Propionibacterium acnes and its phages

Lood, Rolf

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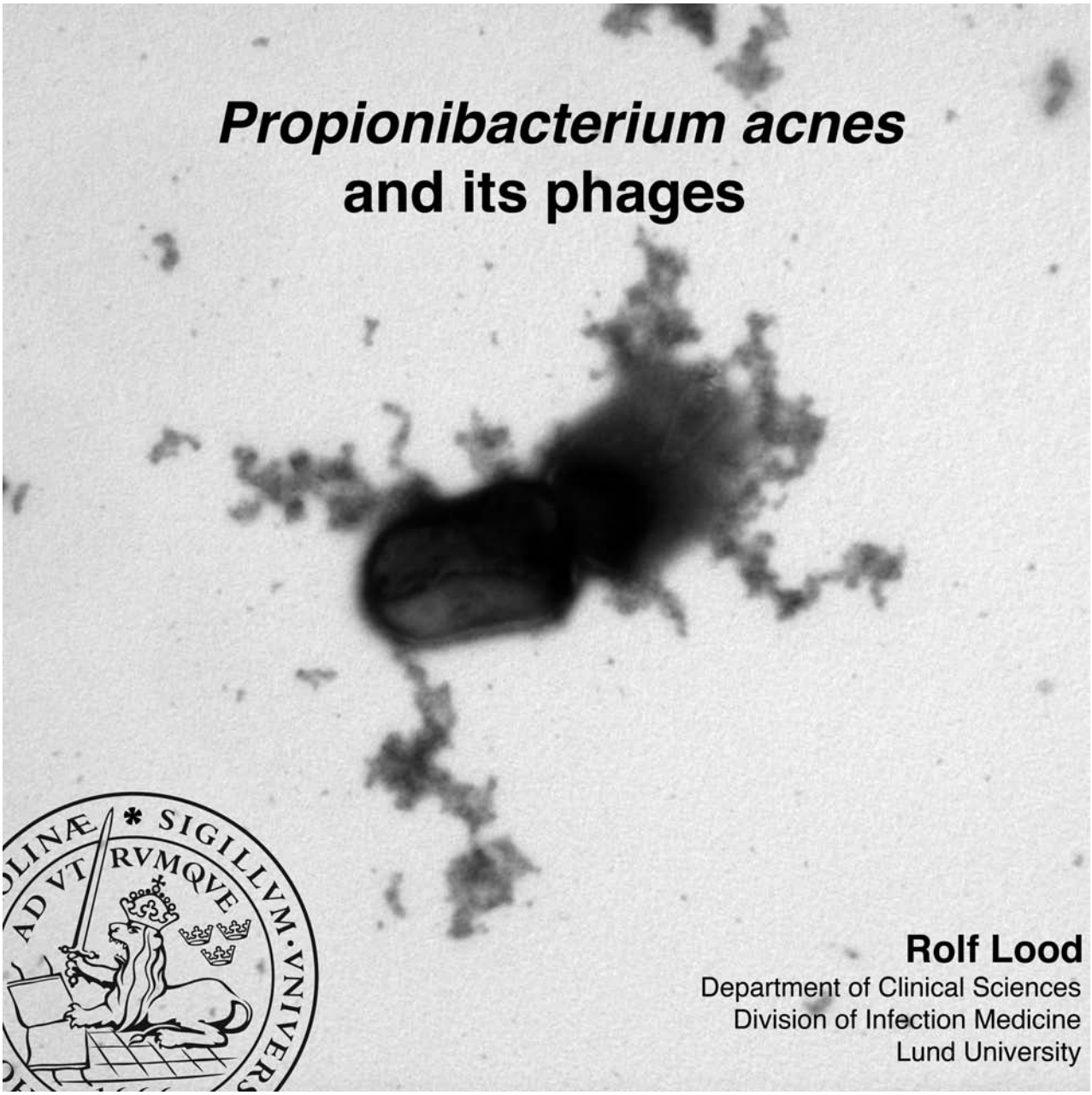

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Doctoral dissertation
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Abstract

Microorganisms are everywhere! They can tolerate many diverse extreme environments, such as the human body. Even though many of us might associate the word microorganism with infections and disease, most are actually either harmless or even beneficial for us. Those commensals often fight off more dangerous bacteria and might also directly benefit their host. The human skin harbors several different microorganisms, with Propionibacterium acnes being one of the most common bacteria. This Gram-positive anaerobe has long been attributed as the cause of acne vulgaris, and is known to cause severe inflammations on orthopedic implants. However, like most bacteria, P. acnes can be infected by specific bacterial viruses, eg. bacteriophages. If those can contribute to the progress of the diseases mentioned is unknown.

In this thesis we have investigated the role of P. acnes as both a pathogen and a commensal, and characterized the phages infecting P. acnes. To undertake those studies, we first had to develop a genetic toolbox to better be able to characterize the bacteria and their phages, since there is a huge lack of molecular tools for the study of P. acnes (Paper I). Furthermore, we found that P. acnes that caused inflammations on orthopedic implants had a higher capacity to form biofilms, than did strains isolated from the skin. Thus, the ability to form biofilm seems to be a characteristic of invasive isolates (Paper II). Even though unwanted on orthopedic implants, we found that colonization by P. acnes on the human skin is beneficial for its host. This is due to that P. acnes secretes a heme-oxygenase that protects our cells against free radicals, and increase the viability of the skin cells (Paper III). Furthermore, we characterized several bacteriophages that could infect P. acnes. Those phages had a high capacity to infect and lyse P. acnes (Paper IV). Finally, the sequencing of two of the phages revealed that the phages were not able to integrate their DNA into its host chromosome, but instead, most likely had a pseudolysogenic relation with their host (Paper V).

In summary, this thesis can conclude that P. acnes is commonly infected by phages, living in a pseudolysogenic relation. Furthermore, colonization by P. acnes might prove both beneficial and harmful for the host, all depending on the site of colonization.

Key words: Propionibacterium acnes, biofilm, bacteriophage, Siphovirus, pseudolysogeny, heme oxygenase, free radicals, recombinant protein expression, vector

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Cover image:
The lysis of *Propionibacterium acnes* infected with bacteriophages, and the subsequent release of the bacteriophages, as visualized using scanning electron microscopy. Maria Baumgarten and Dr. Matthias Mörgelin are acknowledged for the negative staining and electron microscopy, respectively.

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To Mom and Dad

The first to present his case seems right,
till another comes forward and questions him
-Proverbs 18:17

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1 Introduction

1.1 List of papers

Articles included in this thesis

1. **Lood, R***., Garbe, J*., and Collin, M. Development of a genetic toolbox for studies of *Propionibacterium acnes*. *Manuscript in preparation.*

2. Holmberg, A., **Lood, R**., Mörgelin, M., Söderquist, B., Holst, E., Collin, M., Christensson, B., and Rasmussen, M. 2009. Biofilm formation by *Propionibacterium acnes* is a characteristic of invasive isolates. *Clinical Microbiology & Infection* 15(8):787-95.

3. **Lood, R**., Olin, AI, Collin, M., and Allhorn, M. Commensal skin bacteria increase the viability of human cells by protecting them from harmful reactive oxygen species. *Submitted.*

4. **Lood, R**., Mörgelin, M., Holmberg, A., Rasmussen, M., and Collin, M. 2008. Inducible Siphoviruses in superficial and deep tissue isolates of *Propionibacterium acnes*. *BMC Microbiology* 8:139.

5. **Lood, R**., and Collin, M. 2011. Characterization and genome sequencing of two *Propionibacterium acnes* phages displaying pseudolysogeny. *BMC Genomics* 12:198.

Articles not included in this thesis

1. Karlsson, C., Mörgelin, M., Collin, M., **Lood, R**., Andersson, ML., Schmidtchen, A., Björck, L., and Frick, IM. 2009. SufA - a bacterial enzyme that cleaves fibrinogen and blocks fibrin network formation. *Microbiology* 155:238-248.

1.2 Abbreviations

AHL - acyl homoserine lactone
B. acnes - Bacillus acnes
C. acnes - Corynebacterium acnes
C. parvum - Corynebacterium parvum
CAMP - Christie, Atkins, and Munch-Petersen
cfu - colony forming units
CXCL8 - Interleukin 8 (IL-8)
DNA - deoxyribonucleic acid
DNAse - deoxyribonuclease
dsDNA - double stranded deoxyribonucleic acid
dsRNA - double stranded ribonucleic acid
E. coli - Escherichia coli
GFP - green fluorescent protein
ICTV - International committee on taxonomy of viruses
IgA - immunoglobulin A
IL-8 - interleukin 8
kb - kilo bases
kDa - kilo Dalton
LPS - lipopolysaccharide
MAC - membrane attack complex
Mb - mega bases
NCBI - National Center for Biotechnology Information
ORF - open reading frame
P. acidifaciens - Propionibacterium acidifaciens
P. acidipropionici - Propionibacterium acidipropionici
P. acnes - Propionibacterium acnes
P. australiense - Propionibacterium australiense
P. avidum - Propionibacterium avidum
P. cyclohexanicum - Propionibacterium cyclohexanicum
P. freudenreichii - Propionibacterium freudenreichii
P. granulosum - Propionibacterium granulosum

P. humerusii - Propionibacterium humerusii
P. jensenii - Propionibacterium jensenii
P. lymphophilum - Propionibacterium lymphophilum
P. microaerophilum - Propionibacterium microaerophilum
P. propionicus - Propionibacterium propionicus
P. thoenii - Propionibacterium thoenii
PCR - polymerase chain reaction
pfu - plaque forming units
RDE - receptor destroying enzyme
ROS - reactive oxygen species
rRNA - ribosomal ribonucleic acid
RT-PCR - reverse transcriptase polymerase chain reaction
SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD - superoxide dismutase
ssDNA - single stranded deoxyribonucleic acid
ssRNA - single stranded ribonucleic acid
TNF-α - tumor necrosis factor alpha

1.3 Acknowledgements

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Mattias

Thanks for everything! Your supporting leadership, your enthusiasm and for many many interesting discussions where you always have let me come up with my own ideas. I can not imagine any better supervisor for me than you. Also thanks for friendship, not only in the lab but also outside the lab. I have really enjoyed working with you these years. Your ways of supervising and performing science will always serve as an example to me.

My co-supervisors

Inga-Maria

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Maria

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Julia

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Marta
Thanks for taking lots of uncomfortable phone calls for me, reminding me of booking a place to defend my thesis in, and being a great support with the thesis. And of course with all things concerning the party afterwards! I'm looking forward to read your book!

Sara
Thanks for letting me help you in the beginning, making me feel smart. Unfortunately, that did not last long... Also thanks for all encouraging comments and helpful advices!

Jonathan
For showing me that recombinant proteins can be expressed at the first time, I do not envy you at all... Also thanks for being a great friend in the lab, and for the moment at the sunrise...

B14
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My coauthors
Thanks to all my coauthors, for helping me during these four years. Especially thanks to Magnus Rasmussen and Anna Holmberg for introducing me to *P. acnes* and giving great feedback to many of my questions. Also thanks to Anna for letting me do some calculations now and then, making me believe that I actually can math.

My students
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My friends
To all my friends in Charisma, Röke Blås, and Game Over, and to Anders (for lots of poker games!), that have supported me and helped me to think about other things but microbiology, and from time to time got me realizing that there are other things in the world but *P. acnes* to think about.

My family
To mom and dad, who, even though not reading all my papers, and maybe not understood all details, still listened when I tried to explain my work for them, and nodded in the right places! Thanks for all support and for motivating me to continue developing as a researcher in order to be spared from gathering stones at the farm... It really did help!

C
For all intense discussions about the work, and for a much helpful competition. I think we can call it a tie, even though I finished ”a year” before you!

1.4 Populärvetenskaplig sammanfattning

Mikroorganismer finns överallt! De klarar av att överleva i extrema miljöer, extrema pH, extrema temperaturer och kan motstå strålning och gift i doser som vi inte skulle överleva. Framförallt klarar mikroorganismer av att överleva i en specifik extrem miljö - människan. Hundratals olika mikroorganismer bor på och i oss, i ett antal som långt överskrider vårt eget cellantal.

Mikroorganismer är ett samlingsnamn för flera olika typer av små ('mikro') organismer, som bakterier och virus. Många av oss associerar nog dessa med infektioner och olika sjukdomar, vilket också ett fåtal orsakar. Men tvärtemot vad man kan tro är de flesta bakterier ofarliga, och till och med gynnsamma, genom att de hjälper oss att ta upp näring i tarmarna och skyddar oss mot farligare bakterier. Dessa bakterier tillhör vår normalflora och har en stor del i vårt välbefinnande.

Vår hud har flera olika bakterier. Några av de vanligare bakterierna är Propionibakterier, Stafylokocker och Streptokocker. Många av oss kan ha dessa på vår hud utan att de ger några symptom, då de tillhör normalfloran. Men, då tillfälle ges kan även dessa bakterier orsaka opportunistiska infektioner. Detta sker framförallt om vårt försvar mot dem på något sätt är påverkat, genom exempelvis sår (skador eller kirurgiska ingrepp) eller vid nedsatt immunförsvar.

En av de hudbakterier som oftast är inblandad i opportunistiska infektioner är *Propionibacterium acnes*. Bakterien kallades ursprungligen för *Bacillus acnes* eftersom den var stavformad (Bacillus-lik) och isolerades från acne. Fastän bakterien isolerades från acne för mer än 100 år sedan råder det fortfarande tveksamheter kring om *P. acnes* verkligen är orsaken bakom utvecklandet av sjukdomen. Anledningarna till detta är många. Först och främst är vi alla koloniserade av *P. acnes* utan att utveckla några sjukdomar. Därför hävdar många att en isolering av *P. acnes* från huden enbart är en kontaminering av provet. Vidare är *P. acnes* svår att arbeta med av två anledningar. Dels växer *P. acnes* långsamt och behöver leva i en miljö utan syre. Dels finns väldigt få metoder utvecklade för att studera denna bakterie.

Intresset för denna bakterie har på de senaste åren ökat drastiskt. Fortfarande är intresset stort för den potentiella rollen av *P. acnes* i utvecklingen av acne. Men senare forskning har även pekat på att *P. acnes* har en stor roll vid ledimplantatsinflammationer, där ett byte av implantat ofta är nödvändigt för att bli av med infektionen då dessa bakterier ofta är väldigt resistenta mot antibiotika. Detta beror på att bakterien kan bilda ett tjockt lager av socker kring sig (biofilm), som ett pansar som skyddar *P. acnes* mot antibiotikan. Där kan bakterierna ligga i flera månader till år utan att orsaka någon skada, för att sedan skapa en inflammation. Vidare tyder forskning på att *P. acnes* kan vara delaktig i utvecklingen av prostatacancer.

Trots ett ökat intresse för att studera denna opportunistiska bakterie, var möjligheten till detta begränsad, då det saknades flera nöd-

vändiga verktyg för detta. Därför utvecklade vi flera verktyg för att på ett enkelt sätt kunna studera hur delar av bakterien kunde medverka till att utveckla sjukdom (Paper I). Dessa verktyg består av två plasmider, som är cirkulärt DNA. Dessa plasmider är konstruerade till att fungera som fabriker i *P. acnes* och producera olika protein som önskas studeras. Då studier av proteiner i bakterier är en av de grundläggande sätten för att utvärdera hur en bakterie orsakar sjukdom, kommer dessa verktyg att underlätta studier av *P. acnes* i framtiden.

Då *P. acnes* var så vanligt förekommande vid inflammationer kring ledimplantat, isolerade vi flera av dessa stammar och undersökte vad som skiljde dem från de *P. acnes* som vi hade på huden. Vi fann då att de *P. acnes* som hade förmåga att orsaka protesinflammationer alla kunde bilda mer biofilm än de bakterier som isolerades från huden (Paper II). I övrigt hittades inga skillnader mellan de två grupperna av bakterier, och vi kunde fastslå att den troligtvis viktigaste faktorn för att *P. acnes* orsakade protesinflammationer berodde på dess förmåga att bilda biofilm.

Bortsett från att vara förmögen att orsaka sjukdom bär de flesta av oss på *P. acnes* utan att bli sjuka. Därför är en rimlig tanke att dessa bakterier också kan vara nyttiga för oss, men på vilket sätt de potentiellt skulle kunna skydda oss har inte utretts. Vi valde att studera ett protein som *P. acnes* producerar i stora mängder. Detta protein är ovanligt, för det liknar inget hittills undersökt protein i andra bakterier. Därför slogs vi av tanken att detta protein potentiellt kunde vara av stor betydelse för bakteriens interaktion med oss (Paper III). Det visade sig att detta protein, vidare kallat RoxP, kunde skydda våra hudceller mot reaktiva syreradikaler som bildas bland annat av UV-ljus. Hudceller som behandlades med RoxP mådde mycket bättre än celler som inte hade fått RoxP. Detta indikerar att för en frisk person är kolonisering av *P. acnes* viktigt, då detta bidrar till att skydda vår hud. Därför är det viktigt att inte behandla ospecifikt (*eg* antibiotika) mot *P. acnes* om inte denna är upphovet till infektionen.

Men *P. acnes* är inte ensam på spelplanen. Motståndarlaget har en minst lika bred trupp av spelare, och är svurna fiender till *P. acnes*. Mötena dem sinsemellan brukar alltid resultera i vinst åt något av hållen, men kan även ge ett lika-resultat. Det handlar om bakteriofager. Virus som är ofarliga för oss, men som är dedikerade till att eliminera bakterier. Dessa bakteriofager har två principiellt olika spelarstilar. En av dem är anfallaren, som bara vill komma åt bakterien och förstöra för den. Lyckligtvis för bakterien känner den till ett och annat knep för att skydda sig mot dessa tacklingar.

Den andra spelartypen är mer utav en försvarsspelare. Han gillar att komma nära motståndaren och interagera med dem, även tillfälligt hjälpa dem, för att få motståndaren att känna sig ohotad. Sedan, då tillfälle ges, slår försvarsspelaren till med full kraft. Däremot kan bakterierna även klara sig ur dessa situationer genom olika försvar.

Då bakteriofager är kända för att kunna hjälpa bakterierna tillfälligt, var vi intresserade av att undersöka ifall *P. acnes* som kunde orsaka ledprotesinflammationer också i större utsträckning hade hjälp av bakteriofager (Paper IV). Vi fann att *P. acnes* i väldigt stor utsträckning hade

bakteriofager (70%), men att där inte var någon skillnad mellan sjukdomsframkallande *P. acnes* och normalflora. Med andra ord, det verkade inte som om bakteriofagerna samspelade med *P. acnes* i sjukdomsprocessen. Vad vi däremot fann var att bägge lagen hade olika kvaliteter på sina spelare. En del bakteriofager kunde spela anfallare mot nästan alla bakterier med framgång, medan vissa fager enbart kunde dribbla bort ett fåtal motståndare. På samma sätt var kvaliteten i bakterielaget varierande. Medan vissa kunde försvara sig mot nästan alla fagernas anfallare, förutom deras stjärnspelare, borde många av de andra snarare sitta på utbytarbänken då de blev bortdribblade i varje anfall.

Även om det inte verkade som om bakteriofagerna kunde hjälpa *P. acnes*, bestämde vi oss för att granska detta mer, genom att undersöka två av fagerna närmare. Detta bestod i att vi plockade fram deras DNA, och läste av det, för att se om de hade någon förmåga att hjälpa bakterierna (Paper V). Genom detta fann vi att dessa bakteriofager inte verkar kunna samspela med bakterierna. Däremot kom vi fram till att dessa bakteriofager hade en märklig ”spelstil”, då de inte var strikta anfallare, men inte heller strikta försvarare. Inte enbart var deras spelstil annorlunda, dessutom var deras DNA olikt DNA från andra bakteriofager.

Sammanfattningsvis har jag i denna avhandling utvecklat verktyg för att bättre och lättare kunna studera *P. acnes*. Denna bakterie har både positiva och negativa egenskaper. Positiva genom att den hjälper vår hud att må bra, men negativa då den genom att bilda biofilmer medverkar till inflammation av proteser. Men *P. acnes* är inte ensam på spelplanen, utan har motståndare i form av bakteriella virus, så kallade bakteriofager. Dessa bakteriofager är vanliga hos *P. acnes*, men verkar inte bidra till att göra bakterien farligare.

Propionibacterium acnes and its phages

Is there anything of which we can say:
'Look! This is something new'?
It was there already, long ago;
it was there before our time.
-Ecclesiastes 1:10

2 Summary

Microorganisms are everywhere! They can grow in acidic [715] and alkali [516] environments, in high salt concentrations [542], at temperatures exceeding 100°C [200] and below 0°C [577], as well as being highly resistant to radiation [318], and poisons such as arsenic [311]. More importantly, microorganisms can grow on us and in us. Several hundreds of different bacteria colonize us, outnumbering our own cells more than ten times [55, 647].

Even though many of us associate the word bacteria with infections and disease, not all bacteria are bad for us. On the contrary, many bacteria are crucial for us [746], helping us to take up nutrients in the intestine [743], fight off pathogenic bacteria either by themselves [277], or by stimulating host cells to produce antimicrobial agents [302]. Furthermore, commensals (*eg*. the normal flora) can regulate the immune response to certain pathogenic bacteria [544]. Thus, it is important for us to be colonized by bacteria.

The human skin harbors several different bacterial species, mainly belonging to the Gram-positive bacteria *Propionibacterium*, *Staphylococcus* and *Streptococcus* [247]. The colonization of the skin with Gram-negative bacteria as *Pseudomonas* and *Klebsiella* is much lower [129], compared to the Gram-positive bacteria, due to their differences in cell wall structure, and thereby their lower resistance to dry areas [129].

However, even though classified as commensals, several bacteria can act as opportunistic pathogens, causing diseases only when the host immune system is compromised. One of the most prominent skin bacteria regarded as an opportunistic pathogen is *Propionibacterium acnes*.

In this thesis, I have investigated some of the factors from *P. acnes* possibly associated with the development of disease. Furthermore, since *P. acnes* frequently is infected by bacterial viruses, *eg*. bacteriophages, I have also characterized the phages morphologically and genetically.

In the first chapter, *Propionibacterium acnes*, I will discuss some of what is known about this bacterium, before going on to a wider discussion about factors necessary for causing disease (*Virulence factors*). This will be followed by three papers, describing in detail the development of a genetic toolbox to more feasibly study *P. acnes* [417](Paper I), how biofilm formation contributes to the invasive characteristics of *P. acnes* [301] (Paper II), and the characterization of a highly secreted heme oxygenase from *P. acnes* that is beneficial for its host [416] (Paper III).

After having presented ”The bad guys”, the thesis will change focus and take a closer look on ”The good guys” - the enemies of the bacteria, the bacteriophages (*Bacteriophages*), and how they might be used as a novel therapeutic. This will be followed by two papers describing the isolated phages from *P. acnes* in more detail [415, 418] (Paper IV & V).

Part I
The Bad Guys

Research should be conducted at a secluded place, free from the alarm of the unlettered mob, where you can enjoy the philosophical serenity, to which scholars and astute people can get, while the common people, who do not understand such things and do not attach to them their true value, can be kept away.
-free translation of Tycho Brahe, *Astronomiae Instauratae Mechanica* 1598

3 *Propionibacterium acnes*

3.1 Nomenclature

Propionibacterium acnes has historically been classified as *Bacillus acnes* [230], *Corynebacterium acnes* [57], and *Corynebacterium parvum* [452]. The bacterium was first identified 1896 in a sample from acne vulgaris [690], but was not cultivated until the year after [595]. Gilchrist was the first to name the bacterium as *B. acnes* [230] due to its rod-like shape and the site of isolation. Later, in 1923, Bergey *et al.* reclassified the bacterium as belonging to the *Corynebacterium* group due to its morphology [57]. However, this classification did not last many years either, before it was questioned. In 1946, Douglas & Gunter proposed that even though this bacterium shares morphological characteristics of *Corynebacterium*, several of those characteristics are present in the *Propionibacterium* family as well. Thereby they proposed that the bacterium should be classified as *P. acnes* [168]. This classification was validated in 1963 by Moore & Cato [484] and in 1967 when Moss *et al.* compared several *Propionibacteria* with *C. acnes* with respect to their fatty acid composition and their fermentation pattern [491]. Even *C. parvum* was later concluded to be a mixture of different *Propionibacteria*, mainly *P. acnes* and some *P. granulosum* [152].

Table 1: Species of *Propionibacteriaceae*

Species	Habitat	Identifier
P. acidifaciens	cutaneous	Downes & Wades 2009 [170]
P. acidipropionici	classical	Orla-Jensen 1909 [528]
P. acnes	cutaneous	Douglas & Gunter 1946 [168]
P. australiense	cutaneous	Bernard *et al.* 2002 [59]
P. avidum	cutaneous	Eggerth 1935 [184]
P. cyclohexanicum	classical	Kusano *et al.* 1997 [370]
P. freudenreichii subsp. *freudenreichii*	classical	van Niel 1928 [511]
P. freudenreichii subsp. *shermanii*	classical	van Niel 1928 [511]
P. granulosum	cutaneous	Prevot 1938 [553]
P. humerusii	cutaneous	Butler-Wu *et al.* 2011 [106]
P. jensenii	classical	van Niel 1928 [511]
P. lymphophilum	cutaneous	Johnson & Cummins 1972 [328]
P. microaerophilum	classical	Koussémon *et al.* 2001 [367]
P. propionicum	cutaneous	Charfreitag *et al.* 1988 [125]
P. thoenii	classical	van Niel 1928 [511]

3.2 Genetics of the *Propionibacteriaceae family*

Propionibacteria belong to the *Actinobacteria* phylum in the *Actinomycetales* order and the family *Propionibacteriaceae*. The genus *Propionibacterium* consists of many different species (Table 1 and Figure 1), with the most well studied species being *P. acnes*, *P. granulosum* and *P. avidum* that make up a part of the normal flora [546], and *P. freudenreichii* which is a species frequently used in the manufacturing of different cheeses [379]. "Classical" (also called "dairy") *Propionibacteria* are those identified in the dairy industry, while "cutaneous" *Propionibacteria* are those living on the skin as commensals or opportunistic pathogens. Some of the members of the *Propionibacteriaceae* family are also heat and acid resistant, exemplified by *P. cyclohexanicum* that can survive at 90°C for 10 min and grow at pH 3.2 [370].

So far, 6 *Propionibacteria* have been fully sequenced. The first to be sequenced was the *P. acnes* strain KPA171202 in 2004 by Brüggemann *et al.* [93, 95]. The genome was approximately 2.56 Mb, encoded 2333 putative proteins, and had a GC content of 60%. The second *P. acnes* genome from strain SK137 was released six years later, in 2010, with a highly similar genome to KPA171202. This was followed by the release of the genome for strain 266 in 2011 [102]. Furthermore, according to the genome projects listed at NCBI [506] at the end of 2010, 73 more genomes for *P. acnes* are currently during either assembly or in progress for sequencing, indicating that the genomic data available for *P. acnes* shortly will be overwhelming. Other than *P. acnes*, two more *Propionibacterium* species have been sequenced, *P. humerusii* and *P. freudenreichii* subsp. *shermanii* CIRM-BIA1. The genome of the latter was concluded to be approximately 2.62 Mb encoding 2439 putative proteins, and had a GC content of 67% [190]. Even though *P. freudenreichii* is related to *P. acnes*, and shares many characteristic features, this particular strain showed genetically a much less pathogenic potential, as indicated by the absence of endoglyceramidases, sialidases, hemolysins, CAMP-factors and toxins [190]. Since *P. humerusii* just recently was sequenced, a proper annotation is still lacking [106].

3.3 Different types of *P. acnes*

P. acnes can be divided into different types (IA, IB, II, and III), and several methods have been developed to distinguish them from each other, such as the usage of bacteriophages [720] and PCR-based identification [620]. In 1972 Johnson & Cummins started to use antibodies to differentiate between type I and II with agglutination tests [328], and 1975 Cummins found that type II was unable to ferment sorbitol [151]. It was not until 2005 that McDowell *et al.* showed that the differences between type I and II was reflected by specific point mutations in *recA* [457]. They also concluded that some of the *P. acnes* strains used in the study reacted atypical with the antibodies used. However the *recA* sequencing revealed that they belonged to type I, and they were later concluded to belong to a seperate type, IB [692]. Recently, McDowell *et al.* found a fourth *P. acnes* type, type III [456].

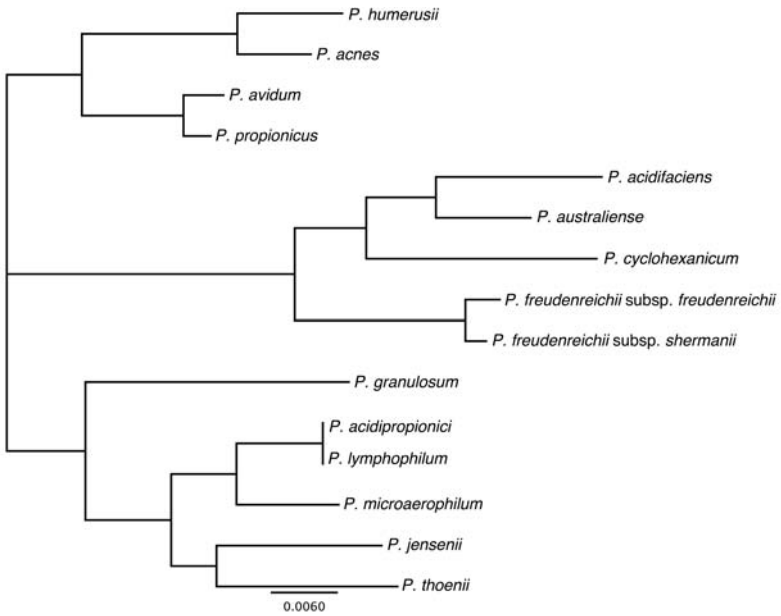

Figure 1: Phylogenetic analysis of the species in *Propionibacteriaceae*

These *P. acnes* strains are morphologically different from other known *P. acnes* types, since they form cells with long filaments that aggregates, rather than forming single coryneformed bacteria [456]. However, even though these four different types of *P. acnes* are well known, there is still little data on the clinical relevance of the different types [94].

3.4 Plasmids in *Propionibacteria*

The first to study plasmids in *Propionibacteria* were Rehberger & Glatz who investigated the presence of plasmids in dairy *Propionibacteria* and found seven distinct plasmids in the strains [568]. They found small plasmids, between 6-10 kb in *P. acidipropionici*, *P. jensenii*, *P. granulosum*, and *P. freudenreichii*. The presence of plasmids in *Propionibacteria* has however varied from 0-38% in other studies [218, 520, 535, 545], indicating that different species may have different carriage rates of plasmids, and only five small plasmids have so far been sequenced (Table 2) [193]. Furthermore, no plasmids have been isolated from *P. acnes*, even though several bacteriophages have been isolated from this species and other *Propionibacteria* (Figure 2). One of the first to isolate bacteriophages from *P. acnes* was Zierdt in 1974 [761]. This was followed by several more studies on phages infecting *P. acnes* [192, 330, 415, 418, 720] (Paper IV & V) and dairy *Propionibacteria* [130, 220, 221, 222, 288].

More details about different phages will be discussed in *Chapter 3 - Bacteriophages*. For those with more interest in the genetics of *Propionibacteria*, the reader is referred to two excellent reviews by Luijk *et al.* [428] and Ventura *et al.* [701].

3.5 Transformation systems in *Propionibacteria*

Several attempts have been done in order to develop transformation systems in *Propionibacteria*. The first successful protocol was developed by Gautier *et al.* in 1995, using purified phage B22 DNA to transform *P. freudenreichii* [219], generating a maximum efficiency of more than 10^5 cfu/μg DNA. However, this DNA was isolated from a bacteriophage infecting *Propionibacteria*. Jore *et al.* found, when developing an *E. coli* - *P. freudenreichii* shuttle vector, that DNA isolated from *E. coli* severely decreased the transformation efficiency to 10-30 cfu/μg DNA, while the same plasmid DNA isolated from a *P. freudenreichii* strain increased the efficiency to more than 10^8 cfu/μg DNA [331]. They concluded that this was due to a restriction-modification system in *Propionibacteria* [331]. In order to increase the transformation efficiency, Cheong *et al.* used *dam*$^-$ *E. coli* strains for the transformation of *P. acnes* and increased the efficiency to approximately 10^4 cfu/μg DNA [127].

The first knock-out in *P. acnes* was demonstrated in 2010 by Sörensen *et al.* [642], where they used homologous recombination to generate knock-out mutants of two co-hemolysin genes. This group, even though using a *dam*$^-$ *E. coli* strain, had to use several μg plasmid DNA in order to get a few colonies [642] indicating that much work still is needed in optimizing a transformation protocol for *Propionibacteria*. Other groups have also developed different genetic tools used to knock-out genes in *P. acidipropionici* [664], to produce 5-aminolevulinic acid using expression vectors [357] and developed shuttle vectors between *P. freudenreichii* and *E. coli* [356]. More recently, we developed a system for the homologous expression of recombinant proteins in *P. acnes* [417] (Paper I), which will facilitate the expression and characterization of proteins from *P. acnes*.

3.6 Morphological characteristics

P. acnes can be identified on agar plates as small (0.5-2.5 mm) circular white to yellow colonies [359, 491]. Under a microscope, they will be visualized as rod-like bacillus with lengths ranging between 0.8 to 2.8 μm and widths ranging between 0.6 to 0.9 μm, even though they are pleomorphic and can have different morphologies [456, 546], see Figure 3.

Table 2: Sequenced plasmids in species of *Propionibacteriaceae*

Species	Plasmid	Size (bp)	ORF
Propionibacterium acidipropionici	pRGO1	6,868	6
Propionibacterium freudenreichii	p545	3,555	2
Propionibacterium freudenreichii	pLME108	2,051	1
Propionibacterium granulosum	pPGO1	3,539	3
Propionibacterium jensenii	pLME106	6,868	10

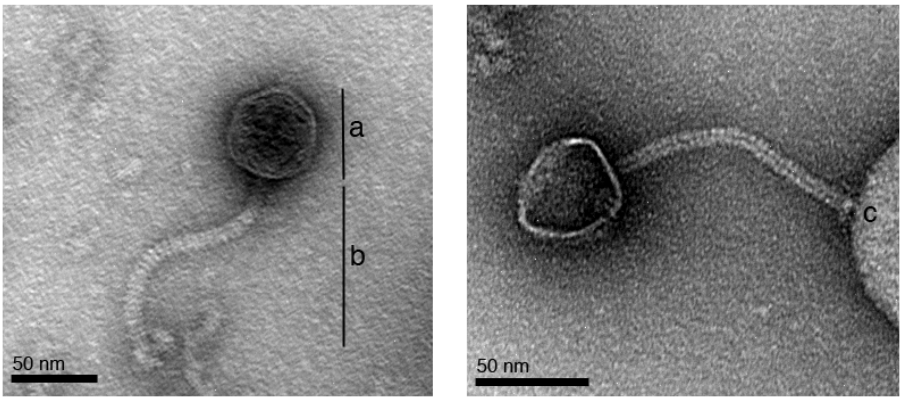

Figure 2: Bacteriophages isolated from *P. acnes*. The phages can be classified as Siphoviruses, due to their icosahedral head (a) and their long non-contractile tail (b). The phages adhere to bacteria with a base plate with attached spikes (c).

3.7 Growth characteristics

The growth of *P. acnes* is optimal at limited oxygen levels (10%) [246], but *P. acnes* can also grow under strict anaerobic conditions and can tolerate 100% oxygen [146] even though it grows slower [145]. The ability of *P. acnes* to live in the presence of oxygen requires the production of several proteins such as catalases and superoxide dismutases [93], which both have been shown to be expressed during oxidative stress [584]. However, even with optimal growth conditions, such as with the addition of Tween 80 [359] and by using complex growth media, *P. acnes* often requires several days of cultivation before reaching the exponential growth phase, and 7-10 days of incubation is often needed for clinical isolates to be visualized on plates [359, 458, 561].

P. acnes can be found on most parts on the skin as a commensal, benefiting its host [416] (Paper III). It can mainly be found in areas rich in sebum [458], including the scalp, forehead and ear, where the number of *P. acnes* is ranging from 10^1-10^4 cfu/cm^2 [639] with the forehead being the most populated area, especially in the pilosebaceous follicles where it outnumbers all other bacteria [216]. *P. acnes* can also be found as a part of the normal flora in the oral cavity and in the large intestine [87]. Even the arms and the legs are populated by *P. acnes*, but at a much lower concentration, ranging from 10^1-10^2 cfu/cm^2. *P. granulosum* is found on the same parts as *P. acnes*, but often at a ten fold lower concentration compared to *P. acnes*. *P. avidum* can also be found in sebum rich areas, but mainly populates the axilla, groin and rectum where it can be found in concentrations up to 10^3 cfu/cm^2 [458].

P. acnes is a Gram-positive bacterium, with a thick peptidoglycan layer outside its cell membrane, even though it stains only weakly Gram-positive [187, 358]. It has several characteristics not ordinarily associated with Gram-positive bacteria. Among others, *P. acnes* has a distinct

peptidoglycan layer and produces phosphatidyl inositol, which normally is a component in eukaryotic cell membranes, and not often found in bacteria [334].

P. acnes can be classified on the basis of fermentation and other biochemical assays. Type I and II can be differentiated due to the fact that type II can not ferment sorbitol. However some strains of type IB share this feature [457]. Furthermore, *P. acnes* is catalase positive [458], indole positive [524] and can degrade gelatin [458, 484, 524]. They are not able to ferment sucrose, maltose, xylose or arabinose [484], but will readily ferment glucose, galactose, glycerol and mannose [561].

3.8 Antibiotic resistance

The first evidence that there existed clinical resistant *P. acnes* strains came in 1979, when Crawford *et al.* reported that clinical *P. acnes* isolates were cross-resistant to clindamycin and erythromycin [148], and Guin *et al.* demonstrated that some *P. acnes* strains were resistant to high concentrations of tetracycline [253]. More than two decades later, Ross *et al.* showed that 50% of patients with acne vulgaris had *P. acnes* isolates that were resistant to the most often used antibiotics, clindamycin and erythromycin, and 20% of the isolates were resistant to tetracycline [589]. Furthermore, by analyzing how this resistance was gained, they showed that resistance to clindamycin and erythromycin mainly was due to three specific mutations in the 23S rRNA [588]. Furthermore, they found that several *P. acnes* strains that were resistant to all macrolide-lincosamide-streptogramin B antibiotics had a resistance gene, *erm(X)*, on the transposon Tn5432, originating from *Corynebacterium striatum* [586]. This transposon was shown to be difficult to mobilize and transfer between different *P. acnes* strains [586]. Ross *et al.* also identified the genetic basis of the resistance to tetracyclines as a point mutation in the 16S rRNA [587]. The resistance to tetracyclines is often associated with resistance to clindamycin and erythromycin [513], but has been shown not to be dependent on any efflux system [526].

Since it might be speculated that high usage of antibiotics may promote the development of resistant *P. acnes* strains, Ross *et al.* conducted a screen for antibiotic resistant *P. acnes* from UK, Spain, Italy, Greece, Sweden and Hungary [589]. They showed that, in Spain, where the usage of antibiotics was high, more than 94% of the *P. acnes* strains exhibited resistance to at least one antibiotic, while it only was 51% in Hungary. However, the higher usage of tetracyclines in UK and Sweden also meant that the highest prevalence of tetracycline resistant *P. acnes* could be found in these countries, with approximately 25% and 15% of the strains being resistant, respectively [589]. Even the mechanism of how the bacteria gained resistance was different between different countries. In 2005 Oprica *et al.* demonstrated that specific mutations in the 23S rRNA were much more common in isolates from Sweden, than in isolates from other countries [526]. Furthermore, no correlation could be seen between different genotypes of *P. acnes*, based on pulse-field electrophoresis, and resistance to antibiotics [525, 526]. Oprica *et al.* also concluded that single persons could have several different *P. acnes* strains colonizing the skin, thereby having strains resistant to several antibiotics [525].

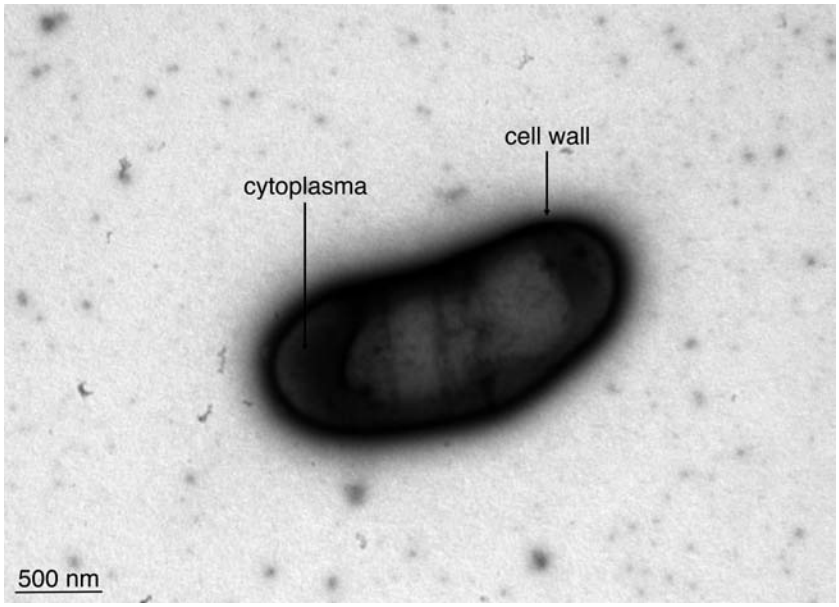

Figure 3: Morphology of *P. acnes* visualized using scanning electron microscopy. *P. acnes* has a thick peptidoglycan layer, building up the cell wall, and thus keeping the cytoplasmic material inside the cell.

3.9 Virulence of *P. acnes*

P. acnes was first examined due to its proposed anti-tumor effects when injected intravenously [267, 740]. However, several side effects were obvious, as reported by Mitcheson *et al.* 1980 [474]. When injecting 350 μg heat-killed *P. acnes* intravenously in a mice, they rapidly developed thrombocytopenia and lost plasma fibrinogen and thereby developed hypocoagulation [270, 474]. This was followed by an increased liver- and spleen weight, more than doubling in weight in one week [378, 474], and a temperature increase to 38-39°C after 2-4 h [122, 270], displaying similar effects as LPS from *E. coli* [472], leading to the expression of several proinflammatory cytokines and chemokines such as CXCL8 and TNF-α from sebocytes [498].

The ability of *P. acnes* to develop hypocoagulation in its host might partly be explained by its ability to bind to and degrade fibronectin [731, 757], fibrinogen, and fibrin [732]. Furthermore, *P. acnes* induces the expression of IL-12 and IL-8 from monocytes [358], influences the differentiation of keratinocytes [11] and stimulates the production of superoxide anions by keratinocytes [242], thereby potentially causing an inflammatory response.

3.10 Acne

P. acnes was first isolated from acne vulgaris, hence the name [690], but still there is a lack of formal evidence supporting that this bacterium causes acne. This is mainly due to that *P. acnes* is present on all persons as a part of the

normal flora, and due to the lack of good models of acne. However some efforts to mimic the pathogenesis of acne have been done and different models have been evaluated [157, 300, 501, 502]. The difficulty of addressing if *P. acnes* is involved in the progress of acne vulgaris might also be attributed to the complex nature of the disease. For further reading in this topic, the reader is recommended three reviews by Toyoda & Morohashi [680], Bojar & Holland [75], and Dessinioti & Katsambas [161].

Even though the contribution of *P. acnes* to acne vulgaris is questioned, the association between acne vulgaris and *P. acnes* is not. Höffler *et al.* showed in 1977 that *P. acnes* was the most frequently isolated *Propionibacteria* from acne vulgaris, and also the most enzymatically active [294], but also that *P. granulosum* only was isolated from patients with acne [223]. However, *Propionibacteria* are not the only bacteria found in acne lesions, since *Staphylococcus* and *Malassezia* are also frequently isolated [388]. Furthermore, Bek-Thomsen *et al.* found that all follicles from normal skin were colonized only by *P. acnes*, while follicles from patients with acne also included *S. epidermidis* and a few other bacteria [52]. Furthermore, Höffler *et al.* showed that the secretion of different enzymes differed between isolates from patients with acne and from normal skin, with the first producing more sialidases [296], and more DNAse and lecithinase, even though these isolates produced less proteolytic enzymes [295]. Even though not yet formally proved, many papers describe the contribution of *P. acnes* in the development of acne vulgaris [719] and putative virulence factors [194] that are supposed to mediate the inflammatory response. It has however recently been shown that some clones of *P. acnes* are associated with severe acne, while other clones are associated with the normal flora [414].

3.11 Prostate cancer

P. acnes has been associated with prostate cancer, with several groups constructing oligonucleotides (primers) for the detection of *P. acnes* in prostate tissue [618, 620]. In 2005 Cohen *et al.* found that 35% of the prostate samples from patients with prostate cancer had infiltration of *P. acnes* [136], which was significantly associated with inflammation. A similar study was performed by Alexeyev *et al.* 2007 using fluorescent *in situ* hybridization to detect *P. acnes* in prostate tissue [15], showing that *P. acnes* can persist for several years in the prostate gland and possibly establish a persistent infection [14, 15]. Recently, Fassi Fehri *et al.* showed that *P. acnes* could be found in more than 80% of cancer prostate tissues, while being absent from healthy prostate and from other cancer tissues [195]. Furthermore, *P. acnes* isolated from cancer prostate tissue were able to alter cell proliferation and cellular transformation, indicating that *P. acnes* might contribute to the development of prostate cancer [195].

3.12 Prosthesis removal - biofilm formation

P. acnes is suggested to have a role in the removal of prostheses due to infection, based on its ability to form biofilms [301] (Paper II). *P. acnes* readily forms biofilms on foreign material

(Figure 4) and when in a biofilm state, the resistance to several antibiotics increases more than 10 folds [562] and also the production of extracellular lipases and quorum-sensing molecules increases [134]. It is estimated that between 2-15% of all revision hip operations are due to infections [40, 375]. However, this number might be vastly underestimated, since Tunney *et al.* 1998 found that by improving the detection method by using ultrasonication and transfer to an anaerobic milieu the detection of bacteria from removed hip prostheses was almost 22% [684]. Besides, 87% of tissue from patients without any culturable bacteria had inflammatory cells, suggesting that also these patients might have had bacterial infection [684].

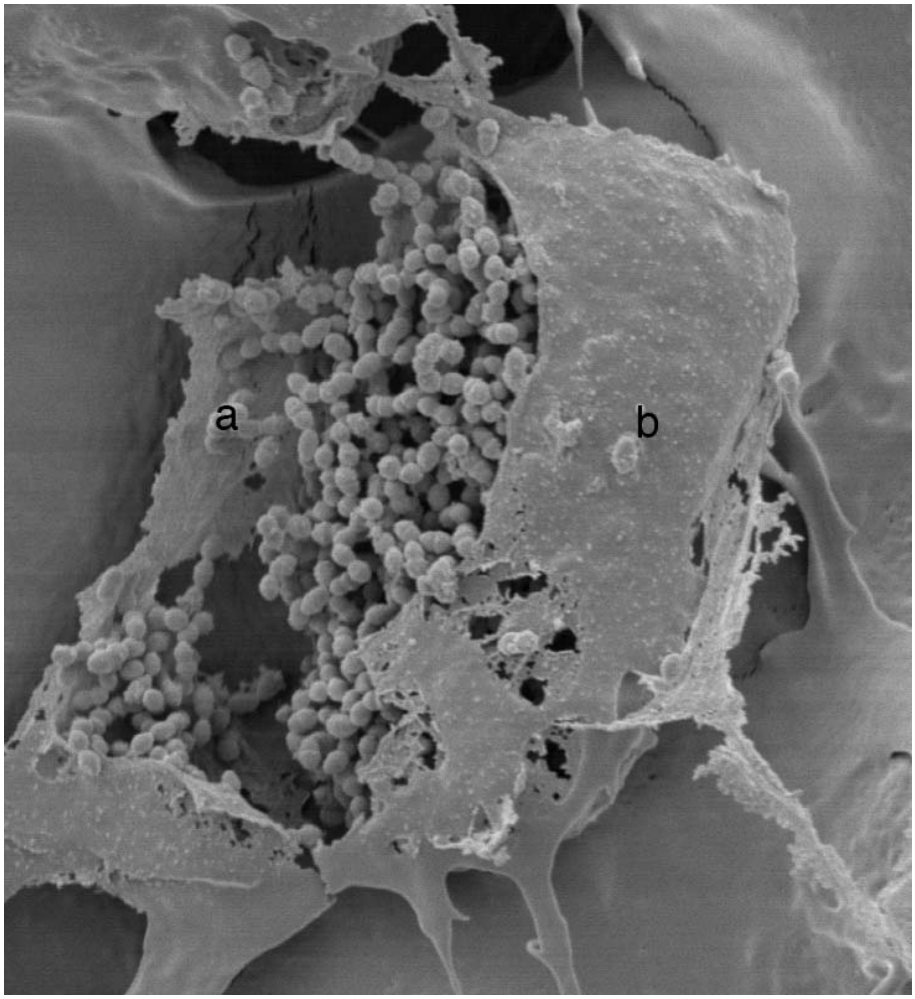

Figure 4: Biofilm formation by *P. acnes*. The bacteria are visible as coryneformed rods (a) inside a biofilm matrix (b).

In a following study in 1999, Tunney *et al.* showed, using immuno-fluorescence microscopy that 63% of the prostheses had either *P. acnes* or *Staphylococcus* [683]. Furthermore, 72% of all prostheses were positive for 16S rRNA amplification using universal primers, and 73% of all patients had infiltration of inflammatory cells in the associated tissue [683]. Further studies confirmed that *P. acnes* could cause several different types of infections, differing between common late chronic infections and much more seldom acute postoperative infections [759]. The median infection occurred 7 months after surgery resulting in joint pain and local inflammation [335]. Males and those that had a history of surgical procedures in the specific joint were concluded having a higher risk of infections [335]. In 2009, Sampedro *et al.* showed that *P. acnes* type I was more often found in biofilms from prostheses than type II or type III [599]. However, there was no significant difference between types and normal skin or infection, suggesting that the different types can form biofilm equally well [599].

P. acnes has also been suggested to have a role in the development of infective endocarditis [637] and inflammations in the cornea [150].

3.13 Characterized proteins from *P. acnes*

Two of the more characterized proteins from *P. acnes* are also two of the most abundant secreted proteins in *P. acnes* [299], lipases and sialidases, two enzymes involved in degrading lipids and carbohydrates, respectively. Since *P. acnes* is thought to be involved in the pathogenesis of acne vulgaris it is not surprising that one of the first proteins to be studied from *P. acnes* was the lipase encoded by *gehA* [473]. This 33 kDa protein was shown to be the major secreted lipase from *P. acnes*, even though the complete genome of *P. acnes* has several proteins annotated as putative lipases [95]. Furthermore, 2009 Iinuma *et al.* showed that *P. acnes* induces the formation of lipids in sebocytes due to increased synthesis of triacylglycerols [315], but as Zouboulis summarizes the finding: "*The role of P. acnes in sebaceous gland function remains uncertain*" [766].

Sialidases from *P. acnes* have also been studied for more than 40 years. Müller described in 1971 that *P. acnes* had enzymes with sialidase activity that could cleave the sialic acid from several plasma proteins, including haptoglobin, α2-macroglobulin, transferrin and IgA [492]. The abundance of sialidases in *P. acnes* was shown a couple of years later when Höffler *et al.* compared the activity of sialidases from different *Propionibacteria* and found that 83% of *P. acnes* had sialidase activity, to be compared with 20% of *P. avidum* and 6% of *P. granulosum* [297]. This conclusion was supported by a further analysis by von Nicolai *et al.* in 1980 that also concluded that 84% of *P. acnes* has sialidase activity, and found this activity in both the cell wall and as a secreted enzyme [510], which nowadays is supported by the genomic information from KPA171202, that suggests that there are both cell wall bound and secreted sialidases [95]. Furthermore, von Nicolai *et al.* purified an enzyme with a molecular weight of 33 kDa from the culture medium, and they concluded that the enzyme had the highest activity against oligosaccharides, rather than to glycoproteins,

indicating that this enzyme is not a virulence factor [510]. However, in contrast to that, Höffler *et al.* found in 1981 that *P. acnes* isolates from patients with acne had sialidase activity in 90% of the cases, compared to strains from the normal skin which only had activity in 73% of the cases [296]. Furthermore, the strains from patients with acne had almost twice as much sialidase activity as compared to the strains from normal skin, indicating that this enzyme might contribute to the development of acne [296]. Nakatsuji *et al.* evaluated this hypothesis on a more molecular level in 2008 when they purified a cell wall anchored sialidase from *P. acnes* [503]. This sialidase was shown to increase the adhesion of *P. acnes* to sebocytes and also increase the cytotoxicity. Mice pre-immunized with the sialidase did not develop any inflammatory response when they were subcutaneously injected with *P. acnes* suggesting that a *P. acnes* vaccine based on sialidases might decrease the inflammatory response caused by *P. acnes* [503].

Other factors from *P. acnes* have so far not gained that much attention. However, lately, secreted CAMP-factors from *P. acnes* have been shown to be expressed at high levels [299], with different levels depending on type of *P. acnes* [692]. Two of the five CAMP-factors were recently knocked-out, but did not seem to affect the virulence of *P. acnes* [642].

4 Virulence factors

4.1 Introduction

The prefix of different bacteria can sometimes be very hard to interpret. Some are called pathogenic bacteria and others normal flora, and even further more are called opportunistic pathogens or facultative pathogens. But what defines the pathogenicity of a microbe? It seems clear that those microbes capable of inducing a pathogenic state have the ability to produce certain proteins or substances that mediates this effect. But what, in this context, distinguish an opportunistic pathogen from a pathogen? And what factors are involved in this pathogenicity?

4.2 Virulence factors

The ability of a microorganism to induce a disease (to be pathogenic) is based on its virulence. The higher virulence, the higher capacity to induce disease. Though this concept is widely accepted, the actual meaning of virulence has been debated for many decades [118], and its meaning has changed from being solely focused on the bacteria to rather focus on the bacteria-host interaction [119]. In the beginning pathogenic bacteria were believed to have different attack and defense mechanisms, which were the only causes to the disease [635]. Thus, one factor of the bacteria was used to protect itself from the hostile host environment, thereby increasing the persistence, and one factor of the bacteria was used to cause damage in the host [762].

The fact that many bacterial virulence factors can be found in mobile genetic elements such as bacteriophages, plasmids and pathogenicity islands [514, 533] and can be differently regulated during the growth by bacterial sigma factors [345] might have contributed to the theory that only the bacteria was important for the virulence. Later, scientists began to understand that bacterial virulence was also dependent on the host [635], and soon it was widely recognized that even though the bacterial proteins and structures were involved in the pathogenesis, they alone were not responsible for the virulence (Figure 1) [716].

Modern scientist have further extended this discussion and proposed the use of a damage-response context to describe virulence [116] and define virulence as a relative capacity of a microbe to cause damage in the host [117]. The focus is no longer only on the microbe, but also the host, and the damage caused to the host might be mediated by the microbe, but also by the host [116]. Furthermore, virulence should not necessary be defined as a factor that affects virulence but not viability [739], since this would exclude several important cell wall structures such as LPS, which also is regarded as a virulence factor [64, 289, 702]. Several other well-known virulence factors include the pneumolysin [406, 444, 590] from *Streptococcus pneumonia*, polysaccharide capsules [551, 655, 672], neuraminidases from Influenza viruses [67, 658], cholera toxin from *Vibrio cholerae* [695], and immunoglobulin modulating enzymes from *Streptococcus pyogenes* [18, 517, 543].

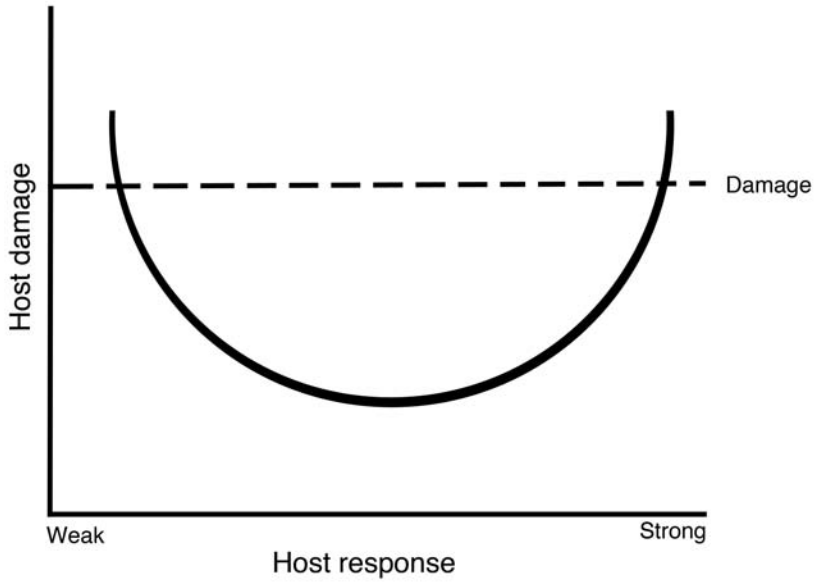

Figure 1: The damage-response curve. The virulence of the bacteria affects the position of the 'damage' line on the y-axis. A weak host response will cause disease, as will a too strong host response. Adapted from Casadevall & Pirofski [119].

Since the focus of this thesis is *Propionibacterium acnes* and its phages, the focus of this chapter will be on a general description of three potential virulence factors characterized in *P. acnes*.

4.3 Triplets in virulence factors

4.3.1 Biofilms

Bacteria cultured in liquid medium in laboratory settings do, in many ways, not represent the normal growth of a bacteria. In nature, nutrients are more scarce, and thus bacteria mostly exist in a stationary phase. This stationary phase is represented by biofilms (Figure 2) [421]. Biofilms were first recognized in 1943 by Zobell [764], but has since then been found on many different locations in nature [488, 602, 738]. Furthermore, biofilms can be found in man [259] and associated with orthopedic implants and teeth, causing severe medical problems [167, 261, 273]. However, many biofilms can be asymptomatic for long periods [259].

A biofilm can be defined as a microbial community with a self-made matrix [82, 262, 421], but even monolayers of bacteria can be called a biofilm [572]. Most often, these biofilms consist of specific different polysaccharides, such as the *Pseudomonas aeruginosa* alginate, PEL and PSL [592, 671], the *Bacillus subtilis* EPS and PGA [81, 646] and the *Staphylococcus epidermidis* N-acetyl glucosamine [519]. Furthermore, biofilms often consist of DNA and pro-

tein [82], thereby stabilizing the matrix further [728]. Also, several protein structures, such as pili and fimbriae, are important for the initial adherence to form biofilms [550, 741].

The biofilm is however not a mishmash of cells and secreted components, but has a highly defined structure [652] with water channels [143] and compartments with bacteria [144]. Those compartments of bacteria can come from the same bacterial clone, but still, due to environmental factors such as oxygen and nutrient gradients, might have different gene expressions, thereby specializing the cells [21, 217, 645, 651]. Those gradients will also enable the co-existence of anaerobes and aerobes within the same biofilm [51].

Except for mixtures of anaerobes and aerobes in biofilms, biofilms often consist of several different microorganisms all influencing the growth of each others [483]. Furthermore, the interaction in biofilms between two or more different microorganisms might be different from that observed in liquid cultures [351, 665], much dependent on the conditions when the biofilms form [368]. Many bacteria form biofilms in order to benefit from the others metabolites [360, 612, 676]. Also, many bacteria can only form biofilms on already existing biofilms. This is the case with dental biofilms, initiated by *Porphyromonas gingivalis*, after which other species can adhere and strengthen the biofilm [744]. This is also the case with *Streptococcus gordonii* that can form biofilms on saliva, while other mouth bacteria, as *Streptococcus oralis*, can not, but instead need a preformed biofilm of *S. gordonii* to adhere [534].

The initial step in biofilm formation is the binding of the microorganism to a foreign material, for example orthopedic implants. Several attempts have been done to change the materials used, in order to decrease the adherence of bacteria, but those attempts have so far not resulted in any biofilm-free implants [142, 569, 570]. This might be due to that bacteria most often, with *Staphylococcus epidermidis* being the exception [698], do not bind directly to the foreign material, but rather bind to a film of plasma protein that forms on the implants [259]. This initial binding is often mediated by different polysaccharides [19, 50], but exactly how this binding is mediated is unknown. Different theories are based on differences in hydrophobicity and charges on the surface [2, 105, 419, 420], but no theory can by itself explain how the biofilm forms, since the actual observations differ from the theoretical [2, 46]. Furthermore, observations suggest that bacteria change their cell walls, and thereby their hydrophobicity, during growth, further complicating any theoretical models [229]. Adhered cells can then spread on the surface, using different suggested models, such as ”rolling” over the surface [385], or by releasing daughter cells [361].

To be able to form such complex structures as biofilms, the bacteria need to collaborate. This is done by communication, by secreting signals. Most bacteria have an autocrine system, where they can both produce a substance and respond to it [109], but *Bacillus subtilis* was recently shown to have a paracrine system regulating biofilm formation [422]. This signaling is mainly mediated by acyl homoserine lactone (AHL) like substances in Gram-negative bacteria, and by peptides in Gram-positive bacteria

[421]. But, even low concentrations of other small molecules, as antibiotics, can trigger the formation of biofilms [298, 750]. Furthermore, signaling is not restricted to signaling between one species, but also exists in mixed biofilms [459, 597]. A similar phenomenon was observed with an *E. coli* strain that even though not producing any AHL, still had a homologous receptor for AHL [694].

One of the largest problems with biofilms is that they are very difficult to eradicate. Even though phages and phage derived proteins have been suggested as a treatment [308, 309], this is not standard procedure today. The problem is mainly due to the higher resistance to different antibiotics and antimicrobial peptides [56, 143, 188, 259, 355, 435, 574]. The increased resistance to antibiotics could partly be explained by the thick polysaccharide layer [650], but also by the existence of non-dividing persister cells in the biofilm [396]. The resistance to different substances is further complicated in mixed biofilms, where one species can secrete enzymes protecting the other [342], or by its mere existence physically hinder the substance from reaching sensitive bacteria [401]. Even though bacteria generally are resistant to antibiotics when the biofilms are formed, pretreatment of implants with antibiotics seems to decrease the adherence of bacteria, thereby also reducing the formation of biofilms [571, 747]. However, the resistance to antibiotics is not only dependent on what species form the biofilm, but also on what material the biofilm is formed on [27, 540].

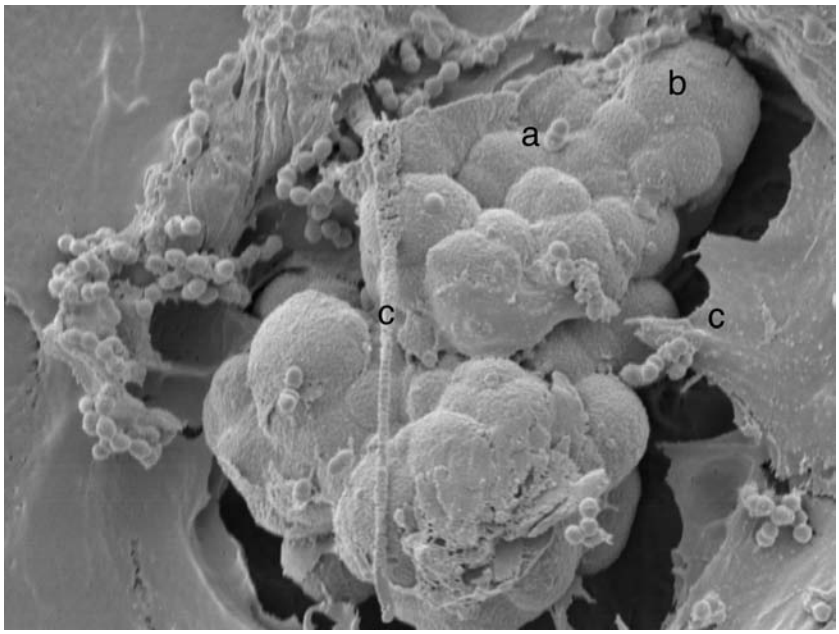

Figure 2: Biofilms. A biofilm formed by *P. acnes* on cement beads (b). Free bacteria (a), as well as biofilm structures (c) can be detected.

4.3.2 Sialidases

Sialidases were first called Receptor-Destroying Enzyme (RDE), since the first observation suggested that this enzyme from *Vibrio cholera* destroyed the receptor site for Influenza viruses [104], which later was concluded to be due to sialidase activity [6, 7]. However, already in 1941 Hirst demonstrated sialidase activity in Influenza virus [292]. A few years later, Gottshcalk named the enzyme "neuraminidase" since it released N-acetylneuraminic acids [239]. Since then, both the word "neuraminidase" and "sialidase" has been used for describing this activity [189].

Sialidases work by cleaving terminal sialic acids from glycoproteins and glycolipids [730]. They usually display some substrate specificity [141], with the *V. cholerae* sialidase being able to cleave 2,3; 2,4; 2,6; and 2,8 α-glycosidic bonds [171], while other sialidases only can cleave certain bonds [240]. Furthermore, sialic acids are not a single substrate, but consist of a family of more than 30 different [581] nine carbon sugars with 2-keto-3-deoxy-5-acetamido-D-glycero-D-galacto-nonulosonic acid structure [705], with the most common sialic acid being N-acetylneuraminic acid (Figure 3) [730]. Sialic acids got their name from the first site of isolation, which was in the saliva (from Greek sialon) [71]. Due to this complexity of sialic acids, some researchers have claimed that: *"Sialic acids are not only the most interesting molecules in the world, but also the most important"* [704].

Not all organisms have the ability to produce sialidases. Most plants do not, neither do most metazoans, but some animals and microorganisms can [494, 581]. However, this ability can differ between species, and also between bacterial isolates [305, 476, 548, 580]. The ability of several different species to produce sialidases has made several researchers to conclude that sialidases have a common ancestor gene [581], since they usually share between 20-30% amino acid identity [305, 581], and have a similar architecture between bacteria and mammals [305, 704, 706]. The sialidases are not biochemically similar, but do share a similar tertiary structure [581], and thereby phylogenetic trees based on primary sequences do not give the full picture [24]. Thus, this implicates that specific bacterial sialidases can be more similar to prokaryotic sialidases on a structural level than to other bacterial sialidases [305]. Sialidases with a lower molecular weight have a rather similar primary and tertiary structure [305, 704], while larger sialidases (>60 kDa) often have both an enzymatic domain and a domain conferring the specificity of the enzyme [305, 673, 704], and are thus not as similar to each other. However, all sialidases have some parts in common. They all have the same catalytic site with seven conserved amino acids, among others an arginine triad [704]. Furthermore, almost all sialidases have Asp-box motifs, even though the exact mechanism of these boxes still is unknown [704]. Many sialidases also need calcium in order to have enzymatic activity [305]. But even though the biochemical part of sialidases is rather well characterized, the biological function of the enzyme is not [704].

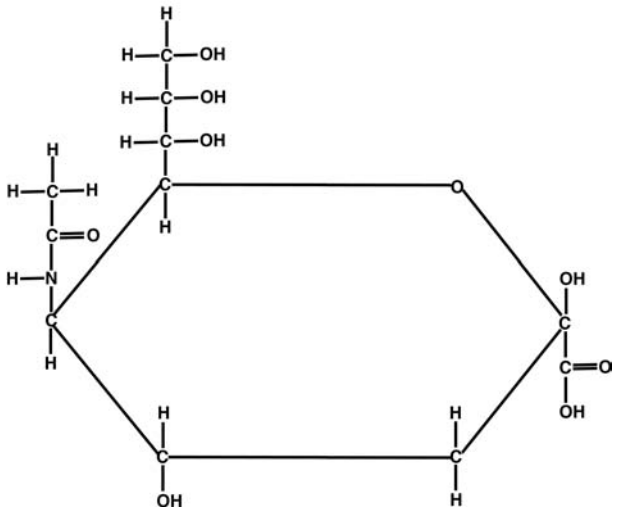

Figure 3: Sialic acids. A representative figure of the most common sialic acid - N-acetylneuraminic acid.

Many microorganisms use sialidases in order to get nutrition [141, 547], and sialic acids can in *E. coli* function as the sole carbon and nitrogen source [445, 547]. The importance of such system is understandable since sialic acids are abundant in the human body. In human serum, there exists 2 mM sialic acids, even though almost all are in a bound format [625], and even a single red blood cell has more than 10 million sialic acid molecules bound to its surface [697]. Due to this abundance, many commensal bacteria use sialic acids as a mean of fast energy, without disturbing the host [304].

However, not only commensal bacteria use sialic acids, but also pathogenic microorganisms [140, 141, 172, 566, 687], where they are suggested to have a role in the pathogenesis [566], by activating and stimulating cells [429, 566]. Even though the sialidases can be both cell wall bound and secreted, it is the latter that is regarded to be the most potent to cause different physiological effects [141], and help to spread the bacteria [140, 493, 495]. So even though both commensals and pathogenic bacteria can secrete sialidases, only some use it as a virulence factor [141]. One of the differences is that commensals often produce sialidases constitutively, while pathogenic bacteria need a stimulus, as free sialic acids or oligosaccharides, to induce their gene expression [141, 303].

Even though commonly regarded as a virulence factor, the main function of sialidases for most bacteria probably is nutritional [305, 496, 673]. However, there is a connection between high levels of sialidase activity and pathogenicity for certain species [141]. Group B streptococci start to produce sialidases late in their exponential phase [471], together with many other different virulence factors, but they do not seem to be necessary to cause disease [497]. How-

ever, this sialidase activity can partly desialylate IgA1 in the saliva, and thereby increase the proteolytic effect [573]. Furthermore, it has lately been shown that *S. pneumonia* sialidase NanA is essential for the adherence to human brain endothelial cells [688], and decreases phagocytosis by neutrophils [153]. However, the most characterized sialidase is from Influenza A viruses, where it is essential for pathogenesis [327]. This Influenza sialidase is also able to increase the adhesion of *Neisseria meningitides* to human cells [563]. In general, sialic acids are removed in order to reveal new receptors [348, 585, 610], but also to increase adhesion due to the decreased negative surface charge [140, 286, 765]. Furthermore, desialylation reveals sites for sialic acid-binding Ig superfamily lectin (Siglec) recognition sites [567], which, when activated, often modulates the immune system [149]. A similar effect can be seen with desialylated IgG that changes its affinity to the Fcγ receptors [337].

Not all microorganisms use sialic acids only for nutrition or revealing adhesion sites, but also for building capsules protecting them from the host immune system [323, 703]. The first bacterial sialic acids were actually identified from an *E. coli* capsule [44], which decreases the phagocytosis [674]. This molecular mimicry benefits the bacteria due to an increased immune tolerance [703]. In *S. pneumoniae*, the sialic acid capsule also lead to an inhibition of the insertion of the MAC-complex in the membrane [708]. At least three different methods to get hold on sialic acids for bacteria exist. Either they can synthetize the molecules themselves, as *E. coli* and *N. meningitidis*, use exogenous sialic acids, as *N. gonorrhoea* [539], or by using trans-sialidases, as *Trypanosoma cruzi* [552].

4.3.3 Protection from reactive oxygen species

Reactive oxygen species (ROS) is a collective name for radicals and non-radicals derived from O_2 [264], and is a substance that all living organisms will encounter. The production of ROS in different tissues in human was first suggested by Gerschman and colleagues [227, 228], and has since then been concluded to be both beneficial and dangerous for the host [131]. A few years after the discovery of ROS, the first superoxide dismutase (SOD) was characterized and suggested to have a protective role against oxidative stress [454]. Furthermore, it was shown that ROS was able to protect human cells from bacteria [31]. When phagocytes become activated by microbes, they will assembly an NADPH oxidase and produce and secrete large amounts of the superoxide anion radical O_2^- [31, 32, 362]. This product is not very reactive in itself, but will produce hydrogen peroxide which is a known mutagenesis factor [249]. Furthermore, hydrogen peroxide can react and produce the highly reactive radical OH^* [49], even though the exact mechanism of this *in vivo* is unknown [469, 724]. This radical is able to react with carbohydrates, lipids, proteins and DNA [249], but will only react where it is formed [137] due to its short diffusion life time of approximately 0.5 μm [468].

All production of ROS from the phagocytes will eventually cause pathogenic conditions in the host if not taken care of correctly [137]. This damage can be direct or indirect, by de-

grading substrates or by activating proteases [137]. Phagocytes will also secrete myeloperoxidases that can convert hydrogen peroxide to hypochlorous acid which is a much more potent radical [362], which will inhibit the effect of several anti-proteases [726] and also activate secreted proteases from the phagocytes [726]. Furthermore, it has been shown that ROS can signal via the immune regulator NF-κB [613], altering the inflammatory response [137], by increasing the expression of the pro-inflammatory cytokines IL-2, IL-6 and TNF-α [306, 398, 619]. However, the addition of SOD and catalase has been shown experimentally to reduce tissue damage due to the activity of free radicals [132, 243, 329, 628]. Furthermore, an *E. coli* deficient in SOD will have a much higher mutagenesis rate on the DNA when exposed to ROS [679]. Those enzymes, together with glutathione peroxidase are well established intracellular proteins that protect the cells from ROS (Figure 4) [66, 137, 213, 266, 624, 679]. However, there are several other known mechanisms of how to protect the cells from free radicals, using antioxidants.

Antioxidants are defined as a substrate that can protect or delay an oxidation of a substrate, even at low concentrations [266]. This effect can be due to a lowered O_2-concentration, a binding of O_2 to stable proteins and binding of otherwise reactive metal ions [263]. Even though mainly thought as being an intracellular protein, several reports have described an extracellular SOD [340, 341, 442], even though these mainly seem to be bound to cells. However, there is a great abundance of antioxidants in the human serum [463]. Inside the cell, reduced glutathione is an important antioxidant [609], however the low extracellular concentration is not enough to generate a good response against free radicals. Furthermore, transferrin is not loaded to more than 20-30% of its capacity with iron, which enables it to bind iron fast before the iron can react and form free radicals [257], since when bound the iron is unable to generate OH^* [28, 256, 257]. Other proteins in the plasma with antioxidant properties are bilirubin [653], ascorbic acid [212, 654], and alpha-1 microglobulin [12, 16, 17]. Furthermore, vitamin C and E are important antioxidants inhibiting oxidation of several substrates [124, 265, 532]. A function similar to transferrin is shared with haptoglobin and hemopexin that will bind free hemoglobin and heme, respectively, before they can react and release free radicals and stimulate oxidation [255, 258, 265, 596].

A free form of heme from damaged hemoglobin or from heme-proteins is a stimulator of oxidation [103, 231, 670], and can generate oxidative stress [258, 669, 670] which has been shown *in vitro* on several molecules [8, 9]. Furthermore, the synthesis of heme can also generate ROS [481]. This is due to the many reactive properties of some of the building substances [169, 291, 523]. Thus it is of importance to degrade free heme before it reacts, which is the function of heme oxygenases [594], that protect cells from oxidative stress by degrading heme to biliverdin (which will generate bilirubin), CO and iron [489].

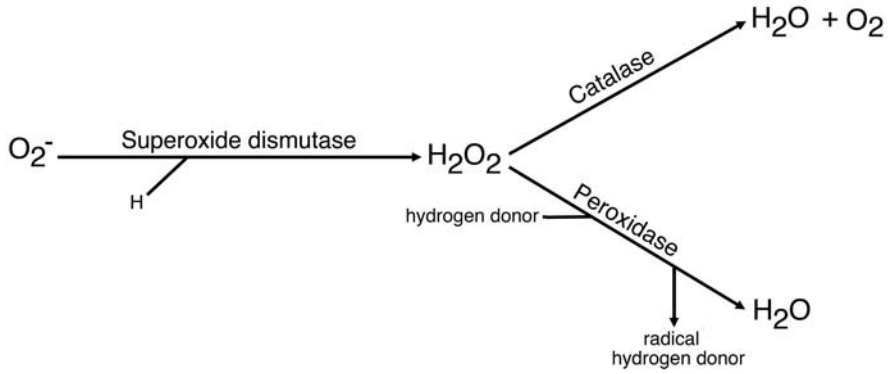

Figure 4: Protective systems against free radicals. Superoxide is dismutated to hydrogen peroxide, and can then by converted to water by either catalases or peroxidases.

The enzyme was discovered in 1968 [675], is inducible by UVA and hydrogen peroxide [353], and will increase the tolerance of oxidative stress to the cells [667]. Heme oxygenases protect from oxidative stress both by decreasing the amount of free heme, but also by the byproducts they form when heme is degraded [25, 352, 353, 354]. Low concentration of CO is anti-inflammatory by downregulating pro-inflammatory cytokines and upregulating IL-10 [490, 529] and protects against oxidative stress [530]. Bilirubin protects cells from hydrogen-peroxide [36] and is one of the most important antioxidant factors in serum [235]. An excess of free iron from the reaction would harm the host. However, a mouse with a heme oxygenase knock-out showed increased levels of iron, compared to the wildtype, indicating that the heme oxygenase has a beneficial role [160]. It has been argued that heme oxygenases can increase oxidation [372]. This is due to the release of small quantities of hydrogen peroxide during the reaction, and due to the abundance of free iron which could benefit a production of OH^* [178, 512, 626].

ROS have lately been gaining interest as signaling molecules [207]. The NADPH oxidase Nox2 is expressed in most cells, even though at much lower level than in phagocytes, and are likely to have a signaling function in those cells [376, 707]. This signaling regulates apoptosis via the JNK-pathway [159, 162, 407], and will furthermore due to the ability to modify proteins [209] affect the faith of the cell [260, 448, 449, 486]. The most probable signaling molecule is regarded to be hydrogen peroxide [656] that can be transported out of the cell using aquaporines [68]. However, data also indicate that O_2^- can function as a signal molecule, even though the mechanism still is unknown [434].

4.4 How to study virulence factors in *P. acnes*

Several putative virulence factors in *P. acnes* have already been studied to some extent, such as the lipase GehA [473], sialidases [503],

and CAMP-factors [299, 642]. However, even though these studies proved successful, the expression of recombinant proteins from *P. acnes* has proven difficult. Miskin *et al.* [473] optimized the expression by lowering the temperature to 27°C and adding 0.45 M sucrose. Nakatsuji *et al.* [503], instead of optimizing the expression, denatured and renatured inclusion bodies, so that the sialidase was soluble and active. Sörensen *et al.* [642] purified recombinant CAMP-factors from *P. acnes*. However, since these proteins only were used for developing antibodies, and their activity was not tested, it is impossible to know if these proteins were active or not.

However, this author has also had problems with recombinant expression of proteins from *P. acnes* and its phages in *E. coli*, where most proteins investigated have formed inclusion bodies. Even though some of these inclusion bodies were able to be dissolved using 6 M guanidine hydrochloride, and refolded to a soluble state, this method is quite unsatisfactory since much of the protein activity might be lost during this harsh purification. Therefore we decided to develop tools to both be able to express recombinant proteins from *P. acnes* in *P. acnes*, and to be able to complement knock-outs (Figure 5) [417] (Paper I).

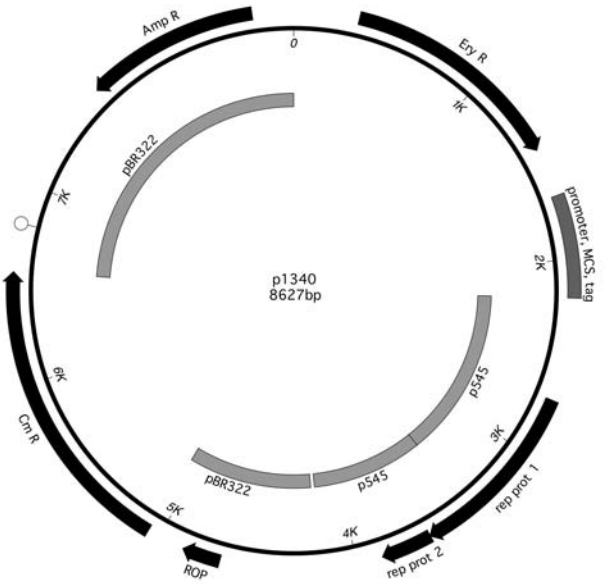

Figure 5: Vector p1340 developed for the recombinant expression of homologous proteins in *P. acnes*. The vector has resistance cassettes for selection in *E. coli* (Amp) and in *P. acnes* (Ery and Cm).

Part II
The Good Guys

You still do not know what you are dealing with, do you?
Perfect organism. Its structural perfection is matched only by its hostility...
I admire its purity; unclouded by conscience, remorse, or delusions of morality
-Ash to Ripley in *Alien* 1979

5 Bacteriophages

5.1 Introduction

Not living, nor dead. Not friends, nor foes. They can be infectious for decades [382] and found wherever their hosts are [722] - in deserts [554], in food [349], in our blood [536], in urine and saliva [33, 749], and in ocean water where they can reach numbers of 10^6 specimens/ml [58]. In fact they are so common in our bodies that more than 1200 different genotypes can be identified from feces only [85]. They are the most common entity found in the world, outnumbering bacteria 10:1 [281, 729], reaching an impressive theoretical number of 10^{31} [281, 662]. Using mathematical models, it has been estimated that 10-20% of all their hosts in the water die every single day due to infections [733]. Furthermore, if placed after each other, they would form a bridge to our second closest star Alpha Centauri, 4.35 light years away - 46 million times [282]! Bacteriophages truly are impressive!

5.2 Classification

5.2.1 Introduction

Bacteriophages, or phages, are viruses that infect bacteria, and are estimated to have been evolved when bacteria diverged from *Eukaryoteae* and *Archeae* [280]. They were first identified by Fredrick Twort 1915 [685] and Felix d'Hérelle 1917 [163]. Even though they both soon realized that phages could be used to treat infectious diseases [659], they did not know that they were working with viruses. It was not until 1942 that phages were recognized as viruses using electron microscopy [430], and phages continued for a long period of time to be the main focus of electron microscopy [23]. Further major scientific discoveries using the phages include the finding in 1952 that DNA is the genetic material [287]. This knowledge was implemented when the first genome ever was sequenced from phage ϕX174 1978 [600], followed a few years later by the sequence of phage λ in 1982 [601]. However, already from the start of phage biology the structural appearance of the phage has been one of the most important features to classify the phages together with the nature of the genomic material [191].

5.2.2 Classification

Phages can be classified into 12 different classes of phages (Table 1). The most common group identified is *Caudovirales*, constituting more than 95% of all known phages [453]. *Caudovirales* are phages with tails, and could be divided into *Myoviruses* (long contractile tails), *Siphoviruses* (long non-contractile tails) (Figure 1) and *Podoviruses* (short non-contractile tails) [438]. In the genomic era, several suggestions have been proposed in order to facilitate the classification of phages without the necessity of electron microscopy. However, so far no golden standard exists and thus the International Committee on Taxonomy of Viruses (ICTV) system is the most widely used [120, 191].

Table 1: Phage families (adapted from Veiga-Crespo & Villa [699] and ICTV [191])

Family	Genetic material	Morphology	Host
Myoviridae	dsDNA	contractile long tail	bacteria
Siphoviridae	dsDNA	non-contractile long tail	bacteria
Podoviridae	dsDNA	non-contractile short tail	bacteria
Tectiviridae	dsDNA	double capsid	bacteria
Plasmaviridae	dsDNA	pleomorphic	bacteria
Corticoviridae	dsDNA	internal lipids	bacteria
Guttaviridae	dsDNA	droplet shaped	archea
Rudiviridae	dsDNA	filamentous; non-enveloped	archea
Lipothrixviridae	dsDNA	filamentous; enveloped	archea
Fuselloviridae	dsDNA	lemon shaped	archea
Microviridae	ssDNA	capsid with spike	bacteria
Inoviridae	ssDNA	filamentous phages	bacteria
Cystoviridae	ssRNA	segmented genome	bacteria
Leviviridae	ssRNA	spherical	bacteria

Some of the proposed methods to classify the phages are similar to the ICTV standard [383], while other methods use a proteomic approach [583]. Even though there is no counterpart to the bacterial *recA* in phages [583], attempts have been made to classify phages based on their capsule genes, since they to some extent are conserved in all known phages [557].

The difficulty by using a genetic approach to classify phages is their modular nature. Phages in the *Caudovirales* family generally consist of several different genetic modules that can be changed between different phages [283], even though phages isolated from the dairy industry are more conserved, potentially due to the selection of the material [98]. However, it is unlikely that modular recombination will take place in modules encoding structural head genes, since such modulations in most cases will lead to defect phages due to the complexity and interactions between those proteins [332]. The transfer of modules is mainly regulated by horizontal gene transfer [114], but there has been theoretical calculations estimating phage mediated transduction to happen in a frequency of 10^{13} times/year [325, 722]. Furthermore, phages have the capacity to load their capsules with up to 4 kb more DNA, enabling the phages to take with them new integrated genes [121]. The module theory was founded more than 30 years ago by Botstein and is still considered valid [78].

5.2.3 Identification of phages

Phages are usually identified using an overlay-plate approach (Figure 2), where the phages are propagated against a selected host bacterium,

and where the number of phages in the sample can be measured [399]. However, the interaction is affected by several parameters as the abundance of specific ions (Ca^{2+} and Mg^{2+}) [399]. Furthermore, all phages are not able to form plaques using ordinary methods [617]. This matter is further complicated by the fact that most bacteria can not be cultured in laboratory environments, and thus phages infecting those species can not be propagated [541]. To circumvent this problem several different methods have been assessed. Transmission electron microscopy and fluorescence microscopy can be used to both detect and determine the amount of phages in an environment [58, 556, 663]. Flow cytometry has also, with success, been used to count phages [441], but for the detection of specific phages antibodies [128], DNA probes [560], PCR and RT-PCR [426, 681] have been the most useful methods.

In order to study phages, common approaches are Denaturing Gradient Gel Electrophoresis [622], to generate genetic fingerprints from a specific environment, or using Shotgun Sequencing [85]. However, a more and more used technique to detect viruses that can not be propagated is by metagenomics of environmental samples [85]. Using this method, it has been shown that the most common detected phage in a sample, only accounts for less than 0.1% of the community, showing an incredible variance amongst the viruses [84]. Furthermore, using this approach, Edwards & Rohwer found that approximately 70% of the sequences found in viral metagenomics are unique, to be compared with 10% for bacterial metagenomics [183], stressing the great variety seen amongst viruses. Further emphasizing this conclusion, Breitbart *et al.* could detect several thousands of different viruses from an environmental sample [86]. Based on a theoretical model, Rohwer *et al.* estimated that there existed 100 million different viruses, and that we so far only have isolated 0.0002% [582].

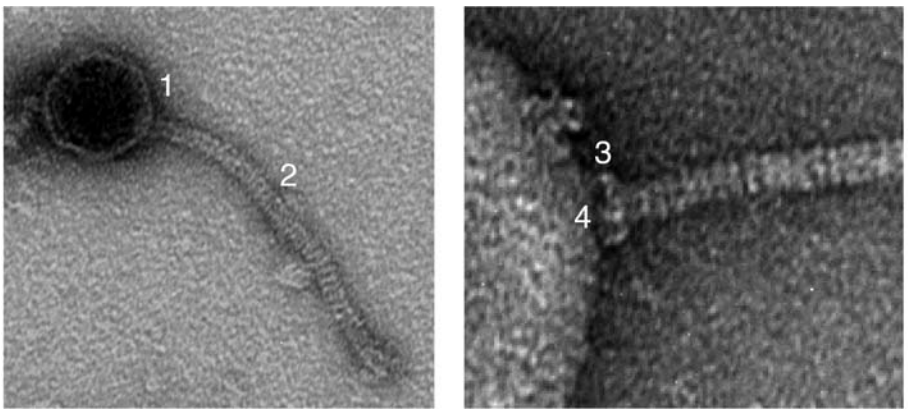

Figure 1: Morphology of Siphoviruses. The phages have an icosahedral head (1) containing all the genetic material. The head is attached to a long tail (2), which ends in a baseplate (3) with attached spikes (4) that can interact and bind to bacterial cells and residues.

5.3 Life cycle

5.3.1 Introduction

Phages can have two in principal different life cycles: the lytic cycle, characterized by Max Delbrück almost 80 years ago [107], and the lysogenic cycle, with phage λ serving as a prototype [386]. The most versatile phages are the temperate phages that are able to either kill the host or to enter a lysogenic cycle, where they may coexist in different forms [433]. This can be either due to the integration of the phage DNA into the host chromosome, or by the existence as a stable replicating plasmid [389, 403, 565]. The other possible life cycle is the lytic cycle, which all phages are capable of. However, the main difference between a temperate phage and a virulent phage is the lack of an integrase and a lack of ability to coexist with its host in the latter [339]. A schematic figure that explains the differences in the life cycles can be found in Figure 3. There also exist a third, much less understood life cycle, termed a pseudolysogenic life cycle, even though it also from time to time is called a persistent infection, chronic infection or carrier-state [549, 722]. This state is characterized by the carriage of phage DNA by the host bacteria, usually during nutritional starvation [722]. Furthermore, it seems like every single phage-bacteria system is, to some part, unique in its life cycle, even though some general aspects still are valid [611].

5.3.2 Attachment

Whether lysogenic or lytic, the phage life cycle always starts with the binding to the host (Figure 4), and the faster the phage binds, the faster they can increase their population [1]. However the interaction of the phage and the bacteria is also influenced by the status of the bacteria [156]. This binding is very specific, and thereby most phages are species or strain specific [5], even though certain phages, as PRD1, can infect several different Gram negative species as long as they express its receptor [241, 522].

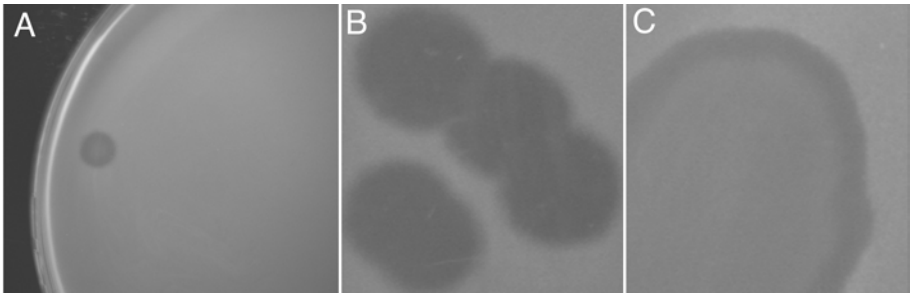

Figure 2: Plaques. Single plaques can be detected as clearings on a lawn of a susceptible host bacterium. All plaques are derived from a single phage. The plaques can be clear (A, B), or have a turbid center with regrowth of resistant bacteria (C).

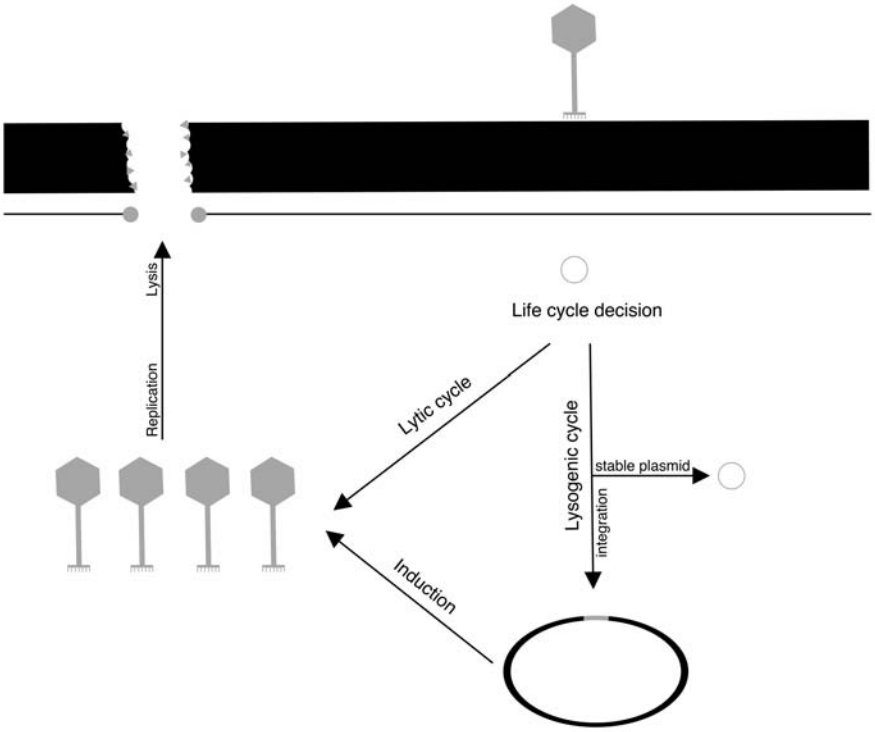

Figure 3: Principal life cycles for bacteriophages. The phages adhere to the bacterial cell wall, and inject their genetic material. The phage can then decide if it should enter a lysogenic cycle or a lytic cycle. In the lysogenic cycle, the phage either integrates its genetic material into its host chromosome, or exist as a stable plasmid. In the lytic cycle, the phage will replicate, degrade the peptidoglycan layer of the host using endolysins, and escape from the host.

The knowledge that phages generally were specific was rapidly used to develop several so called phage typing sets, where the ability of phages to infect certain bacterial strains were used to classify the bacteria [293], with one of the first type-system developed in 1938 for *Salmonella* [147]. Even though this system to some extent still is used today, the importance of phage typing has decreased [638]. Furthermore, the host-range of phages has been shown to depend on which bacterial strain is used for propagation, due to different DNA modification systems in the bacteria. This phenomenon is known as host-induced modifications of the phages [63, 432].

Phages initially bind reversible and then irreversible to a second structure [279, 390, 436, 480, 564, 714]. Bacteriophages infecting Gram positive bacteria usually first bind to sugar moieties in the peptidoglycan layer, followed by an irreversible binding to proteins [26, 482], while phages infecting Gram negative bacteria usually

interacts with LPS [278]. But several different receptors have been identified for different phages, including both proteins [83, 564, 693, 756] and carbohydrates [175, 225]. Since this is the first contact between the phage and the bacterium, inhibition of attachment is one of the most commonly used strategies for bacteria to gain resistance to phages [20, 165], and more than 50 different mechanisms to gain resistance to phages have been characterized [135]. One of the resistance mechanisms is to form capsules, which is known to inhibit the attachment of certain phages [62]. However, phages have counter-measurements to degrade capsules [48], and this polysaccharide hydrolyzing activity has been found in lots of phages infecting a wide diversity of Gram negative bacteria [13, 38, 45, 254, 309, 373, 575, 696, 758]. Even though the phage adhesins bind to very different structures, most of the adhesins have similarities in secondary structure with a homo tetramer and a high percentage of beta structures, leading to a highly stable heat and protease tolerant protein [475, 721].

5.3.3 DNA injection

Once bound to the receptor, the next step of the phage infection is to inject the DNA, but in contrast to many human viruses, the phages leave their structural proteins outside their hosts [392]. This presents another problem, how to get the DNA over the membrane, and also through the thick peptidoglycan layer of Gram positive bacteria, which can be between 20-50 nm thick [65, 374]. This is often solved by phage structural peptidoglycan hydrolyzing enzymes [336, 477, 593], as has been shown for *Lactococcus* phage Tuc2009 that has a cell-wall degrading tail fiber [350].

Even though phage genomes can be very small and constitute less than 12 genes [108], the length of DNA can vary between 4-640 kb [99]. However, the concentration of DNA in the capsule is always approximately the same in different phages, *eg* 450 mg/ml [72]. This high pressure introduced when packaging the DNA is thought to help during the injection [179, 576], since it is known for certain phages (T5) that it is only necessary with a binding to the receptor in order to release the phage DNA [515]. It has also been hypothesized that phage DNA is injected into the bacterium due to proton gradients at the cytosolic membrane [248]. Even though this mechanism in general increases the influx of DNA [79], it seems like there exist several methods for phages. For example phage T5 starts by injecting a small portion of its DNA, then pauses for 4 min until it injects the rest of the DNA [380].

The injection of the DNA is facilitated by phage helper proteins that form a channel for the DNA to pass through the peptidoglycan layer [158, 252]. Those DNA transportation proteins can even cross lipid membranes [74, 199]. It has even been shown that the tape measure protein of the phage, that usually determines the length of the tail, is used as a DNA transporter protein [579, 608]. The DNA is finally injected in the opposite direction as it was loaded [232, 479].

5.3.4 Life cycle decision

It is not entirely known how the decision to enter a lysogenic or a lytic cycle is made, but factors affecting the choice have been identified. What

is known is that the decision is not immediately made after injection, since the fast propagating phage lambda needs 10-15 min before it determines what cycle it should enter [181]. During this time, the phage can ”sense” the status of the bacteria and adopt its cycle to that, using a genetic switch [559]. For example if the bacteria are growing very slowly, due to nutritional limitations, the phage can adapt and prolong the eclipse period (time when the phage produces structural proteins), and enter a pseudolysogenic cycle, since this is the most beneficial state during these circumstances [37, 427]. Furthermore, the decision is influenced by factors such as nutrients, ions, stress and the ratio between the bacteria and the phage [723], where a low number of bacteria with many phages promote a lysogenic decision [180, 649]. There also exist theories that the morphology of the phages influences the choice of life cycle, since *Myoviruses* mainly are lytic and *Siphoviruses* mainly are lysogenic [662], but this is so far not supported by any experimental data.

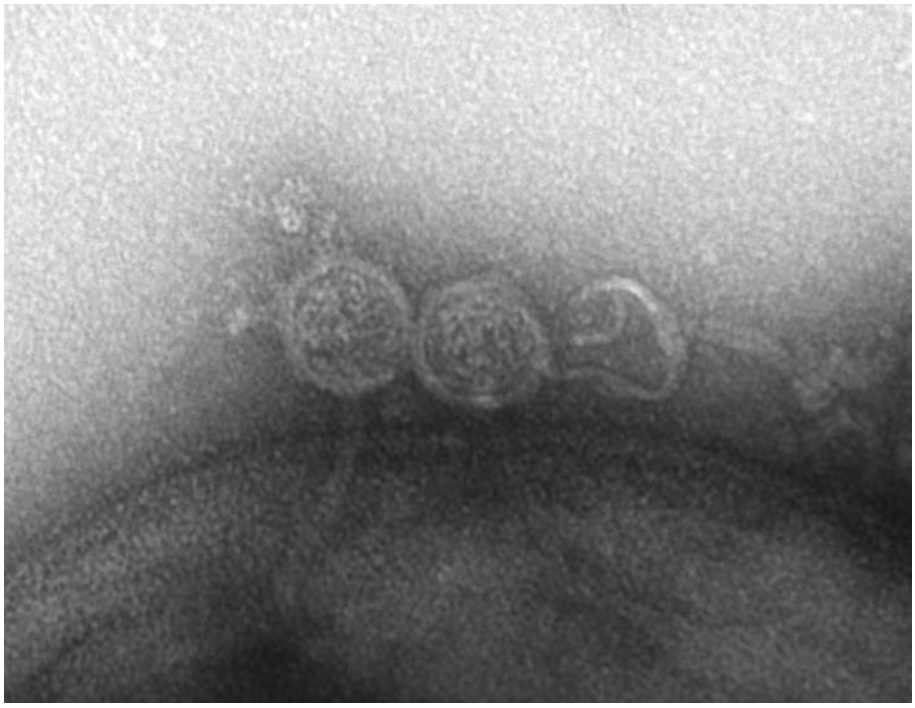

Figure 4: The attachment of bacteriophages to the surface of *P. acnes*. The two phages to the left have still not injected their DNA, which can be detected as white lines in the head. The third phage to the left has already injected the DNA and thus the head is deformed due to the pressure applied using negative staining.

5.3.5 Integration

The ability amongst phages to potentially integrate their DNA was proposed first by Campbell in 1963 [111]. For the temperate phage to integrate it is necessary with an integrase [763] and co-factor proteins assisting in the integration of the DNA [470]. This integration is often in tRNA or mRNA genes [735], even though several other genes also have been identified as sites for the integration [110, 499, 621]. Furthermore, the integration is often very specific for a certain site, with 100-1000 times higher affinity [725], even though less specific phage integrases are known from phage Mu and P2 [41]. When integrated, lysogeny is very stable and the phage can persist in this state in several generations [405], and usually only one gene is active during this state, *eg* the repressor [559]. The function of the repressor is to bind as a dimer [53] to several operator sites on the prophage and regulate the gene expression [559], even to its own promoter [397]. Even though the lysogeny is a stable state, the phage can be activated. This phenomenon is called prophage induction [402, 578]. The induction can be due to different stimuli, but the stimuli need to overcome a threshold value in order to activate the phage. Prophages have been induced with both UV [377] and mitomycin C [711], but both stimuli share the common feature that they activate the SOS-system by damaging DNA, which generates a signal to the phage [404]. However, prophages can also be induced by the SOS-system without any apparent DNA damage. This happens in general less than 10^{-5} per generation and is called spontaneous induction [88]. Furthermore, some bacteria as *Bacillus* can form spores and trap virulent phages for long times. However, since sporulation is triggered by among others nutritional starvation, this state is hypothesized to benefit the phages [319, 344, 462, 485].

5.3.6 Phage release

When temperate phages are induced from their lysogenic cycle, their life cycles are once again similar with the virulent phages, and they start to propagate in their host. This propagation can take place in starving cells [366, 611] and even in newly killed bacteria [22]. The phages start to replicate, which for some phages take place at the cell membrane [320]. At this stage the phages also start producing all their structural proteins and assemble the virions. However, there exist phages infecting Archea that assemble their tail after their host is lysed [271, 272]. Furthermore, the length of the tail is in direct correlation to the length of the tape measure protein of the phage [343].

When the bacteriophage capsule is loaded with phage DNA and all structural proteins are assembled, the next object is to destroy the peptidoglycan layer of the bacteria in order to lyse the bacterium and evade [61] (Figure 5). This must be strictly regulated in order to not lyse the bacterium too early, when the phage is not assembled [712]. This is in part regulated by holins. Holins are often expressed in around 1000 molecules/cell [713] and are integrated in the plasma membrane [251]. Holins are not particularly conserved and are thereby difficult to identify by their primary sequences [713], but they can nevertheless all be divided into three different classes dependent on how many

transmembrane domains they have [713]. The holins are in their turn regulated by anti-holins [755], which are expressed before the holins [70]. Anti-holins and holins often only differ in 2-3 amino acids in the N-terminal part of the protein since the anti-holins starts at an alternative start codon a few bases upstream of the holin [713]. However, this small difference enables the anti-holin to interact with the membrane in a different way than the holin, and thereby inhibit its effect, until enough holins have been expressed [244, 250].

When the holin is assembled the preformed lysins can gain access to the peptidoglycan layer and hydrolyze their substrates [752]. Lysins can have four different activities: N-acetyl-β-D-glucosaminidase, N-acetyl-β-D-muramidase, N-acetylmuramoyl-L-alanine amidase and endopeptidase (Figure 6) [604]. Lysins usually only have one enzymatic domain even though lysins with several different active sites have been characterized [505]. Except for their active domain, which usually is N-terminal, most lysins have a C-terminal that generates the specific binding [203, 285, 412, 423]. Furthermore, lysins from phages infecting Gram negative bacteria are usually considerably smaller than lysins from phages infecting Gram positive bacteria [754]. It has also recently been found that lysins might have signal peptides for secretion and are thereby secreted out of the bacteria [604]. This has experimentally been verified for a phage infecting *Oenococcus oeni*, where the lysin needs to be secreted in order to get activated [605, 606], but several more phage lysins share the same signal peptides and are thereby thought to be secreted [333, 603, 606].

Even though the above described lysis of bacteria is the most common mechanism for the more complex dsDNA phages, other phages employ other as refined methods to evade from their hosts. Smaller phages often only use a single protein [753], interfering with the formation of the peptidoglycan layer [60, 61]. Those peptidoglycan inhibitory proteins of non-lysin origin are commonly called amurins [61]. Filamentous phages employ yet another strategy, since they are being released from the host bacterium without causing lysis [439, 446], by expressing proteins that allows them to be secreted [636] through small channels [196, 591].

5.4 Phage mediated virulence

Since temperate phages have the ability to integrate their DNA into the host chromosome, it is not surprising that bacterial genomes are full of prophages. Studies have found that between 3-10% of the bacterial genome is constituted by phage-like modules [114, 120], but it can be a lot more. For example *S. pyogenes* strain SF370 has more than 16% of its genome covered by prophages [197]. Some of those modules are defective prophages that can not replicate [112], others might be satellite phages that can replicate but do not encode all their structural proteins by themselves, but rather steal them from other phages [120]. Further domains might constitute gene transfer agents that are phage-like particles that assemble bacterial DNA [310].

Even though the prophages are so common, the annotation of them is still mainly performed manually [384]. There have been several attempts to develop software that could detect prophage regions in bacterial genomes, but so

far with limited success [174, 682]. A usual characteristic of prophages is the integrase, repressors and lysins [113, 120], but even the repetition of Gly-X-Y is a characteristic motif often found in phage tail fibers [634], and can be used for the detection of prophages. Furthermore, the functionality of the phage proteins are most often assigned based on similarities to other characterized proteins [531], and secondary structure homologues using Pfam [640].

In order to be accepted by the bacterium, prophages often confer some advantages to their host [99, 182]. This advantage could either be an increased pathogenicity [80] or an increased fitness in a specific environment [700] (Table 2). However, some prophages have been reported to be beneficial for the outcome of pathogenesis, since *E. coli* without a specific phage more often causes disturbances in the gastrointestinal tract [275]. But, it has also been shown that the pathogenic *E. coli* strain O157:H7 has 18 prophages that together constitute more than 50% of the genetic difference between O157:H7 and a non-pathogenic *E. coli* strain [521]. The same phenomenon could be seen in *S. pyogenes* where more than 75% of the genetic variations between pathogenic and non-pathogenic strains can be due to prophages [34].

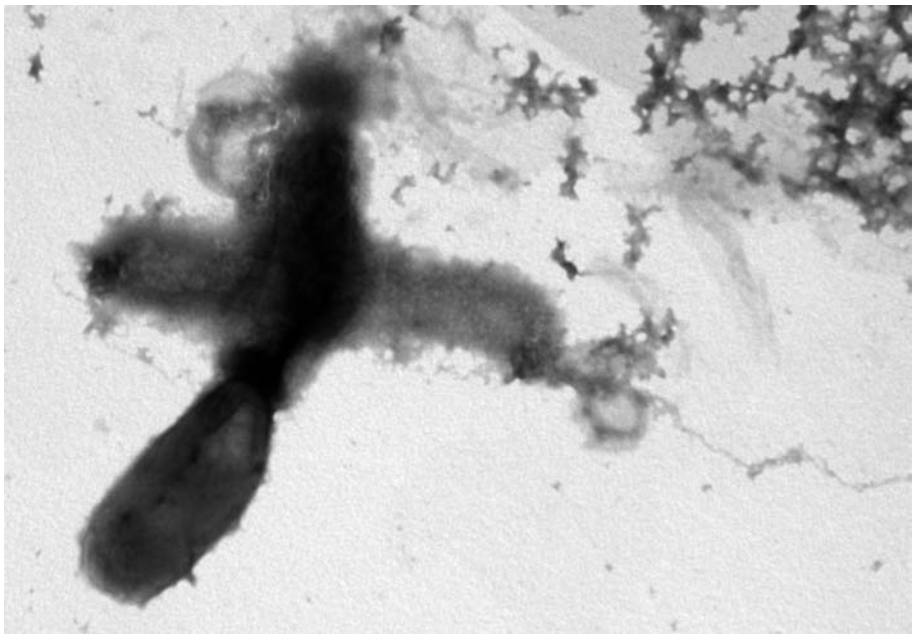

Figure 5: The lysis of bacteria and the subsequent release of phages. *P. acnes* infected with bacteriophages are lysed from within and release a huge amount of cytoplasmic material and phages as it dies.

The ability of phages to mediate virulence factors was shown already in 1926 for *S. pyogenes*, even though it at this time was not concluded that phages were the mediators [115, 214]. The first confirmed phage-toxin-conversion took place in 1951 using the diphtheria toxin [210, 211]. In 1971 it was also formally shown that a pathogenic *E. coli* strain could convert a non-pathogenic strain to a pathogenic strain using phages [633].

The genes encoded by phages that confer advantages to the host bacterium are called morons [332], and are often expressed in the prophage state [460]. Even though the GC-content in those genes usually is different from the rest of the phages, it is not generally believed that the origin of morons is bacterial [154]. Typical phage-encoded genes confer increased adherence [54], increased resistance to antibiotics [686] and can encode superantigens and toxins [97]. Even the prototype temperate phage, phage λ, encodes virulence factors increasing the resistance of the bacterium to serum [39]. However, several *E. coli* prophages also carry the Shiga toxin [709, 710] enabling the *E. coli* strains to be severe food pathogens [338]. Another widely recognized virulence factor encoded by prophages is the cholera toxin [711]. *S. aureus* prophages can carry genes encoding Panton-Valentine leukocidins that can be secreted and kill neutrophils [226]. Prophages in *S. pyogenes* are well known for the abundance of hyaluronidases [313], even though the impact of those is not clarified yet. Furthermore, *S. pyogenes* prophages can carry genes for DNases, SpeA, SpeC, and phospholipases [461], and influence the expression of M-proteins [643]. Prophages have also been found to be able to decrease the phagocytosis [691] and increase intracellular survival in macrophages [201], as well as conferring resistance to macrolides [312, 437], even though the latter mechanism more likely is mediated by a transposon that is packed in the phage during lysis [35]. Furthermore, structural proteins from phages infecting *S. mitis* have been suggested to increase the interaction between the bacteria and platelets, and thereby increase the pathogenic potential of the bacteria [54, 623]. However, the products need not to be harmful for us. For example cyanophages are carrying photosynthesis genes that can be used by the bacteria to grow faster [400, 440].

Table 2: Examples of phage mediated virulence factors

Species	Protein	Effect	Reference
Staphylococcus aureus	leukocidin	kills neutrophils	[226]
Streptococcus pyogenes	hyaluronidase	degrades extracellular matrix	[313]
Streptococcus pyogenes	DNase	escape from NETs	[461]
Streptococcus pyogenes	SpeA	superantigen	[461]
Streptococcus pyogenes	SpeC	superantigen	[461]
Streptococcus mitis	phage protein	binds to platelets	[54]
Vibrio cholerae	AB-toxin	massive diarrhea	[711]

Even when phages are induced, they might still contribute to the virulence for the bacteria, since this induction also triggers the production of the potential virulence factors encoded by the phage [3, 276, 748]. Activators of this process might be environmental, such as cell extracts [90, 91] or hydrogen peroxide from neutrophils [709]. The activating signal can even be antibiotics, thereby enhancing the pathogenesis rather than decreasing it [365, 450]. Activation of prophages can also lead to spread of virulence, mediated by phages, to non-pathogenic strains of the normal flora [89].

5.5 Phages in the industry

5.5.1 Industrial problems with phages

Fermentation, a process to change carbohydrates into alcohols, is often used in several industries to generate among others yogurt and vinegar. This fermentation is processed by bacteria, and is hence sensitive for the activity of phages [73]. It has been estimated that between 0.1-10% of all fermentations are destroyed, depending on the industry [73, 478]. This problem is mainly due to that phages are difficult to eradicate, and investigations have shown the presence of phages in the air of fermentation industries, reaching concentrations of 10^5 pfu/m^3 [509]. Due to this problem, a huge effort has been made to characterize phages infecting lactic acid bacteria, and more than 100 phages have been sequenced [4, 98], mainly being classified as *Siphoviruses* [69]. Several strategies have been assessed in order to solve this problem, among others by using different bacterial rotation schemes [177, 627] and the usage of bacterial strains resistant to most phages [208].

5.5.2 Phage display

The technique for phage display was invented almost 25 years ago [630]. This is a technique where peptides can be displayed on the surface on phages, usually using their capsule proteins [630]. It has so far only been used in phages from *E. coli* [648, 734, 745], but has shown to be a valuable tool in the development of monoclonal antibodies [736] and for vaccination studies [47, 465].

5.6 Phage therapy

5.6.1 Phage therapy using whole phages

When Twort in 1915 first discovered viruses that could infect bacteria he soon realized that phages could be used as antibacterial agents [659]. He was, nevertheless, not the first to identify the antibacterial effect of phages. Already in 1896, Hankin could demonstrate that water from rivers could kill *Vibrio cholerae* [268], but did not conclude that this was due to bacterial viruses. Furthermore, in 1918 d'Hérelle also understood the therapeutic potential amongst phages and used them to treat dysentery [661], and cholera in India [164], where the mortality decreased from 25% to 0% [96]. Soon, many more scientists followed their lead and used phage therapy to treat different bacterial infections [101, 155, 644]. Phage therapy was mainly used to treat dysentery [30], but also against different lung diseases [317] and wounds [760].

Most phage therapies have so far been conducted in former Soviet [464, 629, 657], where they used phages against *S. aureus* and *E. coli*, even during World War II [660], which also was true for Germany [274]. Phage therapy is still being used in some former Soviet states with proposed beneficial effects [364, 598]. However, since most trials were conducted in the former East block, the Western countries had problems to interpret the results [659]. Simultaneously, penicillin was introduced, and phage therapy was in West only suggested to be better than no treatment at all. Due to those coincidences, research in phage therapy was forgotten in West, but still continued in East.

Even though phage therapy was a forgotten subject in parts of the world, it recently started to gain increased attention from the scientific community. The reason for this was mainly the lack of development of new antibacterial drugs from the pharmaceutical industry [508, 666]. Furthermore, some scientists have raised concerns that we might come back to the time before antibiotics were invented [381] due to the increasing resistance seen amongst bacteria. This need for a novel class of antibacterial substances restarted the research in phage biology and the potential of phage therapy. The research was partly done in order to develop veterinary applications [29, 42, 307, 632]. One of the more ambitious phage therapy experiments was performed by Smith & Huggins in 1982 by using an *E. coli* infection model [631]. They could conclude that the presence of phages severely diminished the effect of a bacterial infection [631]. Further studies by other groups could show that phage therapy was effective even against antibiotic resistant bacteria [629], and even had better effect than several antibiotics [464]. Several other groups could conclude that phage therapy could rescue animals from severe infections [42, 451, 641], and again conclude its superiority versus antibiotics, since the animals in the study did not lose as much weight when treated with phages, as did animals treated with antibiotics [742]. However, it is still not concluded if the combination of phages and antibiotics is beneficial, since evidence points at both directions [466, 660].

Still, even though some results were promising, there were also issues that remained to be solved. One of the major reasons that phage therapy was not used even more extensively with success during the first years was due to the lack of basic knowledge of phage biology [371] and due to the fast development of phage resistant bacterial strains [186, 431]. However, this resistance is often expensive for bacteria in the sense that they will have a loss of fitness [186, 631]. This was experimentally shown by Harcombe *et al.* in 2005 when phage resistant *E. coli* was outcompeted by a *Salmonella* strain, not able to outcompete the wildtype *E. coli* [269]. Therefore, a potential development of resistance to phages might be beneficial for a therapeutic purpose [395], and might only be a laboratory problem since the same problem does not seem to exist in *in vivo* assays [431]. Furthermore, this resistance is not gained when the therapy is applied, but already exist in a small fraction of the bacterial community [269]. The potential problem with the development of phage resistant bacterial strains could partly be solved by using phage cocktails [173]. This has

with success been used both for *Salmonella* on chickens [678] and for *E. coli* [668]. Furthermore several products using phage cocktails are becoming commercially available, as PhageBioDerm bandages using phages against *E. coli*, *S. aureus* and *S. pyogenes* [443].

As modern trials with good controls have not indicated any severe side effects of phage therapy [100, 391] it is important to consider the route of administration. Phages are suggested to have a good delivery route in the body [451] and can even cross the brain-blood barrier [668]. Furthermore, they can penetrate the skin and reach the blood stream [346]. This high abundance of phages will start the production of antibodies [133, 387, 451], and we all have several circulating B-cell clones specific for phages [324, 369]. Phages can also rapidly be inactivated by neutrophils secreting hypoclorid acid [198]. However, phages will anyway rapidly be cleared from circulation in a non B-cell mediated way [224] and will be concentrated in the spleen [347].

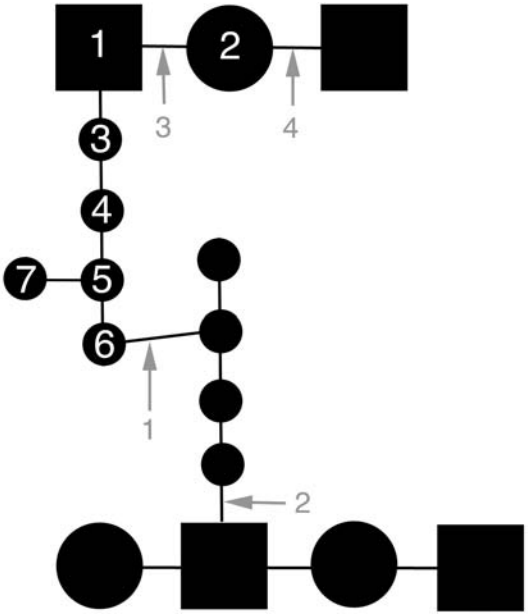

Figure 6: A schematic figure of the petidoglycan layer in *P. acnes*. The glycan chain consist of N-acetylmuramic acid (1) and N-acetylglucosamine (2), and the peptide chain consist of L-alanine (3), D-glutamate (4), L,L-α,*e*-diaminopimelic acid (5), D-alanine (6), and glycine (7). Activities of phage endolysins are visualized as gray arrows. 1 - endopeptidase, 2 - amidase, 3 - N-acetyl-β-D-glucosaminidase, 4 - N-acetyl-β-D-muramidase

In the human body, phages have several proposed functions, unrelated to their antibacterial effect. Phages have been suggested to bind to integrins on human cells [236], thereby inhibiting signaling through NF-κB and thus be immunosuppressive [237, 238]. They also seem to decrease the production of ROS [558, 718] and could protect against other viruses [238, 467] such as herpes simplex [43, 123]. The antiviral activity is suggested to be due to an increase of interferon when stimulated with phages [363], which also is seen when free phage DNA is injected [316, 487]. Furthermore, phages, and in particular holins, have been proposed to have anti-tumor effects [10, 238].

The usage of phages as a therapy has several benefits. Not only are they specific, thereby not affecting the commensals [245]. The commensal flora is not even affected if the therapy is directed to that particular species, potentially due to different susceptibilities [100]. Furthermore, phages can replicate at the site where they are needed the most [699] and are not hindered by biofilms [139]. It has also been reported that phages have effect on intracellular pathogens, by hijacking a bacterium outside the cell. Once the phages are inside the cell, they can kill all resting bacteria [92]. However, what makes phages advantageous is also their weakness as a therapeutic agent. Their high specificity gives a need to identify the infectious agent before treatment [284]. Furthermore, there is a fear that the phages might be able to transport genetic material [326] and even integrate in human cells [614, 615]. The fast destruction of bacteria will also generate a huge amount of free endotoxins from the cell walls that might influence the pathogenesis in an unwanted direction [504].

Even though several issues still remain to be solved considering phage therapy, they are used in several countries [717, 718], and are proposed to be used mainly as veterinary applications to reduce the *Salmonella* and *Campylobacter* burden on chickens [138, 233, 290, 408], against *E. coli* in cattle, in fish farms [500, 538] and to protect fresh fruit from bacteria [393, 394]. However, several more applications are being developed based on the phages, for usage in both medicine and biotechnology.

Except being capable of killing bacterial pathogens, the phages can also be used as specific delivery transportation molecules [133], and deliver DNA or antimicrobial substances [234, 591, 727]. Furthermore, many phages have the ability to degrade biofilms [48] that currently is a huge medical problem. On a more biotechnological level, both luciferase genes [689] and GFP (green fluorescent protein) [215] have been coupled to phages both in order to study phage infection cycles, but also to be able to identify contaminating bacteria [413].

5.6.2 Phage therapy using phage endolysins

Due to the potential drawbacks using whole phages, researchers have started to investigate the potential of using purified phage lytic enzymes. Those enzymes, commonly called lysins, are more correctly called endolysins due to their effect from the inside (Figure 6) [321], but have also been called virolysin [537] even though this word seldom is used. The first lysin ever identified came from *Streptococcus* phage C1, almost 40 years ago, and was identified by

Fischetti [206]. Many years later, the potent effect of this lysin was demonstrated when Loeffler in 2003 lysed more than 10^7 group A Streptococci in less than 5 seconds using no more than 10 ng lysin [409]. The effects seen using those enzymes lead scientists to think of phage lysins as "enzybiotics" [507]. However, so far the only available phage lysin, plyG, is active against *B. anthracis* and is used by the US Center for Disease Control and Prevention in Atlanta [76].

Just as the phages are specific, so are the lysins, even though the specificity is to species rather than to single isolates [205], and even though exceptions exist that have activity to several species [751]. Lysins are quite potent against Gram-positive bacteria even from the outside [203]. Gram-negative bacteria should in theory be resistant to this treatment due to their cell wall structure. However, some data suggest that even Gram-negative bacteria might be affected by lysin treatment [176, 447, 527], and there is ongoing research to increase the efficiency versus Gram-negative bacteria by penetrating the lipid layer [314]. Furthermore, a group has successfully anchored lysins to the cell surface of *E. coli* using a fusion protein with OmpC in order to more easily distribute the lysin and have the ability to express more lysin at the site where it is needed [677]. Another example of increasing the efficiency of phage lysins was demonstrated by Lopez by creating chimeric lysins, with N- and C-terminal parts of the lysin from different phages [424, 425].

Another major concern regarding the usage of phage lysins is the potential development of resistance towards the proteins. Even though resistance to lysins seemed unlikely due to their conserved and essential binding sites [77, 205], this was nevertheless investigated. So far no lysin resistant strains have been identified [203], not even after repeated exposure to low levels of purified enzymes [411, 616]. However it should be mentioned that lysins have a decreased effect against bacteria in stationary phase due to an altered peptidoglycan layer [411, 555]. Furthermore, not even antibodies directed towards the lysins will inhibit their effect [409]. So far, the only bacterial "resistance" known to lysins is sporulation. However, even the spores are sensitive to lysins as soon as they start to germinate [616].

Lysins have been used in several animal models, without displaying any severe side effects, even though the treatment results in a rapid release of endotoxins [185]. This will lead to a release of proinflammatory cytokines, which is higher than compared to treatment with vancomycin [185]. However, some studies also indicate that the levels of proinflammatory cytokines are lower using lysins compared to regular antibiotics [737]. The combination of lysins and antibiotics has been a successful combination in the experimental eradication of *S. pneumoniae* [166]. Furthermore, lysins are able to eradicate antibiotic resistant bacterial strains [409, 410, 411] in biofilms [607], and have been shown to be able to cure several animal models from bacterial infections [126, 322, 507, 518, 616].

These data have made scientists to suggest that lysins could be used as prophylaxis [204] in order to decrease the number of potential pathogens before they can cause disease and thus prevent pathogenic conditions [202]. This therapy would furthermore decrease the risk of secondary viral infections [455].

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