TÉCNICO LISBOA

Engineered lysins as a solution for burn wound infections

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Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The work presented in this thesis was performed at the Laboratory of Gene Technology, in the Faculty of Bioscience Engineering of Katholieke Universiteit Leuven (Leuven, Belgium), during the period of February-July 2020. The thesis was supervised by Prof. Rob Lavigne and Vincent De Maesschalck, taking place within the frame of the Erasmus programme. The thesis was co-supervised at Instituto Superior Técnico by Prof. Ana Azevedo.

A Confidentiality Disclosure Agreement (CDA) has been signed by the Katholieke Universiteit Leuven and Instituto Superior Técnico - University of Lisbon. Therefore, the main goal of the project and the results obtained are confidential (and protected by the CDA) and will not be presented in this document.

Acknowledgments

Making this master thesis come to life feels like a special achievement. It has been five years of effort and immense support from many people. Some of whom have been with me from the beginning of this journey and patient enough to stand by me throughout the whole process. However, along the time and over the last months I was lucky enough to collect a few more on the way, who made this challenging task feel a little less daunting. Every kind word made a difference and I feel very grateful for them.

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Resumo

O desenvolvimento de resistência a antibióticos tem conduzido à ineficácia da maioria dos antibióticos no combate a doenças infeciosas comuns. Entre estas, as feridas por queimadura são comummente infetadas por organismos patogénicos resistentes, como é o caso de *Acinetobacter baumannii*. Mais ainda, a formação de biofilme ocorre frequentemente nestas infeções, interferindo com a eficácia dos tratamentos usados. Contudo, a descoberta do potencial terapêutico das endolisinas levou ao desenvolvimento de uma nova arma antibacteriana: engenharia de lisinas.

Palavras-chave: Resistência a antibióticos, *Acinetobacter baumannii*, queimadura, engenharia de lisinas

Abstract

The development of antimicrobial resistance (AMR) has rendered most of the (small-molecule) antibiotics ineffective against common infectious illnesses. Among these, burn injuries are commonly infected by (multi-)drug resistant pathogens, namely *Acinetobacter baumannii*. Additionally, these infections are often associated with biofilm formation which hinders the efficacy of the current treatments. However, with the discovery of the therapeutic potential of endolysins, a new antibacterial weapon has arisen: engineered lysins.

Keywords: antimicrobial resistance, *Acinetobacter baumannii*, burn injuries, engineered lysins

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List of Abbreviations

- **ABR** - antibacterial resistance
- **AHL** - *N*-acyl homoserine lactone
- **AMP** - antimicrobial peptide
- **AMR** - antimicrobial resistance
- **Bap** - biofilm-associated protein
- **CBD** - cell wall binding domain
- **CDC** - Centers for Disease Control and Prevention
- **CF** - cystic fibrosis
- **CHAP** - cysteine, histidine-dependent amidohydrolases/peptidases
- **DNA** - deoxyribonucleic acid
- **EAD** - enzymatically active domain
- **EDTA** - ethylenediaminetetraacetic acid
- **EPS** - extracellular polymeric substance
- **GlcNAc** - *N*-acetylglucosamine
- **HAI** - hospital-acquired infections
- **HHP** - high hydrostatic pressure
- **IM** - inner membrane
- **LPS** - lipopolysaccharides
- **mDAP** - mesodiaminopimelic acid
- **MIC** - minimal inhibitory concentration
- **MurNAc** - *N*-acetylmuramic acid
- **OM** - outer membrane

- **OMP** - outer membrane permeabilizing
- **PAMP** - pathogen-associated molecular pattern
- **PNAG** - poly-*N*-acetyl glucosamine
- **ppGpp** - guanosine tetraphosphate
- **pppGpp** - guanosine pentaphosphate
- **PRR** - pattern recognition receptors
- **QQ** - quorum quenching
- **QS** - quorum sensing
- **RND** - Resistance-Nodulation-cell Division
- **ROS** - reactive oxygen species
- **SIRS** - systemic inflammatory response syndrome
- **SMAP-29** - sheep myeloid antimicrobial peptide
- **SR** - stringent response
- **WHO** - World Health Organisation

Chapter 1

Introduction

1.1 Antimicrobial resistance

Microbial organisms, such as bacteria, viruses and fungi, are a crucial part of the global ecosystem. The majority of these organisms are essential for the well-being of their hosts, with whom they establish important interactions. The skin, for example, is colonised by a variety of microbial organisms essential for human health [1]. With the discovery and further development of antimicrobial agents and antiviral compounds, infections that used to be fatal could be overcome. However, these days, several of these drugs have lost their potency due to resistance development [2].

Consequently, antimicrobial resistance (AMR) has become one of the major worldwide health threats of the 21st century [2]. The World Health Organization (WHO) defines AMR as the phenomenon that occurs when microorganisms, such as bacteria, viruses and funghi evolve in ways that render the antibiotics used to cure the infections they cause ineffective. It encompasses resistance to antibacterial, antiviral, antiparasitic and antifungal drugs [3]. It is important to analyse the causes that led to this alarming health problem and what are the consequences that derive from it. Finally, there is an urgent need to implement measures that help averting this crisis [4, 5].

1.1.1 Antibacterial Resistance - Causes and Consequences

WHO distinguishes antibacterial resistance (ABR) from antimicrobial resistance (AMR), with the first one being the phenomenon that occurs when bacteria change in response to the use of antibiotics used to treat bacterial infections, rendering them ineffective [3]. Reasons for the development of ABR are found in the bacterium's susceptibility to evolution, as well as human behaviour and misconception of antibiotics' usage [6].

In the presence of antibacterials, bacterial communities are subjected to a selective pressure which prevents proliferation. If a bacterium is able to survive this challenge, however, it will become the dominant genotype amongst the population, turning this selective advantage into a common feature of the population [6]. Antibacterial resistance is acquired through genetic modification, which can be

triggered by multiple factors, such as mutation and gene transfer. In turn, this leads to phenotypic changes, making bacteria resistant to antibacterials [7].

Besides the bacterium's ability to adapt and evolve, human behaviour plays an important role in this problem. The world population boomed over the last decades, with the total world population doubling from 1950 to 1987 [8]. Increasing population numbers has led to the emergence of large population focus points, where people live in closer proximity to each other [9]. In addition, efficient transportation facilitates contact between different population groups. As a result from the demographic and technological evolution over the years, drug-resistant strains are able to spread faster and more widely [6]. Additionally, antibacterials are misused alarmingly frequently, both from the clinician's and the patient's perspective. It is not uncommon to prescribe antibacterials without a thorough diagnosis, in the pursue of a more practical and less time-consuming approach [2]. On the other hand, patients' demand for fast and efficient solutions misguides them into looking for the closest quick fix available, resulting in the prescription of antibacterials [10]. Additionally, the agricultural sector is also of significant importance: excessive usage of antibiotics in crop and animal-farming for growth promotion and disease prevention turns soil and water treatment facilities into the perfect reservoir for the development of new antibiotic resistance genes [2, 11]. The more frequent bacteria are exposed to antibiotics, the more frequent resistance can arise. Therefore, it is of crucial importance to reduce the amount of antibiotics that are being used [4].

This global resistance phenomenon has brought us into a "post-antibiotic" era, as designated by the Centers for Disease Control (CDC) in the USA [12]. Antibiotic resistance has a strong impact on public health, as infections with resistant bacteria lead to longer illnesses, increased mortality and prolonged stays in the hospital [11]. Moreover, antibiotic resistance also leads to economic losses as a consequence of increased health expenses and decrease in productivity due to illness [11].

1.1.2 Antibacterial Resistance - Solutions

With the increasing potential for new virulent and lethal pandemics, a new worldwide health threat is imminent [6]. Even worse, we won't be able to fight this crisis with appropriate weapons if a strict approach to fight AMR is not implemented. For that reason, over the last years, government agencies have been developing long-term plans to combat antimicrobial resistance, collaboratively with national health agencies and welfare agencies [13]. It is worth mentioning that the WHO developed a Global Action Plan in 2014 which addresses AMR by following five strategic objectives in order to reduce mortality due to infectious diseases. By doing so, we are adopting an attitude towards the hindrance of antimicrobial resistance development [11].

Since antimicrobial resistance is a multi-faceted problem, it can be approached by multiple different routes. It is important to consider not only solutions for the problem but also prevention measures. As a matter of fact, prevention should always be the first step in solving this crisis.

As individuals, we can protect ourselves from infection and fight antibacterial resistance by improving hygiene [11] and implement small habits, such as avoiding the unnecessary use of antibacterial

drugs [14]. Respecting instructions of physicians by completing the prescribed course, on the other hand, is equally relevant to over prescription as described earlier. In this way, antibiotic concentrations are held high enough preventing the development of resistance in the presence of subinhibitory concentrations of antibiotic. Furthermore, it is important to educate and motivate the population to follow vaccination programs [15].

In places with extensive use of antibacterials and hence high risk of developing resistance, such as hospitals, some strategies can help in slowing down the evolution of resistance. Two of these strategies comprise cycling and mixing antibacterial drugs. With a cycling approach, a specific class of antibiotic is used preferentially for a period of time, followed by a different class. In the mixing approach, on the other hand, multiple distinct antibiotics are assigned to different patients, with the goal of avoiding potentially resistant bacteria spreading from patient to patient. Furthermore, drugs can also be combined to reduce the risk of resistance development. The increasing resistance of *A. baumannii* to the currently available antibiotics has led to the assessment of combination therapies as promising strategies for *A. baumannii* infections [16]. Moreover, these strategies have been a success in previous trials. A study by McCaughey *et. al* (2013) showed that using a combination of fosfomycin and tobramycin against *Pseudomonas aeruginosa* and *Staphylococcus aureus* could prevent the development of resistance in a larger extent when compared to fosfomycin or tobramycin alone [17]. Nonetheless, it should be kept in mind that it is still not clear whether this type of treatment is always beneficial. Studies have also shown that shortening the duration of treatment might be advantageous, as it reduces selective pressure on commensals and environmental bacteria [18].

With the emergence of antibacterial resistance, researchers have also been focusing on developing new forms of treatment other than antibiotics [19]. Some of the solutions already developed or still being researched involve the use of monoclonal antibodies addressing toxins produced by certain bacteria [20], the use of bacteriophages and the development of vaccines preventing future infections [21]. The development of faster and more efficient diagnostic procedures would indirectly result in slowing down multidrug resistance, since it would avoid treatments with inappropriate antibacterial drugs [15]. In this scope, the use of sequencing methods can help in identifying and profiling resistant microbes, which ultimately allows to choose the best way to treat infections [18].

Another important step is the integration of data regarding resistance prevalence and infection in mathematical models. In this way, the goal is to develop appropriate resistance management approaches for each pathogen [18]. For example, a study on cefixime resistance in *Neisseria gonorrhoeae* was performed, by quantifying both the fitness cost and fitness benefit of resistant strains, which allowed to make predictions about the future prevalence of resistance as a function of how often cefixime is prescribed [22].

Antimicrobial resistance is also of significant importance in burn wound infections. Indeed, several pathogens infecting wounds, such as *P. aeruginosa*, *Acinetobacter baumannii* and *S. aureus* are associated with drug resistance. In fact, studies have shown that 42% to 65% of the total amount of deaths in burn victims are attributable to infections [23]. As a result, burn wound infection is a leading cause of morbidity and mortality in burn wounds [24, 25]. Therefore, antibacterial resistance in burn injuries is

likewise an urgent health concern that needs to be addressed.

1.2 Burn wounds - a global public health problem

1.2.1 Characterization of burn wounds

The skin: more than just a physical barrier

The skin is the largest organ of the human body, comprising 1.8 m^2 of surface area. Being exposed to the external environment, the skin is colonised by a large variety of microorganisms, including bacteria, viruses and fungi [26, 27]. Its primary function, however, is to serve as a physical barrier, protecting the human body against foreign microorganisms [28].

The skin is composed of the epidermis, dermis and a subcutaneous fatty region, as depicted in Figure 1.1. Each of these layers is enriched with multiple distinct structures, such as hair follicles, sweat glands, nerves, blood vessels and lymphatics [29]. The outer layer is designated epidermis and consists of four layers or strata [30]. Keratinocytes are the major cell type present in the epidermis, responsible for creating a barrier against the entry of foreign microorganisms into the organism [28, 30]. Specialized cells of the epidermis include melanocytes, producing the pigment melanin, and Langerhans cells, which are the main skin-resident immune cell [30]. In addition, T-cells can be found in the inner layers of the epidermis [31]. The dermis is anatomically more complex, with greater cell diversity, including dendritic cells, $CD4^+$ T-helper cells and natural killer T-cells [30]. Macrophages, mast cells, fibroblasts and nerve-related cell types are also present in the dermis. The latter is also intersected with blood vessels and nerves, crossing both the dermis and epidermis. Draining lymphatics begin in the dermis and cross the deeper layers of the skin, reaching the lymph nodes spread across the human body, containing important immune cells necessary to combat infection, namely B-cells and T-cells [30].

The immune response of the skin is essential upon wounding/infection and modulates the commensal microbiota that colonise the skin. Keratinocytes have a key role in the detection of pathogens and defense [32]. They express a number of immune receptors, referred to as pattern recognition receptors (PRRs), which sample skin bacteria [32]. These receptors recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) from Gram-negative bacteria and lipoteichoic acids from Gram-positive bacteria [28]. Keratinocytes respond to microbes or tissue damage by releasing a broad range of inflammatory mediators, such as cytokines, chemokines and antimicrobial peptides (AMPs) [32]. Chemokines are a class of small proteins essential in recruiting T-cell and innate effectors to the site of infection, in a process called chemotaxis [33]. Furthermore, cytokines direct the immune response to induce appropriate infection clearance mechanisms [34]. On the other hand, AMPs have the capacity to directly kill bacteria, fungi and enveloped viruses [35]. In addition, AMPs can influence the immune response thereby affecting inflammation. The most relevant families of AMPs in the skin are cathelicidins and defensins [35].

Disruption of the skin can have multiple causes and disturb the regular host-microbiota system. Burn injuries, for example, result in the dysregulation of the host-skin microbiome. Additionally, this

disruption can lead to infections with opportunistic pathogens as well as skin colonisers [36]. Hence, it has become a relevant health concern.

Burn injuries - a complex worldwide phenomenon

A burn wound is an injury present in the skin or other organic tissue caused by heat or other factors including radiation, radioactivity, electricity, friction or contact with chemicals. Annually, approximately 180 000 deaths are caused by burns, with the majority of them occurring in low-income and middle-income countries [24].

Besides the high annual number of deaths caused by burns, wound infection is a leading cause of morbidity and mortality in burn patients [25]. In addition, once patients are affected by a large burn injury, they are admitted to the hospital, becoming at a high risk for developing hospital-acquired infections (HAI) [23] with nosocomial pathogens such as *P. aeruginosa*, *S. aureus* and *A. baumannii*.

Burn injuries can be classified according to the depth of the injury.

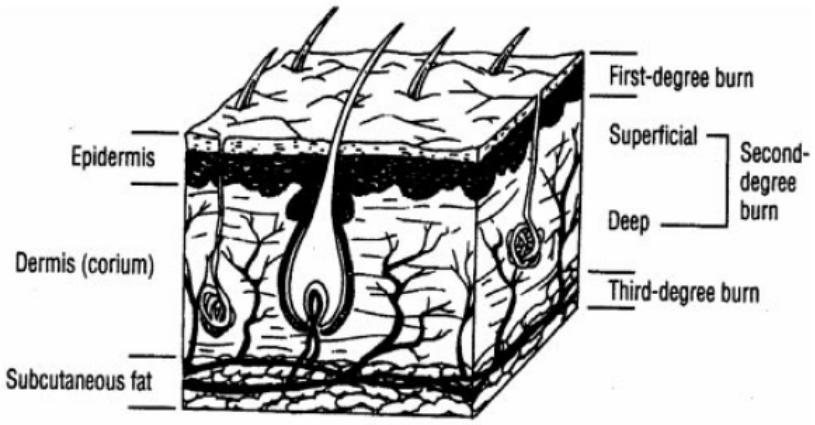

Figure 1.1: Schematic of skin anatomy and classification of burn injuries according to depth of the injury. A first-degree burn only affects the epidermis, while a second-degree burn also impairs the dermis, either superficially or at its deepest level. In a third-degree burn, the epidermis and dermis are destroyed and the innermost layer of the skin, the subcutaneous tissue, is affected. It is commonly referred to as a full thickness burn. Adapted from [37].

A first-degree burn is the least severe. It affects only the epidermis and is characterized by a sensation of pain, itching and a pink colour. A second-degree burn also damages the second layer of skin, the dermis. Depending on the severity, either the most superficial or the deepest part of the dermis is affected. The wound will typically feature blisters and a red colour, as well as a sensation of pain and discomfort. A third-degree burn is the most severe burn injury, characterized by the absence of blisters and a dry aspect, along with a red and white colour. Patients affected with this type of injury will undergo an operative treatment and often experience no pain at all [38]. This is due to the full destruction of the dermis, with its rich network of nerve endings. Once the devitalized tissue is replaced, the patients start to experience the sensation of sharp pain [39].

Burn wounds breach the skin, causing the loss of the human's protective barrier against environmental microbes, along with the exposition of highly nutritive serum. Consequently, the skin barrier

is destroyed and a favourable environment for microbial growth and invasion is created [40]. Hence, burn patients become more susceptible to local and even systemic invasion by opportunistic pathogens [25, 41]. The incidence of wound infections in burn patients is related with the extent and depth of the burn [41]. Likewise, the length of time the wound remains open is relevant [42]. Furthermore, burn injury is typically associated with profound changes in metabolic and host defense mechanisms. In fact, in order to achieve successful wound healing, the metabolic rate of burn patients can surpass twice the normal rate and the lack of capacity to meet these energy requirements can result in impaired wound healing, susceptibility to infection, organ failure and mortality [43]. The state of severe catabolism can last for several years after the infection, which leads to a significant loss of lean body mass as well as a decline of host immune function [44]. The latter triggers a systemic inflammatory response syndrome (SIRS) and subsequent multiple organ failure, in the most severe cases [45]. A common treatment adopted in a severe burn wound is the early excision of the burn, since it will result in the decrease of the release of inflammatory mediators and bacterial colonisation of the wounds. Thus, it is possible to attenuate SIRS and the occurrence of metabolic alteration as well as organ failure [46].

A burn wound can get infected in multiple stages of the healing process. Accordingly, different microorganisms colonise the wound. The microbial population of a burn wound immediately after burning is inexistent [47]. However, commensal Gram-positive bacteria located within sweat glands and hair follicles might survive the heat of the initial injury and colonise the wound, unless topical antimicrobial agents are used [47]. In an initial stage post-burn, other species of Gram-positive opportunistic pathogens, such as *Staphylococcus aureus* and *Enterococcal* species are prevalent in the colonisation of the wound [25]. As time passes, the wound becomes populated by Gram-negative pathogens as well as fungi and yeasts derived from the patient's gastrointestinal or upper respiratory tract flora [25, 48]. Gram-negative pathogens often cause invasive infections as well, which are characterized by a rapid change in burn wound appearance such as separation or discoloration of the eschar. In addition, the pathogen will be detected in blood cultures and inflammation of the surrounding uninjured skin will be observed [37]. It is worth mentioning that burn units present risks associated with sharing equipment and specific types of treatment which might be a cause for targetted wound colonization. For instance, hydrotherapy is frequently associated with Gram-negative pathogens, as it is related to the use of a water source [49].

Pseudomonas aeruginosa is the most frequently found Gram-negative pathogen in a burn wound infection, but other species such as *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiella pneumoniae* are also found in established burn wound infections illustrating the polymicrobial nature of burn wound infections [25]. *Candida* species are the most prevalent fungi in burn wounds, although new fungi are emerging as well. Wound invasion is usually performed by fungi and drug-resistant bacteria, namely, multidrug-resistant *Pseudomonas* and *Acinetobacter* species as well as methicillin-resistant *S. aureus* [25].

Multidrug-resistant strains of bacteria and fungi have become more frequent and increasingly difficult to treat, causing an unanticipated rise in drug-resistant burn wound infections, as well as an increase in sepsis and associated deaths worldwide. Before the post-antibiotic era, *Streptococcus pyogenes* used

to be the leading cause of burn wound infection and a significant cause of death in severely burned patients [50]. Nonetheless, the pathogen was virtually eliminated with the introduction of penicillin G [37]. Currently, although the leading infective bacterium in burn wounds is *S. aureus*, *P. aeruginosa* and *A. baumannii* are two examples of infective microorganisms encountered in burn wound infections with increasing frequency [51]. *P. aeruginosa* remains the main species to be responsible for sepsis and death related to burn infection. However, *A. baumannii* has been observed with increased frequency [52]. This opportunistic pathogen can easily be transferred from patient to patient because of its ability to survive in both dry and wet conditions as well as on both living and non-living objects [25]. Additionally, drug-resistant *A. baumannii* is often associated with biofilms formed in burn wounds, preventing antibacterial activity of topical agents used for burn treatment [53].

1.2.2 Biofilms in burn wounds

After burn injury, colonisation of the wound is facilitated due to disruption of the skin as a physical barrier, which compromises the immune system. Bacterial infection is one of the main complications in burn wounds [25].

Besides appearing as free-living microorganisms, bacteria can also live together in biofilms. These microbial communities are significantly more difficult to eradicate which impacts the treatment of infections associated with biofilm formation. Nonetheless, recent new insights on the physiology of biofilms have broaden the range of alternatives available for the development of anti-biofilm drugs.

The complexity of biofilms: friend or foe?

Biofilms are defined as microbial communities in which bacteria are embedded in a matrix. These communities can be attached to both biotic or abiotic surface and can also be found in submerged or humidified conditions [54]. For example, *Pseudomonas fluorescens* forms biofilm at the air-liquid interface [55], whereas *Escherichia coli* forms submerged biofilms in a static system [56]. Organisms such as bacteria, fungi and protists are able to live in biofilms. Biofilms can cause havoc in many different settings, ranging from industrial piping systems to medical devices, such as catheters and implants. The latter has become an emerging health concern: medical devices colonised by biofilms often cause chronic infections [57].

A. baumannii is a Gram-negative, aerobic, opportunistic pathogen, responsible for a vast number of nonsocomial infections due to its increased antibiotic resistance and virulence [58]. Studies have reported that *A. baumannii* is responsible for up to 26% of total mortality in hospital patients, increasing up to 43% for patients residing in the intensive care unit, in the USA [59]. Widely known for being able to survive in hospital environments despite unfavourable conditions, such as decontamination or surfaces with antimicrobial treatments, its ability to colonise and form biofilms on both biotic and abiotic surfaces remains one of the most relevant causes for chronic infections [60, 61]. In fact, isolates of this species recovered from blood, urine, cerebrospinal fluid, burned skin and catheters were observed to form biofilms [62].

The composition of biofilms determines their structural aspects. Besides, biofilm composition

highly varies from microorganism to microorganism [54]. Bacteria account only for less than 10% of the dry mass, whereas the matrix accounts for over 90%. The matrix is composed mainly of water and different types of biopolymers, known as extracellular polymeric substances (EPS) [63]. EPS include polysaccharides, structural proteins, enzymes, nucleic acids and lipids [63]. Interstitial voids and open water channels intersect the extracellular matrix, functioning as a circulatory system for distribution of nutrients and removal of metabolic waste products [64, 65]. Additionally, the matrix is responsible for holding the bacteria together in a biofilm. Divalent cations like calcium and magnesium aid in maintaining the structural integrity of the biofilm by holding the polymers together through electrostatic interactions between the cations and the negative charges of the LPS, providing binding strength [54, 66].

The self-produced EPS plays an important role in shielding the bacteria from environmental threats, such as shear forces and host immune defenses [65]. Furthermore, the matrix is responsible for retaining water, resulting in biofilm organisms tolerant to drought. Other important functions of the matrix are adhesion and communication among microorganisms [63]. The latter is facilitated by the close proximity in which microorganisms live in a biofilm. This also aids exchange of genetic information between biofilm cells [63]. It should be noted as well that microorganisms living in biofilms often develop particular characteristics such as pathogenicity and tolerance to many antimicrobials [54, 63].

Biofilm formation can be divided into five stages, as depicted in Figure 1.2.

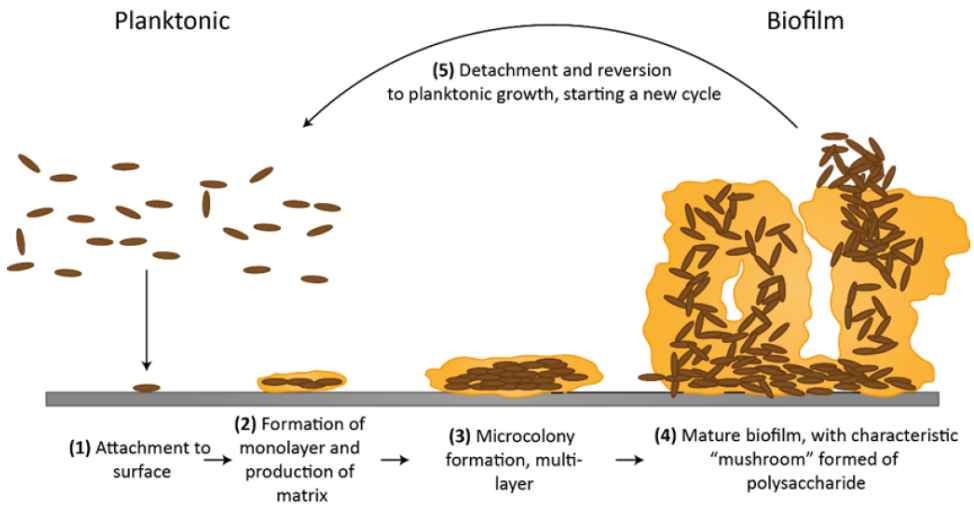

Figure 1.2: Schematic representation of a biofilm formation and development. Biofilm starts with reversible attachment of the plankonic cells (in brown) to a surface (in grey) (1). Bacteria starts to form a monolayer and produce the extracellular matrix (2). At this point, attachment is irreversible and multiple layers have been formed (3). A mature biofilm is obtained (4) and, at last, bacteria start to detach and disperse, allowing for a new cycle to begin (5). Adapted from [54].

The process begins with reversible attachment to a surface or interphase (stage 1). Planktonic bacteria have initial reversible contact with the surface and start to form a monolayer. Consequently, the bacteria produce their characteristic extracellular matrix (stage 2). With the formation of microcolonies, the biofilm acquires a three-dimensional shape. In this phase, attachment becomes irreversible (stage 3). A fully mature biofilm is obtained (stage 4), actively exchanging and sharing products that play a key role in maintaining biofilm architecture and favorable living environment for the resident bacteria. Mature

biofilms have the capacity of tolerating antibacterial treatments as well [67]. Eventually, bacteria will start to disperse into the environment, starting a new cycle of biofilm formation (stage 5) [65].

This process is mainly coordinated by quorum sensing (QS), bacterial cell-to-cell communication used to coordinate gene expression. Besides having a role in biofilm formation, this system is involved in other processes such as symbiosis, virulence, conjugation and motility [54, 68, 69]. QS monitors cell-population density by measuring the concentration of secreted signal molecules, termed autoinducers [70]. When a threshold concentration of autoinducers is achieved, signal transduction cascades are triggered leading to alterations in gene expression and a consequent response in the bacterial population [70].

In Gram-negative organisms, several quorum-sensing systems respond to a class of autoinducers termed acyl homoserine lactones (AHLs). *P. aeruginosa*, for example, features four QS systems, two of them use AHL autoinducers whereas the other two use non-AHL autoinducers [71].

The only QS system of *A. baumannii* is similar to the typical LuxI/LuxR system found in other Gram-negative bacteria [72]. This system regulates several virulence factors such as biofilm formation and surface motility, but also bacterial competence [73]. The QS system of *A. baumannii* is based on an AHL auto-inducer, comprising an enzyme (AbaI) synthesizing the auto-inducer and a receptor protein of the QS system (AbaR) [74]. This receptor protein (AbaR) will bind to the AHL signal molecule, inducing a cascade of reactions. Although five minor AHLs have been detected in culture supernatants of an *A. baumannii* strain, the most predominant AHL molecule is N-(3-hydroxydodecanoil)-L-homoserine lactone (3-OH-C12-HSL) [72]. However, only one AHL synthase gene was identified, which suggests that the synthase has broad specificity and is capable of synthesizing other QS signal molecules [75].

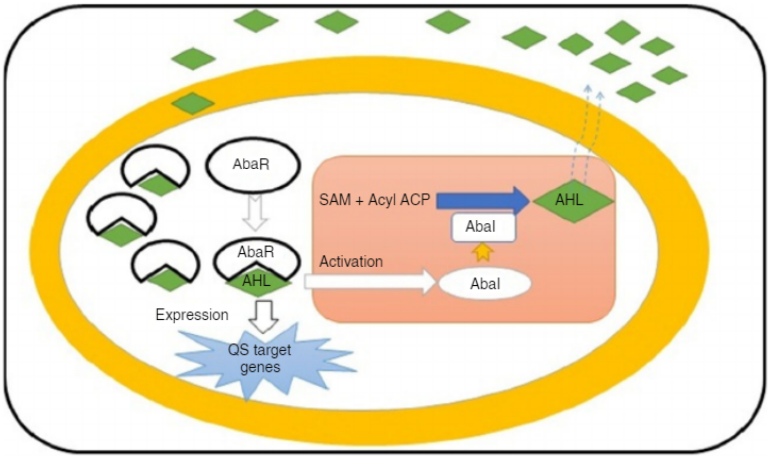

Figure 1.3: Illustration of the quorum-sensing mechanism in *A. baumannii*. In the auto-inducer synthesis process, AbaI uses S-adenosyl methionine (SAM), which binds to the Acyl group of the acyl-carrier protein (ACP), leading to the production of the AHL signal molecule. AHL will bind to the receptor protein, AbaR, triggering a series of reactions, controlled by QS target genes. Adapted from [72].

Biofilms can impact human health both positively and negatively. For instance, biofilms of *Staphyloccoccus epidermis*, a commensal species present in the skin, prevents colonisation of potentially pathogenic bacteria through the stimulation of host-cell immune defenses [65]. Nonetheless, biofilms are also associated with a large number of human infections. According to the National Institutes of Health and the Center for Disease and Prevention of the USA, it is estimated that 65% to 80% of human infections involve biofilm formation [58]. For example, recurrent infections with (drug-resistant) pathogens, known to form biofilms, are difficult to eradicate in patients with cystic fibrosis (CF) [65]. Other examples of diseases reported to be associated with bacterial biofilms include colitis, vaginitis, urethritis, conjunctivitis and otitis [64]. Lastly, bacterial biofilms are often involved in burn wound infection, in which *A. baumannii* is one of the prominent pathogens [23].

Biofilms: tolerant or resistant to antimicrobial agents?

Over the last years, bacterial resistance and tolerance have been introduced as different concepts: bacteria are tolerant when they are able to survive in the presence of an antimicrobial agent, yet incapable of proliferating; resistance, however, relates to the bacteria's capacity to proliferate under the same conditions [76]. It has been widely established that biofilms show elevated tolerance against a significant number of antibacterial agents [54]. In fact, biofilm bacteria are 10 to 1000 fold less susceptible to antimicrobial agents than the same bacteria in a planktonic culture [64]. A study by Walters *et. al* (2002) demonstrated that *P. aeruginosa* biofilms are tolerant to killing by tobramycin (at a concentration of 10 μg/mL) and ciprofloxacin (at a concentration of 1 μg/mL) [77].

Biofilms possess several mechanisms contributing to antibacterial resistance and/or tolerance. As a consequence, biofilms become less susceptible to antibiotics, leading to the persistence of biofilm infections [78]. Some of these mechanisms exploited by biofilm-residing bacteria are depicted in Figure 1.4.

One of the factors attributed to have a role in biofilm antibacterial tolerance is the biofilm's role as a penetration barrier, delaying antibiotic diffusion [78]. This can happen due to chemical reactions between the antibiotic and the extracellular matrix, such as catalytic reactions that lead to the degradation of the antibiotic, or sequestration of the antibiotic by binding to polysaccharides [78–80]. However, antibiotics can still exert their activity when: (i) the biofilm matrix is saturated with antibiotic; (ii) the time required for the antibiotic to penetrate biofilms is shorter than the duration of the treatment; (iii) or when, upon penetration of the biofilm, the reconstitution of the biofilm matrix occurs at a slower rate than the diffusion/reaction of the antibiotic molecules [79]. Nonetheless, it has been reported that antibiotic penetration is hindered only for some antibiotics such as vancomycin (in *S. aureus* and *S. epidermis* biofilms) and chloramphenicol (in all *E.coli* biofilms) [81].

A slow bacterial growth rate comprises another tolerance mechanism adopted by biofilm bacteria, since the conventional small-molecule antibiotics are most effective against metabolically active cells [82]. When a bacterial cell culture becomes starved for a particular nutrient, it slows its growth. This is because starvation induces a cellular response characterized by repression of growth and division [79]. Bacteria's response to nutrient limitation is exerted through a regulatory mechanism designated as

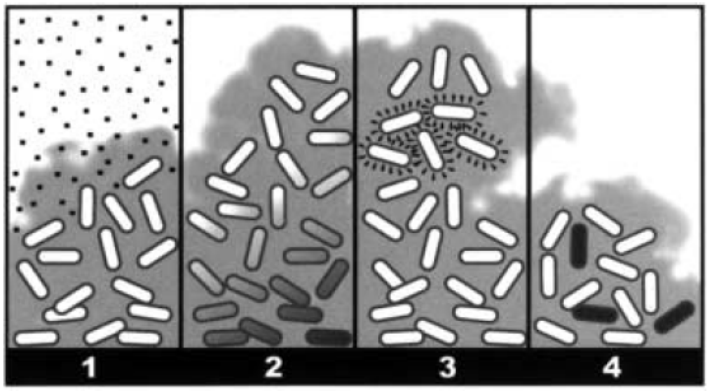

Figure 1.4: Biofilm resistance and/or tolerance mechanisms. 1 - Slow or incomplete antibiotic penetration: its main causes are attributed to chemical reactions that lead to degradation of the antibiotics and sequestration of antibiotic molecules by polysaccharides. 2 - Shaded cells represent zones of slow or non-growing bacteria: nutrient limitation induces cellular growth repression. Conventional antibiotics target growth factors, hence biofilms undergoing reduced metabolic activity become more tolerant to antibiotic treatments. 3 - Marked cells represent adaptative stress response: unfavourable conditions trigger stress responses, which are regulated by specific gene expression. These responses may help in modulating biofilm-associated pathways, responsible for antibiotic resistance. 4 - Dark cells represent persister cells: these cells are resistant to antibiotics, surviving high selective pressures. This phenotype is temporary: once antibiotic pressure decreases, the persister population becomes as equally susceptible to antibiotics as the original cell population. Adapted from [78].

stringent response (SR). Carbon, amino acid and and iron starvation are a few examples that activate the SR by inducing the *relA* and *spoT* gene product to synthesize the nucleotide alarmone, (p)ppGpp [83]. Intracellular concentration of (p)ppGpp changes in response to growth conditions, with its synthesis being controlled by both RelA and SpoT. RelA proteins are monofunctional alarmone synthases whereas SpoT proteins are bifunctional synthases/hydrolases [84]. In growth-favorable conditions, a steady level of these two nucleotides (ppGpp and pppGpp) is maintained. However, in starved cells (p)ppGpp inhibitis synthesis of ribosomal and transfer RNAs. Thus, the ability of cells to produce these macromolecules deeply affects cellular processes such as transcription, replication and translation, with consequences in terms of virulence-induction and persistence [85]. Slow growth has been observed in mature biofilms [86]. In fact, in *E. coli* biofilms under slow-growth conditions, ppGpp normally increases during low nutrient conditions which leads to increased expression of *rpoS*, an alternate sigma factor (σ^{38} or σ^{S}) responsible for controlling multiple genes and operons whose products are involved in stress resistance and protection [87]. This is usually the case for glucose or ammonia starvation in *E. coli*. High levels of ppGpp, upon nutrient limitation, results in the increase of RpoS levels and an adequate stress response [87]. The activity performed by this sigma factor is an example of a stress response regulator expressed by *E. coli*. These type of unique gene-expression patterns, such as stress response regulators, aids in controlling biofilm-specific pathways responsible for antibiotic resistance [79].

Since some biofilms experience reduced metabolic activity, it might justify the enhanced tolerance to treatments with antibiotics that typically target growth factors in planktonic bacteria [88]. A study by Tanaka *et. al* (1999) evaluated the impact of growth rate in antibiotic treatment of biofilm *P. aeruginosa*. Two types of antibacterial agents were tested, namely β-lactams and fluoroquinones. The former

showed weak bactericidal activity to biofilm cells, with greater activity in younger biofilm cells growing in high concentrations of nutrients. The same was not observed for fluoroquinones [89]. Carbapenems, which are a class of β-lactams, act by inhibiting the synthesis of peptidoglycan layer of the bacterial cell wall. Once these molecules enter the bacteria, they permanently acylate PBPs (penicillin-binding proteins), preventing the final crosslinking (transpeptidation) of the rising peptidoglycan layer, disrupting cell wall synthesis [90].

Antibiotic treatment of microbial populations is also hampered by the existence of a persister phenotype among them, confering temporary tolerance for antimicrobials [79]. Persisters are (multi-)drug tolerant cells which adopt a slow-growing or nongrowing rate, by transforming into a dormant state or selectively inactivating biological processes typically targeted by antibiotics [91]. These cells have no acquired resistance through genetic modification, demonstrating the stochasticity of this event in microbial populations [92]. Persister cells are highly tolerant to antibiotics, surviving under high selective pressures [93]. Once antibiotic pressure starts to drop, the surviving persister cells will create a population that is as susceptible as the original cell population, with a similar proportion of persisters [92]. This new bacterial population can then cause a relapsing infection [79]. The persister population of a biofilm has been estimated to constitute approximately 0.1%–10% of all cells in the biofilm [94].

A last example of antimicrobial tolerance mechanisms are processes handling antibiotic-induced oxidative stress. For example, the formation of reactive oxygen species (ROS, usually hydroxyl radicals) is relevant for the effect of antibiotics. Antibiotic-induced ROS production has been described during treatment of *P. aeruginosa* with quinolones [95] and biofilms formed by mutants lacking anti-oxidant systems, such as catalases (*katA* for *P. aeruginosa*) have shown increased sensitivity to antibiotics. These anti-oxidant systems are upregulated by the activation of the SR in biofilms, resulting in the improvement of the antioxidant capacity of biofilms and, consequently, their tolerance to antibiotics [96].

Resistance is commonly attributed to genetic factors. Nonetheless, resistance towards certain classes of antibiotics may also be intrinsic and dependent on innate characteristics of the cell. In fact, the most conventional example of intrinsic antibiotic resistance exhibited by Gram-negative bacteria is attributed mainly to the presence of an outer membrane which is impermeable to many molecules, namely large size molecules, and the expression of multiple MDR efflux pumps that act by reducing the intracellular concentrations of the drug [97].

Efflux pumps are another common escape mechanism from antibiotics. Efflux pumps are transmembrane proteins whose role is to remove specific compounds from within the bacterial cell into the external environment. These substances include antibiotics, detergents, dyes, toxins and waste metabolites [98]. Most efflux pumps in Gram-negative bacteria belong to the RND superfamily, which contemplates the chromosomally-encoded *AdeABC* efflux pump in *A. baumannii* [74, 98]. Studies have reported that biofilm formation in *A. baumannii* requires a certain expression profile of efflux pumps to initiate and maintain biofilm formation [99]. For example, the deletion of the efflux genes *adeG* and *adeJ* help restoring a biofilm phenotype, whereas the mutant *A. baumannii* strains lacking *adeB* exhibited significant defects in biofilm formation [100]. To date, three RND efflux systems have been characterized in *A. baumannii*: AdeABC, AdeFGH and AdeIJK. It has been shown that overproduction of AdeABC

and AdeIJK alters bacterial membrane composition, leading to decreased biofilm formation due to the underexpression of proteins belonging to chaperone-usher pilus assembly systems [100]. These are known to play a major role in the initial stages of biofilm formation, by promoting initial adhesion and surface colonisation but also formation of microcolonies.

Efflux pumps have a multifunctional role in biofilm formation, through four main mechanisms, illustrated in Figure 1.5.

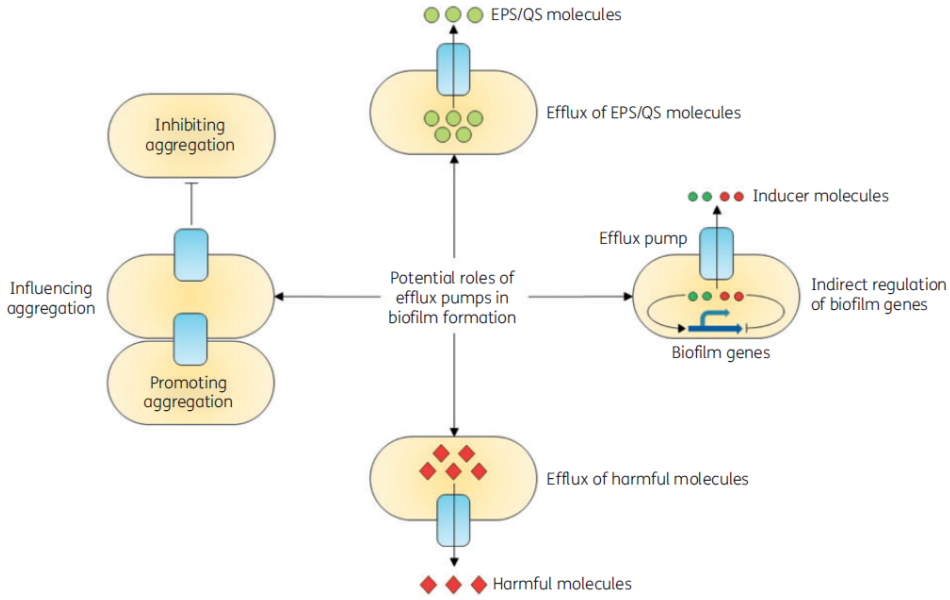

Figure 1.5: Schematic diagram highlighting the four different potential roles of efflux pumps in biofilm formation as suggested from various studies: efflux of EPS and/or QS and quorum quenching (QQ) molecules to facilitate biofilm matrix formation and regulate QS, respectively; indirect regulation of genes involved in biofilm formation; efflux of potentially harmful molecules, such as antibiotics and metabolic waste products; and influencing aggregation through promoting or preventing adhesion to surfaces and other cells. Adapted from [98].

Generally, efflux pumps can contribute to the efflux of EPS and QS molecules to facilitate matrix formation and regulate QS. In *A. baumannii*, a positive correlation was shown to exist between biofilm formation and upregulation of the genes *abaI* and *abeG*. These genes code for an AHL synthase involved in QS and, thus, in biofilm development and a component of the AdeFGH efflux pump, respectively [98]. Thus, the expression profile of *A. baumannii* influences the efflux pumps activated which, in its turn, contribute to QS regulation.

***A. baumannii* - Why its biofilms are so difficult to eradicate**

80% to 91% of all *A. baumannii* bacterial isolates have shown to form biofilms *in vitro*. This biofilm formation rate is substantially higher in comparison to other *Acinetobacter* species (from 5% to 24%) [101]. This opportunistic pathogen was observed to form biofilms in skin and soft tissue infections, but also on hospital equipment such as catheters, rendering *A. baumannii* an important agent in causing nosocomial infections [102].

A. baumannii harbors a multitude of virulence genes contributing to biofilm formation. These genetic elements are controlled and regulated by complex regulatory networks, based on the presence

of antibiotic resistance genes, environmental conditions or cell density [74]. Genes associated with biofilm formation are the *csu* operon, the *pga* locus, *ompA* and *bap* [103].

The majority of *A. baumannii* strains encode a Type I chaperone-usher pilus system, critical for biofilm formation and attachment to abiotic surfaces [74]. The expression of the operon *csu* is regulated by a two-component regulatory system, *bfm*RS. The *bfm*RS system comprises a histidine sensor kinase encoded by *bfmS*, sensing environmental conditions, and a response regulator encoded by *bfmR* [61]. Transcriptional and translational analysis have shown that inactivation of the response regulator results in a decrease of the expression levels of the *csu* operon, with consequent abolition of biofilm formation [60].

The *pga* locus encodes genes for poly-N-acetyl glucosamine (PNAG) synthesis [103]. PNAG is one of the most important polysaccharides in biofilm formation in both Gram-positive and Gram-negative microorganisms. Its important role in biofilm formation was revealed with the creation of a knockout: a deletion mutant of *pgaABCD* in an *A. baumannii* S1 strain (S1δ*pga*) resulted in the loss of a strong biofilm phenotype, which was restored after complementation [104].

OmpA is a prominent porin in Gram-negatives and hence also *A. baumannii* which contributes to passive drug extrusion across the outer membrane, revealing its role in antimicrobial tolerance. This porin couples with inner membrane efflux systems, such as efflux pumps [105]. Additionally, OmpA targets mitochondria upon binding to host epithelial cells. This leads to the release of proapoptotic molecules, resulting in the induction of apoptosis [106]. It has been shown that this outer membrane protein is necessary for the formation of robust biofilms on a polystyrene surface but, more relevantly, essential for biofilm formation on biological surfaces [107]. It is still not clear whether OmpA plays a direct or indirect role in bacterial attachment and biofilm formation. However, OmpA inactivation leads to alterations in the bacterial cell wall, significantly decreasing the minimal inhibitory concentration (MIC) of some antibiotics such as chloramphenicol, most likely due to the destabilization of the outer membrane [108]. This further confirms the hypothesis of OmpA participating in the extrusion of antibiotics from the periplasmic space, coupling with inner membrane efflux systems [105].

The biofilm-associated protein (Bap) plays a role in cell-cell adhesion and is required for acquiring a three-dimensional structure in the biofilm and for water channel formation [109]. Several studies have demonstrated the importance of Bap in mediating biofilm formation and maturation in *A. baumannii*. Bap expression by several *A. baumannii* strains was associated with strong biofilm formation, whereas *A. baumannii* ST92 strain MS3007, which does not encode for the Bap protein, was not able to establish biofilms to the same extent as other strains [103].

To overcome iron limitation, several bacterial species secrete high-affinity iron chelators, called siderophores, as a virulence factor. A specific siderophore encoded by *A. baumannii* is called acinetobactin [110]. The iron uptake system developed by *A. baumannii* helps its proliferation within the host and the progression to infection [111]. Nonetheless, the role of iron as an inducing agent of biofilm formation remains to be enlightened. Different studies have reported contrary conclusions, namely, a study be Gentile *et. al* (2014) with 50 different *A. baumannii* strains revealed that only in 21 isolates, biofilm production was enhanced significantly by iron deficiency, whereas in 12 isolates biofilm formation

was significantly reduced and in the remaining 17 isolates, no effect was noticed. However, a different study (Modarresi *et. al* (2015)) showed that iron concentration does regulate AHL production and, in this way, biofilm formation, since several pathways resulting in biofilm formation are QS-dependent. When the concentration of Fe-(III) in the medium was restricted, the activity of both AHL and siderophore was increased, resulting in robust biofilm [112]. Thus, there is evidence that strongly suggests that iron plays an important regulatory role in biofilm formation.

Several other factors contribute to pathogenicity in *A. baumannii*, such as LPS and capsular polysaccharides [110]. A study by Geisinger *et. al* (2015) showed that capsular polysaccharides increase the antimicrobial tolerance in *A. baumannii*: mutants deficient in the production of these polysaccharides have lower intrinsic resistance to peptide antibiotics, such as colistin, erythromycin and rifampicin. In the presence of chloramphenicol and erythromycin, hyperproduction of capsular polysaccharides is triggered as well [113].

***A. baumannii* biofilms: how to solve the problem?**

A. baumannii is currently considered by the WHO as one of the top priority pathogens to which new antibiotics must be developed, due to its resistance against carbapenems. Carbapenems are a class of β-lactam antibiotics and one of the most commonly used antibiotics for MDR bacterial infections [114]. Antibiotic resistance was mentioned before to be associated with resistance factors that could either be intrinsic such as chromosomally-encoded β-lactamases, drug efflux systems and porins, or acquired through insertion elements and integrons. *A. baumannii* possesses some of these resistance determinants, such as β-lactamases, efflux pumps and aminoglycoside-modifying enzymes [74, 110]. Inactivation of β-lactams antibiotics by β-lactamases is, for example, a major antibiotic resistance mechanism in *A. baumannii*. β-lactamases are divided into four molecular classes: A, B, C and D. Each class has its specific characteristics and confers resistance to a certain group of antibiotics [110]. On the other hand, aminoglycoside-modifying enzymes are the most relevant mechanism by which *A. baumannii* confers resistance to aminoglycosides, another commonly used antibiotic. In fact, these enzymes are typically present on transposable elements and are transferred among pathogenic bacteria [115]

Although the major level of concern addresses carbapenem-resistant isolates, several *A. baumannii* isolates have been reported to be resistant to several other classes of available antibiotics, such as aminoglycosides, fluoroquinolone, polymyxins, and other antibiotics belonging to the β-lactams class of antibiotics [74, 110]. Likewise, it has been observed that drug-resistant *A. baumannii* associated with burn wounds forms biofilms, preventing antibacterial activity of topical agents used for burn treatment [53].

Since bacteria in biofilms are less susceptible to antibacterial treatment than planktonic bacteria, combination therapy may be effective as a treatment option, as mentioned before. Quorum quenching is an alternative viable treatment [110]. The use of AHL analogue molecules to inhibit the quorum-sensing pathway of *A. baumannii* has been proven to be a valid strategy in the attenuation of biofilm formation. However, quorum quenching can also be achieved through the enzymatic degradation of the quorum signal by an AHL lactonase [116]. As an example, an engineered recombinant lactonase has been able

to successfully promote biofilm disruption in *A. baumannii* [116].

An alternative solution is based on a promising novel class of antibacterials with a unique mode of action, which consists of degrading the cell wall of bacterial host and killing the bacteria. These antibacterials agents are bacteriophage-encoded endolysins [79].

1.2.3 Endolysins - A Solution to Antibacterial Resistance

Bacteriophages, also known as phages, are viruses infecting bacteria. Besides being natural predators of bacteria, phages have been used as agents for treating bacterial infections in bacteriophage therapy [79, 110]. Phages initiate infection by injecting its genetic material into the cytoplasm of the host. Then, depending on the nature of the phage and growth conditions of the cell, the phage can follow either a lytic or a lysogenic cycle. The former uses the host cell's machinery to replicate viral proteins and genomic material, with consequent release of new virions and cell lysis [117]. In the latter, the phage genome is integrated into the host DNA resulting in a prophage. The prophage remains dormant in the host cell, without causing immediate cell lysis. The prophage genome is continuously replicated with the host genome, as the host cell divides. In stress conditions, a switch from the lysogenic cycle to the lytic cycle is induced, as prophages extract themselves from the host genome. As virion particles assemble and are packaged with phage DNA, new phages are formed and cell lysis is initiated [117].

In the late stages of their lytic cycle, phages release lytic enzymes that will degrade the peptidoglycan of the bacterial host. Due to their role in contributing to the degradation of the peptidoglycan from within the host cell, leading to eventual cell lysis, these enzymes are termed endolysins [118]. In addition, their unique mode of action has established endolysins as a promising novel class of antibacterials [79]. Endolysins are phage-encoded peptidoglycan hydrolases exploited by the majority of bacteriophages to destroy the cell wall. More specifically, these enzymes degrade the peptidoglycan layer of the host after translocation over the inner membrane through small hydrophobic proteins: holins [119]. Once a critical concentration is reached, holes are created through the cytoplasmic membrane by oligomerization, allowing the endolysins to access the peptidoglycan layer [120]. More recently, it has been discovered that the outer membrane can be disrupted as well with the spanin complex, which consists of a small outer membrane lipoprotein and an integral cytoplasmic membrane protein. These phage-encoded proteins connect the cytoplasmic membrane (inner membrane, IM) and the OM, and their main function is to catalyze fusion of the IM and OM, resulting in destruction of OM, which comprises the last step of lysis [119, 121].

A key difference between Gram-negative and Gram-positive bacteria is the composition of the cell wall. In Figure 1.5, it is possible to notice the main differences in the structure of the cell wall in both types of bacteria. Both Gram-negative and Gram-positive bacteria have peptidoglycan as a common polymer of their cell wall. However, whereas peptidoglycan comprises 30 to 70% of the cell wall of Gram-positives, this is only 10% in Gram-negatives [122]. Furthermore, Gram-negative bacteria cell walls also contain lipopolysaccharide (LPS) and lipoproteins, whereas Gram-positive microorganisms have teichoic acids [122]. In addition, Gram-negative bacteria possess an outer membrane (OM), con-

trary to Gram-positive bacteria. The OM is a lipid bilayer with an inner leaflet composed of phospholipids and an outer leaflet with phospholipids anchored to LPS. Phosphate groups and acidic sugars of the LPS molecules provide the cell surface with a negative charge. Divalent cations (Mg^{2+}, Ca^{2+}) stabilize the OM through ionic interactions with the phosphate groups of adjacent LPS molecules. The peptidoglycan layer of Gram-negative organisms resides subjacent to the OM. Likewise, the surface proteins and carbohydrates usually found in the peptidoglycan layer will be present in the OM. This structure conveys the outer membrane with high asymmetry and, consequently, exceptional impermeability [123, 124]

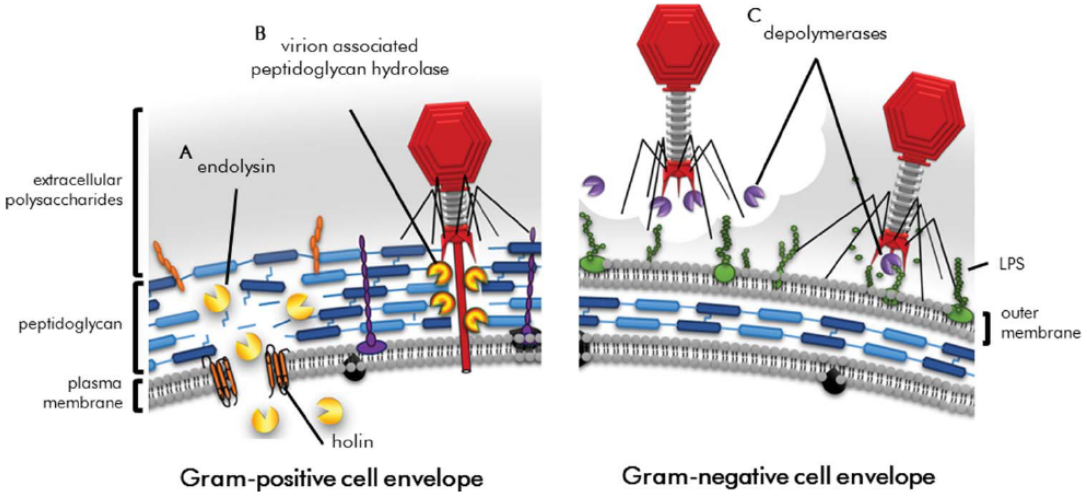

Figure 1.6: Schematic of Gram-positive bacterial cell envelope and Gram-negative bacterial cell envelope. Adapted from [119].

The peptidoglycan layer is composed of linear strands of alternating N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) residues, coupled by β(1-4) linkages, which altogether comprise the glycan polymer in the peptidoglycan. This polymer is covalently linked to a short stem peptide through an amide bond between MurNAc and an L-alanine, the first aminoacid of the peptide component. The remainder of the stem peptide is composed of alternating L- and D-form aminoacids. These are well conserved in Gram-negative bacteria but have variable composition in Gram-positive bacteria [125]. For several Gram-positive bacteria, the third residue of the stem peptide is L-lysine, which is respectively linked to an opposing stem peptide on a separate glycan polymer through an interpeptide bridge. However, Gram-negative bacteria usually contain a mesodiaminopimelic acid (mDAP) residue at position three instead of L-lysins. In this case, mDAP residue cross-links to the terminal D-alanine of the opposite stem peptide, without establishing an interpeptide bridge [125].

The structure of the peptidoglycan is generally well-conserved. Endolysins can be classified according to their catalytic activity site in the peptidoglycan layer [119]. These endolysins can degrade the peptidoglycan with glycosidase, amidase, endopeptidase, or lytic transglycosylase activities. A glycosidase cuts between glycan residues, whereas an amidase hydrolyses the amide bond between the glycan moiety (MurNAc) and the peptide moiety (L-alanine) of the peptidoglycan [119, 125]. Endopeptidases degrade peptide bonds between two amino acid residues and transglycosylases cause degradation of the β(1-4) linkages between N-acetylmuramyl and N-acetylglucosaminyl residues of the peptidoglycan, and form a 1,6 anhydromuramyl residue [125]. Glycosidases are further categorized as

N-acetylmuramidases that cleave the glycan component of the peptidoglycan on the reducing side of MurNAc, or as N-acetylglucosaminidases that cleave the glycan component of the peptidoglycan on the reducing side of GlcNAc [119, 125]

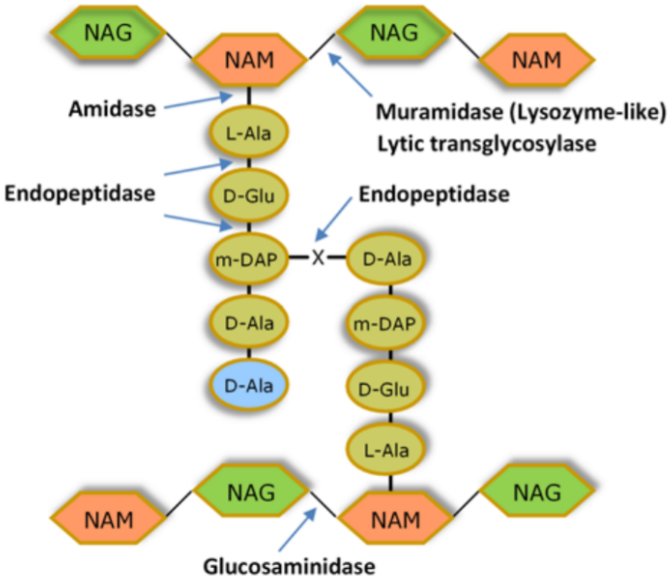

Figure 1.7: Basic structure of the bacterial cell wall peptidoglycan and representation of cleavage sites by endolysins: amidases cleave the amide bond between MurNAc and the peptide moiety (L-alanine); endopeptidases cleave bonds between two amino acid residues; glycosidases cut between the glycan residues MurNAc and GlcNAc (or NAM and NAG, respectively); glycosidases are categorized as muramidases and glucosaminidases: the former cleave the glycan residue on the reducing side of NAM, whereas the latter cleave the glycan residue on the reducing side of NAG. Adapted from [126].

Due to their potential of killing susceptible organisms when applied exogenously, endolysins have been an interesting topic of research over the last decades as potential antimicrobial agents [118]. Furthermore, endolysins contain an important advantage over typical antibiotics which is their specificity for certain peptidoglycans and, consequently, its affinity to a certain bacterial genus, species or even serotype [127]. Application of endolysins as antibacterials was initially limited to Gram-positive organisms, since their cell wall is not protected by an outer membrane, which is the case for Gram-negative species [118]. Nevertheless, different endolysins have been reported to have an intrinsic antibacterial activity against Gram-negative pathogens: *in vivo* studies with *A. baumannii* ATCC 17978 reported over 99% of antibacterial rate following incubation, for 1 hour, with endolysins LysAB3 and LysAB4 (antibacterial activity was calculated with: antibacterial rate (%) = $(N_0\text{-}N_1)/N_0$ x 100%, where N_0 and N_1 are the numbers of colonies in the control culture plates and the experimental culture plates, respectively) [128]; 0.5 mg/ml of endolysin LysPA26 is able to kill up to 4 log units of *P. aeruginosa* D204 in 30 minutes, when incubated with 10^8 exponential cells of host bacteria, in the absence of EDTA. The bacterial cells were more sensitive when treated with 1 mM EDTA, which led to chelation of the divalent cations that stabilize the outer membrane, causing its disruption [129].

Targetting Gram-negative pathogens - A challenge for endolysins

Endolysins can be classified into modular or globular, according to their structure. Modular en-

dolysins are composed of an enzymatically active domain (EAD) and a cell wall binding domain (CBD), attached to a short linker region that typically connects the catalytic domain to the CBD [119, 124]. These interdomain linker sequences can vary in size and create an inherent flexibility to these proteins [125]. Globular endolysins, however, are composed of a single enzymatically active domain. The EAD cleaves a specific bond in the peptidoglycan structure, while the CBD targets the EAD to its substrate by binding peptidoglycan or another cell wall component [124].

The large majority of endolysins derived from phages that infect Gram-positive bacteria typically have a modular structure, with an N-terminal EAD and a C-terminal CBD [119]. Endolysins from Gram-positive infecting phages may also contain more than one catalytic domain [125]. More specifically, double EADs with endopeptidase (cysteine, histidine-dependent amidohydrolases/peptidases - CHAP - domain) and amidase activity, or with endopeptidase and muramidase activity are often found together [125]. LysK, the endolysin encoded by *S. aureus* infecting phage K, for example, consists of a CHAP endopeptidase and an amidase domain [125]. However, endolysins from Gram-negative microorganisms are usually single-domain, globular proteins and lack CBDs [130]. These endolysins will typically consist of a single catalytic domain and have a mass of 15 to 20 kDa [125]. It is hypothesized that the presence of a CBD in endolysins from Gram-positive infecting phages but not in Gram-negative equivalent is due to the high affinity of a CBD for its ligand, with the CBD keeping the endolysin tightly bound to cell debris after cell lysis [131]. By doing so, new potential host cells are prevented from lysis before being actually infected by the phage virions. Since Gram-negative bacteria possess an outer membrane (OM), this risk is not associated with Gram-negative infecting phage, thus rendering a CBD unnecessary in their composition [124].

Despite usually having a globular structure, some endolysins derived from Gram-negative infecting phages have been reported to contain a modular structure, with both a lytic domain and an N-terminal cell wall binding domain (CBD). These modular endolysins have been identified and characterized often in jumbo phages [132]. These are phages with genomes larger than 200 kbp. The first two endolysins found with this composition in a Gram-negative microorganism were named KZ144 and EL188, named for gene products from the corresponding *P. aeruginosa* infecting-phages [133].

The presence of the OM in the cell wall of Gram-negative bacteria makes the exogenous addition of a phage endolysin insufficient to obtain access to the peptidoglycan without an agent or mechanism that allows to translocate the protein across the OM [125]. Several strategies exist to disturb the OM of Gram-negative organisms and promote permeability. For example, applying high hydrostatic pressure (HHP) to a Gram-negative organism has proven to be effective in translocating a lytic enzyme accross the OM [134]. This strategy is mostly used for food-preservation [124]. More interesting for implementation in clinical applications, the integrity of the outer membrane can be disturbed by certain agents that weaken the stabilizing interactions between OM components. These agents are designated outer-membrane permeabilizers (OMPs) [135]. OMPs can be classified according to type of OM permeabilization, namely, physical, chemical or biological [124].

Considering OMPs of chemical origin, two classes can be considered: polycationic agents (polymyxin and its derivatives, for example) which act by competing with the stabilizing divalent cations of the

outer membrane for the negatively charged LPS. The cations are consequently displaced leading the disarrangement of the OM [136]; the other class is represented by chelators with ethylenediaminetetraacetic acid (EDTA) as a commonly present compound. EDTA removes by chelation the stabilizing divalent cations from their binding site in LPS, resulting in the release of a significant proportion of LPS from the cells, and hence OM disruption [136]. Weak organic acids perform similarly to this class of OMPs [135].

Regarding OMPs of biological origin, two permeabilizing strategies can be distinguished. Receptor-mediated uptake of endolysins through the OM is possible with structural engineering. In fact, this strategy has been employed with an outer membrane receptor for FyuA pesticin. Pesticin is a bacteriocin with a modular structure which, when its N-terminal domain binds to the OM protein FyuA as receptor, allows for its uptake across the OM into the periplasm [124, 137]. Artilysins are a more versatile approach, based on a novel type of protein-engineered endolysins. The principle of Artilysins is the fusion of endolysins to outer membrane permeabilizing peptide, which can frequently be introduced in the form of antimicrobial peptides. Several antimicrobial peptides (AMPs) possess outer membrane destabilizing properties, which accounts for the potential of Artilysins [124].

Artilysins - An answer to Gram-negative pathogens

A new class of antibacterials, with the capacity to penetrate the outer membrane, has arisen. These enzymes termed Artilysins covalently combine a highly active bacteriophage-encoded endolysin with an outer membrane-permeabilizing peptide [124]. A more specific class of Artilysins is based on the fusion of a targetting antimicrobial peptide (AMP) with a highly active bacteriophage-encoded endolysin [138]. Produced by a wide variety of organisms, AMPs are quite diverse with respect to their amino acid sequences. Generally, outer membrane destabilizing peptides share an amphipathic conformation, with positively charged aminoacids on one side and negatively charged aminoacids on the other side of the peptide. The overall positive charge of the peptides, however, allows them to accumulate at the polyanionic cell surface of the bacterium, which in Gram-negative bacteria corresponds to the LPS of the outer envelope. [139, 140]. Therefore, Artilysins can be described as engineered-endolysins with LPS-destabilizing properties [141].

Artilysins do not need an active bacterial metabolism to employ their bactericidal effect, since they actively degrade the peptidoglycan layer, resulting in immediate osmotic lysis [142]. Interestingly, a study by Briers *et. al* (2014) has shown that the activity of a series of Artilysins (LoGT-001 to LoGT-014) was enhanced by the presence of a linker of increasing length between the peptide and lysin or even by a combination of both polycationic and hydrophobic/amphipathic peptides [141]. This means that the linker length may influence the Artilysin antibacterial efficacy.

An example of a highly efficient Artilysin active against *P. aeruginosa* is Art175, which consists of a SMAP-29 peptide (sheep myeloid antimicrobial peptide) comprising 29 amino acids fused to the KZ144 endolysins. Art175 has a superior bactericidal effect against persister cells [142]. Remarkably, a more recent study proved that Art175 is equally effective in killing both stationary-phase cells and persister cells of multidrug-resistant *A. baumannii*. Moreover, killing rate could be enhanced with the addition of

0.5 mM of EDTA [143].

Another study by Briers *et. al* (2014) proved that fusion of a peptide with an active endolysin (PVP-SE1gp146) results in highly enhanced antibacterial activity against *P. aeruginosa* [141]. In skin infections, namely, an Artilysin called LoGT-008 demonstrated strong antibacterial activity against both *P. aeruginosa* and *A. baumannnii*, with MICs of 4 and 8 μg/mL respectively.

Endolysins: sustainable solution to AMR?

The development of resistance against endolysins seems unlikely. This is due to several reasons, namely, the continuous co-evolution observed among phages and their respective host bacteria. Several studies have been carried out with the intent to assess potential bacterial resistence against endolysins. A study directed at creating MRSA strains, resistant to a chimeric endolysin (ClyS), revealed an to be an unsuccessful attempt. Conversely, different results were achieved when assessing the MIC of mupirocin, against the same strain. The MIC values increased approximately tenfold over the course of eight days [144]. Likewise, a study with cells of *Streptococcus pneumoniae* repeatedly exposed to the Pal endolysin did not contribute to the development of resistant phenotypes [145].

It is important to keep in mind that endolysins have evolved to recognize and cleave highly conserved structures in the cell wall. Altering these structures could possibly be detrimental to the host bacterium [146]. The fact that several of the endolysins possess two EADs that hydrolyze different bonds in the peptidoglycan is believed to reduce chances for acquiring resistance [147].

More importantly, endolysins can be used in combination with antibiotics to treat antibacterial infections, resulting in a synergistic effect particularly effective against bacterial infections [147]. All in all, endolysins appear to not be significantly susceptible to bacterial resistance strategies.

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