Universidade de Lisboa

Faculdade de Farmácia

Enhancing phage endolysins enzybiotic potential through their combined action with antimicrobial peptides

Doutoramento em Farmácia

(Especialidade de Microbiologia)

Ana Isabel Ricacho Gouveia

Tese orientada pelo Professor Doutor Carlos Jorge Sousa de São José, especialmente elaborada para a obtenção do grau de doutor.

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2026

“Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.”
Louis Pasteur

“Beliefs do not change facts. Facts, if one is rational, should change beliefs.”
Ricky Gervais

“Once we believe in ourselves, we can risk curiosity, wonder, spontaneous delight, or any experiences that reveal the human spirit.”
E.E. Cummings

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Declaração

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I hereby declare that I have developed and written this work in strict accordance with the University of Lisbon's Code of Conduct and Good Practices. In particular, I declare that I have not engaged in any of the varieties of academic fraud which I hereby declare I am aware of, and I have followed the required referencing of phrases, extracts, images and other forms of intellectual work, fully assuming the responsibilities of authorship.

Scientific Outputs

Articles in international peer-reviewed journals

- Gouveia, A., Pinto, D., Veiga, H. *et al.* Synthetic antimicrobial peptides as enhancers of the bacteriolytic action of staphylococcal phage endolysins. *Sci Rep.* 2022; 12:1245. https://doi.org/10.1038/s41598-022-05361-1
- Gouveia, A., Pinto, D., Vítor, J. M. B., & São-José, C. Cellular and Enzymatic Determinants Impacting the Exolytic Action of an Anti-Staphylococcal Enzybiotic. *Int J Mol Sci*. 2023; 25:523. https://doi.org/10.3390/ijms25010523

Oral communications in international meetings

- Gouveia, A., Pinto, D., Veiga, H., Antunes, W., Pinho, M.G., São-José, C. Synergistic Bacteriolytic Action of Phage Endolysins and Antimicrobial Peptides. WMF - World Microbe Forum. An ASM & FEMS Collaboration. June 20-24th, 2021. *Online* Worldwide. (virtual congress).
- Gouveia, A., São-José, C. Unraveling bacterial determinants of tolerance to endolysin lytic action. 6th Viruses of Microbes International Meeting (VoM2022). July 18-22nd, 2022. Guimarães, Portugal.

Oral communications in national meetings

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- Gouveia, A., São-José, C. Understanding Bacterial Tolerance to Antimicrobial Activity of Bacteriophage Endolysins. 13th iMed.ULisboa Postgraduate Students Meeting. July 4-5th, 2022. Faculty of Pharmacy, University of Lisbon. Lisbon, Portugal.
- Gouveia, A., Pinto, D., Vítor, J.M.B., Veiga, H., Antunes, W., Pinho, M., São-José, C. Understanding and overcoming bacterial tolerance: exploring the synergistic action of anti-staphylococcal enzybiotics and antimicrobial peptides. 15th iMed.ULisboa Postgraduate Students Meeting. July 11-12th, 2024. Faculty of Pharmacy, University of Lisbon. Lisbon, Portugal.

Poster communications in national meetings

- Gouveia, A., Pinto, D., São-José, C. Enhancing Endolysins Bacteriolytic Activity Through their Combined Action with Antimicrobial Peptides. 12th iMed.ULisboa Postgraduate Meeting. June 16th, 23rd and 30th and July 7th. Faculty of Pharmacy, University of Lisbon. Lisbon, Portugal. (virtual congress).
- Gouveia, A., Pinto, D., São-José, C. Determinants of bacterial tolerance to endolysin-based enzybiotic. MicroBiotec'21 – Congress of Microbiology and Biotechnology 2021. November 23rd-26th, 2021. Nova University of Lisbon. Lisbon, Portugal. (virtual congress).
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<u>The author of this thesis has also contributed to the following scientific communications</u>

- Gonçalo, R., Pinto, D., Gouveia, A., São-José C. The strong bactericidal action of a chimeric enzybiotic VAL-based against *Staphylococcus aureus*, even in conditions that support bacterial growth. 13th iMed.ULisboa Postgraduate Students Meeting. July 4-5th, 2022. Faculty of Pharmacy, University of Lisbon. Lisbon, Portugal.
- Gonçalo, R., Pinto, D., Gouveia, A., São-José C. A VAL-based enzybiotic displaying strong bactericidal action against *Staphylococcus aureus* in growth supporting media. 6th Viruses of Microbes International Meeting (VoM2022). July 18-22nd, 2022. Guimarães, Portugal.
- Antunes, J.M., Viegas, S.M., Gouveia, A., Perdigão, J., Pimentel, M., São-José, C. Hunting Bacteriophages to Fight WHO Priority Bacteria Displaying Antibiotic Resistance. 15th iMed.ULisboa Postgraduate Students Meeting. July 11-12th, 2024. Faculty of Pharmacy, University of Lisbon. Lisbon, Portugal.

Abstract

With an antimicrobial resistance crisis on the rise, alternatives to traditional antibiotics are needed. Over the past two decades, intensive research has been focused on the development of bacteriophage lytic enzymes as antibacterials. These include endolysins (in their native form or engineered derivatives) and the still underexplored virion associated lysins (VALs). Together, they represent the leading examples of a broader class of protein-based antibacterials known as enzybiotics.

Endolysins are bacteriolytic enzymes produced by bacteriophages (or simply phages), the viruses that infect bacteria. Endolysins are responsible for host cell wall (CW) degradation after viral replication, a key event underlying the lysis of the infected cell for virion progeny release. In the phage infection context endolysins act from within, reaching the CW after being translocated across the cytoplasmic membrane (CM). Importantly, they can also exert they bacteriolytic action from outside the cell (from without), if access to the CW is granted. This is at the basis of the exploration of endolysins as enzybiotics.

Initial research in this field has principally focused on endolysins targeting Gram-positive pathogens, as they were considered better targets for exogenously added endolysins due to the fact they lack an outer membrane (OM) shielding the CW. However, endolysin antimicrobial characterization assays are typically performed in nutrient-depleted, buffered solutions, in which cells are maintained in a "growth-arrested" state. More recently, several studies have revealed that different Gram-positive bacteria maintained in nutrient-rich media, supporting an energized CM and bacterial growth, can display a certain degree of tolerance to endolysin-mediated lysis from without. Yet, the cellular and enzymatic factors regulating endolysins' lytic action and tolerance remain poorly understood. Evidence to date has pinpointed the bacterial membrane potential (or proton motive force, PMF) and certain CW secondary polymers of Gram-positive bacteria as key factors regulating bacterial autolysins and contributing to tolerance against endolysins.

In the natural context of phage infection, endolysins activity is tightly coordinated with that of holins, which are small phage-encoded proteins that lead to CM permeabilization. Holins form "holes" in the CM and in most studied systems they provide the pathway for endolysin passage from the cytoplasm to the CW. Of note, these holes have also the transversal role of causing abrupt and extensive PMF collapse, with consequent cell death. Interestingly, PMF collapse appears to be an important determinant of bacterial susceptibility to endolysins. Indeed, studies have shown that the holin or ionophores mimicking its PMF-dissipation action can significantly enhance the lytic action of endolysins, regardless of whether they access the CW from within or without.

Building on these observations, we proposed that antimicrobial peptides (AMPs) - another class of alternative antimicrobials that frequently act by disrupting the CM and abolishing the PMF - could

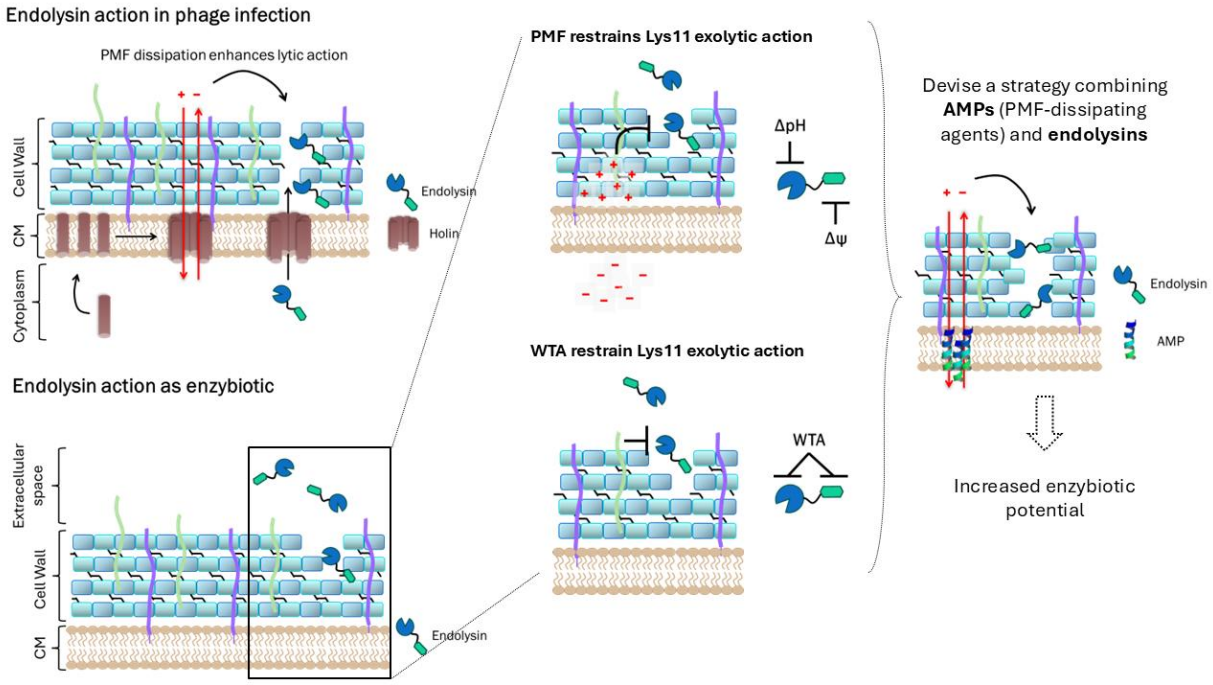
be combined with endolysins, in order to enhance their antibacterial potential. Using as model the high-priority pathogen *Staphylococcus aureus* and anti-staphylococcal endolysins, we demonstrated that in nutrient rich media *S. aureus* cells become much more susceptible to the exolytic action of endolysins in presence of selected AMPs. The combined action yielded a stronger bacteriolytic and bactericidal effect than either agent alone, across a range of *S. aureus* strains, which included methicillin-resistant *S. aureus* (MRSA) from different clonal complexes. The results indicated that AMP-mediated PMF dissipation simultaneously stimulated endolysin binding to the cell surface and subsequent CW degradation.

Using the same model, we next sought to understand in more detail the determinants and mechanisms governing bacterial tolerance to a specific endolysin, Lys11, and how they impacted the enzyme's functional domains. The PMF across the CM has two components: the electrical potential (Δψ) and the proton gradient (ΔpH). In this study we have used selective membrane ionophores to abolish either or both components of the PMF and assess how they impacted Lys11 lytic action. We have shown that the pH and electrical gradients of the PMF affect distinctively the catalytic and binding domains of Lys11. The ΔpH component was preponderant in restraining the endolysin lytic action and its dissipation enhanced CW cleavage primarily via the enzyme's peptidase domain. Interestingly, Δψ elimination boosted Lys11 binding to cells through the enzyme's amidase domain. Thus, the two PMF components appear to regulate distinct functional domains of Lys11. In addition, the drug tunicamycin and a panel of *S. aureus* mutants allowed us to study the role of the major *S. aureus* CW polymers, and their modifications, in tolerance. The negatively charged wall teichoic acids were confirmed as the key CW polymers contributing to tolerance by severely impairing Lys11 association to the CW through its canonical cell binding domain and by restraining the enzyme's CW cleavage activity, thereby restraining endolysin action by a dual mechanism.

In conclusion, this work has contributed to deepening our understanding of how bacterial physiology and CW composition affect the susceptibility of Gram-positive bacteria to the exolytic action of endolysins, providing new insights into the mechanisms of bacterial tolerance. The results open new venues for strategies aiming at increasing the enzybiotic potential of endolysins.

Keywords: Antibiotic resistance; endolysins; antimicrobial peptides; cell wall; membrane potential.

Graphical Abstract



Resumo Estendido

Com o aumento do aparecimento de estirpes bacterianas resistentes a múltiplos antibióticos, há o perigo de haver um regresso a uma era pré-antibióticos se não forem tomadas medidas para mitigar o problema. A descoberta dos antibióticos foi um ponto de viragem na medicina, permitindo reduzir as taxas de mortalidade associada a infeções bacterianas, aumentando também a qualidade e a esperança de vida. No entanto, o aparecimento e desenvolvimento de bactérias resistentes a antibióticos põe em causa a eficácia dos antibióticos. A resistência a antibióticos é considerada pela Organização Mundial de Saúde (OMS) uma das maiores ameaças à saúde humana a uma escala mundial.

Devido à redução da eficácia dos antibióticos no tratamento de infeções bacterianas, é necessário que se desenvolvam terapias alternativas à antibioterapia convencional. Neste sentido, nas últimas duas décadas, o potencial das enzimas líticas derivadas dos bacteriófagos como antibacterianos tem sido intensamente estudado e desenvolvido. As enzimas líticas derivadas dos bacteriófagos incluem as endolisinas (na sua forma nativa ou derivados sintéticos) e, ainda que menos exploradas, as lisinas associadas ao virião (VALs, de *virion associated lysins*), sendo os principais exemplos de uma vasta classe de antibacterianos à base de proteínas, conhecida como enzibióticos.

As endolisinas são enzimas bacteriolíticas produzidas por bacteriófagos (ou, simplesmente, fagos), os vírus que infetam, especificamente, bactérias. As endolisinas são responsáveis pela degradação da parede celular das bactérias hospedeiras, após a replicação viral, permitindo, deste modo, a libertação da descendência viral. No contexto da infeção fágica, as endolisinas são produzidas no citoplasma e translocadas para o compartimento da parede celular através da membrana citoplasmática, atuando assim de dentro para fora da célula hospedeira. Contudo, as endolisinas também podem ter ação bacteriolítica quando aplicadas exogenamente, atuando de fora para dentro, desde que tenham acesso à parede celular. Devido a esta atividade exolítica, as endolisinas são consideradas enzibióticos, tendo grande potencial antibacteriano contra bactérias patogénicas.

Os primeiros estudos que demonstraram o potencial antibacteriano das endolisinas focaram-se principalmente em bactérias patogénicas Gram-positivas. Considera-se que as bactérias Gram-positivas são alvos mais suscetíveis à aplicação exógena das endolisinas, uma vez que não possuem uma membrana externa, que reveste e protege a parede celular (característica das bactérias Gram-negativas). No entanto, têm de ser feitas algumas considerações importantes. A caracterização da atividade lítica das endolisinas é tipicamente realizada em soluções-tampão que não fornecem nutrientes às bactérias, mantendo-as num estado viável, mas não suportando o seu crescimento ativo (estado metabólico e fisiológico desfavorável à divisão celular). Na última década,

vários estudos demonstraram que várias espécies de bactérias Gram-positivas, quando mantidas em meios nutritivos capazes de manter as bactérias num estado de crescimento ativo e com uma membrana polarizada, apresentam um certo grau de tolerância à ação lítica das endolisinas, quando estas atuam de fora para dentro. Ainda assim, os determinantes celulares e enzimáticos que regulam a ação lítica das endolisinas e a tolerância bacteriana a estas ainda são pouco conhecidos. No entanto, os dados disponíveis indicam que o potencial da membrana citoplasmática (ou força proto-motriz, PMF, de *proton-motive force*), e alguns polímeros secundários da parede celular são os principais factores que regulam a ação das autolisinas bacterianas (enzimas que são semelhantes quer estruturalmente quer funcionalmente às endolisinas) e que também estão envolvidos no fenómeno de tolerância bacteriana às endolisinas.

No contexto natural da infeção fágica, a atividade das endolisinas ocorre de forma concertada com a ação das holinas, uma segunda proteína codificada pelos fagos que permeabiliza a membrana citoplasmática. As holinas foram poros ("holes") na membrana citoplasmática que, na maioria dos fagos descritos, funcionam como um canal para as endolisinas passarem do citoplasma para a parede celular. Igualmente importante, é o papel transversal que os canais das holinas têm de dissipar a PMF de forma abrupta e extensa, levando à morte celular. Interessantemente, verificou-se que a dissipação da PMF parece ser um determinante crítico da suscetibilidade bacteriana à ação lítica das endolisinas. De facto, alguns estudos mostraram que a holina, ou ionóforos que mimetizam a sua ação de dissipação da PMF, podem aumentar significativamente a ação lítica das endolisinas, independentemente de estas acederem à parede celular de fora para dentro ou o contrário.

Com base nestes conhecimentos, propusemos que os péptidos antimicrobianos (AMPs, de *antimicrobial peptides*), outra classe de antimicrobianos alternativos que frequentemente atuam por disrupção da membrana citoplasmática, e que, consequentemente, levam ao colapso da PMF, podiam ser combinados com as endolisinas, de forma a potenciar as suas propriedades antibacterianas.

Os AMPs são pequenas moléculas produzidas por praticamente todos os organismos (desde microrganismos a organismos superiores, como animais e plantas), que fazem parte dos sistemas de defesa inatos como mecanismo de proteção contra bactérias, vírus e fungos. Geralmente, estes péptidos são moléculas pequenas, com carga positiva e com propriedades hidrofóbicas ou anfipáticas. Estas características conferem as propriedades antimicrobianas aos AMPs, permitindo que estes se insiram na membrana citoplasmática, levando à sua permeabilização e, consequente, à morte celular.

Deste modo, utilizámos como modelo de estudo a bactéria patogénica *Staphylococcus aureus*, considerada de alta prioridade pela OMS, e a endolisina *anti-S. aureus* Lys11. Foi possível demonstrar que, em meio nutritivo, as culturas de *S. aureus* tornam-se muito mais suscetíveis à ação lítica das endolisinas na presença de AMPs. Utilizámos em particular um AMP derivado do

SMAP-29 (*Sheep myeloid antimicrobial peptide* de 29 aminoácidos), em que a troca de uma arginina por uma lisina resulta num péptido de baixa toxicidade para células humanas. A ação conjunta Endolisina+AMP resultou num efeito bacteriolítico e bactericida superior ao observado com os agentes individualmente. Esta ação antibacteriana aumentada foi observada em diversas estirpes de *S. aureus*, incluindo estirpes resistentes à meticilina representativas de diferentes complexos clonais. Para além disso, os resultados indicam que a despolarização da membrana induzida pelo AMP estimula a ação lítica de Lys11 por duas vias: favorece a ligação da endolisina à parede celular ao mesmo tempo que aumenta a sua atividade de degradação da parede celular.

Utilizando o mesmo modelo, propusemo-nos a estudar e compreender em maior detalhe os determinantes e mecanismos que governam a tolerância bacteriana às endolisinas, em específico à Lys11, e como estes impactam os domínios funcionais da enzima. A PMF é composta por dois componentes: o potencial elétrico (Δψ) e o gradiente de protões (ΔpH). Neste estudo, usámos ionóforos para abolir (anular) seletivamente um ou ambos os componentes da PMF, de modo a avaliar o seu impacto sobre a ação lítica da Lys11. Demonstrámos que o gradiente de pH e o gradiente elétrico afetam de forma distinta os domínios catalíticos e de ligação à parede. O ΔpH tem um papel preponderante na restrição da ação lítica da endolisina, sendo que a sua dissipação potencia a ação de degradação da parede celular, principalmente pela estimulação do domínio de peptidase da enzima. Interessantemente, ao se eliminar o Δψ observou-se um aumento da ligação da Lys11 às células, mediado sobretudo pelo domínio amidase da endolisina. Assim, ambos os componentes da PMF parecem regular de forma distinta os domínios funcionais da Lys11 envolvidos na ligação e clivagem da parede.

Adicionalmente, utilizámos também tunicamicina - um antibiótico que a baixas concentrações inibe a síntese dos ácidos teicóicos da parede (WTA, de *wall techoic acids*) e um painel de mutantes de *S. aureus* para estudar o papel dos principais polímeros da parede celular, e das suas modificações, na tolerância às endolisinas. Confirmámos que os WTA, como têm carga negativa e contribuem para o ambiente aniónico que caracteriza a superfície celular bacteriana, são os principais polímeros da parede que contribuem para a tolerância. Foi possível detalhar que os WTA impedem a ligação de Lys11 à parede mediada pelo seu domínio canónico de ligação, restringindo ao mesmo a atividade catalítica da endolisina. Deste modo, os WTA contribuem para a tolerância bacteriana às endolisinas através de um mecanismo dual. Os resultados suportam a interconexão entre a PMF e os WTA na tolerância às endolisinas.

Os estudos desenvolvidos nesta tese permitiram aprofundar o nosso conhecimento e compreensão sobre como a fisiologia bacteriana e a composição da parede celular podem afetar a suscetibilidade das bactérias Gram-positivas à ação exolítica das endolisinas, fornecendo novas perspectivas sobre os mecanismos de tolerância bacteriana. Desta forma, é possível também desenvolver novas estratégias que visem aumentar o potencial antibacteriano, enquanto enzibiótico, das endolisinas.

Palavras-chave: Resistência a antibióticos; endolisinas; péptidos antimicrobianos; parede celular; potencial de membrana.

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List of Abbreviations and Units

(Those not defined in the text)

ANOVA – Analysis of variance

ATP – adenosine triphosphate

CHAP – cysteine, histidine-dependent aminohydrolase/peptidase

CRISPR – clustered regularly interspaced short palindromic repeats

$DiSC_3(5)$ – 3,3'-Dipropylthiadicarbocyanine iodide

DMSO – dimethyl sulfoxide

DNA – desoxyribonucleic acid

dsDNA – double stranded DNA

DTT – dithiothreitol

EDTA – ethylenediamine tetraacetic acid

eGFP – enhanced green fluorescent protein

FDA – Food and Drug Administration

Gly – Glycine

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

His_6 – Histidine Tag

IPTG – isopropyl β-D-1-thiogalactorpyranoside

L/D-Ala – L/D-Alanine

L/D-Glu – L/D-Glutamic acid

L/D-Ser – L/D-Serine

LB – Lysogeny broth

L-Lys – L-Lysine

Met – Methionine

RNA – ribonucleic acid

Units

h – hours

min – minutes

s – seconds

Da – Daltons

kDa – kilodaltons

M – Molar

mM – millimolar

µM – micromolar

nM – nanomolar

cm – centimetre

µm – micrometre

nm – namometre

ml – millilitre

µl – microlitre

µg/ml – micrograms per millilitre

ng/ml – nanogram per millilitre

CFU/ml – colony forming units per millilitre

° C – Celsius degrees

g – gravitational force

W – watts

kV – kilovolt

Chapter I.

General Introduction

I.1. The Global Crisis of Antimicrobial Resistance

Prior to the discovery of antibiotics, infectious diseases were among the illnesses with the highest morbidity and mortality worldwide[1]. However, with the discovery of penicillin in 1928 by Sir Alexander Fleming and of streptomycin in 1943 by Dr. Selman Waksman a new era in medicine emerged, with the golden age of antibiotic use occurring a few years after World War II[2].

It was S. Waksman and his colleagues that first used the term "antibiotics", to refer to chemical substances produced by microorganisms that inhibited the growth, or killed, other microorganisms. However, this description excluded synthetic substances and biological products of non-microbial origin[3]. In present days, the definition of antibiotic is more inclusive, describing any organic chemical of natural or synthetic origin that inhibits or kills bacteria, by interfering with essential cellular processes[4].

Antibiotic discovery was a turning point in medicine by reducing the high rates of mortality associated to bacterial infections, also increasing the quality and expectancy of life in developed countries[5]. However, since the introduction of the first antibiotics in clinical practice, it became apparent that bacteria could develop resistance to these drugs in a rather fast manner, driving the need for new antibacterial agents. Over the years, this has prompted cycles of modification or discovery of new antibiotic classes and resistance acquirement, leading to a decrease in antibiotic efficiency to treat life-threatening infections[6].

Antimicrobial resistance (AMR) can be defined as the ability of bacteria to survive under the selective pressure of an antimicrobial agent and occurs when an antimicrobial agent becomes less effective in inhibiting bacterial growth[7]. AMR is a naturally occurring, inevitable evolutionary phenomenon, but its rise has been accelerated by the intense use and misuse of antibiotics in many sectors such as human and animal health, and in food production. As result, AMR is nowadays recognized by the World Health Organization (WHO) and other entities as one of the major threats to human health at the global scale[8].

The great majority of antibiotic molecules that have been identified are produced by bacterial species, which often carry resistance genes to the antibiotic they produce as a self-protection mechanism[9,10]. The antibiotic resistance genes are principally involved in (i) inactivation of the antibiotic molecule, either by chemical modification or by degradation[11], (ii) impairment of antibiotic binding to target[12], (iii) alteration of membrane permeability[13] and (iv) increased antibiotic efflux[14]. In nature, resistance determinants spread among bacteria through horizontal gene transfer, which is mostly driven by conjugation (e.g. conjugational plasmids), transformation (uptake of extracellular DNA) and transduction (bacteriophage-mediated transfer)[15]. The already mentioned overuse of antibiotics in many settings has favoured the selection and dissemination of antimicrobial resistance[9].

Nowadays, AMR is a leading cause of human death. In two recent estimates of its global impact, it was found that almost 5 million deaths are associated with bacterial AMR annually, including more than 1 million deaths directly attributable to AMR[16,17]. These numbers are expected to increase if effective solutions to tackle AMR are not found, with some estimates pointing to between 8 and 10 million people dying from AMR-associated infections by 2050[17,18]. This threat combined with a shortage of new antibiotics in the pipeline causes the need for new therapeutic alternatives and solutions. Reduction of usage and the improvement of existing antibiotics should be complemented by other strategies to allow a longer-term resolution. Ideally, these alternative strategies should foster the development of traditional and non-traditional antimicrobial agents with new modes of action that escape current resistance mechanisms, and which minimize resistance development[19,20].

I.2. Reviving Old and Developing New Antibacterial Agents

The golden age of antibiotic (1940s–1970s), launched with the pioneering work of Waksman, led to the discovery of the majority of the known antibiotic classes. The "Waksman approach" to antibiotic discovery was based on bacteria's ability to produce their own antibiotics, with a strong focus on soil *Actinobacteria*, such as the genus *Streptomyces*. This strategy had some limitations as over testing soil microorganisms biased the discovery of antibiotics towards compounds of the same classes[21], with the last class discovered in the late 1980s (daptomycin)[22] (Figure I.1).

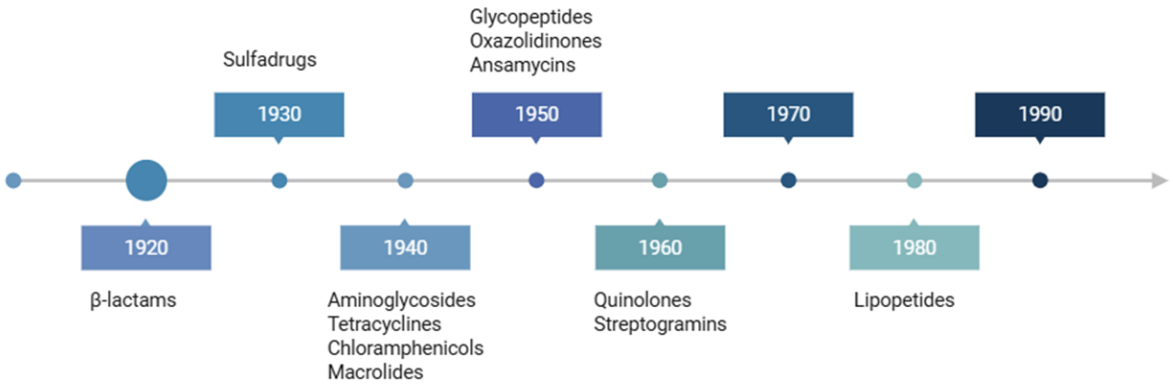

Figure I.1 Timeline of antibiotic discovery.
Sir Alexander Fleming identified the first β-lactam antibiotic, penicillin, in 1928. The golden age of antibiotics started in mid-1940 after the introduction of the Waksman approach, allowing the discovery and identification of most antibiotic classes. The most recent antibiotic class was identified in 1980s, the lipopeptides. Adapted from Ref.[21].

After exhausting the "Waksman approach" to antibiotic discovery, the industry turned to the *in vitro* synthesis of new molecules, based on the known mechanisms of action of previously discovered antibiotics but most often the novelty corresponded just to modifications and improvements of already established molecules. The lack of investment return and the quick emergence of resistance led the pharmaceutical companies to gradually abandoning the research on antibiotics[23].

Since 2017, only 16 new antibacterial compounds were approved for marketing by the regulatory agencies; from these, 13 were traditional antibiotics and 3 were non-traditional agents. However, these compounds lacked innovation, as mostly were derivatives of known classes and none of the newly approved agents had a new mechanism of action or a new molecular target[20]. Therefore, these newly approved agents are not expected to fulfil the needs for sustainably fight drug-resistant bacteria. Currently, only about half of the products being investigated in clinical trials (Phase I to Phase IV) are traditional antibiotics (59%), and the scenario is similar regarding preclinical testing (115 out of 244 products). In fact, the interest in alternative agents, with new modes of action, has been increasing in the last decade. Among the approaches exploring non-conventional antibacterials we can find antimicrobial peptides (AMPs) (both natural and synthetic derivatives) and bacteriocins, bacteriophages and derived lytic enzymes, probiotics, CRISPR/Cas9-based strategies, antibodies, and immune system modulators. Of these, a substantial part that is in clinical or preclinical development corresponds to phage-based products with direct action on bacteria[19,20,24,25].

I.2.1. Antimicrobial Peptides and Bacteriocins

AMPs are a broad class of small peptides, which are synthesized as part of the innate immune system of animals and plants or of the microbial competition weaponry. They have been considered a promising alternative to antibiotics, given that they present antimicrobial activity against a broad range of pathogens, including bacteria, fungi, protozoa and even viruses[26]. Although AMPs can differ strongly in terms of amino acid sequence, they share common properties. These include their small size, the charged nature (most commonly they are positively charged, although there are examples of neutral and negatively charged AMPs), and the amphipathic and/or hydrophobic character[26]. AMPs kill microbial cells primarily by disrupting or permeabilizing the cytoplasmic membrane, although they can also inhibit essential intracellular processes[27]. For example, gramicidin, produced by *Bacillus brevis*, inserts in the membrane of target bacteria to form small channels permeable to monovalent cations, including protons, causing the collapse of the cell energetic state[28]. Since the work described in this thesis involved the use of AMPs, more details on the properties and antimicrobial action of these agents are provided below in section I.4.

Bacteriocins are molecules produced by bacteria which were first identified in 1925 and have since been thoroughly studied. They constitute a diverse group of ribosomally synthesised

(poly)peptides that play a key role mediating microbial population interaction and competition[29]. Many have AMP-like features, being typically cationic and amphipathic, and have high diversity in terms of their amino acidic sequence and length[30]. Bacteriocins can be produced by both Gram-negative and Gram-positive bacteria, although the majority of reported bacteriocins are produced by the latter, particularly by the so-called lactic acid bacteria[31]. Bacteriocins can be classified into three major classes: Class I, composed of post-translationally modified lantibiotics, with less than 5 kDa; Class II, typically comprising unmodified, non-lantibiotic linear peptides with less than 10 kDa; and Class III, covering most bacteriocins from Gram-negative and few from Gram-positive bacteria, corresponding to large thermolabile proteins with more than 30 kDa, and which can be bacteriolytic (bacteriolysins) or non-bacteriolytic[31,32]. Bacteriocins and AMPs differ from conventional antibiotics in various aspects. The formers tend to have a narrower spectrum of activity and higher thermal stability but are more prone to proteolytic degradation. Like AMPs, most bacteriocins act by causing cytoplasmic membrane damage, most often by forming pores[31,33]. For example, the *Lactococcus lactis* nisin, a class I bacteriocin used as biopreservative, induces pore formation after binding to lipid II, an intermediate of bacterial cell wall synthesis[34].

I.2.2. Bacteriophages and Their Lytic Enzymes

Bacteriophages (or phages) are the viruses that infect bacteria. Most of the reported phages kill host cells as result of their replicative cycles, often causing bacteriolysis[35]. They were independently discovered in the early 20th century by Frederick Twort (1915) and Félix d'Hérelle (1917). Soon after their discovery, the concept of phage therapy emerged, that is, the use of phages as therapeutic agents to treat bacterial infections[36]. However, with the widespread use of antibiotics after 1940s, phage therapy was disregarded in most countries of the western world. Nevertheless, phages continued to play a crucial role as models for biological research, building the foundations of modern molecular biology[36,37].

Because of AMR, a remarkable resurgence of phage therapy research occurred in the last few years, spotlighted also by recent high-profile cases of its successful clinical application[37-39]. In addition to the use of natural phages or engineered derivatives as therapies, decades of accumulated knowledge on phage molecular biology allowed the emergence of new strategies that employ specific phage proteins with antibacterial activity[39,40]. Among such proteins are enzymes that can cleave the bacterial cell wall, generally known as phage lytic enzymes or phage lysins[41].

Phage lysins are composed by at least one enzymatically active domain responsible for cleaving a specific bond of the peptidoglycan, which is the major structural component of the bacterial cell wall. The peptidoglycan forms a protective mesh-like sacculus that surrounds the bacterial cytoplasmic membrane and confers rigidity and shape to bacterial cells. One of its major functions is to counteract the intracellular osmotic pressure that otherwise would burst cells[42,43]. Hence,

similarly to what happens with certain traditional antibiotics that block peptidoglycan synthesis, uncontrolled cleavage of the peptidoglycan macrostructure by lysins results in osmotic cell lysis and death. This has set the basis for the growth of a research field exploiting phage lysins as alternative antibacterial agents, and because of their enzymatic nature they are included in the broad group of antibacterial agents called enzybiotics[44].

The mechanisms by which bacteriophages infect and lyse bacterial hosts and how phage lytic enzymes can be explored as antibacterial agents are central to this thesis. For this reason, these subjects will be further discussed below in sections I.5 and I.6.

I.2.3. Probiotics

Probiotics are known as living microorganisms that confer a health benefit to the host, with bacterial strains belonging to *Bifidobacterium* and *Lactobacillus* being the staple probiotic bacteria. Probiotics are employed in different areas, from preservatives of fermented food products to constituents of a healthy microbiota in human and other animals[45].

One major area of interest is the therapeutic potential of probiotics as antimicrobial agents, which relies on several key features: (i) direct effect on the mucosal epithelial cells, by stimulating mucus secretion or by promoting an increase of tight junction protein expression to improve the epithelial barrier[46], (ii) competitive exclusion of pathogenic microorganisms, inhibiting pathogen colonization[47], (iii) production of antimicrobial substances, such as AMPs and bacteriocins, that directly kill pathogens[48,49], and (iv) modulation of the immune response of the host[50]. Furthermore, probiotics can be engineered to produce and deliver antimicrobial compounds as response to external cues[51].

I.2.4. CRISPR/Cas

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and their associated proteins (Cas) constitute an adaptive immune system in bacteria and archaea, which provides defence against invading genetic elements such as bacteriophages, plasmid and other mobile elements. This system uses RNA molecules to guide Cas nucleases to target and cleave the invading DNA at specific sites, neutralizing it[52].

The CRISPR/Cas systems have been harnessed for multiple applications, notably for genome-editing, with the strategies based on the *Streptococcus pyogenes* CRISPR/Cas9 being the most widely used[53], and as such, it has the potential to be used for eradicating antibiotic-resistant pathogens. Interestingly, CRISPR/Cas systems can also be used as antimicrobials by programming them to target antibiotic resistance and virulence genes in bacteria, resensitizing the target pathogens to antibiotics[54]. The application of CRISPR/Cas as alternative to antibiotics is still under

study, mainly due to the low efficiency of the existing delivery systems (typically engineered bacteriophages, synthetic nanocarriers or a combination of both)[55].

I.2.5. Antibodies

Antibodies act against bacterial pathogens either directly, by specifically targeting and neutralizing surface-exposed antigens, bacterial toxins and virulence factors or, indirectly, by activating the complement system and the cellular cytotoxicity of immune cells[56]. They usually have high specificity towards certain antigens of pathogenic bacteria, thus keeping unharmed the beneficial bacteria of the microbiota. Moreover, antibodies action is normally not affected by the antibiotic resistance profile of bacteria, and they can be further improved by engineering strategies to increase affinity and safety[57]. Therefore, antibody-based treatments could offer viable alternatives to fight bacterial infections refractory to conventional antibiotic therapies[58].

In contrast to conventional antibiotics that directly kill or inhibit bacterial growth, antibodies act by neutralizing toxin or virulence factors or by recruiting the human immune systems to attack the pathogens. Thus, bacteria are considered less prone to develop resistance to antibody treatments[59]. Although several antibodies (mostly monoclonal) have been tested in clinical trials for use in bacterial infections, only very few are currently approved by regulatory entities[59,60]. Indeed, a lack of correlation between preclinical (*in vitro* and animal models) and clinical study results is most often observed, which has been mainly attributed to great variations on antigen structure and expression among strain populations and specific types of infection[61]. One solution to improve antibodies therapeutic potential could be antibody-antibiotic conjugates, in which antibodies and antibiotics are combined to improve targeted delivery of the drugs for efficient bacterial cell death[62].

I.2.6. Vaccines

Control of antibiotic-resistant infections should rely not only on the development of alternatives therapies, but also in prevention strategies that diminish the incidence of infections and hence antibiotic consumption. One of the measures used to prevent bacterial infections is vaccination. Vaccines are biological preparations that traditionally are composed of microorganisms with attenuated virulence or of some of their constituents. Upon inoculation in hosts, vaccines will activate an adaptive immune response, stimulating antibodies production by B cells and cell-mediated immunity via T cells. Furthermore, some B and T cells can become memory cells, which rapidly activate the cellular response and production of specific antibodies after re-exposure to the pathogen[63].

Although several vaccines are currently used to protect against pathogens such as *Corynebacterium diphtheriae*, *Clostridium tetani*, *Bordetella pertussis, Streptococcus pneumoniae,*

Neisseria meningitidis and *Haemophilus influenzae*, vaccines targeting top priority antibiotic-resistant bacteria such as *Acinetobacter baumannii*, *Klebsiella pneumonia* and *Staphylococcus aureus*[64] are still unavailable. Therefore, strong efforts are currently in place to develop effective vaccines against these and other bacterial pathogens, with a recent survey reporting 94 active preclinical vaccine candidates and 61 in active clinical development[65]. However, developing vaccines remains challenging as the immune requirements for effective defence and protection against various bacterial pathogens are still poorly understood[66].

Most conventional antibiotics act by inhibiting essential bacterial biosynthetic pathways of the cell wall, protein and nucleic acids metabolism, with only few acting directly in cellular structures such as the cell envelope[67]. In contrast, most of the proposed alternatives to antibiotics (AMPs, bacteriocins, antibodies, phages and derived lytic enzymes) exert their anti-bacterial function by acting directly on different components of the bacterial cell envelope. Therefore, the next section presents relevant characteristics of this cellular structure, namely regarding its composition and key biological functions.

I.3. The Bacterial Cell Envelope

The bacterial cell envelope (BCE) is a complex multilayered structure with some conserved features across all bacterial phyla. It needs to preserve a minimal level of integrity to guarantee cell survival, protecting bacteria from their unpredictable and, often, hostile environments. It secures normal cell physiology and homeostasis, controlling the influx of nutrients and the efflux of waste products to sustain growth[68].

The BCE can be grouped into two main classes based on the number of membranes composing it. Gram-positive (or monoderm) bacteria have a single membrane, the cytoplasmic membrane (CM), whereas Gram-negative and mycobacteria (or diderm) are distinguished by the presence of a second, outer membrane (OM). In Gram-positive bacteria, a cell wall (CW) made of a thick layer of peptidoglycan with associated polymers and proteins surrounds the CM (Figure I.2a). Gram-negative bacteria have a thin layer of peptidoglycan that is encased between the CM and the OM, with the later containing lipopolysaccharide (LPS) (Figure I.2b). Mycobacteria have a particular type of CW, consisting of a network of peptidoglycan and arabinogalactan (Figure I.2c), underneath the mycolic acids rich OM.

In addition to the BCE, bacteria may produce proteinaceous S-layers and polysaccharidic capsules, forming an outermost structure that encases the cells. Protein apparatus like flagella, pili and specialized transport systems may be attached to the BCE, some of which can span and protrude from the cell surface. The specificities of the mycobacterial BCE will not be addressed here.

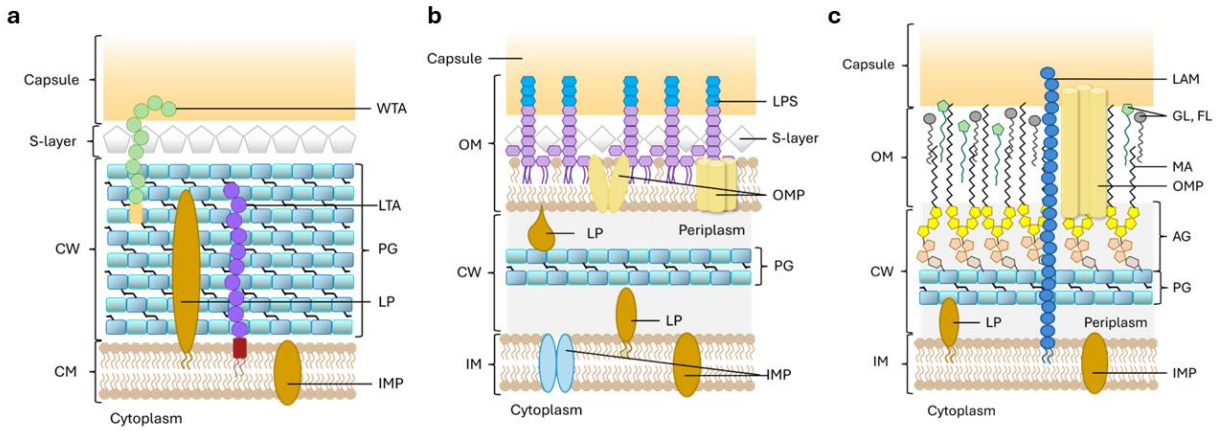

Figure I.2. Schematic representation of the structure and composition of the bacterial cell envelope.
(**a**) Gram-positive bacteria, (**b**) Gram-negative bacteria and (**c**) Mycobacteria. CW, cell wall; CM, cytoplasmic membrane; IM, inner membrane; OM, outer membrane; PG, peptidoglycan; IMP, inner membrane proteins; LP, lipoproteins; LTA, lipoteichoic acids; WTA, wall teichoic acids; OMP, outer membrane proteins; LPS, lipopolysaccharide; AG, arabinogalactan; MA, mycolic acids; LAM, lipoarabinomannan; GL, glycolipids; FL, free lipids. The S-layer and capsule are extracellular structures present in some bacterial species or strains. Adapted from Ref.[69].

I.3.1. The Cytoplasmic Membrane

The CM or inner membrane encloses the cytoplasmic contents of cells, and it is a ubiquitous structure in all cellular life forms. In bacteria, this structure is formed by a semipermeable, symmetrical phospholipid-rich bilayer, which contains embedded proteins, glycolipids, lipoproteins and protein complexes.

The bacterial CM exhibits great diversity in terms of lipid composition. Common lipid components of the CM of both Gram-negative and Gram-positive bacteria are phosphatidylglycerol, phosphatidylethanolamine and cardiolipin. While phosphatidylethanolamine is a zwitterionic phospholipid, both phosphatidylglycerol and cardiolipin have an anionic character, contributing to the global anionic charge of the CM[70]. The anionic nature of the bacterial CM makes it a target for cationic AMPs. The phospholipidic composition of the CM affects diverse properties, such as its permeability through passive diffusion, active solute transport and even protein-protein interactions. Bacterial CM composition and lipid ratios vary not only across species, but also within the same species depending on the environmental conditions in which cells are maintained[71].

The CM has fundamental roles in cell survival such as nutrient uptake, energy production, recognition and response to environmental stimuli, and BCE biogenesis[72]. In bacteria, energy generation depends on protein transporters embed in the CM. These proteins play a central role in maintain ion and solute gradients across the CM. The difference in the concentration and charges of ions on opposite sides of the CM creates an electrochemical gradient of ions, notably of protons. The electrochemical gradient of protons, known as proton motive force (PMF), has two components:

the electrical gradient (Δψ) and the chemical or proton gradient (ΔpH). The Δψ is generated by the difference of charges at both sides of the membrane and in neutralophile bacteria (typically the human pathogens) this results in an electrical potential that is negative inside the cell relative to outside. The ΔpH is created by the difference in proton concentration across the membrane, being typically alkaline inside the cell relative to outside[73]. To maintain PMF homeostasis, bacteria can enhance its counterpart, meaning there is a mutual compensation and transformation between the two PMF components[74,75]. In general, Δψ contributes more to PMF generation than ΔpH due to the production of larger potential energy from charge separation[76].

The PMF drives various cellular processes, including ATP generation, active transport of molecules (import of nutrients and efflux of toxic products, for example), and cell motility (rotation of bacterial flagella). Thus, a stable PMF should be guaranteed, as its irreversible collapse leads to bacterial cell death[77]. Indeed, many antimicrobials, including some discussed above (AMPs, bacteriocins), act precisely by collapsing the PMF and have been put forward as alternatives to fight antibiotic-resistant bacteria[76].

I.3.2. The Cell Wall

Except for the members of *Mycoplasmataceae*, the CM of all bacterial cells is surrounded by a CW, which forms an exoskeleton conferring cell shape and maintaining cell integrity by protecting it from environmental insults[68]. The CW rigidity is also important to withstand the high intracellular osmotic pressure, which otherwise would provoke cell lysis[42]. Furthermore, the CW functions as a scaffold for anchoring proteins and polymers important for cell survival[78,79].

The CW of Gram-positive bacteria is constituted mainly by a thick multilayered peptidoglycan (30-100 nm), to which anionic polymers, proteins and extracellular polysaccharides are attached. In Gram-negative bacteria the CW peptidoglycan is a much thinner (2-6 nm) and simpler structure between the space formed by the CM and the OM, a compartment defined as the periplasm. Here the peptidoglycan serves as a scaffold surface to anchor and stabilize the OM, with several (lipo)proteins tethering the OM to the peptidoglycan, as it is the case of the Braun's lipoprotein Lpp[68].

I.3.2.1. The Peptidoglycan

A. Peptidoglycan Structure and Composition

The peptidoglycan (also known as murein) is a single polymeric macromolecule structure that surrounds the bacterial CM. This structure is composed of several glycan strands of a repeating disaccharide unit and short peptide side chains that cross-link adjacent glycan strands[42].

The chemical nature of the glycan strands is conserved across bacterial species, with the repeating disaccharide being made of N-acetylglucosamine (NAG or GlcNAc) and N-acetylmuramic

acid (NAM or MurNAc) residues, linked by glycosidic bonds β(1 → 4). However, the glycan strands can suffer modifications post-assembly, which include *N*-deacetylation of GlcNAc and *O*-acetylation or *N*-glycolylation of MurNAc (Reviewed in Ref.[80]).

The peptide chains are covalently linked to NAM (MurNAc) via amide bonds and in the mature peptidoglycan they are commonly composed of four (occasionally five) amino acid residues (Figure I.3). Peptidoglycan cross-linking usually occurs between the amino acid residues at positions three and four of complementary peptide chains (3-4 cross-linkage), although 2-4 and 3-3 cross-linking can also occur, with the latter being responsible for resistance to β-lactam antibiotics in certain species[43]. Cross-linking can occur via a direct interpeptide bond (most Gram-negative bacteria and a few Gram-positive species) or via an interpeptide bridge (most Gram-positive bacteria).

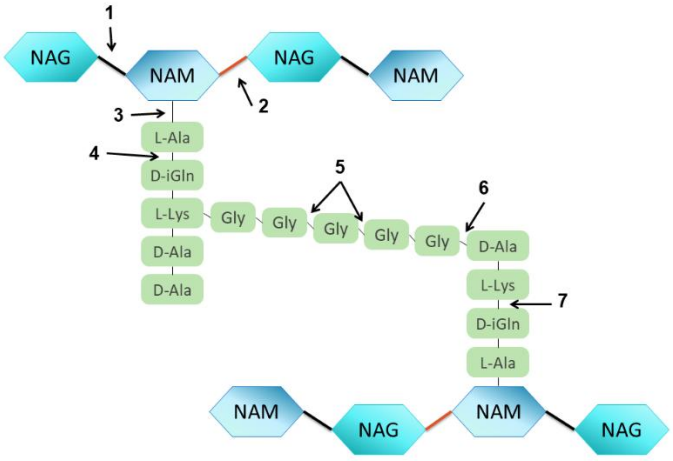

Figure I.3. The bacterial cell wall peptidoglycan.
The peptidoglycan structure found in *S. aureus* is used as example. The cleavage sites of peptidoglycan-degrading enzymes are indicated with arrows: (**1**) N-acetyl-β-D-glucosaminidase; (**2**) N-acetyl-β-D-muramidase and lytic transglycosylase; (**3**) N-acetylmuramoyl-L-alanine amidase; (**4**) L-alanoyl-D-glutamate-endopeptidase; (**5**) Glycyl-glycine endopeptidase; (**6**) D-alanoyl-glycine endopeptidase; (**7**) D-glutamyl-L-lysine endopeptidase. Adapted from Ref.[81].

Peptidoglycan diversity derives from the glycan strand modifications referred to above, but most importantly from variations in the amino acid composition of the peptide stem and interpeptide bridge, as well as the type of cross-linkage. As a result of this variation, more than one hundred peptidoglycan types have been identified[82].

In most bacterial species, the first amino acid of the peptide chain that links to NAM is L-Ala, though a Gly or L-Ser can occur instead. For all bacterial species, D-Glu or its amidated derivative D-isoGlutamine (D-iGln) is observed in the second position. The amino acid at position 3 is the most variable one. Typically, it is meso-diaminopimelic acid (m-DAP) in most Gram-negative bacteria, Bacilli and Mycobacteria or L-Lys in most Gram-positive bacteria. However, in certain species, other amino acids can be observed. Generally, two D-Ala are found at positions 4 and 5 of the peptide

chain, although D-Ser or D-Lac may be encountered in position 5. Besides the variation in the amino acids, some of these can suffer modifications such as amidation, hydroxylation, acetylation, as well as attachment of other amino acids, chemical groups and proteins[82].

The interpeptide bridge found in most Gram-positive bacteria varies not only in amino acid composition, but also in the length and, as mentioned, the stem peptide residue positions that are bridged. The length can range from one to seven residues, being found various amino acids like Gly, L-Ala, L- or D-Ser, L- or D-Glu, among others. Finally, in the same way as the peptide chain, the interpeptide bridge can be modified after its assembly[42].

B. Peptidoglycan Degrading Enzymes

The CW peptidoglycan is targeted by a diverse group of enzymes that cleave its structure. These can be of endogenous or exogenous origin and are here designated peptidoglycan degrading enzymes (PDEs). Those produced by bacteria are involved in key cellular functions, including defence against other bacteria, cleavage of the cell divisional septum, and peptidoglycan metabolism (maturation, turnover and recycling)[83]. Under stress conditions, like nutrient starvation or presence of antimicrobial agents, deregulation of endogenous PDEs can lead to bacterial autolysis, thus the enzymes involved in the process are termed autolysins[77].

PDEs of exogenous origin are produced by virtually all living organisms and they cleave the bacterial peptidoglycan in many different contexts. For example, PDEs like lysozymes make part of the innate immunity system in the animal kingdom[84]. As detailed below (Section I.5.2), phage PDEs play a key role in the viral replicative cycle by causing host cell lysis for virion progeny release.

Independently of their origin, all PDEs can be classified into three major groups based on the type of chemical bond they cleave within the peptidoglycan mesh: (i) glycosidases, (ii) amidases and (iii) peptidases[85,86]. Apart from lytic transglycosylases, all PDEs cleave the peptidoglycan through a hydrolytic mechanism[87]. Glycosidases cleave one of the two glycosidic bons in the glycan strand, being subcategorized into: (i) N-acetyl-β-D-glucosaminidases (or simply glucosaminidases), (ii), N-acetyl-β-muramidases (muramidases or lysozymes) and (iii) lytic transglycosylases (Figure I.3). The N-acetylmuramoyl-L-alanine amidases (amidases) hydrolyze the amide bond between NAM and the first amino acid residue of the peptide stem, while peptidases cleave within the peptide stems or interpeptide bridges (Figure I.3). Peptidases are further subdivided into (i) carboxypeptidases, which remove C-terminal amino acid residues, and (ii) endopeptidases, that cleave internal bonds of the peptide moiety (Figure I.3).

I.3.2.2. Teichoic Acids

The CW of Gram-positive bacteria is highly enriched in other glycopolymers besides the peptidoglycan, namely anionic polymers of which teichoic acids (TA) are notable examples[88]. It is

estimated that TA can account for up to 60% of the CW mass and they contribute largely to the overall anionic charge of the CW, forming an environment described as "a continuum of anionic charge"[79].

TA include wall teichoic acids (WTA), that are covalently linked to the peptidoglycan, and lipoteichoic acids (LTA), which are anchored via lipid domains to the CM. Both TA extend through the peptidoglycan layers, although WTA are thought to protrude further beyond the cell surface than LTA[89,90].

WTA are attached to the NAM residue of peptidoglycan via a phosphodiester link and present variable chemical structure among bacteria. Generally, WTA are made up of a conserved disaccharide linkage unit (normally consisting of NAG and N-acetylmannosamine), followed by, most commonly, a polyribitol or polyglycerol phosphate chain. These chains can be modified with cationic D-alanine esters or by glycosylation reactions, which introduce NAG or other sugar moieties, such as α-galactose or glucose[90,91].

LTA are structurally similar to WTA, they most commonly contain polyglycerol phosphate polymers, and can also be modified by D-alanine ester or sugar substituents. The LTA chain is covalently attached to a glycolipid moiety in the membrane. In most common LTA type, the glycolipid anchor is constituted by two glucose moieties linked to diacylglycerol (DAG), although other sugar residues may connect the glycolipid anchor to the main polymer chain[89]. LTA variation occurs mainly on the nature of the glycolipid anchor, length of polymer chain and the chemical nature of the functional groups that decorate the glycerol phosphate units[92].

Not all Gram-positive bacteria have LTA and WTA. For example, in some *Micrococcus* species, lipomannan polymers (glycolipids that have mannose residues) substitute LTA[93]. *Bacillus subtilis*, a Gram-positive model organism, normally has WTA in the CW composition, but when growing with limitations of phosphate WTA are replaced by teichuronic acids (polymers that contain uronic acid and other carbohydrate residues, such as rhamnose and glucose)[94]. The existence of such anionic polymers' diversity pinpoints a major role of these in Gram-positive bacterial fitness and survival.

A. Teichoic Acids Biosynthesis and Modifications

The biosynthetic pathways of LTA and WTA are distinct, although they have similar sugar-phosphate backbones (Figure I.4).

WTA biosynthesis process was first characterized in *B. subtilis* and in *S. aureus*, and the genes responsible were named as *tag* or *tar* genes, for teichoic acid glycerol or ribitol, respectively. Synthesis of WTA starts with the formation of the disaccharide linkage unit attached to the undecaprenol-phosphate (UndP) lipid carrier, a process conserved across bacterial species that occurs in 3 concerted steps, and which is catalysed by the Tag or TarO/A/B proteins[95]. Polymerization of polyglycerol phosphate WTA is carried out by TagF protein, after the addition of a single glycerol phosphate by TagB[96-98]. WTA composed of polyribitol phosphate have a mono- or di-

glycerol phosphate unit, added by the enzymes TarB (*B. subtilis*) and TarB/TarF (*S. aureus*), respectively[90]. Elongation of polyribitol phosphate chain requires, in some *B. subtilis* strains, the pair TarK/TarL enzymes, and, in *S. aureus* only the TarL enzyme[95,99] (Figure I.4, right side). Once the WTA main chain is assembled, the polymer is glycosylated (TagE or TarQ depending on the *B. subtilis* strains), flipped to the external surface of the membrane by the TagGH/TarGH transporter and then attached to the peptidoglycan by enzymes of the LCP family (TagTUV in *B. subtilis*), with consequent release of UndP[90,100]. In *S. aureus* species, WTA glycosylation is accomplished by TarM and TarS proteins, that add α-NAG and β-NAG residues, respectively[90] (Figure I.4, right side).

WTA were found to be not essential for the survival of *S. aureus*, at least *in vitro*. Mutants in various steps of the WTA biosynthetic pathways were characterized, and *S. aureus* mutants lacking *tarO* or *tarA* genes were reported to have a similar *in vitro* growth rate to the wild-type strain, although sometimes with altered cell morphologies. However, the remaining *tar* genes were found to be essential, as their deletion led to a lethal phenotype, unless the WTA biosynthesis is simultaneously blocked at the level of *tarO* or *tarA* genes[101,102]. At optimal concentrations, the antibiotic tunicamycin is capable of inhibiting TarO without affecting peptidoglycan synthesis in *S. aureus*, being therefore a good tool for reducing the WTA content in the CW without affecting bacterial growth rates. However, at high concentrations, tunicamycin is lethal because it also inhibits peptidoglycan synthesis[79,90,91].

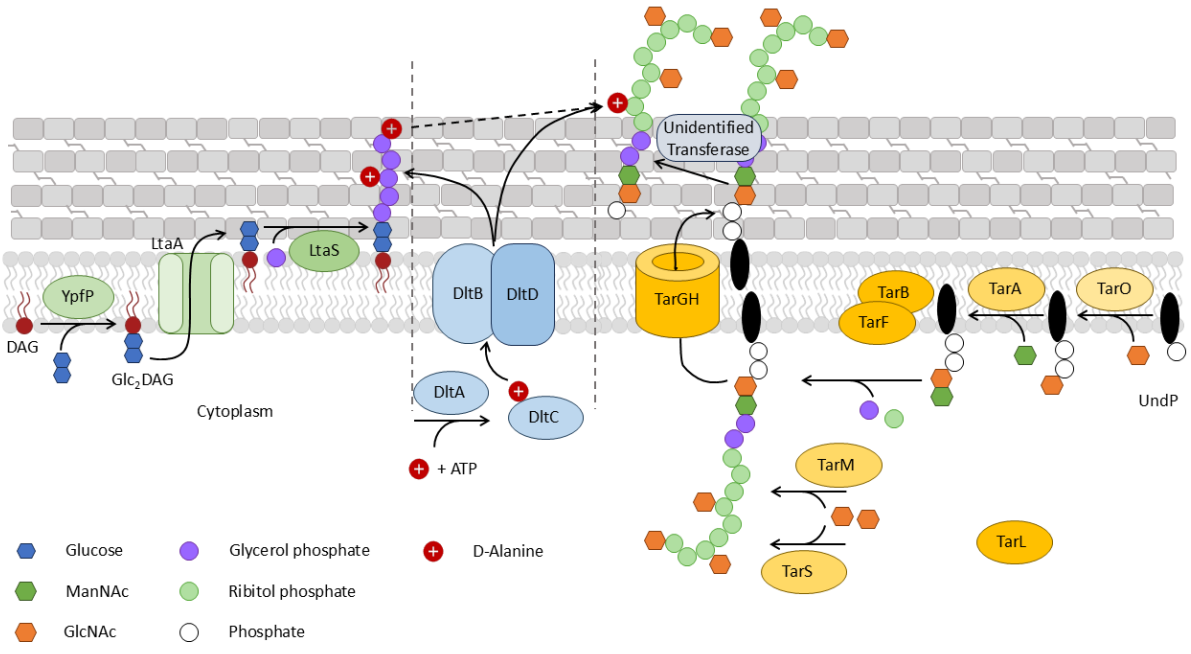

Figure I.4. TA biosynthesis and modification pathways in S. aureus.
LTA (left) and WTA (right) biosynthetic pathways in *S. aureus*. The peptidoglycan structure is depicted in the background. Both LTA and WTA can be modified with D-alanine residues through the dlt pathways, whereas WTA are substituted with α- or β-GlcNAC residues by the glycosyltransferases TarM and TarS, respectively. D-Ala residues were proposed to be transferred from LTA to WTA (dashed arrow). DAG, diacylglycerol; D-Ala, D-alanine; UndP, undecanoprenol-phosphate. Adapted from Ref.[103].

The LTA synthesis pathway varies according to the type of LTA. In the most common type (Type I), the biosynthetic pathway is initiated by the assembly of the glycolipid anchor (Glc_2DAG), which is accomplished by the action of three enzymes, PgcA, GtaB and YpfP[104]. This is followed by the export of Glc_2DAG to the outer leaflet of the membrane, catalysed by the LtaA protein[104]. Polymerization of the LTA occurs by the action of LtaS, which adds multiple units of polyglycerol phosphate (Figure I.4, left side). It was observed that mutations in the *S. aureus* gene *ltaS* caused severe cell division defects (cell elongation, reduced cell diameter, cell bending and abnormally thick septa) or even loss of viability[89,92,105]. However, *S. aureus ltaS* deletion mutants with almost normal growth were successfully obtained that carried secondary mutations suppressing the Δ*ltaS* lethal phenotype[106]. LTA may also be glycosylated, but less frequently than WTA. In *B. subtilis*, LTA can present either NAG or α-galactose residues. Some *Listeria* strains also have LTA modified with α-galactose residues. In the case of some *S. aureus* strains, LTA were found to be glycosylated with NAG[92].

D-alanylation of TA allows for an adjustment of the charge of the cell envelope, as the alanine residues balance the phosphate negative charges of the polymers. The degree of D-alanylation in TA is highly variable, depending on the pH and NaCl concentrations of the growth medium. It is also affected by growth temperature[79]. D-alanylation of both LTA and WTA occurs outside the cell and depends on the D-alanyl lipoteichoic acid (Dlt) pathway. DltA, DltB, DltC and DltD are the proteins involved in the D-alanylation pathway. DltA activates D-alanine ester and transfers this intermediate molecule to the D-alanyl carrier protein, DltC, an intracellular protein[107,108]. Although the mechanism is yet to be fully elucidated, it is proposed that DltC interacts with DltB and DltD, which allow the migration of the D-alanyl moiety across the CM. DltB was proposed to function as both an acyl transferase and a channel, but the exact mechanism remains unclear[109]. DltD, a membrane protein, is hypothesized to transfer D-alanine ester from an unknown intermediate form to LTA[108,110]. WTA D-alanylation is thought to occur via LTA, with D-Ala-LTA serving as the donor[111], however no mechanistic details of such process have been established[108] (Figure I.4, central region).

B. Teichoic Acids Functions

Although the roles and functions of TA remain to be completely unveiled, they can be involved in: (i) control of cell growth and division, (ii) protection against cell damage and (iii) mediation of binding to receptors and surfaces.

Several studies can be found on the effects of WTA and LTA absence in *S. aureus* and *B. subtilis*. In *S. aureus,* a TagO null mutant (no WTA synthesis) shows a decreased level of peptidoglycan cross-linking due to mislocalization of the penicillin-binding protein 4 (PBP4)[112]. Furthermore, both LTA and WTA seem to be relevant for maintaining proper cell division, as mutants deficient in either LTA or WTA synthesis present septal defects and are impaired in cell separation[113-115]. However, LTA appears to play a more critical role, as cell viability is maintained when WTA synthesis is blocked at

initial stages of the biosynthetic pathway, whereas with abolishment of LTA synthesis, cell viability is only possible under specific growth conditions or in presence of compensatory mutations[101,106].

In *B. subtilis*, LTA and WTA appear to be responsible for proper cell division and for cell morphology, respectively. Abolishing WTA synthesis resulted in loss of the typical bacilli cell rod-shape[102,116]. *B. subtilis* carries several homologue genes coding for proteins involved in LTA biosynthesis and, deletion of all genes resulted in a mutant viable but with critical defects in cell morphology and filament formation[116,117]. Moreover, LTA impairment, as in *S. aureus*, results in viability being only possible under specific growth conditions (high salt or sucrose concentrations or low temperatures), and cells present various growth defects[116].

Bacterial strains where both biosynthetic pathways were abolished are not viable, which implies that both LTA and WTA share functions in cell physiology, and the loss of one polymer might be compensated with an overexpression of the other[115,116].

TA are regulators of cation homeostasis, working as scavengers of cations from the medium that may be required for enzyme activity[118]. As such, the degree of D-alanylation of TA "fine tunes" divalent cation homeostasis, namely of Ca^{2+} and Mg^{2+}, with the amount of cations bound being indirectly proportional to the degree of D-alanylation. For example, it was found that WTA lacking D-alanine ester can bind up to 60% more Mg^{2+} ions than those modified[79,119]. Therefore, modifications such as D-alanylation of TA and the glycosylation of WTA affect cell surface properties, such as its charge and hydrophobicity. By modulating the cell surface characteristics, the polymers and their modifications play a role in the protection of the cell against various damaging agents. WTA-deficient *S. aureus* have reduced resistance to β-lactams, an effect that has been attributed to the β-glycosylation of WTA in *S. aureus* and to D-alanylation in *E. faecalis*[113,120,121]. D-alanine esters are thought to neutralize negative charges of the polymers, thus disfavouring interaction with cationic agents[122,123]. Therefore, it is considered that D-alanylation of WTA and LTA confer protection to cells against cationic AMPs, glycopeptide antibiotics and host-defence agents[124].

In the past decade, reports have suggested that WTA can restrict the binding and access of lytic enzymes to the peptidoglycan, possibly due to conformational and/or spatial constraints[125,126] and alterations of the cell surface charge[122]. WTA were also shown to be involved in the control of the localization and catalytic action of endogenous PDEs. In *S. aureus*, it was shown that higher levels of WTA reduced the affinity of autolysins for the peptidoglycan, directing autolysins to regions with low WTA content[127,128], whereas the capacity of these glycopolymers in retaining protons was proposed to impact autolysins activity[129].

As WTA and LTA directly contact the surrounding environment, they have also been implicated in several functions as ligand binders, surface adhesion, biofilm formation, colonization and virulence. In *S. aureus* and *B. subtilis*, WTA are required for initiating phage infection[130-132]. Biofilm formation is intimately related to bacterial cell adhesion to an abiotic surface, as it constitutes the first step in its formation[133]. Stronger negative net charge due to the lack of D-alanine esters in TA

was suggested to prevent adherence of the bacteria to certain surfaces as result of increased repulsive forces[134]. Additionally, LTA seem to be important for the activation of the immune system of infected hosts, as their presence stimulate production of proinflammatory cytokines[135].

The key role of WTA and their D-alanylation or glycosylation in binding to host cells has also been described, for several Gram-positive bacteria. Impairment of D-alanine ester reduces the ability of bacteria to adhere to and colonize host cells. In a similar way, absence of WTA also leads to a diminished colonization of hosts[136]. All these observations support the role of WTA and of D-alanine esters as bacterial virulence factors, as their absence attenuates pathogenicity.

I.3.3. The Outer Membrane and Other External Components

I.3.3.1. The Outer Membrane

The distinguishing feature of Gram-negative bacteria is the presence of an OM surrounding the peptidoglycan layer. The OM, unlike the CM, is an asymmetric lipid bilayer. The inner leaflet is composed of phospholipids, and the outer leaflet consists mainly of LPS[137]. This structure also has lipoproteins, integral proteins, and extracellular glycans attached. The OM is essential for bacterial growth and survival, contacting directly with the surrounding environment and serving as a selective permeation barrier, allowing the entry of nutrient molecules but preventing the entry of damaging compounds[137]. The barrier function of the OM is further supported by the presence of the OM proteins (OMPs), which frequently assume a β-barrel conformation. These OMPs serve most often as porins, allowing passive diffusion of molecules with less than 600 Da and, together with other substrate-specific porins, they determine to great extent the permeability of the OM[138,139].

The LPS molecule has three different moieties: the lipid A, the core oligosaccharide with about 12 sugar units, and the highly variable, strain-specific *O*-antigen polysaccharide[140]. The lipid A region is highly hydrophobic and constitutes the LPS anchor to the OM. The polysaccharide chain, which includes the core and the *O*-antigen, extends beyond the cell surface[141]. It was found that LPS molecules bind to each other avidly, forming a tight and dense structure, that is specially stabilized in presence of divalent cations[137]. Therefore, it functions as a protective barrier to harmful molecules such as some antibiotics. On the other hand, LPS are also a key player in bacteria-host interactions, with the host immune system being activated in the presence of LPS[142]. The *O*-antigen of the LPS is a highly variable region and often built from a repetitive oligosaccharide motif, determining and contributing to the serotype differentiation in Gram-negative bacteria and, frequently, it is a receptor for phages infecting Gram-negative hosts[143].

I.3.3.2. Extracellular Polysaccharides and S-layers

Bacterial cells can be encased in a surface layer (S-layer). Its distinguishing feature from other proteins layers is the ordered and symmetrical matrix, which consists of repeating units of one (occasionally two or more) S-layer protein or glycoprotein (SLP) type. These SLPs self-assemble on the cell surface predominantly at mid-cell, being attached to the cell surface through non-covalent interactions. These interactions are established between the SLP and the TA (or other polymers) in Gram-positive species, and the LPS of the OM of Gram-negative bacteria[144].

The role of S-layers has been the focus of intense research. They are present, in some bacterial species, as the outermost layer directly interacting with the environment, and they have essential roles on bacterial adherence to different substrates, also providing a physical barrier[145].

Some bacterial species produce an outermost layer that span the boundaries of TA, in Gram-positive species, and of LPS, in Gram-negative species. This layer is normally composed by polysaccharides, known in a general manner as extracellular polysaccharides (EPS), and they can be subdivided into capsular polysaccharides or capsule, if covalently anchored to the peptidoglycan or the OM, and into exopolysaccharides or slime layers, if they are loosely associated with the cell surface[146]. The capsules or slime layers generally surround the entire cell. Capsules are found usually in pathogenic bacteria, and have been described as essential virulence factors, being key players in (i) immune evasion, by providing protection against phagocytosis, (ii) prevention of complement-mediated bacterial lysis, (iii) contributing to adherence to surfaces and biofilm formation, (iv) increasing antibiotic resistance and (v) interactions with phages[143,147].

The basic chemical nature of the capsules is species specific, but substantial variation can occur within a single species, as well illustrated by the more than 70 capsular types of *Klebsiella pneumoniae*[148]. The great majority of capsules are composed of polysaccharides, with known exceptions being the capsule of *Bacillus anthracis*, composed of polyglutamate[149], and of *Bacillus megaterium*, which contains both polysaccharides and polypeptides[150]. EPS are polymers made up of repeating oligosaccharide units that may be linear or branched, with a negative net charge due to acidic sugar composition or modifications with charged non-glycosylic moieties (amino acids, pyruvate, O-acetyl groups)[151].

I.4. Antimicrobial Peptides

As referred to above (Section I.2.1), AMPs are ubiquitous small peptides produced by virtually all organisms (from microorganisms to animals and plants)[152]. The great majority of AMPs are bactericidal against a broad range of target bacteria, with some acting also against viruses, fungi and protozoa[27]. Functionally, they have a direct antimicrobial activity by disrupting the CM or by targeting intracellular targets, but they can also present immunomodulatory properties that indirectly

contribute to pathogen elimination[27]. Due to their antimicrobial action, AMPs have gained attention as promising drug candidates to be used alone or in combination with other antibacterial agents[26]. Presently, more than 5000 AMPs have been described, of which about 3300 are natural occurring, and almost 1300 are of synthetic origin[153,154]. When compared to conventional antibiotics, AMPs can act on the CM of various microorganisms, can target intracellular pathogens, and have antimicrobial activity on antibiotic-resistant bacteria, with resistance being thought to occur at a slower pace and with less frequency[155].

I.4.1. Structure and Properties of AMPs

Although AMPs are quite diverse regarding their primary amino acid sequences, they have several features in common, which include their small size (most up to 50 amino acid residues), the charged nature (most frequently, positively charged, but a few negative and neutral peptides exist), and the hydrophobic and amphipathic character[156]. Structurally, AMPs generally fall in one of the following categories: α-helical linear peptides, β-sheet AMPs with disulfide bridges, cyclic peptides and peptides with extended, flexible loop structures[27]. In addition to structural features, AMPs can also be characterized according to other properties such as (i) biosynthesis pathway, which can be ribosomal or nonribosomal; (ii) biological source; (iii) spectrum of activity; (iv) amino acid composition and post-translational modifications, net charge, amphipathicity, hydrophobicity and (v) molecular targets (cell envelope- and intracellular-targeting AMPs)[153].

AMP length, composition, net charge and hydropathic properties are crucial factors for antimicrobial activity, as these parameters contribute to the formation of required secondary structures such as α-helices or β-strands. As peptide length decreases, the likelihood of forming a stable secondary structure diminishes, potentially leading to a loss of function[157]. Additionally, transversing the lipid bilayer typically requires a minimum of 22 residues[158].

As already mentioned, AMPs act by interacting with bacterial membranes, which are structures with an overall negative charge. This explains why the vast majority of AMPs described so far carry a net positive charge of +1 to +9, with a high representation of lysine and/or arginine residues. The initial interaction between an AMP and the membrane is thus primarily electrostatic[159]. Furthermore, several studies have reported a direct correlation between the positive charge of an AMP and its antimicrobial efficacy[160,161].

Hydrophobicity and amphipathicity, which refer to the amount of hydrophobic amino acid residues and to the presence of hydrophobic and hydrophilic regions within a peptide, respectively, play a key role in AMP insertion and accumulation in the bacterial membranes. AMPs typically contain about 50 % of hydrophobic residues. Cationic AMPs with amphipathic α-helical domains are one of the most abundant and widespread classes of these agents[162]. Hydrophobicity controls AMP partitioning into the membrane lipid bilayer and, as it happens with charge, increasing hydrophobicity

of an AMP can increase its activity on microbial cell membranes[163]. However, an increased hydrophobicity is also correlated with toxicity to mammalian cells[164].

I.4.1.1. AMPs Classification Systems

AMPs can be classified according to their secondary structures. There are four major classes (i) α-helical AMPs, with only helical structures (for example, the human cathelicidin LL-37[165]) (Figure I.5a), (ii) β-sheet AMPs, with a minimum of 2 beta-strands, generally containing a disulfide bridge (for example, the protegrin-1[166]) (Figure I.5b), (iii) αβ or mixed structure AMPs, with both α-helices and β-strands (for example, the hBD3 of the β-defensin family[167]) (Figure I.5c), and (iv) extended or non-αβ structure AMPs, which lack both α-helix and β-strands, and generally are further classified as tryptophan-rich, proline-rich and glycine-rich (for example, the lactoferrin B_2[168]) (Figure I.5d).

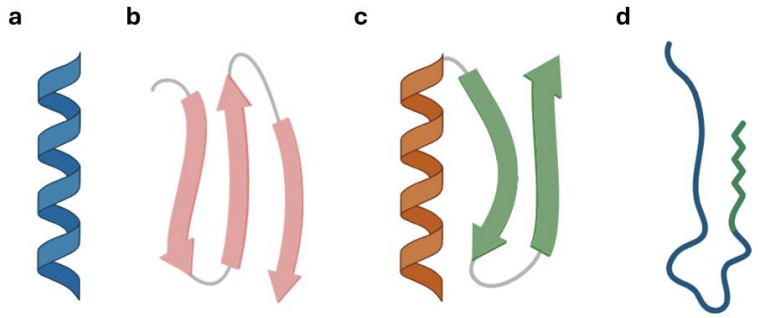

Figure I.5. Different classes of AMPs according to their secondary structures.
(**a**) α-helix peptide structure; (**b**) β-sheet peptide structure; (**c**) αβ or mixed peptide structure; (**d**) extended or non-αβ structure. Adapted from Ref.[169].

Another relevant classification of AMPs is based on sequence and covalent bonding patterns, which was proposed to overcome the lack of 3D structures for the majority of AMPs. This classification, named Universal Classification (UC), is based on the bonds established between the polypeptide chains, defining four universal classes[170]. In this system, (i) Class I or UCLL consists of linear one-chain polypeptides, or two linear chains attached via non-covalent bonds (for example, manganin[171]) (Figure I.6a), (ii) Class II or UCSS is composed of AMPs that form chemical bonds between side chains, which may occur within a single peptide chain or between two different peptide chains (for example, the defensin rattusin[172]) (Figure I.6b), (iii) Class III or UCSB covers AMPs that contain polypeptide chains with chemical interactions between the side chain of one amino acid and the backbone of another amino acid of the same chain, forming a loop structure (for example, daptomycin[173]) (Figure I.6c), and (iv) Class IV or UCBB comprises circular AMPs that have a peptide bond between the amino and carboxyl ends of the polypeptide chain (for example, gramicidin S[174]) (Figure I.6d).

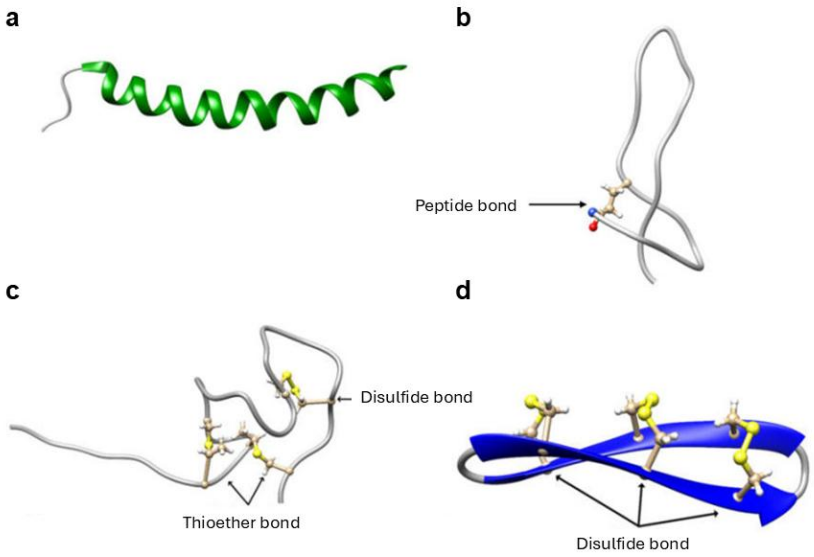

Figure I.6. Different classes of AMPs based on the covalent bonding pattern.
(**a**) Class I or UCLL peptides, consisting of linear peptides; (**b**) Class II or UCSS peptides, AMPs that have chemical bonds between side chains; (**c**) Class III or UCSB, AMPs that form bonds between the side chain of one amino acid and the backbone of a second amino acid of the main chain; (**d**) Class IV or UCBB peptides, comprising circular AMPs. Adapted from Ref.[152].

I.4.2. AMPs Function and Mechanisms of Action

AMPs may have bactericidal and/or bacteriostatic properties, depending principally on their ability to interact with the membrane lipids or with other lipid components of the BCE. As referred to above, in terms of molecular targets AMPs can be classified into two groups: those disrupting the membrane, causing most often cell lysis, and those that interfere with intracellular targets[175].

I.4.2.1. AMP-Mediated Disruption of Cell Membrane

The majority of AMPs are thought to exert their antimicrobial action through the interaction with the bacterial membranes, particularly by inserting into and permeabilizing the CM. This can cause leakage of small ions such as H^+, Na^+ and K^+ with consequent irreversible dissipation of the transmembrane potential, ATP depletion, and cell death. Some AMPs can also cause escape of larger molecules and even induce large-scale membrane disruption and cell lysis[176]. The anionic nature of bacterial membranes facilitates the interaction with most AMPs that are positively charged[177]. However, before reaching the CM or their intracellular targets, AMPs must interact with the external components of the BCE, which may conditionate the effective concentration of the peptides reaching the CM.

The role of the negatively charged, outermost components of the BCE, that is, the TA in Gram-positive and LPS in Gram-negative bacteria has been a matter of debate[178]. From one side, these polymers are viewed as attractors of cationic AMPs. These polymers may entrap the peptide and

prevent peptide association with the CM[178]. From the other side, however, this strong affinity seems also to result in peptide sequestering, probably explaining why AMPs need to saturate the BCE to exert their killing action[179]. Indeed, LPS truncations and other structural changes have been reported to increase susceptibility to AMPs. While for many cases this has been explained by some LPS capacity to retain the peptides, diminishing the amount reaching the CM, for others the higher susceptibility seems to result from an OM destabilization conferred by the defective LPS[180]. Nevertheless, some AMPs appear to be able to transverse the OM by a charge-exchange mechanism, where the positive charges of the AMP compete with the divalent cations bound to LPS. This interaction might disorder LPS structure, with subsequent OM disruption[181,182].

The peptidoglycan has at most a mild negative charge (depending on its amino acid components) and thus it should not strongly compete with the CM for interaction with most cationic AMPs, although synthetic derivatives may bind to peptidoglycan[178]. In addition, the peptidoglycan is believed to allow the diffusion of molecules up to 50 kDa[183], and therefore most AMPs (typically < 5 kDa) should easily cross it to reach the CM.

Many antibacterial AMPs target the CM via initial electrostatic interactions between positively charged peptide segments and negatively charged phospholipid head groups of the CM surface. This is then followed by interactions between the peptide hydrophobic/amphipathic domain and the hydrophobic tails of phospholipids. To explain membrane disruption activity of peptides, several models have been proposed, including the non-pore carpet model, and the transmembrane barrel-stave and toroidal pore models (Figure I.7)[152]. Examples of peptides that likely act via these models are cecropins and indolicidin (carpet), ceratotoxin and some Class II bacteriocins (barrel-stave), and arenicin and magainin 2 (toroidal) (Ref.[152] and references therein). Peptides targeting intracellular components also need to interact with the membrane to transverse it.

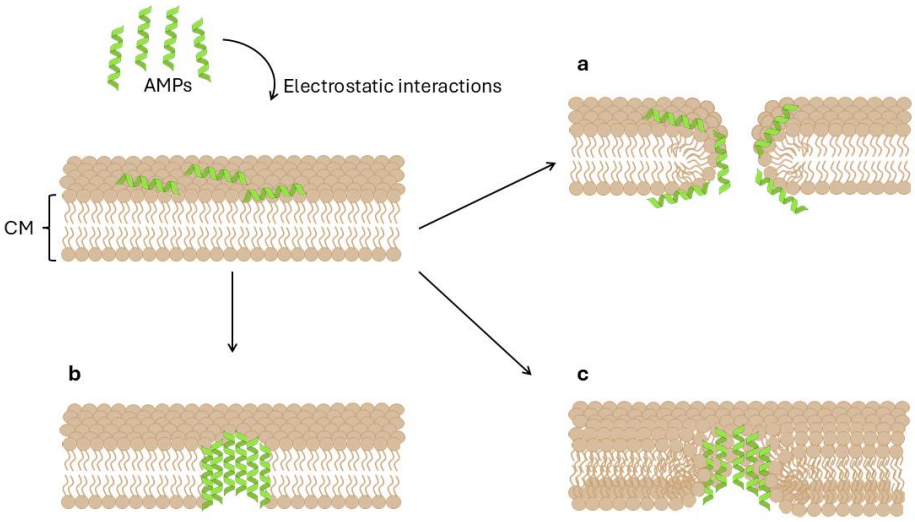

Figure I.7. Models for AMP insertion and disruption of the CM.

(**a**) Carpet model: AMPs align parallel to the membrane surface, covering it like a carpet; at a threshold concentration, the membrane disintegrates in a detergent-like manner. (**b**) Barrel-stave model: AMPs insert perpendicularly into the membrane and aggregate to form a barrel-like pore; hydrophobic sides of the peptides face the lipid core, while the hydrophilic sides line the pore lumen. (**c**) Toroid model: similar to the barrel-stave model, but the lipid layers bend and curve into the pore; both AMPs and phospholipid head groups line the pore. Adapted from Ref.[184].

In the carpet model, AMP contact with the membrane is driven mainly by electrostatic interactions. The hydrophobic surface of the peptide binds to the phospholipid headgroups, being arranged parallel to the membrane surface (Figure I.7a). The AMP covers the entire surface of the membrane and after reaching a threshold concentration, the membrane surface tension is disrupted in a detergent-like manner, leading to micellization and cell death[185].

Formation of a transmembrane pore can be explained by two different mechanisms: the barrel-stave and the toroidal pore models. In the barrel-stave model, the hydrophobic regions of the peptide chains align with the hydrophobic lipidic core of the membrane, whereas the hydrophilic surfaces are oriented towards the centre of the channel, forming an aqueous pore. For the formation of the pore, it is thought that at least 4 peptide molecules need to be aligned (Figure I.7b). The formed channel allows leakage of cytoplasmic contents, leading to membrane PMF collapse and ultimately cell death[26]. On the other hand, according to the toroidal model, AMP insertion into the membrane leads to a local disruption in the bilayer structure. Aggregation of the peptides induces a curvature in the membrane, resulting in a pore lined by both peptides and the phospholipids head groups (Figure I.7c). These channels are transient and less stable than the pores hypothesized by the barrel-stave model; nevertheless, the resulting destabilization of the membrane surface leads to cytoplasmic extravasation and consequent cell lysis[152].

I.4.2.2. AMPs Targeting Intracellular Processes

Beyond their ability to cause membrane damage, some AMPs are also thought to impact bacterial viability through targeting intracellular processes. Although the precise mechanism by which these peptides transverse the CM is not yet elucidated, it is proposed that proline-rich AMPs form transient pores in the membrane[186], whereas certain AMPs can cross the CM through receptor-mediated transport[187]. Once in the cytoplasm, these molecules may inhibit crucial biological processes, including protein and nucleic acid synthesis, cell division and protease activity, and induce degradation of nucleic acids and proteins (Figure I.8)[188]. Nucleic acids-targeting AMPs may act by directly binding to DNA and RNA, or by inhibiting the action of the enzymes involved in their synthesis[189]. AMPs can impair cell division by interfering with components of the divisome such as FtsZ[190]. Protein synthesis-targeting AMPs have effects on transcription, translation, or protein assembly[191]. Cell wall-inhibiting AMPs target lipid II, a precursor of peptidoglycan synthesis[192]. It is

worth noting that, frequently, AMPs do not act by means of a single mechanism of action. They can interfere with both membrane stability and target intracellular process.

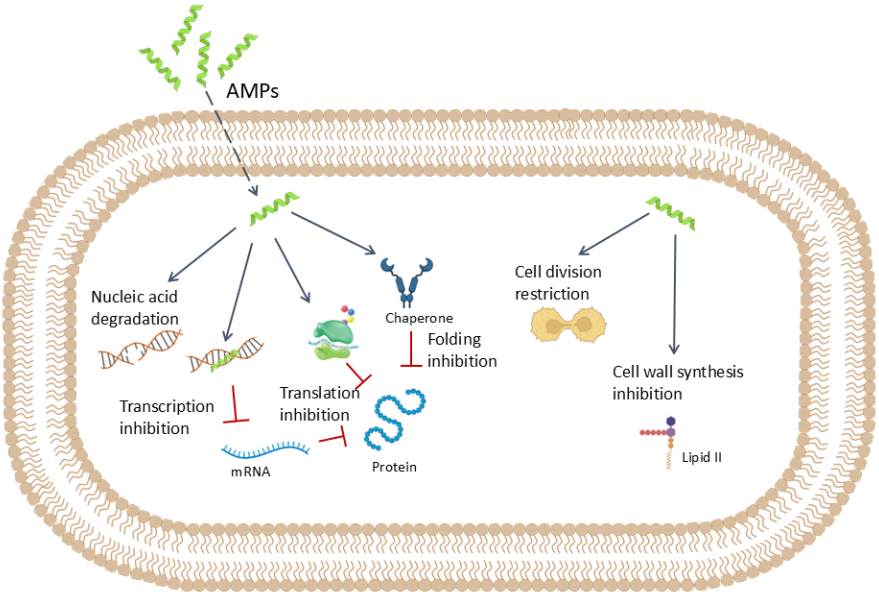

Figure I.8. Models of intracellular antibacterial mechanisms of AMPs.
Some AMPs gain access to the cytoplasm and interact with intracellular components, blocking DNA, RNA and CW synthesis, inhibiting protein folding and enzyme activity or promoting the release of lyases that destroy cell structures. Adapted from Ref.[193].

I.4.2.3. Immunomodulatory Activity of AMPs

AMPs can not only directly kill pathogens (by disrupting the membrane and/or targeting intracellular processes) but can also indirectly contribute to bacterial pathogen killing due to immunomodulatory activities. AMPs can stimulate chemotaxis, increasing the recruitment of neutrophiles, macrophages and other cells of the immune system to the sites of infection and modulate the production of pro- and anti-inflammatory cytokines. Moreover, they can enhance adaptive immune responses, by promoting maturation of dendritic cells and antigen presentation to T cells and induce the activation of cytotoxic T cells[194].

I.4.3. AMPs as Therapeutical Alternatives

The rapid bactericidal activity of AMPs, their broad range of action, their different mechanisms of action allowing efficacy against antibiotic-resistant bacteria, the ability to target and kill bacteria within a biofilm matrix, the low incidence of bacterial resistance, their immunomodulatory properties, and their amenability to engineering strategies are advantages of AMPs that have sustained a vast field of research exploring these agents as alternative antimicrobials[195].

Although AMPs present low incidence of bacterial resistance, some mechanisms that allow bacteria to evade AMP action were already observed. These include proteolytic degradation, alterations in components of the BCE (LPS in the OM of Gram-negative bacteria, TA in the CW of Gram-positive bacteria, and phospholipids of the CM), bacterial cell shielding with a protective capsule, and active efflux[155]. Nevertheless, development of resistance to AMPs appears to occur with much less frequency compared to conventional antibiotics[196].

Despite the referred AMP advantages over conventional antibiotics, there are also several limitations that restrict the clinical applications of AMPs. A major limitation is their stability when administered to infected hosts. AMPs have a short half-life, as they are highly susceptible to proteolytic enzymes circulating in the bloodstream and in tissues. They are also susceptible to inactivation due to pH variation, high salt concentrations and inactivation by binding to plasma proteins, being rapidly eliminated via renal and hepatic systems. Another major obstacle are the high costs of large-scale production, especially ribosomally synthesized AMPs[197]. Furthermore, because most AMPs require a high number of molecules to associate with the BCE for activity, and since they are rapidly eliminated from host bodies, usually they need to be administered at high concentrations. Despite the much higher affinity of AMPs to bacterial membranes, at high doses they frequently present cytotoxic effects on mammalian cells[198].

An ever-growing body of evidence highlights the potential of AMPs, but only a few examples have translated into clinical settings. Gramicidin was the first peptide to be identified, circa 1940, whose antibacterial activity against various Gram-positive bacteria was observed both *in vitro* and *in vivo*, being used mainly for topic applications[199]. In a similar manner, bacitracin was approved by FDA more than 50 years ago for topical application, primarily skin, ear and eye infections[200]. An important example is vancomycin, a glycopeptide produced by *Streptococcus orientalis*, targeting Gram-positive bacteria, including methicillin-resistant strains of *S. aureus* (MRSA), which inhibits peptidoglycan synthesis by binding to the lipid II precursor[201]. Also relevant is the cyclic lipopeptide daptomycin, isolated from *Streptomyces roseosporus*. Daptomycin exerts its bactericidal activity by permeabilizing and depolarizing the CM, having activity against Gram-positive, but not against Gram-negative bacteria[202]. Daptomycin can efficiently kill antibiotic-resistant strains, including MRSA, vancomycin-resistant enterococci and vancomycin-intermediate *S. aureus*, and has been approved in 2003 by the FDA[203]. Against Gram-negative pathogens, both colistin (polymyxin E) and polymyxin B peptides have been approved by the FDA in the 50's. Although with indication for treating carbapenem-resistant infections, the use of these polymyxins has been limited due to nephrotoxic and neurotoxic effect[204,205].

Besides clinical applications, some peptides have been used as food preservatives. As AMPs are sensitive to proteases in the gastrointestinal tract, and at low concentrations they are non-toxic to mammalian cells, most are safe for consumption. For example, nisin was approved as food

preservative in the late 1980's by the FDA[206], for application mainly in cheese and dairy products to inhibit growth of *L. monocytogenes*[207].

Clinical development of AMPs faces several challenges. In the past decades, out of several thousand identified and characterized AMPs, only a fraction was selected for preclinical studies, and out of these, even less have entered clinical trials. To overcome the limitations, there are several strategies that can be followed. These include, *de novo* design and chemical/structural modifications of AMPs assisted by artificial intelligence techniques, fusion with larger functional molecules, and the development of drug delivery systems to turn AMPs more stable, efficient and safe[197]. For example, modifying the structure of the peptide by incorporation of D-amino acids or of non-proteogenic amino acids results in the synthesis of pseudo-peptides and peptide mimetics, that are not prone to proteolysis[208]. Degradation of AMPs by peptidases can also be avoided by cyclization of the AMPs (ligation of the amino and the carboxy terminal with each other, thereby concealing the ends), which can also improve the antimicrobial potential of the AMP[209]. C-terminal amidation occurs naturally for some peptides and it was seen to improve the antimicrobial efficacy of certain AMPs, most likely due to increase the stability of the interaction between the peptide and the membrane, leading to a higher membrane destabilization[210]. Acetylation of the N-terminal is also a common modification observed in cells, and it was observed to contribute to a higher antimicrobial potency[211], but it can also compromise activity as the overall positive charge is reduced[212]. Another strategy is to genetically fuse AMPs to carrier proteins, mimicking the peptide's natural precursor structure and, thereby, protecting the peptide from proteolysis[213].

The bioavailability, stability and controlled release of AMPs can also be enhanced through formulations and drug delivery systems. Different types of nanocarriers, including liposomes, micelles, hydrogels, polymeric or metallic nanoparticles, nanofibers, mesoporous nanoparticles, dendrimers and carbon nanotubes have been described to improve the efficacy of AMPs, reducing host cell cytotoxicity, and preventing AMPs proteolysis[214].

I.5. Bacteriophages

The interest of phages as treatment to bacterial infections faded briefly after the introduction of antibiotics in the western countries, whereas in some eastern countries, such as Georgia and Poland, the therapeutic application of phages continued throughout the years. Due to the uncontrolled rise in antibiotic resistance, the past two decades have seen a remarkable resurgence of interest in the use of phages as antibacterial agents, a movement that has also benefited from the visibility of numerous clinical cases in which phage therapy was successfully applied[36,37]. As therapeutics, phages not only are capable to killing antibiotic-resistant bacteria as they show high specificity in lysing host bacteria, thereby preventing dysbiotic states commonly associated with

antibiotic treatments[215]. Nowadays, phage therapy is based on the use of strictly lytic, tailed phages (see below) and it is considered for difficult-to-treat infections caused by multidrug-resistant bacteria[216,217].

I.5.1. General Features of Bacteriophages

Phages are most often described as the most abundant biological entity on earth (about 10^{31} phage particles), they are ubiquitous throughout ecosystems, and yet only a very small fraction is believed to have been studied[218]. Phages, as other viruses, have been classified based on virion morphology and composition, type of nucleic acid, and lifecycle[219]. Historically, phages were classified according to morphological characteristics of the virus particles, which were basically divided in tailed and non-tailed (Figure I.9). Among the non-tailed, they could be filamentous (e.g. M13), polyhedral (e.g. PRD1, MS2, phi6 and ϕX174) or pleomorphic (e.g. L2)[220]. Tailed phages were further classified into three morphotypes: contractile tail; long, non-contractile tail and short non-contractile tail, which defined the now extinct *Myoviridae* (e.g. T4), *Siphoviridae* (e.g. λ) and *Podoviridae* (e.g. T7) families, respectively. The members of these three families were united under the former order *Caudovirales*[220].

In 2021, with the increase of genomic data, the morphological classification was considered inadequate to capture the phage genomic diversity and phylogenetic relationships. Indeed, the present taxonomy is based on phage genomic data, with all tailed bacteriophages infecting bacteria and archaea being grouped in a single taxon, the class *Caudoviricetes*[221], being distributed by more than 100 families according to the International Committee for Taxonomy of Viruses (ICTV), the entity responsible for overseeing the viruses' nomenclature and classification[222].

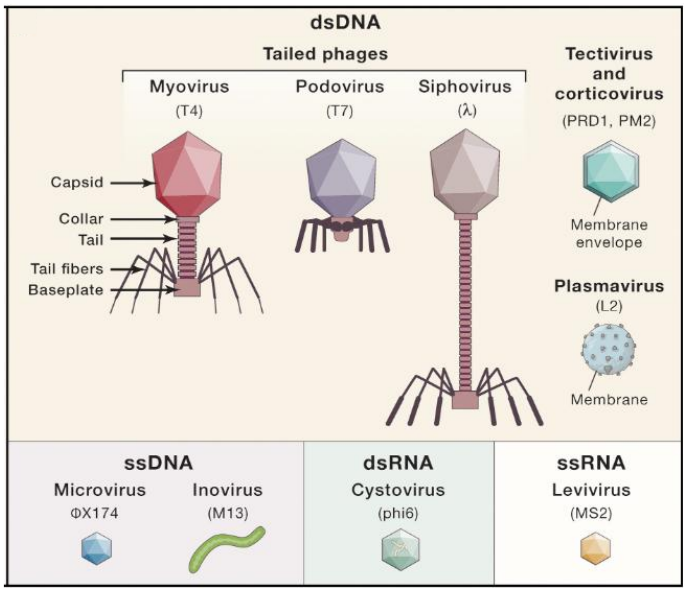

Figure I.9. Major groups of phages infecting Eubacteria based on virion morphology and type of genome nucleic acid.
A representative type of phage for each group is indicated in parenthesis. Adapted from Ref.[223].

Nevertheless, the morphological classification of phages remains an important characteristic trait, and designations as “myophages”, “podophages” and “siphophages” and others are still used to describe newly identified phages.

The phage genome nucleic acid molecule(s), which can be double or single-stranded DNA or RNA, is packed in most phages in a proteinaceous structure, the capsid, and the overall structure is designated as nucleocapsid. In some polyhedral phages, the capsid is lined by an inner (e.g. PRD1) or outer lipid membrane (e.g. phi6), or it can present lipids as part of its constitution (e.g. PM2); some pleomorphic phages, such as the plasmavirus L2 have a circular dsDNA genome surrounded by a lipidic envelope, with no capsid[35,220]. The great majority of phages described so far, more than 90%, carries a tail structure attached to the nucleocapsid, which encases a linear dsDNA genome (tailed phages). The tail plays a crucial role in host receptor recognition and subsequent phage DNA delivery across the BCE, into host cells[69].

As obligatory parasites, phages cannot complete their life cycle outside of a host cell, requiring the biosynthetic machinery of the host to replicate and produce a virion progeny. In tailed and polyhedral phages, the viral offspring is released from infected cells through host cell lysis, when they complete the so-called lytic cycle, whereas in filamentous phages the new viruses are liberated by non-lytic, extrusion mechanisms, defining the chronic infection cycle[224]. However, phage binding (adsorption) to a bacterial host and subsequent viral genome delivery into the cell may not immediately lead to a productive infection. In fact, upon cell entry some phages, designated as temperate, may instead enter a dormant, non-productive state, in which the phage is passed

vertically as the host cell divides[224]. These major phage infection lifestyles are illustrated in Figure I.10.

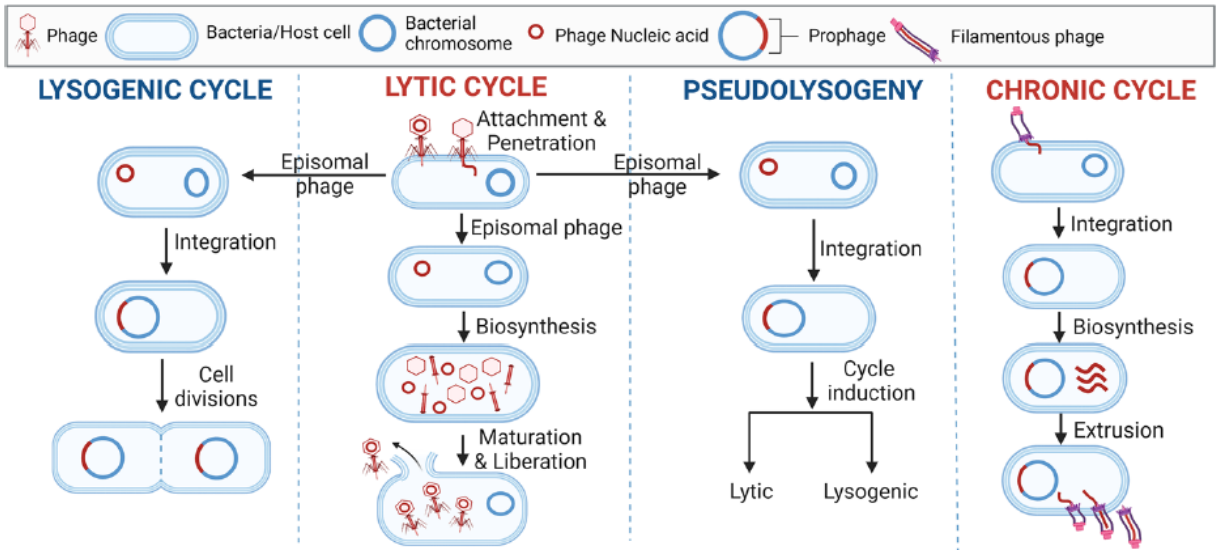

Figure I.10. Illustration of the four major phage lifecycles.
The lytic lifecycle involves active phage replication and lysis of host bacteria for releasing the new virus particles. In the lysogenic cycle, the phage genetic material is instead integrated in the host chromosome, being passed vertically as a resident prophage along with cell division. Bacteria carrying prophages are called lysogens. Upon an environmental trigger, the prophage is induced, and the lytic cycle is activated. In the pseudolysogenic or "carrier" state, the phage genetic material is maintained in the host cell cytoplasm as a non-replicative extrachromosomal element, and upon environmental cues it can either enter the lytic or the true lysogenic pathway. In chronic infection, typical of filamentous phages, the viral progeny is released from infected cell by extrusion mechanisms, without host cell lysis. From Ref.[225].

Virulent or strictly lytic phages replicate only through a productive lytic cycle. After viral genome replication and expression, tens to hundreds of new virus particles are assembled, which are then released by phage-induced host cell lysis (Figure I.10, Lytic cycle panel)[224]. The phages usually employed in phage therapy are strictly lytic *Caudoviricetes*[226].

Depending on the infection conditions, temperate phages can undergo either productive or non-productive infection. Productive infection is either as described for the strictly lytic phages, that is, the new virions are released through host cell lysis (tailed phages) or, alternatively, by a non-lytic mechanism (filamentous phages) (Figure I.10, Chronic cycle panel). In non-productive infection the phage genetic material is either integrated into the bacterial chromosome (lysogenic cycle) or maintained as an extrachromosomal element (pseudolysogeny) (Figure I.10, Lysogenic cycle vs pseudolysogeny panels). The integrated phage DNA is designated as prophage and bacteria carrying functional prophages are called lysogenic. Pseudolysogeny is proposed to occur when infection happens in conditions not supporting growth of the bacterial host (e.g., nutrient-depletion). In these conditions, the extrachromosomal phage element is asymmetrically transferred to one of the daughter cells during division[227,228]. Eventually, and generally in response to environmental factors,

the prophage/phage element can be induced to enter the productive pathway, leading to virion escape from infected bacteria either through cell lysis or extrusion[229].

I.5.2. Virus Entry and Exit in Tailed Phages

Considering the scope of this thesis, from this point onwards we will focus on tailed phages. As seen before, for completing their lytic cycle, tailed phages must successfully breach the different layers of the BCE at least twice: first to deliver their genome to the site of replication/expression within host cells, and then to ensure viral progeny escape from infected cells. Obviously, for genome delivery phages must breach the BCE without compromising cell viability, whereas for effective virion release extensive BCE disintegration (cell lysis) is required. For both situations, tailed phages have evolved specialized functions that allow the controlled breaking of the different layers of the BCE.

I.5.2.1. Crossing the BCE for Genome Delivery

When a phage encounters a suitable host cell through random motions, eventually it will interact with bacterial receptors exposed to the extracellular environment. Receptor-binding proteins (RBPs) located in tail structures (fibers, spikes or baseplates) mediate the specific adsorption to bacterial receptors, which can be integral or associated components of the BCE, including LPS, proteins, TA, peptidoglycan, pili, flagella, capsules and S-layers[69,143,230].

Adsorption is thought to occur in two steps, in which phages first bind reversibly to the host receptors, followed by irreversible attachment. Reversible adsorption, as the name implies, refers to a transient state, in which weak interactions are established between phage RBPs and the cell surface receptors. After this reversible attachment, phages can either dissociate from their host or bind irreversibly to them, which may involve other RBPs and/or other bacterial receptors. The reversible character of the first interaction between phages and their host is thought to facilitate the search and attachment to specific receptors for the irreversible binding[143]. The irreversible attachment triggers conformational changes in the virion structure that results in the protrusion and insertion of virion/tail substructures in the BCE, which will form the channel for conducting the viral DNA into host cells (Figure I.11)[231,232]. It is therefore not surprising that the tail RBP/receptor specific interactions are major determinants of the phage host range[233].

The tail apparatus of *Caudoviricetes* is, indeed, a complex molecular machinery that has evolved to recognize host cells, penetrate the BCE, and deliver the phage DNA (and sometimes proteins) into the host cytoplasm. Tail access to receptors and tail penetration across the BCE may be assisted by enzymatic functions carried in the virion structure (most often in the tail), namely depolymerases that cleave polymers associated or making part of the BCE, and PDEs, known as virion-associated lysins, that promote a local degradation of the peptidoglycan[69].

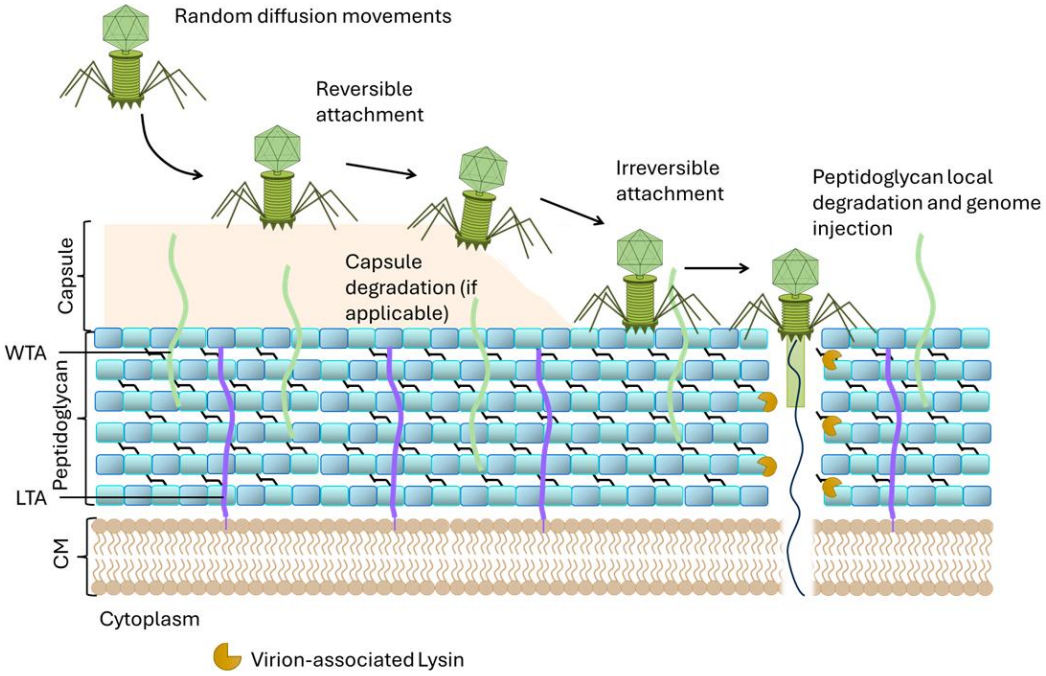

Figure I.11. Phage adsorption and viral DNA channelling across the BCE.
Phage infection starts with the adsorption process, in which phages recognize sensitive hosts through the specific binding of their RBPs to bacterial receptors. Access to bacterial receptors can be hampered by extracellular polysaccharides, which are degraded by phage depolymerases. At least in some phages, irreversible adsorption is followed by the local breakdown of peptidoglycan by the action of virion-associated lysins (depicted as "pacman" symbols in the figure) to facilitate genome injection.

As discussed in Section I.3.3, the EPS in some bacterial species form a capsule layer surrounding the whole cell. This matrix may provide a barrier to phage infection by hindering phage access to receptors in the CW or cell membranes. However, many phages (notably, podoviruses) have evolved RBPs that not only bind to specific EPS components, but also cleave the polymeric structures thanks to the depolymerase activity they carry. The processive depolymerase activity of tail RBPs, that is, the sequential cleavage of polymer bonds without virus dissociation, allows virions to move through the capsule layer and reach other receptors in the BCE for irreversible binding[234]. Phages can also employ depolymerases to degrade and penetrate biofilm matrices for infecting resident bacteria, as well as the O-polysaccharide chains of LPS, a major receptor of phages infecting Gram-negative bacteria[235]. A hallmark of phages carrying depolymerase functions is the formation of turbid halos around the clear center of phage lysis plaques formed in soft agar medium. These halos are thought to result from diffusion of the depolymerase activity (virions and/or free RBPs).

The knowledge accumulated in the field strongly suggests that, regardless of tail morphology, *Caudoviricetes* must eject proteins or deploy tail substructures to extend their tails and span the BCE[232,236]. While crossing the membrane layers of the BCE may be accomplished through mechanical puncturing and/or protein fusion with lipid components, traversing the rigid, and sometimes thick peptidoglycan should present a greater challenge. Indeed, many phages are known

to carry PDEs in the virion structure, which under "normal" infection conditions are thought to locally degrade the peptidoglycan mesh without compromising the overall integrity of the cell wall[237]. However, at very high multiplicities of infection (high virion/bacterium ratios), which are essentially achieved under laboratory conditions, some phages can cause the phenomenon of "lysis from without", in which host cells lyse prematurely (without possibility of phage replication) as result of peptidoglycan degradation at multiple sites[238]. As virion PDEs have this lytic potential, especially when recombinantly produced and added exogenously to bacteria, they are often designated virion-associated lysins, or VALs[239].

VALs are most commonly domains of tail proteins, but they can be located elsewhere on the virion particle. Additionally, they may exist as non-structural elements, either being linked to the phage DNA or as individual proteins enclosed within the capsid, which are released upon nucleocapsid opening[239]. VALs present highly diverse structures, in terms of number of catalytic domains and their organization, molecular weight and oligomeric state. Typically, VALs consist of one or two catalytic domains (CDs), also termed enzymatically active domains (EADs), responsible for peptidoglycan degradation, with a linkage domain to the virion structure (or DNA). Typically, they do not possess a domain for cell binding, as contact to the CW is ensured by tail dynamics and by the tight interaction between RBPs and host cell surface receptors[240]. Most of the described VALs possess a single CD. VALs possessing two CDs appear more frequently in phages infecting Gram-positive bacteria, probably reflecting the thicker peptidoglycan layer in this bacterial group[239]. In terms of CD activities, they are classified according to the chemical bond they cleave within the peptidoglycan mesh. VALs targeting Gram-negative bacteria typically present glycosidase activity, cleaving one of the two glycosidic bonds in the glycan strands, whereas those targeting Gram-positive bacteria can carry an additional activity, frequently an endopeptidase cleaving bonds within the stem peptides or peptide bridges (Figure I.3)[239,240].

I.5.2.2. Overcoming the BCE to Release Phage Progeny

As already mentioned, the second moment when phages need to overcome the different layers of the BCE is at the end of their reproductive cycle, to efficiently release the virion progeny. As opposed to the BCE piercing and local degradation of the peptidoglycan for genome delivery, escape of the newly formed viral descendance involves substantial cleavage of the peptidoglycan and complete disruption of the BCE integrity, with consequent host cell burst or lysis. This event is referred to as "lysis from within" as the phage lysis functions synthesized in the host cell cytoplasm attack the BCE from within. Not surprisingly, host cell lysis is a tightly regulated and temporally programmed process that can impact phage fitness. Lysis before full completion of virion assembly results in reduced phage yield, whereas delayed lysis may compromise the opportunity of infecting new hosts[241].

A. Lysis Mechanisms in Tailed Phages

Tailed phages accomplish bacterial cell lysis through the concerted action of at least two phage-encoded functions: the holin, a protein that forms "holes" in the CM, and the endolysin, responsible for peptidoglycan degradation[69]. Phages infecting Gram-negative bacteria and mycobacteria may encode additional important lysis functions to overcome the outermost layer of the BCE, the OM, namely spanins and AMP-like functions in phages infecting Gram-negative bacteria, and the lipolytic LysB enzyme produced by mycobacteriophages[242-244].

The lysis mechanism of tailed phages has been primarily elucidated thanks to the detailed studies conducted by Ry Young's group on the *E. coli* phage λ, having established the so-called holin-endolysin model[245,246]. According to this model, holin and endolysin genes start being expressed at late stages of phage infection. The endolysin accumulates in the cytoplasm while the holin inserts, accumulates and forms small oligomers in the CM. At a timing inscribed in the holin primary structure, and after reaching a critical concentration in the CM, the holin molecules are triggered to assemble larger oligomers that form non-specific pores (holes) in the membrane. By disrupting CM integrity, these holes lead to irreversible loss of the PMF and, consequently, to cell death. In this model, called canonical model, the holin pores play another essential role as they are large enough to provide a conduit through which the cytoplasm-accumulated canonical endolysin (c-endolysin) gains access to the CW compartment. Once there, the endolysin quickly degrades the peptidoglycan, culminating in osmotic cell lysis (Figure I.12a)[247]. For several years, this endolysin dependence on the holin action for translocation to the CW was viewed as a paradigm feature in the control of host cell lysis. For many phages, lysis timing is also fine-tuned by the action of an antiholin protein that delays the holin trigger (see below).

However, a seminal study published in 2000 on the endolysin Lys44 of the oenococcal phage fOg44 revealed that certain tailed phages utilize a non-canonical lysis pathway, by demonstrating that endolysins can be directed to the CW compartment in a holin-independent manner[248]. In non-canonical systems, endolysins are delivered to the CW by protein export machinery of the bacterial host, notably by the general secretion pathway (the Sec system) (Figure I.12b). To engage the Sec system, the exported endolysins (e-endolysins) may possess a typical signal peptide (SP-endolysins)[248,249], a signal-arrest-release (SAR) sequence (SAR-endolysins)[250] or rely on a chaperone-like protein that directs the lytic enzyme to the secretion apparatus[251].

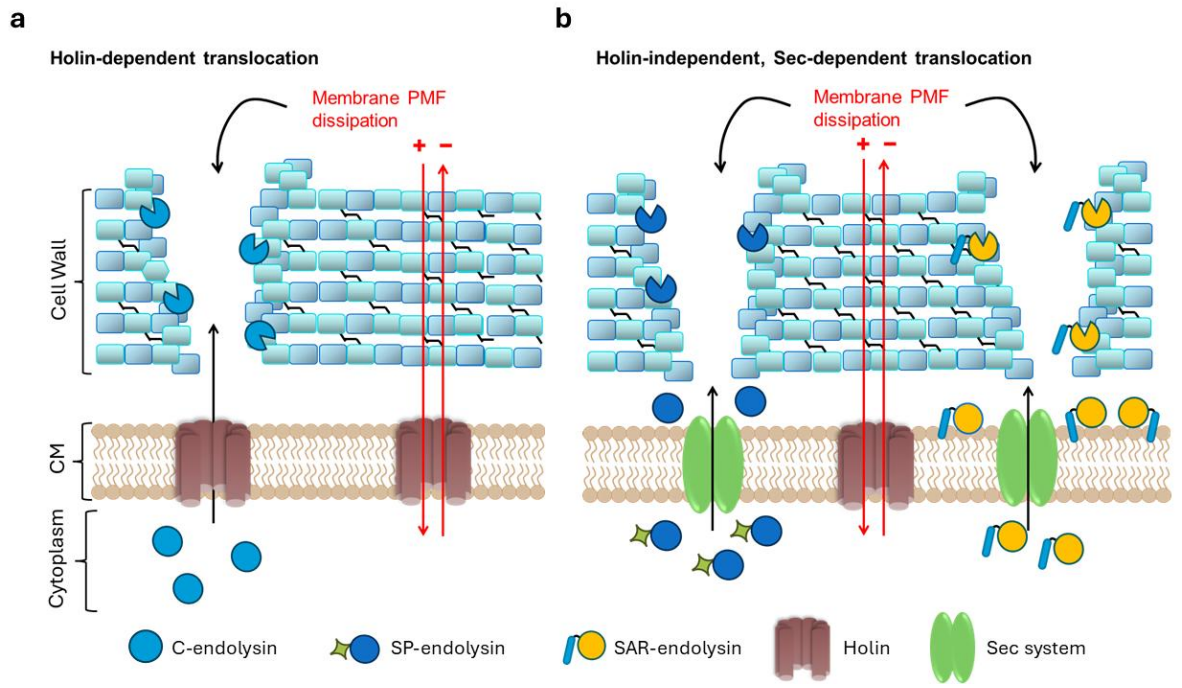

Figure I.12. Models for export and activation of phage endolysins.
(**a**) C-endolysins gain access to the CW through the holin pores, as well-studied in phage λ (canonical lysis model). Recent evidence indicates that holin-mediated collapse of the PMF may enhance the lytic action of c-endolysins (see main text). (**b**) E-endolysins are exported to the CW in a holin-independent manner (non-canonical lysis model). In most known cases, export occurs through the Sec system of the host bacterium, which recognizes endolysins with SP or SAR secretion signals. The "pacman" shape in endolysins represents c- and e-endolysins activation after holin-mediated PMF collapse. CM, cytoplasmic membrane.

Secretion of the SP-endolysin Lys44 was shown to initiate about half time the lysis time, with the SP-less, mature form of the enzyme accumulating in the CW without causing premature lysis. This was an indication that Lys44 was kept inactive in the CW until the proper time for lysis[248]. Such inhibition of Lys44 was observed when the endolysin reached the CW not only from within, but also when added to cultures as a recombinant protein (from without). Restriction of Lys44 lytic action depended on the energized CM, since agents collapsing the PMF immediately activated Lys44 lytic action[252]. As phage fOg44 also encoded a pore-forming holin[253], it was concluded that activation of the e-endolysin pre-positioned in the CW relied on the holin PMF-dissipating action. The fact that agents mimicking the holin-induced PMF loss were able to activate Lys44 bacteriolytic action was consistent with this idea[252].

The role of holins in the activation of e-endolysins was clearly demonstrated for the SAR-endolysins Lyz(P1) and R(21) of coliphages P1 and 21, respectively. In contrast to typical Sec-type signal peptides, the SAR sequence is not cut off after Sec-mediated transport to the periplasm. Indeed, these SAR-endolysins accumulate in the periplasm in a CM-tethered, inactive form. Retention of the SAR domain in the CM is favored by membrane polarization, and it is the holin-mediated dissipation of the PMF that greatly promotes endolysin release from the membrane. The

release of the SAR domain is followed by a refolding of the lytic enzymes that become active to degrade the peptidoglycan[254-256].

The studies on these and other[251] non-canonical systems indicated that, as observed for canonical lysis, holins retained the key role of setting up the lysis timing, since their PMF-collapsing action is responsible for e-endolysin activation. Another idea that emerges from the study of the two major lysis mechanisms is that, regardless of their translocation mode to the CW, in their natural context endolysins only act after the infected host has been killed by the PMF-dissipation function of the holin. This putative transversal role of the PMF on the regulation of endolysins could have an impact on their exploration as enzybiotics and will be further discussed below.

B. General Features of Lysis Players

Holins constitute a very diverse group of proteins, although sharing some characteristics, namely (i) they tend to be small hydrophobic proteins, (ii) they usually have a hydrophilic and positively charged C-terminus and (iii) have at least one transmembrane domain (TMD)[257].

As seen above, holins are considered the clocks of phage infection as they are key players in defining the lysis timing[257]. They are synthesized during the mid to late stage of phage infection and harmlessly accumulate in the CM, where they distribute uniformly. When a critical, genetically defined concentration is achieved, abrupt oligomerization of the holin occurs, initially by formation of membrane “rafts”, which are hypothesized to permeabilize the CM, leading to the collapse of the PMF. PMF dissipation is then thought to induce conformational changes in the holin rafts, which turns them into the final holes[258]. As also said before, in the canonical lysis model these holes are large enough to provide a pathway for c-endolysin access to the CW.

Probably reflecting the fact that the role of holins in non-canonical lysis is restricted to PMF collapse, it was found that some holins associated with SAR-endolysins formed narrow pores incompatible with endolysin translocation, and for this reason they were coined as pinholins. This was first proposed for phage 21[259] and then extended to other phages[244,260]. Like the canonical holins, pinholins are produced at a late stage of phage infection and accumulate harmlessly and uniformly in the CM. The difference between canonical holins and pinholins is that the formed rafts are smaller and in higher number[259]. Despite their reduced size the pinholes allow diffusion of ions and hence collapse the membrane PMF[261]. Therefore, by preserving the PMF-dissipation activity, pinholins also fulfil the role of activating e-endolysins, namely SAR-endolysins, and thus determine the timing of lysis[262].

To fine tune host cell lysis timing in response to infection conditions, phages may encode an inhibitor protein of the holin activity, termed antiholin. The best described antiholin systems are those of coliphages λ and T4[263,264]. In some phages, as prototyped by phage λ, the holin and the antiholin are encoded in the same gene, through the so-called “dual-start motif”[265]. In phage λ, holin translation initiates at the second start codon (Met3) of the gene, whereas the antiholin function

initiates, with somewhat less efficiency, at the first start codon (Met1). This subtle difference makes the antiholin have two additional amino acids in the N-terminus, Met1 and Lys2, which are positively charged (Met1 contributes with a positive charge after being deformylated[266]). These two extra positive charged amino acids prevent insertion of the N-terminal antiholin TMD into the CM. The inhibitory effect of the antiholin arises from its dimerization with the holin protein, delaying hole formation. Notably, PMF dissipation eventually occurs and triggers insertion of the first TMD into the CM, making the antiholin adopt the normal holin topology, thus contributing also to hole formation[266]. For some phages producing pinholins, such as the lambdoid phage 21, the antiholin/holin functions were also described as having the dual-start motif genetic organization, with a mechanism of action resembling that of phage λ counterparts[267].

In many phages the holin and antiholin functions are encoded in separate genes[268-270]. One well-studied example is phage T4. The T4 holin T has a unique topology, possessing only one TMD and a significant C-terminal periplasmic domain of about 160 aa. The PMF-dissipating action of the holin can be modulated by environmental cues, such as the superinfection by other T4 (or T-even) virions. In this scenario, host bacteria activate a lysis inhibition (LIN) state where holin-mediated lysis is delayed[271]. T4 LIN state involves two proteins with antiholin function, RI and RIII, which bind the periplasmatic and cytoplasmatic domains of the T holin, respectively[272]. Antiholin RI is proposed to be the main determinant of the LIN state, whereas RIII contributes to its stabilization. After DNA ejection of T4 superinfecting phages, recently found to be a probable LIN signal[273], the RI antiholin forms a complex with the T4 holin, preventing holin "raft" oligomerization. This inhibits PMF collapse and is thought to facilitate the binding of the cytoplasmic antiholin RIII to the cytoplasmic domain of the holin, forming a structure which is hypothesized to block the formation of the holin hole[272]. Subsequently, the elimination of the LIN state and resume of hole formation is thought to occur once the level of the superinfecting phage DNA in the periplasm is reduced by degradation[273].

As stated before, endolysins are PDEs that are instrumental to achieve host cell lysis at the end of the phage replicative cycle. In its simplest form, the endolysin consists of a single polypeptide chain carrying a CD responsible for peptidoglycan cleavage. However, it may carry more than one CD and additionally a domain involved in CW binding, often referred to as cell wall binding domain (CWBD), or simply cell binding domain (CBD)[274,275]. Endolysins can be classified based on the type of chemical bonds they cleave within the peptidoglycan (Figure I.3). The CD and CBD modules determine the activity range of endolysins, depending on the peptidoglycan bonds and CW ligands they target, respectively. For example, CDs targeting the widely conserved glycosidic and amide bonds will tend to have broader range than those cleaving the most variable interpeptide bridges. Likewise, CBDs may recognize conserved or specific substructures of the peptidoglycan itself, as well as elements of CW-associated polymers characteristic of certain bacterial genus, species or even strains[274-276].

Despite having common functional domains, endolysins present a high structural diversity. The structure and domain architecture of phage endolysins largely differs between those targeting Gram-positive and mycobacteria and those acting on Gram-negative bacteria, possibly to accommodate the differences in the BCE[85,277].

Endolysins of phages infecting Gram-negative bacteria usually correspond to a single, relatively small globular polypeptide ranging from 15 to 20 kDa. Indeed, a survey of more than 2000 endolysins predicted that roughly 90% of those targeting Gram-negative hosts are constituted by a single module corresponding to the CD, therefore lacking a CBD. When present, the CWBD module is more frequently reported at the N-terminus of the protein, but it may also occupy a C-terminal position (Figure I.13)[276,278].

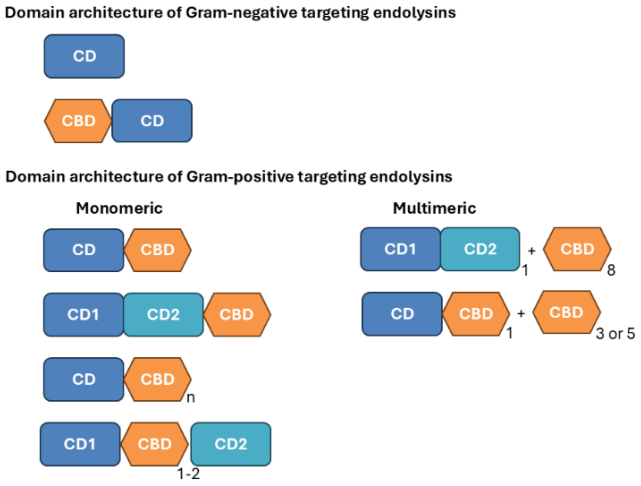

Figure I.13. Domain architecture of studied or most frequently reported Gram-negative and Gram-positive endolysins.
The "n" letter indicates that repeats of CW binding motifs may compose the CBD (usually 2 to 7 copies). The subunits composing the few known hetero-oligomeric endolysins are separated by the "+" sign, with indication of the possible subunit stoichiometries.

Endolysins targeting Gram-positive bacteria typically have a modular architecture, carrying at least one domain responsible for catalytic activity, the CD, linked to a CBD responsible for substrate binding[277]. The different functional domains or modules of these endolysins are connected by flexible linkers, generally composed of amino acids with short and polar side chains. The endolysin modular architecture can vary significantly according to the type, number and relative position of CDs and CBDs (Figure I.13). The most common architecture contains one N-terminal CD linked to a CBD module at the C-terminus. However, it is frequent in endolysins targeting Gram-positive bacteria the presence of two CDs with distinct peptidoglycan specificity. These endolysins are for example commonly found in *Staphylococcus* infecting phages[275,276]. It is also common to observe endolysins equipped with one or two CDs linked to several repeats of the same CW binding motif, as reported for several streptococcal endolysins[279].

Among the most common CBD domains found in studied endolysins are the LysM domain, shown to bind to NAG residues in the peptidoglycan, the SH3 (SH3b) superfamily domain, which as some subtypes specialized in binding the interpeptide bridges of *Staphylococcus* and *Streptococcus* species, the CW_binding_1 domain, which specifically recognizes the choline-containing TA in the CW of *S. pneumoniae* and highly related species of the mitis group, and the CW_7 domain, which binds to streptococcal cell walls in a choline-independent manner (Ref.[276] and references therein). It should be noted that depending on the bacteria/endolysin pair, the presence of a CBD may or may not be essential for enzybiotic applications (see Section I.6.1).

The almost omnipresence of a CBD module in Gram-positive targeting endolysins may fulfil an important role in addition to that in peptidoglycan cleavage. These domains are thought to maintain the endolysins tightly bound to cell debris after lysis and this has been proposed to provide a mechanism to prevent diffusion and subsequent destruction of potential new host cells in the vicinity[280,281]. Such collateral effect is unlikely to occur in Gram-negative systems due to the presence of the OM that shields the peptidoglycan from the endolysins eventually diffusing from burst cells. This may explain why CBDs are seldom seen in Gram-negative targeting endolysins.

Apart from certain pneumococcal endolysins that have long been known to form homodimers upon binding to TA choline residues (Ref.[282] and references therein), endolysins are generally assumed to work as single-polypeptide, monomeric enzymes. However, in recent years the number of endolysins shown to work as multimers has been increasing. The first reported case was the streptococcal endolysin PlyC, which is composed of two different subunits, the dual-CD subunit PlyCA and the CBD subunit PlyCB, which are encoded in two separate genes. The PlyC holoenzyme is a 1:8 complex in which one PlyCA subunit associates with an 8-mer ring made of PlyCB subunits[283,284]. Other homo- or heteromeric endolysins were subsequently discovered, with some exhibiting alternative subunit stoichiometries[285-287]. One interesting and apparently widespread mechanism to generate heteromeric endolysins relies on the production of two polypeptide isoforms from a single endolysin gene thanks to the presence of an in-frame, internal translation start site (iTSS) in the gene. Frequently, this iTSS is located immediately upstream of the CBD coding sequence, driving the independent production of CBD subunits that then associate with the full-length (FL) endolysin polypeptide to generate the fully active enzyme. This was first demonstrated for enterococcal endolysins, which worked as tetrameric enzymes with a 1FL:3CBD stoichiometry[285,288,289]. A wide bioinformatics study revealed that a large proportion of phages infecting Gram-positive bacteria and mycobacteria most likely produce two-polypeptide endolysins through this strategy, and a new 1FL:5CBD enzymatic complex was uncovered[290]. In some endolysins, the iTSS occurs between the CDs in dual-CD endolysins[290-292] and at least in one case the two protein isoforms appear to interact to assemble transient heterodimeric complexes in solution[293]. However, not always the two endolysin isoforms seem to associate to produce a heteromeric enzyme, and in these situations the biological role of this coding strategy is not understood[290].

In addition to the holin/endolysin core functions that target the CM and the CW, phages may encode additional lysis functions to overcome the OM. This third functional class of phage lysis functions may include LysB-like enzymes with activity against lipid components of the mycobacteria OM and AMP-like proteins or spanins that disrupt the OM of Gram-negative bacteria[242-244]. Spanins are by far the most studied example of OM-targeting lysis functions[294].

The name "spanin" was proposed to reflect the property of this class of lysis proteins, which is to span the periplasm to bridge the CM and the OM. In the case of *E. coli* phage T1, the spanin corresponds to a single lipoprotein (unimolecular spanin or u-spanin) that attaches to the inner leaflet of the OM through its N-terminus, while the C-terminal TMD inserts in the CM[295,296]. However, the spanin function is more frequently assembled from two independently produced components, an integral CM protein (inner membrane spanin or i-spanin subunit) and an OM lipoprotein (outer membrane spanin or o-spanin subunit)[294,295].

The prototype of these two-component spanins is that of phage λ, with the Rz and Rz1 proteins corresponding to the i-spanin and o-spanin subunits, respectively. Rz possesses a transmembrane topology in which the N-terminus is oriented to the cytoplasm, and a C-terminal domain faces the periplasm. Rz1 C-terminus also faces the periplasm while being attached to the OM through fatty acyl chains modifying its N-terminus. Rz and Rz1 were found to form a complex linking the CM and OM, thus spanning the entire periplasm[297]. It was shown that in the absence of the spanin complex, host cell lysis is impaired at the end of the infection cycle when cells are not subjected to the shearing forces of culture agitation. In fact, in these conditions the OM can withstand the turgor pressure caused by peptidoglycan degradation, with cells changing from a rod shape to a spherical form[242]. The mechanism by which spanins disrupt the OM barrier is by promoting CM-OM fusion after endolysin-mediated degradation of the peptidoglycan[298]. The available data indicates that the spanin function is the most widely adopted strategy adopted by phages to ensure OM elimination and efficient lysis of Gram-negative hosts. In this view, phage-mediated lysis of Gram-negative hosts occurs in a stepwise pathway that once initiated, it becomes irreversible. The first step is the CM permeabilization by the holin, which allows peptidoglycan degradation by the endolysin action (second step), which in its turn results in the fusion of the CM and OM mediated by the spanin function (final step)[299].

I.5.3. Regulation of Lysis Functions

As referred to above, one key feature that emerges from the study of the lysis mechanisms employed by tailed phages is that regardless of the pathway used for endolysin translocation to the CW compartment, its lytic action is restrained until holin-mediated loss of the PMF. Therefore, the PMF may work as a negative regulator of endolysins lytic action in both canonical and non-canonical lysis. Interestingly, it is for long known the role of the PMF in the regulation of bacterial

autolysins[77,129,300]. Given the sequence and functional relatedness between phage endolysins and bacterial autolysins[275,301], one can expect that the cellular factors regulating autolysins might also be involved in the regulation of endolysins activity, especially of e-endolysins.

Growing evidence suggests that the PMF dissipation action of the holin can also be important to fully sensitize bacteria to the lytic action of c-endolysins. Such function of holins in c-endolysin "activation" could go unnoticed due to the essential role of the holin channel in c-endolysin translocation to the CW. The importance of holin-mediated PMF dissipation in enhancing c-endolysin lytic action was revealed when a cleavable, Sec-type signal peptide was added to a c-endolysin, converting it into an artificial e-endolysin. The artificial e-endolysin was efficiently exported and accumulated harmlessly in the CW during bacterial growth. Peptidoglycan degradation and rapid bacteriolysis only occurred when cells were treated with gramicidin, a CM-acting ionophore mimicking the PMF-dissipation action of the holin[302]. This experiment was a strong support to the notion that the holin PMF-dissipating action can also be important for efficient endolysin lytic action in canonical lysis. Most importantly, additional studies showed that the PMF state can also impact bacterial susceptibility to endolysins when these are applied exogenously as enzybiotics. In fact, it was show that endolysins lytic action from without is greatly enhanced when the cell PMF is abolished by CM-acting drugs (such as ionophores)[285,302,303]. All these observations further highlight the role of the CM energetic state in the regulation of PDEs, including endolysins.

Other than the CM energetic state, the activity of autolysins, and at least some endolysins, is impacted by the presence of TA. It was proposed that TA are key regulators of autolysins in metabolically active cells. The more acidic environment near the extracellular face of the CM is thought to promote a higher proportion of protonated D-alanyl ester of TA, which has been linked to restraint the autolysins activity. Upon PMF-dissipation, the pH in the deeper layers of the CW shifts towards neutrality and destabilization of the protonated D-alanyl ester occur, leading to an activation of autolysins[77,129]. TA were also implicated in the regulation of PDEs by modulating the electrostatic-based binding of the proteins to the bacterial surface[122]. Besides their role in defining the electrical and chemical environment of the CW, WTA were also found to sterically exclude autolysins from older CW regions, directing them to the cross-wall region[127,128]. Furthermore, some reports have directly implicated WTA in bacterial tolerance to the exolytic action of PDEs. WTA were shown to restrict the binding of different PDEs to the CW, including endolysins, probably by sterically shielding or somehow restricting the access of the enzymes to the peptidoglycan[125,126,304]. Conflicting with this however, for some autolysins and endolysins the presence of TA seems to be required or favour their lytic activity[305-307].

Despite the numerous studies pointing to an inhibitory role of the PMF and TA on PDEs acting on Gram-positive bacteria, the mechanisms through which these cellular determinants modulate the lytic action of the enzymes remains largely unclear.

I.6. Exploration of Phage Lytic Enzymes as Enzybiotics

The ability of phage lysins - endolysins and, more recently, VALs - to degrade the CW peptidoglycan from outside and cause cell lysis set the basis for their exploration as potential antibacterial agents, as part of a broader group of lytic enzymes defined as enzybiotics[308]. The potential of phage endolysins as antibacterials against Gram-positive pathogens was first described in 2001[309,310], and over the last two decades, a growing number of studies have supported the hypothesis that native endolysins are capable of antibacterial activity when added from without, both *in vitro* and *in* animal models. Most of the early studies focused on Gram-positive targeting endolysins[311], as the OM of Gram-negative pathogens blocks the access to the CW for most endolysins coming from outside. Nevertheless, in recent years considerable progress has been made in the search and improvement of endolysins to be able to display exolytic activity against Gram-negative bacteria[312].

Lysin-based antibacterials present several strengths, but also some weaknesses, from a therapeutical point of view. First and foremost, lysins can act on bacteria independently of their antibiotic resistance profile because they possess a different mode of action. Lysins target and degrade conserved chemical bonds within the peptidoglycan, and this seems to account for the very low levels of resistance development against lysins[313]. Contrary to conventional antibiotics, endolysins present a much narrower range of action, most often targeting specific bacterial species with minimal impact on the surrounding microbiome[314]. However, this may also be seen as a disadvantage as previous identification of the pathogen is needed to ensure efficacy. Endolysins can also target both dormant and growing cells, not depending on cells presenting an active metabolism, and some can even target cells within a biofilm matrix[315-317]. Furthermore, endolysins can be combined with antibiotics and other antimicrobial agents, which can result in a re-sensitization of resistant bacteria to antibiotics[318].

On the disadvantages side, *in vivo* administration of endolysins may lead to an increased production of pro-inflammatory cytokines due to cellular debris resulting from bacteriolysis[319]. Also, by having a proteinaceous nature, endolysins provoke an immune response in *in vivo* systems, which may decrease endolysins efficacy in long or repeated treatments. However, in a few studies addressing these issues neither major adverse effects nor significant IgG-mediated lysin inactivation were observed[320-323]. The protein nature of endolysins certainly contributes to their instability in biological systems and short circulation half-life. Moreover, most native lytic enzymes lack the ability to target intracellular pathogens[324].

In response to the limitations mentioned above, in the last decade there has been great research investment aiming at improving the antibacterial efficacy and therapeutic features of lysin-based enzybiotics, resorting mainly to synthetic biology and protein engineering strategies, with tenths of pre-clinical and a few clinical studies supporting the key importance of these

approaches[85,325,326]. Interestingly, some studies have shown that another way to take advantage or improve the enzybiotic potential of phage lysins is to combine them with other antimicrobial agents, sometimes resulting in demonstrated synergistic effects. In fact, in clinical settings the combination of different antibiotics, or of these with other agents, has been used to (re)sensitize bacteria and to minimize, or even overcome, antimicrobial resistance[327-330].

I.6.1. Lysin Engineering

When employed as antibacterial agents, phage lysins must exert their lytic activity in conditions significantly different from those found in their natural context of action. The activity of VALs at the initial stages of phage infection is supported by the insertion of tail structures across the BCE, whereas endolysins are naturally designed to attack the CW from within and after host cells have been killed by the holin action (see Section I.5.2). On top of this, in clinical settings lysins will have to act in the complex environments of the body tissues and fluids, in which the proteolytic, immune and excretion systems may negatively affect the performance of the phage PDEs as enzybiotics.

As seen above, the first-generation of lysin-based enzybiotics essentially relied on native endolysins targeting Gram-positive pathogens. They were used in a high number of studies to reveal their antibacterial potential, both *in vitro* and *in* animal models of infection/colonization. However, the same *in vivo* studies also hinted for some efficacy limitations, as most endolysins were only efficient at fighting infection when administered soon after the bacterial inocula (usually 1h post bacterial infection) and at high doses, with modest results obtained against well-established infections[331]. Despite these potential limitations, one of these first-generation lysins, PlySs2 (also known as CF-301 or Exebacase), was the first to reach a phase III clinical trial. This trial tested for the superior efficacy of Exebacase in combination with standard-of-care antibiotics against *S. aureus* in patients with bloodstream infections and/or infectious endocarditis (ClinicalTrials.gov ID: NCT04160468). Unfortunately, and contradicting the positive data of previous phase II studies[322], the clinical trial was stopped because the Exebacase + antibiotics group failed to show improved clinical response when compared to antibiotics-alone group[332].

To enhance features like the bactericidal and spectrum of activity, as well as to improve other characteristics such as production yield, solubility and stability, a second-generation of lysins was developed. The most common strategies followed to generate these new lysins involve the construction of chimeric enzymes (chimeolysins) by domain shuffling or the modification of natural endolysins through domain deletion, mutagenesis, and fusion to other (poly)peptides. In some cases, new lysins were obtained by combining some of these strategies. Fusion of proteinaceous elements to lysins that allow them to overcome the OM was instrumental to extend the enzybiotics field to Gram-negative pathogens (Figure I.14)[85,325].

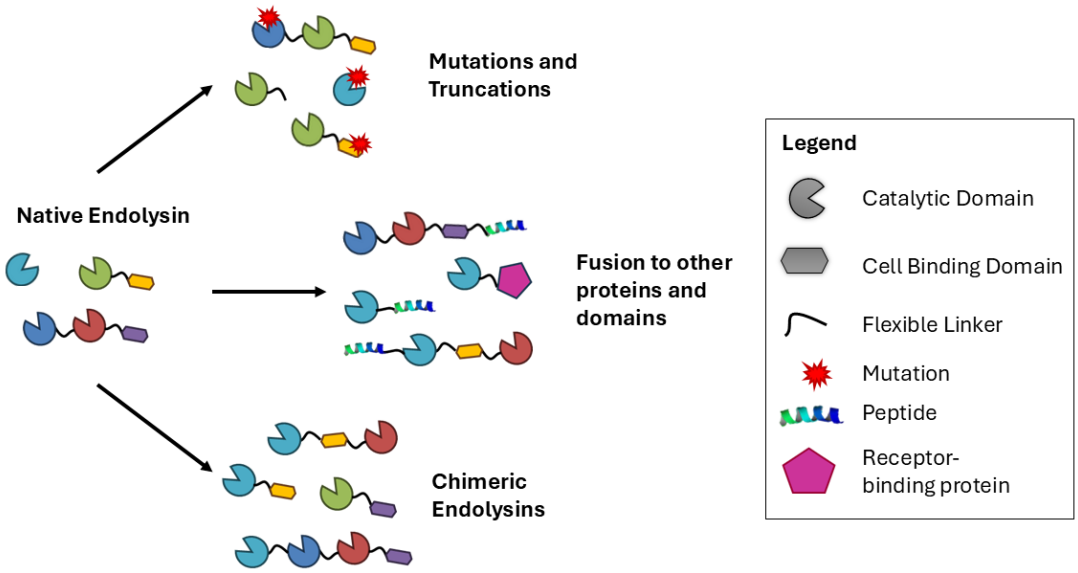

Figure I.14. Engineering strategies used to enhance endolysin features.
Most native endolysins consist of a single catalytic domain or have a modular structure comprising at least one or two catalytic domains (CD) linked to a cell binding domain (CBD) module. The depicted protein modification/engineering approaches have been used to improve lysin properties such as their bactericidal potency, spectrum of activity, solubility and stability. Adapted from Ref.[325].

One of the earliest studies exploring the modular nature of PDEs targeting Gram-positive bacteria employed two pneumococcal lytic enzymes: the autolysin LytA and the phage endolysin Cpl-1. The study demonstrated that in the two chimeolysins resulting from the interchanging of the enzymes' CDs and CDBs, the functional domains maintained their native properties, namely the cleavage specificities of the CDs and the choline dependence or not of the CBDs for conversion into the fully active forms[333]. This work provided evidence that the constituent domains of lysins could be viewed as independent modules, both in folding and function. Swapping domains or fusing single domains to other native PDEs was shown to be an efficient strategy to generate chimeolysins with expanded host ranges, while maintaining the lytic activity of the parental enzymes[334-336]. In addition, the use of CDs and CBDs as independent modules in random combinatorial strategies allowed the successful generation and screening of hundreds of chimeolysins to identify variants with enhanced bactericidal activity in different environments, such as food matrices or blood serum[337-339].

Not only endolysin modules have been used to create chimeric lytic enzymes. VALs have also been explored for such a purpose. In a 2011 study, Paul et al. identified in the genome of the staphylococcal phage K a gene (*orf56*) encoding a VAL, which contained a C-terminal CD of the CHAP family. By isolating and fusing this domain with the CBD of lysostaphin, an anti-*S. aureus* bacteriocin with peptidoglycan degrading activity produced by *S. simulans*, they obtained a chimeric protein (named P128) with much higher bactericidal activity against MRSA and other *Staphylococcus* species than the isolated CHAP CD. In addition, P128 exhibited higher thermostability than

lysostaphin[340,341]. In another study exploring the potential of VALs, the M23 peptidase CD of the VAL Orf73 of *E. faecalis* phage F170/08 was fused to the CBD of the cognate endolysin Lys170. The chimeric enzyme (EC300) exhibited a much higher bacteriolytic activity than the endolysin, especially when target bacteria were challenged in media supporting active cell growth[342].

Alongside chimeolysin technology, protein truncation is also frequently applied to alter the properties of endolysins. As said earlier, typical endolysins targeting Gram-positive bacteria possess one or two CDs cleaving peptidoglycan and a CBD module that generally promotes strong binding of the lytic enzymes to the surface of target bacteria. As such, the deletion of either a CD and/or a CBD could be expected to necessarily lead to a negative impact on bacteriolysis. However, this appears not to be the case for some endolysins and, at least under certain experimental conditions, domain deletions can lead to improved bacteriolytic activity and/or expansion of the spectrum of activity[343-347]. Nevertheless, it has been argued that the conclusions from these types of studies should be critically considered as discrepant results are often seen depending on the methods employed to assess lysin lytic activity[347,348].

As previously mentioned, Gram-negative bacteria are naturally unaffected by the majority of endolysins due to the protection conferred by the OM. For that reason, the activity of endolysins targeting Gram-negative bacteria is frequently studied in the presence of agents that destabilize the OM such EDTA, organic acids (citric and malic acid) or polycationic peptides (poly-L-lysin and poly-L-arginine) (reviewed in Ref.[349]). Although useful to determine lysins' activity, the utility of these agents as partners of the lytic enzymes is limited to specific applications, namely control of infection in the Agrofood industry and bacterial elimination in abiotic materials.

Interestingly, several reports have described endolysins with some intrinsic ability to cross the OM and kill cells (Ref.[350] and references therein). This capacity to overcome the OM is due to the presence of AMP-like subdomains in the endolysin structure, mostly located at the C-terminus, a trait that appears more common than previously thought[276]. The positive and/or amphipathic character of these subdomains are thought to facilitate contact with the negatively charged LPS of the OM and to mediate its translocation for access to the peptidoglycan layer. In some cases, when these regions were produced as synthetic peptides, they were shown to have antibacterial activity[351-353]. Given these observations, it was proposed to fuse endolysins with OM-destabilizing peptides, including those with polycationic AMP-like features, in a manner to increase the antimicrobial potency of the lytic enzymes against Gram-negative pathogens. Such engineered lysins were termed Artilysins® and they were a major breakthrough to expand the use of endolysins against Gram-negative bacteria[354]. One of the first and most studied Artilysins is Art-175, an enzybiotic resulting from the fusion of a derivative of endolysin KZ144 with the polycationic AMP SMAP-29. Art-175 exhibited bactericidal activity against various Gram-negative species, being able to kill bacteria in different growth states (exponential- and stationary-phase cultures and persister cells)[355-357]. As for chimeolysins targeting Gram-positive bacteria, the random combination of endolysin functional

modules and OM-destabilizing peptides enabled the generation of libraries with tens to thousands of Artilysins, with the latter numbers being possible thanks to the development of high-throughput platforms such as VersaTile[358,359].

Other engineering strategies to promote OM crossing are the fusion of lysins with molecules that target specific transport systems or receptors in the OM of Gram-negative bacteria. Lysocins and Innolysins are two examples of this approach. Lysocins result from the fusion of lysins with key segments of bacteriocins that mediate OM translocation as part of the killing mechanism of these agents. These segments target transport systems in the OM, such as FyuA or those dependent on the Tol or Ton translocons, which are responsible for the uptake of the Lysocins into the periplasm[360-362]. Innolysins comprise a different class of fusion proteins generated by adding phage RBPs to endolysins. In this case, it was shown that the targeting of certain cell surface receptors by the RBP moiety promoted OM crossing and subsequent lytic activity of Innolysins[363,364]. In a kind of mixed approach, endolysins were fused to a toxin translocation domain and to an RBP to maximize OM crossing and the spectrum of activity[365].

Despite the multiple approaches to improve the therapeutical potential of lysin-based enzybiotics, only a handful for treating *S. aureus* infections reached human clinical trials (reviewed in Ref.[311]). Current efforts are focused on improving pharmacological properties of lysin-based enzybiotics such as their half-life, biodistribution and immunogenicity, for both topical and systemic applications (third-generation lysins). This is normally achieved using not only protein engineering/modification approaches, but also formulations and delivery strategies adapted to the blood stream or target tissues (reviewed in Ref.[324,366,367]). One common strategy to improve endolysin delivery and serum half-life is endolysin encapsulation in polymeric nanoparticles[368,369]. Endolysin encapsulation, particular in liposomes, is also applied to overcome the OM of Gram-negative pathogens[370].

I.6.2. Lysin Synergisms with Other Antibacterial Agents

In addition to the engineering and technological strategies aiming at improving lysins' antibacterial properties, a significant body of work has been showing that when two different lysins are used together or when an enzybiotic is simultaneously used with another antimicrobial agent, an enhanced antibacterial effect can be observed *in vitro* and *in vivo*. Most reported studies are on synergies between lysins and conventional antibiotics, but it can also be with AMPs (reviewed in Ref.[313,371]).

The interaction between two individual antimicrobial agents can lead to different phenotypic effects: synergism, additivity, antagonism or indifference. The gold standard method to evaluate this interaction is the microdilution checkerboard assay[372,373]. This method allows for a quantitative evaluation of the produced effect, by determining the so-called fraction inhibitory concentration

index (FICI). Synergy is observed when the combined effect of two agents is greater than the sum of the individual effects and results in FICI values ≤0.5. Additive effect is when the combined effect essentially results from the sum of the individual effects, giving a FICI between 0.5 and 1.0. An antagonistic effect occurs if the combined effect of the two antimicrobial is lower than the effect of the most effective agent used alone, resulting in FICI >4.0. Finally, indifference is defined by a FICI between 1.0 and 4.0 and occurs when two drugs act independently without influencing each other's activity[374].

When two lysins targeting different chemical bonds within the peptidoglycan are combined, an enhanced lytic action may be observed, as it can lead to a more unrestrained lytic action of the peptidoglycan bonds. Also, the action of one enzyme may expose the substrate for the other, contributing to the overall cleavage efficiency[375-377]. The efficacy of lysins against bacterial biofilms can be increased with the help of a different class of phage enzymes, which are generally designated as depolymerases. Depolymerases degrade the biofilm matrices, granting endolysin access to the CW[378,379].

The mechanisms underlying the synergistic effect of lysins and antibiotics remains largely unclear. The synergistic killing effect of pairs of antimicrobials might be simply explained by the concurrent action of two antimicrobials with distinct modes of action, which sensitize bacteria to each other[327,371]. The lysin peptidoglycan-degrading activity was proposed to facilitate antibiotic penetration and access to the cellular targets, but it is also plausible that an altered peptidoglycan induced by antibiotic treatment becomes more susceptible to lysin attack. The second hypothesis would be more likely for synergies with antibiotics that inhibit CW synthesis[321,380-382]. Furthermore, inhibition of CW assembly enzymes may lead to unbalances favouring the activity of endogenous bacterial PDEs, namely autolysins, which combined with the lytic action of lysins translates into an accelerated cell lysis[383]. In some cases, lysins may even resensitize bacteria displaying resistance to certain cell envelope-targeting antibiotics[384,385]. Lysin and antibiotic combinations were also reported to be more efficient in removing and eradicating biofilms, most likely because the biofilm-dispersing action of lysins allowed a more efficient antibiotic penetration into the biofilm matrix[386-388]. Other advantages of this combinatorial approach are the prevention or minimization of resistance development and the reduction of antimicrobial dosages and associated toxicity issues[371].

AMPs such as Magainin-2 and its synthetic derivatives, bacteriocins like nisin, and lipopeptide antibiotics such as colistin and daptomycin destabilize the bacterial cell membranes. Bactericidal synergy between these agents and PDEs, including phage-derived lysins, has been frequently reported when applied to Gram-positive[303,389-391] and Gram-negative bacteria[385,392,393]. In the case of Gram-negative bacteria, OM destabilization/permeabilization should be responsible for granting endolysin access to the peptidoglycan, explaining the advantage of the combined treatments. In the

case of Gram-positive bacteria, the preferred explanation for the observed synergies is a facilitated access of the membrane-acting agents thanks to the peptidoglycan-weakening action of the lysins.

In conclusion, current knowledge supports that combining lysins with other antibacterial agents can be a good strategy to further explore the enzybiotic potential of the lytic enzymes and eventually overcome some of their intrinsic limitations.

I.7. Thesis Goals

The main goal of this doctoral project was to further explore the potential of endolysins as enzybiotics, while understanding in more depth the mechanisms governing bacterial tolerance to endolysins in certain conditions.

In the phage infection context, all known endolysins act after holin-mediated cell death due to its PMF-dissipating action. It was previously described that membrane damage (with consequent loss of the PMF) correlates with an increased susceptibility of Gram-positive bacteria to the exolytic action of endolysins. Therefore, we proposed to study the antimicrobial effects that result from combining the action of AMPs and endolysins, as well as to understand how AMPs potentiate the lytic action of endolysins (Chapter II).

Although it is generally accepted that Gram-positive bacteria are more susceptible than Gram-negative bacteria to the lytic action of endolysins from without, research has shown that Gram-positive bacteria can tolerate and/or restrain the lytic action of endolysins when maintained in conditions that support robust bacterial growth. Building on previous studies indicating that tolerance to certain PDEs relies on the PMF and presence of certain CW polymers, we proposed to dissect the mechanisms by which these cellular factors impact endolysin functional domains (Chapter III).

Ultimately, this work aimed at deepening our understanding of the factors that control endolysin lytic action and provide important clues for the design of new powerful enzybiotic antibacterial strategies.

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Chapter II.

Synthetic Antimicrobial Peptides as Enhancers of the Bacteriolytic Action of Staphylococcal Phage Endolysins

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Synthetic Antimicrobial Peptides as Enhancers of the Bacteriolytic Action of Staphylococcal Phage Endolysins

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Author Contributions

C.S.-J. and M.G.P. designed the study.

A.G. executed most of the experimental work. D.P. performed the bactericidal assays with MRSA strains and AMPLys fusions. H.V. performed the Super Resolution Structure Illumination Microscopy experiments. W.A. carried out the Scanning Electron Microscopy imaging.

C.S.-J and D.P. wrote the manuscript with contributions of M.G.P., A.G., H.V. and W.A.

All authors contributed to the analysis and interpretation of data and approved the final manuscript.

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Abstract

Bacteriophage endolysins degrade the bacterial cell wall and are therefore considered promising antimicrobial alternatives to fight pathogens resistant to conventional antibiotics. Gram-positive bacteria are usually considered easy targets to exogenously added endolysins, since their cell walls are not shielded by an outer membrane. However, in nutrient rich environments these bacteria can also tolerate endolysin attack if they keep an energized cytoplasmic membrane. Hence, we have hypothesized that the membrane depolarizing action of antimicrobial peptides (AMPs), another attractive class of alternative antibacterials, could be explored to overcome bacterial tolerance to endolysins and consequently improve their antibacterial potential. Accordingly, we show that under conditions supporting bacterial growth, *Staphylococcus aureus* becomes much more susceptible to the bacteriolytic action of endolysins if an AMP is also present. The bactericidal gain resulting from the AMP/endolysin combined action ranged from 1 to 3 logs for different *S. aureus* strains, which included drug-resistant clinical isolates. In presence of an AMP, as with a reduced content of cell wall teichoic acids, higher endolysin binding to cells is observed. However, our results indicate that this higher endolysin binding alone does not fully explain the higher susceptibility of *S. aureus* to lysis in these conditions. Other factors possibly contributing to the increased endolysin susceptibility in presence of an AMP are discussed.

II.1. Introduction

Antibiotic resistance is currently a major threat to global health and economy, with catastrophic scenarios anticipated if efficient control actions are not taken[1-3]. One of the measures to tackle the problem relies on the development of alternative antimicrobials capable of acting on drug-resistant bacteria, preferentially with new modes of action that minimize the emergence of resistance. Among such alternatives in the pipeline are endolysins[4], which are enzymes (enzybiotics) that destroy the bacterial cell wall (CW)[5].

Endolysins are produced by bacteriophages (phages), viruses that infect bacteria. An endolysin and a holin define the minimal tool set that double-stranded DNA phages use to lyse host bacteria for virion progeny release at the end of infection[6]. The holin forms "holes" in the cytoplasmic membrane that cause cell death through dissipation of the membrane proton-motive force (PMF)[7]. In addition, for phages employing the so-called canonical lysis model, the holin holes also provide the conduit for passage of the cytoplasm-accumulated endolysin to the CW compartment. Once there, endolysins degrade the peptidoglycan, the major structural component of the CW, and at least for Gram-positive bacteria this is usually sufficient to cause osmotic cell lysis[7,8].

Application of endolysins as enzybiotics against Gram-positive bacteria is usually considered facilitated, since these lack the outer membrane that in Gram-negative bacteria and mycobacteria hinders enzybiotic access to the CW[8,9]. However, despite lacking this CW protecting barrier, a few studies have shown that Gram-positive bacteria can also restrict or tolerate endolysin attack to certain extent. This was observed for several bacterial species/endolysin pairs, with endolysin tolerance being favoured in media that supported bacterial growth, but abolished upon membrane PMF collapse by the holin or by ionophores mimicking its action[10-12]. Hence, the hallmark was that to counteract endolysin lytic action, cells needed to be in a competent energetic state, i.e., with an operational PMF. This phenomenon seems to recapitulate the natural context of phage infection, where endolysins only act after the holin-mediated PMF dissipation (lysis mechanisms reviewed in Ref.[8]).

The mechanisms linking the PMF to the bacterial capacity to restrict endolysins lytic action are still not understood, although the importance of the PMF for control of the activity of some bacterial lytic enzymes (autolysins) is well-documented. One of the bacterial CW components proposed to respond to the ionic changes induced by PMF collapse are wall teichoic acids (WTA), which are abundant anionic polymers bound to the CW of many Gram-positive bacteria. Some studies have presented WTA as key elements restricting autolysin activity in a PMF-dependent way (Refs.[13,14] and references therein). Other studies have shown that WTA can be responsible for restricting access of autolysins and endolysins to certain regions of the CW, therefore coordinating their spatial distribution[15,16]. More recently, it was proposed that WTA can protect bacteria from the attack of lytic enzymes by hindering their binding to the CW, with this shielding effect being stronger when cells

are in rich nutritional media[17-19]. For other enzymes however, binding to WTA appears to be a requirement for lytic activity[20].

Current knowledge indicates therefore that susceptibility of Gram-positive bacteria to endolysins lytic action can be highly dependent on the cells physiologic/energetic state, something that could impact the efficacy of the lytic enzymes as antibacterials[21]. We have reasoned that the holin key role in sensitizing bacteria to endolysins could be substituted by the action of antimicrobial peptides (AMPs). AMPs are produced by virtually all living organisms as part of the defence mechanisms against bacteria (and other microbes), and frequently their action involves permeabilization of the bacterial cytoplasmic membrane, with consequent PMF collapse[22,23]. AMPs have been also regarded as promising alternatives to conventional antibiotics due to their particular mechanism of action and immunomodulatory features[23].

By using a PMF-disrupting AMP and a staphylococcal endolysin as models, we have explored the capacity of the AMP to enhance the lytic power of the endolysin against the high priority, Gram-positive pathogen *S. aureus*[24], including methicillin resistant *S. aureus* (MRSA) clinical strains, under growth supporting conditions. We have also focused on the mechanism(s) by which the AMP facilitated endolysin lytic action, considering its multiple effects at the cellular level. Lastly, we have briefly investigated the role of WTA in endolysin tolerance.

II.2. Results

II.2.1. Lytic Action of Endolysin Lys11 is Enhanced in Presence of the AMP R8K

We showed previously that the *S. aureus* ability to counteract the attack of the phage ϕ11 endolysin Lys11 was clearly diminished in presence of the PMF-dissipating agent gramicidin[12]. To have a better understanding of S. aureus capacity to resist Lys11-mediated lysis, cells in rich culture media (TSB) were treated with different concentrations of Lys11 and cell lysis monitored by taking optical density (OD_{600nm}) measurements over time. These assays were carried out in the presence of 0.5 mM $CaCl_2$ (TSBca) as we have recently observed an enhancing effect of calcium ions on the lytic activity of Lys11, as described for related enzymes[25,26]. The two lowest enzyme concentrations tested (25 and 50 nM, about 1.4 and 2.8 μg/ml, respectively) produced no significant cell lysis after 60 min contact, although they could arrest *S. aureus* growth (Figure II.1a). In the same conditions, the other tested enzyme concentrations (100, 250 and 500 nM) reduced culture's OD_{600nm} roughly by 20, 50 and 70%, respectively.

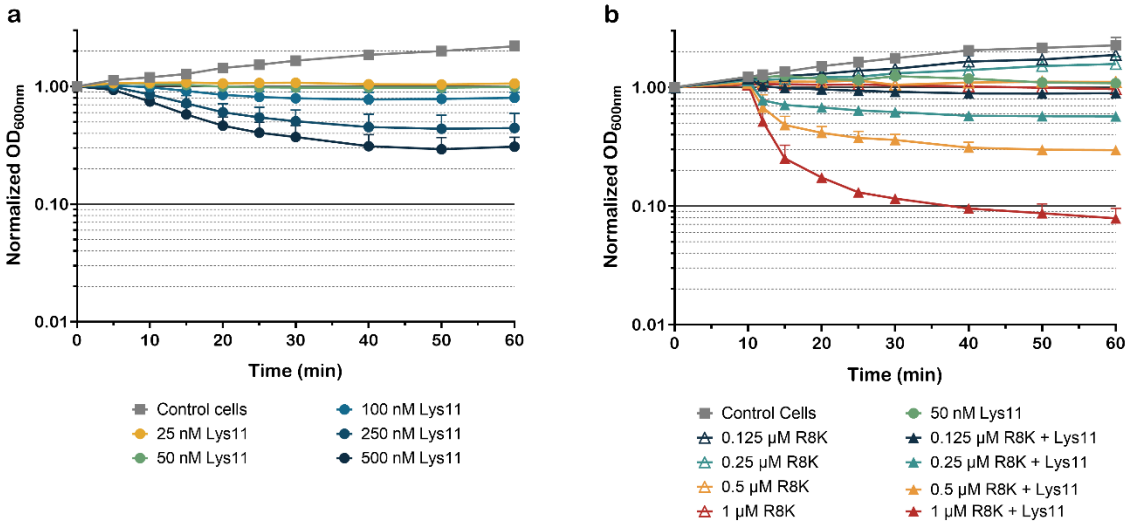

Figure II.1. Lytic activity of the endolysin Lys11 in absence or presence of the AMP R8K.
(**a**) Log phase cells of strain RN4220 were collected in prewarmed fresh TSBca, and lysis monitored by taking OD_{600nm} measurements (OD_{600nm}) after addition of the indicated Lys11 concentrations or of endolysin buffer ("Control cells" curve). (**b**) Log phase cells of strain RN4220 collected in TSBca were treated for 10 min with the indicated concentrations of R8K. Then, 50 nM of Lys11 were added and cell lysis evaluated spectrophotometrically. AMP solvent and endolysin buffer were added to the Control cells. Each curve represents means ± standard deviation from at least 3 independent experiments. For clarity, only mean + standard deviation is represented.

Next, we tested if *S. aureus* cells treated with an AMP would become more prone to Lys11-mediated lysis. For these assays, we selected the peptide SMAP-29(K^8), for simplicity hereafter designated R8K, which corresponds to a low toxicity version of the natural cathelicidin SMAP-29 (sheep myeloid antimicrobial peptide of 29 amino acids)[27], which in turn carries an amphipathic α-helical segment responsible for membrane permeabilization[28]. Exponentially growing *S. aureus* cells recovered in fresh TSBca were pretreated for 10 min with different concentrations of R8K (0.125 to 1 µM) and then supplemented with 50 nM of Lys11, which we have shown previously to cause no lysis (green curve in Figure II.1a). The results showed that *S. aureus* cells became more susceptible to Lys11-mediated lysis as the R8K concentration increased (Figure II.1b). When cells were pre-treated with 1 µM R8K, which corresponded to its minimal inhibitory concentration (MIC) in our experimental conditions, 50 nM of Lys11 were sufficient to clear the culture after about 10 min of contact (time point 20 min in Figure II.1b, ~ 90% cell lysis according to OD_{600nm} measurements). Although at the MIC the R8K peptide caused no visible lysis in absence of Lys11 (Figure II.1b), determination of colony forming units (CFU) revealed between 1 to 1.5 log reduction of cell viability resulting solely from the AMP action (Supplementary Figure SII.1). By using the PMF-sensitive probe $DiSC_3(5)$, we have confirmed that R8K at its MIC caused abrupt membrane depolarization (Supplementary Figure SII.2), which should be responsible for the observed CFU reduction. The determination of CFU counts for the range of R8K concentrations tested suggested a positive

correlation between the bactericidal effect of R8K and its capacity to sensitize cells to Lys11 lytic activity (see below).

The peptide R8K clearly enhanced the bacteriolytic effect of Lys11, but we wondered if the combined action of the two agents translated into a bactericidal gain when compared to the AMP alone. Cells pre-treated with 1 µM R8K were challenged with different Lys11 concentrations (from 12.5 to 500 nM) and cell lysis and viability monitored as before. Cultures were rapidly and massively lysed in presence of R8K and Lys11 (Figure II.2a), even at the lowest enzyme concentration (12.5 nM), whereas the endolysin alone essentially reproduced the absent to moderate lysis observed in Figure II.2a. In fact, when the AMP was present, no obvious dose response to Lys11 could be observed unless the endolysin concentration was progressively lowered from 12.5 to 0.78 nM (~ 0.04 µg/ml), which still could lyse at least 90% of the cultures within 60 min (Supplementary Figure SII.3). The results indicated that R8K-treated *S. aureus* cells were efficiently lysed with minute amounts of Lys11. Reversing the order of R8K and Lys11 additions or treating cultures with both agents at the same time essentially produced the same results, apart from a slight and expected delay on the onset of lysis (due to the time R8K needed to exert its action) and a minor decrease of the lysis rate, especially for the combinations with the lower Lys11 concentrations (Supplementary Figure SII.4).

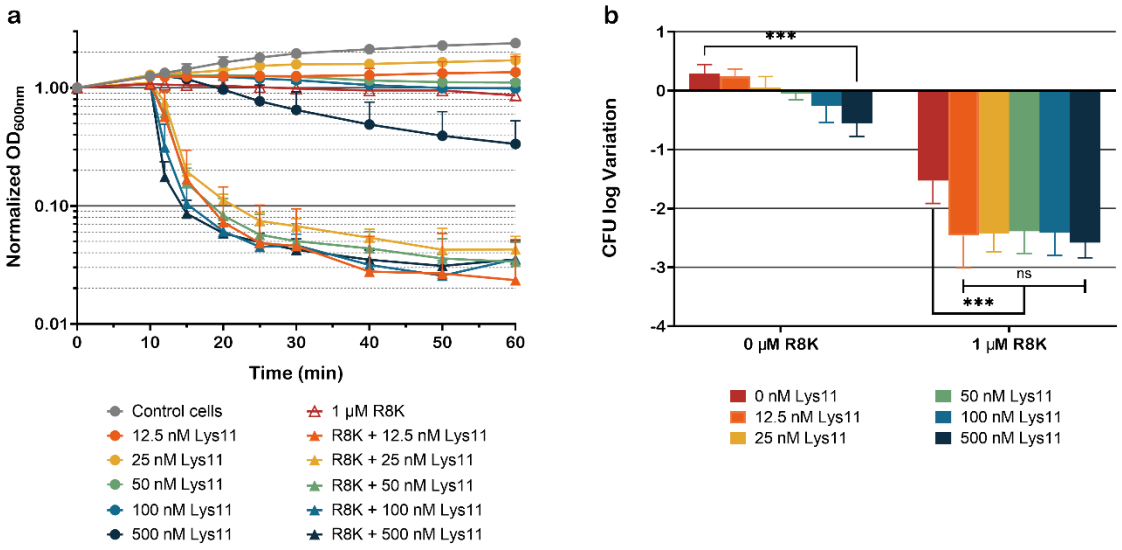

Figure II.2. The enhanced Lys11-mediated bacteriolysis in presence of R8K results in a bactericidal gain.
(**a**) Log phase cells of strain RN4220 collected in TSBca were treated for 10 min with 1 µM R8K or with the peptide solvent. After this treatment, the indicated concentrations of Lys11 were added to the samples and cell lysis evaluated by OD_{600nm} measurements. AMP solvent and endolysin buffer were added to the Control cells. (**b**) Cell viability at time point 60 min of panel (a) was evaluated by CFU counts. For each condition, the results are represented as the log variation of CFU/ml relatively to the cell input. The data of each condition represent means ± standard deviation from at least 3 independent experiments. Asterisks indicate a significant difference of CFU log variations according to one-way ANOVA, followed by Bonferroni post hoc test (***$P < 0.001$). For clarity, only mean + standard deviation is represented in (a).

Cell viability for all conditions at time point 60 min (Figure II.2a) was assessed by CFU counts. As observed before, the peptide reduced cell viability by almost 1.5 log units (Figure II.2b), whereas the endolysin could only produce a discernible impact on cell viability at the highest concentration (500 nM, ~ 0.5 log reduction). In presence of the two agents, CFU counts were reduced by up to 2.5 log units, and basically independently of Lys11 concentration, which agreed with the observed dose independence in lysis at the tested concentrations. Overall, the combination R8K/Lys11 produced a gain in cell death of 1 and 2 log units compared to the isolated action of the AMP and endolysin, respectively, which suggested a synergistic effect resulting from the joint action of the two agents. This AMP effect in sensitizing bacteria to endolysin attack was similarly verified for other endolysin/AMP combinations (Supplementary Figure SII.5) using the endolysin LysK[29] and the AMP vAMP 059, the latter of which was previously shown to kill *S. aureus* due to its membrane-targeting properties[30].

II.2.2. AMP R8K Sensitizes MRSA Strains to Endolysin Attack

The experiments from the previous section were carried out with the laboratory *S. aureus* strain RN4220. Therefore, we wanted to check if similar results were obtained with clinically relevant MRSA strains. To that end, the bacteriolytic and bactericidal assays described above were carried out with 6 MRSA stains representative of 6 clonal complexes[31], using 1 μM R8K and/or 100 nM Lys11. The results confirmed none or very weak lysis as result of the individual action of the two agents, except for strain USA200 that showed lysis with either agent, although higher with the endolysin (Figure II.3). In agreement with the previous results, R8K-treated cultures of these strains were completely cleared in 10 min or less after endolysin addition, apart from strain HGSA146 that took ~ 10 additional minutes to reach an OD_{600nm} reduction of at least 90%. Remarkably, enumeration of cell counts at time point 60 min showed that the bactericidal gain resulting from the combined action of the two agents was substantially higher for some of the clinical strains, with cell death improved by ~ 1.5 log units in strains GRE14 and HGSA146 and up to 3 log units in strains MW2 and USA200, when compared to the R8K induced lethality (Figure II.4a). Overall, the results demonstrated the synergistic bacteriolytic and bactericidal action of the two agents against MRSA strains.

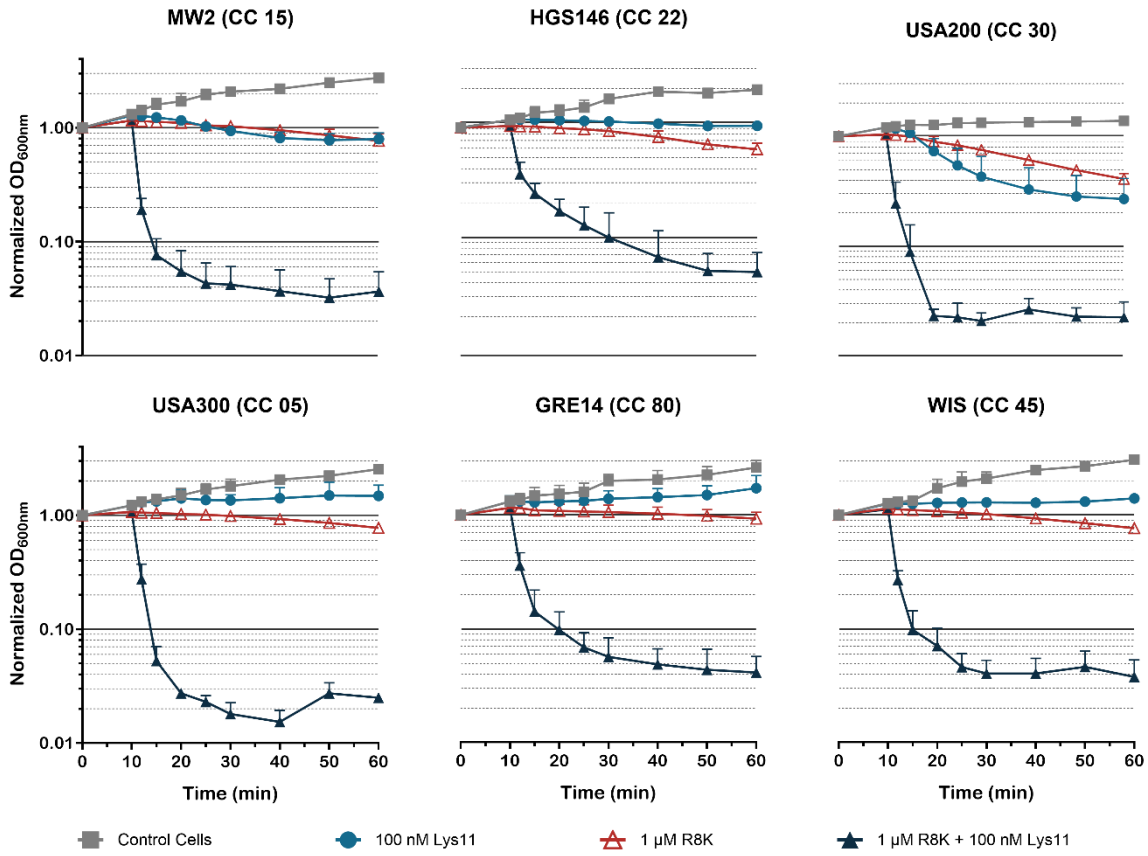

Figure II.3. The AMP R8K makes MRSA strains highly susceptible to Lys11-mediated lysis.
Log phase cells of the indicated MRSA strains were collected in TSBca and treated for 10 min with 1 µM R8K or with the peptide solvent. After this treatment, 100 nM of Lys11 were added to samples and cell lysis evaluated by OD_{600nm} measurements. AMP solvent and endolysin buffer were added to the Control cells. Each curve represents means ± standard deviation from at least 3 independent experiments. For clarity, only mean + standard deviation is represented. Strain designations are indicated above each graph. CC, clonal complex.

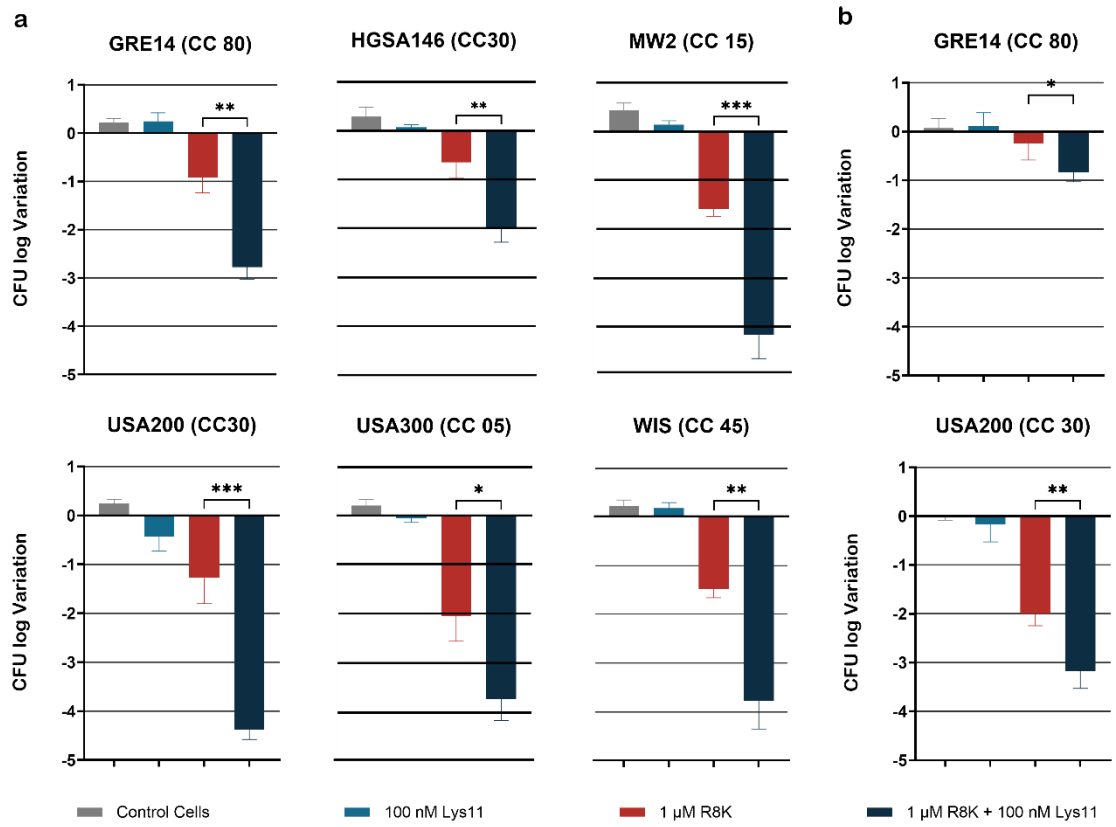

Figure II.4. The combined action of R8K and Lys11 results in enhanced bactericidal action against MRSA strains.

(**a**) Cell viability of MRSA strains at timepoint 60 min of *Figure II.3 was* evaluated for each condition by CFU counts. (**b**) The bactericidal effect of the agents was similarly evaluated against cells of strains GRE14 and USA200 in human blood serum (cell input of ~ 1×10^6 CFU/ml). The results are represented as the log variation of CFU/ml relatively to the cell input. The data represent means ± standard deviation from at least 3 independent experiments. Asterisks indicate a significant difference of the CFU log reduction between the conditions R8K and R8K + Lys11 within each strain, according to independent samples t-Tests (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

AMPs and endolysins may be rapidly degraded, eliminated or have their activity inhibited in complex biological environments, like those found in the human body. Particularly, several AMPs were shown to be susceptible to the presence of salts and/or components present in the human blood serum[32]. The bactericidal action of R8K and Lys11 was assayed individually and in combination in human blood serum against log phase cells of the clinical MRSA strains GRE14 and USA200. These strains were selected because they showed one of the lowest and highest bactericidal gains as result of the combined R8K/Lys11 action in culture medium (~ 1.5 and ~ 3 log units, respectively, Figure II.4a). The assay conditions were as in the experiments of Figure II.3 and Figure II.4a, except that target cells were lowered to a more clinically meaningful concentration (~ 1×10^6 instead of ~ 1×10^8 CFU/ml). In the serum, the endolysin at 100 nM caused none or minor reduction of the CFU counts of GRE14 and USA200, respectively (Figure II.4b), in agreement with its action in culture medium (Figure II.4a). Strain USA200, however, showed to be much more

susceptible to the AMP in these conditions than GRE14 (2 versus 0.2 log reduction, respectively, Figure II.4b). The joint action of the two agents resulted in a bactericidal gain of ~ 1 and ~ 0.5 log units against USA200 and GRE14, respectively. Thus, the bactericidal gain in the serum for the two strains was about threefold lower than that observed in the culture medium. In conclusion, although the combined action of the agents appeared not as effective as in the TSBca, overall, the results in human blood serum were in line with those observed in the bacterial culture medium for the two strains.

II.2.3. *Staphylococcus aureus* Cells Treated with R8K Retain Their Cellular Morphology

Motivated by the results described above, we set to investigate the mechanisms by which R8K facilitated Lys11 lytic action. Under certain conditions, some AMPs, especially at or above the MIC, can induce major damage to the bacterial cell envelope, as observed for some SMAP-29 congeners (reviewed in Ref.[28]). Such action on the cell envelope could explain the R8K effect in making the *S. aureus* CW much more vulnerable to Lys11 attack. Although our previous assays indicated that no substantial cell lysis resulted from the isolated action of R8K at the MIC (Figure II.1b, Figure II.2a), we wanted to check if the AMP was causing major changes in the cell surface and shape of staphylococcal cells. *S. aureus* cells were treated or not with R8K, in the same conditions as the previous experiments, and visualized by ultra-resolution scanning electron microscopy (SEM). We could not identify any obvious changes regarding cell shape and cell surface integrity when comparing untreated with R8K-treated cells (Figure II.5). Hence, there was no indication that the observed R8K/Lys11 synergistic effect could be related with major R8K-induced CW damages.

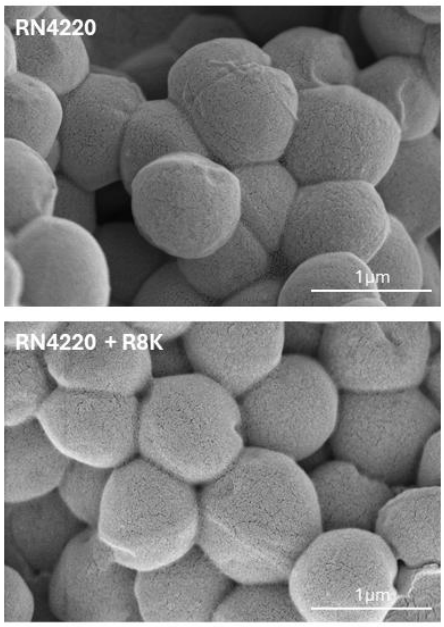

***Figure II.5. The AMP R8K does not change the overall morphology and cell surface features of* S. aureus.**
Log phase cells of strain RN4220 collected in TSBca were treated for 10 min with 1 µM R8K (bottom panel) or with the AMP solvent (top panel) and then visualized by Scanning Electron Microscopy. Scale bar corresponds to 1 µm.

II.2.4. Peptide R8K Enhances Lys11 Binding to *S. aureus* Cells without Affecting the Pattern of Endolysin Distribution on the Cellular Surface

Some studies have linked endolysin tolerance to a deficient binding of the lytic enzymes to cells, caused by WTA (see Section II.1). Therefore, we wondered if the R8K action promoted an increase of Lys11 binding to *S. aureus* cells, which could favor the enzyme's lytic activity. To test this, we have fused the enhanced green fluorescent protein (eGFP) to a non-lytic form of the endolysin and quantified the amount of fusion protein bound to untreated and R8K-treated cells.

Lys11 carries two catalytic domains, CHAP and Amidase_2, followed by a cell binding domain of the SH3_5 family (Supplementary Figure SII.6a). The CHAP peptidase domain was shown to account for almost the entire peptidoglycan-degrading activity of the endolysin *in vitro*[33]. The Amidase_2 domain of the highly related endolysin LysSA12 was also reported to contribute negligibly to CW lytic activity but to greatly enhance endolysin binding to *S. aureus* cells[34]. We have observed a similar role of the Amidase_2 domain in Lys11 binding to cells (data not shown) and have therefore constructed the fusion eGFP-Amidase_2-SH3_5. We confirmed that the fusion, for simplicity henceforth designated eGFP-Ami_{11}-CBD_{11}, had no lytic activity in our assay conditions, both in absence and presence of the AMP R8K (Supplementary Figure SII.6b).

As in the experiments of Figure II.2, *S. aureus* cells were treated or not with 1 µM R8K. Next, eGFP-Ami_{11}-CBD_{11} was added to cells at different concentrations (12.5 to 100 nM). After 10 min

contact, cells were washed to remove free eGFP-Ami$_{11}$-CBD$_{11}$ and the amount of protein associated to the cell population estimated from fluorescent measurements (see II.4.7 in Material and methods for details). The results showed that eGFP-Ami$_{11}$-CBD$_{11}$ binding to R8K-treated cells was favored for all tested concentrations, with the amount of bound protein being three- to four-fold higher when compared to untreated cells (Figure II.6). Cells from the conditions 12.5 nM eGFP-Ami$_{11}$-CBD$_{11}$ with and without R8K were also visualized by fluorescence microscopy and the intensity of the fluorescent signal associated to cells quantified. This approach also indicated higher binding of eGFP-Ami$_{11}$-CBD$_{11}$ to R8K-treated cells, with the fluorescence signal associated with *S. aureus* cells being 1.6 ± 0.2-fold higher when compared to the condition without R8K.

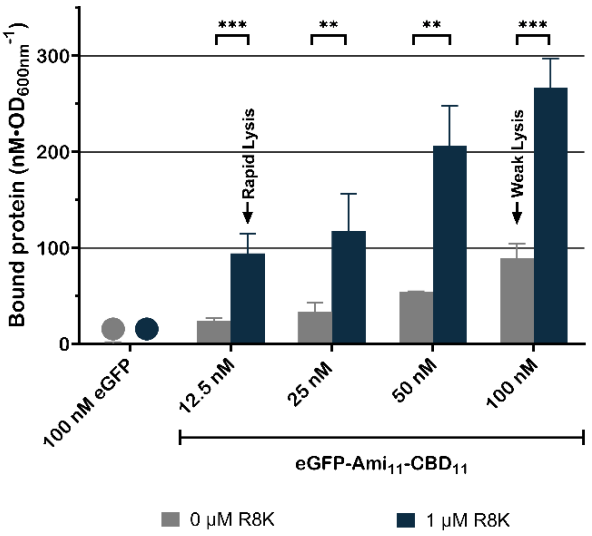

Figure II.6. Endolysin binding to* S. aureus *cells is enhanced upon R8K treatment.
Log phase cells of strain RN4220 collected in TSBca were treated for 10 min with 1 µM R8K or with the peptide solvent. Following this treatment, the indicated concentrations of eGFP-Ami$_{11}$-CBD$_{11}$ were added to the samples and further incubated for 10 min. Cells were washed to discard unbound fluorescent protein and the amount of eGFP-Ami$_{11}$-CBD$_{11}$ associated to cells quantified by fluorometry (data of each sample corrected for the final OD_{600nm} see "II.4.7 in Materials and methods" for details). As a control of unspecific binding, a similar assay was run in parallel with 100 nM eGFP, which consistently originated very low values of cell-associated fluorescence that were incompatible with reliable quantification (noted with circles). For each tested eGFP-Ami$_{11}$-CBD$_{11}$ concentration, the amount of bound protein to R8K-treated cells was three- to four-fold higher. The arrows highlight two conditions showing similar amounts of eGFP-Ami$_{11}$-CBD$_{11}$ bound to cells, which produced very different lysis phenotypes in a Lys11 equivalent context (see Figure II.2a). The data represent means ± standard deviation from at least 3 independent experiments. Asterisks indicate a significant difference between the amount of eGFP-Ami$_{11}$-CBD$_{11}$ bound to untreated cells (0 µM R8K) and to R8K-treated cells, for each protein concentration, according to independent samples t-Tests (**P < 0.01; ***P < 0.001).

In these assays we used eGFP-Ami$_{11}$-CBD$_{11}$ as a proxy of Lys11 binding to cells. We should note though that besides lacking lytic activity, the mass of the fluorescent fusion was about 10 kDa higher than that of the native endolysin. These differences could affect the capacity of eGFP-Ami$_{11}$-CBD$_{11}$ to penetrate through the CW and should be taken into account. Despite this, it is worth noticing that

in absence of R8K the amount of bound protein after incubation with 100 nM eGFP-Ami_{11}-CBD_{11} was not significantly different from that observed after incubation of R8K-treated cells with 12.5 nM eGFP-Ami_{11}-CBD_{11} (arrows in Figure II.6). Yet, with R8K + 12.5 nM Lys11 we observed rapid cell lysis (orange triangles in Figure II.2a), whereas with 100 nM of the endolysin alone only a very weak lytic effect was produced (light blue circles in Figure II.2a). Thus, it appears that for similar amounts of bound endolysin, very different lysis phenotypes are observed depending on the presence or absence of R8K. Hence, although probably contributing to improve lysis, the increased affinity of the endolysin to R8K-treated cells does not seem sufficient to explain the highly enhanced Lys11 lytic activity in presence of the AMP, suggesting that the AMP stimulates endolysin lytic action by additional means. Therefore, we questioned if R8K could be changing the subcellular localization of Lys11, by allowing for example access to CW sites otherwise inaccessible. To answer this question, we have analysed, by super-resolution structured illumination fluorescence microscopy, the pattern of eGFP-Ami_{11}-CBD_{11} localization on the CW of untreated and R8K-treated cells. The analysis revealed that eGFP-Ami_{11}-CBD_{11} distributed around the CW surface, with preferential accumulation at cell junctions, irrespective of the presence or absence of the AMP (Supplementary Figure SII.7). Therefore, the much higher lytic action of Lys11 against R8K-treated cells does not seem to result from an alteration of the endolysin binding pattern to S. *aureus* cells.

II.2.5. *Staphylococcus aureus* Treatment with Antibiotics and Susceptibility to Lys11

We showed that in addition to favoring Lys11 binding to the cells, the AMP R8K also caused cell death, most probably due to its membrane disruption action. In fact, the results suggested that the R8K capacity to sensitize *S. aureus* cells to Lys11 lysis correlated with its bactericidal action (Figure II.1b and Supplementary Figure SII.1). Therefore, we questioned if other agents capable of killing or inhibiting *S. aureus* growth to the same extent as R8K, but exhibiting a different mode of action, would similarly improve Lys11-mediated bacteriolysis. In our assay conditions, treating cultures for 30 min with 50 µg/ml of gentamicin, a bactericidal antibiotic inhibiting protein synthesis, here used at 100 times the reported MIC for strain RN4220[35], resulted in a CFU reduction of 1.23 ± 0.38 log units, which was close to the killing effect of the AMP (~ 1.5 log). Further 50 min incubation reduced cell counts by 3.76 ± 0.09 log units. When *S. aureus* cultures pre-treated 30 min with gentamicin were subjected to the action of 100 nM Lys11 during 50 min, only partial (~ 50% OD_{600nm} reduction) and rather slow lysis was observed when compared to the abrupt and extensive lysis obtained in presence of the AMP (Figure II.7). Therefore, simple cell death cannot reproduce the strong effect of R8K as enhancer of endolysin lytic action.

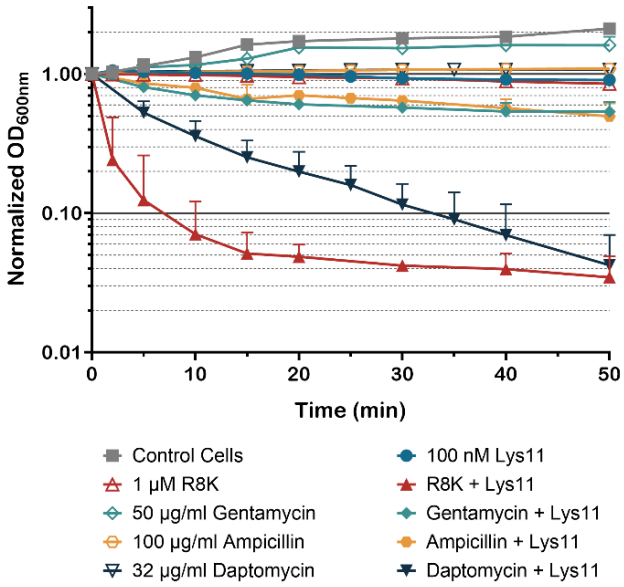

Figure II.7. Endolysin activity against cells treated with antimicrobials with different cellular targets.
Log phase cells of strain RN4220 collected in TSBca were treated with 1 µM R8K, 50 µg/ml gentamicin, 100 µg/ml ampicillin or 32 µg/ml daptomycin (10-min treatment for R8K and 30-min for the antibiotics). Then, 100 nM of Lys11 were added and cell lysis evaluated spectrophotometrically. Antimicrobial solvents and endolysin buffer were added to the Control cells. Each curve represent means ± standard deviation from at least 3 independent experiments. For clarity, only mean + standard deviation is represented.

Some drugs interfering with the cytoplasmic membrane function and its capacity to generate the PMF can also result in CW synthesis impairment[36,37]. To evaluate if a potential inhibitory action of R8K on peptidoglycan synthesis contributed to lysis susceptibility, we have tested the effect of the antibiotic ampicillin, which inhibits CW peptidoglycan synthesis and cell division, on Lys11-meadiated lysis. Under our experimental setting, cell growth halting occurred in less than 30 min with the addition of 100 µg/ml ampicillin (data not shown), which corresponded to about 128 times the reported ampicillin MIC for strain RN4220[38]. Thirty minutes incubation with ampicillin produced only a slight reduction of cell viability (0.23 ± 0.17 log units). Additional incubation until timepoint 80 min (mimicking the 30 min antibiotic incubation plus the 50 min of incubation with the lysin) caused a 0.78 ± 0.18 log reduction of CFU counts. As observed for gentamicin, the addition of Lys11 30 min after ampicillin treatment produced only modest and delayed lysis (Figure II.7), indicating that specific inhibition of peptidoglycan synthesis cannot make cells vulnerable to Lys11 lysis to the extent that R8K does.

The previous results supported that PMF-dissipation promoted by R8K was important to *S. aureus* sensitization to Lys11. Thus, we wondered if an antibiotic sharing this mode of action would be able to enhance the endolysin lytic effect. One such antibiotic is daptomycin[39], which at 32 times its MIC (32 µg/ml) reduced RN4220 CFU counts by 1.43 ± 0.72 and 3.09 ± 0.63 log units, after 30- and 80-min contact, respectively. In contrast to what we observed with the previous antibiotics, the 30 min pre-treatment with daptomycin clearly made strain RN4220 much more vulnerable to Lys11

lytic action, although not as efficiently as with R8K (Figure II.7). Interestingly, the lower capacity of daptomycin in sensitizing cells to Lys11-mediated lysis seemed to correlate with a much slower and less extensive membrane depolarization produced by the antibiotic (Supplementary Figure SII.2). Overall, the results supported the notion that PMF dissipation is a key event to increase *S. aureus* susceptibility to Lys11, and that agents provoking fast and strong membrane depolarization are the best sensitizers.

II.2.6. WTA are Involved in *S. aureus* Tolerance to Lys11

One aspect we started to explore in this work was the identification of cellular determinants of tolerance to endolysins. WTA were previously implicated in tolerance based on their capacity to hamper the binding of some lytic enzymes to target bacteria[17-19], but for some endolysins they were also shown to work as the ligands required for specific recognition and binding to the CW[20]. We have therefore studied the impact of WTA on *S. aureus* susceptibility to Lys11 lytic action and on endolysin binding to cells. WTA synthesis can be severely inhibited in *S. aureus* with low concentrations of tunicamycin, without significantly affecting peptidoglycan synthesis and cell growth. For several *S. aureus* strains, including the laboratory strain RN4220 used here, this specific effect can be obtained with as low as 50 ng/ml of tunicamycin[40,41].

Cells grown in presence of tunicamycin for three generations were challenged with different concentrations of Lys11 (12.5 to 100 nM) to evaluate their susceptibility to lysis. Tunicamycin-treated cells revealed to be significantly more susceptible to Lys11 bacteriolysis, with the highest endolysin concentration (100 nM) decreasing 90% of the culture OD_{600nm} in less than 10 min, and the lowest (12.5 nM) in about 40 min (time points 10 and 40 min in Figure II.8a).

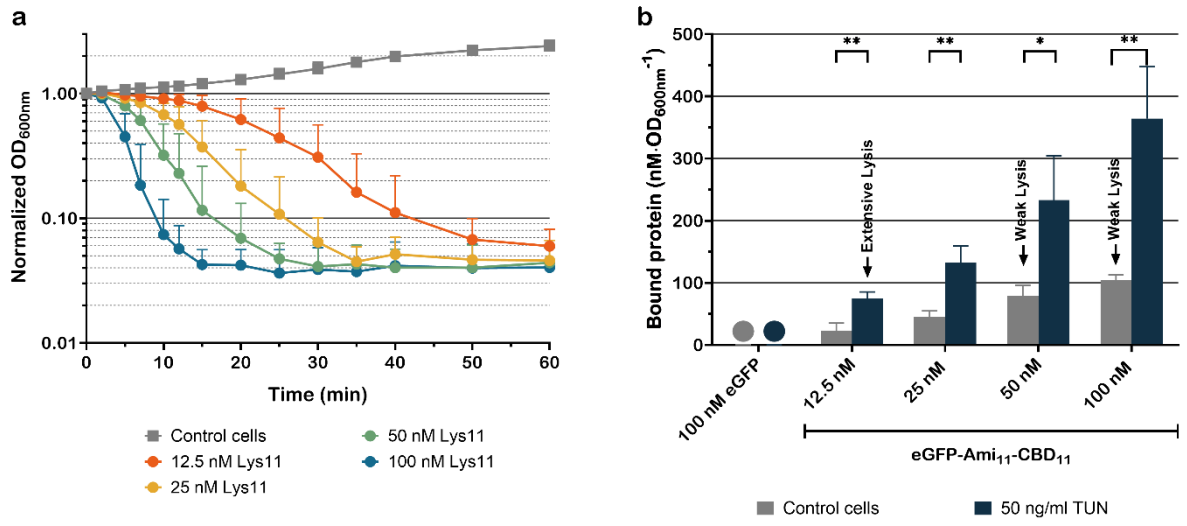

Figure II.8. Endolysin binding and lytic activity are enhanced against S. aureus *cells grown in presence of tunicamycin.*

(**a**) Log phase cells of strain RN4220 grown in presence of 50 ng/ml tunicamycin were collected in TSBca and then incubated with the indicated concentrations of Lys11 or endolysin buffer ("Control cells" curve), and lysis monitored by OD_{600nm} measurements. (**b**) Log phase cells of strain RN4220 grown in absence or presence of tunicamycin (TUN) were collected in TSBca, incubated for 10 min with the indicated concentrations of eGFP-Ami_{11}-CBD_{11}, and the amount of fluorescent protein bound to cells quantified as in Figure II.6. As for the assays of Figure II.6, we could not measure any significant binding of eGFP to cells (noted with circles). For each tested eGFP-Ami_{11}-CBD_{11} concentration, the amount of protein bound to tunicamycin-treated cells was 3 to 3.5-fold higher. The arrows highlight conditions showing similar amounts eGFP-Ami_{11}-CBD_{11} bound to cells, but which produced very different lysis phenotypes in a Lys11 equivalent context (see panel a and Figure II.1a). The data represent means ± standard deviation from at least 3 independent experiments. Asterisks indicate a significant difference between the amount of eGFP-Ami_{11}-CBD_{11} bound to cells grown in absence (0 ng/ml) or presence of tunicamycin (TUN), for each protein concentration, according to independent samples t-Tests (*$P < 0.05$; **$P < 0.01$). For clarity, only mean + standard deviation is represented in (a).

To assess the effect on Lys11 binding, cells grown in the presence of tunicamycin were brought into contact with the same concentration range of eGFP-Ami_{11}-CBD_{11} as in Figure II.6. The fluorescent protein bound 3 to 3.5-fold more effectively to cells grown in presence of tunicamycin (Figure II.8b). Yet, for the same reason explained above, this increased binding seems not to be sufficient to explain the higher bacteriolytic activity of Lys11 towards cells lacking WTA, since for similar amounts of bound protein (arrows in Figure II.8b), obvious lysis is only observed with tunicamycin-treated cells (compare orange curve in Figure II.8a with green and light blue curves in Figure II.1a). Hence, WTA role in endolysin tolerance should go beyond the simple shielding effect in binding. Also, for reasons that are still unknown, we conclude that WTA cannot fulfill their inhibitory role when cells are treated with R8K.

II.3. Discussion

The use of endolysins as antibacterials is based on the idea that the lytic enzymes can efficiently lyse and kill bacteria when added from outside if access to the CW is granted. However, recent studies have shown that this "lysis from without", nicely revealed for a Gram-positive pathogen about 20 years ago[42], may be hampered under certain physiological and growth conditions of target bacteria. The general observation is that energized bacteria actively growing in rich media may tolerate endolysins attacking from without, with the extent of tolerance depending on the endolysin/bacterium pair. Considering the possibility that this capacity of bacteria to fightback the attack of lytic enzymes may negatively impact their performance in a therapeutic context[21], it is crucial to understand the tolerance mechanisms, and how to overcome them, to maximize endolysins enzybiotic potential.

The first studies describing endolysin tolerance recognized a relationship between the PMF and bacterial susceptibility to the lytic enzymes[10-12]. Such direct or indirect role of the PMF in controlling endolysin tolerance is not surprising, if we consider that in the phage lytic cycle endolysins only act

after the abrupt, holin-mediated collapse of the PMF[8]. In addition, it has long been established the role of the PMF in the regulation of autolysins[13,14], which are peptidoglycan-cleaving enzymes structurally and functionally related to endolysins. Therefore, endolysins might also be subjected to the same PMF-dependent mechanisms that control autolysins activity in the bacterial CW. All these observations prompted us to search for fast acting PMF dissipaters with the potential to be used as enhancers of endolysin lytic action in clinical contexts. One obvious possibility were AMPs.

In this work we show that when cultures of *S. aureus* suffer the action of AMPs, endolysins can produce much faster and/or extensive cell lysis (Figure II.2a and Supplementary Figure SII.5). In the assay conditions, Lys11 at the maximum concentration (500 nM) had only a modest impact on cell viability (~ 0.5 log reduction), while the MIC of R8K killed between 1 to 1.5 log units. When acting together, the two agents produced a lethality gain of 1 to 3 log units relative to the AMP alone, depending on the tested *S. aureus* strain (including MRSA). Therefore, although the drastic effect of the AMP in stimulating endolysin-mediated bacteriolysis was essentially transversal to all tested stains, the bactericidal gain resulting from the combined action of the two agents was more variable. Such variation may reflect differences between strains regarding the composition, structure and/or modifications of their cell envelope, which potentially could impact AMP and endolysin activities. The R8K MIC showed minor variation among the strains used in this work, ranging between 1 μM (strains RN4220, HGS146, MW2, USA200 and USA300) and 2 μM (GRE14 and WIS). Thus, the relative susceptibility to R8K does not seem to explain the distinct susceptibility of the strains to the R8K/Lys11 combined action (Figure II.4). It will be interesting to study in *in vivo* models of infection if the AMP/endolysin co-treatment results in therapeutic advantage when compared to the isolated action of the two antibacterials.

Different peptides, including AMPs like SMAP-29, have been fused to endolysins targeting Gram-negative bacteria. With this modification, the endolysins (called Artilysins) gained the capacity to cross the outer membrane of Gram-negative bacteria (reviewed in Ref.[43]). Inspired by this work, Rodríguez-Rubio et al. showed that fusing a polycationic peptide to the endolysin λSa2lys increased its anti-streptococcal activity, probably by improving the enzyme's affinity to the cell surface[44]. Based on the results of this work that showed high bacteriolytic synergy between AMPs and endolysins, we questioned if the fusion of AMPs to endolysins would result in more potent anti-staphylococcal enzybiotics. Hence, we have genetically fused the peptides R8K and vAMP 059 to the C-terminus of both Lys11 and LysK endolysins, generating molecules named AMPLys. Additionally, we fused to the same endolysins the peptide [$K^{2,7,13}$]-SMAP-29(1-17)[45], which for simplicity is hereafter designated as Smap. This peptide is also a derivative of SMAP-29 and, like R8K, it was reported to retain antibacterial activity with lower mammalian cell toxicity, when compared to the native AMP[45]. Smap could be advantageous over R8K due to its smaller size and more positive character. Therefore, 6 anti-staphylococcal AMPLys were successfully cloned, produced and partially purified (Supplementary Table SII.1 and Supplementary Figure SII.8). The bacteriolytic activity of these

AMPLys was compared to that of parental endolysins first by spotting equal molar amounts of the proteins on a dense lawn containing *S. aureus* RN4220 viable cells (Supplementary Figure SII.9a,b). All fusions showed lytic activity against *S. aureus* cells in the spot assay, either with cells incorporated in culture medium or in HEPES buffer. However, the AMPLys exhibited reduced activity when compared with the parental endolysins. The bacteriolytic activity of the anti-staphylococcal AMPLys fusions was also tested against target bacteria in liquid culture medium (Supplementary Figure SII.9c,d). The results confirmed that none of the AMPLys fusions could outperform the bacteriolytic activity of the parental endolysins. In fact, the attachment of an AMP to the endolysins resulted in marked reduction, or even complete abolishment, of enzyme lytic activity in liquid medium. We have generated other AMPLys fusions by changing the AMP position (N- *versus* C- terminal), by varying the length and flexibility of the linker connecting the AMP moiety to the endolysin, and by employing other endolysins. As before, none of the generated AMPLys could show improved bacteriolytic action (Pinto and Gouveia et al, unpublished). Therefore, we could not reproduce the AMP/endolysin bacteriolytic synergy when the two agents were combined in a single molecule (see Chapter IV).

Other studies have explored synergisms resulting from the simultaneous action of CW lytic enzymes and agents damaging the cytoplasmic membrane. Desbois and Coote[46] showed that lysostaphin was synergistically bactericidal in combination with the lantibiotic nisin and lipopeptide antibiotics (colistin, daptomycin and polymyxin B). It was also found that the combination of endolysins with daptomycin, a PMF dissipator antibiotic[39], resulted in better antibacterial activity against *S. aureus* and *Streptococcus pneumonia*, *in vitro* and *in vivo*, when compared to the agents isolated action[47-49]. The authors proposed that the peptidoglycan-degrading activity of the endolysins could be promoting fastest and more efficient membrane insertion of daptomycin. Considering the results presented here, it is also possible that the PMF-depolarizing action of the antibiotic has contributed to endolysins lytic activity. Interestingly, endolysins were also shown to synergize with antibiotics having distinct modes of action, like those inhibiting the CW and protein synthesis[50,51]. Such synergy though was not obvious for the tested antibiotics in our assay conditions, considering their poor stimulatory effect on Lys11 lytic activity (Figure II.7). In any case, the described examples support that synergism between endolysins and other agents deserve further exploration as a source of new antibacterial strategies.

Two key questions that still require further elucidation are: (i) what are the determinants and mechanisms responsible for tolerance, and (ii) what are the exact mechanisms by which AMPs abolish tolerance and strongly stimulate endolysin lytic activity. In agreement with previous reports involving endolysins and autolysins[17-19,29], we verified that WTA were important for *S. aureus* tolerance to Lys11. Based on the observation that in rich media WTA can hinder the binding of peptidoglycan-degrading enzymes to bacterial cells, namely to *S. aureus*, it was proposed that tolerance relied on a WTA-dependent shielding of the CW[17-19]. We have also observed such inhibitory role of WTA on Lys11 binding. However, our data suggest that this shielding effect explains only

partially the function of this CW polymer in the control of lytic enzymes (Figure II.8b). It is possible that in absence of WTA endolysins can not only bind more, but also get deeper into the CW matrix. WTA have been also implicated in the localization and regulation of the peptidoglycan synthetic machinery, influencing for example the level of peptidoglycan cross-linking[52]. Perturbation of these processes due to the lack of WTA could also turn cells more susceptible to endolysins attack.

As stated above, PMF collapse in many Gram-positive bacteria, including *S. aureus*, activates autolysins[13]. A link has been proposed between the ΔpH component of the PMF and WTA in the control of autolytic activity, in which the WTA capacity to retain protons would create an acidic environment in the CW that inhibited autolysins[14]. In this scenario, the PMF-dissipating action of R8K would also cause "deprotonation" of WTA, eventually creating a less acidic and more favourable milieux for Lys11 lytic activity. We can also raise the hypothesis of a destabilization of WTA resulting from the interaction between R8K and WTA. This interaction is likely to occur if we consider the AMP positive charge (between +8 and +9 at culture medium pH) and the anionic nature of WTA[53]. In fact, interaction of cationic AMPs with anionic polymers of the cell envelope, like WTA, is frequently a key step for the subsequent AMP insertion in the cytoplasmic membrane[54]. In any event, it was interesting to note that R8K retained the capacity to stimulate Lys11-mediated lysis of *S. aureus* lacking WTA (Supplementary Figure SII.10), suggesting that the AMP action does not depend on the presence of WTA in the CW.

Interfering with WTA and/or peptidoglycan synthesis with peptides or conventional antibiotics can promote susceptibility to lysis, namely by deregulation of the endogenous autolytic enzymes[55,56]. However, such possible effect resulting from R8K action does not seem to be contributing significantly to lysis in our assay conditions, given the modest effect of ampicillin in sensitizing cells to Lys11 lytic activity (Figure II.7). In any case, our results indicate that all the discussed potential pathways by which the PMF and WTA could be contributing to endolysin tolerance, somehow become non-functional or ineffective upon *S. aureus* treatment with the AMP R8K.

Based on the exposed above and in our results, we tend to believe that the AMP role in abolishment of endolysin tolerance is a multifactorial process, which has as key event the PMF dissipation. In addition to promoting endolysin binding to cells, R8K-mediated collapse of the PMF will certainly change the CW ionic environment, the level of protonation of WTA and their function, and eventually cause deregulation of peptidoglycan synthesis. All these events could collectively compromise the cell capacity to cope with lytic enzymes. Although our SEM analysis did not reveal any major damage in the *S. aureus* cell surface, one cannot exclude the possibility that the AMP also inflicts undetected vulnerabilities that facilitate endolysin access deeper in the CW peptidoglycan mesh. Further studies will be important to elucidate the endolysin tolerance mechanisms and why they suddenly collapse upon AMP action.

II.4. Materials and Methods

II.4.1. Bacterial Strains and Growth Conditions

Unless stated otherwise, *Escherichia coli* strains were routinely grown at 37 °C in LB medium, under aerated conditions. The plasmid expressing eGFP-Ami$_{11}$-CBD$_{11}$ was recovered in *E. coli* strain XL1-Blue MRF' (Stratagene). *E. coli* strain CG61, a BL21 derivative that produces phage T7 RNA polymerase upon thermal induction[57], was used to overexpress Lys11, eGFP and eGFP-Ami$_{11}$-CBD$_{11}$, whereas strain BL21-Gold(DE3) was used to overproduce LysK[29]. For selection of plasmid-bearing cells, LB medium was supplemented with 100 µg/ml ampicillin and/or 40 µg/ml kanamycin. Specific culture conditions for protein expression are explained below. The *S. aureus* lab strain RN4220[58] and the MRSA clinical strains[31] were grown in tryptic soy broth (TSB) medium at 37 °C under aerated conditions. For impairment of WTA synthesis, TSB was supplemented with 50 ng/ml tunicamycin. To minimize variations on RN4220 growth rates, TSB cultures were initiated by diluting 100-fold standardized frozen inocula, which were prepared as follows. Cells were grown until an optical density at 600 nm (OD_{600nm}) of 0.8, collected by centrifugation, and resuspended in half-volume of fresh TSB supplemented with 16% glycerol. Cell suspensions were stored at −80 °C as 200 µl aliquots.

II.4.2. Expression and Purification of Endolysins

The overexpression and purification of Lys11 was carried out as described before[12], with minor modifications. After overnight growth at 28 °C, *E. coli* CG61 expressing Lys11 was 100-fold diluted in LB medium buffered with 0.1 M phosphate buffer pH 7.2 and supplemented with 0.5 M D-sorbitol. For preparation of this medium, twofold concentrated LB with 1 M D-sorbitol and a 0.2 M sodium phosphate buffer solution were prepared and sterilized separately, and then mixed in a 1:1 ratio to reconstitute the medium. Cultures were grown at 28 °C until OD_{600nm} of about 0.5, after which protein synthesis was induced by incubating cultures for 30 min at 42 °C in a shaking water bath. The water bath temperature was rapidly decreased to 16 °C with ice and cultures further incubated for 14–16 h. Cells were recovered by centrifugation (8,000 *g*, 15 min, 4 °C) and resuspended in 1/50 volume of lysis buffer (50 mM HEPES, 500 mM NaCl, 0.1% triton, 10% glycerol, 1 mM DTT, 50 mM Imidazole, pH 7.0) supplemented with 1× Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche Applied Science), 10 mM $MgCl_2$ and 10 µg/ml DNase I. Cells were disrupted by sonication (Vibra Cell, Sonic Materials) with 7 to 10 bursts of 15 s (amplitude 50%, pulse 5, 20–30 W), with 45 s pauses between bursts. Extracts were maintained on ice during sonication. Crude protein extracts were cleared by centrifugation (30,000 *g*, 30 min, 4 °C) and purification was performed by metal chelate affinity chromatography as described previously[12]. Lys11 pure fractions were exchanged to an imidazole-free buffer (same composition of lysis buffer but without imidazole) using HiTrap™ or

HiPrep 26/10 desalting columns (GE Healthcare). Production and purification of LysK was carried out as for Lys11 except for the following two changes: (i) an *E. coli* BL21-Gold(DE3) derivative harboring pET21a::*lysK*[29] was grown until late exponential phase (OD_{600m} ~ 1). At this stage, protein production was induced with 0.5 mM IPTG and cells incubated in a shaking water bath set to 16 °C for 14–16 h; (ii) lysis buffer composition was 50 mM HEPES, 300 mM NaCl, 30% glycerol, 50 mM imidazole, pH 8.0) with the same supplements. Protein fractions were quantified by the Bradford method (Bio-Rad Laboratories), using bovine serum albumin (BSA) as standard. The enzymes were divided in small aliquots and kept at − 80 °C until use.

II.4.3. Antimicrobial Peptides

The peptide SMAP-29(K^8), here designated R8K, with the amino acid sequence RGLRRLG**K**KIAHGVKKYGPTVLRIIRIAG, corresponds to a derivative of SMAP-29 carrying the single R8K substitution[27]. R8K was supplied by the UPF Peptide Synthesis Facility, Universitat Pompeu Frabra, Barcelona, Spain, with acetylated N-terminus and amidated C-terminus (95% HPLC purity). Peptide vAMP 059, with the sequence INWKKWWQVFYTVV[30], was provided by Miguel Castanho Lab (IMM, Universidade de Lisboa, Lisboa, Portugal) with an amidated C-terminus and a free amine N-terminus (> 95% HPLC purity). Stock solutions of 2 mM were prepared from lyophilized peptides in sterile ultra-pure water and stored at − 20 °C as 20-µl aliquots.

II.4.4. AMPs Minimal Inhibitory Concentration (MIC)

Staphylococcus aureus strain RN4220 was grown until an OD_{600nm} of 0.8. Cultures were then diluted in fresh TSB to a cell density of ~ 1×10^6 colony forming units per milliliter (CFU/ml) and distributed into the wells of a 96-well microtiter plate (50 µl per well). Serial twofold dilutions of the AMPs were prepared in TSB and 50 µl added to cells (100 µl final volume). The plates were incubated at 37 °C for 24 h after which they were observed. The recorded MIC corresponded to the lowest AMP concentration inhibiting *S. aureus* growth. MICs were determined in triplicate for each AMP.

II.4.5. Bacteriolytic, Bactericidal and Membrane Depolarization Assays

Unless stated otherwise, *S. aureus* cells were grown until mid-exponential phase (OD_{600nm} ~ 0.4), collected by centrifugation (6,000 *g*, 7 min), and resuspended in half volume of fresh TSB supplemented with 0.5 mM $CaCl_2$ (TSBca) prewarmed at 37 °C. The final OD_{600nm} of cell suspensions was ~ 0.8, which corresponded to a cell density of ~ 1×10^8 CFU/ml. The ability of endolysins to cause cell lysis, either when acting alone or after the indicated treatments with different agents (AMPs, gentamicin, ampicillin or daptomycin), was evaluated under static conditions at 37 °C. Cells impaired in WTA synthesis were similarly prepared, except that TSB/TSBca was supplemented with

50 ng/ml tunicamycin. Lysis was monitored by taking OD_{600nm} measurements at defined time points after endolysin addition, over a period of up to 60 min. Growth controls were similarly prepared and received the equivalent volumes of the agents' solvents.

The bactericidal activity of the endolysin, alone or in combination with other agents, was assessed in terms of the impact on cell viability, expressed as CFU/ml. CFU counts were determined simultaneously with the bacteriolytic assays, for the indicated time points and conditions, by diluting samples in phosphate-buffered saline (PBS) and plating on tryptic soy agar (TSA) plates. Changes in cell viability were expressed as the log variation of CFU/ml relatively to the initial cell input. The bactericidal activity of the endolysin Lys11, isolated or in combination with the AMP R8K, was also assessed in human blood serum (from human male AB plasma, Sigma-Aldrich, Cat. No. H4522). Target cells at OD_{600nm} ~ 0.8 were prepared as described above and diluted 100-fold in the blood serum (~ 1×10^6 CFU/ml) before challenge with the agents. After 50- or 60-min incubation at 37 °C, CFU counts were determined as described above.

The PMF-dissipation action of R8K and daptomycin was confirmed using the membrane potential-sensitive dye $DiSC_3(5)$ (Sigma-Aldrich, Cat. No. 43608) as described elsewhere[59]. Briefly, cells from mid-exponential phase cultures of *S. aureus* strain RN4220 were collected by centrifugation, washed with assay buffer (5 mM HEPES, 20 mM glucose, pH 7.2), and resuspended in the same buffer supplemented with 100 mM KCl and 0.5 mM $CaCl_2$ to a final OD_{600nm} of 0.05. Cells were incubated for 15 min at 37 °C and then distributed through the wells of a black microtiter plate (Greiner Bio-One, Cat. N. 655076). $DiSC_3(5)$ was added to a final concentration of 0.5 µM and the plates incubated in the dark at 37 °C for 30 min to enable dye uptake by the cells and fluorescence quenching. Fluorescence measurements were made using excitation and emission wavelengths of 622 and 672 nm, respectively, to confirm stable fluorescence readings (Varioskan LUX Multimode, ThermoFisher Scientific). The test agents were then added at the indicated concentrations and fluorescence variation measured for additional 50 min. Controls with free $DiSC_3(5)$ dissolved in supplemented assay buffer were similarly analysed to monitor interferences of the agents with the dye.

II.4.6. Scanning Electron Microscopy

Five milliliter sample cultures of *S. aureus* RN4220 at OD_{600nm} ~ 0.8 in TSBca were obtained as described above and incubated with the peptide R8K at its MIC (1 µM) or with its solvent for 10 min at 37 °C. Cells were pelleted (3,000 *g*, 15 min) and resuspended in 1 ml of 0.1 M sodium cacodylate buffer, pH 7.2. Suspensions were again centrifuged (10,000 *g*, 7 min) and cells fixed by resuspension in fixation solution (0.4% glutaraldehyde, 4% paraformaldehyde, 0.1 M sodium cacodylate buffer) and incubation for 60 min at room temperature. Cells were washed three times with 0.1 M sodium cacodylate buffer and dehydrated by covering the cellular pellets with a graded

ethanol series (50, 70, 90 and 100%, 10 min each). Cells were overlaid with tert-butyl alcohol prewarmed at 30 °C, incubated for 60 min at room temperature, and kept overnight at − 20 °C for complete solidification of the tert-butyl alcohol. Samples were dried in a centrifugal vacuum concentrator for 10 min at 37 °C (Genevac™ miVac Centrifugal Concentrator). The dried samples were then mounted in golden sputtered lamellae and sputtered with gold to achieve a final golden layer of ~ 3 nm thickness (Cressington 108 golden sputter). The lamellae were attached to an aluminum stub using a double face copper tape and observed in a Hitachi SU8010 scanning electron microscope, with a beam acceleration of 1 kV, for a working distance of 1.6 mm in beam deceleration mode, to achieve a final resolution of 1.3 nm.

II.4.7. Construction of the eGFP-Endolysin Fusion and Cell Binding Assays

eGFP-Ami$_{11}$-CBD$_{11}$ gene fusion was assembled by overlap-extension polymerase chain reaction (OE-PCR) using the high-fidelity NZYProof DNA polymerase (NZYTech—Genes & Enzymes) and previously described plasmids for *lys11* and *eGFP* templates[12,60]. The Ami$_{11}$-CBD$_{11}$ moiety corresponded to a C-terminal fragment of Lys11 starting at residue P$_{149}$ (GenBank AAL82281.2) and included the putative linker region connecting the CHAP and Amidase_2 domains. The amplified eGFP-Ami$_{11}$-CBD$_{11}$ coding sequence carried 5′ and 3′ N*col* and X*mal* restriction sites, respectively, which allowed its cloning in the expression vector pIVEX2.3d that produced the fusion with an hexahistidine C-terminal tag (Roche Applied Science). A construct expressing the hexahistidine-tagged eGFP was similarly generated. The recombinant plasmids were confirmed by sequencing and transformed into *E. coli* strain CG61 (see above).

For production of eGFP and eGFP-Ami$_{11}$-CBD$_{11}$, the CG61 derivatives were grown in LB medium at 28 °C until an OD$_{600nm}$ of 0.8, after which protein production was induced by temperature up-shift as described above for Lys11. After induction, cultures were transferred to an incubator at 37 °C and grown for additional 180 min. Cells were recovered by centrifugation and resuspended in 1/50 volume of lysis buffer (50 mM HEPES, 300 mM NaCl, 30% glycerol, 50 mM imidazole, 1 mM DTT, pH 8.0) supplemented as above. Cell disruption, protein purification, quantification and storage were as described for the endolysins.

For cell binding assays, *S. aureus* RN4220 at OD$_{600nm}$ ~ 0.8 in TSBca was prepared as described for the bacteriolytic and bactericidal experiments (see above) and treated or not for 10 min with 1× MIC R8K (1 µM) at 37 °C. Cell samples of 200 µl were then put into contact with the indicated concentrations of eGFP or eGFP-Ami$_{11}$-CBD$_{11}$ and further incubated for 10 min at 37 °C. To remove unbound proteins, samples were centrifuged (6,100 *g*, 7 min, room temperature), the supernatant carefully discarded, and cells washed with 1 ml PBS. Cells were pelleted again (8,500 *g*, 5 min, room temperature) and resuspended in 200 µl fresh PBS. The suspensions were transferred to black microtiter plates (Greiner Bio-One, Cat. No. 655076) and fluorescence measured using an excitation

and emission wavelengths of 488 and 507 nm, respectively (Varioskan LUX Multimode, ThermoFisher Scientific). OD_{600nm} was also registered. For quantification of bound protein ($nM \cdot OD_{600nm}$), standard calibration curves were performed for each fluorescent protein. For that, TSB-grown cells at OD_{600nm} ~ 0.4 were washed with PBS and recovered in half volume of the same buffer (OD_{600nm} ~ 0.8). Known concentrations of the fluorescent proteins were serially diluted in this cell suspension and fluorescence measured as above. This procedure accounted for a possible interference of cells in fluorescence measurements. The same protocol was followed to measure the binding to *S. aureus* RN4220 cells grown in the presence of 50 ng/ml tunicamycin (twofold concentrated in fresh TSBca + 50 ng/ml tunicamycin).

II.4.8. Fluorescence Microscopy

For the Super-resolution Structured Illumination Microscopy (SIM) analysis, a *S. aureus* RN4220 culture at OD_{600nm} ~ 0.8 in TSBca was prepared as described above and then divided into two samples. Each culture was incubated at 37 °C for 10 min with R8K MIC (1 μM) or with the peptide solvent, and then for 10 min with 12.5 nM eGFP-Ami_{11}-CBD_{11} at 37 °C. Unbound protein was removed by centrifugation (6,100 *g*, 7 min, room temperature) and the pellets were carefully washed with 1 ml PBS and resuspended in 20 μl fresh PBS. Cells were placed on a thin layer of 1.2% agarose in PBS mounted on a gene frame and imaged by SIM using an Elyra PS.1 microscope (Zeiss) with a Plan-Apochromat 63 × /1.4 oil DIC M27 objective and a 488 nm laser. Images were captured using a Pco.edge 5.5 camera and reconstructed using ZEN software (black edition, 2012, version 8.1.0.484) based on a structured illumination algorithm.

To quantify the intensity of eGFP-Ami_{11}-CBD_{11} signal bound to RN4220 cells treated or not with the AMP R8K, a *S. aureus* RN4220 culture at OD_{600nm} ~ 0.8 in TSBca was prepared as above and divided in two samples. Each culture was incubated at 37 °C for 10 min with R8K MIC (1 μM) or with the peptide solvent, and then for 10 min with 12.5 nM eGFP-Ami_{11}-CBD_{11} at 37 °C. One of the cultures was also incubated for 5 min with the DNA dye Hoechst (1 μg/ml) during the eGFP-Ami_{11}-CBD_{11} incubation period. The samples were then centrifugated (6,100 *g*, 7 min, room temperature) and the pellets were carefully washed with 1 ml PBS and resuspended in 20 μl fresh PBS. The two samples were then mixed and rapidly placed on a microscope slide covered with a thin layer of 1.2% agarose in PBS and imaged by wide-field fluorescence microscopy using a Zeiss Axio Observer microscope with a Plan-Apochromat 100×/1.4 oil Ph3 objective. Images were acquired with a Retiga R1 CCD camera (QImaging) using Metamorph 7.5 software (Molecular Devices). Four independent experiments were performed, two with R8K-treated cells labeled with Hoechst and the other two with the non-treated culture labeled with Hoechst. The intensity of eGFP-Ami_{11}-CBD_{11} fluorescence signal bound to the *S. aureus* cells was automatically determined using eHooke cell imaging analysis

software[61]. For each experiment, between 1100 to 1500 cells were analysed and the result is presented as the mean ± standard deviation of the 4 independent experiments.

II.4.9. Bioinformatics Analysis

Protein similarity searches and identification of conserved domains were carried out with BLAST and CDD tools, which are resources of the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and with Pfam 34.0 (http://pfam.xfam.org/). Protein secondary structures, disordered regions and domain boundaries were analyzed with PSIPRED 4.0, DISOPRED3 and DomPRED (http://bioinf.cs.ucl.ac.uk/psipred/) for prediction of interdomain linker regions.

II.4.10. Statistical Analysis

All data was obtained from repeated assays, with values representing the mean ± standard deviation from 3 to 5 independent experiments. When indicated, statistical significance was evaluated with One-Way ANOVA, followed by Bonferroni post hoc test, or with independent sample *t*-Test.

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II.6. Supplementary Information

Table SII.1. AMPLys fusions derived from Lys11 and LysK endolysins.

The AMPLys fusions were generated by genetically fusing the AMPs R8K, Smap and vAMP 059 to the C-terminus of endolysins Lys11 and LysK. By employing standard molecular techniques, the *E. coli* codon-optimized coding sequences of the AMPs were genetically fused to the 3' end of the endolysin genes, using the PGGS linker coding sequence between the two elements. A hexahistidine tag was present at the C-terminus of all constructs for immunodetection and affinity purification purposes.

Lysin	AMP[a]	AMP position	Protein ID	Linker sequence between Lysin and AMP	Linker sequence between AMP and His_6 tag
Lys11	R8K	C-terminal	Lys11_R8K	PGGS	PGGGS
	Smap	C-terminal	Lys11_Smap	PGGS	GPGGGS
	vAMP 059	C-terminal	Lys11_vAMP059	PGGS	PGGGS
LysK	R8K	C-terminal	LysK_R8k	PGGS	PGGGS
	Smap	C-terminal	LysK_Smap	PGGS	GPGGGS
	vAMP 059	C-terminal	LysK_vAMP059	PGGS	PGGGS

[a]R8K, RGLRRLGKKIAHGVKKYGPTVLRIIRIAG;
Smap, RKLRRLKRKIAHKVKKY;
vAMP 059, INWKKWWQVFYTVV

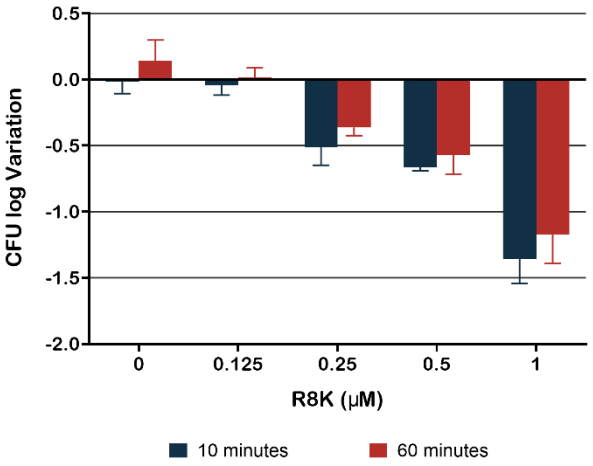

Figure SII.1. Bactericidal activity of the peptide R8K.

Log phase cells of *S. aureus* strain RN4220 were collected in fresh TSBca (~1x10^8 CFU/ml) and incubated at 37 °C for 10 or 60 min with the indicated concentrations of R8K. After the incubation, cell viability was assessed by CFU counts. For each condition, the results are represented as the log variation of CFU/ml relatively to the cell input. The data represent means ± standard deviation from at least 3 independent experiments.

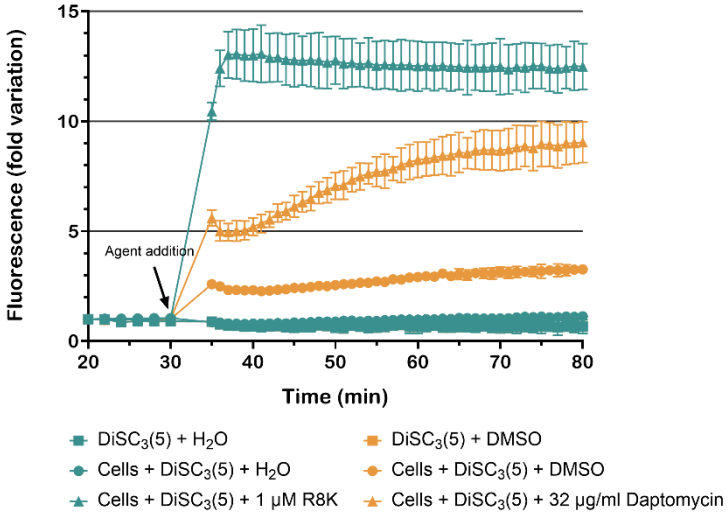

Figure SII.2. R8K causes fast and extensive depolarization of the cytoplasmic membrane.

Cells of *S. aureus* strain RN4220 loaded with the PMF-sensitive probe $DiSC_3(5)$ (see II.4.5 in Materials and Methods) were treated with 1× the MIC of R8K (1 µM) or with 32× the MIC of the membrane-depolarizing antibiotic daptomycin (32 µg/ml). Changes in fluorescence were monitored following the addition of these agents or their solvents (arrow). The effect of the solvents (water and DMSO) on the fluorescence of free $DiSC_3(5)$ was also controlled. The data of each curve represent means ± standard deviation from at least 3 independent experiments.

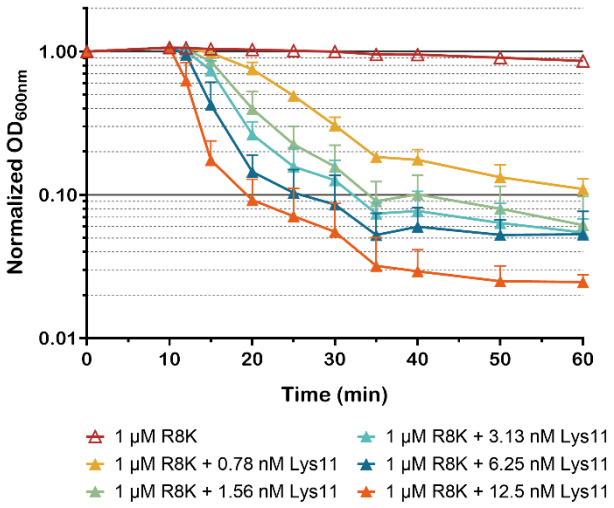

Figure SII.3. Lysis of R8K-treated S. aureus cells in response to decreasing concentrations of Lys11 endolysin.
Log phase cells of strain RN4220 collected in TSBca were treated for 10 min with 1 µM R8K, after which the indicated concentrations of Lys11 were added to samples and cell lysis monitored by OD_{600nm} measurements. The data of each curve represent means ± standard deviation from at least 3 independent experiments. For clarity, only mean + standard deviation is represented.

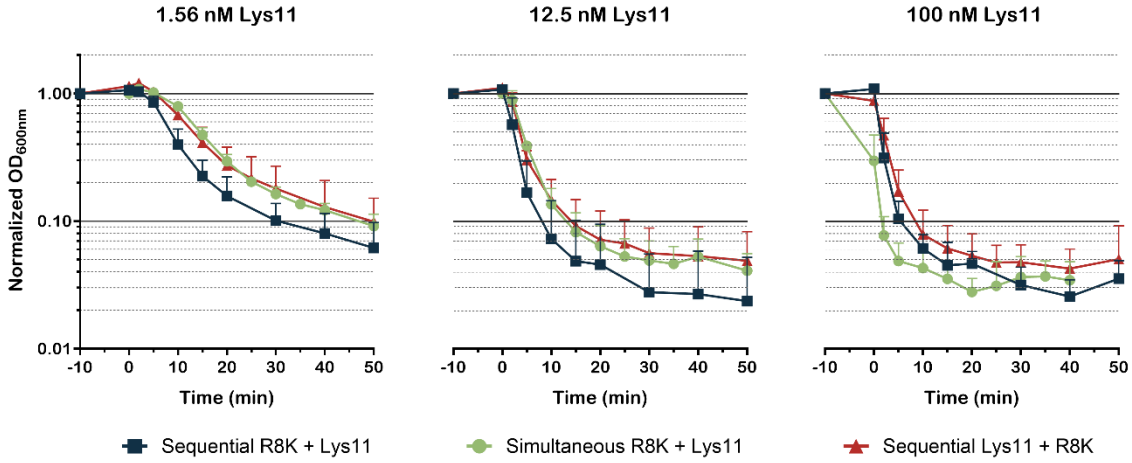

Figure SII.4. Inverse order or simultaneous addition of R8K and Lys11 also results in enhanced bacteriolysis.
Log phase cells of strain RN4220 collected in TSBca were treated sequentially either with 1 µM R8K (10 min) followed by Lys11 (50 min with the indicated concentrations) or with the reverse order (10 min with Lys11 followed by 50 min with 1 µM R8K). In a third condition cells were treated simultaneously with R8K and Lys11 (both added at time 0 min). Cell lysis was monitored by OD_{600nm} measurements in all conditions. The data of each curve represent means ± standard deviation from at least 3 independent experiments. For clarity, only mean + standard deviation is represented.

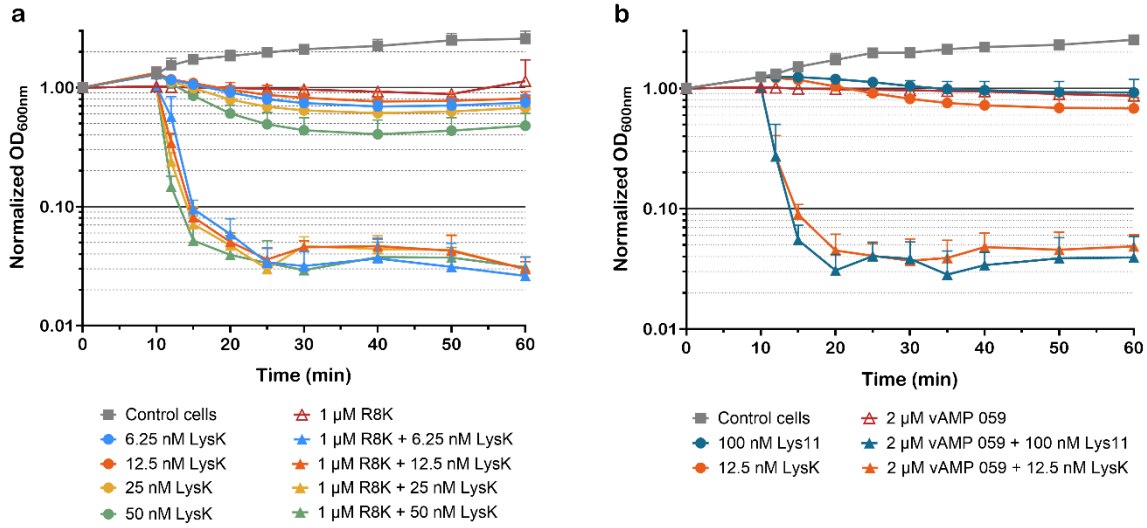

Figure SII.5. Increased endolysin lytic activity in presence of an AMP is observed for different AMP/endolysin combinations.

(**a**) Log phase cells of strain RN4220 collected in TSBca were treated for 10 min with 1 µM R8K or with the AMP solvent. After this treatment, the indicated concentrations of the endolysin LysK were added to samples and cell lysis evaluated spectrophotometrically. AMP solvent and endolysin buffer were added to the Control cells. (**b**) Cells as in (a) were treated for 10 min with 2 µM of the peptide vAMP 059 (MIC) or its solvent. After this period, the indicated concentrations of Lys11 or LysK were added to samples. Cell lysis monitoring and control as in (a). The data of each curve represent means ± standard deviation from at least 3 independent experiments. For clarity, only mean + standard deviation is represented.

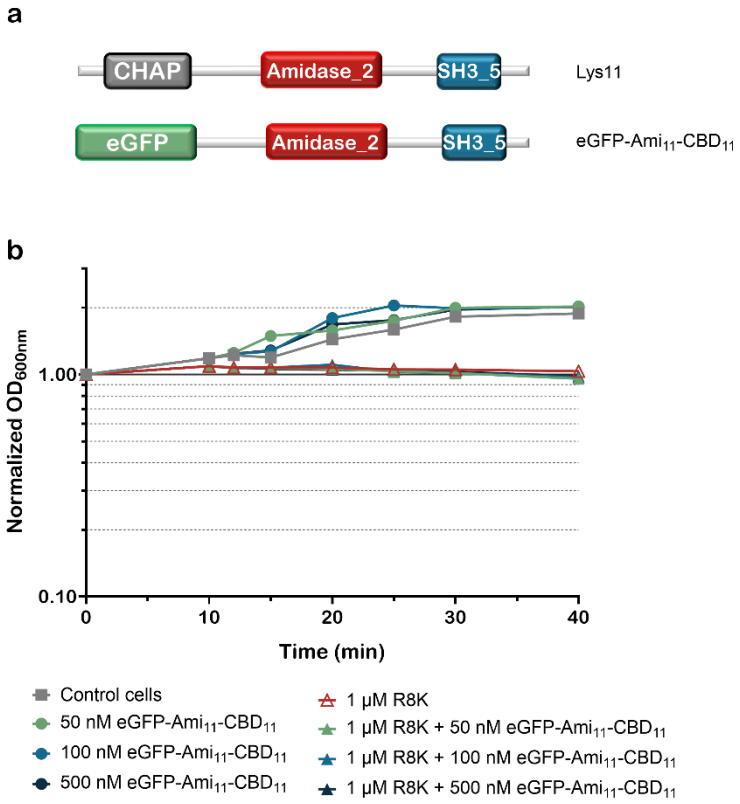

Figure SII.6. Lys11 and the derived fusion eGFP-Ami$_{11}$-CBD$_{11}$.
(**a**) Lys11 displays the following domain architecture from N- to C-terminus (Ref.[33] and Pfam analysis): a CHAP domain (pfam05257) with peptidase activity, an Amidase_2 domain (pfam01510), and a cell binding domain (CBD) of the SH3_5 family (pfam08460). For construction of the fusion eGFP-Ami$_{11}$-CBD$_{11}$, the CHAP domain was substituted by eGFP. (**b**) The fusion eGFP-Ami$_{11}$-CBD$_{11}$ does not show lytic activity. Log phase cells of strain RN4220 collected in TSBca were treated for 10 min with 1 µM R8K or with the peptide solvent. After this treatment, the indicated concentrations of eGFP-Ami$_{11}$-CBD$_{11}$ were added to the cells and lysis was evaluated spectrophotometrically. AMP solvent and endolysin buffer were added to the Control cells. Data of each curve are representative of 2 to 3 assays.

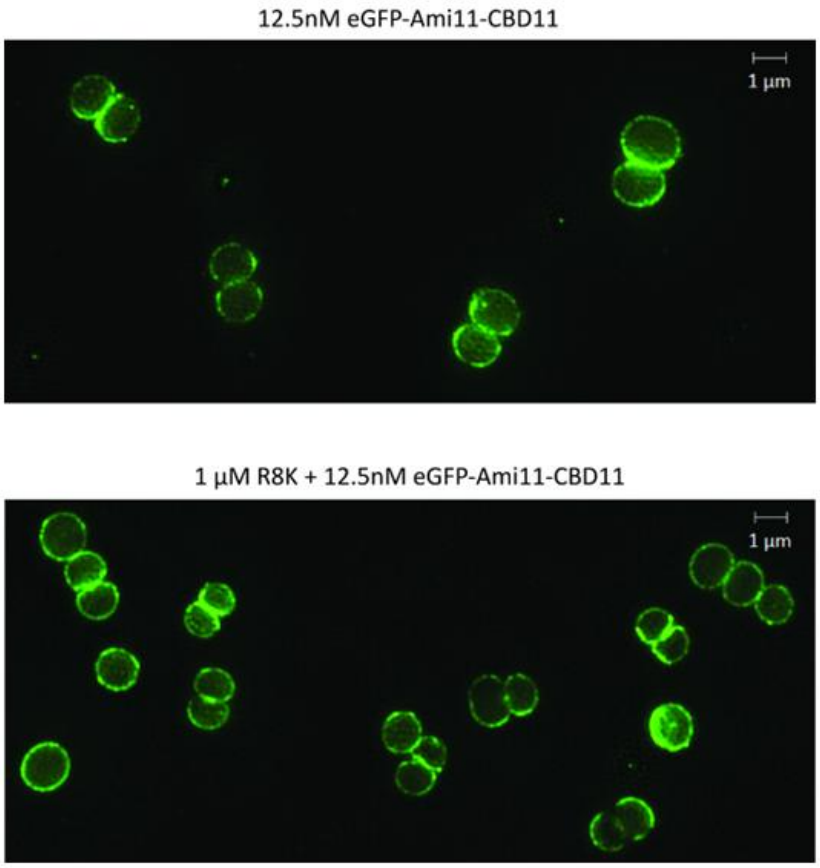

Figure SII.7. Pre-treatment with the AMP R8K does not alter eGFP-Ami$_{11}$-CBD$_{11}$ distribution pattern on S. aureus cell surface.

Super-resolution Structured Illumination Microscopy (SIM) images of eGFP-Ami$_{11}$-CBD$_{11}$ binding to *S. aureus* RN4220 cells treated (bottom panel) or not treated (top panel) for 10 min with 1 µM R8K. Scale bar corresponds to 1 µm.

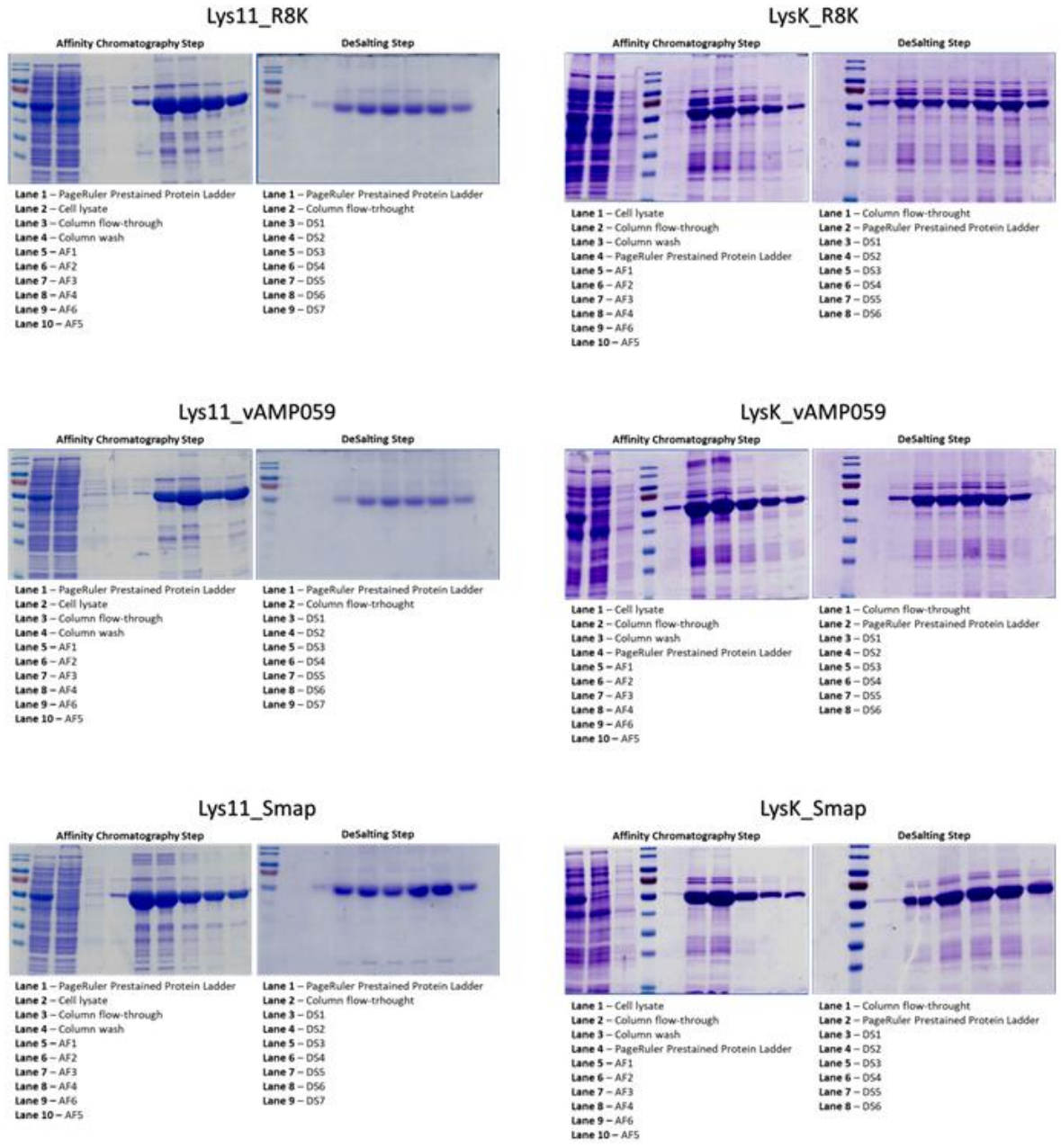

Figure SII.8. SDS-PAGE analysis of anti-staphylococcal AMPLys production and purification steps.
AF(n), Affinity chromatography fractions; DS(n), Desalting fractions (for removal of imidazole).

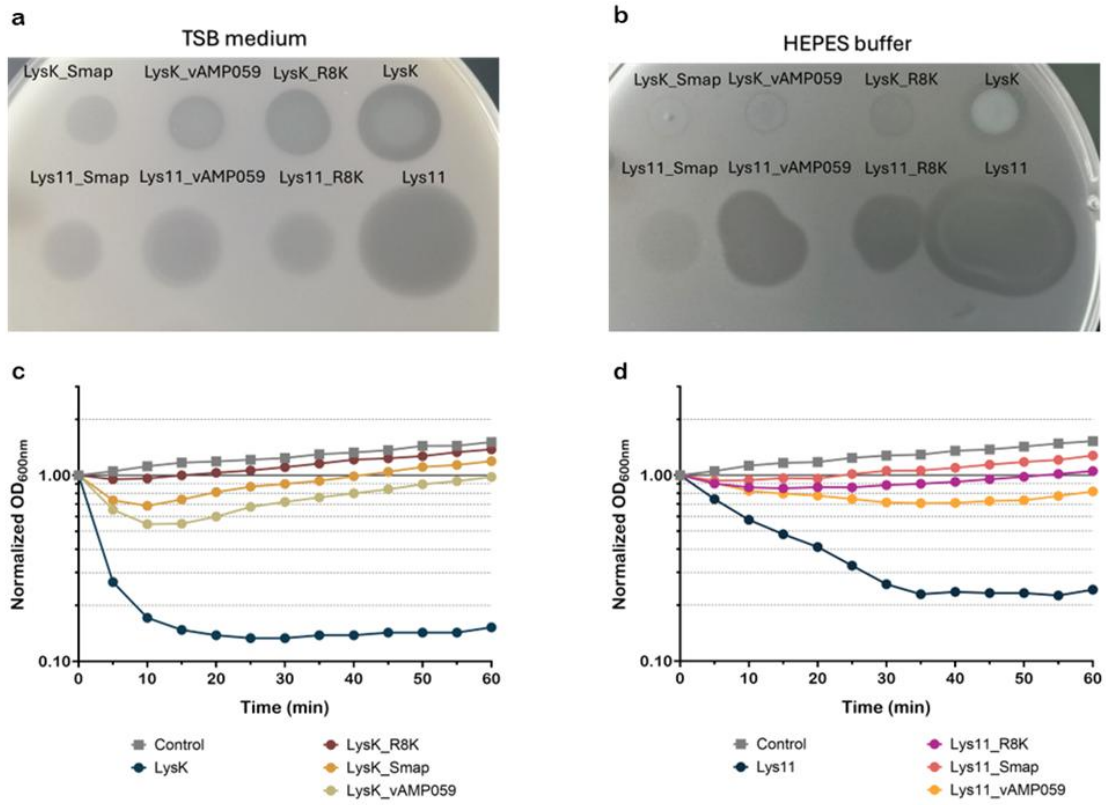

Figure SII.9. Bacteriolytic activity of LysK and Lys11 native endolysins and derived AMPLys fusions.
S. aureus RN4220 cultures were grown until an OD_{600nm} of about 0.8 and cells recovered in 1/100 volumes of either TSBca medium or HEPES-based buffer (50 mM HEPES, 150 mM NaCl, 0.5 mM $CaCl_2$, pH 7.2). A 300 µl-sample of the concentrated cell suspension was incorporated in 10 ml of agarized (0.7 %) TSB (**a**) or HEPES buffer (**b**) and poured into a petri dish to form dense lawns of viable cells. Ten microliters of the lytic agents at 25 µM were spotted on the lawn (250 pmol of each protein per spot) and the formation of lysis halos evaluated after overnight incubation at 37 ºC. Lytic activity of LysK and its AMPLys fusions (**c**) or of Lys11 and its AMPLys derivatives (**d**) was also tested in TSB liquid cultures. Briefly log phase cells of strain RN4220 were collected in TSBca and challenged with 500 nM of each lytic protein. Lysis was monitored by taking OD_{600nm} measurements at regular intervals.

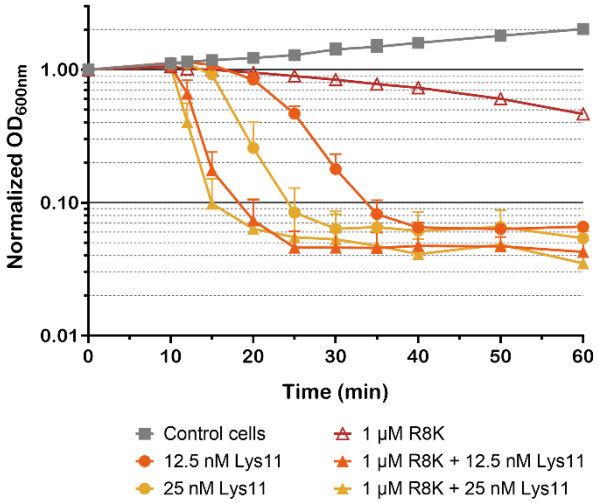

Figure SII.10. R8K stimulates Lys11-mediated lysis of tunicamycin-treated* S. aureus *cells.
Log phase cells of strain RN4220 grown in presence of 50 ng/ml tunicamycin were collected in TSBca and then treated for 10 min with 1 µM R8K or with the peptide solvent. After this treatment, the indicated concentrations of Lys11 were added to the cells and lysis was evaluated spectrophotometrically. AMP solvent and endolysin buffer were added to the Control cells. The data of each curve represent means ± standard deviation from at least 3 independent experiments. For clarity, only mean + standard deviation is represented.

Chapter III.

Cellular and Enzymatic Determinants Impacting the Exolytic Action of an Anti-Staphylococcal Enzybiotic

This chapter contains data published in:

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Cellular and Enzymatic Determinants Impacting the Exolytic Action of an Anti-Staphylococcal Enzybiotic

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Keywords: endolysin; lysin; enzybiotic; peptidoglycan hydrolase; cell wall; antibiotic resistance; membrane potential; proton motive force; wall teichoic acids; staphylococcus aureus

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Author Contributions

Conceptualization, C.S.-J.; methodology, A.G., D.P., J.M.B.V. and C.S.-J.; validation, A.G., D.P. and C.S.-J.; formal analysis, A.G.; investigation, A.G., D.P., J.M.B.V. and C.S.-J.; resources, J.M.B.V. and C.S.-J.; data curation, A.G. and C.S.-J.; writing—original draft preparation, A.G.; writing—review and editing, A.G., D.P., J.M.B.V. and C.S.-J.; visualization, A.G. and C.S.-J.; supervision, C.S.-J.; project administration, C.S.-J.; funding acquisition, C.S.-J. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

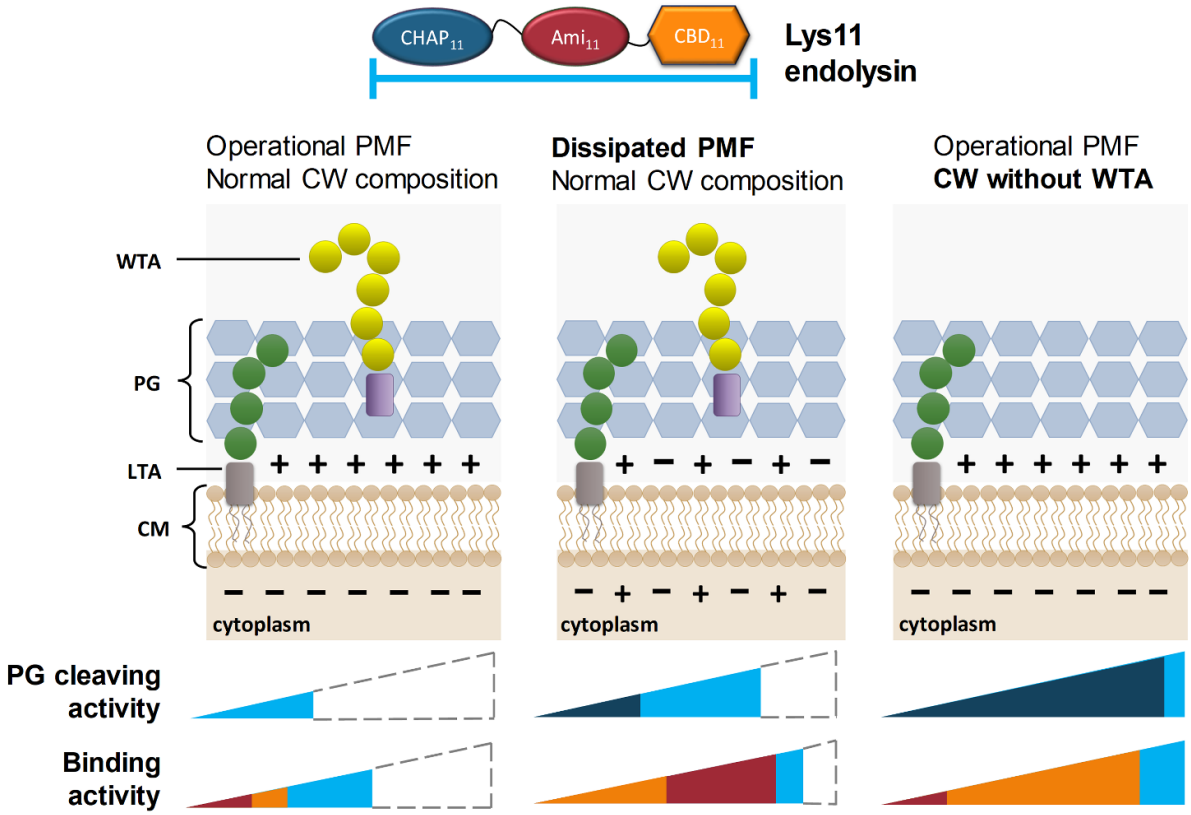
The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abstract

Bacteriophage endolysins are bacteriolytic enzymes that have been explored as potential weapons to fight antibiotic-resistant bacteria. Despite several studies support the application of endolysins as enzybiotics, detailed knowledge on cellular and enzymatic factors affecting their lytic activity is still missing. The bacterial membrane proton motive force (PMF) and certain cell wall glycopolymers of Gram-positive bacteria have been implicated in some tolerance to endolysins. Here, we studied how the anti-staphylococcal endolysin Lys11, a modular enzyme with two catalytic domains (peptidase and amidase) and a cell binding domain (CBD_{11}), responded to changes in the chemical and/or electric gradients of the PMF (ΔpH and Δψ, respectively). We show that simultaneous dissipation of both gradients enhances endolysin binding to cells and lytic activity. The collapse of ΔpH is preponderant in the stimulation of Lys11 lytic action, while the dissipation of Δψ is mainly associated with higher endolysin binding. Interestingly, this binding depends on the amidase domain. The peptidase domain is responsible for most of the Lys11 bacteriolytic activity. Wall teichoic acids (WTA) are confirmed as major determinants of endolysin tolerance, in part by severely hindering CBD_{11} binding activity. In conclusion, the PMF and WTA interfere differently with the endolysin functional domains, affecting both the binding and catalytic efficiencies.

Keywords: endolysin; lysin; enzybiotic; peptidoglycan hydrolase; cell wall; antibiotic resistance; membrane potential; proton motive force; wall teichoic acids; staphylococcus aureus

Graphical Abstract



III.1. Introduction

Antimicrobial resistance (AMR) is a leading cause of death worldwide[1], and its continued rise constitutes a major threat to human health and the global economy[2-4]. As innovation regarding conventional (small-molecule) antibiotics has been mostly relying on the modification of established classes[5], truly alternative antimicrobials are needed, preferentially more pathogen-specific and with new modes of action that minimize resistance development. Among such alternatives are bacteriophage (phage) lytic enzymes, namely endolysins and engineered derivatives[6]. These enzymes destroy the bacterial cell wall (CW) and are viewed as a promising novel class of antimicrobials (enzybiotics)[7,8]. Some have already reached clinical trials[9].

At the end of bacterial infection, phages employ endolysins to degrade a major structural component of the host CW, the peptidoglycan, resulting in osmotic cell lysis for virion progeny release. Another phage-encoded protein instrumental to lysis is the holin, a protein that causes a fatal dissipation of the proton motive force (PMF) by forming "holes" in the host cytoplasmic membrane. The holin channels can have a dual role: to provide a pathway for the passage of the endolysin from the cytoplasm to the CW and/or to activate the lytic action, through PMF dissipation, of endolysins (pre)positioned in the CW compartment[10,11]. The PMF across the cytoplasmic membrane, which is crucial for cell survival, consists of two components: the electrical potential (Δψ) and the proton gradient (ΔpH)[12]. To maintain PMF homeostasis, bacteria can tune the relative contribution of Δψ and ΔpH in response to changes in growth conditions[13,14].

In addition to peptidoglycan, other glycopolymers make part of the CW of Gram-positive bacteria, among which teichoic acids (TA) are perhaps the best-studied ones[15]. These can be covalently linked to the peptidoglycan mesh or be attached to the membrane via a lipidic anchor, being designated wall teichoic acids (WTA) and lipoteichoic acids (LTA), respectively. TA play several functions in bacterial physiology, including protection against antibacterial agents (e.g., bacteriocins, antimicrobial peptides and certain antibiotics) and the control of endogenous enzymes involved in peptidoglycan synthesis, cleavage and cell division. These functions can be modulated by the decorations of the glycopolymers such as glycosylation and the incorporation of D-alanine esters, the latter of which are thought to mask negatively charged sites in TA[16,17].

The capacity of endolysins to cleave the CW peptidoglycan when exogenously added to bacteria is at the basis of their intense exploration as enzybiotics. It is usually considered that Gram-positive bacteria are more susceptible to endolysin attack than Gram-negative and mycobacteria because these have an outer membrane that hinders enzyme access to the peptidoglycan[10,18]. However, it has been observed for different Gram-positive bacteria growing in nutrient-rich environments that they can display different levels of tolerance to endolysins. The mechanisms responsible for this tolerance are still poorly understood, but they were shown to rely on an operational PMF and on the presence of certain CW glycopolymers. Studies have demonstrated that agents that dissipate both

PMF components, such as membrane ionophores, holins and cationic peptides, can render Gram-positive bacteria much more susceptible to endolysin attack[19-21]. In fact, the membrane- and peptidoglycan-acting agents can act synergistically to promote cell killing[22-24]. On the other hand, WTA were shown to greatly restrict the action of lytic enzymes, including endolysins, at least in part by hindering their binding/access to the CW peptidoglycan[24-27].

This study aimed at increasing our knowledge on the determinants of endolysin tolerance, using as a model the endolysin Lys11 that targets an important Gram-positive pathogen in the context of AMR, *Staphylococcus aureus*. Lys11 has one of the most common domain architectures found in staphylococcal endolysins, displaying from N- to C-terminus a CHAP peptidase domain ($CHAP_{11}$), an Amidase domain (Ami_{11}) and an SH3-like cell binding domain (CBD_{11})[28]. We used selective membrane drugs to decompose the relative contribution of each PMF component to tolerance. *S. aureus* mutants were also employed to study if, in addition to the WTA, other CW components and/or modifications played a role in endolysin susceptibility. The impact of these cellular cues was evaluated on the whole enzyme and its individual functional domains, monitoring lytic and cell binding activities. We show that the two PMF gradients produce distinct effects in endolysin functional domains, impacting their binding and peptidoglycan cleavage activities. We also provide a clearer view of WTA as restrictors of endolysin binding.

III.2. Results

III.2.1. The ΔpH Component of the PMF Has Major Contribution to Endolysin Tolerance

As mentioned in Section III.1, the membrane PMF integrates the Δψ and ΔpH components, i.e., the electrical and proton gradients, respectively. Gramicidin, which collapses both gradients by forming channels in the membrane[12,29], is amongst the PMF-dissipating agents previously shown to significantly increase *S. aureus* susceptibility to the lytic action of endolysin Lys11[21]. To understand the relative contribution of each PMF component to endolysin tolerance, *S. aureus* cells in rich culture media were treated with gramicidin, nigericin or valinomycin, before being challenged with Lys11. Nigericin promotes the electroneutral exchange of K^+ for H^+, whereas valinomycin promotes K^+ influx under an external excess of this cation. Hence, the two latter agents were employed as selective ionophores dissipating ΔpH and Δψ, respectively[12,29,30]. We confirmed the expected effect of the three ionophores on the membrane potential and cell viability, using the membrane depolarization sensitive dye $DiSC_3(5)$[24] and by determining colony-forming units per millilitre (CFU/ml), respectively (Supplementary Figure SIII.1).

After 10 min treatment of *S. aureus* with each ionophore, Lys11 was added to cells, and lysis was monitored by following the culture optical density at 600 nm (OD_{600nm}). In agreement with previous observations, gramicidin treatment clearly turned cells more susceptible to Lys11 lysis. For the tested Lys11 concentration (100 nM), culture OD_{600nm} decreased by almost 90% in gramicidin-treated cells, within 40 min, whereas for the same contact time, they were only reduced by ~40% in the presence of the endolysin alone (Figure III.1a). Nigericin also provoked an obvious stimulation of Lys11-mediated lysis, although it was somewhat slower and less extensive compared to gramicidin (Figure III.1b). Valinomycin was less effective in potentiating endolysin lytic action, with cultures lysing at a much slower pace (~60% lysis after 60 min, Figure III.1c). In the assay conditions, the ionophores inhibited cell growth without causing visible cell lysis (Figure III.1).

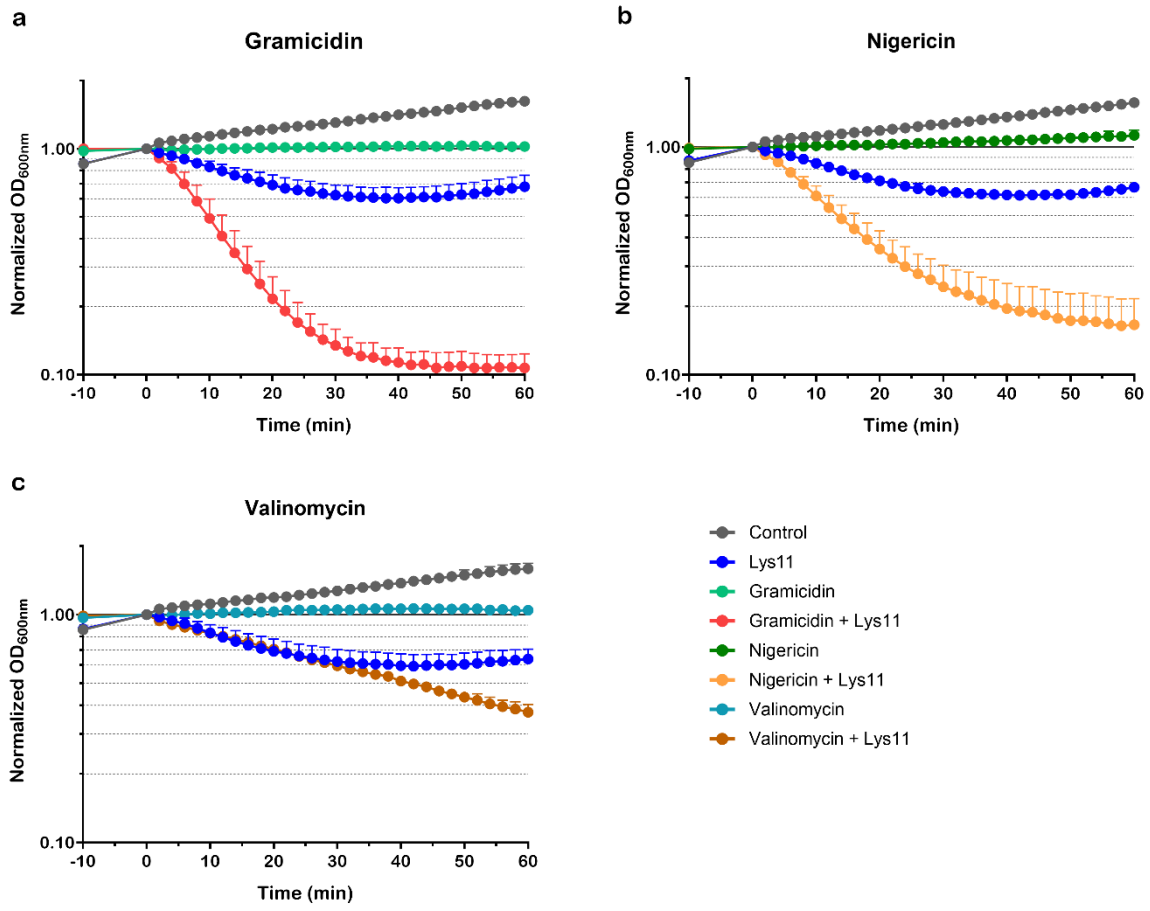


Figure III.1. Bacteriolytic activity of endolysin Lys11 is enhanced upon PMF dissipation.
Log phase cells of *S. aureus* strain RN4220 were collected in TSB medium supplemented with 0.5 mM $CaCl_2$ (TSBca) and treated for 10 min with 30 µg/ml gramicidin (**a**), 10 µM nigericin (**b**) or 20 µM valinomycin (**c**). Cells treated with valinomycin were first supplemented with 200 mM KCl. After ionophore treatment, 100 nM Lys11 was added to cells, and lysis was monitored by following the optical density at 600 nm (OD_{600nm}). Ionophore solvents and endolysin buffer were added to "Control" curves. Endolysin buffer was added to cells treated with the ionophore only. Time points −10 and 0 min indicate the time of ionophore and endolysin addition, respectively. OD_{600nm} values were normalized at t = 0 min. Each curve represents means ± standard

deviation from at least 4 independent experiments. For clarity, only the mean + standard deviation is represented.

Taken together, the results indicated that although maximum endolysin susceptibility is achieved upon dissipating both PMF components, the pH gradient across the membrane seems to have a preponderant role in antagonizing Lys11 lytic action.

III.2.2. Dissipation of Both PMF Components Stimulates Endolysin Binding to Cells

In Section III.2.1, we showed that the dissipation of both PMF components (gramicidin) or of the ΔpH only (nigericin) turned *S. aureus* cells significantly more prone to lysis by Lys11. Since previous studies (see Section III.1) have shown that tolerance to lytic enzymes could be associated with a deficient binding of the proteins to the cell surface, we questioned whether the higher cell susceptibility to Lys11 in the presence of the ionophores correlated with an increased endolysin binding to cells. To address this question, we fused different Lys11 domains to the enhanced green fluorescent protein (eGFP) and measured their binding to *S. aureus* cells treated with the ionophores. The constructed fusions were eGFP-Ami_{11}-CBD_{11}, eGFP-Ami_{11} and eGFP-CBD_{11} (Supplementary File SIII.1 and Figure SIII.2 and Figure SIII.3). Note that although an amidase activity has been attributed to Ami_{11} in studies with purified CW[28], in Lys11 and some related enzymes, the amidase domain was shown to contribute poorly to exolytic activity against intact cells. However, it was found to significantly enhance endolysin binding to the *S. aureus* surface when associated with the cell binding domain[24,31]. Therefore, the binding of eGFP-Ami_{11}-CBD_{11} was assumed here to be a proxy of Lys11 binding, whereas the two other fusions were intended to provide information regarding the relative contribution of Ami_{11} and CBD_{11} to the binding. Since Lys11 derivatives carrying $CHAP_{11}$ as the sole catalytic domain can cause significant cell lysis in certain conditions (see Section III.2.6), measuring by the same approach the possible contribution of this domain to binding was not straightforward, and therefore it was not investigated in this study.

S. aureus cells were exposed or not to the ionophores as described above and then incubated with the eGFP fusions. After removing the unbound protein, the amount of eGFP fusion associated with cells was quantified by fluorimetry as described in Gouveia et al.[24] (see Section III.2.5). The binding of eGFP-Ami_{11}-CBD_{11} was enhanced after collapsing both PMF gradients with gramicidin, resulting in ~2-fold more bound protein when compared to untreated, control cells (Figure III.2a). Selective dissipation of either ΔpH (nigericin) or Δψ (valinomycin) also promoted eGFP-Ami_{11}-CBD_{11} binding to cells, although it was not as pronounced as gramicidin (~1.4-fold increase). The fact that nigericin was not as effective as gramicidin in stimulating endolysin binding could contribute to the lower Lys11 lytic performance in response to nigericin when compared to gramicidin (Figure III.1a,b).

The fusion eGFP-Ami$_{11}$ bound poorly both to control and nigericin-treated cells (Figure III.2b). Remarkably, its binding was drastically increased upon gramicidin and valinomycin treatment (~23- and ~20-fold increase relative to control cells, respectively). Finally, eGFP-CBD$_{11}$ showed moderate binding to control cells (Figure III.2c), which was about 1/3 of that measured with eGFP-Ami$_{11}$-CBD$_{11}$ in the same condition (Figure III.2a). Cells treated with any of the ionophores appeared to register some increase in eGFP-CBD$_{11}$ binding (Figure III.2c), although it was not considered statistically significant probably due to the relatively large standard deviations.

Overall, the results indicated that PMF dissipation favored endolysin binding to cells, which could at least partially explain the higher Lys11 lytic action in these conditions. This increased endolysin binding appears to derive in great part from a stimulation of Ami$_{11}$ binding activity in response to PMF collapse, particularly the elimination of the Δψ component (Figure III.2b). Yet, the isolated Ami$_{11}$ bound poorly to energized cells (Figure III.2b, Control), while it stimulated binding when associated with CBD$_{11}$ (seen when we compare the binding of eGFP-Ami$_{11}$-CBD$_{11}$ and eGFP-CBD$_{11}$ to control cells). This hints at either a cooperative binding of Ami$_{11}$ and CBD$_{11}$ when in the same polypeptide chain or an indirect role of Ami$_{11}$ as an enhancer of the proper CBD$_{11}$ conformation for binding.

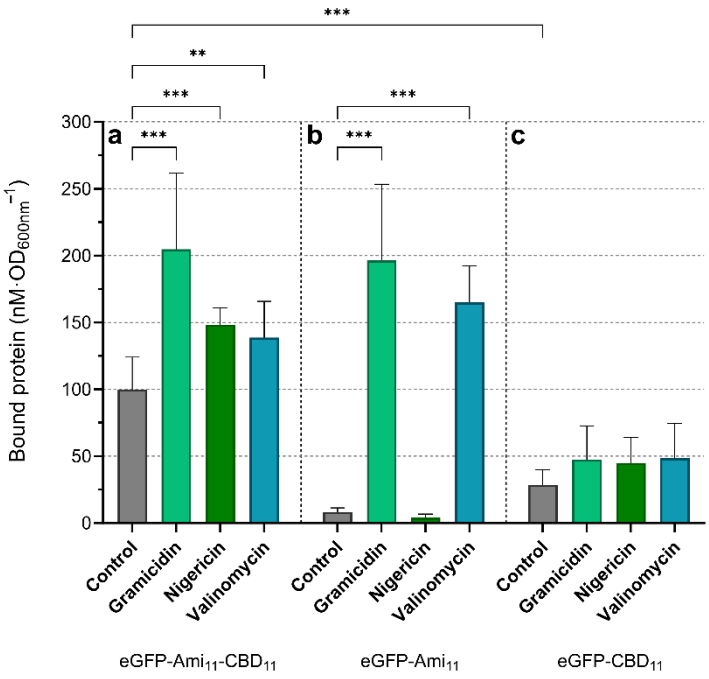


Figure III.2. Lys11 binding to* S. aureus *cells is enhanced by collapsing the PMF.
Cells of strain RN4220 harvested in TSBca were treated with gramicidin, nigericin, valinomycin or ionophore solvent ("Control") as in Figure III.1. Following treatment, 100 nM of eGFP-Ami$_{11}$-CBD$_{11}$ (**a**), eGFP-Ami$_{11}$ (**b**) or eGFP-CBD$_{11}$ (**c**) was added to samples and further incubated for 10 min. After removal of free protein, the amount of eGFP fusions bound to cells was quantified by fluorimetry (see Section III.4.5). The data represent means ± standard deviation from at least 6 independent experiments. Asterisks denote significant differences according to two-way ANOVA test, followed by Tukey post hoc test (***P* < 0.01; ****P* < 0.001).

III.2.3. PMF Dissipation Simultaneously Favours Endolysin Binding and Peptidoglycan Cleavage

The ensemble of results from Sections III.2.1 and III.2.2 suggested that the collapse of both PMF gradients potentiated Lys11 bacteriolytic action in two different ways: by favoring endolysin binding to cells and by stimulating peptidoglycan cleavage. The latter effect was mainly inferred after the dissipation of ΔpH (nigericin), which clearly improved Lys11 bacteriolytic action (Figure III.1b) while causing only a moderate increase in eGFP-Ami_{11}-CBD_{11} binding (Figure III.2a). To further support this dual effect, we devised an experiment in which depolarized and energized cells would bind similar amounts of eGFP-Ami_{11}-CBD_{11}, followed by an evaluation of the corresponding lysis profiles with Lys11. The incubation of untreated cells with 500 nM of eGFP-Ami_{11}-CBD_{11} resulted in amounts of bound protein similar to those obtained after the incubation of gramicidin-treated cells with 100 nM of the fluorescent protein (Figure III.3a). Yet, in the corresponding lysis assays with Lys11, the condition gramicidin plus 100 nM endolysin produced much faster and more extensive cell lysis (Figure III.3b). This was another indication that the enhancement of endolysin binding in response to PMF collapse is not sufficient to explain the highest Lys11 lytic action and that a stimulation of peptidoglycan cleavage should also be occurring. This increased cleavage may result from an effect produced either in the substrate (peptidoglycan) and/or in the enzyme's catalytic activity upon PMF collapse.

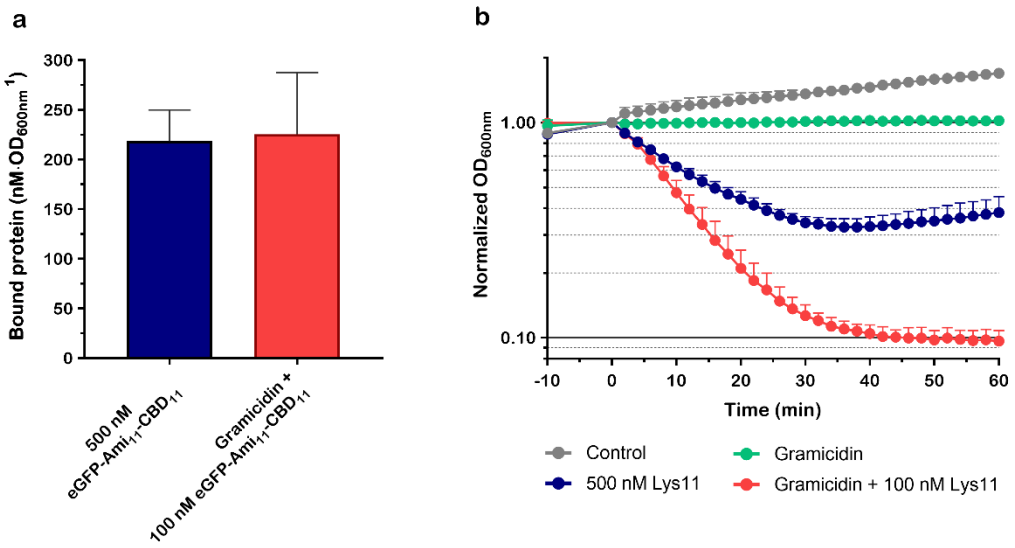


Figure III.3. PMF dissipation enhances Lys11 lysis by simultaneously favouring endolysin binding to cells and peptidoglycan cleavage.

(**a**) Cells of strain RN4220 were untreated or treated with gramicidin and then incubated with 500 and 100 nM of eGFP-Ami_{11}-CBD_{11}, respectively, which resulted in similar amounts of fluorescent protein bound to cells (no significant differences of bound protein according to Student's t-test). The data represent means ± standard deviation from at least 9 independent experiments. (**b**) In the corresponding lysis assay, 500 and 100 nM of Lys11 were added to cells that had been untreated or treated with gramicidin, respectively. Despite

the similar endolysin binding inferred in (**a**), the condition gramicidin + 100 nM Lys11 causes more extensive and faster cell lysis. The data represent means ± standard deviation from at least 5 independent experiments.

III.2.4. WTA is the Key CW Glycopolymer Contributing to Endolysin Tolerance

As referred to in Section III.1, CW glycopolymers and their modifications can impact the action of peptidoglycan-degrading enzymes. Under conditions supporting bacterial growth, WTA were shown to hinder the binding of lytic enzymes to the CW surface[25-27], including Lys11[24]. In this work, we wanted to test if LTA, the other major glycopolymer associated with the *S. aureus* CW, could also play a role in cell susceptibility to Lys11 lysis. In addition, we sought to investigate the possible influence of major TA modifications. For this, we used an *S. aureus* mutant disabled in LTA synthesis (Δ*ltaS*) that is still capable of almost normal growth due to the presence of a suppressor mutation in the GdpP phosphodiesterase[32]. For the TA modifications, we used a double mutant Δ*tarM*Δ*tarS*, which is simultaneously impaired in α-O-GlcNAcylation and β-O-GlcNAcylation of WTA[33], and a Δ*dltA* mutant that cannot perform D-alanylation of TA[34]. All mutants were derivatives of the *S. aureus* strain RN4220 used throughout this work, except the Δ*dltA* mutant that was derived from strain SA113.

By growing cells in the presence of an inhibitor of WTA synthesis (tunicamycin), we confirmed that in our experimental conditions, the *S. aureus* WTA work as an important determinant of Lys11 tolerance[24]. A less than 10 min contact of Lys11 with tunicamycin-grown cells was sufficient to cause more than 90% cell lysis (Figure III.4a). The Δ*ltaS* mutant was constructed in a RN4220Δ*spa* background, which exhibits normal LTA production but lacks protein A (SpA) in the CW surface[32]. The growth of the mutant RN4220Δ*spa*Δ*ltaS* was unaffected in the presence of Lys11 (Figure III.4b), therefore displaying even higher endolysin tolerance than strain RN4220 (Figure III.4a). Curiously, the intermediate strain RN4220Δ*spa* seemed more susceptible to Lys11 attack than RN4220, with culture OD_{600nm} decreasing almost 70% within 40 min (Figure III.4b). This hinted at some antagonizing effect of SpA toward Lys11 lytic action, something we did not explore in this work. Nevertheless, the increased tolerance of the mutant RN4220Δ*spa*Δ*ltaS* to Lys11 was an indication that the *S. aureus* LTA should not work as restrictor of the endolysin bacteriolytic activity.

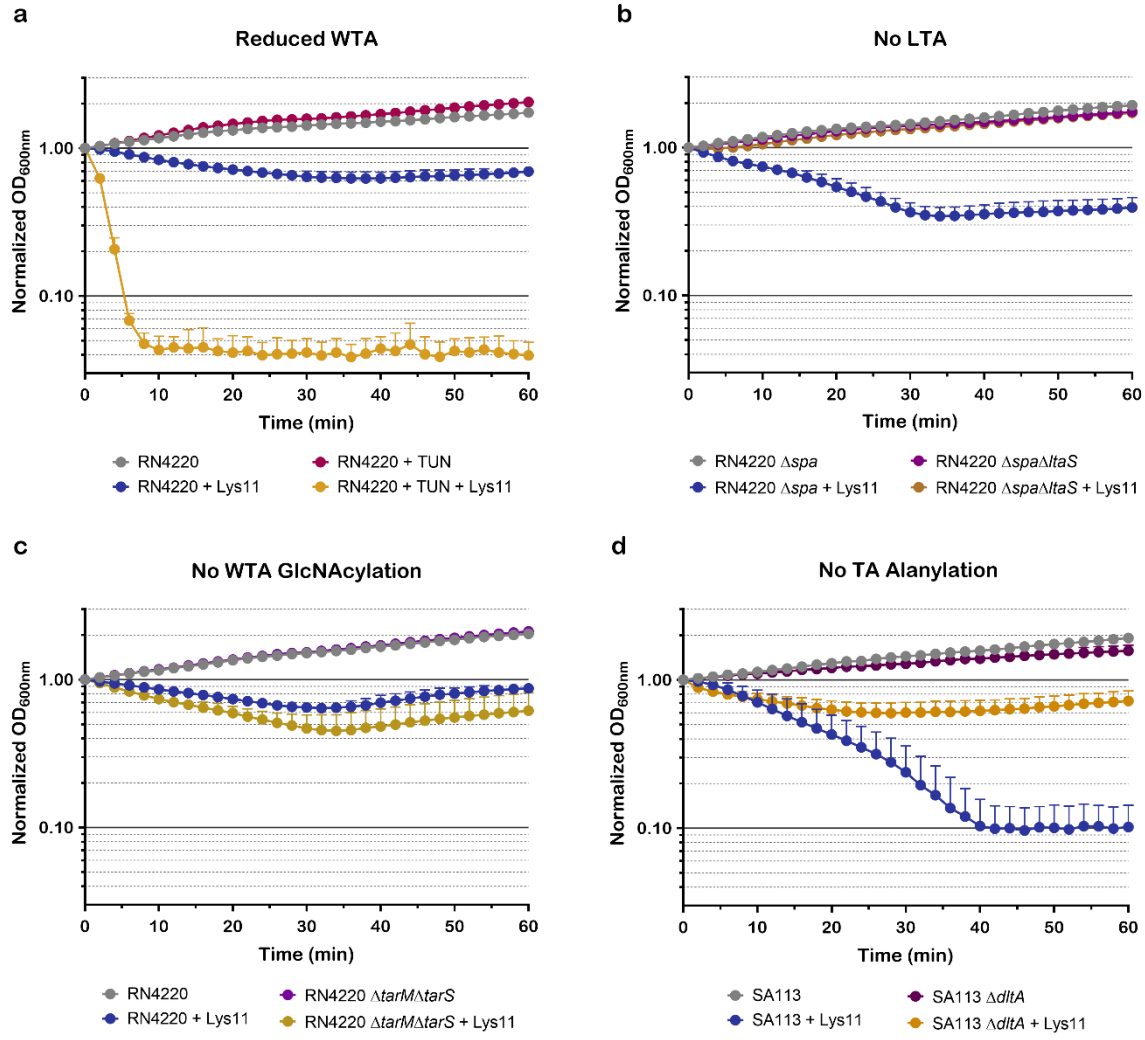


Figure III.4. Contribution of CW glycopolymers and their modifications to Lys11 tolerance.
Log phase cells of the indicated *S. aureus* strains and derived mutants were collected in TSBca and then challenged with 100 nM Lys11. Bacteriolysis was followed by taking OD_{600nm} measurements. (**a**) Cells grown in absence or presence of 50 ng/ml tunicamycin (TUN), which reduces WTA synthesis without affecting growth. (**b**) Mutant Δ*spa*Δ*ltaS* is impaired in LTA synthesis, whereas the control strain Δ*spa* has normal LTA production. (**c**) The mutant Δ*tarM*Δ*tarS* lacks the α- and β-O-GlcNAcylation modification of WTA. (**d**) The Δ*dltA* mutation causes no alanylation of TA. Each curve represents means ± standard deviation from 5 independent experiments. For clarity, only the mean + standard deviation is represented.

Regarding the mutants affected in TA modifications, we found that the lack of α- and β-O-GlcNAcylation of WTA resulted, at most, in a slight increase in *S. aureus* susceptibility to Lys11 lytic action (Figure III.4c), indicating that these substitutions have no major role in the endolysin antagonistic action of WTA. The Δ*dltA* mutant (no TA alanylation) had much lower endolysin susceptibility than the parental strain SA113 (Figure III.4d). Interestingly, SA113 was previously described as a low-WTA *S. aureus* strain[35], which should explain its considerably higher vulnerability to Lys11 lysis compared to strain RN4220. Despite this, the results obtained with the Δ*dltA* mutant

indicated that normal TA D-alanylation favours Lys11 lysis, instead of conferring protection, at least in strain SA113.

Overall, the assays with *S. aureus* cells affected in TA composition confirmed WTA as the major CW glycopolymer contributing to endolysin tolerance.

III.2.5. WTA Drastically Hinders Binding Mediated by CBD_{11}

Next, we studied how WTA interfered with the binding efficiency of the three eGFP fusions used in section III.2.2, by quantifying their association with cells grown in the presence or absence of tunicamycin.

In agreement with previous observations[24], the fusion eGFP-Ami_{11}-CBD_{11} bound approximately three times more efficiently to cells with diminished WTA content (Figure III.5). The binding of eGFP-Ami_{11} was again minimal and not significantly augmented by reducing the WTA level in the CW. Notably, eGFP-CBD_{11} binding to cells with low WTA content was ~7-fold higher than the binding to cells with normal WTA production (Figure III.5). Taken together, the results indicated that CBD_{11} has a dominant role in endolysin binding to cells with low WTA. In agreement with this, the binding of eGFP-Ami_{11}-CBD_{11} to cells with inhibited WTA synthesis was only ~1.4-fold higher than that of eGFP-CBD_{11}.

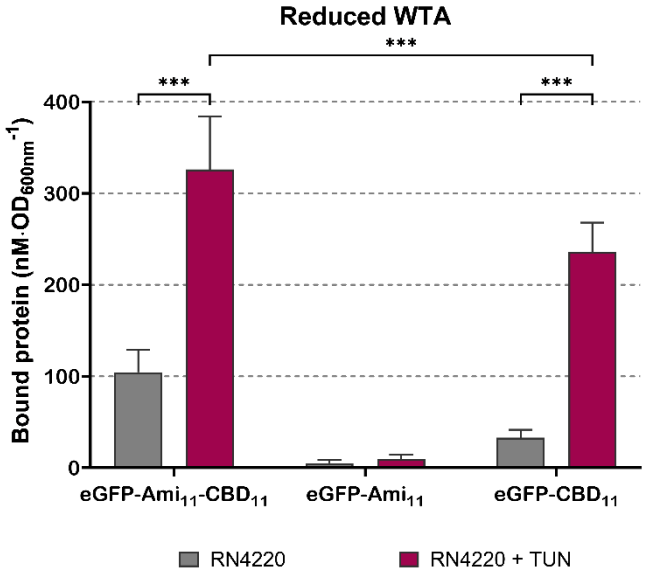


Figure III.5. WTA primarily affects CBD_{11} binding.
Log phase cells of strain RN4220 grown in absence ("RN4220") or presence of 50 ng/mL tunicamycin ("RN4220 + TUN") were collected in TSBca and, after 10 min incubation with the indicated eGFP-endolysin fusions, the amount of fluorescent protein associated with cells was determined as in Figure III.2. The data represent means ± standard deviation from at least 7 independent experiments. Asterisks denote a significant difference, according to two-way ANOVA test, followed by Tukey post hoc test (***$P < 0.001$).

In summary, the results strongly suggested that WTA restricts Lys11 association to the CW mainly by interfering with the CBD_{11} binding activity. Also, the binding auxiliary role of the amidase domain seems to be more relevant for Lys11 association with cells with normal WTA content (Figure III.2a,b).

III.2.6. Lys11 Catalytic Domains Respond Differently to Cell Tolerance Determinants

Finally, we wanted to understand how the Lys11 catalytic domains (CDs), $CHAP_{11}$ and Ami_{11}, responded in terms of lytic activity to the changes in the cell membrane energetic state (using the ionophores) and to the WTA reduction in the CW (achieved with tunicamycin). For that, we produced Lys11 derivatives having each CD fused to the cell binding domain, i.e., $CHAP_{11}$-CBD_{11} and Ami_{11}-CBD_{11} (Supplementary File SIII.1 and Figure SIII.2 and Figure SIII.3).

The lytic action of Lys11 and its CD deletion mutants was again tested at 100 nM. As seen in Section III.2.1, at this concentration, Lys11 stopped *S. aureus* growth and caused some cell lysis. In contrast, the single-CD mutants could not affect culture growth, with the corresponding curves being essentially indistinguishable from that of the control cells (Figure III.6a). To verify that $CHAP_{11}$-CBD_{11} and Ami_{11}-CBD_{11} retained peptidoglycan degrading activity, they were serial diluted and spot-tested on a dense lawn of viable *S. aureus* cells incorporated in a buffered, soft agar matrix (see Section III.4.4). From our experience, this is one of the most sensitive assays to reveal lysin lytic activity. In these conditions, $CHAP_{11}$-CBD_{11} appeared to be just slightly less bacteriolytic than Lys11, as judged by the somewhat clearer lysis halos of the latter (Figure III.6b). In contrast, an obvious Ami_{11}-CBD_{11} lytic effect could only be observed at the two highest spotted concentrations (5 and 2.5 μM). As already noted, a reduced peptidoglycan cleaving activity of the amidase domain of Lys11 and some related endolysins was previously described[28,31,36]. In fact, we have observed some peptidoglycan-cleaving activity for the construct Ami_{11}-CBD_{11} when tested against CW fragments (Supplementary Figure SIII.4), although this is not reflected on intact cells. Thus, the results indicated that the single-CD mutants could not elicit obvious cell lysis of liquid cultures, despite displaying significant ($CHAP_{11}$-CBD_{11}) or residual (Ami_{11}-CBD_{11}) peptidoglycan-degrading activity in the spot assay.

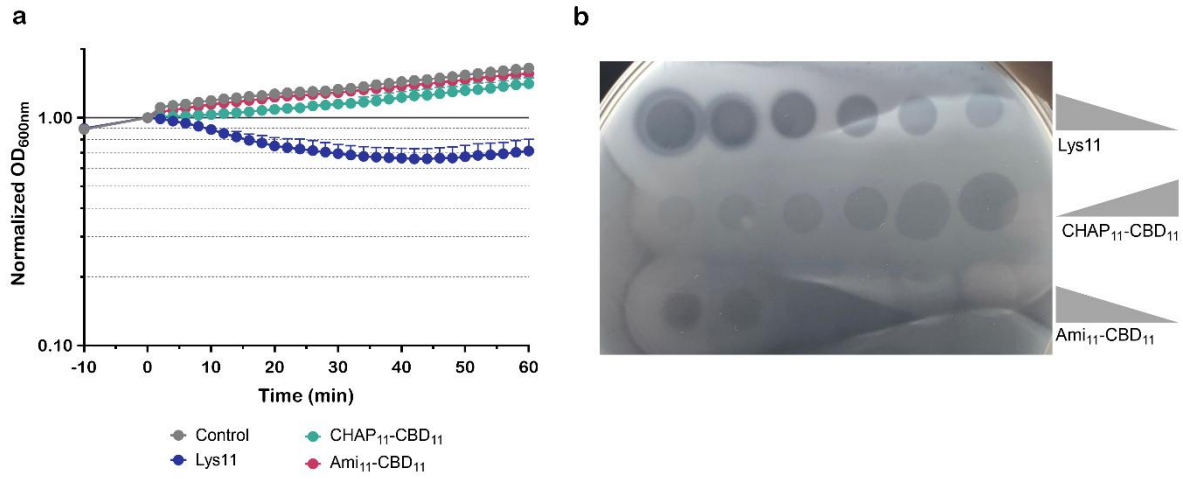


Figure III.6. Lytic action of single-CD mutants of Lys11.
(**a**) Cells of strain RN4220 in TSBca were incubated for 10 min with ionophore solvent, and then 100 nM of the indicated lysin variants was added. Cell lysis was monitored by following culture OD_{600nm}. Each curve represents means ± standard deviation from 6 independent experiments. For clarity, only mean + standard deviation is represented. (**b**) The lytic activity of Lys11, $CHAP_{11}$-CBD_{11} and Ami_{11}-CBD_{11} was evaluated by spotting 10 µl of 2-fold serial dilutions of the proteins on a dense lawn of *S. aureus* RN4220 (see Section III.4.4). Lysin concentrations ranged from 5 to 0.16 µM. Note that the whitish opacity around lysis halos is due to the high glycerol concentration (30%) present in the lysin storage buffer, which is also serially diluted like the proteins.

When $CHAP_{11}$-CBD_{11} was tested against cells treated with gramicidin, which affects both PMF components, slow but steady cell lysis could be observed, with culture OD_{600nm} being reduced by ~40% after 60 min (Figure III.7a). In the same conditions, no cell lysis could be measured with Ami_{11}-CBD_{11} (Figure III.7a), even when its concentration was increased to 4 µM (Supplementary Figure SIII.5). Therefore, the poor lytic performance of the single-CD mutants in liquid cultures remained unaffected (Ami_{11}-CBD_{11}) or showed some stimulation ($CHAP_{11}$-CBD_{11}) after PMF collapse, contrasting with Lys11 that lysed ~90% of the cells within 40 min, as seen before. As expected, the ΔpH and Δψ selective ionophores (nigericin and valinomycin, respectively) failed to significantly potentiate the lysis by the single-CD mutants, with $CHAP_{11}$-CBD_{11} only causing a slight drop in the OD_{600nm} curve when compared to those of Ami_{11}-CBD_{11} and cells with the ionophore only (Figure III.7b,c).

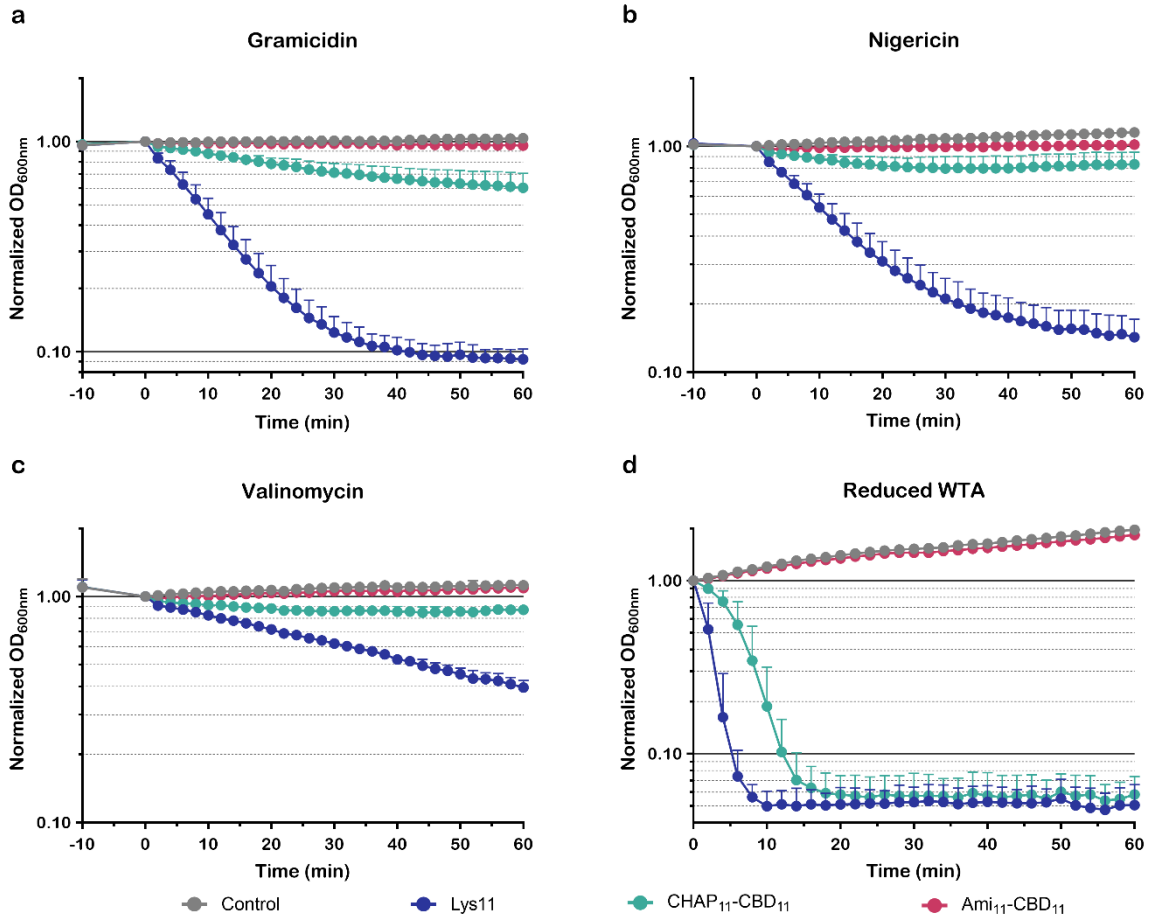


Figure III.7. The catalytic domains of Lys11 respond differently to cell tolerance signals.
Cells of strain RN4220 in TSBca were treated with gramicidin (**a**), nigericin (**b**) or valinomycin (**c**). Following ionophore treatment, 100 nM of the indicated proteins were added, and cell lysis was monitored. (**d**) Cells of strain RN4220 grown in presence of tunicamycin were collected in TSBca, they were challenged with 100 nM of the indicated proteins, and then cell lysis was similarly monitored. In each panel, the "Control" curves correspond to cells with ionophore or tunicamycin only (no protein added). Each curve represents means ± standard deviation from at least 4 independent experiments. For clarity, only the mean + standard deviation is represented.

Hence, the results indicated that the two Lys11 CDs are required for efficient lysis and effective stimulation of the endolysin lytic action in response to PMF dissipation. Nevertheless, the spot (Figure III.6b) and lysis assays (Figure III.7a-c) denoted a preponderant role of $CHAP_{11}$ in Lys11 lytic action, which is further supported by the high peptidoglycan-degrading action observed with crude CW fragments (Supplementary Figure SIII.4). The fact that in the tested conditions $CHAP_{11}$-CBD_{11} appeared to inefficiently respond to PMF dissipation could in part result from its poor binding to cells, due to the lack of the Ami_{11} stimulatory role in binding (eGFP-Ami_{11}-CBD_{11} versus eGFP-CBD_{11}, Figure III.2). In fact, when the concentration of $CHAP_{11}$-CBD_{11} was doubled (200 nM), the gramicidin enhancing effect on its lytic action became more evident, while 5-fold more protein (500 nM) still produced almost no lysis in the absence of the ionophore (Figure III.8a). In addition, in the

corresponding binding assays with eGFP-CBD_{11}, these two conditions resulted in similar amounts of protein bound to cells (Figure III.8b). Thus, as observed above for Lys11 (Figure III.3), it can be inferred that for the same amount of bound $CHAP_{11}$-CBD_{11} peptidoglycan cleavage is favored when cells are depolarized. Moreover, the equivalent binding in the two conditions hinted again at some improvement in the CBD_{11} binding efficiency to depolarized cells (see Figure III.2c).

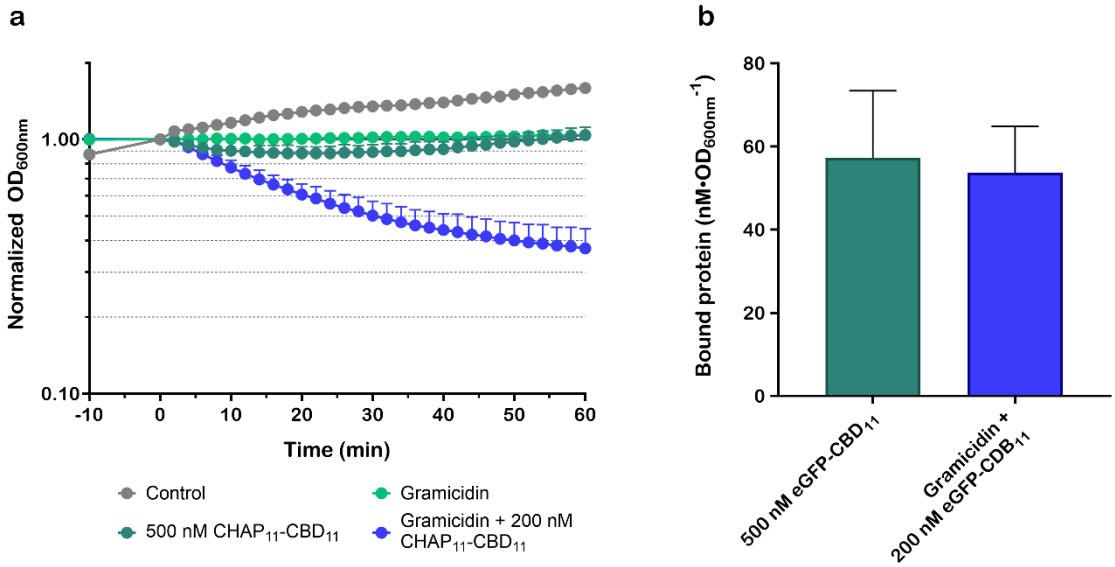


Figure III.8. PMF dissipation with gramicidin facilitates peptidoglycan cleavage by $CHAP_{11}$-CBD_{11}.
(**a**) Cells of strain RN4220 were untreated or treated with gramicidin and then incubated with 500 and 200 nM of $CHAP_{11}$-CBD_{11}, respectively. Only the condition gramicidin + 200 nM Lys11 caused obvious cell lysis. The data represent means ± standard deviation from at least 5 independent experiments. (**b**) In the corresponding binding assay with eGFP-CBD_{11}, the two conditions resulted in similar amounts of fluorescent protein bound to cells (no significant difference according to Student's t-test). The data represent means ± standard deviation from 7 independent experiments.

Finally, we tested the single-CD mutants on cells grown in the presence of tunicamycin (low WTA content). Although lysis mediated by Ami_{11}-CBD_{11} remained undetectable in this condition, $CHAP_{11}$-CBD_{11} lytic action was drastically improved, being able to reduce culture OD_{600nm} by ~90% in about 15 min (Figure III.7d). This result confirmed that the poor lytic performance of $CHAP_{11}$-CBD_{11} against depolarized cells, but with normal WTA content (Figure III.7a), did not derive from a major intrinsic defect of the $CHAP_{11}$ catalytic activity in the single-CD construct. Nevertheless, lysis by Lys11 was still the fastest (90% OD_{600nm} reduction in ~5 min) (Figure III.7d), hinting again at a catalytic contribution of the Ami_{11} domain that is not apparent with the Ami_{11}-CBD_{11}. Note that as seen above, the Ami_{11} domain seemed to have only a moderate contribution to the binding to cells with reduced WTA content (Figure III.5).

III.3. Discussion

An increasing body of literature supports that Gram-positive bacteria under growth-supporting conditions can restrict to a certain extent the exolytic action of endolysins, something that might have implications in the application of native endolysins and engineered derivatives as enzybiotics[37]. This work aimed at contributing to our understanding of the endolysin tolerance phenomenon by studying in more detail previously known determinants, the PMF and WTA, and by investigating new potential factors. As a model system, we used the bacterium *S. aureus* and the modular, dual-CD endolysin Lys11. The major findings are schematically summarized in Figure III.9.

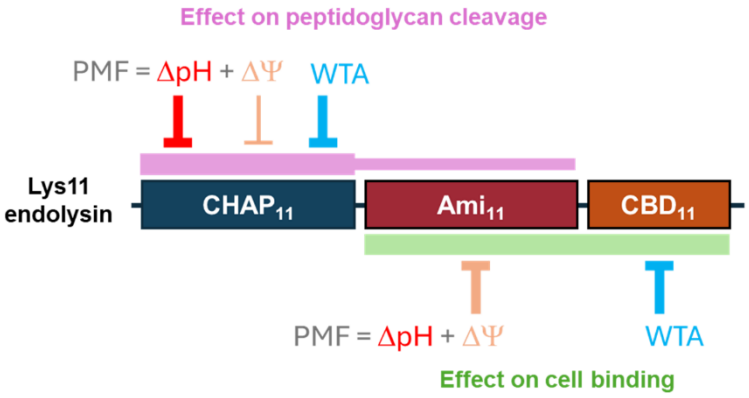


Figure III.9. Schematic summary of the major findings of this work.
The $CHAP_{11}$ domain is responsible for most of the endolysin's peptidoglycan cleavage activity, which is mainly restrained by the tolerance determinants ΔpH and WTA. An antagonist effect of WTA toward Lys11 cleavage activity was also inferred in a previous work[24]. The domains Ami_{11} and CBD_{11} cooperate in endolysin binding to the CW, being primarily counteracted by the tolerance determinants Δψ and WTA, respectively.

We started by dissecting the relative contribution of the two gradients that compose the PMF, ΔpH and Δψ, to endolysin tolerance. In our experimental conditions, the highest susceptibility to the Lys11 lytic action was observed in the presence of gramicidin, suggesting that both gradients of the PMF contribute to tolerance. Still, Lys11 lytic performance was clearly higher against cells treated with nigericin compared to valinomycin, indicating that the proton gradient (ΔpH) has a preponderant role in controlling susceptibility to Lys11. During bacterial growth, the pH gradient is generated by membrane pumps that extrude protons[14], creating a relatively low pH in the CW environment[38]. This acidification has been proposed as one of the mechanisms inhibiting the enzymatic activity of bacterial autolysins[39,40], and anionic polymers like WTA are thought to play an important role in this ΔpH inhibitory effect by retaining the extruded protons in the CW compartment[41,42]. Considering the structural and evolutionary relationship between bacterial autolysins and phage endolysins[43,44], it is not surprising that ΔpH also exerts control on the phage lytic enzymes, as we have shown here for Lys11.

The Lys11 deletion analysis indicated that most of the enzyme's peptidoglycan cleavage activity relies on the $CHAP_{11}$ domain, which is majorly restrained by the tolerance determinants ΔpH and WTA. Still, an auxiliary role of the Ami_{11} domain in peptidoglycan cleaving could be inferred from certain experiments. Such preponderant role of the CHAP CD in cutting the peptidoglycan, while the amidase CD seems to have a preeminent role in endolysin binding, is a consistent feature of this type of staphylococcal endolysin[31,45]. The full-length endolysin performed always better than the single-CD mutants, either under conditions supporting tolerance (cells growing in rich media) or favoring lysis (collapsed PMF or WTA deficiency), indicating that the native enzyme with its two CDs and a CBD is optimized for lytic activity. Our results agree with a previous study reporting that Lys11 constructs bearing either the endopeptidase or amidase domain directly fused to the CBD failed to induce visible lysis against heat-killed *S. aureus*[46]. However, in certain experimental conditions, structurally related staphylococcal endolysins were shown to display normal, or even superior, lytic activity, after the deletion of the amidase CD[45,47]. Therefore, for related endolysins, the outcome of combining or deleting functional domains can vary significantly, probably depending on the intrinsic features of the individual domains.

In agreement with previous suggestions[24], the data here presented showed that the PMF can influence endolysin affinity to the cell surface, which will naturally affect its lytic performance. The binding assays with the different eGFP-endolysin fusions showed that when Ami_{11} and CBD_{11} are in the same polypeptide chain, they contribute to maximizing binding both to normal and depolarized cells. The amount of eGFP-Ami_{11}-CBD_{11} (the proxy of Lys11) that bound to cells with collapsed PMF was essentially doubled relative to normal cells (gramicidin-treated versus control cells). Interestingly, eGFP-Ami_{11} bound poorly to energized cells, but its binding was drastically increased after the abolishment of the Δψ gradient. Thus, during phage infection, it is likely that Ami_{11} favors Lys11 binding to the CW in response to the PMF collapse accomplished by the holin function.

In neutrophilic bacteria like *S. aureus* and *Bacillus subtilis*, the Δψ component of the PMF corresponds to an asymmetric distribution of ions across the membrane, with a net accumulation of cations outside the cell and anions inside[14]. The ionic environment of the CW together with its charged constituents determines the electrostatic binding properties of the bacterial surface. The collapse of the PMF can contribute to a more negative charge of the bacterial cell surface, resulting in an increased capacity to bind positively charged probes or molecules[39,41]. Our recombinant Lys11 and the fusion eGPF-Ami_{11}-CBD_{11} have similar charge distributions and predicted positive net charges (+4.8 and +3.4, respectively) at pH 7.0, which is close to the medium pH in the assays. Therefore, the increased endolysin binding could be at least partially explained by an enhancement of the electrostatic interactions with the more negatively charged cell surface after PMF dissipation. Notably, the Ami_{11} module and its upstream linker contribute with most of the endolysin basic amino acids, having individual net charges of +7.3 and +2.8, respectively (Supplementary Figure SIII.2). The $CHAP_{11}$ and CBD_{11} modules have net charges of −6.3 and +0.4, respectively, whereas the eGPF

moiety in the fluorescent fusions has a net charge of −7.7. Thus, the cationic character of Ami_{11} could be at the basis of its highest responsiveness to Δψ collapse with respect to cell binding.

A recent study with two endogenous peptidases of *S. pettenkoferi* that have the same functional domains, but very distinct isoelectric points, showed that the affinity of the enzymes was governed by the protein and bacterial surface charges, with the relative lytic activities essentially correlating with the binding efficiencies[48]. In fact, modifications that increase lysin net positive charge have been proposed as a strategy to improve their association with the negatively charged bacterial surface and, with this, enhance exolysis (reviewed in Ref.[45]). However, such an approach does not always produce the desired effect[49], and in some cases, the neutralization of specific basic residues in the enzymes may actually increase processivity and lysis kinetics by allowing their faster dissociation from the CW[50]. In other words, excessive binding affinity may also be detrimental to lysis kinetics by restricting enzyme movement on the CW[50].

In this study, we evaluated the possible contribution of LTA and WTA modifications to Lys11 tolerance. LTA were proposed to have an inhibitory action against certain staphylococcal autolysins[51,52], whereas in some *S. aureus* genetic backgrounds, the presence of LTA seems to favor autolytic activity[53]. Hence, we questioned if LTA played any relevant role in Lys11 susceptibility. The *S. aureus* mutant disabled in LTA production used in this work showed complete tolerance to Lys11, whereas the parental strain, with normal LTA synthesis, presented some cell lysis in the same conditions. Therefore, the *S. aureus* LTA does not seem to contribute to Lys11 tolerance; on the contrary, it appears to facilitate Lys11 lytic action. The *S. aureus* LTA occupies the inner layers of the CW and is likely to preferentially accumulate in the site of its synthesis, the division septum[54]. The WTA tend to be excluded from this place and accumulates in the older regions of the CW[55]. Considering the key role of the WTA in Lys11 tolerance and that the nascent peptidoglycan at the septum might be more exposed to endolysin attack, a possible explanation for the lower susceptibility of the LTA mutant to Lys11 could be a compensatory accumulation of WTA[56], namely in the cell cross-wall. In agreement with this hypothesis, we observed a reduced Lys11 binding to the *lta*S mutant (Supplementary Figure SIII.6a).

The studies with *S. aureus* mutants deficient in WTA GlcNAcylation and TA D-alanylation indicated that these substitutions do not contribute significantly to Lys11 tolerance. The lack of α- and β-O-GlcNAcylation was previously reported to have little or no impact on *S. aureus* susceptibility to lysostaphin (only half-reduction in its MIC) and autolysin activity[33]. Yet, the substitution of TA with cationic D-alanine esters has been shown to affect the activity of bacterial autolysins, lysostaphin and cationic antibacterial peptides. D-alanylation balances the negative character of WTA and LTA, and this was proposed to directly impact the binding of the referred agents to the CW and/or to change the local concentrations of cations that modulate their activity (reviewed in Refs.[16,17,57]). On this basis, and considering the predicted positive net charge of Lys11, one could expect higher endolysin binding to cells lacking TA D-alanylation and eventually increased exolysis. However, in

contrast to this prediction, Lys11 binding to the D-alanylation mutant was in fact diminished (Supplementary Figure SIII.6b) when compared to parental strain and the susceptibility to Lys11 lysis was clearly reduced (Figure III.4d). This suggests that besides the altering of the electrostatic properties of the cell surface, the lack of D-alanylation may produce other effects that negatively impact Lys11 lytic action; these could be, for example, changes in cation availability in the CW and TA conformation[58-60].

The assays with *S. aureus* cells having altered CW glycopolymers therefore confirmed the WTA as a major determinant of Lys11 tolerance. Here, we uncovered more details of the WTA function as inhibitor of Lys11 binding to cells by showing that it hinders primarily the binding activity of CBD_{11}. In fact, when cells have low levels of WTA, the Ami_{11} domain is almost dispensable both for binding and cell lysis. Yet, the available data indicate that the inhibitory action of WTA goes beyond a simple shielding effect that restricts endolysin binding to the cell surface[24]. WTA might also exclude endolysins from certain regions of the CW and contribute to the inhibition of catalytic activity due to their interplay with the PMF[42,57].

III.4. Materials and Methods

III.4.1. Bacterial Strains and General Growth Conditions

Escherichia coli and *S. aureus* strains used in this work are listed in Table III.1. Unless stated otherwise, *E. coli* and *S. aureus* were grown at 37 ºC, under aerated conditions, in Lysogeny Broth (LB, NZYTech—Genes & Enzymes) and tryptic soy broth (TSB, BIOKAR Diagnostics) media, respectively. When necessary for strain/plasmid selection, LB was supplemented with 100 µg/ml ampicillin and/or 40 µg/ml kanamycin and TSB with 120 µg/ml spectinomycin. Specific growth conditions for protein production in *E. coli* are described in Section III.4.3. To generate a phenotype of impaired WTA synthesis without significantly affecting growth rate, *S. aureus* was cultured in presence of 50 ng/ml tunicamycin[24].

Table III.1. E. coli *and* S. aureus *strains used in this study*

Strains	Relevant Features	Reference/Source
E. coli		
XL1-Blue MRF'	Cloning strain for recovering of plasmid constructs	Stratagene
CG61	Protein expression strain; produces phage T7 RNA polymerase upon thermal induction. Used to produce all Lys11 variants. Selection with 40 µg/mL kanamycin	Ref.[61]
S. aureus		
RN4220	Prophage-cured, restriction-deficient mutant of strain 8325-4	Ref.[62]
RN4220Δ*tarM*Δ*tarS*	Derivative of RN4220 lacking α- and β-O-GlcNAcylation due to *tarM* and *tarS* deletion	Ref.[33]
RN4220Δ*spa*	In-frame deletion in *spa* coding for protein A	Ref.[63]
RN4220Δ*spa*Δ*ltaS* (supressor strain 4S5)	Derivative of RN4220Δ*spa* lacking LTA as result of *ltaS* deletion. Carries a mutation suppressing the Δ*ltaS* lethal phenotype	Ref.[32]
SA113	Mutant strain of 8325, with an agr^- background and 11-bp deletion in *rsbU*	Ref.[64]
SA113Δ*dltA*	Derivative of SA113 lacking D-alanylation of TA due to *dltA* deletion. Selection with 120 µg/mL spectinomycin	Ref.[34]

III.4.2. Generation of Endolysin Variants

Plasmids expressing Lys11 derivatives were constructed following standard recombinant DNA techniques. The endolysin variants produced and purified in this work are indicated in Table III.2. Previously described derivatives of the expression vector pIVEX2.3d (Roche Applied Science, Mannhein, Germany) carrying Lys11 and eGFP coding sequences[21,24] were the basis for generating new endolysin variants (Supplementary File SIII.1 and Figure SIII.2). Genes expressing domain deletion mutants or domain fusions to eGFP were assembled by PCR or overlap-extension PCR, using plasmids carrying *lys11* and *eGFP* as templates, and suitable primers (Table SIII.1). All coding sequences were inserted in pIVEX2.3d with *NcoI* and *XmaI* restriction sites, allowing variants to be tagged at the C-terminus with a hexahistidine tail. The recombinant plasmids, selected in presence of 100 µg/ml ampicillin, were confirmed by sequencing before transformation of E. coli expression strain CG61.

Table III.2. Variants of endolysin Lys11 used in this study

Lys11 Variant	Features	Reference/Source
Lys11	3-domain endolysin: $CHAP_{11}$, Ami_{11} and CBD_{11}	Ref.[21]
$CHAP_{11}$-CBD_{11}	Lys11 lacking Ami_{11}. Deletion encompassing residues 151 to 360 of Lys11	This work
Ami_{11}-CBD_{11}	Lys11 lacking $CHAP_{11}$. Deletion encompassing residues 2 to 178 of Lys11	This work
eGFP-Ami_{11}-CBD_{11}	Ami_{11}-CBD_{11} (P_{149} to S_{481} of Lys11) fused to the C-terminus of eGFP	Ref.[24]
eGFP-Ami_{11}	Ami_{11} (P_{149} to M_{360} of Lys11) fused to the C-terminus of eGFP	This work
eGFP-CBD_{11}	CBD_{11} (D_{361} to S_{481} of Lys11) fused to the C-terminus of eGFP	This work

III.4.3. Protein Production and Purification

Two previously described protein production conditions[24] were applied according to the group of proteins, with group A including Lys11, $CHAP_{11}$-CBD_{11} and Ami_{11}-CBD_{11} and group B including eGFP-Ami_{11}-CBD_{11}, eGFP-Ami_{11} and eGFP-CBD_{11}. Irrespective of the protein group, the different *E. coli* CG61 derivatives were grown overnight at 28 ºC and on the next day 100-fold diluted either in phosphate-buffered LB medium supplemented with 0.5 M D-sorbitol (group A) or in regular LB (group B). Cultures were grown at 28 ºC until mid/late exponential phase, and then protein production was induced by temperature upshift (30 min at 42 ºC in a shaking water bath). Cultures from group A proteins were then incubated at 16 ºC for 14–16 h, whereas those of group B were incubated for 3 h at 37 ºC. After cell disruption[24], Lys11 deletion mutants and eGFP fusions were purified by metal chelate affinity chromatography, as reported previously for Lys11 and eGFP-Ami_{11}-CBD_{11}, respectively[24], except that all buffers contained 30% glycerol to minimize protein precipitation (Figure SIII.3). Protein quantification and storage were also as described before[24].

III.4.4. Bacteriolysis Assays

S. aureus cell lysis as a result of lysin treatment in liquid culture medium was studied essentially as before[24]. Briefly, cells from exponentially growing cultures were collected by centrifugation and resuspended in fresh, pre-warmed TSB supplemented with 0.5 mM $CaCl_2$ (TSBca) to an initial OD_{600nm} of ~0.8 (cuvette with slit and light path of 0.5 and 1 cm, respectively), which corresponded to about 1×10^8 CFU/ml. Cells were challenged with Lys11 (or its derivatives) after 10 min treatment with the ionophores gramicidin (30 µg/ml, Sigma-Aldrich, Saint Louis, Missouri, USA, Cat. No. G5002), nigericin (10 µM, Sigma-Aldrich, Cat. No. N7143), valinomycin (20 µM, Sig-ma-Aldrich, Cat. No. V0627) or the corresponding ionophore solvents. These were DMSO (gramicidin and valinomycin) and ethanol (nigericin). Cells treated with valinomycin were also supplemented with

200 mM KCl. The ionophore treatments were defined based on previous work[30], with the drug concentrations adjusted to the minimum required to inhibit *S. aureus* growth in our experimental conditions. To monitor cell lysis, the OD_{600nm} of cultures in 96-well microplates (200 µl final volume per well) was measured at regular time points after addition of the different agents (Epoch 2 microplate reader, Biotek Instruments, Inc., Winooski, Vermont, USA). The impact of gramicidin, nigericin and valinomycin on the PMF was confirmed using the membrane potential-sensitive dye $DiSC_3(5)$ (Sigma-Aldrich, Cat. No. 43608), as reported previously[24].

S. aureus cell lysis in soft agar medium was studied using a spot assay. Cultures at an OD_{600nm} of ~0.8 were centrifuged, and cells were resuspended in 1/100 volumes of incorporation buffer (50 mM HEPES, 150 mM NaCl, 0.5 mM $CaCl_2$, pH 7.2). A 300 µl sample of this suspension was added to 10 ml of soft agar incorporation buffer (0.75% agar) and poured into a Petri dish. Soft agar incorporation buffer was prepared by mixing equal volumes of 2x incorporation buffer and a solution of 1.5% agar (both solutions equilibrated at 50 ºC before mixing). After solidification and drying, 10 µl drops of lysin dilutions in incorporation buffer were spotted onto the dense lawns of viable S. aureus cells. Plates were incubated overnight at 37 ºC, and formation of lysis halos was analysed.

III.4.5. Binding of eGFP-Endolysin Fusions to Cells

The binding of eGFP-endolysin fusions to *S. aureus* cells in different conditions was studied exactly as described in Gouveia et al.[24]. Cells of strain RN4220 set to an OD_{600nm} of ~0.8 in fresh TSBca were treated or not with ionophores (see Section III.4.4), and then 200 µl cell samples were incubated with the indicated concentrations of fluorescent proteins for additional 10 min. After washing cells with PBS for removal of unbound protein, the fluorescence associated with cells was measured in black microtiter plates (Greiner Bio-One, Kremsmünster, Austria, Cat. No. 655076) with excitation and emission wavelengths of 488 and 507 nm, respectively (Varioskan LUX Multimode, ThermoFisher Scientific, Waltham, Massachusetts, USA). OD_{600nm} was also registered. The amount of eGFP-endolysin fusion associated with cells, expressed in $nM \cdot OD_{600nm}^{-1}$, was calculated by performing standard calibration curves with each fluorescent protein as described previously[24]. The same method was employed to measure the binding of eGFP fusions to *S. aureus* RN4220 cells grown in the presence of 50 ng/ml tunicamycin.

III.4.6. Bioinformatics Analysis

Protein-conserved domains, domain boundaries and putative linker regions were defined using InterPro: https://www.ebi.ac.uk/interpro/ (accessed on 1 January 2023), CD-search: https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 1 January 2023) and the AlphaFold structure prediction for LytO (same as Lys11): https://alphafold.ebi.ac.uk/entry/Q2FX77 (accessed on 1

January 2023). Theoretical molecular masses, pI values and net charge at pH 7 (z) of recombinant proteins were determined with the Prot pi Protein tool: https://www.protpi.ch/Calculator/ProteinTool (accessed on 1 October 2023), using ExPASy as the data source of pKa values.

III.4.7. Statistical Analysis

Data are represented as the mean ± standard deviation from at least 3 independent experiments and were analysed using GraphPad Prism version 10.0.2 (Boston, Massachusetts, USA). Data normality was confirmed with Kolmogorov–Smirnov test. For multiple group comparisons, the significance of the data differences was analysed with a two-way ANOVA test, followed by Tukey's post hoc test. Two group comparisons were made using Student's *t*-test. Differences were considered statistically significant when the calculated adjusted *p* value was below the alpha level of 0.05.

III.5. Conclusions

In summary, the results of the present study reinforce the notion that the roles of the PMF and WTA in endolysin tolerance are most likely multifactorial and interconnected. By influencing the electrochemical environment of the CW, the PMF directly impacts endolysins by interfering with their binding and catalytic activities while probably also modulating the charge/conformation of WTA. On the other hand, WTA not only hinder endolysin binding but are also likely to contribute to the inhibitory role of the ΔpH component of the PMF by retaining protons in the inner layers of the CW. When thinking about the natural context of the action of endolysins, that is, during phage infection, it is relevant to note that the lytic enzymes only act after complete PMF dissipation mediated by the holin[10] and that this event can also stimulate endolysin lytic action from within[21]. This, associated with the fact that PMF collapse is also linked to the activation of bacterial autolysins[57,65], confirms the pivotal role of the PMF in the control of many peptidoglycan-degrading enzymes.

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III.7. Supplementary Information

III.7.1. Supplementary File SIII.1

Features of the endolysin Lys11 and derivatives used in this study

Relevant features of Lys11 and its derivatives are denoted in the corresponding primary sequences with coloured lettering and shading.

Theoretical molecular masses, pI values and net charge at pH 7 (z) of Lys11 and its variants were determined with the Prot pi Protein tool (https://www.protpi.ch/Calculator/ProteinTool), using ExPASy as the data source of pKa values.

- **Protein domains**, according to InterPro (https://www.ebi.ac.uk/interpro/):

  CHAP (IPR007921), here designated $CHAP_{11}$

  Amidase (IPR002502), here designated Ami_{11}

  SH3-like (IPR003646), here designated CBD_{11}

  eGFP (IPR000786)

- Linkers were defined based on an integrated analysis with InterPro (for domain boundaries) and AlphaFold structure prediction of LytO (same as Lys11, https://alphafold.ebi.ac.uk/entry/Q2FX77).

- PGGGSHHHHHH is the vector-borne C-terminal tail containing the hexahistidine tag.

- "***E***" denotes a Q_2E substitution introduced in the native Lys11 sequence resulting from the use of the restriction enzyme *Nco*I recognition sequence in the cloning procedures.

Endolysin Lys11

>Lys11 (55.21 kDa, pI = 8.49, z =+4.79)

M*E*AKLTKNEFIEWLKTSEGKQFNVDLWYGFQCFDYANAGWKVLFGLLLKGLGAKDIPFANNFDGLATVYQNT
PDFLAQPGDMVVFGSNYGAGYGHVAWVIEATLDYIIVYEQNWLGGGWTDGIEQPGWGWEKVTRRQHAYDFP
MWFIRPNFKSETAPRSVQSPTQAPKKETAKPQPKAVELKIIKDVVKGYDLPKRGSNPKGIVIHNDAGSKGATAE
AYRNGLVNAPLSRLEAGIAHSYVSGNTVWQALDESQVGWHTANQIGNKYYYGIEVCQSMGADNATFLKNEQAT
FQECARLLKKWGLPANRNTIRLHNEFTSTSCPHRSSVLHTGFDPVTRGLLPEDKRLQLKDYFIKQIRAYMDGKI
PVATVSNESSASSNTVKPVASAWKRNKYGTYYMEESARFTNGNQPITVRKVGPFLSCPVGYQFQPGGYCDYTE
VMLQDGHVWVGYTWEGQRYYLPIRTWNGSAPPNQILGDLWGEISPGGGSHHHHHH

Lys11 catalytic domain deletion mutants

>CHAP11-CBD11 (31.89 kDa, pI = 5.83, z = -5.72)

M*E*AKLTKNEFIEWLKTSEGKQFNVDLWYGFQCFDYANAGWKVLFGLLLKGLGAKDIPFANNFDGLATVYQNT
PDFLAQPGDMVVFGSNYGAGYGHVAWVIEATLDYIIVYEQNWLGGGWTDGIEQPGWGWEKVTRRQHAYDFP
MWFIRPNDGKIPVATVSNESSASSNTVKPVASAWKRNKYGTYYMEESARFTNGNQPITVRKVGPFLSCPVGYQ
FQPGGYCDYTEVMLQDGHVWVGYTWEGQRYYLPIRTWNGSAPPNQILGDLWGEISPGGGSHHHHHH

>Ami11-CBD11 (35.15 kDa, pI = 8.93. z = +6.61)

MVELKIIKDVVKGYDLPKRGSNPKGIVIHNDAGSKGATAEAYRNGLVNAPLSRLEAGIAHSYVSGNTVWQALDE
SQVGWHTANQIGNKYYYGIEVCQSMGADNATFLKNEQATFQECARLLKKWGLPANRNTIRLHNEFTSTSCPH
RSSVLHTGFDPVTRGLLPEDKRLQLKDYFIKQIRAYMDGKIPVATVSNESSASSNTVKPVASAWKRNKYGTYY
MEESARFTNGNQPITVRKVGPFLSCPVGYQFQPGGYCDYTEVMLQDGHVWVGYTWEGQRYYLPIRTWNGSAP
PNQILGDLWGEISPGGGSHHHHHH

eGFP fusions to Lys11 domain

>eGFP-Ami11-CBD11 (65.17 kDa, pI = 7.99, z = +3.40)

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSR
YPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYN
SHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHM
VLLEFVTAAGITLGMDELYKPNFKSETAPRSVQSPTQAPKKETAKPQPKAVELKIIKDVVKGYDLPKRGSNPKGI
VIHNDAGSKGATAEAYRNGLVNAPLSRLEAGIAHSYVSGNTVWQALDESQVGWHTANQIGNKYYYGIEVCQSM
GADNATFLKNEQATFQECARLLKKWGLPANRNTIRLHNEFTSTSCPHRSSVLHTGFDPVTRGLLPEDKRLQLK
DYFIKQIRAYMDGKIPVATVSNESSASSNTVKPVASAWKRNKYGTYYMEESARFTNGNQPITVRKVGPFLSCPV
GYQFQPGGYCDYTEVMLQDGHVWVGYTWEGQRYYLPIRTWNGSAPPNQILGDLWGEISPGGGSHHHHHH

>eGFP-Ami11 (51.65 kDa, pI = 8.10, z = +3.33)

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSR
YPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYN
SHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHM
VLLEFVTAAGITLGMDELYKPNFKSETAPRSVQSPTQAPKKETAKPQPKAVELKIIKDVVKGYDLPKRGSNPKGI
VIHNDAGSKGATAEAYRNGLVNAPLSRLEAGIAHSYVSGNTVWQALDESQVGWHTANQIGNKYYYGIEVCQSM
GADNATFLKNEQATFQECARLLKKWGLPANRNTIRLHNEFTSTSCPHRSSVLHTGFDPVTRGLLPEDKRLQLK
DYFIKQIRAYMPGGGSHHHHHH

>eGFP-CBD11 (41.50 kDa, pI = 6.01, z = -7.19)

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSR
YPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYN
SHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHM
VLLEFVTAAGITLGMDELYKDGKIPVATVSNESSASSNTVKPVASAWKRNKYGTYYMEESARFTNGNQPITVRK
VGPFLSCPVGYQFQPGGYCDYTEVMLQDGHVWVGYTWEGQRYYLPIRTWNGSAPPNQILGDLWGEISPGGGS
HHHHHH

III.7.2. Supplementary Figures

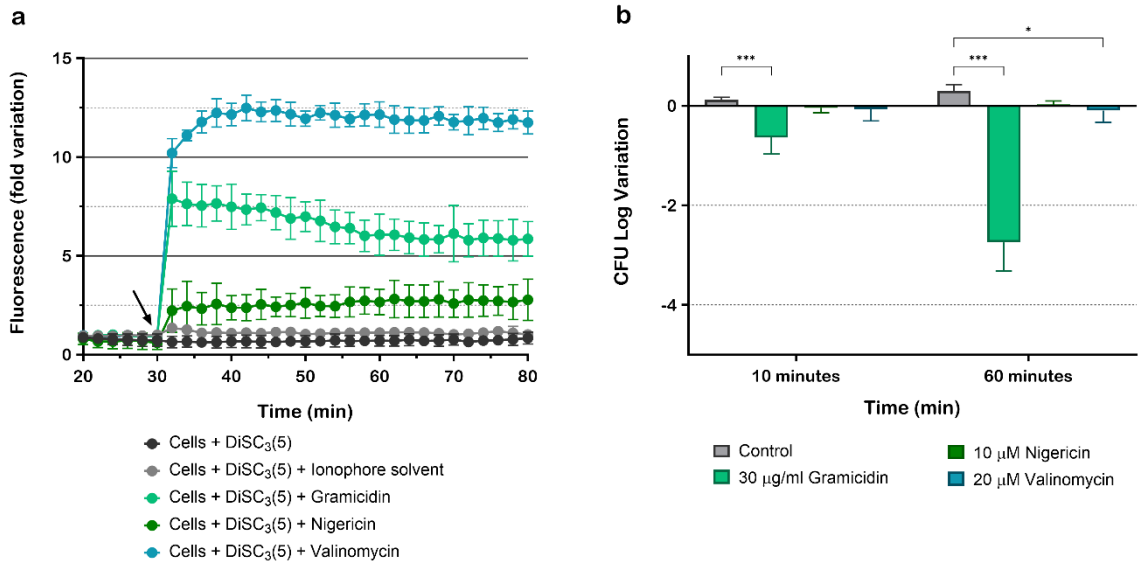

Figure SIII.1. Effect of ionophore on* S. aureus *membrane potential and cell viability.
(**a**) The expected effect of selected ionophores on the membrane potential of *S. aureus* cells was confirmed using the potentiometric fluorescence probe $DiSC_3(5)$, as described previously[24]. $DiSC_3(5)$ was added to polarized cells of strain RN4220, which uptake the dye and quenched its fluorescence. $DiSC_3(5)$-loaded cells were then treated with 30 µg/ml gramicidin, 10 µM nigericin, 20 µM valinomycin or ionophore solvent (indicated with an arrow), and the increase in fluorescence as result of membrane depolarization monitored by fluorimetry. At the tested concentrations, valinomycin and gramicidin caused an abrupt and marked rise in fluorescence, while nigericin produced only a slight increase, showing that the latter ionophore essentially worked as an electroneutral carrier. The data of each curve represent means ± standard deviation from at least 3 independent experiments. (**b**) Log phase cells of *S. aureus* RN4220 collected in fresh TSBca (see Section III.4.4) were incubated at 37 ºC for 10 or 60 min with the indicated concentrations of ionophores or solvent volume equivalents ("Control"). After incubation, cell viability was assessed by CFU counts. The results confirmed that significant lethality is only observed upon dissipation of both gradients of the PMF ("Gramicidin"). For each condition, the results are represented as the log variation of CFU/ml relatively to the cell input. The data represents means ± standard deviation from at least 3 independent experiments. Asterisks denote a significant difference, according to two-way ANOVA, followed by Tukey post-hoc test (***P <0.001).

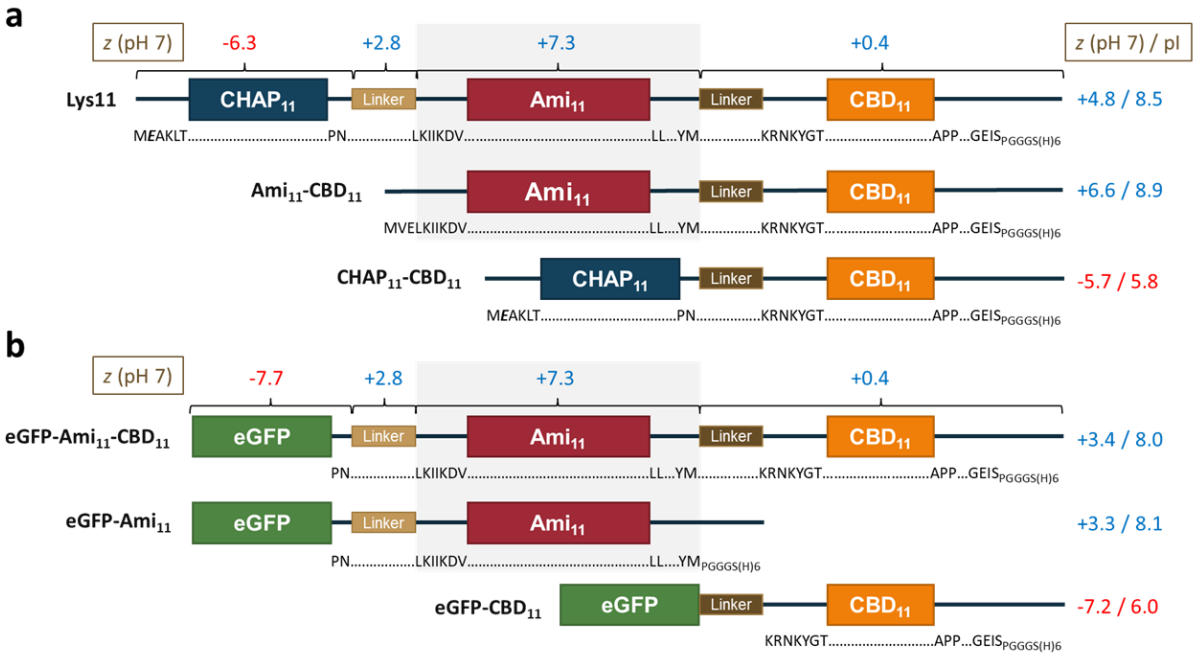

Figure SIII.2. Schematic representation and features of the endolysin Lys11 variants used in this study (not drawn to scale).
Relevant modules of deletion mutants (**a**) and eGFP fusions (**b**) are depicted with indication of the predicted net charge (z value) at pH 7. The z value and isoelectric point (pI) of the full-length constructs are also indicated on the right side. The z and pI values were predicted with the Prot pi Protein tool available at https://www.protpi.ch/Calculator/ProteinTool, using ExPASy as the data source for pKa values.

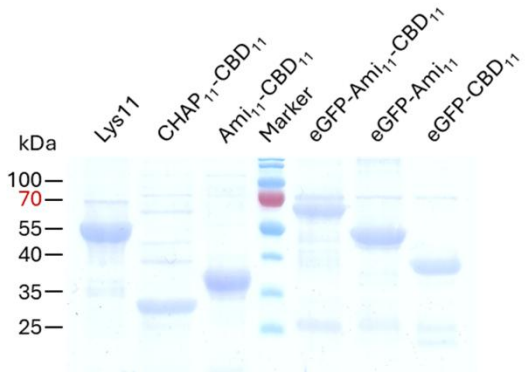

Figure SIII.3. SDS-PAGE analysis of the purified Lys11 variants used in this work.
The His_6-tagged recombinant proteins were purified by metal chelate affinity chromatography, followed by a desalting step to remove imidazole from the pure fractions. Each lane was loaded with 5 µg total protein. Molecular weight marker: PageRuler Prestained Protein Ladder (ThermoScientific).

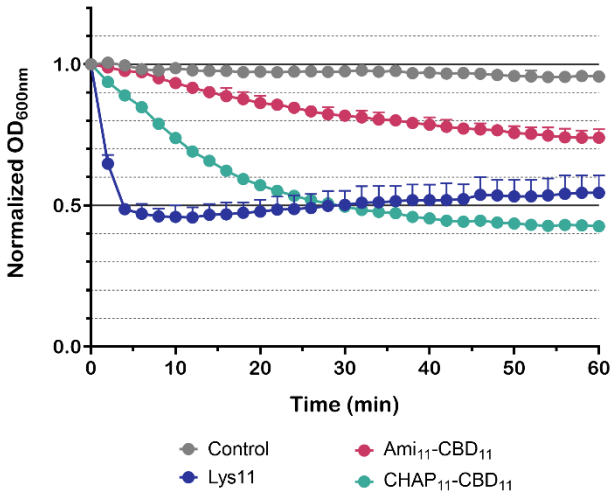

Figure SIII.4. Peptidoglycan-degrading action of Lys11 and single-CD mutant derivatives on crude CW.
The CW fragments were prepared according to the protocol described in Wu et al[66]. Briefly, *S. aureus* RN4220 cells were grown in TSB until mid-exponential phase (OD_{600nm} ~ 0.6). Cells from a 300 ml culture were harvested by centrifugation, washed once with deionized water and suspended in 4% SDS. The suspension was boiled at 95 °C for 30 min, incubated overnight with agitation at room temperature, and further boiled for 10 min. The SDS-insoluble CW material was collected by centrifugation at 11,700 *g* for 15 min at 4°C. The pellet containing cell wall fragments was extensively washed with deionized water to remove SDS before being resuspended in 1 ml sterile deionized water and stored at 4 °C until further use. The fragments were exposed to 500 nM of the single-CD mutants and of Lys11 and changes in OD_{600nm} monitored spectrophotometrically. In these conditions, peptidoglycan-cleaving activity was observed for all Lys11 constructs, with the relative activities being Lys11 > $CHAP_{11}$-CBD_{11} > Ami_{11}-CBD_{11}, confirming the preponderant role of the CHAP domain to the peptidoglycan-digesting activity of Lys11.

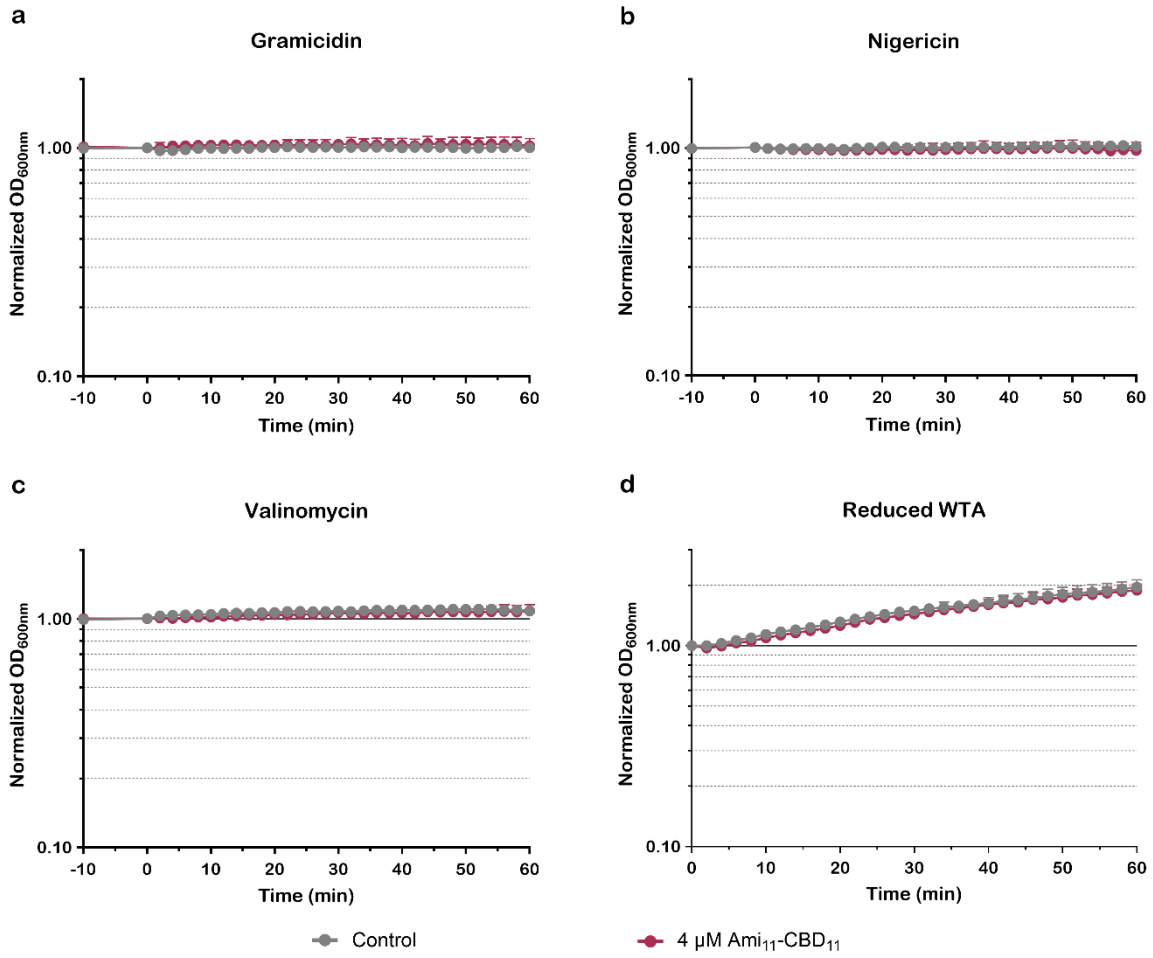

Figure SIII.5. The endolysin variant Ami$_{11}$-CBD$_{11}$ displays no lytic action in liquid cultures.
S. aureus cells with affected PMF or with low WTA remain refractory to bacteriolysis mediated by Ami$_{11}$-CBD$_{11}$. Cells of strain RN4220 in TSBca were treated with gramicidin (**a**), nigericin (**b**) or valinomycin (**c**). Following ionophore treatment, 4 µM of Ami$_{11}$-CBD$_{11}$ were added, and cell lysis monitored. (**d**) Cells of strain RN4220 grown in presence of tunicamycin were collected in TSBca, challenged with 4 µM of Ami$_{11}$-CBD$_{11}$, and lysis similarly monitored. In each panel, the "Control" curve corresponds to cells with ionophore or tunicamycin only (no protein added). Each curve represents means ± standard deviation from 4 independent experiments. For clarity, only the mean + standard deviation is represented.

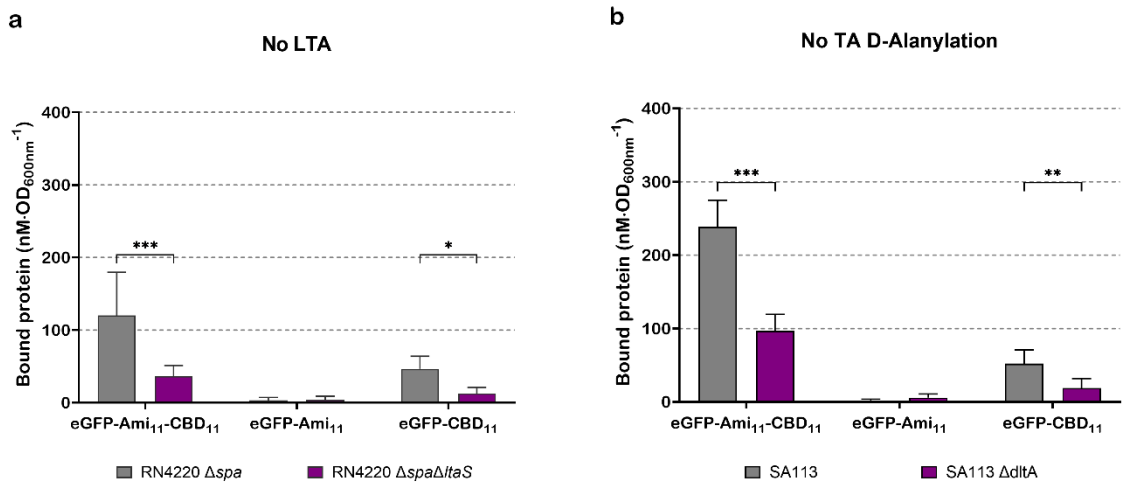

Figure SIII.6. LTA and TA substitution with D-Alanine esters appear to contribute to Lys11 binding to cells.
Log phase cells of (**a**) strains RN4220 Δ*spa* and RN4220 Δ*spa*Δ*ltaS* or (**b**) strains SA113 and SA113 Δ*dltA* were collected in TSBca, incubated for 10 min with 100 nM of the indicated eGFP-endolysin fusions, and the amount of fluorescent protein associated with cells determined as in Figure III.2. The data represents means ± standard deviation from at least 7 independent experiments. Asterisks denote a significant difference, according to Two-Away ANOVA test, followed by Tukey post-hoc test (*p<0.1; **p<0.01; ***p>0.001).

III.7.3. Supplementary Table

Table SIII.1. Sequence of primers used for the generation of Lys11 derivatives.

Variant	Template	Protein region	Primer name	Primer 5'→3' sequence[1,2]	Reference
Lys11 variants obtained by PCR Amplification					
Lys11	Phage phi11 genome	Full Lys11 endolysin	Lys11Fw-Nco	CGA**CCATGG**AAGCAAAATTAACTAAAAATGAGTTT	Ref. [21]
			Lys11Rv-Xma	GTC**CCCGGG**ACTGATTTCTCCCCATAAGTCA	Ref. [21]
Ami$_{11}$-CBD$_{11}$	pIVEX2.3d::Lys11	Ami$_{11}$-CBD$_{11}$ region	Ami11-Fw-Nco	ACCTA**CCATGG**TAGAACTTAAAATCATCAAAGATGT	This work
			Lys11Rv-Xma	GTC**CCCGGG**ACTGATTTCTCCCCATAAGTCA	Ref. [21]
eGFP-Ami$_{11}$	pIVEX2.3d::eGFP-Ami11-CBD11	eGFP-Ami11 region	eGFP-Nco-Fw	ATATAG**CCATGG**TGAGCAAGGGCGAGGAGCT	Ref. [24]
			Ami11-Rv-Xma	TATTAT**CCCGGG**CATGTACGCCCTAATCTGCTTGA	This work
Lys11 variants obtained by Overlap Extension-PCR Amplification					
CHAP$_{11}$-CBD$_{11}$	pIVEX2.3d::Lys11	CHAP$_{11}$ module	Lys11Fw-Nco	CGA**CCATGG**AAGCAAAATTAACTAAAAATGAGTTT	Ref. [21]
			CHAP11-Rv1	TTCGGACGGATAAACCACATAG	This work
		CBD$_{11}$ module	CBD11_Fw2	TTTCCCTATGTGGTTTATCCGTCCGAATGATGGTAAAATACCGGTTGCCA	This work
			Lys11Rv-Xma	GTC**CCCGGG**ACTGATTTCTCCCCATAAGTCA	Ref. [21]
eGFP-Ami$_{11}$-CBD$_{11}$	pUC19::LacIeGFP	eGFP module	eGFP-Nco-Fw	ATATAG**CCATGG**TGAGCAAGGGCGAGGAGCT	Ref. [24]
			eGFP-Rv2	CTTGTACAGCTCGTCCATGC	Ref. [24]
	pIVEX2.3d::Lys11	Ami$_{11}$-CBD$_{11}$ region	Lys11_Ami11_SH3b-Rw	TCTCGGCATGGACGAGCTGTACAAGCCGAATTTTAAAAGTGAGACAGC	Ref. [24]
			Lys11Rv-Xma	GTC**CCCGGG**ACTGATTTCTCCCCATAAGTCA	Ref. [21]
eGFP-CBD$_{11}$	pUC19::LacIeGFP	eGFP module	eGFP-Nco-Fw	ATATAG**CCATGG**TGAGCAAGGGCGAGGAGCT	Ref. [24]
			eGFP-Rv2	CTTGTACAGCTCGTCCATGC	Ref. [24]
	pIVEX2.3d::Lys11	CBD$_{11}$ module	Lys11_SH3b_Fw	TCTCGGCATGGACGAGCTGTACAAGGATGGTAAAATACCGGTTGCCAC	This work
			Lys11Rv-Xma	GTC**CCCGGG**ACTGATTTCTCCCCATAAGTCA	Ref. [21]

[1]Restriction site sequences are in bold: CCATGG, NcoI; CCCGGG, XmaI

[2]Underlined sequences denote the complementary region of primers allowing Overlap Extension-PCR

Chapter IV.

General Discussion

AMR is an emergent global challenge that connects human, animal, and environmental health sectors, being recognized under the One Health framework. The injudicious use of antibiotics in these sectors, principally in human medicine, has accelerated the emergence, establishment and dissemination of resistant bacteria, giving rise to infections increasingly difficult to treat. While controlling antibiotic use is crucial, there is also an urgent need for novel antimicrobial agents to address this problem.

IV.1. Endolysins action from within (phage infection) and from without (enzybiotic): parallels and contrasts

Landmark studies from V. Fischetti and colleagues showed, more than 20 years ago, that phage endolysins could eliminate Gram-positive human pathogens in animal models of upper respiratory tract colonization[1,2]. This was a demonstration that endolysins, as lytic enzymes capable of degrading the CW of host bacteria to release phage progeny ("lysis from within"), could also be used to induce "lysis from without", not only *in vitro*, but also *in vivo*. This was a key finding for the establishment of the (endo)lysin therapy field. Historically, the field was first driven by studying endolysins targeting Gram-positive bacteria. These bacterial cells were considered more susceptible to the exolytic action of the enzymes due to the more exposed peptidoglycan. Indeed, later expansion of the field to Gram-negative systems required endolysins to be modified, or used in conjunction, with OM-permeating agents[3]. However, a growing body of evidence has been accumulating in the last years supporting that endolysins added exogenously to Gram-positive bacteria do not exactly recapitulate the lytic efficiency observed in the phage infection context. In fact, in some conditions, these bacteria can present some tolerance or even completely restrict the peptidoglycan degrading action of endolysins reaching the CW from outside. The goal of this thesis was to increase our knowledge on the cellular and enzymatic factors involved in endolysin tolerance and explore this knowledge to propose strategies aiming at improving the exolytic action of endolysins, and thus their enzybiotic potential.

Fernandes and São-José[4] demonstrated that Gram-positive bacteria maintained under conditions supporting an energized CM and cell division could counteract the lytic activity of canonical endolysins reaching the CW either from within (when endowed with an artificial secretion signal) or from without (when added to cells as purified proteins). Such capacity to fightback the endolysins was severely impaired when the membrane PMF was dissipated by the holin function or by the membrane depolarizing agent gramicidin. Thus, it could be inferred that after holin-mediated PMF collapse, phage-infected cells are much more susceptible to the lytic action of endolysins when compared to growing cells challenged with the same amount of endolysin from without. In fact, the

same study has estimated for a specific endolysin – LysSPP1 targeting *B. subtilis* – that the amount of enzyme needed to lyse from without an exponentially growing culture was 75 to 100-fold higher than that needed to lyse an equivalent cell population from within as result of phage infection. In agreement with a role of the PMF in controlling the endolysin, in presence of gramicidin the amount of LysSPP1 needed to cause similar exolysis could be reduced about 60-fold[4]. Thus, in essence canonical endolysins seem to be subjected to the same regulatory mechanisms that restrain exported endolysins in non-canonical lysis models, in which full activation of the lytic enzymes pre-positioned in the CW only occurs after (pin)holin-mediated PMF dissipation (see section I.5.2). Overall, these results indicated that "healthy" bacteria can exert some control over canonical endolysins that impacts their lytic action and, potentially, their enzybiotic performance. In line with this observation, a few studies have reported increased bactericidal action of endolysins when working together with nisin, a bacteriocin known to cause PMF dissipation of susceptible bacteria[5-7]. All these data indicate that the membrane PMF is a general determinant of endolysin tolerance and that agents abolishing the PMF can potentiate the lytic action of endolysins.

With these observations in mind, in Chapter II we have explored the PMF-collapsing action of AMPs, another class of non-traditional antibacterials, to potentiate the exolytic action of anti-staphylococcal endolysins. We have tested two AMPs, both reported to have anti-staphylococcal activity and low toxicity to mammalian cells[8,9]. As anticipated, the two AMPs could greatly sensitize growing bacteria to the lytic action of the endolysins and, for the AMP/endolysin combination studied in detail (R8K/Lys11), a bactericidal gain of up to 3 logs resulted from the joint action of the two agents. This increased bactericidal activity was also observed in human blood serum. Therefore, our work showed that selected AMPs could be used to enhance the enzybiotic potential of endolysins for treating human infections caused by Gram-positive pathogens, an antibacterial strategy that is clearly underexplored.

One important observation of this study was that the R8K enhancing effect on Lys11 lytic action was not merely attributed to the killing activity of the AMP, but instead to its PMF-dissipation action. In fact, when we tested the capacity of gentamycin (inhibits protein synthesis), ampicillin (blocks peptidoglycan synthesis) and daptomycin (PMF-dissipator) to sensitize *S. aureus* to Lys11-mediated lysis, only daptomycin clearly turned cells more vulnerable to Lys11, even if gentamycin produced the same cell killing effect. Therefore, simple inhibition of bacterial growth, or even cell death, may not be sufficient to increase bacterial susceptibility to endolysin bacteriolysis; sudden collapse of the PMF appears to be the key sensitizing event, akin of the holin role in the phage infection context.

Previous studies showed that CM targeting agents (nisin and lipopeptide antibiotics, including daptomycin) synergized with enzybiotics such as the bacteriocin lysostaphin or the endolysins Cpl-1 and PlySs2 (also known as CF-301 or Exebacase) to eliminate pathogens like *S. aureus* and *S. pneumoniae*, *in vitro* and *in vivo*[10-13]. In these studies, the preferred hypothesis for explaining the observed synergisms was that the degradation of the peptidoglycan-mesh by the enzybiotics could

assist in a faster and more efficient insertion of the PMF-dissipating agents into the CM. However, the results obtained in Chapter II and Chapter III are more consistent with an alternative explanation, in which the PMF dissipation caused by the CM-targeting antimicrobials boosted the bacteriolytic action of the enzybiotics. According to our studies, such enhancement of the lytic action derives both from an increased binding and peptidoglycan cleavage activity of the endolysins in response to collapse of the PMF, which works as a major determinant of endolysin tolerance. Supporting also this alternative view, we have seen that the bacteriolytic synergy between the AMP R8K and the endolysin Lys11 could be observed irrespective of the order of addition of the agents (Supplementary Figure SII.4). Still, treating cells with Lys11 before R8K addition produced a slightly slower lysis compared to the inverse order (seen for endolysin lower concentrations). Therefore, the results suggest that peptidoglycan degradation promoted by the endolysin did not significantly improve AMP penetration through the peptidoglycan mesh to reach the CM. Indeed, it is not expected that AMPs and lipopeptides, typically under 5 kDa, will become sequestered by the peptidoglycan mesh, which acts as a molecular sieve for compounds larger than 50 kDa[14]. Teichoic acids, as major determinants of the electrostatic properties of the cellular surface, seem to have a more preponderant role in controlling the interaction of these antimicrobial agents with Gram-positive bacteria[15,16]. Nevertheless, a recent study presented evidence supporting that peptidoglycan degradation by an endolysin can facilitate the penetration and action of the human antimicrobial peptide LL-37 against *Enterococcus faecalis*[17].

In Chapter III, we studied in more depth how the PMF impacted the exolytic action of Lys11, our model anti-staphylococcal endolysin. For this purpose, we employed ionophores that abolished either or both gradients composing the PMF (ΔpH and Δψ) and assessed how they affected Lys11 binding to cells and its lytic action. As expected, dissipation of both PMF gradients with gramicidin strongly enhanced Lys11 staphylolytic action. Yet, gramicidin was not as effective as peptide R8K in stimulating lysis (Figure III.1a and Figure II.2a). This suggests that the AMP contributed to lysis by other means besides leading to PMF collapse. Although in our experimental conditions R8K appeared to provoke neither significant cell lysis *per se* (except for strain USA200), nor major alterations of the *S. aureus* cell surface features (strain RN4220), it is possible that the AMP caused changes in the CW that facilitated endolysin action and/or penetration.

Selective dissipation of each component of the PMF revealed ΔpH as the preponderant gradient counteracting Lys11 lytic action. The pH gradient results from the accumulation of protons outside the CM, which generates a low pH environment in the inner layers of the CW. The negatively charged WTA are thought to retain the protons in the CW and thus assist in the maintenance of this acidic milieu, which was proposed to restrain the action of bacterial autolysins[18-20]. It is likely that the ΔpH also works as a negative regulator of endolysins, given that they are closely related, both in structure and function, to bacterial autolysins. The ΔpH appeared to exert its inhibitory role mainly on the Lys11 peptidase domain, which was found paramount for the enzyme peptidoglycan cleavage

activity. An interesting antibacterial approach against *S. aureus* (as probably other Gram-positive pathogens) could be then the combined action of endolysins and compounds targeting the ΔpH compatible with therapeutic use. Indeed, usage of the PMF components as drug targets has been limited due to toxicity issues[21].

When acting from the outside of Gram-positive bacteria, endolysins face a CW decorated with secondary polymers, such as WTA and LTA, which can interfere with their lytic action. Several studies have established a correlation between the presence of WTA and an inhibition of the activity of lytic proteins[22-24]. In these studies, WTA were found to exert a shielding-like effect, inhibiting either the overall binding of the enzymes to the cell surface or excluding them from certain regions of the CW. In Chapter II and Chapter III, we have studied the binding properties of Lys11 using as proxy the fluorescent eGFP-Ami_{11}-CBD_{11} fusion, which carries the two domains involved in binding, Ami_{11} and CBD_{11}. The fusion could bound around the entire CW surface, with some images suggesting a preferential accumulation at cell junctions (Supplementary Figure SII.7). Total eGFP-Ami_{11}-CBD_{11} binding to *S. aureus* cells with reduced WTA content (grown in presence of tunicamycin) was at least 3-fold higher. Such increase in binding was essentially mediated by CBD_{11}, since association of eGFP-Ami_{11} was unaffected by the reduction of WTA levels (Chapter III). CBD_{11} belongs to the recently proposed structural family SH3_P1[25], which includes the CBDs of the previously annotated family SH3_5 (Pfam database entry PF08460) of the SH3 superfamily. The CBD of lysostaphin is a well-studied representative of this family, that was shown to target the pentaglycine cross-link of the *S. aureus* peptidoglycan[26,27]. Therefore, WTA probably work as a physical barrier that hinders CBD_{11} access to the peptidoglycan. However, our studies have shown for the first time that the inhibitory role of WTA appears to go beyond a simple shielding effect, since for the same amount of bound Lys11, lysis is much faster and extensive in WTA-depleted cells (Supplementary Figure SII.8). Although we have not studied if WTA reduction changed the pattern of eGFP-Ami_{11}-CBD_{11} fusion distribution on the cell surface, it is tempting to propose that WTA also work as restrictors of the Lys11 peptidoglycan cleavage activity, in this case by inhibiting $CHAP_{11}$, the key domain responsible for cutting the peptidoglycan. An indirect support to this hypothesis emerges from the fact that addition of 500 nM of eGFP-Ami_{11}-CBD_{11} to normal cells and of 100 nM of eGFP-CBD_{11} to WTA-depleted cells results in almost the same amount of bound protein (~200 nM·OD_{600nm}^{-1}, Figure III.3a and Figure III.5, respectively). Yet, in the corresponding lysis assays with Lys11 against normal cells (Figure III.3b) and with $CHAP_{11}$-CBD_{11} against tunicamycin-treated cells (Figure III.7d), lysis is much faster and extensive in the latter condition. Whether this WTA inhibitory effect is direct or indirect remains unknown. However, considering the postulated role of WTA in maintaining the ΔpH-derived protonation of the CW (see above), it is possible that the effect is indirect. In total alignment with the results presented in this thesis, studies from J.S. Dordick's lab showed that when cells of species such as *C. difficile*, *S. aureus* and *B. cereus* are maintained under growth-supporting media (*e.g.* BHI, TSB, LB) they are very refractory to the action of lytic enzymes[23,24,28]. Such cell tolerance depends

on the presence of WTA in the CW, which were shown to impair lysin binding. Interestingly, the WTA-mediated protection was drastically decreased when cells were washed and resuspended in non-nutritional media such as PBS buffer (also seen for Lys11, results not shown). The authors proposed that the proper WTA conformation conferring protection to the lytic enzymes could be lost when cells are changed to PBS. However, while cell viability is maintained for some time in buffered solutions like PBS, the PMF is expected to be affected due to the stopping of cell metabolism in absence of nutrients. Thus, considering the abovementioned link between the PMF and the proton-binding capacity of WTA, it is possible that the observed effect results from a progressive deprotonation of the WTA when cells are transferred to PBS. In any case, most likely cell tolerance/susceptibility to the exolytic action of enzymes is a multifactorial process involving an interplay between the cell energetic state, CW polymers and peptidoglycan reparations mechanisms.

In summary, from the exposed above it seems clear that when working as enzybiotics against Gram-positive bacteria, endolysins face specific challenges compared to their action in the natural context of phage infection. Specifically, when acting from outside endolysins face cells with an operational PMF that generates a CW environment unfavourable for lytic activity, whereas at the end of phage infection the lytic enzymes act after PMF dissipation mediated by the holin channels. In addition, endolysins attacking from without are more likely to experience the inhibitory role of WTA as these polymers are thought to be enriched in the outermost fibrous layer of the cell wall, extending beyond the peptidoglycan mesh[15,29].

IV.2. Tolerance of Gram-positive bacteria to the exolytic action of endolysins: is it universal and problematic?

The studies presented in this thesis contributed to understand how the PMF and WTA can restrain the (exo)lytic action of endolysins against Gram-positive bacteria, by showing that these tolerance determinants can impact both the cell binding capacity and the peptidoglycan cleavage efficiency of the enzymes' functional domains. However, one picture that emerges from the results here reported and from previous literature, is that although these determinants appear to operate in different systems, the level of observed tolerance varies significantly depending on the bacterium/endolysin tested, and on the testing conditions. For example, tolerance of *B. subtilis* strain 168 against LysSPP1 was clearly evidenced only when cells were challenged with a relatively low concentration of the endolysin (2.5 μg/ml), whereas for the pair *S. aureus* RN4220/Lys11 tolerance could be clearly seen with an enzyme concentration of 20 μg/ml[4]. For other bacterium/lytic enzyme combinations[6,23,28] or growth conditions (*e.g.*, vigorous aeration[4]), bacteria could fully survive

or even grow in presence of 20 to 150 μg/ml of the lytic enzymes. These data strongly suggest that the intrinsic properties of specific bacteria and endolysins will dictate the level of tolerance.

In line with these previous observations, we have seen for the same bacterial strain, in this case *S. aureus* strain RN4220, different levels of tolerance to the related endolysins Lys11 and LysK. For the same experimental conditions (cells growing under static conditions in fresh TSB medium), RN4220 was more tolerant to Lys11 lytic action than to LysK. Interestingly, in the presence of a PMF-dissipating AMP, the difference between the two enzymes is attenuated as both cause similar lysis profiles at identical protein concentrations (Supplementary Figure SII.3 and Figure SII.5a). The high bacteriolytic synergy resulting from the combined action of Lys11 and R8K on the lab strain RN4220 was essentially replicated in a panel of 6 MRSA strains, each representing a different clonal complex. However, in absence of the peptide, different levels of tolerance to Lys11 bacteriolysis were observed, with strains showing growth in presence of the endolysin (strain GRE14), no growth but also no significant cell lysis (strain WIS), or significant cell lysis (strain USA200) (Figure II.3). Although we have not studied the reasons behind these variations, they might result from differences in the overall structure of the peptidoglycan, namely the level of peptidoglycan cross-linking and modifications of the glycan strands, variations in the CW content of WTA, and differences in the type/level of modifications of CW polymers. For instance, MRSA *S. aureus* strains USA300 and MW2 are described has having higher levels of WTA in the CW compared to the laboratory strain SA113[30], and this could explain the higher susceptibility of the later strain (Figure III.4d) – and eventually of strain USA200 (Figure II.3) – to Lys11. However, impairment of D-Alanine modification of TA in SA113 (Δ*dltA* mutant) turned the strain much less susceptible to Lys11 lytic action (Figure III.4d). These examples illustrate how changes in CW composition/modification can affect the exolytic capacity of endolysins. In addition, known adaptations of antibiotic-resistant *S. aureus* strains include the thickening of the CW[31], something that could also contribute to a higher tolerance to the bacteriolytic action of endolysins, as more layers of peptidoglycan need to be degraded to cause lysis.

One question that remains largely unanswered is whether the tolerance determinants we have studied *in vitro* can have a significant impact on the exolytic action of endolysins when these are applied as enzybiotics in *in vivo* systems. We showed that at least in an *ex vivo* assay (human blood serum), tolerance to Lys11 was still seen and a bactericidal gain resulted from the R8K/Lys11 combined action (Figure II.4b). The *in vitro* evaluation of the antibacterial action of endolysins is typically performed by two methods: the turbidity reduction assay and the killing assay[32]. These assays provide important information about the mode of action of endolysins. The turbidity reduction assay measures the decrease of the optical density of bacterial suspensions, which reflects cell burst due to the peptidoglycan-degrading activity of the endolysin. Determination of cell viability allows the quantification of the killing effect resulting from the enzyme action. However, retrospective analysis of the literature reveals that the turbidity and killing assays are mostly performed with cells washed and resuspended in nutrient-depleted, buffered solutions. As showed by Vazquez et al. [33], the ionic

strength and tonicity (osmolarity) of these buffers can have great influence on the killing effect of lysins. For example, buffers with low ionic strength may favour the electrostatic interactions of positively charged lysins with the CW and hypotonic solutions can enhance bacteriolysis by promoting higher cell turgor pressure. In addition, although cell viability is maintained in these environments, cell division stops, and metabolic activity progressively shuts down, which is expected to negatively impact the PMF-dependent tolerance mechanisms. Thus, it is likely that in these conditions the antibacterial performance of lytic enzymes is artificially increased. For these reasons, it has been argued that these *in vitro* assays may not be good predictors of the enzybiotics efficacy in the complex environments of *in vivo* systems, not only because of factors that can directly affect lysins stability/activity, but also because these environments often support bacterial growth[32,33]. Ideally, the bactericidal action of the enzybiotics should be confirmed in conditions mimicking as close as possible the media in which they are intended to be used. In our studies, the bacteriolytic and bactericidal action of the endolysins were tested in TSB culture medium supplemented with 0.5 mM calcium chloride, which essentially is an isotonic medium with physiologic salt concentrations. Review of the *in vivo* studies using animal models also shows that in most cases good efficacy is observed only when the lytic enzymes are administrated to animals soon after bacterial challenge, typically 1 hour post bacterial infection, suggesting a limited action of the enzybiotics when bacteria resume robust growth *in vivo*[32]. All these observations can be viewed as indirect evidence that the tolerance mechanisms might be operating at least under certain *in vivo* infection contexts.

None of the assays used to assess endolysins bactericidal activity has standard guidelines approved by the reference international entities responsible for the standardization of antimicrobial susceptibility testing (CLSI in the USA and EUCAST in Europe). Evaluation of the antibacterial efficacy of traditional antibiotics is usually done by determining the minimum inhibitory concentration (MIC) by CLSI/EUCAST standardized methods. MIC evaluation provides the information regarding the minimum concentration of an agent needed to completely inhibit visible bacterial cell growth, accounting for both bacteriostatic and bactericidal effects. MIC determination is performed in nutrient rich media (Mueller–Hinton medium for most bacteria), therefore allowing for bacterial robust growth. This may explain why some lysins displaying high bacteriolytic action in the previously described assays are not amenable to standard MIC determinations[34,35], hampering the assessment of endolysins therapeutic potential and prediction of *in vivo* efficacy. A recent study proposed a modified version of the broth microdilution method for endolysin MIC determination, ensuring generation of accurate and reproducible susceptibility data, with the endorsement of the CLSI Subcommittee on Antimicrobial Susceptibility Testing[36].

In conclusion, current knowledge strongly suggests that tolerance to the exolytic action of native endolysins is a common trait of Gram-positive bacteria when thriving in media that support cell growth, with the level of tolerance being determined in great extent by the specific properties of target bacteria and of the lytic enzymes.

IV.3. Implications for Enzybiotic Development

Although the antibacterial potential of endolysins against Gram-positive pathogens has long been demonstrated *in vitro*, their strong activity is often not reproduced under *in vivo* conditions. To try to bridge this gap, and to generate antibacterials with higher efficacy *in vivo* or in complex matrices, there are some engineering strategies available to improve lysins activity from without. The diversity of lysin functional domains and architectures opens the possibility to create genetically engineered lytic enzymes, with not only increased antibacterial properties, like the killing efficiency and spectrum of activity, but also with other improved features, such as solubility and stability[37,38]. Shuffling and fusion of CDs and CBDs of different origin has been the most common engineering strategy to generate novel enzybiotics with enhanced characteristics[39-41]. Perhaps not surprisingly, when large combinatorial libraries of lysin functional domains are screened in more stringent conditions (complex matrices like blood serum and milk), usually the best performing hits are non-natural, chimeric or mutated enzymes, frequently with functional domains, such as the M23 CD (see below), which are not commonly found in native endolysins[42-44]. One possible interpretation is that these non-natural lysins are much less susceptible to the tolerance mechanisms that counteract the lytic action of native endolysins.

Previous studies have suggested that modulation of endolysins net charge to favour the electrostatic interactions with the surface of target cells could improve their antibacterial action. Most bacterial surfaces have a negative charge, and thus the common approach has been to increase the net positive charge of lytic enzymes, under the rational that higher lysin binding should translate into higher lytic activity (reviewed in Ref. [39]). A recent study investigated how the cell surface charge properties affected the lytic action of two lytic enzymes of *Staphylococcus pettenkoferi*, which were closely related in terms of functional domains but had very different isoelectric points (5.7 vs 10.3). The conclusions were that electrostatic interactions favouring association of the enzymes to the cell surface resulted in general in higher bacteriolytic activity[45]. This was in part corroborated by the results obtained in this thesis, but we showed that binding capacity is not the sole determinant of bacteriolytic action, since tolerance determinants also affect peptidoglycan cleavage activity. In addition, at least in one endolysin, increasing its net positive charge did not result in better lysis kinetics[46].

The strong binding of the endolysins to the CW debris of Gram-positive bacteria after phage-mediated lysis has been put forward as a possible mechanism that prevents diffusion of the lytic enzymes, which otherwise could be harmful for uninfected host cells in the vicinity[47,48]. In agreement with this view, in Chapter III we showed that Lys11 presented higher binding to *S. aureus* cells with dissipated PMF, which is a hallmark of the holin action at the end of phage infection. Interestingly, this increased binding appeared to result from a notorious enhancement of the binding activity of the enzyme's amidase domain in response to the collapse of the Δψ component of the PMF. While

high binding capacity of an exogenously added endolysin will likely contribute to faster initial cell lysis, if the enzyme then becomes tightly associated with the cleaved CW it will hinder its ability to introduce new cuts in other regions of the peptidoglycan network. In a scenario of very high affinity, it has been postulated that endolysins may work as "single-hit" enzymes. At least for lysostaphin, it was shown that tunning down this high affinity could result in higher processivity and lysis efficacy[49]. Quite interestingly, the elimination of the amidase domain in some endolysins analogous to Lys11 was shown to result in improved lytic performance[50,51]. Based on our results, it is tempting to speculate that such deletion produced lytic enzymes with a balanced CW affinity for several binding-release cycles, thus becoming "multiple-hit" enzymes. This was not observed, though, with our amidase-deletion construct $CHAP_{11}$-CBD_{11} that was always outperformed by the native endolysin Lys11 in our experimental conditions (Figure III.6 and Figure III.7), probably due to the very low binding mediated just by CBD_{11} (Figure III.2c). However, when we performed the same deletion analysis with LysK, which carries a CBD with much higher binding activity, the resulting $CHAP_K$-CBD_K outperformed the native endolysin in terms of lysis extension and cell killing effect (unpublished data). These results suggest that, depending on the intrinsic features of endolysins functional domains, a reduction of binding affinity may translate into better bacteriolytic performance.

The studies here presented indicate that the peptidoglycan cleavage efficiency of endolysin CDs can be impacted by the level of protonation and amount of WTA in the cell wall. A strategy to overcome these tolerance determinants could be equipping endolysins with CDs that, in theory, are better adapted to degrade the peptidoglycan of "healthy" bacteria from without. One source of these CDs could be VALs, which are peptidoglycan degrading functions carried in the phage virus particle, often in the tail structure, which assist on tail tube penetration and phage DNA injection across the CW (Section I.5.2.1). A common CD found in phage VALs, including in staphylococcal phages, is the M23 endopeptidase domain (Pfam database entry PF01551, Ref. [52]). This domain is also present in the well-known bacteriolysin-type bacteriocins lysostaphin, enterolysin A and zoocin A, which are secreted by producing bacteria to kill from without competitors disputing the same niche[52]. As referred to above, in screening protocols chimeric endolysins containing the M23 domain are frequently isolated as the best performers in complex environments and, for at least one of these chimeras, EC300, it was demonstrated its lower sensitivity to the restraining action of the PMF[6] and high efficacy *ex vivo*[53].

Based on the accumulated data indicating that compromising the PMF turns cells more susceptible to endolysins, we had proposed to fuse into single agents selected AMPs and endolysins (AMPLys), with the goal of obtaining modified endolysins with enhanced antibacterial characteristics. Several different AMPLys fusions were produced during this work (examples in Supplementary Figure SII.8), but unfortunately none of the obtained AMPLys exhibited better bacteriolytic properties than the parental endolysin (Supplementary Figure SII.9). This result was somewhat unexpected, as many reports demonstrated that fusing peptides with AMP-like features to endolysins can give rise to

enzybiotics with much higher bactericidal action, even in *in vivo* assays. It is true however, that this strategy has been principally applied to endolysins targeting Gram-negative pathogens, whose principal barrier to the exolytic action of endolysins is an OM protecting the peptidoglycan layer. Thus, it is likely that in these cases the key function of the peptide moiety is just to facilitate OM crossing, and not insertion in the CM for PMF dissipation[54]. Nonetheless, at least one study reported that the AMP moiety fused to a Gram-negative targeting endolysin was able to permeabilize the OM and depolarize the CM[55]. In the case, the AMP was fused to the lysin through a specific linker that emerged from a screening of a variety of linkers, and the authors were able to demonstrate that the linker moiety is crucial for the antibacterial activity.

Most probably, in our AMPLys fusions, AMP penetration and accumulation in the CM was inhibited by steric hindrance, in addition to possible negative impacts on the more complex structure of endolysins targeting Gram-positive bacteria. Also, due to the thicker peptidoglycan layer of Gram-positive bacteria, it is likely that the linker region in these fusions needs to be adjusted, allowing the AMP to reach the CM. A flexible and longer linker may accommodate the translocation of the AMP across the peptidoglycan towards the CM. Moreover, AMP action often relies on the accumulation of a threshold concentration in the CM, something that might be difficult to attain when the peptide is fused to an endolysin.

IV.4. References

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Chapter V.

Concluding Remarks and Future Perspectives

The fundamental idea that drove all the work presented in this thesis was that the exolytic power of endolysins towards energized Gram-positive bacteria is much weaker than the enzymes' lytic action taking place from within at the end of phage infection, after holin-mediated PMF dissipation. This notion was built from previous studies showing that the membrane PMF of actively growing bacteria, together with CW secondary polymers like WTA, somehow antagonized endolysin attack from outside, conferring to cells some tolerance to the lytic enzymes. Thus, one of the major goals of this project was to devise a strategy to enhance endolysins action from without by targeting one of the tolerance determinants, the PMF. By using as model anti-staphylococcal endolysins, we showed that the PMF-dissipating action of AMPs can be used to greatly improve the exolytic action of endolysins, with the combined action of the two agents translating into a higher bactericidal effect. We have also obtained data supporting that conventional antibiotics targeting the membrane PMF, such as daptomycin, can also fulfil the role of enhancing endolysins exolytic action. To prove that these strategies can be extended to other Gram-positive bacterial species, it would be interesting to expand the range of tested endolysins and AMPs/antibiotics. An important step for validation of these approaches is to test them *in vivo*, using animal models of infection such as *Galleria mellonella*, zebrafish or mice. Further developments include the study of the scalability of production, formulation, and pharmacokinetics and pharmacodynamics studies.

Most known human pathogens can produce biofilms, resistance structures that actively increase bacterial survival under hostile conditions and contribute to the failure of antibiotic treatments. In this way, the anti-biofilm potential of AMPs and endolysins conjoint action should be evaluated, both in static and dynamic models. The advantage of such approach in biofilm eradication is that neither agent requires actively dividing bacteria to exert their antimicrobial action. In this case, endolysins could also be combined with enzymes capable of degrading the extracellular matrix that composes the biofilm, such as phage-derived depolymerases.

Another important goal of this work was to study in more depth how the PMF and CW secondary polymers contributed to bacterial tolerance against endolysins. By using ionophores targeting both or either component of the PMF (Δψ and ΔpH), we demonstrated for the first time that each PMF gradient can impact endolysin binding and cleavage functions by distinctively interfering with the enzyme's functional domains. For the endolysin studied in detail, Lys11, the ΔpH has a major role in restraining Lys11 peptidoglycan cleavage mediated by the CHAP peptidase domain, whereas Δψ appears to mostly impair endolysin binding to cells assisted by the amidase domain. Our studies confirmed WTA as key tolerance determinants and provided details on how they antagonize endolysins lytic action. WTA confer protection by acting mainly at two levels: i) inhibition of Lys11 association to the staphylococcal cell surface by interfering with the binding activity of the CBD, and ii) by repression the CHAP-mediated peptidoglycan cleavage. Our results strongly indicate an interdependence of the ΔpH and WTA to provide endolysins tolerance. As already discussed, WTA are thought to retain protons in the inner layers of the CW, thus contributing to the ΔpH-mediated

endolysins restriction. Therefore, any drug or strategy capable of removing WTA from the CW or inhibiting their synthesis should also turn bacteria much more susceptible to the exolytic action of endolysins, something that deserves further exploration in the future.