Mechanisms of aminoglycoside resistance in mycobacteria

Advisor
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Reading

Objectives
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:
- presence of aminoglycoside modifying enzymes (aminoglycoside nucleotidytransferases (ANT), aminoglycoside acetyl transferases (AAC), aminoglycoside phosphotransferases (APH)).
- altered structure of the target (ribosomal proteins, ribosomal RNA)
- altered membrane permeability
- extrusion of the aminoglycosides 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 looses 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 particular 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)

- Böttger E.C. et al. (2001) Structural basis for selectivity and toxicity of ribosomal

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**Practical work**

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

**Material and experimental protocols**

**Experiment 1**

**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**

1. Streaking
2. Incubation
3. Growth
Experiment 2
Determination of the antibiotic resistance phenotype of mycobacteria

Material: Agar plates (1 each – LB, LB streptomycin (Sm), LB- kanamaycin (Kan), LB-paromomycin (Par), LB-gentamicin (Gm), LB-amikacin (Am), LB-tobramycin (Tob)), LB-broth, sterile Eppendorf tubes, µl-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 µl of the bacterial suspension with 900 µl of LB-Tween broth in sterile Eppendorf-tubes (10⁻¹ dilution).
- use a new tip for preparing each dilution
- transfer 100 µl of the 10⁻¹ dilution into another tube containing 900 µl of broth. This results in the 10⁻² dilution.
- transfer 100 µl of the 10⁻² dilution into a third tube containing 900 µl 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 µl 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>LB</th>
<th>Sm</th>
<th>Kan</th>
<th>Par</th>
<th>Gm</th>
<th>Am</th>
<th>Tob</th>
<th>resistance phenotype</th>
<th>genotype</th>
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<td>A</td>
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</tbody>
</table>

Strain | LB | Sm | Kan | Par | Gm | Am | Tob | resistance phenotype | genotype |
--------|----|----|-----|-----|----|----|-----|-----------------------|----------|
A       |    |    |     |     |    |    |     |                       |          |
B       |    |    |     |     |    |    |     |                       |          |
C       |    |    |     |     |    |    |     |                       |          |
D       |    |    |     |     |    |    |     |                       |          |
E       |    |    |     |     |    |    |     |                       |          |
F       |    |    |     |     |    |    |     |                       |          |
Control |    |    |     |     |    |    |     |                       |          |

Experiment 3: 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: 2 h
- 2nd day: 1.5 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

<table>
<thead>
<tr>
<th>aminoglycoside</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$R_3$</th>
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<td>kanamycin A</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>kanamycin B</td>
<td>$NH_3^+$</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>amikacin</td>
<td>OH</td>
<td>OH</td>
<td>L-AHBA</td>
<td>H</td>
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<tr>
<td>tobramycin</td>
<td>$NH_3^+$</td>
<td>OH</td>
<td>$R_3$</td>
<td>CONH$_2$</td>
</tr>
<tr>
<td>dibekacin</td>
<td>$NH_3^+$</td>
<td>H</td>
<td>H</td>
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</table>
Phylogenetic tree of slow-growing mycobacteria

![Phylogenetic tree of slow-growing mycobacteria](image)

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

*Mycobacterium* grown of Lowenstein-Jensen

![Mycobacterium grown of Lowenstein-Jensen](image)