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Development and Synthesis of Plant-Derived Recombinant Biopharmaceutical Proteins Active Against Gram-Negative Pathogenic Bacteria

DOCTORAL DISSERTATION

Natural Sciences,
Biochemistry (N 004)

Vilnius 2023

The work presented in this doctoral dissertation has been carried out during doctoral studies (2015–2019) at the Institute of Biotechnology, Life Sciences Centre of Vilnius University in a collaboration with Nomads UAB, during 2015–2023.
The dissertation is defended on an external basis.

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Augaluose susintetintų farmacinių rekombinantinių baltymų preparatų, aktyvių prieš gramneigiamas patogenines bakterijas, kūrimas

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CONTENTS

LIST OF ABBREVIATIONS

AMR – antimicrobial resistance
BAM – β-barrel assembly machinery
BeYDV – bean yellow dwarf virus
C55-OH – undecaprenol
C55-P – undecaprenyl phosphate
CF – cystic fibrosis
CFU – colony forming units
ColM – colicin M
CPA – common polysaccharide antigen
DHF – dihydrofolic acid
DNase – deoxyribonuclease
dpi – days post infection
FDA – Food & Drug Administration
GlcNAc – N-acetylglucosamine
GRAS – generally recognized as safe
IM – inner membrane
IN – intranasal
IP – intraperitoneal
IUTD – intrinsically unstructured T domain
IV – intravenous
kTHB – kinked three-helix bundle
LPS – lipopolysaccharide
MDR – multi-drug resistant
MurNAc – N-acetylmuramic acid
OM – outer membrane
PABA –para-aminobenzoic acid
PBP – penicillin binding protein
PDB – protein data bank
PFD – pore-forming domain
PFP – pore-forming protein
PG – peptidoglycan
PTLB – phage tail-like bacteriocin
RNase – ribonuclease
rRNase – ribonuclease
THF – tetrahydrofolic acid;
TLR4 – Toll-like receptor 4
TMV – tobacco mosaic virus
tRNase – ribonuclease
WHO – World Health Organization
WWI/II – World War I/II

INTRODUCTION

All life forms have one essential goal – to survive. Bacteria are no exception, whether they are beneficial or harmful to the surrounding environment. It should come to us as no wonder: when bacteria are threatened of their existence by some "anti-living" matter – antibiotics, they try their best to survive. Antibiotic resistance is ancient, therefore, natural phenomenon started long before human mass-produced antibiotic era (Dcosta et al. 2011). The serendipitous discovery of penicillin in the beginning of the twentieth century fundamentally changed the way of dealing with bacterial infections. However, excessive exploitation and misuse of antibiotics have accelerated the resistance process to such extent that now it is one of the leading public health threats of the 21st century (Murray et al. 2022). The overuse can be nicely illustrated by the following numbers: the worldwide stock of penicillin in 1941 was just a few milligrams compared to 4000 kg in 1945 (Bud 2007). The future predictions are horrendous and apocalyptic – the death toll caused by bacterial infections is expected to rise to 50 million people per year in 2050 from 1.3 million deaths reported in 2019 (Murray et al. 2022). Gram-negative bacteria belonging to the genus *Pseudomonas* and *Stenotrophomonas* are just a few representatives of pathogens readily acquiring resistance to all known antibiotics. In such context, the development of a new generation of antimicrobial substances is needed, and nature may provide a source of solutions.

Bacteriocins are one of the most promising alternatives to antibiotic treatment and the prevention of bacterial infections. Although the first bacteriocin was identified almost 100 years ago (Gratia 1925), the clinical applications of bacteriocins are just beginning to gain momentum. Bacteriocins are antibacterial proteins encoded by bacteria and represent an arsenal of weapons designed to inhibit and eliminate phylogenetically related rivals. Four main classes of bacteriocins have been shown to be produced by gram-negative bacteria: modular, phage tail-like, lectin-like, and microcins. Modular bacteriocins usually share common tri-domain architecture – translocation, receptor-binding, and cytotoxic domains. The killing is performed by cytotoxic domain and varies from pore-forming or blocking of peptidoglycan synthesis to enzymatic cleavage of nucleic acids – DNA, tRNA, rRNA (Ghequire & De Mot 2014). Modular bacteriocins can offer tailored medicine experience because they, in contrast to antibiotics, are highly selective towards killing bacterial species. At first glance, it may seem not much of an advantage, but, for instance, this trait limits the likelihood of misbalancing natural healthy gut microbiota (Francino 2016). Additional

blend of advantages, such as low-toxicity for mammalian cells, high activity in the nanomolar range, specific mechanisms of action, biodegradability, and most importantly, activity against multidrug resistant bacteria, make bacteriocins attractive candidates for therapeutical use both for humans and animals (Soltani et al. 2021). Plant-based expression system offer an attractive production platform as multiple bacteriocins from a range of gram-negative bacterial species have been already successfully synthesized and recovered with high yield in *Agrobacterium*-mediated transient expression system in tobacco (Schulz et al. 2015, Schneider et al. 2018, Denkovskienė et al. 2019).

This thesis focuses on identification, characterization, production of bacteriocins from gram-negative bacteria and exploring their possible applications to treat infections in humans and animals.

The goal of this study was to identify and develop active antimicrobials against pathogenic gram-negative bacteria. To achieve the goal, the following **objectives** were defined:

1. To identify putative bacteriocins by mining genome sequences of *Pseudomonas* and *Stenotrophomonas* species, to clone them, express *in planta* and purify to homogeneity.
2. To determine the efficacy of cloned bacteriocins both *in vitro* and *in vivo* experiments.
3. To develop superior chimeric bacteriocins.

Scientific novelty and practical value:

The data obtained from this work provide the initial groundwork for developing plant-made bacteriocins as future non-antibiotic pharmaceuticals. It was clearly demonstrated that all tested bacteriocins – six pyocins from *Pseudomonas* and two stenocins from *Stenotrophomonas* – can eradicate respective bacterial species *in vitro*. Moreover, selected most active pyocins confirmed their potential as antimicrobials *in vivo* by rescuing infected *Galleria mellonella* larvae. Domain swapping experiments with pore-forming pyocins resulted in superior chimeric pyocin S5-PmnH and demonstrated the near endless possibilities of engineering bacteriocins with modified or broadened activity spectra. Newly designed chimeric PyoS5-PmnH efficiently performed as topical treatment in murine keratitis model of disease caused by *P. aeruginosa.* Furthermore, intranasally administered S5-PmnH efficiently eradicated lung colonization by *P. aeruginosa* in murine model of disease. Highly encouraging results with chimeric pyocin S5-PmnH led us to file patent application with the European Patent office under the following title "Chimeric bacteriocins and methods for the control of *Pseudomonas*". All

results taken together, we propose that plant-produced bacteriocins should be considered as a viable alternative to antibiotics for the control of pathogenic gram-negative bacteria.

The major findings presented for defense in this thesis:

- Bacteriocins from *P. aeruginosa* and *S. maltophilia* can be expressed at high yields in plants and are fully functional.
- Modular pyocins S5, M, M4 and lectin-like pyocins L1, L2, L3 effectively kill planktonic and biofilm-forming *P. aeruginosa* bacteria *in vitro* and reduce bacteremia in *G. mellonella* model *in vivo.*
- Chimeric pyocin S5-PmnH demonstrates broadened activity spectrum in comparison to PyoS5.
- Chimeric pyocin S5-PmnH shows high efficacy in topical treatment of two unrelated murine models of disease caused by *P. aeruginosa* – keratitis and lung infection.
- Two newly identified bacteriocins from *S. maltophilia* – SmaltM and SmaltM2 exhibit *in vitro* antimicrobial activity.

1. REVIEW OF LITERATURE

1.1. Antibiotic resistance in global priority pathogens

The term "antibiotic" comes from Greek roots ἀντι anti, "against" and βίος bios, "life" literally translating to "opposing life". The very start of antibiotics in a far-reaching sense can be traced to 1877, when L. Pasteur wrote "La vie empêche la vie" (Life prevents life), meaning he was well aware of phenomenon of antagonism between organisms. Twelve years later (in 1889), the use of words "antibiosis" and "antibiotic substance" to narrate anti-living processes in a broad sense was first introduced by French bacteriologist J. P. Vuillemin (Brunel 1951). Although penicillin was serendipitously discovered by A. Fleming in 1928, the terms "antibiotic" and "antibiotic agent" were first used in the present sense by S. Waksman only in 1942 (Waksman 1947). This mismatch in the timeline can be elucidated by the fact that Fleming's 1928 paper was forgotten for ten years and it was only in 1940, when scientists from Oxford – H. Florey and E. Chain – managed to extract and obtain a stable form of penicillin as we have it today (Brunel 1951). The commercialization of antibiotics led us to a new era of modern medicine. Before that, pneumonia and flu, tuberculosis, and gastrointestinal infections were the main leading causes of death. Moreover, bacterial infections caused more deaths during World War I than battle wounds (Browne et al. 2020). The "golden era" of antibiotics ranged from 1930s to 1960s and provided us with an illusion that the problem of bacterial infections is solved. The over-reliance and improper use of antibiotics came at a cost – the rise of resistant bacteria. A brief timeline of antibiotic resistance is provided in **Figure 1**.

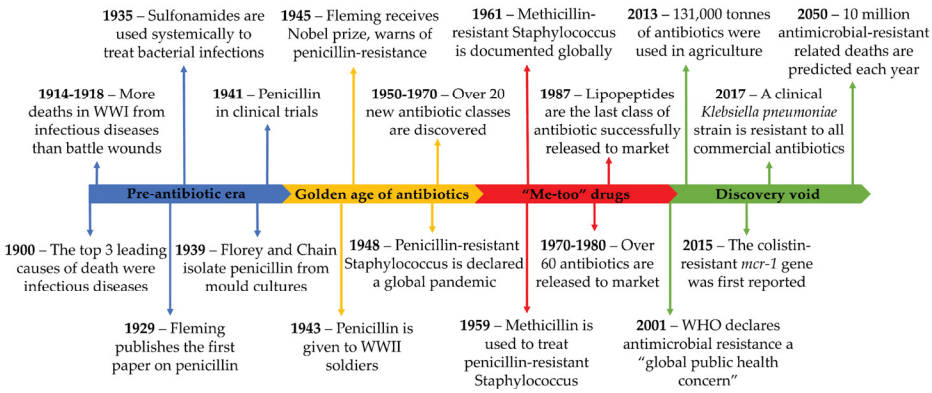


Figure 1. A summary of events in the antibiotic-resistance timeline, adapted from (Browne et al. 2020). WHO – World Health Organization; WWI/II – World War I/II.

Today, one of the greatest global public health threats of the 21st century is growing antimicrobial resistance (AMR). Concerns for a looming AMR crisis may have been temporarily overshadowed by the COVID-19 pandemic, but many experts worry that pandemic-induced increases in antibiotic exposures, hospitalizations, and diverted resources have only accelerated its arrival (George et al. 2022). Eight years ago, Review on Antimicrobial Resistance commissioned by the UK Government estimated what is likely to happen based on high-level scenarios. The results showed that AMR could lead to 10 million people dying every year and a reduction of Gross Domestic Product by 2–3.5% – the cost for the world would be ~100 trillion USD (O'Neill 2014). While the numbers of deaths attributed to antimicrobial resistance for the past decade were around 700 000 per year (Ghosh et al. 2019), the first exhaustive analysis of global impact of antimicrobial resistance in 2019 reveals alerting numbers (Murray et al. 2022):

- resistance was directly responsible for 1.27 million deaths (more deaths than HIV/AIDS or malaria);
- resistance was associated with 4.95 million deaths.

Albeit these predictions were criticized by some (Cox & Worthington 2017; de Kraker et al. 2016), numerous groups of scientists, researchers, and WHO come to an agreement that AMR is an urgent challenge requiring global cooperation. The unanimous effort from governments, policy makers, pharmaceutical companies, healthcare workers, agricultural workers, and the general public is essential for preventing apocalyptic post-antibiotic era scenario (Murray et al. 2022).

The overuse and misuse of antibiotics and an absence of effective new antibiotics released to the market are the main driving forces for causing significant concerns in healthcare institutions and global spread of antibiotic-resistant bacteria. In addition, other important factors, commonly referred to as "socioeconomic determinants", also significantly contribute to AMR. These factors include over-prescribing of antibiotics, poor community hygiene and sanitation, poor infection control in hospitals and clinics, over-use of antibiotics in livestock and fish farming, and accumulation of antibiotics in the environment. Antibiotic consumption is also affected by such socioeconomic determinants as income, access to education, demographic structure, and density of general practitioners (Collignon & Beggs 2019).

Bacterial pathogens have been extremely successful in causing infections worldwide (**Figure 2**) owing to their various virulence and resistance features. ESKAPE pathogens, which include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter*

baumannii, *Pseudomonas aeruginosa*, *Enterobacter* spp., are the main players and culprits of public health emergency (M Campos et al. 2020). However, more pathogens, such as *Clostridium difficile* and *Salmonella* (belongs to *Enterobacteriaceae* family), are keen to join this emergency pathogen group, changing the initially proposed acronym ESKAPE to ESCAPE (*E. faecium*, *S. aureus*, *C. difficile*, *A. baumannii*, *P. aeruginosa*, and *Enterobacteriaceae*) (De Rosa et al. 2015).

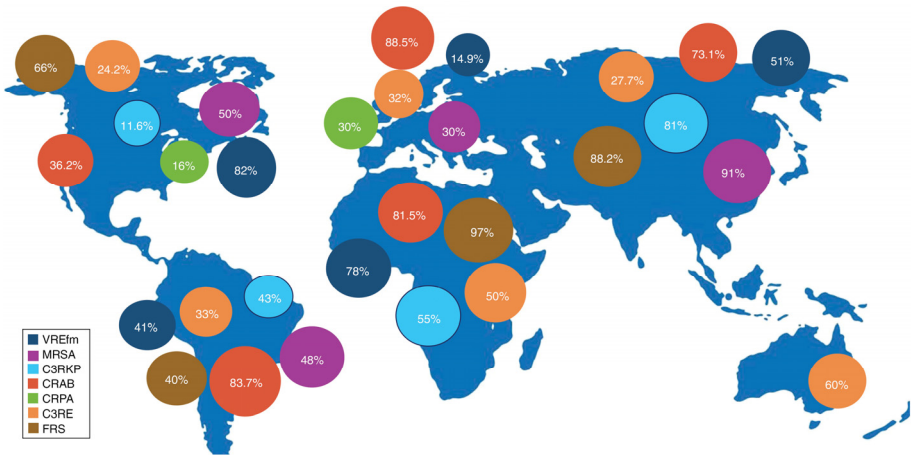


Figure 2. Distribution of antimicrobial resistance rates among the major bacterial pathogens across the world, adapted from (M Campos et al. 2020). C3RE – Third-generation cephalosporin-resistant *Enterobacter* spp.; C3RKP – Third-generation cephalosporin-resistant *Klebsiella pneumoniae*; CRAB – Carbapenem-resistant *Acinetobacter baumannii*; CRPA – Carbapenem-resistant *Pseudomonas aeruginosa*; FRS – Fluoroquinolone-resistant (or nonsusceptible) *Salmonella*; MRSA – Methicillin-resistant *Staphylococcus aureus*; VREfm – Vancomycin-resistant *Enterococcus faecium*.

Antimicrobial agents can be divided into groups based on the mechanism of antimicrobial activity. The main groups are: agents that inhibit cell wall synthesis, depolarize the cell membrane, inhibit protein synthesis, inhibit nucleic acid synthesis, and inhibit metabolic pathways in bacteria (**Figure 3**).

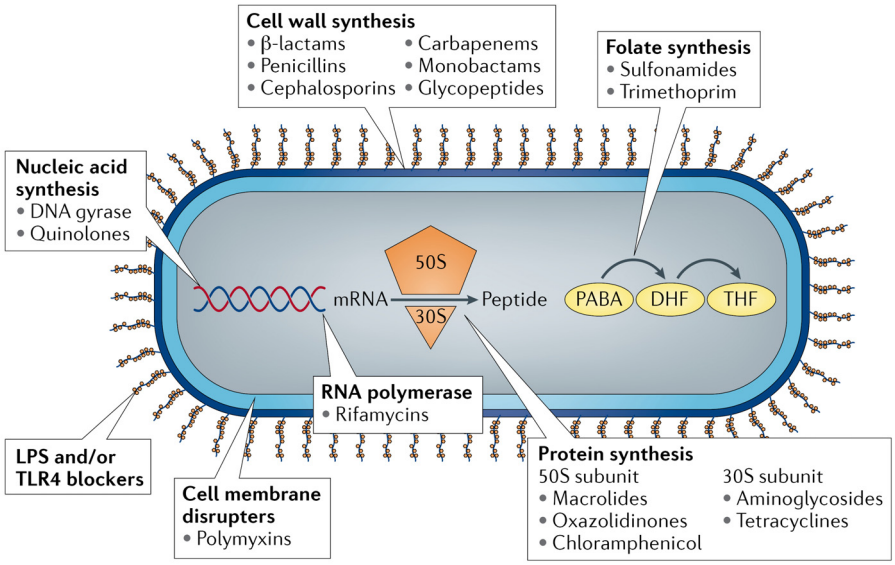


Figure 3. Sites of antibacterial action, adapted from (Brown 2015). Antibiotics can be classified by their mechanism of action. DHF – dihydrofolic acid; LPS – lipopolysaccharide; PABA – para-aminobenzoic acid; THF – tetrahydrofolic acid; TLR4 – Toll-like receptor 4.

1.1.1. Resistance, AMR, tolerance, and persistence

All living beings have one essential goal – survival. Microorganisms, in this case bacteria, are no exception. Bacteria over time evolved to avoid killing by antimicrobial agents through various strategies (**Figure 4**). AMR arises when an organism acquires certain genes from other species or piles up mutations in its genome. These strategies can be divided (Blackman et al. 2022; Reygaert 2018):

1. Natural, which can be subdivided into (**Figure 4A**):
    a. intrinsic – always expressed in the species, meaning bacteria are naturally resistant to antimicrobials.
    b. induced – when bacteria activate certain genes after exposure to clinical amounts of antibiotics.
2. Acquired (**Figure 4B**). Resistance is borrowed from the surrounding environment and can occur through two distinct processes:
    a. *de novo* genetic mutation(s) during replication or DNA transfer.
    b. horizontal gene transfer – DNA from the biosphere containing an antibiotic resistance gene can be transferred by horizontal gene transfer into a recipient by several paths: cell-

to-cell conjugation; transformation by naked DNA (on plasmids or as linear DNA) that is released by dead cells; phage-mediated transduction (Andersson & Hughes 2010).

3. Situational (**Figure 4C**). Resistance depends on changes in surrounding environment e.g., in biofilms or within intracellular environments, where pathogen come into favorable conditions for temporary resistance. It is important to note here that situational resistance is not AMR as no genetic changes occur and the resistance is transient (Blackman et al. 2022).

It is important to clearly acknowledge the distinction between these three forms of resistance – natural, acquired, and situational – when developing new therapeutics, together with mechanisms by which pathogens can avoid killing as well as their capacity to gain and pass their resistance (Blackman et al. 2022).

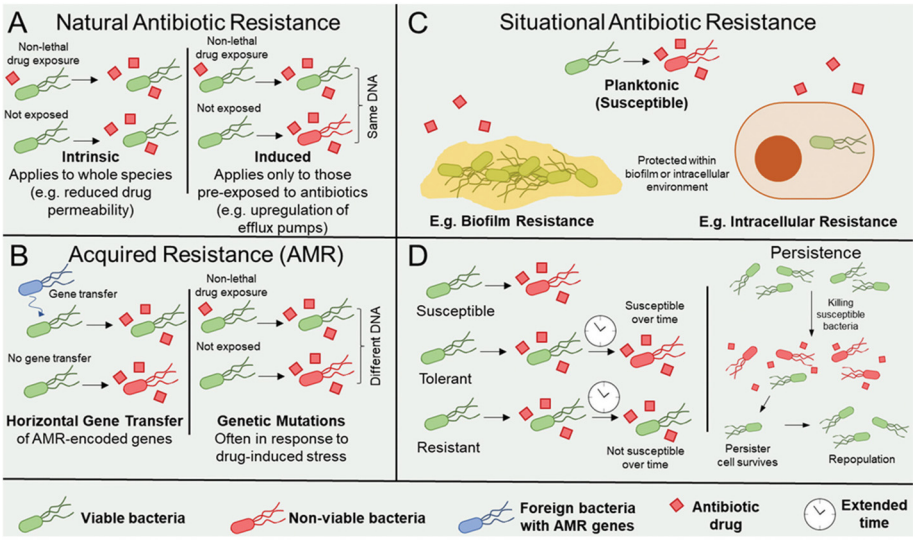


Figure 4. Illustrative outline of various ways that an organism can be resistant to an antibiotic **(A–C)** and the concept of tolerance and persistence **(D)**, adapted from (Blackman et al. 2022). Green – alive, red – dead bacteria, blue – a foreign resistant (sub)species, red squares – antibiotics.

Also, it is important to stress microbes' different possible responses towards antimicrobial treatment – tolerance, resistance, and persistence (**Figure 4D**). Higher resistance projects lower effectiveness of antimicrobial, while higher tolerance elongates exposure time for antimicrobial. Persistence describes the presence of small subset of bacterial population that are transiently tolerant to antibiotic treatment, despite any additional resistance

mechanisms (Van den Bergh et al. 2022). However, there is mounting evidence that surviving persister cells have greater susceptibility for genetic mutations, suggesting that resistance is heritable, therefore persisters are able to generate greater numbers of antimicrobial-resistant mutants (Barrett et al. 2019).

When AMR is acquired, it is driven through such biochemical aspects as antibiotic inactivation, enzymatic modifications of antibiotics, target bypass, decreased antibiotic penetration and activation of efflux pump (Pulingam et al. 2022) (**Figure 5**). Because of differences in their structure, gram-negative bacteria employ larger arsenal of mechanisms to inactivate antimicrobials (Varela et al. 2021).

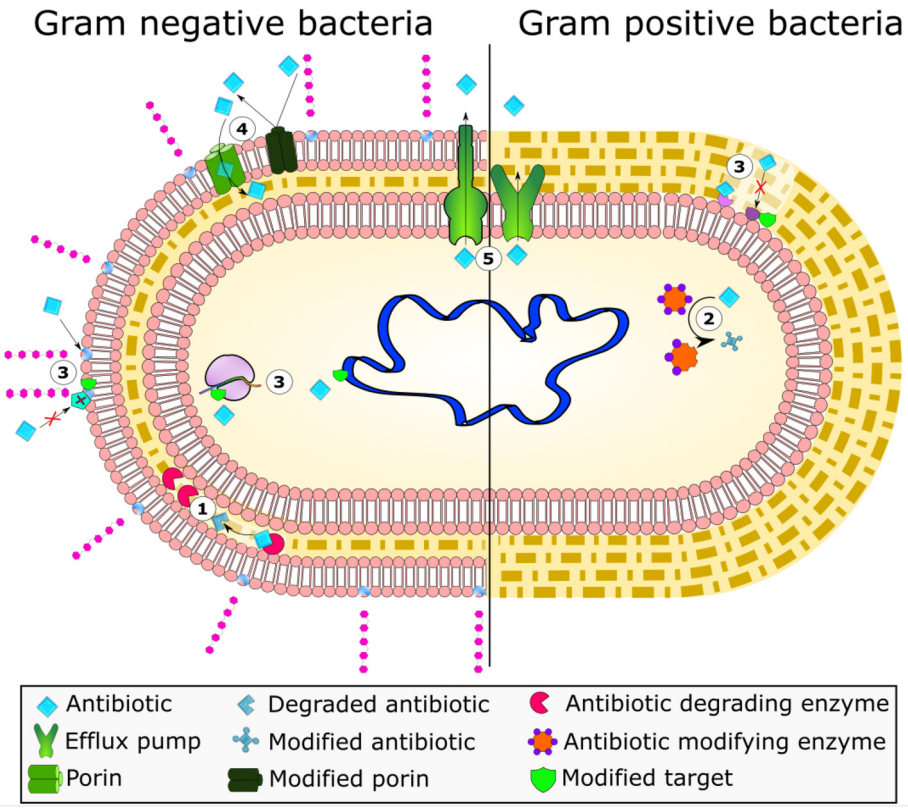


Figure 5. Bacterial mechanisms of resistance to antimicrobial agents, adapted from (Varela et al. 2021). The common mechanisms of antibiotic resistance in bacteria are: 1 – enzymatic hydrolysis, 2 – enzymatic modifications of antibiotics by group transfer and redox process, 3 – modifications of antibiotic targets, 4 – reduced permeability to antibiotics by modifications of porins, 5 – active extrusion of antibiotics by membrane efflux pumps.

1.1.2. Biofilms

Microbial biofilms present an organized communal multicellular way of life for bacterial population (single or multispecies) where organisms are embedded in an extracellular matrix (Ciofu et al. 2022) that is composed mainly of water and polymeric substances, such as polysaccharides, proteins, and extracellular DNA (Serra & Hengge 2021). Extracellular matrix can be metaphorically described as the "house of biofilm cells" and act as large-scale scaffold for bacterial attachment (Serra & Hengge 2021). Biofilm formation on both biotic and abiotic factors can be advantageous as well as having negative impacts (Mukherjee et al. 2022). Exploring the positive sides of biofilm – there is increasing evidence that biofilms are of utmost importance to humans and animals for proper functioning (Sentenac et al. 2022). However, this formation of communities has a profound negative effect in medical settings, mainly because of increased antibiotic resistance within the biofilms (Mah 2012); there are reports with 1000-fold increase of resistance (Ceri et al. 1999). In addition to medical settings, biofilm significantly contributes to the fouling of biomedical devices, bioreactors, food, and paper processing equipment, membranes, and pipelines. The estimated costs in damage by biofilms are $1–$2 billion/year in corrosion and $94 billion/year in healthcare (Conrad & Poling-Skutvik 2018). Moreover, most microorganisms can form biofilm on a wide range of surfaces *i.e.*, biological and inert surfaces, such as breast implants, ventricular shunts, tissue fillers, ventricular-assisted devices, contact lenses, catheters, joint prostheses, urinary catheters, orthopedic implants, pacemakers, mechanical heart valves, defibrillators, vascular grafts, endotracheal tubes, voice prostheses (Rather et al. 2021).

Figure 6 presents biofilm formation cycle that can be divided into four major steps (Ciofu et al. 2022):

1. Adherence to surfaces or aggregations in tissues;
2. Proliferation;
3. Maturation and development of 3D biofilm structure;
4. Dispersal.

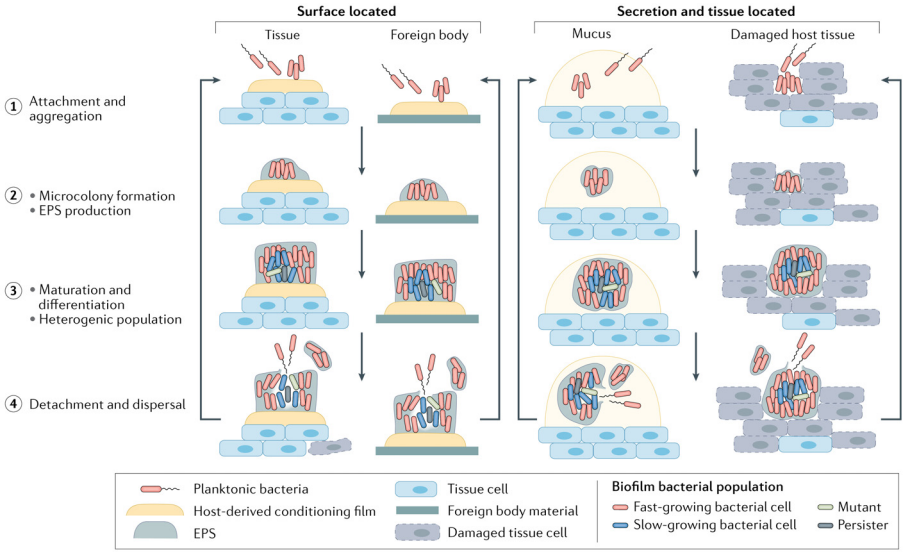


Figure 6. Biofilm formation, adapted from (Ciofu et al. 2022). EPS – extracellular polymeric substance.

1.1.3. Global priority pathogens

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a ubiquitous gram-negative, rod-shaped, asporogenous, monoflagellated bacterium belonging to the class Gammaproteobacteria, and is characterized in part by its high nutritional versatility. Consequently, *P. aeruginosa* can persist in diverse environments including water and the rhizosphere, as well as in the human body. *P. aeruginosa* is an opportunistic pathogen and can cause diseases in plants and animals, including humans. In the latter (**Table 1**), it can cause life-threatening chronic infections (pneumonia, ventilator-associated pneumonia, catheter-related infections, burn wound infections, and sepsis) especially in patients with a weakened immune system. In addition to nosocomial infections, *P. aeruginosa* is very dangerous for cystic fibrosis sufferers. Cystic fibrosis (CF), or mucoviscidosis, is an autosomal recessive genetic disease most common in Northern Europe and the USA. The disease is caused by a mutation in the gene encoding the CFTR protein. CFTR is a membrane permeability regulator, and its malfunction causes the production of abnormally viscous and sticky mucus, which accumulates instead of leaving the site of production. The lungs of patients with CF become prone to infections. When *P. aeruginosa* colonizes the lungs of CF patients and chemotherapy is unsuccessful, the infection becomes chronic and lung function deteriorates irreversibly (Malhotra et al. 2019). *P. aeruginosa* is also a frequent culprit in one of the most serious eye infections – keratitis, that often leads to corneal blindness (Teweldemedhin et al. 2017).

Table 1. Infections caused by *P. aeruginosa* in humans, adapted from (Veetilvalappil et al. 2022).

Organ	Disease
Eye	Keratitis, blepharitis, conjunctivitis and dacryocystitis
Lungs and bronchi	Cystic fibrosis, ventilator-associated pneumonia, community-acquired pneumonia
The skin and nails	Green nail syndrome, toe-web intertrigo, folliculitis and ecthyma gangrenosum
Ear	Perichondritis, otitis externa (swimmer's ear)
Nose	Bacterial sinusitis
Urinary bladder	Uncomplicated and complicated urinary tract infections
Blood	Septicemia
Bones	Osteomyelitis
Heart	Endocarditis

Stenotrophomonas maltophilia

S. maltophilia is a motile, rod-shaped, non-fermentative, gram-negative obligate aerobe that is closely related to the genus *Pseudomonas* (Brooke 2012). The genus *Stenotrophomonas* name comes from Greek for "a unit feeding on few substrates" (Denton & Kerr 1998). *S. maltophilia* strains are found in a wide range of aquatic and soil environments, including extreme ones, although in nature it is usually associated with plant rhizospheres. This bacterium has historically been regarded as nonpathogenic, but over the last 3 decades it has emerged as a pathogen and global gram-negative multidrug-resistant organism (MDR) that is most commonly associated with infections in severely immunocompromised and debilitated individuals. According to the WHO, *S. maltophilia* is currently one of the leading MDR pathogens in hospitals (Gupta et al. 2018), along with ESCAPE pathogens. Moreover, in medical centers enrolled in the SENTRY Program (Gales et al. 2019), *S. maltophilia* has been reported to be most frequently isolated from patients with pneumonia (55.8%) and bloodstream infections (33.8%). Less frequently, it has been associated with endocarditis, urinary infections, biliary sepsis, bacteremia, and osteomyelitis (Brooke 2012). Recently, cooperativity has been inferred between *S. maltophilia* and *P. aeruginosa* in CF patients (McDaniel et al. 2020). Furthermore, both species have also been associated with keratitis (Dantam et al. 2020). The treatment of *S. maltophilia* infections is challenging since the bacteria are naturally resistant to many broad-spectrum antibiotics, such as quinolones, cephalosporins, carbapenems, and aminoglycosides, furthermore, they easily acquire new resistances via horizontal gene transfer and mutations (Sánchez 2015). Clinical and environmental isolates of *S. maltophilia* are phenotypically and taxonomically similar, indicating that the low susceptibility to various antibiotics is evolutionary and does not result from patient treatment with antibiotics (Gil-Gil et al. 2020)

1.2. Alternatives to conventional antibiotics

There is an undeniable need to research novel antibiotic compounds and new strategies to combat bacterial infections (Browne et al. 2020). The successful combat against MDR bacteria leans on three major approaches – discovery of new antibiotics, developing new antibiotic adjuvants and screening the alternatives to antibiotics (Liu et al. 2019). One of the ways to check the novelty of newly created antimicrobial is to look over if it follows these criteria:

1. belongs to a novel chemical class and interacts with a new target.
2. works via new mechanisms or binding to new target sites.
3. and/or is biochemically modified to re-sensitize a previously resistant pathogen.

The end of "golden era" (1930s-1960s) of antibiotics marks the sharp decline of number of discovered novel classes of antibiotics. Even what is more important, the newly introduced antibiotic into the market rapidly generates resistance (Wang et al. 2020a). Also, double membrane of gram-negative bacteria especially hardens the task of designing novel antimicrobial. Indeed, there are three kinds of interrelated reasons for the slowdown in the development of newer antimicrobial agents: 1) economic; 2) scientific research; 3) regulatory. However, multidisciplinary advancements in biotechnology, genetic engineering, synthetic biology have expanded horizons for drug innovations and alternative therapies that can replace conventional antibiotics (Ghosh et al. 2019). **Table 2** summarizes various promising approaches that have been adopted as substitutes to classical antibiotics. Following are more "exotic" alternatives not included in the **Table 2**: antibacterial fatty acids (Casillas-Vargas et al. 2021), stem cells (Rello et al. 2019), secondary plant metabolites (Keita et al. 2022), antisense peptide nucleic acids (Lee et al. 2019), traditional Chinese medicines (Pang & Zhu 2021), non-biological complex drugs (Blackman et al. 2022). Potential adjunct approaches such as immune stimulation, immune suppression could prove beneficial for antibiotic therapy (Czaplewski et al. 2016). Artificial intelligence could also prove as a useful tool mining drug repositories and identifying antibacterial compounds that are structurally distant from known antibiotics (Stokes et al. 2020).

Table 2. An overview of current alternatives to antibiotics, their main advantages, and disadvantages, adapted from (Czaplewski et al. 2016; Ghosh et al. 2019; Rello et al. 2016, 2019).

Strategy	Advantages over conventional antibiotics	Possible disadvantages
Bacteriocins	• Specificity towards pathogenic strains of bacteria • Low toxicity for mammalian cells • High activity in the nanomolar range	• Expensive large-scale production • Susceptible to proteolysis • Resistance development
Antimicrobial peptides	• Not prone to resistance development • Broad-spectrum activity is an advantage, depending upon application	• Expensive large-scale production • Susceptible to proteolysis • Toxicity
Synthetic mimics of antimicrobial peptides	• Ease of synthesis • Not prone to resistance development • Broad-spectrum activity is an advantage, depending upon application	• Toxicity • Route of administration
Innate defense regulatory peptides	• Work by modulating the immune system • No resistance development as no direct antimicrobial activity	• Expensive large-scale production • Susceptible to proteolysis
Lysins	• Specificity towards pathogenic strains of bacteria • Amenable to genetic engineering • Not prone to resistance development	• Production • Lack of sufficient knowledge
Phage therapy	• Self-replicating pharmaceuticals • Selective towards specific strains of bacteria • Amenable to genetic engineering	• Immunogenicity • Pharmacokinetics • Release of bacterial endotoxins • Inadequate preparations – failure to remove endotoxins and pyrogenic substances • Resistance development

Strategy	Advantages over conventional antibiotics	Possible disadvantages
CRISPR/Cas9	• Can be tuned for a variety of antimicrobial applications • Reversal of antibiotic usage • Specificity towards pathogenic strains	• Expensive large-scale production • Toxicity
Probiotics	• Easy availability	• Used mostly for intestinal infections
Antibodies	• Selective towards specific strains of bacteria • Do not damage the microflora	• High cost of production • Poor shelf life
Vaccines	• Can target a combination of virulence factors	• Not as fast acting as antibodies • A phase III trial using a vaccine against *C. difficile* toxoid was recently terminated by Sanofi
Antimicrobial polymers	• Some can re-sensitize drug-resistant bacteria to conventional antibiotics	• Moderate activity against bacterial pathogens
Inhibitors of Bacterial Virulence	• Negligible propensity to induce resistance • First-in-class drug CAL-02 was introduced for clinical trials	• High cost of production
Fecal Transplant Therapy	• Very high cure rate (~90%) for the treatment of *C. difficile* infections	• Investigation of the efficacy against MDR *Enterobacteriaceae* pathogens in humans is limited
Predatory bacteria	• Minimal inflammatory response • Low toxicity • Negligible propensity to induce resistance	• Kill only gram-negative bacteria • Still at early-stage of research

1.3. Bacteriocins

Microorganisms in their natural settings have diverse and complex relationships between each other, ranging from nutritional collaboration to warfare among competitors (Ghequire et al. 2013). The examples of such interactions include not only competition between unrelated organisms (e.g., fungi and bacteria (Frey-Klett et al. 2011)), but also between distant relatives (e.g., members of different bacterial genera (Garbeva et al. 2011)) and even between close relatives (e.g., at inter- and intra-species levels (Liu et al. 2022)). Colonization of ecological niches implies a never-ending struggle for space, nutrients and competitive advantage. These intense interactions lead to the evolution of diverse arsenal of biological weapons (Riley 2011). Antagonistic interactions between microorganisms are typically of two types, those that depend on physical or close contact and those that are contact-independent. Contact-dependent inhibition examples include: (i) type VI secretion system, where toxins are transported by a needle that punctures the outer membrane (OM) and (ii) bacteria that projects toxin to its prey's surface receptor. Contact-independent attacks are being mediated by a tremendous variety of diffusible inhibitory mediators, including antibiotics and protein toxins. (Francis et al. 2021). Protein toxins are collectively referred to as bacteriocins and, in essence, are used as very potent weapons to target phylogenetically related bacteria that occupy the same ecological niche. Narrow activity spectrum and production in ribosomes distinguish majority of bacteriocins from classical antibiotics (Riley 2011). Bacteriocins not only play significant roles in microbial biodiversity, but also are key players in maintenance of stable coexistence of populations in niches such as gastro-intestinal tract (Dicks et al. 2018).

The discovery of bacteriocins dates back to almost 100 years ago, when Belgian scientist André Gratia described a remarkable antagonism between two strains of *Escherichia coli* (Gratia 1925). In other words, Gratia showed that *E. coli* strain V released a substance that was able to kill another *E. coli* strain. Gratia's discovered substance was named "colicin" according to the species name of organism that originally produced them (Kim et al. 2014). Similar terminology was endorsed for most bacteriocins discovered thereafter: *Pseudomonas* produces pyocins, *Klebsiella* – klebicins, *Stenotrophomonas* – stenocins, *Salmonella* – salmocins, *Yersinia pestis* – pesticins, *Clostridium difficile* – diffocins, *Bacillus thuringiensis* – thuricins, etc. To date, all major prokaryotic lines and archaea are believed to produce at least one bacteriocin (Meade et al. 2020). **Table 3** presents classification of bacteriocins based on cell wall type of producing organism. Representatives from Archaea domain were also included.

Table 3. Classification of bacteriocins.

Producer	Class	Molecular mass, kDa	Examples	Source
Gram-negative bacteria	Modular bacteriocins	20-80	Colicins, S-pyocins, klebicins, stenocins	(Calcuttawala et al. 2022; Denkovskienė et al. 2019)
	Microcins	<10	Microcin C7, microcin B17, colicin V	(Beis & Rebuffat 2019; Collin & Maxwell 2019)
	Phage tail-like bacteriocins	20-100	R-, F-pyocins	(Scholl 2017)
Gram-positive bacteria	Class I – Lantibiotics	<5	Nisin, mersadicin, lacticin 3147, cinnamycin	(Hsu et al. 2004; Hullin-Matsuda et al. 2016; Ryan et al. 2021; Viel et al. 2021)
	Class II – Non-lantibiotics	<10	Leucocin A and pediocin PA-1	(Derksen et al. 2008)
	Class III – Bacteriolysins, Non-lytic bacteriocins Tailocins	>10	Lysostaphin, zoocin A, Diffocin, monocin	(Bastos et al. 2010; Curtis & Mitchell 1992; Gebhart et al. 2012; Lester & Simmonds 2012)
	Class IV – contains lipid or carbohydrate parts	2-10	plantaricin S, leuconocin S	(Lewus et al. 1992; Savadogo et al. 2006)
Archaea	Halocins	>5	Halocin H4, S8, C8	(Kumar et al. 2021)
	Sulfolobicins	~20	SulAB	(Besse et al. 2015)

Bacteriocins present a group of antimicrobials with different structures, modes of action, mechanisms of biosynthesis and self-immunity, as well as gene regulation (Zimina et al. 2020). There are surprisingly vast number of descriptions of bacteriocins throughout scientific literature. Most of them do not cover all possible variants of bacteriocins, while usually prioritizing the small molecular weight bacteriocins from gram-positive bacteria. To the best of my knowledge, (Chikindas et al. 2018) description seems to be most appropriate – "bacteriocins are ribosomally-produced multi-functional

substances of a proteinaceous nature, with pronounced antimicrobial activity at certain concentrations".

1.3.1. Bacteriocins from gram-positive bacteria

Although bacteriocins are produced by both gram-positive and gram-negative bacteria, the lion's share of reports is dedicated to bacteriocins from gram-positive bacteria, and particularly from lactic acid bacteria (Hassan et al. 2012). **Table 3** presents current gram-positive bacteriocin classification into three classes (Simons et al. 2020):

- Class I, historically named lantibiotics, includes small peptides (<5 kDa) that undergo extensive post-translational modifications. A common feature of this class is the presence of unusual amino acids, such as lanthionine and/or methyllanthionine that form multiple ring structures. The result of such structures is stability to heat, pH and proteolysis. The members of this class either kill other bacteria by pore-formation or by inhibiting essential enzymes for the targeted bacteria. Lantibiotics (containing lanthionine rings), lipolanthines (containing avionin moiety and an N-terminal guanidine fatty acid), linear azol(in)e-containing bacteriocins, thiopeptides (containing thiazole and other dehydro amino acids with a pyridine/piperidine core), bottromycins (containing macrocyclic amidine), sactibiotics (containing sulphur-α-carbon linkages), lasso peptides (consisting of an N-terminal macrolactam ring), glycocins (containing S-linked glycopeptides), head-to-tail cyclized (peptide bond that links the N- and C-termini of the core peptide) bacteriocins are the representatives of this class (Acedo et al. 2018)
- Class II, or non-lantibiotics, small post-translationally unmodified bacteriocins that do not contain unusual amino acids in their structure. These peptides are also like Class I small (<10 kDa), heat-stable and mainly act to destabilize and permeabilize bacterial membranes. YGNG-motif-containing bacteriocins, linear two-peptide bacteriocins (containing double glycine-type N-terminal leaders), leaderless bacteriocins (are not produced as precursors with an N-terminal leader) belong to this class (Acedo et al. 2018)
- Class III bacteriocins are large proteins (>30 kDa), sensitive to higher temperatures and include bacteriolysins, non-lytic large bacteriocins, phage-tail-like bacteriocins. Their antibacterial potential is linked to enzymatic activities.

Most of these bacteriocins are secreted by diverse transporters belonging to a large family of ATP-binding cassette (ABC) transporters, while

some use the general sec-dependent pathway (Zheng & Sonomoto 2018). To date, no stress-inducible production of gram-positive bacteriocins has been documented. The majority of them are produced constitutively and are auto-regulated with the cell-density regulatory model being the most common (Kleerebezem & Quadri 2001).

1.3.2. Bacteriocins from Archaea

Bacteriocin analogues in Archaea are known as archaeocins. They are also ribosomally-synthesized antimicrobial peptides/proteins as weapons to help its producer in microbial warfare and are classified into:

- Sulfolobicins (membrane associated), produced by *Sulfolobales*;
- Halocins (secretory), produced by *Halobacteriales*.

Most of the archaeocins remain stable and functional at high salt concentrations (10–35% NaCl), high temperatures (up to >100 °C), alkaline and dehydrating conditions (Matarredona et al. 2020). These extraordinary properties may suggest the potential to apply archaeocins in diverse biotechnological processes. Examples include using halophilic archaea in the biological treatment of saline wastewaters, textile dyeing, oil and gas production, tanneries and drinking water treatment processes (Xiao & Roberts 2010).

1.3.3. Bacteriocins from gram-negative bacteria

Three main classes of bacteriocins have been shown to be produced by gram-negative bacteria (**Table 3**): modular bacteriocins, microcins and phage tail–like bacteriocins. Additional fourth class of distinct lectin-like bacteriocins come mainly from *Pseudomonas* species (Ghequire et al. 2018c). These ribosomally synthesized proteins and peptides are being more and more studied, especially colicins (*E. coli*) and pyocins (*P. aeruginosa*) serving as model systems for studying receptor binding, cell import mechanisms and toxin-immunity interactions (Chassaing & Cascales 2018).

Bacteriocin biosynthesis pathways in gram-negative bacteria differ depending on producing organism, but they all have one common determinant – are induced under stress conditions such as DNA-damaging treatment by UV light or mitomycin C or under nutrient limitation, oxygen starvation, stationary phase of growth (Simons et al. 2020). Modular bacteriocins – colicins, pyocins – are secreted by a non-specific mechanism, where bacterial membrane lysis is involved. In short, modular bacteriocin operon encodes lysis proteins that can promote the extracellular release of bacteriocins (Lloubes et al. 2013).

1.3.3.1. Modular bacteriocins

Modular bacteriocins from gram-negative bacteria are ~20-80 kDa proteinaceous toxins and most of them share a common tri-domain architecture – the central R-domain is involved in binding to specific receptors located in the outer membrane (OM), the N-terminal T-domain with IUTD (intrinsically unstructured T domain) is in charge of translocation across the OM to periplasm, while the C-terminal cytotoxic domain presents the active center of bacteriocin and displays its cytotoxicity (**Figure 7A**). All to date identified modular bacteriocins have single cytotoxic domain at their C-terminus with exception of recently identified bacteriocin with dual-toxin architecture from *Pseudomonas synxantha* (**Figure 7B**) (Ghequire et al. 2017a).

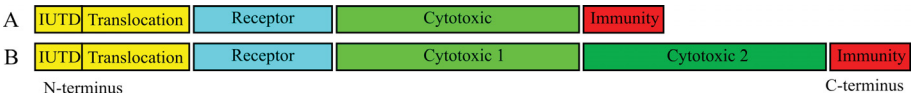


Figure 7. Schematic representation for modular bacteriocin domain organization. **(A)** Bacteriocins with single cytotoxic domain. **(B)** Bacteriocin with dual cytotoxic domains. IUTD – intrinsically unstructured T domain.

Cell death is ensured by cytotoxic domain acting in different localizations of the cell:

- Periplasm – through depolarization of the cell by an ionophore (pore-forming) or enzymatic degradation of lipid-II precursor for peptidoglycan synthesis (peptidoglycan-degrading).
- Cytoplasm – enzymatic cleavage of nucleic acids (DNA, tRNA, rRNA).

Self-preservation for bacteriocin producers is guaranteed by immunity proteins. The coding sequences of these antidotes are usually located just after bacteriocin genes. Bacteriocins acting in periplasm are disarmed by either an inner membrane (IM) anchored periplasmic protein or by an integral membrane protein (Ghequire et al. 2017b). Nucleases in cytoplasm are inhibited by co-expressed cytoplasmic immunity protein (Kleanthous & Walker 2001).

1.3.3.2. Journey into bacterial cell

Gram-negative bacteria have a complex cell envelope that is comprised of (Francis et al. 2021):

- asymmetric outer membrane with lipopolysaccharide in the outer leaflet and phospholipids in the inner leaflet;
- intervening periplasm;
- energized inner membrane.

The outer membrane of gram-negative bacteria is a robust layer of defense shield against such environmental insults as the immune systems of plants and animals, bile salts in the human gut and several classes of antibiotics (Francis et al. 2021). Every bacteriocin face a formidable task of translocating across one or both gram-negative bacterial cell membranes in order to induce cell death. The translocation is accomplished by parasitizing host proteins involved in nutrient and metabolite trafficking (Atanaskovic & Kleanthous 2019). While bacteriocins from *E. coli* parasitize β-barrel shaped protein in the OM that is normally involved with the import of nutrients and metal ions such as vitamins, sugars and Fe^{3+} (Kim et al. 2014), Achilles' heel for *Pseudomonas* are exclusively receptors involved in the uptake of iron-siderophore complexes (Grinter et al. 2013).

The outer membrane is not energized and there is no ATP in the periplasm, thus bacteriocins take advantage of periplasmic energy transducing systems (Szczepaniak et al. 2020) and are divided into two groups (**Figure 8**):

- Group A bacteriocins exploit Tol-Pal system, which straddles the three layers of the cell envelope and is composed of IM proteins (TolA, TolQ, and TolR), a periplasmic protein (TolB), and an OM lipoprotein (Pal). Group A bacteriocins generally require additional OM proteins, such as porins OmpF, OmpC to reach Tol proteins in the periplasm (Housden et al. 2010)

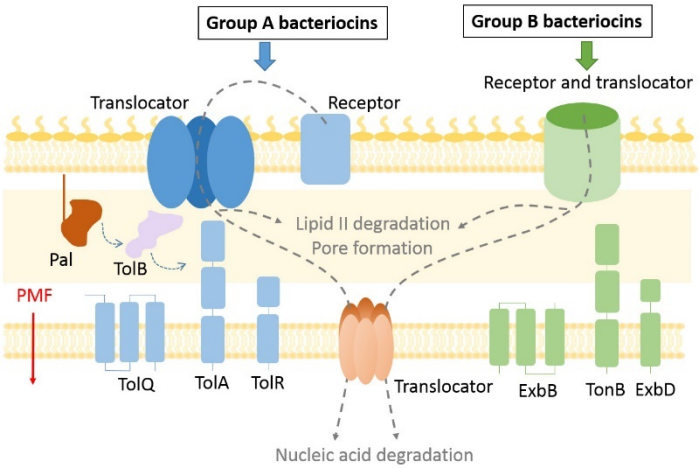


Figure 8. Import pathways for modular bacteriocins, adapted from (Atanaskovic & Kleanthous 2019). Bacteriocins use outer membrane receptors for import into bacterial cell. Group A bacteriocins use Tol-Pal system to enter periplasm, while Group B bacterocins make use of TonB system. Lipid-II precursor degrading bacteriocins stay in periplasm, pore-forming bacteriocins are inserted into IM and nucleases cross the IM through distinct protein translocator.

- Group B bacteriocins use TonB-dependent transporters, composed of three IM proteins (TonB, ExbB, and ExbD).

The Tol-Pal (peptidoglycan-associated lipoprotein) assembly is fundamentally important as it coordinates separation of daughter cells and helps to maintain OM integrity by facilitating accumulation of peptidoglycan (PG)-binding lipoprotein Pal at division sites (Webby et al. 2022). It is noteworthy to mention, that the name Tol (tolerance) derives from experiments where mutations in *tol-pal* genes engendered *E. coli* tolerance towards colicins and bacteriophages (Nagel de Zwaig & Luria 1967).

Another periplasmic energy transducing system TonB is defined as a molecular motor that is specific to gram-negative bacteria. It is primary responsible for generating energy required for nutrient transport through OM to periplasmic space (Celia et al. 2020). Ton ensures conveying ferric siderophores, heme, or Fe^{3+} as well as vitamin B12, nickel complexes, and carbohydrates (Krewulak & Vogel 2011). The TonB system was first characterized when analyzing *E. coli* B cells resistance to phage T1, hence the ton name is derived from T-one (Weidel 1958).

1.3.3.3. Microcins

Historically, the name microcins has been attributed to ribosomally-produced antimicrobial peptides from *Enterobacteriaceae*, mainly *E. coli* (Arnison et al. 2013). Microcins are low-molecular-weight (up to 10 kDa), and mainly differ from modular bacteriocins by their resistance to temperature, extreme pH, and proteases. They are chromosomally- or plasmid-encoded and in many cases undergo post-translational modifications (Bosák et al. 2021). Microcins have a relatively broad spectrum of activity, showing intra-species activity. For instance, microcin J25 from *E. coli* is broadly active against important human foodborne infectious agents, including pathogenic *E. coli* and *Salmonella* isolates (Vincent et al. 2004). While *Enterobacteriaceae* is prominent producer of microcins (so far, there are 15 characterized microcin types (Parker & Davies 2022)), pseudomonads are not equipped with elaborate tools for post-translational modification of ribosomally synthesized peptides (Arnison et al. 2013). However, there are reports of microcin activities from environmental *Pseudomonas,* such as *P. syringae* (Metelev et al. 2013) and *P. antarctica* (Lee et al. 2017)

1.3.3.4. Phage tail-like bacteriocins (PTLBs)

The first phage tail-like bacteriocins (PTLBs) were identified in *P. aeruginosa* in 1954 (Jacob 1954). PTLBs are very large (2×10^6–1×10^7 Da) protein structures that resemble, and are evolutionarily related to, the tail

structures of various bacteriophages (Scholl 2017). These high-molecular-weight bactericidal protein particles consist of eight to fourteen different polypeptide subunits. They can be grouped into two major classes:

- the R-type PTLBs that are related to contractile *Myoviridae* phage tails.
- the F-type PTLBs, that are related to non-contractile *Siphoviridae* phage tails.

PTLBs are functional stand-alone units, missing an accompanying phage head structure (Patz et al. 2019). They function to kill competing bacteria and firstly bind to target cell via receptor-binding proteins. The death of competitor cell is achieved by disrupting membrane gradients (Scholl 2017).

1.3.3.5. Lectin-like bacteriocins – reinforcement to antibacterial armamentarium from *Pseudomonas*

An interesting addition to protein antibiotics is lectin-like bacteriocins. The absence of enzymatic or pore-forming toxin-immunity pair characteristic for modular bacteriocins suggests novel mode of killing – without bacteriocin import (Ghequire et al. 2018c).The first one identified was $LlpA_{BW}$ (Putidacin A) from rhizosphere isolate *Pseudomonas putida* BW11M1 (Parret et al. 2003) and it represents *Pseudomonas*-specific antibacterial proteins not related to any known bacteriocin (Ghequire et al. 2013). Llps exhibit remarkable similarity with a family of mannose-binding lectins from monocotyledonous plants (McCaughey et al. 2014). The sequence similarities of Llps to these lectins are localized in monocotyledonous plants mannose-binding lectin (MMBL) domains. So far, there have been identified two subclasses of lectin-like bacteriocins (Llps):

- LlpAs are composed of a tandem of B-lectin (Pfam PF01453) domains followed by short carboxy-terminal extension (Ghequire et al. 2013). The carboxy-terminal lectin domain selectively interacts with D-rhamnose, which is present in a common polysaccharide antigen (CPA) in the lipopolysaccharide (LPS) layer in *Pseudomonas* cells (Lam et al. 2011). The amino-terminal domain binds to outer membrane protein insertase BamA (Ghequire et al. 2018d). BamA protein is an essential component in so called β-barrel assembly machinery (BAM), that is responsible for the integration of new proteins into the outer membrane (Noinaj et al. 2015). The killing mechanism of LlpAs remains unclear.

- LlpBs consist of a single B-lectin domain and a short carboxy-terminal extension. *llpB* genes are absent in *P. aeruginosa* genomes and are found mainly in plant- and soil-associated *Pseudomonas* isolates (*P. libanensis, P. psychrophile, P. putida, P. syringae, P. viridiflava*) . Interestingly, no strain was isolated that harbors both *llpA* and *llpB* genes (Ghequire & De Mot 2019). The killing mechanism behind LlpBs remains also elusive.

1.3.4. Pyocins – weapons from *Pseudomonas*

François Jacob was the first one to identify bacteriocin from *P. aeruginosa* (Jacob 1954). By analogy with colicins, bacteriocin was named pyocin – after the species name part of *P. pyocyanea* (former name of *P. aeruginosa*). Pyocins are produced by 90% of strains and most of them produce more than one pyocin (Michel-Briand & Baysse 2002). While most bacteria encode their bacteriocins on plasmids, *Pseudomonas* bacteriocins are chromosomally encoded (Ghequire & De Mot 2014). There are four types of pyocins: modular (S-type), phage tail-like (R- and F-type), microcins, and lectin-like. S-type bacteriocins are soluble, protease sensitive and are encoded by a single gene (usually in tandem with self-immunity gene), whereas phage-tail-like genes are organized in clusters, resulting in very large protein structures (2×10^6–1×10^7 Da) (Scholl 2017). All to my knowledge currently characterized pyocins (excluding microcin type) are presented in **Table 4**.

Table 4. Characteristics of *Pseudomonas* pyocins, adapted from (Atanaskovic et al. 2020; Ghequire & De Mot 2014; Ghequire et al. 2017a, 2018c,a; Ghequire & De Mot 2019; McCaughey et al. 2016a; Scholl 2017).

Pyocin	Species	Strain	Mode of action	Size, AA	Cytotoxic domain(s)	Receptor(s)
Modular bacteriocins						
PyoAP41	*P. aeruginosa*	PAF41-2	DNase (HNH)	777	PF12639	FhaC?
PaeM1		JJ692	Lipid II degradation	289	PF14859	FiuA
PaeM4		BL01	Lipid II degradation	342	PF14859	HxuC
PyoS1		NIH-H	DNase (HNH)	618	PF12639	?
PyoS2		PAO1	DNase (HNH)	689	PF12639	FpvAI
PyoS3		P12	DNase	766	PF06958	FpvAII
PyoS4		PAO1	tRNase	764	PF12106	FpvAI
PyoS5		PAO1	Pore-forming	498	PF01024	FptA, CPA
PyoS6		CF-PA39	rRNase	571	PF09000	?
PyoS7		BWHPSA018	rRNase	642	PF09000	?
PyoS8		VRFPA07	DNase (HNH)	772	PF12639	?
PyoS9		BL04	DNase (HNH)	421	PF12639	?
PyoS10		PABL056	DNase	517	PF06958	?
PyoS11		LESB58	tRNase	662	PF11429	?
PyoS12		PA7	tRNase	740	PF11429	?
PyoSD1 (S13)		PA62	tRNase	591	PF11429	?
PyoSD2		MSH10	tRNase	662	PF11429	?
PyoSD3		PA7	tRNase	740	PF11429	?
PyoG		PAO1	Nuclease**	640	PF06958	Hur

Pyocin	Species	Strain	Mode of action	Size, AA	Cytotoxic domain(s)	Receptor(s)
PmnH	*P. synxantha*	BG33R	Lipid II degradation	462	PF14859, PF01024	FiuA
PflM	*P. fluorescens*	Q8r1-96	Lipid II degradation	271	PF14859	FiuA
SyrM	*P. syringae*	DC3000	Lipid II degradation	276	PF14859	FiuA
Phage-tail-like bacteriocins						
R-type	*P. aeruginosa*	PAO1	Membrane potential dissipation	***	-	LPS
F-type	*P. aeruginosa*	PAO1	Membrane potential dissipation	***	-	LPS
Lectin-like bacteriocins						
PyoL1	*P. aeruginosa*	C1433	stress-activated killing system[1]	256	PF01453	CPA and BamA
PyoL1		62		256		
PyoL1		BWHPSA007		269		
$LlpA_{BW11M1}$	*P. mosselii*	BW11M1		276		
LlpA1	*P. protegens*	Pf-5		280		
$LlpA_{Pss642}$	*P. syringae*	642		290		
$LlpB_{PfluA506}$	*P. fluorescens*	A506		185		CPA
$LlpB_{PspUW4}$	*Pseudomonas* sp.	UW4		193		

[1] – the actual mechanism is not known; ? – the actual receptor is not known; ** – the specific mode of action not determined; *** – 2×10^6–1×10^7 Da.

1.4. Models and mechanisms for modular bacteriocin import into bacteria

The cell envelope of gram-negative bacteria is comprised of outer membrane, inner membrane, and the intervening periplasm. The OM provides an impermeable barrier to the environment, thus, various substances, including large (20-80 kDa) modular bacteriocins, must find a route across OM. What is fascinating is that these very large toxins are exploiting relatively small OM β-barrel proteins for their import. Years of work combining crystallography together with isothermal titration calorimetry, confocal fluorescence microscopy, and *in vivo* cross-linking has shed some light on this process (Guérin & Buchanan 2021). **Table 5** presents all known structures of modular bacteriocins from various bacterial species. Although translocation of modular bacteriocins across outer membrane (their associations with OM receptors, translocator proteins and components of the Tol or Ton systems) is well understood, but how these toxins (nucleases) cross the cytoplasmic membrane is unknown (Atanaskovic et al. 2022), since no bacteriocin has yet been trapped and structurally characterized within its IM translocon (Housden et al. 2021). The research in revealing import mechanisms is of great importance for the design of new protein antibiotics.

Table 5. Reported crystal structures of modular bacteriocins.

Bacteriocin	Mode of action	Organism	PDB identifier	Source
Pyocin M	Lipid-II degradation	*P. aeruginosa*	4G75	(Barreteau et al. 2012b)
Pyocin AP41	DNase	*P. aeruginosa*	4UHQ	(Joshi et al. 2015)
Pyocin S2*	DNase	*P. aeruginosa*	5ODW	(White et al. 2017)
Pyocin S5	Pore-forming	*P. aeruginosa*	6THK	(Behrens et al. 2020)
Pyocin S8*	DNase	*P. aeruginosa*	6W0V	(Turano et al. 2020)
Klebicin C	rRNase	*K. pneumoniae*	7NNA	(Housden et al. 2021)
Colicin M	Lipid-II degradation	*E. coli*	2XMX	(Helbig et al. 2011a)
Colicin A*	Pore-forming	*E. coli*	1COL	(Parker et al. 1992)

Bacteriocin	Mode of action	Organism	PDB identifier	Source
Colicin B*	Pore-forming	*E. coli*	1RH1	(Hilsenbeck et al. 2004)
Colicin S4	Pore-forming	*E. coli*	3FEW	(Arnold et al. 2009)
Colicin Ia	Pore-forming	*E. coli*	1CII	(Wiener et al. 1997)
Colicin N	Pore-forming	*E. coli*	1A87	(Vetter et al. 1998)
Colicin D*	tRNase	*E. coli*	1V74	(Graille et al. 2004)
Colicin E1*	Pore-forming	*E. coli*	2I88	(Elkins et al. 1997)
Colicin E2*	DNase	*E. coli*	3U43	(Wojdyla et al. 2012)
Colicin E3	RNase	*E. coli*	1JCH	(Soelaiman et al. 2001)
Colicin E5*	tRNase	*E. coli*	2DJH	(Yajima et al. 2006)
Colicin E7*	DNase	*E. coli*	7CEI	(Ko et al. 1999)
Colicin E9	DNase	*E. coli*	5EW5	(Klein et al. 2016)
Syringacin M	Lipid-II degradation	*P. syringae*	4FZL	(Grinter et al. 2012)
Pectocin M2	Lipid-II degradation	*P. brasiliense*	4N58	(Grinter et al. 2014)

* – Cytotoxic domain

1.4.1. Model for pore-forming pyocin S5 import into *Pseudomonas aeruginosa*

Pyocin S5 (PyoS5, NCBI reference sequence WP_003115311) belongs to S-type modular bacteriocin class and delivers its cytotoxic pore-forming domain across the outer membrane to depolarize the cell and kill it. Immunity to PyoS5-producing cells is achieved by a small membrane-localized immunity protein ImmS5 (Ling et al. 2010).

The structure shows that PyoS5 is an elongated (36 Å on the short axis and 195 Å on the long axis), α-helical protein that contains 17 helices (**Figure 9A**). High number of helices may suggest the need to forcibly unfold during the transport through the cell envelope, as lower energy is needed to unfold α-helices than β-sheets (Brockwell et al. 2005). The structure of PyoS5 consists

of three ordered domains. The translocation domain (T, red) consists of residues 40-194, while receptor domain (R, grey) comprises residues 195-314. The core structural motif for both translocation and receptor domains is kinked three-helix bundle (kTHB) and both these domains are structurally similar to each other, but do not share high sequence identity (~12%) (Behrens et al. 2020). The carboxy-terminal cytotoxic domain (C, black) includes residues 315-498 and uses 10-helix bundle fold that is common for pore-forming colicins (Cascales et al. 2007)

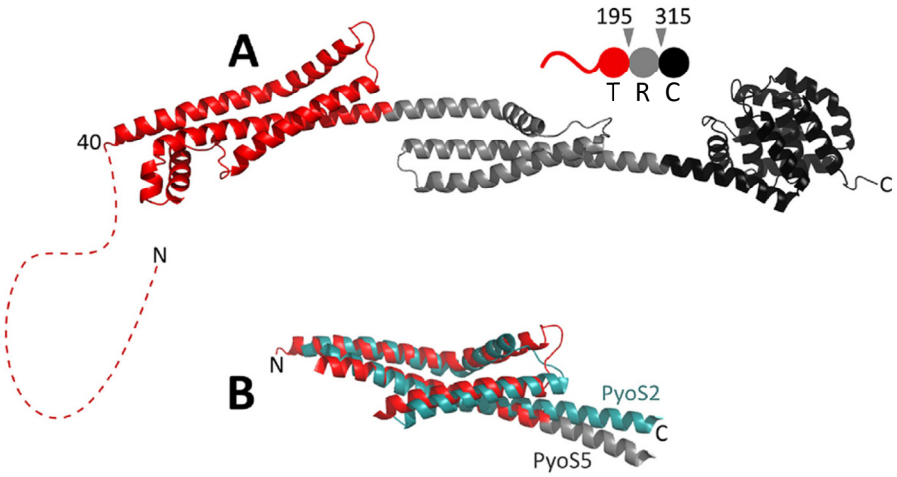


Figure 9. Crystal structure of PyoS5, PDB identifier 6THK, residues 40 to 505, adapted from (Behrens et al. 2020). **(A)** The translocation domain (T) is in red (residues 40-194), receptor domain (R) is in grey (residues 195-314), and the cytotoxic pore-forming domain (C) is in black (residues 315-498). Residues 2-39 are not resolved and represent IUTD (intrinsically unstructured T domain). **(B)** Structural alignment of PyoS5 kTHB motif-containing domains (red and gray) with that from PyoS2 (teal).

Not long ago, the structure of N-terminal domain of nuclease pyocin S2 (PyoS2) bound to outer membrane protein FpvAI was reported (White et al. 2017). The structural superposition shows that kTHB motif of receptor domain of PyoS5 is structurally similar to its counterpart in PyoS2 (**Figure 9B**). Moreover, sequence similarity of kTHB motif-containing domains to domains of pyocins S1, SD1, SD2, S3, SD3, and S4 may propose that kTHB-motif is common among pyocins (Behrens et al. 2020).

S-type pyocins generally hijack TonB-dependent receptors by a “Trojan Horse” stratagem, mimicking the iron–siderophore complexes, to secure entry into target cells. To date, four outer membrane receptors for pyocins entry

have been identified – the type I ferripyoverdine transporter FpvAI for PyoS2 (White et al. 2017), the type II ferripyoverdine transporter FpvAII for PyoS3 (Baysse et al. 1999), ferripyochelin transporter FptA for Pyocin S5 (Behrens et al. 2020) and HxuC heme receptor for Pyocin M4 (Ghequire & Öztürk 2018). The outer membrane receptors FpvAI, FpvAII, FptA, HxuC facilitate the binding and transport of iron-bound siderophores into the periplasm (Cornelis & Dingemans 2013).

Figure 10 presents unifying model for TonB-dependent pyocin import based on data for PyoS5 (Behrens et al. 2020) and PyoS2 (White et al. 2017). Initially, PyoS5 binding to CPA helps to orient pyocin horizontally with respect to the membrane plane. This initial surface association aids N-terminal IUTD to find its transporter (in this case FptA) and allows PyoS5 reorientation in such manner that N-terminal kTHB domain can engage the FptA transporter. The described mechanism is called "fishing pole" model and has been proposed for modular bacteriocins from *E. coli* as well (Zakharov et al. 2004). After "bait is catched", TonB channel allows IUTD to thread through FptA pore and interact with translocation machinery. This interaction results in energized TonB complex (together with ExbB-ExbD) that generates proton-motive force (PMF) to pull pyocin through transporter into periplasm (Behrens et al. 2020).

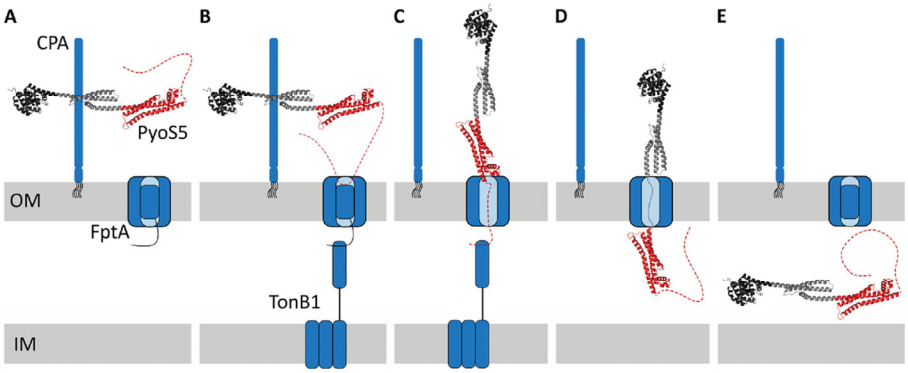


Figure 10. Model of Pyocin S5 import, adapted from (Behrens et al. 2020). **(A)** PyoS5 binds to CPA through receptor domain. **(B)** At first, outer membrane translocator FptA is contacted by IUTD and then bound by translocation domain of PyoS5. **(C)** Interactions between FptA and TonB1 possibly remove FptA plug domain, allowing the IUTD to thread through the receptor and reach the periplasm. When in periplasm, IUTD of PyoS5 binds to TonB1 through the Ton-box motif. The formation of the PyoS5-TonB1complex energizes Ton system. **(D)** Energy generated by the Ton system promotes transport of PyoS5 through the outer membrane. **(E)** Ultimately, PyoS5 is translocated into periplasm.

1.4.1.1. Pore-forming mechanism

Currently, in contrast to pore-forming colicins, there is no pore-forming mechanism proposed for pyocins. However, killing domain of PyoS5 shares 46% homology with that of pore-forming colicin Ia (Parret & De Mot 2000), so it is logical to hypothesize that PyoS5 is forming pores in similar manner to colicins, a model system for studying the process of pore-formation (Prieto & Lazaridis 2011).

Pore-forming proteins (PFPs) are produced by many pathogenic bacteria and can be classified into two groups according to secondary structure of their membrane-spanning elements – α-helices (α-PFPs) or β-barrels (β-PFPs). All PFPs are water-soluble monomers; however, these proteins share several unique strategies enabling them to undergo unexpected metamorphosis from a soluble protein to a transmembrane protein (Peraro & Van Der Goot 2016). This metamorphosis in colicins (belonging to α-PFP class) is camouflaged in a three-layer bundle of 10 α-helices of pore-forming domain (PFD) (**Figure 11A**). The hydrophobic segment formed by helices 8 and 9 is positioned in the central layer, therefore, sequestered from aqueous environment by the other eight helices. This "inside-out membrane protein fold" (hidden hydrophobic core in the PFD) makes ten-helix domain water-soluble (Lakey & Slatin 2001). Despite the lack of data of exact structure and stoichiometry of these pores, hydrophobic helical hairpin (formed by helices

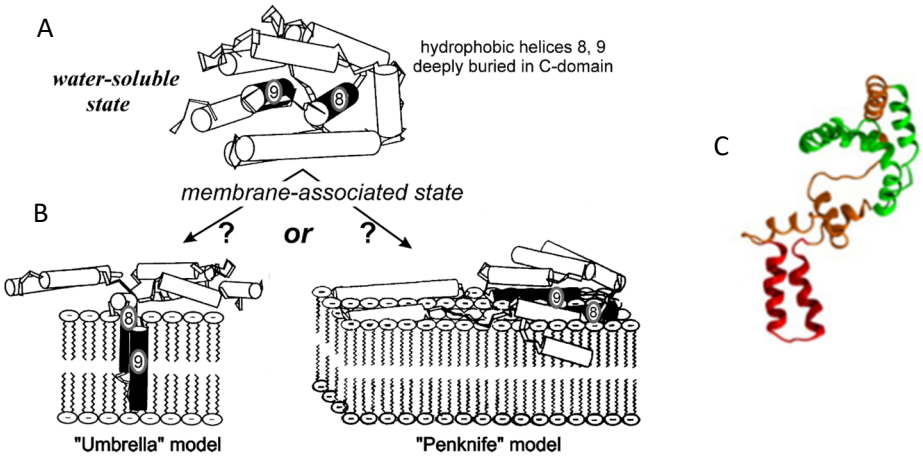


Figure 11. (A), (B) The "umbrella" and the "penknife" models for membrane-bound state of colicin A cytotoxic domain, adapted from (Möbius et al. 2005). **(C)** The unwound cytotoxic domain of colicin Ia, adapted from (Rajapaksha et al. 2015). Hydrophobic helices 8 and 9 are shown in red, membrane penetrating helices 2–5 – in green, other helices – in orange.

8 and 9; **Figure 11C**) is thought to act as anchor by inserting itself into the inner membrane and producing non-specific voltage-gated channel that leads to membrane depolarization and ultimately – cell death (Peraro & Van Der Goot 2016). Two main models have been offered for the membrane-bound state: penknife and umbrella (Bermejo et al. 2013) (**Figure 11B**). The main difference between them is orientation of the hydrophobic hairpin with respect to the plane of membrane – perpendicular ("umbrella") or nearly parallel ("penknife"). Again, there is no unified model confirmed, mostly because these pores are not able to form stable oligomeric structures that are amenable for structural analysis (Iacovache et al. 2010). However, there are data favoring "umbrella" model (Pulagam & Steinhoff 2013), "penknife" model (Massotte et al. 1993) as well as data suggesting both models exist in equilibrium (Prieto & Lazaridis 2011).

Be membrane-bound state model as it may, but the fact of pore-formation in IM is undisputable. **Figure 12** proposes model for colicin Ia channel open-close events and water filled pore formation pathway (Rajapaksha et al. 2015):

a) Pore-forming domain gets absorbed on the lipid bilayer.
b) Hydrophobic helices 8 and 9 insert themselves into the lipid bilayer.
c), d) Charged residues of colicin Ia are driven by external transmembrane electric field and induce a distortion in the lipid bilayer. Water starts to form conical intrusions.
e) Water intrusions outgrow large hydrophilic pores, therefore allowing charged colicin Ia residues to translocate.
f) Ion channel is formed, having four helices in the lipid bilayer and the helices 2-5 (green) in the trans side of the lipid bilayer.

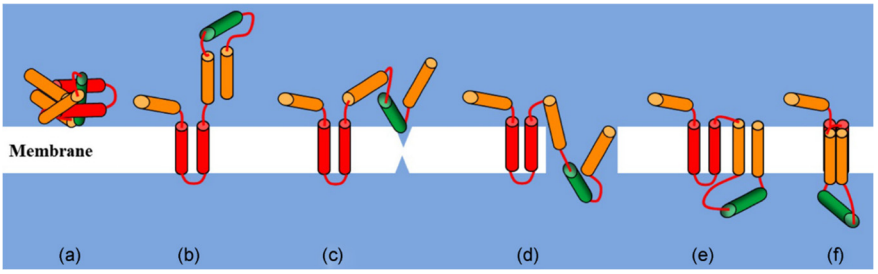


Figure 12. The model for the channel formation by colicin Ia, adapted from (Rajapaksha et al. 2015). **a** – adsorption onto the lipid bilayer, **b** – colicin Ia in "umbrella" conformation, hydrophobic hairpin (red, helices 8, 9) inserts into the lipid bilayer, **c-d** – formation of water wire and translocation of the helices 2-5 (green) across the lipid bilayer, **e** – membrane is spontaneously repaired after translocation of helices 2-5 and helices 1, 6, 7, 8, 9 subsequently insert into the lipid bilayer, **f** – formation and activation of ion channel.

1.4.2. Import and mode of action of lipid-II degrading colicin M

Unfortunately, mechanisms of lipid-II degrading pyocins (together with pore-forming pyocins) import into *Pseudomonas* are yet to be revealed. But luckily, lipid-II degrading bacteriocins from various species (*Burkholderia, Escherichia, Klebsiella, Pectobacterium, Pseudomonas, Stenotrophomonas*) share high sequence similarity, especially in C-terminus toxicity domain (Ghequire et al. 2018b). The first identified bacteriocin of this subclass was from *E. coli* – colicin M (Fredericq 1951), and now, correspondingly, all lipid-II degrading bacteriocins from other bacteria species are often called colicin M-type (ColM-type) bacteriocins. ColM and ColM-type bacteriocins develop their lethal activity in bacteria by inhibiting peptidoglycan biosynthesis through hydrolysis of the last peptidoglycan precursor, lipid II, into two dead-end products, resulting in cell lysis (Chérier et al. 2021).

Figure 13 presents import of ColM and its enzymatic disruption of peptidoglycan precursor. Initially, ColM hijacks the FhuA iron receptor and translocates through the outer membrane. As for class B bacteriocin, the energy needed for OM translocation is generated by both the TonB machinery and the proton motive force of the inner membrane (Cascales et al. 2007). There is still no bulletproof evidence, but data from several research groups let us hypothesize with confidence that ColM is unfolded during translocation process (Helbig et al. 2011b; Hullmann et al. 2008). Once in the periplasm, ColM interacts with chaperone FkpA – protein assisting in folding and refolding of outer membrane proteins as well as preventing aggregation of misfolded periplasmic derivatives (Stull et al. 2018). FkpA extends its responsibilities as chaperone, as it not only refolds imported ColM, but is also essential for the activity of imported ColM (Hullmann et al. 2008).

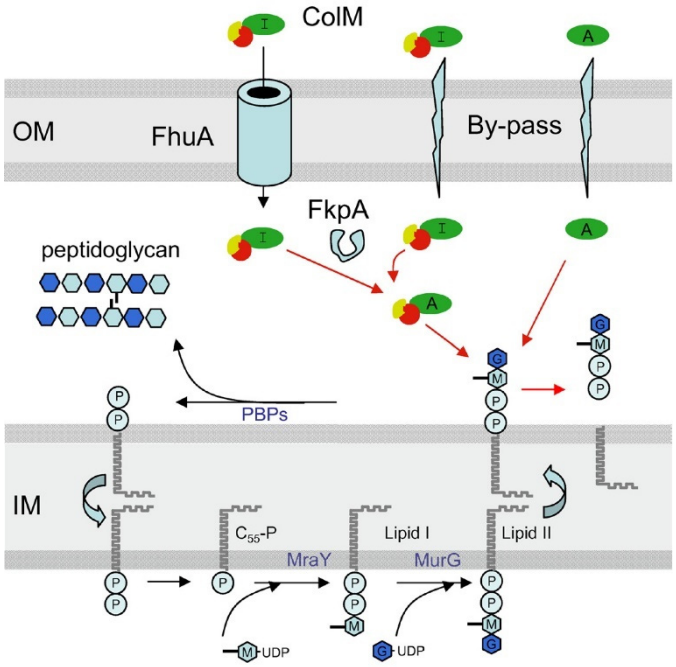


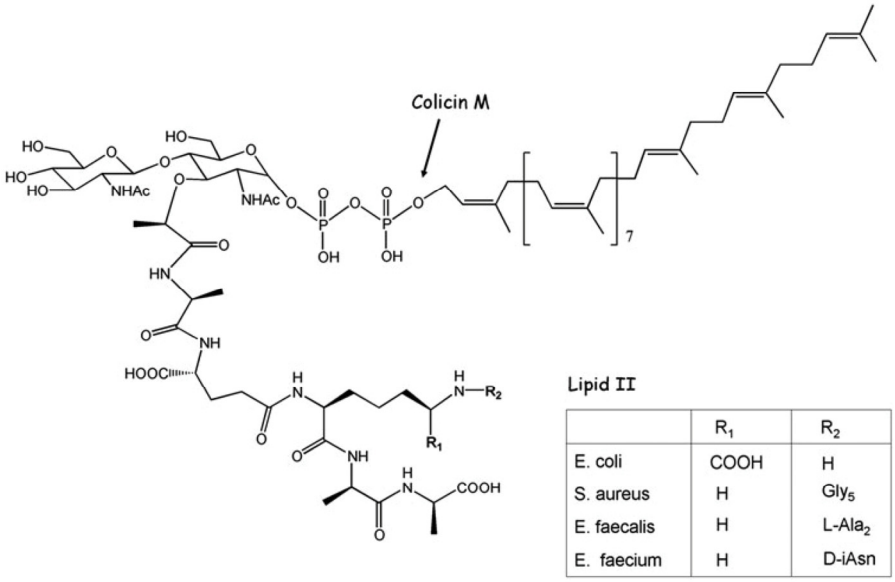
Figure 13. Synthesis of peptidoglycan lipid-linked intermediates and ColM mode of action, adapted from (Barreteau et al. 2010). The MraY and MurG catalyzed reactions yield in lipid II, which is translocated into periplasmic place. Thereafter, disaccharide-peptide is polymerized and incorporated into the growing peptidoglycan. Intruding ColM cleaves lipid II intermediate thus leading to PG synthesis arrest. The receptor, translocation and cytotoxic domains of ColM are represented in red, yellow and green, respectively. ColM reaches periplasm through FhuA receptor and periplasmic FkpA changes ColM from inactive (I) to active confirmation (A). Full ColM delivered to periplasm through osmotic shock ("by-pass") also is activated by FkpA, but "by-passed" cytotoxic domain is active without FkpA help.

Peptidoglycan (murein) is an essential component of almost all bacteria with its main function to preserve cell integrity by withstanding the turgor (Vollmer et al. 2008). The glycan strands are made up of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues linked in β-1,4 bonds. Moreover, linear glycan chains are cross-linked by short peptides (Chérier et al. 2021). PG synthesis is complex three-stage process, which takes place (Blanot & Mengin-lecreulx 2017):

1. In cytoplasm – nucleotide precursors are synthesized;
2. In plasma membrane – lipid intermediates (lipid-linked precursors, lipid I and lipid II) are synthesized and lipid II is translocated to the outer side of the membrane;

3. In the extracytoplasmic space – PG polymerization and maturation reactions.

Undecaprenyl phosphate (C55-P) is a key lipid involved in plasma membrane step forming lipid-linked precursors –lipid I (C55-PP-MurNAc-pentapeptide) and lipid II (C55-PP-MurNAc(-pentapeptide)-GlcNAc). After lipid II is translocated toward the outer leaflet of the inner membrane, PG building block (disaccharide peptide) moiety is incorporated into the growing polymer by the PBPs (Penicillin Binding Proteins) and the lipid carrier is recycled into a pyrophosphorylated form (C55-PP) (Chérier et al. 2021). In the scenario when ColM has intruded periplasmic space, lipid II is hydrolyzed (**Figure 14**) to 1-PP-MurNAc(-pentapeptide)-GlcNAc and unrecyclable undecaprenol (C55-OH). That leads to eventual arrest of this essential cell-wall polymer synthesis and ultimately cell lysis.



	R_1	R_2
E. coli	COOH	H
S. aureus	H	Gly_5
E. faecalis	H	L-Ala_2
E. faecium	H	D-iAsn

Figure 14. Chemical structure of lipid II, adapted from (Barreteau et al. 2012a). R1 and R2 moieties vary among bacterial species.

1.5. Applications of Bacteriocins

1.5.1. Bacteriocins from gram-positive bacteria

As it was mentioned before, the vast amount of research has been done on bacteriocins produced by lactic acid bacteria, mostly because to their U.S. Food & Drug Administration (FDA) granted GRAS (Generally Recognized as Safe) status and immediate use in food industries (Juturu & Wu 2018). The first identification of antibacterial substance from dairy starter culture bacterium *Lactococcus lactis* subsp. *lactis* was in 1920s (Rogers & Whittier 1928; Whitehead 1933). Later, this substance was named Nisin (Group N *Streptococcus* Inhibitory Substance IN) (Mattick et al. 1947). Nisin belongs to class I (lantibiotic) bacteriocin and is composed of 34 amino acid-long cyclic polypeptide chain with a molecular weight of 3354 Da (Ng et al. 2020). Nisin is the most prominent bacteriocin, as it was the first one to get an approval from US FDA in 1988 (for its application in cheese industry) and is currently used in more than 50 countries (Juturu & Wu 2018). However, more than 30 years later, only two more bacteriocins – pediocin, and Micocin® (the combination of carnocyclin A, carnobacteriocin BM1, and piscicolin 126 bacteriocins) – both from gram-positive bacteria – were approved by FDA for use as food preservatives and anti-spoilage agents, and are commercially available in the US and Canada (Naskar & Kim 2021).

There is an upward trend for investment in bacteriocin research for potential applications in food, medicines and livestock (Ng et al. 2020), but all examples used in practice come almost solely with bacteriocins produced by gram-positive bacteria. Bacteriocins used in food preservation include nisin, enterocin, pediocin, leucocin, lactoccocin, carnocyclin, carnobacteriocin, piscicolin, sukacin, aureocin, mycocin, bacteriocin 7393A, bacteriocin 7293B and bacteriocin CAMT2 (Ng et al. 2020). Also, bacteriocins have various biomedical applications and are used in catheter coatings, oral tablets, chewing gum, aquaculture dry sprays, hydrogels scaffolds and food packaging (Naskar & Kim 2021). Moreover, numerous studies have been carried out for bacteriocins concerning human health problems, such as urinary tract infection, skin infection, diarrhea, dental carries, lung infection, bloodstream infection, mastitis, respiratory tract infection and cancer. Following bacteriocins are being applied in the infectious disease treatment for humans: nisin, lacticin, salivaricin, subtilosin, mersacidin, enterocin, gallidermin, epidermin, and fermencin (Ng et al. 2020). The example list of bacteriocins employed in livestock is shorter and encompasses nisin, lacticin, garvicin, and macedocin (Ng et al. 2020).

From all this huge pool of bacteriocins and their diverse potential applications, to date, only few from lantibiotic class were able to progress into clinical trials – microbisporicin (NAI- 107, Naicons SRL and Sentinella Pharmaceuticals), mutacin 1140 (MU1140 Oragenics, United States) and duramycin (Moli1901, AOP Orphan Pharmaceuticals and Lantibio) (Soltani et al. 2022).

1.5.2. Bacteriocins from gram-negative bacteria

As it was mentioned before, gram-negative bacteria produce four classes of bacteriocins – modular bacteriocins, phage tail-like bacteriocins, lectin-like bacteriocins and microcins. All representatives have demonstrated their antimicrobial *in vitro* potential against both planktonic state and bacterial formed biofilms (Ghequire et al. 2018d; Redero et al. 2020; Rendueles et al. 2014; Smith et al. 2012; Zihler et al. 2009) Since the ultimate goal is to use bacteriocins as therapeutic agents in treating various infections in humans and animals, the **Table 6** summarizes current results available from *in vivo* models of infection to date. However, currently there is no data from lectin-like bacteriocins, as they have yet to be tested *in vivo.*

Table 6. Effectiveness of gram-negative-produced-protein antibiotics in *in vivo* challenge models, adapted from (Behrens et al. 2017)

Model host	Protein	Infection type	Treatment route	Challenge organism	Source
Modular bacteriocins					
Mouse	Pyocin S2	Lung infection	IN	*P. aeruginosa*	(McCaughey et al. 2016b)
	Pyocin AP41				
	Pyocin S5				
	Pyocin L1				
Mouse	Pyocin SD2	Lung infection	IN	*P. aeruginosa*	(McCaughey et al. 2016a)
Mouse	Pyocin S5	Sepsis	IV	*P. aeruginosa*	(Six et al. 2021)
	Pyocin AP41				
Mouse	Pyocin S5-PmnH	Keratitis	Topical	*P. aeruginosa*	(This study)
		Lung infection	IN		
Mouse	Lysocin PyS2-GN4	Bacteremia	IP	*P. aeruginosa*	(Heselpoth et al. 2019)
Mouse	Klebicin KvarIa	Gastrointestinal tract infection	Oral	*Klebsiella quasipneumoniae*	(Karaliute et al. 2022)
Mouse	Combination of colicin E9 and Ia	Gastrointestinal tract infection	Oral	*E. coli*	(Carpena et al. 2021)
Pig	Colicin E1	Postweaning Diarrhea	Oral	*E. coli*	(Cutler et al. 2007)
Phage-tail-like bacteriocins					
Mouse	Pyocin R2	Wound infection	Topical	*P. aeruginosa*	(Alqahtani et al. 2021)
Mouse	Pyocin R1	Lung infection	IN	*P. aeruginosa*	(Redero et al. 2020)

Model host	Protein	Infection type	Treatment route	Challenge organism	Source
Mouse	Pyocin R2	Peritonitis	IP, IV	*P. aeruginosa*	(Scholl & Martin 2008)
Rabbit	AvR2-V10.3 (engineered R-type pyocin)	Diarrhea and intestinal inflammation	Orogastrically	*E. coli*	(Ritchie et al. 2011)
Mouse	Enterocoliticin	Gastrointestinal tract infection	Oral	*Yersinia enterocolitica*	(Damasko et al. 2005)
Microcins					
Mouse	microcin J25 (MccJ25)	Enteritis (small intestine infection)	IP	*E. coli*	(Yu et al. 2020)
Broiler Chicken	microcin J25 (MccJ25)	Gastrointestinal tract infection	Orally	*E. coli* and *Salmonella*	(Wang et al. 2020b)
Mouse	microcin J25 (MccJ25)	Peritonitis	IP	*Salmonella* Newport	(Lopez et al. 2007)
Unknown and unidentified bacteriocins					
Mouse	Unknown Pyocin78-C2	Sepsis	IV	*P. aeruginosa*	(Merrikin & Terry 1972)
Mouse	Unknown pyocin P10	Peritonitis	IP	*P. aeruginosa*	(Haas et al. 1974)

IV – intravenous; IP – intraperitoneal; IN – intranasal.

The first patent for bacteriocin colicin E1 was filed as early as 1990 (**Table 7**). Many patents have since been granted for infection treatment applications with modular bacteriocins. However, currently the closest to commercialization are US FDA GRAS granted colicins (GRN No. 593, GRN No. 676, GRN No. 775) and salmocins (GRN No. 824) for application on fruits, vegetables, egg products and meat.

Table 7. List of patents for controlling gram-negative pathogens.

Patent	Filed	Title	Inventor	Applicant
22163290.4 (pat. pending)	2022	Chimeric bacteriocins and method for the control of *Pseudomonas*	Š. Paškevičius, A. Misiunas, A. Razanskiene	Nomad Bioscience Gmbh, UAB Nomads
EP3980442A1	2020	Klebicins for the control of *Klebsiella*	E. Denkovskiene, A. Misiunas, A. Razanskiene	Nomad Bioscience Gmbh, UAB Nomads
WO2021053242A3	2020	Bacteriocins for control of *Salmonella enterica*	Simone Hahn, Tobias Schneider, Anett Stephan, Steve Schulz, Anatoli, Giritch, Yuri Gleba	Nomad Bioscience Gmbh
US10201168B2	2016	Colicins for the control of EHEC	A. Giritch, S. Hahn, S. Schulz, A. Stephan, Y. Gleba, F. Jarczowski	Nomad Bioscience Gmbh
WO2016046218A1	2015	Pulmonary administration of pyocins for treating bacterial respiratory infections	D. Walker, L. McCaughey	University of Glasgow

Patent	Filed	Title	Inventor	Applicant
US9139622B2	2013	*Citrobacter freundii* antibacterial agents and their use	R. M. Q. Shanks, D. E. Kadouri	University of Pittsburgh
US20150164984A1	2013	Colicins for Treating Bacterial Infections	D. Walker, K. Smith	University of Glasgow
US20100261258A1	2010	Modified bacteriocins and methods for their use	D. M. Scholl, S. R. Williams	AvidBiotics Corp
US20080286236A1	2007	Inhibition of *Yersinia pestis*	D. M. Gebhart, D. M. Scholl	AvidBiotics Corp
US20060229244A1	2004	Engineered bacteriocins and bacteriocin combinations and methods for treating bacterial based infections	R Dorit, M. Riley	R Dorit, M. Riley
US5043176A	1990	Synergistic antimicrobial compositions	N. L. Bycroft, G.S. Byng, S. R. Good	Haarmann and Reimer Corp

1.5.2.1. Advantages and limitations

Bacteriocins have high potential to be used as the replacement for antibiotics in the future as their major advantages include low-toxicity for mammalian cells, high activity in the nanomolar range, specific mechanisms of action (Soltani et al. 2021), biodegradability, and selective killing (Ghequire & De Mot 2019). Moreover, the eligibility of some bacteriocins for large-scale production in plants was shown recently, that may encourage commercialization (Schulz et al. 2015a). At first glance, the selective killing may seem not much of an advantage, because the bacteria responsible for the infection need to be identified prior to selecting necessary active bacteriocin. But eventually microflora stays healthy and intact as only the infection-causing species, or even one strain of bacteria, is targeted and eliminated. This goes in contrast with broad-spectrum antibiotics – their administration can result in disruption of co-evolved communities that are integral to human

health and cause gut dysbiosis (Francino 2016). Moreover, the narrow killing spectrum reduces the possibility for bystander microorganisms to develop resistance (Behrens et al. 2017). On the other hand, combinatorial therapies of bacteriocins and antibiotics can broaden antimicrobial spectra, enhance inhibition or killing activities and/or result in antibiotic dose reduction.

However, besides many advantages, bacteriocins exhibit some limitations. The primary issue is shared together with antibiotics – bacteria can develop resistance against bacteriocins (de Freire Bastos et al. 2015). Secondly, the pharmacokinetic parameters (e.g., bioavailability, stability, solubility in physiological conditions, and susceptibility to enzymatic proteolysis) and route of administration play important role for *in vivo* efficacy of bacteriocins (Soltani et al. 2021). For instance, some bacteriocins are susceptible to enzymatic and pH degradation in the gastrointestinal tract and small intestine when administered orally. On the other hand, switching from oral administration to parenteral may offer the possibility to avoid proteolytic degradation of bacteriocins in the gastrointestinal tract (Benítez-Chao et al. 2021). Excellent data from efficacy experiments in murine models when bacteriocin is administered in such routes as intranasal, intragastric, intraperitoneal, subcutaneous, topical is encouraging (Benítez-Chao et al. 2021).

The encapsulation of bacteriocins is a potential approach to overcome these limitations and maximize the use of protein antibiotics. Film coatings, liposomes, nanofibers, and nanoparticles are the main methods proposed and utilized for protection from degradation (Chandrakasan et al. 2019)

. Nanoencapsulation (metal nanoparticles, chitosan, nanofibers, and liposomes) are of special interest, because bacteria do not develop rapid resistance against nanomaterials (Naskar et al. 2020) although, molecular mechanisms of bacterial resistance to metal and metal oxide nanoparticles have been already reported (Salas Orozco et al. 2019).

Furthermore, combinatorial therapies with bacteriocins may broaden activity spectra and reduce the concentration of antibiotics needed for successful infection treatment. Recent data from (Budiardjo et al. 2022) offer a potential roadmap for designing bacteriocins to employ them to plug the exit of antibiotics from bacteria (**Figure 15**). Also, various other encouraging results were obtained when bacteriocins were combined with other bacteriocins, antibiotics, phage lysins and other antimicrobials/stressors, such as naturally-derived plant essential oils (Mathur et al. 2017).

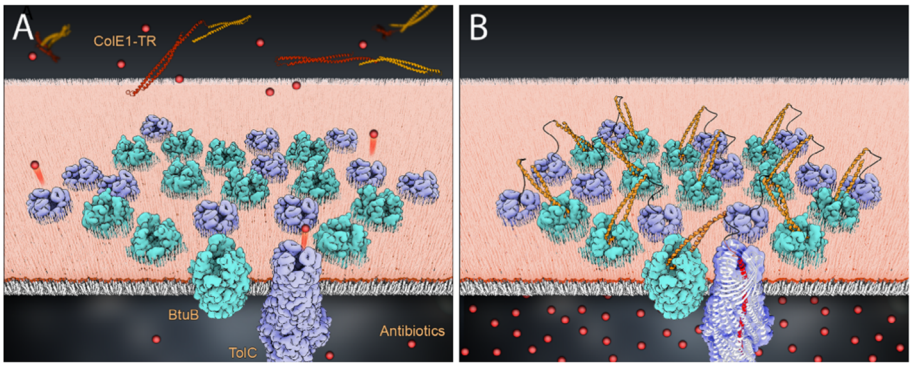


Figure 15. Model of colE1-TR inhibition of efflux, adapted from (Budiardjo et al. 2022). **(A)** Bacteriocin ColE1-TR is not bound to outer membrane and antibiotics are effluxed. **(B)** After binding, the colicin E1 fragments prevent some antibiotic efflux.

Even if we stay optimistic and do find and develop an arsenal of various antimicrobials against bacteria, this quote by Hans Zinsser from his 1935 book "Rats, Lice and History" will still be true: "Infectious disease is one of the great tragedies of living things – the struggle for existence between different forms of life. Man sees it from his own prejudiced point of view; but clams, oysters, insects, fish, flowers, tobacco, potatoes, tomatoes, fruit, shrubs, trees, have their own varieties of smallpox, measles, cancer, or tuberculosis. Incessantly, the pitiless war goes on, without quarter or armistice – a nationalism of species against species." (Khardori et al. 2020).

1.6. Plant molecular farming

Recombinant proteins are exogenous ("foreign") proteins produced in the host of choice and are mostly used for disease treatment in humans or animals. The odyssey of recombinant protein production starts in 1977 in *E. coli*, when the first functional recombinant peptide somatostatin was synthesized (Itakura et al. 1977). Few years later, in 1982, the first recombinant protein for treatment, human insulin, was commercially produced also in *E. coli*, and the list of expression systems, with its own advantages and limitations, have expanded over the years and now encompass insects, mammalian cells, whole animals, yeast/filamentous fungi and plants (Burnett & Burnett 2020). Not surprisingly, the demand for recombinant proteins is rising steadily, with a market valued at USD 800 Million in 2019 and is projected to reach USD 1,600 Million by 2027 (Mitsui & Yamada 2021).

The first successful expression of recombinant antibodies in plants (Hiatt et al. 1989) demonstrated proof-of-principle for future plant molecular

farming. Since then, plant bioproduction platforms have expanded and can be classified as (Xu et al. 2018):

- *in vitro* cell culture systems, including cell suspensions, hairy roots, moss protonema;
- aquatic plants, including duckweed and microalgae;
- whole plants using both stable and transient expressions.

Again, each bioproduction platform has its advantages and drawbacks, but generally, plant production system offers such potential advantages as low maintenance cost, easy scalability, no human pathogen contamination and ability to assemble complex proteins with eukaryotic-like post-translational modifications (Webster & Thomas 2012). The main barrier of using plants worldwide as hosts for manufacturing therapeutic proteins is the lack of established regulatory approval (Shanmugaraj et al. 2020). This may be explained by the fact that the first plant-derived protein pharmaceutical (β-glucocerebrosidase) was approved for commercial use in humans just 10 years ago (Mor 2015).

Nicotiana genus is considered as a "workhouse" of whole plant bioproduction platform due to their growth rate and easy genetic manipulation. Tobacco plants *Nicotiana benthamiana* and *Nicotiana tabacum* are routinely used for both stable and transient expression of recombinant proteins (Shanmugaraj et al. 2020). Stable expression is achieved in transgenic plants with constitutively expressed transgenes whereas transient expression is realized using *Agrobacterium tumefaciens* binary vectors: standard non-replicating vectors or replicating viral vectors (Werner et al. 2011). Although transgenic plants can produce a wide variety of proteins of interest, but it is a time-consuming and costly process, whereas transient expression offers high expression yields on a rapid timescale (**Figure 16**) (Gleba et al. 2014). Viral expression vectors generally produce higher levels of recombinant protein in comparison with common nonviral plant expression vectors (Marillonnet et al. 2004). The most prominent representatives of viral vector-based expression platforms are magnICON® (Icon Genetics), Geneware® (Kentucky BioProcessing LLC) based on a tobacco mosaic virus (TMV) and geminiviral expression system based on a bean yellow dwarf virus (BeYDV) (Arizona State University) (Xu et al. 2018).

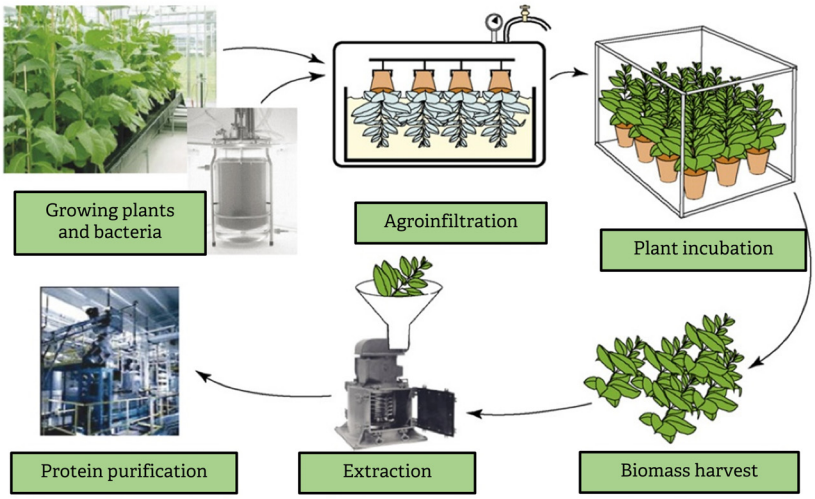


Figure 16. General scheme for transient recombinant protein production in plants, adapted from (Gleba et al. 2007).

2. MATERIALS AND METHODS

2.1. Bacterial strains and cultures

Unless otherwise stated, *P. aeruginosa* strains were prepared by culturing in Lysogeny Broth (LB) medium (Roth) or Casamino Acids (0.5% Bacto™ Casamino acids, 5.2 mM K_2HPO_4, 5 mM $MgSO_4$) medium (BD Bacto) at 37 °C under shaking conditions (200 rpm); overnight (16 hours) cultures were prepared by inoculation from frozen stocks. The bacterial strains used in this study were obtained from the following sources: Leibniz Institute DSMZ-German Collection of Microorganisms, BCCM/LMG Bacteria Collection, and ATCC Bacteriology Collection. Clinical strains have been isolated at Lithuanian University of Health Sciences, Kaunas clinics. *Stenotrophomonas* strains from soil were kindly donated by Prof. Dr. E. Sužiedėlienė (Vilnius University, Lithuania)

2.2. Construction of pyocin and stenocin expression vectors

The pyocins PaeM4, PaeM, S5, L1, L2, and L3 coding sequences (NCBI Reference Sequences ERY59288, ERZ09841.1, WP_003115311, CDG56231.1, ERX71449.1, and ERZ0 4935.1, respectively) were optimized for expression in the host plant *Nicotiana benthamiana* and synthetized by GenScript (USA) while stenocins SmaltM, SmaltM2 (NCBI Reference Sequences WP_058981958 and WP_143570807, respectively) were optimized and synthetized by by Thermofisher Scientific (USA). Sequences were inserted as *Bsa*I-*Bsa*I fragments in pICH29912 or pICH26201, assembled TMV-based magnICON® vectors. Obtained plasmids were used to transform *A. tumefaciens* GV3101.

2.3. Construction of chimeric pyocins

The open reading frames encoding for PmnH (*P. synxantha* EIK72868), Pflu095 (*P. fluorescens* WP_016979095), Pflu373 (*P. fluorescens* WP_014717373), Pflu794 (*P. fluorescens* WP_081041794), Pflu618 (*P. fluorescens* WP_034155618) and Pput259 (*P. putida* WP_098964259) optimized for expression in *N. benthamiana* were synthetized by Thermofisher Scientific (USA). Chimeric proteins were constructed as follows: N-terminal end of pyocin S5 (coding sequence of 1–310 aa containing receptor binding and translocation domains of this pyocin) was amplified with sequence-specific primers flanked by *Bsa*I recognition sites. Cytotoxic domains of all non-*P. aeruginosa* putative bacteriocins were amplified with sequence specific primers flanked with *Bsa*I recognition sites. Each killing domain fragment was paired with S5 fragment and both

fragments were inserted in *Bsa*I digested pICH29912, assembled TMV-based MagnICON vector. Obtained plasmids were used to transform *A. tumefaciens* GV3101.

2.4. Bacteriocin expression in plants

Nicotiana benthamiana plants were grown in a growth chamber at 25 °C with a 16 h light and 8 h dark photoperiod. Four-to-six-week-old plants were used for vacuum infiltration with recombinant *A. tumefaciens*. All plant experiments were performed in compliance to relevant institutional, national, and international guidelines and legislation.

Agrobacterium strains were inoculated from frozen stocks in 4 ml LB medium containing 50 µg/ml rifampicin and 50 µg/ml kanamycin and cultivated at 28 °C with shaking at 220 rpm. Overnight cultures were diluted 1:1000 starting from OD_{595} = 1.0 in tap water and supplemented with 0.05% Silwet L77 (Kurt Obermeier). *Agrobacterium* suspension was poured into a desiccator vessel, connected to a vacuum pump. The entire leaf system of a plant was then submerged into the suspension. Agroinfiltration was achieved by applying (till pressure of 200 mbar) and releasing vacuum through the pump. Plant leaves were harvested 5–6 days post agroinfiltration.

2.5. Agar disk-diffusion assay

Overnight *P. aeruginosa* cultures grown in CAA medium were equalized till OD_{595} = 1.0 in CAA and diluted 100×. Sterile cotton swab was briefly submerged in diluted microbial suspension, removing the excess of liquid by pressing it against the container wall. The swab was used for evenly streaking bacteria on plates containing growth medium with CAA solid agar (1.5%). 6 mm diameter sterile Whatman discs were placed on soft-agar and 0.3–30 µg of chimeric pyocins were spotted to paper disks. The plates were incubated overnight at 37 °C and bacteriocin inhibition zones were observed.

2.6. Antimicrobial activity evaluation in liquid culture for bacteriocins

Overnight *P. aeruginosa* cultures were diluted to OD_{595} = 0.3 in iron-deficient Casamino Acids (CAA) medium (BD Bioscience) up to 1.2 ml. Lyophilized purified bacteriocins were resuspended in CAA medium, added to diluted bacterial suspension and incubated for 5.5–6.5 hours at 37 °C with shaking (200 rpm). The antimicrobial activity of bacteriocins was evaluated by determining cell numbers of bacterial test culture. Serial dilutions of 10, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were made, plated on LB agar plates, incubated overnight at 37 °C and the CFU calculated.

2.7. Infection of *Galleria mellonella* larvae

Overnight *P. aeruginosa* strains were grown in CAA medium and diluted in 0.8% NaCl in order to achieve a concentration of ~5 x 10^4 CFU/ml. Volumes of 10 µl of *P. aeruginosa* culture (~500 CFU) and 10 µl of pyocins solution were injected into the hemocoel of fifth instar *G. mellonella* larvae (Livefood UK) in proximity of the left and/or right prolegs. Bacteriocins were injected three hours post infection with *P. aeruginosa*. Injected larvae were incubated at 37 °C in 9 cm Petri dishes without food for up to 5 days. Caterpillars were considered dead when they displayed no movement in response to mechanical stimulus to the head, leading to distinct changes in color from cream to dark brown/black. Twenty larvae were used per each treatment point.

2.8. *Ex vivo* porcine corneas as model for bacterial keratitis

Preparation of porcine corneas. Porcine eyes were acquired from the nearest slaughterhouse. Enucleated eyes were stored at -70 °C. Before the start of experiment, eyes were transferred to 4 °C for 1 h, then to room temperature for 1–2 h until the eyeballs were completely defrosted. Then eyeballs were individually placed in sterile plastic containers and submerged for 5 min in 2.5% Povidone-iodine (Betadine 100 mg/ml; EGIS Pharmaceuticals PLC), then twice washed with sterile PBS. The corneas were excised with sterile surgical blade No. #12. The excised corneas were stored in Minimum Essential Medium (MEM) supplemented with Non-Essential Amino Acids, l-Glutamine (2 mM), penicillin (200 U/ml) + spectinomycin (25 µg/ml) until further use at 4 °C up to two weeks.

Cornea infection and pyocin treatment. P. aeruginosa was grown overnight from frozen stock. Next morning, the culture was diluted 100-fold by fresh LB medium and grown until OD_{595} = ~ 0.6 (~ 6 h). Bacteria were collected by centrifugation and resuspended in PBS. The dissected corneas were placed on agarose-gelatin (0.5% each) solid support in a 6-well culture plate containing 800 µl MEM with antibiotics (100 U/ml penicillin, 25 µg/ ml spectinomycin), then three horizontal and 3 vertical scratches were made using a sterile 25-gauge needle. 3 × 10^4 CFU of *P. aeruginosa* ATCC 19660 strain or 0.4 × 10^4 CFU of PAO1 strain were applied to cornea and incubated for 16–20 h in 37 °C CO_2 incubator (20% CO_2). 5 corneas were used for each experimental point. After incubation with *P. aeruginosa*, the corneas were visually inspected for opacity. The corneas which were clear and without signs of infection were considered as non-infected and discarded from further study.

5 μg of S5-PmnH (in 5 μl of PBS) or 5 μl of PBS were applied to infected corneas and incubated for additional 16–20 h in 37 °C CO_2 incubator.

Homogenization and CFU counting. Prior the homogenization the corneas were washed 3 times for 10 min in 50 ml sterile PBS with occasional agitation. Each cornea was chopped in 4 equal parts, trying to get rid of sclera. 2 parts were placed in Precellys 24 tissue homogenizer (Bertin technologies) tubes CKMix50-7 ml and 2 ml PBS was added. Homogenization was performed at following conditions: 6500 rpm 20 s., 5 cycles, 3–5 min breaks on ice between the cycles. The obtained homogenate was transferred into 15 ml Falcon tube and briefly spun to sediment the big debris, then the supernatant recovered into new tube and centrifuged at full speed for 10 min to pellet all bacteria. Bacteria were resuspended in 200 μl sterile PBS, serially diluted and plated on LB-agar plates.

2.9. Murine keratitis model

Animals. The inbred mice of C57BL/6 strain of both sexes in equivalent numbers were used for the research. 2–6 months old, adult female and adult male mice were purchased from the Vilnius University vivarium of laboratory animals. The animals throughout the period of the experiment were given standard chow and drinking water *ad libitum*. Animals were housed in the individual plastic cages in a 12 h light/dark cycle at 21–23 °C. All regulated procedures on living animals were approved by The Lithuanian Ethics Committee of Biomedical Research (Protocol no. B1-442) and were carried out in accordance with the European Union legislation of OECD (directive 2010/63/EU). The study was carried out in compliance with the ARRIVE guidelines. The experiments were carried out in Biological Research Center of Lithuanian University of Health Sciences.

Pseudomonas aeruginosa keratitis induction and treatment. For *P. aeruginosa* infection and keratitis induction the mice were anesthetized by Ketamine and Xylazine 90:9 mg/kg intraperitoneal injection. The cornea of the left eye of each mouse was visualized under a stereoscopic microscope, and three 1 mm scratches were made using a sterile 25-gauge needle. A 10 μl aliquot containing 4×10^6 cells of *P. aeruginosa* ATCC 19660 (cytotoxic strain) or PAO1 ATCC 15692 (invasive strain) was applied to the corneal surface. Depending on the experiment, the treatment was started 30 min or 6 h post infection. 10 μl aliquot containing 0.14 mg of tobramycin or 20 μg of S5-PmnH (both containing 0.5% hydroxypropyl methylcellulose (HPMC)) or PBS containing 0.5% HPMC was applied as one drop of substance to each eye. HPMC is used as artificial tears and was used in order to thicken the tears film and prolong the presence of the applied product on the surface of the

cornea. The treatment was continued for five days twice daily. Mice were euthanized by cervical dislocation 1, 3, 5 days post infection, and the eyeballs were collected and homogenized for viable bacteria count.

Assessment of clinical score. The eyes were examined and photographed with a dissection microscope equipped with a digital camera at 1, 3 and 5 dpi to monitor the disease progression. At 1, 3 and 5 dpi disease severity was visually graded by using an established corneal damage scale: 0, the pupil was partially or fully covered by clear or slight opacity; + 1, the anterior segment was partially or fully covered by slight opacity; + 2, the pupil was partially or fully covered by dense opacity; + 3, the entire anterior segment was covered by dense opacity; and + 4, corneal perforation.

Histopathology. One randomly chosen mice of each study group was used for histopathology experiments. Enucleated eyes were preserved in 10% formaldehyde. Eyes were paraffin-embedded, cut into 3-μm-thick sections, deparaffinized, rehydrated and used for preparation of hematoxylin/eosin-stained samples. All samples were observed with the Eclipse TE2000-U microscope (Nikon, Tokyo, Japan).

2.10. Murine lung colonization and treatment

Animals. The studies were performed in Evotec facility under UK Home Office Legislation and Guidelines, with relevant Establishment, Project, and Personal license authorities in place. Local ethical committee approval was in place for this model and all studies were prepared and conducted in keeping with the ARRIVE Guidelines. CD1 male mice were supplied by Charles River (Margate, UK) and were specific pathogen free. Mice were 11 to 15 g on receipt at the facility and were allowed to acclimatize for at least 7 days. Mice were housed in individual ventilated cages always exposing the mice to HEPA filtered air. Mice had free access to food and water and were provided with aspen chip bedding. The room temperature was 22 ± 1 °C, with a relative humidity of 60% and maximum background noise of 56 dB. Mice were exposed to 12-h light/dark cycles.

Mice infection and pyocin treatment. *Pseudomonas aeruginosa* ATCC 27853 was cultured on cystine-lactose-electrolyte-deficient (CLED) agar at 37 °C under aerobic conditions for approximately 16–24 h. 20 ml of Mueller Hinton broth was inoculated with a single well isolated colony and cultured overnight at 37 °C with shaking at 300 rpm. The overnight broth was diluted 1:100 in Mueller Hinton broth and 100 ml was cultured in a baffle flask for ~ 6 h at 37 °C with shaking at 300 rpm until the broth OD was ~ 0.6. 20 ml of culture was centrifuged at 2465 g for 10 min and washed in PBS. The pellet was suspended in PBS and the OD_{600} adjusted to 0.67 (~ 2.6×10^8 CFU/ ml).

The study inoculum was prepared from this by appropriate dilution with PBS. The inoculum concentration was confirmed by quantitative culture on *Pseudomonas* Selective Agar (PSA). The inoculum concentration for the study was 1.47×10^7 CFU/ml (5.87×10^5 CFU/mouse). Mice were infected under temporary inhaled 3% isoflurane anesthesia by intranasal (IN) instillation with 40 µl of the inoculum suspension split as 20 µl/nostril. 1 h post infection 6 mice were sacrificed in order to evaluate the pre-treatment burden in lungs. Treatments were administered IN once at 1 h post infection (50 µl split to 25 µl/nares). Three treatment groups consisting of six mice each were administered S5-PmnH (2.5, 25 and 250 µg). The fourth group of mice (n = 6) was administered 200 µg Tobramycin (40 mg/ml injection solution, Hospira UK Ltd, diluted 10 times). The study was terminated at 5 h post infection for all animals. The clinical conditions and weights were assessed, and animals were immediately euthanized using an overdose of pentobarbitone. Following confirmation of death, the lungs were excised and homogenized in ice cold sterile phosphate buffered saline using a Precellys bead beater. The homogenates were quantitatively cultured onto PSA agar and incubated at 37 °C for 24 h before colonies were counted.

3. RESULTS AND DISCUSSION

3.1. Expression of pyocins in plants

For pyocin expression in plants, we chose pICH29912, the assembled magnICON® TMV-based transient expression vector (**Figure 17A**). Extracts of *N. benthamiana* leaves transfected with *A. tumefaciens* harboring Pyocin S5, PaeM and PaeM4 constructs and harvested at 5–7 dps (days post spraying) showed distinct bands of expected molecular weight in SDS-PAGE (56 kDa, 32 kDa and 39 kDa, respectively) (**Figure 17B**, left panel). All three pyocins were expressed in *N. benthamiana* at very high levels, comprising between 30% and 50% of total soluble leaf protein. The expression of L1, L2 and L3, the three lectin-like pyocins was further attempted and was also successful, although giving lower expression levels of 10% to 30% of total soluble leaf protein. Detected supplementary SDS-PAGE bands corresponded to expected molecular weights: 28.4 kDa for L1, 28.4 kDa for L2 and 30.3 kDa for L3. Among the three lectin-like pyocins, L3 was expressed most efficiently. Although L1 and L2 have a high degree of identity (85%), the expression level of L1 was significantly superior to the expression level of L2 (**Figure 17B**, right panel).

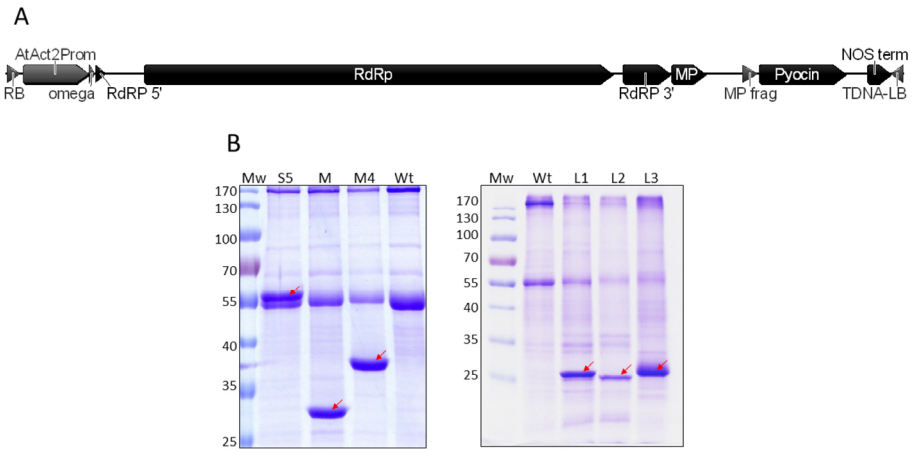


Figure 17. Pyocin expression in plants. **(A)** Schematic presentation of T-DNA region with pyocin expression cassette. RB – right T-DNA border, AtAct23Prom – *A. thaliana* actin promoter, RdRp – RNA-dependent RNA polymerase, MP – truncated TMV movement protein, LB – left T-DNA border. **(B)** Expression of pyocins in *N. benthamiana* leaves. Solutions containing 5 µg of protein were resolved in 12% polyacrylamide gel for Coomassie staining. Mw – PageRuler Prestained protein ladder (ThermoFisher Scientific Baltics), Wt – crude extract of nonsprayed *N. benthamiana leaves*, S5, M, M4, L1, L2, L3 – extracts of *N. benthamiana* leaves, sprayed with pyocin expression constructs (pyocin S5, PaeM, PaeM4 and lectin-like pyocins L1, L2, L3). Bands corresponding to recombinant pyocins are marked by arrows.

Thus, the non-antibiotic antibacterial proteins pyocins can be expressed at high levels in the plant *Nicotiana benthamiana*, the standard manufacturing host for multiple biopharmaceuticals currently undergoing clinical trials. We believe that the plant-based process we use can be a competitive alternative to pyocin production by *E. coli* fermentation in terms of manufacturing economics and scalability. The expression levels reached in this study were up to 50% of the plant leaves' total soluble protein without process optimization, meaning that pyocins are not toxic to plants. In future studies, industrial procedures to enhance yield, including optimizing transfection or inducing expression in transgenic hosts, could be adopted to further increase process efficiency and lower the cost of pyocin manufacturing. These efforts have yet to be conducted. In contrast, attempts to express bacteriocin proteins in bacterial hosts have sometimes met with general toxicity challenges, even in when using expression hosts other the homologous species natively producing the bacteriocins (Diaz et al. 1994; Medina et al. 2011). This problem is most pronounced with bacteriocins with DNAse and RNAse activity, but even bacteriocins that do not express well in bacterial hosts could be successfully expressed in plants by introducing introns in the toxic domains of these proteins (Schulz et al. 2015b). Thus, plants are excellent hosts for manufacturing not only phage endolysins (Oey et al. 2009b,a; Starkevič et al. 2015) and colicins (Schulz et al. 2015b) but also pyocins. Manufacturing based on transient expression in plants has already been brought to GMP compliance, and several biopharmaceuticals produced transiently in plants are currently undergoing clinical studies, such as anti-Ebola therapeutic antibodies (Qiu et al. 2014) (Prevail trial, 2016) and vaccines against non-Hodgkin's lymphoma (Bendandi et al. 2010; Tuse et al. 2015).

3.2. Activity of plant-produced pyocins in agar drop plate assays

Purified pyocins were tested at first in the agar drop plate assay with 12 *P. aeruginosa* strains. We used iron-deficient CAA medium for S5, PaeM and PaeM4 pyocins, which are known (S5 and PaeM) or expected (PaeM4) to use the iron receptor. For lectin-like pyocins, LB medium was used. All six plant-expressed pyocins formed inhibition zones on several *P. aeruginosa* strains. Inhibition zones were of different width and haziness, depending on the strain and the pyocin. PaeM4 formed large inhibition zones on almost all tested strains. Pyocins S5 and L3 also demonstrated wide ranges of activity, while pyocins PaeM, L1 and L2 had narrower specificity spectra (**Figure 18A and 2B**).

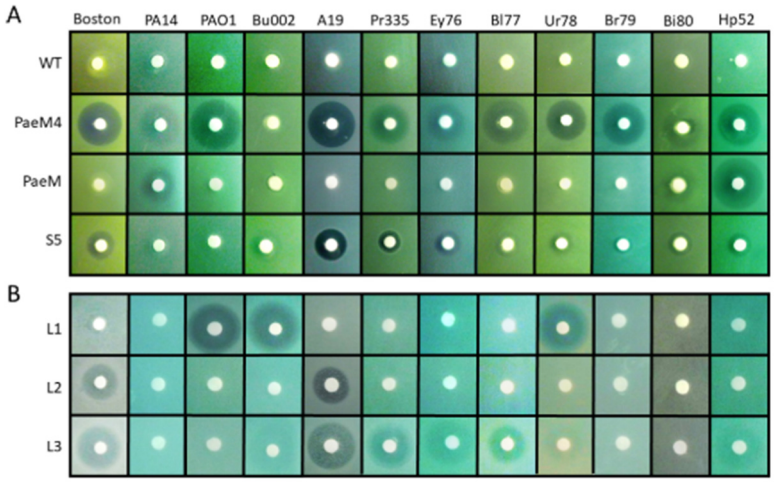


Figure 18. Agar drop plate assay on different *P. aeruginosa* isolates with plant-produced pyocins. Aliquots of 10 µg of purified pyocins were spotted on *P. aeruginosa* agar lawn and incubated overnight. **(A)** *P. aeruginosa* agar lawn grown on CAA medium. **(B)** *P. aeruginosa* agar lawn grown on LB medium.

To confirm initial results that showed that PaeM4 has significantly wider specificity than PaeM, and that the above-mentioned results were not biased by our initial choice of strains, we conducted a larger activity study. In this secondary inhibition screen, 100 clinical isolates of *P. aeruginosa* were tested in the agar drop plate assay using purified plant-made pyocins.

Pyocin S5 formed inhibition zones on 40% of strains tested, while PaeM inhibited 26% of the strains. PaeM4 inhibited 53% of the strains, demonstrating again the widest range of control among the three bacteriocins. By using only three pyocins we could target up to 68% of all tested clinical isolates of *P. aeruginosa* (**Figure 19**).

Figure 19. Sensitivity of clinical isolates to three plant-expressed pyocins. Venn diagram describing pyocin susceptibilities of clinical *P. aeruginosa* isolates. Left – susceptibility of all 100 tested isolates, right – susceptibility of 21 antibiotic-resistant isolates.

We then looked more closely at the antibiotic resistance spectrum of the clinical isolates to ascertain whether pyocins were equally active against antibiotic susceptible and resistant strains. Twenty-one isolates out of the 100 tested were known to be resistant to at least three antibiotics. It appeared that 43% (9 of 21) of the resistant strains were targeted by pyocin S5, 24% (5 of 21) were sensitive to PaeM, and 58% (12 of 21) were targeted by PaeM4 (**Figure 19**). We then looked at pyocin sensitivity of carbapenem-resistant strains. Twenty-one strains in the tested panel were carbapenem resistant, and 14 of these strains (67%) were sensitive to at least one of the three tested pyocins. These numbers are very close to the sensitivity of all 100 of the strains tested in general and suggest that antibiotic resistance in *P. aeruginosa* strains does not influence their sensitivity to pyocins.

Thus, plant-expressed pyocins, like the earlier reported plant-produced *E. coli*-analogues colicins, are fully functional antibacterials. Furthermore, our results allow us to envisage the use of pyocins to treat infections caused by carbapenem-resistant and even multi-drug resistant *P. aeruginosa* serotypes. We show that simple mixture of three pyocins, applied at low concentrations, are highly and broadly active against 68 of the 100 pathogenic *P. aeruginosa* isolates tested. These studies can be extended in the future to include additional pyocins, which collectively could allow for even higher pathogen coverage.

3.3. Pyocin susceptibility assays in liquid nutrient medium

We next evaluated the susceptibility of *P. aeruginosa* strains to plant-expressed pyocins in liquid culture assays. For this experiment, we selected three *P. aeruginosa* strains demonstrating different pyocin sensitivities. The A19 strain was chosen because in agar drop plate assays it demonstrated sensitivity to the highest number of pyocins (S5, PaeM4, L2 and L3). PAO1 strain is a producer of pyocin S5 and is immune to this pyocin; it cannot be targeted by PaeM but is sensitive to PaeM4 and L1. The hospital pneumoniae isolate HP52 is sensitive only to *P. aeruginosa* colicin M homologs, PaeM and PaeM4. Before starting the experiment, we determined the minimum inhibitory concentration (MIC) of pyocins S5 and PaeM4 for the A19 strain in order to approximate the treatment concentration. An MIC of 0.1 μg/ml was determined for pyocin S5 and 0.6 μg/ml for PaeM4. Thus, we used 5 μg/ml as the treatment dose, which is 5 to 10-fold the determined MIC. In this experiment, the A19 strain was found highly susceptible to PaeM4 and S5. Five μg/ml of pyocins added to CAA medium reduced bacterial CFU counts by several orders of magnitude: PaeM4 treatment achieved CFU reduction of almost three orders of magnitude, S5 treatment achieved CFU reduction of

five orders of magnitude, and a mixture of both pyocins reduced the CFU count by as much as 5.5 $\log_{10}$ (**Figure 20A**). Treatment of strain HP52 with PaeM and PaeM4 yielded similar results for both pyocins with slightly better efficacy *in vitro* for PaeM. Addition of 5 µg/ml of PaeM reduced bacterial CFU by about two orders of magnitude (**Figure 20B**). The CFU reduction achieved by a mixture of both pyocins had no cumulative effect and was similar to that obtained by either pyocins alone (2.4 $\log_{10}$). *P. aeruginosa* PAO1 strain is sensitive to L1 and PaeM4, with L1 forming clear inhibition zones and PaeM4 forming hazy inhibition zones in the agar drop test assay. The sensitivity assay in liquid PAO1 culture demonstrated less than 1 $\log_{10}$ CFU reduction after treatment with 5 or 50 µg/ml of PaeM4. L1 treatment was more effective, providing >2-log CFU reduction when 50 µg/ml pyocin was used for the treatment. Exposure of bacteria to a mixture of both pyocins yielded similar results as PaeM4 treatment alone (**Figure 20C**).

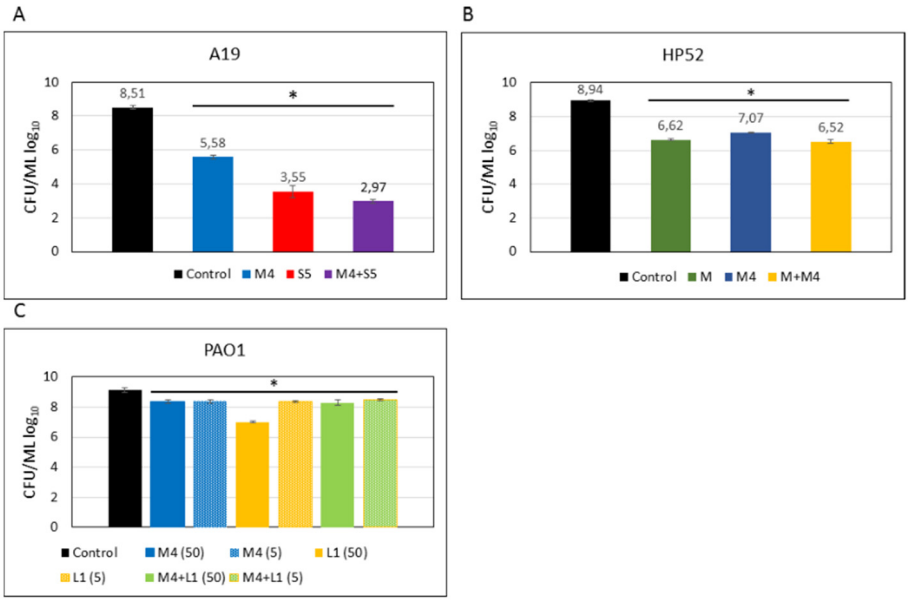


Figure 20. Pyocin antibacterial assays in liquid culture. *P. aeruginosa* strains were cultivated in CAA medium, treated with 5 µg/ml or 50 µg/ml of one or several pyocins. The antimicrobial activity of pyocins was evaluated by determining cell numbers of bacterial test culture. Data are the mean ± SD of three independent experiments. * Denotes statistical significance ($p \leq 0.001$) for comparison of treatment with antimicrobials versus control by a one-way ANOVA test with Bonferroni correction applied.

3.4. Pyocin activity in *Galleria mellonella* challenge assays

Prior to administration of pyocins, we sought to determine the minimal lethal dose for the larvae of *P. aeruginosa* strains PAO1 and A19 (**Figure 21**). It

appears that as little as ~2 CFU of *P. aeruginosa* PAO1 are sufficient to kill all the larvae in 18 hours (LD_{100} = ~2 CFU). Strain A19 is less virulent, with ~200 CFU needed to achieve complete killing of the insects after 18 hours (LD_{100} = ~200 CFU). However, when incubation was extended to 24 hours, all larvae were dead also after infection with ~2 CFU of A19, demonstrating the high sensitivity of *Galleria mellonella* to *P. aeruginosa* infection in general.

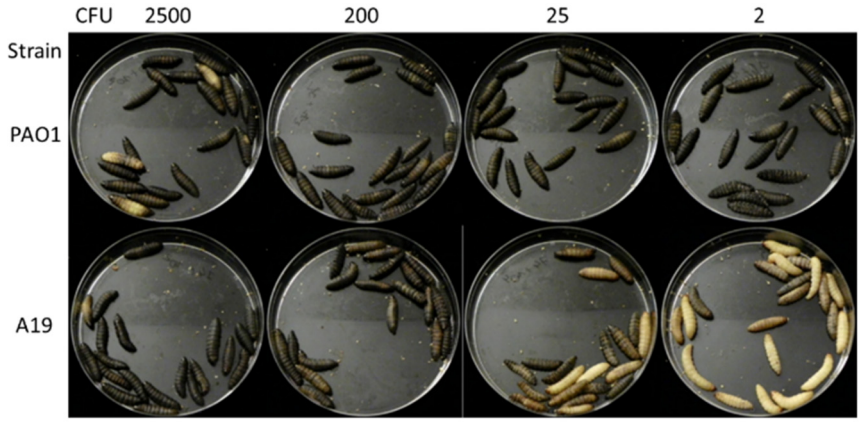


Figure 21. Survival of *Galleria melonella* larvae after injection with *P. aeruginosa* PAO1 or A19. Fifth-instar *G. mellonella* larvae were injected in the left hind proleg with different amounts of *P. aeruginosa* PAO1 and A19 bacteria. The image was captured 18 hours post infection. Healthy larvae are cream colored, and darker pigmentation indicates infection. Dead larvae can be recognized from their dark brown-black color.

Next, we tested the ability of PaeM4 to rescue larvae from infection with *P. aeruginosa* PAO1 and A19 strains (**Figure 22**). 500 CFU of each strain were used separately for infection, which corresponds to about 250-times the LD_{100} (18 h) for PAO1 and 2.5-times the LD_{100} (18 h) for A19. Pyocins were injected three hours after infection with *P. aeruginosa*. Larvae infected with the A19 strain were treated with 10 µg each of pyocins S5, PaeM4, L2 and L3. All larvae in the control group not treated by pyocins were dead in 18 hours, as expected. Pyocin S5 treatment rescued all larvae from infection (100% efficacy). PaeM4 treatment rescued 90% of larvae and L2 treatment rescued 75% of larvae. Treatment with pyocin L3 prolonged the survival of the larvae significantly (all dead at 96 h vs 18 h for the control larvae), but the treatment did not rescue the animals as did treatment with the other pyocins (**Figure 22A**). The results obtained with L2 are somehow surprising because this pyocin was only slightly active in reducing CFU counts in liquid medium experiments.

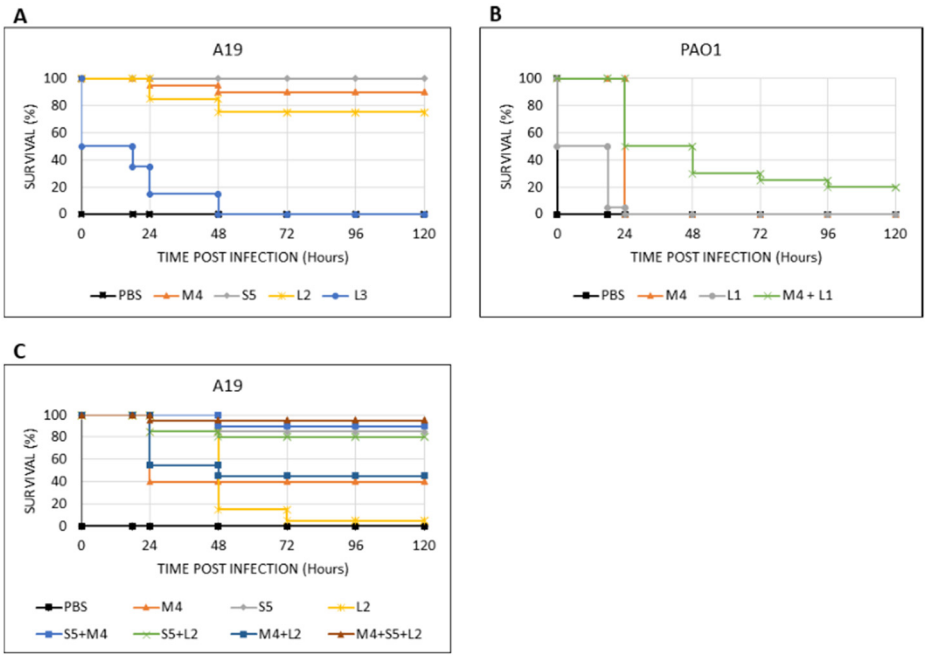


Figure 22. Survival of larvae pre-infected with *P. aeruginosa* PAO1 or A19 and treated therapeutically with pyocins. *G. melonella* larvae were infected with 500 CFU of *P. aeruginosa* A19 or PAO1 and treated with pyocins 3 hours after infection. **(A)** Larvae infected with A19 strain and treated with 10 µg of the indicated pyocin. **(B)** Larvae infected with PAO1 strain and treated with 10 µg of the indicated pyocin. **(C)** Larvae infected with A19 strain and treated with 1 µg of the indicated pyocin. Killing curves were plotted and estimation of differences in survival analyzed by the Kaplan-Meier method using XLSTAT software.

Pyocins PaeM4 and L1 were used in PAO1 larval challenge experiments. In this experiment, neither of two pyocins were able to rescue larvae from infection completely. However, both pyocins significantly extended the survival of the larvae. PaeM4 completely protected all larvae for 24 hours post infection, but all larvae succumbed to infection after 48 hours. L1 protected 50% of larvae for 18 hours, but almost all animals were dead after 24 hours. However, when both pyocins PaeM4 and L1 were used in a mixture, the survival was significantly improved in comparison with either pyocin applied alone: 50% of larvae survived 48 hours post infection and 20% of larvae survived until the end of experiment (Day 5) (**Figure 22B**). We repeated the larval challenge experiments with ten-fold lower doses of pyocins and strain A19. After treatment with 1 µg PaeM4, 40% of larvae survived. At the same low dose, pyocin S5 alone rescued 85% of the larvae, while pyocin L2 did not rescue the larvae but did prolong their survival. Cocktails of

pyocins did lead to improved survival, with 95% of larvae protected and alive at 120 hours post exposure when all three pyocins were used (**Figure 22C**).

We repeated the larval challenge experiments with tenfold lower doses of pyocins and strain A19. After treatment with 1 μg PaeM4, 40% of larvae survived. At the same low dose, pyocin S5 alone rescued 85% of the larvae, while pyocin L2 did not rescue the larvae but did prolong their survival. Cocktails of pyocins did lead to improved survival, with 95% of larvae protected and alive at 120 hours post exposure when all three pyocins were used (**Figure 22C**).

In general, the results obtained are very encouraging. The difference in survival rates between the two tested strains of *P. aeruginosa*, PAO1 and A19, reflects the different virulence of these strains. The extreme sensitivity of wax moth larvae to *P. aeruginosa* requires complete killing of all bacteria, which is hardly achievable in real life. However, the *Galleria mellonella* model provides an excellent tool for pre-screening antimicrobial candidates and should help reduce the number of experiments with mammalian species. Relative to screening inhibitory activity *in vitro*, the larval model may be more closely aligned with the mouse model for *P. aeruginosa* infection and control, which in turn might be more relevant to how humans may be treated with pyocins in a clinical setting.

3.5. Broadening activity spectrum for pyocins

Successful *in vitro* and *in vivo* experiments with PyoS5, PaeM, PaeM4, PyoL1, PyoL2, and PyoL3 allowed us to hypothesize that the breadth of activity and potency of pyocins might be further improved by engineering the proteins by swapping their receptor/translocation and activity domains. The idea and attempt to construct new hybrid molecules are not novel, but, as far as we know, previous attempts to develop chimeric antimicrobials relied on combining domains of phylogenetically and/or functionally unrelated proteins. Several most significant attempts include endolysins with added cell-penetrating-peptide to make them able to cross outer membrane of gram-negative bacteria (Briers & Walmagh 2014), “hybrid lysin” consisting of receptor binding domain of pesticin fused to the N-terminus of T4 lysozyme (Lukacik et al. 2012), and PyS2-GN4 “lysocin” comprised of pyocin S2 receptor binding and outer membrane translocation domains fused to the GN4 lysin (Heselpoth et al. 2019). Albeit all these hybrid examples were functional and active, practical use of these chimeric bacteriocins is limited mainly because of the narrow activity spectrum. Therefore, we went for slightly different path and endeavored to construct chimeric molecules by swapping functional domains of closely related bacterial species with the same

mechanism of antibacterial activity, namely chimeras of porin-porin type. Our goal was to broaden activity spectrum of bacteriocin PyoS5, which demonstrated superior activity compared to all other pyocins, by modifying its killing domain to avoid its recognition by the immunity protein.

3.6. Identification of *Pseudomonas* putative pore-forming bacteriocin sequences

The only known pore forming bacteriocin of *P. aeruginosa* was first detected in PAO1 strain (Parret & De Mot 2000). Pyocin S5 binds highly conserved ferripyochelin FptA receptor (Elfarash et al. 2014). The exact prevalence of clinical *P. aeruginosa* strains producing S5 is unknown, but it was demonstrated in the bacteriocin prevalence study of catheter *P. aeruginosa* isolates that about 25% of these strains contained pyocin S5 coding gene (Snopkova et al. 2020). Thus, these 25% of catheter isolates should be in theory resistant to pyocin S5. We have tested pyocin S5 and S5 immunity protein gene presence in 25 *P. aeruginosa* isolates in our in-house collection and found very similar results, 6 strains (or 24%) contained pyocin S5 and immunity protein coding gene sequences. As expected, the presence of pyocin S5 coding gene in these strains correlated with the resistance to the antimicrobial activity of pyocin S5. Thus, we speculated that if the immunity of S5 producing strains could be overcome by using cytotoxic domain other than S5, the spectrum of the chimera would be significantly broadened, and it could be able to target more than half of strains.

Towards this goal, we constructed six chimeric S5 pyocins, in which the pore-forming domain of S5 was replaced by pore-forming domains of putative bacteriocins from *Pseudomonas* species other than *P. aeruginosa*. Putative pore-forming bacteriocins from genus *Pseudomonas* have been retrieved from NCBI by BLAST search using as query pore-forming domain of pyocin S5 (pfam01024). After the analysis of BLAST results, we selected six most divergent putative pore forming bacteriocins from different Pseudomonas species: Pflu095 (*P. fluorescens* WP_016979095), Pflu373 (WP_014717373 from strain A506), Pflu794 (WP_081041794 from strain ATCC 17400), Pflu618 (WP_034155618 from strain H16), Pput259 (WP_098964259 from *P. putida* strain FDAAR-GOS_376) and PmnH from *P. synxantha* strain BG33R. PmnH is the only one of selected proteins that has been published previously. It has unusual architecture as it harbors two cytotoxic domains, colicin-M like domain and pore-forming domain. So far, only activity of its pore-forming domain has been demonstrated.

3.7. Construction and plant expression of chimeric pyocins

Pore-forming domains of six selected *Pseudomonas* putative porins were used for the construction of the chimeric proteins. All chimeric proteins contain identical N-terminal end of first 309 a.a. of pyocin S5 including translocation, FptA binding and CPA binding domains. The S5 fragment was fused to the cytotoxic domain of the putative pore-forming bacteriocins.

3.8. *In vitro* activity of chimeric pyocins

As the next step we analyzed the activity of S5 chimeras in agar disc diffusion assay against a panel of 25 *P. aeruginosa* strains (from culture collections and clinical isolates) (**Table 8**). Two chimeric proteins, S5-Pflu095 and S5-PmnH demonstrated broadened activity spectrum in comparison to pyocin S5. Both chimeras formed inhibition zones on the lawns of all six S5-producing strains. The inhibition zones on HP6 and HP7 lawns were significantly larger and clearer than those formed by S5. These chimeric proteins also demonstrated activity similar to S5, with only small variations, on all other remaining *P. aeruginosa* strains. S5-PmnH chimeric protein was selected for further experiments in murine models for topical treatment of two unrelated models of disease caused by *P. aeruginosa*: keratitis model and lung infection model.

Table 8. Activity of chimeric pyocins on panel of *P. aeruginosa* strains as determined in agar disc-diffusion assay. *Activity detected only with 30 μg of protein, **activity detected with 3 μg of protein, ***activity detected with 0.3 μg of protein. Pyocin S5 killing and immunity protein genes-containing strains are in bold.

	Colicin E1-like				**Colicin A-like**		
	S5	**S5-Pflu095**	**S5-Pflu373**	**S5-Pflu794**	**S5-PmnH**	**S5-Pflu618**	**S5-Pput259**
Boston	***	***	***	*	***	**	***
PA14	–	**	**	–	***	–	–
PAO1	–	***	***	–	***	**	**
Bu002	***	***	***	–	***	**	**
A19	***	***	***	***	***	***	***
Pr335	***	***	***	**	***	**	***
EY76	***	***	***	–	***	–	–
BL77	***	**	**	–	**	*	*
UR78	***	***	***	–	***	*	*
BR79	***	**	**	–	**	–	*
BI80	***	***	***	–	***	*	*
HP1	***	***	***	–	***	**	***
HP6	*	***	***	*	***	*	**
HP7	**	***	***	–	***	**	**

	Colicin E1-like				Colicin A-like		
	S5	**S5-Pflu095**	**S5-Pflu373**	**S5-Pflu794**	**S5-PmnH**	**S5-Pflu618**	**S5-Pput259**
HP40	***	***	***	*	***	**	**
HP41	***	***	***	–	***	**	**
HP52	***	**	**	–	**	*	*
HP75	**	*	**	–	**	–	–
ATCC 29260	***	***	***	–	***	*	*
12-35708	–	–	–	–	–	–	–
13-18499	***	**	**	–	**	*	*
12-29165	–	–	–	–		–	–
ATCC 19660	–	*	–	–	**	–	*
NCTC 13437	**	*	*	–	*	–	–
NCTC 13921	–	***	**	*	***	*	*

3.9. Efficacy of S5-PmnH in *P. aeruginosa* keratitis models of disease

Two types of *P. aeruginosa* strains can be isolated from keratitis cases, cytotoxic *P. aeruginosa* strains are mainly causing keratitis related to contact-lens wear, while invasive strains are mostly causing disease in post-surgical complications. We aimed to investigate if both type of strains could be targeted by S5-PmnH in disease models. Cytotoxic and invasive strains can be distinguished by genotyping the effector protein coding genes of their type III secretion systems (TTSS). Invasive strains were found to possess both exoS and exoT genes whereas cytotoxic strains appeared to have lost exoS but presented exoT and exoU genes. We selected for our experiments cytotoxic strain ATCC 19660 (exoT, exoU) and invasive strain PAO1 (exoY, exoT, exoS). Both strains are pyocin S5 producers and immune to pyocin S5, but both are sensitive to S5-PmnH.

3.10. S5-PmnH treatment can reduce *P. aeruginosa* bacterial numbers in porcine corneas *ex vivo*

We first investigated the possibility to use S5-PmnH for eradication of *P. aeruginosa* colonizing the cornea in an *ex vivo* model, the dissected porcine corneas. Porcine corneas were colonized with invasive strain PAO1 or cytotoxic strain ATCC 19660. S5-PmnH MIC determined by agar dilution method against PAO1 was 4 μg/ml (0.07 nmol/ml) and against ATCC 19660 was 32 μg/ml (0.57 nmol/ml). In order to obtain *P. aeruginosa* colonization, porcine corneas were incubated with 3×10^4 CFU of *P. aeruginosa* ATCC 19660 or 0.4×10^4 CFU of *P. aeruginosa* PAO1 for 16 to 20 h. Then 5 μg of S5-PmnH were applied to the cornea and incubated for additional 16–20 h. At

the end of the experiment PAO1 burden in untreated corneas reached an average of 7.6 $\log_{10}$ CFU/cornea, while S5-PmnH-treated corneas the burden was only an average of 10 CFU per cornea, demonstrating 6.6 $\log_{10}$ reduction. In ATCC 19660 colonized corneas S5-PmnH treatment reduced CFU number by 5.3 $\log_{10}$ (**Figure 23**). Thus, in *ex vivo* porcine corneas, S5-PmnH efficiently reduced *P. aeruginosa* colonization by both strains.

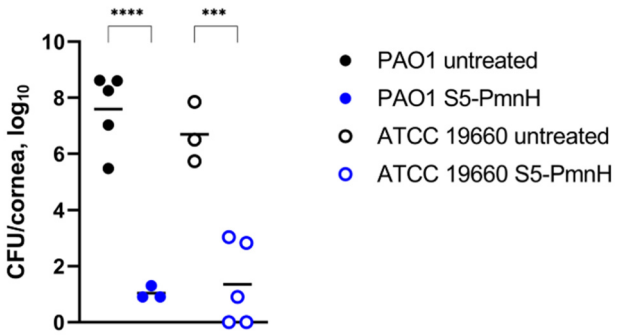


Figure 23. CFU counts in *ex vivo* porcine corneas, infected with *P. aeruginosa* ATCC 19660 or PAO1 and treated with S5-PmnH. 3×10^4 CFU of *P. aeruginosa* ATCC 19660 strain or 0.4×10^4 CFU of *P. aeruginosa* PAO1 strain were applied to cornea and incubated for 16–20 h at 37 °C. Then 5 µg of S5-PmnH were applied to cornea and incubated for additional 16–20 h. Statistical significances of the quantitative data were analyzed using GraphPad Prism software by the 1-way repeated measures ANOVA and Bonferroni's' correction for multiple comparisons. Mean is indicated by horizontal bar. ***$P \leq 0.001$, ****$P \leq 0.0001$ vs vehicle-treated mice.

3.11. S5-PmnH efficiently kills bacteria and prevents acute disease in murine keratitis models

3.11.1. Infection by cytotoxic strain ATCC 19660

For induction of keratitis the mice were anaesthetized, the cornea of left eye was scratched with a sterile needle and *P. aeruginosa* ATCC 19660 (4×10^6 CFU) was applied to the corneal surface. The treatment by S5-PmnH, tobramycin or PBS (mock-treatment) was started 30 min post infection or 6 h post infection. When the treatment was started 30 min after the infection, no viable *P. aeruginosa* were isolated from infected eyes in both S5-PmnH and tobramycin-treated groups on 1, 3 or 5 dpi. By contrast, the bacterial burden in infected and untreated eyes reached 6–7 $\log_{10}$ CFU/cornea (**Figure 24a,** left panel).

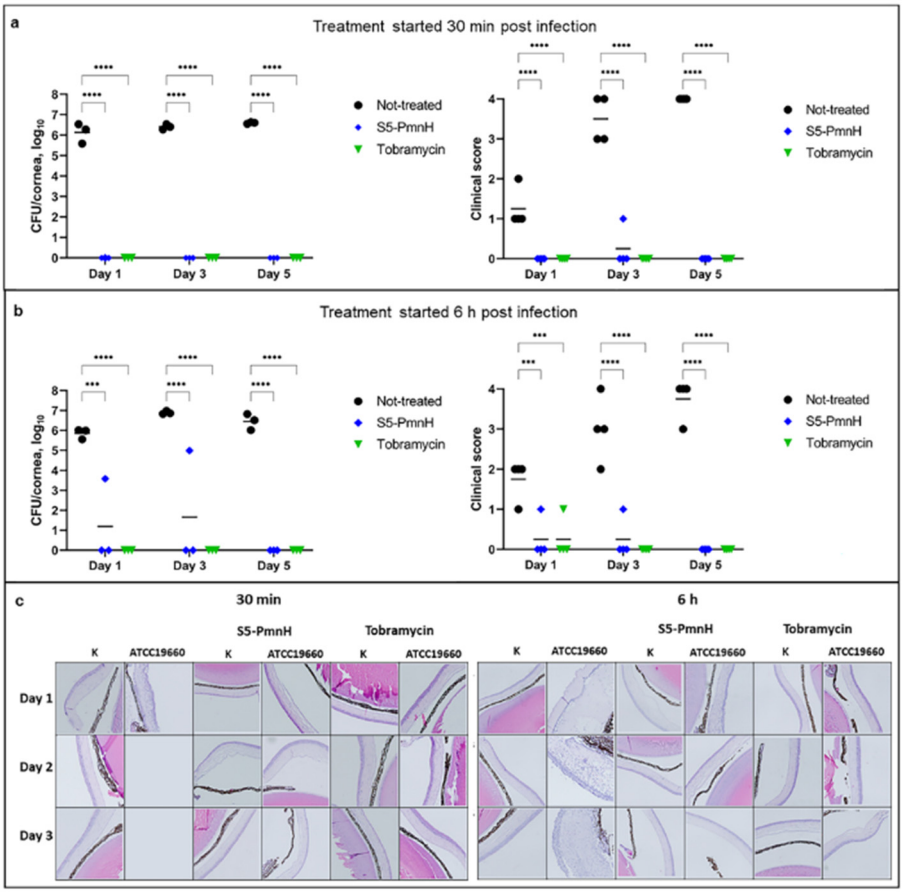


Figure 24. Mice cornea infection by cytotoxic strain ATCC 19660 and treatment by S5-PmnH or tobramycin. **(a)** CFU counts and clinical scores of mice eyes, when treatment started 30 min post-infection. **(b)** CFU counts and clinical scores of mice eyes, when treatment started 6 h post infection. **(c)** Hematoxylin–eosin staining of cornea sections. Left panel – treatment with pyocin started 30 min post-infection. Uninfected eyes – no observed microscopic aberrations. Infected control eyes: day 1 – strong corneal inflammation, days 3 and 5 – the histology was not possible because of disrupted structure of the eye. Weak edema in the corneal stroma is observed in all infected eyes treated with S5-PmnH and with tobramycin. Right panel – treatment with pyocin started 6 h post infection. Uninfected eyes: no marked cornea aberrations. Infected untreated eyes: day 1 – thinning of corneal epithelium, thickening of stroma, acute inflammation, days 3 and 5 – acute suppurated inflammation of cornea. S5-PmnH-treated infected eyes: day 1 – acute inflammation of cornea, days 3 and 5— no marked aberrations, week edema of corneal stroma. Tobramycin-treated infected eyes: day 3 and 5 – slight thickening of epithelium. Statistical significances of the quantitative data were analyzed using GraphPad Prism software by the 2-way repeated measures ANOVA and Dunnett's correction for multiple comparisons. Mean is indicated by horizontal bar. ***P ≤ 0.001, ****P ≤ 0.0001 vs vehicle-treated mice.

The visual inspection by microscope of infected untreated eyes revealed acute disease signs: slight to dense opacity of cornea at 1 dpi, and dense opacity and sometimes cornea perforation at 3 dpi. At 5 dpi all the mice had cornea perforations. No signs of disease were observed in majority of samples treated by S5-PmnH or tobramycin (**Figure 24a**, right panel). The histopathology examination of infected and mock-treated eyes revealed strong corneal inflammation at 1 dpi, and disrupted structure of the eye at 3 dpi and 5 dpi. Only weak oedema in the corneal stroma was observed in all infected eyes treated with PyoS5-PmnH and with tobramycin (**Figure 24c**). Thus, both S5-PmnH and tobramycin completely eradicated *P. aeruginosa* ATCC 19660 and prevented the disease when the treatment was started almost immediately, 30 min, after infection.

We repeated the experiment with delayed treatment time, allowing the infection to establish for 6 h. Similar to the previous experiment, the average CFU burden in infected mock-treated eyes reached 6.1 to 6.6 $\log_{10}$ CFU/cornea. In the tobramycin-treated group of mice, no viable bacteria were isolated on 1, 3 or 5 dpi. In the S5-PmnH-treated group viable bacteria were isolated from one mouse at 1 dpi (3.6 $\log_{10}$ CFU/cornea) and from one mouse at 3 dpi (5 $\log_{10}$ CFU/cornea). No viable bacteria were isolated from neither of three mice at 5 dpi (**Figure 24b,** left panel). The clinical score evaluation of infected and mock-treated eyes revealed very similar picture to the previous experiment: the opacity of cornea started at 1 dpi and 3 out of 4 mice had cornea perforations at 5 dpi. Only mild disease signs were observed in two S5-PmnH-treated and one tobramycin-treated mice at 1 and 3 dpi and all corneas were completely clear at 5 dpi (**Figure 24b,** right panel). The histopathology examination of mock-treated eyes revealed the thinning of corneal epithelium, thickening of stroma, acute inflammation at 1 dpi and acute suppurated inflammation of cornea at 3 dpi and 5 dpi. PyoS5-PmnH-treated infected eyes at 1 dpi presented signs of acute inflammation of cornea and no marked aberrations at 3 and 5 dpi, just weak oedema of corneal stroma was observed. Tobramycin-treated infected eyes at 3 and 5 dpi showed slight thickening of epithelium (**Figure 24c,** right panel). Thus, here again S5-PmnH treatment was efficient in eradicating *P. aeruginosa* from infected corneas and in preventing the establishment and progress of disease.

3.11.2. Infection by invasive strain PAO1

Next, we examined the efficacy of S5-PmnH for treatment of cornea infection by invasive *P. aeruginosa* PAO1. The infection and treatment procedures were similar to the previous experiment and treatment was started 6 h post infection. The control group of infected mice suffered from severe disease and

were euthanized at 3 dpi. At 1 dpi, bacterial burden in mock-treated group of mice reached 6.26 $\log_{10}$ CFU/cornea. The S5-PmnH and tobramycin treatment reduced burden by average 1.04 $\log_{10}$ CFU/cornea and 1.42 $\log_{10}$ CFU/cornea, respectively. At 3 dpi no viable bacteria were isolated from all three tobramycin-treated mice, and from two S5-PmnH-treated mice. The cornea from the third S5-PmnH-treated mouse contained 2.38 $\log_{10}$ CFU of viable bacteria. By contrast, burden in the control group of mice reached 6.73 $\log_{10}$ CFU/cornea. At 5 dpi, no bacteria were isolated from two tobramycin-treated and one S5-PmnH-treated mice. One mouse from the tobramycin treated group contained 2.8 $\log_{10}$ CFU/cornea. The two remaining mice from the S5-PmnH treated group contained 5.14 $\log_{10}$ and 3.34 $\log_{10}$ CFU/cornea (**Figure 25a,** left panel).

Clinical examination revealed stronger disease symptoms compared to the cytotoxic strain ATCC 19660. Mock-treated eyes presented severe disease signs at 1 dpi (clinical score 2–3). Several S5-PmnH-treated and tobramycin-treated eyes presented mild clinical scores (1–2) starting from 1 dpi to the end of experiment (**Figure 25a,** right panel)

Histological examination of uninfected eyes revealed no marked aberrations, although weak edema in corneal stroma was observed in most samples. Infected S5-PmnH-treated eyes presented signs of acute inflammation of cornea at 1 dpi and 3 dpi. Infected tobramycin-treated eyes presented signs of acute inflammation of cornea at 1 dpi, thinning of cornea epithelium and strong edema of the corneal stroma at 3 dpi and local inflammation of cornea, thinning and degeneration of cornea epithelium at day 5 (**Figure 25b**).

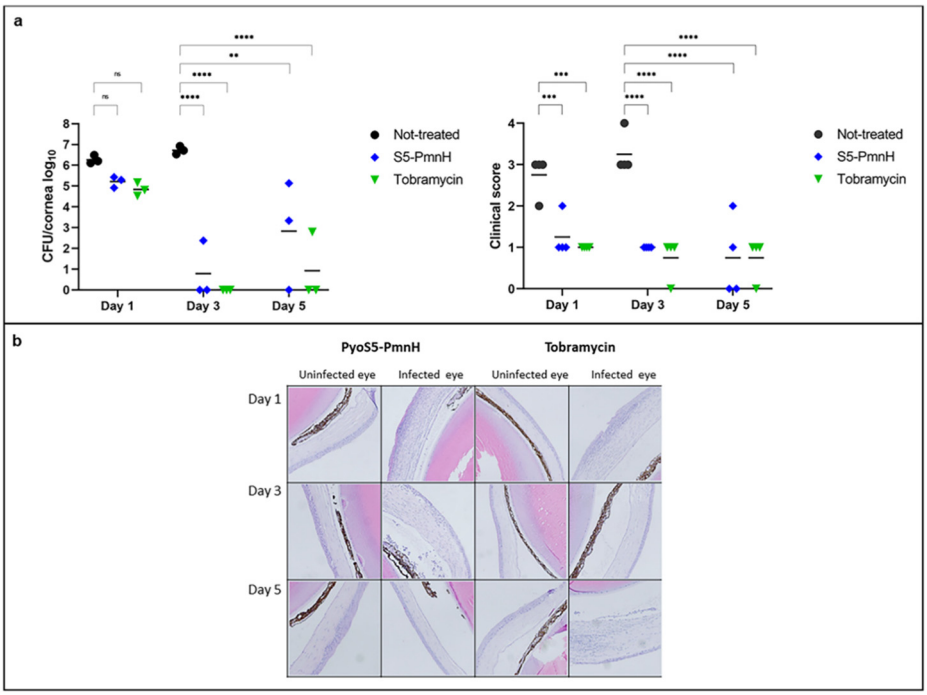


Figure 25. Mice cornea infection by invasive strain PAO1 and treatment by S5-PmnH or tobramycin. The corneas of left eye of mice were infected with 4×10^6 CFU of *P. aeruginosa* PAO1 strain. The treatment with 20 µg of S5-PmnH or 140 µg of tobramycin started 6 h post-infection and was applied twice daily. **(a)** CFU counts in mice corneas and cornea clinical scores at 1, 3 and 5 dpi. **(b)** Hematoxylin–eosin staining of cornea sections. Uninfected eyes: no marked aberrations, weak edema in corneal stroma observed in most samples. Infected S5-PmnH-treated eyes: days 1 and 3 – acute inflammation of cornea, day 5 – no aberrations. Infected tobramycin-treated eyes: Day 1 – acute inflammation of cornea, local cornea lesions, Day 3 – thinning of cornea epithelium, strong edema of the corneal stroma; day 5 – local inflammation of cornea, thinning and degeneration of cornea epithelium. Statistical significances of the quantitative data were analyzed using GraphPad Prism software by the 2-way repeated measures ANOVA and Dunnett's correction for multiple comparisons. Mean is indicated by horizontal bar. **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 vs vehicle-treated mice. Not significant (ns) P > 0.05.

Chimeric pyocin S5-PmnH demonstrated efficacy in both cytotoxic and invasive *P. aeruginosa* models of eye infection. In cytotoxic keratitis model, the treatment completely eliminated all bacteria one day post infection, if treatment was started 30 min after infection. When treatment was delayed for 6 h, viable bacteria were isolated only from one mouse at 1 dpi and one mouse at 3 dpi. Visual inspection and histological examination of eyes of all treated mice in both experiments did not reveal any significant lesions, with no

marked difference from non-infected eyes. By contrast, very strong keratitis symptoms were observed in all infected and untreated eyes at 3 and 5 dpi. Thus, S5-PmnH treatment efficiently eradicated *P. aeruginosa* from cornea infected by the cytotoxic strain *P. aeruginosa* ATCC 19660 and prevented the establishment and progress of the disease. A similar experiment was performed using the invasive *P. aeruginosa* strain PAO1 with treatment starting 6 h after infection. Although both treatments (S5-PmnH or tobramycin) were less efficient in eradicating bacteria, the progress of disease was greatly reduced in all treated mice. Taken together, these studies clearly demonstrate the efficacy of chimeric pyocin in this disease model, comparable to that of the standard of care antibiotic tobramycin. Further experiments are necessary to evaluate the efficacy of S5-PmnH against bacteria growing as biofilms and against bacteria which have been internalized by eukaryotic cells.

3.12. S5-PmnH efficiently eradicates lung colonization by *P. aeruginosa* in a murine model of disease

Pseudomonas aeruginosa is also a frequent cause of lung infections, including hospital-acquired pneumonia and ventilator-associated pneumonia. This pathogen has a worsening global trend towards more likely displaying MDR phenotypes (Jean et al. 2020). In addition, *P. aeruginosa* can cause chronic lung infections in patients with cystic fibrosis (CF) and non-CF bronchiectasis. Acquisition of *P. aeruginosa* is associated with increased morbidity and mortality in patients with CF, and is an important factor in the development and progression of CF respiratory disease (Maurice et al. 2018; Planquette et al. 2013; Sadikot et al. 2005). Patients with CF are at very high risk of developing infections with multidrug-resistant *P. aeruginosa*, owing to the frequent and often prolonged courses of oral, intravenous, and aerosolized antibiotics that are used to treat the chronic lung disease of CF (AbdulWahab et al. 2017).

Mice were infected intranasally (IN) with *P. aeruginosa* ATCC 27853 strain. One hour later, 2.5, 25 and 250 µg/mouse of S5-PmnH were administered IN once to both nares of the mouse. 5 h later mice were euthanized and lung burden of *P. aeruginosa* ATCC 27853 was evaluated. 5 h post infection, *P. aeruginosa* ATCC 27853 burden in the mock-treated mice reached 1.24×10^7 CFU/g of lung tissue, corresponding to an increase of 1.53 $\log_{10}$ CFU/g from 1 h post infection. S5-PmnH administered IN at 2.5 µg/mouse reduced lung burden by 2.1 $\log_{10}$ CFU/g, administered at 25 µg/mouse by 2.31 $\log_{10}$ CFU/g and administered at 250 µg/mouse by 2.66 $\log_{10}$ CFU/g. The bacterial burden was reduced to below the level of stasis (pre-treatment) in all S5-PmnH treatment groups: in 2.5 µg/mouse group by 0.58 $\log_{10}$ CFU/g, in 25 µg/ mouse group by 0.78 $\log_{10}$ CFU/g and in 250 µg/mouse group by 1.13 $\log_{10}$ CFU/g. Increased

reduction in burden was observed with higher dose levels of S5-PmnH, however the differences were not statistically significant. Tobramycin administered IN once at 200 µg/mouse reduced bacterial burden by 2.75 $\log_{10}$ CFU/g compared to vehicle, corresponding to 1.23 $\log_{10}$ CFU/g below the level of stasis. Higher variability was observed in this group compared to S5-PmnH or vehicle treatments (**Figure 26**).

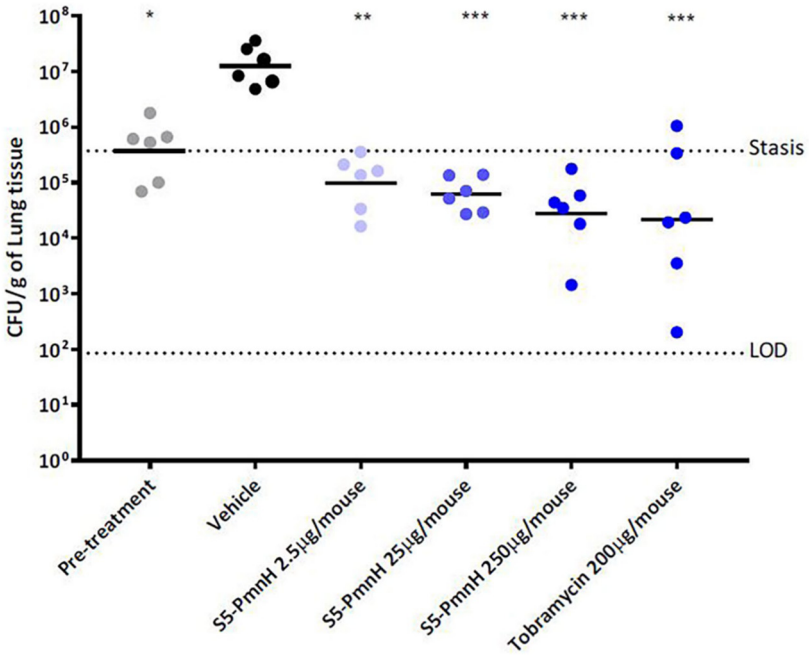


Figure 26. Scatterplot of terminal lung burden following IN infection with *P. aeruginosa* ATCC 27853. The data from the culture burdens were analyzed using appropriate non-parametric statistical models (Kruskal– Wallis using Conover-Inman to make all pairwise comparisons between groups) with StatsDirect (v. 3.3.3). The geometric mean burden of each treatment is indicated by the horizontal bar. *P ≤ 0.05, **P ≤ 0.0005, ***P ≤ 0.0001, compared to vehicle control. LOD – limit of detection.

In our validated mouse lung colonization model, one single application of chimeric pyocin S5-PmnH, even at the lowest concentration used, 2.5 µg, reduced bacterial burden in the lungs below the level of stasis. In a similar study, pyocin S5 had been shown successfully to eradicate lung colonizing *P. aeruginosa* in mice with superior activity compared to pyocins L1, S2 and AP41 (McCaughey et al. 2016c). The results obtained between the two studies cannot be compared directly because of differences in experimental protocols and *P. aeruginosa* strains used. Our study has limitations, as we only performed short time bacteremia evaluation. However, the successful results

obtained with chimeric pyocin S5-PmnH used in our study back up the possibility to use porin type bacteriocins for efficiently targeting bacteria in lungs and demonstrate the endless possibilities of engineering bacteriocins with modified or broadened activity spectra.

3.13. Identification and analysis of novel modular bacteriocins – stenocins – from opportunistic pathogen *Stenotrophomonas maltophilia*

In light of recent publications discussing the cooperativity between *S. maltophilia* and *P. aeruginosa* in both cystic fibrosis patients (McDaniel et al. 2020) and keratitis infection (Dantam et al. 2020) we decided to mine *S. maltophilia* genome for putative bacteriocins. Two peptidoglycan-degrading stenocins SmaltM and SmaltM2 were identified and their killing activity confirmed on *Stenotrophomonas* strains.

Putative amino acid sequences for the newly identified bacteriocins were retrieved from the NCBI by a BLAST search using ColM as a query (Pfam PF14859). These bacteriocins, SmaltM (*S. maltophilia*, WP_058981958.1) and SmaltM2 (*S. maltophilia*, WP_143570807.1) belong to a subset that contains the ColM domain (type M), a toxin module that blocks the biosynthesis of peptidoglycan. To the best of our knowledge, this is the first time that these types of bacteriocins have been identified and verified in genomes of *Stenotrophomonas*. We called them stenocins SmaltM and SmaltM2, where the capital letter "S" stands for the *Stenotrophomonas* genus, "malt" comes from the host species name, and the last capital letter is assigned according to the most related colicin. The only other characterized antimicrobials from *S. maltophilia* are phage tail-like bacteriocins called maltocins P28 (Liu et al. 2013) and S16 (Chen et al. 2019).

SmaltM and SmaltM2 sequence alignment with Colicin M and other Colicin M-like (lipid-II-degrading) bacteriocins from *Burkholderia*, *Escherichia*, *Klebsiella*, *Pectobacterium*, *Pseudomonas* revealed all of them to share sequence similarity, especially in C-terminus toxicity domain (**Figure 27**). Five residues are essential for full ColM cytotoxicity: Asp-226 (113 *), Tyr-228 (115 *), Asp-229 (116 *), His-235 (122 *), and Arg- 236 (123 *) (* represents numbering in Figure 27, ClustalW alignment) (Barreteau et al. 2010) . These surface-exposed amino acids are highly conserved among stenocins and their homologues. All aligned bacteriocins (**Figure 27**) fall in three groups depending on the level of matching residues responsible for ColM cytotoxicity:

1. All 5 amino acid analogues are conserved for KvarM, PaeM, PecM1, PecM2, PflM, and PsyM.

2. 4 out of 5 amino acids are conserved. KaerM contains Ser, KpneM – Phe and PaeM4 – Gln instead of His-235 (122 *).
3. His-235 (122 *) amino acid is absent, meaning that BurM1, BurM2, and both stenocins SmaltM and SmaltM2 contain 5 residues between Asp-229 (116 *) and Arg-236 (123 *) instead of 6 residues like in ColM. Moreover, both burkhocins contain additional mismatch in key residues: BurM1 contains Lys instead of Asp-229 (116 *) and BurM2 contains Phe instead of Tyr-228 (115 *)

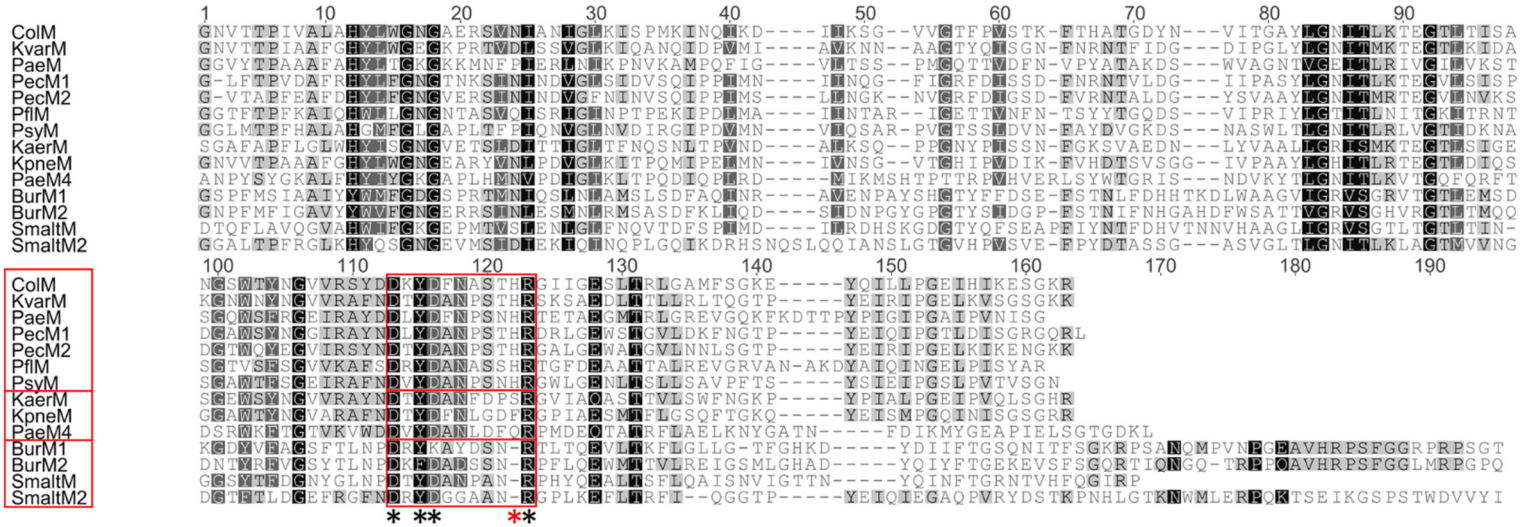


Figure 27. Multiple sequence alignment of stenocins and their homologues. Clustal W aligment of C-terminus, cytotoxic domains of *B. ambifaria* BurM1 (EDT37562), BurM2 (ABI85884), *E. coli* ColM (AAA23589, 2XMX), *K. aerogenes* KaerM (WP_015367360), *K. pneumonia* KpneM (EWD35590), *K. variicola* KvarM (CTQ17225), *P. aeruginosa* PaeM (ERZ09841, 4G75), PaeM4 (ERY59288), *P. carotovorum* subspp. *carotovorum* PecM1 (YP_003017875), *P. carotovorum* subspp. brasiliensis PecM2 (WP_010276821, 4N58) P. *fluorescens* PflM (EIK70389), *P. syringae* PsyM (WP_011103200, 4FZL), *S. maltophilia* SmaltM (WP_058981958) and SmaltM2 (WP_143570807). Asterisks indicate essential amino acids for ColM activity. Red asterisk indicates changes in SmaltM and SmaltM2 as compared to ColM. Parentheses contains accession numbers (GenBank and RCSB PDB for solved structures).

3.14. Stenocins' antimicrobial activity

The antibacterial activity of stenocins SmaltM and SmaltM2 was evaluated in an agar disc diffusion assay against a panel of 69 various bacterial strains from the genera of *Stenotrophomonas*, *Pseudomonas*, and *Burkholderia*. Iron-deficient Casamino Acids (CAA) medium (BD Bioscience) was used for the assay, as it is known that ColM-type bacteriocins exhibit enhanced antibacterial activity under iron-limited growth conditions by hijacking an existing ferrichrome-type transporter to gain access to target cells. For SmaltM, 5 out of the 19 *Stenotrophomonas* strains tested showed clear lysis zones and 2 strains generated turbid zones. SmaltM2 presented a clear lysis zone for 1 *Stenotrophomonas* strain and generated turbid zones for 5 strains. (**Table 9**). No sensitivity to stenocins was found among strains of other tested genera.

Table 9. Antibacterial activities of crude extracts, containing stenocins. Inhibition effects were evaluated according to the annular radius of growth inhibition zones. Radius of lysis zone: +++ , > 6 mm; T, turbid; reduced cell density compared with cell lawn.

***Stenotrophomonas maltophilia* group**		**Growth inhibition by**	
Strain Designation	Isolation	SmaltM	SmaltM2
12-30593	Hospital	+++	–
11-12535	Hospital	T	–
12-11325	Hospital	–	T
14-1590	Hospital	–	T
2-12-28 #50	Soil	–	T
2-4-11 #34	Soil	+++	–
2-8-21 #44	Soil	+++	–
2-9-23 #46	Soil	+++	–
2-12-31 #53	Soil	T	T
6-7-1 #150	Soil	+++	T
ATCC13637	Hospital	–	+++

Both stenocins lack one amino acid in their active center in comparison to ColM and other ColM-like bacteriocins (**Figure 27**). This "missing" residue could explain the low average activity and low pathogen coverage for both stenocins. One of the ways to improve it could be the site-directed mutagenesis, where the "missing" His-235 (122*) is introduced or even 6

“core” residues identical to those from ColM are introduced to both stenocins instead of their original sequences. This hypothesis is based on the measured activity data of ColM mutants. ColM was completely unable to inhibit cell growth after His-235 was mutated to Ala (Barreteau et al. 2010). At the same time, in another study, antibacterial activity was observed for ~7–26% of the tested strains for burkhocins BurM1 and BurM2, which are missing histidine and additional “core” mismatches (Ghequire & De Mot 2015). This indicates that histidine is not an essential amino acid in burkhocins. In the same study, however, BurM2 generated mostly turbid halos, not clear lysis zones. This brings back our idea of re-introducing the “missing” histidine into the active site for antimicrobial activity improvement.

OVERVIEW OF RESULTS

In this thesis, bacteriocins active against gram-negative pathogens were studied as a potential alternative to antibiotics. We demonstrated that plant-expressed pyocins from *Pseudomonas* and stenocins from *Stenotrophomonas*, like their *E. coli* analogues colicins reported earlier (Schulz et al. 2015a), can be expressed at high yields in plants and are fully functional. To the best of our knowledge, we were the first to describe peptidoglycan-degrading pyocin PaeM4 and the first to identify, report and describe peptidoglycan-degrading stenocins. Furthermore, we have found that by swapping pore-forming domains of *Pseudomonas* bacteriocins we can create highly active molecules with a broader spectrum of activity.

This work started with the analysis of six different pyocins – PyoS5, PaeM, PaeM4, PyoL1, PyoL2, and PyoL3. The activity of plant-made pyocins was confirmed in the agar diffusion, liquid culture susceptibility and biofilm assays, and in the *Galleria mellonella* animal infection model. Pyocin S5 demonstrated superior activity compared to all other pyocins. We decided to try to broaden the activity spectrum of this pyocin, by modifying its killing domain in order to avoid its recognition by the immunity protein. The goal was achieved by fusing receptor-binding and translocation domains of PyoS5 with pore-forming domain of bacteriocin PmnH from *P. synxantha*. *In vitro* experiments confirmed our hypothesis, as S5-PmnH chimera demonstrated broadened activity spectrum in comparison to pyocin S5. Ignited by these successes, we decided to go further and test S5-PmnH efficacy in murine models for topical treatment of two unrelated models of disease caused by *P. aeruginosa*: keratitis model and lung infection model.

S5-PmnH exhibited high efficacy in topically treating *P. aeruginosa*-induced eye infection comparable to that of the standard of care antibiotic tobramycin. S5-PmnH completely eliminated infection by cytotoxic P. aeruginosa strain ATCC 19666 as no viable bacteria were isolated at 5 dpi. S5-PmnH performed less efficiently when eyes were infected with invasive strain PAO1 – no viable bacteria were isolated from 1 mouse out of 3 at 5 dpi; the two remaining mice contained 5.14 $\log_{10}$ and 3.34 $\log_{10}$ (burden in the control group reached 6.73 $\log_{10}$). Lung infection model also produced promising results. A single application of chimeric pyocin S5-PmnH, at low concentration as 2.5 μg in validated mouse lung colonization model, reduced bacterial burden in the lungs below the level of stasis.

In the future, bacteriocins are expected to be a suitable alternative to antibiotics due to their many benefits, such as low toxicity for mammals, strong activity even at very low concentrations, specific modes of action

(Soltani et al. 2021), biodegradability, and the ability to target specific bacteria without harming others (Ghequire & De Mot 2019). Initially, the specific targeting of bacteria may appear to be a drawback as the type of bacteria causing the infection must be determined before selecting the appropriate bacteriocin. However, this results in the preservation of a healthy microflora as only the harmful bacteria are targeted and eliminated. By contrast, broad-spectrum antibiotics can disrupt the balance of microorganisms important for human health and lead to gut dysbiosis (Francino 2016).

Bacteriocins have limitations, including the risk of bacteria developing resistance (de Freire Bastos et al. 2015), and factors affecting efficacy such as pharmacokinetics and route of administration (Soltani et al. 2021). Unprotected bacteriocins will degrade in the digestive system when taken orally, but parenteral administration can avoid this issue (Benítez-Chao et al. 2021). Encapsulation (Chandrakasan et al. 2019) and combination therapies with other bacteriocins, antibiotics, phage lysins, and other antimicrobials/stressors can enhance their use and reduce antibiotic concentration needed for treatment.

Therefore, this study highlights the need for further development and improvement of *in vivo* models that can evaluate the effectiveness of bacteriocins as antimicrobial agents and assess any potential toxicity and side effects. This is crucial for determining their potential as therapeutic agents against infections caused by drug-resistant microorganisms.

CONCLUSIONS

1. Pyocins from *P. aeruginosa* and stenocins from *S. maltophilia* can be expressed at high yields in plants and are fully functional.
2. Pyocins S5, M, M4, L1, L2, L3 effectively kill planktonic and biofilm-forming *P. aeruginosa* bacteria *in vitro* and reduce bacteremia in *G. mellonella* model *in vivo.*
3. Chimeric pyocin S5-PmnH demonstrates broadened activity spectrum in comparison to PyoS5.
4. Chimeric pyocin S5-PmnH shows high efficacy in topical treatment of two unrelated murine models of disease caused by *P. aeruginosa* – keratitis and lung infection.
5. Two newly identified bacteriocins from *S. maltophilia* – SmaltM and SmaltM2 are active *in vitro* and have potential to be further improved as antibacterial protein biologics against *S. maltophilia* infections.

PUBLICATIONS

The list of publications and contributions forming the basis of this thesis:

1. **Paškevičius Š**, Starkevič U, Misiūnas A, Vitkauskienė A, Gleba Y, Ražanskienė A. 2017. Plant-expressed pyocins for control of Pseudomonas aeruginosa. *PLoS One*. 12(10):e0185782.
   Conceived and conducted the experiments in bulk, analyzed and prepared the results for publication, reviewed the manuscript.
2. **Paškevičius Š**, Dapkutė V, Misiūnas A, Balzaris M, Thommes P, et al. 2022. Chimeric bacteriocin S5-PmnH engineered by domain swapping efficiently controls Pseudomonas aeruginosa infection in murine keratitis and lung models. *Sci. Rep.* 12(1):5865.
   Conceived and conducted the experiments in bulk, analyzed and prepared the results for publication, reviewed the manuscript.
3. **Paškevičius Š**, Gleba Y, Ražanskienė A. 2022. Stenocins: Novel modular bacteriocins from opportunistic pathogen Stenotrophomonas maltophilia. *J. Biotechnol.* 351(April):9–12.
   Designed the study, conceived and conducted the experiments in bulk, analyzed and prepared the results for publication, wrote original draft and revised the publication.
4. Patent. **Paškevičius Š.**, Misiūnas A., Ražanskienė A. 2022, 22163290.4 (pat. pending), "Chimeric bacteriocins and method for the control of *Pseudomonas*", Nomad Bioscience Gmbh, UAB Nomads.

THESIS MATERIAL WAS PRESENTED IN THE FOLLOWING CONFERENCES[1]:

1. **Paškevičius Š**., Dapkutė V., Misiūnas A., Thommes P., Sattar A., Gleba Y., Ražanskienė A. Oral presentation "Chimeric Pyocin Efficiently Eradicates *Pseudomonas aeruginosa* in Mouse Keratitis and Lung Colonization odels", 32nd European Congress of Clinical Microbiology & Infectious Diseases, 2022, Lisbon, Portugal
2. **Paškevičius Š**., Schneider T., Ražanskienė A., Hahn S., Gyrych A. and Gleba Y. Oral presentation "LEGOCINS™: Chimaeric Bacteriocins Engineered by Domain Shuffling", Plant-Based Vaccines Antibodies and Biologics, 2019, Riga, Latvia
3. **Paškevičius, Š**., Starkevič, U., Misiūnas, A., Dapkutė, V., Vitkauskienė, A., Gleba, Y., Ražanskienė, A. Poster presentation "Plant-Expressed Pyocins as Weapons to Combat Pathogenic Bacteria", Our well-being and our microbes, 2017, Auckland, New Zealand
4. **Paškevičius, Š**., Starkevič U., Misiūnas A., Vitkauskienė A., Gleba Y., Ražanskienė A. Poster presentation "Plant-Expressed Pyocins", Plant-Based Vaccines Antibodies and Biologics, 2017, Albufeira, Portugal

[1]All presentations in the conferences were presented by the first authors.

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CURRICULUM VITAE

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RESEARCH EXPERIENCE

2011-current	***Research Scientist***, JSC "NOMADS". Participation in projects: · 2020-2022, Lithuanian Business Support Agency, grant 01.2.1-LVPA-K-856-01-0015, "Generation of novel non-antibiotic antimicrobial substances for treatment of *P. aeruginosa* and *Klebsiella* infections" · 2018-2022, Lithuanian Business Support Agency, grant J05-LVPA-K-03-0011, "Antimicrobial substance for treatment of *Klebsiella* infections" · 2016-2018, Lithuanian Business Support Agency, grant J05-LVPA-K-01-0035, "Antimicrobial substance for treatment *Pseudomonas aeruginosa* infections"
2011-2016	*Research Assistant*, Department of Eukaryote Gene Engineering, Vilnius University Institute of Biotechnology. Participation in project: · 2013-2015, Research Council of Lithuania, "Analysis of the Expression of Anthocyanin Pathway Genes in Horticultural Plants"
2010-2012	Professional practices funded by Research Council of Lithuania: · 2010 autumn · 2011 summer
2008-2011	*Trainee*, Department of Eukaryote Gene Engineering, Vilnius University Institute of Biotechnology

EDUCATION

2015-2019	**PhD studies in Biochemistry**, Department of Eukaryote Gene Engineering, Vilnius University Institute of Biotechnology
2011-2013	Master of Science, Biochemistry, thesis topic: "Construction of Biologically Contained Agrobacteria. Programmable Cell Death", supervisor Dr. Aušra Ražanskienė, Vilnius University

2007-2011	Bachelor of Science, Biochemistry, thesis topic: "Transient Expression of Pharmaceutical Proteins in Plants", supervisor Dr. Aušra Ražanskienė, Vilnius University
CONFERENCES	
2022	**Paškevičius Š.,** Dapkutė V., Misiūnas A., Thommes P., Sattar A., Gleba Y., Ražanskienė A**.** Oral presentation "Chimeric pyocin efficiently eradicates *Pseudomonas aeruginosa* in mouse keratitis and lung colonisation models", 32nd European Congress of Clinical Microbiology & Infectious Diseases, Lisbon, Portugal
2019	**Paškevičius Š.**, Schneider T., Ražanskienė A., Hahn S., Gyrych A. and Gleba Y. Oral presentation "LEGOCINS™: Chimaeric Bacteriocins Engineered by Domain Shuffling", Plant-Based Vaccines Antibodies and Biologics, Riga, Latvia
2017	**Paškevičius, Š.**, Starkevič, U., Misiūnas, A., Dapkutė, V., Vitkauskienė, A., Gleba, Y., Ražanskienė, A. Poster presentation "Plant-Expressed Pyocins As Weapons to Combat Pathogenic Bacteria", Our well-being and our microbes, Auckland, New Zealand
2017	**Paškevičius, Š.**, Starkevič U., Misiūnas A., Vitkauskienė A., Gleba Y., Ražanskienė A.. Poster presentation "Plant-Expressed Pyocins", Plant-Based Vaccines Antibodies and Biologics, Albufeira, Portugal
2011	Poster presentation, "Transient Expression of Pharmaceutical Proteins in Plants", International Conference "Advances in Plant Biotechnology in Baltic Region", Kaunas, Lithuania
2010	Oral presentation, "Transient Expression of Pharmaceutical Proteins in Plants", International Student's Conference of Natural Sciences COINS2010, Vilnius, Lithuania
COURSES	
2022	CodeAcademy "Python beginner course", Vilnius, Lithuania
2021	Lithuanian University of Health Sciences Centre For Postgraduate Studies training program "Keeping, care and usage requirements for animals used for scientific and educational purposes", Kaunas, Lithuania

2019	EMBO-FEBS Lecture Course “The new microbiology”, Spetses, Greece
AWARDS	
2016	*3rd place* at Three Minute Thesis (3MT) competition, Vilnius University, Lithuania
2014	*Best Presentation Award* **Paškevičius, Š.**, Ražanskienė, A. „Construction of biosafe Agrobacteria. Programmable cell death“, Life Science Baltics 2014, International Students Conference, Vilnius.
TEACHING	
2016-2018	Supervisor for bachelor thesis for Viktorija Dapkutė
2014-2016	Supervisor for master thesis for Justina Stankevičiūtė
2015-2016	Supervisor for bachelor thesis for Matas Raišys
FOREIGN LANGUAGES	
	English (fluent), French (basics), Russian (basics)

SANTRAUKA

1. Santrumpų sąrašas

AR – aptikimo riba
DAA – dauginis atsparumas antibiotikams
dpp – dienos po purškimo
dpu – dienos po užkrėtimo
KFV – kolonijas formuojantis vienetai
ND – nestruktūrizuotas domenas
PGL – prieš-gydyminis lygis
SN – standartinis nuokrypis
TMV – tabako mozaikos virusas

2. Įvadas

Visos gyvybės formos turi vieną esminį tikslą – išgyventi. Ne išimtis ir bakterijos, nesvarbu, ar jos naudingos, ar kenksmingos jas supančiai aplinkai. Tad nieko nuostabaus, jog bakterijos bet kuria kaina stengsis išvengti jų egzistencijai grėsmę keliančių medžiagų – šiuo atveju antibiotikų. Atsparumas antibiotikams yra natūralus reiškinys, prasidėjęs gerokai prieš masinės gamybos antibiotikų erą (Dcosta et al. 2011). XX a. pradžioje netikėtai atrastas penicilinas iš esmės pakeitė požiūrį į bakterines infekcijas – susidarė klaidingas įspūdis, jog bakterinių infekcijų problema išspręsta amžiams. Pernelyg dažnas ir netinkamas antibiotikų vartojimas paspartino antibiotikų atsparumo procesą taip, kad dabar jis yra vienas pagrindinių XXI a. grėsmių visuomenės sveikatai (Murray et al. 2022). Nuolatos didėjantį vartojimą vaizdžiai iliustruoja tokie skaičiai: 1941 m. pasaulinės penicilino atsargos buvo vos keli miligramai palyginti su 4000 kg 1945 m. (Bud 2007) ir su 2012 m. vien Jungtinėse Amerikos Valstijose suvartotomis ~12 tonų antibiotikų (O'Neill 2015). Ateities prognozės yra siaubingos ir apokaliptinės – tikėtina, kad 2050 m. bakterinių infekcijų sukeltų mirčių skaičius iš 2019 m. užregistruotų 1,3 mln. mirčių padidės iki 50 mln. žmonių per metus (Murray et al. 2022). Gram-neigiamos bakterijos, priklausančios *Pseudomonas* ir *Stenotrophomonas* gentims, yra tik keli patogenų, lengvai įgyjančių atsparumą visiems žinomiems antibiotikams, atstovai. Tokios aplinkybės primygtinai reikalauja naujos kartos antimikrobinių medžiagų kūrimo.

Bakteriocinai yra daug žadanti alternatyva antibiotikams. Bakteriocinai yra bakterijų koduojami antibakteriniai baltymai žudantys filogenetiškai

gimininga bakterijas. Gram-neigiamos bakterijos gamina keturias pagrindines bakteriocinų klases: modulinius, panašius į fagų uodegas, panašius į lektinus ir mikrocinus. Moduliniai bakteriocinai pasižymi bendra tri-domenine architektūra ir yra sudaryti iš translokacijos, receptoriaus ir citotoksinio domenų. Citotoksinis domenas užtikrina bakteriocino gebėjimą galabyti bakterijas ir jo veikimo mechanizmas varijuoja nuo porų formavimo ar peptidoglikano sintezės blokavimo iki fermentinio nukleorūgščių – DNR, tRNR, rRNR – skaldymo (Ghequire & De Mot 2014).

Moduliniai bakteriocinai gali būti lengvai panaudojami personalizuotoje medicinoje, nes jie, priešingai nei antibiotikai, yra labai selektyvūs naikinant bakterijų rūšis. Ši savybė sumažina natūralios ir sveikos žarnyno mikrobiotos išbalansavimo tikimybę (Francino 2016). Tokie privalumai kaip mažas toksiškumas žinduolių ląstelėms, didelis aktyvumas nanomoliariniame diapazone, specifiniai veikimo mechanizmai, biologinis skaidumas ir, svarbiausia, aktyvumas prieš antibiotikams atsparias bakterijas, daro bakteriocinus patraukliais kandidatais terapiniam naudojimui tiek žmonėms, tiek gyvūnams (Soltani et al. 2021). Augalinė raiškos sistema siūlo patrauklią bakteriocinų gamybos platformą, nes daugybė bakteriocinų iš įvairių gram-neigiamų bakterijų rūšių jau buvo sėkmingai susintetinti ir išgauti su didele išeiga *Agrobacterium* tarpininkaujamoje laikinosios raiškos sistemoje tabake (Denkovskienė et al. 2019; 2022a; Schneider et al. 2018; Schulz et al. 2015a).

Šioje disertacijoje daugiausia dėmesio skiriama modulinių bakteriocinų iš gram-neigiamų bakterijų identifikavimui, charakterizavimui, gamybai ir jų galimo pritaikymo žmonių ir gyvūnų infekcijoms gydyti tyrimui.

Pagrindinis **darbo tikslas** yra sukurti aktyvius antimikrobinius preparatus prieš patogenines gram-neigiamas bakterijas. Tam pasiekti išsikelti šie **uždaviniai**:

1. Nustatyti numanomas bakteriocinų sekas *Pseudomonas* ir *Stenotrophomonas* rūšių genomuose, jas klonuoti į raiškos vektorius, ištirti raišką augaluose ir išgryninti iki homogeniškumo.
2. Nustatyti bakteriocinų veiksmingumą *in vitro* ir *in vivo* eksperimentuose.
3. Sukurti pranašesnius chimerinius bakteriocinus.

Mokslinis naujumas ir aktualumas:

Nepaisant to, jog pirmasis bakteriocinas nustatytas beveik prieš 100 metų (Gratia 1925), klinikinis bakteriocinų taikymas tik pradeda įgauti pagreitį. Šioje disertacijoje gauti duomenys „išlieja pamatus“ augaluose susintetintų bakteriocinų, kaip būsimų vaistinių preparatų, kūrimui. Aiškiai įrodyta, kad visi tirti bakteriocinai: šeši piocinai iš *Pseudomonas* ir du

stenocinai iš *Stenotrophomonas* sugeba naikinti atitinkamas bakterijų rūšis *in vitro.* Be to, atrinkti aktyviausi piocinai patvirtino savo, kaip antimikrobinių medžiagų, potencialą *in vivo*, sėkmingai gydydami infekuotas *Galleria mellonella* lervas. Atlikus poras formuojančių piocinų domenų sumainymo eksperimentus, sukurtas pranašesnis chimerinis piocinas S5-PmnH ir tuo pačiu parodytos beveik neribotos galimybės kurti bakteriocinus su modifikuotais ar išplėstais aktyvumo spektrais. Naujai sukurtas chimerinis S5-PmnH demonstravo puikų efektyvumą kaip išorinis vaistas gydant pelėse *Pseudomonas. aeruginosa* sukeltą keratitą. Be to, intranazaliniu būdu vartojamas S5-PmnH veiksmingai naikino *P. aeruginosa* infekciją pelių plaučiuose. Džiuginantys chimerinio piocino S5-PmnH rezultatai paskatino mus pateikti Europos patentų biurui paraišką pavadinimu: „Chimeriniai bakteriocinai ir metodai *Pseudomonas* kontrolei“. Apibendrinus visus rezultatus, manome, jog augaluose pagaminti bakteriocinai turėtų būti laikomi perspektyvia antibiotikų alternatyva patogeninių gram-neigiamų bakterijų kontrolei.

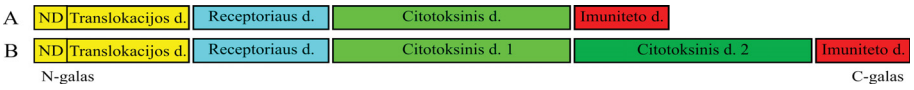
3. Metodai

Šiame darbe panaudoti įvairūs metodai – antibakterinio aktyvumo tyrimai *in vitro* (difuzijos į agarą metodas, skystoje terpėje ir bioplėvelėse), *G. mellonella* lervų infekcija, *ex vivo* kiaulių ragenos infekcijos modelis, *P. aeruginosa* sukeltas keratitas ir plaučių infekcija pelėse. Difuzijos į agarą metodas leido atsirinkti perspektyviausius augaluose susintetintus ir išgrynintus bakteriocinus. *G. mellonella* lervų modelis padėjo atsirinkti būsimų antimikrobinių preparatų kandidatus, taip sumažindamas eksperimentų skaičių su žinduoliais. *Ex vivo* kiaulių ragenų modelis leido patikrinti bakteriocinų galimą panaudojimą gydant bakterinį keratitą. Galiausiai, eksperimentai su pelėmis – tiek gydant bakterinį keratitą, tiek gydant sukeltą plaučių infekciją atskleidė, jog tirti bakteriocinai yra veiksmingi gydant išoriškai. Visi naudoti metodai detaliai aprašyti atskirose publikacijose.

4. Darbo objektai

Šis darbas atliktas su gram-neigiamų bakterijų koduojamais moduliniais bakteriocinais. Moduliniai bakteriocinai yra ~20-80 kDa dydžio baltyminiai toksinai ir, kaip jau minėta įvade, jų bendras bruožas yra trijų

domenų struktūra – centrinis receptoriaus domenas jungiasi su specifiniais išorinėje bakterijų membranoje esenčiais receptoriais, N-galinis translokacijos domenas su nestruktūrizuotu domenu (ND, *angl. IUTD – intrinsically unstructured T domain*) yra atsakingas už bakteriocino pernešimą per išorinę membraną į periplazmą, o C-galinis citotoksinis domenas yra bakteriocino aktyvusis centras, atsakingas už bakterijų naikinimą (**1A pav.**). Visi iki šiol nustatyti moduliniai bakteriocinai į ląstelę perneša vieną C-galo citotoksinį domeną, išskyrus neseniai nustatytą *Pseudomonas synxantha* koduojamą bakteriociną PmnH su dviguba toksino struktūra (**1B pav.**) (Ghequire et al. 2017a).



1 paveikslas. Modulinių bakteriocinų domenų išsidėstymo schema. **(A)** Bakteriocinai su vienu citotoksiniu domenu. **(B)** Bakteriocinai su dviem citotoksiniais domenais. ND – nestruktūrizuotas domenas, d. – domenas.

Už ląstelių mirtį yra atsakingas citotoksinis domenas, veikiantis įvairiose ląstelės vietose:

- Periplazmoje – depoliarizuoja ląstelę suformuodamas porą vidinėje ląstelės membranoje arba fermentiškai skaidydamas peptidoglikano sintezei būtiną pirmtaką – lipidą-II.
- Citoplazmoje – fermentiškai skaido nekleorūgštis – DNR, tRNR, rRNR.

Bakteriocinus gaminančių bakterijų savisauga yra užtikrinama sintetinant priešnuodį – imuniteto baltymą.

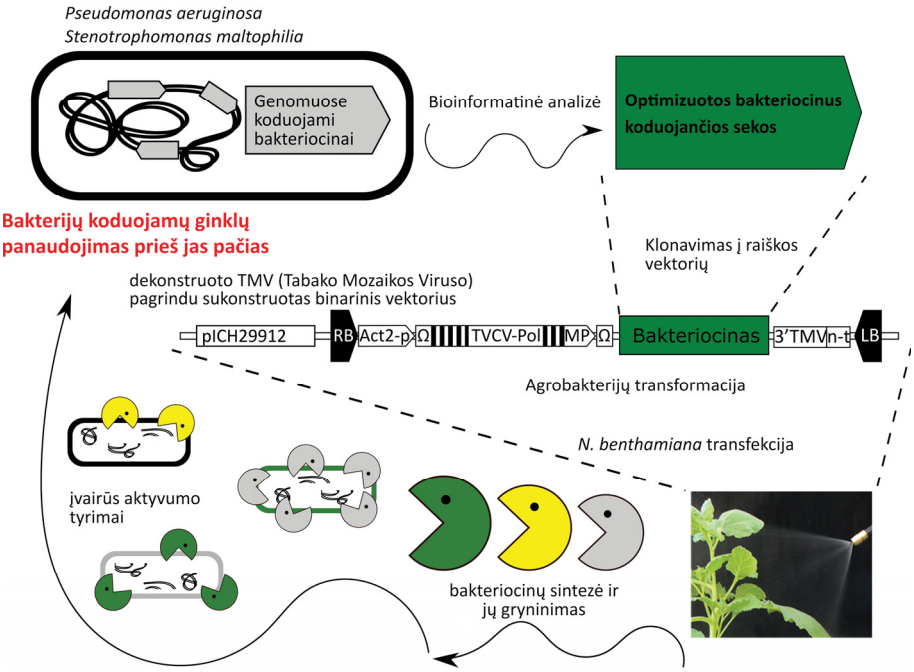
Šių priešnuodžių koduojančios sekos paprastai eina iškart po bakteriocino genų. Periplazmoje veikiančius bakteriocinus nuginkluoja arba vidinėje membranoje įtvirtintas periplazminis baltymas, arba integralusis membranos baltymas (Ghequire et al. 2017b). Citoplazmines nukleazes slopina tuo pačiu metu pagaminamas citoplazminis imuniteto baltymas (Kleanthous & Walker 2001).

Gram-neigiamios bakterijos yra apgaubtos kompleksišku ląstelės apvalkalu, kurį sudaro (Francis et al. 2021):

- asimetrinė išorinė membrana su lipopolisacharidais išorinėje dalyje ir fosfolipidais vidinėje dalyje;
- periplazma;
- energizuota vidinė membrana.

Gram-neigiamų bakterijų išorinė membrana atstoja tvirtą gynybinį skydą, apsaugantį nuo nepalankaus aplinkos poveikio, o taip pat ir nuo dalies antibiotikų (Francis et al. 2021). Kiekvienam bakteriocinui, norinčiam nužudyti bakteriją, tenka kirsti vieną arba netgi abi gram-neigiamų bakterijų ląstelių membranas.

Ankstesni tyrimai atskleidė, jog augaluose susintetinti kolicinų šeimos bakteriocinai gali būti veiksmingos antibakterinės medžiagos prieš visus pagrindinius enteropatogeninius *Escherichia coli* kamienus (Schulz et al. 2015a). Šių rezultatų įtakoje, į kolicinus panašių bakteriocinų tyrimus išplėtėme iki piocinų ir stenocinų kuriuos atitinkamai gamina *P. aeruginosa* ir *Stenotrophomonas maltophilia* kamienai. Šio darbo bendra eksperimentų eiga pavaizduota **2 pav.**

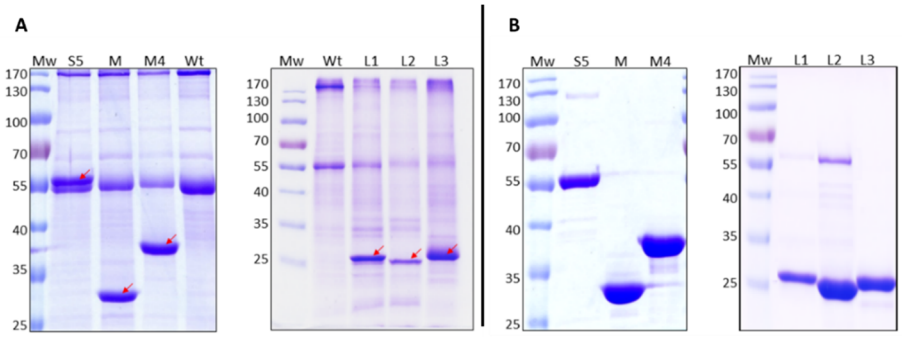


2 paveikslas. Bakteriocinų tyrimų schema.

5. Rezultatai

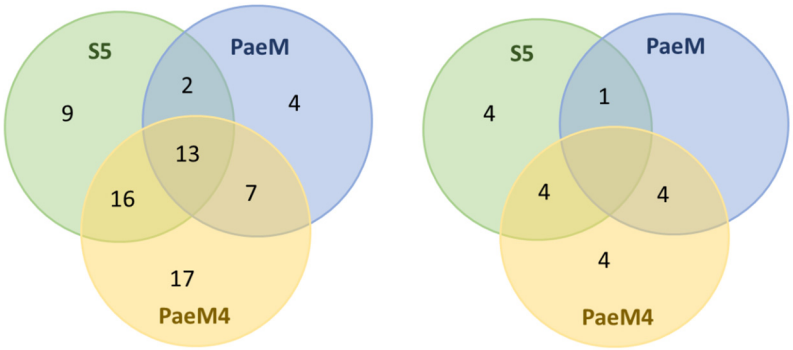
Piocinų S5, M, M4, L1, L2, L3 kova su *P. aeruginosa* *(1 publikacija)*

Piocinų gebėjimas gydyti *P. aeruginosa* infekcijas ne kartą pademonstruotas gyvūnų modeliuose (McCaughey et al. 2016c; Scholl & Martin 2008; Smith et al. 2012). Mes susintetinome poras formuojantį piociną S5, peptidoglikano sintezę blokuojančius piocinus PaeM ir PaeM4 ir į lektinus panašius piocinus PyoL1, PyoL2, PyoL3 labai efektyvioje augalų laikinosios raiškos sistemoje ir išgryninome juos iki homogeniškumo. Remdamiesi *in vitro* ir *in vivo* (*G. mellonella* infekcijos modelyje) gautais rezultatais, parodėme, jog šiuos piocinus galima naudoti atskirai arba kombinacijose kovojant su *P. aeruginosa*. Pirmiausia, minėtų bakteriocinų koduojančios sekos buvo optimizuotos sintezei tabake *Nicotiana benthamiana*, įterptos į plazmidę pICH29912 (TMV pagrindu sukurtas magnICON® vektorius) ir galiausiai sukonstruotos plazmidės panaudotos *A. tumefaciens* transformacijai. 4–6 savaičių amžiaus augalų lapai nupurkšti 1:1000 praskiestais rekombinantiniais *A. tumefaciens* kamienais. Transformuoti augalų lapai buvo surenkami 5–10 dpp (dienos po purškimo) ir naudojami dviejų pakopų chromatografijai. Iš pradžių išgrynintų (**3 pav.**) piocinų aktyvumas buvo patikrintas difuzijos į agarą metodu prieš 12 skirtingų *P. aeruginosa* kamienų.



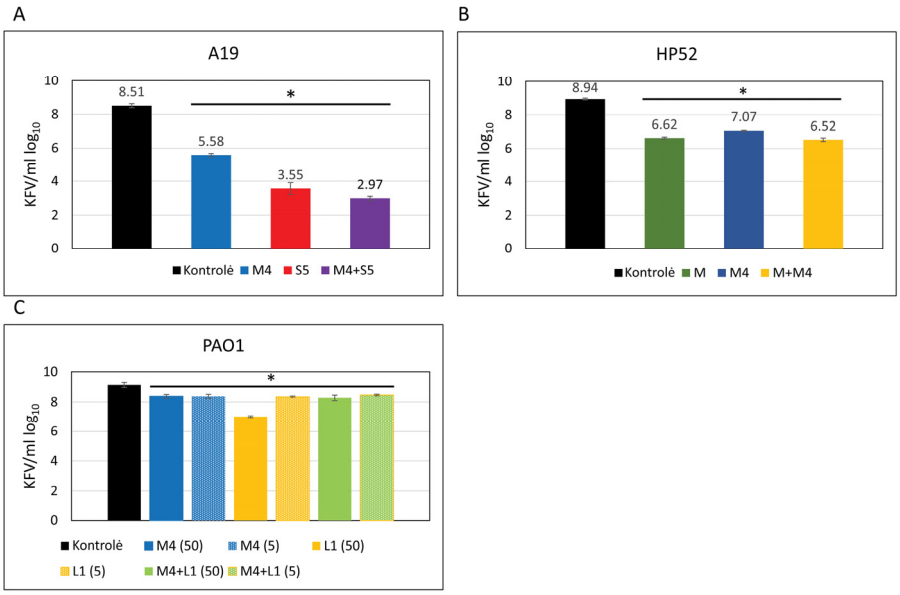
3 paveikslas. Piocinų raiška augaluose. Denatūruojančios elektroforezės su natrio dodecilsulfatu poliakrilamido geliai (12 %). **(A)** Neapdoroti mėginiai iš *N. benthamiana* lapų. **(B)** Išgryninti piocinai. Mw – markeris, dydžiai nurodyti kilodaltonų tikslumu, Wt – nepurkštų *N. benthamiana* lapų mėginiai, S5, M, M4, L1, L2, L3 – *N. benthamiana* lapų mėginiai, purkšti agrobakterijomis su piocinų raiškos konstruktais (piocinas S5, PaeM, PaeM4 ir į lektinus panašūs piocinai L1, L2, L3), rodyklėmis pažymėtos juostos atitinka tikslinius piocinus.

Visi piocinai buvo aktyvūs ir visi tirti kamienai demonstravo jautrumą bent vienam iš piocinų. Sekančio eksperimento metu ištyrėme PyoS5, PaeM ir PaeM4 aktyvumus prieš 100 klinikinių kamienų. Parodėme, jog trijų piocinų mišinys veikia plačiai – prieš 68 iš 100 tirtų patogeninių kamienų (**4 pav., kairė**). 21 iš 100 tirtų kamienų buvo atsparūs antibiotikams. 43 % (9 iš 21) kamienų rodė jautrumą piocinui S5, 24 % (5 iš 21) – PaeM, o net 58 % (12 iš 21) buvo jautrūs PaeM4 (**4 pav., dešinė**).



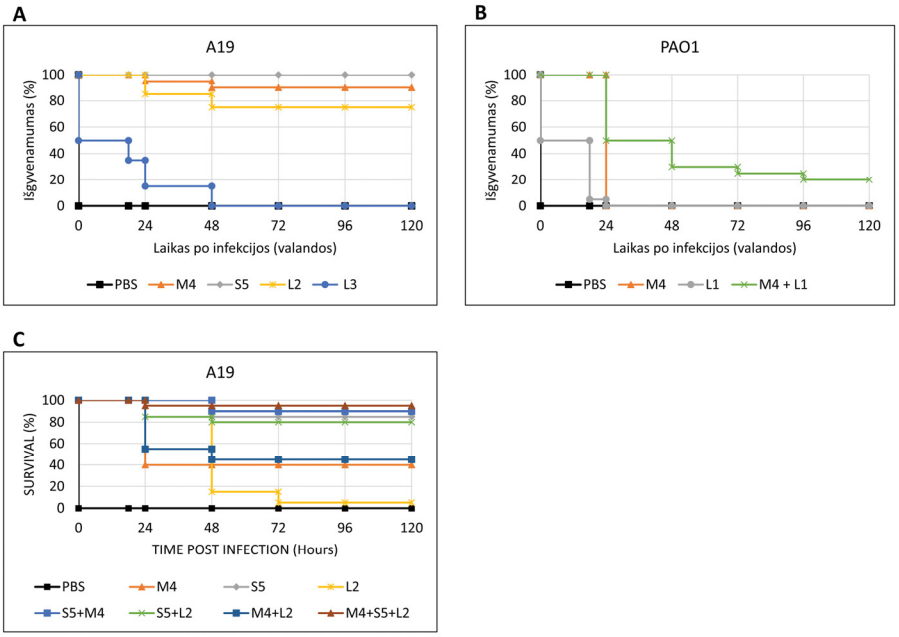
4 paveikslas. Venn‘o diagramos vaizduojančios klinikinių kamienų jautrumą trims augaluose susintetintiems piocinams. Kairėje – visų 100 tirtų kamienų jautrumas, dešinėje – 21 antibiotikams atsparių kamienų jautrumas.

Toliau įvertinome piocinų aktyvumą skystose kultūrose prieš tris atrinktus kamienus – PAO1, A19, HP52 (**5 pav.**). Geriausi rezultatai pasiekti su A19 kamienu (**5A pav.**) – PyoS5 ir PaeM4 kokteilis sumažino KFV (Kolonijas Formuojantys Vienetai) skaičių net per 5,5 eiles.



5 paveikslas. Piocinų aktyvumai skystoje kultūroje. **(A)** Aktyvumai prieš A19 kamieną. **(B)** Aktyvumai prieš HP52 kamieną. **(C)** Aktyvumai prieš PAO1 kamieną. Pateikti duomenys yra trijų nepriklausomų eksperimentų vidurkis ± SN (standartinis nuokrypis). * žymi statistinį reikšmingumą (P≤0,001) lyginant piocinų aktyvumą su kontrole pagal vienpusį ANOVA testą su Bonferroni korekcija.

Panašūs rezultatai pasiekti ir tiriant piocinų aktyvumą prieš vienos dienos senumo *P. aeruginosa* bioplėveles – piocinas S5 stipriausiai naikino A19 kamieno suformuotą bioplėvelę ir sumažino KFV skaičių beveik trimis eilėmis. Įtikinami *in vitro* rezultatai leido mums pereiti prie *in vivo* eksperimentų – patikrinti piocinų gebėjimą išgydyti *G. mellonella* lervas nuo *P. aeruginosa* sukeltos infekcijos (**6 pav.**). A19 kamienu užkrėstos lervos buvo gydytos su 10 µg piocinų S5, M4, L2 ir L3. Gydymas su S5, M4 ir L2 išgelbėjo 75–100 % lervų, o gydymas su PyoL3 gerokai pailgino lervų išgyvenamumą, tačiau galiausiai gyvūnų neišgelbėjo (**6A pav.**). Kitame eksperimente, kai infekcijai sukelti naudotas PAO1 kamienas, nė vienas iš dviejų tirtų piocinų negalėjo visiškai išgydyti lervų nuo infekcijos, tačiau abu piocinai gerokai prailgino lervų išgyvenamumą (**6B pav.**). PaeM4 visiškai apsaugojo visas lervas 24 valandas po užsikrėtimo, o PyoL1 apsaugojo 50 % lervų 18 valandų. Naudojant PaeM4 ir PyoL1 mišinį išgyvenamumas buvo gerokai didesnis – 50 % lervų išgyveno 48 val. po užsikrėtimo, o 20 % lervų išgyveno iki eksperimento pabaigos (5 dienas).



6 paveikslas. *P. aeruginosa* užkrėstų *G. mellonella* lervų gydymas piocinais. **(A)** Lervos, užkrėstos A19 kamienu ir gydytos nurodytu piocinu. **(B)** Lervos, užkrėstos PAO1 kamienu ir gydytos nurodytu piocinu arba nurodytų piocinų mišiniu. Išgyvenamumo kreivės nubraižytos ir analizuotas naudojant Kaplan-Maier metodą su XLSTAT programine įranga.

Pagrindiniai publikacijos 1 teiginiai:

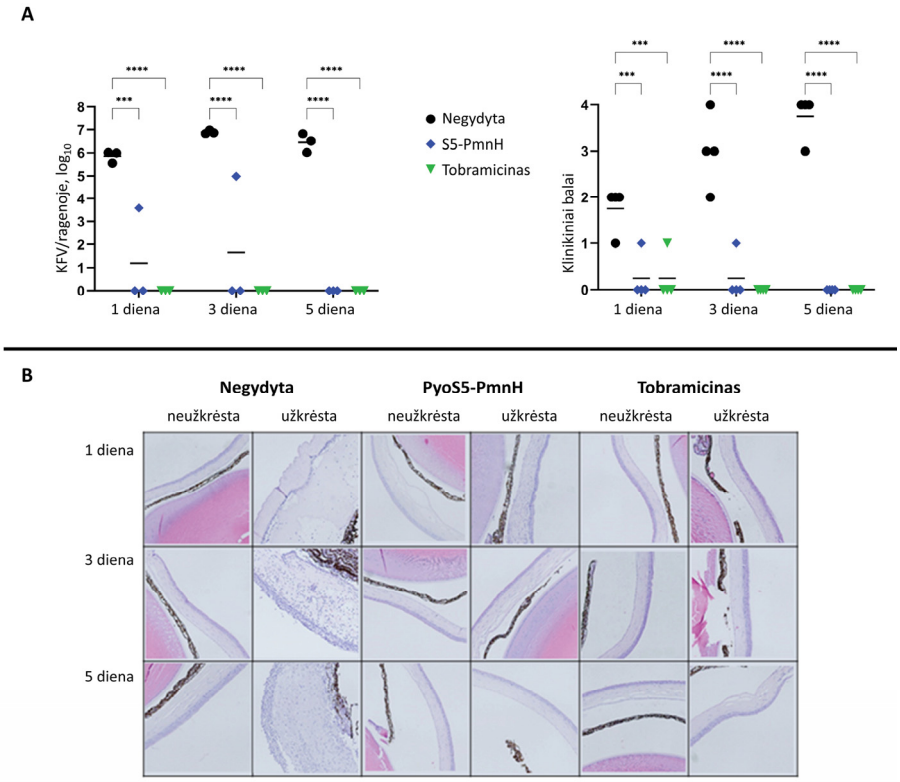
- Piocinai yra efektyviai sintetinami augaluose ir yra funkcionalūs.
- Piocinai yra veiksmingi tiek prieš planktonines, tiek prieš bioplėvelę sudarančias bakterijas.
- Piocinai yra aktyvūs *in vivo*, naudojant *G. mellonella* gyvūnų infekcijos modelį.

Chimerinis bakteriocinas S5-PmnH veiksmingai gydo *P. aeruginosa* infekcijas pelių modeliuose (2 *publikacija*)

Viena iš pagrindinių kliūčių bakteriocinų pritaikomumui medicinoje yra ribotas jų aktyvumo spektras. Nors poras formuojantis piocinas S5, lyginant su visais kitais piocinais, pasižymėjo geresniu aktyvumu mažinant bakterijų skaičių *in vitro* ir *in vivo*, jis buvo aktyvus tik prieš 40 % tirtų klinikinių kamienų (1 publikacija). Bakterijos geba apsaugoti save nuo susintetintų piocinų žudančio poveikio kartu su bakteriocinu sintetindamos ir

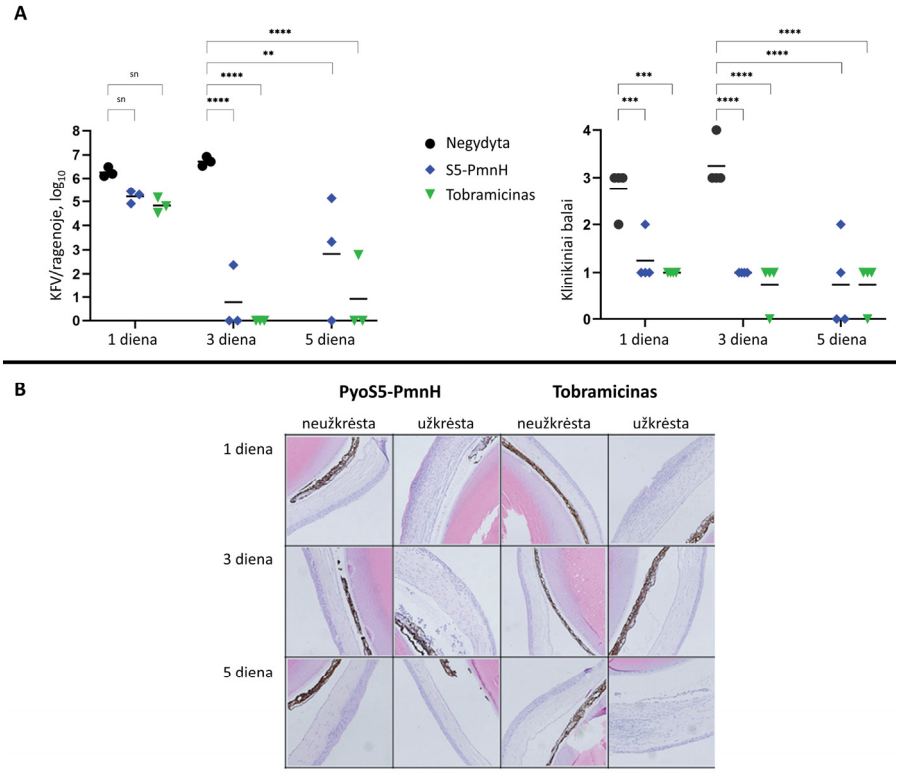
imuniteto baltymą. Tokiu būdu, viena iš priemonių išplėsti S5 aktyvumo spektrą būtų modifikuoti jo žudymo domeną, kad jo neatpažintų imuniteto baltymas. Kitos *Pseudomonas* rūšys (ypač *P. fluorescens*) koduoja ne vieną į bakteriocinus panašų poras formuojantį baltymą. Naudodamiesi GenBank duomenų baze, atrinkome spėjamai poras formuojančių bakteriocinų sekas iš įvairių *Pseudomonas* rūšių ir galiausiai atrinkome šešis kandidatus. Tada panaudojome šių bakteriocinų citotoksinius (poras formuojančius) domenus chimerinių baltymų konstravimui, sujungdami juos su piocino S5 receptorių jungiančiu ir translokacijos domenais. Atlikę sukonstruotų chimerų aktyvumo tyrimus *in vitro*, atrinkome geriausiai veikiantį chimerinį baltymą S5-PmnH, kuris, palyginti su piocinu S5, pasižymėjo platesniu aktyvumo spektru. Be to, S5-PmnH aktyvumą ištyrėme dviejose skirtinguose gyvūnų ligų modeliuose – išoriškai gydant *P. aeruginosa* sukeltą keratitą pelėse (**7, 8 pav.**) ir inhaliaciniu būdu naikinant *P. aeruginosa* infekciją pelių plaučiuose (**9 pav.**).

Dviejų tipų *P. aeruginosa* kamienai yra sutinkami keratito ligos atvejais – citotoksiniai *P. aeruginosa* kamienai dažniausiai sukelia keratitą, susijusį su kontaktinių lęšių nešiojimu, o invaziniai kamienai dažniausiai sukelia ligą po chirurginių komplikacijų. Mes ištyrėme, ar abiejų tipų kamienai yra jautrūs mūsų sukurtam chimeriniam baltymui. S5-PmnH sugebėjo visiškai išgydyti citotoksinę infekciją (*P. aeruginosa* ATCC 19660, 6 val. trukmės infekcija) – praėjus 5 dienoms po užkrėtimo (dpu) nei vienoje iš trijų tirtų pelių nebuvo išskirta gyvybingų bakterijų (**7A pav.**).



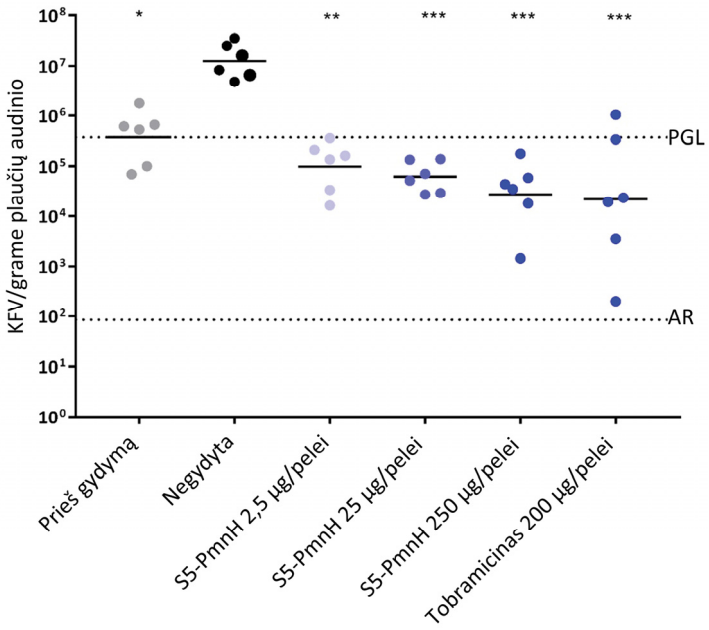
7 paveikslas. Pelių ragenos užkrėtimas citotoksiniu *P. aeruginosa* kamienu ATCC 19660 ir po to sekantis gydymas chimeriniu bakteriocinu S5-PmnH arba antibiotiku tobramicinu. **(A)** KFV skaičius ir pelių akių klinikiniai balai. **(B)** Ragenos pjūvių dažymas hematoksilinu-eozinu. Duomenys analizuoti taikant dvipusį ANOVA testą su Dunnett korekcija daugkartiniams palyginimams su GraphPad (versija 9.0.0). Horizontalia juosta pažymėtas kiekvieno gydymo būdo efektyvumo geometrinis vidurkis. ***P ≤ 0,001, ****P ≤ 0,0001 lyginant su kontrole.

Invazinio keratito infekcija gydyta šiek tiek su mažesniu efektyvumu – 5 dpu iš vienos gydytos pelės nebuvo išskirta nė viena bakterija, o likusiose dviejose pelėse izoliuota atitinkamai 5,14 $\log_{10}$ ir 3,34 $\log_{10}$ KFV/ragenoje (kontrolinės pelių grupėse bakterijų skaičius siekė 6,73 $\log_{10}$ KFV/ragenoje) (**8A pav.**).



8 paveikslas. Pelių ragenos užkrėtimas invaziniu *P. aeruginosa* kamienu PAO1 ir po to sekantis gydymas chimeriniu bakteriocinu S5-PmnH arba antibiotiku tobramicinu. **(A)** KFV skaičius ir pelių akių klinikiniai balai. **(B)** Ragenos pjūvių dažymas hematoksilinu-eozinu. Duomenys analizuoti taikant dvipusį ANOVA testą su Dunnett korekcija daugkartiniams palyginimams su GraphPad (versija 9.0.0). Horizontalia juosta pažymėtas kiekvieno gydymo būdo efektyvumo geometrinis vidurkis. **P ≤ 0,01 ****P ≤ 0,001, ****P ≤ 0,0001 lyginant su kontrole. sn – statistiškai nereikšmingas P > 0,05.

Kitos ligos modelyje, pelių plaučiai buvo infekuoti ir kolonizuoti su *P. aeruginosa* ATCC 27853 kamienu intranazaliniu būdu (**9 pav.**). Gydymas pradėtas praėjus 5 val. po infekcijos. Visose S5-PmnH gydytose grupėse bakterijų kiekis (KFV) sumažėjo iki žemesnio nei prieš pradedant gydymą lygio: per 0,58 eilę 2,5 µg/pelei grupėje, 0,78 eilę 25 µg/pelei grupėje ir 1,13 eilę 250 µg/pelei grupėje. Didesnis KFV sumažėjimas fiksuotas vartojant didesnes S5-PmnH dozes, tačiau skirtumai nebuvo statistiškai reikšmingi. Tobramicinas sumažino bakterijų kiekį per 1,23 eiles, bet šioje grupėje pastebėtas didesnis išsibarstymas lyginant su S5-PmnH grupėmis.



9 paveikslas. Pelių plaučių užkrėtimas *P. aeruginosa* kamienu ATCC 27853 ir po to sekantis gydymas chimeriniu bakteriocinu S5-PmnH arba antibiotiku tobramicinu. Duomenys analizuoti taikant atitinkamus neparametrinius statistinius modelius (Kruskal-Wallis naudojant Conover-Inman visiems poriniams grupių palyginimams atlikti) su StatsDirect (versija 3.3.3). Horizontalia juosta pažymėtas kiekvieno gydymo būdo efektyvumo geometrinis vidurkis. *P ≤ 0,05, **P ≤ 0,0005, ***P ≤ 0,0001, palyginti su kontrole. PGL – prieš-gydyminis lygis, AR – aptikimo riba.

Pagrindiniai publikacijos 2 teiginiai:

- Sukurtas chimerinis bakteriocinas S5-PmnH demonstruoja platesnį aktyvumo spektrą lyginant su PyoS5.
- S5 PmnH efektyviai naikina bakterijas ir apsaugo nuo *P. aeruginosa* sukelto keratito pelių modelyje.
- S5 PmnH efektyviai naikina *P. aeruginosa* sukeltą plaučių kolonizaciją pelių ligos modelyje.

Bakteriocinai SmalM ir SmaltM2 prieš *S. maltophilia* (*3 publikacija*)

S. stenotrophomonas yra dar vienas vis daugiau dėmesio klinikinėje praktikoje sulaukiantis dauginio atsparumo antibiotikams (DAA) patogenas. Paskatinti ankstesnių publikacijų (1 ir 2) rezultatų, taikėme panašų metodą *S. maltophilia* moduliniams bakteriocinams nustatyti ir apibūdinti. Šioje

publikacijoje sėkmingai pristatomi du nauji nariai – pirmieji modulinių bakteriocinų grupės atstovai: stenocinai SmaltM ir SmaltM2. Pirmiausia, atlikę BLAST paiešką, kaip užklausą naudojant ColM (Pfam PF14859, abu stenocinai priklauso pogrupiui, kurie turi ColM domeną (M tipo), toksino modulį, blokuojantį peptidoglikano biosintezę), nustatėme numanomas aminorūgščių sekas iš NCBI. Antra, parodėme, kad šie bakteriocinai taip pat veiksmingai sintetinami tabako augale – *N. benthamiana*. Stenocinų SmaltM ir SmaltM2 antibakterinis aktyvumas įvertintas naudojant difuzijos į agarą testą prieš 69 įvairius *Stenotrophomonas*, *Pseudomonas* ir *Burkholderia* genčių kamienus. SmaltM atveju, 5 iš 19 tirtų *Stenotrophomonas* kamienų parodė skaidrias lizės zonas, o 2 kamienai pasižymėjo drumstomis zonomis. SmaltM2 vienam *Stenotrophomonas* kamienui demonstravo aiškią lizės zoną, o 5 kamienams – drumstą zoną (**1 lentelė**). Kitų tirtų bakterinių genčių kamienai nedemonstravo jautrumo stenocinams.

Pagrindiniai publikacijos 3 teiginiai:

- Nustatyti du nauji bakteriocinai – stenocinai SmaltM ir SmaltM2, kaip pirmieji *S. maltophilia* modulinių bakteriocinų grupės atstovai.
- Stenocinai yra sintetinami augaluose dideliais kiekiais ir yra funkcionalūs.

1 lentelė. Stenocinų SmaltM ir SmaltM2 antibakterinis aktyvumas. Antibakterinis poveikis vertintas pagal lizės zonų spindulį: +++ , > 6 mm; T – drumstas, sumažėjęs ląstelių tankis.

S. maltophilia		**Antibakterinis aktyvumas**	
Kamieno identifikacinis numeris	Aptikimo vieta	SmaltM	SmaltM2
12-30593	Ligoninė	+++	–
11-12535	Ligoninė	T	–
12-11325	Ligoninė	–	T
14-1590	Ligoninė	–	T
2-12-28 #50	Dirvožemis	–	T
2-4-11 #34	Dirvožemis	+++	–
2-8-21 #44	Dirvožemis	+++	–
2-9-23 #46	Dirvožemis	+++	–
2-12-31 #53	Dirvožemis	T	T
6-7-1 #150	Dirvožemis	+++	T
ATCC13637	Ligoninė	–	+++

REZULTATŲ APTARIMAS

Šiame darbe buvo tiriami bakteriocinai, veikiantys prieš gram-neigiamus patogenus, kaip alternatyvūs antibiotikams antimikrobiniai preparatai. Parodėme, jog *Pseudomonas* piocinai ir *Stenotrophomonas* stenocinai, kaip ir anksčiau aprašyti jų analogai kolicinai iš *E. coli* (Schulz et al. 2015a), gali būti gaminami augaluose dideliu kiekiu ir yra visiškai funkcionalūs. Kiek mums žinoma, mes pirmieji aprašėme peptidoglikaną skaidantį piociną PaeM4 ir pirmieji nustatėme, pranešėme ir aprašėme peptidoglikaną skaidančius stenocinus. Be to, nustatėme, kad sukeitus *Pseudomonas* bakteriocinų poras formuojančius domenus, galima sukurti labai aktyvias molekules, pasižyminčias platesniu aktyvumo spektru. Šis darbas prasidėjo nuo šešių skirtingų piocinų – PyoS5, PaeM, PaeM4, PyoL1, PyoL2 ir PyoL3 – analizės (1 publikacija). Augaluose susintetintų piocinų aktyvumas buvo patvirtintas, difuzijos į agarą metodu, skystose kultūrose ir bioplėvelių tyrimuose bei *G. mellonella* gyvūnų infekcijos modelyje. Sėkmingi *in vitro* ir *in vivo* eksperimentai leido mums iškelti hipotezę, jog piocinų aktyvumo platumas ir stiprumas gali būti dar labiau patobulinti pasitelkiant genų inžineriją, sukeičiant vietomis jų receptoriaus/translokacijos ir citotoksinius domenus. Idėja sukurti naujas hibridines molekules nėra nauja, tačiau, kiek mums žinoma, ankstesni bandymai sukurti chimerinius antimikrobinius preparatus buvo grindžiami keičiant filogenetiniu ir (arba) funkciniu požiūriu nesusijusių baltymų domenus. Keletas įsimintiniausių pavyzdžių apima endolizinus su prijungtu ląsteles skverbiančiu peptidu, skirtu palengvinti gram-neigiamų bakterijų išorinės membranos kirtimui (Briers et al. 2014), „hibridinį liziną“, sudarytą iš pesticido receptoriaus domeno, sujungto su T4 lizocimo N-galu (Lukacik et al. 2012) ir PyS2-GN4 „lizociną“ sudarytą iš piocino S2 receptoriaus ir translokacijos domenų sujungtų su T4 lizocimo N-galu (Heselpoth et al. 2019). Nors visi šie hibridiniai pavyzdžiai buvo funkcionalūs ir aktyvūs, praktinis šių chimerinių bakteriocinų panaudojimas yra ribotas dėl jų siauro aktyvumo spektro. Todėl pasirinkome šiek tiek kitokį kelią ir stengėmės sukonstruoti chimerines molekules, sukeičiant vietomis gimininguų bakterijų rūšių funkcinius domenus, turinčius tą patį antibakterinio aktyvumo mechanizmą, t. y. porino-porino tipo chimeras (2 publikacija). Mūsų tikslas buvo išplėsti bakteriocino PyoS5 aktyvumo spektrą, modifikuojant jo žudymo domeną, taip kad jo nebegalėtų atpažinti pats PyoS5 imuniteto baltymas. Tikslas pasiektas sujungus receptoriaus/translokacijos domenus su poras formuojančiu domenu PmnH iš *P. synxantha*. *In vitro* eksperimentai patvirtino mūsų hipotezę – S5-PmnH chimera pasižymėjo platesniu aktyvumo spektru, lyginant su piocinu S5. Ši sėkmė mus paskatino imtis ir ištirti S5-PmnH veiksmingumą dviejose nesusijusiuose *P. aeruginosa* sukeliamuose ligų modeliuose: keratite ir

plaučių infekcijoje. S5-PmnH veiksmingai gydė *P. aeruginosa* sukeltą akių infekciją (gydymo veiksmingumas panašus į standartinio antibiotiko tobramicino). Savaime suprantama, jog būtina atlikti daugiau eksperimentų, siekiant įvertinti S5-PmnH veiksmingumą prieš bakterijas, kolonizuojančias ragenos paviršių kaip bioplėvelės, ir prieš bakterijas, internalizuojančias ragenos epitelio ląsteles. Pelių plaučių kolonizacijos modelyje vienkartinis chimerinio piocino S5-PmnH panaudojimas, net ir mažiausios naudotos koncentracijos – 2,5 μg, sumažino bakterijų skaičių žemiau negu prieš prasidedant gydymui.

Atsižvelgdami į naujausias publikacijas, kuriose aptariamas *S. maltophilia* ir *P. aeruginosa* „bendradarbiavimas“ tiek cistine fibroze sergančių pacientuose (McDaniel et al. 2020) tiek keratito infekcijose (Dantam et al. 2020) nusprendėme paieškoti naujų bakteriocinų *S. maltophilia* genomuose (3 publikacija). Identifikuoti du peptidoglikaną skaidantys stenocinai SmaltM ir SmaltM2 ir patvirtintas jų antibakterinis aktyvumas prieš *Stenotrophomonas* kamienus.

IŠVADOS

1. *P. aereuginosa* piocinai ir *S. maltophilia* stenocinai gali būti gaminami augaluose dideliais kiekiais ir yra visiškai funkcionalūs.
2. Piocinai S5, M, M4, L1, L2, L3 veiksmingai naikina planktonines ir bioplėveles sudarančias *P. aeruginosa* bakterijas *in vitro* ir efektyviai kovoja su bakteremija *G. mellonella* modelyje *in vivo*.
3. Chimerinis piocinas S5-PmnH pasižymi platesniu aktyvumo spektru, palyginti su PyoS5.
4. Chimerinis piocinas S5-PmnH yra efektyvus išoriškai gydant du nesusijusius *P. aeruginosa* sukeltų ligų modelius – keratitą ir plaučių infekciją.
5. Du naujai identifikuoti *S. maltophilia* bakteriocinai – SmaltM ir SmaltM2 – yra aktyvūs *in vitro* ir gali būti toliau tobulinami kaip antibakteriniai baltyminiai vaistai nuo *S. maltophilia* infekcijų.

PUBLIKACIJŲ SĄRAŠAS

Publikacijų, kurių pagrindu parengta ši disertacija, sąrašas:

1. **Paškevičius Š**, Starkevič U, Misiūnas A, Vitkauskienė A, Gleba Y, Ražanskienė A. 2017. Plant-expressed pyocins for control of Pseudomonas aeruginosa. *PLoS One*. 12(10):e0185782.
   Parengiau tyrimų planą, atlikau didžiąją dalį eksperimentų, analizavau ir parengiau rezultatus publikavimui, redagavau publikacijos rankraštį.
2. **Paškevičius Š**, Dapkutė V, Misiūnas A, Balzaris M, Thommes P, et al. 2022. Chimeric bacteriocin S5-PmnH engineered by domain swapping efficiently controls Pseudomonas aeruginosa infection in murine keratitis and lung models. *Sci. Rep.* 12(1):5865.
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4. Patentas. **Paškevičius Š.**, Misiūnas A., Ražanskienė A. 2022, 22163290.4 (pat. svarstomas), “Chimeric bacteriocins and method for the control of *Pseudomonas*“*,* Nomad Bioscience Gmbh, UAB Nomads.

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TYRIMŲ PATIRTIS	
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KONFERENCIJOS	
2022	Žodinis pranešimas, **Paškevičius Š.,** Dapkutė V., Misiūnas A., Thommes P., Sattar A., Gleba Y., Ražanskienė A**.** “Chimeric pyocin efficiently eradicates *Pseudomonas aeruginosa* in mouse keratitis and lung colonisation models“, 32nd European Congress of Clinical Microbiology & Infectious Diseases, Lisabona, Portugalija
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