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Accumulation of a bactericidal antibiotic by tolerant bacteria and insights into bacterial persistence

Mahnaz Sabeti Azad

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Accumulation of a bactericidal antibiotic by tolerant bacteria and insights into bacterial persistence.

Thèse de doctorat de l'Université Paris-Saclay
préparée à l'Université Paris-Sud

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Abbreviation

BF: Bright Field

CCCP: Carbonyl Cyanide m-ChloroPhenyl hydrazone

Cm: Chloramphenicol

Cy3: Cyanine 3

Cy5: Cyanine 5

DiBAC 4-(3): bis-(1,3-dibutylbarbituric acid)-trimethine oxonol

DMSO: DiMethyl SulfOxide

DNA: DeoxyriboNucleic Acid

EDPI, II: Energy-Dependent Phase I, II

FACS: Fluorescence-activated cell sorting

GFP: Green Fluorescent Protein

LB : Luria-Bertani broth

LPS: LipoPolySaccharide

MIC: Minimum Inhibitory Concentration

MS channels: Mechanosensitive channels

MscL: Mechanosensitive channels of large conductance

MscS: Mechanosensitive channels of small conductance

Neo-Cy5: Neomycin-Cyanine5

OD: Optical Density

PCR: Polymerase Chain Reaction

PDMS: Polydimethylsiloxane

PI: Propidium Iodide

PMF: Proton Motrice Force

rRNA: ribosomal RNA

STORM: Stochastic Optical Reconstruction Microscopy

TA: Toxin–Antitoxin

TIRF: Total Internal Reflection Fluorescence microscopy

TR: Texas Red

tRNA: transfer RNA

VBNC: Viable but nonculturable

Chapter I Introduction

1. Aminoglycoside: The ribosome as an antimicrobial target

The ribosome is a highly complex machine; it is nature's largest and most complex enzyme. Functional sites of ribosomal RNA (rRNA) are a target of about fifty percent of antibiotics, which are used clinically. These antibiotics block or alter synthesis of proteins. Knowing the importance of rRNA for decoding the messenger RNA (mRNA) and performing protein synthesis, it makes sense why most inhibitors of ribosome aim for rRNA-rich parts on both 30S and 50S subunits of ribosome¹(Fig1.1).

Figure 1.1 Antibiotics target the functionally important regions of ribosome¹. Two ribosomal subunits are displayed: 30S subunit (left) and 50S (right). rRNAs (in yellow and grey) and ribosomal proteins (in bronze and blue) are shown. In each subunit, the sites of antibiotic binding, which has been determined by biochemical and genetic techniques are indicated. Then details were obtained by X-ray crystallography.

Here, in this work, I will focus on a family of antibiotics called aminoglycosides, which are composed of amino modified sugars with large structural diversity.

1.1.Discovery

The aminoglycosides were the first significant therapeutic drugs, which were produced *via* fermentation pathways in bacteria. In 1943 streptomycin was discovered at Selman Waksman's laboratory from a soil bacterium: *Streptomyces griseus*². Streptomycin is the earliest modern agent used against *Mycobacterium tuberculosis*. The work of the same team led to the discovery of neomycin in 1949 from another species of soil bacteria, *Streptomyces fradiae*³. This discovery was of strong importance and Selman Waksman received the Nobel Price of Medicine in 1952.

Subsequently, kanamycin was discovered by Hamao Umezawa in 1957, and gentamicin in 1963 by Marvin Weinstein's group at Schering-Plough Corporate. Since then other aminoglycoside related compounds have been isolated through fermentation or semisynthetic methods. Although it is long time that these antibiotics are used, they still have a vital role in the treatment of infectious diseases worldwide. These bactericidal compounds are chemically stable with a broad spectrum of action except on anaerobic bacteria⁴.

From 1940s till now aminoglycosides are most commonly used worldwide, due to their high efficacy and low cost⁵.

Most common side effects of aminoglycosides are nephrotoxicity (impairment of kidney function) and ototoxicity (impairment of the organs of hearing and balance)⁶. However, they are still largely used to treat bacterial infection worldwide. The recent emergence of infections due to Gram-negative antimicrobial resistance bacterial strains has encouraged physicians to strengthen usage of aminoglycosides. Considering the effect of various dosage of aminoglycosides on toxicity combined with the retained activity against most Gram-negative bacterial isolates make

this class of antibiotics still valuable in today's clinical practice ⁷. Aminoglycosides are probably the most effective treatment for *P. aeruginosa* chronic infections ⁸.

1.2. Structure of aminoglycosides

Aminoglycosides are composed of amino modified sugar. Here we can see two main groups of aminoglycosides with their respective representatives, neomycin and gentamicin (figure 1.2). These two groups differ by their linkage between rings II and III.

Figure 1.2 Examples of aminoglycoside chemical structures. On the left are some aminoglycosides of the neomycin group (4–5 ring II–ring I, ring II–ring III linkages), which can be directly compared to the gentamicin components on the right (4–6 ring II–ring I, ring II–ring III linkages). Rings are numbered and the chemical structure of variable positions indicated. Ring II is made of deoxystreptamine, which is very conserved ⁹.

The neomycin group includes paromomycin, neomycin, ribostamycin and neamine. Ribostamycin contains all rings except ring IV while neamine lacks both rings III and IV. In this study I focused on the neomycin. The neomycin family shares a common core of rings I and II, which are conserved among aminoglycosides.

1.3.Sites of attachment in the ribosome

Aminoglycoside are one of the most characterized antibiotic family, which bind both subunits of the ribosome causing miscoding or inhibiting translocation and ribosome recycling^{10, 11}. The well-characterized aminoglycosides from the neomycin family have been shown to accelerate both forward reaction rates (GTPase activation and accommodation) in tRNA selection and to reduce the rate of cognate and near-cognate tRNA dissociation from A site^{11, 12}.

Aminoglycosides are found to be translocation inhibitors^{13, 14} and increase back-translocation^{15, 16, 17}. Majority of aminoglycosides bind to 30S ribosomal subunit at an internal loop in helix 44 (h44) which mainly forms the decoding site¹⁸. This interaction has been studied by chemical probing (biochemical approaches)^{19, 20} and by complex of aminoglycoside and a small RNA fragments mimicking h44 in ribosome (structural approaches)^{9, 21, 22, 23, 24}.

Mechanism of decoding and the action of aminoglycosides on the decoding center have been the topic of studies by ribosomal X-ray crystallography^{25,26,18,27,28}. The binding of aminoglycosides places nucleotides A1408, A1492, and A1493 in a conformation that is different from that observed in the absence of the drug (Figure 1.3).

Figure 1.3 Rearrangements of rRNA helices h44 and H69 in the near-cognate state upon binding of the aminoglycoside paromomycin (PAR) ²⁹. Paromomycin (green) is bound into the decoding site on 16S rRNA. The tRNA is displayed in red and mRNA in yellow. The differences between complexes formed with near-cognate tRNA in presence and absence of paromomycin are highlighted in dark and light blue.

Besides, the antibiotic disturbs the destacking of A1492 and A1493 (nucleotides critical for A-site tRNA binding ³⁰) from h44 into a position where they interact with the codon-anticodon helix (Figure 1.4). An X-ray study of the *Escherichia coli* ribosome demonstrated that neomycin has a second binding site in helix 69 (H69) of 23S rRNA³¹.

Figure 1.4 Overview of binding sites of neomycin in ribosome ³². **(a)** Neomycin (Neo) in dark green in 30S subunit, helix44 (blue) and neomycin in H69 (light blue) of the 50S subunit (gray). tRNA (green), mRNA (pink). **(b)** Neomycin interactions with the H69. Dashed lines display neomycin and rRNA

contacts < 3.5 Å. Ring II of neomycin is positioned at the major groove, which is facing G1921, G1922 and U1923. The rings I, III and IV are mainly in contact with the backbone.

The work in the last ten years revealed the aminoglycosides' action at the decoding center and Helix 69 in ribosome yet the interplay between these two proximal sites remains unknown³². Based on some recent and unexpected results on the 70S crystal structures in complex with mRNA and tRNAs, a new model was proposed for the recognition of the codon-anticodon helix by the decoding center with new insights into the interaction of paromomycin^{29, 33}. In this model the antibiotic partially induces a conformation of rRNA around the decoding center like that observed for cognate tRNA.

1.4.Mechanism of uptake

Aminoglycoside need to be transported through the cytoplasmic membrane to reach their target, ribosomes. Aminoglycoside accumulation in the cytoplasm is dependent on proton motive force (PMF) in cells³⁴. It is suggested that accumulation of aminoglycosides by bacteria growing in aerobic conditions occurs in three successive steps^{5, 34}.

1.4.1. The accumulation theory (3 successive steps)

1.4.1.1. Step1: Electrostatic binding

First the drugs would bind to anionic sites situated at the outer membrane instantly and in a reversible way. It was also proposed that in Gram-negative bacteria lipopolysaccharide (LPS) would directly interact with the polycationic aminoglycosides thereby helping their uptake ³⁵. Therefore, a self-promoted uptake mechanism for aminoglycosides was favored in terms of hypothesis rather than a mechanism involving porins ³⁶ because studies showed that mutants which are porin-deficient, are not always more resistant to aminoglycosides ³⁷ (Figure 1.5). Interestingly, a recent study showed that the subpopulation of cells with lower expression of outer membrane porin C (ompC) are more tolerant to kanamycin³⁸.

Figure 1.5 Gram-negative bacteria cell wall envelope ³⁷. (a) Porin pathway (not likely involved in aminoglycoside uptake) (b) Self-promoted aminoglycoside uptake in which polycations and chelators can dislocate divalent cations from LPS.

In this mechanism, the antibiotic interacts with the outer cell wall and this interaction increases the permeability of this cell wall to the antibiotic. This step would be followed by two irreversible energy dependent phases termed energy-dependent phase I and II (EDPI and EDPII, respectively).

1.4.1.2. Step 2: Energy-dependent phase I (EDPI)

The first step is followed by EDPI that varies in its duration and rate depending on the antibiotic concentration used and the energy level of bacterial cell. It was also observed a relation between the growth rate of bacteria prior to the addition of the antibiotic ³⁹. Exponential phase bacterial cells were found to be very susceptible to aminoglycoside due to the higher PMF. On the contrary, the stationary phase cell populations are less sensitive to aminoglycosides. EDPI corresponds to the entrance of aminoglycoside into the cytoplasm. EDPI can be blocked by inhibitors of electron transport such as carbonyl cyanide-m-chlorophenyl-hydrazone (CCCP). The mechanism of transport of aminoglycosides across the cytoplasmic membrane is poorly characterized and may involve quinone-linked redox energy or other components of the electron transport chain or both. Aminoglycosides may also enter by nonspecific membrane channels created by low-level intrinsic misreading or some imperfections in the cytoplasmic membrane, mainly in the area of growth where the membrane is being made ⁴⁰. In fact, to date much remains unknown about the phase EDPI.

1.4.1.3. Step 3: Energy-dependent phase II (EDP II)

The third phase of aminoglycoside uptake also remains unclear. It requires energy from the electron transport chain and ATP hydrolysis however has diffusion kinetics ⁴¹. The phase EDPII requires aminoglycoside sensitive ribosomes, and inhibitors of protein synthesis such as chloramphenicol can abolish EDPII ⁴².

It is supposed that miscoding by aminoglycosides that entered the cytoplasm during EDPI produces misfolded proteins. Mistranslated proteins tend to be misfolded and expose

hydrophobic regions that interact with membranes or bind to other cellular components, which end up in membrane and lead to membrane permeabilization ⁴³.

This permeabilization has been observed by the leakage of amino acids, nucleotides or K⁺ ^{44,45,46}. Bacteria observed after treatment with streptomycin ⁴⁷ had a collapsed appearance in scanning electron images.

1.4.2. Iron–sulfur clusters affect aminoglycosides' accumulation

Iron–sulfur clusters are in many biological systems, components of electron transfer proteins in cellular process such as central metabolism and respiration. Studies show that Fe-S clusters in bacterial cells are needed for aminoglycoside bactericidal activity ⁴⁸. Fe-S proteins increase aminoglycoside killing by boosting their uptake ⁴⁹.

Cells in which substitute machinery are favored instead of major Fe-S cluster biosynthesis machinery, are not able to mature respiratory complexes I and II in efficient manner and consequently they have lower PMF. Likewise, the cells become transitionally resistant to aminoglycoside under iron limitation condition. In those cells both respiratory complexes are down-regulating and cells are using alternative machinery ⁴⁸.

Another recent study showed the role of the small regulatory RNA (sRNA), which is called RyhB. It has a major role in iron homeostasis in *E. coli*. Authors showed that in iron deficient conditions, RyhB induces phenotypic resistance to gentamicin. This phenotype is due to

the inhibition of respiratory complexes. It has been suggested that RyhB plays a role in antibiotic stress adaptation ⁵⁰.

1.4.3. Aminoglycoside uptake in presence of Silver

Silver can destabilize the membrane either by disrupting intrinsic membrane proteins (directly) or by its effect on ribosomes (indirectly), which would lead to misfolded proteins that would finally go to the inner membrane. Consequently, membrane's permeability increases and it may lead to aminoglycoside uptake in higher levels ⁵¹ (figure 1.6).

Figure 1.6. Model for the role of silver in aminoglycoside uptake ⁵¹. Silver augments aminoglycoside uptake. Silver can disrupt cells' membrane by changing membranes' proteins. It can also target ribosomes, which result in synthesis of misfolded protein.

Silver potentiates aminoglycoside toxicity by enhancing their uptake ⁵². Herisse *et al.* showed that silver increased toxicity of aminoglycoside by a PMF independent mechanism. Besides, silver enhanced aminoglycoside uptake by mutant with lower PMF level such as mutants for complex I and II or Fe-S cluster biosynthesis. In contrast, silver effect on aminoglycoside toxicity has been shown to be remaining dependent on translation ⁵². Treating

cells with chloramphenicol which is a bacteriostatic antibiotic through inhibition of translation, prevented silver to increase aminoglycoside toxicity. It was proposed that silver destabilizes the membrane in a protein translation dependent, manner and it allows aminoglycoside to enter to cytoplasm more efficiently. This fact settles with the proposition of Morones *et al.* ⁵³, which suggested that silver's interaction with ribosome and misfolded proteins would disturb inner membrane and destabilize it. Hence, regardless of cause or origin, both studies suggested that increase in misfolded protein levels leads to membrane permeability.

On the contrary, the role of ROS to silver toxicity was more debatable. Morones *et al.* assumed that silver ions increase the toxicity of gentamicin because silver produces ROS in cells ⁵³. However, in this study they did not show that ROS production is increased in the presence of the combination of silver with gentamicin ⁵¹. Results from Herisse *et al.*, disfavor this hypothesis of increase of ROS production. Authors showed that silver potentiated gentamicin toxicity even in anaerobic conditions. In addition, they tested mutants with anti-ROS activities in comparison with wild-type *E. coli* strain. Authors showed similar sensitivity to silver potentiating effect for the different strains ⁵².

1.5. Consequences of the presence of aminoglycosides in bacterial cells

Aminoglycosides go through the bacterial membranes and unfortunately (for bacteria) have affinity to ribosomes. As bacteria had hundreds of million years to evolve, they respond to antibiotic presence to increase their chance of survival. It is important to understand the consequences of aminoglycoside in the cells in order to be able to combine different factors to decrease the chance of antibiotic failure.

1.5.1. Membrane damage and response

Once in the cytoplasm, the bactericidal effect of aminoglycosides is thought to result from misreading of mRNA and misfolding of proteins and especially membrane proteins^{54,43,55}. A hallmark response of this stress is the upregulation of heatshock proteins, for instance the heat shock inclusion body associated proteins IbpA and IbpB, that may help bacteria to resist stress. However, some of these misfolded proteins would go into the membrane due to their hydrophobic characters, and destabilize further the membrane, allowing for enhanced entry of aminoglycoside^{56,57}.

In *P. aeruginosa*, when cells go through a membrane stress it triggers an adaptive response. This response is controlled by a 2-component regulator (AmgRS). It has been suggested that this response can be caused by translational misreading which is induced by aminoglycosides. This response may help protect cells from aminoglycoside-induced mistranslated proteins that would disrupt the membrane⁵⁸.

It has been reported that two-component system activation by aminoglycoside trigger antibiotic-mediated cell death⁵⁴. Activated sensor histidine kinase (CpxA) in *E. coli* phosphorylates transcriptional regulatory protein (CpxR), which consequently leads to upregulation of genes involve in envelope stress-response, such as the periplasmic protease (*degP*).

1.5.2. Chaperonin

In 2013, the Goltermann team tested a long overdue study on the possible effects of chaperonin action on aminoglycoside susceptibilities. Chaperonins are proteins which assist the folding of large protein molecules. They demonstrated that chaperonin GroEL/GroES overexpression decreases the level of misfolded cytosolic proteins, which is caused by aminoglycosides⁵⁹ (Figure 1.7).

Figure 1.7 Model for effect of chaperone on early aminoglycoside exposure in bacterial killing or survival⁵⁹. An initial uptake of aminoglycoside across envelope and binding to ribosomes result in miscoded peptide (orange stars). Misread polypeptides are substrates of several chaperones. Limited chaperone capacity results in protein misfolding, leading to cell death due to an increase of membrane permeability. On the contrary, higher capacity of chaperones, in particular GroEL/GroES, correct the misfolding in those proteins and increases bacterial survival.

During aminoglycoside treatment, overexpression of chaperonin protected the bacterial membrane potential, rescued cell growth, whereas inhibition of chaperonin expression sensitized bacteria. In *Escherichia coli*, chaperonins support bacteria in handling early exposure to aminoglycosides. Chaperonins fight aminoglycoside-induced protein misfolding and promote short-term tolerance.

1.5.3. Biofilm induction

It has been reported that aminoglycosides induce the formation of biofilm. Biofilms are adherent aggregates of bacterial cells formed on surfaces. Biofilms help bacteria to resist antibiotic treatment and play a role in bacterial persistence in the case of chronic infections. It has been shown that sub-inhibitory concentrations of aminoglycoside induces the biofilm formation in certain strains such as *P. aeruginosa* and *E. coli*⁶⁰.

Aminoglycosides are commonly used to treat infections caused by the pathogen *P. aeruginosa*. However, clinical isolates of *P. aeruginosa* often acquired resistance to aminoglycosides and may also have an inducible resistance system to tobramycin. This antibiotic is produced by *Streptomyces tenebrarius*, which is a soil bacterium. Taking into account that both bacteria coexist in soil raised an interesting hypothesis. The development of resistance in *P. aeruginosa* to tobramycin can be an adaptive response to the presence of this antibiotic in its natural environment. *P. aeruginosa* makes a larger biofilm when the cells are treated with tobramycin. However, larger biofilm in this case was due to higher number of cells within the biofilm community rather than an increase in extracellular matrix production of biofilm. This response seemed to be regulated by the *arr* gene (aminoglycoside response regulator). The *arr* is

an inner-membrane phosphodiesterase and its substrate is cyclic di-guanosine monophosphate (c-di-GMP), which is a second messenger in bacteria known for cell surface adhesiveness regulation. In the absence of *arr* gene, *P. aeruginosa* cells are not forming biofilm in presence of tobramycin (figure1.8).

Figure1.8 c-di-GMP role in biofilm formation in presence of aminoglycosides ⁶⁰. In *P. aeruginosa* aminoglycosides may directly interact with Arr or indirectly through changing level of c-di-GMP.

It is proposed, in this model, that antibiotic induces the activity of the Arr protein. However, the details of its activity are yet to be determined.

1.5.4. Oxidative stress

It was proposed that aminoglycosides eventually trigger hydroxyl radical formation which leads to cell death ^{54,61,62}. It had been suggested that oxidative stress triggered by membrane protein traffic, mistranslation and misfolding of membrane proteins is the main reason for cell death after aminoglycoside treatment. Authors suggested that a two-component system for stress-response in the cell envelope is a key player in this process and a redox-responsive two-component system, as well, was shown to have a connected role ⁵⁴(Figure1.9). However, it

should be reminded that ram (ribosome ambiguity mutations) *E. coli* strains that have single amino-acid mutations in the ribosomal proteins S4 and S5, which cause comparable levels of misreading as aminoglycosides, are viable ⁶³. It is therefore unclear whether miscoding leads to cell death *via* oxidative stress or not.

In line with this observation, it was shown that Fe-S proteins promote aminoglycosides induced cell death without the involvement of the reactive oxygen species (ROS) response ⁴⁸. Keren *et al.*, also proposed that ROS is not involved in antibiotic mediated cell death ⁶⁴. More recently, a study that supports the ROS response with more robust assays was reported ⁶⁵.

Figure 1.9 Suggested mechanism of hydroxyl radical formation and cell death⁵⁴. (A) Misfolded membrane proteins are carried to membrane translocation complexes (e.g., SecYEG) by chaperone proteins (SecB). Misfolded proteins are present in the membrane or translocated to the periplasm (B) Misfolded proteins lead to phosphorylation of CpxA. (C) Activated CpxA phosphorylates CpxR, which upregulates expression of envelope stress-response proteins (DegP). (D) CpxA also activates ArcA, which regulates respiratory and metabolic genes. These changes in cell's state would cause free radical formation, which leads to cell death.

It has been shown that, unlike *E. coli*, in *Vibrio cholerae* SOS response is triggered by sub-minimal inhibitory concentrations of aminoglycosides. *E. coli* is well protected against this kind of oxidative stress because of its efficient oxidative stress response through the RpoS general stress response sigma factor. However, in *V. cholera*, the SOS response is triggered by aminoglycoside mediated indirect DNA damage through oxidized guanine residues. Aminoglycoside mediated SOS induction is preserved among other Gram-negative pathogens such as: *Klebsiella pneumoniae* and *Photobacterium luminescens* ⁶⁶.

A year later, the same team through genetic screening methods found several genes, which cause destabilization of the RNA polymerase complex. These genes are involved in SOS response in sub-inhibitory concentration of aminoglycosides ⁶⁷.

1.5.5. Alarmone

Misfolded proteins in the bacterial membrane, as a result of aminoglycoside lethal dosage treatment, trigger production of ROS. ROS induces lysyl-tRNA synthetase (LysU) expression and production of dinucleotide alarmone metabolite called diadenosine tetraphosphate (Ap4A). It is proposed that Ap4A might inhibit intracellular proteostasis, which cause membrane disruption leading to cell death (Figure 1.10).

The activation of alarmone Ap4A increased aminoglycoside accumulation in the cells by membrane disruption. It has been suggested that blocking Ap4A degradation or increasing its synthesis can be an approach to increase aminoglycoside lethality.

Figure 1.10 Model for Ap4A alarmone action in aminoglycoside killing ⁶⁸. Aminoglycoside causes the production of misfolded proteins and membrane disruption and ROS generation. ROS induces the expression of LysU, which results in production of Ap4A. Ap4A then somehow inhibits intracellular proteostasis in cell (remained to be explored further), which cause more disruption in cell membrane.

1.6. Phenotypic suppression by aminoglycosides

Streptomycin can reverse the mutant phenotypes of many nonsense and missense mutations in *E. coli* and in bacteriophage T4. This phenomenon has been called phenotypic suppression, since the mutant phenotype returns after removal of the drug ⁶⁹.

Aminoglycoside-induced misreading at sense codons ⁷⁰ on the other hand, according to recent study, tobramycin is a suppressor of premature termination codons. As we know, the molecular basis of many genetic diseases such as cystic fibrosis is premature translation terminations. It has been demonstrated that tobramycin exhibits read-through ability on premature termination codons ⁷¹. This result is in line with the global therapeutic strategy, which consists of using aminoglycosides to suppress the termination of protein synthesis and restore full-length functional proteins.

1.7. Resistance mechanisms

In bacteria, resistance to aminoglycosides is often due to enzymatic inactivation by phosphotransferases, acetyltransferases and nucleotidyltransferases (adenylyltransferases) ^{72, 73}. Other mechanisms include ribosomal alterations and increase of membrane permeability. Aminoglycoside-resistant strains often appear due to possessing plasmid-borne genes encoding aminoglycoside-modifying enzymes ⁷⁴. Furthermore, many of these genes are associated with transposons, which help fast diffusion of drug resistance across species boundaries ⁷⁵.

1.7.1. Adaptive resistance

Adaptive resistance to antibiotics is well documented where a change in the environment will lead to up or down regulation of genes that will confer to the cell an advantage to survive.⁷⁶ It should also be mentioned that the capacity of a bacterial population to adapt to environmental changes does not only rely on the presence of stimuli. It can also be inherent to the phenotypic heterogeneity present in populations ³⁸. It happens that some cells have, at the exact moment of the stress, a molecular composition that result in low levels of resistance to drugs. All together, these strategies can have important outcome during clinical treatment rendering some infections difficult to cure.

It has been shown that sub-inhibitory and inhibitory concentrations of aminoglycosides in *Pseudomonas aeruginosa* and other aerobic Gram-negative bacilli induced adaptive resistance. This kind of resistance has been shown to be the result of down-regulation of antibiotic uptake at EDP II phase ⁷⁷.

2. Persisters

2.1. Discovery

More than 70 years ago, Hobby and Bigger observed that it is not possible to sterilize bacterial cultures by bactericidal antibiotics. Hobby observed that a small part of a *Staphylococcus* population was not killed upon penicillin treatment ⁷⁸. Few years later Bigger focused on few bacterial cells that survive very high antibiotic treatments. He named this subpopulation of bacteria ‘persisters’⁷⁹. In his pioneering work, he suggested that these cells were provisionally non-dividing. In addition, he discovered that these persisters created offspring, which were sensitive to penicillin as the original culture.

Persisters can be defined as the antibiotic tolerant subpopulation of bacteria in the sensitive population. Persistence is like a dormancy physiological state and antibiotic persistence is a fascinating example of heterogeneity at single cell level, which is not due to their genetic background. This heterogeneity may be the cause of failure of antibiotic therapy ⁸⁰.

It has been found that uropathogenic strains of *E. coli* (UPEC) are the major cause of bacterial urinary tract infections. These cells can survive after antibiotic treatment due to phenotypic heterogeneity. This study showed the importance of host pathogen response in occurrence of heterogeneity in cell population ⁸¹.

Several recent studies show that the line between the states of persistence and resistance seems not as clearly defined as previously thought. Persisters’ offspring had a stronger propensity to exhibit genetic mutations giving antibiotic resistance ^{82,83}.

2.2. Terminology: Antibiotic Resistance vs. Tolerance vs. Persisters

Recently, researchers working in the field of antibiotic persistence gathered to publish a “consensus statement” defining clear definitions and guidelines for research in this field ⁸⁰. Here, we give a brief description of these definitions.

Antibiotic resistant cell: a cell that tolerates antibiotic treatment by possessing a resistance gene (such as an efflux pump). Resistant bacteria grow in the presence of antibiotic concentrations that stop the growth of sensitive bacteria.

Antibiotic tolerant cell: a cell that survives antibiotic treatment and regrow after antibiotic elimination. Tolerance is a term to describe homogenous population and its factors can be genetical or environmental. Tolerance factors help the cell population to survive antibiotic treatment, which is lethal for antibiotic sensitive bacteria (Figure 1-2-1).

Antibiotic persistent cell: a cell that is tolerant to a bactericidal antibiotic in a population of cells that are susceptible. After treatment of a susceptible population, the biphasic killing curves observed indicate the presence of two subpopulations. A first one consists of cells that are killed fast (the majority) and a second one corresponding to persister cells that survive or are killed at a much slower rate (Figure 1-2-1).

Figure 1-2-1 Profiles of antibiotic killing kinetics of resistant, tolerant and persister cells ⁸⁴. The time of addition of a bactericidal antibiotic is indicated with a red star. A homogeneous population of resistant bacteria is shown with blue line. Resistant bacteria population continues its growth. A homogeneously tolerant bacterial population showed in light grey takes longer time to be killed by an antibiotic than a susceptible population shown in dark grey line. In heterogenous populations, the presence of persisters is shown by a biphasic kill curve (yellow). After antibiotic treatment, there is a period of rapid killing of susceptible cells followed by a slower decrease in colony forming units (CFU).

2.3.Importance and danger

Antibiotic resistance is a one of the major causes of failure in antibiotic treatment. However, in case of infection with non-resistant bacteria, persisters escape antibiotic treatment. After stopping antibiotic therapy against an infection caused by susceptible bacteria, persisters step out of their dormant states and cause infection relapse. Understanding the persistence mechanism has a great importance because it will lead us to find the solution for increasing efficacy of the current antibiotic therapy.

In a resistance evolutionary study, it has been demonstrated that bacterial persistence rapidly adapts to drug treatment frequency. Authors observed that the rates of entering to the persister state can be considered in the context of ‘bet-hedging’ theory. It has been suggested that persistence is a main component of the evolutionary cell response to antibiotics stress that is essential to be considered in both diagnostic testing and treatment design in studies against antibiotic tolerance⁸⁵. It should be mentioned that in this study tolerance appeared because of single point mutations in specific genes.

In the following part I presented two main examples of medical complications caused by persisters. During infection the environments that are inhabited by bacteria, such as biofilms and macrophages, have shown to be related with the formation of persisters⁸⁶.

2.3.1. Persisters in biofilm

The presence of persister cells in the biofilm is now considered as a major cause of biofilm antibiotic resistance. Persisters are less than one percent of a bacterial population in stationary-phase cultures and biofilms. In the presence of antibiotics, persisters do not multiply. When the antibiotic is not in presence of persisters anymore, persisters regenerate the original, non-persister antibiotic tolerant population⁸⁷. There are factors, which induce persister formation such as the lack of nutrients, a situation that experience bacteria trapped in the biofilm⁸⁸. We can say that biofilms constitute a favorable environment for persister generation.

Therefore, because biofilms induce formation of persister sub-populations this makes the eradication of persisters more difficult.

2.3.2. Persisters in macrophage

Macrophages are responsible for phagocytosis as a part of our immunity system. These big eaters engulf and digest anything that does not have the type of proteins specific to healthy body cells or look like foreign substances such as microbes. At the same time pathogens can manipulate host immune responses and decrease the chance of pathogen clearance ⁸⁹. For example, many pathogens, including *M. tuberculosis*, *Legionella pneumophila* and *Salmonella spp.*, avoid degradation following phagocytosis by host immune cells. It has been found that these pathogenic bacteria inhibit the maturation of phagosomes ⁹⁰.

It has been shown that by fluorescent single-cell analysis, it is possible to identify *Salmonella* persisters in infected macrophage. Persisters are nonreplicating cells formed instantly after bacterial uptake into the macrophages. It has been suggested that persistence was induced in the cells because of firstly the acidic environment of the vacuole in macrophages and lack of nutrition. These conditions also induce virulence gene expression in *Salmonella* ⁸⁶. However, level of acidity and lack of nutrition may vary in different macrophages cells or within different phagosomes, thus inducing the formation of persisters for some bacteria and not for others. The exact mechanisms of how these environments induce the formation of persisters are still a matter of debate ⁸⁹.

It has been shown as well that the 14 toxin-antitoxin modules play a role in the persister formation of *Salmonella* in macrophages ⁸⁶.

2.4. Why do they become persister?

2.4.1. Dormancy and type I vs. type II persister

Dormancy is a state of a bacterium in which bacteria does not grow and it has very low activity in comparison with growing exponential phase or stationary phase cells. Dormant bacteria are most likely tolerating many antibiotics because their metabolism level is low, and they stop growing⁸⁷. However, tolerance and persistence may be caused without dormancy. It has been found that being in non-growing state or inactive metabolically do not assure persister formation, because most “dormant” subpopulations were not persisters. In this study authors claimed that dormancy is not enough for persister formation⁹¹. According to the study of formation of persisters of *salmonella* in macrophages, these non-growing cells in the macrophage maintain an active state⁹².

Presence of persisters in a given population can be viewed as a protection strategy⁹³. When a population goes through a stressful condition, persisters may survive and allow the survival of the community. Moreover, it has been suggested that persisters may be a strategy by which bacteria can flee programmed cell death which could be induced by antibiotic treatment. When an antibiotic interacts with its target in cells, it leads to inhibition of cell growth and like that the cell avoids programmed cell death⁹⁴.

It has been proposed that there are two types of persisters that enter to dormancy state either in the stationary phase (type I) or during growth (type II)⁹⁵.

2.4.2. Toxin and anti-toxin modules

Genetic basis of antibiotic persistence was explored for the first time by Moyed and Bertrand ⁹⁶. They isolated mutants of *Escherichia coli*, which produce a higher percentage of persisters. Years later, Balaban and colleagues ⁹⁵ used a strain carrying *hipA7* allele, which was one of these gain-of-function alleles to track persisters in a microfluidic device. They showed that *hipA7* persisters are the non-growing cells. Later, HipA was found to be a toxin and with its cognate antitoxin HipB making a toxin–antitoxin (TA) module.

Among all the 10 type II TA modules in the model bacteria *E. coli*, HipA is one of the most studied toxins and it has been proven to be involved in persister formation.

In exponentially growing *E. coli* cells, the deletion of 10 mRNA endonucleases (Δ TA10 strain : Δ mazF, Δ chpB, Δ relBE, Δ dinJ/yafQ, Δ yefM/yoeB, Δ hipBA, Δ prfF/yhaV, Δ yafNO, Δ mqsRA, Δ hicAB) decreased bacterial persistence ⁹⁷. However, 5 years later, it was shown that the lower persister formation in Δ TA10 strain was due to the presence of the ϕ 80 bacteriophage ⁹⁸. Reconstruction of the original Δ TA10 strain which was not contaminated by bacteriophage ϕ 80 showed that these 10 TA modules do not affect persister formation in nutrient-rich conditions ⁹⁹. In minimal medium, the Δ 10TA mutation reduces tolerance of ciprofloxacin but not that of ampicillin. It has also been shown that persister formation in Δ 10TA strain is not affected by complete inhibition of protein synthesis (chloramphenicol or tetracycline treatment) ¹⁰⁰. How mRNA interferases are connected to persister formation remains unclear.

We know the role of TA modules is to increase the heterogeneity in the population in response to different source of stresses ¹⁰¹. It has been suggested that the activation of these TA modules in a fraction of the population will affect the cell growth, which is leading to bacterial

persistence⁹⁵. As TA modules dysregulation may lead to growth arrest it has been suggested that this is the reason why mutations in TA modules have been easily isolated by genetic screens for high persistence phenotypes¹⁰² (Figure1-2-2).

Figure1-2-2 persister cells frequency can increase due to stress responses¹⁰¹. Persisters exist in a heterogenous population and after activation of stress responses their frequency increases.

Among different sources of stress such as osmotic stress, pH stress, NaCl stress and stringent response only the last one led to TA-dependent persister formation¹⁰⁰. This phenotype is specific to fluoroquinolones. No effect was detected on tolerance to ampicillin¹⁰⁰ and no equivalent study has done on aminoglycoside.

Stringent response helps pathogens survival in the host during hostile conditions. Under amino acid starvation or intoxication by HipA toxin, uncharged tRNA in the ribosomal A site promotes the accumulation of a messenger nucleotide called guanosine pentaphosphate or tetraphosphate ((p)ppGpp). This molecule binds to several proteins ranging from DNA replication, protein transcription to various metabolic pathway proteins¹⁰³. (p)ppGpp augmentation in this condition is through RelA, which detects uncharged tRNA in the ribosome

leading to increase of persister cells in the population. (p)ppGpp activates specific type I and II TA modules which leads to persister formation (for instance, type I toxin HokB). Similarly, the SOS response can trigger activation of specific type I and type II TA modules that contribute to increase in number of persister cells in cell population ¹⁰¹ (Figure 1-2-3).

Figure 1-2-3 TA modules activated by stress responses lead to bacterial persistence ¹⁰¹. Starvation and excess of HipA toxin lead to the presence of uncharged tRNA in the ribosomal A site, which leads to accumulation of (p)ppGpp through RelA and formation of persisters. (p)ppGpp and SOS response activate specific type I and type II TA modules that lead to persister formation.

Considering a critical step in persister formation, the stringent response pathway may be considered as a potential target to discover a new class of antimicrobial agents active against persisters ¹⁰³.

2.4.3. ATP level

As persisters are non-growing cells, it has been hypothesized that they have low level of translation. An interesting method has been established by Shah and his colleagues in 2006 to sort out cells with low level of translation. They expressed an unstable GFP gene under control of ribosomal RNA promoter: *rrnB* P1. They sorted out dim cells and cells for GFP positive signal. The dim cells were much more tolerant to antibiotics and also exhibited a gene expression profile different from cells in exponential or stationary phases ¹⁰⁴. They concluded that persisters represent a distinct state of bacterial physiology, which is different from both exponential and stationary forms.

As I mentioned before it has been suggested that in certain conditions, mis-regulation of toxin-antitoxin modules lead to increasing the level of (p)ppGpp in the cells. As high level of ppGpp is known to be an inhibitor of *rrnB* P1, it was assumed that all the pieces of the puzzle are coming together. However recent study of Shah *et al.*, in 2017 showed that *rrnB* P1 promoter can report persister status independently of ppGpp ¹⁰⁰. They suggested that apart from (p)ppGpp there is another known effector on ribosomal RNA promoter which is ATP. This is in agreement with the known *rrnB* P1 activity which is positively controlled by ATP ^{105, 106} (figure 1-2-4).

On the other hand, another study suggested that the persister reporter RpoS is also an ATP reporter. Proteolysis of RpoS by ClpXP is inhibited at lower ATP levels. It appears that *rrnB* P1, as a persister reporter, is coregulated by ATP and ppGpp. It seems that persisters in a fast-growing culture are cells that go in a stationary-like state. ATP seems like a decent candidate for a general cause of tolerance, since most bactericidal antibiotics kill by attacking energy-dependent targets ¹⁰⁰.

Figure 1-2-4 The model suggesting that lacking ATP leads to persister formation¹⁰⁰. A decrease in ATP reduces the antibiotic target activity, which leads to antibiotic tolerance.

2.5.Aminoglycoside persisters

As our lab focused on the action mechanism of aminoglycoside, here, I focused on the studies on the persisters of this family of antibiotics. On the other hand, very few antibiotics can kill stationary phase cells. Aminoglycosides are known to be able to kill both growing and nongrowing cells, this fact makes these antibiotics good candidates for further investigation on formation of persisters in stationary phase cell population.

It has been shown that specific metabolic compounds can cause the killing of both *E. coli* and *S. aureus* persisters with aminoglycosides¹⁰⁷. This phenomenon is specific to aminoglycoside in either aerobic and anaerobic conditions in planktonic cells as well as cells in biofilm. These metabolites increase the PMF level in cells, which boost aminoglycoside uptake in cytoplasm. This study presented a strategy for persister eradication based on metabolism and showed the importance of the metabolic environment in efficiency of the mechanism of action of antibiotics (Figure 1-2-6).

Figure 1-2-6 Mechanism of persister eradication *via* the action of metabolites ¹⁰⁷. Eradication of persisters is through catabolism of carbon sources, which leads to generation of NADH. The production of NADH is independent from the PPP, EDP or TCA cycle. NADH is oxidized in the electron transport chain of membrane, which contributes to increase of PMF. Higher PMF in such cells facilitates aminoglycoside uptake.

The genetic basis of tolerance of *E. coli* to aminoglycoside was studied by transposon sequencing (Tn-Seq) in order to understand deeply the mechanism of tolerance to aminoglycoside ¹⁰⁸. The constructed library cells was screened by treating stationary-phase cell culture with a lethal dose of gentamicin. Among the mutant library, there were 105 disrupted genes or promoter regions found to have more than 5-fold decrease in gentamicin tolerance and 37 genes in which the cells were more than 5-fold tolerant. These data showed that mechanisms of persister formation to aminoglycoside are linked to various and redundant cellular pathways.

Functional cluster analysis in the Tn-seq library suggested that lack in motility and synthesis of amino acid drastically reduced the number of persisters, which are tolerant to gentamicin. However, in these mutants, the population of persisters was decreased and there was no change in MIC values.

Amino acid auxotrophs, for instance auxotrophs for serine, threonine, tryptophan and glutamine led to lower gentamicin tolerance. This phenomenon cannot be reestablished by adding the missing amino acids to the cell culture. Also, supplying these amino acids to wild-type *E. coli* cells caused sensibilization of the stationary-phase cells, probably because of inhibition of amino acid synthesis. The serine biosynthesis disruption had the highest effect on gentamicin survival. In *serA* mutant, gentamicin uptake increases enormously. However, in this study they used gentamicin Texas-red as a fluorescently tagged antibiotics for FACS analysis and they were not able to analyze cells with microscope imaging.

As it has been mentioned in the first part of the introduction, gentamicin uptake is directly correlated to PMF level. Apparently, in the absence of serine biosynthesis, energy level of cells is higher. Amino acid and other small molecule biosynthesis are related to central carbon metabolism, and it is possible that amino acid synthesis changes the flow of carbon metabolism, which can change consequently the energy state in the cells.

However, the most unexpected finding in this screen was that the genes related to motility strongly affected gentamicin tolerance in *E. coli*. The activation of motility stimulated the formation of persisters tolerant to gentamicin. However, deletion of flagellar genes did not affect gentamicin uptake, which created another mystery in persister world.

Some of the genes that were identified in the screen of this Tn-seq library had previously been shown to disturb gentamicin sensitivity, for example the genes which are involved in respiration and oxidative phosphorylation.

3. Mechanosensitive channels

3.1.Introduction

Bacterial cells are responding to vibration, touch, gravity or changes in osmolarity. In general, they sense the changes in bilayer cytoplasmic membrane. As the osmotic and turgor pressure on the membrane are essential for cell integrity, the immediate detection and response to such stress is vital to cells. The Mechanosensitive (MS) channels have been proposed to have major roles in the adaptation to osmotic stress. The main role of these channels is to balance cells' osmotic pressure during hyperosmotic shock. Bacterial MS channels are universal, and they are considered as a model for higher organism. However, bacterial MS channels are molecules and ion non-specific in contrast to the mammalian ones. These pores are changing their conformation from a closed state to an open state. These changes range from 6 to 30 Å in diameter for mechanosensitive channels of large conductance (MscL)¹⁰⁹ and about ~6 Å for mechanosensitive channels of small conductance (MscS)¹¹⁰.

3.1.1. Solute concentrations in cytoplasm and osmoregulation in bacterial cells

Bacterial cells accumulate the solutes in their cytoplasm which they need for running their metabolism. Though, they accumulate the solutes in their cytoplasm well beyond the concentrations that might be required for metabolism. Most bacteria accumulate potassium and glutamate preferably¹¹¹. The concentration of different metabolites can reach up to millimolar levels, which can lead to an osmolarity of 400 mOsm when cells are cultivated into a standard medium which has an osmolarity of 240 mOsm. Although it is hard to measure the turgor

pressure in cells, its net value is estimated to be about 4 atm (Figure1-3-1). When cells are growing, the turgor pressure is required for water intake and cell expansion. Bacterial cells increase of turgor pressure is regulated by elasticity of peptidoglycan and mechanosensitive channel actions (Figure1-3-1).

Figure 1-3-1 turgor pressure and osmolarity of *E. coli*¹⁰⁹. *E. coli* grow in a medium, which has 240 mOsm (Minimal medium or LB containing 5 g/L NaCl). Cytoplasm make 10 atm turgor pressure toward the cytoplasmic membrane. The medium pressure is of about 6 atm toward the outer membrane and 4 atm towards the inner membrane. MS channels will gate if there is a change of this equilibrium even by a small increase of 0,1 atm of outward pressure.

3.1.2. How bacteria adapt to changes and water movements?

Bacterial cells are capable of surviving a rapid hypotonic shock thanks to the presence of MS channels and the nature of the cell wall. Peptidoglycan are consisting of oligosaccharides of different lengths, which are linked together by short peptides. The length difference and the

incomplete peptide bond make a mesh like cell wall with holes of different sizes. Peptidoglycans have a very dynamic structure, when cells size increases the peptide bonds break and new cell walls materials insert into this structure.

3.1.3. Expansion of the cell and activation of MS channels

When cells reach to the maximum of turgor pressure that they can tolerate, MS channels are activated. It has been found that the MscL and MscS are activated 3 to 5 seconds after cells reach the activation threshold tension.

3.2. Discovery of mechanosensitive channels

The discovery of pressure activated channels on spheroplasts of *E. coli* was reported for the first time in 1987 by the patch-clamp electrical recording technique. These channels were proven to be activated by positive or negative pressure, they have voltage dependence and large conductance ¹¹².

In 1994 the membrane capability of replying to the osmolarity changes was studied by patch-clamp on *E. coli* envelope. They tested the candidate mechanosensitive channels and suggested that two channels, MscL and MscS, are important in osmoregulation. They solubilized and fractionated the membrane and tested the MscL *in vitro*. This was the first research which lead to predicting the coding gene for MscL, which they describe as a very different porin with a very hydrophobic core ¹¹³.

As the null mutant of *mscL* did not change the cell phenotypes in further electrophysiological experiments it had been suggested that probably other membrane proteins such as YggB are involved in mechanosensation by bacteria. It has been shown that YggB and

MscL are essential in controlling cell pressure and these channel open when necessary to prevent a dramatic shock that would otherwise lead to cell death ¹¹⁴.

For many years there were only two main MS channels to be known (MscL and MscS). However, in *E. coli* there are other MscS-type genes such as *mscK* (*kefA*), *ybdG*, *ybiO*, *yjeP* (*mscM*) and *ynaI*. The two channels MscK and YbdG play minor roles in preserving cell integrity. It has been found that the YbiO channel expression is specific to NaCl and RpoS-dependent ¹¹⁵.

It has been shown that expression of *mscL* and *mscS* is up-regulated when cells are entering into the stationary phase as well as in bacteria grown in high osmotic media ¹¹⁶. This is going to protect the bacteria against hypo-osmotic shock. The up-regulation is proven to be triggered by the stress sigma factor RpoS.

3.3. Structure and gating of mechanosensitive channels

The first MS channel that has been studied by the X-Ray crystallography was MscL from *Mycobacterium tuberculosis* (TbMscL). TbMscL consists of five identical subunits which are positioned symmetrically to create a channel ¹¹⁷. Each subunit contains two transmembrane helices called TM1 and TM2. The TM1 helices are making the trans-membrane channel and the TM2 helices form the peripheral part of the channel. TM1 and TM2 are connected by a periplasmic loop. The N-terminal (S1) and C-terminal (CP) of the protein are situated in the cytoplasm ¹¹⁸ (Figure1-3-2). The *E. coli* homolog EcoMscL ¹¹⁹ and several other homologs ¹²⁰ are suggested to be a non-selective pore of ~30 Å in diameter. These channels open when the turgor pressure in the cytoplasm is lytic.

A functional model was suggested where the five amino-terminal helices (S1) make a bundle when the channel is closed. Open and close conformations in this model have been tested by substituting cysteines for other residues ¹²¹. The S1 segments are crosslinking between each other, keeping the channel in the closed confirmation. When there is tension in the membrane, the transmembrane barrel expands and pulls the channel apart through the S1 and TM1 linker. The interaction and the crosslinking between the S1 and TM2 maintain the channel in the open conformation.

Figure 1-3-2 Structure and gating of MscL ¹¹⁰. (a) MscL structure of *Mycobacterium tuberculosis*. This channel consists of 5 monomers. Each monomer (left) is composed of two α -helical trans-membrane domains called TM1 and TM2, cytoplasmic N- terminal (S) and C-terminal regions and a central periplasmic loop domain which links the TM1 to TM2. The five TM1 domains make a tightly packed hydrophobic bundle which makes the gate of the pore (right). (b) 3D construction of MscL from *E. coli* in the membrane (top) and from above (middle). The channel is shown in its closed (left) and open (right) states. Gray balls indicate lipid phosphate of outer and inner membrane. The monomer configuration is shown at the bottom.

The conformation changing in the five subunits of MscL channels can be describe as follow. The pore is in the closed conformation resting (C). When the turgor pressure increases it leads to a Closed-Expanded (CE) conformation. This conformation is assumed to occur firstly at one of the subunits' S1 domain. The remaining subunits' disruption happens very quickly which leads to the fully open state (O) ¹²¹ (Figure 1-3-3).

Figure 1-3-3 Iris-like gating of MscL ¹²¹ .At left the different stages of gating of MscL is displayed. The channel is closed in the conformation (C). When there is tension in the membrane, it turns into the Closed-Extension (CE) form. The pentamer channel reaches the open conformation (O) through the series of monomer configuration changes (S1, S3...) At the right is a typical single current trace of MscL recorded at -50 mV.

Another study confirmed the gating model and suggested that MscL channels are opening in an iris-like expansion and flattening by membrane tension. In this study they find that the bridging between Cys 20 and Cys 36 of TM1 helices lead to stabilized open channel ¹²².

It has been shown that MscL channels have the tendency to cluster in the membrane. These clusters can be consisting of tens to hundreds of channels. Through the various methods

such as patch-clamp electrophysiology, microscopy (fluorescence and atomic force) in hand with mathematical modeling, it has been proposed that the MscLs self-assembled and packed tightly together while they are active. The channel activity is altered by the presence of neighboring proteins, showing the importance of protein-protein interactions in the membrane. Authors claimed that clustering of the MscLs is an intrinsic property of this channel and it is probably because of lipid-mediated protein-protein interactions ¹²³.

3.4. Visualization of MscL

In order to localize MscL channels using fluorescence microscopy, each monomer has been fused with the one copy of the green fluorescent protein (GFP). The fused gene *mscL-gfp* was then expressed in live *E. coli* cells. It has been demonstrated that the MscL-GFP were correctly folded and that the GFP did not disturb the intact folding nor the functionality of the channel. The channels were located at the cytoplasmic membrane. This method is a non-invasive tool for studying the localization and expression regulation of this channel. Although the fused MscL-GFP channel shares the same electrophysiological characteristics as the wild-type channel, MscL-GFP requires more pressure to open ¹²⁴.

Thanks to recent study by quantitative photo-activated localization microscopy (qPALM) microscopy it has been shown that there is a linear relationship between the number of the MS channels (MscL, MscS and MscK and MscK) and cell survival following an hypotonic shock. This time authors tagged each monomer by fluorescent protein mEos3.2. These labeled channels were functional, and the microscopy imaging shows that the channels are distributed evenly in the membrane. However authors found that overexpression of the MscL-mEos2.3 caused formation of clusters in the membrane and decreased channel mobility ¹²⁵ (figure1-3-4).

Figure 1-3-4 MscL form clusters when all monomers were labeled by mEos3.2 in *E. coli*¹²⁵. (a) When plasmid-derived MscL-mEos3.2 expressed in the presence of native chromosomal MscL subunits, not all the subunits of one channel are tagged. In this case MscL channels formed less clusters. (b) If all the subunits are labelled in the cells, it can reduce the rotational freedom of mEos3.2 and lead to formation of artificial MscL cluster.

At this point it remains unclear if the clustering is an intrinsic property of the channels or if it originates from the side effect of the fluorescent protein tag.

3.5. Number of channels in the cells

We know the importance of osmoprotection in bacterial cells. This task is assured in bacterial cells by the redundant MS channels. The number of MS channel in each cell has been studied by the quantitative study of the expression of MscL in different media and growth phases. Researchers used both quantitative fluorescence microscopy and quantitative Western blots analysis to calculate the number of channels at the single cell level. Authors confirmed again the functionality of the MscL-GFP fusion protein and its native expression as well. Authors showed that expression of MscL is intensively depending to the environmental conditions (media richness, carbon sources and stress factors) and also it is differing from cell to cell. Overall, Authors estimated that the number of channels per cell is much higher than the modeling estimation¹²⁶.

Figure 1-3-5 Cell level counts of MscL in different conditions with different techniques ¹²⁶. Two different strains of *E. coli* were tested in different media (rich LB and minimum media with different carbon sources) under different growth phase. These channels were counted by different techniques, (A) Western blots with MscL antibody and (B) Western blots with GFP antibody and fluorescence microscopy imaging.

A recent study showed, by modifications in gene expression, that each cell needs between 500 to 700 channels to have up to 80% of a population to survive large changes in osmotic pressure. This number agrees with the previous Western blotting counting of the average MscL copy number in wild-type *E. coli* cells. Authors observed that cells with less than 100 channels have zero chance of survival after a hypo-osmotic shock ¹²⁷.

3.6. Aminoglycoside interaction with MscL

In early studies of aminoglycoside accumulation, it has been observed that streptomycin induces the potassium efflux ¹²⁸. This leakage preceded the decrease in cell viability and remained unexplained. Possible explanations were local distortion in the growing membrane or synthesis of abnormal channels leading to membrane damage.

Bacterial MS channels have been suggested to be a perfect drug targets due to their central role in the physiology of bacteria and also the lack of identified homologs in higher organisms ¹²⁹. In a high-throughput screening for new target for MscL, an aminoglycoside dihydrostreptomycin (DHS) was one of the hits among the compound library.

It has been found that MscL is necessary for streptomycin-induced potassium efflux that has been previously described. DHS also increases MscL activity in electrophysiological studies in a direct manner. This result showed a new mode of action of this antibiotic. This antibiotic enters into the cytoplasm through MscL's large pore. This phenomenon has been tested *in vivo* in the study by MscL expression and antibiotic accumulation and also by *in vitro* patch clamp experiments ¹³⁰.

In a persister study it has been proposed a role of MS channels in persisters' eradication. It has been reported that bacterial persisters and also normal growing cells can be eradicated extremely fast by aminoglycoside treatment under hypoionic shock. The eradication of persister upon the hypoionic shock (e.g. only water) is only specific to the aminoglycoside family of antibiotics such as streptomycin and kanamycin (no effect was observed in β -lactams or quinolones antibiotic treatment).

This phenomenon has been shown to be PMF-independent. CCCP, rifampicin and cold treatment were used to show that antibiotic accumulation is unrelated with the PMF level in the cells. It has been verified that the bactericidal activity of this antibiotic was still through its activity on the 30S ribosomal subunit. No persister eradication was observed in the streptomycin resistant cells after the hypoosmotic shock and streptomycin treatment ¹³¹.

As the MS channels activated during such shocks it has been hypothesized that these channels are responsible for antibiotic accumulation. The diameter of an active MS channel is as large as 30 Å. It can easily allow aminoglycosides (~500 Dalton) to enter into the cytoplasm. To prove this hypothesis single MS channel mutants were tested and unexpectedly no changes were observed in the survival ratio. This was probably due to the redundancy action of MS channels. Two or multiple channel mutants were not tested in this study because the viability in these mutants is decreasing¹³¹.

A direct interaction of DHS with MscL was still lacking. In a recent genetic screening study (cysteine library), biochemical and mutational approaches and molecular dynamics simulation (drug docking and single mutation study) were used to understand how DHS interacts directly with MscL. This study demonstrated that DHS binds to MscL at a specific site and that changes the conformation of the channel let the potassium and glutamate out and the antibiotic into the cells.

DHS enters into the cells by changing the MscL pores probably through a binding pocket (L19 and I25 contribute to DHS binding), open the channel allowing the antibiotic to go through these channels into the cytoplasm. The changing on the MscL makes the K⁺ and glutamate to leak out. (Figure 1-3-6)¹³².

Figure 1-3-6 Interaction model of streptomycin to MscL ¹³⁰. When streptomycin is in the media it interacts with MscL channel which leads to activation of MscL. This channel gating allows K⁺ leakage. Streptomycin keeps the MscL open and the channel becomes a pathway for the entry of the antibiotic into the cytoplasm.

3.7.Voltage-gated channel vs. mechanosensitive channels

The MS channels are designed to respond when the membrane undergo changes due to an increase of the turgor pressure however there are not the only ion channels to respond to such trigger. It has been shown that voltage-gated channels are also sensitive to the physical environment of the membrane and their structure is influenced by the transmembrane voltage and the pressure on its surface as well ¹³³.

Interesting example of the activation of voltage-gated channels by mechanosensation has been reported where calcium fluxes are triggered by simply touching the bacteria ¹³⁴. (Figure 1-3-7)

On the other hand, other study showed that the MscL is gating after the hyperpolarization of the membrane as well. Although the MS channels do not contain the voltage sensor domain that makes the voltage gated channels open with voltage.

Figure 1-3-7 Calcium gated channels are induced by mechanosensation ¹³⁴. (a) In a microfluidic channel cells are continuously imaged in the liquid media and during the mechanical stimulation by physical contact with a piece of 1% low-melt agarose pad on the top of the cells. Cells in green are expressing a reporter gene (cpGFP-based calcium sensors) which shows the activation of calcium gated channels. (b) Single-cell calcium recordings before and after mechanical stimulation. Each color is for traces recorded for single cells before and after stimulation.

It is interesting that the channels without the voltage sensor domain can sense voltage modulation as well. This phenomenon is due to fact that a given typical channel, which is situated in the polarized membrane will see its properties to be modified by the local parameters such as electric charges and the dielectric permeability. So, changes in the voltage can affect the channel configuration as well. The MscL channel has also been demonstrated to have the ability to respond to changes in the voltage inside the membrane ¹³⁵.

4. Microfluidic systems

4.1. Advantages of microfluidic devices for bacterial cell culture

In experimental sciences, *in vitro* experiences are advantageous in comparison with *in vivo* ones because they are simpler and faster to carry out. In recent years thanks to technological advancements, researchers and engineers tried to develop techniques and devices, which help us to make better and more realistic *in vitro* test conditions. Microfluidic devices for cell culture are one of these advantageous techniques. With such devices, it is possible to analyze biological phenomenon in real time with higher precision. Some tests and analysis, which were impossible can now be performed and far more data can be driven with for instance real time analysis.

The main advantage of microfluidics for studying real time response of bacterial cells to a chemical or change in the media is the possibility to inject extremely small volumes of a solution while cells are trapped in channels filled with media on the microfluidic device. Moreover, the used of small volumes can be advantages in case of the limited amounts of chemicals or materials that can be available because for instance of costs or difficulty to produce. Moreover, in microfluidic devices it is possible to study cell-to-cell interaction.

4.2. Microfluidic in persister study

In persisters study, real time microscopy of bacterial cells placed in microfluidic devices is an extraordinary tool to understand more about the nature of this subpopulation of bacteria.

Persisters existence was known from the forties, however, it took sixty years to see persister formation using microscopy. Balaban *et al.*, combined microfluidics and microscopy to do single-cell level studies of *E. coli* persisters⁹⁵ (Figure 1-4-1). In this study, authors identified non-growing cells prior to antibiotic treatment thanks to their capacity to survive a normally lethal ampicillin treatment and observed these cells to regrow after subsequent removal of the antibiotic.

Figure 1-4-1: Example of a microfluidic device used for studying persister cells⁹⁵. Cells were trapped and grew into narrow channels. Microfluidics allowed for solution exchange as indicated at the bottom of the fluorescence microscopy images. At the time of addition of a media containing ampicillin (orange rectangle), most of the cells lysed except persisters (red arrow). After removing the antibiotic with an exchange of the solution *via* the microfluidic system, persisters resumed growth.

Persisters form typically small subpopulations within normal cell populations with frequencies of occurrence ranging from 10^{-6} to 10^{-1} . On the other hand knowing heterogeneity in a cell population is one of the main factor for persister formation, it is essential to develop a device suitable for such study¹³⁶. In recent years, microfluidics combined with fluorescent reporters (fused with Toxin-Antitoxin promoters or metabolic reporters) were used to characterize persister formation^{97, 137}.

While microfluidics gives us advantages to study single cell formation of persisters, in some studies, percentages of persister formation in the test tube and colony formation units on plates differed from the number of persisters formed in microfluidic devices. For example, it has been shown that indole induced persister formation and CFU survival percentage were much smaller in classical microbiological conditions than what was observed in microfluidic and fluorescence microscopy conditions¹³⁸. This phenomenon could be due, for instance, to differences in cell to cell interaction, that occur in the environment of the microfluidic devices which differ from test tube experiments. However, the reason for such differences remains to be clarified.

New study showed that it is possible to combine microfluidic device and super resolution microscopy ¹³⁹. Super-resolution microscopy is a technique, which provide single-molecule precision and important data about the dynamics of fluorescent proteins or dyes in a cell with for instance single-particle tracking experiments. As we developed a fluorescently tagged aminoglycoside (Neo-Cy5), we are very enthusiastic to be able to track down the antibiotic in interaction with bacteria into a microfluidic device using for instance super-resolution microscopy.

Chapter II: Objective of thesis

In our lab, we generate fluorescently tagged neomycin with Cyanine 5 (Neo-Cy5) by click chemistry. The main objective of my thesis project was first to fully study and characterize this antibiotic conjugate and study in more depth the interaction of this antibiotic with bacterial cells at single-cell level. I aimed to study aminoglycoside accumulation in susceptible fast-growing cells and less tolerant strains, as well as cells that are transitionally tolerant to antibiotics: bacterial persisters.

In aminoglycoside interaction with bacteria, there is a lot which remains unclear. For instance, we do not know how aminoglycoside antibiotics pass through the cytoplasmic membrane to reach their target. Potential transporters of aminoglycoside in the cell membrane remain to be identified.

In aminoglycoside studies, for tracking down the uptake levels of aminoglycosides in bacterial cells, fluorescently-tagged aminoglycoside have been widely used as gentamicin tagged with the Texas Red (TR) dye. However, Texas Red enters into cells even without gentamicin fused to it. One of the advantages of Neo-Cy5 is that Cy5 is not interacting with bacterial cells. It is then possible to track down the antibiotic by fluorescence microscopy and define the antibiotic accumulation patterns. Other advantages of Cy5, is that it is suitable for super-resolution fluorescence microscopy.

Previous work in the laboratory demonstrated the bactericidal activity of Neo-Cy5. In this study, first and foremost, a first objective was to verify that the bactericidal activity and accumulation properties of Neo-Cy5 are PMF dependent in comparison with the non-tagged neomycin. After this step, the objective was to establish protocols, which would be suitable for

this conjugate to perform fluorescence microscopy and FACS analysis. The goal was to have a robust method that would combine these two tools in order to interpret FACS data in terms of accumulation patterns.

After studying the different steps of antibiotic accumulation in susceptible wild type cells, the objective was to test Neo-Cy5 on mutant strains that are less tolerant to aminoglycosides and see if we could obtain insights on the tolerance mechanism.

A final step in applying our method would be to investigate aminoglycoside uptake by persister cells. The goal was also to study Neo-Cy5 interaction with bacterial cells in more clinically relevant test conditions such as persisters, which are formed in macrophages and in biofilm. However, I would not explore this final objective because of surprising results that I got in the process of protocol adjustment, which led my project to a new direction.

While I was preparing bacterial cells for FACS analysis, I noticed that physical manipulation of bacterial cells such as centrifugation followed by pipetting or cells agitation, stimulate the mechanism of mechanosensation and activation of mechanosensitive channels. Stimulation of these channels lead to enormous cytoplasmic accumulation of Neo-Cy5. This is worth to mention that such manipulations are so common and frequently used in cell preparation in microbiology. I therefore established a method to handle cell preparation without triggering the mechanosensitive channels and study the effect of mutants for such channels on Neo-Cy5 accumulation.

With a powerful tool such as Neo-Cy5, I wanted to go further and combine microfluidics and fluorescence microscopy to capture aminoglycoside accumulation in fast-growing cells and persister cells in real time imaging.

Chapter III Result: Neo-Cy5 newly tagged aminoglycoside

1. Introduction

First essential experiments before using Neo-Cy5 as a tagged aminoglycoside were to prove if adding the fluorescent dye was not changing the characteristics of these antibiotics. In the laboratory, we did the series of experiments to prove that Neo-Cy5 is an active aminoglycoside.

First, we measured the minimal inhibitory concentration (MIC) of our conjugate. We proved that tagged antibiotic is bactericidal in slightly higher concentration than non-tagged one.

Then we tested the fluorescence characteristics of Neo-Cy5 to check if the Cy5, which is conjugated to the neomycin, has a different excitation and emission wavelength. In our case, the peaks of Neo-Cy5 excitation and emission were not changed.

As I mentioned in the introduction, aminoglycoside accumulation in bacterial cells involves two energy dependent steps: EDPI and EDPII. As it has been studied when cells have lower level of energy, which means a weaker proton gradient cross their membrane (lower PMF), it affects aminoglycoside accumulation. In order to check if Neo-Cy5 enters into cells in the same energy dependent manner, I used a treatment with Carbonyl cyanide m-chlorophenylhydrazone (CCCP). CCCP is a chemical inhibitor of oxidative phosphorylation. The result shows that CCCP inhibits the bactericidal activity of Neo-Cy5 *via* inhibition of antibiotic accumulation.

At last, I performed a control experiment where I tested a possible interaction of the dye Cy5 with the cells by fluorescence microscopy imaging combined with FACS. These results showed that the dye alone did not accumulate inside the cells nor it strongly interacted with

membranes. Therefore, I conclude that the antibiotic entry into the cells was only due to the neomycin properties and Cy5 by itself was not capable of entering into the cells.

In our lab, it has been tested if our conjugate was still interacting with the decoding site of 16S rRNA in 70S ribosomes. Footprinting analysis on ribosomal particles showed that Neo-Cy5 interacts with the decoding site of the small ribosomal subunit in a similar way as neomycin or Neo-U also with a slightly decreased affinity (data shown in the unpublished manuscript in chapter 10).

Aminoglycosides cause miscoding *in vitro*. Since Neo-Cy5 binds the ribosomal decoding site on the small subunit, our lab has also shown with a dual-luciferase gain-of-function assay, that Neo-Cy5 derivative induces misreading in a similar way as neomycin. Translation activity was monitored by the activity level of wild-type renilla luciferase, which served as internal standard. A second gene coding for a mutant of the firefly luciferase was used. Indeed, miscoding restores a wild-type amino acid in a position critical for the activity of the enzyme. Using this system it was found that Neo-Cy5 induced protein synthesis inhibition in a similar manner as neomycin as well as miscoding (data shown in the unpublished manuscript in chapter 10).

2. MIC measurement

Here in order to verify the bactericidal activity of Neo-Cy5 or Neo-Cy3, after each synthesis, I have measured the MIC of each conjugated antibiotic in comparison to the parental drug neomycin. Although it is known that MIC value can be different according to the media in which the cells are growing, I tested the MIC in the minimum media MOPS-G. These results complemented previous data from our lab where the MIC was tested in media M9-G and LB. We observe that MIC is higher in M9 media. The main difference between these two media is that

M9 contains higher concentration of phosphate. I have tested two neomycin conjugates one with Cy5 and the other one with Cy3 that have different spectral properties (table 3.1).

	Neomycin	Neo-Cy5	Neo-Cy3
M9 medium	2.4 μM	10.0 μM	ND
MOPS medium	1 μM	8 μM	4 μM
LB medium	3.2 μM	ND	ND

Table 3-1 MIC comparison of neomycin and different neomycin conjugates in different media.

3. Excitation and emission

Another step of verification of Neo-Cy5's characteristics was to test if Cy5 has changed its fluorescence properties while conjugated to neomycin. I measured the excitation and emission of Neo-Cy5 and Cy5 with a spectrofluorometer (figure 3-1). As recorded data showed, optical excitation and emission wavelength of Cy5 did not changed whether it is bound to neomycin or not.

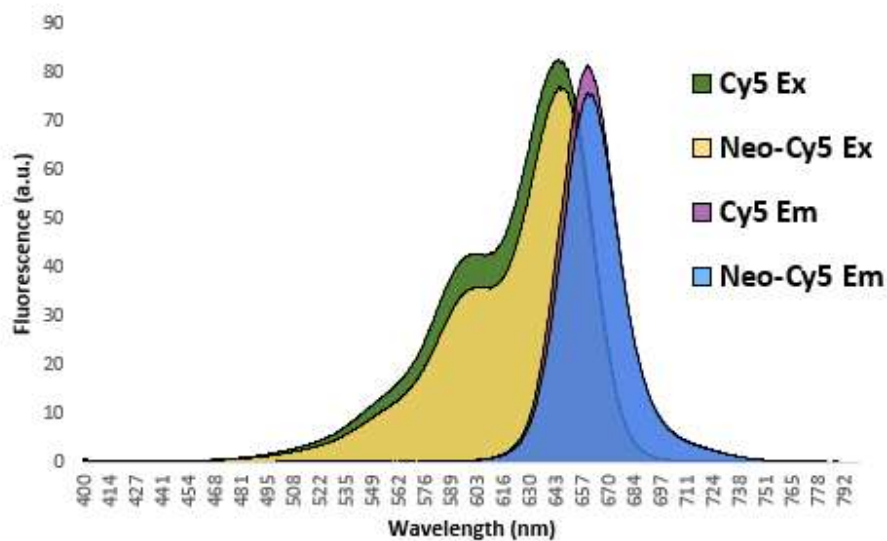


Figure 3-1 Excitation and emission spectra of Cy5 and Neo-Cy5. Data were recorded on a spectrofluorometer for solutions at 1 μ M in Tris-HCl buffer (pH 7.6). Optical properties are identical.

4. Uptake inhibited by CCCP

As I mentioned in the introduction, aminoglycoside accumulation in bacterial cells is known to be dependent on PMF. Here I tested by survival assay the effect of the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) on Neo-Cy5 induced drug killing (Figure 3-2).

The results showed that addition of CCCP protected cells from the drug-induced killing. This data proved that like aminoglycosides, the accumulation of Neo-Cy5 is PMF dependent and it was inhibited by CCCP, which is an uncoupling agent of the proton gradient of inner membrane.

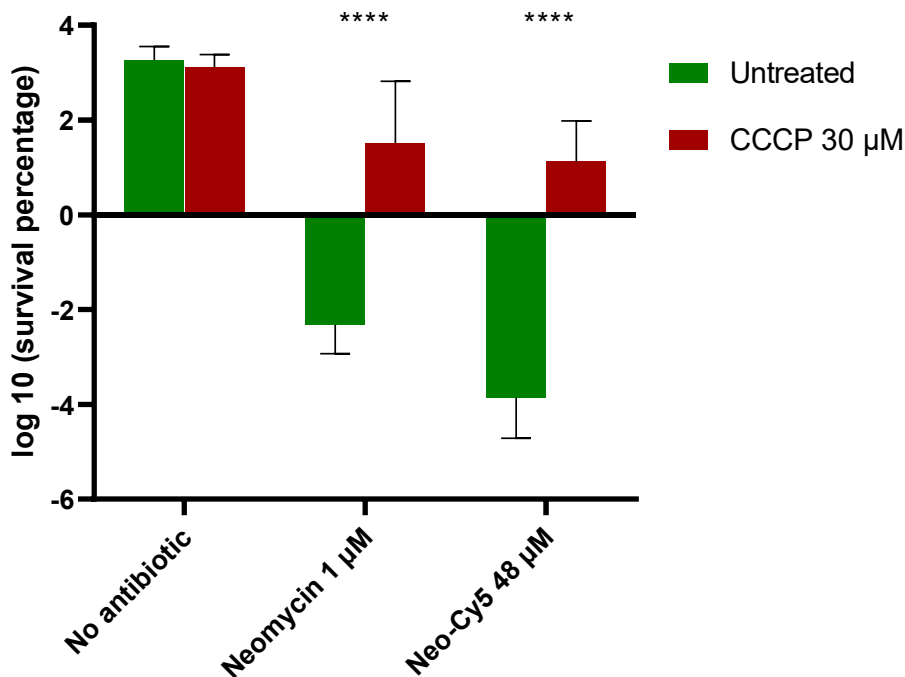


Figure 3-2 Activity of Neo-Cy5 is PMF dependent. Survival of *E. coli* MG1655 cells after a 4.5-h treatment with 1.5 μM neomycin or 48 μM Neo-Cy5 with and without 30 μM CCCP (2-h incubation prior to addition of the antibiotic). Plotted are values relative to untreated control culture. Error bars are standard errors of the means and were calculated from three independent experiments. Significant differences, calculated by the Mann–Whitney test, are indicated (****P < 0.0001).

5. Cy5 interaction with cells

To prove the advantages of Neo-Cy5 in the microscopy and the FACS experiments, I wanted to show that Cy5 alone was not interacting with the cells. Here I used three different methods to perform this control.

5.1. MIC measurement for Cy5

Some fluorescent dyes have bactericidal activities due to their photoactivation properties and production of singlet oxygen during illumination. As I define the MIC of Neo-Cy5 by measuring of growth rate (excitation at 500nm or 400nm) in the microplate reader, I wanted to test if during this experiment I was not photo-activating Cy5 and verify that the bactericidal effect of Neo-Cy5 is solely due to the binding of neomycin to the ribosome.

I measured the MIC of Cy5, and the result showed that Cy5 did not show any phototoxicity in the conditions of illumination of the experiments as cells were growing normally.

5.2. FACS

As a control experiment, I analyzed the cells treated with Cy5 (32 μ M) or Neo-Cy5 at the same concentration for 5 min at 37°. Cells were then washed on the membrane and analyzed by FACS (Figure 3-3).

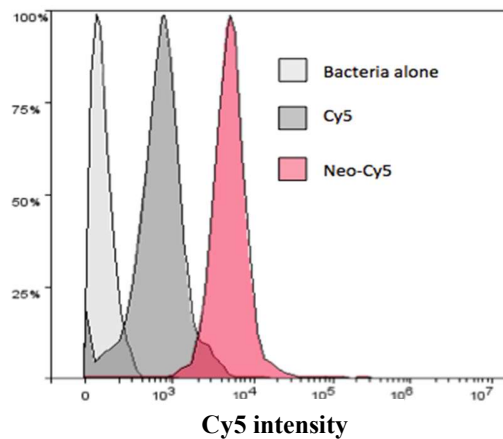


Figure 3-3 Cy5 vs Neo-cy5 interaction in FACS analysis. *E. coli* MG1655 cells exposed to 32 μ M Cy5 or Neo-Cy5 for 5 minutes at 37°C. Cells were washed on a 0.45 μ m-pore-size HAWP membrane filter (mixed cellulose esters, Merck Millipore). Binding of Neo-Cy5 was clearly distinct from levels of cellular autofluorescence or residual binding of the dye Cy5.

This result confirms that Cy5 is not interacting in the same manner as Neo-Cy5. There is an interaction of Cy5 with the membrane as it is demonstrated in figure 3-3. Cy5 signal from the cells treated with Cy5 is higher than the control sample of non-treated cells. I decided to do microscopy experiments to observe the possible non-specific interaction.

5.3. Microscopy

In this experiment, I tested if I can observe the interaction of Cy5 to the membrane by fluorescent TIRF microscopy. The *E. coli* cells were treated with 32 μM Cy5 then washed by filtration and observed by bright field and fluorescence imaging (figure 3-4). Conditions of acquisition are identical to the conditions that will be used after for Neo-Cy5 imaging with bacteria (see later in the text).

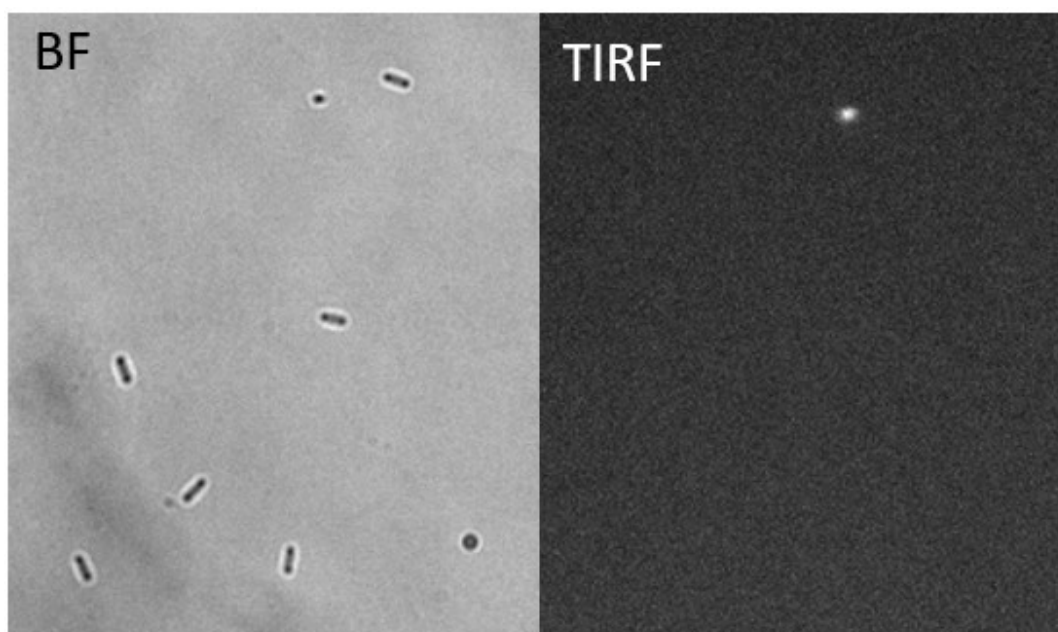


Figure 3-4 Cy5 does not enter live *E. coli* cells. Left: transmitted light image of a field of *E. coli* MG1655 cells exposed to 32 μM Cy5 for 1 hour at 37°C. Cells were washed on a 0.45 μm -pore-size HAWP membrane filter (mixed cellulose esters, Merck Millipore). Right: fluorescence image of the same field of view obtained with an electron-multiplying gain of 50 and an exposure of 50 ms.

This result proved that Cy5 by its own is not interacting strongly with the membrane and we verified here that using the acquisition parameters that will be described later in the text for monitoring Neo-Cy5 interaction with the cells, we did not observe any signal with Cy5. This result validates our imaging protocol of Neo-Cy5 with *E. coli* cells.

6. Conclusion

Overall in this chapter, I showed the results, which prove that Neo-Cy5 is an active aminoglycoside. It has kept its bactericidal activity. The MIC measurements showed that the position on neomycin in which the Cy5 is fused is not changing the bactericidal activity.

These results are in agreement with the footprinting analysis on ribosomal particles and miscoding level measurement *in vitro*, which showed that Neo-Cy5 binds ribosomal A site and has miscoding activity (data show in chapter 10).

I showed that Neo-Cy5 accumulation by cells is depend on the PMF level of the cells. I proved as well that the dye Cy5 is not toxic for bacterial cells and it is not interacting strongly with cell membrane.

After these control experiments, I moved forward to observe aminoglycoside accumulation at single cell level.

Chapter IV Results: Characterization of aminoglycoside uptake in bacterial cells

1. Introduction

In this chapter I present the results that I obtained from treatment of *E. coli* cells with Neo-Cy5. I captured the kinetics of accumulation of Neo-Cy5 in the cells by fluorescence microscopy. To do this, I elaborated a method to wash the excess of Neo-Cy5 from cells without stimulating Neo-Cy5 accumulation. The stimulated aminoglycoside uptake is more elaborated in chapter V.

I captured different stages of antibiotic accumulation in cell and by the parallel experiment of survival CFU counting; I found that bacterial cells could survive under a low concentration of antibiotic present in their cytoplasm. This phenomenon has been already studied which is called phenotype suppression by low dosage of aminoglycoside treatment.

I also observe that the cell concentration is very critical to the killing kinetics. I did control experiments and I present here the efficiency of Neo-Cy5 accumulation and killing curve under given conditions.

Then to confirm my results on a much larger cell population, I analyzed the treated cells by FACS. I run a control test by the inhibitor of EDPI and EDPII, chloramphenicol. As it has been mentioned in the introduction, EDPII is a step of aminoglycoside accumulation in which drug uptake increases due to mistranslated proteins in the cells. Chloramphenicol is inhibiting protein synthesis in the cells, which arrests the production of mistranslated polypeptides. By FACS experiments, I was able to distinguish the EDPI and EDPII in the cell population.

Before aminoglycosides penetrate into the cells, molecules interact with the cells' membrane. I found the saturation point of membrane by testing different concentration of Neo-Cy5 to make sure that in my experimental conditions, the membrane was saturated. The positively charged Neo-Cy5 molecule can interact with the negatively charged membrane in specific and non-specific manners. By running different control experiments, I established a method that guaranteed the observation of strongly interacting Neo-Cy5 molecules with the membrane.

I studied the accumulation of Neo-Cy5 on the membrane in much more depth by super resolution microscopy and found that attachment of Neo-Cy5 to the membrane is not homogenous as it forms several clusters all-around of cell. Interestingly, I observed that cell's septum and the new poles experienced stronger localization of Neo-Cy5.

To investigate more in the different stages of Neo-Cy5 accumulation I marked the cells by propidium iodide (PI) in different time point after Neo-Cy5 treatment. PI has been presented in biological tests as a marker of dead cells. In dead cells that have holes in the membrane, PI is entering into the cytoplasm and binds to DNA. However, according to the FACS analysis, the dead cells detected by the killing curve experiment were not stained by PI.

Finally, I treated the cells, which expressed the unstable GFP under control of ribosomal RNA promoter with Neo-Cy5 in order to find a link between the physiological state of bacteria and the level of Neo-Cy5 accumulation. Unfortunately, I did not find a direct link between the Neo-Cy5 accumulation level in the cytoplasm and the expression of ribosomal RNA by microscopy analysis (data not shown).

2. Neo-Cy5 accumulation in bacterial cells

2.1. Microscopy

Thanks to Neo-Cy5, this is a first time that we can monitor in detail the interaction of a member of aminoglycoside family at single cell level by fluorescence microscopy.

2.1.1. Accumulation kinetics and killing curve

Here I show the results that I obtained after treating the cells with 32 μ M Neo-Cy5 at 37°C and collecting cells at different time points (2, 5, 10, ..., 80, 120 min). For each point, I washed the cells on a nitrocellulose filter (see material and methods) and observed under the microscope the cells placed on an agar pad (Figure 4-1-A).

This concentration of antibiotic is more than 3 times the MIC. I observed that during the first 30 minutes of incubation, Neo-Cy5 had a peripheral accumulation on the bacteria. After 40 min of incubation, I observed that Neo-Cy5 signal became detectable inside the cytoplasm in a heterogeneous manner; there are cells, which are far brighter than others are. I measured the Neo-Cy5 accumulation in these cells and this heterogeneity in the cytoplasmic accumulation continued all along the experience (Figure 4-1-B).

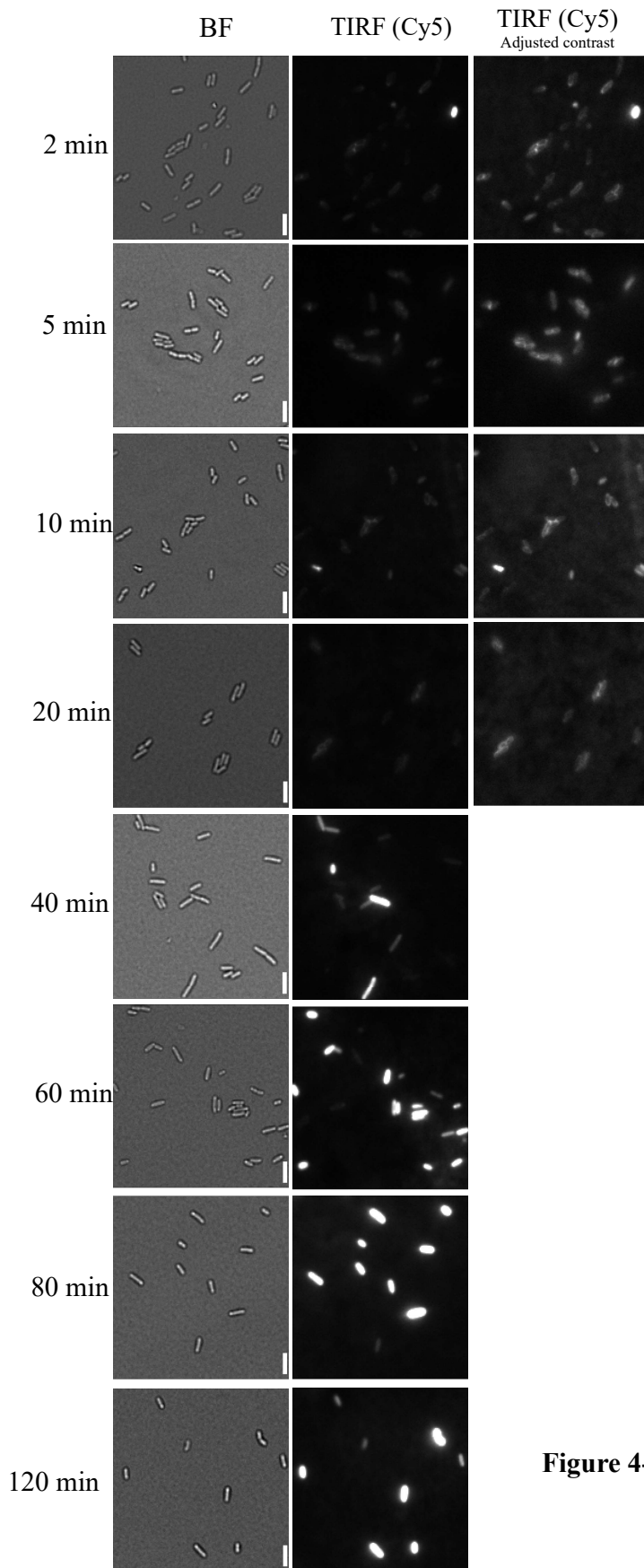


Figure 4-1-A

B

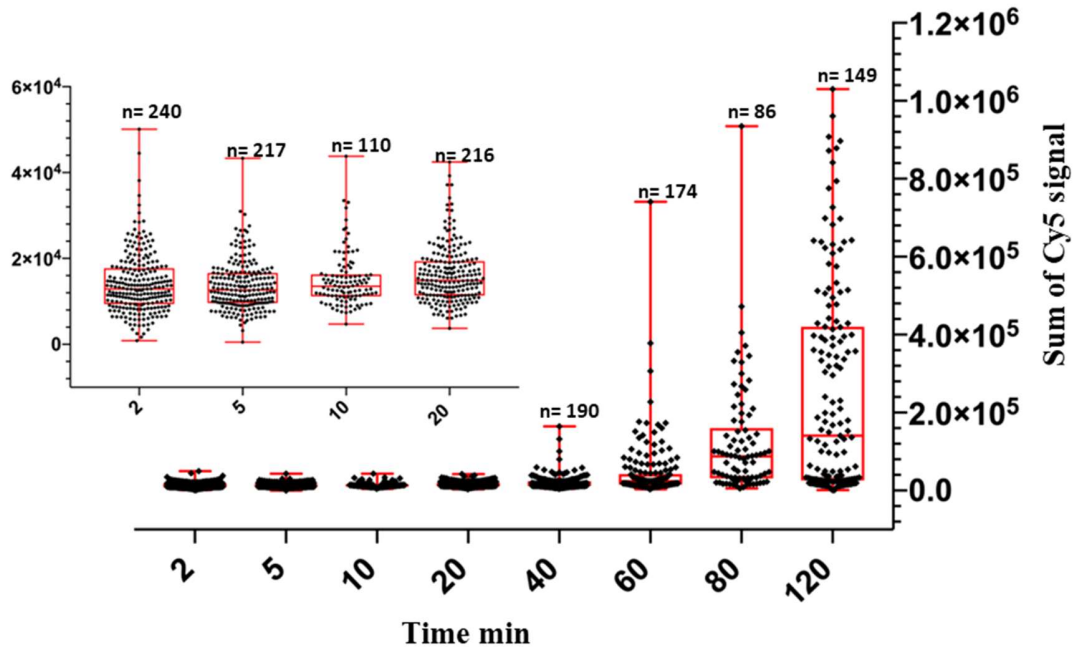


Figure 4-1. Kinetics of Neo-Cy5 accumulation in fast growing *E. coli* cells. A) Fast growing cells at OD: 0.02 were incubated at 37 °C with Neo-Cy5 (32 μ M). At each time points, cells were washed on filter and then observed directly on agarose pad containing MOPS-G on the slide. Left column are images in bright field. In the middle column are displayed the TIRF images for Cy5 (gain 10, exposure time 10 ms), for the first 4 time points the contrast was adjusted for better visual representation (gain 10, exposure time 10 ms), right column. B) For each time point, the corresponding images were analyzed by microbeJ (ImageJ plug-in to analyze bacterial cells) for Cy5 signals. Analyzed bacterial cells number in each time point is indicated. First 4 time points' plot are shown in smaller scale on the left.

At the same time, for each time point I plated the cells on LB or MOPS-G agar plates and counted the CFU in a survival test (Figure 4-2). This result showed that in the first 40 min of incubation when the antibiotic was not detected in TIRF imaging inside the cytoplasm, the CFU was not decreasing. However, after that time when the antibiotic cytoplasmic accumulation became visible, the survival count decreased.

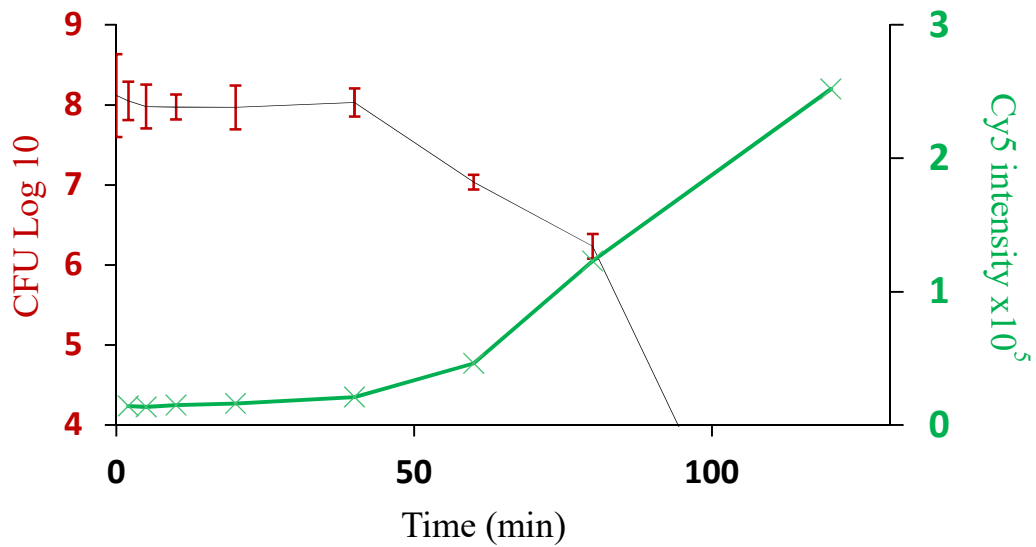


Figure 4-2 Neo-Cy5 killing curve. *E. coli* cells were treated with Neo-Cy5 (32 μ M) at 37°C and at each time point cells were washed on filter and divided in two aliquots, one aliquot diluted in series and spotted on LB or MOPS-G plates for CFU counting. The second aliquot was used for microscopy. Sum of Cy5 fluorescence intensity in each bacterium measured by ImageJ.

2.1.2. Cells with low accumulation sustain the growth

As it has been explained in figure 4-1-A, at certain time points, levels of accumulation were very heterogeneous at the single cell level. I was interested to analyze in more details these time points. Therefore, I performed time-lapse experiments to better characterize growing cells after 40 min and 60 min of incubation with Neo-Cy5. The goal was to monitor the faith of each cell in link with the accumulated levels of Neo-Cy5 (Figure 4-3-A).

The results confirmed that the cells with lower accumulation of Neo-Cy5 are those who grew whereas those with higher accumulation did not. Interestingly in some cases, I observed that the cells are growing even though they have cytoplasmic accumulation (Figure 4-3-B).

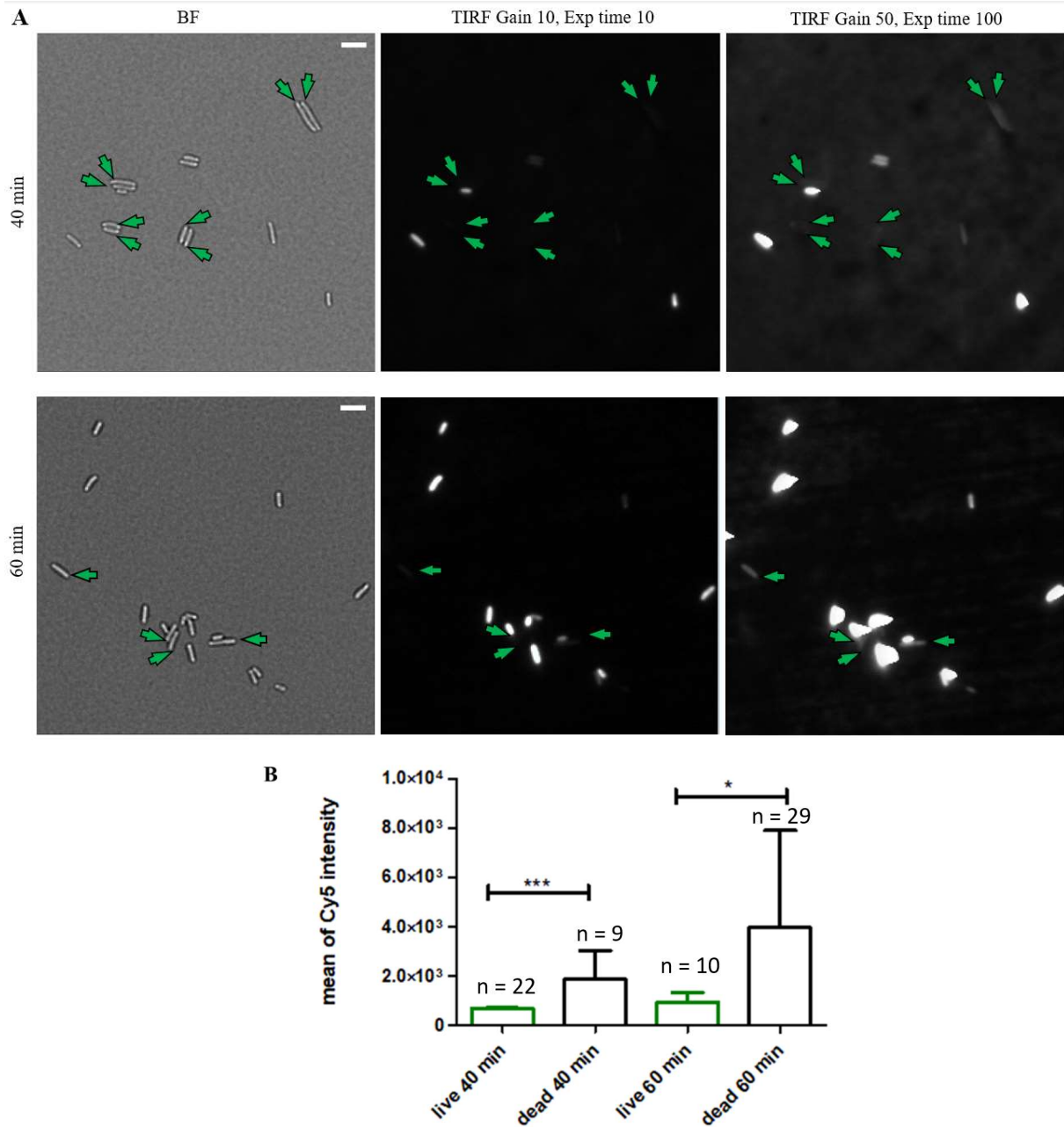


Figure 4-3. *E. coli* bacterial cells survive with a low amount of Neo-Cy5 in their cytoplasm. A) *E. coli* cells at OD: 0.02 were treated by 32 μ M Neo-Cy5 at 37°C for 40 min top panel and 60 min for bottom panel. Cells showed by green arrows continued to grow according to the time-lapse results. B) Cy5 intensities of dead and live cells measured by ImageJ for each time point. Scale bar 5 μ m.

Low concentrations of aminoglycoside in the cytoplasm caused low rates of miscoding in the cells, which were not lethal as cells continued to divide. This result is related to a phenomenon that I mentioned in the introduction called phenotypic suppression.

2.1.3. Effect of cell dilution on antibiotic action

In the process of creating and adjusting the protocol of cell treatment by Neo-Cy5, I observed an interesting fact about the cell to antibiotic ratio.

When I measured MIC of Neo-Cy5 for *E. coli* in MOPS-G according to the standard protocols, I treated the cells with Neo-Cy5 at the final OD of 0.02. In one of the microscopy experiments, I used this time exponential cells at the OD ~0.2 or 0.3 (Figure 4-4-A). The concentration of 32 μ M of Neo-Cy5 (3x MIC) was not lethal for cells at DO: 0.2 even after 120 min incubation at 37°C.

I measured then the MIC of neomycin for the cells at 2 different OD (0.2 and 0.02). (Table 4-1). I confirmed that changing the ratio of antibiotic molecule per cell is critical for the lethality.

	MIC Neomycin	MIC Neo-Cy5
OD: 0.2	1.5 μ M	16 < MIC < 32 μ M
OD: 0.02	1 μ M	8 < MIC < 16 μ M

Table 4-1 MIC measurements at two different optical density of cells. MIC increases when cell have higher OD.

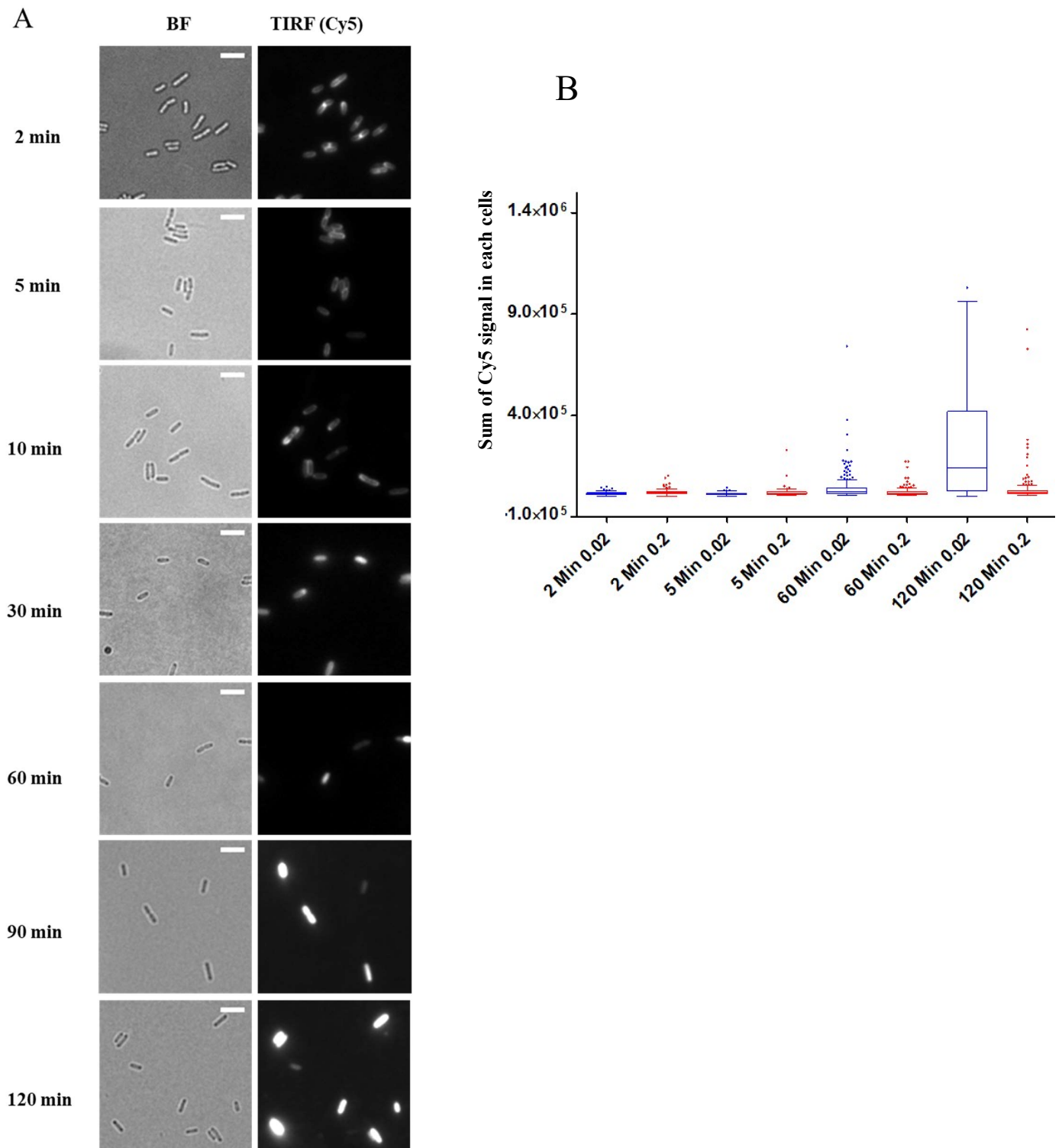


Figure 4-4 Effect of cell density on antibiotic lethality. A) Cells at OD: 0.2 were treated with 32 μ M Neo-Cy5 at 37°C. At each time points, cells were washed on filter and put on agar pad directly. B) Comparing the Neo-Cy5 accumulation in two different OD (0.2 or 0.02) at different time points. Cells with higher OD have lower accumulation.

Following the microscopy imaging, I measured Neo-Cy5 accumulation for the cells at ODs 0.2 and 0.02. I observed that the cytoplasmic accumulation of Neo-Cy5 in cells at OD 0.2 was very low when compared to the OD 0.02 cells (Figure 4-4-B). The strong signal for cells at an OD of 0.02 after 120 min is the result of EDPII cytoplasmic uptake in link with the killing curve Figure 4-2. This indicates that EDPI levels of Neo-Cy5 accumulation were weak and not sufficient to trigger strong EDPII uptake. These results are in link with the known effect of the inoculum size and MIC ¹⁴⁰.

2.2. FACS

FACS is a very powerful tool. It analyses very large population of bacterial cells in few minutes. One of the aims of this study was to test if it is possible to analyze Neo-Cy5 accumulation in bacterial cells by an efficient technique such as FACS.

2.2.1. Neo-Cy5 accumulation in the fast-growing cells

I analyzed Neo-Cy5 (32 μ M Neo-Cy5 at 37°C) accumulation in fast growing cells by FACS. As I showed in the previous chapter in figure 3-3, Cy5 alone is not interacting with the cells in the same way as Neo-Cy5. Therefore, I establish a protocol to record the Neo-Cy5 accumulation by FACS.

2.2.2. Neo-Cy5 accumulation in cells treated by chloramphenicol

I analyzed the cells incubated with 32 μ M Neo-Cy5 at 37 °C and for several time points, I took an aliquot of cells, washed them to remove the excess of antibiotic and eliminate the non-

specific membrane attachment of antibiotic. Then I analyzed them by FACS (Figure 4-5). I detected that the accumulation is increasing when the cells are incubated longer with Neo-Cy5.

As I wanted to distinguish the membrane accumulation from EDPI and EDPII by FACS analysis, I used chloramphenicol (Cm). This antibiotic binds bacterial ribosomes and halts protein synthesis in the cells. The bacteria treated with chloramphenicol are becoming insensitive to neomycin or Neo-Cy5 because of non-functional ribosomes. Therefore, in cells treated with chloramphenicol prior to the addition of aminoglycoside, EDPI will be reduced and EDPII abolished. EDPII occurs after miscoding events. Because ribosomes are already inhibited by chloramphenicol, this step is not detected in those cells.

I treated the cells with chloramphenicol before adding Neo-Cy5 and I analyze the cells at different time points. In comparison with the control cells (treated only by Neo-Cy5), I showed that we can capture EDPI and EDPII in those cells in FACS analysis (Figure 4-5).

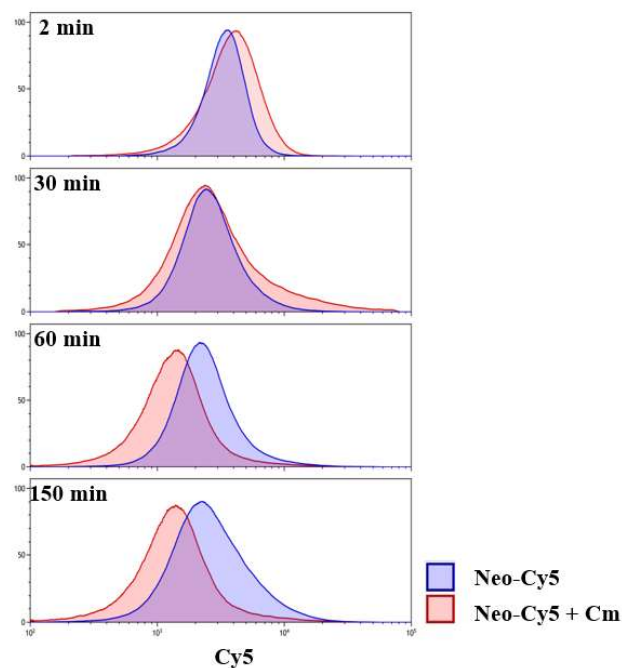


Figure 4-5 EDPI and EDPII inhibition by chloramphenicol treatment. FACS analysis of cells treated with Neo-Cy5 at different time points in blue and the cells treated with chloramphenicol prior to Neo-Cy5 in red. At 60 min and 150 min, EDPII occurred in the cells treated solely by Neo-Cy5; however, the accumulation was not increasing in cells pre-treated with chloramphenicol.

It was confusing for me how the cells analyzed after 2 min had a higher (1.5 times) Neo-Cy5 signal comparing to the other time points and especially the point at 30 min. I hypothesized that as cells are in the fast-growing phase at 2 min incubation, majority of cells are dividing and the cells are analyzed when their septum is not fully segregated. The microscopy images also showed that there are more cells attached together in 2 min and 20 min time points than longer time. Maybe this is the reason for which the signal intensity is higher by a factor of about 1.5 (ratio of fluorescence intensity at 2 min vs 30 min) at the beginning. After all, comparing the cells with or without chloramphenicol treatment I conclude that FACS can be an efficient tool to analyze EDPI and EDPII in aminoglycoside studies.

3. Interaction of Neo-Cy5 with the membrane

One of the long-lasting questions in the study of aminoglycoside is how these molecules enter inside the cells. Up to now a single channel, the mechanosensitive channel MscL has been reported by which aminoglycoside would go through the cytoplasmic membrane. Here I explore the interaction of Neo-Cy5 with the membrane hoping to find more data on this subject.

3.1. Pole accumulation

Interestingly when I analyze the images of fast-growing cells which were treated for a very short time with Neo-Cy5, I found that at cell division the cells' septum were marked by Neo-Cy5 much more than other parts of bacteria (Figure 4-6). I recorded a time-lapse to confirm this fact at the moment of cell division the septum became much brighter, however why the new poles have strong Neo-Cy5 localization remains to be explained.

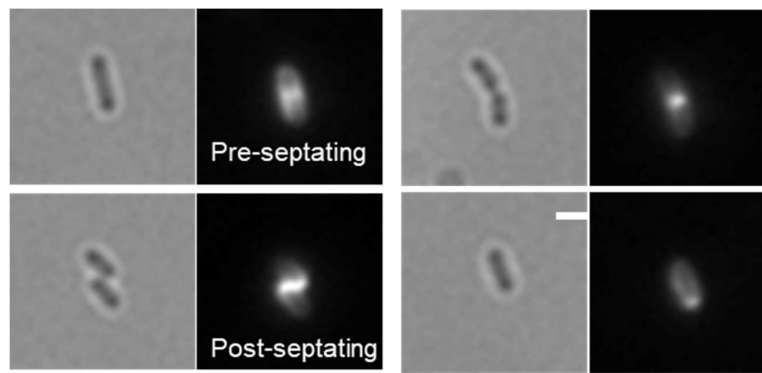


Figure 4-6 Septum accumulation of Neo-Cy5. When cells were treated for short amount of time with a non-lethal concentration of Neo-Cy5, before cell division cell septum became brighter in Cy5 signal. After cell division, new poles were much brighter in Cy5 signal than old poles.

3.2. Super resolution microscopy

As Cy5 is a dye, which is suitable for super resolution microscopy, I did an experiment in order to investigate polar accumulation patterns (Figure 4-7). I confirmed that cells have a peripheral accumulation pattern after few minutes of incubation with Neo-Cy5 and I observed that the peripheral binding is not homogeneous. I found that there is formation of clusters of Neo-Cy5 on the membrane. These clusters interested me because maybe Neo-Cy5 accumulation at these spots may be related to the mechanism of entry into the cytoplasm. There is a possibility that these clusters may correspond to aggregation of Neo-Cy5 on the membrane. However, I did

not observe these clusters in the cytoplasm (data not shown). Therefore, it is unlikely that these clusters may originate from Neo-Cy5 aggregation.

Another hypothesis was that these clusters are the MscL channels, which probably have affinity to Neo-Cy5. It is interesting to note that some data suggest that MscL has the tendency to form clusters *in vitro* and *in vivo* ¹²⁵. We have performed one experiment with the *E. coli* strain lacking the gene coding for MscL. We observed that clusters were still present on the cellular membrane. A more detail analysis with the comparison of the number of clusters for the wild-type and mutant strains is required to conclude if there was a decrease of the number of clusters. It is indeed possible that MscL only accounts for a fraction of the observed clusters; the other fraction could possibly correspond to other membrane channels that would also bind aminoglycosides.

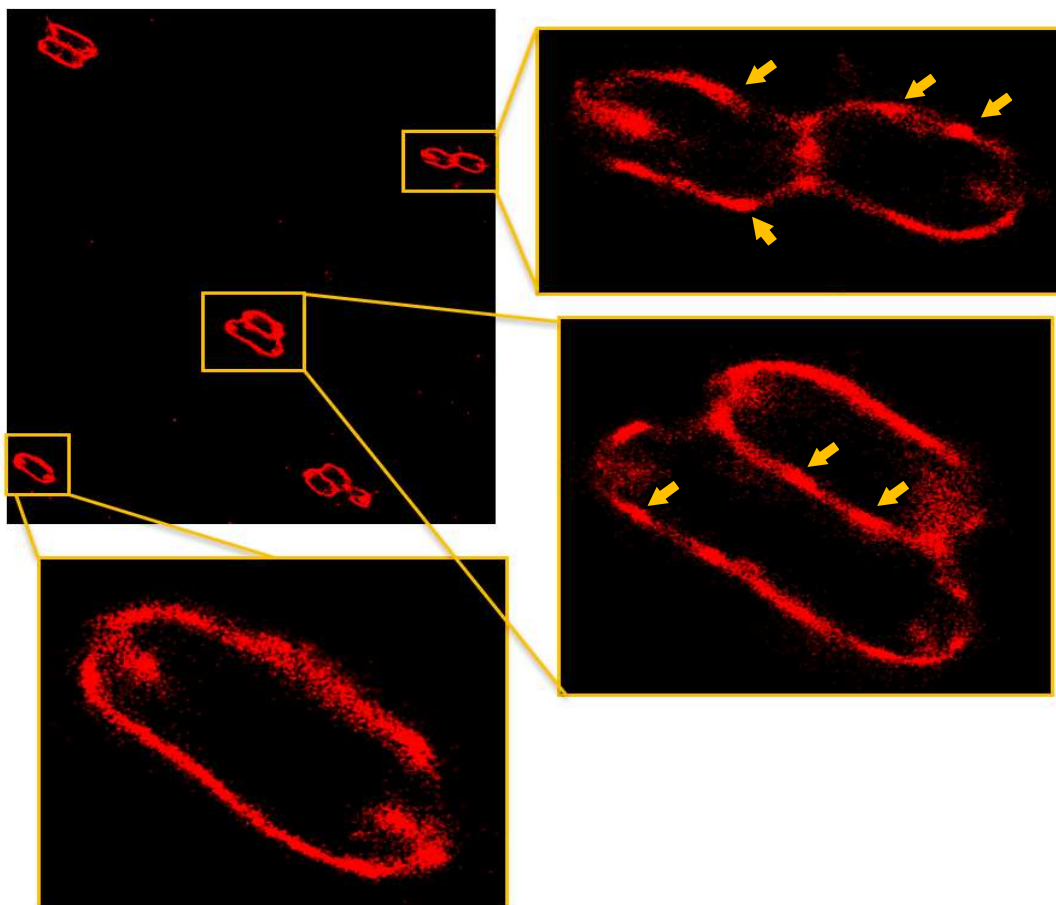


Figure 4-7 Super resolution images of *E. coli* cells treated with tagged aminoglycoside. Cells at OD 0.02 were treated by 4 μ M Neo-Cy4 for 2 min at 37°C, then washed on the filter. Cells were placed directly on agar pad for imaging. Note that once on the microscope there was a delay of about 15-20 minutes before acquisition of these images. Peripheral accumulation of antibiotic is not homogenous and there are Neo-Cy5 clusters detected on the membrane (indicated by yellow arrows).

3.3. Number of cell washes

In order to observe specific interaction of antibiotic with membrane, I added the additional steps to wash the cells and eliminate the unspecific Neo-Cy5 attachment to the cell membrane. However, to make sure that I am successfully eliminating the excess of Neo-Cy5 from the membrane, I did a control test. The results showed that by this method of washing the cells on the membrane, three washing steps are necessary in order to remove the non-specific Neo-Cy5 membrane attachment (Figure 4-8).

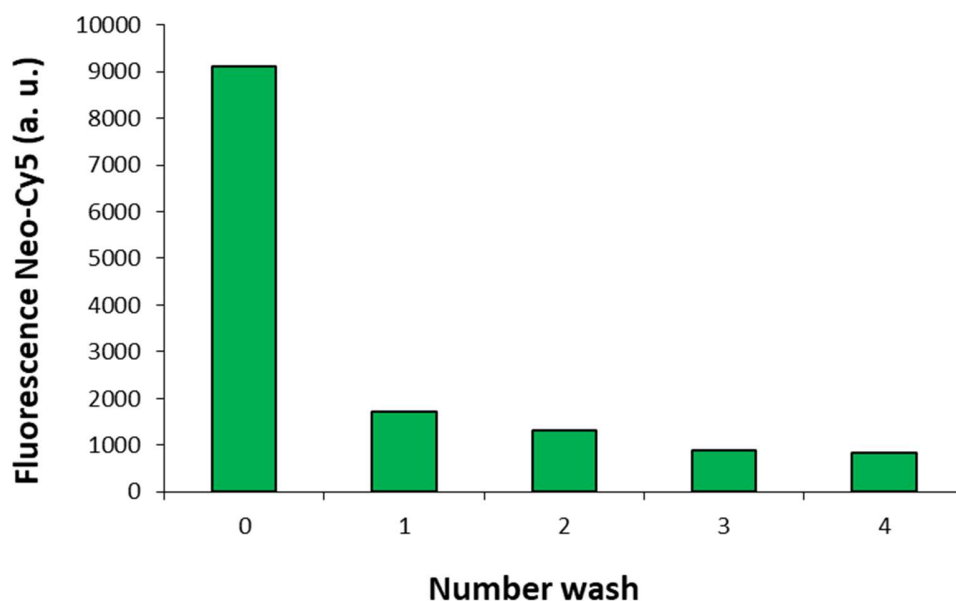


Figure 4-8 Removing Neo-Cy5 excess by filtration. *E. coli* MG1655 cells (OD600 :0.02) were incubated 5 minutes with 32 μ M Neo-Cy5 at 37°C then filtered on a 0.45 μ m-pore-size HAWP membrane filter (mixed cellulose esters, Merck Millipore). Cells were washed or not with 100 μ L MOPS-G medium (kept at 37°C), recovered from the filter and immediately analyzed by FACS. The average of Neo-Cy5 signals for each sample was measured. Three washing steps were required to remove the excess of Neo-Cy5.

3.4. FACS experiment Neo-Cy5 saturation of membrane

I tested the interaction of cells with Neo-Cy5 at 32 μM (more than 3 times the MIC). This concentration is lethal to the fast-growing cells diluted to an OD of 0.02. As described above in the text, the Neo-Cy5 to cell ratio is very critical in order to observe the cells in conditions of drug induced killing. Here, I performed a test by FACS to measure at which concentration of Neo-Cy5 (incubated for a few minutes) saturation of the membrane would be reached.

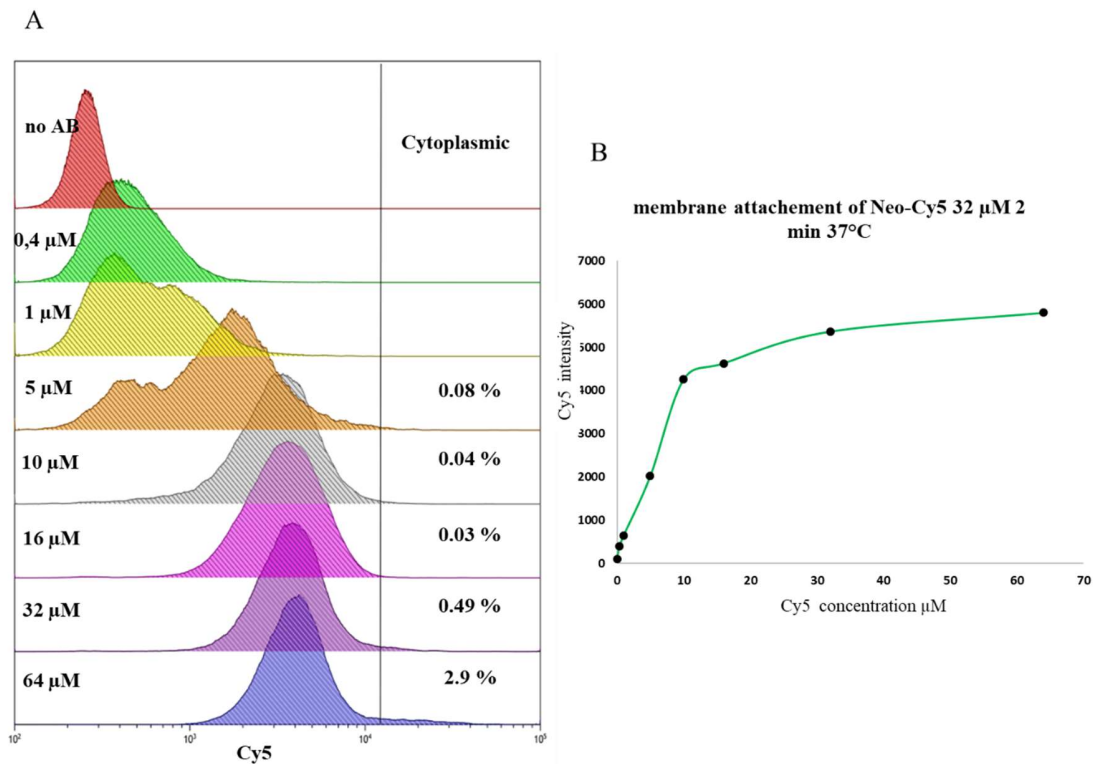


Figure 4-9 Membrane saturation by Neo-Cy5. A) FACS analysis of cells at OD 600nm: 0.02 treated with different concentrations of Neo-Cy5 and then washed on the filtered and analyzed by FACS. The cytoplasmic accumulation is on the right part of the panel as percentage. B) Periplasmic Cy5 signals plotted for each concentration. From 32 μM and above Neo-Cy5 binding reached saturation.

I tested a series of different concentration of Neo-Cy5 on the cells and the results demonstrated that the membrane is almost reaching saturation at 32 μM of Neo-Cy5 (Figure 4-9). Interestingly increasing the concentration of Neo-Cy5 up to 64 μM did not change the membrane

attachment of Neo-Cy5 in fast growing cells but stimulated, at the highest concentration, a second population of cells with cytoplasmic accumulation. This is in agreement with the dose dependent response of aminoglycoside described in the literature.

Here I take a moment to explain that at higher concentrations of Neo-Cy5 it is possible to decrease the time in which Neo-Cy5 will enter into the cytoplasm. Yet, I chose to test the killing curve and other microscopy imaging at 32 μM (and not higher). Firstly because of high cost of Neo-Cy5 and secondly, while performing the experiment I needed enough time to analyze cells at each time point and faster killing rate with higher concentrations of Neo-Cy5 would not have been an advantageous step to my protocol.

4. Neo-Cy5 accumulation combine with marker for dead cells

After aminoglycosides cause miscoding in the cells it has been suggested that misfolded proteins induce holes in the membrane, which leads to cell leaking and EDPII followed by cell death.

Here I used the dye PI which a cell marker reporting on cell death. PI is suggested to enter into the cells through holes present in dead cell's membranes. After 40 min of incubation according to Neo-Cy5 (32 μM) killing curve we know that cells are starting to die and the dead cell number in the population is increasing in this condition. After 120 min, the microscopy data and the CFU survival test have shown that majority of cell population is dead. However, the PI could not enter into most of the cells, even after 120 min incubation with Neo-Cy5 (Figure 4-10).

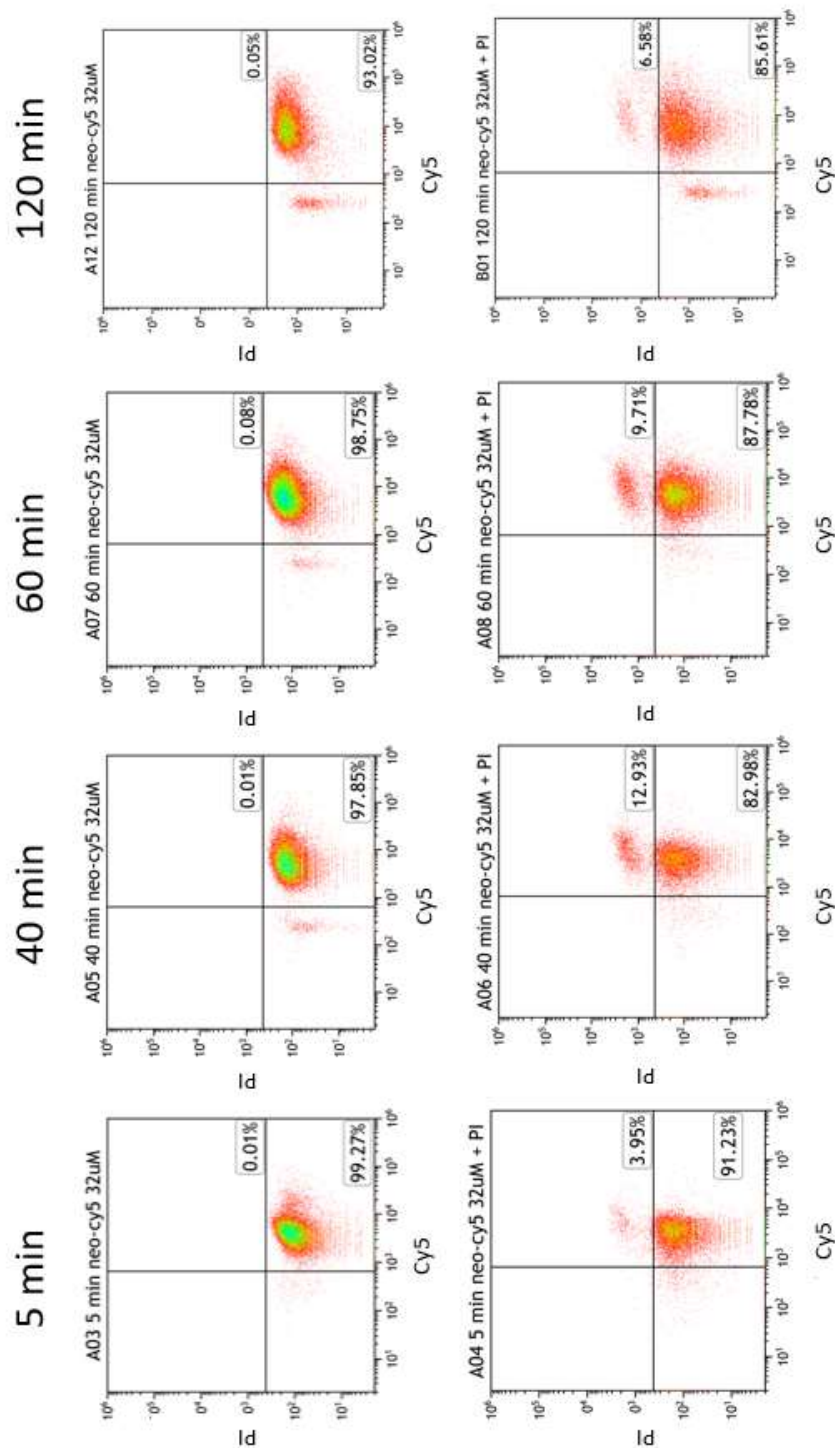


Figure 4-10 Kinetics analysis of accumulation of Neo-Cy5 and PI by FACS. *E. coli* cells (OD 600nm: 0.02) treated by Neo-Cy5 at 32µM at 37°C left panel or treated by Neo-Cy5 and also PI at right. Cells were analyzed at different time points. Positive signal percentages of Cy5 and PI are indicated for each experiment.

There are many studies, which are criticizing PI as a marker for dead cells. Knowing that PI is not the best marker for dead cells, here I suggested that maybe the holes created as a result of Neo-Cy5 induced miscoding are not large enough to let PI enter into the dead cells. Other hypothesis can be that formation of holes on the membrane is not the main reason why aminoglycosides are bactericidal.

5. Conclusion

In this chapter, I established a protocol to characterize the accumulation of Neo-Cy5 in live bacteria by fluorescence microscopy, super-resolution imaging and FACS analysis. I performed killing curves of Neo-Cy5 at Neo-Cy5 concentration above the MIC and evaluated the effect of cell density. This result was complementary to the microscopy kinetic imaging, which showed that as soon as Neo-Cy5 enters into the cytoplasm it starts its bactericidal actions. However, I showed that low concentration of Neo-Cy5 into the cytoplasm is not lethal and cells were able to tolerate and continue to grow according to the time-lapse analysis. I also found that the cell density is critical when we are studying the action mechanism of an antibiotic.

By FACS analysis, I characterized the kinetics of Neo-Cy5 accumulation in the cells and by using chloramphenicol treatment, which reduces EDPI and abolishes EDPII; I was able to show that FACS analysis when combined to TIRF imaging can reveal the phases EDPI and EDPII.

I studied the binding of Neo-Cy5 to the membrane. I ensured that in my protocol, the membranes are saturated by Neo-Cy5. In addition, I made sure that there is not non-specific

binding of Neo-Cy5 to the cell membrane using several washing steps guaranteeing that in both, microscopy and FACS analysis there are only specifically bound antibiotic molecules.

The super-resolution study demonstrated that Neo-Cy5 forms clusters on the membrane that may help us to find more about the mechanism of aminoglycoside penetration into the membrane.

At the end, I characterized further EDPII and the theory of formation of the holes on the membrane by a marker for dead cells, PI. The data do not allow to conclude that the holes are formed at the end of EDPI which leads to EDPII because even in the cell population in which majority of cells are dead, PI did not stain more than 6% of the cells. It is possible that the size of the holes or the membrane damages generated by the action of aminoglycosides is not compatible with an entry of the dye PI explaining the absence of staining. It also remains possible the membranes remain intact even though the cells are dead. I concluded that maybe the formation of the holes in membrane is not the most important cause, which leads to cell death in aminoglycoside treatments.

Chapter V results: Role of mechanosensitive channels on aminoglycoside accumulation

1. Introduction

In this chapter, I presented the unpredicted results that I obtained during establishing the protocol for cell treatment with Neo-Cy5. The protocol that has been used in our laboratory for fluorescence microscopy for incubated cells with Neo-Cy5, was followed by several washing steps of the cells. These repetitive washing steps, necessary for reducing the background, consisted of centrifugation of the cells and changing of cell media.

When I wanted to study the Neo-Cy5 accumulation by FACS experiment, I repeated this cell washing protocol and I analysed the cells several times by FACS. The results confirmed the microscopy analysis that has been done before. In this protocol very short incubation of cells (2min) with a sub MIC concentration of Neo-Cy5 (0.4 μ M) leads to high cytoplasmic accumulation in some cells. The cytoplasmic subpopulation of the cells entirely showed PI accumulation as well. At that point I concluded that the subpopulation of the cells with higher PMF accumulate the Neo-Cy5 in their cytoplasm even in a sub-MIC concentration. Presence of Neo-Cy5 in the cytoplasm is lethal and will kill the cells that will become PI positive. These results, on the diluted cells showed that as cells are diluted, they have higher cytoplasmic concentration. I hypothesized that dilution may increase the PMF in the cells. However, the membrane potential reporter dye (DiBAC4-(3)) did not show any difference in PMF level of the cells diluted in different media with different carbon sources.

FACS analysis of Neo-Cy5 is an easy method to analyse aminoglycoside accumulation. In a regular protocol to analyse cells with FACS, normally it is not necessary to remove the excess

of fluorescent dye because FACS detector is capturing only the signals from each particle (bacteria), as suggested to us, by our colleague of the FACS platform. As I eliminate the washing steps in my protocol, I observed that there was no cytoplasmic accumulation in cells even though the test condition was kept the same (Neo-Cy5 concentration and time of incubation). I doubted that the cell washing steps perturbed cells and triggered accumulation of Neo-Cy5 in the cells artificially. Therefore, I took one-step back and observed the Neo-Cy5 accumulation of cells by fluorescence microscopy however this time I did not wash the cells by centrifugation, I only spread the cells on the agar pad on a slide in a way to cover a large surface. Using this protocol, I had minimum of background (excess of Neo-Cy5 in the media). I did a series of experiments to confirm that indeed the washing steps (centrifugation and several times changing of media) were triggering Neo-Cy5 accumulation. Then I changed the washing protocol, I used a filter device to wash the cells on a 0.45 μm filter. I recovered the cells from filter into media and analysed the cells *via* microscopy or FACS. All the results that have been presented in chapter IV are following this protocol, which does not trigger artificial accumulation of Neo-Cy5.

I tested the effect of centrifuge and cell resuspension method (other mechanosensation factor) by FACS experiments. I found that centrifugation of the cells on its own is not triggering the Neo-Cy5 accumulation. After centrifugation, the way that cells are resuspended is important. If the cells are resuspended by agitation, it triggers cytoplasmic accumulation in a subpopulation of the cells; however, the very mild tapping of the tube is not triggering any cytoplasmic accumulation.

As I mentioned in the introduction, the mechanosensitive (MS) channels have been reported to have affinity to the aminoglycoside streptomycin. Here my observations clearly showed that mechanosensation factor is triggering Neo-Cy5 accumulation. To investigate this

phenomenon, I used the strains *ΔmscL* and *ΔmscS* from the *E. coli* Keio Knockout Collection. I transferred these mutations into the MG1655 *E. coli* strain by P1 bacteriophage.

The MIC of these 2 strains for neomycin was slightly higher. This can suggest that aminoglycosides may naturally go through these channels, even though there is no centrifugation in MIC measurement.

I ran a test on the *ΔmscL* strain by FACS in comparison with the wild type strain. The results confirmed that MS channels (at least MscL) were activated *via* centrifugation and resuspension. This activation leads to Neo-Cy5 accumulation in the cytoplasm in a sub-population of bacteria.

Finally, when I first centrifuged wild-type and *ΔmscL* cells and added Neo-Cy5 after stopping the centrifugation, I observed that WT cells lysed very easily, in contrast to the *ΔmscL* cells, which were resisting.

2. Mechanosensation and Neo-Cy5 accumulation

Here I present the results of Neo-Cy5 accumulation in the cells after washing steps with centrifugation. At first, I present the Neo-Cy5 accumulation patterns in *E. coli* by centrifugation cell washing protocol. Then I present more experiments in order to understand the reason behind the cell's accumulation patterns.

2.1. Cells washing protocol

In microscopy analysis of Neo-Cy5 accumulation in live bacteria, in order to remove the excess of Neo-Cy5 in the cell culture and non-specific Neo-Cy5 binding to cell membrane, it is necessary to wash the cells after treatment. In our lab, a protocol had been established which

consisted of cell centrifugation at 8000 g for 5 min and then changing the media for a fresh one. This step was repeated a minimum of 3 times to have the least amount of background in the fluorescence microscopy imaging.

2.1.1. Accumulation patterns by microscopy and FACS

I present here results, which were obtained previously in our laboratory. *E. coli* cells at OD 600nm of 0.3 were treated by 0.4 μ M (sub-lethal) for 2 min at 37°C. After washing cells by centrifugation, cells were put on agar pad and analysed by fluorescence microscopy (figure 5-1). Cells have 3 different accumulation patterns: peripheral, cytoplasmic and accumulation at the cell septum in the dividing cells.

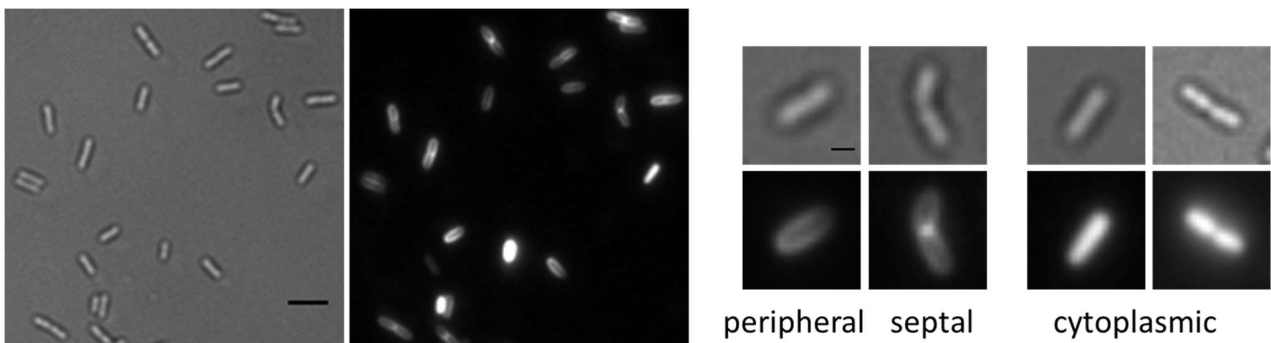


Figure 5-1 Accumulation of Neo-Cy5 in exponential phase cells. Cells were incubated in MOPS-G media for 2 min with sub-MIC concentration of Neo-Cy5 at 37°C and then cells were washed 3 times by centrifugation and changing the media. There are two distinct Neo-Cy5 accumulation patterns: peripheral and cytoplasmic. Cytoplasmic sub-population is less than 10%. Septum of cells in division have a higher Neo-Cy5 signal. Scale bar 5 μ m.

I repeated the same protocol and analysed the cells by FACS (figure 5-2). The two Cy5 positive peaks correspond to the peripheral (lower) and cytoplasmic (higher) accumulation levels. The percentage of each peak depends on the test conditions.

2.1.2. Dilution effect

It has been studied by fluorescence microscopy in our lab that dilution of exponential phase cells influences the cytoplasmic accumulation subpopulation. The more diluted the cells are, the higher the percentage of cells with cytoplasmic accumulation is when using the centrifugation protocol. Here, I confirm this result by FACS analysis. Cells at OD: 0.3 have lower sub-population of cytoplasmic accumulation (6%). When cells are diluted 10 times, the cytoplasmic sub-population increases to 44 % and when cells are diluted 100 times, there are 66 % of the cells with cytoplasmic Neo-Cy5 uptake (Figure 5-2).

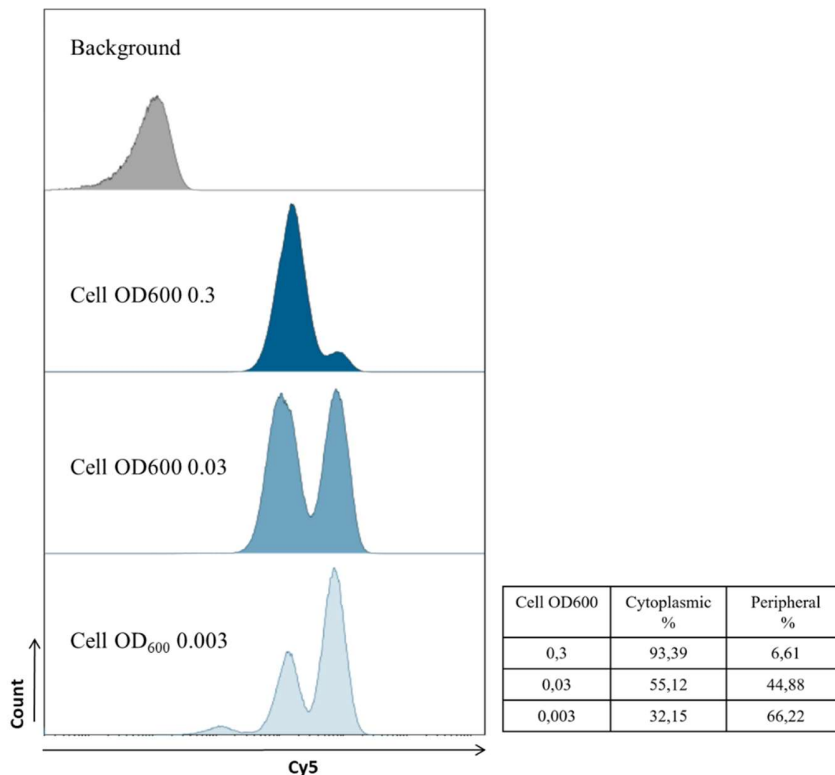


Figure 5-2 Analysis of Neo-Cy5 accumulation patterns by FACS. Exponentially phase cells at OD: 0.3 were diluted 10 times and 100 times. Each cell culture was then treated with sub-MIC concentration of Neo-Cy5 for 2 min at 37°C. Cells were then washed 3 times by centrifugation for changing the media. Cells were then analysed by FACS for Cy5 signal. The peak with lower fluorescence intensity is showing the peripheral binding of Neo-Cy5 and the second higher peak is showing the cytoplasmic uptake. The percentage of each peak is shown in the table.

As it has been mentioned in the introduction, increase of aminoglycoside lethality by cell dilution has been reported before; however, the reason of such an observation remains unknown.

2.1.3. PMF measurement in diluted cells

I hypothesized that the effect of cell dilution on cytoplasmic accumulation is due to increase of PMF in the population. When the PMF is higher, there is more accumulation of Neo-Cy5 in the cytoplasm as the aminoglycoside accumulation is PMF dependent. When the cells are diluted there are more nutrients, which may lead to an increase of PMF in the cell population. Here, I tested to see if the PMF is changing while the cells are diluted in media (MOPS) with different sources for carbon. I used the negatively charged dye DiBAC4-(3) [bis-(1,3-dibutylbarbituric acid)-trimethine oxonol], which can reveal the polarization level of the membrane. DiBAC4-(3) signal increases when the cells are depolarized. Signal is weaker when cells have normal potential and decreases further when cells are hyperpolarized. The FACS analysis for DiBAC4-(3) showed no changes in the cell population PMF while they are diluted in media with different carbon sources (figure 5-3). This dye has been used for measuring the PMF in *E. coli*, I may conclude that there is no changing in the level of PMF in the cells diluted in different media or maybe DiBAC4-(3) is not sensitive enough to show a small PMF variation.

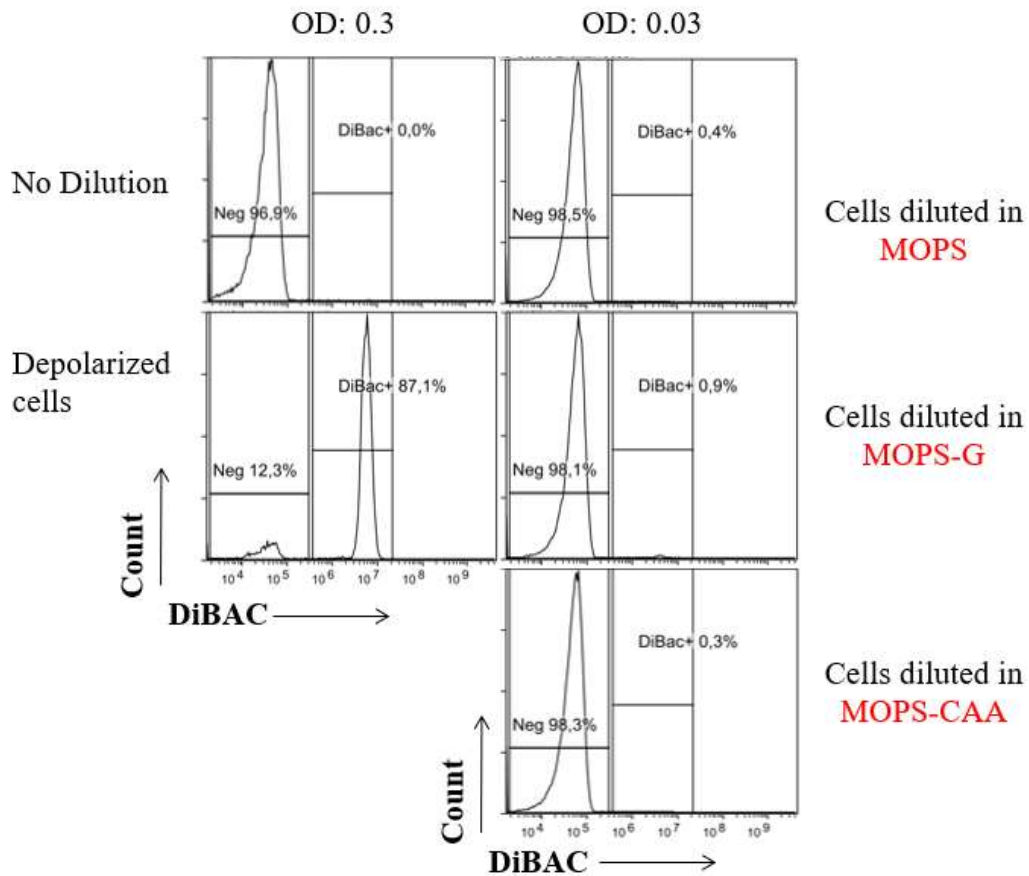


Figure 5-3 Measuring membrane potential in the diluted cells in different media and comparing to non-diluted cells. Exponential cells were diluted in MOPS media with or without different carbon sources then treated with DiBAC4-(3). As a control for positive signals, cells were treated with ethanol 60% in order to depolarize the cell then washed and treated by DiBAC4-(3). G: Glucose, CAA: casamino acid.

2.1.4. Different Neo-Cy5 concentrations and time of incubation

Here I presented the results of testing different Neo-Cy5 concentrations and different time of incubation with Neo-Cy5 by the centrifugation washing protocol.

Cells at OD 0.3 were treated at 37°C with different concentrations of Neo-Cy5 (figure 5-4-A). Cytoplasmic subpopulation peak is slightly increasing while the peripheral accumulation is very heterogeneous in the lower concentration and becomes more homogenous in higher ones.

When cells were incubated longer with 0.4 μM of Neo-Cy5, the cytoplasmic population was not increasing, I conclude that the cytoplasmic accumulation is independent of the time of incubation prior the centrifugation and washing steps. This also suggests that the cytoplasmic uptake is rapid and only driven by the centrifugation/washing protocol (Figure 5-4-B).

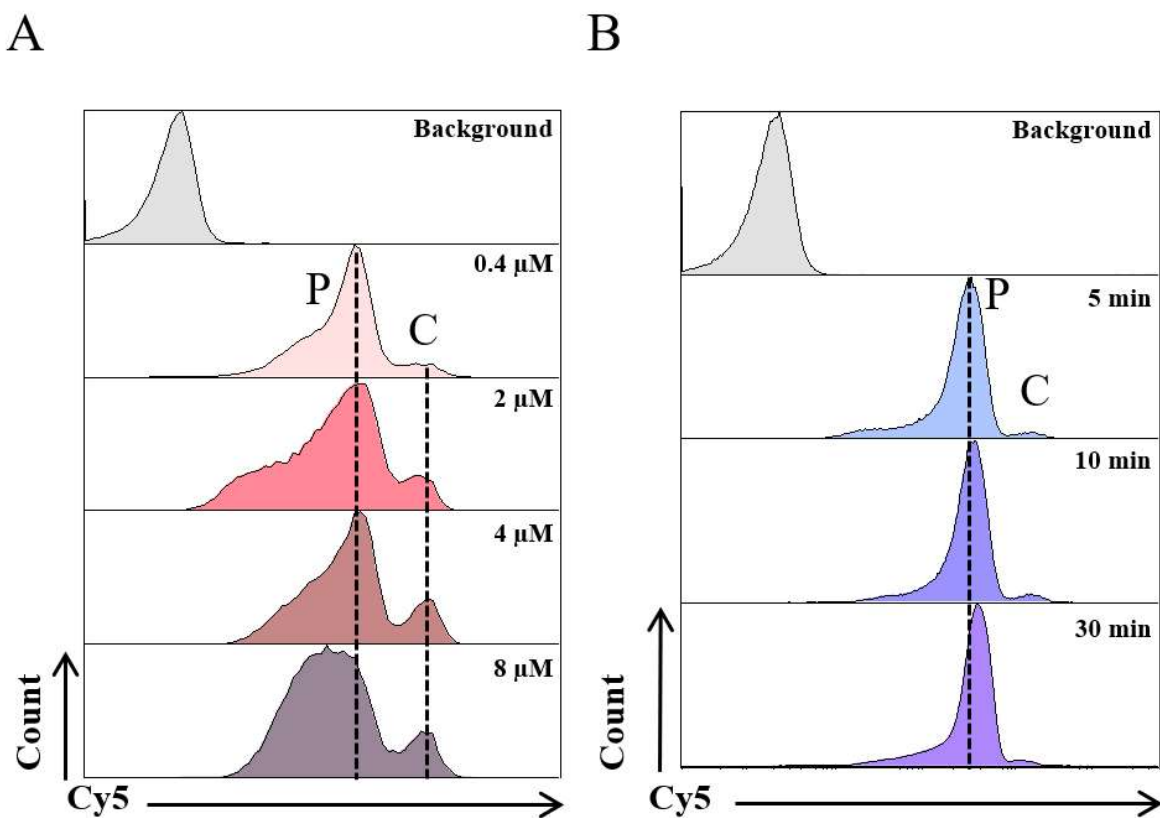


Figure 5-4. Patterns of Neo-Cy5 accumulation in exponential phase cells. A) Exponential cells OD: 0.3 were treated with different concentrations of Neo-Cy5 for 2 min then washed by the centrifugation protocol. Cells were then analysed by FACS for Cy5 signals. B) Exponential cells OD: 0.3 were treated by 0.4 μM of Neo-Cy5 for different time of incubation then subjected to the same protocol of analysis. P: Peripheral, C: Cytoplasmic accumulation.

2.1.5. PI and cytoplasmic accumulation of Neo-Cy5

Knowing that Neo-Cy5 is an active antibiotic, I assumed that cells with high cytoplasmic accumulation are dead. It has been shown in our lab by time-lapse microscopy that cells with cytoplasmic accumulation are non-growing.

Here I treated the cells with neo-Cy5 and PI to prove that cells with cytoplasmic accumulation of Neo-Cy5 suffer membrane damage and PI enters into their cytoplasm. FACS results showed that cells with peripheral accumulation of Neo-Cy5 are PI negative and the cytoplasmic population of cells have positive PI signals (Figure 5-5).

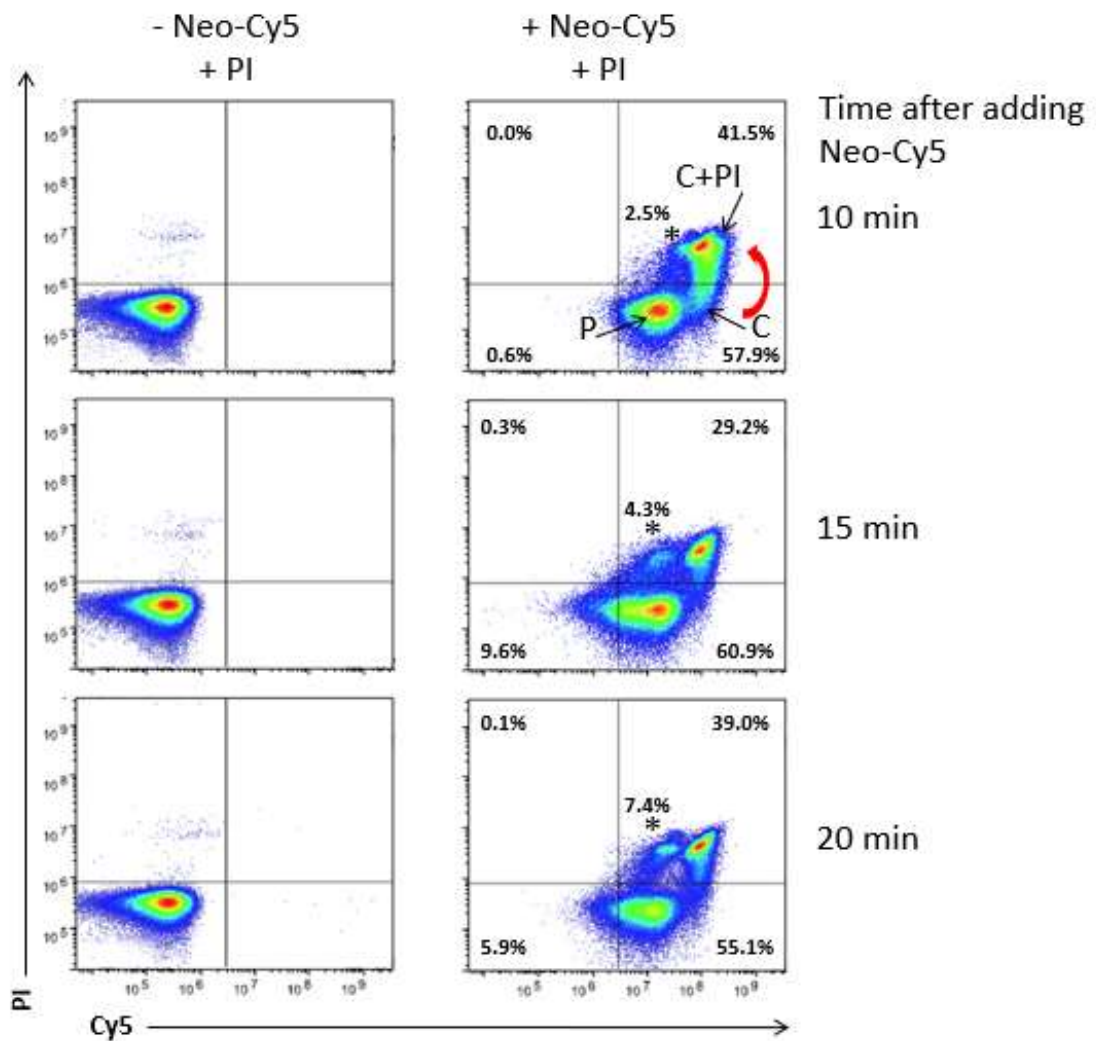


Figure 5-5. Cells with cytoplasmic accumulation are PI positive. Exponential cells were diluted 10 times then treated with 0.4 μ M of Neo-Cy5 at 37 °C for indicated durations. Cells were then washed by centrifugation then incubated with PI (15 min) and analysed by FACS for Cy5 and PI signals.

2.2. Effect of washing steps

Once I had different result from non-washed cells (no cytoplasmic signals with such intensity), I assumed that the washing steps, which consisted of centrifugation and media changing, triggered mechanosensation in the bacterial cells. This led to cytoplasmic accumulation Neo-Cy5 in high levels (even when the cells were treated with sub-MIC concentration).

I did a series of experiments by FACS analysis to find which step of my protocol leads to cytoplasmic accumulation.

2.2.1. Centrifugation and cell resuspension methods

Cells were treated with sub-MIC concentration of Neo-Cy5 at 37°C. After centrifugation and changing the media cells were resuspended either by tapping very delicately on the tube or by pipetting up and down several times. The result showed that cells, which were resuspended by pipetting contained higher subpopulation of cells with cytoplasmic accumulation (Figure 5-6). When the cells were mixing by up and down pipetting it triggered mechanosensation activation in cells. I also observed that when the experiment was performed with different cells cultures in same day or different days, the peripheral and cytoplasmic peaks were shifting in the different experiments.

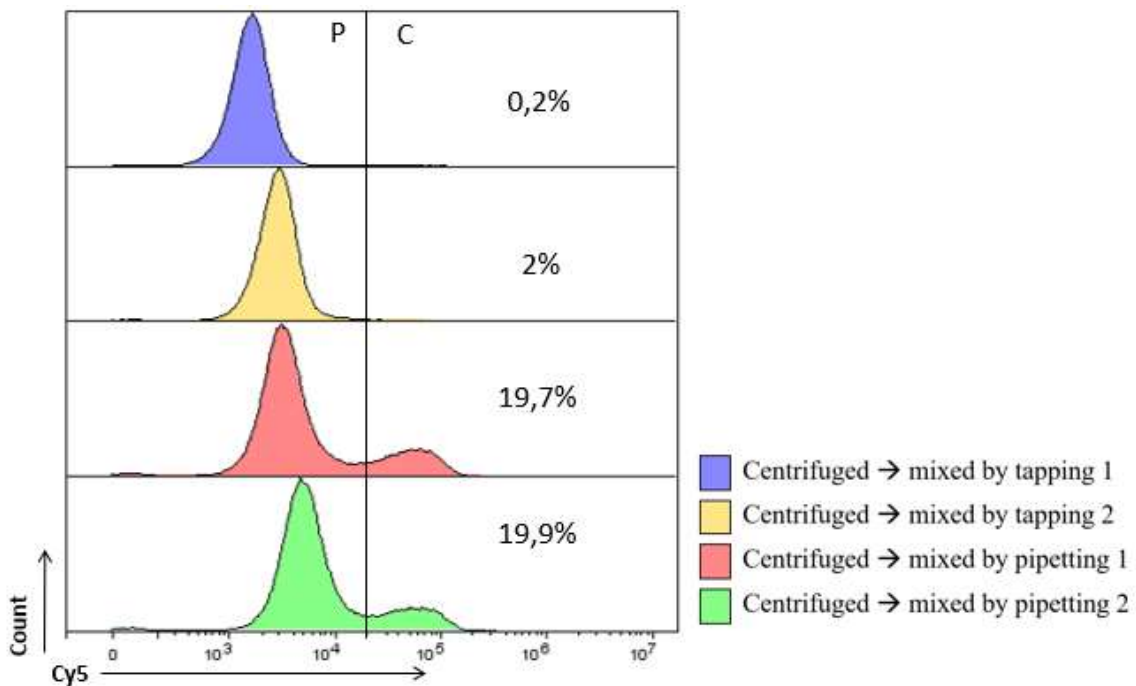


Figure 5-6. Resuspension methods trigger mechanosensation in cells and change cytoplasmic accumulation of Neo-Cy5. Cells were grown at OD: 0.3 then diluted 10 times. Cells were incubated at 37°C with sub-MIC concentration of Neo-Cy5 for 2 min. Then cells were centrifuged at 8000g for 5 min and resuspend in the new media either by tapping gently on tube or mixed by pipet. Each experiment was done two times. The cytoplasmic percentage (indicated in the figure) changed slightly and the peak of peripheral Neo-Cy5 binding varied as well. C: cytoplasmic, P: peripheral.

I also showed that if the cells are not centrifugated and only mixed by pipet the effect is not the same. There is no cytoplasmic accumulation in the cells without centrifugation. Cytoplasmic accumulation occurred only for cells mixed by pipetting (Figure 5-7). As I found that the resuspension methods trigger mechanosensation, for further study, I resuspend the cells with same strength by the shaker with a tube holder to keep the shaking step equal all long the experiments.

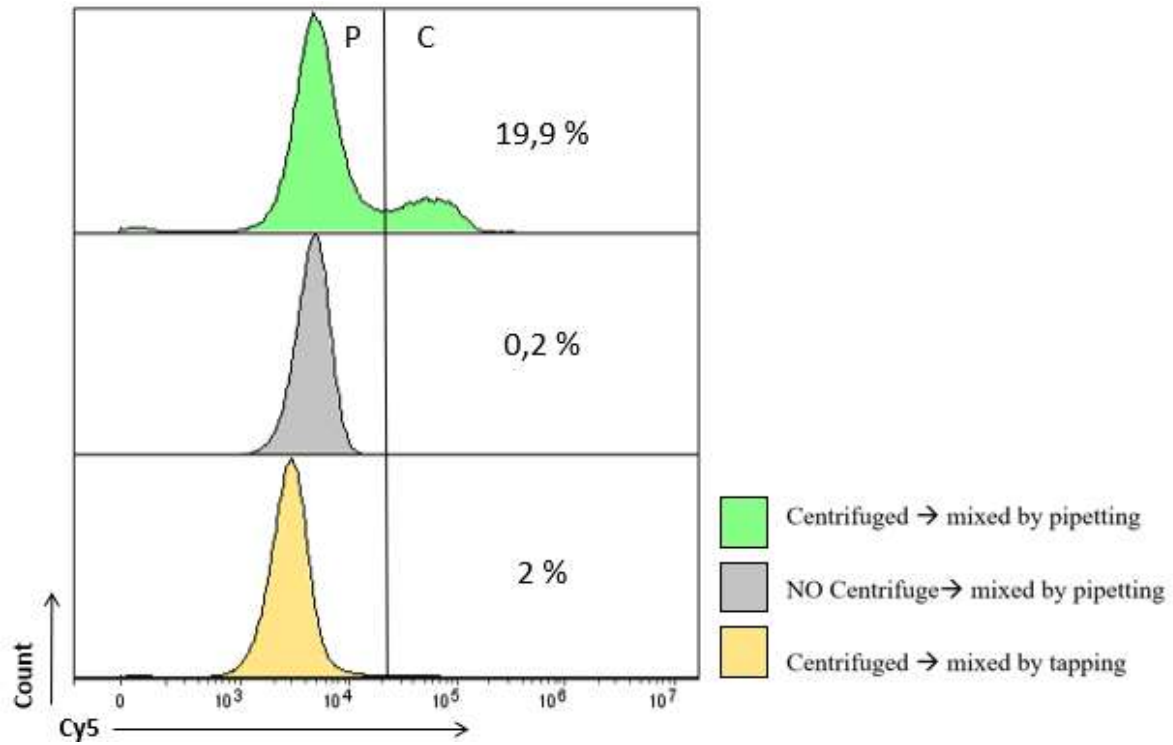


Figure 5-7. Centrifugation should be followed by pipet mixing the cells to trigger accumulation of Neo-Cy5 in the cytoplasm. Cells were grown at OD: 0.3 then diluted 10 times. Cells incubated at 37°C with sub MIC concentration of Neo-Cy5 for 2 min. Then cells were centrifuged (or not as indicated) at 8000g for 5 min and resuspended in the new media either by tapping gently on tube or mixed by pipet. The cytoplasmic percentages are indicated in the figure. C: cytoplasmic, P: peripheral.

2.2.2. Rifampicin treatment

Rifampicin is an antibiotic, which inhibits DNA-dependent RNA polymerase. Cells treated with rifampicin see their levels of mRNA to rapidly drop with the subsequent halt of protein synthesis. Here I tested to see if rifampicin treatment can inhibit the accumulation of Neo-Cy5 in the cells while its accumulation is triggered by centrifugation and washing steps. Rifampicin treatment stopped accumulation of Neo-Cy5 in the cytoplasm (Figure 5-8).

Here, I supposed that Neo-Cy5 cytoplasmic accumulation by activation of mechanosensation in bacteria is disabled by rifampicin treatment or that rifampicin competes with Neo-Cy5 for entry. I do not know what the mechanism is, which leads to such result.

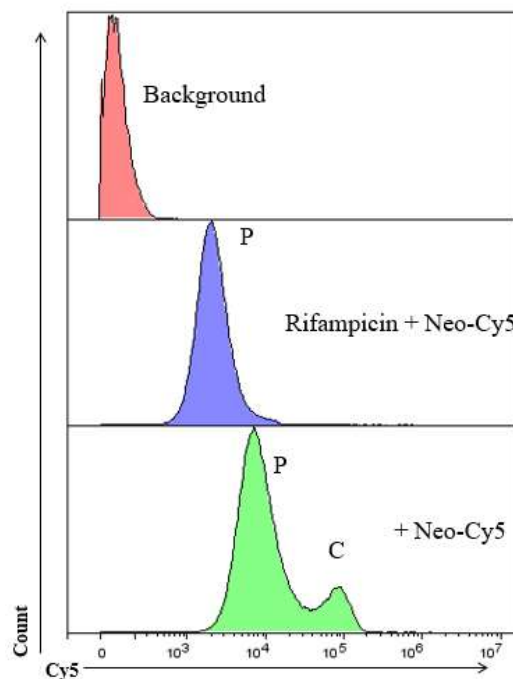


Figure 5-8 Rifampicin treatment inhibits accumulation of Neo-Cy5 in the cytoplasm induced by centrifugation washing steps. Exponential phase cells (OD: 0.3) were diluted 10 times. Cells incubated with 30 μ M rifampicin for 10 min were then incubated with sub-MIC concentration of Neo-Cy5 for 2 min and washed with centrifugation protocol before FACS analysis. P peripheral, C cytoplasmic accumulation.

3. Mechanosensitive channels role in aminoglycoside accumulation

I have tested here the effect of one of mechanosensitive channels in *E. coli* MG1655, as these channels have been reported to have affinity to streptomycin.

3.1. Strain construction

I transferred the gene deletion mutation for two mechanosensitive channels: *mscL* and *mscS* (which were replaced by kanamycin resistance gene) from the strains of the *E. coli* Keio Knockout Collection by P1 bacteriophage transduction into MG1655 *strain*. Then I delete the kanamycin resistance gene in the strain by inducing the thermosensitive plasmid coding for Flp recombinase. The strains were then verified by PCR (figure 5-9).

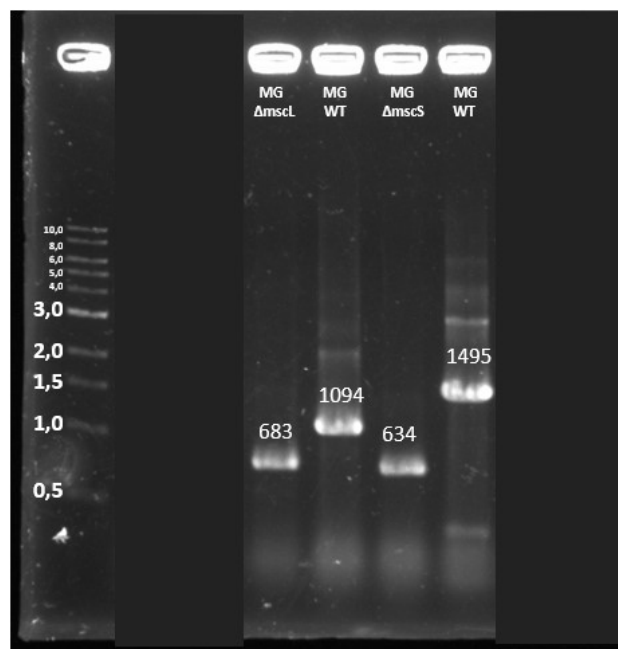


Figure 5-9. Verification of the construction of the mutants *mscL* and *mscS* by PCR. Single-colony PCR after mutant construction in comparison with WT strain with the same pairs of specific DNA primers. The bands and their sizes corresponding to the amplified DNA fragments are displayed.

3.2. MIC of *ΔmscL* and *ΔmscS*

First, I measured the MIC of the two mutant strains *ΔmscL* and *ΔmscS* for neomycin (experiments with triplicates). Both strains had a slightly higher MIC than the WT strain (MIC for WT was 0.5 μ M neomycin comparing to MIC of *ΔmscL* and *ΔmscS* which was 1 μ M). In the protocol of measuring MIC, there is no step of centrifugation, which can suggest that these channels are involved in the neomycin accumulation in a natural way.

3.3. Cytoplasmic accumulation of Neo-Cy5 in *ΔmscL* strain

I incubated the WT and *ΔmscL* strains with Neo-Cy5 (sub-MIC concentration) and then washed the cells by centrifugation and used the tube shaker device to resuspend the cells in the same manner all long the experiment. I observe that the fraction of cells with WT cytoplasmic accumulation of Neo-Cy5 was much higher than that of the *ΔmscL* strain (figure 5-10).

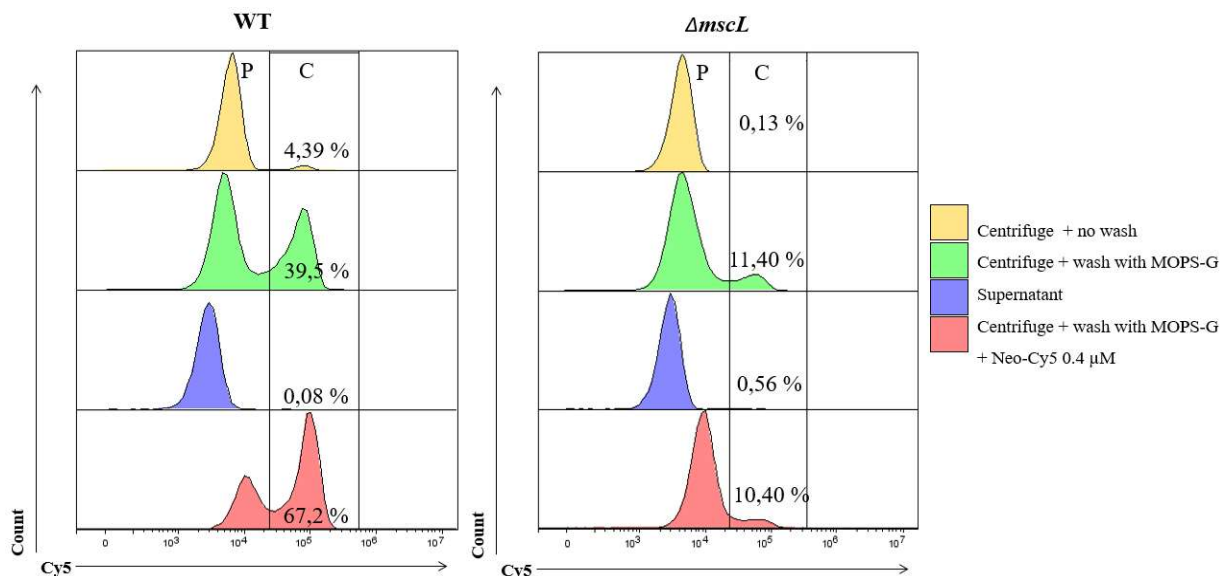


Figure 5-10. *ΔmscL* has smaller percentage of cells with Neo-Cy5 cytoplasmic accumulation comparing to WT. Cells in exponential phase of growth were diluted 10 times and incubated with sub-MIC concentration of Neo-Cy5 for 2 min at 37°C. Cells were centrifuged, washed with MOPS-G or MOPS-G with Neo-Cy5 or not washed. Then cells, as well as supernatant of the cells washed with MOPS-

G, were analysed by FACS. Percentage of the cells with cytoplasmic accumulation of Neo-Cy5 is indicated for each experiment. P: Peripheral, C: Cytoplasmic.

In this experiment I washed the cells either with MOPS-G media or with MOPS-G containing sub-MIC concentration of Neo-Cy5. The WT cells continue to accumulate Neo-Cy5 in their cytoplasm in contrast with the *ΔmscL* strain where the percentage of cytoplasmic subpopulation did not increase. I also analysed the supernatant of cells after the first wash to verify if cells were eliminated in the analysis by the washing steps (figure 5-10).

With these preliminary results, I suggest that the mechanosensitive channel MscL is activated by centrifugation and shaking during cell resuspension and that its activation leads to stimulation of cytoplasmic uptake of Neo-Cy5. The percentage of cytoplasmic accumulation of Neo-Cy5 in *ΔmscL* population is not zero, probably due to the presence of other MS channels in this mutant.

3.4. Neo-Cy5 action on cells in which MS channels were already triggered

I observed that centrifugation and shaking of cells activates the MS channel MscL in the cells, which were incubated with Neo-Cy5. Next, I tested what can happen if I first activate the channels by centrifugation and shortly after, I add Neo-Cy5 (sub-MIC concentration) to the cells. I tested WT and *ΔmscL* cells. Quite interestingly, WT cells were lysed by this protocol and *ΔmscL* cells survived (figure 5-11). This result showed that there is a connection between mechanosensation in bacteria cells and centrifugation, which activates MscL leading to cell death in the presence of sub-MIC concentration of Neo-Cy5 (0.4 μ M).

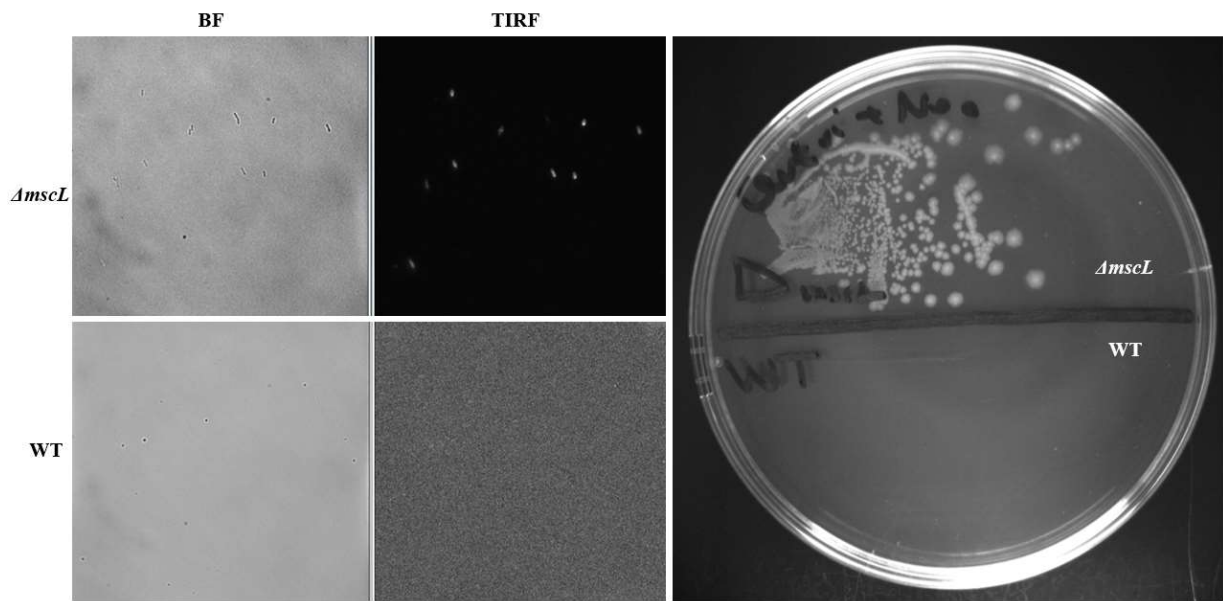


Figure 5-11. Cells with MscL are lysed due to Neo-Cy5 sub-MIC treatment after centrifugation. Exponential WT and *AmscL* cells were diluted 10 times and centrifuged for 5 min at 8000 g. 30 seconds after cells were resuspended in the MOPS-G media with Neo-Cy5 (0.4 μ M) by strong shaking. Shortly after cells were put on agar pad and observed by fluorescence microscopy (left) or plated on LB agar (right).

4. Conclusion

In this chapter I presented the role of the MS channel MscL in Neo-Cy5 cytoplasmic accumulation in bacterial cells. While adjusting the protocol for FACS analysis I discovered that the activation of this MS channel by centrifugation followed by physical pressure on cells resulted in stimulation of Neo-Cy5 uptake. The results showed that Neo-Cy5 entered into the cytoplasm for a subpopulation of cells (depending on cell dilution). The cytoplasmic subpopulation of cells was marked by PI, which indicated that there are damages in the cell membrane. *AmscL* cells showed lower percentage of cytoplasmic Neo-Cy5 accumulation comparing to the WT cells after centrifugation. Other result showed that WT cells lysed

(probably exploded) if they were treated by Neo-Cy5 shortly after centrifugation. Meanwhile the *ΔmscL* cells survived, which demonstrates the role of these channels.

I suggest that centrifugation and shaking cells leads to opening the cells' MS channels and increases the accumulation of Neo-Cy5 in the cytoplasm. This phenomenon is happening for a sub-population of cells, which increases according to the cells' dilution. The effect of dilution on activation of MS channels remains to be explored further.

Chapter VI Aminoglycoside persistence, tolerance and persister recovery

1. Introduction

As it has been presented in the introduction, persisters form an antibiotic tolerant sub-population within an antibiotic susceptible population. Persisters are resisting to the presence of a lethal dose of antibiotic. Fluorescently tagged antibiotics are used to measure antibiotic uptake in persister studies. However, up to now, for aminoglycoside family, there were no active tagged antibiotic by which it is possible to do single cell microscopy analysis. Since in our laboratory Neo-Cy5 has been developed we were able to observe the patterns of antibiotic accumulation in persisters by fluorescence microscopy for the first time.

In persister studies, Gentamicin-Texas Red was used as a fluorescently tagged aminoglycoside to track down antibiotic accumulation in cells by FACS analysis ¹⁰⁷. However, it has been shown in our lab that Neo-Cy5 accumulation patterns can be peripheral or cytoplasmic, an information that cannot be extracted from FACS analyses. I showed in chapter 4 that cells can have different level of Neo-Cy5 cytoplasmic accumulation. Also, I present in this chapter that peripheral Neo-Cy5 binding varies in some mutants that are suspected to have higher PMF level ¹⁰⁸. These mutants are involved in aminoglycoside tolerance. Knowing the importance of patterns of aminoglycoside accumulation in the cells, I think that it is essential to analyse persister cells by microscopy analysis. It has been suggested that persisters have lower accumulation of aminoglycoside ¹⁰⁷, however, for understanding the mechanism of tolerance of aminoglycoside persisters it is critical to know if they have lower cytoplasmic or peripheral accumulation.

Here, first I present experiments that visualized the accumulation of Neo-Cy5 in persisters by fluorescence microscopy imaging. These persisters were yielded by treatment of very high dosage of ofloxacin (fluroquinolone) antibiotic. According to my time-lapse observation, persisters have only peripheral accumulation of Neo-Cy5. These results would agree with the fact that persisters have low level of PMF and as aminoglycoside, accumulation is PMF-dependent it cannot enter into the cytoplasm of cells with low PMF level.

In a more specific study of neomycin persisters, I used a common protocol to study persisters. I treated stationary cell population with high concentration of Neomycin to reach a plateau of persisters. Persisters cells which were yielded after such treatment were only about 0.05% of the population. I treated this population, which contained mostly dead cells and persister, by Neo-Cy5 (sub-MIC) and analysed by FACS. Beside fluorescence intensity signals of cells, FACS gave us data on cell size and cell granularity as well. In order to find out the physical characteristic(s) of neomycin persisters, I attempted to sort out different cell categories according to their size and their Neo-Cy5 intensity.

This experiment principally is not very complicated, however due to FACS contaminations by bacteria I could not finalise my results. Here I present the preliminary results in which no contamination had been detected. I found that persisters are more frequent in the cell category with lower Neo-Cy5 accumulation and smaller cell size. However, they were found, but at lower frequency, in different cell category as well for size and Neo-Cy5 accumulation levels.

It has been reported that there are more persisters in sub-population of cells with lower level of ribosomal RNA ¹⁰⁴. Shah and his colleagues used an unstable GFP protein (asv) under control of *rrnBP1* ribosomal RNA promoter. The fact that the GFP is an unstable protein; it gives us a real time marker for energy level and state of each bacteria. The cells with different signal of

GFP had been sorted out and treated by high dosage of antibiotic. More recently Shan and his colleagues demonstrated that after ofloxacin and ampicillin treatment, there are more persisters in the sub-population with low GFP level ¹⁰⁰.

I constructed a strain with a chromosomal insertion of *gfp(asv)* gene under control of short promoter of *rrnBP1*. This time I tested to see if the sub-population with lower level of GFP was also richer in the antibiotic neomycin.

In this experiment I analysed by FACS the expression of GFP in the exponentially phase cells. I had again hard time to sort out the cells due to contamination of the FACS machine. However, the percentage of persisters surviving neomycin treatment is very low (less than 10^{-4} percent) and it was practically very difficult to study.

According to the analysis of a Tn library for aminoglycoside tolerance mutants carried out by Shah and his colleagues in 2015, there are hundreds of genes which play a role in increasing and decreasing tolerance to aminoglycosides ¹⁰⁸. Authors chose newly found and unexpected mutants within the biosynthesis of flagella and amino acid synthesis for further study. These mutants showed less tolerance to gentamicin. Knowing the importance of aminoglycoside accumulation patterns and different level of Neo-Cy5 binding to membrane, I decided to test Neo-Cy5 accumulation in the mutant for the flagellar transcriptional regulator, *ΔflhC* and a mutant for an enzyme in the serine biosynthesis pathway, *ΔserA*. I used our developed method, which combines fluorescence microscopy with FACS analysis to study the patterns of Neo-Cy5 accumulation in those less tolerant mutants.

I did a series of FACS and microscopy experiences using different concentrations of Neo-Cy5 and different incubation time for each mutant. I observed that *ΔserA* accumulation of Neo-

Cy5 is higher than the one measure for $\Delta flhC$ and WT however, with differences when compared to the study by Shan et al., 2015. Antibiotic accumulation varies in different mutants according to the Neo-Cy5 concentration and time of incubation. The microscopy observation agreed with the FACS results and confirmed that $\Delta serA$ has higher Neo-Cy5 binding to the membrane in comparison with $\Delta flhC$ and the wild-type cells for a short incubation time.

Persister recovery is a subject, which is not much studied. We do not know which are the factors that make persisters to get out of the hibernation state and turn into fast-growing cells. Here I present effects of cell culture dilution in shortening the transitioning time of neomycin persister cells to normal growing cells. I demonstrated that when cell culture is more diluted, persister recovery is faster. It seemed that there is a factor or chemical in the supernatant of dead cells mixed with persister and when this factor is more diluted, it allowed persisters to wake up earlier and when it is more concentrated it keeps the persisters in the dormant state. Before searching for an unknown quorum-sensing factor, I tested the effect of this supernatant on aminoglycoside resistant cells in comparison with susceptible cells and as there were no effect on resistant cells. So remaining neomycin in the cell culture keeps persister in dormant state. This effect was surprisingly specific of supernatant of neomycin and not the one of ofloxacin.

2. Ofloxacin persister time-lapse

To observe persisters' Neo-Cy5 accumulation, I performed the experiment on stationary phase cells, which have a higher percentage of persister cells and treated this population with a high dosage of ofloxacin. Ofloxacin is a fluoroquinolone antibiotic, which inhibits DNA gyrase and DNA replication in bacterial cells. It had been shown that ofloxacin induces persister formation *via* activation of TisB toxin^{141 142}. Using the stationary phase cells and ofloxacin treatment about 1.6% of cells were persisters. I washed the cells with PBS to remove the excess

of antibiotic then I incubated cells with Neo-Cy5 at sub-MIC concentration (0.4 μM) and recorded a time-lapse by fluorescence microscopy (figure 6-1).

In this protocol there is no marker for persister cells. However, by performing time-lapse experiment, I was able to capture the persister growth under the microscope and observe the Neo-Cy5 accumulation in persister cells. In this study I found that persister have peripheral accumulation of Neo-Cy5. This accumulation was heterogenous among persisters.

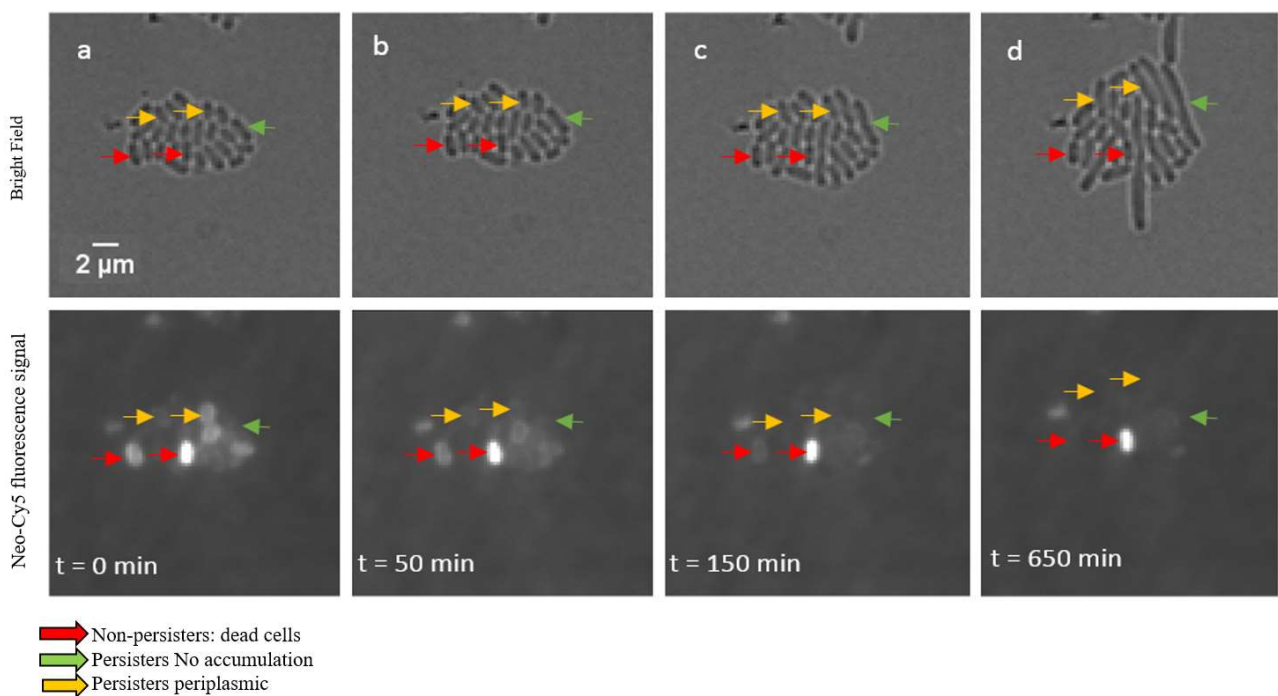


Figure 6-1 Persisters for ofloxacin have peripheral accumulation of Neo-Cy5. Stationary phase cells were treated with ofloxacin, washed with PBS and treated with a sub-MIC concentration of Neo-Cy5. At time zero of time-lapse in the field there is a mixture of persisters with dead cells. As time passes persisters start to grow and that is how we identified them. Persisters have peripheral accumulation (yellow arrow) or no accumulation (green arrow). Non-persister cells do not grow (red arrow) and correspond to dead cells or VBNCs.

In this experiment I used a sub-MIC concentration of Neo-Cy5, as my only marker for persister cells was cell growth. Higher concentration of Neo-Cy5 would not have let persisters to exit the dormant state.

3. Neomycin persister

In the last decade, there were so many advances in persister studies. All agreed that it is not possible to generalize the naming of persister to all non-growing cells, which tolerate any antibiotic. Persister study is becoming more and more specific to the testing conditions such as type of antibiotic or carbon source in cell culture. As my research is on aminoglycoside persister, I established a protocol to make persisters by neomycin treatment however the percentage of neomycin persister was extremely low in exponential phase as well as stationary phase cell population. In order to study persister of neomycin by microscopy I had to find sub-populations of cells, which are richer in persister cells and sort them out. Then I would be able to have at least few persister in the field of imaging on the microscope.

3.1. Sorting out neomycin persister from stationary phase population

In order to find a sub-population rich in persisters, I sorted out cells by the FACS sorter device according to cell size and Neo-Cy5 signal intensity. I treated stationary phase cells with high concentration of neomycin for long enough to kill all non-persister cells. In this condition, the percentage of persister cells was 0.05%. After elimination of the excess of neomycin by several washing steps, I treated the cells with a sub-MIC concentration of Neo-Cy5. Next, I sorted out cells according to their size and their Neo-Cy5 signal intensity in different categories, A to I (figure 6-2). After sorting, cells were collected in a 96-well microplate and persister growth was measured on a plate reader monitoring the optical density for 48h.

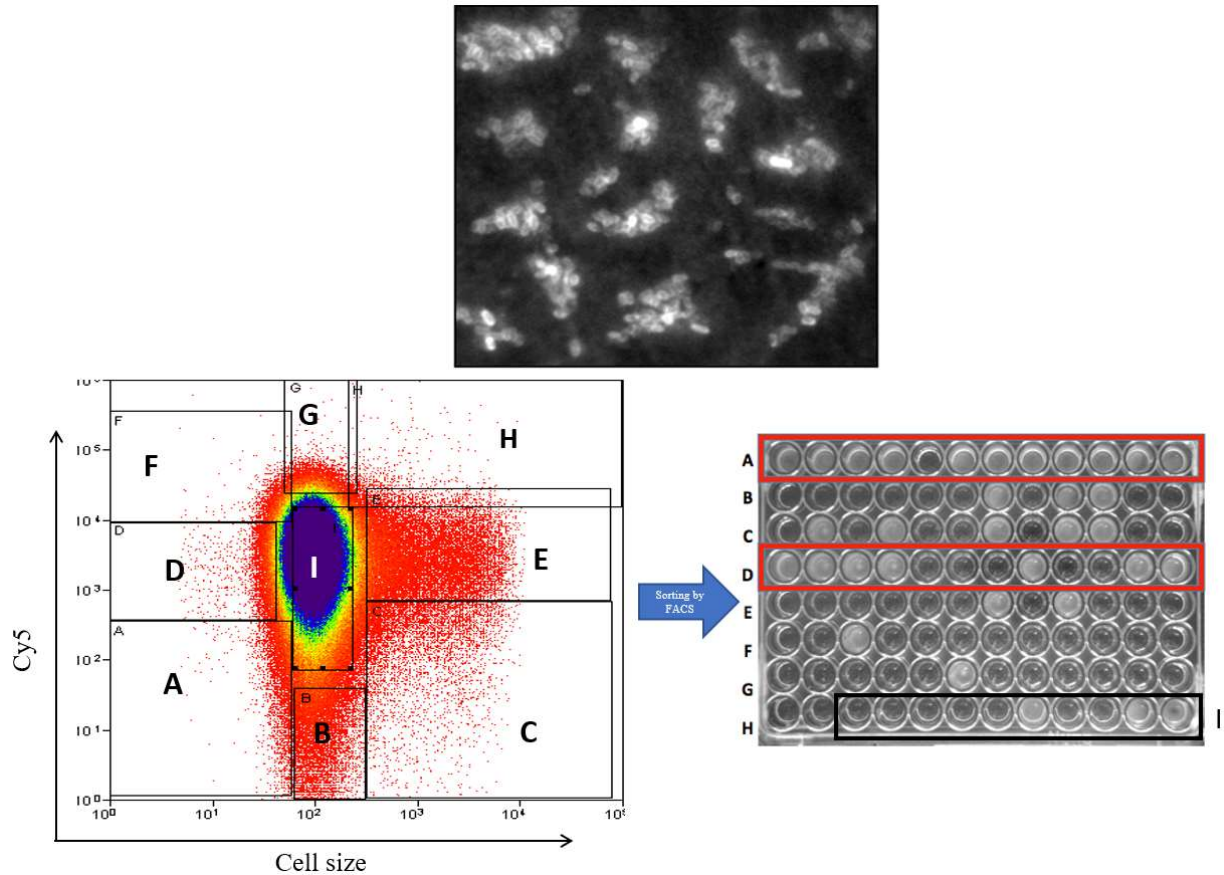


Figure 6-2 Sorting cells with FACS according to their size and Neo-Cy5 signal. *E. coli* stationary phase cells were treated by 500 μM neomycin for 6 hours to kill all the non-persister cells. Then cells were washed to eliminate the excess of neomycin. Cells were next treated with a sub-MIC concentration (0.4 μM) of Neo-Cy5. Cells were observed by fluorescence microscopy (top). Same cells analysed with FACS and sorted out into a 96-well plate filled with rich media (500 cells in each well). Cells were categorized in 9 groups according to their cell size and Cy5 signal intensity (A to I). After collecting cells, the plate was incubated at 37°C for 48h. Group A was 6 times and group D was 3 times richer in persisters in comparison to whole population.

Due to the difficulties that I had faced because of FACS device contaminations, unfortunately, I could not finalise these results. However, the preliminary results confirmed that cell population in which the cells are smaller in size and lower in Neo-Cy5 accumulation, was 6 times richer in persister population.

3.2. Sorting out cells with lower ribosomal RNA expression

In fast growing cell population, the sub-population of cells with lower expression of rRNA had been shown to make more persister cells after treating with a lethal concentration of antibiotic^{104,100}. I constructed a strain in which the unstable green fluorescent protein, *gfp*(*asv*) gene had been inserted into the chromosome under the control of ribosomal RNA promoter *rrnBP1* (figure 6-3-A). Instability of GFP protein gives us this advantage to be able to monitor the activity of the ribosomal RNA promoter. Controls showed that when cells reached the stationary phase, the GFP signals decreased as expected (figure 6-3-B).

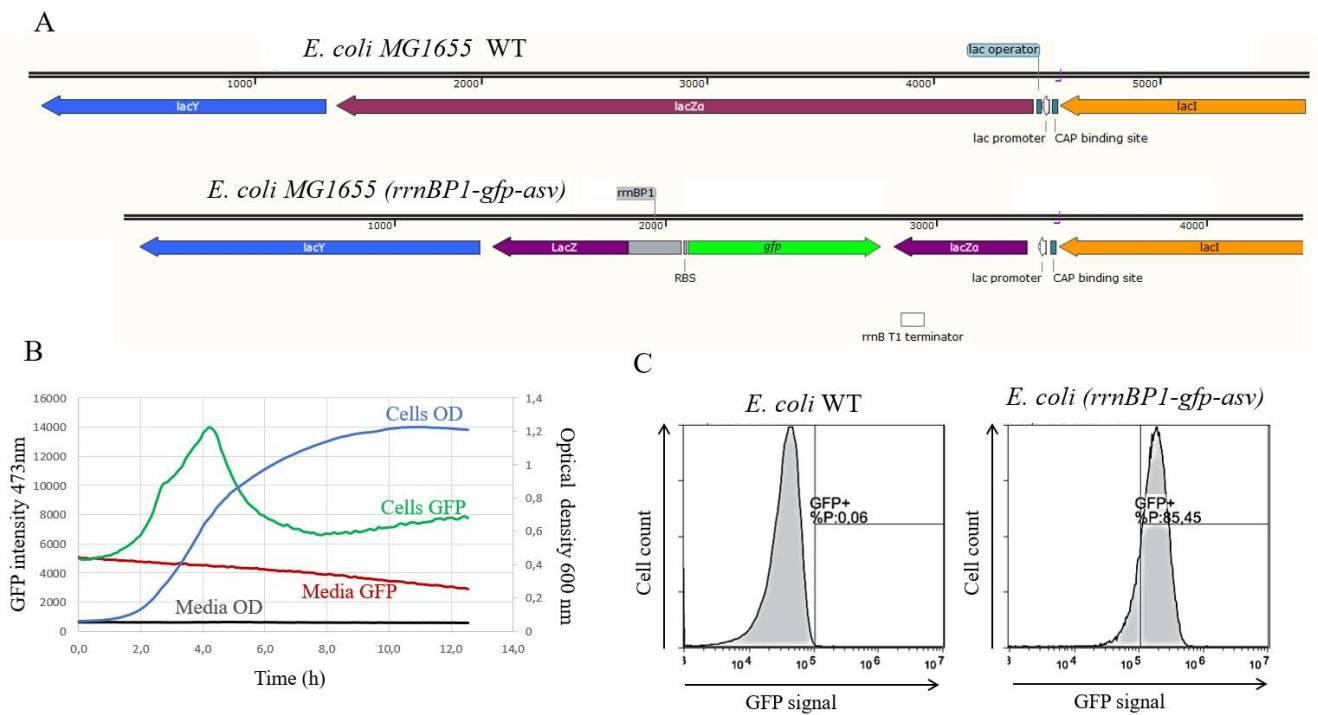


Figure 6-3. Ribosomal RNA expression as a marker for persister rich sub-population. A) chromosome insertion of *gfp*(*asv*) with *rrnBP1* in *lacZ* gene in *E. coli* MG1655. B) Kinetics measurement of GFP(ASV) expression. C) GFP fluorescence levels measured by FACS in WT and cells with coding GFP(*asv*) in exponential phase of growth.

By FACS analysis, I sorted out the dim cells and GFP positive cells from an exponential phase cell population (figure 6-3-C). Then I treated these two sub-populations with a lethal dose of neomycin to select persisters. In exponential phase cells, the percentage of persisters of neomycin was about 10^{-4} %. Because of FACS contamination and the low percentage of persisters in this condition, unluckily I was not able to prove that the dim cell sub-population is richer in persister cells.

4. Mutant of less tolerant to aminoglycoside

In Tn-seq analysis for a study of aminoglycoside tolerance by Shan *et al.*, authors analysed two aminoglycoside less tolerant strains: $\Delta serA$ and $\Delta flhC$. The *flhC* gene is coding for a flagellar transcriptional regulator and *serA* for D-3-phosphoglycerate dehydrogenase, which catalyses the first step of the phosphorylated L-serine biosynthesis pathway. In this study, stationary cells of mutant and WT strains were treated by a lethal dose of gentamicin-TR followed by FACS analysis. Interestingly, they found that only in $\Delta serA$, gentamicin accumulation was increased and accumulation levels by the strain $\Delta flhC$ were the same as WT. As we know the advantages of Neo-Cy5 to gentamicin-TR, (TR dye alone accumulates in the cells) and with Neo-Cy5 we can define the accumulation patterns by fluorescence microscopy. Therefore, I combined the two techniques of microscopy and FACS to study Neo-Cy5 accumulation patterns in those less tolerant mutants.

At first, I repeated the same protocol and treated the stationary phase cells with high concentration of Neo-Cy5 (128 μ M) for 3 hours at 37°C and then analysed the cells by FACS. On the contrary to the equivalent test performed with Gentamicin-TR, I did not detect any remarkable difference between the accumulation level of mutants *AflhC* and *AserA* when comparing to WT (Figure 6-4).

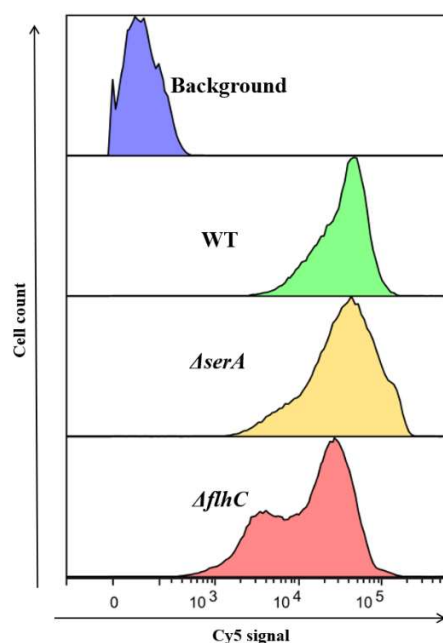


Figure 6-4. FACS analysis of Neo-Cy5 accumulation by less tolerant mutants and WT *E. coli* strains. Stationary phase cells were treated with 128 μ M of Neo-Cy5 for 3h at 37 °C. Then cells were washed and analysed by FACS.

Next, in order to characterize better the interaction of Neo-Cy5 with these mutants we investigated its binding to cell membrane. It has been reported that PMF level in these mutants is higher¹⁰⁸. Hyperpolarized membrane may stimulate peripheral binding as well as cytoplasmic accumulation. I did a series of FACS and microscopy experiences for different Neo-Cy5 concentrations and different incubation times for each mutant. To analyse the peripheral binding by fluorescence microscopy, I treated stationary phase cells shortly (2 min) with Neo-Cy5 at 32 μ M. I observed that Neo-Cy5 accumulation was only peripheral and higher in *AserA* in

comparison with $\Delta flhC$ and WT (figure 6-5). When cells were incubated longer time (150 min) with Neo-Cy5, mutants and WT have the same peripheral accumulation levels. These results were confirmed by FACS analysis (figure 6-5).

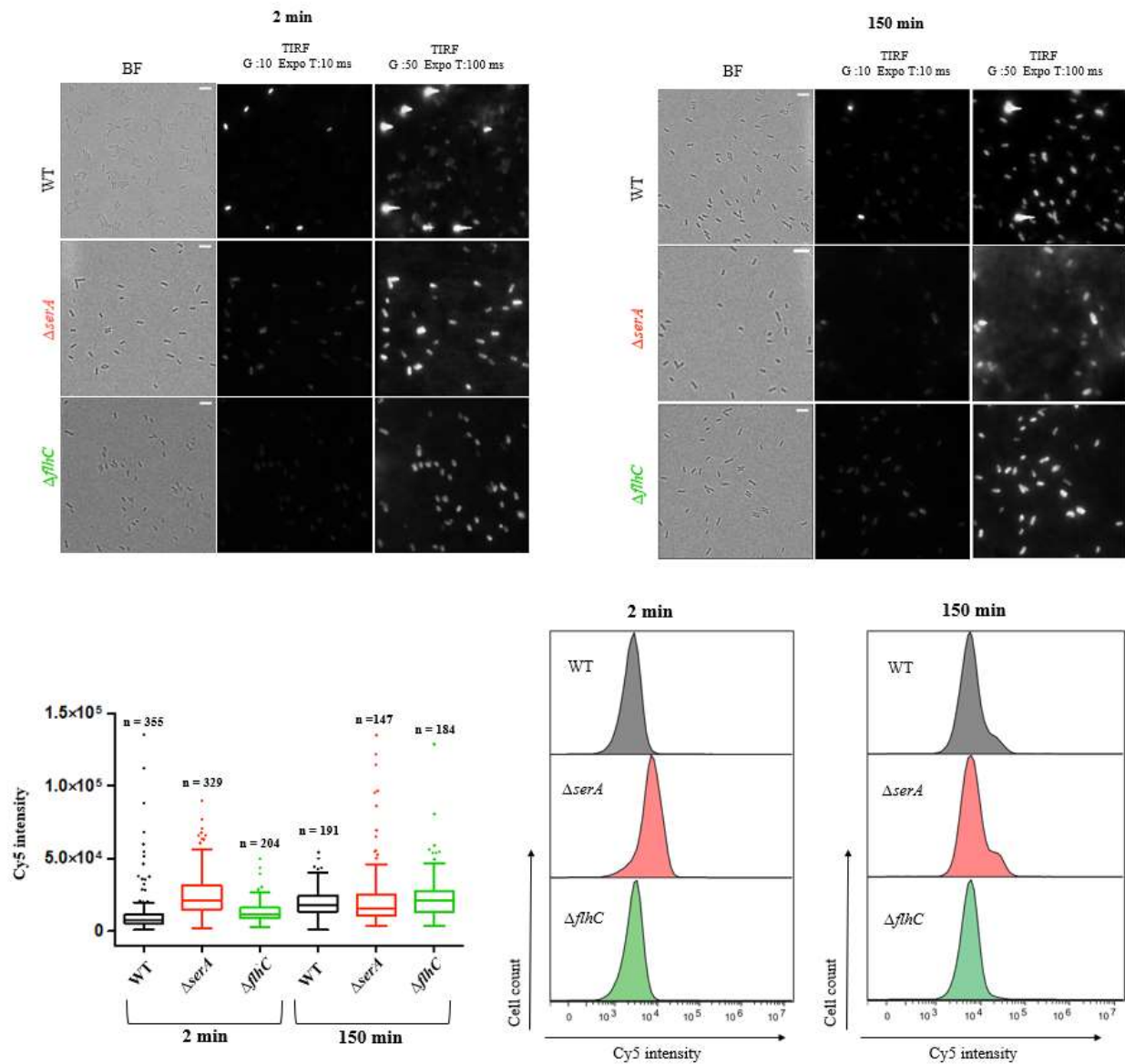


Figure 6-5 Analysis of membrane accumulation of Neo-Cy5 in aminoglycoside less tolerant mutants. Stationary phase cells treated with 32 μ M Neo-Cy5, incubated at 37°C for 2 min or 150 min. Cells were washed and analysed by microscopy (top and bottom left) or FACS (bottom right). For more clarity microscopy images are presented for two different settings. G: Gain, Expo T: Exposure Time.

Later, I tested the kinetics of accumulation of Neo-Cy5 concentration (128 μM). By FACS analysis I demonstrated that antibiotic accumulation evolves in the different strains according to the Neo-Cy5 concentration and time of incubation and further microscopy imaging are required to fully conclude on the accumulation pattern in these mutants (figure 6-6). Interestingly again for $\Delta serA$ strain at 2 min incubation level of incubation was higher than WT.

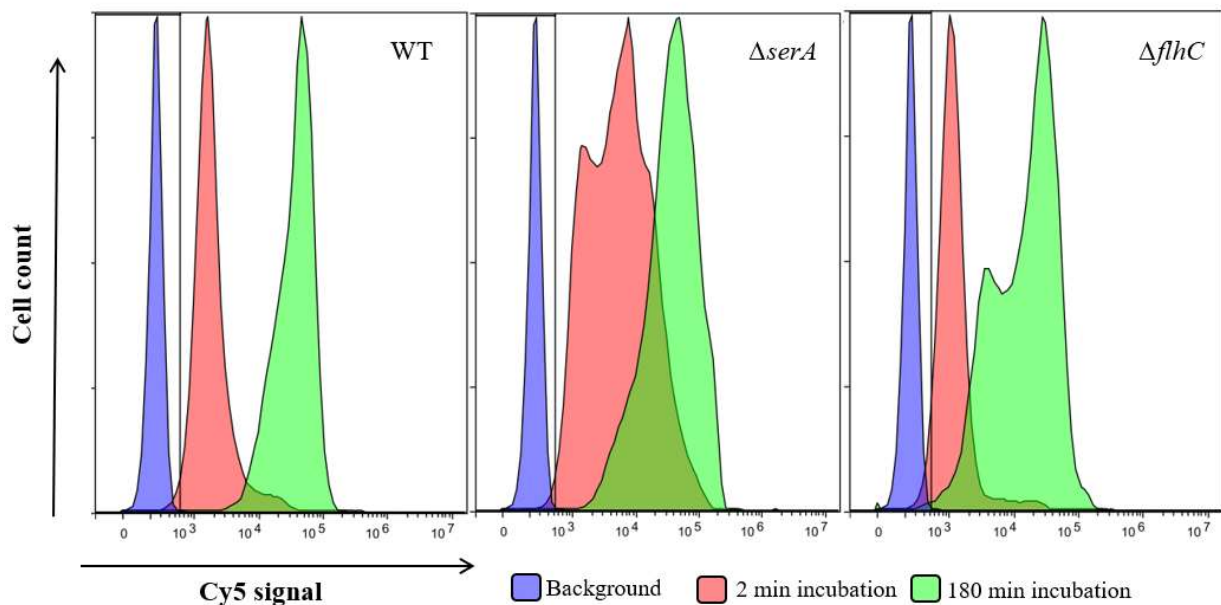


Figure 6-6. Kinetics of accumulation of Neo-Cy5 (128 μM) in less tolerant cells. Stationary cells of mutant and WT were treated by 128 μM Neo-Cy5 at 37°C. After 2 min and 180 min, cells were washed and analysed by FACS.

5. Dilution effects on persister recovery time

Persisters remain in dormant state as far as they sense the stress factor, such as antibiotics, in their environment. In the absence of antibiotic and in presence of a carbon source, persisters grow into an antibiotic susceptible population. Here I presented the effect of cell culture dilution

in shortening the transitioning time of neomycin persister cells to normal growing cells. After treatment of a cell culture with a high dose of antibiotic in order to yield persisters, cells were washed several times in order to eliminate the excess of antibiotic and to initiate the regrowth of persisters. I observe that when the cell culture (consisting of dead cells and persisters) was more diluted after the washing steps, persisters recovery time was shorter. This phenomenon was specific to neomycin persisters and not observed with ofloxacin persisters (figure 6-7).

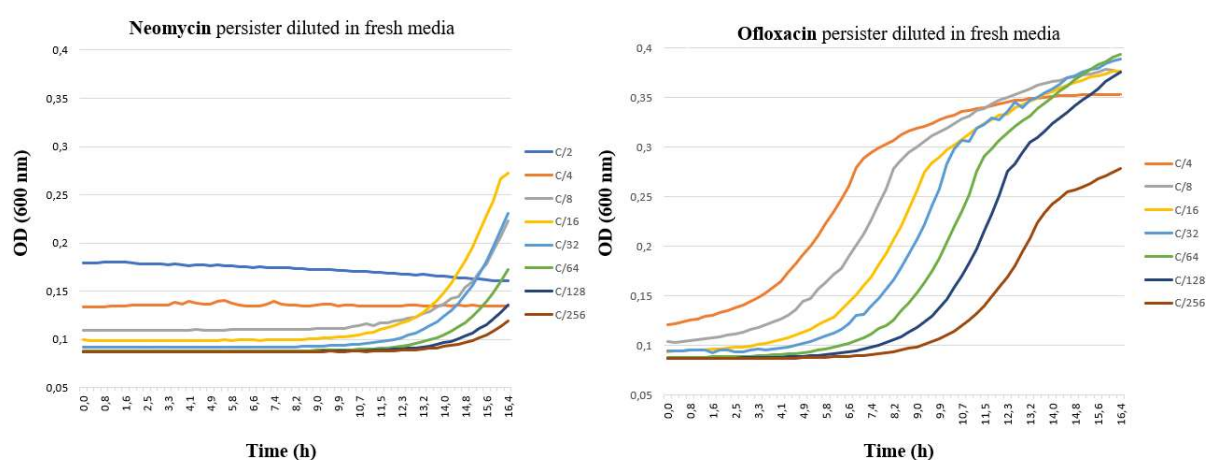


Figure 6-7. Neomycin persisters exit the dormant state when cells were diluted. Stationary cells treated with a concentration of neomycin of 500 times the MIC (left) or ofloxacin (right) at 37°C for 6 hours. Cells were then washed and diluted in fresh MOPS-G media. Cells were diluted in series: 2, 4, 8, ... ,128, 256 times (C: Cell concentration). Then cells' OD was measured using a plate reader for 16 hours.

I showed that persisters recovery was not limited due to the limitation of carbon source and the ratio of glucose per cells. Persisters for a given dilution started to grow at the same time and glucose concentration played only a role in the level of cell density at the stationary phase (Figure 6-8).

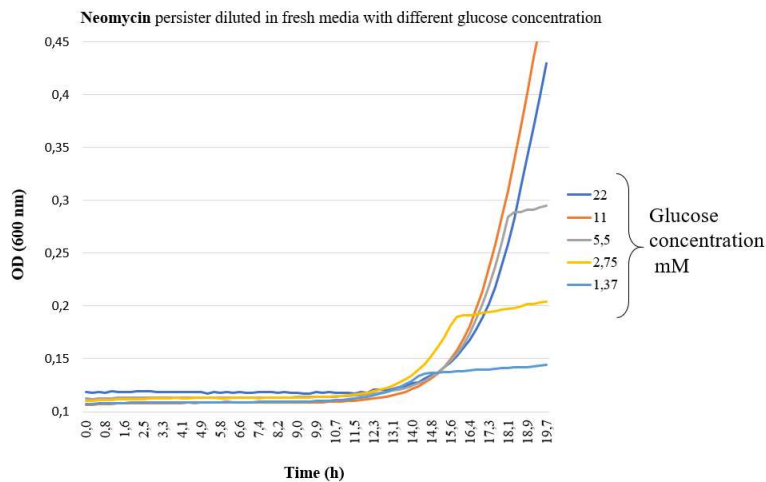


Figure 6-8. Persisters were not in carbon source (glucose) limitation during their recovery. Neomycin persisters diluted 16 times in MOPS media containing different glucose concentrations. The persister growth optical density (OD) at 600 nm was measured with a plate reader for 20h.

It seemed that there was a factor or chemical in the supernatant of dead cells and persisters that was present after treatment with neomycin. When this factor was more diluted, it allowed persisters to wake up earlier and when it was more concentrated it maintained persisters in the dormant state. When persisters were diluted in this supernatant they did not wake up at all. I called this media: pre-incubated media, which is a fresh MOPS-G media that was in contact with dead cells and persisters only for 15 min (Figure 6-9-A). Yet again, this result was specific to persisters of neomycin and not ofloxacin (figure 6-9-B). On the other hand, the supernatant of neomycin persisters had this potential to perturb growth of non-persister cells as well as persisters of ofloxacin (figure 6-9-C).

I tested the effect of pre-incubated media of neomycin persister on resistant cells to neomycin in comparison with susceptible cells. There was no effect on neomycin resistant cells (figure 6-10). I conclude that remaining neomycin in the cell culture keeps persisters in a dormant state.

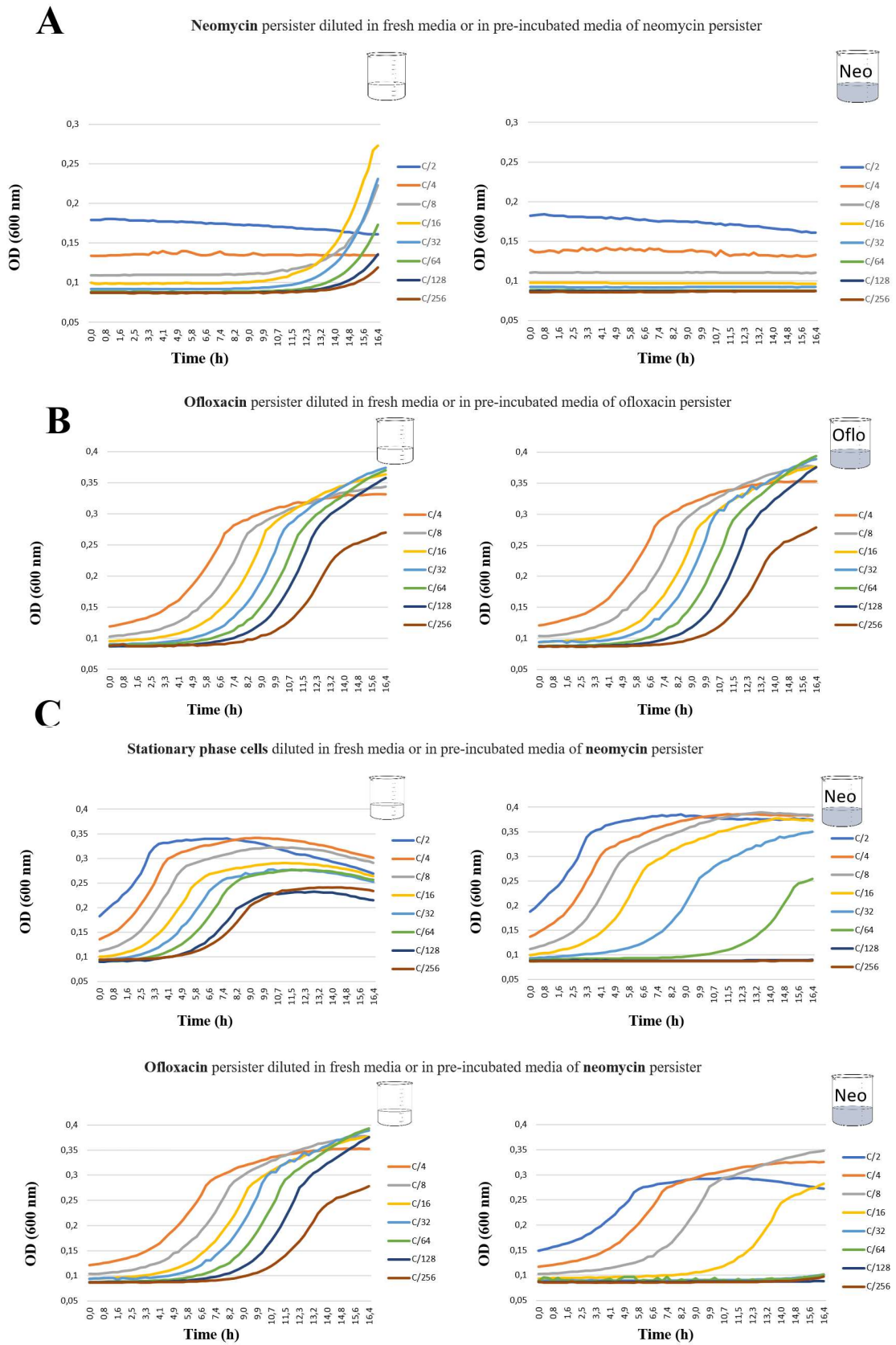
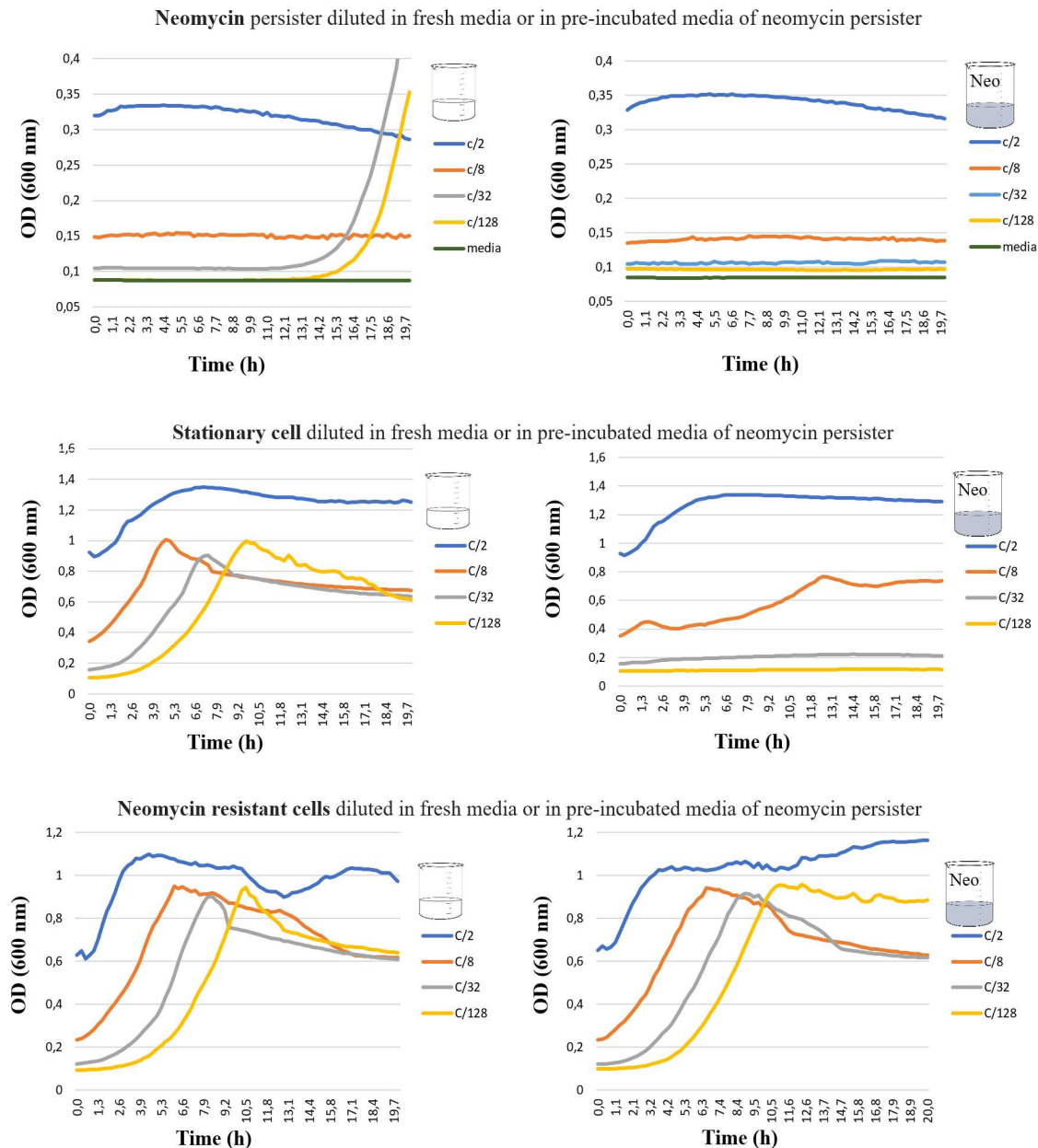


Figure 6-9 Pre-incubated media with neomycin persisters perturbed cell growth. In left column, cells

diluted in fresh MOPS-G media (indicated by a transparent beaker). In right column cells were diluted in pre-incubated media of either neomycin or ofloxacin persister (indicated by a grey beaker) A) Neomycin persisters were diluted in fresh media **or** pre-incubated media with neomycin persister. B) Ofloxacin persisters were diluted in fresh media **or** pre-incubated media with ofloxacin persister. C) Stationary phase cells and ofloxacin persisters were diluted in fresh media or pre-incubated media with neomycin



persisters.

Figure 6-10 Pre-incubated media with neomycin persisters did not perturb cell growth of neomycin resistant cells. In left column cells diluted in fresh MOPS-G media (indicated with transparent beaker).

In right column cells were diluted in pre-incubated media of neomycin persister (indicated with grey beaker). Pre-incubated media with neomycin persister kept persisters of neomycin in dormant state (top) and perturbed growth stationary phase of neomycin susceptible cells (middle). However, it had no effect on neomycin resistant cells (bottom).

6. Conclusion

In this chapter, I investigated the accumulation of Neo-Cy5 in persisters and mutants, which are less tolerant to aminoglycosides. I showed by time-lapse fluorescence microscopy that persisters have peripheral accumulation of aminoglycosides. I studied accumulation patterns of Neo-Cy5 in the less tolerant mutants $\Delta serA$ and $\Delta flhC$ by FACS. Contrary to the study by Shan *et al.*, I did not find a difference between the accumulation levels of tagged aminoglycoside in these mutants when compared to levels of accumulation by WT strains. I found that $\Delta serA$ had only slightly higher peripheral accumulation levels in short incubation time.

In this chapter, I demonstrated the effect of the supernatant of aminoglycoside persisters and cell dilution in the recovery of persisters. Neomycin, which remained in the media, probably originated from the dead cells and contributed to inhibit persisters' growth. This phenomenon had an important effect on the persister recovery study. In such study, washing steps and cell dilution must be kept constant in order to have the same percentage of persisters that resume growth in different analysis. I showed that the remaining traces of neomycin in the cell culture maintained persisters in the dormant state. However, how dormant cells sense the presence of antibiotic in the media remains a mystery.

Chapter VII results: Microfluidic

1. Introduction

In order to be able to observe the accumulation of Neo-Cy5 at single cell level by fluorescence microscopy, I used a microfluidic device. Such a device would allow us to capture Neo-Cy5 interaction with bacterial cells in real time. In microfluidic channels, we can wash away the access of Neo-Cy5 by changing the media while bacterial cells stay trapped in the channels. Other advantages of microfluidics are that we use very small quantities of pricy Neo-Cy5.

Another application, which is planned here, is to keep persister cells in the dormancy state with a media lacking carbon sources and incubate cells with Neo-Cy5. Next, the media will be exchanged for a fresh media containing a carbon source allowing for persisters to resume growth. This step will be recorded in real time imaging.

Here I presented the design of the microfluidic system and its fabrication as well as specific required surface treatment.

2. Design

I used a mold of a microfluidic circuit, which has been developed in the laboratory of Bruno Le Pioufle at ENS Paris-Saclay. Using this mold, I made a Polydimethylsiloxane (PDMS) circuit, which reproduces exactly the design of the mold. I bonded the PDMS circuit on the coverslip by surface activation *via* plasma treatment. This circuit has 2 input and 2 output channels (figure 7-1-A). The outer input (input 1) is for loading the bacterial cells. The cells are trapped in the channels (Figure 7-1-B, C). The second input is for loading media containing Neo-Cy5. Neo-Cy5 enters to the bacterial chambers through 1-micron wide connector channels.

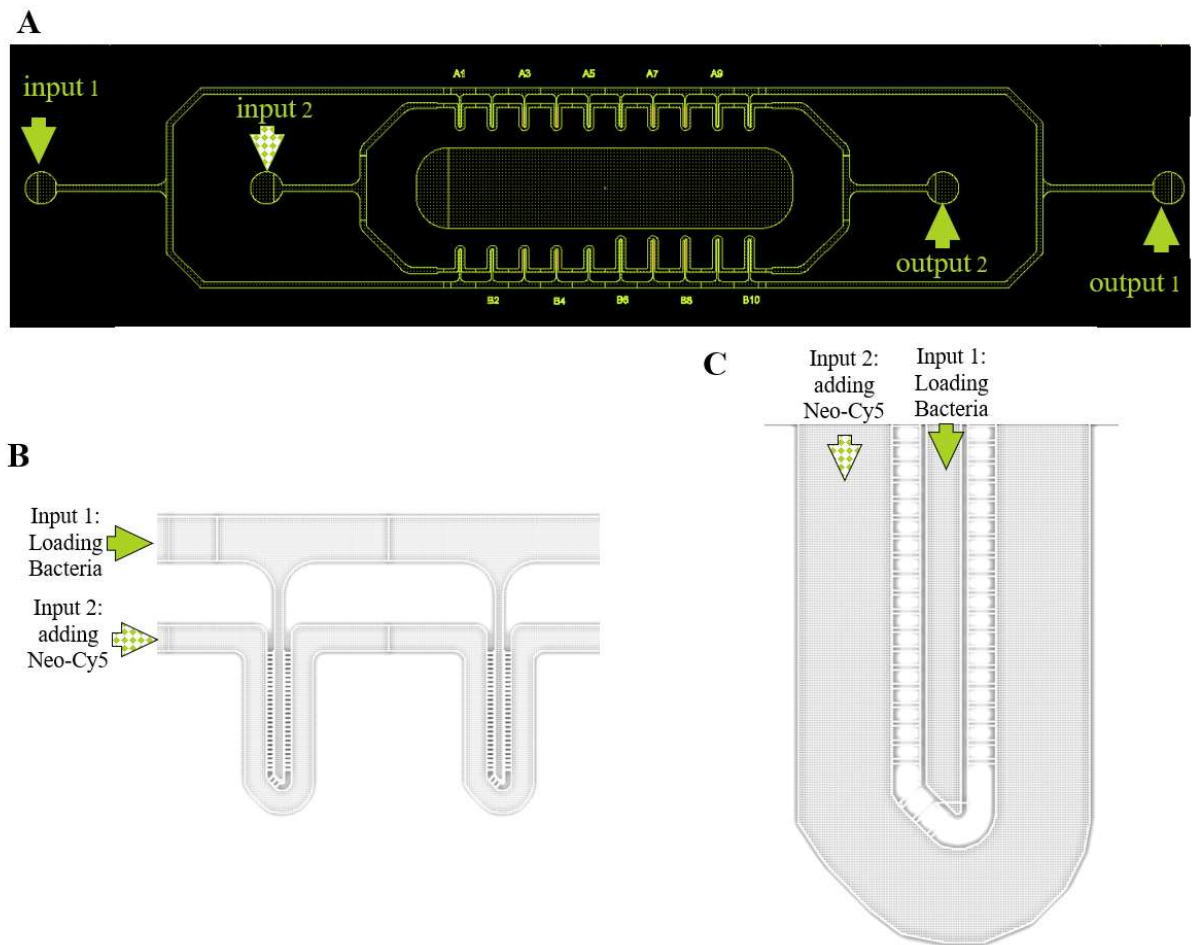


Figure 7-1. Design of microfluidic device. A) plan of the circuit. It has 2 inputs and 2 outputs. B) Bacterial cells were loaded in the circuit from outer input 1 and the media containing Neo-Cy5 was added from input 2. C) Cells were trapped in the channels and the solution of antibiotic arrived into contacts with the bacteria *via* a 1-micron wide channel connector.

Aminoglycosides are known to easily attach non-specifically to surface such as glass. Therefore, I ran a pilot test on this circuit, and I observed that indeed Neo-Cy5 was attaching to the surface of the coverslip during TIRF microscopy; I was not able to observe the bacteria due to the very high background level (figure 7-2).

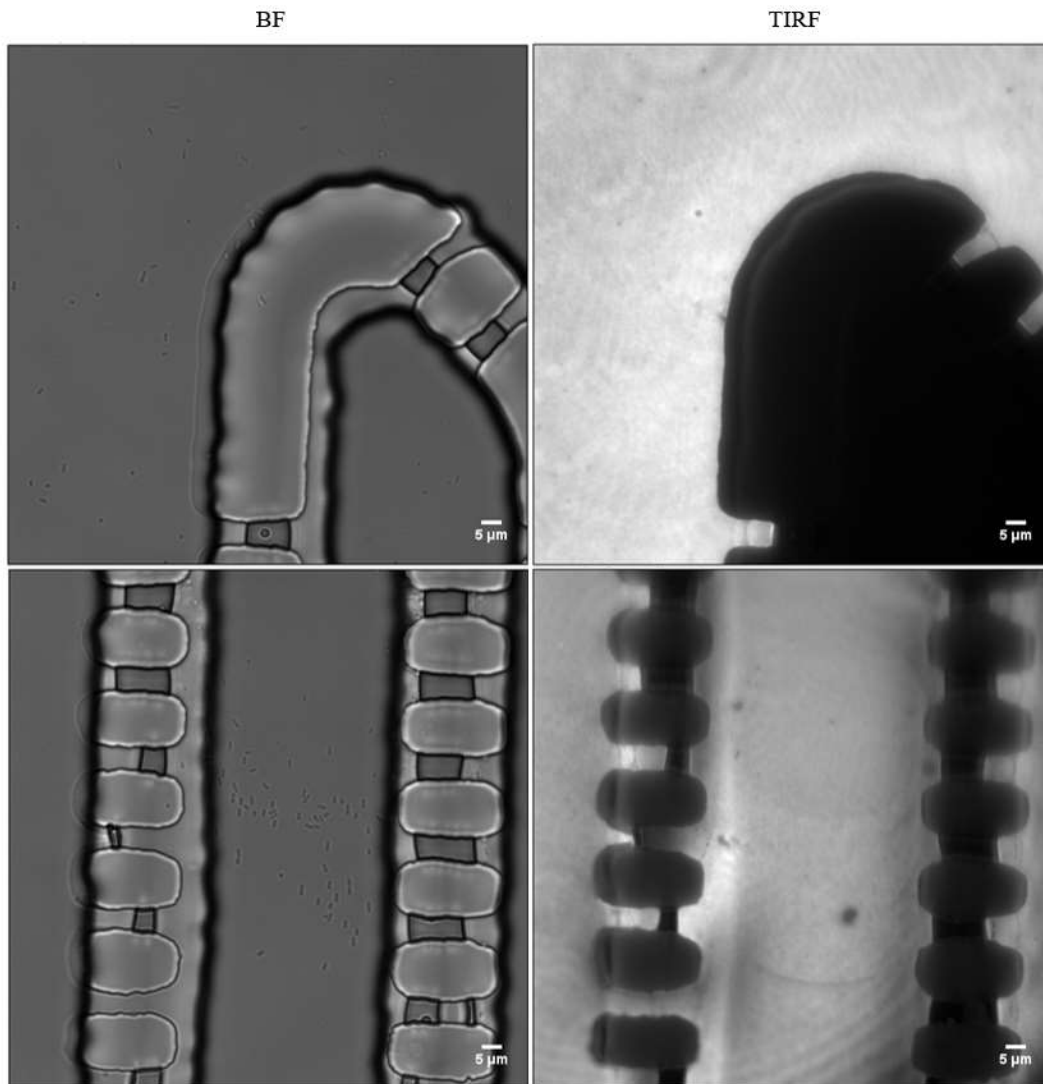


Figure7-2. Neo-Cy5 binds to hydrophilic surface causing a very high background. Left panel: Bright field images of microfluidic channels. Bacteria are visible. Right panel: fluorescence microscopy images of the same field of view. Due to high background level, Neo-cy5 interaction with bacteria is not visible.

3. Coating

Neo-Cy5 is a positively charged molecule and I expected that it would attach to the surface of the microfluidic circuit especially the glass. However, I was surprised by the higher than expected level of background. To tackle this problem, I ran a test to understand its cause.

For making a microfluidic circuit, PDMS needs to be bonded to the glass coverslip. Both hydrophobic surfaces were treated by plasma. Plasma treatments eliminate hydrocarbon groups on both surfaces and leave behind OH groups and make the surface hydrophilic. This enables solid Si–O–Si covalent bonds to form between the two surfaces (Figure 7-3-A). However, inside walls of the channels and chambers, the surface remains hydrophilic. I had the assumption that the hydrophilic surfaces in the circuit would lead to a very high attachment of Neo-Cy5 to the surface.

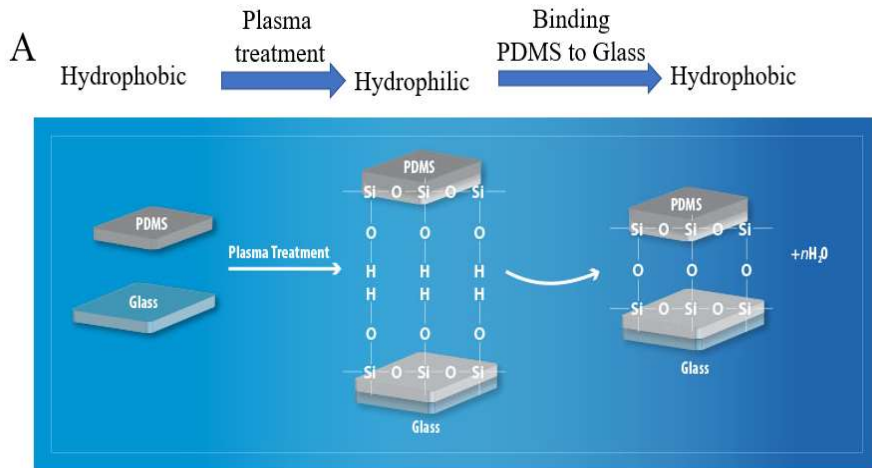
I ran a test on the coverslip with plasma treatment based on the principle of contact angle measurement. The shape of water drops on a hydrophobic or hydrophilic surface is visually analyzed. (Figure 7-3-B).

I tested the hydrophobicity of coverslips, which were treated by plasma and then kept them either in water or in contact with air and made a comparison with non-treated coverslips. I concluded that it takes 60 days after plasma treatment for a glass surface to become hydrophobic if it was kept in contact with air (Figure 7-3-C).

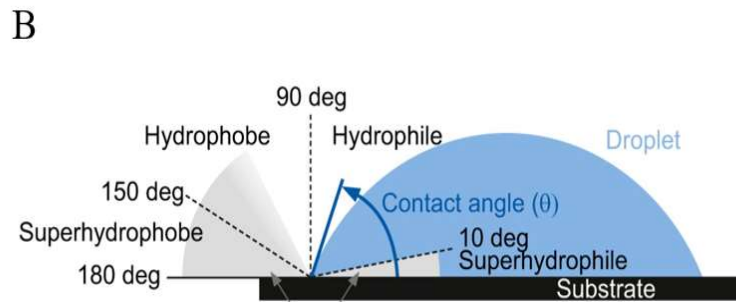
I also ran a test to see how much the attachment of Neo-Cy5 increases due to the plasma treatment of coverslip. I measured the intensity of Neo-Cy5 in a coverslip by spectrophotometer and confirmed that Neo-Cy5 bind excessively to hydrophobic glass surface (Figure 7-4-B).

To make a coverslip and PDMS surface (walls of channel) hydrophobic after plasma treatment I treated inside the microfluidic circuit by mPEG-Silane, which binds to the -OH of both surfaces. The PEG-silanized surface is hydrophobic (Figure 7-4-A). I confirmed lower Neo-Cy5 attachment level of PEG-silanized surface when comparing to non-treated one with a spectrophotometer and fluorescence microscopy. Neo-Cy5 attachment is decreasing 6-fold on a

plasma treated surface after mPEG-Silane treatment and I was able to observe bacterial accumulation with lower background (Figure 7-4-C).



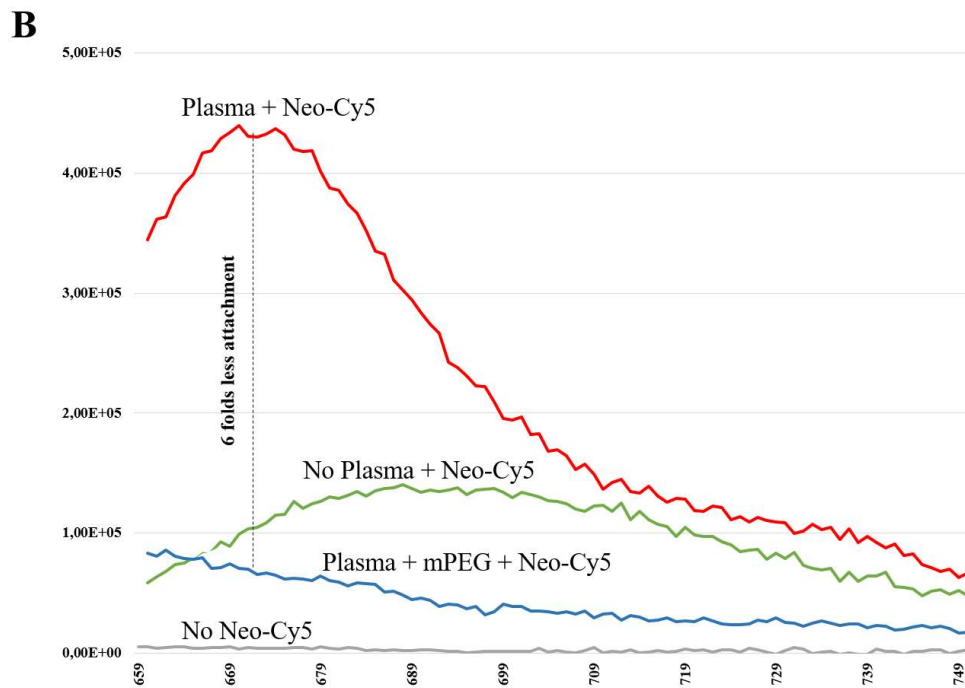
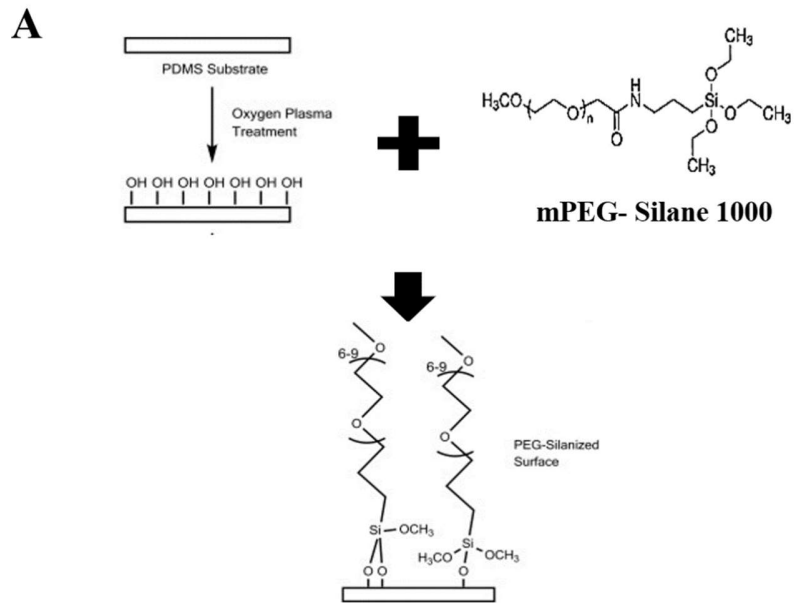
Principle of contact angle measurement



C

	3 days after plasma treatment	8 days after plasma treatment	15 days after plasma treatment	60 days after plasma treatment
Coverslip treated with plasma keep in water				
Coverslip treated with plasma keep in contact with air				
Control (not treated coverslip)				

Figure7-3. Plasma treatment makes the surface hydrophilic for a long time. A) Coverslip and PDMS become hydrophilic after plasma treatment, which leads to free -OH groups. This allows forming covalent bonds between the PDMS and the glass. B) Principle of contact angle measurement. C) Evolution of the hydrophilic property of a coverslip after plasma treatment as a function of time.



C

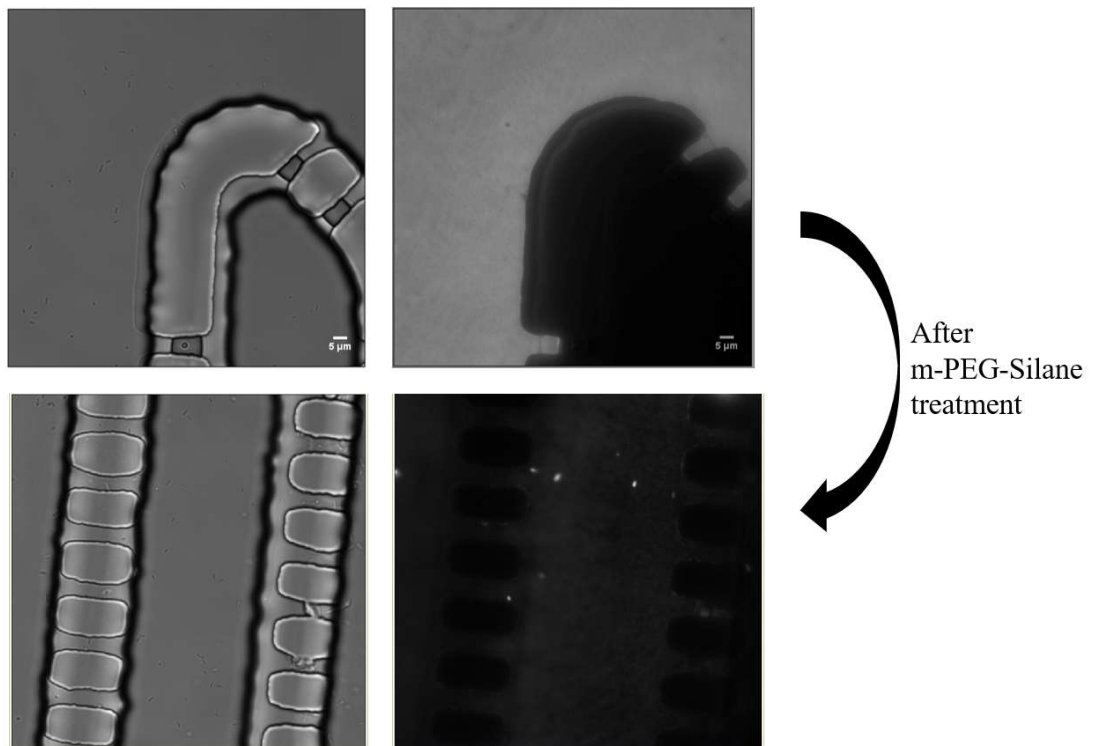


Figure 7-4. m-PEG Silane treatment turns a hydrophilic surface into a hydrophobic one and decreases Neo-Cy5 attachment. A) Formation of m-PEG-silanized surface. B) Measurement of Cy5 signals on a spectrophotometer on a coverslip either treated by plasma and m-PEG-Silane or not. Then coverslips were treated with Neo-Cy5 (0.4 μM) and washed several times before measurement. C) Bright field (left) and TIRF (right) microscopy images. Comparison of Neo-Cy5 attachment to the surface, before m-PEG-Silane treatment (top) and after (bottom). Bacteria that accumulated Neo-Cy5 are visible on the device with the m-PEG-silanized surface (bottom).

4. Conclusion

Here I presented the microfluidic circuit design that I planned to use for real time Neo-Cy5 accumulation and persister recovery microscopy imaging. I treated the channels' wall to be hydrophobic right after plasma treatment. I showed the proof of principle of how to prepare a microfluidic device suitable for Neo-Cy5 accumulation study.

However, due to time limitation I could not ran more test to record a time-lapse of the entry of Neo-Cy5 in the fast-growing cells as well as Neo-Cy5 accumulation in persisters and their recovery.

Chapter VIII: Discussion and Perspective

In this study, I presented the newly made fluorescently-tagged aminoglycosides with preserved bactericidal properties. I used these conjugates to track down the interaction of aminoglycosides at single cell level by fluorescence microscopy. I combined fluorescence microscopy and FACS analysis to measure aminoglycoside accumulation in the cells at different time points to capture the kinetics of antibiotic penetration.

This study showed that there are two accumulation patterns for Neo-Cy5 in cells: in the first step there is a peripheral accumulation, which corresponds to specific binding to cell membrane. We also find that peripheral accumulation levels can be different, which again showed the importance of defining aminoglycoside accumulation patterns by our methodology.

Next, there is cytoplasmic accumulation in which the antibiotic is entering into the cytoplasm. According to microscopy time-lapse study, low levels of cytoplasmic accumulation are tolerated by cells and did not cause cell death. Further study needs to investigate the nature of tolerance in some cells despite the presence of aminoglycoside in the cytoplasm, which causes a low level of miscoding and misfolded proteins in those cells.

Using FACS analysis, I used an inhibitor of EDPI and EDPII and proved that with this technique we can distinguish different steps of aminoglycoside accumulation. Moreover, I found that in a dead cell population, after aminoglycoside treatment only a small sub-population of cells was accumulating PI (marker of dead cells due to membrane damage). This result can give us hints on the fact that maybe the membrane damage is not finally the major reason why cells are killed after aminoglycoside treatment.

During protocol adjustment, I found that aminoglycosides can enter into the cytoplasm as a result of mechanosensation and activation of mechanosensitive channels. This was the first time that we observed that mechanical manipulation of cells leads to opening of mechanosensitive channels causing massive cytoplasmic accumulation of an aminoglycoside. This unpredictable result may lead us to a better understanding of the mechanism of aminoglycoside entrance into the cytoplasm. Moreover, my results showed that the activation of MS channels occurred in a sub-population of cells. The extent of this sub-population was found to vary according to cell concentration and dilutions. How cell concentration can influence the mechanosensation in the bacterial cells remains unknown.

In order to investigate more the interaction of MS channels and aminoglycosides, we can explore and track down Neo-Cy5 interaction with the membrane in cell in which MS channel monomers will be fused with fluorescent proteins. By combining STORM and PALM super-resolution microscopy we might have more information in the role of MS channels in entrance of aminoglycoside into the cytoplasm. It will be also interesting to study the effect of Neo-Cy5 on MscL using the patch-clamp technique.

Prior to this study, it was only possible to know the levels of accumulation of aminoglycosides and not the corresponding patterns. I applied our method to investigate aminoglycoside accumulation in two mutants of *E. coli*, which are less tolerant to aminoglycoside and defined their patterns of accumulation. These results differ from previously published data

108.

After studying aminoglycoside accumulation in fast growing cells, I studied accumulation of Neo-Cy5 by persisters. By fluorescence microscopy, I showed that persister cells have

peripheral accumulation of Neo-Cy5. Thereby in these cells, there is no strong cytoplasmic accumulation.

While studying persisters of neomycin, I found that sub-MIC concentrations of neomycin can be sensed by neomycin persisters, which prevents them from exiting the dormant state. However, it is uncanny, how in dormant cells, low concentration of aminoglycoside has been sensed. We can think about a hypothesis that this phenomenon involves quorum sensing mechanism and a receptor on cell membrane by which cells can sense antibiotic stress in cells' environment.

Finally, I developed a coated microfluidic system, which is adapted to our antibiotics for studying in real time drug accumulation by persister cells.

Chapter IX: Material and methods

Medium compositions

LB: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl

MOPS : 1.32 mM K₂HPO₄, 9.52 mM NH₄Cl, 0.523 mM MgCl₂, 0.276 mM K₂SO₄, 0.01 mM FeSO₄•7H₂O, 5 μM CaCl₂, 50 mM NaCl, 40 mM MOPS, 4 mM Tricine, 0.003 μM (NH₄)₆(MO₇)₂₄•4H₂O, 0.4 μM H₃BO₃, 0.03 μM CoCl₂(II)•6H₂O, 0.01 μM CuSO₄(II)•5H₂O, 0.08 μM MnCl₂•4H₂O, 0.01 μM ZnSO₄•7H₂O

MOPS supplemented with 0.4 % (w/v) glucose (G) or with 0.2 % (w/v) glucose and 0.2 % casamino acid (CAA).

Strains used in this study

Escherichia coli strains

MG1655: Wild type strain

F⁻, lambda⁻, ilvG⁻, rfb-50, rph-1

MG1655 Δ lacZ :rrnBP- *gfp*(*asv*)

Single mutants:

MG1655 Δ serA

MG1655 Δ flhC

MG1655 Δ mseL

MG1655 Δ mseS

MIC measurement

The MICs were determined using a liquid culture assay. Aliquots of an overnight culture of *E. coli* MG1655 or mutant $\Delta mscL$ and $\Delta mscS$ were diluted into medium containing 2-fold serial dilutions of antibiotic to an OD_{600nm} of 0.04. Growth was measured by turbidity at each concentration following 16 h of incubation at 37 °C. Measurements were performed on 200 μ L samples in a 96-well microtiter plate with transparent lid using an InfiniteM200pro (TECAN). For Neo-Cy5, absorbance was measured at 400 nm or 500 nm instead of 600 nm to minimize any Cy5 excitation.

Treatment of cells with CCCP

E. coli cells MG1655 from an overnight culture in MOPS supplemented with 0.4 % (w/v) glucose (MOPS-G) were diluted into the same medium. Cells were grown for 2 h at 37 °C with or without 30 μ M CCCP before the addition of the antibiotic. The number of live cells, at the time of CCCP addition, was estimated as 5×10^7 colony-forming units per milliliter (CFU/mL). After a 4.5-h incubation at 37 °C with neomycin or Neo-Cy5, cell survival was measured in CFU/mL. The concentration of Neo-Cy5 stock solution was estimated from the absorbance at 649 nm measured in 10 mM Tris-HCl, pH 7.5.

Neo-Cy5 uptake

For the liquid culture, MOPS supplemented with 0.4% glucose (MOPS-G) was used. For experiments involving single-deletion mutant of genes *serA* and *flhC* MOPS contained 0.2% glucose and 0.2 % of casamino acids. Overnight cultures were diluted to an OD₆₀₀ of 0.037 with fresh medium and grown until the OD₆₀₀ reached 0.2. Cell (diluted or not according to the

experiment) was incubated with indicated concentration of Neo-Cy5 (or Cy5) dissolved in milliQ water. Cells were incubated in a heat block at 37 °C without shaking.

To remove Neo-Cy5 not tightly bound or internalized 2 methods were used:

First method: washing cells by filtering. Cells were filtered on HAWP mixed cellulose esters (MCE) membranes 0.45 µm-pore-size (Merck Millipore) mounted on a micro-sample filtration Minifold II (Schleicher & Schuell) and washed (typically 3 times unless it was mentioned otherwise) with MOPS-G prewarmed at 37°C.

Second method is by centrifugation of cells and then changing the supernatant by fresh media. Cell suspension in the fresh media was done by different methods such as mixing cells by pipetting or shaking with a tube holder tray at maximum for 1 min.

Immediately after, the section of the membrane containing cells was placed into an Eppendorf tube containing MOPS-G and cells were recovered by gentle up and down pipetting. Cells were immediately placed on 1% agarose pad for imaging by fluorescence microscopy, super-resolution microscopy or analyzed by FACS or spotted on MOPS-G or LB plate for CFU survival test.

In the indicated experiments cells were incubated with 25 µg/mL chloramphenicol for 2 min at 37°C or with rifampicin at 30µM for 10 min at 37°C prior to Neo-Cy5 addition.

In the experiment with PI, cells were incubated with 30 µM PI dissolved in DMSO for 10 min at 37 °C before analyses.

Fluorescence microscopy

Fluorescence images were taken with an EMCCD camera (Photometrics) through a 63X TIRF objective (Zeiss, NA 1.43) mounted on an inverted Zeiss Axio Observer Z1 microscope.

Image acquisition was done with the Metamorph software package (Molecular Devices). Cy5 illumination was performed using a 642-nm laser (0.002 kW/cm², Roper Scientific) and a filter set was used for fluorescence excitation and emission (Chroma Technology; ET 532/640 nm Laser Dual Band; excitation filter 530/20 nm and 638/25 nm, dichroic mirror 585/70 nm and 650 nm (long pass), and emission filter 580/70 nm and 700/100 nm). Neo-Cy5 uptake was typically observed with an electron-multiplying gain of 1 or 50 and an exposure of 10 to 100 ms. Image analyses were performed using ImageJ.

FACS analysis

To measure Cy5 fluorescence, cells were analyzed with a BD AccuriTM C6 flow cytometer (BD Biosciences). Excitation was performed with a 640-nm diode laser (14.7 mW), and emission was detected through a 675/25 nm band pass filter.

PMF measurement

Cells in different cell culture and different concentration were incubated with 10 µg/mL DiBAC4-(3) dissolved in water or DMSO at 37°C for 2 min in dark. Then cells were analyzed by FACS directly.

Strain construction

gfp(asv) gene under control of *rrnBP1* short promoter (kindly provided by Christophe Beloin lab) inserted in *lacZ* gene position in *E. coli* MG1655 by conjugation of plasmid containing *rrnBP-gfp(asv)* gene surrounded by upstream and downstream homologous region for *lacZ* gene. After induction of double homologous recombination, constructed colonies were selected and verified by PCR.

Single-deletion MG1655 strains

E. coli single-deletion strains from the Keio collection ¹⁴³ were transferred in the *MG1655* background by P1 transduction and validated by PCR. Kanamycin resistance was then eliminated by thermosensitive plasmid coding for Flp recombinase.

Persister formation

Stationary phase cells in MOPS-G-CAA media were incubated for 16h at 37°C in a shaker incubator then treated by either 500µM of neomycin or 5µg/mL ofloxacin for 6h at 37°C with agitation at 220 rpm. Then cells were centrifugated and washed two times with MOPS without any carbon source or PBS. Then cells were plated for CFU survival test or incubated with Neo-Cy5 at 0.4 µM for 2 min and washed by centrifugation for elimination of the excess of Neo-Cy5 and then analyzed by microscopy or FACS.

Persister recovery assay (persister pre-incubated media preparation)

Stationary phase cells in MOPS-G-CAA media were grown for 16h at 37°C in a shaker incubator then treated by either 500µM of neomycin or 5µg/mL ofloxacin for 6h at 37°C with agitation at 220 rpm. Then cells were centrifugated and washed twice with MOPS without any carbon source.

Cells were incubated in fresh MOPS media for 15 min then cells' supernatant separated by centrifugation and called persister pre-incubated media. Cells now diluted either with the persister pre-incubated media or with fresh media and persister growth was measured in a 96-well microtiter plate with transparent lid using an InfiniteM200pro (TECAN) at 600nm.

Microfluidic circuit coating

After bonding the PDMS and coverslip together or coverslips alone after plasma treatment were treated 2h with a solution of mPEGsilane (600 MW) 2mM in ethanol/H₂O/acetic acid 94.8/5/0.2 v/v/v. Then it was washed by ethanol followed by milliQ water extensively.

Chapter X Manuscript of submitted paper

Please contact Dominique.FOURMY@i2bc.paris-saclay.fr for requesting the paper manuscript.

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Chapter XII Résumé en Français

Les aminoglycosides forment une famille d'antibiotiques composés de sucres modifiés par des amines qui ciblent les ribosomes bactériens. Quelques exemples de cette famille sont la néomycine, la gentamicine et la streptomycine. Les aminoglycosides ont été les premiers médicaments thérapeutiques importants, qui ont été produits par des voies de fermentation dans les bactéries. En 1943, la streptomycine a été découverte dans le laboratoire de Selman Waksman à partir d'une bactérie du sol : *Streptomyces griseus*¹. La streptomycine est le plus ancien agent moderne utilisé contre *Mycobacterium tuberculosis*. Depuis les années 1940 jusqu'à aujourd'hui, les aminoglycosides sont utilisés dans le monde entier, en raison de leur grande efficacité et de leur faible coût².

Lorsque les aminoglycosides se lient aux ribosomes, ils perturbent la lecture du code génétique ou inhibent la synthèse des protéines, ce qui entraîne la mort des cellules. Les aminoglycosides s'avèrent être des inhibiteurs de translocation^{3, 4} et augmentent la rétro-translocation^{5, 6, 7}. La majorité des aminoglycosides se lient à la sous-unité ribosomale 30S au niveau d'une boucle interne de l'hélice 44 (h44) qui forme principalement le site de décodage⁸.

Bien que la découverte de ces antibiotiques remonte à plus d'un demi-siècle, il existe de nombreux faits sur le mécanisme d'action des aminoglycosides qui restent inconnus. Les aminoglycosides doivent être transportés à travers la membrane cytoplasmique pour atteindre leur cible, les ribosomes.

L'accumulation d'aminoglycosides dans le cytoplasme dépend de la force motrice des protons (PMF) dans les cellules. Il est suggéré que l'accumulation d'aminoglycosides par les

bactéries se développant dans des conditions aérobies se produit en trois étapes successives ⁹ (figure 1).

Dans un premier temps, les médicaments se liaient aux sites anioniques situés sur la membrane extérieure de manière instantanée et réversible. Il a également été proposé que dans les bactéries Gram-négatives, les lipopolysaccharides (LPS) interagissent directement avec les aminoglycosides polycationiques, ce qui favoriserait leur absorption ¹⁰. Auparavant, un mécanisme d'absorption auto-promu pour les aminoglycosides était favorisé en termes d'hypothèse plutôt qu'un mécanisme impliquant les porines ^{11, 12}, cependant, une étude récente a montré que la sous-population de cellules ayant une expression plus faible de la porine C de la membrane externe (ompC) sont plus tolérantes à la kanamycine ¹³. Dans ce mécanisme, l'antibiotique interagit avec la paroi cellulaire externe et cette interaction augmente la perméabilité de cette paroi cellulaire à l'antibiotique. Cette étape serait suivie de deux phases irréversibles dépendantes d'énergie, appelées phases dépendantes d'énergie I et II (EDPI et EDPII, respectivement).

Dans l'EDPI, l'antibiotique pénètre dans le cytoplasme et atteint les ribosomes, ce qui entraîne un mauvais décodage du code génétique et la production de protéines mal repliées. L'EDPI dépend du niveau d'énergie cellulaire. On a également observé une relation entre le taux de croissance des bactéries avant l'ajout de l'antibiotique et le niveau d'accumulation ¹⁴. Les cellules bactériennes en phase exponentielle se sont avérées très sensibles à l'aminoglycoside en raison d'un niveau de PMF plus élevé. Au contraire, les populations de cellules en phase stationnaire sont moins sensibles aux aminoglycosides. L'EDPI correspond à l'entrée de l'aminoglycoside dans le cytoplasme. L'EDPI peut être bloquée par des inhibiteurs du transport d'électrons tels que le cyanure de carbonyle m-chlorophénylhydrazone (CCCP). Cependant, le

mécanisme par lequel les aminoglycosides traversent les membranes et entrent dans le cytoplasme est inconnu à ce jour (Figure 1).

La troisième étape est la phase II dépendante de l'énergie (EDPII), au cours de laquelle l'antibiotique pénètre dans le cytoplasme en plus grande quantité en raison des dommages causés à la membrane par l'EDPI. Cette étape d'absorption des aminoglycosides reste également floue. Elle nécessite une synthèse des protéines donc de l'énergie et possède une cinétique de diffusion¹⁵. La phase EDPII nécessite des ribosomes sensibles aux aminoglycosides, et les inhibiteurs de la synthèse des protéines tels que le chloramphénicol peuvent abolir EDPII¹⁶.

On suppose qu'un décodage du code génétique perturbé par les aminoglycosides qui sont entrés dans le cytoplasme pendant l'EDPI produit des protéines mal repliées. Les protéines mal traduites ont tendance à être mal repliées et à exposer des régions hydrophobes qui interagissent avec les membranes ou se lient à d'autres composants cellulaires, ce qui aboutit à une perméabilisation de la membrane¹⁷ (Figure 1).

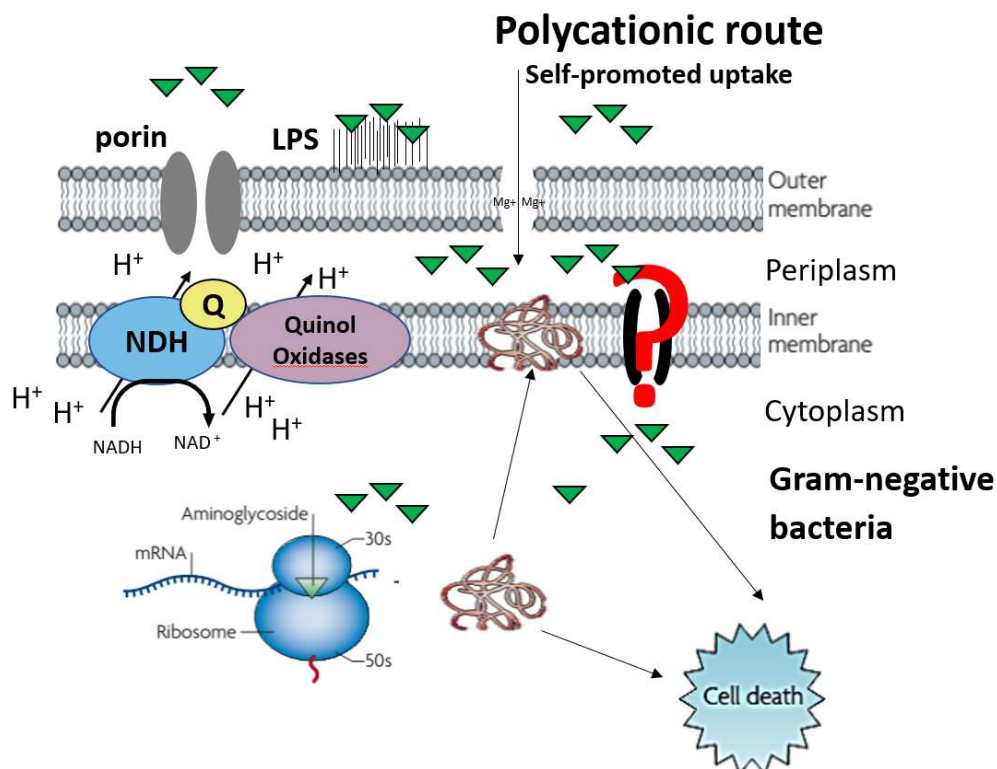


Figure 1. Mécanisme proposé d'absorption et d'action des aminoglycosides chez les bactéries Gram-négatives. Dans un premier temps, l'aminoglycoside (représenté par des triangles verts) pénètre dans l'espace périplasmique par la voie des pores et par une voie auto promue en se fixant à des poches anioniques et par déstabilisation du LPS. La deuxième étape de l'absorption est l'EDPI, au cours de laquelle l'antibiotique pénètre dans le cytoplasme par une voie inconnue. Cette étape dépend du niveau d'énergie bactérienne (PMF) qui est produit par la chaîne d'électrons située dans la membrane interne. Une fois que l'antibiotique pénètre dans le cytoplasme, il se lie au ribosome, ce qui entraîne la synthèse de protéines mal codées. Il est suggéré que ces protéines mal codées et mal repliées perturbent l'intégrité de la membrane, ce qui conduit à la dernière étape de l'accumulation d'antibiotiques (EDPII), à savoir la diffusion d'antibiotiques en plus grande concentration dans le cytoplasme, ce qui entraîne la mort cellulaire.

En ce qui concerne l'interaction des aminoglycosides avec les bactéries, beaucoup de choses restent floues. Par exemple, nous ne savons pas comment les antibiotiques aminoglycosides traversent la membrane cytoplasmique pour atteindre leur cible. Les transporteurs potentiels des aminoglycosides dans la membrane cellulaire restent à identifier.

L'échec de l'antibiothérapie est l'un des principaux problèmes qui menacent le monde entier et la compréhension du mécanisme d'action exact des antibiotiques peut nous amener à nous attaquer à ce problème. Outre le problème évident causé par la résistance aux antibiotiques, en cas d'infection par des bactéries non résistantes, les bactéries persistantes et tolérantes peuvent également échapper au traitement antibiotique, ce qui diminue l'efficacité de la thérapie antibiotique.

Pour plus de clarté, j'ai présenté ici une brève description des cellules bactériennes résistantes, tolérantes et persistantes aux antibiotiques.

Cellule résistante aux antibiotiques : cellule qui tolère le traitement antibiotique en possédant un gène de résistance (comme une pompe d'efflux). Les bactéries résistantes se

développent en présence de concentrations d'antibiotiques qui arrêtent la croissance des bactéries sensibles. (Figure 2)

Cellule tolérante aux antibiotiques : cellule qui survit au traitement antibiotique et qui repousse après l'élimination de l'antibiotique. La tolérance est un terme qui décrit une population homogène et ses facteurs peuvent être génétiques ou environnementaux. Les facteurs de tolérance aident la population cellulaire à survivre au traitement antibiotique, qui est mortel pour les bactéries sensibles aux antibiotiques (figure 2).

Cellule persistante aux antibiotiques : cellule qui tolère un antibiotique bactéricide dans une population de cellules sensibles. Après traitement d'une population sensible, les courbes de mortalité biphasique observées indiquent la présence de deux sous-populations : une première composée de cellules qui sont tuées rapidement (la majorité) et une seconde correspondant aux cellules persistantes qui survivent ou sont tuées à un rythme beaucoup plus lent (figure 2).

Après avoir arrêté l'antibiothérapie contre une infection causée par une bactérie sensible, les cellules persistantes sortent de leur état de dormance et provoquent une rechute de l'infection. La compréhension du mécanisme de la persistance revêt une grande importance car elle nous permettra de trouver la solution pour accroître l'efficacité de l'actuelle antibiothérapie.

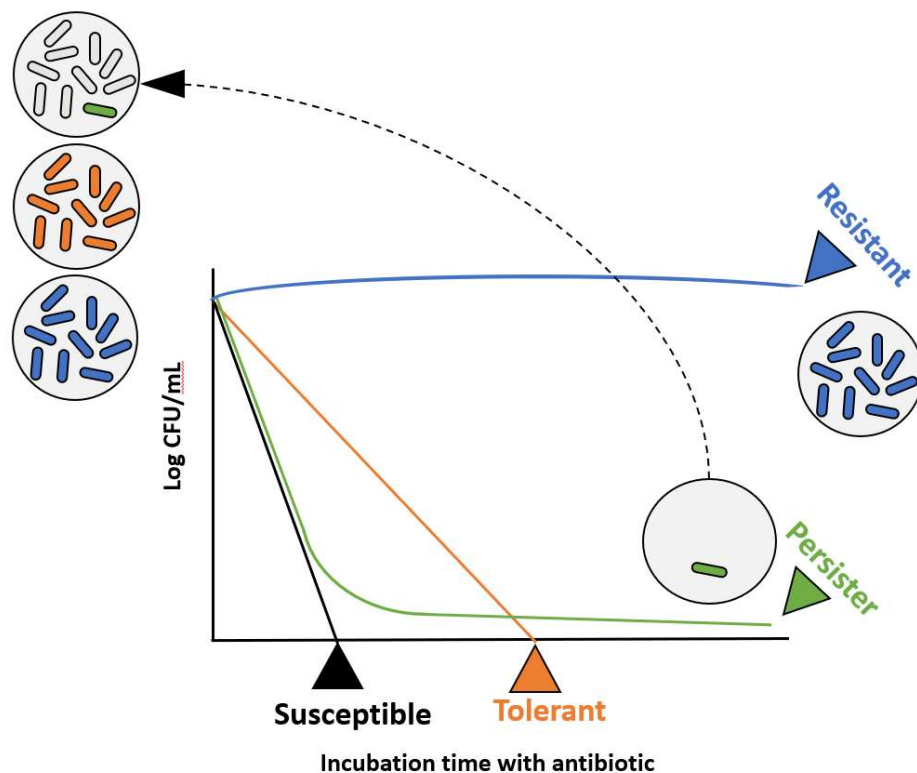


Figure 2. Comparaison de la résistance, de la tolérance et de la persistance aux antibiotiques et de la réponse des populations de cellules bactériennes aux antibiotiques bactéricides. Dans la population bactérienne sans résistance (en haut), les bactéries sensibles (en gris) sont rapidement tuées par le traitement antibiotique. Dans la sous-population à persistance (en vert), elles résistent au traitement antibiotique et peuvent régénérer une population sensible aux antibiotiques. Les bactéries tolérantes (en orange) sont tuées plus lentement par le traitement antibiotique et les bactéries résistantes (bleu) résistent à l'antibiotique.

L'objectif de ce projet de doctorat est de créer de nouveaux outils pour étudier l'interaction des aminoglycosides avec les bactéries. Dans notre laboratoire, nous générons de la néomycine marquée par un fluorophore, la cyanine 5 (Neo-Cy5). L'objectif principal de mon projet de thèse était d'abord d'étudier et de caractériser complètement ce conjugué antibiotique et d'étudier plus en profondeur l'interaction de cet antibiotique avec les cellules bactériennes au niveau de la cellule unique. Je voulais appliquer cette méthodologie pour étudier l'accumulation d'aminoglycosides dans les cellules sensibles à croissance rapide puis dans des bactéries

tolérantes, ainsi que dans les cellules qui sont transitoirement tolérantes aux antibiotiques : les bactéries persistantes.

Dans de précédentes études sur les aminoglycosides, pour suivre les niveaux d'absorption des aminoglycosides dans les cellules bactériennes, l'aminoglycoside était marqué avec le colorant Texas Red. Cependant, le Texas Red pénètre dans les cellules même sans que l'aminoglycoside y soit fusionné. L'un des avantages de la Neo-Cy5 est que le Cy5 n'interagit pas avec les cellules bactériennes. Il est alors possible de suivre l'antibiotique par microscopie de fluorescence et de définir les schémas d'accumulation de l'antibiotique. Un autre avantage du Cy5 est qu'il convient à la microscopie de fluorescence en super-résolution.

Nous avons d'abord vérifié si le Neo-Cy5 conservait ses propriétés bactéricides. Nous avons mesuré la concentration minimale inhibitrice (CMI) de notre conjugué. J'ai prouvé que l'antibiotique marqué est bactéricide à une concentration légèrement supérieure à celle de l'antibiotique non marqué. (Tableau 1)

	Neomycin	Neo-Cy5	Neo-Cy3
M9 medium	2.4 μ M	10.0 μ M	ND
MOPS medium	1 μ M	8 μ M	4 μ M
LB medium	3.2 μ M	ND	ND

Tableau 1 : Comparaison de la CMI de la néomycine et de conjugués de la néomycine dans différents milieux.

Nous avons utilisé ces conjugués pour suivre l'interaction de l'aminoglycoside au niveau de la cellule unique par microscopie à fluorescence. Nous avons combiné la microscopie à fluorescence et l'analyse par tri cellulaire activé par fluorescence (FACS) pour mesurer

l'accumulation des aminoglycosides dans les cellules à différents moments afin de saisir la cinétique de la pénétration des antibiotiques.

Afin de vérifier si la Neo-Cy5 pénètre dans les cellules de la même manière de façon dépendante de l'énergie comme la néomycine, j'ai utilisé un traitement au cyanure de carbone m-chlorophénylhydrazone (CCCP). Le CCCP est un inhibiteur chimique de la phosphorylation oxydative. Le résultat montre que le CCCP inhibe l'activité bactéricide de la Neo-Cy5 par inhibition de l'accumulation de l'antibiotique (figure 3).

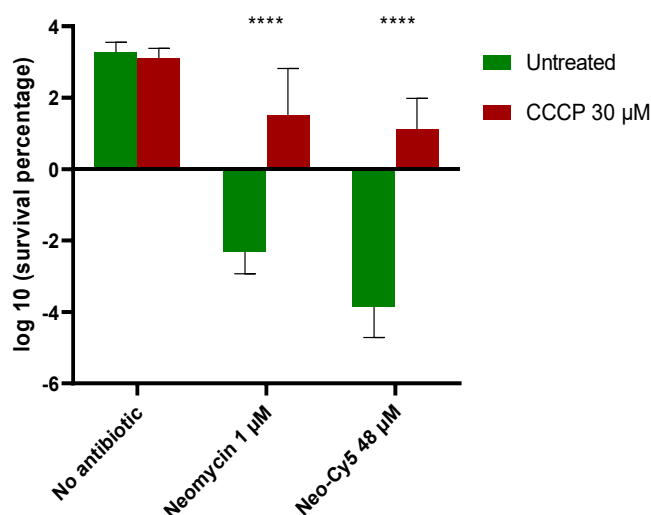


Figure 3 L'activité de Neo-Cy5 est dépendante de la PMF. Survie des cellules *E. coli* MG1655 après un traitement de 4,5 heures avec 1 µM de néomycine ou 48 µM de Neo-Cy5 avec et sans 30 µM CCCP (incubation de 2 heures avant l'ajout de l'antibiotique). Les valeurs indiquées sont des valeurs relatives à la culture témoin non traitée. Les barres d'erreur (erreur standard de la moyenne) et ont été calculées à partir de trois expériences indépendantes. Des différences significatives, calculées par le test de Mann-Whitney, sont indiquées (****P < 0,0001).

Ensuite, nous avons observé l'accumulation d'antibiotiques marqués dans des bactéries à croissance rapide par microscopie à fluorescence et analyse FACS. Selon nos expériences en microscopie à fluorescence avec différents temps d'incubation avec l'antibiotique, il y a deux phases d'accumulation du médicament dans les cellules. Dans la première étape, il y a une

accumulation périphérique, qui correspond à une fixation spécifique sur la membrane cellulaire. Ensuite, après 20 minutes d'incubation avec la Neo-Cy5, il y a une accumulation cytoplasmique dans laquelle l'antibiotique entre dans le cytoplasme. Selon l'étude des temps de passage au microscope, de faibles niveaux d'accumulation cytoplasmique sont tolérés par les cellules et n'ont pas causé la mort cellulaire. (Figure 5)

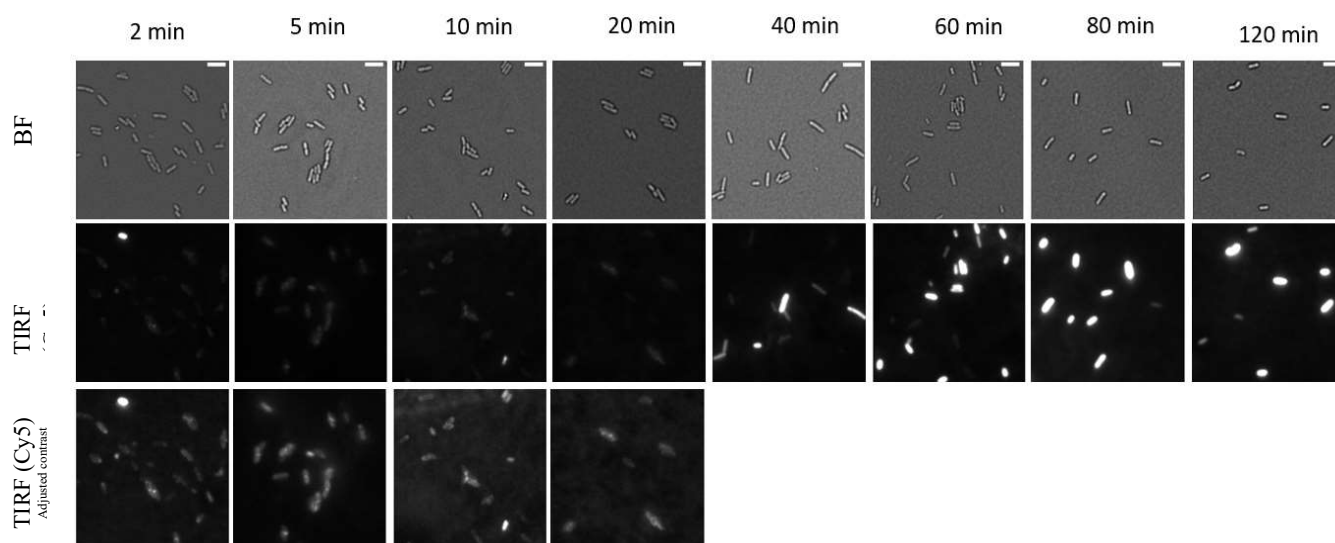


Figure 4. Cinétique de l'accumulation de la Neo-Cy5 dans les cellules d'*E. coli* à croissance rapide. Les cellules à croissance rapide (DO : 0,02) ont été incubées à 37 °C avec de la Neo-Cy5 (32 μ M). À chaque point de la cinétique, les cellules ont été lavées sur un filtre puis observées directement sur un bloc d'agarose contenant du MOPS-G sur la lame. La colonne du haut contient des images en champ clair. Dans la colonne du milieu sont affichées les images TIRF pour le Cy5 (gain 10, temps d'exposition 10 ms), pour les 4 premiers points temporels le contraste a été ajusté pour une meilleure représentation visuelle (gain 10, temps d'exposition 10 ms), colonne du bas.

Dans cette même expérience (en même temps), pour chaque point de temps, j'ai placé les cellules sur des géloses LB ou MOPS-G et j'ai compté les CFU dans un test de survie (figure 5). Ce résultat a montré que dans les 40 premières minutes d'incubation, lorsque l'antibiotique n'était pas détecté dans l'imagerie TIRF à l'intérieur du cytoplasme, la CFU ne diminuait pas. Cependant, après cette période, lorsque l'accumulation cytoplasmique de l'antibiotique est devenue visible, le nombre de cellules survivantes a diminué. De plus, selon l'analyse FACS avec un inhibiteur de l'EDPI et de l'EDPII, nous observons que cela correspond au schéma d'accumulation que nous avons observé par microscopie à fluorescence et comptage de la CFU. Nous avons également prouvé qu'avec cette technique, nous pouvons distinguer différentes étapes d'accumulation des aminoglycosides.

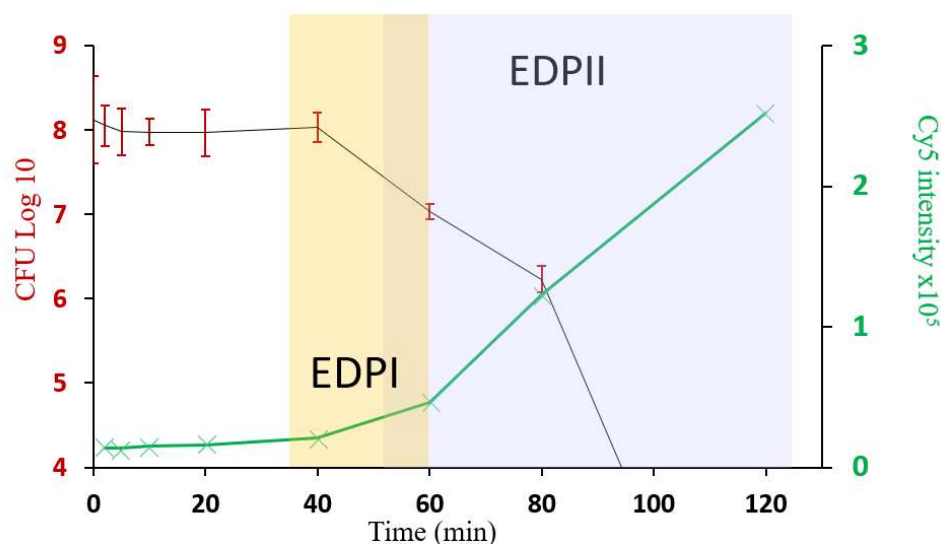


Figure 5 Courbe de mortalité de la Neo-Cy5. Les cellules de *E. coli* ont été traitées avec la Neo-Cy5 (32 μ M) à 37°C et à chaque point les cellules ont été lavées sur un filtre et divisées en deux aliquotes, une aliquote diluée en série et étalée sur des boîtes LB ou MOPS-G pour le comptage des CFU. La seconde aliquote a été utilisée pour la microscopie. Somme de l'intensité de la fluorescence Cy5 dans chaque bactérie mesurée par le logiciel ImageJ.

Après avoir étudié les différentes étapes de l'accumulation des antibiotiques dans les cellules sauvages sensibles à croissance rapide, l'objectif était de tester la Neo-Cy5 sur des

souches mutantes moins tolérantes aux aminoglycosides et de voir si nous pouvions obtenir des informations sur le mécanisme de tolérance. J'ai étudié les modèles d'accumulation de la Neo-Cy5 chez les mutants les moins tolérants *ΔserA* et *ΔflhC* par le FACS et microscopie à fluorescence. Contrairement aux études précédentes, je n'ai pas trouvé de différence entre les niveaux d'accumulation d'aminoglycoside marqué chez ces mutants et nous arrivons à cette conclusion que chez ces mutants, une létalité plus élevée n'est pas due à une accumulation plus importante d'antibiotique.

J'ai également étudié l'accumulation de la Neo-Cy5 chez les bactéries persistantes. Par microscopie à fluorescence, nous avons montré que les cellules persistantes ont une accumulation périphérique d'aminoglycoside. Une dernière étape dans l'application de notre méthode consisterait à étudier l'absorption des aminoglycosides par les cellules persistantes. L'objectif était également d'étudier l'interaction de la Neo-Cy5 avec les cellules bactériennes dans des conditions de test plus pertinentes sur le plan clinique, comme les bactéries persistantes, qui se forment dans les macrophages et dans les biofilms. Cependant, je n'ai pas voulu explorer cet objectif final en raison des résultats surprenants que j'ai obtenus lors du processus d'ajustement du protocole, qui a conduit mon projet dans une nouvelle direction.

Au cours des étapes d'ajustement du protocole, nous avons découvert que les aminoglycosides peuvent entrer dans le cytoplasme par suite de la mécanosensation et de l'activation des canaux mécanosensibles (MS). Alors que je préparais des cellules bactériennes pour l'analyse FACS, j'ai remarqué que la manipulation physique des cellules bactériennes, telle que la centrifugation suivie d'un pipetage ou l'agitation des cellules, stimulent le mécanisme de mécanosensation et d'activation des canaux mécanosensibles.

Il a été proposé que les canaux mécanosensibles jouent un rôle majeur dans l'adaptation au stress osmotique. Le rôle principal de ces canaux est d'équilibrer la pression osmotique des cellules lors d'un choc hypo-osmotique. Ces pores modifient leur conformation, passant d'un état fermé à un état ouvert. Ces changements vont de 6 à 30 Å de diamètre pour les canaux mécanosensibles de grande conductance (MscL)¹⁸ et environ ~6 Å pour les canaux mécanosensibles de petite conductance (MscS)¹⁹. (Figure 6)

Figure 6. Structure et changement de conformation du canal MscL¹⁹. Structure de MscL de *Mycobacterium tuberculosis*. Ce canal est constitué de 5 monomères. Chaque monomère (à gauche) est composé de deux domaines trans-membranaires hélicoïdaux α appelés TM1 et TM2, de régions cytoplasmiques N-terminales (S) et C-terminales et d'un domaine central en boucle périplasmique qui relie TM1 à TM2. Les cinq domaines TM1 forment un faisceau hydrophobe très serré qui constitue la porte du pore (à droite).

Il a déjà été démontré que ces canaux ont une affinité avec un aminoglycoside, la streptomycine, et c'est ici la première fois que nous avons observé que la manipulation mécanique des cellules conduit à l'ouverture du canal mécanosensible provoquant une accumulation cytoplasmique massive. Ce résultat imprévisible pourrait nous amener à mieux comprendre le mécanisme d'entrée des aminoglycosides dans le cytoplasme. Pour prouver ce fait, nous avons testé le mutant où le gène *mscL* est absent et nous avons observé que l'antibiotique

entre dans le cytoplasme de beaucoup moins de cellules pour le mutant que pour les cellules de type sauvage (Figure 7).

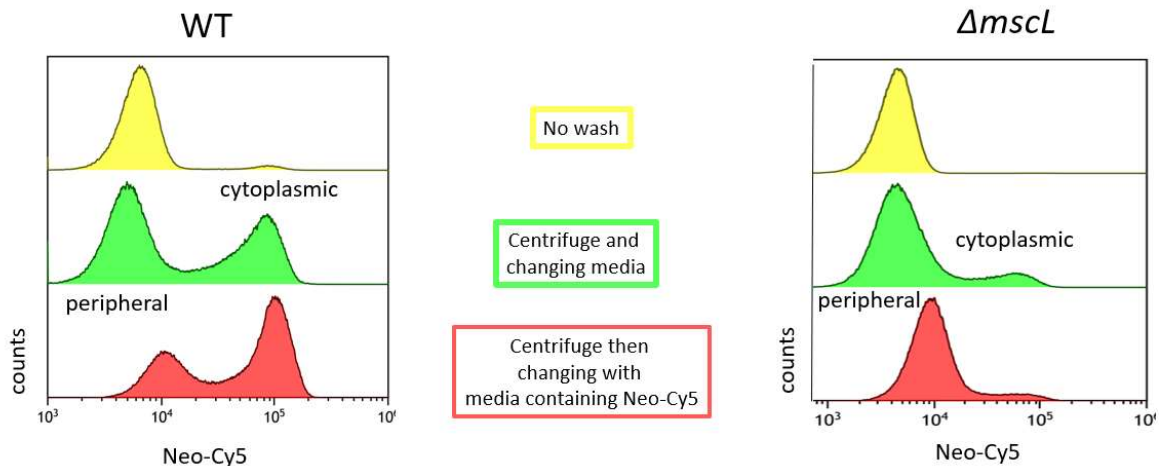


Figure 7. *ΔmscL* présente un pourcentage plus faible de cellules avec accumulation cytoplasmique de Neo-Cy5 par rapport aux cellules de type sauvage (WT). Les cellules en phase de croissance exponentielle ont été diluées 10 fois et incubées avec une concentration sous-CMI de Neo-Cy5 pendant 2 minutes à 37°C. Les cellules ont été centrifugées, lavées avec du MOPS-G ou du MOPS-G avec de la Neo-Cy5 ou non lavées. Ensuite, les cellules, ainsi que le surnageant des cellules lavées avec MOPS-G, ont été analysées par FACS.

La stimulation de ces canaux conduit à une énorme accumulation cytoplasmique de Neo-Cy5. Il convient de mentionner que ces manipulations sont si courantes et fréquemment utilisées dans la préparation des cellules en microbiologie. J'ai donc établi une méthode pour manipuler la préparation des cellules sans déclencher les canaux mécanosensibles et étudier l'effet des mutants pour ces canaux sur l'accumulation de Neo-Cy5. (Figure 6)

Grâce à notre méthodologie, nous disposons d'un outil puissant qui nous permet de déterminer les schémas d'accumulation des aminoglycosides. Avant cette étude, il était seulement possible de connaître les niveaux d'accumulation et non les "patterns" correspondants. Nous avons appliqué la méthode pour étudier l'accumulation d'aminoglycosides chez deux mutants d'*E. coli*, qui sont moins tolérants aux aminoglycosides, et avons défini leur modèle d'accumulation.

Avec un outil puissant tel que la Neo-Cy5, je voulais aller plus loin et combiner la microfluidique et la microscopie à fluorescence pour capturer l'accumulation d'aminoglycosides dans les cellules à croissance rapide et les cellules persistantes en imagerie en temps réel. J'ai initié la mise au point un système microfluidique où les surfaces ont été traitées afin d'empêcher la fixation non spécifique de l'antibiotique fluorescent. Ce système adapté à nos antibiotiques, permettra d'étudier en temps réel l'accumulation de médicaments par les cellules persistantes.

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Title: Accumulation of a bactericidal antibiotic by tolerant bacteria and insights into bacterial persistence.

Keywords: bacterial persistence, aminoglycosides, microscopy, microfluidics, flow cytometry

Abstract:

Aminoglycoside (AG) is a family of antibiotic which target bacterial ribosome. Few examples of this family are neomycin, gentamicin and streptomycin. When these antibiotics bind to ribosomes, they cause miscoding or inhibit protein synthesis which consequently leads to cell death. Although discovery of these antibiotics was more than half a century ago, there are many facts about AGs' action mechanism which remain unknown. AG accumulation in the bacterial cells happens in three steps. First step is cell membrane attachment. This step is driven by an electrostatic interaction with the cationic AGs. Second step is an energy dependent phase I (EDPI). In EDPI, the antibiotic enters into the cytoplasm and reaches ribosomes, causing miscoding and production of misfolded proteins. EDPI depends on cellular energy level, however, to date the mechanism by which AGs pass through membranes and enter cytoplasm is unknown. The third step is energy dependent phase II (EDPII) in which the antibiotic enters into the cytoplasm in larger amount due to damages in the membrane that resulted from EDPI. The aim of this PhD was to create new tools to study the interaction of AGs with bacteria and apply the methodology to study fast growing bacteria as well as persister cells. We have made fluorescently-tagged AGs with preserved bactericidal properties. We used these conjugates to track down the interaction of AG at single cell level by fluorescence microscopy. We combined fluorescence microscopy and fluorescence-activated cell sorting (FACS) analysis to measure AGs accumulation in the cells at different time points to capture the kinetics of antibiotic penetration. This study showed that there are two accumulations patterns for the drug in cells: in the first step there is a peripheral accumulation, which corresponds to.

specific binding to cell membrane. Next there is a cytoplasmic accumulation in which the antibiotic is entering into the cytoplasm. According to microscopy time laps study, low levels of cytoplasmic accumulation is tolerated by cells and did not cause cell death. Using FACS analysis, we used an inhibitor of EDPI and EDPII and proved that with this technique we can distinguish different steps of AGs accumulation. During protocol adjustment steps we found that AGs can enter into the cytoplasm as a result of mechanosensation and activation of mechanosensitive (MS) channels. These channels have already been shown to have affinity to AG and here this is a first time that we observed that mechanical manipulation of cells lead to opening of MS channel causing massive cytoplasmic accumulation. This unpredictable result may lead us to a better understanding of the mechanism of AG entrance into the cytoplasm. After studying AG accumulation in fast growing cells, we studied AG tolerance for non-growing cells, which are called persisters. Persisters are antibiotic tolerant sub-population among susceptible bacterial cell population. Persisters are non-growing, dormant cells which tolerate high concentrations of antibiotic. In the absence of antibiotic, they exit this dormant state and grow into an antibiotic susceptible population. By fluorescence microscopy we showed that persister cells have peripheral accumulation of AG. Thanks to our methodology, we have a powerful tool by which we can determine the patterns of AG accumulation. Prior to this study, it was only possible to know the levels of accumulation and not the corresponding patterns. We applied the method to investigate AG accumulation in two mutants of *E. coli*, which are less tolerant to AG and defined their pattern of accumulation. Finally, we developed a coated microfluidic system, which is adapted to our antibiotics for studying in real time drug accumulation by persister cells.



Titre : Etude de l'accumulation d'un antibiotique bactéricide par des bactéries tolérantes en lien avec la persistance bactérienne.

Mots clés : persistance bactérienne, aminoglycosides, microscopie, microfluidique, cytométrie en flux

Résumé:

Les aminoglycosides (AG) sont une famille d'antibiotiques qui ciblent le ribosome bactérien. À titre d'exemple il s'agit de la néomycine, la gentamicine et la streptomycine. Quand ces antibiotiques se fixent au ribosome, ils provoquent des erreurs de lectures ou inhibent la synthèse des protéines, ce qui conduit à la mort cellulaire. Même s'ils ont été découverts il y a plus d'un demi-siècle, de nombreux aspects de leur mode d'action restent inconnus. L'accumulation des AG dans les bactéries se passe en trois étapes. La première consiste en une interaction électrostatique avec la membrane. La deuxième est une phase I énergie-dépendante (EDPI). Les antibiotiques rentrent dans le cytoplasme, atteignent les ribosomes, ce qui cause des erreurs de lecture et donc la production de protéines mal repliées. EDPI dépend du niveau énergétique de la cellule et le mécanisme d'entrée à travers les membranes reste inconnu. La troisième étape est la deuxième phase énergie-dépendante (EDPII), où l'antibiotique pénètre dans le cytoplasme en grande quantité par des membranes endommagées lors de la phase I. Le but de cette thèse était de créer de nouveaux outils afin d'étudier l'interaction des AG avec les bactéries et d'appliquer la méthodologie à des bactéries en phase rapide de croissance ou bien en état de persistance. Nous avons synthétisé des conjugués fluorescents des AG aux propriétés bactéricides. Avec ces conjugués nous avons analysé l'interaction des AG avec les bactéries à l'échelle de la cellule unique par microscopie de fluorescence. Nous avons combiné cette technique avec la cytométrie de flux (FACS) pour évaluer la cinétique d'accumulation. Cette étude démontre qu'il y a deux types d'accumulation : une à la périphérie avec interaction à la membrane et une deuxième où l'antibiotique est localisé dans le cytoplasme. Notre analyse démontre aussi que de faibles

niveaux d'antibiotiques dans le cytoplasme sont tolérés et n'inhibent pas la croissance cellulaire. En utilisant un inhibiteur des phases EDPI et EDPII nous démontrons que cette technique permet de distinguer les différentes étapes de l'accumulation. Au cours d'ajustements du protocole, nous avons découvert que les AG peuvent entrer dans le cytoplasme par mechano-sensation et activation de canaux mécanosensibles (MS). Ces canaux sont connus pour avoir une affinité pour les AG. Ici pour la première fois nous montrons qu'une manipulation mécanique ouvre les canaux et stimule une entrée massive d'antibiotiques. Ce résultat inattendu pourrait permettre de mieux comprendre le mécanisme d'entrée des AG dans le cytoplasme. Après avoir étudié l'accumulation des AG dans les cellules en croissance nous avons étudié la tolérance aux AG pour les bactéries en phase de dormance : les cellules persistantes. Elles forment une sous-population parmi une population sensible. Elles sont en dormance et tolèrent de fortes doses d'antibiotiques. En absence d'antibiotique elles sortent de l'état de dormance pour reformer une population sensible à l'antibiotique. Par microscopie de fluorescence, nous montrons que les cellules persistantes ont une accumulation périphérique d'AG. Grâce à notre méthodologie, nous avons un outil performant pour identifier les différents états d'accumulation des AG. Avant cette étude il était seulement possible de connaître les niveaux d'accumulation mais la localisation de l'antibiotique demeurait inaccessible. Nous avons avec cette méthode étudié deux mutants d'*E. coli*, qui sont moins tolérants aux AG et identifié leurs caractéristiques d'accumulation. Enfin, nous avons développé un système de microfluidique adapté à l'étude de nos conjugués fluorescents pour étudier en temps réel l'accumulation par les cellules persistantes.

