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MARILIIS HINNU

In vitro methods for studying the mechanisms of ribosome-targeting antibiotics

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In vitro methods for studying the mechanisms of ribosome-targeting antibiotics

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Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia

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LIST OF PUBLICATIONS

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I. Construction of the reporter system used for detecting chloramphenicol, design and analysis of experiments using the reporter, co-writing the manuscript.

II. Study design, all experimental procedures, data analysis, and writing the manuscript.

III. Study design, construction of the mistranslation reporters, all experimental procedures, data analysis, and writing the manuscript.

ABBREVIATIONS

AG	aminoglycoside
AHT	anhydrous tetracycline
AMI	amikacin
AmpR	ampicillin resistance
AST	antimicrobial susceptibility test
AZI	azithromycin
CAM	chloramphenicol
MHB	cation-adjusted Müller-Hinton broth
cDNA	complementary DNA
CFTS	cell-free translation system
CLSI	Clinical and Laboratory Standards Institute
DAP	diamino pimelic acid
DDS	drug delivery system
DMSO	dimethyl sulfoxide
ERY	erythromycin
EtOH	ethanol
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
FISH	fluorescence *in situ* hybridization
FRET	fluorescence/Föster resonance energy transfer
GFP	green fluorescent protein
HPLC	high performance liquid chromatography
HUS	hemolytic uremic syndrome
iSc	mScarlet-I
KAN	kanamycin
KanR	kanamycin resistance
LB	lysogeny broth
LPS	lipopolysaccharide
MAM	macrolide arrest motif
MBC	minimum bacterial concentration
MDR	multi-drug resistance
MIC	minimum inhibitory concentration
NTS	non-typhoid *Salmonella*
PAMP	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PCL	polycaprolactone
PEO	polyethylene oxide
PhR	phenol red
PTC	peptidyl-transferase centre

QS	quorum sensing
RF	release factor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
StrR	streptomycin resistance
T3SS	type-3 secretion system
TLR	toll-like receptor
UPEC	uropathogenic *Escherichia coli*
UTI	urinary tract infection
WHO	World Health Organization

1. INTRODUCTION

Antibiotics are antibacterial drugs that have saved countless lives. Now, about 100 years since the discovery of first modern antibiotics, we are facing a threat of common infections becoming deadly again. This is because infectious organisms are becoming increasingly resistant to antibiotics. According to the World Health Organization (WHO) antimicrobial resistance is one of the top 10 global public health threats, and misuse of antibiotics is a major cause for it. One approach to prevent antibiotic resistance crisis is to optimize antibiotic therapy, and for that we need a thorough understanding of antibiotic mechanisms of action during infection. Additionally, novel antibiotics are needed to tackle drug-resistant infections.

After the discovery of first antibiotics in the turn of the last century many new classes of antibiotics were discovered, and the middle of 20th century is considered the "golden age" of antibiotic development. Main targets of antibiotics are bacterial cell wall/membrane, replication, transcription, translation, and metabolism. However, the mechanisms of action were not known initially – first antibiotics were screened in large-scale laboratory animal studies and clinical studies in humans. Understanding of how antibiotics work came long after the commercialization and large-scale clinical use. For some antibiotics the exact mechanisms are still not completely clear. A combination of extensive *in vitro* and *in vivo* studies is necessary to give a full understanding of antibiotic action and resistance mechanisms.

Ribosome is the centre of protein biosynthesis in cells, a fundamental process for life. Therefore, the ribosome is also a major target for several antibiotic classes, such as macrolides, aminoglycosides, tetracyclines etc. Knowledge on the different translational processes has evolved gradually since the 1960s together with the molecular working mechanisms of ribosome-binding antibiotics. The initial studies on antibiotic mechanisms were limited by available equipment, were laborious and often required the use of radiolabelled substrates and toxic chemicals. Current methods allow to carry out comprehensive studies on living cells, yielding in large amounts of data and a deeper network of knowledge on antibiotic actions.

The aim of this thesis was to develop methods, which would allow to improve the estimation of antibiotic treatment efficacy. The thesis is focused on the studies of ribosome-targeting antibiotics. The literature part gives an overview of bacterial infections and treatment strategies, describes antibiotics relevant for the thesis, as well as *in vitro* methods for studying these antibiotics. Experimental part of the thesis focused on developing methods, which help to reveal antibiotic's availability, activity, and mechanisms of action in different environments, keeping in mind the biological relevance of these assays. One strategy was based on the development of fluorescent bioreporters (I, III), which allow to study individual bacteria during antibiotic treatment. These bioreporters were used for studying

drug release from antibiotic-containing nanofibers intended for wound treatment (I). Other reporters were used to estimate translational fidelity changes in host-mimicking environments and during antibiotic treatment (III). We also questioned the hypothesis whether the addition of main blood buffer component bicarbonate to standard medium used in standard antimicrobial susceptibility test (AST) improves the predictability of clinical outcome (II).

2. LITERATURE REVIEW

2.1. Bacterial infections

Bacterial infections have accompanied us since the beginning of humankind and have had a large impact on the course of history. For example, plagues, unambiguous term for different epidemic infections, have killed more than half of the human population at times (Prescott et al., 2005c). Of course, people back then were not able to prove the cause of the disease, but instead blamed God or magical "bad air" for it. An ancient Ayurvedic medical textbook has described leprosy, fever and other infectious diseases as highly contagious through direct contact with an infected person, although the disease itself is considered a curse for bad deeds (Susruta & Bhishagratna, 1911, p. 42). Many bacterial infections are described in Hippocratic Corpus, a collection of medical teachings from the 5th century BC. External causes from mostly bad quality of water, food, and air are accounted for the diseases in these texts (Pappas et al., 2008).

While several scholars throughout following history noted that infectious diseases are spread via invisible creatures, real breakthroughs in infection biology did not come before the 19th century. In human medicine, first it was found that sterilization and hygiene maintenance during surgical procedures reduced the mortality of patients from infections significantly (Prescott et al., 2005c; Sakai & Morimoto, 2022). Groundbreaking direct evidence was presented by Robert Koch, who after discovering the causative agents of anthrax, tuberculosis, and cholera, presented his thesis now known as Koch's postulates. Shortly, he claimed that: a) the microbe is always present in the diseased, but not in the healthy individual, b) microbe isolated from the infected person causes the same disease in a healthy host (Antonelli & Cutler, 2016; Prescott et al., 2005c). While the postulates are not accurate in every case, they were still revolutionary for modern infection biology and medicine. From what we know today, deviations from Koch's postulates come from host specificity (no animal models), polymicrobial infections, difficulties in isolating pathogens (non- or difficultly culturable), asymptomatic carriage, infecting a healthy host does not always result in infection, one pathogen can cause a variety of symptoms, and opportunistic pathogens (Antonelli & Cutler, 2016).

We live side-by-side with a wide range of microorganisms, which are indispensable for life on Earth. Only a small fraction of microbes is known to cause disease, hence be pathogenic. Primary, or dedicated pathogens are able to cause an infection irrespective of host's immune state, whereas opportunistic pathogens are able to cause infection only when the host's immune system is compromised (Alberts et al., 2002; Prescott et al., 2005a). Bacterial pathogenicity is determined by several complex factors arising from both the bacterium and the host. An infectious disease will develop when a sufficient number of bacteria capable of invading and propagating within the susceptible host and evading immune defence mechanisms result in tissue damage due to the microbe or by the host's

inflammatory response (Casadevall & Pirofski, 2001; Prescott et al., 2005a). However, it is important to note that pathogenicity is relative, and each individual relationship between host and microbe is unique (Casadevall & Pirofski, 2001).

Before infection a pathogenic bacterium must first maintain a reservoir – a natural living environment, where it can spread to new hosts. Other humans, animals and arthropods, and environment (soil, water) act as reservoirs. Pathogens are then transmitted by water or dust particles via air, by direct contact with a contaminated reservoir, material or food, or via animate vectors (Prescott et al., 2005b). Opportunistic pathogens are commonly benign microbes, such as those part of the normal microbiota, that can cause an infection only when host's immune functions are compromised (Alberts et al., 2002). First step of bacterial infection is entry to the susceptible host followed by colonization, which requires the bacterium to adhere to and reproduce in a niche within the host. Sometimes the internalization of the pathogen is facilitated by host-dependent mechanisms, such as the presence of wounds, or endocytosis by the host cells. Many bacterial pathogens then invade the host cells and tissues, and after reaching circulatory system the bacterium can access all organ systems. The disease that will manifest is a sum of effects produced by the bacterium, such as cell damage and toxin production, and by the host's immune response, for example fever (Prescott et al., 2005a). The infection process is summarized in Figure 1.

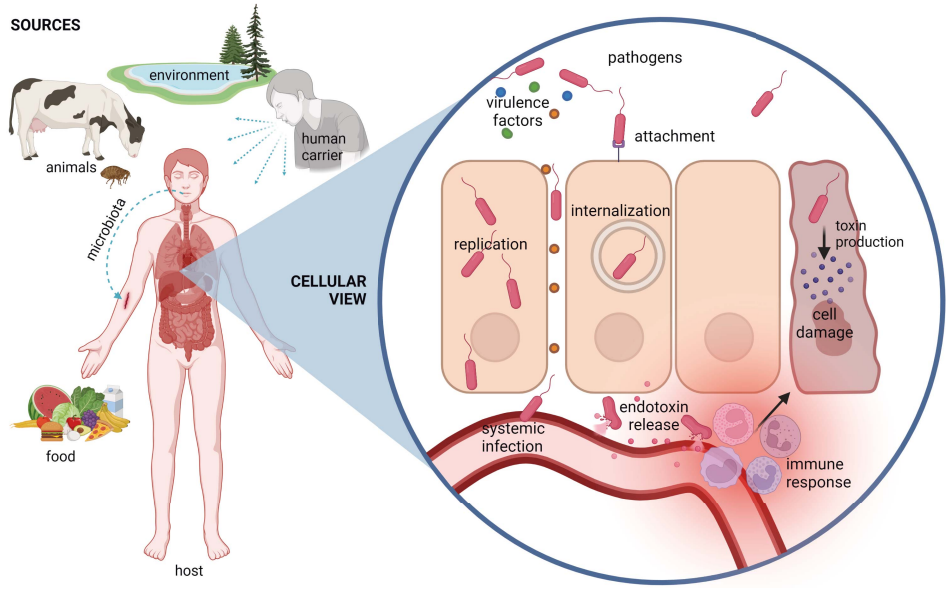

Figure 1. *Simplified schema of infection process. The pathogens enter the susceptible host via any of the natural openings of the body or via damaged epithelium (wounds) by contaminated organisms or materials. Virulence factors expressed by pathogens enable them to establish an infection in host tissues, damage them, and result in disease manifestation.*

An important determinant of pathogenicity are virulence factors. They are specific products or structural components that help the pathogen to survive and thrive within the host. Virulence factors, either vital for pathogenicity or contributory, can for example facilitate the attachment, invasion, promote the pathogen's growth or help to avoid eradication by immune system (Casadevall & Pirofski, 2001). Almost all virulence factors in bacteria are secreted or expressed on the cell surface. Virulence factor genes are often organized into pathogenicity islands, which are located either on mobile genetic elements, and can spread via horizontal gene transfer to other bacteria within or outside of the species, or spread via bacteriophages (Finlay & Falkow, 1997).

Host defence mechanisms are important contributors to infectious disease development. The epithelium provides first line of defence against bacterial infection. In addition to physically blocking the bacterial entry into the body, shedding during tissue renewal and mucosal clearance help to eliminate bacteria attached to the epithelium. Protective enzymes and acidic pH in different mucosal tissues restrict bacterial growth by creating a hostile environment. Healthy microbiota also contributes to host susceptibility to infections by providing colonization resistance (direct competition for host resources, immune priming) (Peterson, 1996).

Cellular immune response can be broadly categorized into innate and adaptive immunity, which are interdependent and use similar mechanisms (Hancock et al., 2012). Innate immunity provides initial recognition of any foreign bodies, entering the host as phagocytic cells, mainly polymorphonuclear neutrophils, capture and kill the potential pathogens (Peterson, 1996). Pattern recognition receptors on the immune cells recognize pathogen-associated molecular patterns (PAMPs), which are microbe-specific proteins, polysaccharides, lipids, or viral nucleic acid sequences. Receptor-PAMP pairing activates cellular defence mechanisms, such as phagocytosis, production of reactive oxygen species, antimicrobial peptides and proinflammatory cytokines, latter of which aggravates immune cell response even further (Hancock et al., 2012).

Adaptive immunity, facilitated mainly by B and T lymphocytes, is activated by a combination of signals from the innate immune system and antigen recognition. Antigens are introduced to adaptive immune system by antigen-presenting cells, such as dendritic cells or macrophages, which are part of the innate immunity. This leads to clonal expansion of B and T lymphocytes, which carry receptors recognizing the specific antigen. B lymphocytes produce antigen-specific antibodies, which block and/or opsonize the pathogen for complement system or phagocytosis-mediated killing. T lymphocytes can kill the infected host cells or up- or downregulate the immune response. While the adaptive immune response takes initially longer to react to a pathogen than innate immune response, it can provide a life-long protection to specific pathogens by keeping memory B and T lymphocytes (Hancock et al., 2012).

Symptoms of the infectious disease vary between the pathogens, and depend on the bacterial localization in the body, mechanisms they use and the immune response of the host. General symptoms include fever, pain and swelling, and

while some pathogens present more distinct symptoms, in many cases they overlap (Reller et al., 2022). Some bacteria cause acute infections with sudden onset of symptoms and complete eradication after the disease, whereas others, especially those able to invade host cells, can become chronic, sometimes lasting a lifetime with alternating states of acute disease, variable contagiousness levels, and asymptomatic carriage (Young et al., 2002).

Nutrition, sanitation, and treatment improvements during the last century have led to massive decreases in both spread and mortality rates of bacterial infections. Average life expectancy worldwide has increased from about 30 years in 1800 (Riley, 2001) to almost 73 years in 2019 (Issifou & Pewitt, 2022). According to The Global Burden of Disease 2019 study about 20% of all deaths are still caused by communicable diseases, mostly by bacterial infections (about 10 million), with ranges from about 54% in low to about 6% in high sociodemographic index countries (Institute for Health Metrics and Evaluation, 2022). Acute lower respiratory tract infections, diarrhoeal diseases and tuberculosis are among the top 10 causes of overall death (Institute for Health Metrics and Evaluation, 2022; Issifou & Pewitt, 2022; World Health Organization, 2017).

Surveillance report on antibiotic use in Europe in 2022 found that on average about 2% of people receive antibiotics at any given moment (range 0.9 to 3%, depending on the country) (European Centre for Disease Prevention and Control, 2023). A study in the UK found that about every third primary-care patients received at least one antibiotic prescription per year (Shallcross et al., 2017). WHO has issued a list of priority pathogens for research and antibiotic development, which in addition to being highly virulent with high mortality rates are also showing concerning levels of multi-drug resistance (MDR). *Mycobacterium tuberculosis* is considered number one priority, and *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae* spp. (incl. *Escherichia coli* and *Salmonella*) are considered critical priority pathogens (World Health Organization, 2017). Clinically most problematic pathogens (in addition to *M. tuberculosis*) due to high levels of resistance are colloquially known as ESKAPE pathogens: ***E****nterococcus* (vancomycin resistant), ***S****taphylococcus aureus* (methicillin resistant, MRSA), ***K****lebsiella pneumoniae,* ***A****. baumanii,* ***P****. aeruginosa,* ***E****nterobacteriaceae* spp. (De Oliveira et al., 2020). The following paragraphs will give an overview about *E. coli* and *Salmonella*, the main model pathogens used in the experimental part of the thesis, as well as general treatment strategies for bacterial infections.

2.1.1. Escherichia coli

E. coli is a gram-negative 2–4 µm-long rod-shaped bacterium that is most numerous facultatively anaerobic commensal residing in the mucous layer of colon in humans and animals. *E. coli* normally exists in mutual benefit with the host, and only causes disease when the host is immunocompromised or the epithelial barrier is broken. Species can be subdivided into serovars, depending on antigenic properties, ultimately affecting the behaviour and virulence of pathogens. In the

presence of certain virulence factors, *E. coli* can cause either diarrhogenic or extraintestinal infections. *E. coli* is a major cause for fatal diarrheal disease, especially in children living in developing countries. It is also the main cause of urinary tract infections (UTIs), gram-negative septicaemia, and neonatal meningitis (Croxen & Finlay, 2010; Kaper et al., 2004; Levinson et al., 2022; Pottinger et al., 2022). However, some *E. coli* serovars, such as the Nissle 1917, a probiotic sold under the trademark Mutaflor, are known to provide protection against other enteropathogens (Sonnenborn, 2016).

Pili, capsule, endotoxin, and exotoxins are the main *E. coli* virulence factors that are responsible for ability to cause a disease in humans (Levinson et al., 2022). These affect many cellular processes of the host, such as cell signalling, protein synthesis, ion balance, mitochondrial function, cell division, and cytoskeletal function (Kaper et al., 2004). Genomes of pathogenic isolates can be up to 1 Mb larger than those of commensals due to virulence factor genes (Croxen & Finlay, 2010). *E. coli* strains are categorized into serovars depending on their O-, K- and H-antigens. O-antigen refers to the outer membrane lipopolysaccharide (LPS), which differs based on the sugar composition. There are more than 150 types of O-antigens in *E. coli* (Levinson et al., 2022; Pottinger et al., 2022). *E. coli* has more than 50 types of H-antigen, which is a protein located on the flagellum of motile strains, and more than 80 types of K-antigens, which refer to the cell surface polysaccharide capsule or mucous layer, important for evading the host's immune system (Kaper et al., 2004). Importantly, adhesins are important virulence factors, which also have antigenic properties, however they are not used for serotyping (Pottinger et al., 2022).

The pathogenesis of *E. coli* starts with the adhesion to host surfaces. This is often facilitated by adhesins, such as rod-like pili (5–10 nm diameter), long and flexible fibrillae (2–4 nm diameter), or outer membrane proteins (Kaper et al., 2004). Pili attach to specific receptors on the host cell surface, commonly sugar residues, glycolipids or glycoproteins. Most *E. coli* express hair-like type 1 pili, which bind to D-mannose residues present on many epithelial cell types. P pili bind digalactosides, which are common on kidney cells and certain erythrocyte types. Different pili types can exist simultaneously in one bacterium, and the expression of each type depends on environmental conditions (Pottinger et al., 2022). The main component of flagella, flagellin, binds to Toll-like receptor 5 (TLR5), causing interleukin 8 (IL-8) activation and inflammatory response (Kaper et al., 2004).

Toxins and other proteins, which are secreted using different secretion systems or autotransporters, can affect a wide variety of host's cellular processes. Different enterotoxins increase the concentrations of intracellular messenger molecules, such as cAMP, cGMP, and Ca^{2+}, which lead to ion imbalances and water secretion to the intestine, resulting in diarrhoea. Shiga toxin cleaves ribosomal RNA of epithelial or endothelial cells, disrupting protein synthesis and killing the cells, resulting in bloody diarrhoea – dysentery. Some toxins block cell division, disrupt membrane potential of mitochondria or change the structure of the host cells, leading to necrosis (Kaper et al., 2004; Levinson et al., 2022). α-hemolysin forms

a pore in the plasma membrane of the host cell, leading to leakage of cellular contents and cell death (Pottinger et al., 2022).

E. coli intestinal infections vary in severity from mild and self-limiting to fatal. It is contracted by ingestion of food or water contaminated with faeces. Some serovars have infectious doses as low as 100 organisms, whereas some enterotoxigenic strains have high infective dose $10^6 \ldots 10^8$ organisms. Intestinal infections can be categorized based on their invasive properties and toxin production, which is in correlation with the type of disease. Enterotoxin producing isolates cause watery diarrhoea, while invasive and cytotoxic strains cause dysentery, which is inflammatory diarrhoea characterized by fever, stomach cramps, vomiting and the presence of blood, mucous, and leukocytes in stool (Kaper et al., 2004; Khalil et al., 2021; Pottinger et al., 2022). Diarrhoea is a result of several mechanisms, such as active ion secretion, increase of intestinal permeability, increased fluid secretion, inhibited absorption, inflammation of the intestines and disruption of absorptive structures due to the effacement of microvilli. Enterotoxigenic strains utilize fimbrial or fibrillar colonization factors, and express heat-labile (LT) and/or heat-stable (ST) toxins, which produce the diarrhoea. Enteroinvasive serovars reside inside epithelial cells, and rarely disseminate past submucosa, however they can induce apoptosis of macrophages (Kaper et al., 2004). Certain Shiga-toxin producing strains can cause potentially fatal haemolytic uremic syndrome (HUS) – haemolytic anaemia and thrombocytopenia accompanied by kidney damage. HUS can develop in 5–15% of patients infected with Shiga-toxin producing strains, or even higher in epidemic situations with certain serovars. In some cases of HUS, neurological symptoms, such as seizures and brain damage also appear (Michael et al., 2022).

Uropathogenic *E. coli* (UPEC) is the most common cause for urinary tract infections (UTIs). The source of UTI is the microbiota of the host, however serovars responsible for infection are distinct from commensal *E. coli* strains. They commonly harbour type 1 and P pili, a capsule, produce certain toxins, and are resistant to serum (Kaper et al., 2004). Vital attributes for UPEC are iron acquisition and ability to grow in urine. During infection process attachment to the bladder epithelial cells is followed by invasion, as well as apoptosis and exfoliation of epithelial cells (Kaper et al., 2004). In uncomplicated cases the infection is confined within the bladder, resulting in often self-limiting cystitis characterized by frequent and sometimes painful urination. Some serovars, however, can ascend to kidneys by downregulating type 1 pili and upregulating P pili and flagella. This results in detachment and migration from bladder to kidneys and causing pyelonephritis, symptoms of which include fever, malaise, and pain in the flank area. Secreted toxins will cause kidney damage, and by penetrating endothelial cells the bacteria can cause a systemic infection (Kaper et al., 2004; Pottinger et al., 2022). UTIs have high, up to 55% recurrence rates and one explanation is UPEC's invasion into deeper layers of bladder epithelium, where they form quiescent intracellular reservoirs inaccessible to immune system and antibiotic actions from which bacteria can reactivate during epithelial renewal (Blango et al., 2014; De Nisco et al., 2019).

E. coli is a most common cause for hospital-acquired sepsis and a major cause for meningitis and sepsis in newborns. Systemic infection is facilitated by two virulence factors: the endotoxin LPS, and the capsule (Levinson et al., 2022). LPS, which is part of the outer membrane of gram-negative bacteria, binds to TLR4 and can cause septic shock, a combination of cytokine action, complement activation and coagulation cascade, and can result in death of the host (Kaper et al., 2004; J. W. Wilson et al., 2002). Antiphagocytic capsular polysaccharide K1 is an important virulence factor in neonatal meningitis caused by *E. coli* acquired from the mother's birth canal during birth (Levinson et al., 2022).

Opportunistic infection occurs when *E. coli* gains access to body sites, which are normally sterile, and tissue damage occurs due to bacterial activity. Mucosal and skin traumas enable access to soft tissues, whereas aspiration gives access to lungs. As with other infections, pili are major contributors to these infections (Pottinger et al., 2022).

2.1.2. Salmonella

Salmonella spp. are closely related to *E. coli.* Most common clinical manifestations of this Gram-negative rod-shaped facultatively anaerobic bacterium are gastroenteritis (food poisoning), bacteraemia and enteric fever. There has been some confusion with *Salmonella* nomenclature throughout history, however currently most common method divides *Salmonella* into two species: *Salmonella enterica* and *Salmonella bongori*, which are further categorized into subspecies and serovars. *Salmonella enterica* subsp. *enterica* contributes to about 99% of *Salmonella* infections in humans and animals, whereas other subspecies as well as *S. bongori* are mainly found in nature and cold-blooded organisms, rarely causing disease in humans (S.-K. Eng et al., 2015). Therefore, this section will focus only on serovars of *Salmonella enterica* subsp. *enterica.*

Almost all *S. enterica* strains are pathogenic, and the illness depends on both host susceptibility and the serovar. Human salmonellosis can be divided into two groups: systemic typhoid and mainly gastroenteritis-causing non-typhoid *Salmonella* (NTS) infections. Overall fatality rate of typhoid infection is estimated to be around 1%, and as high as 25% for invasive NTS. Pathogens are typically ingested orally with contaminated food or water (S.-K. Eng et al., 2015; MacLennan et al., 2014), but typhoid-causing pathogens can also spread via direct contact with infected people or chronic carriers (Coburn et al., 2007). Genomic alterations have also been found in some NTS strains, which allow the pathogens to spread primarily between humans (MacLennan et al., 2014). The infectious dose for *Salmonella* is relatively high: upwards of even as high as 10^5–10^6 bacterial cells. Gastric acid as well as colonization resistance by healthy microbiota are important host defence mechanisms (Coburn et al., 2007; Pottinger et al., 2022).

S. enterica harbours two large pathogenicity islands in its chromosome (Alberts et al., 2002). There are about 2600 serovars identified, based on the LPS, capsular and flagellar structures (S.-K. Eng et al., 2015; Pottinger et al., 2022).

The flagellar antigen of some species can alternate between phase 1 and phase 2, which can help the pathogen to escape host's immune response (Levinson et al., 2022). *Salmonella* has multiple types of pili, which help the bacterium to bind to eukaryotic cells. Due to flagella most strains are motile (Pottinger et al., 2022). Importantly, flagella are also strong inducers of inflammatory processes within the hosts. Immune response on the other hand induces flagellin production furthermore (Coburn et al., 2007). While capsular antigen is generally less important in *Salmonella* spp., typhoid fever-causing S. serovar Typhi has Vi antigen, a surface polysaccharide, which can reduce the infective dose, interfere with complement deposition, induce uptake by macrophages and promote intracellular multiplication (Pottinger et al., 2022). Another important contributor to pathogenicity is the ability to synthesize siderophore enterochelin. This helps *Salmonella*, as well as *Escherichia* and other pathogens, to acquire iron, an essential nutrient, from the host's proteins (Peterson, 1996).

Gastroenteritis-causing NTS reside mainly in animal reservoirs. Serovars Enteritidis and Typhimurium are the most common causative agents of NTS, and infections occur world-wide. The disease results in non-bloody diarrhoea, stomach cramps, nausea and vomiting, head- and muscle aches (S.-K. Eng et al., 2015). The diarrhoea is caused by both the invasion and transcytosis of enterocytes as well as inflammatory response, most importantly neutrophil recruitment resulting in epithelial necrosis, oedema, and changes in vascular permeability. While several enterotoxins have been described, their role in the disease is unclear (Coburn et al., 2007; Pottinger et al., 2022) NTS symptoms start usually after only 6–72 h of ingestion (Coburn et al., 2007; S.-K. Eng et al., 2015), and usually last less than 10 days. While in most cases self-limiting, young children, elderly and immunocompromised people are more susceptible to severe symptoms. Complications can include cholecystitis, pancreatitis and appendicitis, cellulitis, pneumonia, UTIs, endocarditis, and meningitis. NTS patients are unlikely to become chronic carriers, as the primary reservoir is animals (S.-K. Eng et al., 2015). Serovar Typhimurium causes a systemic typhoid-like disease in mice, therefore it is commonly used to study typhoid infection in a mouse model (Coburn et al., 2007).

Pathogenicity of NTS relies on its ability to persist inside the host cells. Interestingly, *Salmonella* can induce its own phagocytosis by normally non-phagocytic human host cells to gain intracellular access. For this they utilize type III secretion systems, encoded by the genes located on pathogenicity islands. T3SS are multichannel proteins that inject effector molecules into intestinal epithelial cells through the membrane. These effector molecules activate signal transduction pathway, which in turn causes actin cytoskeleton rearrangements in the host cell, resulting in an epithelial cell membrane protrusion ("ruffle") that engulfs the bacterium, resembling phagocytosis. Intracellular *Salmonella* resides inside a vacuole, and by utilizing the T3SS it blocks the normal immune response, the fusion of lysosome and action of harmful enzymes. This allows the bacterium to survive and replicate intracellularly (S.-K. Eng et al., 2015). When the *Salmonella* multiplies in the vacuole, it continues into deeper tissues of the intestine, where

it induces strong inflammation and is phagocytosed by immune cells. There it uses a second secretion system, which induces apoptosis of the immune cells and further spread of the pathogen within the tissue. It is also possible that instead the bacterium becomes dormant with a potential for relapsing infection (Pottinger et al., 2022).

Salmonella bacteraemia occurs when bacteria enter the bloodstream by passing the intestinal barrier. Any *Salmonella* serovar can cause bacteraemia, and bacteraemia can occur in 5 to 10% of patients, usually in those with chronic diseases or children. It results in high fever without a rash, and can lead to septic shock, which is usually more indolent than in case of septicaemia with other Gram-negative rods. Bacteraemia can cause infection of other organ systems, most commonly osteomyelitis, pneumonia and meningitis (S.-K. Eng et al., 2015; Levinson et al., 2022). Invasive NTS was responsible for 80 thousand deaths in 2019 (Institute for Health Metrics and Evaluation, 2022).

Enteric fever, or (para)typhoid fever, is caused by *S. enterica* serovars Typhi and Paratyphi, which are strictly human pathogens. It affects mostly people in developing countries and is related to high morbidity and mortality. After an incubation period of a week or more, it starts with a headache, abdominal pain and digestive issues (mild diarrhoea or constipation), followed by slowly developing high fever, which can persist upwards of a month, if left untreated. Additional symptoms include muscle pain, enlargement of spleen and liver, and a rash. About 15% get complications like pancreatitis, hepatitis, cholecystitis, and intestinal haemorrhage. The ability of *Salmonella* to survive in macrophages results in relapse in about 10% of patients. Some patients will become chronic carriers, where bacteria persist and are shed with stool from an asymptomatic human host for more than a year after acute infection (S.-K. Eng et al., 2015). According to WHO *S.* Typhi was responsible for 1.5 million deaths in 2015 (World Health Organization, 2017), however Global Burden of Disease study from 2019 estimates this number to be much lower, around 150 thousand deaths (Institute for Health Metrics and Evaluation, 2022).

After surviving gastric acidity and out-competing host's microbiota *S.* Typhi cells are phagocytosed and translocated through the intestinal epithelium, where they systemically spread via the reticulo-endothelial system. Bacteria then maintain a reservoir inside host cells mainly within the spleen and liver, gallbladder, as well as gut epithelium (Coburn et al., 2007; Levinson et al., 2022), mesenteric lymph nodes, and bone marrow. At these sites *S.* Typhi continues to multiply, spreading the infection into new macrophages. Eventually the pathogens reach the bloodstream, where LPS triggers a slowly increasing fever. Through systemic infection other organ systems can be infected, or the gut reinfected. In uncomplicated cases the symptoms resolve in about 3 weeks (Pottinger et al., 2022). The initial infection process is similar between typhoid and NTS. The main differences, however, are the presence of Vi (capsular) antigen in the Typhi serovar, which limits phagocytosis by neutrophils, reducing the acute inflammatory response, and extended multiplication of *S.* Typhi in macrophages, instead of

killing the host cell. *S.* Typhi is able to inhibit oxidative damage by macrophages (Pottinger et al., 2022).

2.1.3. Treatment

Historically infections have been empirically treated with plant extracts, honey, soil, minerals, moulds, and other natural products, or even bloodletting, restrictive diet and praying (Aminov, 2010; Mohr, 2016). Before antibiotics were widely available pneumonia, tuberculosis, and gastroenteritis were the top 3 causes of death (Sakai & Morimoto, 2022). The discovery of many different antibiotics during the 20th century might be the most life-saving medical advancement in history. Still prevention strategies, such as improvements of wastewater treatment systems, hygienic practices, food and water treatment practices, and general education on the topic, as well as improved nutrition are major contributors on limiting the spread of infectious agents (Pottinger et al., 2022).

The first step of modern treatment strategy is the correct diagnosis. Bacterial isolation is not necessary in mild infections, as frequently the most likely pathogen can be determined based on symptoms. However, it is important to isolate the pathogen in case of serious infections and when the initial antimicrobial therapy is ineffective. Bacterial identification can be done via microscopy or culturing patient samples on selective media, such as urine for UTI, blood in case of bacteraemia, or stool in case of gastroenteritis. Culturing enteropathogens from the faeces is not routinely done due to large numbers of bacteria naturally present. Immunoassay and PCR-based methods have been used instead to detect virulence factors of *E. coli*, however even these methods can fail to identify the correct causative agent and are relatively expensive. For *S.* Typhi it is possible to identify the organism via culturing, biochemical reactions, or detection of antibodies in serum. Whole-genome sequencing and other molecular typing methods are used to confirm the findings later (Pottinger et al., 2022). Identification of *Salmonella* requires attention as it has a potential to become endemic, and the source should be clarified (Levinson et al., 2022).

If antibiotic treatment is necessary, antimicrobial susceptibility testing (AST) is not routinely carried out in individual patients. The choice of an antibiotic is in majority of cases empirical and based on epidemiological data. However, AST is carried out in the context of local surveillance and monitoring of outbreaks (Riddle et al., 2016). AST is required for critically ill patients, although antibiotic therapy is initiated before the AST results arrive (World Health Organization, 2022). AST-s are discussed in the paragraph 2.3.2.

Current guidance of antibiotic therapy is directed towards reducing and refining antibiotic use in general to fight the emergence of resistant infections. According to WHO AWaRe (Access, Watch, Reserve) antibiotic use recommendations mild infections, such as gastroenteritis or UTI in otherwise healthy patients can be treated without antibiotics – only symptomatic treatment, such as pain relief is recommended. Patients should still be clearly informed of any danger signs. If antibiotic therapy is necessary, there are many things to consider:

which antibiotic to prescribe, the dose and the duration of treatment, the route of administration. Patient-derived circumstances should also be considered for choosing the antibiotic treatment, such as patient's comorbidities, concomitant medications, allergies, as well national regulations (World Health Organization, 2022).

Antibiotics are used as prophylaxis only in certain cases: patients undergoing high infection risk traumas, invasive surgical procedures, such as prosthetic implant surgery, immunocompromised patients undergoing immune-suppressing therapy, and people who are in contact with known patients with communicable infections. Often the prophylactic treatment is a single dose of antibiotic (Leekha et al., 2011). Topical treatment should be preferred in case of wound infections, which are often polymicrobial (World Health Organization, 2022). In case of moderate infections, empiric treatment with a narrow-spectrum antibiotic against the suspected pathogen should be used. In critically ill patients, antibiotic therapy should be started as soon as possible with a broad-spectrum antibiotic, possibly in high doses. While single agents are usually preferred, combinations of antibiotics can be used in case of a) serious infections, when there is known synergism between the antibiotics, b) critically ill patients, esp. hospital-acquired infections, c) polymicrobial infections, d) prevention of resistance selection, esp. in prolonged treatment plans. Once microbiology results become available the spectrum of antibiotics should be narrowed (Leekha et al., 2011).

Oral antibiotics should be preferred in moderate infections. In case of invasive infections, antibiotics with good absorption and bioavailability, i.e. yielding high serum concentrations, are recommended, such as fluoroquinolones or metronidazole. Critically ill patients are usually administered intravenous antibiotics, however they should be switched to oral dosage forms as soon as the patient is stable enough, as parenteral administration has complication risks. Switch to oral therapy is not recommended if the infection requires high drug levels in serum or cerebrospinal fluid. In general, shorter treatment durations (up to a week) are preferred for uncomplicated mild infections, and longer treatment durations (4–6 weeks) are recommended for more serious and invasive infections. Treatment efficacy is assessed by both clinical, e.g. fever or leukocyte levels, and microbiological parameters, such as blood culture in case of sepsis (Leekha et al., 2011).

Clinical breakpoints, issued by relevant agencies, categorize different bacteria into susceptible or resistant based on laboratory susceptibility to specific antibiotic, and guide the choice of an antibiotic. Clinical breakpoints are determined by a committee of experts mainly based on available dosage forms, susceptibility distributions, pharmacokinetic and pharmacodynamic data, and clinical evidence. If a breakpoint value for a certain antibiotic has not been given for a bacterial group, the antibiotic is considered unsuitable for the treatment of systemic infections with pathogens belonging to the group (European Committee on Antimicrobial Susceptibility Testing, 2021). For *Enterobacteriales* breakpoint values have been given to following antibiotics: majority of penicillins, cephalosporins, carbapenems, monobactams, fluoroquinolones, aminoglycosides, some tetracyclines, and some other agents of various classes. Macrolides are not indicated for this

group of pathogens, however there is a special note that azithromycin can be used for treatment of typhoid fever (The European Committee on Antimicrobial Susceptibility Testing, 2023).

Treatment of infections caused by Enterobacteriaceae

Enterobacteriaceae most commonly cause acute gastroenteritis, which is usually self-limiting, and does not require microbiological analysis nor drug treatment. Only oral rehydration, electrolyte replacement therapy, and in some cases zinc tablets are recommended, as majority of acute gastroenteritis cases are caused by viral pathogens not susceptible to antibiotics (Riddle et al., 2016; World Health Organization, 2022). Antibiotic therapy is recommended only if the patient has risk factors for severe disease development (e.g. immunocompromised patients), or in case of dysentery. First choice antibiotics are fluoroquinolone ciprofloxacin or macrolide azithromycin. Antibiotic treatment should be reconsidered if the symptoms do not improve in 48 hours. If the symptoms last longer than 2 weeks, microbiological analysis should be done to identify the pathogen, as protozoal parasites (e.g. *Entamoeba histolytica*, *Giardia intestinalis*) are often the cause of persistent diarrhoea (World Health Organization, 2022).

Antibiotic treatment should be started as soon as *Salmonella* infection is suspected. In severe cases, blood cultures should be taken to confirm the diagnosis. Fluoroquinolone ciprofloxacin is the first choice of antibiotic. If there is a high risk for fluoroquinolone resistance, azithromycin or ceftriaxone are prescribed (World Health Organization, 2022). Use of antibiotics in case of Shiga-toxin producing *E. coli* strains is debated, as it can increase the risk of Shiga-toxin release and HUS development. In mild cases fluid balance, nutrition and hypertension is controlled, however up to 60% of patients can require dialysis and erythrocyte transfusion. Using antimotility agents can worsen the disease and increase the risk of HUS (Michael et al., 2022; Pottinger et al., 2022).

Sepsis is a life-threatening disorder often caused by *Enterobacteriaceae.* It has non-specific symptoms and is characterized by organ dysfunction due to dysregulation of host response to infection. Sepsis is assessed and classified mainly according to respiratory rate, blood pressure, mental status, as well as blood parameters, such as platelet count or bilirubin concentration, which all reflect the stage of organ failure. Septic shock is the most severe form of sepsis, that is often associated with increased mortality. Microbiological analysis is carried out depending on the suspected primary site of infection, but always includes blood cultures, although bacteraemia and sepsis are not interchangeable. In addition to administering antibiotics, other symptomatic treatment is carried out, such as fluid resuscitation and organ support. The source of infection must also be controlled, for example by surgical removal of infected tissue. If bacterial sepsis is suspected, intravenous antibiotic treatment should be initiated as soon as possible (World Health Organization, 2022).

Meningitis is another severe life-threatening infection, which is considered of bacterial origin, unless proven otherwise. *E. coli* and *Salmonella* (both Typhoid

and NTS) are often the cause of bacterial meningitis in children. Intravenous antibiotic treatment should be initiated as soon as possible, even before carrying out laboratory tests, such as cerebrospinal fluid analyses. Ampicillin, cephalosporins, aminoglycoside gentamicin and their combinations are first line of treatment. Corticosteroids are recommended in some cases to reduce the risk of neurological complications. (World Health Organization, 2022)

Non-antibiotic therapies

More traditionally surgical treatment, such as abscess drainage and wound lavage, is used for the treatment of infections. Even invasive surgery, such as cholecystectomy is carried out to remove chronic typhoid infection (Levinson et al., 2022). If patients receive immunosuppressive drugs, dose reduction or discontinuation of the latter might be necessary for infection treatment. Immunoglobulin therapy can be used to neutralize toxins produced by the pathogen. Probiotics are used in some intestinal infections to restore the normal microbiota. Often these alternative therapies are recommended based on clinical experience, and often lack strong evidence (Leekha et al., 2011).

A review by (Czaplewski et al., 2016) listed possible alternative science-based therapies to antibiotics. Candidates with best clinical potential were found to be:

a) antibodies that inactivate the pathogen, its virulence factors, or toxins
b) probiotics for restoring the intestinal microbiota
c) bacteriophages (wild-type and engineered)
d) phage lysins that target the cell wall of the bacterium
e) immunomodulation
f) novel vaccines

Other potential alternative therapies, which currently have less supportive evidence, are antimicrobial peptides, also named as host defence peptides, or antibiofilm peptides. Selective immune suppression to reduce host's systemic inflammatory response, antibacterial and antiresistance nucleic acids, liposomes to bind toxins secreted by pathogens, metal chelation have also been mentioned (Czaplewski et al., 2016). A potential strategy for infectious disease treatment is blocking quorum sensing (QS), which is used by bacteria to communicate between cells, and has a vital role in regulating virulence. QS is facilitated by small molecules *N*-acyl homoserine lactones, peptides and other molecules. Analogues of QS molecules, which block binding sites of natural ligands, QS-molecule degrading enzymes, and agents inhibiting QS molecule synthesis can be used to block biofilm formation and virulence gene expression (Hancock et al., 2012; Nigam et al., 2014).

Another approach is to improve antibiotic treatment efficacy by changing the drug formulation or the drug delivery system. For example, it is possible to use

antibiotic resistance breakers, such as enzyme inhibitors, membrane permeabilizers, efflux pump inhibitors, etc. in conjunction with antibiotics. Additionally, nanoparticles carrying antibiotics could improve antibiotic treatment (Cavaco et al., 2022). Indeed, nanoparticles can improve the bioavailability, stability, and physicochemical properties of antibacterial drugs, or have independent antibacterial effects. Antibiotic-conjugated nanoparticles are promising strategies for treatment of infections caused by *M. tuberculosis* and other MDR pathogens (Kumar et al., 2018).

2.2. Ribosome-targeting antibiotics

Translation is a fundamental process in all living organisms, where genetic information in the form of RNA is translated into proteins, which act as effector molecules. Translation takes place in the ribosome. The ribosome is one of main targets of antibiotic action since the translation process is crucial for life. Prokaryotic ribosome consists of 3 ribosomal RNAs (rRNA; 16S, 23S, 5S) and ~54 ribosomal proteins, which form two subunits: 30S (small) and 50S (large). Initiation requires interaction of Shine-Dalgarno sequence on the messenger RNA (mRNA) with the complementary sequence on the 16S rRNA, as well as initiation factors IF1, IF2, and IF3 (Ramakrishnan, 2002; D. N. Wilson, 2014).

During translation initiation the two subunits together with mRNA being translated form one large 70S ribosome. Initiator transfer RNA (tRNA), fMet-tRNA, together with the start codon are positioned in the P-site. During elongation cycle elongation factor Tu (EF-Tu) complex with GTP brings aminoacylated tRNA (aa-tRNA) (EF-Tu:GTP:aa-tRNA complex, i.e. ternary complex) into the A-site of the ribosome. If the tRNA anticodon and mRNA codon match, GTP is hydrolysed, and a peptide bond forms between the amino acids attached to tRNAs located in the A- and P-site. With the assistance of GTP hydrolysis EF-G helps to translocate the tRNAs in A- and P-sites per one codon to E- and P-sites, respectively, to make room for a new ternary complex in the ribosome. The growing polypeptide exits through the tunnel in the 50S subunit, until a stop codon reaches the A-site. Release factors RF1 and RF2 are responsible for recognizing the stop codon, hydrolysing the peptidyl-tRNA bond and releasing the polypeptide into the cytoplasm, where the protein is folded. The 70S ribosome disassembles into subunits, and the components get recycled for new translation initiations (Ramakrishnan, 2002; D. N. Wilson, 2014). Translation cycle is shown schematically in Figure 2.

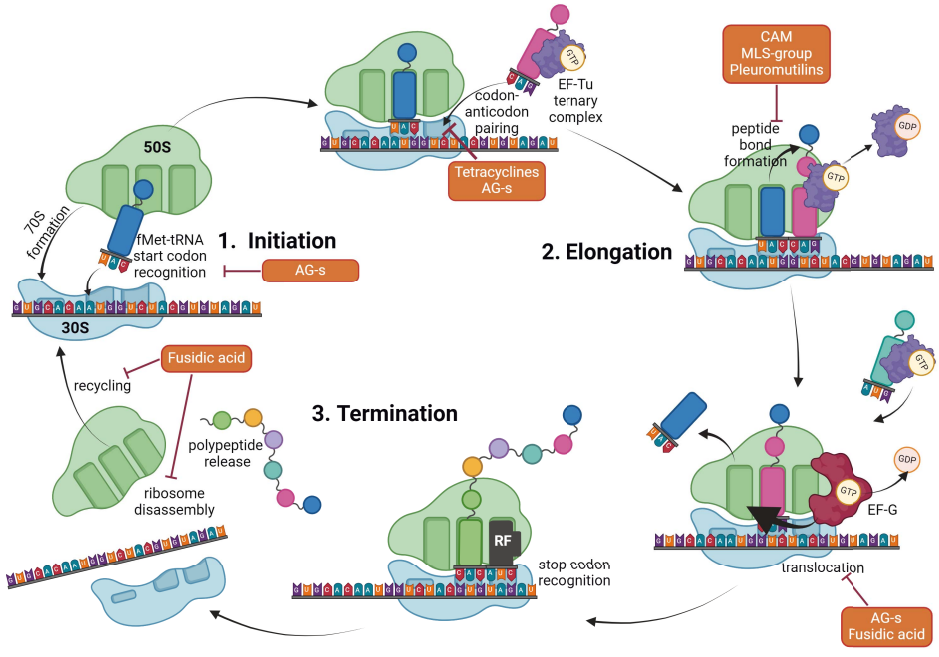

Figure 2. *Overview of prokaryotic translation cycle. Targets of some commonly used antibiotics (orange boxes) are shown. RF – release factor, AG-s – aminoglycosides, CAM – chloramphenicol, MLS – macrolide-lincosamide-streptogramin.*

Maintaining the correct expression of genetic sequences is required for normal function of the organism. It is estimated that the translational error rate is approximately $10^{-3}…10^{-4}$ per codon. There are several proofreading steps involved in maintaining translational fidelity. First, the tRNA must be correctly paired with the respective amino acid during aminoacylation by the aa-tRNA synthetase (aaRS). There are several quality control steps involved in aminoacylation, based on hydrolysis of labile non-cognate bonds. Some aaRSs even compete with EF-Tu for tRNA binding and check the correct amino acid-tRNA pair again. Secondly, the correct ternary complex of the mRNA codon at the A-site must bind to the ribosome and correct codon-anticodon base pairing must occur. The recognition is essentially based on binding affinity differences: the correct aa-tRNA binds an order of magnitudes stronger. The correct pairing will produce a geometrical rearrangement in the 30S ribosome, which leads to conformational change in the ternary complex and GTP hydrolysis. After hydrolysis the aa-tRNA is released into the 50S ribosome A-site, where it can easily disassociate in case of codon-anticodon mismatch. If a codon-anticodon mismatch manages to translocate into the P-site, the correct ribosomal functioning can be hampered, resulting in amplification of errors (Reynolds et al., 2010). Increasing the translation rate decreases the fidelity of translation and increasing translational accuracy has a fitness cost on bacteria (Kurland, 1992), therefore there is an evolutionally acquired optimal fidelity rate.

Majority of ribosome-targeting antibiotic classes are summarized in table 1. Several ribosomal targets are schematically shown in Figure 2. Most of known antibacterials, including phenicols, macrolides and aminoglycosides, which are relevant for this thesis, target the elongation cycle. A few antibiotics, albeit with limited clinical applications, target specifically elongation. Some antibiotics interfere also with the termination and recycling of the ribosome, although their effect is mostly during elongation phase (D. N. Wilson, 2014). The following paragraphs will describe ribosome-targeting antibiotics relevant for this thesis: chloramphenicol, aminoglycosides, and macrolides.

Table 1. *Overview of the majority of ribosome-targeting antibiotics. Antibiotics relevant for this thesis are highlighted. G+ = Gram-positive; G– = Gram-negative*

Ribosomal target	**Antibiotic class**	**Example drugs**	**Main mechanism of translation inhibition**	**Main indications**	**Reference**
50S	Phenicols	**Chloramphenicol**, thiamphenicol	Blocks peptidyl transferase reaction of specific amino acid sequences	Broad spectrum, topically in eye infections, bacterial brain infections (meningitis), systemically in life-threatening infections resistant to other drugs	(Crowe-McAuliffe & Wilson, 2022; Yunis, 1988)
	Macrolides	Erythromycin, **azithromycin**	Blocks peptidyl transferase reaction of specific peptide sequences	Widely used, mostly against G+ and some specific intracellular pathogens (sexually transmitted infections, typhoid)	(Hooda et al., 2019; Unemo et al., 2019; Vázquez-Laslop & Mankin, 2018b)
	Lincosamides	Clindamycin, lincomycin	Blocks peptidyl transferase reaction by blocking A site	Mostly G+ infections of moderate severity	(Spížek & Řezanka, 2017)
	Streptogramins	Dalfopristin, quinupristin, pristinamycin	Bind to PTC, inhibit tRNA binding to both A and P site, stop elongation	Active against G+ and some G-, used rarely for life-threatening infections and complicated skin infections	(Mast & Wohlleben, 2014)
	Oxazolidinones	Linezolid	Inhibits tRNA binding to A-site, amino acid sequence specific, blocks initiation	Pneumonia, skin and soft-tissue infections, G+ infections	(Crowe-McAuliffe & Wilson, 2022; Zahedi Bialvaei et al., 2017)
	Pleuromutilins	Retapamulin	Binds to P site, inhibits peptidyl transferase reaction and P-site interactions	Superficial skin infections by *S. aureus* and *S. pyogenes*	(Yang & Keam, 2008)

Ribosomal target	Antibiotic class	Example drugs	Main mechanism of translation inhibition	Main indications	Reference
30S	Amino-glycosides	Kanamycin, **amikacin**, streptomycin	Interfere with tRNA binding to A-site, induce misreading	Broad spectrum, used in case of severe infections	(Aguirre Rivera et al., 2021)
	Tetracyclines	Tetracycline, doxycycline	Inhibits tRNA binding to A-site	Broad spectrum, widely used in developing countries, systemically for respiratory infections, in Western countries used mostly topically	(Roberts, 2003)
70S	Tuberactino-mycins	Viomycin, capreomycin	Lock tRNA in the ribosome and inhibit translocation	Tuberculosis	(Holm et al., 2019)
EF-G	Steroid fusidane	Fusidic acid	Inhibits translocation by binding EF-Tu, inhibits termination	Narrow spectrum against G+ bacteria, mostly used topically in dermatology, systemic use for bone/joint infections	(Fernandes, 2016)
EF-Tu	Elfamycins	Kirromycin, aurodox	Blocks peptidyl transferase reaction by lock EF-Tu:GDP complex in the ribosome	Active mostly against gram-positives, clinical trials for *Clostridoides difficile*	(Parmeggiani & Swart, 1985; Prezioso et al., 2017)
aaRS		Mupirocin	Inhibits isoleucyl-tRNA sythetase	Topically for staphylococcal and streptococcal infections	(Ward & Campoli-Richards, 1986)

2.2.1. Chloramphenicol

Chloramphenicol (CAM) is a broad-spectrum bacteriostatic antibiotic first isolated in the end of 1940s from *Streptomyces venezuelae*, but due to its simple structure it is produced synthetically. It was the first broad-spectrum antibiotic used systemically, classically for the treatment of typhoid fever. While it has saved countless lives, it is in rare cases hemotoxic, causing potentially fatal bone marrow suppression, due to which it has been largely substituted with more potent drugs with less side effects. It is still used in low-income countries for bacterial meningitis. It is used in developed countries exceptionally as topical ophthalmic preparations or in case of life-threatening infections, if there are no other options due to resistance, or as combination therapy in case of infections in the brain, as it passes well into the brain tissue (Fisch & Bryskier, 2005). CAM is active against majority of bacterial pathogens, except for most *Pseudomonas aeruginosa* isolates, mycobacteria, *Acinetobacter* spp. And *Nocardia*. Due to its excellent tissue and intracellular distribution, it is suitable for the treatment of infections in deep tissues and those caused by intracellular pathogens. It also has a weak antiplasmodial effect (Fisch & Bryskier, 2005).

The structure of CAM is simple compared to other antibiotics. Phenicols are formed by dichloroacetic acid, an aminopropanediol chain and an aromatic nucleus (Figure 3A). The propanediol group is necessary for the biological effectivity. CAM has a nitro-group attached to the aromatic nucleus, which might be responsible for the toxic side effects. CAM has two stereoisomers, but only D-form is biologically active (Fisch & Bryskier, 2005; Yunis, 1988). Ester forms, such as CAM-succinate and CAM-palmitate, are used due to improved chemical properties, but these forms are inactive *in vitro*, and only acquire antimicrobial properties after enzymatic activation *in vivo.* While it is possible to synthesise many derivatives of CAM, only thiamphenicol, which has a methyl-sulfonyl group instead of the nitro group, has been used in humans. CAM is used orally, by injection or topically (Fisch & Bryskier, 2005).

CAM binds to the 50S ribosomal subunit onto the 23S rRNA at the peptidyl-transferase centre (PTC), where the incoming tRNA's aminoacyl moiety positions. It was thought to block peptide bond formation by inhibiting the aa-tRNA from binding to the A-site (D. N. Wilson, 2014). More recent data show that translation is arrested in specific sites, which is affected by the second to last, or penultimate (–1), amino acid on the growing polypeptide attached to peptidyl-tRNA (Figure 3B). The size of this amino acid plays a role in drug binding to the ribosome, as an optimally sized amino acid (most often alanine) locks CAM to its binding site. As some alanines are still efficiently translated, additional nascent peptide context might also affect antibiotic binding (Crowe-McAuliffe & Wilson, 2022). CAM's antibacterial effect during treatment is time-dependent (Estell et al., 2020).

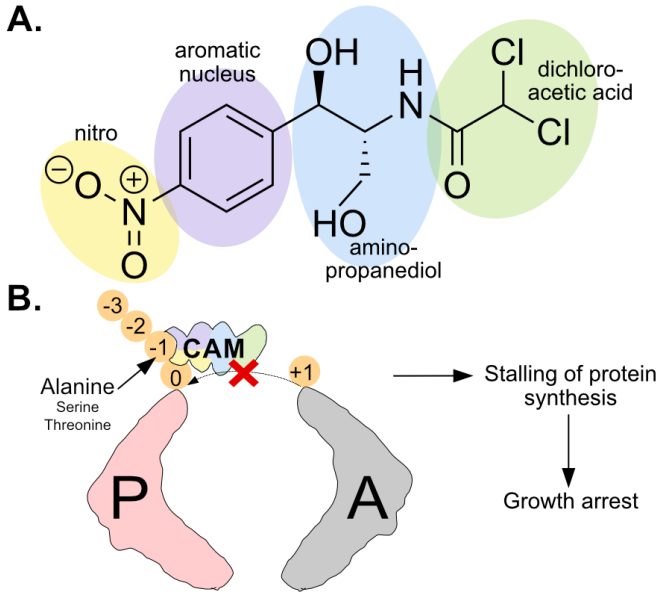

Figure 3. *Chloramphenicol's (CAM) molecule and mechanism of action on the 50S ribosome. A. Molecular structure of CAM. B. CAM binds to the peptidyl-transferase centre, depending on the favourable amino acid in the –1 position of the growing polypeptide, stalling the ribosome, and inducing growth arrest. P – P-site tRNA, A – A-site tRNA.*

Resistance to CAM, while currently not a severe issue in the clinics of the western world, develops rapidly. The main mechanism is the production of acetyl-transferases, which inactivate CAM. This resistance gene, possibly combined with resistances to other antibiotics, is usually carried on a plasmid, and can therefore spread between bacteria. Other mechanisms for CAM resistance are reduced permeability or increased efflux (Fisch & Bryskier, 2005). Specific resistance genes (e.g. CAM acetyltransferase *cat*) can be activated via the regulation of mRNA secondary structure. CAM induces ribosomal stalling in specific regions of the leader peptide, and the stalled ribosome opens the reading frame that is located upstream of the resistance gene, which allows the downstream gene to be translated (Crowe-McAuliffe & Wilson, 2022).

Toxicity is the main obstacle limiting the clinical usage of CAM. There are two types of hematotoxicity related to CAM. First is dose-dependent reversible bone marrow suppression, which affects mainly erythrocytes. The second, is a dose-independent (even when administered topically) bone marrow aplasia resulting in pancytopenia, which can lead to fatal haemorrhage and/or infection. The incidence is about 1 in 20–60 thousand, but with more than 50% mortality rate (Fisch & Bryskier, 2005). The pancytopenia can develop in a couple of weeks to even 4 months after the treatment. CAM-metabolites, such as nitroso-CAM and dehydro-CAM, have been found to be highly toxic, inducing DNA damage and death of myeloid cells even at low concentrations. There might be a genetic

predisposition in some individuals for the high production of these toxic intermediates, and therefore the occurrence of CAM-induced aplastic anaemia. Another well-known side effect is Gray baby syndrome in neonates, which results from immature enzymatic inactivation of CAM. The reason behind these side effects in humans, as well as those of other ribosome-targeting antibacterials, might be due to affecting mitochondrial protein synthesis (Fisch & Bryskier, 2005; Yunis, 1988).

2.2.2. Macrolides

Macrolides were discovered in the 1950s. Erythromycin (ERY) was the first clinically used macrolide. It was isolated from *Saccharopolyspora erythraea*, and several other macrolides were discovered from *Streptomyces* spp. Some macrolides have been developed semi-synthetically. The potency and spectrum of activity varies between the different macrolides (Bryskier & Bergogne-Bérézin, 2005). ERY has a moderate spectrum of activity, whereas its derivatives, such as azithromycin (AZI), have a wider spectrum. Macrolides are used for treating respiratory tract infections, sexually transmitted infections, skin and soft tissue infections, and other infections caused by intracellular pathogens. They are known to downregulate virulence factors of some pathogens. Macrolides are also used for treating infections caused by protozoa. Interestingly, macrolides also present anti-inflammatory properties (Roberts, 2008).

Structurally macrolides consist of a 14- to 16-atom lactone (macrolactone) ring (Figure 4A), which is accompanied by side groups (Tenson & Mankin, 2006). ERY's poor chemical and pharmacological properties led to the discovery and development of other macrolides, some derived semi-synthetically from ERY (Bryskier & Bergogne-Bérézin, 2005). For example, AZI is a semi-synthetic derivative of ERY with much improved pharmacologic properties (Parnham et al., 2014) As opposed to many other antibiotics, such as beta-lactams or fluoroquinolones, macrolides achieve high intracellular concentrations, making them a good choice for treatment of intracellular pathogens (Bryskier & Bergogne-Bérézin, 2005). AZI has excellent tissue distribution and is especially known to accumulate in phagocytes, particularly in lysosomes impairing their function, and achieving high concentration at infection sites (Nujić et al., 2012; Parnham et al., 2014). 400- to 800-fold serum macrolide concentrations have been detected in macrophages, and 5- to 100-fold serum concentrations in tissues (Zimmermann et al., 2018).

Macrolides are often grouped together with chemically distinct lincosamides, streptogramins (MLS group antibiotics), and sometimes also with oxazolidinones, due to overlapping binding sites on the 50S ribosome and similar mechanisms of action and resistance (Roberts, 2008). Ketolides are a newer generation of macrolides, intended to overcome resistance to macrolides. They feature a keto group instead of a sugar residue, as well as an alkyl-aryl side chain, which is responsible for the bactericidal action of ketolide (Beckert et al., 2021).

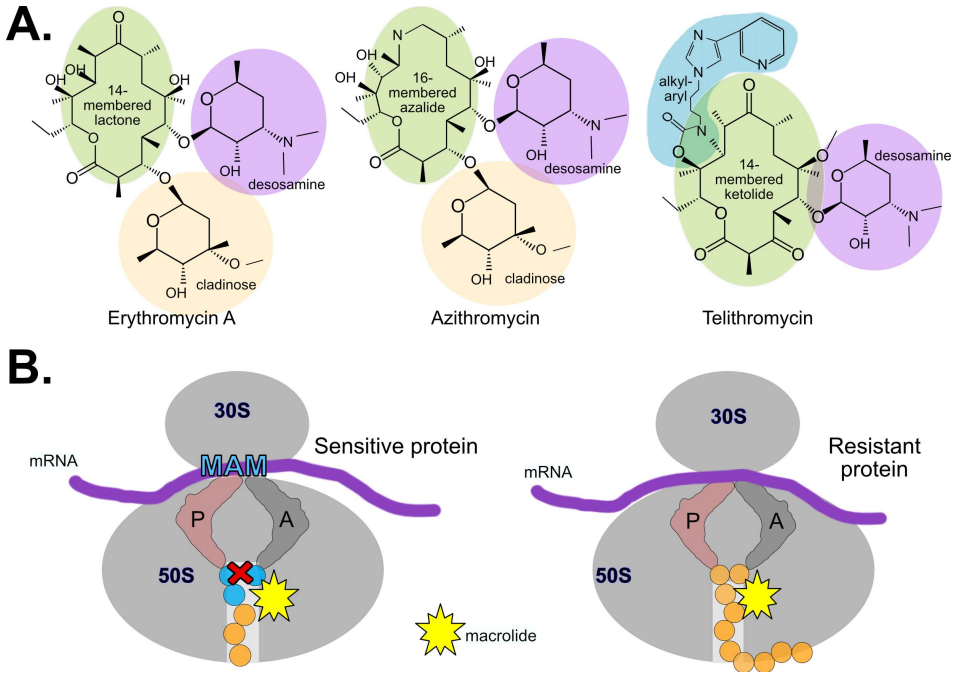

Figure 4. *Macrolide chemical structure and mechanism on the ribosome. A. Macrolides compose of a macrolactone ring with sugar residues and other side chains B. Macrolide (yellow star) binds into the 50S ribosome nascent peptide exit tunnel near the PTC, specifically blocking the peptidyltransferase reaction of certain amino acid sequences termed macrolide arrest motifs, or MAM-s. The translation of some proteins, which do not have MAM-s, continues.*

Macrolides bind to the nascent peptide exit tunnel at the 50S ribosome, near the PTC (Tenson & Mankin, 2006). Macrolides inhibit the synthesis of certain amino acid sequences, called macrolide arrest motifs, or MAMs, so the synthesis is not haltered globally in all proteins, but rather in a subset of proteins. Macrolide-bound ribosome fails to polymerize a MAM amino acid sequence and will stall. If a protein lacks MAM, its translation will carry out normally. Specific MAMs depend on the macrolide structure (Vázquez-Laslop & Mankin, 2018b). Macrolides' working principle on translation is shown schematically in Figure 4B. Macrolides inhibit ribosomal assembly indirectly by translation inhibition (Siibak et al., 2009).

Some macrolides, such as ERY and AZI, are bacteriostatic, whereas some, e.g. telithromycin, are bactericidal. The cidality depends on binding affinity and kinetics of the macrolide with the ribosome – slowly disassociating macrolides are cidal, whereas fast-disassociating macrolides are mainly bacteriostatic (Svetlov et al., 2017). However, the number of ribosomes, which depends on the growth phase, also affects the cidality, therefore slower growing cells, with less ribosomes, are killed by macrolides more easily (Łapińska et al., 2022). This is controversial to many other antibiotics, which preferably kill fast growing cells

(R. H. Eng et al., 1991; Tuomanen et al., 1986). Interestingly, AZI has also been shown to inhibit QS in *P. aeruginosa*, decreasing biofilm formation and sensitizing the bacteria to itself (Hoffmann et al., 2007).

Soon after ERY's discovery resistance to the drug was reported. Interestingly, macrolides take advantage of the MAMs and translational frameshifting in regulating the expression of their *erm* resistance genes, which code for rRNA methyltransferases and are one of the most important resistance mechanisms for macrolides. They methylate the macrolide binding site, reducing the affinity of macrolides. The expression of some of these methyltransferases is regulated by macrolide presence – it presents a leader peptide in front of the open reading frame (ORF) of the methyltransferase gene, which alters the secondary structure of the mRNA depending on the macrolide-induced stalling in the leader peptide, turning the translation of the downstream gene "ON" or "OFF" (Beckert et al., 2021). Efflux, inactivation of the drug or target site mutations also confer resistance to macrolides (Roberts, 2008).

Macrolides are relatively safe antibiotics. Main side effects of macrolides are interactions with other drugs, which are also substrates for the cytochrome P450 liver enzymes. Due to this, macrolides can inhibit the metabolism of these drugs, e.g. antiepileptics, leading to toxicity (Bryskier & Bergogne-Bérézin, 2005). AZI does not significantly interact with the cytochrome P450 enzymes and has less drug interactions than other macrolides, e.g with anticoagulant warfarin (Parnham et al., 2014; Westphal, 2000). Additionally, macrolides have been associated with mild gastrointestinal side effects and potentially fatal cardiac arrhytmias and fainting in predisposed individuals (Bryskier & Bergogne-Bérézin, 2005). Due to its low metabolism AZI has a long half-life of 50 to 70 h. Therefore, it can be used once per day for relatively short treatment courses of 3 to 5 days, which would still lead to antibacterial drug concentrations in tissues for up to 10 days. For some indications one-time high oral dose of AZI is prescribed. Overall, this leads to better treatment compliance in patients (Lode et al., 1996; Parnham et al., 2014)

Immunomodulatory properties of macrolides were noticed in the 1970s, when it was found that long-term administration of ERY reduced the 10-year mortality rate of diffuse panbronchiolitis, a severe progressive inflammatory lung disease, from 90% to 10%. The effect remained despite the presence of ERY-resistant infections in those patients, and a low dose of ERY. The anti-inflammatory effect is employed in case of chronic lung diseases, such as cystic fibrosis, chronic obstructive pulmonary disease, asthma, etc. (Pollock & Chalmers, 2021). The benefit of AZI's immunomodulatory effects has even been studied in case of treating COVID-19 infections (Echeverría-Esnal et al., 2021). Macrolides have also been used topically in case of chronic eyelid inflammation, and periodontitis. While the exact immunomodulatory mechanisms remain unclear, macrolides most importantly reduce the neutrophil counts, their adhesion, as well as regulate the levels of several inflammatory cytokines. There have been some reports of eosinophil-count lowering effects (Zimmermann et al., 2018).

2.2.3. Aminoglycosides

Aminoglycosides (AGs) are bactericidal broad-spectrum antibiotics of natural or semi-synthetic origin. They were among the first clinically used antibiotics after the introduction of streptomycin in 1944. Streptomycin was isolated from *Streptomyces griseus*. AGs were outplaced from clinical use in the 1980s due to availability of potentially safer antibiotics, such as cephalosporins or fluoroquinolones (Krause et al., 2016). However, due to increasing resistance to the other antibiotics, as well as a fast effect, synergy with beta-lactams, chemical stability and low cost, AGs are still widely used (Lopez-Novoa et al., 2011). Structurally, AGs are characterized by dibasic aminocyclitol, such as 2-deoxystreptamine, connected with amino sugars via glycosidic bonds (Krause et al., 2016). Amikacin (AMI) is the most widely used semisynthetic AG (Ramirez & Tolmasky, 2017). Structures of some AGs are shown in Figure 5A.

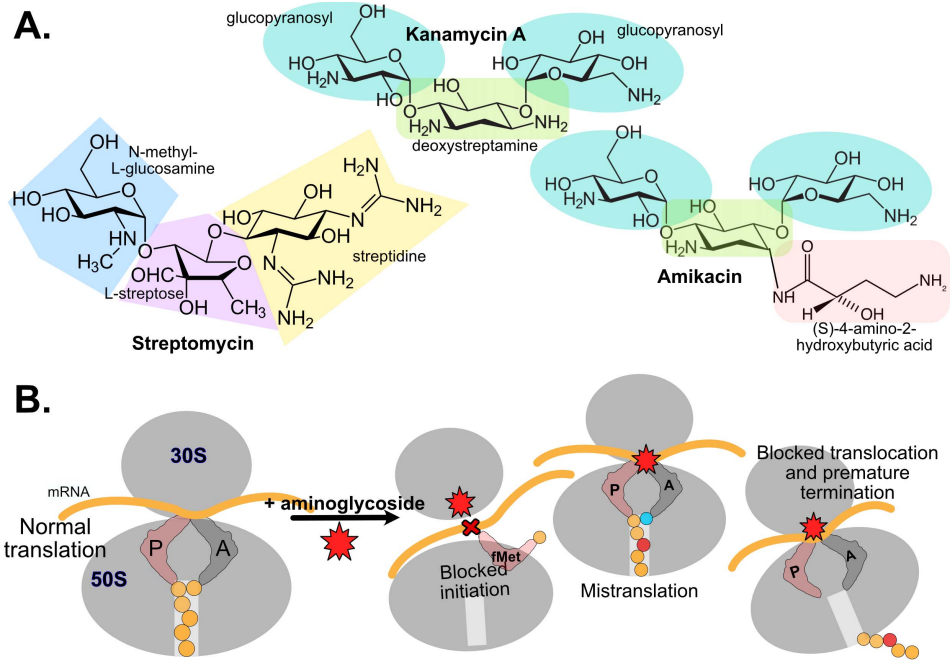

Figure 5. *Aminoglycosides. A. Chemical structures of aminoglycosides, which consist of amino sugar residues linked with glycosidic bonds. Streptomycin was the first aminoglycoside discovered. Amikacin is a derivative of kanamycin. B. General mechanism of action on the ribosome. Aminoglycosides bind to the 16S rRNA in the small ribosomal subunit near the A-site. They inhibit protein synthesis by blocking initiation and translocation, and by causing erroneous protein synthesis.*

To enter the cells, first the cationic AG binds electrostatically to the negatively charged bacterial membrane, displacing magnesium ions. This destabilises the bacterial membrane integrity, leading to enhanced permeability. The entry into

the cytoplasm is a slow and energy-dependent process. AGs bind with high affinity to the small ribosomal subunit 16S rRNA A-site decoding centre, changing its conformation in a manner that resembles the ribosome in case of codon-anticodon pairing. AGs inhibit translocation and subunit splitting, cause erroneous protein synthesis, damage to the cell membrane, some block elongation or inhibit initiation (Figure 5B). While the exact mechanism is not known, all AGs quickly kill the bacterial cells (Krause et al., 2016; D. N. Wilson, 2014).

AGs are particularly effective against *Enterobacteriaceae* and other Gram-negatives, as well as having good activity against Gram-positives, such as *Staphylococcus aureus* (including problematic methicillin-resistant *S. aureus*, MRSA), or even *Mycobacterium tuberculosis*. They are not active against anaerobes, as well as streptococci, enterococci and a few other species. Due to poor absorption from the gut AGs are administered by injection or inhalation. Infrequent high dosing is possible due to dose-dependent killing and is associated with better patient outcomes and less toxic side effects. They distribute broadly into tissues, which enables their use during deep tissue infections. AGs are excreted via urinary tract, so they can be used for treating UTIs. Combination therapy with beta-lactams is often used empirically in case of serious infections, and resistance is suspected, or in case of MDR. Because paromomycin can bind to eukaryotic ribosomes, but not absorbed from the gut of the host, it is sometimes used as antiprotozoal agent (Krause et al., 2016).

AGs have a narrow therapeutic window, where effective doses are close to toxic doses. The main toxicity manifests in the kidneys and in the internal ear (cochleovestibular apparatus). In the kidneys the tubular epithelial cells die, inflammation occurs, which leads to obstruction of tubules, change of water and solute transport and altered glomerular filtration. As with CAM, action on mitochondria is involved in toxicity. Laboratory parameters, such as urine protein and leukocyte levels, serum creatinine and electrolyte levels, as well as drug concentrations in blood should be monitored to detect kidney damage. Nephrotoxicity can occur in 3–25% of patients despite monitoring. The toxicity potential of different AGs varies – gentamicin appears to be most nephrotoxic, whereas AMI and tobramycin are tolerated the best (Lopez-Novoa et al., 2011; Veyssier & Bryskier, 2005). Ototoxicity presents first in vestibular damage with coordination and balance issues, which usually subsides, while the more serious cochlear damage, beginning with tinnitus and sudden loss of hearing, is irreversible. The cochlear damage can occur during therapy or months later. Incidence of ototoxicity can be up to 20% in the presence of risk factors (Veyssier & Bryskier, 2005).

Main resistance mechanisms include enzymatic inactivation of the drug, increased efflux, and target site modification. The latter is difficult to achieve by a chromosomal mutation, as nearly all prokaryotes encode several copies of rRNA. Instead, the target site is modified by specific methyltransferases, which prevent drug binding. Enzymatic inactivation of the drug is facilitated by acetylating, phosphorylating, or adenylating enzymes, which modify the amino or hydroxy groups of the AG, decreasing the binding affinity of the drug (Krause et al., 2016).

AMI is refractory to many modifying enzymes (Ramirez & Tolmasky, 2017). Efflux is used by several pathogens, which present intrinsic resistance to AGs (Krause et al., 2016). *E. coli* uses AcrAB-TolC efflux pump complex for multi-drug resistance of mainly lipophilic and amphiphilic drugs, which does not apply to AGs, as they are hydrophilic. Instead AcrB homologue AcrD is used as a transport protein to specifically pump out AGs in conjunction with the AcrA and TolC (Aires & Nikaido, 2005; Z. Zhang et al., 2023) Studies published in 2014 and 2015 found that about 90% or more of *E. coli, Klebsiella* spp*., and Pseudomonas aeruginosa* isolates from intensive care units were susceptible to AGs. Often AG resistance factors coincide with the resistance to other antibiotics (Krause et al., 2016).

2.3. *In vitro* methods for studying ribosome-targeting antibiotics

First antibiotics were screened from laborious animal infection models, which were soon switched to *in vitro* screening assays (Aminov, 2010). Methods for studying ribosome-targeting antibiotic effects and binding properties vary a lot depending on the research question. The methods can be broadly categorized as those based on i) cell-free systems (containing purified components of the translation system), and ii) those based on intact living cells. Structural biology methods on drug-bound and drug-free ribosomal particles provide single molecule resolution information about antibiotic interactions and induced changes. However, structural methods are not in the scope of this thesis, and are extensively discussed elsewhere (Lin et al., 2018; Paternoga et al., 2023; D. N. Wilson, 2014; L. Zhang et al., 2021). Living cell methods can be subdivided into bulk culture and single cell methods.

Cell-free methods provide detailed information on direct interaction of the drug with translation components, however in addition to being laboursome they can fail to reproduce the complexity of a real cell environment. Whole living cell methods provide more complex information about antibiotic responses, take into account the drug's ability to pass into the cells, and the physiological changes they introduce. However, living cell methods do not give information on direct effects on the ribosome, but rather on the global response within the cells. *In vivo* experiments would give even more complex information on the antibiotic behaviour within the host organism, effects of immune system on the pathogen and the effects of the pathogen on the host, however for the purpose of this thesis will not be covered. The following paragraphs will give an overview of classical cell-free methods, as well as living cell methods based on bulk culture analysis and those based on single cell analysis.

2.3.1. Cell-free methods

Cell-free translation systems (CFTS), sometimes also called *in vitro* translation systems, were discovered in the 1950s, when several groups noticed that translation occurs even after cell membranes have disintegrated. In simple systems only protein biosynthesis and the factors that block it, e.g. antibiotics, are studied. More complex systems combine both transcription and translation. CFTS based on different cell extracts are also available commercially (Szaflarski et al., 2012). Cell extracts or purified ribosomal components can be used for *in vitro* translation. The translation products are visualized by incorporation radioactively or fluorescently labelled amino acids, which are then purified or separated by gel electrophoresis, or by using luciferase or Green fluorescent protein (GFP) mRNA as a template (Osterman, Bogdanov, et al., 2016).

Simple CFTS-s are based on the synthesis of protein homopolymers, such as poly(U)-dependent polyphenylalanine synthesis. The components for the system are obtained from bacterial extracts by centrifugation and a subsequent sucrose gradient purification. Both intact 70S ribosomes and vital cofactors (initiation factors, aminoacyl-tRNA synthetases, elongation factors) are purified from the extracts. Free ribosomes and other components necessary for translation (ATP/GTP, amino acids, cofactors) are mixed in a buffer with poly(U) mRNA as a template. Poly(U) does not require a Shine-Dalgarno sequence to initiate translation. With this system up to 300 amino acid polyphenylalanine can be synthesised, allowing to analyse the whole protein synthesis pathway. This method allows to measure for example misincorporation of radiolabelled amino acids, such as near-cognate lysine instead of Phe, in the presence of antibiotics (Szaflarski et al., 2012). Poly(A), poly(C) and polyinosinic acid (poly(I)) in combination with the respective radiolabelled amino acids have also been used to study misincorporations caused by AGs (Davies & Davis, 1968).

CFTS-s allow to rapidly screen protein synthesis inhibitors for their mechanism of action or to determine the inhibitory concentration. GFP is often used in these systems due to simple quantification by measuring fluorescence (Szaflarski et al., 2012). Both poly(U) and GFP methods were used for example to study misincorporation induced by AG streptomycin. Total GFP and active GFP fractions were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and native PAGE, respectively (Szaflarski et al., 2008). In a similar assay, edeine, an antimicrobial peptide inhibiting translation initiation was found to induce misreading, whereas pactamycin (aminocyclitol) did not. Instead, studies with defined ribosomal complexes, where specific translation components are added one by one, revealed that pactamycin inhibited translocation, disproving that it is an initiation inhibitor (Dinos et al., 2004).

Classically drug-ribosome interactions have been studied by analysing the resistance mutations of ribosomal genes (Tenson & Mankin, 2006). Other classical approaches for studying drug-ribosome binding include equilibrium dialysis, gel filtration and filter binding, which often use radiolabelled compounds. Another tactic is to develop fluorescent antibiotic derivatives, however, this can change

drug characteristics significantly, and lead to false conclusions. Other classical methods like footprinting and crosslinking are time-consuming and laborious (Llano-Sotelo et al., 2009). These classical studies showed that most antibiotics bind close to known functional centres of the ribosome, such as the PTC or decoding centre. Nuclear magnetic resonance and crystallography methods have provided detailed views of drug-ribosome interactions on atomic level (Lin et al., 2018; Paternoga et al., 2023).

In equilibrium dialysis method two chambers with a semi-permeable membrane are used. Principally one chamber is filled with radiolabelled antibiotic and ribosome or individual ribosomal subunits. Unbound radiolabelled antibiotic diffuses to the other chamber and after reaching the equilibrium the radioactivity of solutions from each compartment is measured by scintillation to estimate the concentration of antibiotic-ribosome complexes (Le Goffic et al., 1979). By using tritium-labelled kanamycin in equilibrium dialysis and filtering methods, ultimately measuring radioactivity from the filter, it was shown that both ribosomal subunits have binding sites for AGs (kanamycin, neomycin, gentamicin, but not streptomycin), the AG molecules per ribosome correlate positively with the drug concentration, and these drugs inhibit mainly translocation (Misumi et al., 1978). Later studies have found that AG-binding to RNA is often non-specific due to electrostatic interactions (Liang et al., 2006).

Two-phase partitioning can also be used for studying drug-ribosome binding. In a dextran-polyethylene glycol (PEG) phase system ribosomes will stay in the dextran phase. A (radiolabelled) antibiotic is added to the system with ribosomal components and the system is separated by centrifugation. The antibiotic can then be quantified from each phase using a suitable method, such as scintillation, fluorimetry, or UV-spectrophotometry. The advantage of this method over equilibrium dialysis is that it is much faster, the polymer solution increases the stability of biological products, and there is no unspecific binding to a membrane, which can occur in equilibrium dialysis. AGs, CAM and tetracycline binding constants were determined to verify this method (Le Goffic et al., 1980).

Classical chemical footprinting is used to study RNA (or DNA) complexes, including antibiotic binding. Ribosomes are first incubated with the antibiotic, after which the sample is treated chemically, e.g. with dimethyl sulphate or kethoxal, to modify the ribonucleotides unprotected from the bound antibiotic with chemical probes. Complementary DNA (cDNA) is produced from the rRNA by reverse transcription with radiolabelled substrates, resulting in “footprints”, or blank spots, on sequencing gels in antibiotic-bound sequences (Moazed & Noller, 1987; Tijerina et al., 2007). This method revealed, for example, the binding sites of AGs and tetracycline on the 16S rRNA (Moazed & Noller, 1987). Toeprinting assay is principally a similar method to study antibiotic-stalled mRNA-ribosome complexes. The ribosome itself halts reverse transcription of the mRNA. Sequencing allows to estimate the length of the mRNA unprotected by the ribosome. The 3’ end of the cDNA is located 15–16 nucleotides from the P site. Translation initiation inhibitors either prevent ribosome binding to mRNA or arrest ribosome at the start codon. Inhibitors of peptidyl transferase reaction,

translocation, or tRNA binding will arrest the ribosome at the start codon, or at a random codon, if the drug is added after translation initiation. Drugs binding to the nascent peptide tunnel are expected to inhibit translation after a short peptide has been produced (Orelle et al., 2013; Osterman, Bogdanov, et al., 2016; Vázquez-Laslop & Mankin, 2018a).

Fingerprinting method can be used for studying drug binding to specific ribosomal proteins in two-dimensional (2D) gel electrophoresis, which separates proteins depending on their charge. Proteins are made visible with staining, resulting in 2D electropherogram, or a "fingerprint", where each spot represents a single ribosomal protein. The whole process takes days to complete (Kaltschmidt & Wittmann, 1970). To study drug binding, a photoreactive group and a radiolabel is added to the drug, which is then incubated with purified ribosomes. Drugs are covalently bound, or crosslinked, to their binding site by irradiation. The proteins and rRNA are separated, 2D gel electrophoresis is run and the gels are exposed via radiographic methods. Overlapping spots on the 2D electropherogram and radiography plate represent the drug-protein binding. This method together with high performance liquid chromatography (HPLC) was used to reveal the binding site of ERY (Arévalo et al., 1988).

High-throughput approach for studying drug-ribosome binding is to incorporate fluorescent labels to ribosomal proteins of interest. Drug binding would change the fluorescence. This method was validated using AG antibiotics and fluorescently labelled S12 ribosomal protein. While the production of fluorescently labelled ribosomal components is laborious, the method can be used for the large-scale screening of antibiotics once the components have been obtained (Llano-Sotelo et al., 2009). Recent advances, such as single-molecule fluorescence resonance energy transfer (FRET) allow to follow the conformational change and translation kinetics of a single ribosome in the presence of antibiotics (Vázquez-Laslop & Mankin, 2018a). FRET is a fluorescence-based technique, which enables to measure interactions between different fluorescently labelled structures. In case of interaction fluorophores come close, so that light emitted from the first fluorophore excites the second fluorophore. This approach has been used to monitor translational and conformational changes during AG treatment in purified ribosomes (Ermolenko et al., 2007; Wang et al., 2012).

2.3.2. Bulk culture methods

In the early days of antibiotic research potential drugs were screened on a large-scale in animal models, such as rabbit syphilis infection model tested by Paul Ehrlich and colleagues (Aminov, 2010). However, a much simpler screening method, a gold standard for antimicrobial susceptibility tests (AST-s) used today, was introduced by Alexander Fleming. The screening method involved determining antibiotic inhibition zones of pathogenic bacteria on solid medium (Aminov, 2010). Currently, the most important bulk culture-based method for studying antibiotic effects is based on testing growth inhibition per standardized protocols on solid or in liquid medium.

Agar diffusion test is a standard method based on diffusion of antibiotic into an agar medium from a solid vehicle containing a specific quantity or a concentration gradient of the antibiotic. Agar medium is covered with bacterial lawn, antibiotic vehicle is added, and the plate is incubated for a certain time to produce visible bacterial growth. Exact medium composition and incubation parameters depend on the pathogen to be tested. The zone around the antibiotic vehicle where bacteria do not grow after incubation reflects the bacterial sensitivity to the drug. The antibiotic can be loaded to a filter paper disc, resulting in a growth inhibition zone around the disc. The size of the inhibition zone allows to estimate, if a bacterium is sensitive or resistant to the drug based on standard inhibition zone diameters issued by relevant agencies (The European Committee on Antimicrobial Susceptibility Testing (EUCAST) in Europe, Clinical and Laboratory Standards Institute (CLSI) in USA). Standardized discs are available commercially. Several discs can be used on one plate in parallel (Matuschek et al., 2014). ETEST® strips are commercially available plastic strips containing a defined concentration gradient of an antibiotic (*ETEST®*, n.d.). The marking on the strip allows to determine the minimum inhibitory concentration (MIC), or the lowest antibiotic concentration that inhibits visible growth.

Serial dilution method is a laboratory standard test for determining the MIC. The method involves diluting the antibiotic in a 2-fold series in the medium and visually checking the growth of bacteria after incubation. MIC is the lowest concentration of the antibiotic, which produces no visible growth of the bacterial culture. The preparation of the inoculum is standardized (Andrews, 2001; EUCAST, 2003). To determine whether the bacterium is resistant or susceptible to the antibiotic, the MIC is compared with values given in clinical breakpoint tables issued by relevant agencies. Epidemiological and clinical data, pharmacokinetic and pharmacodynamic parameters are taken into account when constructing these annually revised tables (European Committee on Antimicrobial Susceptibility Testing, 2021; The European Committee on Antimicrobial Susceptibility Testing, 2023). It has been found that 64% of patients respond to treatment with antibiotics, which the infection-causing pathogen is resistant to according to MIC (Doern & Brecher, 2011). Minimum bactericidal concentration (MBC) is the minimum concentration that kills >99.9% of the initial inoculum used for MIC assay and is determined subsequently after MIC test by subculturing bacteria from concentrations exceeding the MIC in fresh medium without antibiotics. An antibiotic is considered bacteriostatic, if it kills less than 99% of the initial inoculum (Pankey & Sabath, 2004; Reimer et al., 1981).

Developing novel rapid diagnostic tools is part of the WHO's global action plan on antibiotic resistance (World Health Organization, 2015). Novel rapid AST methods are based on either determining genetic or phenotypic resistance. Genetic resistance detection methods are based on detecting resistance genes by nucleic acid amplification or next generation sequencing methods, or by detecting resistance proteins. This method relies on sequence databases and known resistance genes. Phenotypic AST methods use a variety of methods, including microscopy, flow cytometry, biochemical reactions, nanoscale movement detection, etc. These

methods aim to detect bacterial viability or cell damage and produce AST results within a few hours, instead of 2–3 days necessary for standard culture-based AST-s. Only a few methods and indications have been approved for clinical use so far (Banerjee & Humphries, 2021; van Belkum et al., 2020).

AST-s do not provide information about mechanisms of action. A classical method for studying translational kinetics or alterations caused by antibiotics is done by radiolabelled amino acid incorporation assays. For pulse-labelling, cells are incubated shortly with a radiolabelled amino acid precursor (pulse), sometimes followed by an excess of unlabelled amino acid (chase). Radioactivity of the cells or cell extracts is then measured to estimate the amount of newly translated proteins. The samples taken in short intervals allow to calculate the rate of protein synthesis (Lodish et al., 1995). The method was, for example, used in classic studies for CAM and streptomycin (Kirschmann & Davis, 1969). Similar approach by using radiolabelled ribonucleotides and detecting incorporation into rRNA can be used for monitoring ribosome assembly in the presence of translation-inhibiting antibiotics (Champney, 2020; Champney & Miller, 2002). Initial studies on streptomycin uptake were conducted by adding radiolabelled streptomycin to growing cultures and measuring radioactivity of washed cells. It was found that streptomycin uptake occurs in several steps – fast initial uptake, followed by a plateau, then a second slower uptake. Addition of CAM prevented the secondary uptake of streptomycin. The authors hypothesized that the secondary uptake is a result of membrane damage (Anand et al., 1960).

A significant amount of knowledge on how ribosomal antibiotics work has come from ribosome profiling, or Ribo-seq, studies. Ribo-seq is based on detecting ribosomal distribution across mRNA in a bacterial cell. Cell extracts are treated with RNase to remove RNA, which is not covered by the ribosome. The ribosome protects about 30 nucleotides on its mRNA. Intact ribosomes are then purified in a sucrose gradient, and whole RNA is extracted. These mRNA footprints are then converted into cDNA, which are deep sequenced. Deep sequencing is a quantitative measurement in a library of mRNA fragments. The fragments are then aligned and mapped to genome sequences. For studying antibiotics ribosome footprints of treated and untreated cells are compared. Ribosome-binding drugs are expected to arrest translation in specific sites on the open reading frame, indicating its mechanism of action (Ingolia et al., 2009; Vázquez-Laslop & Mankin, 2018a).

Bioreporters are genetically engineered cells, which produce a measurable signal in response to some kind of environmental change, such as the presence of antibiotics. The signal can be a colorimetric change, fluorescence, or luminescence. Classical bioreporters responding to translation inhibitors utilize stress promoters, gene fusions or riboswitches to induce reporter genes (Bianchi & Baneyx, 1999; Cheng Vollmer & Van Dyk, 2004; Mehdizadeh Aghdam et al., 2016; Melamed et al., 2012; Osterman, Komarova, et al., 2016; Shapiro & Baneyx, 2007). Alternative approach is to mutate the reporter gene, so it is inactive during normal translation. Signal is only produced when translation is altered, such as by amino acid substitution or frameshift, which restores the reporter protein (Kramer &

Farabaugh, 2007; Thompson et al., 2002, 2004). Dual-reporters, expressing two reporter genes, are useful when testing translational inhibitors, as they allow to monitor general translation inhibition in parallel to reporter gene induction (Fan et al., 2017; Osterman et al., 2012).

Simplest reporter systems are based on the β-galactosidase assay. β-galactosidase is an enzyme encoded by the *lacZ* gene, which is responsible for the hydrolysis of lactose. In classical bioreporters the *lacZ* gene is expressed upon treating the cells with antibiotics, which results in a chromogenic change of synthetic β-galactosidase substrates, such as *o*-nitrophenyl-β-D-galactoside (ONPG) turning from colourless to yellow. The colour change is induced by cell lysates or within the cells after permeabilization (Bianchi & Baneyx, 1999). Luminescence-based systems use firefly and/or *Renilla* luciferase genes, *Fluc* and *Rluc*, respectively. Luciferase released from cells by lysis oxidates luciferin (*Fluc*) or coelenterazine (*Rluc*) in the presence of cofactors into a luminescent product, which can be measured by luminometry (Smale, 2010). Currently, fluorescence genes are probably used the most, as fluorescence can be measured directly from live cells. Many are based on the Green fluorescent protein (GFP), and its derivatives. However, a wide range of fluorescent proteins of any excitation and wavelength combination and special characteristics, such as redox state or growth phase indicating fluorescent proteins, have been developed. FPbase, a comprehensive fluorescent protein database, currently lists more than 920 proteins (Lambert, n.d., 2019). Fluorimetry allows to measure fluorescence signal from bulk cultures, however methods like fluorescence microscopy and flow cytometry allow to detect signals from individual cells.

Current advanced omics-based methods are powerful tools to monitor global changes within bacteria upon antibiotic exposure. These methods can also be used to screen new potential drugs for their mechanism of action. Transcriptomics, or gene expression profiling, uses microarray (Boshoff et al., 2004; Hutter et al., 2004) or next generation sequencing (RNA-Seq) (O'Rourke et al., 2020) based methods to determine transcriptome signatures responding to different drug classes. On average >1200 genes were found to be differentially expressed after the treatment with translation inhibitors in *E. coli,* and 30S and 50S binding drugs share 45% similarity, clustering separately from antibiotics with other targets (O'Rourke et al., 2020).

Proteomics, or protein profiling, is another approach, which helps to understand antibiotic actions within cells. Earlier studies used radioactive labelling, followed by 2D-PAGE to separate the proteins. By comparing the electropherograms of cells treated with ribosome-targeting antibiotics and cells subjected to heat or cold stress it was found that these antibiotics induce either cold or heat shock response (VanBogelen & Neidhardt, 1990), which has been useful for developing bioreporters responding to translation inhibitors (Bianchi & Baneyx, 1999). Current improved technologies use combination of 2D gel electrophoresis or liquid chromatography to separate the proteins, followed by mass spectrometry analysis to identify the proteins. In some earlier studies only differentially expressed proteins were excised from gels and detected (Bandow et al., 2003),

whereas whole proteome studies allow to cluster different antibiotics based on their mechanism of action (Yu et al., 2020). Proteomics can be used for detecting proteins responsible for persistence or resistance (Sulaiman & Lam, 2020). Mass spectrometry has been used to study translational accuracy during antibiotic treatment (Garofalo et al., 2019). Proteomics also allows to rapidly identify bacteria: matrix-assisted laser desorption ionization time-of-flight (known as MALDI-TOF) mass spectrometry is method widely used in the clinics for identifying infection-causing bacteria. This method can in some cases be modified for rapid detection of antibiotic-resistant isolates (Vrioni et al., 2018).

Metabolomics, or metabolic profiling, is another omics approach, which focuses on the study of metabolites. For the analysis metabolites are extracted and analysed using HPLC followed by mass spectrometry. This method helps to investigate how different antibiotics change the biochemistry and metabolic pathways, giving additional layer of information about the mechanisms of action. Different antibiotic classes have found to induce different metabolic profiles, with most significant changes in tricarboxylic acid cycle, nicotinamide adenine dinucleotide, amino acid, and nucleotide metabolites (Schelli et al., 2017)

2.3.3. Single cell methods

Many of the methods described for bulk culture can be modified to provide single cell resolution. These methods are most often based on the measurement of fluorescence in single cells using microscopy or flow cytometry. Single cell methods allow to detect population heterogeneity and help to explain why genetically identical bacteria have different reactions to antibiotics, and how to optimize antibiotic therapy. Single cell methods are rapidly developing, and the wider use of them is largely limited by available equipment. As the technologies are becoming available more widely, more comprehensive knowledge on antibiotic effects on individual bacteria will come soon.

Fluorescence in single cells can be produced by either the expression of reporter genes (described in more detail in paragraph 3.3.2), by using specific fluorescent dyes or antibodies, or fluorescence *in situ* hybridization (FISH) techniques. Fluorescent dyes, which bind to nucleic acids, can be used to determine bacterial viability after antibiotic exposure, as some dyes can penetrate only dead cells (e.g. propidium iodide), whereas some penetrate all cells (e.g. Syto9) (Moyes, 2009). Some dyes or fluorescent reporters can be used to monitor cell division after antibiotic exposure – bacteria are loaded with fluorescent probes before antibiotic treatment, and the fluorescence will dilute out with subsequent divisions, whereas cells unable to divide will remain fluorescent (Marro et al., 2022; Roostalu et al., 2008). Dyes can be used to measure oxidative stress, membrane potential, and other physiological changes (Imlay, 2015; Suller & Lloyd, 1999). Using dye-labelled tRNA-s it is possible to monitor translation rates after antibiotic exposure in single cells (Aguirre Rivera et al., 2021). Antibodies and FISH are usually used for identifying bacteria, especially in polymicrobial communities. Antibodies targeting antibiotics can be used to visualize antibiotic localization around single

bacteria during cell culture infection (Mu et al., 2016) or to label bacterial surface molecules, such as pili (Wright et al., 2007). Intracellular structures can be labelled after permeabilization (Wessel et al., 2023). FISH can be used to study mRNA levels of single genes (Skinner et al., 2013), and could be used after antibiotic exposure, however the method's use in bacteria is limited so far. Aptamers, which become fluorescent after ligand and subsequent organic dye binding show more potential in studying RNA in bacteria (van Gijtenbeek & Kok, 2017).

Fluorescence-activated cell sorting allows to separate single bacterial cells from a phenotypically heterogeneous culture based on their fluorescence level, e.g. reporter signal, and further analyse these subpopulations with other analysis methods, such as growth assays, transcriptomics, or proteomics. These subpopulations can then be investigated for their susceptibility to different antibiotics (Personnic et al., 2019), or how genes are differentially expressed in these subpopulations after antibiotic treatment (Peyrusson et al., 2020). Time-lapse microscopy and microfluidics allow to estimate the growth rate of single cells without using fluorescent probes (Marro et al., 2022). Raman microspectroscopy allows to create metabolic images of single cells after antibiotic exposure (Hong et al., 2018), however the method is not widely used.

Single cell omics is still a developing field. This is based on separating bacterial cells by microfluidics into droplets, which act like individual reaction containers. Nucleotide sequences can then be amplified within each droplet, individual cells can be barcoded and analysed via next generation sequencing to obtain genome or transcriptome data. Thousands of single cells must be analysed to make statistically significant observations (Liu et al., 2019). Recent single-cell transcriptome study revealed that antibiotics with different mechanisms cluster separately and there is always a small subpopulation of cells that differentially express mobile genetic elements, which might promote antibiotic tolerance and resistance (Ma et al., 2023).

Single cell proteomics and metabolomics methods in bacteria are still largely limited by technology. Nanoscale Secondary Ion Mass Spectrometry, or NanoSIMS shows particular potential to study metabolic activity in single cells (K. M. Davis & Isberg, 2016). NanoSIMS has been used to study antibiotic localization in single *M. tuberculosis* during infection (Greenwood et al., 2019), however due to the limited availability of this technology there are only a few studies published using this technology on bacteria.

3. AIMS OF THE STUDY

The overall aim of the thesis was to develop methods, which would allow to improve the estimation of antibiotic treatment efficacy in patients. The study was focused on ribosome-binding antibiotics. Different traditional and novel methods were combined.

The 1st article is focused on studying local drug delivery systems intended for wound treatment. Current standard methods are far from mimicking wound environment. Therefore, the specific aim was to develop a suitable drug release model for nanofibrous antibacterial wound dressings. We used CAM as the model drug and compared two different electrospun nanofibrous mats. The objectives were:

1) to compare drug release kinetics from the mats using traditional drug release assays into buffer solution and drug release into semisolid hydrogel
2) to develop a fluorescent *E. coli* reporter strain to detect CAM in the environment and to use the reporter strain to measure CAM release from the mats into agar hydrogel
3) to find a suitable method that would allow to differentiate the mats with different drug release profiles

The 2nd article is focused on questioning whether the addition of bicarbonate to testing media improving the accuracy of AST-s. The specific aim was to understand if bicarbonate, the main blood buffer component, makes the AST environment biologically more relevant. We hypothesized that the effect in changing the MIC-s was just an effect caused by increased pH due to addition of bicarbonate. We used a macrolide antibiotic AZI as an example drug. The objectives were:

1) to study how the MIC of AZI in *S. enterica* Typhimurium changes with bicarbonate with different buffering systems
2) to measure pH-change of the AST medium containing bicarbonate with or without buffering

The specific aim of the 3rd article was to develop a fluorescence-based reporters that would allow to measure the effects of translation inhibitors in infection environments. We studied the effects of amikacin and azithromycin on stop codon readthrough and translational frameshifting in *E. coli.* The objectives were:

1) To compare stop-codon readthrough and frameshifting levels induced by the antibiotics
2) To compare stop-codon readthrough and frameshifting levels in different growth environments and in different genetic backgrounds of *E. coli*
3) To test the reporters in cell culture infection model

4. METHODS

4.1. Construction of reporter strains (I, III)

All plasmids were constructed using the circular polymerase extension cloning (CPEC) protocol (Quan & Tian, 2014). Low copy number pSC101 with ampicillin resistance (AmpR) was used as the plasmid vector. *GFPmut2* was used and the control protein, followed by reporter gene *mScarlet*, the brightest red fluorescent protein available at the time. *mScarlet* was switched to faster maturing *mScarlet-I* (*iSc*), which harbours a mutation T74I (Bindels et al., 2016). In mistranslation reporters both reporter proteins were transcribed on a single mRNA from two different promoters: a constitutive Ptet and a heat-shock promoter PdnaK1, which was added to the system to increase reporter signals during AG treatment. For the ribosomal stalling reporter responding to CAM, the area between the *GFPmut2* and *iSc* features a transcription attenuation-based regulatory trpL2Ala region together with a terminator and constitutive T5 promoter from plasmid pRFPCER-TrpL2A (Osterman et al., 2012).

Stop codons or frameshift mutations were introduced into the 6th codon of the *iSc* gene for the mistranslation reporters. To produce red fluorescence reporter cells have to either ignore the stop codon or shift the reading frame ±1 nucleotide to restore the original *iSc* open reading frame. Frameshift motifs are based on known slippery sequences (Caliskan et al., 2015). Plasmid with unmutated *iSc* was used as the positive control. Empty vector plasmid without fluorescence genes or a plasmid with *GFPmut2* without *iSc* (ΔiSc) was used as the negative control for measuring autofluorescence. Mistranslation and ribosomal stalling reporter plasmids are listed in Table 2. Mistranslation plasmids are available through Addgene (accession numbers 208183…208195). The reporter system is shown schematically in Figure 6.

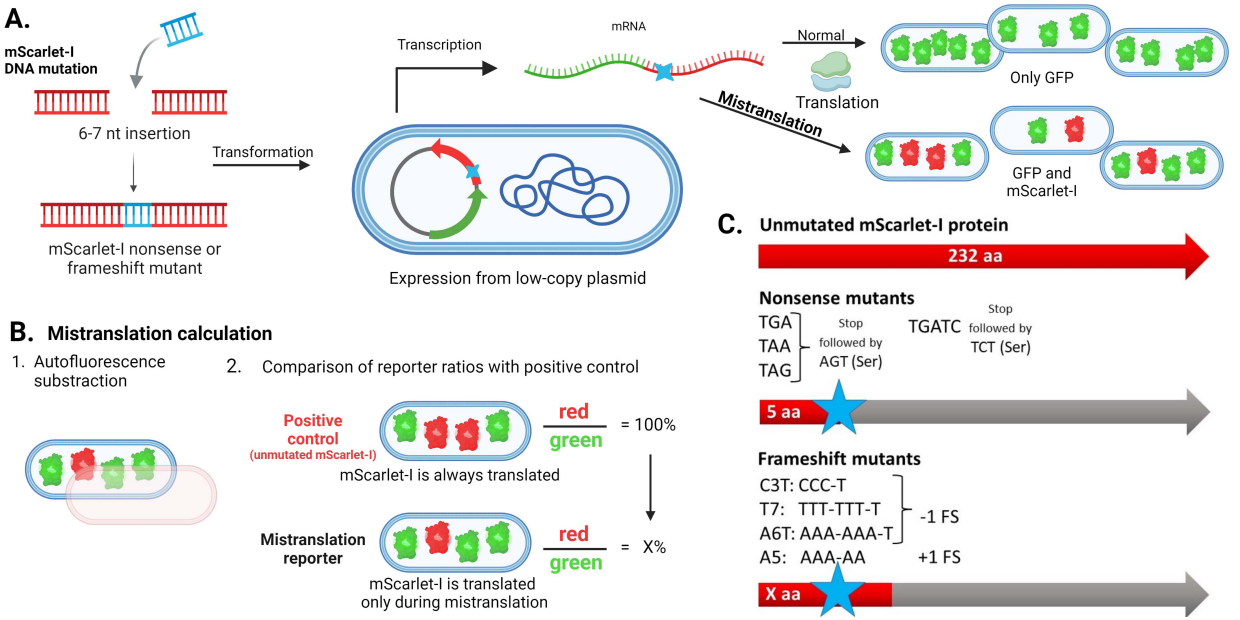

Figure 6. *Schematic representation of the mistranslation reporters' working principle. A. Frameshift or stop-codon mutation is introduced into the mScarlet-I gene. Red fluorescence is only produced when a stop-codon readthrough or frameshifting event occurs in the mutation site of mScarlet-I. B. Mistranslation is calculated as the percentage of red/green fluorescence ratio of the red/green fluorescence ratio of the positive control, where unmutated mScarlet-I is always translated. C. Different stop-codon variants and frameshift sequences were introduced into the 6th codon of the mScarlet-I gene. +1/–1 FS indicates the frameshift direction, which can be detected with these reporters.*

Table 2. *List of plasmids used.*

Plasmid name	**Abbr.**	**Description (mScarlet-I reporter primary DNA sequence when applicable)**
Mistranslation reporters		
Reporter control plasmids		
pSC101-AmpR EV	EV	pSC101 empty vector, negative control, autofluorescence
pSC101-GFPmut2 (ΔmScarlet)	ΔiSc	No mScarlet-I gene; red negative control, constitutive GFP, mScarlet negative, red autofluorescence
pSC101-GFPmut2-mScarlet-I	GFP+iSc+	constitutive GFP & mScarlet-I, positive control; ATGGTGAGCAAGGGC GAGGCA…225aa…TGA
pSC101-GFPmut2-mScarlet-I 6Trp7Ser	6Trp	constitutive GFP & mScarlet-I with Trp and Ser codons in the mistranslation mutation site, positive control ATGGTGAGCAAGGGC**TGGAGT**GAGGCA… 225aa…TGA
Nonsense reporters		
pSC101-GFPmut2-mScarlet-I TAA	TAA	stop-codon readthrough iSc, constitutive GFP ATGGTGAGCAAGGGC**TAAAGT**GAGGCAGTG
pSC101-GFPmut2-mScarlet-I TAG	TAG	stop-codon readthrough iSc, constitutive GFP ATGGTGAGCAAGGGC**TAGAGT**GAGGCAGTG
pSC101-GFPmut2-mScarlet-I TGA	TGA	stop-codon readthrough iSc, constitutive GFP ATGGTGAGCAAGGGC**TGAAGT**GAGGCAGTG
pSC101-GFPmut2-mScarlet-I TGATC	TGATC	stop-codon readthrough iSc, constitutive GFP ATGGTGAGCAAGGGC**TGATCT**GAGGCAGTG
Frameshift reporters		
pSC101-GFPmut2-mScarlet-I A4	A4	–1 frameshift iSc, constitutive GFP ATGGTGAGCAAGGGC**AAAA**GAGGCAGT…. 76aa…TAA
pSC101-GFPmut2-mScarlet-I A5	A5	+1 frameshift iSc, constitutive GFP ATGGTGAGCAAGGGC**AAAAA**GAGGCAGTGA
pSC101-GFPmut2-mScarlet-I A6T	A6T	–1 frameshift iSc, constitutive GFP ATGGTGAGCAAGGGC**AAAAAAT**GAGGCAGT
pSC101-GFPmut2-mScarlet-I C3T	C3T	–1 frameshift iSc, constitutive GFP ATGGTGAGCAAGGG**CCCCT**GAGGCAGTGAT
pSC101-GFPmut2-mScarlet-I T7	T7	–1 frameshift iSc, constitutive GFP ATGGTGAGCAAGGGC**TTTTTTT**GAGGCAGT
Ribosomal stalling reporter for detecting CAM		
pSC101-GFPmut2-term-trpL2Ala-mScarlet-I	CAM bio-reporter	Constitutive GFP, iSc transcribed only during ribosomal stalling

All plasmids were transformed into *E. coli* DH5α chemical competent cells via heat shock based method, and plasmids were purified from overnight cultures grown in lysogeny broth (LB) medium using commercial plasmid purification kits. Constructs were verified by sequencing. Mistranslation reporter plasmids were transformed into *E. coli* laboratory strains via heat shock and into clinical isolates via electroporation. The strains used are listed in Table 3. 15% glycerol stocks were made from overnight cultures, flash frozen in liquid nitrogen and stored at –80 °C. 8% dimethyl sulfoxide (DMSO) stocks were prepared from exponential cultures (OD_{600} ~0.5) in cation-adjusted Müller Hinton II broth (MHB), flash frozen in liquid nitrogen and stored at –80 °C.

Table 3. *List of bacterial strains used.*

Name	**Comments**	**Source or reference**
E. coli laboratory strains		
DH5α	Standard laboratory strain	
MG1655	Standard laboratory strain	(Hayashi et al., 2006)
BL21 DE3	Standard laboratory strain for protein production	Stratagene, USA
E. coli clinical isolates		
CFT073	Uropathogen; isolated before 1989	(Mobley et al., 1990)
Nissle	Also known as Nissle 1917 and EcN; Probiotic; isolated in 1917	Mutaflor®, Germany
DSM1103	Clinical isolate; EUCAST quality control strain for susceptibility testing of bacteria to antibiotics; isolated in 1946	DSMZ, Germany
Staphylococcus aureus		
DSM2569	Clinical isolate from a wound; EUCAST quality control strain for susceptibility testing of bacteria to antibiotics	DSMZ, Germany
Salmonella enterica serovar Typhimurium *strains*		
SL1344	Used for mouse typhoid infection model, resistant to streptomycin (StrR)	(Claudi et al., 2014; Hoiseth & Stocker, 1981)
SL1344 *acrB*-R717Q	Efflux mutant strain resistant to AZI (StrR)	(Hinnu et al., 2022)

4.2. Construction of azithromycin-resistant mutant (II)

AcrAB-TolC is a major efflux pump in *Enterobacteriaceae* and is often responsible for multi-drug resistance (Weston et al., 2018). AcrB-R717Q mutation in *Salmonella* and *E. coli* confers resistance to AZI in the clinics by increasing efflux of the drug (Hooda et al., 2019). We introduced this mutation in *Salmonella enterica* serovar Typhimurium SL1344 (Claudi et al., 2014), which is used for mouse typhoid infection model. SL1344 is resistant to streptomycin (StrR). The point mutation was introduced using dual-negative selection genome editing method (Cianfanelli et al., 2020).

Shortly, a suicide vector plasmid pFOKT (kanamycin-resistant; KanR) was constructed with Gibson assembly to include the DNA fragment containing the point mutation and 700 bp complementary sequence up- and downstream of the mutation site of the native gene. The plasmid was transferred into diaminopimelic acid (DAP)-dependent *E. coli* donor strain JKe201 for conjugation. Overnight LB-cultures of the donor strain and recipient *Salmonella* SL1344 were mixed, collected on filter, which was then incubated on LB agar for 6 h for the conjugation. Cells were retrieved from filter and *Salmonella* was selected overnight for StrR (90 µg/ml) and KanR (50 µg/ml) on DAP-free LB-plates. Several colonies were then combined and incubated for 4 h in LB liquid medium and streaked on salt-free LB-plates containing 20% sucrose and 0.5 µg/ml anhydrotetracycline (AHT) and incubated at 28 °C for 24 h. AHT induces the expression of two genes in the pFOKT plasmid: one converts sucrose into a toxic product and the other is a restriction enzyme causing double-stranded breaks in a recognition site. This allows to select for clones, which have lost the plasmid. Colonies were screened by PCR for desired mutation and verified via sequencing.

4.3. Drug release studies of nanofibers (I)

CAM was used as the model drug to test release from two different nano- or microfibrous mats consisting of polycaprolactone (PCL) or polyethylene oxide (PEO) in combination with PCL (PCL/PEO), resulting in mats with different drug release kinetics. The nanofibers were produced via electrospinning. Drug release was first tested in a dissolution system, which is a standard method for studying drug release from various drug delivery systems (DDS), mainly oral DDS-s, such as tablets or capsules etc. (Azarmi et al., 2007). For the modified dissolution test, we inserted 4 cm^2 CAM-containing nanofibers into phosphate buffered saline (PBS) and the system was kept at 37 °C with constant agitation. The drug concentration in the buffer was constantly monitored via UV/Vis spectrometry.

Alternatively, CAM release into agar was tested. First, nanofiber mats were put on LB-agar plates and kept at 37 °C for a specific time. Zones of LB-agar around the nanofibers were cut out, CAM was extracted from agar with 96% ethanol (EtOH), and EtOH was evaporated. CAM residues were dissolved in EtOH and CAM concentration was determined with HPLC. Additionally, the

drug release from nanofibers into 0.5% agarose hydrogel was monitored at 37 °C with UV imaging at four alternating wavelengths: 525 nm, 280 nm, 255 nm, and 214 nm. For studying the antimicrobial properties of the nanofibers, LB-agar plates were spread with an even layer of *Staphylococcus aureus* standard strain DSM2569 and nanofibers were added to the plates for specific time intervals. After subsequent 24 h of incubation at 37 °C inhibition zones were measured.

Combination of drug release kinetics and antimicrobial activity was assessed with a bioreporter disc diffusion assay. CAM bioreporter responds to CAM-induced ribosomal stalling. *E. coli* MG1655 with CAM bioreporter plasmid was spread evenly with a sterile cotton swab on a MOPS minimal agar medium plates supplemented with 0.4% (w/V) glucose and incubated for 10 h. Then the nanofibrous mats were added to agar plates and incubated further. The plates were scanned with a fluorescence scanner Amersham Typhoon (GE Healthcare Europe GmbH, Freiburg, Germany) every hour until 6 h. The fluorescence images were analysed with ImageJ software (Abramoff et al., 2004).

4.4. Azithromycin susceptibility tests (II)

AZI susceptibility was determined via standard microdilution assay (Andrews, 2001) in cation-adjusted Müller Hinton II broth (CA-MHB) with or without 25 mM $NaHCO_3$ (sodium bicarbonate) and 100 mM buffers (HEPES, TRIS, MOPS) adjusted to pH 7.4, in ambient or 5% CO_2 atmosphere. Additionally, as different buffers can cause different biological effects (Ferreira et al., 2015), MIC-s were determined in a range of phosphate buffers (pH 6…8.5). Modified media were prepared and adjusted to pH 7.2 with HCl directly prior to the experiment. AZI dihydrate 15 mg/ml stock solution was prepared in 96% EtOH and stored at 4 °C for up to a week. AZI susceptibility was tested for *Salmonella* SL1344 wild-type (wt) and *acrB*-R717Q. AZI was serially diluted into 96-well plates, exponential phase bacteria were added to yield a ~$5*10^5$ cells/ml at the start of the experiment and the plates were incubated statically at 37°C in ambient or 5% CO_2 atmosphere for 18 h. MIC was determined via visual inspection as the lowest concentration which inhibited any bacterial growth.

4.5. pH studies of bicarbonate-added media (II)

The pH-change was monitored in different buffered CA-MHB media with indicator dye phenol red (PhR). PhR gradually changes colour of the media from yellow at pH 6.2 to red at pH 8.2. In culture media color change is reflected in the changes at wavelengths 415 and 560 nm (Held, 2018). In our experiments PhR was used in a final concentration of 15 mg/L. Absorbance at 415 nm, 560 nm, and 600 nm was measured with BioTek SynergyMx (BioTek Instruments, Inc., USA) at 37 °C in ambient air. For CO_2 incubation only endpoint measurements were recorded. Absorbance data were first normalized according to absorbance at 600

nm, then normalized absorbance of media without PhR was subtracted, and the 415 nm to 560 nm absorbance ratio was calculated. A calibration curve was constructed based on media adjusted to pH 5 to 9 and used as the basis for pH calculations.

4.6. Studies of mistranslation reporters (III)

Spontaneous mistranslation was measured in 3 laboratory *E. coli* strains: DH5α, MG1655 and BL21 DE3; and 3 clinical isolates: CFT073, DSM1103, Nissle. Mistranslation was measured in standard rich MHB medium and in human urine. Urine was collected from 6 healthy volunteers (3 male, 3 female), prefiltered through 0.45 µm filter, sterile filtered through 0.22 µm filter, and pooled in equal volumes before experiment. 3 different batches were analysed. Bacteria were streaked from glycerol stocks onto LB-agar plates with 100 µg/ml ampicillin (Amp100) for selection and incubated overnight. Next day a single colony from each reporter was picked with a sterile toothpick and inoculated into 100 µl of indicated medium with Amp100. Plates were incubated aerobically using a microtiter plate shaker-incubator at 750 rpm (Cole-Parmer™ Stuart™, UK) or microtiter plate reader Synergy Mx at 37 °C for 24 h. Precultures were diluted into fresh medium to about 10^6 CFU/ml and incubated in the same conditions for another 18 h. Cultures were diluted into sterile filtered PBS and analyzed immediately with flow cytometry (Attune™ NxT Acoustic Focusing Flow Cytometer (Thermo Fisher Scientific, USA)). Filters used: green fluorescence: λ_{ex} 488 nm/λ_{em} 530/30 nm; red fluorescence: λ_{ex} 561 nm/λ_{em} 585/16 nm (MHB experiments) or 590/40 (urine experiments)). OD_{600}, green fluorescence (λ_{ex} 485/9 nm/λ_{em} 510/9 nm, gain 80) and red fluorescence (λ_{ex} 569/13.5 nm/λ_{em} 600/17 nm, gain 100) were recorded with platereader in MHB experiments.

Mistranslation was analysed after treatment with two different antibiotics: aminoglycoside amikacin (AMI) and macrolide AZI. Aminoglycoside experiments were done with *E. coli* MG1655 in M9 minimal medium with 0.2% glucose. 3 ml M9 precultures were started from DMSO stocks, grown aerobically at 37 °C to OD_{600} ~0.5, and then diluted to OD_{600} 0.1. The diluted preculture was added to a 96-well plate containing serially diluted AMI in a final concentration range of 8…0.5 µg/ml. The plate was incubated aerobically at 37 °C and ~1 µl samples for flow cytometry were taken every hour until 6 h, at 18 h, and analysed immediately with flow cytometry. Alternatively, a few samples were analysed microscopically on 1% agarose in PBS pads. Only non-treated and 2 µg/ml AMI, and TGA/TAG stop-codon readthrough reporters together with positive (GFP+iSc+) and negative control (ΔiSc) were analysed. Microscopy samples were grown in 3 ml cultures for 4 h. AMI-treated samples were concentrated 20X before microscopy analysis.

AZI was studied in uropathogenic *E. coli* CFT073 in MHB medium. 25 µg/ml AZI stock solution was prepared in 96% EtOH. Precultures were started from

single colonies on LB-agar (with Amp100) plates, as in spontaneous mistranslation experiments. The overnight culture was diluted 100X into fresh medium and incubated for 3 h, then diluted 10X into medium containing serially diluted AZI in a concentration range 32…0.5 µg/ml. Plates were incubated aerobically at 37°C for 4 h. Plates were chilled on ice and 20% final concentration of glycerol was added and plates were frozen at –80 °C until analysis with flow cytometry (green fluorescence: λ_{ex} 488 nm/λ_{em} 530/30 nm; red fluorescence: λ_{ex} 561 nm/λ_{em} 585/16 nm).

To calculate mistranslation, first red autofluorescence was subtracted from all reporters' red fluorescence signal. In platereader experiments the fluorescence is first normalized according to OD_{600} and then autofluorescence is subtracted. Then the ratio of red and green fluorescence was calculated. The red-green ratio of the positive control (GFP+iSc+) was considered to be 100%, and mistranslation reporter's red-green fluorescence was calculated as a percentage of the positive control. For statistical analyses an unpaired t test with Welch correction assuming individual variance for each group (α=0.05) was carried out. No multiple comparison corrections were made.

4.7. Macrophage infection with mistranslation reporter and microscopy (III)

UPEC strain CFT073 carrying T7 frameshift reporter plasmid was used for infecting mouse macrophage-like J774 cells according to protocol (Kerkez et al., 2021) with minor modifications. Namely, bacterial cells from a colony grown overnight (<20 h) on LB-agar (containing 100 µg/ml carbenicillin) were used for. Multiplicity of infection was 50 bacterial cells per 1 macrophage (MOI-50). Bacteria were added to 96-well cell culture plates suitable for microscopy, centrifuged, and allowed to phagocytize for 1 h. cell culture medium (RPMI 1640 supplemented with 10% fetal bovine serum) was removed and replaced with the same medium containing 100 µg/ml gentamicin for 1 h to eliminate non-phagocytized bacteria. The medium was then replaced with medium containing final concentration of 5 µg/ml gentamicin and serial dilutions of AZI. Plates were incubated overnight in a 5% CO2 incubator. Next day cells were fixed with 4% formaldehyde in 1X PBS for 20 min. Fixation solution was removed, cells were washed stained for 15 min with 100 ng/ml DAPI in 1X PBS containing 0.1% Triton X. Cells washed thrice with 1X PBS, after which kept in 1X PBS.

Cells were imaged with Zeiss LSM900 confocal microscopy system with 20X magnification. Green (Ex 488 nm: 1.8%; Em 500–560 nm), red (Ex 561 nm: 2.8%; Em 576–700 nm), and blue (Ex: 405 nm: 3.4%; Em 400–500 nm) fluorescence was recorded. Fluorescence levels were adjusted during image processing for presentation purposes in an equal manner.

5. RESULTS AND DISCUSSION

5.1. Drug release from nanofibers is less drastic in semisolid than in liquid systems (I)

Electrospun nanofibrous mats are relatively novel DDS-s, which are intended for local drug delivery like antibacterial dressings for infected wound treatment. There are no standardized assays for analysing such materials. Traditional Pharmacopoeial assay for solid DDS-s involves dissolving the material in a liquid system, which is far from mimicking local therapy. Therefore, in this study we compared drug (CAM) release in the dissolution assay with drug release into semisolid agar/agarose gel.

First, in a dissolution assay with liquid buffer solution we found that CAM is released much faster from PCL/PEO mat compared to PCL-only mat (Figure 7). This is because PCL/PEO is more hydrophilic and with better wetting properties than the hydrophobic PCL (Preem et al., 2017). Both mats showed fast initial burst release. The PCL/PEO mat released ~90% of the CAM within 15 minutes, while PCL mat released only 30% of the CAM within the 60 minutes of the assay, and in a previous study 60% within 80 minutes (Preem et al., 2017), indicating prolonged release.

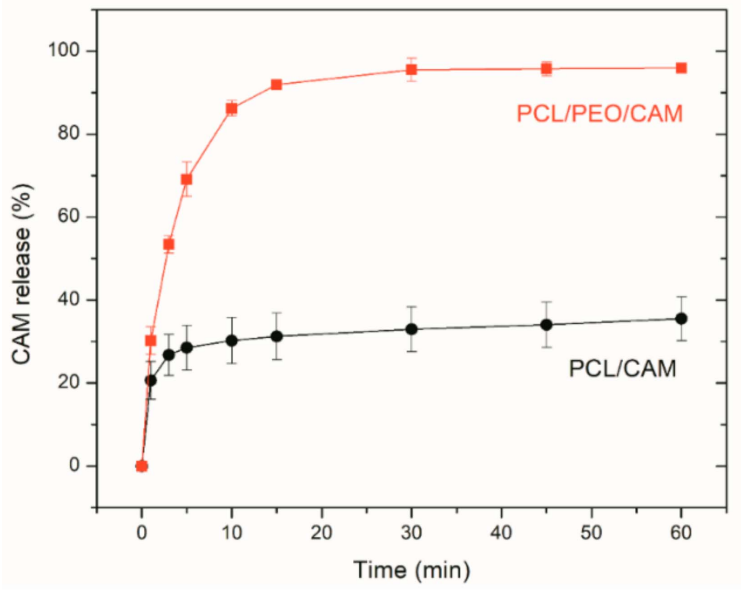

Figure 7. *CAM release from PCL (black) and PCL/PEO (red) nanofibrous mat in a dissolution assay into PBS. CAM is released much faster from PCL/PEO mat compared to PCL-only mat. Mean ± SD (N ≥ 3).*

Next, antimicrobial activity was estimated in a disc diffusion assay after adding the nanofibers to LB-agar plates covered with *S. aureus* for different time intervals (Figure 8A) and measuring the inhibition zone after incubation. This method showed differences between PCL and PCL/PEO mats within first few minutes, confirming the burse release effect, after which the differences between the two mats were insignificant. Majority of the CAM was released from either of the mats within 30 minutes. The amount of CAM released into the LB-agar was also quantified with HPLC (Figures 8B to 8D). PCL mat was found to release less CAM in all tested time intervals. Most of the CAM was found in the zone right below the area where the mat was placed. Despite this, differences in the amount of CAM found in the agar even after 30 or 180 minutes of treatment, biological effects were relatively similar (Figure 8A), indicating a concentration well above the MIC even with the PCL mat.

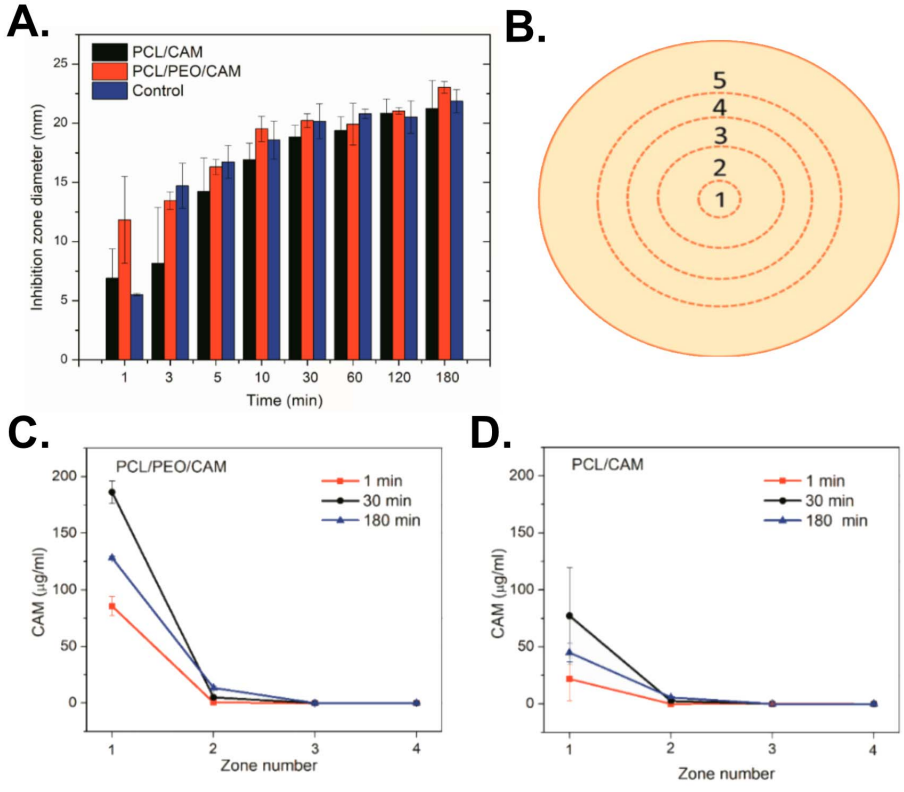

Figure 8. *CAM release into LB-agar estimated according to S. aureus inhibition zones (A) and by extracting CAM from zones around and underneath the nanofiber (B–D). Mean ± SD (N ≥ 3).*

Next, drug release was assessed in semisolid hydrogels. One approach was to use a bioreporter, which features *GFPmut2* as a control protein for expression and *mScarlet-I* as the reporter protein for ribosomal stalling. Red fluorescence is only produced when ribosomal stalling occurs in the regulatory region, e.g. due to

antibiotics, such as CAM (Figure 9A). In a disk diffusion assay with a bioreporter, a red signal will be produced most in a region, where there are enough bacteria with stalled ribosomes still capable of translation, resulting in a red fluorescent circle around the inhibitory area (Figure 9B). Image analysis from the disk diffusion assay showed minor differences between the two mats (Figure 9C), still confirming that PCL/PEO mats release CAM faster, resulting in a maximal red fluorescence, hence CAM-induced ribosomal stalling, further away from the mat.

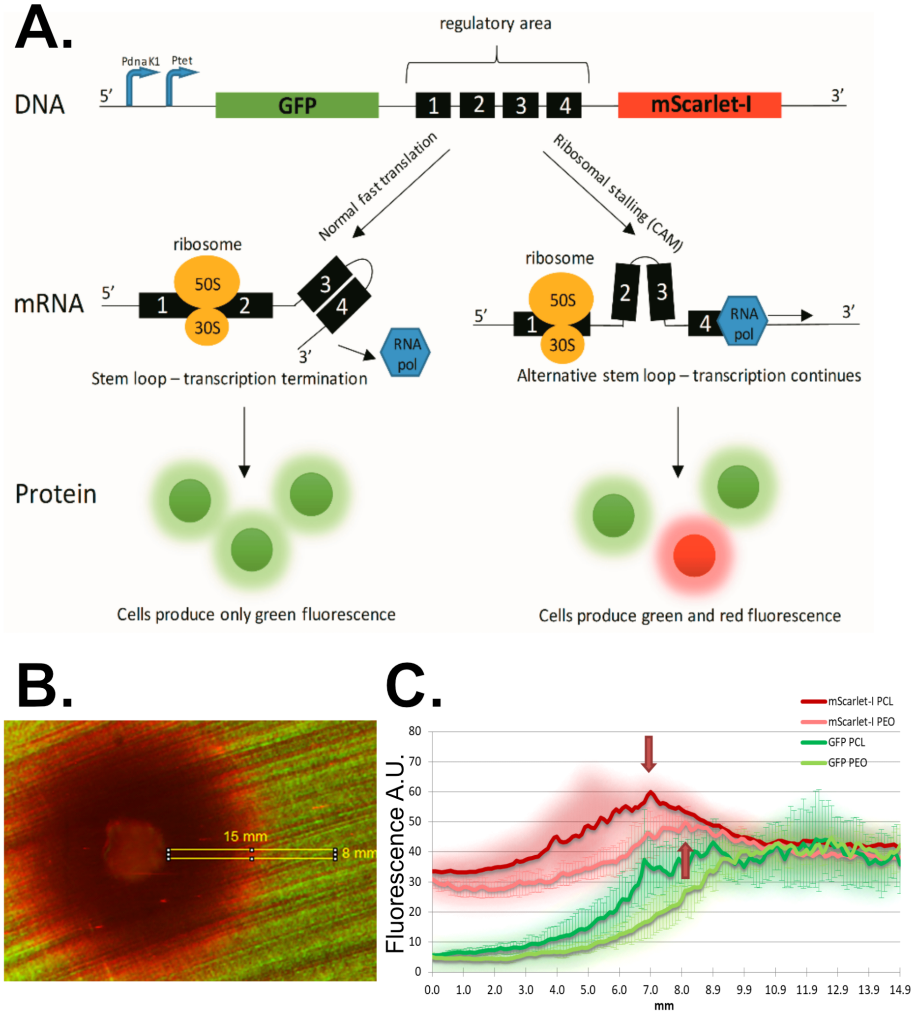

Figure 9. *Bioreporter analysis of CAM-releasing nanofibers. A. Schematic of the working principle of the bioreporter. B. Red and green fluorescence overlay image of the bioreporter disk diffusion assay including a PCL/PEO nanofiber (grey disk). The analysis area is marked with yellow. C. Fluorescence values obtained from reporter disk diffusion assay with ImageJ analysis of grey values. After 6 h of nanofiber exposure PCL/PEO (in figure marked as PEO; pink line) induces reporter signal maximum (red arrow) further from the disk than with PCL-only (dark red line) mat. Mean±SD (N=3). B and C are modified figures from (Sagor, 2020).*

In summary, we developed methods, which are suitable for testing drug release from nanofibers intended for local wound therapy. We found vast differences between drug release from two mats in a standard liquid system, but the differences were modest when drug release was measured in semisolid hydrogels, which are probably more relevant as topical drug release models. We developed a fluorescent reporter to detect CAM and estimated CAM release in agar hydrogel. While the developed methods allow to estimate drug released from the nanofibers, differences between the tested mats remained modest. Whether these methods reflect clinical effects need to be investigated further.

Overall, this study highlights the need for standardized assays for testing novel pharmaceutical DDS-s. All the assays showed that PCL mat releases the drug slower than the PCL/PEO mat. The standard dissolution assay into a liquid buffer overestimated the differences of drug release profiles between the two nanofibrous mats. Drug release into semisolid vehicles and biological assays resulted in minor differences between the two mats, with most of the differences in early timepoints. However, as these nanofibers are intended for topical treatment, it is still unknown how and if these nanofibers will result in different treatment outcomes. As CAM is a time-dependent antibiotic (Estell et al., 2020), it is plausible that a slower release might be more efficient in eradicating infection than the fast release mat, if it is able to maintain a concentration above the MIC in the wound for a longer duration. The assays used are still far from mimicking an infection environment, so additional studies are necessary to draw conclusions about the treatment efficacy.

5.2. Bicarbonate alters bacterial sensitivity to antibiotics due to pH change (II)

It has been hypothesized that the addition of bicarbonate to AST media improves the clinical predictability of these assays (Ersoy et al., 2017; Farha et al., 2018, 2020; Meerwein et al., 2020). Macrolides, such as AZI, were shown to have a significantly lower MIC in media with bicarbonate. We tested the hypothesis on *Salmonella* and AZI. For measuring the pH during incubation in microtiter plates we used PhR (Figure 10A). Indeed, we found that bicarbonate lowers the MIC 6 to 16 times, depending on the condition, however the MIC correlates strongly with the pH of the medium at the end of incubation (Figure 10B). The pH effect on macrolide efficacy has been described previously (Barry et al., 1988; Butler et al., 2001; Hardy et al., 1988; Pruul & McDonald, 1992; Retsema et al., 1991). pH rises in ambient atmosphere already within a few hours of incubation (Figure 10C) before any visible cell growth in a MIC assay. None of the tested buffers were able to maintain equal pH between the medium with or without bicarbonate (Figure 10D). In conditions, where the pH is maintained neutral (HEPES buffer+5% CO_2), bicarbonate has no effect on AZI's MIC (Figure 10B).

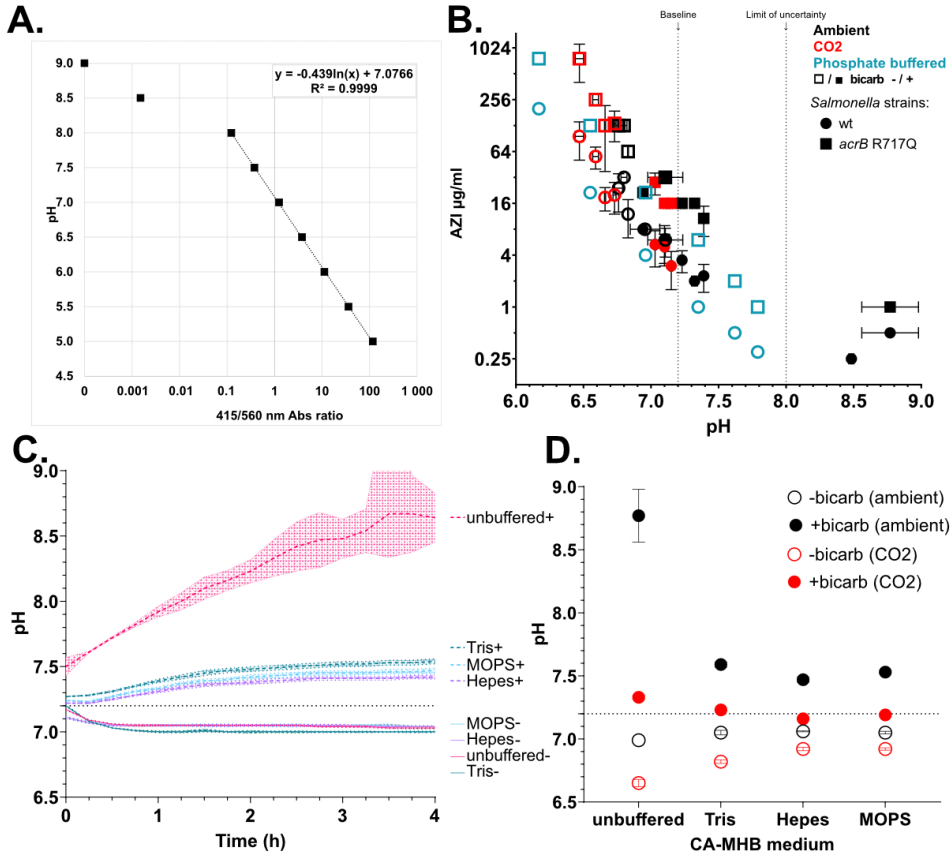

Figure 10. *Medium's pH and AZI concentration are in correlation. A. Calibration curve used as the basis of pH calculations. Based on the averages of 3 technical replicates. B. Salmonella's susceptibility to AZI correlates with the pH of the medium at the end of the MIC experiment. The efflux-efficient acrB mutant is ca 4-fold less susceptible to AZI throughout the tested pH conditions. Baseline is the pH at the start of the experiment and limit of uncertainty is pH > 8 due to pH indicator PhR's range. C. The pH of the test media changes from baseline (black dotted line) within first few hours of incubation despite buffering. D. None of the tested buffers were able to maintain an equal pH between media with and without bicarbonate. Smallest pH differences between ± bicarbonate were in media with buffer and 5% CO_2 incubation. Mean±SD (N≥3).*

We also tested an efflux-efficient *acrB* mutant in parallel and found that the difference between WT and the mutant strain remained about 5-fold throughout tested conditions (Figure 10B, squares). If efflux would have been impacted by bicarbonate, as implied by some authors (Farha et al., 2018), the difference between the two strains would diminish, however this is inconsistent with our data. Instead, we hypothesize that the alkaline pH, which deprotonates AZI's amines (McFarland et al., 1997), allows easier access of the drug into the bacterial cell (Butler et al., 2001; Retsema et al., 1991). Additionally, our results are in line

with a buffer composition impact, as shown by the slight shift of susceptibility in phosphate buffered MHB (Figure 10B).

This study shows the importance of controlling a simple matter, the pH of the AST media, as some antibiotics, e.g. macrolides, are extremely sensitive to pH change, which can result in false interpretations. The idea of the "bicarbonate effect" got carried away and resulted in several other articles, which also neglected bicarbonate's effect on the pH, resulting in false interpretations. The pH change only occurs when liberal gas exchange between the atmosphere and the medium is allowed, such as in a well of a non-hermetically closed microtiter plate. If a medium is stored in a tightly closed tube, the pH remains relatively unchanged. The research is also an important reminder for clinical practice, who might prepare a large batch of microdilution plates for MIC test in advance, which can result in a changed pH during storage. Additionally, adjusting pH during medium preparation might fail to maintain a pH during storage, and addition of an adequate buffer should be considered by advisory authorities, such as EUCAST, to effectively control the pH effect in clinical laboratories.

5.3. Higher mistranslation in *E. coli* laboratory strains and during growth in urine (III)

We developed a dual-fluorescent reporter that allows to measure stop-codon readthrough (nonsense mistranslation) and translational frameshifting. The reporter's working principle is shown schematically in Figure 6 in the paragraph 4.1. Our reporters showed in *E. coli* MG1655 that TGA is the most commonly misread stop codon, and TAA is the strictest stop codon. On average, the stop codon reporters had about 1% readthrough level in MHB medium. Tested frameshift sequences, however, showed about 0.5 to 4% frameshifting rates in MHB, depending highly on the specific sequence (Figure 11A). Interestingly, we noticed that mistranslation levels of individual reporters remained relatively similar in different rich (MHB) and defined minimal media (M9 with 0.2% glucose), indicating a conserved mechanism for maintaining an optimal level of mistranslation in standard conditions.

We compared different laboratory and clinical strains in the rich MHB medium. We discovered that the laboratory strains are up to 2 times more prone to stop-codon readthrough and frameshifting than clinical isolates (Figures 11B and 11C).

We also investigated mistranslation in human urine, a clinically relevant environment for uropathogens, most common of which is *E. coli* (Croxen & Finlay, 2010). We found that mistranslation can increase up to 4 times in urine compared to MHB (Figure 11). Mainly frameshifting was induced in urine, however a slight increase can also be seen in stop codon readthrough levels. The induction levels varied significantly between individual reporters. The uropathogenic CFT073 was able to maintain fidelity more in general than other strains, except for a few frameshift sequences, which still showed high induction rates. As urine is known to be slightly acidic (Cook et al., 2007), increased

mistranslation might come from the pH change (H. Zhang et al., 2020). Other urine components or the lack of them might also contribute to the changed translational fidelity.

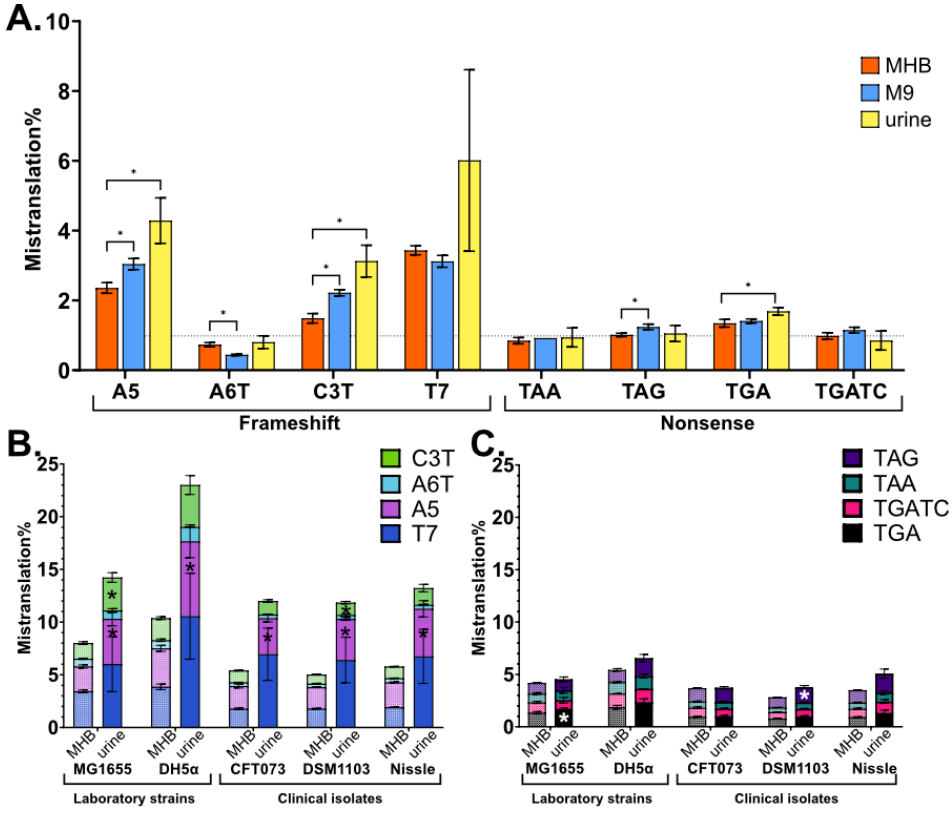

Figure 11. *Stop codon readthrough and frameshifting levels in different growth media. A. Standard media have a small effect on changing translational fidelity in MG1655. Frameshifting (B) and stop codon readthrough (C) are higher in laboratory strains than in clinical strains. Mistranslation in increased further during growth in human urine. Means±SD (N=3) are shown. *P value of the difference to MHB medium <0.05.*

In conclusion, we showed that our reporters can effectively be used for measuring stop-codon readthrough and translational frameshifting in various media and *E. coli* strains. Different standard media have a small effect on mistranslation levels, whereas mistranslation, mainly frameshift, is elevated in human urine. This indicates that cells in a "comfortable" environment have an optimal mistranslation level, which is well tolerated. Indeed, it has been shown that increasing/decreasing translational fidelity reduces the growth rate of bacteria (Kurland, 1992). Experiments in urine reflect that when a bacterium encounters a new niche, increased mistranslation levels result in heterogeneous proteomes, which can increase the chance of survival in the new environment (Ribas de Pouplana et al., 2014).

5.4. Amikacin slightly increases stop-codon readthrough (III)

After measuring mistranslation in different media and strains, we focused on measuring mistranslation caused by antibiotics. Aminoglycosides, are known to increase mistranslation (Davies & Davis, 1968; B. D. Davis et al., 1986; B. D. Davis, 1987; Kohanski et al., 2008; Thompson et al., 2002; Gromadski & Rodnina, 2004). We tested how aminoglycoside amikacin's (AMI) affects stop codon readthrough and frameshifting levels with our reporter system in MG1655 in M9 minimal medium supplemented with 0.2% glucose. We found that AMI increased stop codon readthrough levels about 1.5 times and slightly increased frameshifting levels (Figure 12A and 12B). Reporter response correlated with aminoglycoside concentration best after 4 h of treatment. We were able to detect increase in stop-codon readthrough with fluorescence microscopy (Figure 12C). Interestingly, in microscopy experiments we noticed the appearance of halos and shades near the poles of some bacteria Figure 12D), which might indicate that cells become leaky after antibiotic action and can lose their fluorescence.

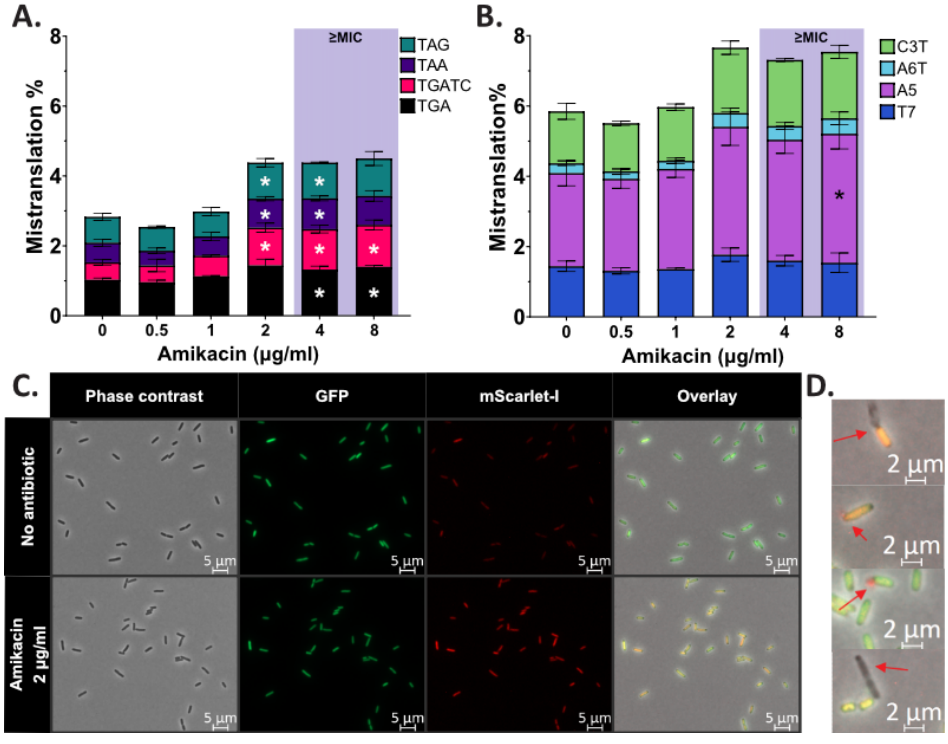

Figure 12. *Amikacin-induced mistranslation in MG1655 after 4 h of treatment in M9 medium. Flow cytometry analysis reveals that amikacin induces stop-codon readthrough (A) and translational frameshifting (B). Means ±SD (N=3) are shown. Mistranslation increase can also be detected in microscopy analysis (C). Microscopy analysis revealed appearance of halos near bacterial poles (D), which might indicate leakiness. *P value of the difference to medium without antibiotics <0.05.*

Based on our results we can conclude that our reporter system can be used to measure stop codon readthrough and translational frameshifting during aminoglycoside treatment. A definitive limitation of our reporter set is the lack of a miscoding reporter. Miscoding reporter could be constructed by substituting one by one the 67th to 69th amino acid residues of the *mScarlet-I* gene, which correspond to the chromophore (Bindels et al., 2016). This tactic, however, would require extensive validation. Previous *lacZ*-based reporters have shown that AGs induce preferably amino acid substitution (Thompson et al., 2002) with mainly third position miscoding resulting in cognate or near-cognate pairing (Garofalo et al., 2019; Gromadski & Rodnina, 2004) and error clusters (Wohlgemuth et al., 2021). Aminoglycoside-induced cell death is still not completely clear, but they are thought to cause damage to the cell membrane, which promotes the autocatalytic uptake of the drug and complete translational arrest by blocking translation initiation (Montie & Patamasucon, 1995; Schlessinger, 1988). It is up to debate whether the membrane damage is caused by mistranslated membrane proteins or a direct action of the drug on the membrane.

5.5. Azithromycin increases mistranslation and causes heterogeneity (III)

Macrolides are known to use programmed frameshifting to induce their resistance gene *ermC* (Gupta et al., 2013). We tested our reporter system in the presence of macrolide antibiotic AZI in *E. coli* CFT073 in MHB medium. Interestingly, we found that AZI induces both stop codon readthrough and frameshifting to highest observed level. Stop codon readthrough (Figure 13A) and frameshift (Figure 13B) increased up to about 4 times. The maximal induction was achieved at the AZI concentration 8 μg/ml, which corresponds to the MIC determined according to the standard protocol (Andrews, 2001).

Interestingly, we found that AZI induces a significant loss of red or both red and green fluorescence signals at high AZI concentrations (Figure 13C). The cause for this phenomenon is unclear. If the mistranslated membrane proteins indeed damage the membrane, it is possible that the fluorescent proteins leak out of the cells. It is unclear why iSc is more prone to the loss of signal. During the analysis of flow cytometry data, the cells are gated according to GFP, therefore the “dark” population, lacking both red and green fluorescence, is excluded from the analysis. This strategy still includes the GFP+/iSc- in the analyses, so this can cause some bias in the results. However, we expect that both the reporters and the positive control contain a comparable number of cells with lost red fluorescence.

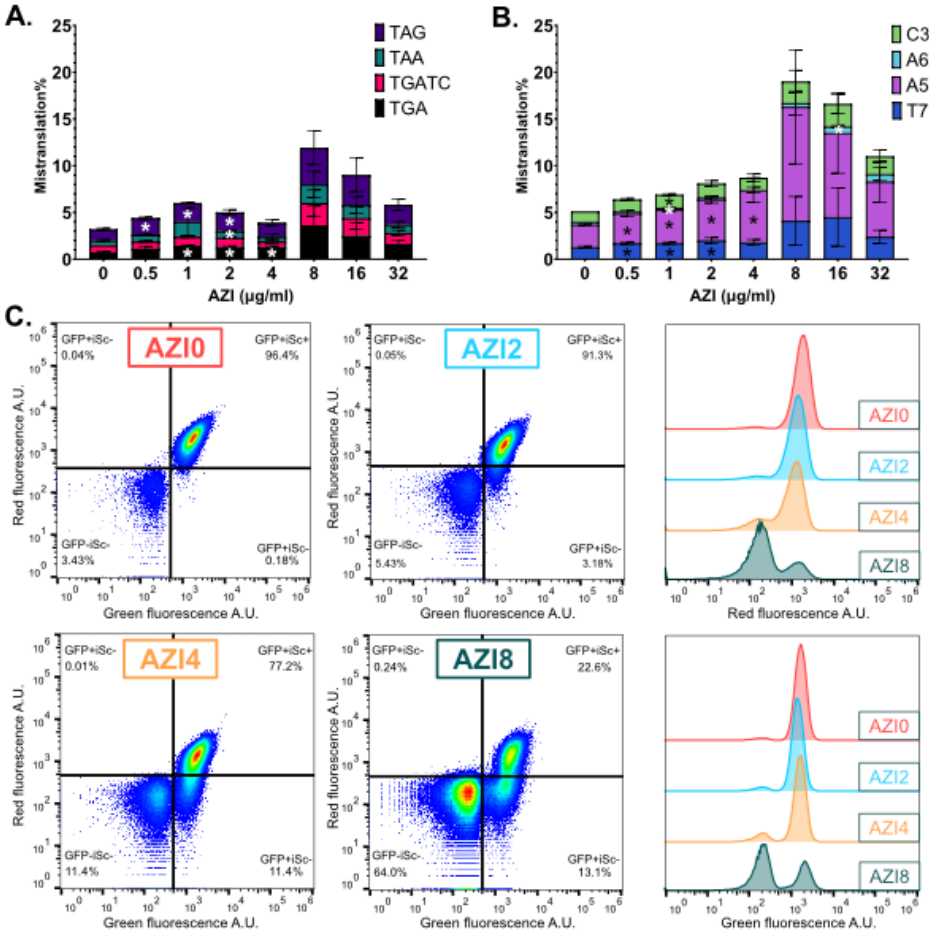

Figure 13. *Flow cytometry analysis of CFT073 after 4 h of treatment with AZI in MHB medium. Stop codon readthrough (A) and frameshifting (B) increase up to 4 times at 8 µg/ml of AZI. Means ± SD (N=3) are shown. C. Green and red fluorescence dot plots and respective histograms of ungated cells show an appearance of cells with lost red or both fluorescence signals at higher AZI concentrations. Representative plots from a single experiment. *P value of the difference to medium without antibiotics <0.05.*

Interestingly, we saw much higher stop codon readthrough and frameshifting levels with bacteriostatic AZI than with bactericidal aminoglycoside AMI. Indeed, high stop codon readthrough levels have been reported for macrolides other than AZI (Thompson et al., 2004). Gentamicin was found to induce high levels of amino acid misincorporations (Thompson et al., 2002), which cannot be detected with our reporters. Therefore, it is difficult to compare the two antibiotic classes. However, we hypothesize that high mistranslation alone is not the cause of cell death. Instead, aminoglycosides might have a quick and more direct effect on causing membrane damage (B. D. Davis, 1987; Montie & Patamasucon, 1995). Bacteriostatic macrolides, on the other hand, probably do not damage the

membrane directly, but rather specifically kill slow-growing cells with a low number of active ribosomes resulting in the loss of membrane integrity, while the more active cells with many ribosomes are able to overcome antibiotic's lethal effects and stay alive (Łapińska et al., 2022). Additionally, disassociation kinetics from the ribosome plays a role in macrolide cidality (Svetlov et al., 2017). Slower growth of bacteria during infection (Claudi et al., 2014) might also contribute to the clinical efficacy of AZI.

5.6. Mistranslation reporters can be used in a cell culture infection model (III)

We tested whether the mistranslation reporters can be used in an intracellular infection and AZI treatment model. UPEC strains are known to cause intracellular infection in the bladder epithelium (Blango et al., 2014; Mulvey et al., 2001; Wright et al., 2007). We used UPEC strain CFT073 with frameshift reporter T7 to introduce intracellular infection in mouse macrophage cell line J774. We were able to detect both green and red fluorescence signals in untreated samples using confocal microscopy (Figure 14), indicating that the reporters can be used to estimate mistranslation during intracellular infection. AZI treatment at 30 µg/ml increased the red fluorescence signal inside the macrophages, however, the red fluorescence did not overlap with the green fluorescence signal (Figure 14). Green fluorescent cells in this sample seemed to be outside of the macrophages.

The macrophage infection experiments prove that the reporters can be used to detect spontaneous mistranslation with our reporters. AZI, however, produces red fluorescence inside the macrophages, which does not overlap with the control protein GFP signal. This can indicate either very high mistranslation levels inside the macrophages and/or loss of membrane integrity, which leads to leakage of proteins, such as GFP. These results together with the previous findings of AZI-induced heterogeneity from flow cytometry experiments highlight the complexity and limitations of using such fluorescence-based reporters to study bacterial response to antibiotic treatment.

In summary, the III study showed that it is possible to measure mistranslation using the developed dual-fluorescent reporter system. The reporter system used GFP as the control protein for translation capacity, and red iSc as the reporter protein for mistranslation. The clear advantages of this system before previously published similar reporter systems (Fan et al., 2017; H. Zhang et al., 2020) are: i) independent translation of both fluorescent proteins (opposed to fusion proteins), ii) use of red fluorescence with low autofluorescence background, and iii) usability in infection models. The reporter set revealed that mistranslation levels vary significantly depending on the genetic context and specific *E. coli* stain. In general, stop-codon readthrough was around 1%, but frameshift motifs tested produced variable levels between 0.5 and 4%. Surprisingly, standard laboratory media had a small effect on translational fidelity, whereas human urine significantly increased translational frameshifting. Laboratory strains presented

significantly higher mistranslation levels than clinical isolates. Bactericidal AG AMI increased preferably stop-codon readthrough, but only at a modest level, whereas bacteriostatic macrolide AZI significantly increased mistranslation levels, which hints that bacteria can tolerate high mistranslation levels. Single cell studies revealed that part of the population lose one or both fluorescence signals upon antibiotic treatment. The reporters maintained their fluorescence during intracellular macrophage infection, but GFP signal was lost after AZI treatment, in contrast to lost red signal in *in vitro* analyses. This overall complicates the use of such reporters for studying antibiotic effects, as well as raises questions whether systems based on cytoplasmic fluorescent proteins are ideal for studying antibiotics, which can affect membrane integrity.

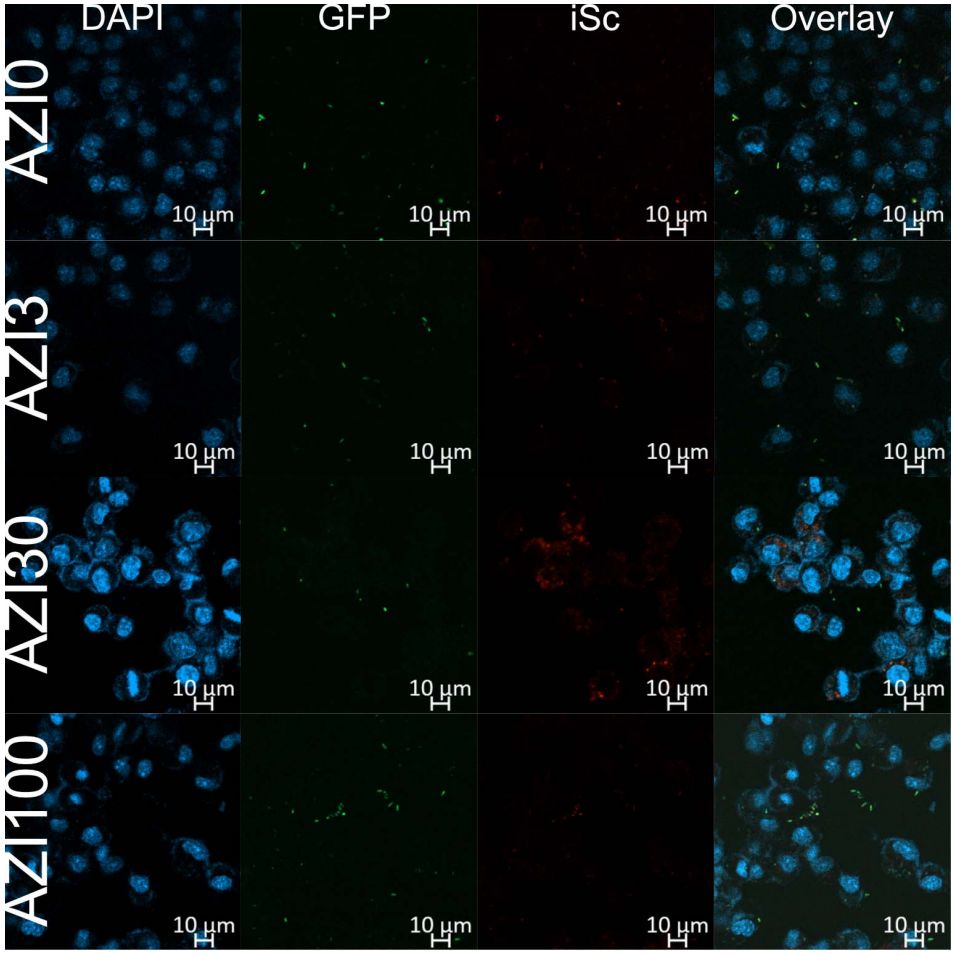

Figure 14. *Microscopy images of macrophage infection and AZI treatment with CFT073 frameshift reporter T7. Nuclei of macrophages are stained with DAPI (blue), and bacterial reporter cells can be detected via GFP (green) signal. Number after AZI indicates the concentration of in µg/ml. Both GFP and iSc can be detected during infection.*

6. CONCLUSIONS AND SUMMARY

Standard *in vitro* assays for testing antibiotic effects are far from infection-mimicking environments. The drug behaves very differently inside the human body compared to aqueous solution. The biological activity of the antibiotic depends on many factors, beginning from those originating from the pharmaceutical formulation, affecting the release and absorption of the drug, its distribution and metabolism in the body, and excretion, as well as pharmacodynamic parameters. Additionally, antibiotic treatment efficacy is affected by the complicated interplay of pathogen-derived factors and host's immune system. Therefore, in order to better understand the antibiotic's effects in the patient's body and improve the current treatment options, it is important to take all these factors into account.

In this thesis methods were developed and studies were carried out to estimate the effects and characteristics of translation inhibitors and testing environments were made more biologically relevant. Based on the results the following can be concluded:

- The work proved that drug release profiles from nanofibers intended for local therapy differ significantly depending on the analysis method. Drug dissolution assay overestimated the differences of CAM release between PCL and PCL/PEO mats. Release assays into semisolid hydrogel, including bacterial susceptibility assays, still showed differences between the two mats, however the differences were small and seen only in early timepoints. In conclusion, dissolution-based assay intended mainly for oral drugs is unsuitable for testing nanofibrous mats intended for local therapy, and standardized methods are necessary to estimate such drug delivery systems.
- It was shown that bicarbonate's effect comes solely from elevated pH in insufficiently buffered media. To maintain the neutral pH, both addition of a buffer and CO_2 incubation are necessary. When the pH is maintained, bicarbonate does not affect AZI's potency. Therefore, the addition of bicarbonate to AST media does not improve the clinical predictability. However, this does not mean that AST conditions are perfect. They could still be improved by changing the environmental conditions to be more biologically relevant.
- A sensitive fluorescence-based method was developed to measure translational fidelity in living bacterial cells in different environments. The reporter system consists of red iSc as the reporter protein and GFP as the control protein for expression. The reporter set was used to estimate translational fidelity in different strains, growth environments, as well as changes in fidelity caused by translation inhibitors and intracellular macrophage infection.
- Translational fidelity rates remain similar in different standard laboratory media, whether rich or minimal. Growth in human urine, however, increases translational frameshifting rates. This proves that mistranslation maintains at an optimal level in normal conditions, but if cells encounter a new niche or a

stressful condition, higher mistranslation levels are tolerated to increase the population survival.

- Laboratory strains have higher stop codon readthrough and translational frameshifting rates than clinical isolates. This indicates that antibiotic studies should be performed on clinical isolates, which are more relevant in infection context.
- Bacteriostatic macrolide AZI increased the stop codon readthrough and frameshifting rates about 4 times, the highest observed level in our studies. AZI-treated samples also presented presumably dead cells with lost fluorescence. Bactericidal aminoglycoside AMI moderately increased stop codon readthrough rates. These results hint that high mistranslation alone probably does not kill bacteria, and it is plausible that direct effects on the membrane and/or ribosome are instead responsible for antibiotic-induced cell death.
- The developed reporters can be used in intracellular infection models, however their usability in case of AZI treatment is complicated due to high levels of heterogeneity and low signals. This proves that fluorescent reporters need to be extensively characterized before using them in infection models. Loss of fluorescence is a significant obstacle of using fluorescence-based reporters.

In summary, careful consideration must go into testing the effects of antibiotics, both from novel pharmaceutical drug delivery systems, as well as during the study of molecular effects of the free drugs on bacteria. The choice of medium can significantly alter the results. Still, we are far away from mimicking host environments, which would add layers of complexity to antibiotic assays. Hopefully improvements in clinically relevant assays will be available soon, allowing to optimize antibiotic therapies and prevent the spread of antibiotic resistance.

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8. SUMMARY IN ESTONIAN

In vitro meetodid ribosoomiga seonduvate antibiootikumide toimemehhanismide uurimiseks

Ribosoomis toimuv valkude biosüntees ehk translatsioon on eluks hädavajalik protsess. Seetõttu on ribosoom ka üks peamisi antibakteriaalsete ainete sihtmärke bakterites. Kuigi palju antibiootikume võeti kliinilisse kasutusse juba antibiootikumide kuldajastul 20. sajandi keskel, ei ole nende bakterirakke tappev või infektsiooni raviv mehhanism tänaseni täielikult teada. Translatsiooni häiriv toime bakteri ribosoomis võib toimuda mistahes translatsiooni etapis – initsiatsioonil, translokatsioonil, peptiidsidemete moodustamise takistamisel, translatsiooni ebatäpsuse suurendamisel jne. Teadmised antibiootikumide toimetest bakterirakkudele täienevad ajas koos tehnoloogia arengu ja kättesaadavuse paranemisega. Kuna resistentsus (olukord, kus antibiootikum enam ei toimi) erinevatele antibiootikumidele on üha kasvav probleem, on väga oluline tõhustada olemasolevaid ja töötada välja uusi antibakteriaalseid ravimvorme ja raviskeeme.

Antibiootikumide toimeid on võimalik uurida *in vitro* ehk katseklaasis või nakkusmudelites koos loomarakkude või -kudededega (*ex vivo*) või elusloomades (*in vivo*). Kliinilised katsed inimestel on rangelt reguleeritud, töömahukad ja kulukad ning neid viiakse läbi vaid pärast põhjalikke uuringuid *in vitro* ja *in vivo* loomkatsetes. Antud doktoritöö keskendub *in vitro* katsemudelitele. Klassikaliselt on translatsiooni inhibiitoreid uuritud rakuvabades süsteemides, kus katseklaasi lisatakse ainult translatsiooni komponendid, nagu ribosomaalsed alaühikud, tRNA-d, kofaktorid jne. Sellised katsed annavad infot antibiootikumide täpsete seondumiskohtade kohta ribosoomil ja võimaldavad jälgida toimeid konkreetsete translatsiooni etappide kohta, kuid on sageli töömahukad ja vastavad vähestele küsimustele. Klassikalised elusatel bakterirakkudel tehtavad uuringud hõlmavad antibiootikumide põhjustatud kasvu takistamise uurimist, s.t viiakse läbi antimikroobsete ainete tundlikkuse määramise test (*antimicrobial susceptibility test* – AST). Teine levinud võimalus on uurida antibiootikume bakteriaalsete reportersüsteemide abil, kus geneetiliselt muundatud bakterid tekitavad vastusena antibiootikumi toimele mingi mõõdetava signaali, näiteks keskkonna värvimuutuse või fluorestsentsi. Kuna on teada, et ka geneetiliselt ühetaolises populatsioonis esineb heterogeensust, eriti antibiootikumide toimel, on tänapäeval suundumus kasutada üksikraku meetodeid.

Eesmärgid

Käesolevas doktoritöö eesmärk oli täiustada antibiootikumide *in vitro* katsemudelite tingimusi nii, et need oleksid sarnasemad bioloogilise keskkonna ehk inimorganismiga. On teada, et tingimused standardsetes laborikatsetes erinevad oluliselt bakterite elutingimustest infektsiooni ajal ning taoliste laborikatsete tulemused võivad erineda suurel määral kliinilistest tulemustest. Muutes katsetingimusi infektsioonikeskkonnaga sarnasemaks on võimalik paremini mõista

antibiootikumi ja bakteri käitumist organismis ning suurendada seeläbi kliiniliste tulemuste etteennustatavust. Doktoritöös uuriti ribosoomiga seonduvate antibiootikumide toimeid bakteritele erinevates katsetingimustes. Üheks strateegiaks oli antibiootikumile vastavate fluorestseeruvate bioreporterite väljatöötamine ja kasutamine erinevates katsetingimustes (I, III), mis võimaldaks uurida raviaine vabanemist uudsetest ravimvormidest, näiteks krooniliste haavade lokaalseks raviks mõeldud antibiootikume sisaldavatest nanofiibritest (I), kus standardsed raviaine vabanemise testid on ebatõhusad. Reporterite abil uuriti, kuidas eri katsetingimused ja antibiootikumid mõjutavad translatsioonil tekkivaid vigu (III). Kontrolliti ka hüpoteesi, et bikarbonaadi, olulise verepuhvri komponendi lisamine AST söötmele muudab testi kliiniliselt täpsemaks (II).

Meetodid

Antibiootikumi vabanemise uurimine nanofiibritest (I). Mudelraviaine CAM-i vabanemist uuriti kahest erineva polümeerse koostisega elektrospinnitud nanofiibermatist: polükaprolaktoonist (PCL) ja polüetüleenoksiidi (PEO) segust PCL-iga (PCL/PEO). Raviaine vabanemise mõõtmiseks kasutati esmalt standardmeetodit tahkete ravimvormide uurimiseks (dissolutsioonitest), kus raviaine vabanemist jälgitakse vesikeskkonnas. Seejärel uuriti raviaine vabanemist pooltahkes agarsöötmes ilma bakteriteta ja koos bakteritega, et näha, kuidas nanofiibritest vabanev CAM takistab bakterite kasvu, ja reporterite abil uuriti, kuidas erineb kahe erineva mati bioloogiline mõju bakteritele.

Reporterbakterite geneetiline modifitseerimine. Töötati välja kahel fluorestseeruval valgul põhinev reportersüsteem. Roheline fluorestseeruv valk (GFP) näitab translatsiooni üldtaset ja punane fluorestseeruv valk mScarlet-I (iSc), mille signaal tekib vaid ribosoomi peatumisel klooramfenikooli (CAM) toimel (I) või translatsioonivea (stopp-koodoni ülelugemise või raaminihke) tekkel mutatsioonikohas (III). GFP ja iSc suhet kasutatakse vigade esinemise sageduse hindamiseks. Plasmiidkandjal olev reportersüsteem viidi erinevatesse *Escherichia coli* tüvedesse. Antibiootikumide toimel tekkivad fluorestsentsi muutused sõltuvad ainult bakterirakkude sees olevast antibiootikumi tasemest.

Translatsioonivigade uurimine (III). Reportereid kasutati translatsioonivigade taseme mõõtmiseks erinevates *E. coli* labori- ja kliinilistes tüvedes, erinevates standardsöötmetes ja ka uriinis. Lisaks uuriti antibiootikumide amikatsiini (AMI) (aminoglükosiid) ja asitromütsiini (AZI) (makroliid) mõju translatsiooni täpsusele. Hiire makrofaagide rakuliiniga rakusiseses infektsiooni- ja AZI-ravi mudelis hinnati reporterite kasutatavust nakkusmudelites.

Fluorestsentsi mõõtmine. Bakterite punase ja rohelise fluorestsentsi mõõtmiseks kasutati erinevaid meetodeid. Pooltahkel söötmel raviaine vabanemise uurimiseks kasutati fluorestsents-skaneerijat (Typhoon) (I). Vedelkultuuri fluorestsentsitasemete mõõtmiseks kasutati plaadilugeja-fluoromeetrit (Biotek) (III). Üksikrakkude fluorestsentsi mõõtmiseks kasutati voolutsütomeetriat või laivälja mikroskoopiat (III). Rakukultuuri infektsiooni analüüsiks kasutati konfokaalmikroskoopiat (III).

Bikarbonaadi mõju uurimine antibiootikumi toimele (II). Naatriumvesinikkarbonaadi (bikarbonaadi) lisamise mõju AST standardsöötmele testiti *Salmonella enterica* serotüübi Typhimurium tundlikkuse määramisel makroliidantibiootikumile AZI-le. Tundlikkuse määramiseks kasutati standardmeetodeid (Andrews, 2001). Kasutati puhverdamata söödet ja erinevaid puhvreid (TRIS, HEPES, MOPS) tavaatmosfääris või 5% CO_2 juures. Enne katset kohandati kõik söötmed HCl-ga pH-le 7.2. Minimaalne inhibeeriv kontsentratsioon (MIC) määrati pärast inkubatsiooni 37 °C juures kui kõige madalam antibiootikumi kontsentratsioon, mis inhibeeris nähtavat bakterite kasvu. pH muutuse jälgimiseks söötmes kasutati indikaatorvärvi fenoolpunane.

Tulemused ja arutelu

I Dissolutsiooni testiga, kus testitakse raviaine vabanemist puhverlahusesse, leiti kahe nanofiibermati vahel suured erinevused: PCL/PEO matt vabastas CAM-i oluliselt kiiremini ja suuremas ulatuses kui PCL matt. Kuigi ka pooltahkes agar- või agarooskeskkonnas raviaine vabanemise meetodid tuvastasid, et PCL/PEO matt vabastas raviainet kiiremini, oli kahe erineva mati vahel erinevusi ainult vahetult pärast kokkupuudet matiga ja hilisemates ajapunktides olid vahed statistiliselt ebaolulised. Antud uurimistöö näitab, et dissolutsiooni test ei ole parim meetod nanofiibermattide testimiseks ja sellega võib ülehinnata raviaine vabanemise bioloogilisi mõjusid. Siiski on vajalik ühtselt aktsepteeritud ja reguleeritud standardmeetodite väljatöötamist taoliste uudsete ravimvormide, nagu peamiselt lokaalseks haavade raviks ette nähtud nanofiibrite analüüsimiseks.

II Uurimistöö tulemused lükkasid ümber bikarbonaadi kui „maagilise“ AST täpsust suurendava koostisosa lisamise kasulikkuse. Nähtud antibiootikumide tundlikkust suurendav efekt tulenes söötme pH tõusust bikarbonaadi toimel. Ükski testitud puhvritest ei suutnud hoida pH-d ühtlasena bikarbonaadiga ja bikarbonaadita söötme vahel. Kui pH oli bikarbonaadiga ja bikarbonaadita võrreldavas söötmes sama, ei olnud bikarbonaadil mõju antibiootikumitundlikkusele. Bakteri tundlikkus AZI-le oli tugevas seoses söötme pH-ga inkubatsiooniperioodi lõpus. Katsed fenoolpunasega näitasid, et pH-tõus toimus tavaatmosfääris paari esimese tunniga. Selleks, et söötme pH-d säilitada oli vajalik nii puhvri lisamine kui ka CO_2-inkubatsioon. Antud uurimistöö näitab, kui lihtne on jõuda valejäreldusteni, unustades ära lihtsa, kuid olulise aspekti: pH kontrollimise söötmes. Kuna pH võib oluliselt mõjutada antibiootikumide toimet, peaksid ka regulatiivsed asutused kaaluma puhvrite lisamist standardprotokollidesse.

III Töötati välja tundlik fluorestsentsil põhinev translatsioonivigade reporterite komplekt. Kasutatud reportersüsteem näitas, et AZI suurendas translatsioonivigu umbes 4 korda. Huvitaval kombel põhjustas AZI bakteripopulatsioonis olulist heterogeensust – osa baktereid kaotas AZI toimel punase fluorestsentsi või nii punase kui rohelise fluorestsentsi. AMI tõstis stopp-koodoni

läbilugemist vaid vähesel määral. Laboritüvedes oli translatsioonivigade tase kõrgem kui kliinilistes isolaatides. Standardsöötmed ei avaldanud olulist mõju translatsiooni täpsusele, kuid uriinis olid raaminihked oluliselt suurenenud. Reportereid on võimalik kasutada rakusisese infektsiooni ajal translatsioonivigade taseme mõõtmiseks, kuid nende kasutatavus samas mudelis translatsiooni inhibiitorite mõju uurimiseks on küsitav. Kuna AZI on bakteriostaatiline, ehk kasvu pidurdav, ja AMI on bakteritsiidne, ehk baktereid surmav, siis antud töö vihjab, et suurenenud translatsioonivead üksi ei põhjusta rakkude surma, nagu varasemalt aminoglükosiidide puhul on arvatud. Lisaks leiab kinnitust, et translatsiooni täpsus on tavatingimustel reguleeritud optimaalsele tasemele, kuid bioloogilises keskkonnas, nagu uriin, võib talutav translatsioonivigade määr oluliselt tõusta, suurendades tõenäoliselt bakterite ellujäämise tõenäosust ja kohanemist ebasoodsas keskkonnas.

Järeldused

Doktoritöö toob esile, kui olulised on antibiootikumide uurimisel keskkonnatingimused, seda nii uudsetest ravimvormidest toimeaine vabanemise uurimiseks, kui ka molekulaarsete toimete uurimiseks bakterirakkudes. Söötme ja keskkonna valik võib oluliselt muuta tulemusi. Siiski ei suudeta veel täielikult jäljendada keskkonnatingimusi peremeesorganismis infektsiooni ajal, kuid see annaks kõige täpsema arusaamise antibiootikumide toimete kohta kehas. Loodetavasti on taolised kliiniliselt asjakohased katsed standardtingimustel lähitulevikus saadaval, andes võimaluse antibiootikumravi optimeerimiseks ja antibiootikumresistentsuse ohjamiseks.

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PUBLICATIONS

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Professional employment:

2015–2022	Tiido & Partners Language Agency, freelance medical translator
2019	University of Tartu, Institute of Pharmacy, visiting lecturer in Pharmaceutical Technology and Physical Pharmacy
2019–2022	University of Tartu, Institute of Technology, EPIC-XS project manager
2020–2021	University of Tartu, Institute of Technology, junior researcher in Molecular Microbiology
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Research fields:

Antibiotic and infection studies, biotechnology, molecular microbiology, laboratory animal models (CERCS classification: T490 Biotechnology; B230 Microbiology, bacteriology, virology, mycology; B510 Infections).

Research projects:

2019–2022	RITA1/02-75-01: Routs for development and spread of antibiotic resistance and resistance containment measures
2019–2022	H2020-WIDESPREAD-2018-2020/GA: 857518: Molecular Infection Biology Estonia – Research Capacity Building
2019–2023	PRG335: Elucidating protein synthesis-related processes underlying heterogeneity of bacterial populations during infection
2021–2023	MOBERA23: The mechanisms of action of macrolide antibiotics
2022–2026	PRG1507: Development of biorelevant assays for the development of multifunctional antimicrobial wound dressings for the treatment of wound infections

List of publications:

Murina, V., Kasari, M., Takada, H., **Hinnu, M.**, Saha, C. K., Grimshaw, J. W., Seki, T., Reith, M., Putrinš, M., Tenson, T., Strahl, H., Hauryliuk, V., & Atkinson, G. C. (2019). ABCF ATPases Involved in Protein Synthesis, Ribosome Assembly and Antibiotic Resistance: Structural and Functional Diversification across the Tree of Life. Journal of Molecular Biology, 431(18). https://doi.org/10.1016/j.jmb.2018.12.013

Preem, L., Bock, F., **Hinnu, M.,** Putrinš, M., Sagor, K., Tenson, T., Meos, A., Østergaard, J., & Kogermann, K. (2019). Monitoring of Antimicrobial Drug Chloramphenicol Release from Electrospun Nano- and Microfiber Mats using UV Imaging and Bacterial Bioreporters. Pharmaceutics, 11(9), 487. https://doi.org/10.3390/pharmaceutics11090487

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2017 Laboratory animal licence for rats and mice (FELASA accredited). Obtained from course: "The use of animals in research: course for persons carrying out procedures (Functions A & D)". University of Helsinki, Finland.

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Teaching:

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Murina, V., Kasari, M., Takada, H., **Hinnu, M.**, Saha, C. K., Grimshaw, J. W., Seki, T., Reith, M., Putrinš, M., Tenson, T., Strahl, H., Hauryliuk, V., & Atkinson, G. C. (2019). ABCF ATPases Involved in Protein Synthesis, Ribosome Assembly and Antibiotic Resistance: Structural and Functional Diversification across the Tree of Life. Journal of Molecular Biology, 431(18). https://doi.org/10.1016/j.jmb.2018.12.013

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Hinnu, M., Putrinš, M., Kogermann, K., Bumann, D., & Tenson, T. (2022). Making Antimicrobial Susceptibility Testing More Physiologically Relevant with Bicarbonate? Antimicrobial Agents and Chemotherapy, 66(5). https://doi.org/10.1128/AAC.02412-21

Hinnu, M., Putrinš, M., Kogermann, K., Kaldalu, N., & Tenson, T. (2024). Fluorescent reporters give new insights into antibiotics-induced nonsense and frameshift mistranslation. Scientific Reports, 14(1), 6883. https://doi.org/10.1038/s41598-024-57597-8

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