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Phage–derived Endolysins as Potential Antibacterials

A Study of Peptidoglycan Hydrolase and Mycolylarabinogalactan Esterase Enzymes

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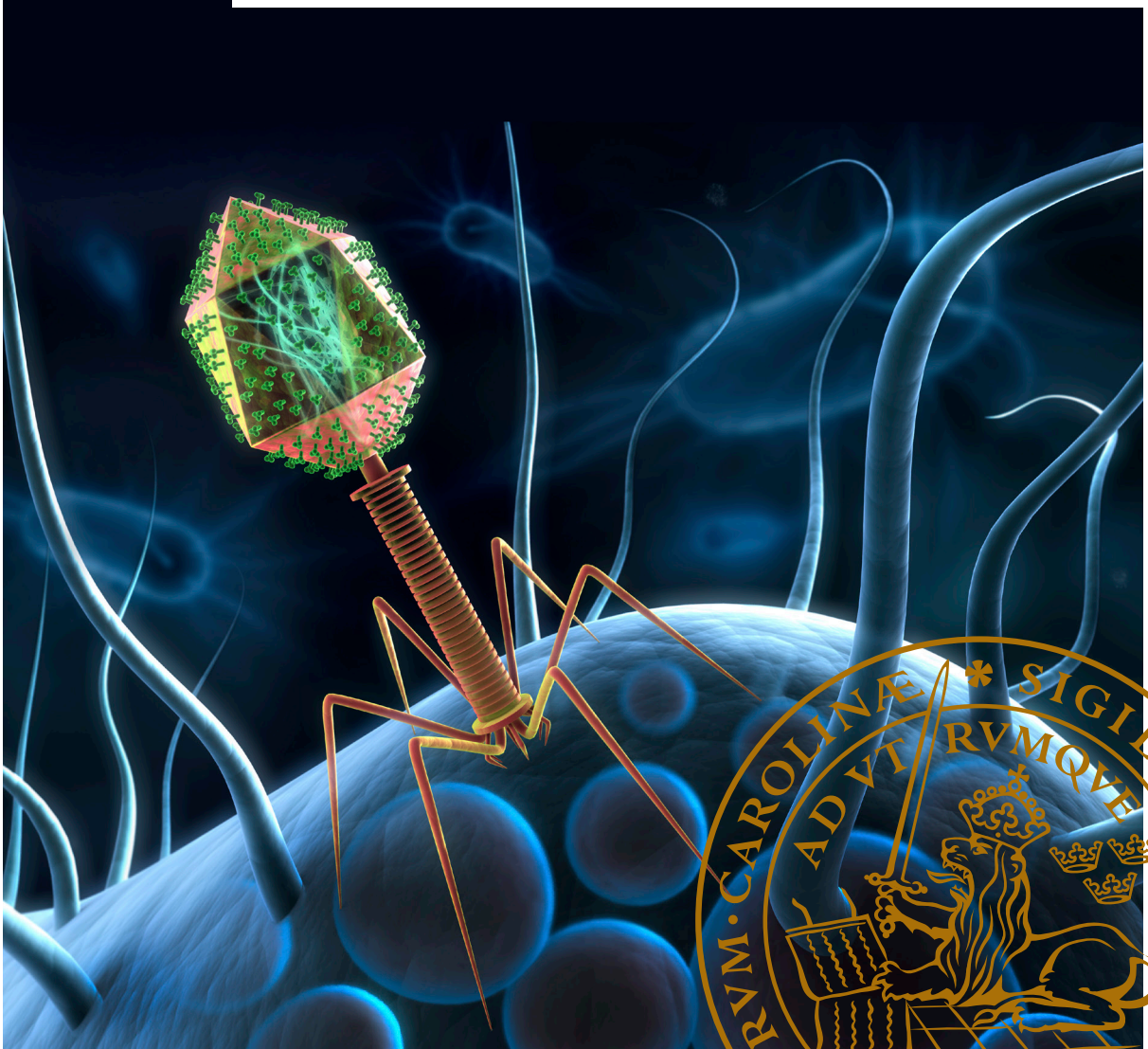

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Adel Elsayed Attia Abouhmad

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DOCTORAL DISSERTATION

2019

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Abstract

Bacteriophages, or phages, are viruses that infect bacteria, at the end of their lytic cycle produce a set of enzymes called endolysins to lyse host cells from within facilitating the release of the viral progeny. Due to their lytic activity, endolysins have gained great interest as potential antibacterials targeting both Gram–positive and –negative bacteria, especially in the actual context of increasing rates of antibiotics resistance. This approach relies on the observation that, external application of recombinant endolysins (enzybiotics) can efficiently lyse target bacteria from without. The current thesis explores the potential of two groups of endolysins, peptidoglycan hydrolase and mycolylarabinogalactan esterase as potential antibacterials. The peptidoglycan hydrolases hydrolyze glycosidic and amide bonds in the peptidoglycan layer of the bacterial cell wall, while mycolylarabinogalactan esterases hydrolyze the ester bond between mycolylarabinogalactan and peptidoglycan in mycobacterial cell wall. The current thesis approach was accomplished through development of novel strategies for immobilization, increasing the spectrum of activity, improving stability and characterization of novel enzymes.

Different strategies for immobilization of the well–known peptidoglycan hydrolase, lysozyme from T4 bacteriophage and its antibacterial activity was studied. Immobilization of the T4 lysozyme (T4Lyz) to wound dressing gauze in a single facile binding step was achieved through engineering the endolysin with a cellulose binding module (CBM) as a fusion tag. T4Lyz–CBM–immobilized gauze retained antibacterial activity against Gram–positive *Micrococcus lysodeikticus* (3.8 Log_{10} reduction) and Gram–negative *Escherichia coli* and *Pseudomonas mendocina* with 1.59 and 1.39 Log_{10} reduction, respectively.

In another approach, the antibacterial activity and storage stability of the T4Lyz as well as Hen Egg White Lysozyme (HEWL) were enhanced via covalent immobilization to tailored positively charged aminated cellulose nanocrystals (Am–CNC). Am–CNC–lysozyme conjugates retained muralytic activity of 86.3% and 78.3% for HEWL and T4Lyz, respectively. Am–CNC–T4Lyz conjugates also showed enhanced bactericidal activity with MIC (minimum inhibitory concentration) values of 62.5, 100, 500 and 625 µg/ml against *M. lysodeikticus, Corynebacterium* sp., *E. coli* and *P. mendocina*, respectively. The Log_{10} reduction of the tested bacteria occurred in a relatively shorter time as confirmed by time kill study using Alamarblue® as metabolic indicator dye. Transmission electron microscopy revealed altered membrane morphology of the cells treated with the conjugates. The immobilized preparations further exhibited enhanced storage stability at 4 and 22 °C.

The second part of the study dealt with lysin B (LysB), a mycolylarabinogalactan esterase produced by mycobacteriophages that infect mycobacterial cells, which possess a unique cell wall structure with a thick mycolic acid layer. In this work, the genome database of mycobacteriophages was explored to find and categorize LysB enzymes based on similarity to LysB–D29, the only LysB with available crystal structure. Comparative structural analysis of some novel mycobacteriophage LysB enzymes resulted in homology modeling of 30 LysB proteins different in their similarity to LysB–D29. Structure alignment showed that LysB enzymes are not true lipases due to the lack of the lid domain which was confirmed by testing the esterase activity of LysB–D29 against *para*–nitrophenyl butyrate (*p*NPB) in presence and absence of surfactant. Our results showed that unlike true lipases, LysB–D29 has higher enzymatic activity in the absence of Triton X–100 as a surfactant and hence doesn't require interfacial activation. Moreover, some LysB homologs with different degree of similarity to LysB–D29 were cloned and recombinantly expressed in *E. coli* BL 21 (DE3) expression host. Characterization of their kinetic parameters for the hydrolysis of *para*–nitrophenyl ester substrates showed LysB–His_6 enzymes to be active against range of substrates (C4–C16), with a catalytic preference for *para*–nitrophenyl laurate (C12). Moreover, LysB–His_6 enzymes have the highest catalytic activity at 37°C, and some divalent metal ions e.g. Ca^{2+} and Mn^{2+} enhance the catalytic activity. The mycolylarabinogalactan esterase activity for hydrolysis of mycolylarabinogalactan—peptidoglycan complex as substrate for the LysB–His_6 enzymes was confirmed by LC/MS. Extracellular application of LysB–His_6 against *Mycobacterium smegmatis* resulted in marginal antibacterial activity. However, combining LysB–His_6 enzymes with half MIC (1 µg/ml) of colistin (outer membrane permealizer) enhanced the antibacterial activity of LysB–His_6 enzymes against *M. smegmatis*.

Key words: Antibacterial, Endolysin, Nanocrystals, Immobilization, Mycolylarabinogalactan esterase

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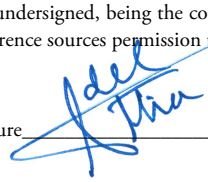
Signature  Date 2019-08-27

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MADE IN SWEDEN

Dedicated to

my parents,
my wife Marwaa,
my kids Mohammad, Mariam and Khadija

Table of Contents

Abstract

Bacteriophages, or phages, are viruses that infect bacteria, at the end of their life cycle produce a set of enzymes called endolysins to lyse host cells from within, facilitating the release of the viral progeny. Due to their lytic activity, endolysins have gained great interest as potential antibacterials targeting both Gram–positive and –negative bacteria, especially in the actual context of increasing rates of antibiotics resistance. This approach relies on the observation that external application of recombinant endolysins (enzybiotics) can efficiently lyse target bacteria from without. The current thesis explores the potential of two groups of endolysins, peptidoglycan hydrolase and mycolylarabinogalactan esterase as potential antibacterials. The peptidoglycan hydrolases hydrolyze glycosidic and amide bonds in the peptidoglycan layer of the bacterial cell wall, while mycolylarabinogalactan esterases hydrolyze the ester bond between mycolylarabinogalactan and peptidoglycan in mycobacterial cell wall.

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The mycolylarabinogalactan esterase Lysin B (LysB) is produced by mycobacteriophages that infect mycobacterial cells that possess a unique cell wall structure with a thick mycolic acid layer. The genome database of mycobacteriophages was explored to find and categorize LysB enzymes based on similarity to LysB–D29, the only LysB with available crystal structure. Comparative structural analysis of some novel mycobacteriophage LysB enzymes resulted in homology modelling of 30 LysB proteins differing in their similarity to LysB–D29.

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Popular Summary

Ensuring good health and well–being is one of the 17 sustainable development goals adopted by United Nations Member states. Sustainability of mankind is dependent to a great extent on our ability to prevent and cure diseases. The current dissemination of antibiotic resistance puts the future efficacy of current antibiotics under question. The misuse and overuse of existing antibiotics has led to the evolution of superbugs that are resistant to nearly all available antibiotics. Indeed, catastrophic scenarios are predicted indicating severe human and economic losses if we fail in finding new treatments with tens of million deaths per year and costs ascending to trillions of USD by 2050. Moreover, this threat is also associated with a very limited pipeline of new effective therapies from the pharmaceutical industry. Concerted efforts are thus required to tackle antimicrobial resistance and to discover new antibiotics and alternatives.

Among the various alternatives are bacteriophage derived enzymes, endolysins. Bacteriophages or simply phages are abundant in the environment and are considered as the natural enemy of bacteria and can help in eradicating pathogenic bacteria. The phages inject their own genetic code into a bacterial cell, turning it into a phage factory until the virus progeny bursts out of the cell by the action of the endolysins on the bacterial cell envelope. Endolysins have rapid onset of action and high potency (i.e. active at a very low concentration), and do not provoke resistance.

Despite their efficiency, endolysins are active mainly against Gram–positive bacteria. The high lipid content in the outer layer of both Gram–negative and mycobacteria protects them from the action of endolysins making them ineffective. Therefore, new strategies are being developed to extend the action of endolysins against Gram–negative and mycobacteria, for example binding of endolysins to tailored nanoparticles or using compounds that destabilize the outer layer of bacterial cell wall to grant access to the endolysins.

This thesis presents studies on different endolysins with potential antibacterial activity. The well–known endolysin from T4 bacteriophage was genetically modified to allow it to bind easily to a wound dressing gauze with retention of significant antibacterial activity. The same enzyme was also bound to biodegradable cellulose nanocrystals and used to kill both Gram–positive and –negative bacteria. Furthermore, new endolysins produced by bacteriophages infecting mycobacteria were identified in databases, and some of them were produced by recombinant DNA and tested for their activity to be a foundation for their application against the pathogenic *Mycobacterium tuberculosis* that causes the lung disease, tuberculosis.

Arabic Summary (**الملخص العربي**)

يعتمد نجاح وتقدم البشرية على قدرتها المستقبلية في الوقاية والعلاج من الأمراض والحد من إنتشارها. يحد الإنتشار الحالي لمقاومة البكتريا للمضادات الحيوية من فاعليتها المستقبلية مما يضع المضادات الحيوية اليوم على المحك. إن سوء إستخدام وإفراط إستخدام المضادات الحيوية الحالية قد أدى إلى ظهور ميكروبات مقاومة لجميع المضادات الحيوية المتوفرة تقريبًا. في الواقع، يتنبأ العلماء بسيناريوهات كارثية نتيجة مقاومة البكتريا للمضادات الحيوية مما قد يؤدي إلى خسائر بشرية واقتصادية جسيمة تقدر بعشرات الملايين من الوفيات كل عام وتكاليف تصل إلى تريليونات من الدولارات الأمريكية بحلول عام2050 إذا فشلنا في إيجاد علاجات جديدة. وعلاوة على ذلك، يرتبط هذا التهديد بمحدودية إكتشاف وإنتاج مضادات حيوية جديدة أمنة وفعالة في مواجهة هذه البكتريا. نتيجة لذلك، هناك حاجة ماسة لإكتشاف مضادات حيوية جديدة وأيضا البحث عن بدائل فعالة.

أحد هذه البدائل هو الإنزيمات المشتقة من البكتريوفاج (الفيروس المعدي للبكتريا) والتي تسمى أيضًا الإندوليسين. يعتبر البكتريوفاج والمعروف أيضًا باسم الفاج العدو الطبيعي للبكتيريا ويمكن أن يساعدنا في القضاء على البكتيريا المسببة للأمراض. يغزو البكتريوفاج البكتيريا ويحقن الشفرة الوراثية الخاصة به ويسخر الخلية البكتيرية ويحولها إلى مصنع لإنتاج جيل جديد من الفيروسات حتى تنفجر الخلية البكتيرية في نهاية المطاف من خلال عمل الإندوليسين محررة الجيل الجديد من الفيروسات.

يستخدم البكتريوفاج في نهاية دورته التكاثرية داخل البكتريا إنزيمات الإندوليسين. وتعتبر وظيفة هذه الإنزيمات تحطيم الببتيدوغليكان الموجود في جدار الخلية البكتيرية مما يؤدي إلى إطلاق السلالة الفيروسية. تعد هذه الانزيمات سريعة في عملها، ولا تسبب مقاومة ويمكن إستخدامها بتركيزات منخفضة جدًا (نانومولار). على الرغم من كفاءتها، تنشط الإندوليسين بشكل أساسي ضد البكتيريا موجبة الجرام. بيمنا البكتريا سالبة الجرام يحميها جدارها الخلوي ذو المحتوى العالي من الدهون مما يثبط من عمل الإندوسيلين ويجعلها غير فعالة. لذلك، يتم تطوير إستراتيجيات جديدة لتوسيع نطاق عمل الإندوليسين ضد البكتريا سالبة الجرام والميكوبكتريا (البكتريا المسببة لمرض السل). للتغلب على هذه المشكلة يتم تحميل هذه الإنزيمات على أجسام نانونية أو بإستخدام بعض المركبات التي تزعزع إستقرار الطبقة الخارجية وتمنح وصول الإندوليسين إلى الجدار الخلوي للبكتريا.

تمثل هذه الأطروحة أمثلة للتطبيقات المختلفة للإندوليسين التي لها نشاط مضاد للجراثيم. من بينها، ربط الإندوليسين المستخلص من بكتريوفاج T4 إلى شاش تضميد الجروح وإختبار فاعلية هذا الشاش المحمل بالإنزيم ضد البكتيريا المختلفة. في دراسة أخري تم تحمل الإنزيم نفسه علي أجسام نانونية متناهية الصغر مشتقة من السليلوز وأثبتت كفائتها في قتل كل من البكتيريا موجبة وسالبة الجرام. تم البحث أيضًا في قواعد البيانات لتحديد خصائص طائفة جديدة من الإنزيمات لها نشاط ضد ميكروب الميكوبكتريا وتم توصيف مجموعة من الإنزيمات لها القدرة والفاعلية علي قتل هذه البكتريا حيث تم إختبارها معمليا علي بكتريا *Mycobacterium smegmatis*وأعطت نتائج واعدة.

Populärvetenskaplig sammanfattning

Mänsklighetens framgång och framsteg beror på dess förmåga att förebygga och bota sjukdomar. Den nuvarande spridningen av antibiotikaresistens ifrågasätter den framtida effekten av dagens antibiotika . Missbruk och överanvändning av befintliga antibiotika hade lett till utveckling av superbakterier som är resistenta mot nästan alla tillgängliga antibiotika. Katastrofala scenarier förutspås som leder till allvarliga mänskliga och ekonomiska förluster om vi inte lyckas hitta nya behandlingar, med tiotals miljoner dödsfall per år och kostnader som stiger till biljoner USD 2050. Dessutom är detta hot också förknippat med en mycket begränsad pipeline av nya effektiva terapier från läkemedelsindustrin. Därför krävs det snabbt nya antibiotika och alternativ.

Ett av dessa alternativ är fag rellaterade enzymer som också kallas endolysiner. Fager, även kända som bakteriofager, finns i överflöd i naturen och betraktas som bakteriernas naturliga fiende och kan hjälpa oss att utrota patogena bakterier. De landar på ytan av en bakterie och injicerar sin egen genetiska kod. Detta kapar bakteriecellen och förvandlar den till en fag–fabrik, tills virusavkommorna så småningom sprids ut ur cellen, genom endolysiners verkan.

Endolysiner som också kallas enzymbiotika (enzymbaserad antibiotika) används av bakteriofager i slutet av deras replikationscykel för att bryta ned peptidoglykan i bakteriecellväggen vilket resulterar i frisläppandet av den virala avkomman. Endolysiner verkar snabbt, framkallar inte resistens och är potenta (aktiva i en mycket låg koncentrationer).

Trots deras effektivitet är endolysiner huvudsakligen aktiva mot grampositiva bakterier. Det höga lipidinnehållet i det yttre skiktet av både gramnegativa och mykobakterier skyddar dem från verkan av endolysiner vilket gör dessa ineffektiva. Därför utvecklas nya strategier för att utöka effekten av endolysiner mot gramnegativa och mykobakterier, till exempel bindning av endolysiner till skräddarsydda nanopartiklar eller användning av föreningar som destabiliserar det yttre skiktet vilket ger åtkomst för endolysinerna.

Denna avhandling ger exempel på olika tillämpningar av endolysiner med potentiell antibakteriell aktivitet. Av dessa, bindning av endolysin från T4–bakteriofag till ett sårförband, och testning av aktiviteten hos detta gasbinde–immobiliserade enzym mot olika bakterier. Samma enzym var också bundet till biologiskt nedbrytbara nanokristaller av cellulosa och användes för att döda både Gram–positiva och–negativa bakterier. Vi sökte också i databaserna för att identifiera och karakterisera nya endolysiner som kan verka på mykobakterier. Slutligen testas nya endolysiner från mycobacteriofager (fager som infekterar mycobacteria) för deras aktiviteter med

potentiell modifiering för applicering mot den patogena *Mycobacterium tuberculosis* som orsakar TB; lungsjukdomen.

List of Publications

The thesis is built on the following papers and manuscripts, listed and referred on the thesis as follows

I. **Adel Abouhmad**, Gashaw Mamo, Tarek Dishisha, Magdy A. Amin and Rajni Hatti–Kaul. T4 lysozyme fused with cellulose–binding module for antimicrobial cellulosic wound dressing materials. *Journal of Applied Microbiology*, 2015, 121, 115–121.

II. **Adel Abouhmad**, Tarek Dishisha, Magdy A. Amin and Rajni Hatti–Kaul. Immobilization to positively charged cellulose nanocrystals enhances the antibacterial activity and stability of hen egg white and T4 lysozyme. *Biomacromolecules* 2017, 18, 1600–1608.

III. Ahmed H. Korany, **Adel Abouhmad**, Walid Bakeer, Tamer Essam, Magdy A. Amin, Rajni Hatti–Kaul and Tarek Dishisha. Comparative structural analysis of different mycobacteriophage derived mycolyl–arabinogalactan esterases (lysin B). (Manuscript)

IV. **Adel Abouhmad**, Ahmed H. Korany, Carl Grey, Tarek Dishisha, Magdy A. Amin and Rajni Hatti–Kaul. Exploring the enzymatic and antibacterial activities of novel Mycobacteriophage lysin B (LysB) enzymes. (Manuscript)

Conference presentations

Adel Abouhmad, Gashaw Mamo, Tarek Dishisha, Magdy A. Amin and Rajni Hatti–Kaul. "Antimicrobial cellulose and chitosan nanoparticles with immobilized lysozyme." Poster presentation; *Industrial Biotechnology Meeting the Challenges*" 12–13 September 2013, Lund, Sweden.

Adel Abouhmad, Ahmed H. Korany, Carl Grey, Tarek Dishisha and Rajni Hatti–Kaul. "Exploring enzymatic and antitubercular activities of novel LysB enzymes." Poster presentation; "*Viruses of Microbes*" 09–13 July 2018, Wroclaw, Poland.

Adel Abouhmad, Ahmed H. Korany, Tarek Dishisha and Rajni Hatti–Kaul. "Structural insights and characterization of some mycobacteriophage derived LysB enzymes." Poster presentation; "*4th International Hands–on Phage Biotechnology*" 17–21 June 2019, Braga, Portugal.

My contribution to the papers

The overall idea for the thesis was generated by Prof. Rajni Hatti–Kaul and Dr. Tarek Dishisha.

I. I performed all the experiments, data analysis, writing the first draft of the manuscript and was involved in editing the final and proofreading version. Dr. Gashaw Mamo introduced the information about molecular biology tools. Dr. Tarek Dishisha helped in cell cultivation and protein expression. The whole work was done under the supervision of Prof. Rajni Hatti–Kaul.

II. I designed and performed all the experiments, data analysis, writing the first draft of the manuscript and was involved in editing the final and proofreading version. Prof. Rajni Hatti Kaul revised the manuscript.

III. I designed and performed all wet lab experiments, involved in the data analysis and writing the first draft of the manuscript. Ahmed H. Korany performed the computational part of the manuscript including sequence and structure alignments, substrate docking and homology modelling. The work was supervised by TD, who also revised the manuscript. Rajni Hatti–Kaul was involved in the revision of the manuscript.

IV. I designed and performed all experiments including gene cloning, protein expression and purification, enzyme characterization and antibacterial activity experiments. Ahmed H. Korany performed the enzyme docking part of the work. Dr. Carl Grey helped to run the LC/MS experiment. I performed the data analysis and writing the first draft of the manuscript which is currently under revision. Prof. Rajni Hatti–Kaul and Dr. Tarek Dishisha supervised the work.

Abbreviations

MDR	Multidrug Resistant
mAGP	Mycolylarabinogalactan–peptidoglycan
T4Lyz	Lysozyme from bacteriophage T4
HEWL	Hen Egg White Lysozyme
AMPs	Antimicrobial Peptides
QS	Quorum Sensing
GIT	Gastrointestinal Tract
BALOs	*Bedellovibrio* And Like Organisms
LPS	Lipopolysaccharides
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Cas	CRISPR–associated
pmf	proton motive force
***m*–DAP**	*meso*–diaminopimelic acid
CNC	Cellulose nanocrystals
Am–CNC	Aminated cellulose nanocrystals
EAD	Enzymatically Active Domain
CBD	Cell Wall Binding Domain
CBM	Cellulose Binding Module
CFUs	Colony Forming Units
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
***p*NPB**	*para*–Nitrophenyl Butyrate

1. Introduction

The first instance of antibiotic resistance was recognized by no other than Alexander Fleming, who reported that bacteria can overcome the action of penicillin and develop resistance after prolonged exposure to the antibiotic. Although antibiotics have been used in clinical practice since the 1940s, there has been an immense overuse and misuse in both humans and animals, which has resulted in dissemination of antibiotic resistance nearly in all bacterial pathogens. Several pathogens have become resistant to all known antibiotics and are named multidrug–resistant (MDR) [1]. MDR bacteria are rapidly emerging as one of the greatest threats to the humankind. In Europe, about 400,000 people were infected by MDR bacteria in 2007. In United States, the mortality rate due to MDR bacterial infections is approximately 23,000 people per year, while globally the estimated number is expected to rise to 10 million by 2050 [2]. Consequently, our healthcare faces enormous challenge since conventional antibiotics are becoming ineffective in treating simple bacterial infections [1]. Therefore, there is an urgent demand to develop new antimicrobials besides additional approaches to preserve the value of existing ones. There is also a need for alternative antimicrobials with novel mechanisms of action to decrease the chance of development of resistance.

Among the most promising alternatives or complements to conventional antibiotics are phage–derived endolysins [3, 4]. Endolysins are enzymes that degrade peptidoglycan (endolysin A/ peptidoglycan hydrolases) or mycolylarabinogalactan–peptidoglycan (endolysin B/ mAGP esterases) layer in the bacterial cell wall at the end of the phage replication cycle inside the bacterial cells, resulting in release of the phage progeny. By virtue of their natural function as potent antibacterials, endolysins have been coined ‘enzybiotics’ i.e. enzyme–based antibiotics. External application of endolysins to Gram–positive bacteria results in osmotic lysis and bacterial cell death, also termed as “lysis from without”. This mechanism of action without the need to penetrate the bacterial cell make endolysins overcome a majority of possible resistance mechanisms (e.g. efflux pump and decreased membrane permeability) that have a major role in development of bacterial resistance [5]. Moreover, some endolysins harbor more than one enzymatically active domain that hydrolyze different bonds in the peptidoglycan which is also believed to decrease the chance of provoking bacterial resistance [6]. In different animal models of bacterial infections,

endolysins have confirmed their efficacy *in vivo* which has led to development of few leads in various phases of preclinical and clinical trials [7]. Recently, endolysins are ranked as appropriate alternative class of antibacterials with the greatest potential due to their clinical impact and technical feasibility [3]. Their development is thus a promising approach to meet the need for new antibacterials as MDR bacteria are emerging and spreading whilst the antibiotic development pipeline is significantly diminished.

1.1 Scope of the thesis

The aim of the current thesis is to explore phage–derived enzymes as a potential alternative and complement to conventional antibacterials. Two classes of endolysins are studied – one a well–known peptidoglycan hydrolase – lysozyme, the other is mycolylarabinogalactan esterase. All the endolysins in this study have been recombinantly produced and purified. In case of lysozyme, novel immobilization approaches have been developed and their effect on its antibacterial activity has been investigated. On the other hand, since mAGP esterases are not well explored enzymes, focus was more on more fundamental studies including bioinformatics analysis and enzyme activity characterizations as well as their antibacterial activity.

The thesis contains four papers, two of which are published.

Paper I deals with cloning, expression and production of a chimeric protein T4 lysozyme (T4Lyz) fused with cellulose binding module (CBM). The muralytic as well as antibacterial activity of the chimeric T4Lyz–CBM was determined in both native and heat–denatured forms and compared with T4Lyz alone. Also, the CBM tag was used to immobilize the enzyme to a cellulosic wound dressing gauze which was further characterized for its antibacterial activity.

Paper II explores the use of cellulose nanocrystals (CNCs) as carrier for lysozyme immobilization with enhanced antibacterial activity, stability and extended spectrum. Different preparations of T4Lyz and hen egg white lysozyme bound to CNC with varying zeta potentials were made using different chemistries for immobilization. The muralytic and antibacterial activities of the nanoconjugates were assessed with different techniques. The study showed that immobilizing lysozyme to positively charged aminated cellulose nanocrystals significantly improved the antibacterial activity of the preparation.

In **Paper III,** structural, bioinformatics as well as modelling tools were employed to explore and group endolysin B enzymes from mycobacteriophages according to similarity to LysB–D29, the enzyme with a known crystal structure. Subsequent docking studies of different *para*–nitrophenyl ligands (C4 – C18) to the 3D models

were performed to predict the potential enzymatic activity of each of the 3D homology models.

Paper IV reports cloning and expression of selected novel LysB enzymes as well as kinetic parameters for the hydrolysis of *para*–nitrophenyl ester substrates with variable carbon chain length (C4–C16). The enzymes were also characterized for their lipase activity for hydrolysis of different Tweens as substrates. The mycolylarabinogalactan esterase as well as the antibacterial activity of the recombinant enzymes were also determined.

The following chapters represent the background of the research area besides our contribution with the results obtained during the thesis work. Chapter 2 describes the discovery of antibiotics as well as the emergence of resistance problems. Chapter 3 gives an overview of possible alternatives to the conventional antibiotics. Chapters 4 and 5 deal with bacteriophages and endolysins, respectively, especially those studied in this thesis. Chapter 6 describes the potential of endolysins and the technical considerations for their application as antibacterials. The thesis is finally concluded with concluding remarks and future perspectives in Chapter 7.

2. Antibiotics Discovery and Development of Resistance

2.1 Antibiotic era

While antimicrobial agents have been used throughout history, the onset of the gilded age of antibiotics is considered to have begun in 1928 with the discovery of penicillin by Alexander Fleming [8]. A decade later, penicillin was introduced to the public and became widespread as a lifesaver, especially for Gram–positive infections. Nonetheless, the first resistance to penicillin was reported by 1945 [9].

During 1940s, a new class of antibiotics that comprised protein translation inhibitors (e.g. tetracyclines and chloramphenicol) was discovered, and soon after their release into the market, resistant bacterial strains were observed [10].

This era with profound discoveries of new antibiotics continued until the discovery of three new classes of drugs, glycopeptides (vancomycin in 1953), rifamycins (rifampicin in 1957) and quinolones (ciprofloxacin in 1961), against which bacteria developed resistance soon after their availability in the market [11].

From that time, discovery of new antibiotics was ceased until 1986 when a lipopeptide, daptomycin was discovered [12]. Although resistance to daptomycin was observed a year later, it was still approved for use by the FDA until 2003 [13]. Renovation of the old antibiotics has been done through derivatization of the old molecules which led to new approved antibiotics, such as tigecycline in 2005 (a glycylcycline derived from tetracycline) [14] and ceftaroline in 2010 (5^{th} generation cephalosporin) [15], and occasionally both have been met with the emergence of resistant bacterial strains [16, 17]. Another revolution of derivatization of old antibiotics led to new antibiotics to be recently approved such as Tedizolid in 2014 (an oxzaolidinone derivative) [18], Dalbavancin and Oritavancin in 2014 (2^{nd} generation glycopeptides) [19], Delafloxacin in 2017 (a fluoroquinolone derivative) [20], Eravacycline and Omadacycline in 2018 (tetracycline derivatives) [21] and Plazomicin in 2018 (2^{nd} generation aminoglycosides) [22]. Among the last new class of antibiotics to be approved was the diarylquinolines (Bedaquiline) [23] for treatment of multidrug–resistant (MDR) *Mycobacterium tuberculosis* in 2012.

Recently, Pretomanid a nitroimidazooxazines derivative was approved by FDA targeting adult patients with extensively drug resistant, treatment–intolerant or nonresponsive multidrug resistant pulmonary TB in combination with Bedaquiline and linezolid [24]. Apparently, new antibiotics cannot be developed quickly enough to be considered a viable therapeutic option to combat the resistance problem [25].

2.2 Mode of action of antibiotics

Antibiotics act via targeting cellular processes or structures that are crucial for survival. For the bacterial pathogens, antibiotics can be either bactericidal (that cause bacterial cell death) or bacteriostatic (that arrest the bacterial cell growth, metabolism and reproduction). Antibiotics target the bacterial cells through one of the following mechanisms:

- Inhibition of peptidoglycan biosynthesis by preventing cell wall cross–linking or via interacting with/inhibiting cell wall precursors (β–lactams, β–lactamase inhibitors, glycopeptides, polypeptides, cycloserine, fosfomycin, isoniazid, ethambutol, teixobactin).
- Disruption of cell membrane permeability and integrity resulting in ion leakage and membrane depolarization followed by cellular death (polymyxins, ionophores).
- Inhibition of DNA (fluoroquinolones, novobiocin) or RNA (rifamycin) synthesis.
- Inhibition of RNA translation and protein synthesis through interaction with the 30S ribosomal subunit (glycylcyclines, furanes, aminoglycosides, tetracyclines) or 50S ribosomal subunit (macrolides, ketolides, chloramphenicol, lincosamides, oxazolidinones, streptogramins, pleruromutilins).
- Antimetabolite activity that blocks enzyme–catalyzed reactions essential for bacterial cell metabolism, as for folic acid synthesis inhibitors (sulphonamides, trimethoprim, dimethyl sulfones) and ATP synthase inhibitors (diarylquinolines).

2.3 Bacterial resistance to antibiotics

Despite the discovery and introduction of different classes of antibiotics with different mechanisms of action tackling different targets in the bacterial cells,

bacteria have evolved different resistance mechanisms to combat the effect of the antibiotics. As it is a survival battle between bacteria and antibiotics, sooner or later after introduction of a new antibiotic we will discover a resistant bacterial strain. Bacterial resistance to antibiotics can occur in two different ways [26].

Intrinsic (natural) bacterial resistance that occurs when inherent features in the bacteria abolish the effect of the antibiotic [27]. It is the kind of resistance that is inherently/naturally acquired by the bacteria without being genetically resistant. This happens when some bacteria are resistant to particular type of antibiotics rather than others. Inherent resistance is considered as an innate characteristic of the bacteria that can be transmitted vertically to the progeny. Moreover, such kind of resistance is considered as consistently inherited characteristics of genus/species of bacteria and is to be predicted once the genus/species is mentioned [28]. An example of inherent bacterial resistance is the resistance of Gram–negatives to several antibiotics active against Gram–positives including vancomycin, and most β–lactams. This pattern of resistance in Gram–negatives might be due to the presence of the outer membrane that acts as a permeability barrier which is absent in Gram–positives or lack of antibiotic transporter system or the target site [29].

On the other hand, acquired bacterial resistance is caused by the selective pressure imposed by the application of an antibiotic [10]. Bacteria acquire those mechanisms through mutations or horizontal gene transfer. In mutational resistance, a subset of bacterial cells develops mutations (nucleotide(s) substitutions/single nucleotide polymorphisms, insertions, deletions, or frameshifts) in genes affecting the activity of the antibiotic, promoting/restoring the cell survival in the presence of the antibacterial molecule [30]. Therefore, a resistant mutant arises, the antibiotic eradicates the susceptible bacteria and the resistant strains dominate. On the other hand, horizontal gene transfer occurs via uptake of new piece of DNA, through transformation (uptake of naked DNA), conjugation (direct bacteria–bacteria contact), or transduction (bacteriophage DNA) [31]. Generally, acquired resistance confers antibiotic resistance via one of the following mechanisms; 1) decrease of the antibiotic uptake, 2) modification of the drug target through decrease of its affinity, 3) activation of efflux pumps mechanisms to extrude the drug extracellularly, 4) enzymatic degradation of the antibiotic molecule and 5) drastic changes in vital metabolic pathways (**Table 1**).

Table 1
Different mechanisms of acquisition of antibiotic resistance in bacteria [32].

Antibiotic	Mechanism of resistance
Chloramphenicol	Reduced uptake into the bacterial cell
Tetracyclines, Aminoglycosides	Active efflux pump
β–lactams, Lincomycin, Erythromycin	Decreased affinity to the drug target
β–lactams, Fusidic acid	Detachment from the target via protein binding
β–lactams, Erythromycin	Enzymatic inactivation via hydrolysis
Lincomycin, Aminoglycosides, Chloramphenicol	Enzymatic inactivation via derivatization
Sulphonamide/Trimethoprim	Metabolic circumvention of the inhibited reaction
Sulphonamide/Trimethoprim	Overproduction of drug target (titration)

3. Alternatives to Antibiotics

The misuse and overuse of antibacterial agents have led to a critical situation of drug resistance with urgent needs for new more efficient antibacterials with novel mechanisms of action. The endeavors to control the use of antimicrobials to halt the rise of antibacterial resistance have been various and to some degree successful, and yet might be hard to implement. Taking into account the decrease in investments for development of new antibiotics by pharma companies and the rapid increase in the resistance rate altogether raise the question: if the time for the antibacterials is off? In this chapter we will shed the light on some therapeutic strategies as alternatives to conventional antibiotics. A summary of innovative strategies with future promise as antibiotic alternatives are listed in **Table 2**.

3.1 Bacteriophage (phage) Therapy

3.1.1 Wild Type Bacteriophages

Phages are viruses that infect and propagate within bacteria [33]. Since phages can select between mixed bacterial populations, lytic phages can be exploited as an alternative therapy with high selectivity towards pathogenic bacteria only [34].

In Eastern European countries and the former Soviet Union, phage therapy was considered as a successful therapy even before the discovery of antibiotics [35, 36]. On the contrary, in the rest of the world the discovery of antibiotics limited the usage of phages for treatment and prevention of bacterial infections [37]. As an example, researchers at Hirszfeld Institute of Immunology and Experimental Therapy (HIIET) (Wroclaw, Poland) and at Eliava Institute of Bacteriophage, Microbiology, and Virology (EIBMV) (Tbilisi, Georgia) are actively and successfully using phage cocktails for treating different bacterial infections [38–41]. Currently, the interest in phage therapy has been rekindled due to the incapacitated status of the antibacterials. Phage therapy can be used for treatment of both Gram–positive and –negative bacterial infections including multidrug resistant *Staphylococcus aureus*, *Shigella*, *Salmonella*, *Acinetobacter* and *Pseudomonas aeruginosa* [42]. Phage therapy has considerable advantages, but the significance, concern and efficacy of its usage as is

the case with any treatment option exists. Of these concerns, bacterial resistance to phages that had been reported [43], use of phage cocktails instead of single selected phage due to the lack of rapid diagnostic platforms [44], endotoxin release during perpetration of cell lysate as a contaminant during phage purification process, pharmacokinetics, phage stability and storage stability [45] and the last but not the least is the immunogenicity against phages [46].

3.1.2 Engineered Bacteriophages

Engineering phages to gain new properties and overcome existing obstacles opens a new era for promising therapeutic applications. Many concerns linked with immunogenicity, spectrum and strain coverage, resistance development, stability, pharmacokinetic and pharmacodynamic issues could be addressed [3]. As a proof of concept, T7 bacteriophage was enzymatically engineered to produce biofilm–degrading–enzymes that upon contact with pathogenic *E. coli* induces both cell lysis and biofilm clearance [47]. Phasmids, the plasmids carrying an origin of replication from a phage and can be packed in capsids, are engineered to express antimicrobial peptides/toxins that lead to bacterial cell death upon contact with the pathogen [48]. In another study, phasmids are engineered to deliver small regulatory RNAs inside drug resistant pathogens rendering them susceptible to conventional antibiotics [49]. Recently, engineered mycobacteriophages were tested and showed efficacy in eradicating MDR *Mycobacterium abscessus* causing respiratory and skin infections in an immunocompromised patient [50]. To our knowledge, this is the first therapeutic usage of genetically engineered phages in humans. Bacteriophage–derived enzymes (endolysins) as possible alternative to antibacterials will be discussed in detail in Chapter 5.

3.2 Antimicrobial Peptides (AMPs)

AMPs as well as host defense peptides are produced by multicellular organisms as a first line defense mechanism against pathogen invasion [51–53]. They are versatile, acting as antibacterial, antifungal, antiprotozoal, antiviral, anticancer molecules [54]. These peptides are amphiphilic with a net positive charge, their cationic domain interacts with the negatively charged bacterial cell surface, while the hydrophobic domain interacts with the lipid layer of the cell membrane resulting in dismantling of the cell membrane followed by cell death [55, 56]. The specificity and selectivity of AMPs towards bacterial cells is attributed to the target net surface charge, which is anionic allowing for interaction with AMPs, in contrast to the mammalian cell surface which is Zwitterionic and hence not interacting with AMPs [57]. Moreover, some AMPs has the ability to inhibit the growth of intracellular bacteria. NZX a

novel nontoxic derivative of plectasin (fungal defensin–like AMP) showed 45% inhibitory capacity against intracellular *M. tuberculosis* infecting primary human macrophages with at a therapeutic concentration (50 μM) 6 days post treatment [58]. Despite their potential for broad–spectrum activity, it was disappointing that AMPs had failed clinical trials for systematic administration [3]. Low efficacy and safety are the main underlying reasons for failure of AMPs in clinical trials [59].

Another group of AMPs produced by bacteria are called Bacteriocins that act as a defense mechanism against other bacteria within the same population through preventing competitions and promoting survival [60]. Bacteriocins are ribosomally synthesized peptides and released extracellularly either in a modified condition through posttranslational modifications or as native unmodified peptides [61]. Bacteriocins are produced by both Gram–positive and –negative bacteria with high potential activity against drug resistant clinical isolates [62]. Bacteriocins have versatile mechanisms of actions such as targeting the cell membrane, inhibition of peptidoglycan biosynthesis via binding to lipid II (Nisin), binding to pore–forming receptor mannose phosphotransferase system (Lactococcin A), and affecting DNA, RNA and protein translation and metabolism (Microcin B17, thiopeptides) [63–69]. Unlike AMPs, bacteriocins are selective in their action targeting only particular bacterial strains, as in the case of thuricin, the bacteriocin that targets only *Clostridium difficile* without any effect on the commensals [70]. The major advantage of bacteriocins is their stability towards harsh conditions of heat, UV and pressure giving them the benefit of large–scale industrial application as the case for Nisin, the globally used food preservative. However, bacterial resistance to Bacteriocins has been reported, still slow but approaching [71, 72].

Another class of AMPs are innate defense regulatory peptides (synthetic peptides) and host defense peptides (natural peptides) with no antibacterial mechanism of action. They act through antiendotoxin and immunomodulatory activities via enhancing expression of anti–inflammatory chemokines and cytokines and reducing the expression of proinflammatory cytokines. Addressing the host response as a target might have an increased risk of side effects making it quite difficult for potential application [73–77]. To overcome the problems encountered with AMPs, Synthetic Mimics of Antimicrobial Peptides (SMAMPs) have been designed to imitate the action of AMPs and overcome toxicity, protease instability and the cost of AMPs. There are three categories of SMAMPs: peptidomimetic oligomer, small molecules and polymeric mimics of AMPs [78, 79]. The protease degradation has been overcome through modification of the peptide backbone but keeping the substantial cationic and amphiphilic structures. These modifications resulted in oligomeric compounds (oligoureas, β–peptides, α–AA peptides and peptoids), with retention of the secondary structure required for the antibacterial activity [80–85].

3.3 Antibodies

Antibodies that identify a specific structure in the pathogens (e.g. toxin, virulence factor, etc.), then bind to and inactivate it, are considered as promising alternative therapeutics with high clinical impact. They can be used directly to treat existing bacterial pathogens through adherence to their surface or indirectly through neutralizing their toxins. They are considered to be of low risk with high technical feasibility. Currently, several antibodies against *Bacillus anthracis*, *C. difficile*, *P. aeruginosa* and *S. aureus* are in different stages of clinical trials. A few of them have been recently approved by FDA and released to the market [86–93].

3.4 Antivirulence Antibacterials (Pathoblockers)

In contrast to antibiotics, pathoblockers aim to deactivate the bacterial pathogens via inhibition of expression of virulence factors, thus hindering the interaction between the pathogen and its host. Since pathoblockers do not display any bactericidal activity, there is a low tendency for resistance development. To establish an infection, the bacterial pathogen must adhere to the surface of the target host cell surface through specific carbohydrate binding proteins (lectins and adhesins) [94, 95]. Thus, targeting these receptors with glycomimetics has been under investigation since the past two decades. The biphenyl mannosides have been identified to block FimH, the lectin responsible for adhesion of uropathogenic *E. coli* to the urinary tract causing urinary tract infections [96–98]. Another scenario is targeting the bacterial toxins with pathoblockers; CAL02 is a broad–spectrum liposome–based antitoxin targeting both Gram–positive and –negative bacteria including ESKAPE (***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa,* ***E****nterobacter* spp.) pathogens. CAL02 neutralizes pore–forming toxins, enzymes, and toxin–effector virulent adjuncts that play a crucial role in the severity and progress of pathogenicity as in bacteremia, pneumonia, and sepsis [99]. Another prime target for pathoblockers is the bacterial signaling system (Quorum sensing; QS) the system that is responsible for bacterial communication and usually associated with bacterial biofilm formation. After bacterial colonization, production of virulence factors and establishing sessile communities is a function of bacterial population density which is governed by QS signals. Hence, interrupting QS process can enhance the bacterial susceptibility to the immune system and antibiotics [31, 100]. Targeting QS via enzymes [101, 102], antibodies [103] and receptor antagonists [104, 105] is a promising approach to inhibit QS associated virulence factors and inflammatory mediators.

3.5 Probiotics

Probiotics are living microorganisms that when administered properly in adequate amounts, promote the health benefits to the host organism by improving its intestinal microbial balance [106]. Probiotics are considered a new strategy to promote health and prevent infections of the urogenital, intestinal and even skin in both humans and animals. Harboring more than 1000 bacterial species including *Eubacterium* sp., *Bacteroides* sp., *Bifidobacteria*, *Lactobacilli*, *Fusobacterium* sp., *Peptococcus* sp., *Clostridiodes* sp., *Streptococcus,* the human gut is a highly complex environment that determines the health of the host significantly through food digestion, production of metabolites or even toxic compounds [107]. There is a great versatility in the gut microenvironment between individuals, and to some extent it can be altered with ingestion of antibiotics [108]. Usually broad–spectrum antibiotic treatment ends up with disturbance of the harmony of the gut microbiota, favoring the growth of drug–resistant strains resulting in recurrent secondary bacterial infections for instance *C. difficile* induced colitis. Hence, promoting the gut microbiota with beneficial probiotics could be an alternative strategy to antibiotics [109]. The approach ruling administration of probiotics to restore the gut microbiota balance, nourishing the commensals and competitively excluding the pathogens is the key for treating different gastrointestinal infections as pseudomembranous colitis caused by *C. difficile* and *Helicobacter pylori* [110–112]. Another approach to treat gastrointestinal tract (GIT) bacterial infections and dysbiosis is fecal transplant therapy, in which the microbiome from a healthy individual is transferred into a gut diseased patient. Although the exact mechanism is unrevealed yet, it is used for treatment of *C. difficile* associated infections [113].

3.6 Predatory Bacteria

Predatory bacteria represent an interesting alternative to antibiotics. Despite different species of predatory bacteria being identified, *Bedellovibrio* and related organisms (BALOs) are considered as promising strains [114]. BALOs are deltaproteobacteria that are obligately predators of Gram–negative bacteria such as pathogenic *E. coli*, *Salmonella* and *Pseudomonas* for energy and nutrients. BALOs degrade cells by a variety of hydrolytic enzymes (DNases and proteases), allowing them even to penetrate the biofilm layer [115–117]. Moreover, since bacteria living in a biofilm can be 1000 times more resistant to antibiotics than the planktonic cells, BALOs have a therapeutic advantage over the antibiotics themselves. BALOs can gain access to mixed bacterial communities that antibiotics cannot penetrate such as polymicrobial infection as in cystic fibrosis and catheterized patients [118]. With regard to BALOs–host interactions, BALOs have unique lipopolysaccharide (LPS)

structure which is less toxigenic than *E. coli* and have low affinity to LPS receptors in human immune cells indicating their potential application for treatment of bacterial infections [114].

3.7 CRISPR/CAS

Clustered regularly interspaced short palindromic repeats (CRISPR) together with CRISPR–associated (Cas) proteins encode for the response of prokaryotes that capture pieces of DNA from phages integrating them as new spacers in the CRISPR loci. Consecutively, the CRISPR array will be processed and transcribed into short CRISPR RNAs that guide Cas nucleases to destroy target DNA sequence [119]. The discovery of these RNA guided nucleases opened a new era of biotechnological applications through genome editing that extends to the field of antimicrobial therapy via developing programmable antimicrobials selectively targeting pathogenic strains only [120, 121]. Phasmids were used as carriers to deliver pre–programmed Cas9 targeting virulent genes that specifically kill virulent MRSA (methicillin–resistant *S. aureus*) strains when the target gene is present in the chromosome, hence preventing horizontal transfer of resistance. The latter approach was also confirmed in a murine skin model, when MRSA viable cells decreased from 50 to 11.2% which was significantly different from all other treatment conditions [122].

3.8 Antibiotic Degrading Enzymes

The rampant use of broad–spectrum antibiotics resulted in disruption and alteration of the gut microbiota. Exposure of gut microbiota to such antibiotics can result in development of resistance and drive *C. difficile* associated colitis and antibiotic associated diarrhea. A promising strategy is to limit the selective pressure of antibiotic residuals excreted into the gut on the microbiota by antibiotic degradation [123, 124]. SYN–004 (Ribaxamase), an engineered β–lactamase enzyme, currently in phase II clinical trials is designed to degrade excess β–lactam antibiotics in the upper GIT before the antibiotic has a chance to disrupt the gut microbiome. It is administered orally concomitantly with intravenous administration of β–lactam antibiotics [125, 126].

Table 2
Different antibiotic alternatives strategies with their advantages and constraints [3, 127, 128].

Strategy	Advantages	Constraints
Phage Therapy	• Selectivity and specificity towards the target strain • Simple, rapid with low cost of production • Can be used for detection, prevention and treatment of pathogens • Susceptible to genetic engineering	• Resistance development • Stability • Pharmacokinetics • Contamination with endotoxin • Immunogenicity • Lag time till diagnosis • Narrow host range
Phage–derived enzymes (Endolysins)	• High specificity for target organism • Natural, nontoxic agents • Metabolism independent activity • Rapid onset of action • Effective against biofilms • Active against drug resistant strains • Do not provoke bacterial resistance • Susceptible to engineering • Synergy with other antibacterial agents	• Immunogenicity • Gram–negative bacteria • Intracellular bacteria • *In vivo* kinetics and short half–life • Stability
Natural AMPs	• Broad spectrum • Low immunogenicity • Low target–based resistance • Rapid onset of bactericidal action	• Toxicity • Cost, expensive large–scale production • Sensitivity to proteases • Formulation; suitable mainly for topical applications
SMAMPs	• Protease resistant • Easily designed and synthesized	• Toxicity • Formulation, suitable mainly for topical applications
Antibodies	• Strain specific • Do not affect the normal flora • Considered as safe with low risk	• Stability • Cost
Pathoblockers	• Strain specific • Do not affect the normal flora • Synergy with antibiotics	• Resistant strains were reported
Probiotics	• Availability • Maintain healthy gut commensals • Prevent gut colonization	• Targeted mainly for GIT infections • Should be administered in a mixture rather than as single strain
Predatory Bacteria	• Active against wide range of Gram–negatives • Low immunogenicity • Low toxicity • Low target–based resistance • Active against bacteria in biofilm	• Data about interaction with host and host microbiota are scarce
CRISPR/Cas	• Specific against virulent strains only	• Expensive • Still under development
Antibiotic degrading enzymes	• Low toxicity • Maintain healthy gut microbiota	• Formulation • Targeted mainly for GIT infections • Selectivity and specificity

4. Bacteriophages and Host Bacterial Cell Envelope Targeted by Endolysins

4.1 Bacteriophages: general features and life cycle

In 1917, the term bacteriophage was conceived by Felix d`Herelle who independently confirmed the discovery of bacteriophages by Frederick William Twort, and experimented the possibility of phage therapy [129]. Phages are predominant as a biological entity with more than 10^{31} particles on the planet, the estimated number of phage infection is up to 10^{25} per second resulting in annual production of 3.7×10^{30} particles, indicating that the phage population is not only large but also highly dynamic [130–132]. As abundant and diverse biological entities, phages are environmental key players responsible for (**a**) horizontal gene transfer of bacterial DNA released after host cell lysis, (**b**) circulation of dissolved particulate organic matter through cell lysis, (**c**) biodiversity modulation of bacterial population by governing the number of dominating bacteria, and (**d**) lysogenic conversion of temperate phages [133].

According to the type of nucleic acid, DNA or RNA single stranded or double stranded, phages were classified into six groups. The International Committee for Taxonomy of Viruses (ICTV) had classified viruses into 7 orders, 103 families, 455 genera and 77 families with unassigned order; bacteriophage presently constitute 20 families [134]. More than 90% of phages described in the literature are tailed phages with linear double–stranded DNA enclosed in an icosahedral capsid, comprising the order *Caudovirales* [135], which includes three families based on the tail morphological features: (1) *Siphoviridae* (61%) with long, non–contractile tails, (2) *Myoviridae* (25%) with contractile tails, and (3) *Podoviridae* (14%) with short tail (**Figure 4.1**) [136].

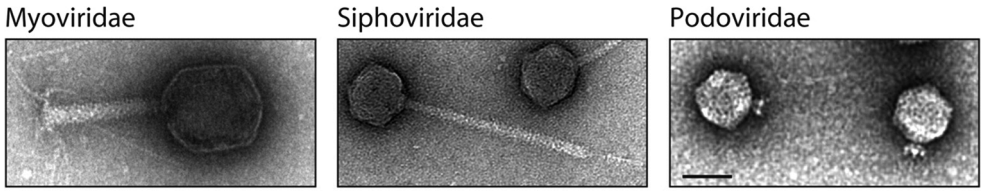


Figure 4.1

Viruses of the order Caudovirales. Transmission electron micrographs of T4–like virus, HK97 and P22 representing the families Myoviridae, Siphoviridae, and Podoviridae, respectively [137].

Phages have distinct four life cycles: lytic, lysogenic, pseudolysogenic and chronic infections [138]. The phage infection cycle starts with its adsorption to the host cell surface. This occurs via specific interactions between phage receptor binding proteins and variety of cell surface components including lipopolysaccharides, teichoic acid, proteins, peptidoglycan, pili and flagella [139]. At the beginning, the adsorption is a reversible process then turns into an irreversible mode when the phage undergoes conformational changes. Immediately after adsorption, the phage delivers its genetic material into the bacterial host cell through ejection or endocytosis–like mechanism.

The outcome after delivery of the genetic material to the host cell depends on the nature of the phage life cycle. In lytic cycle, the bacterial cell machineries are enforced to amplify the viral DNA and synthesize viral proteins by which phage capsids are assembled and then packed with the amplified viral DNA. At the end, the host cell lysis occurs with the aid of lytic enzymes releasing the viral progeny [138]. In the lysogenic cycle, the phage DNA is integrated into the bacterial genome. The phage genetic material, called a prophage, gets transmitted to daughter bacterial cells during cell division and can be maintained for many generations until encountering an event such as UV radiation or certain chemicals that causes its release and proliferation of new phages via lytic cycle [140]. In pseudolysogenic cycle, the viral DNA exists in the bacterial host cell as an independent episome, a phage carrier state. The bacterial host cell acts a carrier to the phage and the episome is clustered asymmetrically during the cell division allowing the phage to multiply only in a fraction of the population [133, 138]. The last form of phage infection is the chronic state in which the virions are released spontaneously from the host cell without cell lysis via budding or extracellular extrusion (**Figure 4.2**).

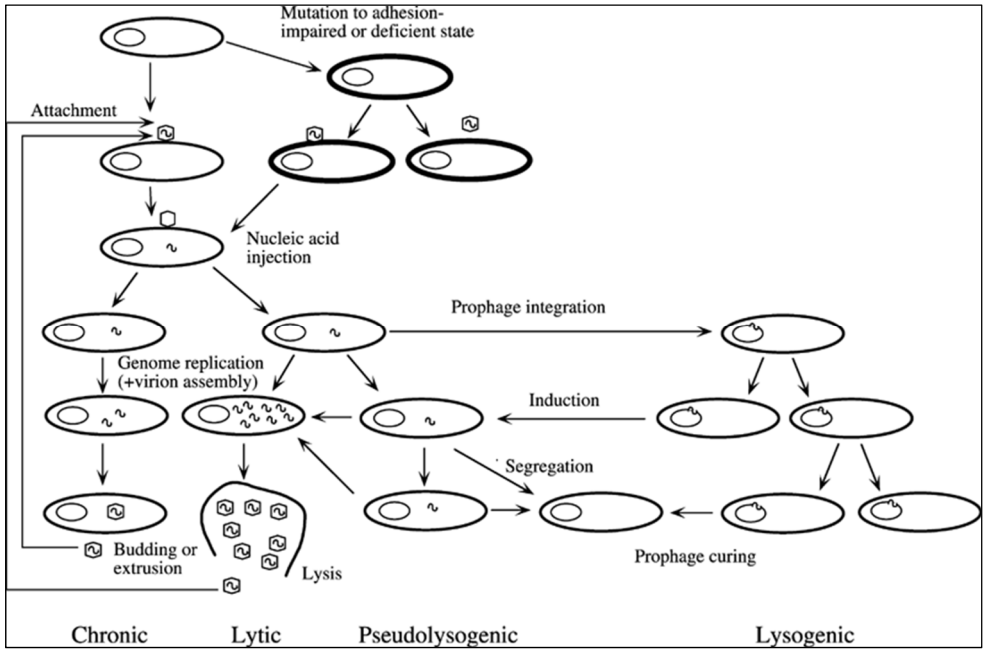


Figure 4.2
Different types and outcomes of phage life cycles [138].

4.1.1 Mycobacteriophages

Mycobacteriophages are viruses that infect mycobacteria e.g. *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* (*Mbt*). All mycobacteriophages are double stranded DNA–tailed phages and morphologically classified in the order *Caudovirales*. Generally, mycobacteriophage genomes are characteristically mosaic with only few genes being conserved and shared between individual phage genomes when compared on the amino acid level [131, 141]. The isolation and characterization of the first mycobacteriophage was in 1940s, while now around 15 500 mycobacteriophages have been isolated, among which 1790 have been fully sequenced and their sequences are available online [142]. Since mycobacteriophages target a particular group of bacteria including the highly pathogenic and deadly bacteria (*Mtb*), studying the endolysins produced by these phages is crucial to develop novel lysins active against mycobacteria.

4.2 Bacterial Cell Envelope

The complexity and the multilayered structure of the bacterial cell envelope stands as an armor protecting the cell from any unfavorable environmental conditions or predatory attacks. It is also responsible for maintaining the cell integrity, osmotic balance and supporting the cell homeostasis. Moreover, the cell envelope acts as a barrier for phages to gain access inside the cell to initiate the infection and at the end of the lytic cycle for the release the virion progeny. The bacterial cell envelope is mainly composed of cell wall and cytoplasmic membrane, however in Gram–negative and mycobacteria an additional outer membrane is also present. The major differences between the cell envelope of Gram–positive and –negative bacteria and mycobacteria are illustrated in **Figure 4.3**.

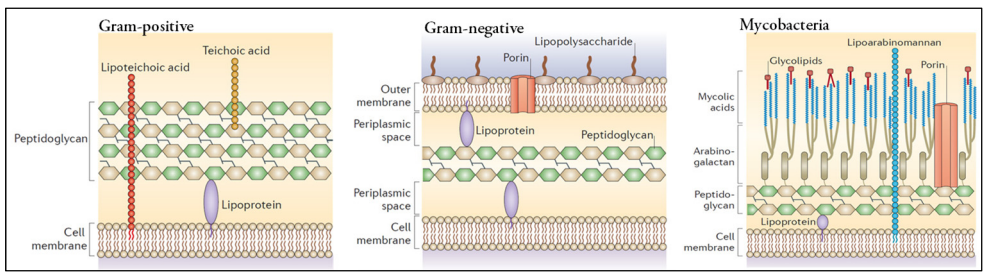


Figure 4.3
Schematic structure of cell walls of Gram–positive and –negative bacteria and mycobacteria [143].

4.2.1 Bacterial Cell Membrane

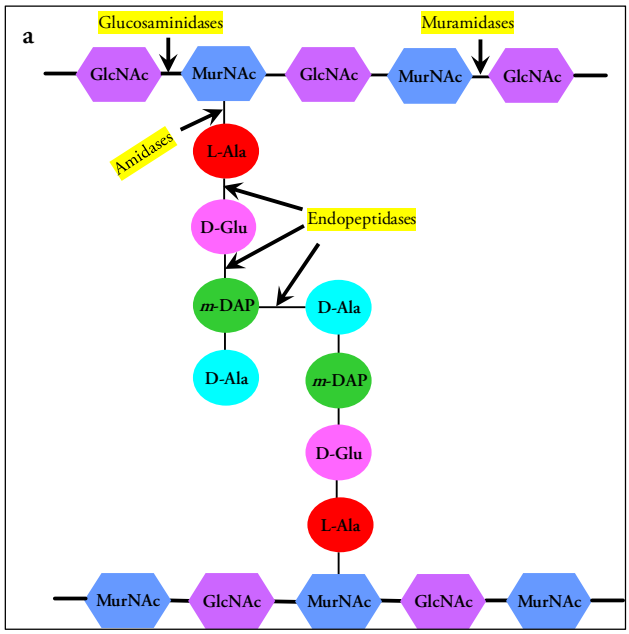
The cell membrane is the common structure in all bacteria and is composed of proteins embedded in a lipid matrix of phospholipids, with two fatty acid chains. The distinction in the concentration and charge of ions on both sides of the cell membrane creates proton motive force (pmf) which is required for generation of adenosine triphosphate (ATP), glucose transport, chemotaxis control and bacterial autolysis [144, 145].

4.2.2 Bacterial Cell Wall

The cell wall is composed of a major polysaccharide backbone known as peptidoglycan, which imposes strength, flexibility, mechanical stability, and rigidity to the bacterial cell [146]. Peptidoglycan is a heteropolymer composed of linear glycan strands that are crosslinked via short peptide bridges. The glycan strands are composed of repeating units of the disaccharide β–1,4–linked N–

acetylglucosamine–N–acetylmuramic acid. The glycan strands can be modified to promote the cell wall stability and resistance against enzymes, e.g. deacetylation of N–acetylglucosamine and N–acetylmuramic acid residues as in *Bacillus* species or O–acetylation of muramic acid residues as in *Micrococcus* and *Streptococcus* and N–glycolylation of muramic acid residues as in Actinomycetales [147]. The peptide chains that crosslink the glycan strands together via transpeptidation reactions imparts flexibility to the peptidoglycan. The type of the peptides as well as the way they crosslink vary greatly and are even considered as a basis for classification of peptidoglycans. The peptides are composed of five amino acids with L– and D–configurations, linked to the lactyl groups of N–acetylmuramic acid by an amide linkage. The second amino group of the diamino acid present at position two or three crosslink the peptide stems together. The peptidoglycan chemotype in Gram–negative bacteria is A1γ type in which the stem peptide is composed of L–Alanine (Ala)–D–iso glutamic acid (iGlu)–meso (*m*)–diaminopimelic acid (*m*–DAP)–D–Ala–D–Ala (**Figure 4.4a**). On the other hand, in Gram–positive bacteria iGlu is amidated to isoglutamine (iGln) and *m*DAP is replaced by L–Lysine (Lys) (**Figure 4.4b**) [148]. Moreover, peptidoglycan is also subjected to surface modifications through functionalization with proteins or glycopolymers. In Gram–positive bacteria, peptidoglycan is covalently linked to teichoic acid, a glycopolymer that is linked to C6 of every ninth N–acetylmuramic acid residue (in *B. subtills* and *S. aureus*) via phosphodiester bond.

Gram–negative bacteria differ from Gram–positive ones by the presence of an outer membrane (**Figure 4.3**), which establishes a compartment i.e. the periplasm. The peptidoglycan has anchored lipoproteins covalently linked via its C–terminal Lysine or Arginine residues to the *m*–DAP, while the fatty acid part is inserted into the inner leaflet of the outer membrane [149]. The presence of peptidoglycan and teichoic acid in Gram–positive bacteria as well as LPS in Gram–negative bacteria impose the negative charge on the cell surface.



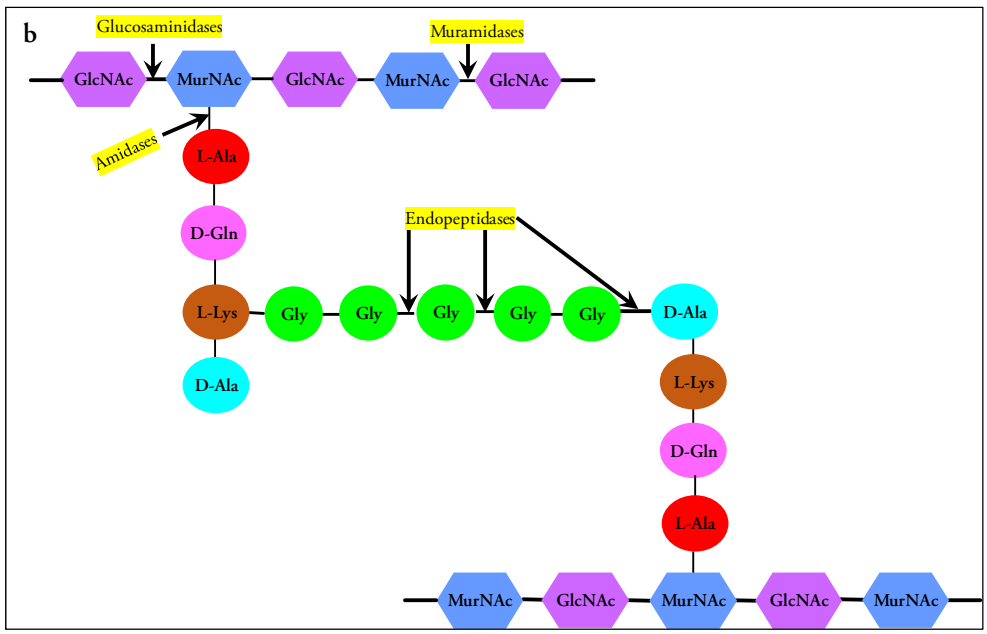


Figure 4.4

Schematic structure of bacterial peptidoglycan and the cleavage sites of different endolysins. (a) peptidoglycan of Gram–negative bacteria, (b) peptidoglycan of Gram–positive bacteria [33, 150]. GlcNac: N–acetylglucosamine, MurNac:–N–acetylmuramic acid, L–Ala: L–Alanine, D–Glu: D–Glutamic acid, *m*–DAP: *meso*–diaminopimelic acid, D–Gln: D–Glutamine, L–Lys: L–Lysine and Gly: Glycine.

4.3 Structure of Mycobacterial Cell Envelope

4.3.1 General Overview

The mycobacterial cell envelope is a complex structure with unique features that make it rather distinct from Gram–positive and –negative bacterial cell envelopes. The presence of the extensive network of peptidoglycan in the mycobacterial cell envelope categorizes mycobacteria as a Gram–positive bacterium, however it has been linked to Gram–negative bacteria due to the presence of covalently linked mycolic acids intercalating with different lipids forming a symmetric lipid bilayer like the outer membrane of the Gram–negative bacteria (**Figure 4.3**). Mycobacteria have a cell wall of chemotype IV containing arabinan, galactan, mycolic acid altogether linked to peptidoglycan via ester bond forming mycolylarabinogalactan–peptidoglycan complex (mAGP) [151]. The major difference between the mycobacterial and Gram–negative outer membrane is that the mycobacterial outer membrane is entirely connected to the peptidoglycan layer of the cell wall (**Figure 4.3**). Subsequently, the presence of mycolic acids is crucial for the integrity of the mycobacterial outer membrane making it a good target for antibacterials targeting mycobacteria [152]. Mycobacterial outer membrane, named as mycomembrane, has been visualized with cryo–electron microscopy to be only 8 nm thick, which is only 15% thicker than outer membrane of Gram–negative bacteria [153, 154]. Different models have been proposed to configure the fold of the mycolic acid in the mycomembrane. Hoffmann et al. proposed two models: in the first one the longer meromycolate chain of the mycolic acids extends into the outer leaflet, and a second in which the base of the mycolic acids remains in the periplasm with only the ends extending into the inner leaflet [153]. On the other hand, Zuber et al. suggested that the longer chains of the mycolic acids fold to stay within the inner leaflet [154].

4.3.2 Mycobacterial Outer Membrane

The major characteristic feature of the mycobacterial outer membrane is the presence of mycolic acids, which are α–alkyl, β–hydroxy C_{60}–C_{90} fatty acids, the saturated α–branch contains C_{20}–C_{25} in average, while the main chain meromycolic acid moiety (the β–hydroxy branch) averages C_{60} and can contain double bonds, cyclopropane rings, and oxygen functions according to the species (**Figure 4.5**). The outer membrane contains two types of lipids, non–extractable lipids that are covalently linked to the peptidoglycan that comprises mAGP, and extractable lipids and lipoglycans: phosphatidylinositol mannosides, phthiocerol dimycocerosates, phenolic glycolipids, a variety of acyltrehaloses, lipoarabinomannan (which is similar to lipoteichoic acid in Gram–positive bacteria), trehalose monomycolates (TMM),

trehalose dimycolates (TDM) (the cord factor which is one of the virulence factors of *Mtb*) (**Figure 4.6**). The extractable lipids are located in the outer leaflet of the outer membrane and associated with the mycolic acid part of the mAGP complex. The less–packed lipids of this outer leaflet are more disordered than the closely packed inner mycolic acids, creating a gradient of decreasing fluidity (and permeability) from the outside to the inside of the cell wall.

The high lipid content of the cell wall makes it impermeable to hydrophilic compounds, although porins such as MspA allow the passage of small molecules such as glucose [155]. Notably, the cell wall is significantly less permeable to hydrophobic compounds than would be predicted, due to the decreasing permeability towards the inner cell wall; however hydrophobic compounds do traverse the cell wall considerably more easily than the hydrophilic ones [156]. The impermeability of the cell wall imparts the resistance of mycobacteria to different classes of antibiotics, adding to the complication of treating mycobacterial infections. It is also considered to be a barrier to mycobacteriophage–induced lysis. Additionally, mycobacteria are resistant to drastic conditions including sunlight exposure, drying, alkaline conditions, and to many disinfectants, making it difficult to prevent transmission in overpopulated areas [156].

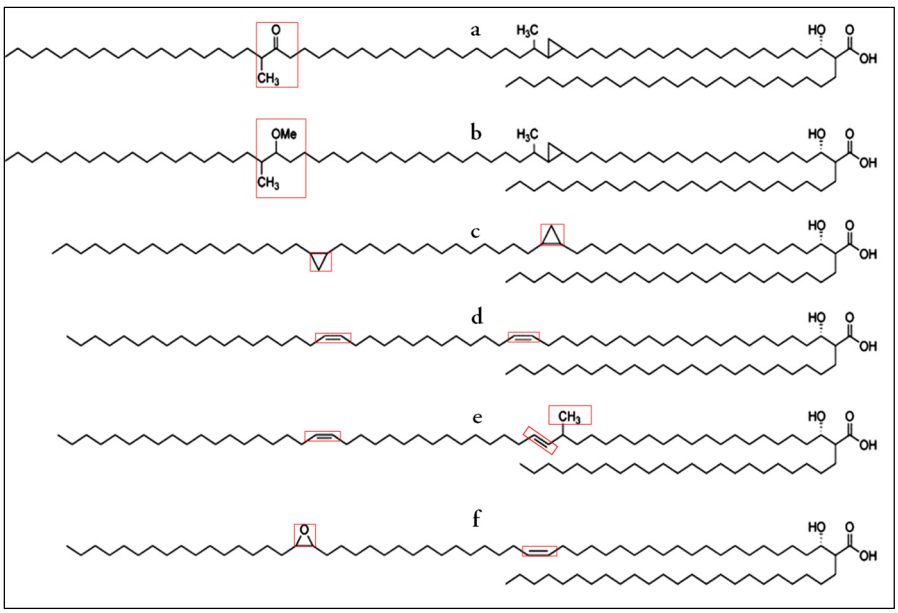


Figure 4.5

Different types of mycolic acids in which meromycolic acid moiety is modified with a) keto group, b) methoxy group, c) cyclopropane rings, d) double bonds, e) methyl group and double bonds, and f) epoxy group and double bond [157].

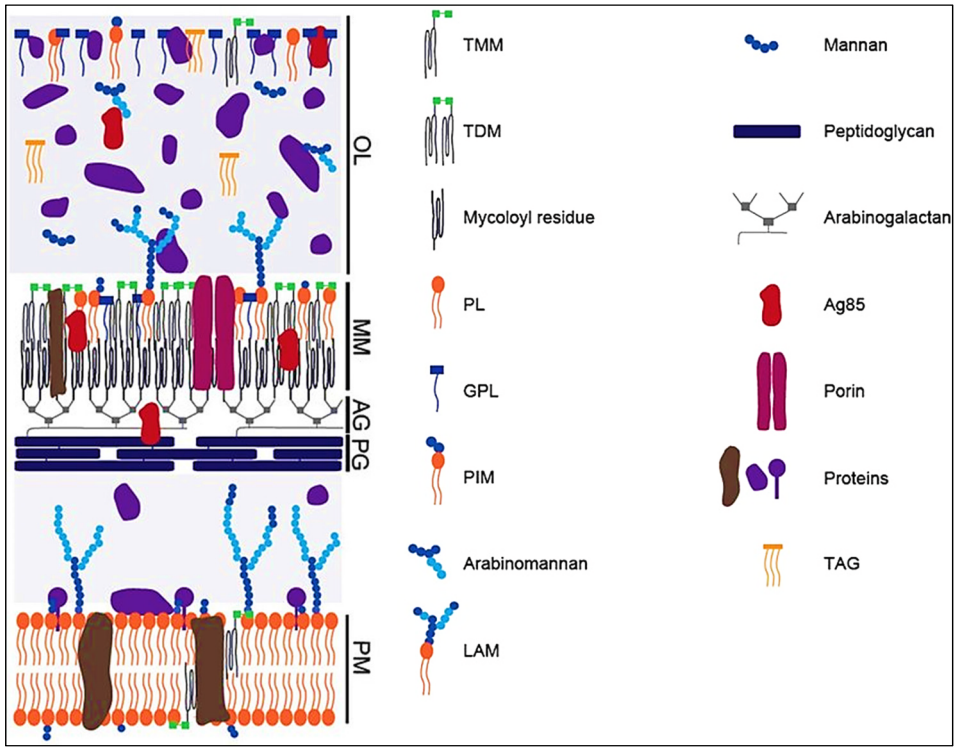


Figure 4.6

M. smegmatis cell envelope. The plasma membrane (PM) is separated from the cell wall by a periplasmic space, peptidoglycan is covalently linked to AG which is esterified by mycolic acids contained in the inner leaflet of the mycomembrane. The outer leaflet of the mycomembrane is composed of extractable lipids including phospholipids, trehalose mycolates, glycopeptidolipids, and lipoglycans. The outermost layer comprises mainly proteins, small amount of carbohydrates and few lipids. PM: plasma membrane; TMM: trehalose monomycolates; TDM: trehalose dimycolates; GPL: glycopeptidolipids; PL: phospholipids; PIM: phosphatidyl–myo–inositolmannosides; LAM: lipoarabinomannans; TAG: triacylglycerols; Ag85: antigen 85 [158].

4.3.3 Modifications in Mycobacteria Cell Wall

4.3.3.1 Modifications in Peptidoglycan Structure

The peptidoglycan of the mycobacteria has unique features that provide rigidity and resistance to the cells towards osmotic pressure. Although peptidoglycan of Gram–negative and mycobacteria belongs to A1γ type, mycobacterial peptidoglycan is approximately 75% cross linked compared to 20–30% in the Gram–negative *E. coli* [159]. Albeit *m*–DAP is cross–linked to D–Alanine in another tetra–peptide moiety, one–third of the cross–linking occurs between two *m*–DAP moieties (**Figure 4.7**) which is responsible for the additional rigidity of the peptidoglycan. Adding to that, the D–Glutamic acid and *m*–DAP in the stem peptide chain are often amidated to D–Glutamine and NH_2–*m*–DAP. The modification expands also to the glycan

strands in which the muramic acid residues are N–glycolylated instead of N–acetylation. This modification adds extra alcohol group promoting more hydrogen binding, hence enhancing the cell wall stability and strengthening the peptidoglycan mesh network. N–glycolylation of muramic acid residues also contributes to the resistance of the mycobacteria to the hydrolytic action of lysozyme [156].

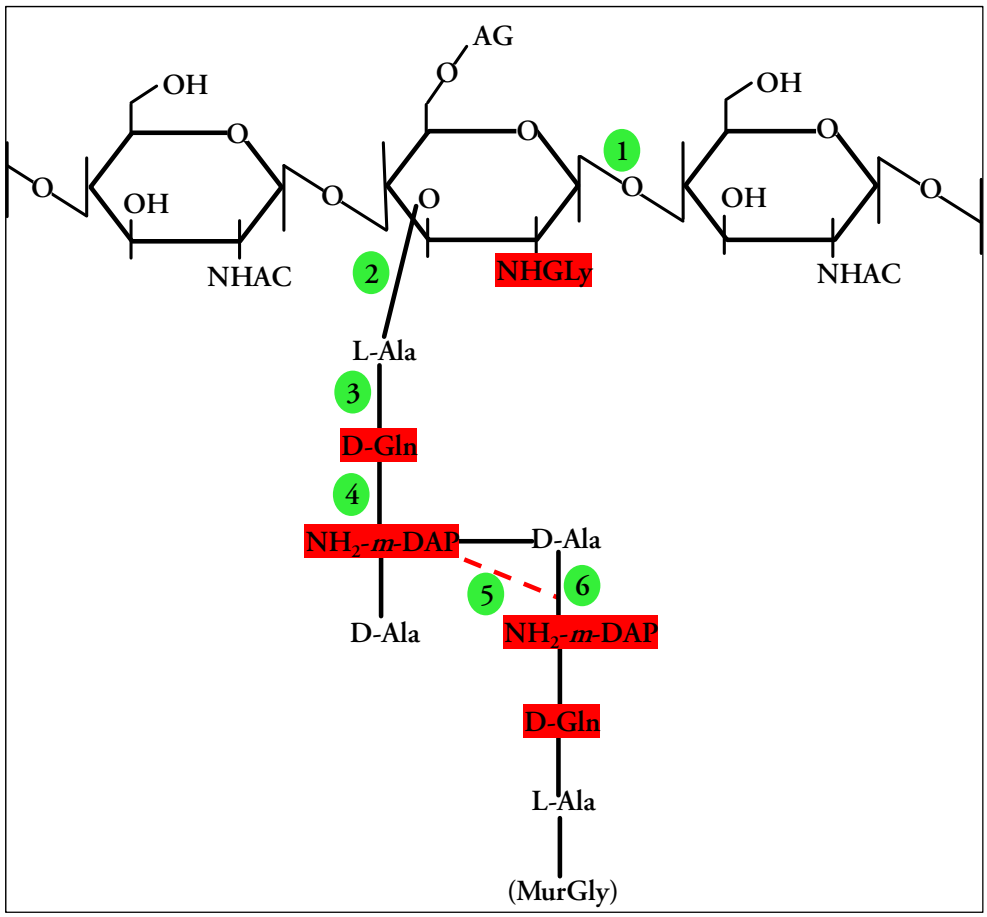


Figure 4.7

Schematic presentation of peptidoglycan in mycobacteria and target bonds by LysA enzymes. Modifications in peptidoglycan structure are highlighted in red, while the target bonds are highlighted in green. Mycobacteriophage LysA enzymes contain domains that target the highlighted positions, GH119, GH25 and TG domains cleave at position 1, Ami–2A and Ami–2B cleave at position 2, N1 domain acts at position 3, N5 domain acts at position 4, N2, N3 and M23 are predicted to act on position 5 and 6 [160].

4.3.3.2 Arabinogalactans

Attached to the 10–12% of N–glycolyl muramic acid moieties are chains of arabinogalactan (AG) composed of the furanose forms of arabinose (Ara*f*) and galactose (Gal*f*). The galactan comprises the core which is covalently linked to the

C6 of N–glycolyl muramic acid via a diglycosylphosphoryl bridge and extends as a chain of approximately 30 alternating 5– or 6–linked α–D–Gal*f* residues [161]. Arabinin chains branch from the C5 of some 6–linked Gal*f* residues and forms 5–linked α–D–Ara*f* extensions. A 3,5–linked α–D–Ara*f* divides the chain into two branches followed by three more 5–linked Ara*f* residues and ending with the non–reducing terminal pentaarabinofuranosyl structure (**Figure 4.8**). This structure includes a 3,5–linked α–D–Ara*f* branching into two 2–linked β–D–Ara*f* units. Approximately two–thirds of the pentaarabinofuranosyl units are esterified by mycolic acids [162].

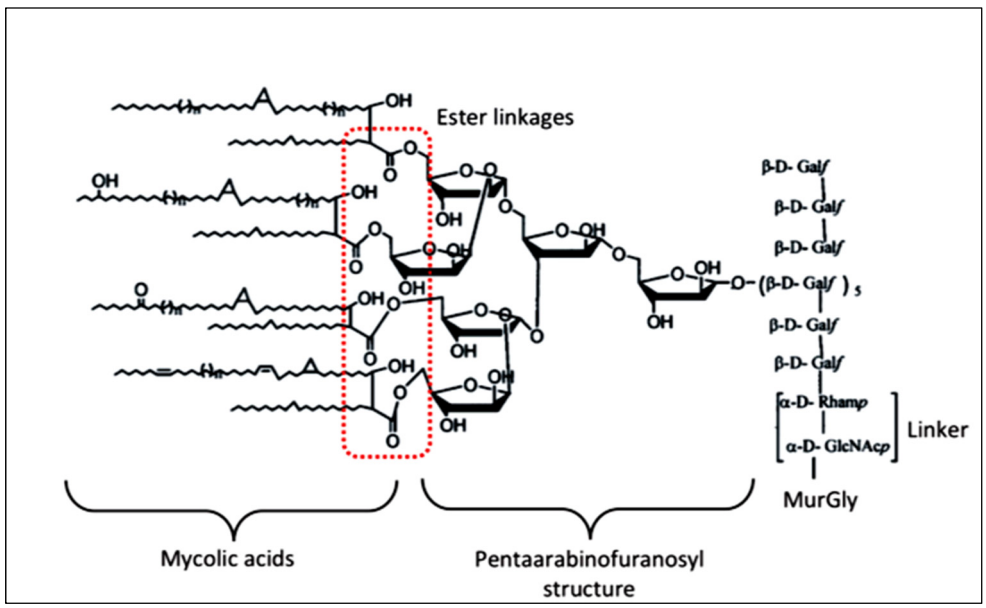


Figure 4.8
Arabinogalactan chain with branching mycolic acids [163].

5. Phage–derived Endolysins

5.1 Endolysins

Endolysins are peptidoglycan hydrolyzing enzymes secreted at the end of the phage lytic cycle to enzymatically degrade the peptidoglycan layer "**from within**" and can kill the host cell when applied externally "**from without**" by creating a high osmotic pressure within the cell (≈ 50 atmospheres for Gram–positive and 5 for Gram–negative) [150, 164]. The term Enzybiotics (Enzyme based antibiotics) was coined to describe the enzymatic and antibacterial activities of endolysins when applied externally to achieve lysis from without [165].

5.2 Structures and Enzymatic Activities of Endolysins

5.2.1 Endolysin Structures

Endolysins can have either globular or modular architecture. Endolysins from phages infecting Gram–negative bacteria have unique globular structure with relatively small single enzymatically active domain (EAD) degrading the peptidoglycan layer. However, endolysins from phages infecting Gram–negative bacteria with modular structure, especially among phages with large genomes are growing in number e.g. lysins from *Pseudomonas* phages (KZ144 and EL188) [7, 150]. On the other hand, endolysins from phages infecting Gram–positive bacteria share the modular structure in which one or two N–terminal EADs are connected via flexible linkers with varying lengths to a cell wall binding domain (CBD) which is responsible for recognition of a specific binding site on the surface of the cell wall [7]. In contrast to Gram–positive endolysins, Gram–negative modular endolysins have different orientation with N–terminus CBD and one or two C–terminus EADs (**Figure 5.1**) [166–168]. Up to date, there are 13 CBD and 24 EAD types identified that are clustered in 89 different combinations, suggesting the high versatility among endolysins. CBDs are composed of tandem repeats of amino acid motifs that bind to peptidoglycan ligands or secondary cell wall structures e.g. teichoic acid and neutral polysaccharides that make CBD binding strain or close to species specific.

The function of CBD is proposed to be irreversible binding to the insoluble cell wall in the cell debris after lysis preventing possible lysis of adjacent cells by the action of diffused Gram–positive endolysins, allowing the phage progeny to start a new infection. Since Gram–negative bacteria have an outer membrane that effectively prevents the diffusion of the endolysin extracellularly, the corresponding endolysins are globular without any need for the irreversible binding of the CBD. In **Paper I**, the globular T4 lysozyme was fused through its C–terminus with cellulose binding module (CBM) from *Cellulomonas fimi* and its antibacterial activity against Gram–positive and –negative bacteria, was tested.

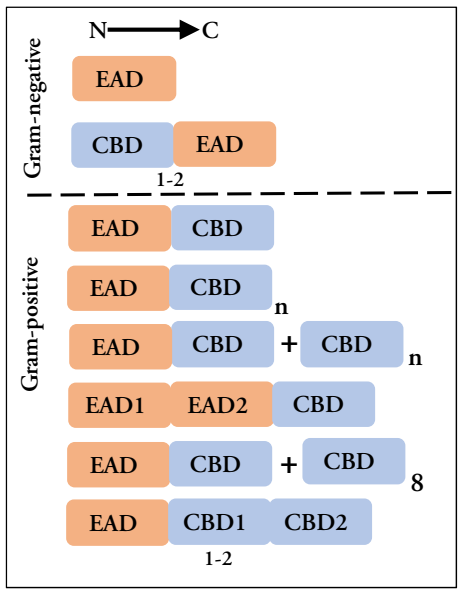


Figure 5.1
Domain architecture of endolysins from phages infecting Gram–positive and –negative bacteria. CBD, cell wall binding domain; EAD, enzymatically active domain. The "n" letter stands for the variable number of cell wall binding motifs that may compose the CBD (2–7 copies), which might be present in tandem repeats or as heterooligomers represented by the sign (+) [4].

5.2.2 Enzymatic Activity

According to the target site in the peptidoglycan structure (glycosidic, amide and peptide bonds), endolysins can be classified into 5 major groups [169] (**Figures 4.4, 4.7**):

1. Glycosidases (N–acetyl–β–D–glucosaminidases, glucosaminidases) (EC 3.2.1.52) cleave N–acetylglucosamine –β– (1–4)– N–acetylmuramic acid glycosidic bond in the glycan strands releasing glycopeptides with at least one

N–acetylglucosamine–N–acetylmuramic acid disaccharide attached to its corresponding peptide.

2. Lysozymes or muramidases (**endo** N–acetyl–β–D–muramidases) (EC 3.2.1.17) attack N–acetylmuramic acid –β– (1–4)– N–acetylglucosamine glycosidic bond resulting in a hydrolysis product with a terminal reducing N–acetylmuramic acid residue. There are four classes of lysozyme, three of which (phage T4 lysozyme, Hen Egg White lysozyme and Goose Egg White lysozyme) have the same structure fold containing the catalytic and the substrate binding sites, and cellosyl – a *Chalaropsis* lysozyme with different structure features.
3. Lytic transglycosylases (**exo** N–acetyl–β–D–muramidases) (EC 3.2.1.17) that catalyze intramolecular transglycosylation reaction attacking N–acetylmuramic acid –β– (1–4)– N–acetylglucosamine glycosidic bond resulting in formation of 1,6–anhydro–N–acetylmuramic acid –containing disaccharide peptide. Since the reaction is carried out in the absence of water, lytic transglycosylases are not hydrolases in contrast to lysozyme.
4. Amidases (N–actylmuramoyl–L–alanine amidases) (EC 3.5.1.28) hydrolyzing the amide bond between N–acetylmuramic acid and L–Alanine releasing the stem peptide free from the glycan strands.
5. Endopeptidases (EC 3.4. X.X) hydrolyze the LD, DD and DL peptide bonds in the peptidoglycan. They can act on the peptide stem (e.g. L–alanoyl–D–glutamate endopeptidases, γ–D–glutaminyl–L–lysine endopeptidases) or on the peptide bridge (e.g. D–alanyl–glycyl endopeptidase).

5.3 Measurement of Endolysin Activity

Since measuring the peptidoglycan hydrolase activity of endolysins is not the same as measuring the antibacterial activity, the activity of endolysins can be classified into enzymatic (muralytic) and antibacterial activities.

5.3.1 Measurement of Muralytic Activity of Endolysins

5.3.1.1 Turbidity Reduction Assay

Reduction in the optical density (turbidity) of cell suspension upon addition of endolysins can be used as a spectrophotometric method to access the peptidoglycan

hydrolase activity. Following the decrease in the optical density of the cell suspension with time (usually minutes), △OD/min is used to determine the reaction rate and quantify the specific activity after subtraction from negative control (buffer instead of enzyme mixed with the cell suspension). An example for the turbidity reduction assay is represented in **Figure 5.2.** The slope of the curve was used to calculate the specific activity of both enzymes. In **Paper I**, the lytic activity of HEWL, T4Lyz and T4Lyz fused with CBM (T4Lyz–CBM) were tested against lyophilized *Micrococcus lysodeikticus* and chloroform treated *E. coli* B cells; the specific activities are represented in **Table 5.1**.

Table 5.1

Lytic activities of T4Lyz, T4Lyz–CBM and HEWL against chloroform treated E. coli B cells and lyophilized M. lysodeikticus.

Enzyme	Chloroform treated *E. coli* B cells	Lyophilized *M. lysodeikticus*
T4Lyz	8.3×10^{8} U/mg	73 600 U/mg
T4Lyz–CBM	4×10^{7} U/mg	24 750 U/mg
HEWL	38 100 U/mg	39 450 U/mg

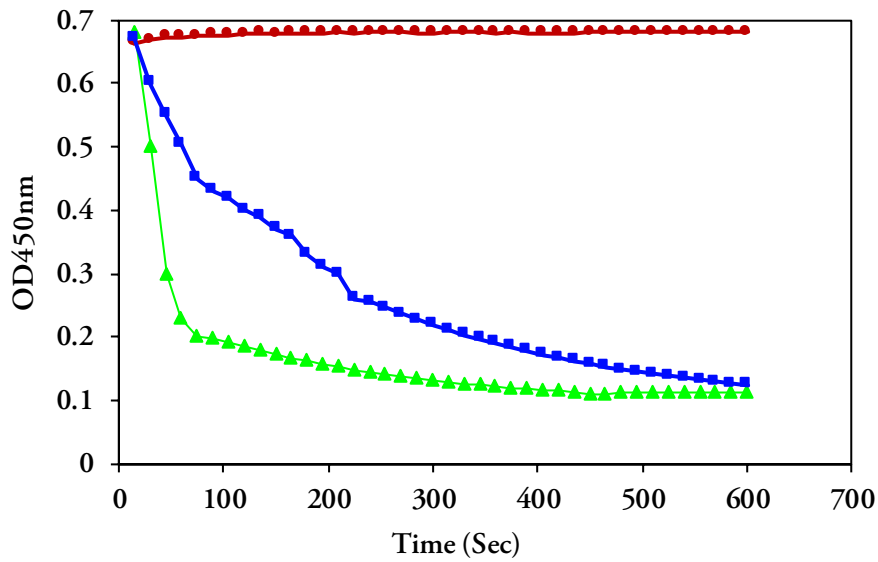


Figure 5.2

Turbidity assay for muralytic activity of HEWL and T4Lyz. The assay was performed in 96–well microtiter plate against lyophilized *Micrococcus lysodeikticus*. 180 µl of 0.3 mg/ml cell suspension in 10 mM sodium phosphate buffer, pH 7.4 was mixed with 20 µl HEWL and T4Lyz, OD_{450nm} was monitored as a function of time. Symbols indicate (●) negative control, (▲) HEWL and (■) T4Lyz [170].

5.3.1.2 Agar Lysoplate Assay (plate lysis assay)

This assay is used to determine the peptidoglycan hydrolase activity of endolysins against autoclaved lyophilized *M. lysodeikticus* cells suspended in 1.5 % (w/v) agar. The zone diameter of the cell lysis is related to the concentration of the lysozyme in the wells and can be used to deduce a standard curve to calculate the specific enzymatic activity (**Figure 5.3**).

Figure 5.3
Lysoplate assay of different concentrations of HEWL. 0) Blank (buffer), 1) HEWL 1 mg, 2) HEWL 0.1mg, 3) HEWL 10 µg, 4) HEWL 5 µg, 5) HEWL 2.5 µg, 6) HEWL 1.25 µg. 80 µl of HEWL were added to the wells punched in 1.5% agar autoclaved with 0.3% *M. lysodeikticus* cells, and the plates were incubated at 30°C for 12 h and examined for lysis [171].

5.3.1.3 Hydrolytic Activity against Glycol Chitin

Besides its muramidase activity, lysozyme can hydrolyze β– (1–4) glycosidic linkages of N–acetylglucosamine homopolymer present in glycol chitin to produce aldehydes as reducing end groups which can be oxidized by potassium ferricyanide and the reaction followed at 420 nm.

In **Paper II**, HEWL and T4Lyz were immobilized to cellulose nanocrystals using different immobilization techniques and the retained enzymatic activity was determined with different assays (**Table 5.2**). A standard calibration curve ($\triangle A_{420nm}$) with different concentrations of the free lysozymes was prepared and the retained hydrolytic activity of immobilized lysozymes was calculated (**Figure 5.4**).

Table 5.2
Retained enzymatic activity of HEWL and T4Lyz after immobilization to cellulose nanocrystals measured using different assays.

Enzyme preparation	% Retained activity determined by Lysoplate assay	Glycol chitin
HEWL		
Free enzyme	100	100
Adsorbed (pH7.4)	88.8 ± 1.3	92.0 ± 2.2
Coupled to EDC activated CNC	90.3 ± 1.7	95.0 ± 0.7
Coupled to Am–CNC	97.4 ± 2.1	98.0 ± 0.4
T4Lyz		
Free enzyme	100	100
Adsorbed (pH7.4)	60.0 ± 1.6	72 ± 2.7
Coupled to EDC activated CNC	86.0 ± 0.4	89 ± 0.5
Coupled to Am–CNC	98.0 ± 1.8	95 ± 2.1

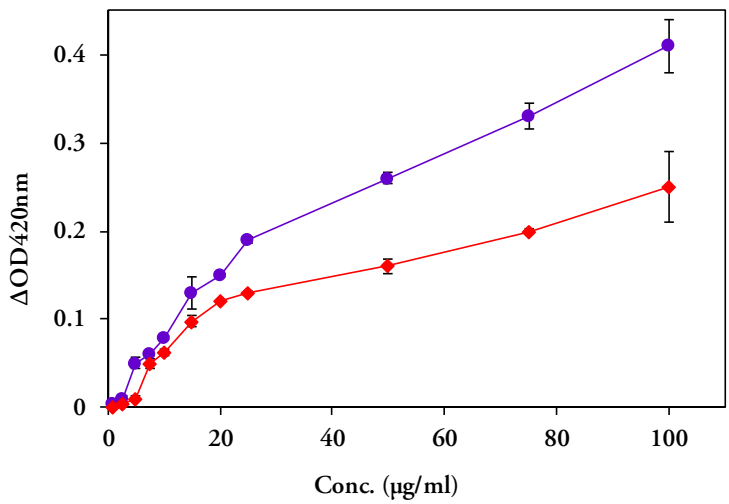


Figure 5.4
Hydrolytic activity assay of different concentrations of free HEWL and T4Lyz against Glycol Chitin as a substrate. Symbols refer to (●) HEWL and (♦) T4Lyz. One ml of 0.05% Glycol Chitin in 0.1 M acetate buffer; pH 4.5 was added to 0.5 ml enzyme and incubated at 40°C for 30 min. Afterwards, 2 ml of color reagent (0.5 g/l of potassium ferricyanide in 0.5 M sodium carbonate) were added and the mixture was immediately boiled for 15 min in a water bath. After cooling, the OD420nm was measured versus water as a blank; the absorbance difference (ΔA420nm) was used as a measure of lysozyme hydrolytic activity [171].

5.3.2 Measurement of Antibacterial Activity of Endolysins

5.3.2.1 Reduction in Number of Colony Forming Units (CFUs)

The change in the CFUs is frequently used to express the antibacterial activity of endolysins. The endolysin is mixed with a live bacterial suspension generally in buffer

and incubated for a certain period. Later on, samples are taken, diluted, plated on agar plates and incubated for a certain period of time and the corresponding CFUs are counted. In **Paper I**, the viable count plating assay showed the antibacterial activity of T4Lyz and T4Lyz–CBM on different bacteria to be dose–dependent. The highest potency was obtained against *M. lysodeikticus* with 98% bactericidal activity (1.7 $\log_{10}$ reduction) using 10 µg/ml of T4Lyz and 97.5% (1.61 $\log_{10}$ reduction) of T4Lyz–CBM (**Figure 5.5a**). Against *E. coli* and *P. mendocina*, the bactericidal activity was 99.96% (3.398 $\log_{10}$ reduction) and 95% (1.301 $\log_{10}$ reduction), respectively, with 200 µg/ml of native T4Lyz–CBM (**Figure 5.5b**). The heat–denatured T4Lyz–CBM showed no antibacterial activity against the Gram–positive *M. lysodeikticus* even at enzyme concentration up to 200 µg/ml, but retained its activity against the Gram–negative bacteria with a bactericidal activity of 94% (1.22 $\log_{10}$ reduction) with 100 µg/ml for *E. coli* and 91% (1.04 $\log_{10}$ reduction) with 200 µg/ml for *P. mendocina* (**Figure 5.5c**). In comparison, the bactericidal activity of native and heat denatured T4Lyz (200 µg/ml) against *E. coli* was 97.5% (1.602 $\log_{10}$ reduction) and 87% (0.888 $\log_{10}$ reduction), respectively (**Figure 5.5d**).

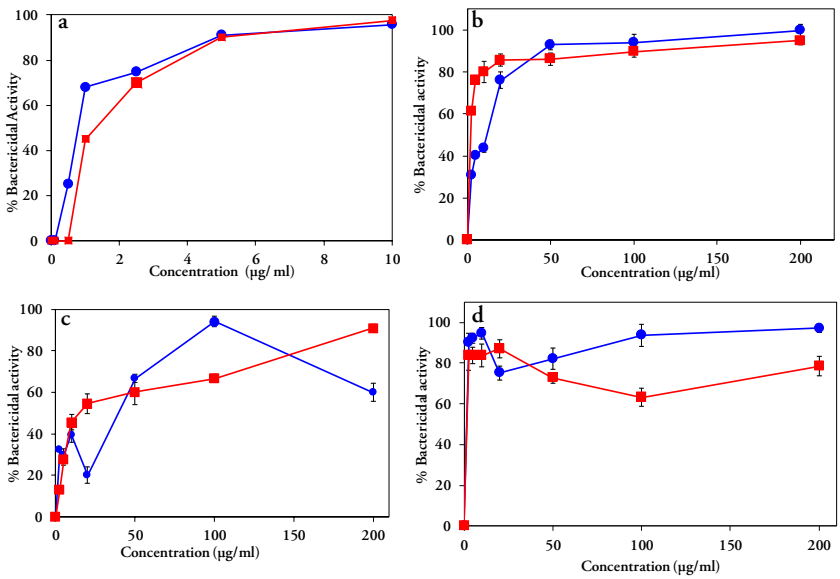


Figure 5.5

Bactericidal activity of native and heat–denatured T4Lyz and T4Lyz–CBM. (a) native T4Lyz (•) and T4Lyz–CBM (■) tested against *M. lysodeikticus*, (b) native T4Lyz–CBM tested against *E. coli* (•) and *P. mendocina* (■), (c) heat–denatured T4Lyz–CBM tested against *E. coli* (•) and *P. mendocina* (■) and (d) native (•) and heat–denatured T4Lyz (■) tested against *E.coli*.

5.3.2.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concertation (MBC)

MIC and MBC are the classical methods to express the antibacterial activity of a given compound. In general, a 2X serial dilution of the endolysin is performed in a 96–well microtiter plate in a growth medium (usually Muller Hinton Broth). A fixed volume of cells with a predetermined CFU/ml (~$1x10^6$ CFU/ml) is added to the plates which are incubated overnight at the optimum bacterial growth temperature, and the wells are examined for growth or growth inhibition. The lowest concentration of the endolysin that inhibits the bacterial growth is considered as the MIC. For MBC, aliquots are withdrawn from clear wells (with no growth) and plated on agar culture media, incubated overnight and examined for growth, the lowest concentration that showed no growth is the MBC. In **Paper II**, determination of MIC and MBC of free HEWL and T4Lyz against Gram–positive and –negative bacteria showed T4Lyz to be more potent than HEWL. However, none of them showed MBC against Gram–negative bacteria; the action was only bacteriostatic, and this might be due to the nature of the outer membrane of the Gram–negative bacteria that limits access of the enzyme to the peptidoglycan layer (**Table 5.3**). Furthermore, this assay can be used to detect any synergistic activity between endolysins and conventional antibiotics. In P**aper IV**, a synergistic activity between endolysin B enzymes and the outer membrane permealizers colistin and protamine sulfate against *Mycobacterium smegmatis* was detected.

Table 5.3
MIC and MBC (µg/ml) of HEWL and T4Lyz against different Gram–positive and –negative bacteria.

	M. lysodeikticus		***Corynebacterium* sp.**		***E. coli***		***P. mendocina***	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
HEWL	200	500	375	800	>1250	>1250	>1250	>1250
T4Lyz	100	250	275	650	750	>1250	1000	>1250

5.3.2.3 Time Kill Assay

This method is used to determine the time point (end point) for the endolysin antibacterial activity. The MBC of the endolysin is mixed with the bacterial load and incubated, samples are frequently collected at predetermined time points, the cell viability is monitored either through determination of CFU/ml or via addition of a metabolic indicator dye. In **Paper II**, Alamarblue® (AB) a metabolic redox indicator dye was used to visualize the reading of checkerboard assay with no effect on the bacterial growth. As a result of the bacterial growth, AB is converted to the reduced form with a color change from purple to pink. It has been confirmed that viable

count plating assay (CFU/ml) is comparable and well correlated with AB assay for determination of bacterial cell viability as well as in time kill kinetic studies.

5.4 Mycobacteriophage Endolysins

Previous studies of mycobacteriophage genomes have led to the identification of two key players responsible genes to complete the cell lysis: Lysin A (LysA) and Lysin B (LysB) enzymes.

5.4.1 Endolysin A (LysA)

Mycobacteriophage LysA are peptidoglycan hydrolases that have been predicted to target nearly every bond in the peptidoglycan structure of the mycobacterial cell wall. These include N–glycolyl–β–D muramidases, N–glycolyl muramic acid–L–alanine amidases, *m*–DAP–*m*–DAP (LD) endopeptidase, L–Ala–D–glutamate (LD) peptidase, D–Glu–*m*–DAP (DL) peptidases, and D–Ala–*m*–DAP (DD) endopeptidase (**Figure 4.7**). LysAs are highly modular and diverse, composed of at least two EADs at the N–terminus and a C–terminal CBD, respectively, with the majority containing an extra central catalytic domain that is usually with a peptidase activity [160]. There are at least 26 different endolysin organizations (Org–A to Org–Y) with distinctive domain combinations. Some follow a different pattern of domain organization presenting two CBDs (Org–D, H, T, and V) or none (Org–L), and others do not present the amidase or glycosidase catalytic domains (Org–C, H, M, T, and Y) [172]. EADs that are present at the N–terminal or central domains of LysAs encompass N–glycoyl–β–D–muramidases belonging to glycoside hydrolase families GH25 or GH19. GH25 has a muramidase activity, while GH19 has a chitinase domain and has been found mainly in cluster A of LysAs. Moreover, the transglycosylase (TG) activity (pfam06737) included in the lysozyme–like superfamily is restricted to cluster A, where the TG is the only EAD, flanked by conserved N–terminal peptidase and C–terminal CBD [172]. The limitation of TG to cluster A mycobacteriophage, suggests that this TG activity may be specific for N–glycolylated peptidoglycan. The amidase–2A (Am2A) conserved domain (pfam01510) that belongs to peptidoglycan–recognition proteins (cd06583) are over–represented among 224 mycobacteriophages LysA that were analyzed [160]. In the same study, a total of six sequence variants of peptidases have been proposed: the M23 peptidase domain (pfam01551) and N1–N5 N–terminal domains. The N1 domain encodes L–Ala–D–Gln peptidase activity, that attacks mainly type A1γ peptidoglycan, suggesting substrate specificity (**Figure 5.6**).

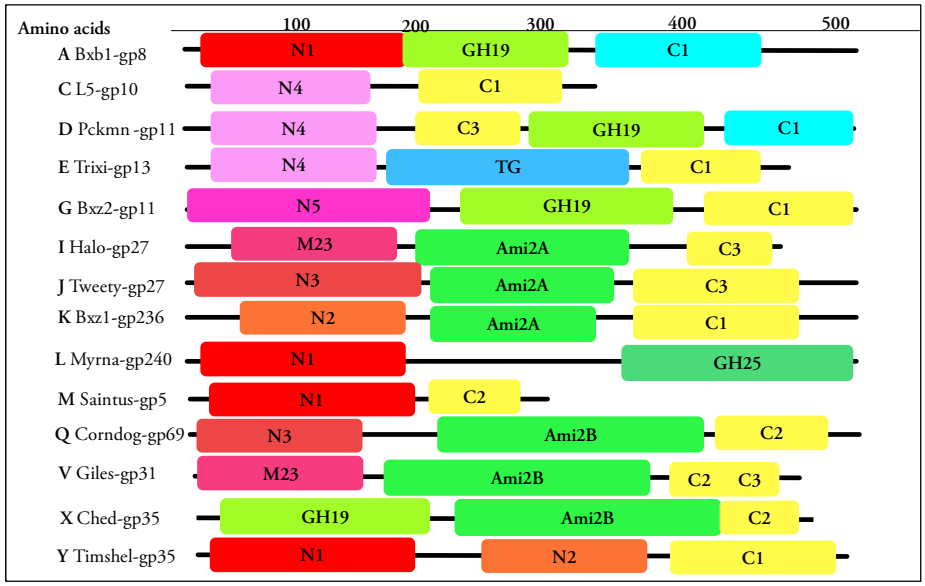


Figure 5.6

Schematic presentation of modular architecture of mycobacteriophage LysA enzymes with different domain organizations that are colored differently. The predicted domains are putative peptidases (red and pink shades): N1–N5 and M23, Enzyme active domains (green shades): GH19 (lytic glycoside hydrolase family 19), GH25 (lytic glycoside hydrolase family 25), TG (lytic transglycosylase), Ami2A (Amidase) and Ami2B (Amidase) and cell wall binding domains (yellow color) C1—C3 [160].

5.4.2 Endolysin B (LysB)

LysB genes are involved in cell lysis, because of their linkage to *lysA* as well as demonstration of the mycolylarabinogalactan esterase activity of LysB–D29, –Ms6, –TM4, –L5, –Bxz2 and –Bxb1 [173–175]. LysB homologs have been bioinformatically identified in the majority of completely sequenced mycobacteriophage genomes and are located downstream of *lysA* gene and separated from it by no more than four intervening genes (**Figure 5.7**). The presence of *lysB* downstream of *lysA* in the lysis module strongly supports the role of LysB in cell lysis.

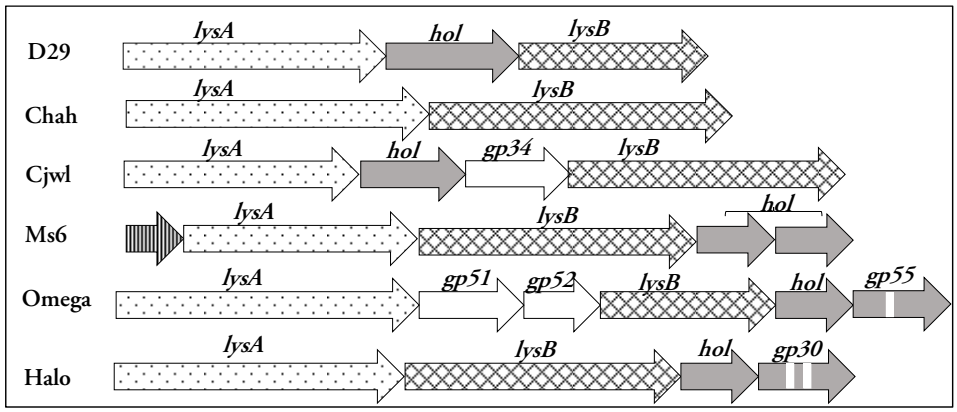


Figure 5.7
Lysis cassette of some mycobacteriophages from different clusters [171]. LysB, Holin, LysA, chaperon and unknown function [172].

5.4.2.1 Structure of LysB Enzymes

Sequence alignment of LysB proteins shows that they are globular and highly diverse with only three residues being completely conserved. Among 1790 mycobacteriophage genomes that have been deposited in the database, the crystal structure of only LysB from Mycobacteriophage D29 has been determined [160]. According to the percentage similarity to LysB–D29, LysB homologs are grouped into 7 groups, ranging from 100–89% similarity (group 1) to 30% similarity (group 7) (**Paper III**). Multiple alignments of different LysB amino acid sequences revealed the following (**Paper III**):

- LysB proteins vary greatly in length from 244 (LysB–BabyRay) to 346 (LysB–Dylan) residues.
- Domains of the 29 representatives of LysB proteins were highly diverse ranging from no conserved motifs (LysB–Obama12 and LysB–Enkosi) to enzymes with up to seven different motifs (LysB–MrMagoo). The majority (25 LysBs including LysB–D29) have two combined domains: PE–PPE (PF08237) and Cutinase (PF01083). However, LysB–Palestino and–Omega have either the PE–PPE or Cutinase –motif, respectively.
- With regard to conserved residues, Serine and Aspartate in the catalytic triad are absolutely conserved in contrast to the third member (Histidine) with weak conservation. Additionally, the pentapeptide G[DA]–Y[F]–S–Q–G[S] and the GNP motif are highly conserved. Surprisingly, among all LysB sequences two hypervariable regions were found, region–1 (the N–terminal extra residues) and region–2 (the C–terminal mobile loop).

The crystal structure of LysB–D29 was determined at 2.0 Å resolution and showed the typical fold of α/β hydrolases with a remarkable structural similarity to *Cryptococcus* cutinase–like protein. The catalytic triad [Ser82–Asp166–His240], which is closely similar to those in other members of the α/β hydrolase family, is located at the edge of the central β–sheet between the α/β sandwich and the linker domain [160]. To understand the difference between LysB and other α/β hydrolases, the crystal structure of LysB–D29 was used as a query in Dali server (http://ekhidna2.biocenter.helsinki.fi/dali/) to search for similar α/β hydrolase members. The retrieved structures were in an opened conformation as they were co–crystallized with inhibitors (especially for lipases which have a lid domain). The common features and differences in their secondary structures and 3D surfaces are summarized in **Table 5.4** (**Paper III**).

Table 5.4
Relative members of the α/β hydrolase family to LysB–D29 (**Paper III**).

Name	Pdb ID	Z–score	RMSD	Aligned residues	Total length	(%) Similarity to LysB–D29
Penicillium purpureogenum Acetylxylan esterase	1G66	18.9	2.2	162	234	22
Fusarium solani cutinase	1XZM	16.4	2.6	156	230	20
Humicola insolens cutinase	4OYL	16.2	2.6	155	194	21
Trichoderma reesei cutinase	4PSE	14.2	2.6	148	254	22
Human pancreatic lipase	1LPB	8.6	2.8	147	465	15
Pseudomonas cepacia lipase	1YS1	9.1	3.1	143	364	16
Candida rugosa lipase	1Lpo	7.3	3.4	100	549	12

Furthermore, the structural alignments showed LysB–D29 to have common features with esterases, cutinases and lipases. LysB–D29 has a typical α/β fold (consisting of five central parallel β–sheets winged by two α–helices on each side) similar to esterases and cutinases, however it lacks the first two short N–terminal α–helices found in all cutinases (**Figure 5.8**). LysB–D29 exhibits low similarity to lipases, therefore several different features were observed. LysB–D29 is relatively shorter by 100–300 amino acid residues than the aligned lipases, which can be attributed to the higher number of parallel β–sheets of the central α/β fold in lipases (6, 8 and 10 for *P. cepacia* lipase, human pancreatic lipase and *Candida rugosa* lipase, respectively). Moreover, lipases (except for *Candida antarctica* lipase B) have lid domains covering the active site when in closed conformation which is a missing feature in LysB–D29.

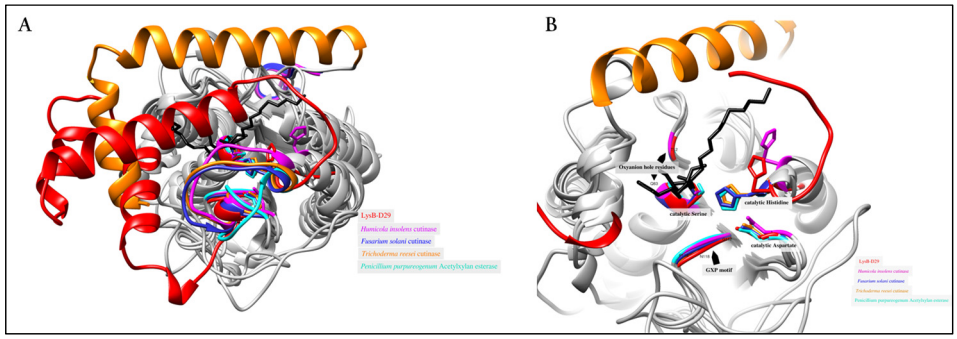


Figure 5.8

Three-dimensional structural alignment of 3D structures of LysB-D29 (red color) and its relative members of the α/β hydrolase family including: *Fusarium solani* cutinase (blue color), *Humicola insolens* cutinase (purple color), *Trichoderma reesei* cutinase (orange color), *Penicillium purpureogenum* acetylxylan esterase (cyan color). (A) Total alignment: showing the lid domain of *T. reesei* cutinase (orange color), linker domain of LysB-D29 (red color). (B) Focus view: showing catalytic triad residues, oxyanion hole residues and GXP residues (each is shown in its color), co-crystalized inhibitor molecule (black), and the rest of protein (gray color) (**Paper III**).

On the other hand, LysB–D29 shares high conservation of the GXP motif with lipases where X accounts for Asparagine in LysB–D29, Lysine in *Candida rugosa* lipase and Threonine in both *P. cepacia* lipase and human pancreatic lipase, respectively. However, the position of this motif is poorly conserved in *C. rugosa* lipase and human pancreatic lipase in comparison to LysB–D29, *P. cepacia* lipase, *Penicillium purpureogenum* acetylxylan esterase and *Trichoderma reesei* cutinase where this motif is located at the end of the fourth β–sheet just adjacent to the catalytic serine (35, 24, 42 and 28 residues, respectively, downstream the catalytic Serine) (**Figure 5.9**) (**Paper III**). The long amino acid sequence between the catalytic Aspartate and Histidine corresponding to the linker domain in LysB–D29 (73 residues) was found to be comparable to that of human pancreatic lipase and *C. rugosa* lipase (87 and 108, respectively) and much longer than *P. cepacia* lipase (22 residues) (**Paper III**).

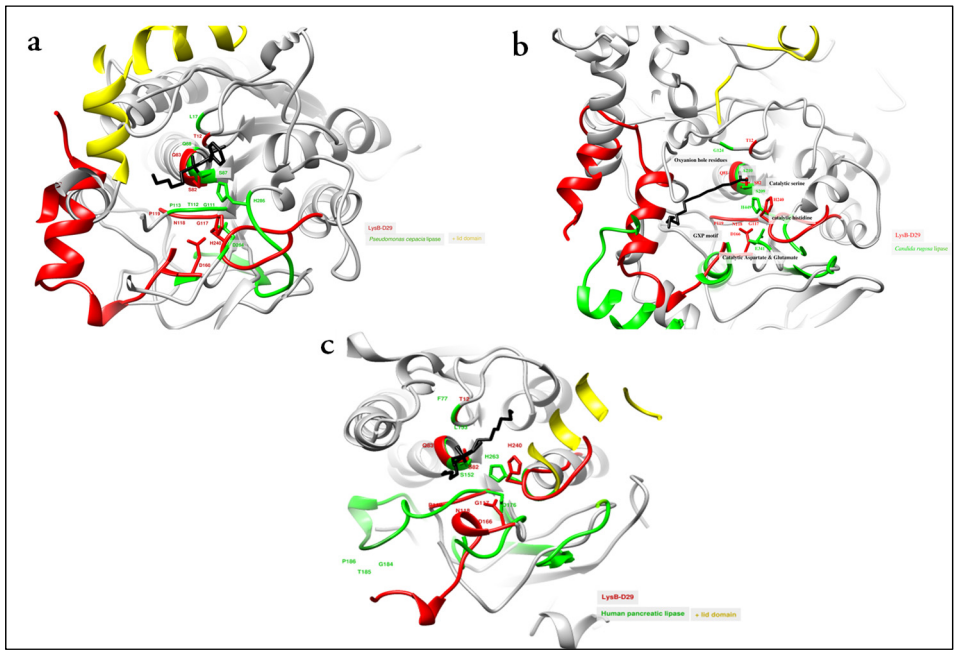


Figure 5.9

Three-dimensional structural alignment of (a) LysB-D29 to *P. cepacia*, (b) alignment of LysB-D29 to *C. rugosa*, (c) alignment of LysB-D29 to Human pancreatic lipase, showing the catalytic triad residues, oxyanion hole residues, GXP residues in red color (LysB-D29) & green color (aligned lipase enzyme), and co-crystalized inhibitor molecule (black color)(**Paper III**).

Lacking the lid domain (like all true cutinases) gives LysB enzymes the advantage of being activated by default without the need for interfacial activation (as in the case of lipases) prior to reaction with a fatty molecule [176]. In contrast, Grover and coworkers concluded that the increased activity of LysB–Ms6 and –Bxz2 enzymes against *para*–nitrophenyl butyrate (*p*NPB) in the presence of surfactants is due to the conformational changes in the lid domain that keeps the active site in the open form [174]. This assumption is in contradiction to the knowledge that enzymes with lid domains exhibit detectable activity only on partially soluble substrates like *p*NPB at substrate concentrations exceeding the solubility limit or in the presence of surfactants (oil/water interface) where the active site is opened by moving the lid out [177]. Both LysB–Ms6 and –Bxz2 enzymes showed specific activity exceeding 0.1 and 1.5 U/mg, respectively, against *p*NPB at concentration of 1 mM which is below the solubility limit without addition of any surfactants [174]. Additionally, the esterase activity of LysB–D29 on different concentrations of short chain *p*NPB and long chain *para*–nitrophenyl palmitate (*p*NPP) substrates showed activity pattern close to that of esterases as they act instantly on their substrates and their activity reaches a plateau at substrate concentrations below the solubility limit (**Paper IV**). Finally, the esterase activity of LysB–D29 against 1mM *p*NPB was tested in the presence and absence of Triton X–100 as surfactant. Surprisingly, the esterase

activity of LysB–D29 was higher (1.6 U/mg) in the absence of Triton X–100 than 0.93 U/mg in the presence of Triton X–100, supporting the hypothesis that LysB enzymes do not have lid domain and are thus not true lipases (**Paper III**).

Structural alignment of LysB–D29 with its 3D homology models revealed almost identical pattern of their secondary structures except for a twelve residues–long loop of the linker domain (loop–5) extending from Serine232 to Asparagine243 in LysB–D29 (**Figure 5.10**). Loop–5 is a hypervariable region in terms of length and its residues and contains the catalytic Histidine residue. In many LysB models, structural alignment illustrated great translocation of the catalytic Histidine from its aligned position in D29, and far away from the two other catalytic residues (Serine and Asparagine) whose positions were well conserved (Paper **III**).

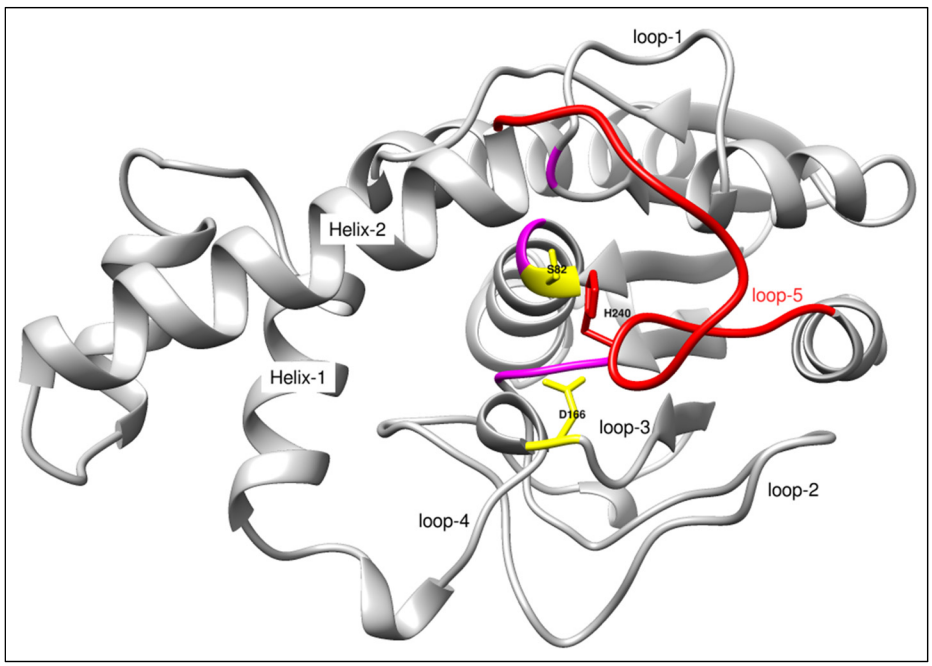


Figure 5.10
Crystal structure of LysB–D29. Loops forming the hydrophilic opening [Loop–1(10–21) and loop–5 (231–244)]. Loops and helices forming the hydrophobic opening [Helix–1 (211–230), Helix–2 (179–190), loop–3 (172–178) and loop–4 (162–171)]. Catalytic residues (yellow color), linker domain (red color), oxyanion hole residues (pink color) and rest of the protein (gray color) (**Paper III**).

5.4.2.2 Activity of LysB Enzymes

In **Paper IV**, esterase and lipase activities of recombinant LysB–D29, –Omega, –Saal and –Obama12 enzymes were evaluated against *para*–nitrophenyl esters and Tween

substrates. Kinetic studies with *para*–nitrophenyl esters with variable carbon chain lengths revealed highest catalytic efficiency against C12 ester *para*–nitrophenyl laurate (*p*NPL) (**Table 5.5**).

Table 5.5
Kinetic parameters of the activities of LysB–His_6 enzymes against *para*–nitrophenyl esters with variable carbon chain length (**Paper VI**).

LysB–	*p*NPB	*p*NPO	*p*NPL	*p*NPM	*p*NPP
		K_m (μM)			
D29	422.6	42.3	19.6	24.08	37.7
Omega	618.8	193.3	280.8	98.72	24.4
Saal	4172.12	1519.25	1476.39	956.7	2833.32
Obama12	1268.5	2666.8	3000	1266.63	800
		Vmax (U. mg^{-1})			
D29	122.3	9.85	7.55	3	2.73
Omega	111.8	15.7	79.8	22.3	1.45
Saal	1.49	0.247	0.425	0.414	0.4
Obama12	0.470	0.165	0.17	0.119	0.084
		K_{cat} (min^{-1})			
D29	716.5	57.68	44.28	17.63	14.53
Omega	704.11	452.51	503.84	140.78	9.13
Saal	11204.83	1855.22	6373.97	3107.73	3364.4
Obama12	3461	1215.69	1256.84	814.35	622.07
		K_{cat}/K_m (μM^{-1}. min^{-1})			
D29	1.7	1.36	2.26	0.732	0.38
Omega	1.14	2.34	2.41	1.43	0.374
Saal	2.68	1.22	4.31	3.24	1.18
Obama12	2.72	0.455	0.42	0.642	0.77

Recombinant LysB–His_6 enzymes are active over a wide range of temperature (with optimum of 37 °C against *para*–nitrophenyl esters and 30 °C against Tweens) and pH 7.4–8. While Mn^{2+}, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} ions increased both the esterase and lipase activities of some LysB–His_6 enzymes, Zn^{2+} ions were shown to reduce the esterase activity (**Paper VI**).

The natural substrate of LysB enzymes is mAGP, which is composed of mycolic acids esterified to a non–reducing terminal of the penta–arabinosyl motif in the arabinogalactan (**Figure 4.8**) [174, 175, 178]. In **Paper IV**, the hydrolytic activity of LysB–D29, –Omega, –Saal and –Obama12 enzymes against the isolated mAGP substrate was evaluated by LC/MS in negative–ion mode. LysB–His_6 treated mAGP showed a peak with the same retention time as the mycolic acid standard. On the other hand, there was no peak in *Rhizopus oryzae* lipase treated mAGP indicating that only LysB–His_6 enzymes have the ability to hydrolyze such a complex substrate (**Figure 5.11**). The *m/z* values (**Figure 5.12**) of the mycolic acid confirmed that when

LysB enzymes applied externally have the ability to hydrolyze mAGP as reported for LysB– Ms6, –Bxz2 and –D29 enzymes [174, 175, 178].

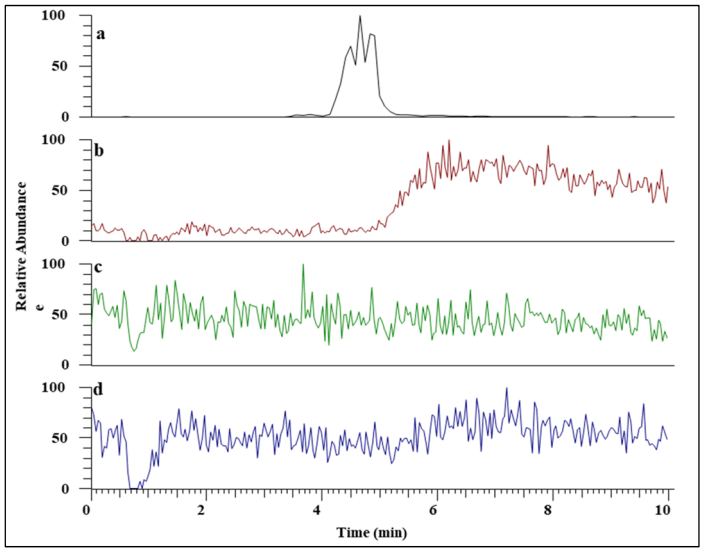


Figure 5.11
HPLC chromatogram for a) mycolic acid standard, b) mAGP treated with LysB–D29, c) mAGP treated with *Rhizopus oryzae* lipase, and d) mAGP treated with buffer as negative control (**Paper VI**).

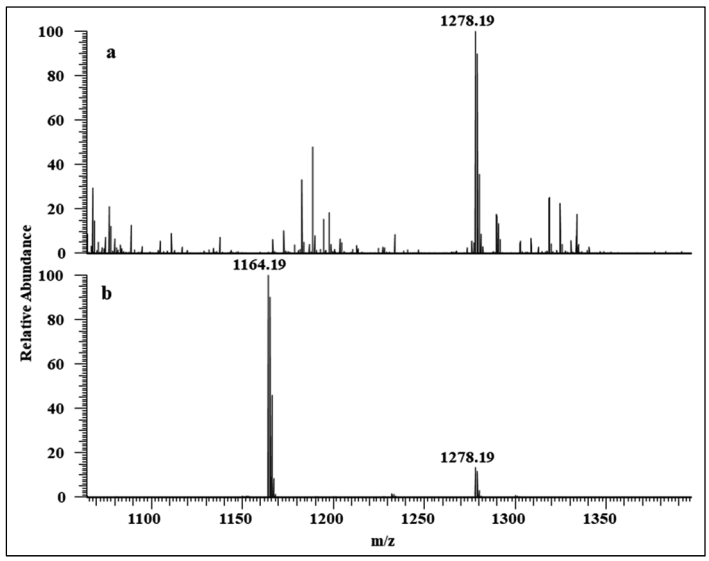


Figure 5.12
MS spectrum full scan of a) mAGP treated with LysB–D29 and b) mycolic acid standard (**Paper VI**).

The importance of LysB in cell lysis has been proven earlier as LysB completes the lysis of the host mycobacterium, a Giles*Δlys*B mutant mycobacteriophage is viable, but defective in the normal timing, progression, and completion of the host cell lysis

[175]. Recently, cryo–electron microscopy of mycobacteria cells infected with Ms6 mutant with a *lysB* deletion (Ms6Δ*lysB*) revealed that in the absence of LysB, Ms6Δ*lysB* phage particles are trapped in deformed incompletely lysed cells, while at the same time point cells infected with the wild type Ms6wt are completely lysed [179].

In paper **IV**, recombinant LysB–His_6 enzymes did not exhibit significant antibacterial activity either alone or in combination with anti–TB drugs. This might be attributed to the hypothesis that Tween 80 as a surfactant is required not only for removal of *M. smegmatis* cell clumps and aggregates due to surface hydrophobicity but also for promoting the antibacterial activity of LysB enzymes [174]. Our findings also agree with the hypothesis that LysB enzymes exert higher antibacterial activity in presence of Tween 80 due to the liberated oleic acid after hydrolysis with LysB enzymes [174]. The difference in the catalytic activity and the effectiveness in the lysis might be attributed to the high diversity among LysB enzymes [172]. Moreover, the poor permeability nature of the mycomembrane acts as a hindrance for LysB to access its target when applied externally to *M. smegmatis* cells. Nevertheless, half MIC values of colistin and protamine sulfate (outer membrane permealizers) resulted in higher Log_{10} reduction of *M. smegmatis* treated with 100 µg/ml LysB–His_6 enzymes However, with this combination we could not detect MIC/MBC levels (**Table 5.6**).

Table 5.6

Log_{10} reduction of *M. smegmatis* after treatment with 100 µg/ml of LysB–His_6 enzymes alone and in combination with colistin (1 µg/ml) and protamine sulfate (10 µg/ml), respectively (**Paper VI**).

LysB–	Alone	plus 1 µg/ml Colistin	plus 10 µg/ml Protamine
D29	1.1	3	1.8
Omega	1.32	3.45	2.1
Saal	1.44	3.1	1.9
Obama 12	1.36	4	2.8

6. Endolysins as Antibacterials

Due to their ability to degrade vital components in the bacterial cell wall, interest in exploring the endolysins as alternative to conventional antimicrobials has increased during the past two decades [150, 180]. The use of endolysins to combat bacterial infections has some advantages, for example so far no cases of bacterial resistance have been reported even after repeated therapy [181, 182]. Endolysins can be identified and used from both temperate and lytic phages, and the modularity of endolysins allows for enzyme engineering for achieving enhanced properties (stability, solubility, activity, broadening the spectrum, etc. …). The 'lysis from without' of target bacteria by endolysins has been demonstrated mainly against Gram–positive bacteria, as the mycomembrane of mycobacteria and outer membrane of Gram–negative bacteria hinders their access to the peptidoglycan [183]. However, recently a remarkable progress has been made in the search and development of endolysins with killing activity extending to Gram–negative bacteria, and efforts are ongoing against mycobacteria [184–186].

6.1 Protein Engineering of Endolysins

In order to overcome limitations of endolysins such as narrow host range, low solubility and reduced *in vivo* activity, many aspects for engineering endolysins have been reported [180, 187], including mutagenesis, truncation, domain swapping (chimeras) and artilysation [7]. Point mutation has been applied to enhance the lytic activity and increase plasma half–life as in the case of Cpl–1, pneumococcal phage Cp–1 endolysin. Cpl–1 is a dimer and introducing a disulfide bridge resulted in increased lytic activity against *Streptococcus pneumoniae* cells and prolonged half–life in mice as a result of the increased molecular weight [188]. On the other hand, truncation mutagenesis approach was applied to enhance the antibacterial activity of streptococcal phage NCTC11261 endolysin (PlyGBS) that infects Group B *Streptococci* resulting in mutants with upgraded activity (18–28 fold) compared to the full–length PlyGBS [189]. Another approach is to develop chimeric endolysins by domain swapping and/or combining different EADs and CBDs. The major advantage of domain swapping is that it is not restricted to bacteria of the same species or genus. Moreover, it can be used to generate customized endolysins, and as

a result the spectrum of activity of some endolysins can be extended. For example, swapping the CBDs of the endolysins (PlyPSA and Ply118) encoded by *Listeria monocytogenes* phage changed the serovar specificity [190]. Domain swapping was also used to generate chimeras with enhanced antibacterial activity as in the case of the chimeric endolysin (Ply187AN–KSH3b). Ply187AN–KSH3b is a chimeric protein combining CHAP (Cysteine, Histidine–dependent amidohydrolases/peptidase) domain of Ply187 and the SH3b of LysK that has enhanced antibacterial activity against MRSA that is higher than the full length Ply187 [191].

The outer membrane in bacterial cells acts as a semi–permeable membrane (cutoff ~600 Da via nonspecific porins) that armors the peptidoglycan layer. Moreover, the outer membrane is stabilized through ionic interactions of divalent cations (e.g. Mg^{2+} and Ca^{2+}) and the phosphate groups of adjacent lipopolysaccharides (LPS) molecules and by hydrophobic stacking of the lipid A moiety of LPS molecules. As a result, the outer membrane acts as an impermeable hurdle for hydrophobic and hydrophilic compounds ≥ 600–650 Da that cannot cross via the non–specific porins [184]. To overcome the hindrance in Gram–negative bacteria, the endolysin is fused with outer membrane permeabilizing peptide (OMP) leading to destabilization of the outer membrane, followed by passage of the fusion protein, resulting in peptidoglycan degradation and eventually cell lysis. These fusion proteins are called 'Artilysins'; the OMP–peptide can be polycationic, hydrophobic or amphipathic peptides, to be able to interfere with the ionic and hydrophobic stabilizing forces of the outer membrane [192, 193]. Art–175 is an Artilysin that is composed of fusion of SMAP–29, an α–helical amphipathic AMP to the N–terminus of mutated KZ144 endolysin where three cysteines were mutated to serine to avoid aggregation via oligomer formation and to enhance the structural stability and antibacterial activity. Art–175 is effective against all tested *P. aeruginosa* strains (79), including multidrug–resistant isolates, with a MIC of (0.3 μM) similar to conventional antibiotics with no provoked resistance upon exposure to sublethal doses in contrast to control antibiotics. Moreover, Art–175 did not raise cross–resistance related to 13 prevalent resistance mechanisms, including the recently emerging mcr–1 colistin resistance [193]. Different detailed endolysins engineering approaches are well discussed by Gerstmans et al [7].

6.2 Formulations of Endolysins

Most of the research and development on endolysins has been directed towards their application as antibacterials against Gram–positive bacteria for external infections. Endolysins had shown promising antibacterial activity in different animal models as well as in food control, which has led to development of endolysins that are in pre–

clinical and phase II clinical trials [7]. Meanwhile, variety of global companies and startups are developing endolysins active against Gram–positive pathogens for commercial applications as pharmaceutical and cosmetics (ContraFect, Lysando, Micreos, IntronBiotechnology, GangaGen, Hyglos) [7]. Lysando is also currently formulating an artilysin as a wound care spray (Medolysin) to combat infections caused by Gram–negative pathogens.

As bacteria can colonize and infect the majority of human body, endolysins need to be formulated in a way to reach the target site of infection. Bacterial infection is a major challenge in wound care, for topical application antimicrobial wound dressings are of great value for treating wound infections. In **Paper I,** a chimeric protein (T4Lyz–CBM) composed of endolysin T4Lyz fused with cellulose binding domain (CBM) from *Cellulomonas fimi* was immobilized on a cellulosic wound dressing gauze. The binding was irreversible and the (T4Lyz–CBM)–immobilized gauze retained antibacterial activity against Gram–positive *M. lysodeikticus* (3.8 Log_{10} reduction) and Gram–negative *E. coli* (1.59 Log_{10} reduction) and *P. mendocina* (1.39 Log_{10} reduction).

On the other hand, the chimeric endolysin P128 was formulated in a hydrogel for treating staphylococcal nasal infections. When tested under physiological conditions mimicking the anterior nares, P128 hydrogel decreased the cell number of nasal staphylococcal isolates by 2–4 Log_{10} reduction [194]. In another study, subcutaneous application of 50 µg recombinant LysB–D29 to murine model infected with *Mycobacterium ulcerans* led to 1 log_{10} reduction in the bacterial burden 16 days post–infection which was associated with high levels of IFN–γ and TNF in the draining lymph node [195].

For systemic infections (bacteremia, meningitis, osteomyelitis and endocarditis) endolysins have been formulated as injections and its efficacy was evaluated in different animal models [196–199]. Recombinant CF–301 and SAL200 that are currently in clinical phase II trials are formulated as injection tackling systemic staphylococcal infections [200]. For respiratory tract infections, endolysin Cpl–1 had been formulated in an aerosol dosage form and evaluated for its efficacy in murine models infected with *Streptococcus pneumonia*. Cpl–1 aerosol rescued the mice from the fatal pneumococcal respiratory and blood infections [201].

Nanoparticles are considered as carriers for antimicrobial peptides and enzymes with extended properties. Since their size is proportionate to that of the bacterial cells, and their surface can be tailored, nanoparticles can be a potential platform for extra– and intracellular delivery of the antimicrobials with enhanced efficacy and low risk of resistance. In Paper II, T4Lyz and HEWL were immobilized to cellulose nanocrystals with different immobilization techniques. Only when covalently immobilized to aminated (positively charged) CNC (Am–CNC), T4 Lyz and HEWL retained 78.3% and 86.3% muralytic activity against *M. lysodeikticus* and

chloroform treated *E. coli* B cells, respectively. Since the driving force for the enhanced activity is the surface charge as was confirmed by the zeta potential results, the MIC and MBC values of Am–CNC–T4L and Am–CNC–HEWL was much lower when compared with the free enzymes and other immobilization methods (Table 6.1). A time–kill study using Alamarblue® (AB) as an indicator for cell viability confirmed that T4Lyz and HEWL immobilized to Am–CNC has faster killing rates than the free enzymes, for example Am–CNC–T4Lyz showed bactericidal activity within 4 hours compared to 8 hours for the free enzyme (Figure 6.1). The antibacterial activity was confirmed by transmission electron microscopy, which revealed altered cell membrane morphology and cell debris with cytoplasmic aggregates after treatment with Am–CNC–lysozyme, while pristine Am–CNC did not show any bactericidal effect (Figure 6.2).

In another study, solid lipid nanoparticles (SLN) were used as a carrier for LysB–MS6 enzyme to develop host directed approach against mycobacterial infections. The system was evaluated for its antibacterial activity in rat peritoneal macrophages infected with *M. smegmatis*. SLN–LysB–MS6 eradicated 50% of the intracellular *M. smegmatis* 1–hour post–infection when compared with infected non–treated macrophages [202]. In some cases, nanoparticles–loaded antibacterials have higher activity compared to the free antibacterial agent. Mesoporous silica particles (MSPs) loaded with the antimicrobial peptide NZX showed higher bactericidal activity against intracellular *M. tuberculosis* than the free peptide in murine lung models [203].

Table 6.1
Minimum Inhibitory Concentrations (MIC; µg/ml) and Minimum Bactericidal Concentrations (MBC; µg/ml) of free and immobilized HEW and T4 Lysozymes against different bacteria after 24 h at 37°C (or 30°C) in Brain Heart Infusion Broth.

	M. lysodeikticus		*E. coli*	
	MIC[b]	**MBC**[c]	**MIC**	**MBC**
CNC blank [a]	>1250	>1250	>1250	>1250
HWEL preparation				
Free	200	500	>1250	>1250
Coupled to EDC activated CNC	250	800	>1250	>1250
Coupled to Am–CNC	125	500	650	1000
T4L preparation				
Free	100	250	750	>1250
Coupled to EDC activated CNC	250	400	>1250	>1250
Coupled to Am–CNC	62.5	175	500	750

[a] Cellulose nanocrystals for different immobilization methods were used as blanks.

[b] MIC (µg/ml) is the lowest concentration of free or immobilized lysozyme preparations that inhibited the growth of the test microorganism (no visible growth at the end of the experiment; OD_{620nm} of the test well equal to the OD_{620nm} of the blank).

[c] MBC (µg/ml) is the lowest concentration of the lysozyme preparations that killed 99.9% of the test inoculum.

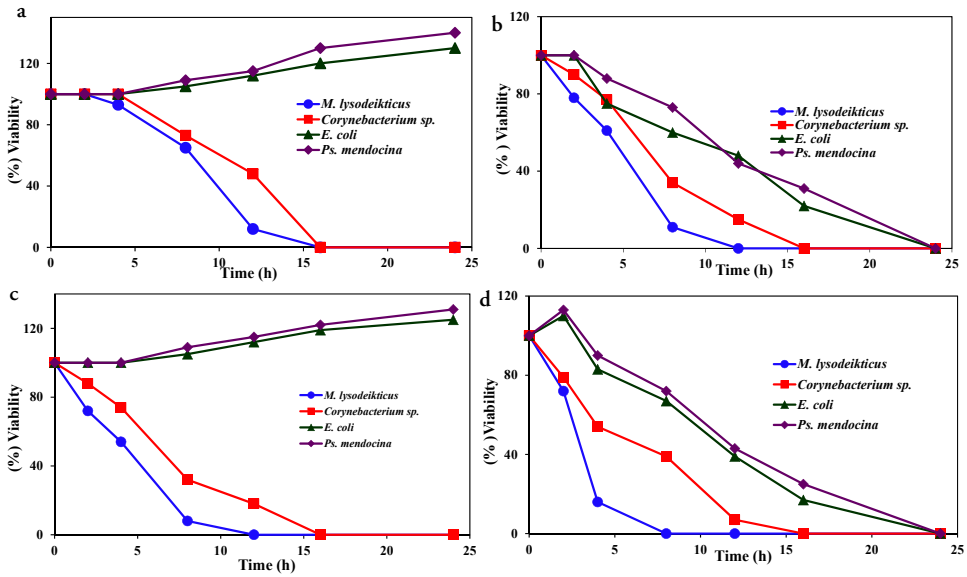


Figure 6.1

Time–kill study with Alamar Blue assay for (a) free HEWL, (b) Am–CNC–HEWL, (c) free T4L, and (d) Am–CNC–T4L against *M. lysodeikticus*, *Corynebacterium* sp., *E. coli*, and *P. mendocina*. After treating the bacterial cultures for 24 h with free and immobilized lysozymes, samples (100 µl) were collected at different time points, mixed with 1× Alamarblue®, incubated for 4 h at 37 °C (or 30 °C), and the absorbance was read at 560 and 595nm [171].

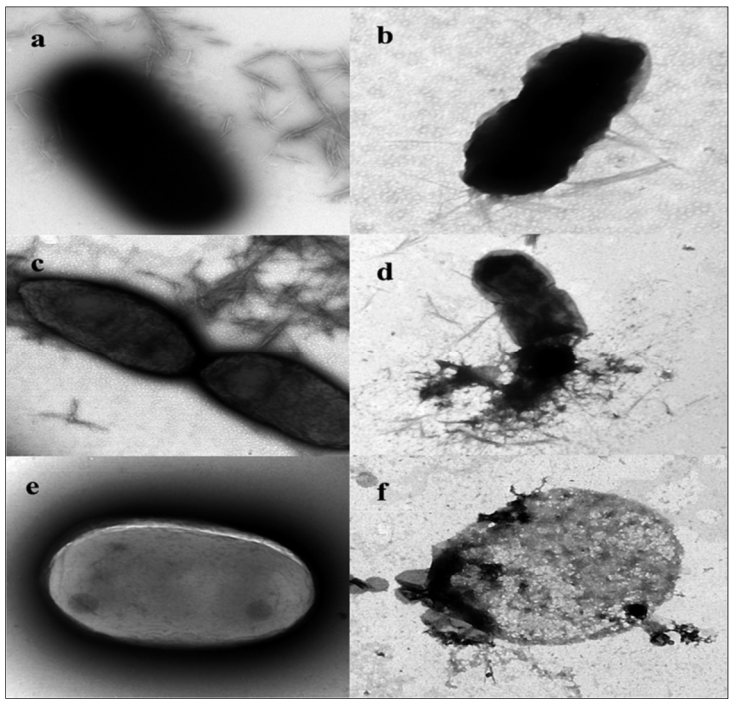


Figure 6.2
TEM images of different microorganisms treated with pristine CNC and Am–CNC–T4Lyz, respectively. (a) *E. coli* cells treated with pristine Am–CNC as negative control, (b) *E. coli* cells treated with Am–CNC–T4Lyz, (c) *P. mendocina* cells treated with pristine Am–CNC as negative control, (d) *P. mendocina* cells treated with Am–CNC–T4Lyz, (e) *Corynebacterium* sp. cells treated with pristine Am–CNC as negative control, and (f) *Corynebacterium* sp. cells treated with Am–CNC–T4Lyz. The scale bar is 500 nm [171].

7. Conclusions and Future Perspectives

As bacterial resistance to antibiotics is becoming a major public health threat and very few novel classes of antibacterial agents have been discovered, there is a critical need to explore and develop alternative strategies against bacterial pathogens, especially MDR Gram–negative bacteria and *Mycobacterium tuberculosis* [204]. Several alternative strategies have been proposed, of these endolysins are considered as a promising alternative with encouraging advantages over other alternatives. The studies presented in the current thesis concern two different endolysins – peptidoglycan hydrolases represented by lysozyme and mycolylarabinogalactan esterases represented by LysB.

Studies with lysozyme were focused on engineering of the enzyme by genetic fusion (**Paper I**) and immobilization (**Paper II**), respectively. Fusion of T4Lyz with cellulose binding module as an immobilization tag represents a facile single step irreversible binding to cellulosic wound dressing material with retained enzymatic and antibacterial activities (**Paper I**). Such an approach can even be applied to other antimicrobial enzymes and further protein engineering may be performed to improve activity and stability, if needed. Immobilization of lysozyme to cellulose nanocrystals using different chemistries was further investigated as an alternative form of enzyme formulation that could potentially be included in creams or gels. We demonstrated that the lysozyme coupled to CNCs tailored with positively charged amino groups could provide an efficient antibacterial agent. The combination of the positive charge on the nanocrystals and the antibacterial activity of the lysozymes in the conjugates improved the antibacterial activity as well as extended the spectrum to include Gram–negative bacteria (**Paper II**). Again, this is a generic approach for engineering other antimicrobial enzymes. Testing the conjugates against pathogenic isolates would be interesting. Moreover, formulating them as therapeutic dosage forms (e.g. gel, creams, spray) and testing their bioavailability, stability, storage conditions will open a new era for application of immobilized endolysins as alternative to antibiotics.

In case of LysB, we limited our studies to increasc our understanding of this group of enzymes prior to evaluating their potential as antimycobacterial agents (**Paper III**

and **IV**). High diversity of LysB enzymes at the amino acid sequence and structure levels was revealed. Multiple sequence and structural alignments of LysB enzymes showed that LysB enzymes to be intermediary between esterases and lipases. They resemble the esterases in not requiring interfacial activation for their activity, and like lipases possess long acyl binding site and deep–shaped active sites that can act on long chain substrates (**Paper III**). As multiple sequence and structural alignments showed many LysB enzymes with extra N–terminus domain with lower similarity to LysB–D29, it would be interesting to determine the crystal structure of such homologs to give us a deep insight about the function of these domains and to search the databases for more LysB enzymes that have not been annotated yet.

Characterization of the four LysB enzymes clearly demonstrates that these enzymes are lipolytic enzymes hydrolyzing wide range of substrates and exhibit mAGP hydrolase activity. However, when applied externally to *M. smegmatis* cells, LysB–His_6 enzymes showed marginal antimycobacterial activity indicating inaccessibility to the mycolic acid layer that was confirmed by enhancement in the activity on combining outer membrane permealizers with the enzymes (**Paper VI**). This implies that application of LysB enzymes needs to be integrated with the enzymes and antimicrobial peptides acting on other layers of the cell wall in order to inactivate the mycobacteria. Protein engineering could be an interesting approach for these enzymes including domain swapping and mutagenesis to generate chimeras with enhanced properties. Such work has already been initiated in our laboratory with some positive results (unpublished data).

While external application of endolysins is relatively simple and a promising alternative to the antibiotics in use, treatment of intracellular pathogens is more complicated. For example, in case of *M. tuberculosis* that is located inside the lung macrophages, several aspects need to be considered such as risk of proteolytic digestion of endolysins, targeting the endolysin to the site of infection, intracellular delivery of the endolysin. Hence, engineering of endolysins and development of a formulation providing stable, active molecule is highly crucial.

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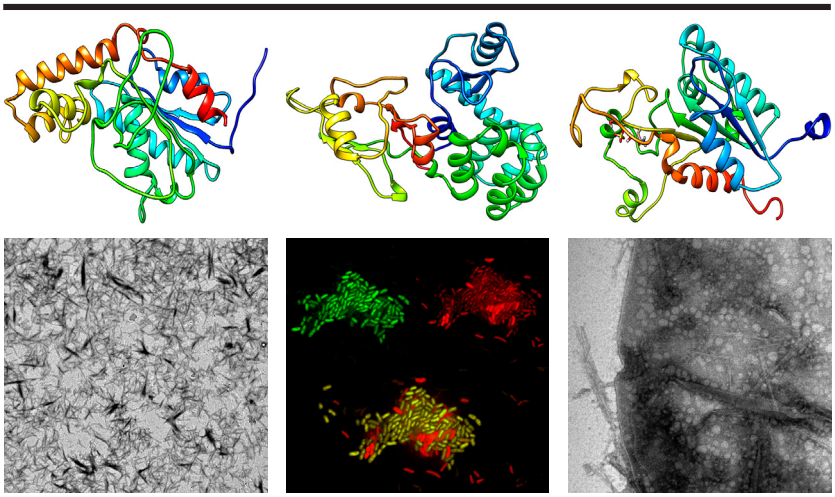

Phages are viruses that infect bacteria, at the end of their life cycle produce a set of enzymes called endolysins to lyse host cells from within, facilitating the release of the viral progeny. Due to their lytic activity, recombinant endolysins (also named enzybiotics) have gained great interest as potential antibacterials especially in the actual context of increasing rates of antibiotics resistance. The current thesis explores the potential of two groups of endolysins, peptidoglycan hydrolase and mycolylarabinogalactan esterase as potential antibacterials.

Different strategies for immobilization of the well-known peptidoglycan hydrolase, lysozyme from T4 bacteriophage and its antibacterial activity was studied. Immobilization of the T4 lysozyme (T4Lyz) to wound dressing gauze in a single facile binding step was achieved through engineering the endolysin with a cellulose binding module (CBM) as a fusion tag. In another approach, the antibacterial activity and storage stability of the T4Lyz as well as Hen Egg White Lysozyme (HEWL) were enhanced via covalent immobilization to tailored positively charged aminated cellulose nanocrystals (Am–CNC).

The mycolylarabinogalactan esterase Lysin B (LysB) is produced by mycobacteriophages that infect mycobacterial cells that possess a unique cell wall structure with a thick mycolic acid layer. The genome database of mycobacteriophages was explored to find and categorize LysB enzymes. Moreover, LysB homologs were cloned and recombinantly expressed in *E. coli* BL 21 (DE3) expression host. The mycolylarabinogalactan esterase activity as well as the antibacterial activity against *Mycobacterium smegmatis* cells were tested.

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