped with 70 % ethanol after completion of the experiment

Experiences gained

- basic microbiology techniques
- determination of antibiotic resistance
- mechanisms of antibiotic resistance in bacterial pathogens
- model organisms for ribosomal antibiotic resistance

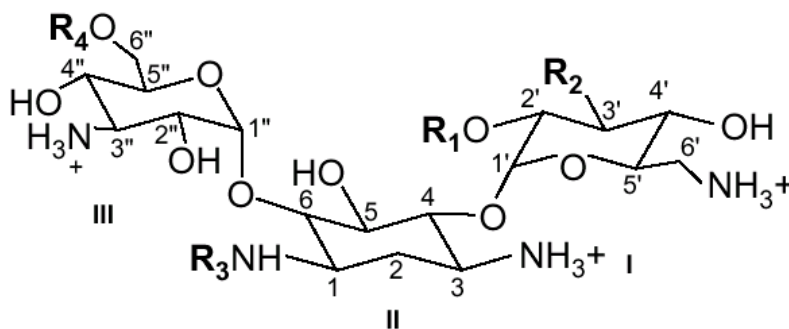
Timing

- 1st day: 3.5 h
- 2nd day: 3.5 h

Reporting

- description of colony morphology
- description of antibiotic resistance phenotypes and genotypes
- determine minimal inhibitory concentrations
- discussion of putative resistance mechanisms

Example of 2-deoxystreptamine aminoglycosides



aminoglycoside	R ₁	R ₂	R ₃	R ₄
kanamycin A	OH	OH	H	H
kanamycin B	NH ₃ ⁺	OH	H	H
amikacin	OH	OH	L-AHBA	H
tobramycin	NH ₃ ⁺	OH	R ₃	CONH ₂
dibekacin	NH ₃ ⁺	H	H	H

Phylogenetic tree of slow-growing mycobacteria

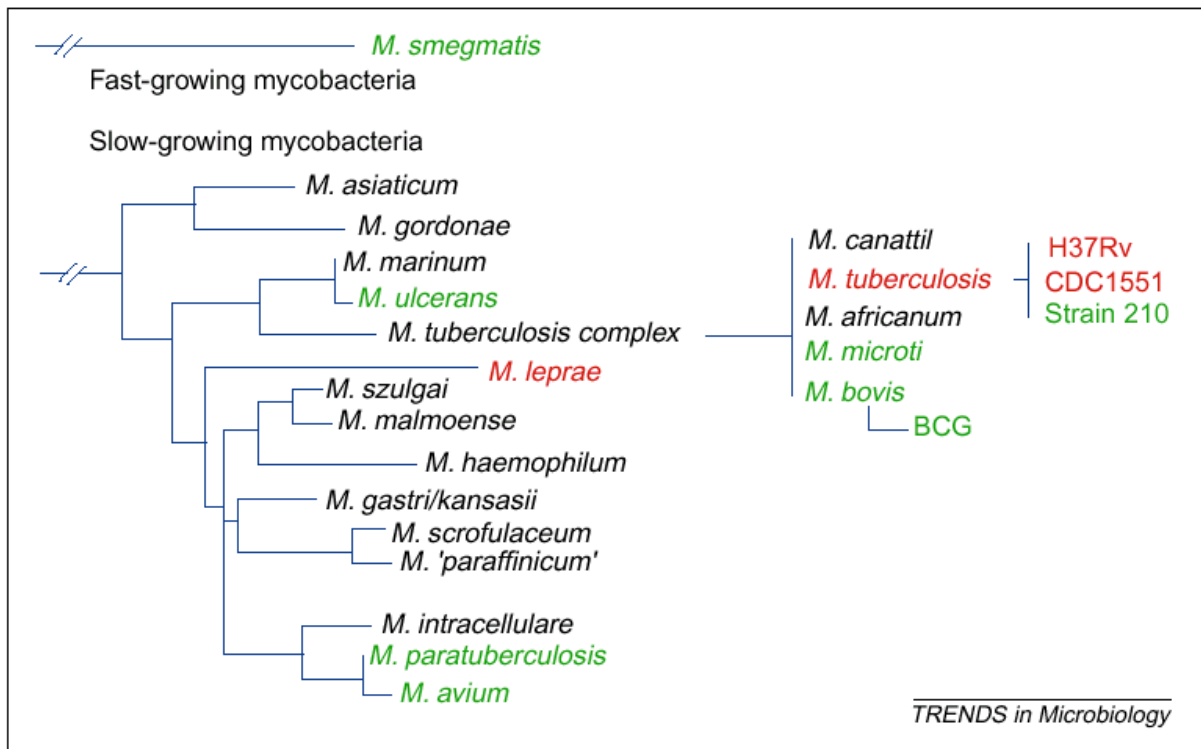


Fig. 1. Phylogenetic tree of selected mycobacteria, based on 16S rRNA sequences², including those species whose genome sequences are in progress (green) or completed (red).

Mycobacterium grown of Lowenstein-Jensen