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BACTERIAL PROPAGATION HOST CHANGES THE HOST RANGE OF A *STAPHYLOCOCCUS AUREUS* SPECIFIC BACTERIOPHAGE VB_SAUP_EBHT WITHOUT ALTERING THE PHAGE GENOME

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Abstract

As an answer for the emergence of antibiotic resistance within pathogenic bacteria, the research and use of bacteriophages in treatment of infections has become a promising solution. This treatment is called phage therapy and it employs the capacity of phage to infect and kill its host. Bacteriophages recognise their host bacteria by their tail fibre proteins that determine their host range. In order to provide successful phage therapy, it is important to understand the interactions between the phage and its host. Bacteria have multiple ways to create resistance mechanisms against phages, which is one of the challenges concerning phage therapy. A yet unpublished research revealed that changing the production host caused a change in the host range of vB_SauP_EBHT, a *Staphylococcus aureus* specific lytic phage, without altering the phage genome. Here, the phage produced in the original host #6662 is referred to as ϕEBHT and the host range mutant phage, produced in strain #6433 is referred to as mEBHT.

The aim of this work was to perform a comparative proteomics analysis for purified phage particles produced in the two different bacteria hosts. The samples were prepared for proteomics analysis with ion exchange chromatography, for which the run conditions were first optimized. The comparison of proteomics data between purified ϕEBHT and mEBHT phage particles was used to find proteins originating from the propagation hosts that are present in purified phage samples and have the potential to change the vB_SauP_EBHT host range. The host range difference was observed initially with two strains. Therefore, a collection of 77 *S. aureus* strains from human and pig origin, as well as 20 coagulase negative *Staphylococcus* strains were tested to discover how extensive the host range difference is between the phages.

In the proteomics analysis 17 proteins originating from the host, with different abundancies between the two purified phage particles, were identified. Except for two enzymes, the identified proteins were part of regular upkeeping metabolism of the host and originated from the used host strain. The two enzymes, which were not part of regular bacterial metabolism, belonged to the class of M42 metallopeptidases. BlastP tool revealed a 98 % identity between these two peptidases, found from both samples, and they seemed the most prominent to cause altered host range. Based on the host range screening results, the phages differed by broadness of the host ranges and the infection efficiencies. The screening results revealed that ϕEBHT infected 40 % of the tested strains when mEBHT was able to infect 28 % of the strains. In conclusion, the chosen host strain for phage propagation can lead to an altered host range of the phage. To determine whether the M42 class metallopeptidases are involved in changing host range of phage vB_SauP_EBHT, further studies would be required.

Keywords Bacteriophage, *Staphylococcus aureus*, MRSA, phage-host interactions, phage host range, proteomics

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Työn nimi Faagin tuottoon käytetty isäntäbakteeri muuttaa *Staphylococcus aureus* -spesifin bakteriofagin vB_SauP_EBHT: isäntäkirjon muuttamatta faagin genomia

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Tiivistelmä

Vastauksena patogeenisilla bakteereilla yleistyvään antibioottiresistenssiin, bakteriofagien tutkimuksesta ja käytöstä on tullut lupaava ratkaisu bakteeritulehdusten hoitoon. Faagiterapia, kuten tätä hoitomuotoa kutsutaan, hyödyntää faagien kykyä infektoida ja tappaa isäntäbakteerinsa bakteeritulehdusten hoidossa. Bakteriofagit tunnistavat isäntäbakteerinsa häntäkarvaproteiinien avulla, jotka samalla määrittävät sen isäntäkirjon. Jotta voidaan tarjota toimivaa faagiterapiaa, on tärkeä ymmärtää millaisia vuorovaikutuksia bakteriofagien ja niiden isäntäbakteereiden välillä vallitsee. Bakteereilla on monenlaisia tapoja kehittää vastustuskyky bakteriofageja vastaan, mikä on yksi suurimmista faagiterapiaan liittyvistä haasteista. Toistaiseksi julkaisemattomassa tutkimuksessa paljastui, että vaihtamalla *Staphylococcus aureusta* infektoivan vB_SauP_EBHT -faagin tuottokantaa, kyseisen faagin isäntäkirjo muuttuu, vaikka faagin genomi pysyy identtisenä. Tässä työssä alkuperäisessä isäntäkannassa #6662 tuotettuun faagiin viitataan nimellä ϕEBHT ja isäntäkirjomutantti-faagiin, joka tuotettiin kannassa #6433, viitataan nimellä mEBHT.

Tämän työn tarkoituksena oli verrata puhdistettujen ja eri isäntäkannoissa tuotettujen faagipartikkeleiden proteomiikka-analyysin tuloksia keskenään. Näytteet valmisteltiin proteomiikka-analyysiä varten puhdistamalla faagit ioninvaihtokromatografialla, jota varten optimoitiin puhdistusolosuhteet. ϕEBHT ja mEBHT proteomiikka-analyysin vertailutuloksia tarkastelemalla pyrittiin löytämään sellaisia proteiineja, jotka ovat peräisin faagin tuottoon käytetystä isäntäkannasta ja joissa on eroavaisuuksia näytteiden välillä. Hypoteesi oli, että näiden proteiinien vertailu paljastaisi sellaisia proteiineja, jotka mahdollisesti pystyisivät muuttamaan vB_SauP_EBHT -faagin isäntäkirjoa. Ero näiden kahden faagin isäntäkirjojen välillä huomattiin kahdella aluksi kahdella kannalla, minkä vuoksi yhteensä 77 humaani ja sika *S. aureus* -kantaa, sekä 20 koagulaasi negatiivista Staphylococcus kantaa testattiin, jotta voitiin selvittää kuinka laaja ero ϕEBHT ja mEBHT faagien isäntäkirjojen välillä todellisuudessa on.

Proteomiikka-analyysissä löytyi yhteensä 17 proteiinia, jotka olivat peräisin isäntäbakteerista ja joiden määrä faaginäytteiden välillä vaihteli. Lukuun ottamatta kahta entsyymmiä, nämmä löydetyt proteiinit olivat osa isäntäkannan tavallista ylläpitoaineenvaihduntaa. Kaksi entsyymiä, jotka eivät osallistu bakteerin ylläpitoaineenvaihduntaan, kuuluivat M42 metallopeptidaasien ryhmään. BlastP -työkalun avulla voitiin tunnistaa 98 % yhtäläisyys näiden peptidaasien välillä, minkä lisäksi kyseiset entsyymit vaikuttivat todennäköisimmiltä vaihtoehdoilta aiheuttamaan muutoksia faagin isäntäkirjoon. Isäntäkirjotestaus vahvisti, että ϕEBHT ja mEBHT faagien isäntäkirjojen ja infektiotehokkuuksien välillä on eroa. Isäntäkirjoanalyysistä saatiin tulokseksi, että ϕEBHT infektoi 40 % testatuista kannoista verrattuna mEBHT -faagiin, joka infektoi kannoista 28 %. Lopuksi voidaan todeta, että faagin tuottoon käytetty isäntäkanta voi aiheuttaa muutoksia isäntäkirjossa. Jotta voidaan varmistaa, onko muutoksen takana M42 ryhmän metallopeptidaasit, tarvitaan lisää tutkimuksia.

Avainsanat Bacteriofagi, *Staphylococcus aureus*, MRSA, faagi-isäntä vuorovaikutukset, faagin isäntäkirjo, proteomiikka

Preface

This work was completed as part of Bacteriophage Therapy project at the Medical Department of University of Helsinki, in order to understand an aspect of phage-host interactions that has not been witnessed earlier. I want to thank my thesis supervisor Silvan Scheller for supervising and guiding me through the project and making me start the writing process already early on. For great advising and making this master's thesis possible, I want to thank Saija Kiljunen who gave me the opportunity, support, and encouragement needed to complete this work. I am grateful for Katarzyna Leskinen, who taught me how to analyze proteomics data and taught how to write about the process. Mikael Skurnik I want to thank for giving the opportunity to work in the Phage Therapy group and for interest in this project. Furthermore, I want to thank the whole phage therapy team. Especially Sheetal Patpatia, who participated in the initial discovery of the host range difference, provided the annotated bacterial genomic data for this work and for all the support, she gave throughout my thesis work and those years we have worked together. Matti Ylänne, Annette Asplund, Anna Kolsi and Outi Lyytinen I want to thank for support, encouragement, and unforgettable moments in the lab. Finally, I want to thank my family and friends for support and encouragement. Especially Adriana Delgado, for support and for helping me with forming decent English sentences.

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Table of Contents

1 Introduction 1
2 Literature review 2
2.1 *Staphylococcus aureus* 2
2.1.1 *S. aureus* genomics 4
2.1.2 Antibiotic resistance in *S. aureus* 5
2.1.3 *S. aureus* impact on health and agriculture 6
2.2 Bacteriophages 9
2.2.1 Bacteriophage structure 10
2.3 Bacteriophage classification system 11
2.3.1 Bacteriophage life cycles 12
2.4 Phage – host interactions 18
2.4.1 Host receptor proteins 18
2.4.2 Co – evolution 20
2.4.3 Prophages 20
2.4.4 Satellite phages 21
2.4.5 Phage infection efficiency 22
2.4.6 Phage resistance 24
2.5 Phage applications 25
2.5.1 Phage therapy 25
2.5.2 Vaccine development 27
2.5.3 Biocontrol 28
3 Aim of the study 29
3.1 General aim 29
3.2 Hypothesis 30
4 Materials and methods 31
4.1 Bacteriophages used in the study 31
4.2 Bacterial strains used in the study 31
4.3 Phage production 32

4.4 Phage titration 33
4.5 Ultrafiltration 34
4.6 Ion exchange chromatography 35
4.7 Proteomics 36
5 Results 37
5.1 Phage production for purification and host range screening 37
5.2 The effect of production host on vB_SauP_EBHT host range 38
5.3 Optimization of ion exchange chromatography conditions for phage purification 39
5.4 Ion exchange purification for proteomic study 41
5.5 Proteins identified from purified phage samples 43
6 Discussion 47
6.1 Future research 49
6.2 The suitability of the experimental set up of this work 50
6.3 Importance of the results for phage therapy 50
7 Conclusion 51
8 References 52

APPENDICES

APPENDIX 1: *Staphylococcus* –strains, host range screening and efficiency of plating results

APPENDIX 2: Examples of host range screening results with ϕEBHT and mEBHT, with different infectivity results and plaque morphologies.

APPENDIX 3: A list of proteins identified from purified phage samples

List of abbreviations

AMR	Antimicrobial resistance
BlastP	Basic Local Alignment Search Tool
CA-MRSA	Community associated methicillin resistant *S. aureus*
CC398	Clonal complex 398
CRISPR/Cas	Clustered regularly interspaced palindromic repeats
CV	Column Volume
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
ECDC	The European Centre for Disease Prevention and Control
EOP	Efficiency of plating
GT	Generalized transduction
HA-MRSA	Healthcare associated methicillin resistant *S. aureus*
HGT	Horizontal Gene Transfer
HPLC	High Purity Liquid Chromatography
ICTV	International Committee on Taxonomy of Viruses
IEC	Ion Exchange Chromatography
KEGG	Kyoto Encyclopedia of Genes and Genomes
LA-MRSA	Livestock-associated methicillin resistant *S. aureus*
LPS	Lipopolysaccharide
LC-MS/MS	Liquid Chromatography – Mass Spectrometer/Mass Spectrometer
MGEs	Mobile genetic elements

MRSA	Methicillin -resistant *Staphylococcus aureus*
ncbi	National Center for Biotechnology Information
OmpC	Outer membrane porin C
PHASTER	PHAge Search Tool Enhanced Release
PFU	Plaque forming units
PICIs	Phage inducible chromosomal islands
PLEs	PICI –like structures
R-M	Restriction modification
RT	Room temperature
SaPI	S*taphylococcal* Pathogenicity Island
SCC	*Staphylococcal* chromosomal cassette
ssDNA	single stranded DNA
ssRNA	single stranded RNA
TCA –cycle	Citric acid cycle
WTA	Wall teichoic acid
WHO	World Health Organization

1 Introduction

The spread and development of antibiotic resistance within bacteria is a major concern towards health and modern medical practices according to the World Health Organization (WHO). The emergence of antibiotic resistance mechanisms within human pathogens causes multiple problems, from more difficult treatment procedures to increased death rates and cost of treatment (Cassini et al., 2019, WHO, 2020). Attempts to solve this threat require development of novel antibiotics or alternatives for them. With this in mind, researchers follow up a 100-year-old treatment named phage therapy. This solution uses bacteriophages, viruses that infect and kill bacteria and are referred to as phages, in eradication of pathogenic bacteria. What makes phage therapy attractive as a solution is the possibility to target antibiotic resistant bacteria, since antibiotic resistance does not prevent bacteriophages from infecting their host. Some countries of the former Soviet Union region and Poland have phage therapy as a legal treatment method, in contrast to Western Europe and USA, where the discovery of antibiotics overdrew phage therapy research (Altamirano and Barr, 2019).

A key feature for a successful phage therapy is finding a phage that is able to infect the pathogen causing infection. The phage needs to recognize the bacterial cell as a host before it can infect the cell. Due to the rather narrow host range of bacteriophages, determined by their tail fiber proteins, phage therapy does not affect the normal microbiota, as antibiotics do. Regardless of the advantages provided by a narrow host range, this feature can make it also difficult to find a suitable phage for phage therapy treatment. Furthermore, it is crucial to understand how the phage host specificity is determined and how it might change during the treatment, when aiming to provide as effective phage therapy as possible. For this reason, it is important to understand those mechanisms of how bacteria use to protect themselves against phages and create phage resistance, which can further affect the

treatment outcome of phage therapy (Hyman, 2019, Pires et al., 2020, Torres-Barcelo, 2018).

A yet unpublished research done at the Phage Therapy group, revealed a surprising finding, where the host range of a bacteriophage vB_SauP_EBHT changed as a result of switching the propagation host from a clinical *Staphylococcus aureus* strain #6662 into a *S. aureus* pig isolate #6433. An even greater surprise was when sequencing revealed that the phage genomes were identical between the phage samples produced in these two different hosts. The vB_SauP_EBHT phage propagated in #6662, is called in this work ɸEBHT, and the vB_SauP_EBHT phage propagated in #6433, is called mEBHT. This change in the host range was noticed after changing the propagation host to strain #6433, which lead the phage to not infect the #6662-host strain anymore. To confirm the sequencing result that ɸEBHT and mEBHT have identical genome, the phages were re-isolated and sequenced again, which led to the same conclusion; mEBHT phage did not infect the original host strain #6662 but the sequence was identical to the one of ɸEBHT. There is not previous research on phages that describes a similar case, making this a completely novel finding.

The initial difference in the host range of a *S. aureus*-specific bacteriophage vB_SauP_EBHT was discovered at the Phage Therapy Unit, University of Helsinki. Eventually, the work done with the vB_SauP_EBHT –phage led to performing a set of experiments described in this thesis to discover the phenomena behind the host range difference between vB_SauP_EBHT –phage samples produced in two different host strains and with identical genome.

2 Literature review

2.1 *Staphylococcus aureus*

Staphylococcus aureus is a common inhabitant of the skin and nasal passages of humans and livestock. The current estimation is that 30 % of the human population

are carrying it in their nasal passages and on skin. *S. aureus* is a gram-positive bacterium with a cocci-shaped cell-structure and the individual cells arrange in forms, often described as grape-like clusters (Taylor and Unakall, 2020, Wertheim et al., 2005). Despite being a part of normal flora, *S. aureus* causes infections that are more difficult to treat in healthcare system and communities as well as infections and production loss in the livestock industry (Lozano et al., 2016, Wertheim et al., 2005). In farming, *S. aureus* can contaminate food products with its toxins, which is one of the most common sources of food-born poisonings (Dinges, 2000, Kadariya et al., 2014). Additionally, *S. aureus* is a zoonotic bacterium, meaning serotypes found in animals can transmit to humans and communities. (European Food Safety et al., 2017, van Rijen et al., 2008)

S. aureus is a nosocomial pathogen, commonly causing infections in hospital settings, for example for immunocompromised patients such as diabetics, burn wound patients and patients undergoing cancer treatment (Alrabiah et al., 2016). WHO has listed it as one of the ESKAPE bacteria, a group of bacteria that includes human pathogens, which by acquiring antibiotic resistance genes, have become an urgent threat for health which needs an immediate solution. The ESKAPE bacteria includes *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp* (Mulani et al., 2019). In humans, *S. aureus* causes a variety of different symptoms, ranging from mild to severe, including skin, soft tissue and blood stream infections. For example, the toxic shock staphylococcal superantigen is responsible for causing toxic shock syndrome, a condition that causes fever and shock symptoms. Endocarditis, osteomyelitis, pneumonia, surgical wounds, and intravascular device infections are additional examples of problems caused by *S. aureus* (Capparelli et al., 2010, Diekema et al., 2001).

2.1.1 *S. aureus* genomics

The family of *S. aureus* includes an assortment of different strains for which there are multiple classification systems. A common way for *S. aureus* classification is dividing strains into Staphylococcal clonal complexes (SCCs). This classification uses a distinct quality of *S. aureus*, namely its highly clonal core genome. The division of SCCs into lineages is based on their sequence types. Regardless of this stable core genome, *S. aureus* cells are able to acquire mobile genetic elements (MGEs) from the environment. The quantity of these MGEs, which can vary between 15-20 % of the full genome depending on the strain, reflects how adaptive the organism is. MGEs can bring a variety of advantages and qualities to the receiving cell, including virulence and fitness-increasing factors (Haaber et al., 2017). Alongside the SCC - classification system, the sequence similarity of the repeat region of *Staphylococcus* protein A gene functions as the basis for another classification system. This system, called *spa* typing, is a method characterizing a single locus of *S. aureus* genome, and therefore it is considered preferable over more laborious and expensive methods when addressing outbreak cases (Koreen et al., 2004). Furthermore, categorizing *S. aureus* strains based on their origin can sometimes best serve a purpose, such as when studying the transmission of *S. aureus* strains between animals and humans or the transmission of strains from hospitals to communities (Davis et al., 2018). *S. aureus* strains commonly infecting a certain host, such as humans, can be further examined based on the characteristics of these strains. For example, the strains causing infections in humans belong to 10 different SCCs (Goerke et al., 2009, Lindsay et al., 2012).

In contrast to the advantages provided by the great adaptability of the *S. aureus*-genome, certain situations might render it disadvantageous. To illustrate this, *S. aureus* utilizes horizontal gene transfer (HGT) to survive in a changing environment and to adapt new genes. HGT is favorable for exchanging genetic material, but while it may benefit the bacteria by enabling the adaptation of fitness-increasing factors, it also makes *S. aureus* vulnerable for lytic phage infections. This is the case when a *S.*

aureus cell has lost the Clustered regularly interspaced palindromic repeats defense system (CRISPR/Cas), which degrades foreign DNA. In this situation, the cell receives efficiently new genetic material and the possible fitness-increasing factors that come with it. However, as a trade-off, it cannot use CRISPR/Cas to protect itself if infected by a lytic bacteriophage (Li et al., 2015).

2.1.2 Antibiotic resistance in *S. aureus*

The most significant health threat comes from *S. aureus* strains that are resistant to antimicrobials, especially if the resistance mechanisms are effective against multiple different antibiotics. Traditionally, doctors treat infections caused by *S. aureus* with the group of β -lactam antibiotics, which inhibit the synthesis of peptidoglycan structures, e.g., penicillin and methicillin (Haaber et al., 2017, Pandey et al., 2013). However, already in 1947, the first penicillin resistant strains emerged, and by 1961 the first *S. aureus* strains that became resistant towards methicillin were identified. These came to be known as methicillin resistant *S. aureus* (MRSA). When antibiotic treatment does not heal *S. aureus* infections, the infections become more severe and both the cost and length of the treatment increase (Wertheim et al., 2005, Zschach et al., 2018).

After the emergence of MRSA -strains, the used antibiotics changed from methicillin to, for instance, vancomycin, daptomycin, and linezolid. Due to *S. aureus*'s effective capacity for acquiring antibiotic resistance genes, researchers were able to find strains with resistance genes towards linezolid shortly after the introduction of this antibiotic. Although development of bacterial resistance towards antibiotics is a natural phenomenon, the rate in which resistance mechanisms are acquired is amplified due to human action. The current data indicates that the emergence of antimicrobial resistant (AMR) strains is due to excessive and unregulated use of antibiotics in healthcare and agriculture. In the global north, this manifests in the prescription of antibiotics in uncertain diagnoses, and in the global south in self-treatment of infections with antibiotics. Similarly, within the food industry and

agriculture, the use of antibiotics as a preventative strategy contributes to the emergence of AMR -strains (Chaw et al., 2018, Chokshi et al., 2019). However, the absence of environmental pressure caused by antibiotics causes the obtained resistance mechanisms to be a fitness-decreasing quality for the bacterium. More specifically, the upkeeping of these AMR-genes takes resources from other qualities that would be more useful in an environment without the presence of antibiotics (Jensen et al., 2006, Scott et al., 2007, Zahid et al., 2008).

HGT is therefore responsible for the adaptation of bacteria to certain environmental conditions, for instance to the presence of antibiotics. Most commonly, the adaptation of genetic material happens through phage-mediated transduction, since bacteriophages are the most common gene-transferring agents. Conjugation is a gene-transfer mechanism, where the genetic material transfers between two bacterial cells via direct physical contact. Finally, transformation is an event where the bacterial cell absorbs the extracellular genetic material from the environment (Cafini et al., 2017, Emamalipour et al., 2020).

2.1.3 *S. aureus* impact on health and agriculture

Infective *S. aureus* –strains belong into three categories, based on the origin of the strain and on the circumstances in which they cause problems. Livestock-associated MRSA –strains (LA –MRSA) are strains mainly prevalent in pig, cow, and sheep industries. Health care associated MRSA –strains (HA –MRSA) are those strains most commonly found in healthcare settings. Lastly, community associated MRSA –strains (CA –MRSA) typically cause infections within human communities. However, this origin-based classification of the strain loses significance as a result of the decreasing differences between the three groups and of the spread of strains from one host to another. (Goerke et al., 2009, Faria et al., 2005). The first MRSA -strains identified were associated with healthcare settings due to the environment, which contains high dosages of antibiotics. The emergence of CA-MRSA started in the 1980s, and by the 1990 MRSA had spread to all continents. CA-MRSA evolved independently from

the LA-MRSA, which only became more common after 2005. Before 2005, LA-MRSA and its related problems were not known or recognized (Breitbart et al., 2004, Waldron and Lindsay, 2006).

Currently, hospital practices increase the spread of strains from hospital to community, through the movement of personnel, visitors and recovering patients in and out of hospitals. The recently popularized practice of home treatment also leads to the spread of hospital-acquired pathogens to communities. Tackling the problem caused by MRSA requires a 'One Health' approach where veterinarians, farmers, doctors, and environmentalists are included in finding a solution. The 'One Health' approach is a strategy that takes into consideration all the different aspects that influence the spread of AMR -strains and tries to include the stakeholders required to tackle the problem (Aminov, 2010, Bal et al., 2016, D'Accolti et al., 2019).

The effect of HA-MRSA in hospital settings.

An estimation from 2019 was that approximately 5 – 15 % of all hospital patients got infections by pathogens colonizing the hospital environment (Caini et al., 2012). *S. aureus* is one of the most common infection-causing bacteria in western hospitals, and its ability in adapting AMR-genes and other fitness-improving characteristics is a major concern. The bacterial presence in healthcare environments causes majority of healthcare-associated Staphylococcal infections (D'Accolti et al., 2019, Bal et al., 2016). The European Centre for Disease Prevention and Control (ECDC) reports that MRSA causes over 170 000 hospital infections and 380 million euros extra costs for hospitals per year (Köck et al., 2014). In USA, *S. aureus* is responsible for 60 % of bloodstream infections and MRSA causes approximately 50 % of surgical wound infections (Capparelli et al., 2010).

The emergence and distinctiveness of CA-MRSA.

The traditional idea of *S. aureus* as a nosocomial pathogen, which causes infections in health care settings and immunocompromised patients, changed during the 90's. At that time, definite genetic MRSA-lineages able to infect healthy adults were described in literature and they were found from every continent. Commonly, these

infections included skin and soft tissue infections. The lineages were classified as a distinct group called CA –MRSA. Today, these MRSA –strains also increasingly cause nosocomial infections, which were traditionally a result of the presence of highly antibiotic resistant strains in hospital settings. As an example, the spread of Panton-Valentin leucocidin genes among MRSA –strains is a consequence of the emergence of CA-MRSA. (Breitbart et al., 2004, Waldron and Lindsay, 2006)

Implications of LA-MRSA on food production, animal welfare and human health

Bacterial contaminations found from food raised attention to the presence of LA-MRSA in farms (Bal et al., 2016, Voss et al., 2005). A bacterial colonization can affect both the animals and the end products of the farm. For example, bovine mastitis, a mammalian gland infection caused by *S. aureus*, can result in clinical or subclinical conditions. In some countries, bovine mastitis is a nuisance for even 50 % of milk production units and results in clinical signs in animals, milk abnormalities, production loss and lower milk quality (Gruet et al., 2001, Leitner et al., 2003). The most common lineage of LA-MRSA is the Clonal complex 398 (CC398), which has especially colonized pigs. Typically, it has poorly colonized humans, although an increasing number of humans colonized by CC398 strains suggests that it is adapting to new hosts. In parts of Central Europe, even 40 % of MRSA strains that infect humans belong actually to the CC398 lineage (Berning et al., 2015, Feld et al., 2018, Kalupahana et al., 2019). Additionally, approximately 50 % of people working in the pig industry carry LA-MRSA strains. For instance, in Danish pig farms, LA-MRSA colonization has increased from 16 % to 88 % in animals. Similarly, nasopharyngeal swabs containing MRSA CC398 taken from 39 North German hospital patients had increased from 14 % to 29 % between 2008 and 2012 (European Food Safety et al., 2017, Feld et al., 2018). Finally, studies show that farms that do not use antibiotics have a smaller MRSA colonization when compared to farms that utilize antibiotics in their farming practices (Harper et al., 2010).

2.2 Bacteriophages

Viruses that use bacteria as host cells to produce a new generation of viruses are bacteriophages, phages in short. These biological entities are most abundant in the biosphere, and the estimation of their quantity is 10^{31} phage particles. (Bergh et al., 1989) A distinct characteristic of bacteriophages is their lack of metabolic machinery, making them completely dependent on host metabolism (Harada et al., 2018). Frederick Twort first discovered phages in 1915, but only two years later, in 1917, Félix d'Herelle confirmed their capacity to kill bacteria. Phages were studied for curing bacterial infections until the discovery of antibiotics, which overthrew phage research in Western Europe and North America. In the former Soviet Union region and Poland, phage research has continued since its discovery until today, and phages have been applied broadly to the treatment of infections (Altamirano and Barr, 2019, Pires et al., 2020).

Bacteriophages are an important part of the ecosystem, as they participate in the regulation of bacteria quantity and in the structure of bacterial populations, bacterial evolution, and host metabolism reprogramming, together with the transfer of genes between bacterial cells (Paez-Espino et al., 2016, Fazzino et al., 2020). The host range of bacteriophages is narrow, ranging from phages, which infect only some strains from a certain bacterial species, to phages, which can infect various strains of a host species alongside close relatives of that species. Regardless of the narrow host range of phages, studies show that they can have a significant effect on microbial communities through direct and indirect ways. More specifically, the direct effect of phages on a specific host comes through the lysis of the host cell, which leads to the reduction of bacteria quantity. Indirectly, the host cell lysis and freeing of cell content change the molecular composition of the environment, which can further affect the uptake of molecules and the metabolism of the bacteria population (Fazzino et al., 2020).

2.2.1 Bacteriophage structure

Based on the published 4500 phage genomes, 96 % of known bacteriophages belong to the class of tailed phages (Hendrix et al., 1999). These tailed bacteriophage particles consist of head capsid, genome, tail and tail fibers. Except from the phage genome, these parts consist of structural proteins and functional proteins. An example of a structural protein is the head capsid proteins, which protect the genetic material of the phage. The head capsid consists of head capsid proteins, structured most commonly into an icosahedral form. Small phages usually have their head capsid structured from a single type of head capsid protein, whereas bigger phages can have additional proteins with a variety of functions. The other proteins present in a phage particle are functional proteins. For instance, tail fiber proteins and neck proteins are examples of functional proteins, which help in the host recognition process and in the injection of the genetic material inside the host cell (Figure 1).

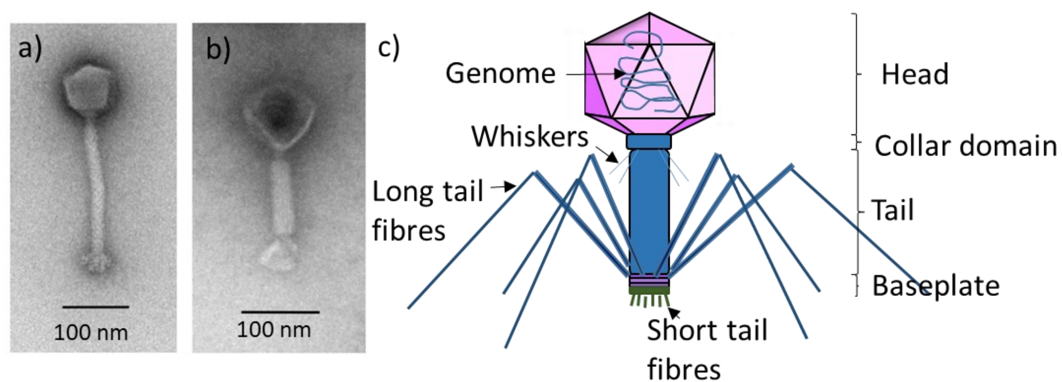

Figure 1. Structure of tailed bacteriophage. The pictures a and b present negatively stained electron micrographs of a *S. aureus* specific *Twortlikevirus* named vB_SauM-fRuSau02. a) Picture of vB_SauM-fRuSau02 particle with a non-contractile tail. b) Picture of vB_SauM-fRuSau02 with a contracted tail (Leskinen et al., 2017). c) A general structure of a Myovirus particle showing the different structures phage particle consists of.

The tail of tailed phages is a tunnel composed of proteins. It functions as a route for the phage genome to travel from the phage head capsid to the cytoplasmic space of

the host cell. Some tailed phages, for example *Myoviridaes*, contract their tail during the injection of phage genome inside the host cell. This structural change in the tail morphology is possible due to a cylindrical sheath protein that does not exist in phages with a non-contractile tail e.g., *Siphoviridaes* (Arnaud et al., 2017, Gonzalez-Garcia et al., 2015). The tail fiber proteins are attached through a base plate or a tail tip to the tail. The phage tail fibers are divided into short tail fiber proteins and long tail fiber proteins, both of which recognize molecular receptor structures on the host cell surface and induce attachment of the particle on the cell wall. Some phages, such as the *Salmonella enterica* specific phage P22, have a tail spike structure instead of tail fiber proteins. This structure differs from the tail fiber proteins by presenting both adhesion and receptor degrading activities. Tail spikes have endorhamnosidase activity, which cleaves glycosidic linkages in the *Salmonella* O-antigen (Andres et al., 2010, Steinbacher et al., 1997).

Finally, the phage genetic material is packed inside the head capsid and can be either double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), double stranded RNA (dsRNA) or single-stranded RNA (ssRNA). From these alternatives, dsDNA is the most common form of genetic material within the group of tailed phages. Correspondingly, practically all known *S. aureus*-specific phages are tailed phages and thus have genome composed of dsDNA. (ICTV, 2011)

2.3 Bacteriophage classification system

In the International Committee on Taxonomy of Viruses (ICTV, 2011), phages infecting *S. aureus* are under the order of *Caudovirales*. The *Caudovirales* group is traditionally determined based on similarities in morphology of the viral particles, how the DNA is packed inside the head capsid and on the method of assembly of virions. Recently, the classification of phages shifted from morphology-based methods to a multidimensional approach, which compares phylogenetic, single genome and proteome data. (Ackermann, 1998, Ackermann, 2003, Barylski et al., 2020, Rohwer and Edwards, 2002) The order of *Caudovirales* is further divided into 9

families, which are *Podoviridae, Siphoviridae, Myoviridae, Ackermannviridae, Autographiviridae, Chaseviridae, Demerecviridae, Drexlerviridae, Herelleviridae*. Due to the rapid evolvement of phage classification, many older publications have a simpler way to classify *Caudovirales* into *Sipho*-, *Podo*- and *Myoviridae*. The constant research in the field also provides new information on the topic, leading to possible future changes to the current classification system (ICTV, 2011). Furthermore, the *S. aureus* phages known today mostly belong to the group of *siphoviridae*, phages with a long non-contractile tail and a lysogenic lifecycle (Barylski et al., 2020, Deghorain and Van Melderen, 2012).

2.3.1 Bacteriophage life cycles

2.3.1.1 Lytic phages

The role of lytic bacteriophages in nature and the initiation of infection

Generally, phages are divided into three major groups based on their life cycle. These groups are lytic, lysogenic and pseudolysogenic lifecycles. All of them have a different role in nature and additionally differ in the events of the life cycle, although some steps are similar among these groups (Ackermann, 1998, Dennehy and Abedon, 2021). The group of phages that kill their host directly after infection are lytic phages. These phages have a great impact on the microbial quantity in biosphere and act as an environmental factor driving bacterial evolution forward. The lytic phage life cycle includes the following steps: attachment, penetration, biosynthesis, maturation, and release of the phage particle (Figure 2)(ICTV, 2011).

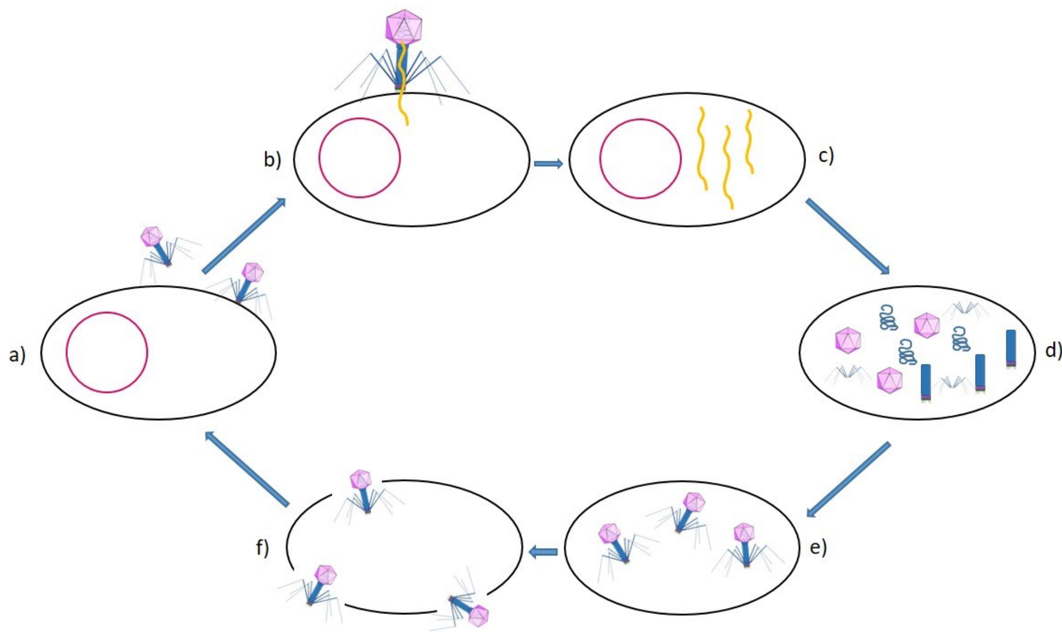

Figure 2. Lytic life cycle of a phage. a) Phage particles recognize receptor molecules from the host cell surface and attach there. b) The phage particle uses enzymes to make a hole into the host cell surface and injects its genetic material inside the host cell. c) The phage hijacks the bacterial protein synthase to replicate the phage DNA d) Phage genome is translated into structural proteins. e) Phage structural proteins are assembled, and the genetic material is packed inside the phage head capsid. f) The host cell wall is broken by the endolysins and a new generation is released. These phages are ready to start the cycle from beginning.

In the attachment phase, the long and short tail fibers of phage recognize molecular structures on the host cell surface. The tail fibers then bind the phage particle on the surface, enabling the continuation of the phage infection to the penetration step. In the penetration step, the phage punctures the host cell wall, resulting in a hole on the host cell surface. Through this hole, the phage injects its genetic material into the host's cytoplasmic space (ICTV, 2011).

Phage genome translation and the assembly of new phage particles.

Here, after the phage takes over the host metabolic machinery, this metabolic machinery transcribes and translates the phage dsDNA into proteins. (Gonzalez-Garcia et al., 2015, Marti et al., 2013, North and Davidson, 2021) The phage genes belongs into early, middle, and late expression genes and each group have a distinct function. The early genes encode for proteins needed in the beginning of the lytic cycle. These proteins usually participate in the transcription of phage genes and in

some instances in modification of host DNA. Take the case of the T4 phage, which uses small early proteins in disrupting the metabolic functions of the host and can destabilize host transcribed mRNA, which is further degraded by the own processes of the cell (Chevallereau et al., 2016, Howard-Varona et al., 2018). After the translation of phage genes into ready proteins, the proteins assemble into virions and the phage DNA is packed inside the head capsid by terminases. The late genes are generally responsible for directing the assembly and DNA packaging stages. Finally, holins and endolysins break the host cell wall, these are enzymes that degrade the peptidoglycan structure and are expressed during the maturation process (Feiss and Rao, 2012, Shao and Wang, 2008).

Phage additional genes that aid in the infection and lysis process.

Moreover, phages have certain genes that influence the broadness of the host range. Phages with a narrow host range express genes with qualities that are not present in broad-host-range phages, and vice versa. More specifically, generalist phages tend to express genes that allow them to employ host transcription and translation machinery across a broad range of various hosts. In contrast, specialist phages express genes that enable the phage to escape the restriction/modification mechanisms of the host (Howard-Varona et al., 2018). To accomplish host metabolism take-over and get the host machinery to encode phage proteins, some bacteriophages degrade host chromosomal DNA. This degraded DNA is then used in building phage genome while simultaneously degrading the template for encoding the host proteins which otherwise could be used in encoding anti-phage genes (McKitterick et al., 2019).

Some phages utilize genes originating from the host, which has metabolic functions to favor and increase the translation and transcription of the phage genome. These genes are auxiliary metabolic genes, and they include genes from most of the central carbon metabolism pathways. The function of these genes can participate in mediating for example phosphate and nitrogen metabolism as well as in synthetization of nucleic acids (De Smet et al., 2016).

2.3.1.2 Temperate phages

Unlike the lytic phages, temperate phages have two possibilities of life cycles, after infection of the host cell and injection of its genome. Temperate phages can either enter the lytic life cycle similarly to lytic phages, which result in directly killing the host cell. However, the phage genome can also be integrated as part of the host genetic material. The temperate phage genome, which is integrated in the DNA of the host or as an extra-chromosomal structure, is referred to as prophage. The phage DNA remains in the host cell and is passed on to the daughter cells during the host cell division. This process continues until e.g., a stress factor for the host cell induces the replication of the phage DNA, leading to the activation of lytic life cycle (Figure 3) (De Paepe et al., 2014, Feiner et al., 2015, Nanda et al., 2015).

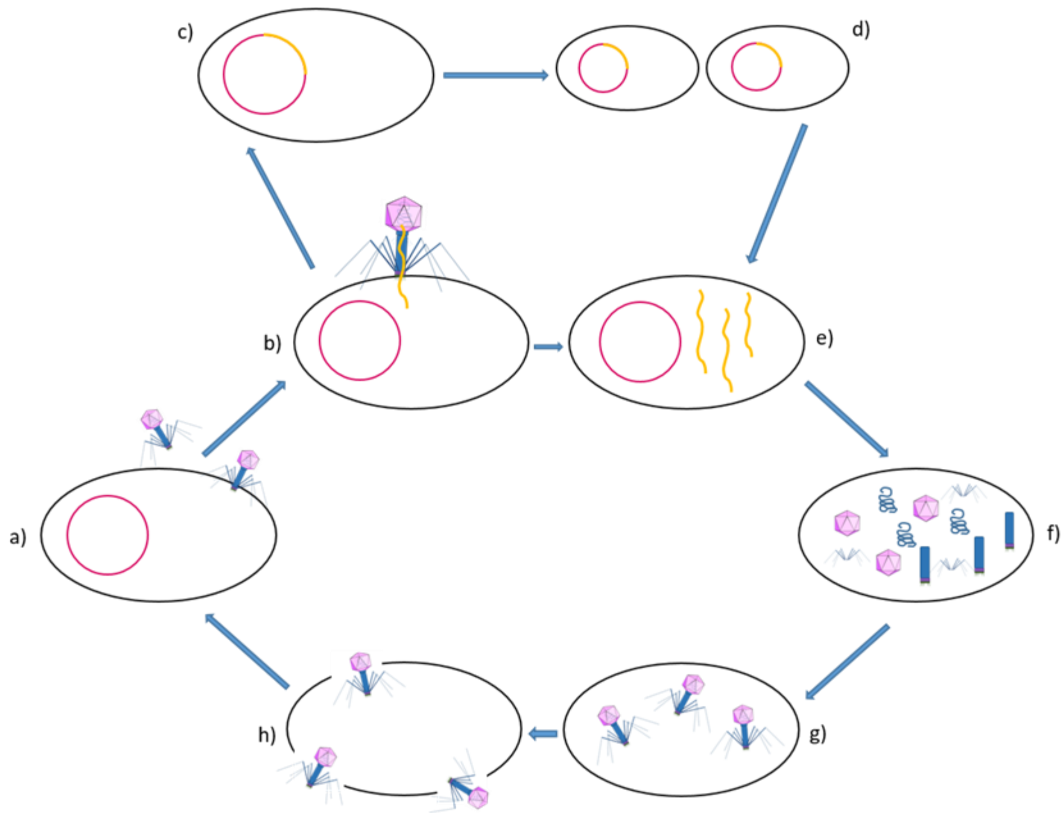

Figure 3. Lysogenic life cycle of a phage. a) Bacteriophage recognizes receptor molecules from host cell surface. b) The phage attaches on the cell wall, breaks a hole in it and injects its genetic material inside the host cell. c) Phage genetic material is inserted either in the host chromosome or as extra-chromosomal prophage. d) During cell division, the bacterial metabolic machinery replicates the prophage, which is then passed forward to the daughter cells. e) – h) Lytic life cycle steps where the phage DNA is replicated, and translated into proteins, which are further assembled into maturated phage particles and released through the lysis of host cell.

The lysogenic life cycle starts similarly to the lytic cycle, with the phage tail fibers recognizing the receptor molecules on the host cell surface, after which the phage injects its genetic material inside the host cell. Here, integrases assist in integrating phage genome to the host genetic material as part of the host genome or as a non-chromosomal structure. Integrases are enzymes that determine the integration site where the phage DNA is incorporated inside the host cell. Integrase are divided into seven major and six minor groups, and phage encoded virulence factors were found to correlate with these integrase groups. Those phages with a similar integrase were

associated with the spread of certain virulence factors (Goerke et al., 2009). The prophage remains in latent state by repressing the transcription of its lytic genes, and the lysogenic life cycle ends when the phage enters the lytic cycle. The activation of the lytic life cycle happens by inducing replication of the prophage genes into phage particles. Usually, the driving force to switch the life cycle is a condition that is stressful for the host cell. These stress factors include UV radiation, certain antibiotics e.g., fluoroquinolones as well as physiological factors e.g., change in pH level and temperature. Exposure to them can lead to DNA damage, which further results in excision of the prophage, mediated by excisionases (Egilmez et al., 2021, Feiner et al., 2015, Nanda et al., 2015).

Impact of lysogeny on bacterial populations and individual cells.

It has been confirmed in a study conducted by Davies et al 2016, that lysogeny plays an important role in preserving phage genomes in the environment and in bringing fitness to host cells when compared to strains with no prophages. Altogether, they observed that a certain prophage increases the probability for a mutation in a certain loci of bacterial genome, allowing favorable conditions for natural selection to occur. Mutations, which improve the host fitness and originate from temperate phages, have been connected to, for example, biofilm-forming *P. aeruginosa* strains causing lung infections (Davies et al., 2016). Temperate phages can also participate in improving the host pathogenicity by providing their hosts with toxin genes. These toxin genes are part of the phage genome, and as prophages the bacterial host can express and utilize these toxins (De Smet et al., 2016, Waldron and Lindsay, 2006).

As concluded earlier, bacteriophages are one of the main agents in gene transfer between *S. aureus* strains. These transduction particles are formed when some of the host DNA is packaged inside the phage head capsid by either generalized transduction or specialized transduction. Generalized transduction (GT) allows the packaging of bacterial DNA, while specialized transduction is more limited and thus allows only the packaging of specific sets of phage genes (Cafini et al., 2017, Emamalipour et al., 2020). Antibiotic resistance genes are not the only genes adopted from phages by bacteria. Virulence factors, such as Panton-Valentin leucocidin and

toxic shock syndrome toxin genes, can be acquired from temperate phages by *S. aureus* -cells (Meric et al., 2015, Sung et al., 2008). The genetic exchange between host and phage results in the host being able to adapt new qualities, although it is possible for phages to adapt new qualities as well. Through the analysis of prophages identified from 386 *S. aureus* strains, Goerke et al. (2009) classified *S. aureus*-specific phages into ten distinct groups, each group of phages present in different CC types. Thus, a connection was found between CC types and the presence of phages belonging to specific integration gene clusters (Goerke et al., 2009).

2.3.1.3 Pseudolysogenic phages

The phenomenon of pseudolysogeny was first described by Twort and later on explained more precisely by Romig and Brodetsky (1961), by using *Bacillus* and its phages, as a model organism. Pseudolysogenic life cycle is a state, where the phage nucleic acids are present in the cytoplasm of the host cell in a non-active form (Romig and Brodetsky, 1961). It differs from the earlier described phage life cycles by not actively interacting with the host cell. The DNA is not transcribed nor translated as in the lytic cycle, but neither is it integrated to the host genome. The role of pseudolysogeny is maintaining the phage genetic material in environment for extended periods of time (Ripp and Miller, 1997). Most commonly, pseudolysogeny have been connected to take place when the host cell is under nutrient deficient conditions (Ripp and Miller, 1998).

2.4 Phage – host interactions

2.4.1 Host receptor proteins

Receptors are molecular structures on the host cell surface that phages recognize and utilize during infection of the host cell. The primary functions of these molecules relates to the bacterial metabolism rather than for phage recognition. (Kortright et al., 2020, Le et al., 2014) The cell wall of gram -positive bacteria consists mostly of

peptidoglycan structure to which teichoic acid molecules are attached. Bacteriophages can recognize both of these structures as their receptors. The *Bacillus subtilis* specific phage SPP1 adheres to teichoic acid molecules, in contrast to the *Listeria monocytogenes* infecting phage A511, which adheres to the peptidoglycan structure itself (Godinho et al., 2018, Wendlinger et al., 1996).

S. aureus, being a gram –positive bacteria, is infected by phages that recognize these teichoic acid and peptidoglycan structures from its cell wall. It is possible that certain phages can recognize a variety of host cell wall molecules as a receptor instead of being dependent on a single molecular structure. The genus of *Twortlikeviruses*, a genus under the family of *Myoviridae*, is a class of phages with a broad host range and a large genome size and includes the *Staphylococcus* phage K and ϕSA012. These phages were identified with several receptor-binding proteins. The ability to recognize different molecular structures leads to a broader host range and is also present within the group of phages, which infect gram-negative strains (Takeuchi et al., 2016).

Phages infecting gram-negative bacteria often employ protein structures or lipopolysaccharides (LPS) as their receptor. It is also possible for bacterial flagella and pili to function as receptors, which is the case for example for *Campylobacter jejuni* phage CP220 (Letarov and Kulikov, 2017, Baptista et al., 2008). Some phages require for a stable attachment two different receptors, the primary receptor, and the secondary receptor. Only the attachment to the secondary receptor makes the attachment irreversible and the genome injection possible. This double receptor system was studied in the coliphage T4, where the protein gp37 on the long tail fiber binds the LPS or the outer membrane porin C (OmpC) in a reversible manner. The first binding further activates a second, irreversible, binding by the gp12 protein on the short tail fibers to the LPS structure (Kortright et al., 2020, Takeuchi et al., 2016, Riede, 1987).

2.4.2 Co – evolution

The development of all host-parasite interactions are based on a continuous arms race between the host and the parasite. The same phenomenon is present in the microbial world between bacteriophages and their host bacteria (Laanto et al., 2017). Throughout history, phages have evolved improved ways of taking over their host cells; meanwhile bacteria constantly keep developing mechanisms to protect themselves from phages (Boon et al., 2020, Lenski and Levin, 1985). A continuous improvement in defense and invasion capabilities has also a cost on the coevolution by constraining the extent where the evolution can proceed (Laanto et al., 2017). For phages, these limitations derive from not having the capacity to use deletions and insertions to mutate their genetic material. In opposition to bacteria that are structurally more complex and have broader range of mechanisms to mutate their genome. Additionally, the receptors on the bacteria cell surface are primarily used for other purposes than as phage receptors. Thus, blocking the synthesis of proteins, used as receptors by phages, can affect the metabolism of the bacteria (Lenski and Levin, 1985). In environment, however, these interactions discovered in laboratory settings might not apply, at least as an exact model. When the bacteria and the phage exist in a more complex environment, plenty of more factors contribute to these interactions (Laanto et al., 2017).

2.4.3 Prophages

Lysogenic phages have been studied to enter the lysogenic cycle, especially under conditions that do not provide sufficient growth for the infected host. Prophages can be a significant part of the bacterial genome. For instance, the *E. coli* O157 serotype strains, which are well-known human pathogens, can have 15 to 18 prophages in their genome. Human pathogens, with a high number of prophages, can utilize a broad variety of pathogenic properties. Moreover, the advantages prophages bring for their hosts goes beyond the increased pathogenicity. A bacterial population can benefit from a lysis of a single cell by a temperate phage. The released phage particles

can spread beneficial genes to other cells in the population, which further increases the chance for survival of the whole population (Bossi et al., 2003, Touchon et al., 2016). Additionally, the individual cell that carries the prophage benefits from it. Prophages protect their host from other infecting phages by blocking the recognition of the receptor proteins or by blocking the injection of phage genome. For example, phage sensitive strains, to which a *Staphylococcal* pathogenicity island (SaPI) region was transferred by phage infection, became resistant to this particular phage. The experiment implies that there is a link between prophages and phage resistance (Bondy-Denomy et al., 2016, McCarthy et al., 2012). Prophages play a crucial role in *S. aureus* infections. They foster several qualities that make the *S. aureus* infections difficult to treat, even though the strains seem antibiotic susceptible *in vitro*. Furthermore, prophages promote biofilm formation, increased hemolysin production and higher growth rate of the bacterial cell. In addition, they contain immune evasion cassettes, which host cells can utilize in causing an infection. All of previously listed qualities provided by prophages make these infections harder to treat (Li et al., 2020, McCarthy et al., 2012).

In conclusion, the beneficial qualities phages provide to their host gives an advantage for survival of both the host and therefor the prophage as well. While prophages can help their hosts by providing new qualities, they can also evolve and adopt new genes from their host or another phage infecting their host (Brüssow et al., 2004, Wertheim et al., 2005).

2.4.4 Satellite phages

In all classes of viruses occurs a phenomenon where a parasite virus captures and utilizes molecules from the host organism. This type of employment of structural molecules that originates from another organism is associated with outcomes including the ability to cause more severe illness, to provide virulence factors as well as enabling the host strain to further resist phage infections. Satellite phages, as these pirate viruses are referred to as, require help from another phage to be able to enter

the lytic life cycle (Barth et al., 2020, Christie and Calendar, 1990). An example of such phage is the coliphage named P4, which is classified as a *Peduoviridae*, a subclass under the *Myoviridae* family (Christie and Calendar, 1990, ICTV, 2011). The P4 phage is dependent on the P2 type helper phage, which enables the phage particles assembly, as well as plays an important role on the determination of the P4 phage host range. Additionally, the P2 type helper phage is responsible for packing the phage DNA inside the head capsid. The interaction between these two phages results in a particle that consists of P2 tail, 1/3 of the phage P2 head capsid and full P4 genome (Christie and Calendar, 1990).

Other similar viral parasites have been discovered from Firmicutes and are referred to as phage inducible chromosomal islands (PICIs). SaPIs are an example of PICIs and are known for their capacity to provide *Staphylococcal* strains with additional virulence factors. Furthermore, PICI –like elements (PLEs) found in *Vibrio cholerae* are their own distinguish class of satellite phages. Unlike for example the P4 phage, PLEs can completely block the production of its helper phage rather than partially restricts the helper phage production. Another quality that separates PLEs from PICIs and P4 is that its helper phage, ICP1 has its own replication machinery required for the initiation for phage DNA replication (Barth et al., 2020).

2.4.5 Phage infection efficiency

Phage infection efficiency describes how fast the phage infection takes place and how efficiently it kills the infected host cell. The capacity of a phage to infect a host cell does not mean that it is always an efficient infection. Some phages lyse their host in a short period of time, whereas others lyse their hosts more slowly. The growth phase of bacteria is one important factor affecting the phage infection efficiency. The efficiency of replication of the host genome is equivalent to the efficiency in which the phage genome replicates. Hence, the replication of the phage genome slows down during, for example, the latent phase of cell growth. The phage infection becomes also more efficient if it is easy for the phage to find a suitable receptor for

attachment. Thus, the quantity of phage receptor proteins on the host cell surface correlates with the infection efficiency. The more receptor proteins there are on the cell surface, the faster the phage infection can happen. Regarding the quantity of receptor proteins, bacteria can protect itself from phage infection through quorum-sensing driven reduction of receptor molecules on the cell surface (Hoyland-Kroghsbo et al., 2013, Qin et al., 2017). As an example, some *S. aureus* strains have developed a way of escaping lytic phage infection by altering wall teichoic acids (WTA) polymers to prevent phage infection. WTA polymers, which are required for phage infection and absorption, may be a key component in determination of *Podoviridae* host range among certain *S. aureus* strains. Since this is a quality observed in a very narrow range of *S. aureus* strains, as well as among other *Staphylococcal* species, it is thought of as a prevention tool that is adapted by bacteria in an environment with a high quantity of *Podoviridae* (Li et al., 2015).

Moreover, the structure of the host population can affect how well the phage is able to find a host cell and infect it. The denser the bacterial population is, the easier it is for the new phage generation to find hosts (Hoyland-Kroghsbo et al., 2013, Qin et al., 2017). Outside of the laboratory environment, the weak phage infections, mainly by broad-host-range phages, are not fully understood. Unlike narrow-host-range phages, these generalists might infect a wide array of hosts, but with a reduced infection efficiency (Howard-Varona et al., 2018).

Lastly, kinetics of phage infection affects how efficiently the host cells lyse and are divided commonly into three stages: First, the time it takes a phage particle to find a host and infect it. Secondly, what is the maturation rate of the particles and, thirdly, the time it takes for the new generation of phages to excite from host cell (Howard-Varona et al., 2017, Shao and Wang, 2008). The initiation of the infection depends on two parameters that are host cell density and the phage specific absorption rate. The maturation lasts until there is enough of material for assembling phage particles and until the assembly is complete. Simultaneously with the maturation, holin and endolysin genes are expressed, and the lysis time is used to describe the time it takes for a new phage generation to be released. The three aspects of phage infection

kinetics together determine the phage burst size, referring to the amount of new phage particles released during cell lysis (Shao and Wang, 2008).

2.4.6 Phage resistance

The mechanisms, which protects bacteria from phage infection, can be of two different types, active or passive. Passive phage resistance mechanisms include, for example, mutations in the phage-binding cell surface receptors. Here, the environmental pressure, caused by the presence of phages, results in favoring those bacterial cells that are not infected by phages, which shapes the bacterial community (Laanto et al., 2020, Wright et al., 2019). In active form of phage resistance, the host uses defense mechanisms to degrade the phage genetic material that has been inserted inside the cell. There are variety of different active mechanisms, which include e.g. clustered regularly interspaced short palindromic repeats paired with *cas* -genes (CRISPR/Cas) system and restriction modification (R-M). CRISPR/Cas is the major system with which most bacteria species defend themselves against phages and they have been identified from approximately 50 % of studied bacterial genomes (Zschach et al., 2018, Bondy-Denomy et al., 2013). In nature, R-M in bacteria have developed to protect bacterial cells from DNA viruses. The R-M mechanism has two aspects, the host DNA methyl transferase protects specific host DNA sequences through methylation process, whereas restriction endonuclease cleaves the foreign phage DNA (Goerke et al., 2009, Laanto et al., 2020).

The *S. aureus* has high percentage of its genome being accessory genes, which can be even quarter of the full genome length. These accessory genes are likely to contain mechanisms with which the bacteria fight against phage infections. *S. aureus* utilizes mainly R-M against phages (Goerke et al., 2009, Zschach et al., 2018). For survival from the lytic phage infections, when the R-M or CRISPR/Cas systems are not widely utilized, some *S. aureus* strains have developed more alternatives for its protection, mainly against *Podoviridae*. As an example, the modification of WTAs, by the host, can protect the cell from the lytic infection by disabling the infecting phage from

recognizing the WTA as a receptor. SaPIs are another mechanism for *S. aureus* to protect itself against infecting bacteriophages. They disturb the process of packaging phage DNA by being packed inside the phage capsid instead of the DNA of the infecting phage. Regardless of disturbing the packaging process, it is possible for a small part of the infecting phage to be produced. Meaning that the phage load decreases, instead of a complete destruction of the infecting phage. The summary of all this is, that the infected host should prevent the virus from hijacking the host metabolic machinery to efficiently resist the phage infection (Tormo et al., 2008, McKitterick et al., 2019).

2.5 Phage applications

2.5.1 Phage therapy

Phage therapy means the use of bacteriophages in treatment of bacterial infections, for example, through topical application of the phage, inhalation or by injections. The significance of phage therapy is, in its potential to treat AMR-infections. AMR infections are a constantly increasing problem according to World Health Organization and one of the biggest health threats towards humankind. Phage therapy is an approved treatment method in Russia and Georgia in comparison to Western Europe and North America, where it can be given for patients as compassionate treatment, under the conditions determined in the Helsinki Declaration (WMA, 2018). Here, it is stated that phage therapy can be given for a patient to whom the treating physician has confirmed that antibiotics or other form of treatment does not work and the patient agrees to receive the treatment (Altamirano and Barr, 2019). In order for phage therapy to be an official treatment in Western Europe and USA, a set of challenges needs to be overcome. These challenges include designing phage therapy to meet the legal frameworks of medicinal products and designing production conditions to match the standards of good manufacturing practices. Equally importantly, successful double-blinded clinical trials need to be

conducted for affirming safety, efficiency, and suitability of phage therapy for use (Altamirano and Barr, 2019, Luong et al., 2020).

Advantages of phage therapy

Advantages of phage therapy include the ability to target specifically antibiotic resistant pathogens and even more specifically, the strain causing an infection. Phages have a narrow host range, which is most commonly determined by their tail fiber -proteins or tail spike structures. Due to this narrow host range, phages have potential to be used for treating bacterial infections without destructing the healthy human microbiota. On the contrary, antibiotics, which target a broad range of pathogens, also kill the healthy microbiota during treatment (Altamirano and Barr, 2019, Luong et al., 2020). In a similar manner as antibiotic resistance, bacteria develop resistance mechanisms towards bacteriophages, which is seen especially in *in vitro* studies. When becoming resistant towards phages, bacteria often lose some of their virulence factors as a tradeoff. The bacteriophage resistance mechanisms affect, for example genes, which are responsible for pilus motility or LPS formation. The loss of virulence factors means that immune system can more efficiently kill and remove the bacteria cells (Gibson et al., 2019, Rohde et al., 2018).

The design and construction of phage therapy products used in treatment can improve the treatment outcome. Phage cocktails, composed of a few different phages, can prevent the drawbacks of phage resistance. On this occasion, even if the pathogen would develop a resistance towards one of the phages used in treatment, it is likely that other phages in the cocktail would keep infecting that pathogen and the phage therapy product would continue to be functional (Gibson et al., 2019, Roach et al., 2017, Rohde et al., 2018). For designing a suitable product, the phages used for therapy purposes should be chosen and characterized carefully. Lytic phages are preferred due to their life cycle, which results in killing their host immediately, and due to their ability to self-replicate at the infection site for as long as there is host bacteria present. Temperate bacteriophages, on the other hand, are not used in phage therapy, since they are more likely to transfer genetic material from bacteria to another, possessing a risk for further spreading AMR genes or other virulence

factors. From a practical point of view, bacteriophage product can be faster and cheaper to produce compared to the development of novel antibiotics, which brings an additional benefit for phage therapy (Hyman, 2019, Loc-Carrillo and Abedon, 2014, Nale and Clokie, 2021, Torres-Barcelo, 2018).

Developmental targets for phage therapy.

Regardless the advantages, phage therapy still has its disadvantages, even though some of them are possible to overcome with careful design of the phage product. One of the biggest issues is the narrow host range of phages and the need for a large phage collection to cover a great part of pathogenic bacteria. When a suitable phage is discovered quickly, phage therapy can be given for the patient within a relatively short time. However, if a lytic phage that can infect the target strain is not found, phage therapy is not a possible option. Therefore, in order to prevent such scenarios, it is beneficial to have vast phage collections and possibly a functional international network to overcome this problem (Caflisch et al., 2019, Gibson et al., 2019).

Application of the phage therapy product to the infection site brings additional challenges. Since phages are external particles introduced to the body, patients own immune system can recognize them as such, removing them from the body. Elimination of phages reduces the number of phages on the infection site and therefore can decrease the efficiency of the treatment. Here, also the site of the infection affects how well the phage is able to find the target bacteria. Phages were shown in experimental studies to be able to survive in human bodies. However, if the quantity of phages, which survive to the target site, is not high enough, the efficiency of phage therapy can be reduced (Dabrowska et al., 2005, Vinner et al., 2017).

2.5.2 Vaccine development

The importance of vaccination is the long-lasting protection they give against a variety of diseases, such as anthrax and polio. Designing a vaccine product, which is safe and gives a long-lasting effect, is causing majority of the problems related to vaccine development (Prisco and De Berardinis, 2012). Bacteriophage-based

vaccination products are a rather modern approach for development of vaccines, and they are one of the most promising preventative strategies in the fight against diseases. These phage-based vaccines fall under two categories, which are phage display vaccines and phage DNA vaccines (de Vries et al., 2020). Phage display vaccines are based on a technology where the phage capsid proteins are genetically engineered to include protein structures, which are not part of the original phage particle. Here, the phage particle is presenting an antibody on its capsid and when introduced to body, the phage particle becomes an antigen and eventually this creates a cell response against it. Phage display vaccine -design also has as an advantage the capacity to present multiple antibodies on its capsid, creating a multicomponent vaccination, which can create an immune response against multiple antibodies. This method was successfully used to create a multicomponent vaccination against anthrax by Shivachandra et al. 2006 (Gamkrelidze and Dąbrowska, 2014, Shivachandra et al., 2006). Phage DNA vaccine, on the other hand, is a method used for vaccine development where a eukaryotic expression cassette is inserted in phage genome. These phages are then injected to body, where they are eaten by immune cells. Human immune cells then express the genes in the particular expression cassette, which finally leads to a cell response (Harada et al., 2018).

2.5.3 Biocontrol

Biocontrol is essential especially in the food industry, due to the huge losses bacterial contaminations causes economically, production wise and from the point of view of animal wellbeing. Avoiding these losses is crucial due to the growing population, which increases the work that needs to be done to fight hunger (Raymaekers et al., 2020). Phages and their proteins can be utilized in the detection and elimination of bacterial contaminated food products as well as in prevention of contaminations. The tail fiber proteins, which are stable and highly specific, detects of bacteria from food products. In this method, the tail fibers capture pathogen structures from the sample and PCR is then used to detect these structures. Vidas UP® already markets this

application. Its advantages include stability and sensitivity, which can be higher than those of antibodies and antibody-based methods can (Santos et al., 2018).

Endolysins, which phages uses to break the host cell after progeny, are explored due to their ability to kill bacteria and their potential for novel antibiotics. These could be applied to for example food products to eliminate bacteria from them. Since endolysins are enzymes, which break peptidoglycan structures through hydrolysis, they have been mainly studied to be used against gram-positive bacteria. For gram-negative bacteria, endolysins are not effective since the peptidoglycan is protected under LPS. However, the use of endolysins in eradication of bacteria from food products is considered to be rather safe option. That is because endolysins cannot facilitate the transfer of virulence factors or resistance genes between bacterial cells. Regardless of the benefits of endolysin in biocontrol, this form of biocontrol is not yet conducted on a bigger scale (Chang, 2020). Additionally, whole phage particles and phage cocktails have been applied in food products varying from meat to milk and different plants by introducing them into the target product. This is done to minimize the losses bacterial contaminations cause by eradicating pathogens from the product (Raymaekers et al., 2020).

3 Aim of the study

3.1 General aim

The aim of this work was to verify an initial discovery, where the host range of a *S. aureus* specific bacteriophage vB_SauP_EBHT changed after the production host, a clinical isolate #6662, was substituted with a pig isolate #6433. The peculiarity of this finding was that the vB_SauP_EBHT phage produced in #6662, called ϕEBHT, and the vB_SauP_EBHT phage produced in host #6433, called mEBHT, had identical genome, regardless of their difference in their host ranges. To understand this phenomenon, the host range differences were verified with a broad range of *Staphylococcus* strains, which were used for finding how extensive the host range differences actually were.

The phenomenon was further studied through a proteomics analysis, for which the ϕEBHT and mEBHT phage particles were purified with ion exchange chromatography. The aim of the proteomics analysis was to find if there is host-originating proteins present in purified phage particles, which could alter the phage host range without affecting the phage genome. In a successful phage therapy treatment, the chosen phages infect the pathogen effectively, if the phage used for eradication of bacteria suddenly changes its host specificity or infectivity, the treatment outcome turns out to be different from expected. By understanding the mechanisms behind these phenomena, it is possible to design and produce a phage therapy product in a way that minimizes the possibility for such undesirable outcomes and functions as desired.

3.2 Hypothesis

The hypothesis behind the host range difference between phage particles ϕEBHT and mEBHT was that a protein originating from the used propagation host would cause it. Mutations in the phage genome were not behind the host range difference, since sequencing conducted earlier, confirmed the genomes to be identical. More specifically, the hypothesis was that the host-originating protein or proteins attached the phage particle could come from a prophage residing in the genome of the propagation host or alternatively be a satellite phage that affects the host range of vB_SauP_EBHT. This work focused on optimizing purification conditions for ion exchange chromatography and verifying the host range difference with a set of *Staphylococcus* strains. In addition to comparing the proteomics data of purified ϕEBHT and mEBHT phage particles to each other as well as comparing proteins present in purified phage particles to the genomes of the used propagation hosts.

4 Materials and methods

4.1 Bacteriophages used in the study

The phage studied in this work is a *Podoviridae* with a 17 471 base pair genome, officially named as vB_SauP_EBHT. This phage was isolated from a German wastewater sample, against *S. aureus*, as part of yet unpublished work (GenBank accession number: MT926124) (GenBank, 2020). Here, the name ϕEBHT was used to differentiate the original phage from the host range mutant mEBHT, although they both have an identical genome sequence and are thus the same phage (Figure 4).

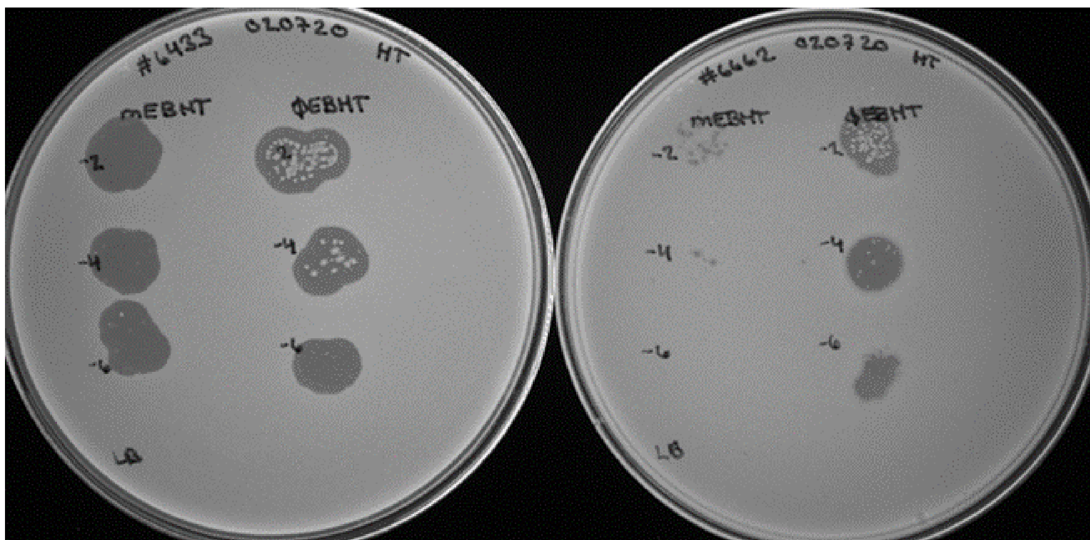

Figure 4. The host range difference between ϕEBHT and mEBHT. Infectivity of both phages was tested with spot assay (Sambrook et al., 2001) on both propagation host strains, *S. aureus* #6662 and #6433. Dilution series 10^{-2}, 10^{-4} and 10^{-6} was used to detect the phage infection efficiency and Luria-broth was used as negative control.

4.2 Bacterial strains used in the study

For the ϕEBHT –phage, *S. aureus*-strain isolated from a clinical sample by DSMZ, was used as host (Strain designation MHH 639507) (DSMZ). In this study, it was referred to as #6662, based on its storage number given at the time of receiving and storing it

at the University of Helsinki. The mEBHT phage was propagated in a *S. aureus* pig-isolate -strain 19A2, isolated by Annamari Heikinheimo (Heikinheimo et al., 2016). Here, it was referred to as #6433 based on the identification number given at the University of Helsinki. All strains were grown in LB –media at +37 °C. The strains and results are listed in Appendix 1.

In total, 109 *Staphylococcus* strains were tested for the host range analysis of ϕEBHT and mEBHT phages. At the beginning of the project, it was known that both phages infect the *S. aureus* –strain #6433, but only the original ϕEBHT –phage is able to infect the strain #6662 with similar efficiency. For host range screening, a variety of different *S. aureus* strains, including 56 clinical *S. aureus* isolates from HUSLAB, were chosen (Leskinen et al., 2017). The other 21 *S. aureus* –strains were isolated from healthy carrier pigs by Annamari Heikinheimo (Heikinheimo et al., 2016).

To study if either of the phages were able to infect other *Staphylococcus* species, 30 clinical coagulase negative isolates, received from HUSLAB, were tested (Leskinen et al., 2017). Both human and pig *S. aureus* as well as coagulase negative isolates were chosen for the screening, to accomplish a comprehensive view of the host range.

4.3 Phage production

Both phages ϕEBHT and mEBHT were produced under similar conditions, except for their host strains, which were #6662 for ϕEBHT and #6433 for mEBHT. The phages were prepared as 10 ml liquid cultures, where total of 10^8 plaque forming units (PFU) of phage was mixed with 400 µl of overnight culture (in Luria –broth) of host strain and 9.6 ml Luria –broth. The samples were then incubated while shaking at +37 °C for 3.5 hours until the bacteria were lysed. After lysis, the lysate was treated with 667 µl of chloroform for 15 minutes while shaking at room temperature (RT). Lysates were centrifuged at 3500 rpm, for 15 min at RT (Heraeus Megafuge 1.0). Finally, the supernatant was filtered through 0.22 µm pore sized filter (Minisart® Syringe Filter, Polyethersulfone, Pore Size 0.22 µm, ⌀ 28 mm, sterile, Sartorius).

4.4 Phage titration

Phage concentration was determined with a double-layer titration method described by Sambrook et al (2001). In this method, the host strain and the phage is mixed with 0.4 % Luria soft-agar to obtain an even bacterial layer on a Petri dish, on which the phage infection can be observed as clear areas called plaques, where the phage has killed the host. Here, the host strains were grown in 1.3 ml of broth until it reached exponential growth phase, determined by measuring A_{600} nm absorbance value with DMS Cell Density meter (Laxco, Inc). Measured A_{600} values between 0.25 and 1.0 were indicating exponential growth phase and thus accepted for further use. A dilution series of the bacteriophage was prepared to obtain 25 – 200 single plaques per plate (1:10 dilutions in Luria –broth). Host strain and 50 µl of phage dilution were mixed with 3 ml of 0.4 % Luria soft agar, temperated to 55 °C, respectively. The amount of host strain mixed into soft agar was determined with equation:

$$\frac{45}{A_{600}} = \mu l\ bacteria \tag{1}$$

Where A_{600} = Absorbance value (nm) measured with DMS Cell Density Meter

µl = result, the volume of bacteria culture inoculated into 0.4 % Luria soft agar

Soft agar, including phage and host strain, was then poured on 1.5 % Luria –agar plate. As a negative control, 50 µl of Luria broth was added into the soft agar, instead of the phage dilution, and poured on the plate. The plates were let to solidify, after which they were transferred into an incubator for overnight growth at 37 °C. The phage concentration was calculated based on the number of plaques on the plate, the dilution factor and volume of phage dilution.

$$Phage\ titer\ (\frac{PFU}{ml}) = a \times b \times \frac{1000}{c} \tag{2}$$

Where a = number of plaques on plate

b = phage dilution factor

c = the volume of phage dilution

The host range screenings were completed with spot assay, also described by Sambrook et al (2001). In this method, host bacteria were grown and the A_{600} measurement as well as the amount of host/3 ml of Luria soft agar (0.4%) was done as in the double layer method, except no phage dilution was added. The soft agar with the host strain was poured on a 1.5 % Luria -agar plate and let solidify for approximately 30 min. Phage dilution series was prepared in LB -broth. After the plates were solid, 5 µl drops of each phage dilution and 5 µl of Luria –broth as a negative control were added on the soft-agar layer. The drops were left to dry at RT for approximately one hour, after which the plates were transferred into +37 °C, for overnight (Sambrook et al., 2001). The efficiency of plating (EOP) was calculated by comparing phage titers in test strains (Equation 2) to the titer with the original host, which value was set to be 1 (Equation 3).

$$EOP = \frac{A}{B} \tag{3}$$

Where: A = the phage titer on the test strain

B = the phage titer on the original host strain

4.5 Ultrafiltration

Ultrafiltration was used for preliminary purification and exchanging the phage into SM-buffer (100 mM NaCl, 10mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5). Ultrafiltration steps were completed with Vivaspin 6 or 20 ultrafiltration units with a 100 kD cut off filter membrane (Sartorius). The sample was first concentrated to 1/10 of starting volume, after which it was washed twice with one total volume (V) of SM-buffer. Phage sample in SM-buffer was then concentrated to approximately 1 to 1.3 ml final volume. Centrifugations were done at room temperature with 3000 rpm (Heraeus Megafuge 1.0).

4.6 Ion exchange chromatography

Phage purification was done with ion exchange chromatography (IEC), where an ion exchange column (CIMmultus QA, volume 1 ml) was attached to ÄKTA Purifier High Purity Liquid Chromatography system (HPLC). To verify the separation capacity of the column, a BioRad AE protein standard (~70 µl) was run through the column. The optimization of run conditions was completed only with ϕEBHT phage (sample V 1 ml, 10^{10} PFU/ml), because the mEBHT and ϕEBHT phages function the same way in anion exchange chromatography. For optimization, the phage sample was run through the column with linear salt gradient. Run conditions were adjusted to 1 ml/min and pressure limit to 1.8 MPa according to column instructions. The IEC run started by running 7 column volumes (CV) of Buffer A (20 mM Tris-Cl, pH 7.5) through the column. In second step, B –buffer (20 mM Tris-Cl, pH 7.5 – 1 M NaCl), concentration was linearly increased from 0 % B -buffer to 55 % B –buffer during 20 CV. After reaching 55 % B –buffer concentration, the concentration was kept stable for 2 CV. At the end of the program, B –buffer concentration was increased to 100 % to wash all bound material from the column.

In order to find out the B –buffer concentration where the phage was eluted, a drop test was conducted for several fractions where the UV 260 nm curve rose over the base line. In between the runs, the system was rinsed first with 5 to 10 CV of 100 % B-buffer, then with 10 CV of 1 M NaOH and again with 5 to 10 CV of 100 % B-buffer. After the wash with 100 % B -buffer and 1 M NaOH solution the column was balanced with 0 % B -buffer and the system was ready for a new run. The purification of the ϕEBHT and mEBHT phages for proteomics analysis with IEC was based on results obtained from the optimization process with linear gradient. The final run program is described in Table 1.

Table 1. Ion exchange chromatography program for phage purification. In the injection step, the sample is injected in the system and rinsed with 5 column volumes (CV) of 0 % B -buffer. In the step 1, 8 CV of 0 % B -buffer was run through the column to wash unbound proteins. During the step 2, the lightly bound proteins are washed with 15 % B-buffer. In the step 3, the phage was eluted at 25 % B –buffer and the length of this step was 10 CV.

Step	Length	Salt Concentration	Description
Inject	5 CV	0 % B	Injection of sample
Step 1	8 CV	0 % B -buffer	Wash unbound proteins
Step 2	8 CV	15 % B -buffer	Wash lightly bound proteins
Step 3	10 CV	25 % B -buffer	Elution of the phage

Peak fractions from the elution step (Table 1) were collected and the buffer containing salt, was changed into SM –buffer with Vivaspin 6 ultrafiltration units, with 100 kD cut off filter membrane at 3000 rpm, RT. After wash, the samples were concentrated into final volume of 300 µl, from which the phage titer was determined with double-layer method and the protein concentration with Qubit™ Fluorometer and Qubit™ Protein Assay Kit (Invitrogen by Thermo Fisher Scientific) according to manufacturer's instructions.

4.7 Proteomics

In order to identify proteins present in ϕEBHT and mEBHT samples, liquid chromatography-Mass spectrometry-Mass spectrometry (LC/MS-MS) analysis was conducted at Proteomics Unit, Institute of Biotechnology, University of Helsinki. The ϕEBHT and mEBHT phage samples, purified with IEC and ultrafiltration, were digested with trypsin and the proteomics data was obtained by method described in Leskinen et al. (2017). The ϕEBHT and mEBHT amino acid sequences were annotated by using as references the annotated sequence of vB_SauP_EBHT (GenBank accession number: MT926124) as well as the amino acid sequences of host strains #6662 and #6433. The host strain sequences were assembled with A5 pipeline and Sheetal Patpatia (unpublished results) annotated genes with Rapid Annotation using

Subsystem technology version 2.0 (Aziz et al., 2008). For more precise analysis of host-originated proteins, identified from purified ϕEBHT and mEBHT phage samples, a cut-off value in read coverage was set to 4.00. The proteins with a read coverage above the cut-off value were compared with Basic Local Alignment Tool (BlastP, 2020) against host amino acid sequences. Furthermore, from complete host sequences prophage-originating proteins were identified by using Phage Search Tool Enhanced Release (PHASTER) provided by Wishart lab, University of Alberta, 2015 (Arndt et al., 2016, Zhou et al., 2011).

5 Results

5.1 Phage production for purification and host range screening

The bacteriophages ϕEBHT and mEBHT were produced for IEC purification and proteomics analysis as well as for completing the host range screening. The produced ϕEBHT lysates had titers between $1*10^{10}$ PFU/ml and $6.4*10^{10}$ PFU/ml. These samples were concentrated and purified with ultracentrifugation into 1.3 ml in SM -buffer, where the phage titers were between $8*10^{10}$ PFU/ml and $2.8*10^{11}$ PFU/ml. The yields after pre-purification for ϕEBHT were between 9.6 % and 17.6 %. Production of the phage mEBHT resulted in lysates with titers between $4*10^{10}$ PFU/ml and $5.4*10^{10}$ PFU/ml. The mEBHT lysates were pooled and the phage titer after concentration and pre-purification with ultrafiltration unit was $1.0*10^{11}$ PFU/ml in approximately 1 ml of SM -buffer. The yield of mEBHT phage production and purification was 10.6 %. The produced and pre-purified phage samples were suitable for IEC purification, which was needed in order to get rid of all particles present in the samples and that originated from, e.g., LB -broth or lysed bacterial cells that might have remained in the samples. For host range screening, unpurified ϕEBHT and mEBHT with a titer of approximately 10^{10} PFU/ml were used.

5.2 The effect of production host on vB_SauP_EBHT host range

The aim of the host range screening for ϕEBHT and mEBHT phages was to confirm the initial observation that there is a difference between the phage host ranges and to study how broad the difference is within a broad range of *Staphylococcus* strains. From the 109 tested *Staphylococcus* –strains, the original ϕEBHT infected 44 (40.4 %) in contrast to mEBHT which infected 31 strains (28.4 %). Results are presented in Appendix 1. The biggest difference in the host ranges were in the clinical *S. aureus* –strains. Here, 53.6 % of the 56 strains were infected by ϕEBHT and 32.1 % of the strains with mEBHT. Additionally, mEBHT infected 10 of the clinical *S. aureus* strains with lower EOP than ϕEBHT. From pig-MRSA-isolates, 12 were infected with ϕEBHT and 11 with mEBHT. The host range of the phages differed only by one strain named, #6286, which was infected by ϕEBHT, but not by mEBHT. It can also be noted that in this strain ϕEBHT had a low EOP, 3.7 $*10^{-6}$, compared to the original host strain #6662. Neither phage was able to infect the coagulase negative *Staphylococcus* -strains.

The efficiency of plating (EOP) was determined to study how the efficiency of phage infection differs between the *Staphylococcus* strains screened in this work. The EOPs of the infected strains varied between 1.48 $*10^{-6}$ and 3.7. There were 10 strains in which ϕEBHT phage had a higher EOP than in #6662. mEBHT had a higher EOP in six strains than in #6433, suggesting that the phage infects them more efficiently than its host strain #6433. Additionally, 34 of the tested strains had lower EOPs than the strain #6662 infected with #6662 and 25 of the strains had lower EOP than strain #6433 infected with mEBHT. The morphology of the plaques was smaller in some hosts when compared to #6662 or #6433 (Appendix 1). The host range screening confirmed that there is a significant difference between host ranges of ϕEBHT and mEBHT and that this difference is not only present within the two strains #6662 and #6433 (Appendix 2). Thus, the ϕEBHT and mEBHT phages were purified with anion exchange chromatography in order to do a proteomics analysis for pure phage samples to find out if this change occurs due to a host-originating protein.

5.3 Optimization of ion exchange chromatography conditions for phage purification

The optimization of IEC -purification conditions, needed to be completed for each phage individually based on the pI -value of the phage head capsid protein. The pI -value of vB_SauP_EBHT was 5.5, which meant that using pH 7.5 buffer-solutions was suitable for anion exchange chromatography. To determine at which point of the IEC run ϕEBHT phage was eluted, the fractions A1-A3, A8, A13-B13, B5 and waste flow (Figure 5) were titrated with drop test. The drop test showed that there was phage present in fractions A13-B13, B5 and waste flow, indicating that the phage was eluted at approximately 20 % B-buffer concentration. The chromatogram of the optimization run, with titrated fractions marked, is presented in Figure 5.

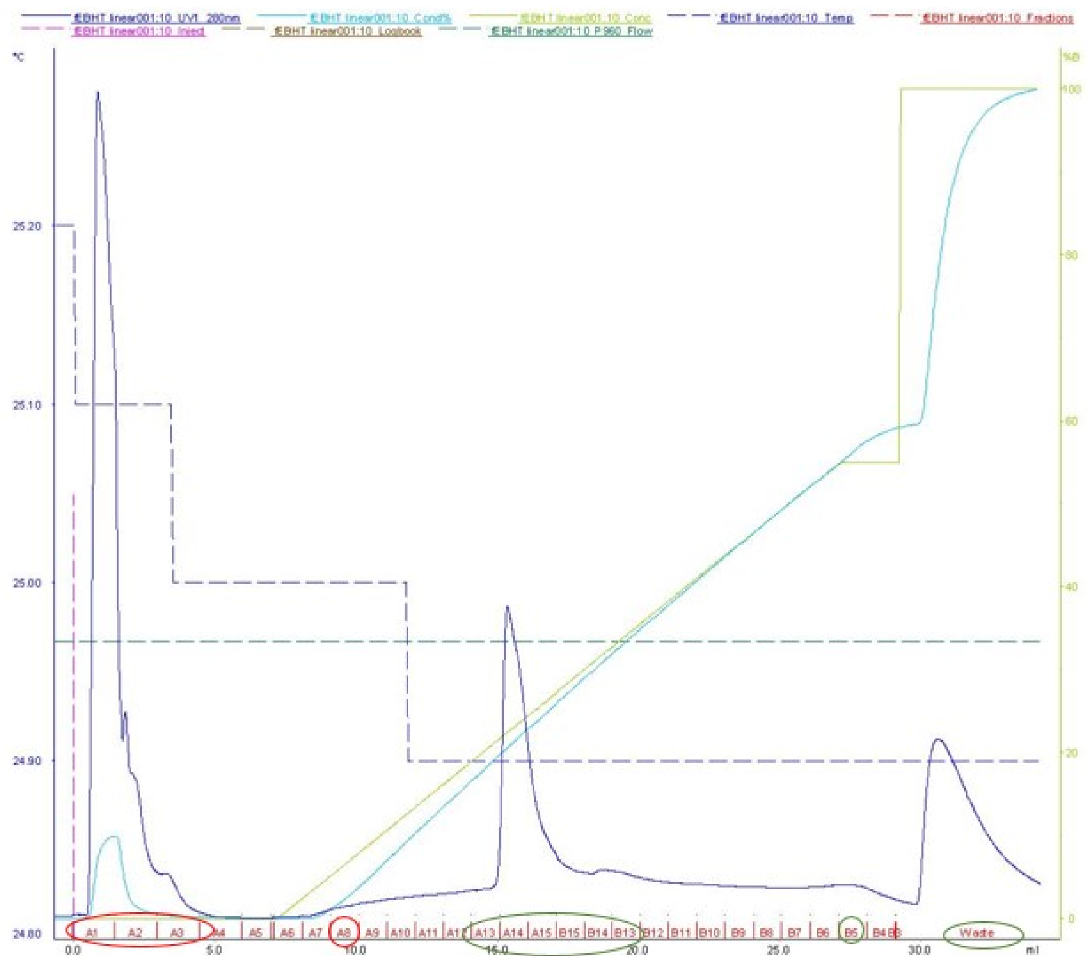

Figure 5. Optimization of ϕEBHT phage with linear salt gradient IEC program. On the left y -axis, is UV1 curve measured at 280 nm and on the right y -axis the B -buffer concentration is presented as percentages. X -axis shows the fractions collected during the run. B-buffer concentration was increased from 0 % to 55 %. In the chromatogram, the UV1 curve at 280 nm is shown as the dark blue line and the B-buffer concentration (%) with green line. The turquoise line represents the conductivity. Tested fractions where no phage was eluted, marked with red and tested fractions with eluted phage marked with green.

In order to calculate the yield of purified phage product, the fractions A13, A14 and A15 were combined from the optimization run (Figure 5) and the buffer was changed back to SM –buffer by ultrafiltration, resulting in $9*10^9$ PFU/ml of phage (sample volume of 1 ml). The yield of this ultrafiltration – IEC – ultrafiltration process with the ϕEBHT phage was 90 % of the $1*10^{10}$ PFU/ml (sample volume 1 ml) starting amount. The linear optimization run resulted in a single peak that indicated phage elution and the result were suitable for designing the run conditions for vB_SauP_EBHT purification.

5.4 Ion exchange purification for proteomic study

Ion exchange chromatography was used as a preparative purification for proteomics analysis. A three-step gradient program for ϕEBHT and mEBHT purification was designed based on the optimization of IEC -conditions during a linear gradient run. The step gradient IEC-run showed a single peak at the elution step (Table 1) in all performed runs. Here, the B -buffer concentration used for phage elution was 25 % and an example of a preparative purification run is presented in Figure 6.

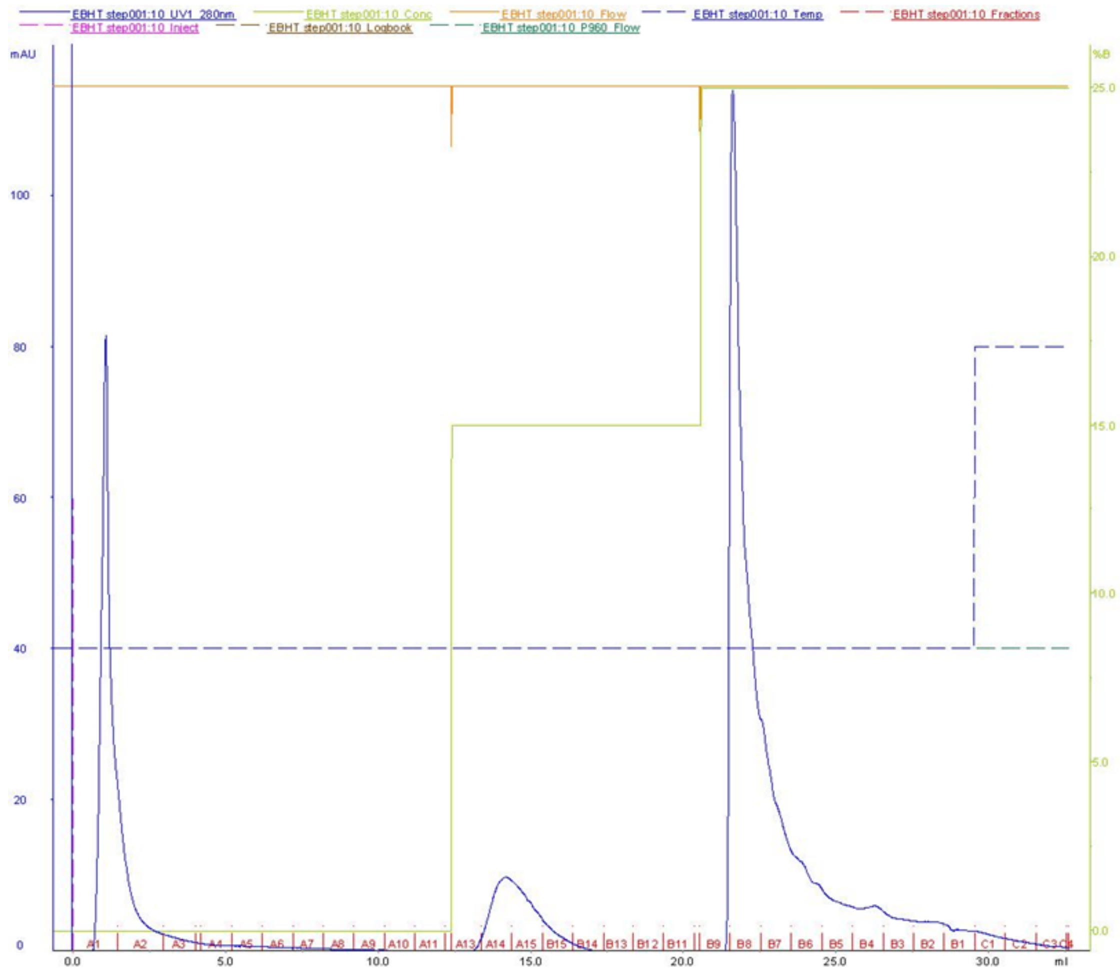

Figure 6. An example of a gradient chromatography run with phage mEBHT. The left y -axis presents the UV1 curve measured at 280 nm and on the right y -axis is the B -buffer concentration presented as percentages. X -axis show the fractions collected during the run. The peak in fractions A1 and A2 were an indication for proteins that did not bind to the column and were washed off during the first step of the run. The second step of the run with 15 % B -buffer washed off proteins, which were weakly bound to the column and are seen as the peak in fractions A14, A15 and B15. The peak at fractions B8, B7 and B6 was the eluted phage, and these fractions were collected for proteomics analysis. In the chromatogram, the dark blue line represented the UV1 value at 280 nm and the green line represented the B-buffer concentration (%).

After the IEC runs, the fractions containing the phage peak were pooled and the buffer was exchanged into SM -buffer. After the purification process, the ϕEBHT had a titer of $8.7 * 10^9$ PFU/ml in 300 µl and the mEBHT phage had a titer of $6.9 * 10^9$ PFU/ml in 300 µl. The protein concentrations measured with Qubit were 95.6 ng/µl for ϕEBHT and 94.4 ng/µl for mEBHT. The target protein concentration for

proteomics analysis was 20 µg of protein, thus after the preparative purification with IEC there was enough of both phages for proteomics analysis.

5.5 Proteins identified from purified phage samples

Proteomics analysis was completed in order to find host-originating proteins that were present, differed between the IEC -purified ϕEBHT and mEBHT phage particles and have the potential to change the host range of vB_SauP_EBHT. As a result of the proteomics analysis, 12 proteins originating from the vB_SauP_EBHT genome and 9 host bacterium-originated proteins were identified from ϕEBHT sample. For mEBHT phage particle, 10 proteins found were originated from vB_SauP_EBHT genome and 8 from the host strain #6433. A list of the proteins identified in the proteomics analysis and their read coverages are presented in Appendix 2. A cut off –coverage value of 4.00 was used for protein identification.

Host-originating proteins found from purified ϕEBHT phage particle

Host-originating proteins identified from purified ϕEBHT particle with a 100 % identity were aconitate hydratase (EC 4.2.1.3), deblocking aminopeptidase (EC 3.4.11.-), 6,7-dimethyl-8-ribityllumazine synthase (EC 2.5.1.78), M42 glutamyl aminopeptidase, and Inosine-5'-monophosphate dehydrogenase (EC 1.1.1.205) / CBS domain. Additionally, the proteomics analysis found host-originating proteins with a read-coverage value below the cut-off value, and these are listed in Appendix 2. Both deblocking aminopeptidase and M42 glutamyl peptidase enzymes belong to the class of metallopeptidases, which are able to cleave the N-terminal end of amino acid residues of peptides and protein. Aconitate hydratase belongs to the category of hydro-lyases and catalyzes the interconversion of citrate into iso-citrate in the citric acid cycle (TCA cycle). 6,7-dimethyl-8-ribityllumazine synthase is an enzyme which is involved in synthesizing riboflavin by catalyzing the reaction between 1-deoxy-*L*-glycero-tetrulose 4-phosphate and 5-amino-6-(*D*-ribitylamino)uracil. Inosine-5'-monophosphate dehydrogenase is an oxidoreductase involved in transferring H- ions

from inosine to NAD^+ and $NADP^+$, helping in the purine metabolism (Kanehisa and Goto, 2000).

Host-originating proteins found from purified mEBHT phage particle

Host-originating proteins identified from purified mEBHT particle included deblocking aminopeptidase (EC 3.4.11.-), Bacterial non-heme ferritin (EC 1.16.3.2), M42 glutamyl aminopeptidase, and glutamine synthetase type I (EC 6.3.1.2). The host-originating proteins that had a read-coverage value lower than the cut-off value are listed in Appendix 2. The purified mEBHT phage particle contained two host-originating enzymes, which were not found from the purified ϕEBHT particle: bacterial non-heme ferritin (EC 1.16.3.2) and glutamine synthetase type I (EC 6.3.1.2). Bacterial non-heme ferritin is an oxidoreductase. Glutamine synthase is involved in incorporating nitrate into *L*-glutamate forming *L*-glutamine, which is an essential part of nitrogen metabolism. Both of these enzymes are found widely from all kingdoms of life (Kanehisa and Goto, 2000).

BlastP aligment

The host-originating proteins present were cross-referenced against the host strain not used for phage propagation. More specifically, the purified ϕEBHT phage particle was aligned with BlastP –tool against the #6433 -host strain amino acid sequence and the host-originating proteins present in purified mEBHT phage particle were aligned with BlastP tool against the host strain #6662 amino acid sequence. The alignments were done in order to see if there were differences between the amino acid sequences of the proteins found in the phage particles and the proteins in bacterial genome (Table 2).

Table 2. List of host-originating proteins, which could affect the host range. In table a) proteins found from ϕEBHT –sample and BLAST results compared against host bacterium #6433 amino acid sequence. In table b) proteins found from mEBHT –sample and BLAST results compared against host bacterium #6662 amino acid sequence.

a)	**ϕEBHT**	**Sequence compared against #6433**					
	Function	Query cover	E-value	Identity	Positives	Gaps	Read-Coverage
	Aconitate hydratase (EC 4.2.1.3)	100 %	0,00	897/901(99%)	898/901(99%)	0/901(0%)	41,77
	Deblocking aminopeptidase (EC 3.4.11.-)	100 %	0,00	339/343(99%)	342/343(99%)	0/343(0%)	22,02
	6,7-dimethyl-8-ribityllumazine synthase (EC 2.5.1.78)	98 %	3,00E-107	150/152(99%)	152/152(100%)	0/152(0%)	29,34
	M42 glutamyl aminopeptidase	100 %	0,00	350/358(98%)	355/358(99%)	0/358(0%)	19,28
	Inosine-5'-monophosphate dehydrogenase (EC 1.1.1.205)	100 %	0,00	488/488(100%)	488/488(100%)	0/488(0%)	15,71
b)	**mEBHT**	**Sequence compared against #6662**					
	Function	Query cover	E-value	Identity	Positives	Gaps	Read-Coverage
	Deblocking aminopeptidase (EC 3.4.11.-)	100 %	0,00	339/343(99%)	342/343(99%)	0/343(0%)	4,29
	Bacterial non-heme ferritin (EC 1.16.3.2)	100 %	0,00	165/166(99%)	166/166(100%)	0/166(0%)	14,46
	M42 glutamyl aminopeptidase	100 %	0,00	350/358(98%)	355/358(99%)	0/358(0%)	19,69
	Glutamine synthetase type I (EC 6.3.1.2)	100 %	0 %	445/446(99%)	445/446(99%)	0/446(0%)	54,81

The host-originating proteins in ϕEBHT phage particle were all found from the #6433-host bacterium genome, but the proteins had none to eight amino acid difference in the sequences. The biggest difference between the host-originating protein in ϕEBHT phage particle and the #6433 -host amino acid sequence was in the M42 glutamyl aminopeptidase (Table 2). Aconitate hydratase (EC 4.2.1.3) and deblocking aminopeptidase (EC 3.4.11.-) differed by four amino acids while 6,7-dimethyl-8-ribityllumazine synthase (EC 2.5.1.78) differed with two amino acids. The BlastP comparison showed that only the Inosine-5'-monophosphate dehydrogenase present in ϕEBHT sample had 100 % identity with the #6433 strain amino acid sequence.

The host-originating proteins present in mEBHT phage particle were aligned against the #6662-host strain amino acid sequence. All of the proteins were present in the #6662 –host genome, and revealed one to eight amino acid differences between the compared sequences (Table 2). Deblocking aminopeptidase (EC 3.4.11.-) had a four amino acid difference, whereas bacterial non-heme ferritin (EC 1.16.3.2) and Glutamine synthetase type I (EC 6.3.1.2) differed between the sequences by one amino acid. M42 glutamyl aminopeptidase had an eight amino acid differences between the amino acid sequence of the protein in purified phage particle and in the bacterial genome.

PHASTER analysis

PHASTER database was searched to identify prophage-originating proteins from the host sequences. The prophage-originating proteins were then compared to the proteins identified in purified phage particles in order to find out if any of these proteins were of prophage-origin. The analysis revealed that only inosine-5'-monophosphate dehydrogenase, which was found exclusively from the purified ϕEBHT phage particle, originated from a prophage present in the genome of the #6662 host strain. The same prophage was not present in the #6433 host genome.

Proteins originating from the vB_SauP_EBHT -phage

The proteomics analysis conducted in this work covered phage-originating proteins alongside with host-originating proteins (Appendix 2). The proteins present in purified ϕEBHT and mEBHT phage particles and which originated from vB_SauP_EBHT –genome, were identified as structural phage proteins. All proteins originating from the vB_SauP_EBHT phage had similar level in the abundance of coverage between the two purified phage particles. However, two of the phage-originating proteins were found only from the ϕEBHT sample. The proteins were glycyl-glycine endopeptidase ALE-1 precursor and putative encapsidation protein. The difference was likely due to low read coverages, which were under the cut-off value. The low abundance of coverages in ϕEBHT sample were 2.13 for and 2.83, thus it is possible that they were under the detection level in the mEBHT sample and did not show in the analysis.

6 Discussion

Host range screening of 109 *Staphylococcus* strains confirmed the initial discovery that changing the propagation host of phage vB_SauP_EBHT, also changed the host range of the phage while its genome remained unaffected. The two phages, ϕEBHT propagated in strain #6662 and mEBHT propagated in strain #6433 were confirmed to have an identical genome but different host range. Confirmation that the host range difference was more extensive and not limited only to the #6662 and #6433 strains indicated that the chosen propagation host can have more impact on the host range of phage than what was thought before. Such phenomenon has not been known to exist and thus, has not been described in literature discussing phage biology or phage-host interactions before. The study of phage biology and phage-host relationship has led to the discovery of many molecular and metabolic principles of cell biology, for example, DNA as the genetic material and that mutations in DNA sequence occure randomly (Hershey and Chase, 1952, Luria and Delbrück, 1943). In

a similar manner, this novel finding of host range mutation of the vB_SauP_EBHT phage broadens the understanding of phage-host interactions.

To understand what other mechanisms than mutation in the phage genome, a prophage or a satellite phage could be responsible for changing the vB_SauP_EBHT host range, a proteomics analysis for purified ϕEBHT and mEBHT phage particles was conducted. The proteomics analysis revealed two potential host-originating peptidases with potential to cause the change in the host range. The peptidases were found from both host strains but had differences in their amino acid sequences and abundances between the strains. The two enzymes were deblocking aminopeptidase (EC 3.4.11.-) and M42 glutamyl peptidase. Both of these enzymes belong to the class of metallopeptidases, which are able to cleave the N-terminal end of amino acid residues of peptides and proteins. BlastP analysis against National Center for Biotechnology Information (ncbi) database confirmed them as M42 aminopeptidases, but further details of their enzymatic actions were not possible to obtain from Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000, ncbi). The most likely mechanism of action in which the peptidases could alter the host range is by modifying either the phage tail fibers or the host receptor protein.

This study concluded that host range mutation was not likely caused by a prophage or a satellite phage. Enzymes found to differ in their abundances and amino acid sequences between the original and host range mutant were not of prophage origin, except for the Inosine-5'-monophosphate dehydrogenase identified by PHASTER from sample ϕEBHT. The lack of prophage-originating proteins in both ϕEBHT and mEBHT particles also suggests that the host range was not changed due to a prophage. In addition, if the change in the host range would have occurred due to a satellite phage, several proteins from another phage genome would have been identified from the samples. The satellite phages that have been characterized, e.g., P4 and PLEs, utilize several proteins from their helper phages, ranging from replication initiators to structural proteins (Barth et al., 2020, Christie and Calendar, 1990). Since Inosine-5'-monophosphate dehydrogenase was the only protein found

from the purified ϕEBHT sample, and it was additionally found through BlastP alignment to be present in the #6433 genome. This finding suggests that it was not attached into the mEBHT phage particle during the host cell lysis. It is likely that this protein was an impurity found from the ϕEBHT sample that the purification process did not remove.

6.1 Future research

For clarification of the relationship between the phage production host and the host range, it would be important to study more in depth the potential proteins identified in this work, together with the reactions they catalyze as well as the parts of the phage particle and the host cell where they interact. Especially, it would be important to characterize the M42 metallopeptidases found in both phage samples, since their activity seems the most likely to cause host range deviation. For example, it is possible to use cryo-electron microscopy to place the host-originating proteins on the phage particle and see whether their position is, for instance, at the tail fibers. Confirming the locations of the proteins of interest would provide further knowledge on how they potentially affect the host range. Whether the host range differs due to metallopeptidases cannot completely be verified based on the data obtained through the proteomics analysis in this work. The M42 group of metallopeptidases has not been studied enough to understand the reactions catalyzed by this group of enzymes nor how the enzymes in this group differ from each other (Kanehisa and Goto, 2000, ncbi). To prove if metallopeptidases were responsible for changing the host range of vB_SauP_EBHT, the peptidases in host genome of #6662 could be replaced with peptidases in the host bacterium #6433, and vice versa. After changing the peptidases between strains #6662 and #6433, the vB_SauP_EBHT could be produced in both genetically engineered strains and their host ranges could be screened and compared to the results obtained in this work.

6.2 The suitability of the experimental set up of this work

The experimental set up was suitable for preparing the samples for proteomics analysis and for identifying proteins that might change the host range. In an article written by Hietala et al. (2019) a set of purification -methods were tested for their efficiency and suitability for phage purification. A purification process where the phage was first pre-purified with ultrafiltration, then with ion exchange chromatography and finally the IEC purified phage was changed to SM-buffer with ultrafiltration was concluded to be suitable for phage purification. The experimental set up for preparing ϕEBHT and mEBHT phage samples for proteomics analysis followed this process and was suitable for the purpose. For each individual phage, the purification conditions require individual optimization, as concluded in the article (Hietala et al., 2019). This was also the case for vB_SauP_EBHT, for which the optimization resulted in purification conditions that succeeded in separating phage particles from the LB -broth. Finally, preparative purification with IEC lead to two samples with purified phage particles for which the proteomics analysis was successfully conducted.

6.3 Importance of the results for phage therapy

Finally, alongside with understanding biological events, phage research that concentrates in development of phage therapy can help in solving the urgent threat that antibiotic resistant bacteria pose. The development of phage therapy to be a considerable and efficient solution requires thorough understanding of the interactions between bacteriophages and bacteria and this study was able to add new knowledge on these phage-host interactions (Rohde et al., 2018, Torres-Barcelo, 2018).

7 Conclusion

The present study investigated phage-host interactions, specifically whether the host range of the vB_SauP_EBHT phage changes due to proteins originating from propagation host. The research suggests, as it was thought in the initial hypothesis, that there was differences in the proteomics of vB_SauP_EBHT phage produced in host #6662 and #6433, but concluded that the host range did not change because of a satellite phage or a prophage. The study proposes that the most likely reason for the change in the host range are differences in metallopeptidases found from both purified phage particles, since these enzymes digest proteins and peptides. Specifically, metallopeptidases could digest tail fiber proteins of the phage or the host receptor proteins to the extent that the infection efficiency of the phage is reduced, or they recognize different receptors.

However, the experiments conducted in this thesis are not alone enough to confirm the hypothesis. More experiments are required to prove whether the hypothesis presented here is true. Altogether, it is likely that the variations in the proteome of the phage particle can affect the phage infection efficiency alongside with the phage genome and host defense systems. A discovery of a novel mechanism affecting host range of a bacteriophage, without altering the phage genome, broadens our understandings on the interactions between phages and their hosts.

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APPENDIX 1. *Staphylococcus* –strains, host range screening and efficiency of plating results.

Species	Strain code	Origin	Publication	ϕEBHT	Titer	EOP ϕEBHT [1]	mEBHT	Titer	EOP mEBHT[2]
S. aureus	#6662	Hannover Medical School	DSMZ	+	5,40E+09	1,00E+00	+	1.2E+10	1,50E+00
S. aureus	#6433	Pig	Heikinheimo et al. 2016	+	1,60E+06	2,96E-04	+	8,00E+09	1,00E+00
S. aureus	#6472	HUSLAB	Leskinen et al. 2017	+ (*)	1,14E+10	2,11E+00	+(*)	2,20E+07	2,75E-03
S. aureus	#6469	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#6466	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#6465	HUSLAB	Leskinen et al. 2017	+ (*)	4,20E+08	7,78E-02	+(*)	3,20E+08	4,00E-02
S. aureus	#6462	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#6457	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5849	HUSLAB	Leskinen et al. 2017	+	6,00E+07	1,11E-02	+	2,80E+07	3,50E-03
S. aureus	#5850	HUSLAB	Leskinen et al. 2017	+	4,00E+05	7,41E-05	+	3,40E+05	4,25E-05
S. aureus	#5851	HUSLAB	Leskinen et al. 2017	+	2,00E+09	3,70E-01	+	1,60E+05	2,00E-05
S. aureus	#5852	HUSLAB	Leskinen et al. 2017	+	2,00E+09	3,70E-01	+	3,00E+05	3,75E-05
S. aureus	#5853	HUSLAB	Leskinen et al. 2017	+	1,60E+07	2,96E-03	-		
S. aureus	#5854	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5855	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5856	HUSLAB	Leskinen et al. 2017	+	1,00E+04	1,85E-06	-		
S. aureus	#5857	HUSLAB	Leskinen et al. 2017	+	4,00E+04	7,41E-06	-		
S. aureus	#5858	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5859	HUSLAB	Leskinen et al. 2017	+ (*)	5,60E+07	1,04E-02	+	1,20E+08	1,50E-02
S. aureus	#5860	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5861	HUSLAB	Leskinen et al. 2017	+	6,00E+04	1,11E-05	-		
S. aureus	#5676	HUSLAB	Leskinen et al. 2017	+	1,60E+05	2,96E-05	-		
S. aureus	#5677	HUSLAB	Leskinen et al. 2017	+	8,00E+03	1,48E-06	-		
S. aureus	#5678	HUSLAB	Leskinen et al. 2017	+	1,08E+08	2,00E-02	-		
S. aureus	#5679	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5680	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5681	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5682	HUSLAB	Leskinen et al. 2017	+ (*)	9,40E+09	1,74E+00	-		
S. aureus	#5683	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5684	HUSLAB	Leskinen et al. 2017	+ (*)	6,20E+09	1,15E+00	+ (*)	1,40E+09	1,75E-01
S. aureus	#5685	HUSLAB	Leskinen et al. 2017	-			-		

[1] EOP = 1 for host strain #6662 infected with ϕEBHT

[2] EOP = 1 for host strain #6433 infected with mEBHT

(*) is used to mark those strains where the phage plaque morphology was smaller than in host strains #6662 and #6433

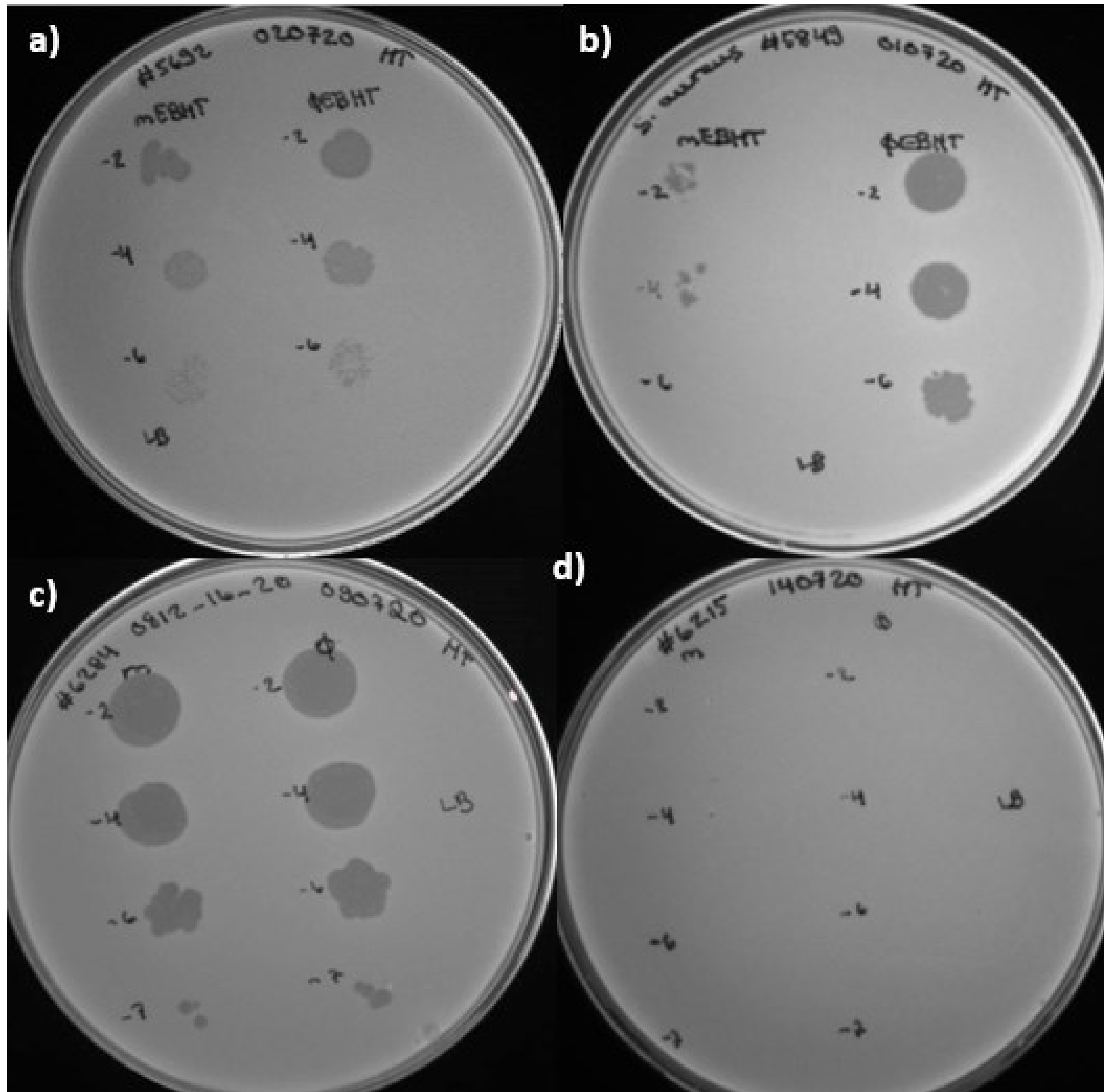
EOP < 1

EOP > 1

S. aureus	#5686	HUSLAB	Leskinen et al. 2017	+	1,00E+09	1,85E-01	+	4,40E+09	5,50E-01
S. aureus	#5687	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5688	HUSLAB	Leskinen et al. 2017	+	1,60E+08	2,96E-02	+	2,00E+05	2,50E-05
S. aureus	#5689	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5690	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5691	HUSLAB	Leskinen et al. 2017	+ (*)	1,56E+07	2,89E-03	+ (*)	1,14E+07	1,43E-03
S. aureus	#5692	HUSLAB	Leskinen et al. 2017	+ (*)	1,18E+10	2,19E+00	+ (*)	1,36E+10	1,70E+00
S. aureus	#5693	HUSLAB	Leskinen et al. 2017	+	1,80E+09	3,33E-01	+	1,60E+10	2,00E+00
S. aureus	#5694	HUSLAB	Leskinen et al. 2017	+	4,80E+08	8,89E-02	+	8,00E+08	1,00E-01
S. aureus	#5695	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5696	HUSLAB	Leskinen et al. 2017	+	1,82E+07	3,37E-03	-		
S. aureus	#5697	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5698	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5699	HUSLAB	Leskinen et al. 2017	+	8,20E+07	1,52E-02	+	2,80E+05	3,50E-05
S. aureus	#5700	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5701	HUSLAB	Leskinen et al. 2017	+ (*)	3,40E+06	6,30E-04	-		
S. aureus	#5702	HUSLAB	Leskinen et al. 2017	+ (*)	1,34E+07	2,48E-03	-		
S. aureus	#5703	HUSLAB	Leskinen et al. 2017	+	4,00E+08	7,41E-02	+	4,00E+05	5,00E-05
S. aureus	#5704	HUSLAB	Leskinen et al. 2017	+	2,00E+04	3,70E-06	-		
S. aureus	#5705	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5530	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5531	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5526	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5527	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5528	HUSLAB	Leskinen et al. 2017	-			-		
S. aureus	#5516	HUSLAB	Leskinen et al. 2017	+	1,24E+08	2,30E-02	+	3,80E+05	4,75E-05
S. aureus	#5511	HUSLAB	Leskinen et al. 2017	+	1,40E+09	2,59E-01	+	6,00E+06	7,50E-04
S. aureus	7065_6_10P	Pig	Heikinheimo et al. 2016	-			-		
S. aureus	7936_6_10	Pig	Heikinheimo et al. 2016	+ (*)	6,40E+05	1,19E-04	+ (*)	8,08E+05	1,01E-04
S. aureus	7936_16_20	Pig	Heikinheimo et al. 2016	-			-		
S. aureus	1333_6_10	Pig	Heikinheimo et al. 2016	-			-		
S. aureus	1057_11_15	Pig	Heikinheimo et al. 2016	-			-		
S. aureus	7502_1_5P	Pig	Heikinheimo et al. 2016	-			-		
S. aureus	6161_1_5	Pig	Heikinheimo et al. 2016	+	1,00E+10	1,85E+00	+	4,00E+07	5,00E-03
S. aureus	6161_6_10P	Pig	Heikinheimo et al. 2016	-			-		
S. aureus	3582_11_15	Pig	Heikinheimo et al. 2016	+ (*)	6,40E+05	1,19E-04	+ (*)	5,84E+05	7,30E-05
S. aureus	0812_1_5	Pig	Heikinheimo et al. 2016	+	2,00E+10	3,70E+00	+	2,00E+10	2,50E+00
S. aureus	0812_6_10	Pig	Heikinheimo et al. 2016	+	8,00E+09	1,48E+00	+	1,40E+10	1,75E+00
S. aureus	0812_11_15	Pig	Heikinheimo et al. 2016	+	8,00E+09	1,63E+00	+	8,60E+04	1,08E-05

S. aureus	0812_16_20	Pig	Heikinheimo et al. 2016	+	8,00E+09	1,48E+00	+	4,00E+09	5,00E-01
S. aureus	0250_1_5	Pig	Heikinheimo et al. 2016	-			-		
S. aureus	0250_6_10	Pig	Heikinheimo et al. 2016	+	2,00E+04	3,70E-06	-		
S. aureus	0250_11_15	Pig	Heikinheimo et al. 2016	-			-		
S. aureus	0250_16_20	Pig	Heikinheimo et al. 2016	-			-		
S. aureus	0186_11_15	Pig	Heikinheimo et al. 2016	+	5,40E+06	1,00E-03	+	4,20E+05	5,25E-05
S. aureus	6672_1_5	Pig	Heikinheimo et al. 2016	+	4,48E+05	8,30E-05	+	2,96E+05	3,70E-05
S. aureus	6672_6_10	Pig	Heikinheimo et al. 2016	+	9,04E+05	1,67E-04	+	4,40E+04	5,50E-06
S. aureus	6672_11_15	Pig	Heikinheimo et al. 2016	+	9,20E+05	1,70E-04	+	5,40E+04	6,75E-06
S. intermedius	#6209	HUSLAB	Leskinen et al. 2017	-			-		
S. intermedius	#6210	HUSLAB	Leskinen et al. 2017	-			-		
S. intermedius	#6211	HUSLAB	Leskinen et al. 2017	-			-		
S. intermedius	#6212	HUSLAB	Leskinen et al. 2017	-			-		
S. intermedius	#6213	HUSLAB	Leskinen et al. 2017	-			-		
S. lugdunensis	#6214	HUSLAB	Leskinen et al. 2017	-			-		
S. lugdunensis	#6215	HUSLAB	Leskinen et al. 2017	-			-		
S. lugdunensis	#6216	HUSLAB	Leskinen et al. 2017	-			-		
S. lugdunensis	#6217	HUSLAB	Leskinen et al. 2017	-			-		
S. lugdunensis	#6218	HUSLAB	Leskinen et al. 2017	-			-		
S. epidermidis	#6219	HUSLAB	Leskinen et al. 2017	-			-		
S. epidermidis	#6220	HUSLAB	Leskinen et al. 2017	-			-		
S. epidermidis	#6221	HUSLAB	Leskinen et al. 2017	-			-		
S. epidermidis	#6222	HUSLAB	Leskinen et al. 2017	-			-		
S. epidermidis	#6223	HUSLAB	Leskinen et al. 2017	-			-		
S. haemolyticus	#6224	HUSLAB	Leskinen et al. 2017	-			-		
S. haemolyticus	#6225	HUSLAB	Leskinen et al. 2017	-			-		
S. haemolyticus	#6226	HUSLAB	Leskinen et al. 2017	-			-		
S. haemolyticus	#6227	HUSLAB	Leskinen et al. 2017	-			-		
S. haemolyticus	#6228	HUSLAB	Leskinen et al. 2017	-			-		
S. saphrophyticus	#6229	HUSLAB	Leskinen et al. 2017	-			-		
S. saphrophyticus	#6230	HUSLAB	Leskinen et al. 2017	-			-		
S. saphrophyticus	#6231	HUSLAB	Leskinen et al. 2017	-			-		
S. saphrophyticus	#6232	HUSLAB	Leskinen et al. 2017	-			-		
S. saphrophyticus	#6233	HUSLAB	Leskinen et al. 2017	-			-		
S. pseudointer	#6234	HUSLAB	Leskinen et al. 2017	-			-		
S. pseudointer	#6235	HUSLAB	Leskinen et al. 2017	-			-		
S. pseudointer	#6236	HUSLAB	Leskinen et al. 2017	-			-		
S. pseudointer	#6237	HUSLAB	Leskinen et al. 2017	-			-		
S. pseudointer	#6238	HUSLAB	Leskinen et al. 2017	-			-		

APPENDIX 2. Examples of host range screening results with ϕEBHT and mEBHT, with different infectivity results and plaque morphologies.



a) Small plaque morphology of ϕEBHT and mEBHT phages in a Clinical *S. aureus* isolate #5692.

b) A clinical *S. aureus* isolate #5849 in which the EOP with phage mEBHT is lower than with phage ϕEBHT.

c) A positive result where *S. aureus* pig isolate 0812_16_20 is infected with both phages.

d) A negative result where clinical *Staphylococcus* isolate #6215 that is not infected with ϕEBHT or mEBHT.

APPENDIX 3. A list of proteins identified from purified phage samples and the read-coverage values.

a)	**Proteins originating from EBHT phage**	Read coverage	
	Protein name	**ϕEBHT**	**mEBHT**
	EBHT_rev_-_CHAP_domain_protein_CDS_translation	65,48	27,69
	EBHT_rev_-_tail_fibre_protein	103,54	157,30
	EBHT_rev_-_Glycyl-glycine_endopeptidase_ALE-1_precursor_CDS_translation	1,83	NA
	EBHT_rev_-_hypothetical_protein_CDS_translation_pI_3	104,37	52,17
	EBHT_rev_-_hypothetical_protein_CDS_translation_pI_4	48,23	43,97
	EBHT_rev_-_hypothetical_protein_CDS_translation_pI_6	97,75	108,82
	EBHT_rev_-_hypothetical_protein_CDS_translation_pI_9	1,79	2,73
	EBHT_rev_-_major_capsid_protein_CDS_translation	102,12	146,15
	EBHT_rev_-_putative_encapsidation_protein_CDS_translation	2,13	NA
	EBHT_rev_-_putative_lower_collar_protein_CDS_translation	2,09	1,62
	EBHT_rev_-_putative_upper_collar_protein_CDS_translation	101,98	81,98
	EBHT_rev_-_tail_protein_CDS_translation	22,56	22,79
b)	**Proteins originating from the host**	Read coverage	
	Protein name	**ϕEBHT**	**mEBHT**
	Aconitate hydratase (EC 4.2.1.3)	41,77	NA
	Elongation factor Tu (Bacteria)	NA	0,00
	alpha-ketoacid dehydrogenase subunit beta [Staphylococcus]	NA	0,00
	Chain O, Crystal Structure Of Holo Glyceraldehyde-3-Phosphate Dehydrogenase 1 (Gapdh1)	2,82	NA
	isoleucine--tRNA ligase [Staphylococcus aureus]	2,40	NA
	Deblocking aminopeptidase (EC 3.4.11.-)	22,02	NA
	Deblocking aminopeptidase (EC 3.4.11.-)	NA	4,29
	MULTISPECIES: ornithine carbamoyltransferase [Staphylococcus]	NA	0,00
	Hypothetical protein SA1_55657 [Staphylococcus aureus subsp. aureus PSP1996]	1,67	NA
	Bacterial non-heme ferritin (EC 1.16.3.2)	NA	14,46
	dihydroorotase [Staphylococcus aureus subsp. aureus JH9]	1,74	NA
	6,7-dimethyl-8-ribityllumazine synthase (EC 2.5.1.78)	29,34	NA
	M42 glutamyl aminopeptidase, cellulase	NA	19,69
	M42 glutamyl aminopeptidase, cellulase	19,28	NA
	hyperosmolarity resistance protein Ebh [Staphylococcus aureus]	NA	2,37
	Glutamine synthetase type I (EC 6.3.1.2)	NA	54,81
	Inosine-5'-monophosphate dehydrogenase (EC 1.1.1.205) / CBS domain	15,71	NA