Aus der Kinderklinik und Kinderpoliklinik im Dr. von Haunerschen Kinderspital
der Ludwig-Maximilians-Universität München
Direktor: Prof. Dr. med. Dr. sci. nat. Christoph Klein

Identification and evaluation of bacteriophage genes involved in the virulence of *Enterococcus faecalis*

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Eva Kristina Holzapfel

aus Bad Honnef

2023

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:	Prof. Dr. med. Johannes Hübner
Mitberichterstatter:	Prof. Dr. med. Christine Josenhans PD Dr. med. Sophia Horster PD Dr. med. Dimitrios Franggoulidis
Mitbetreuung durch den promovierten Mitarbeiter:	Dr. Luis Felipe Romero Saavedra
Dekan:	Prof. Dr. med. Thomas Gudermann
Tag der mündlichen Prüfung:	12.01.2023

Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel **„Identification and evaluation of bacteriophage genes involved in the virulence of *Enterococcus faecalis*“** selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 12.01.2023	Eva Kristina Holzapfel
_____	_____
Ort, Datum	Doktorandin Eva Kristina Holzapfel

Contents

Index of tables and figures

Tables

Figures

List of abbreviations

ALA	Alanine
AI-2	Autoinducer-2 (4,5-dihydroxy-2,3-pentanedione)
BHI	Brain heart infusion
BHIA	Brain heart infusion agar
BLAST	Basic Local Alignment Search Tool
bp	base pair
c	mutant for complementation
$CaCl_2$	calcium chloride
CFU	colony forming unit
DMEM	Dulbecco Modified Eagle Medium
DNA	Deoxyribonucleic acid
EB	Electroporation Buffer
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
E. faecalis	*Enterococcus faecalis*
e.g.	exempli gratia / for example
ELISA	Enzyme-linked immunosorbent assay
et al.	et alii / et aliae
FAO	Food and Agriculture Organization of the United Nations
FBS	Fetal Bovine Serum
FW	forward
g	gram
GLY	Glycine
GM17	M17 media + glucose 0.5 %
kb	kilobase
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
LB Agar	Luria/Miller Agar
LB Broth	Luria/Miller Broth

LEU	Leucine
LPS	lipopolysaccharide
LS	Lysozyme Solution
M	molar mass
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
min	minute
mL	mililiter
mM	millimolar
MMC	Mitomycin C
MRSA	methicillin-resistant *Staphylococcus aureus*
NaCl	sodium chloride
Na_2HPO_4	disodium hydrogen phosphate
NCBI	National Center for Biotechnology Information
ng	nanogram
nm	nanometer
OD	Optical density
OD 595 nm	Optical density at 595 nm
OD 600nm	Optical density at 600 nm
ON	Overnight
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
Pg	picogram
pp (1-7)	Prophage 1-7
ppX	Prophage 1/5/7
ppGpp	guanosine tetraphosphate
pp5ΔEF2143	phage 5 with deletion of the *ef2143* gene
pp5ΔEF2144	phage 5 with deletion of the *ef2144* gene
rDNA	ribosomal Deoxyribonucleic acid
rpm	runs per minute
RV	reverse

SER	Serine
STSB	Tryptic soy broth with 0.5 M sucrose
TBE	Tris Borate Ethylenediaminetetraacetic acid
THR	Threonine
TNF-α	Tumor necrosis factor α
TSA	Tryptic soy agar
TSA-S	Tryptic soy agar + 5 % sheep blood
TSB	Tryptic soy broth
TSBg	Tryptic soy broth + glucose 1 %
TYR	Tyrosine
UK	United Kingdom
UP-water	ultra-pure water
UV light	ultraviolet light
V	Volt
VAL	Valine
VRE	Vancomycin-resistant enterococci
WHO	World Health Organization
WT	wild type
xg	relative centrifugal acceleration
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µF	microfarad
µg	microgram
µL	microliter
µM	micromolar
Δ	deletion mutation
Ω	Ohm

I. Introduction

I.1. Enterococci

I.1.1 General characteristics about enterococci

Enterococci (from the Greek enteron: intestine, coccus: berry)[1] are gram-positive, catalase-negative and facultative anaerobic bacteria, which have a wide temperature growth range from 10 °C to 45 °C with maximum growth at 35 °C[2]. They belong to the lactic acid bacteria[3]. Enterococci grow in broth containing 40 % of bile salts and 6.5 % NaCl[4]. The ovoid or spherical bacteria are usually arranged in pairs or short chains, and their highest abundance is in the human gut[5,6]. Normally 10^5-10^7 enterococci per gram of feces are found in humans and animals, which is a very small percentage of all bacteria found in feces (10^{10}-10^{12})[7]. Besides the human gut, smaller numbers of enterococci are also found sometimes in vaginal and oropharyngeal secretions as well as on the skin[7]. Also, soils, foods (e.g. dairy product), terrestrial and aquatic vegetation can be colonized by enterococci[5,7,8].

Until 1984 enterococci were included to the group of Streptococci before they were placed in their own genus, after revealing low identity in DNA-DNA and DNA-rDNA hybridization between these two groups[1]. Nowadays there are more than 40 different species of enterococci known[4]. Numerous studies have shown that the two most clinically important enterococcal species are *Enterococcus faecalis* and *Enterococcus faecium*[9]. In general, the other species of enterococci are rarely responsible for healthcare-associated infections[9]. In the 1990s 80 to 90 % of all clinical isolates from enterococci were *E. faecalis* and up to 10 to 20 % *E. faecium*[7]. In the last decades the proportion of *E. faecium* infections have increased strongly, e.g. up to 43.2 % in Poland in 2011[9]. Nevertheless, *E. faecalis* is still responsible for most of the serious and sometimes even life-threatening healthcare-associated infections caused by enterococci[9]. Taking into account the aforementioned information this thesis will focus especially on *E. faecalis*.

I.1.2. Pathogenicity of enterococci

E. faecalis and *E. faecium* are used as probiotic bacteria to treat diarrhea or improve host immunity as described later in more detail (see chapter I.1.3.)[8]. In contrast to this, other studies show that up to 14 % of healthcare infections associated with common pathogens are caused by enterococci[10]. Other investigations have revealed a high virulence of the enterococcal species in medical settings with a mortality rate up to 61 %[6]. That is why these microorganisms present a dual behavior; they are not only harmless bacteria living primarily in the human gut but are also associated with sometimes even life threatening infections[11]. Beside endocarditis and bacteremia they can also cause urinary tract infections, meningitis, surgical wound infections, hepatobiliary and neonatal sepsis as well as intra-abdominal, pelvic and soft tissue infections[6,12]. These infections are mostly of endogenous origin starting by translocation of enterococci trough the intestinal cell barrier[6]. Another characteristic that makes enterococci as successful nosocomial pathogen is that they can survive for a long time on hospital environmental surfaces like doorknobs, bed rails or medical equipment, especially indwelling medical devices[13]. Moreover, enterococci are also able to withstand many chemicals, such as disinfectant in the hospitals e.g. chlorine or alcohol preparations[12,14].

I.1.3. Enterococci as probiotics

By definition of the WHO/FAO, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host"[15]. Apart from products of the pharmaceutical industry, probiotics are mainly found in dairy products and in some other food or beverages like granola bars and in some extend in meat[3]. But they not only play an important role in food and its fermentation, but also in the human body[3]. Next to improvement of host immunity and balance of the intestinal microbiome, probiotics are claimed to have benefits in case of irritable bowel syndrome, diarrhea, or even to reduce cholesterol levels[3,16].

While some strains of enterococci are among the most common pathogens associated with healthcare-associated infections, other strains of enterococci are well-known probiotics[3,10]. Lactic acid bacteria which also include enterococci are, besides from Bifidobacteria, the best studied and mostly used probiotics[17]. Probably the best characterized representative of probiotic enterococci is *E. faecalis* Symbioflor 1, which was introduced in the 1950s and since then, has been used as a probiotic without being responsible for infections[16]. A reduced recurrence rate in chronically bronchitis and sinusitis has been observed using *E. faecalis* Symbioflor 1[18].

I.1.4. *Enterococcus faecalis*

I.1.4.1. Antibiotic resistance of *E. faecalis*

The era of antibiotics began with Alexander Fleming's discovery in 1928, that the fungus *Penicillium notatum* killed his cultured bacteria[19]. In the last decades the use of antibiotics has increased in hospitals worldwide, as well as in animal and food industry and other areas of life[6,14]. Through this huge consumption, many bacteria (*E. faecalis* among them) have acquired resistances against antibiotics, with some becoming intrinsically resistant to many or all of them[20].

In general *E. faecalis* is intrinsically resistant to penicillin, cephalosporins and other beta-lactams[20]. Furthermore, they are usually resistant to aminoglycosides, clindamycin (a lincosamide), and quinopristin / dalfopristin (both streptogramins)[20]. Moreover, sporadic resistances against linezolid have been described in the literature[20].

Acquired resistances are increasing fast and are a huge challenge when it comes to treat bacterial infections[12]. Just 15 years after using Vancomycin for the first time in 1972, resistant enterococci were already reported[6]. The *E. faecalis* V583 was the first strain found in the United States with resistances against Vancomycin. It was the first fully sequenced enterococcus and has been used as model strain ever since[21,22].

Through the rise of antibiotic resistances not only treatment failure increased up to 20 %, but also mortality rate doubled up to 52-61 % (depending on the patient population)[6].

Nowadays up to 7 % of all *E. faecalis* strains are resistant to Vancomycin[14,23]. Studies have shown that enterococci have the potential to develop resistances against all clinically used antibiotics[14,20]. Therefore, the development of new therapeutical options besides antibiotics is very important to treat multidrug-resistant *E. faecalis* infections.

I.1.4.2. *E. faecalis* V583

E. faecalis V583 is a model strain used in research after revealing the first resistance against Vancomycin in 1987 in a human blood stream infection in Saint Louis, Missouri[21]. *E. faecalis* V583ΔABC used in this study and named *E. faecalis* V19 is a derivative of *E. faecalis* V583 which is cured of the three natural plasmids A, B and C[24]. This bacterial strain was constructed to avoid technical problems related to present replicating plasmids[24].

I.2. Bacteriophages

I.2.1. General characteristics of bacteriophages

In 1915 and 1917 Frederick Twort and Felix d´Hérelle coined the term "bacteriophage" (from now on referred as phages), which are viruses that can infect bacteria for example by integrating into their chromosome[25,26]. Thus, they confer to their bacterial hosts important biological properties, and even some of the harmless bacteria can become more virulent when they get infected[11,25]. For example, phages enlarge not only the stock of genetic diversity to the bacteria but can also be vectors to transfer different genes like antibiotic resistance or virulence genes[11]. Scarlet fever is a well-known example of how bacteriophages can enhance virulence of their host[27]. Infection of *Streptococcus pyogenes* with bacteriophage T12 gives the host the ability to produce exotoxin A, the disease-causing toxin[27].

With up to 10^{30} particles, studies have shown that phages, which can be silently present in their host for long periods, are numerically the most common biological systems on earth[25,28,29]. So it is not surprising that we can find phages in our food, for example in dairy

products, in our oral cavity, in the gut, on our skin, as well as in the whole environment[28]. Interestingly, although phages can be found everywhere where prokaryotes are found, not every individual bacteria carries phages[25,26].

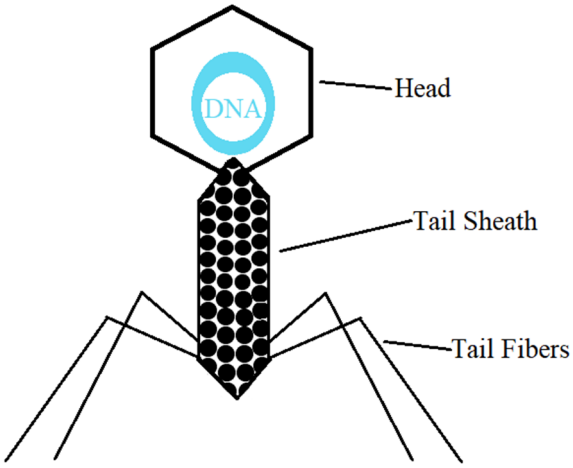

Figure 1. Simplified scheme of the typical morphology of a bacteriophage. Head, Tail Sheath and Tail Fibers are shown in black; the DNA of the bacteriophage is shown in blue. More details about the morphology of bacteriophages are given in the text.

The typical morphology of phages is illustrated in figure 1. The head consists of nuclear proteins at the outside and contains the phage DNA encoding for important proteins and enzymes of the phage[30]. The tail is used for infection and ensures the introduction of the phage's DNA into the host, while the tail fibers help the phage to hold on the host during infection process[30]. To this date phages are classified in 13 families with 39 genera not only after their phenotype, but also due their different properties[26]. With a size between 3.5 kb until 6000 kb, phages can contribute up to 20 % of a bacteria's genome[25,29]. Thus, interactions with cellular activities get more complex the more phages a genome contains[29].

I.2.2. Lysogenic and lytic life cycle of temperate bacteriophages

Bacteriophages can be subdivided in two different groups: Lytic or temperate phages[26]. While virulent phages are replicating in the lytic life cycle and are not integrated into the

bacteria's DNA, temperate phages can switch between a lytic and a lysogenic cycle as it is shown in figure 2[26].

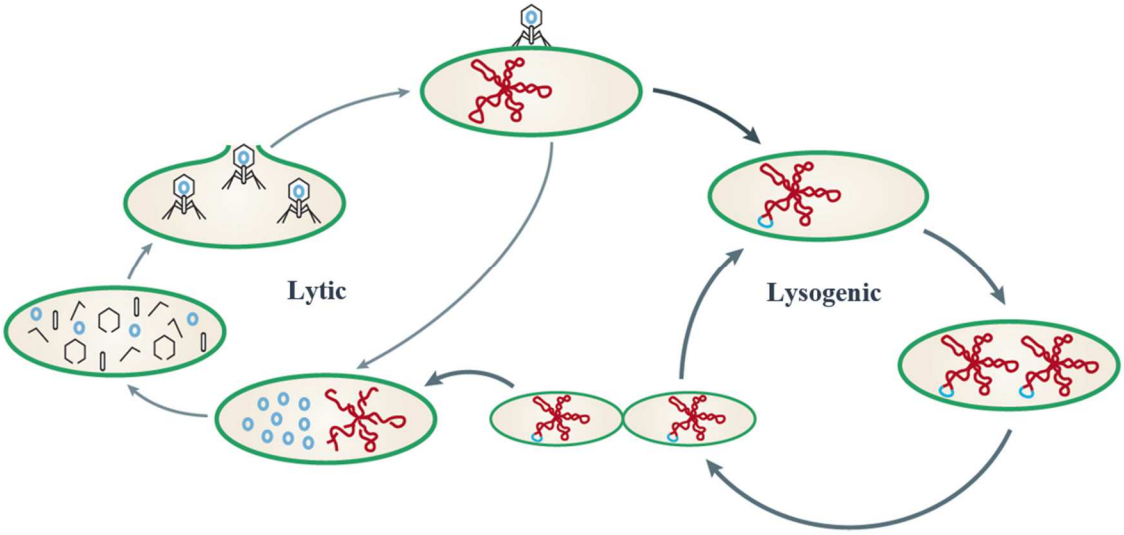

Figure 2. Lytic and lysogenic life cycle of bacteriophages[26]. Phages are shown in black and blue, the hosts DNA is shown in red. While phages replicate separately from the hosts DNA in the lytic life cycle, they are integrating into the hosts chromosome in the lysogenic life cycle.

Lysogeny is characterized by the existence of prophages and their replication together with the hosts DNA[31]. It is not only a survival strategy for the virus, there are also advantages for the bacterial host[28]. An increase in pathogenicity of the bacteria and their protection against viruses are two of the advantages of having a phage integrated in their genome[25,32]. Also, phages in a lysogenic life cycle can ensure persistence of the infection for a lifetime[31].

On the other hand, the lytic life cycle is characterized by replication of the phage genome separately from the host bacterial DNA, producing new virulent phage particles and eventually destruction/lysis of the bacteria to release new virons[31].

The regulation between the two life cycles depends on different conditions in and around the phages and its host[31]. During the early phase of an infection, phages prefer the lytic life cycle because the number of susceptible bacteria is very high[31]. This changes during the end of an infectious cycle when the goal is to keep the infection continuing[31]. The process to switch

between the two life cycles is controlled by the balance between the "repressor protein cl" and its antagonist, the "regularory protein cro"[31].

I.2.3. Bacteriophages and their contribution to virulence

Already in 1927 it was understood that phages play an important role in the pathogenicity and the evolution of their bacterial hosts[33]. For example, phages can influence the bacteria's properties depending on the infection status, and they have been shown to possess virulence genes e.g. exotoxins, which are more robust against external influences than the ones from the bacteria[32,33].

Horizontal gene transfer is perhaps the best known example of genetic variation and thus also possibility to increase virulence of bacteria containing a phage[34]. The transfer of genes between different host bacteria was discovered more than 75 years ago and is still relevant for today's research[35]. A distinction is made between general and special transduction[35]. While general transduction includes parts of the hosts chromosome, special transduction includes a hybrid molecule of phages and bacterial genes[35]. The probably most well-known problem of horizontal gene transfer is transfer of resistances of bacteria against antibiotics[35].

Quorum sensing plays an increasingly important role in the virulence of phages[36]. This well-characterized communication system of gram-positive and gram-negative bacteria was first described about 25 years ago by Fuqua *et al.* and is still an important research topic[37]. Small extracellular chemical signal molecules called autoinducers enable bacteria to analyse and adapt to their environment, and thus increases their pathogenicity of bacteria substantially[38,39]. In 2014 Hargreaves *et al*. published, the first quorum sensing genes found in a phage genome[36]. Activation via quorum sensing can increase biofilm production and thus contribute to pathogenicity[38]. This has been shown for induction of the quorum sensing molecule autoinducer-2 (4,5-dihydroxy-2,3-pentanedione, from now on AI-2) for *E. faecalis* V19 containing prophage 5, as described in more detail in chapter I.2.6.[11].

Nevertheless, in addition to horizontal gene transfer and quorum sensing it is also known that bacteriophages are influencing the virulence of bacteria in many other ways[40,41]. They

can for example induce the production of cytokine TNF-α, a proinflammatory protein involved in autoimmune reactions, inflammation or host defence[40,42]. Besides, phages are known to assist the transfer of bacterial pathogens to humans as it was e.g. demonstrated by the faecal-oral transmission of phage-encoded cholera-toxin[33].

I.2.4. History of phage therapy

Already shortly after the discovery of bacteriophages in 1915 (and long before the discovery of antibiotics), phage therapy was conceived as a possible treatment strategy for bacterial infections[43]. Phage discoverer d'Hérelle tested phage suspensions for wound recovery on himself and in his patients in 1919[44]. The first study mentioning phage therapy was published in 1921 by Bruynoghe and Maisin, describing the reduction of skin blows and their signs of inflammation caused by staphylococci using injections of phage suspensions within 48 hours[44]. It was also published, that d'Hérelle used intravenous phage therapy against cholera in 1931 in India and started a preventive anti-cholera study in fountain systems[43]. The successes achieved through this study was so overwhelming that the government requested to treat the entire population with it[43].

During and after world war II studies about phage therapy became less important in many countries mainly due to the increasing and uncomplicated availability of antibiotics to treat bacterial infections[44]. Many years later with the increasing number of infections with multidrug-resistant bacteria worldwide, phage therapy gained popularity again starting in the 1970s[26,43]. Several promising studies showing success using phages against orthopedic infections, cholera or infections caused by *E. faecalis* were published[43,44]. Other interesting diseases on which research is still focused are skin ulcerations and wound prophylaxis, methicillin resistant *Staphylococcus aureus* (MRSA) and other multidrug-resistant bacteria, burns, eye-, respiratory-, gastrointestinal- or urinary tract infections, as well as chronic otitis, sepsis, among others[43].

I.2.5. Phage therapy nowadays

With huge amounts of antibiotics used worldwide, the number of multidrug-resistant bacteria has been greatly increased becoming an challenging problem in almost all medical settings[45]. In 2014 around 50 000 persons in Europe and the United States died from infections caused by antimicrobial resistant pathogens[45]. According to current projections, this number could increase up to 10 million deaths around the world every year in 2050 if antibiotics continue to be used to such an extent as they are used today[45].

Phage therapy is a promising way to treat bacterial infections and to decrease the amount of antibiotic resistances by using targeted applications of specific phages[43,46]. There are different goals of phage therapy[47]: The first would be to enable phage therapy in everyday clinical settings to treat bacterial infections[47]. The second would be to control antibiotic resistances[47]. The third could be to use phages in prophylactic medicine e.g. as probiotics to modulate the gastrointestinal flora[47].

In order to achieve the above-mentioned goals, there are currently different approaches pursued in phage research[44]. In some cases, a cocktail of several phages given together has been used with the aim of decreasing the risk of bacterial resistance to the phages[44]. Another approach reduces possible side effects and therefore tries to keep the number of different phages as low as possible[44]. It was shown that the use of phages combined with antibiotics had a synergistic effect[47]. A significant reduction of the bacterial pathogens in these cases highlights the relevance of this combined approach[47].

In some countries like Georgia and Poland phage therapy is already used for many patients to treat a variety of bacterial infections[43]. Successes in treating osteomyelitis and lung infections with phages have been documented[48]. A promising success was the treatment of a patient in California in 2016 with intravenous phage therapy[49]. The story received huge publicity as the patient was in coma due to infection with multidrug-resistant *Acinetobacter baumannii* and woke up only three days after intravenous phage therapy with a cocktail containing four different phages[49].

In general, for phage therapy lytic phages are mostly used because they can effectively kill bacteria at the end of a phage infection cycle[44]. For temperate phages there is concern that they may transfer virulence factors to the bacterial host and may eventually worsen the patients' health[44,47].

Although there are already several successful examples of using phages as therapy for bacterial infections, they are currently not the first choice[46]. In Germany, there are no approved phage-drugs to this date[46]. Nevertheless, some promising cases in which patients were treated with phages in German hospitals are also found in the literature[46]. To understand more about phages and their possible benefit in medicine, it is necessary to investigate potential virulence factors of phages, which is one of the focuses of this thesis.

I.2.6. Bacteriophages of *E. faecalis* V19

The presence of one or more prophages has been demonstrated in many *E. faecalis* clinical isolates and this seems to have different advantages regarding to their pathogenicity in contrast to bacteria without bacteriophages integrated into their chromosome[50]. Polylysogeny is not specific for the clinical isolates of *E. faecalis* V19, which harbors seven prophages[11,32,51]. The prophages of *E. faecalis* V19 are named V583-pp1 to V583-pp7 (from now on called pp1 to pp7) and they all have different characteristics[32].

Pp2 seems to be part of the core genome as it is found in all *E. faecalis* isolates and lacks an integrase in its genome[32]. All prophages except pp2 are putatively able to excise from the chromosome under specific conditions[32]. For instance, pp6 is only able to excise from a strain lacking pp3 and pp5. This shows, that pp6 is suppressed in the presence of pp3 and pp5, which have similar gene organisation[32]. After excising from the chromosome pp1, pp3, pp5 and pp7 are able to replicate their own genome and form infectious particles, while this property has not been demonstrated for pp4 and pp6[32].

Pp1, pp3, pp4, pp5 and pp6 are shown to have all necessary functions to exhibit a lytic life cycle, while pp7 needs to use pp1 as helper[32]. On the other hand, pp2 seems to be unable to enter a lytic life cycle[32]. Also, pp1, pp4, and pp6 promote the binding of the bacterial host to

human blood platelets, and thus playing an important role in infections like endocarditis caused by *E. faecalis*[32].

Of particular interest is pp5 (genes *ef2083-ef2145*) with a size of 43.0 kb. Rossman *et al.* showed in several experiments that pp5 plays an important role in the pathogenicity of *E. faecalis* V19, perhaps by carrying specific virulence factors in its genome[11]. A reduced dispersal of biofilm was observed in *E. faecalis* V19 lacking pp5, while the same strain containing pp5 produced significantly more biofilm after the exposure of the bacteria to the universal signaling molecule AI-2[11]. In addition, 28 genes of pp5 were upregulated after the induction of the *E. faecalis* V19 with AI-2[11]. Also, higher TNF-α production by RAW 264.7 cells was demonstrated when the cells were exposed to culture supernatants of *E. faecalis* V19 containing pp5 compared to bacteria cured of pp5[11].

A summary of the most relevant properties of pp1-pp7 from *E. faecalis* V19 are listed in table 1 below.

Table 1. Characteristics of the prophages present in *E. faecalis* V19[32].

	Phage size (kb)	**Ability to excise from Chromosome**	**form infectious particles**	**fulfill lytic life cycle**	**bind human blood platelets**
pp1	38.2	+	+	+	+
pp2	14.6	-	-	-	-
pp3	47.3	+	+	+	-
pp4	39.0	+	-	+	+
pp5	43.0	+	+	+	-
pp6	36.0	*pp3^{-}, pp5^{-}	-	+	+
pp7	12.0	+	+	**pp1^{+}	-

*pp3^{-}, pp5^{-}: strain lacking pp3 and pp5

**pp1^{+}: pp1 need as helper phage

I.2.6.1. The *ef2143* gene from *E. faecalis* V19, a putative toxin

Bacterial toxins are virulence factors associated with host cell death, and dysregulated immune responses[40,52]. They are important for survival and pathogenicity of the bacteria[40]. Some of these toxins are encoded by bacteriophages integrated into the bacteria, causing important infections in humans, such as sepsis in cholera or diphtheria infections[53]. Not only the fact that toxins play key roles in the origin of sepsis, but also that gram-positive bacteria such as *E. faecalis* are among the most common cause of sepsis, demonstrate that toxins of gram-positive bacteria are important proteins to study[40].

The *ef2143* gene was selected for this study for various reasons. As Rossmann *et al.* have shown and as is already described in chapter I.2.6, pp5 (*ef2083 – ef2145*) seems to play an important role in the virulence of *E. faecalis* V19 by carrying virulence factors in its genome[11]. The genetic information of the *ef2143* gene present in the pp5 from *E. faecalis* V19 was compared by basic local alignment search tool (BLAST) to other gram-positive bacteria[54]. The results showed that the *ef2143* gene is most likely a putative toxin[54]. Considering that the *ef2143* gene encodes a putative toxin and in addition because it is part of the pp5, EF2143 seems to be an important and interesting protein of *E. faecalis* V19 to study[54].

I.2.6.2. The *ef2144* gene is a lipoprotein

Lipoproteins are anchored in the plasma membrane and have been described to perform various functions in the host-pathogen interaction and other different cellular activities[41,55]. These proteins have been shown to be particularly important for virulence in gram-positive bacteria[55]. Examples of the functions influenced by lipoproteins are inflammatory processes, adhesion, antibiotic resistances and colonization of the host[41]. Due to exposed location in the plasma membrane, lipoproteins are possible vaccine candidates and alternative targets for drug development that makes them interesting proteins to study[56].

E. faecalis V19 contains 90 different lipoprotein-encoding genes[56]. While over 90 % of these lipoprotein-encoding genes are located in the chromosome of *E. faecalis* V19, only five of

them are in a bacteriophage-related region[56]. Lipoprotein EF2144 is one of these and, in addition, also part of the genome of pp5, which has been suspected to play an important role in the virulence from *E. faecalis* V19[11,56]. Since not only lipoproteins but also bacteriophages are associated with the virulence of bacteria, especially this combination makes lipoprotein EF2144 interesting to study[11,56].

I.3. Biofilms

I.3.1. General characteristics about biofilms

Biofilms are a complex three-dimensional formation of microorganisms living in a community with cell-to-cell interaction between each other or attached to a surface[57,58]. They are producing extracellular substances in which they are enmeshed and can display different phenotypes regarding their properties when living in this community[59]. While the initial stage of a biofilm is to establish an infection, later stages comprise the maturation and eventual dispersal of a subfraction of the biofilm to disseminate the settled bacterial cells as shown in figure 3[11].

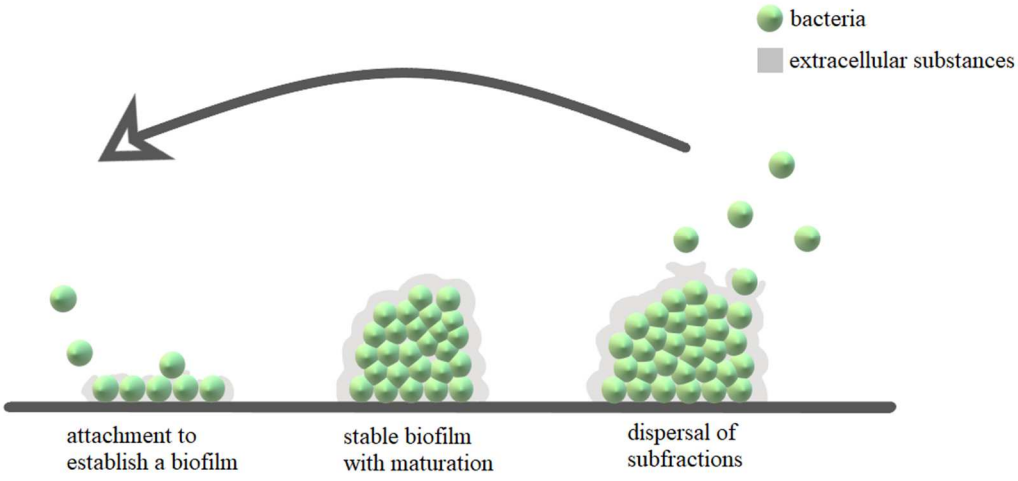

Figure 3. Simplified scheme of the microbial biofilm lifecycle. Bacteria are shown in green while the extracellular substances they are enmeshed in are shown in light grey. While bacteria are attaching to a surface and between each other in the early stage of a biofilm, dispersal of subfractions takes place in the later stages to spread the infection.

Already in the seventeenth century Van Leeuwenhoek wrote about “animalcules” on his teeth, giving the first hint for the existence of biofilms[59]. In 1978 Bill Costerton coined the term “biofilm” suggesting that bacteria stick to different surfaces like medical devices (but also hulls of ships or tubing of water systems or air condition devices)[60].

Nowadays it is clear that biofilms are a huge problem associated with infections in medicine, and that its formation plays an important role for the virulence of certain bacteria[57]. Biofilms are frequently found on indwelling medical devices (for example on central or peripheral venous catheters or implants such as artificial heart valves or stents), and these are often the reason for urinary tract infections, endocarditis, central nervous system infections or bloodstream infections[11,57,58]. A major challenge in the clinical setting is the removal of established biofilms, as bacteria in biofilms are resistant to 10 - 1000 times more concentrated antibiotics than bacteria not being part of a biofilm[58]. With this background and the knowledge of rising resistances against antibiotics used in hospitals worldwide, it is clear that biofilms are a really important research topics[57].

I.3.2. Biofilms by enterococci

Most enterococci are strong biofilm producers[13,61]. About 25% of catheter-associated urinary tract infections are caused by enterococci, mostly *E. faecalis* or *E. faecium*, due to established biofilms on the catheters[62]. The proportion of *E. faecalis* producing biofilms varies worldwide and most biofilms in which *E. faecalis* was found consist not only of this single bacterial species but are polymicrobial[58,62]. Considering only Europe in the early 2000s, the importance of *E. faecalis* producing biofilms varied between 57 % in 2001 in Spain, and 100 % in 2003 in the UK[58]. Not only the high number of infections caused by biofilm formation through enterococci are a huge problem, but also the decreasing response of antibiotic therapies to these infections[62]. For example, the proportion of enterococci with resistances to vancomycin increased from 0 % in the mid-1980s to around 5 % for *E. faecalis,* and to over 80 % in 2007 for *E. faecium*[62] (although this varies between countries or time periods). There is a clear distinction between resistances towards antibiotics of planktonic enterococci and those enmeshed in a biofilm [62].

First, antibiotics are less able to penetrate the extracellular substances of biofilms, and this phenomenon seems to increase with the age of the biofilm[62]. The bacteria in a biofilm can therefore easily survive significantly higher concentrations of antibiotics[58]. Important is that the bacteria themselves recognize the bacterial density via quorum sensing[38,39]. Signal molecules, which are then released via this communication system, play an essential role in biofilm formations, as Rossmann *et al.* showed for AI-2 in *E. faecalis* V19 biofilm formations[11]. The detachment of a subfraction from a biofilm, as well as the release of bacteriophages from *E. faecalis* V19 to infect other bacteria are stimulated by high AI-2 concentrations (e.g. more than 100 µM)[11].

Second, studies with vancomycin-resistant enterococci (VRE) showed the existence of persister cells in biofilms[62]. These cells with the presence of guanosine tetraphosphate (ppGpp) appear to have a selective advantage for some bacteria over certain antibiotics and are currently being further studied[62].

Third, it has been demonstrated that not only the gene expression differs between planktonic enterococci and those in a biofilm, but also that the rates of horizontal gene transfer are higher in biofilms[62]. For example, enterococci present in a biofilm showed an increased expression of genes responsible for higher resistance to antibiotics[62]. With this they are also serving as a pool for genes for horizontal gene transfer e.g. of virulence genes[62].

II. Objectives and context of this work

Enterococci are responsible for many infections in different clinical settings[11], and especially *E. faecalis* plays a major role and can lead to life-threatening disease[9]. In particular, increasing resistances of bacteria to currently available antibiotics is a growing and serious problem worldwide[20]. This has led to a great deal of interest into research of alternative therapeutics[43]. A special focus has been placed on phages, which are viruses that can integrate into the genome of bacteria and therefore, can change the properties of the bacteria[25]. For example, specific virulence factors can be transferred via horizontal gene transfer[35].

The aim of this work was to identify and evaluate bacteriophage genes contributing to the virulence of *Enterococcus faecalis*. The two genes *ef2143* and *ef2144* from *E. faecalis* V19 studied in this thesis are not only interesting because of their properties per se: EF2143 as putative toxin and EF2144 as lipoprotein, but also because they are also part of the bacteriophage 5 integrated into *E. faecalis* V19[54,56]. The following objectives have been pursued in this work:

- Generation of two mutants with deletion of about 80% of the genes *ef2143* and *ef2144*. In addition, complementation of the two deletion mutants was planned through three silent single point mutations of the genes *ef2143* and *ef2144*.
- Investigation of the differences and similarities between wild type *E. faecalis* V19 and the four designed mutants regarding growth behaviour.
- Analysis of whether a pp5 release is possible after deletion of about 80% of genes *ef2143* and *ef2144* as well as further studies on whether pp5 can be transduced into other *E. faecalis* strains.
- Analysis of the role of pp5 and the different mutants in biofilm formation of *E. faecalis* and their behaviour upon stimulation with autoinducer-2.
- Investigations if the absence of the proteins EF2143 and EF2144 affects the inflammatory response by measuring the cytokine TNF-α production after stimulation of peripheral blood mononuclear cells

- Investigate if the absence of the proteins EF2143 and EF2144 affects the virulence of *E. faecalis* by using the animal model *Galleria mellonella.*

III. Materials and methods

III.1. Materials

III.1.1. Bacterial strains

All bacterial strains used in this study are listed in table 2.

Table 2. Bacterial strains used in this study.

Species	Strains	Abbreviation	Description	References
E. coli	Top 10	Top 10	Cloning host for the different plasmids used	Invitrogen, USA
E. faecalis	V583ΔABC	V19	*E. faecalis* V583 derivative cured from its natural plasmids A, B and C	[63]
E. faecalis	V19ΔEF2143	ΔEF2143	*E. feacalis* V19 with 83,6 % of the gene *ef2143* deleted from its genome.	This study
E. faecalis	V19ΔEF2144	ΔEF2144	*E. feacalis* V19 with 80 % of the gene *ef2144* deleted from its genome.	This study
E. faecalis	V19cEF2143	cEF2143	*E. feacalis* V19 with three single point silent mutations in the gene *ef2143*. Used as complementation of the strain ΔEF2143	This study
E. faecalis	V19cEF2144	cEF2144	*E. feacalis* V19 with three single point silent mutations in the gene *ef2144*. Used as complementation of the strain ΔEF2144	This study
E. faecalis	V19pp-	pp-	*E. faecalis* V19 cured from all seven phages	[32]
E. faecalis	V19pp3+	pp3+	*E. faecalis* V19 containing only pp3	Chiara Lincetto, LMU, Germany

III.1.2. Genes studied

The sequences of the genes *ef2143* and *ef2144* from *E. faecalis* were retrieved from the NCBI database (National Center for Biotechnology Information, {http://www.ncbi.nlm.nih.gov/gene/}) and are listed in table 3 below.

Table 3. Genes of interest in this study.

Protein name	Accession number	Abbreviation	Gene length (bp)
Hypothetical protein	NP_815806.1	EF2143	468
Lipoprotein	NP_815807.1	EF2144	861

III.1.3. Primers

All primers used in this study were bought from Eurofins (Germany) and are listed in table 4 below.

Table 4. Primers used in this study.

N°	Primer Name	5´-3´sequence	Restriction site	Use
1	EF2143_11_BamHI	accaGGATCCATACTAATTGGGCCACTT	BamHI (GGATCC)	To introduce the restriction site in the construction by PCR with oligo EF2143_44_PstI
2	EF2143_22_XhoI	agcaCTCGAGTTCAGGATAATTAGCCAT	XhoI (CTCGAG)	To introduce the restriction site to make the deletion by PCR with oligo EF2143_55_FW
3	EF2143_33_XhoI	agcaCTCGAGACAATTGGGGTATATGAA	XhoI (CTCGAG)	To introduce the restriction site to make the deletion by PCR with oligo EF2143_66_RV
4	EF2143_44_PstI	accaCTGCAGCATAAATACGAATACGAA	PstI (CTGCAG)	To introduce the restriction site in the construction by PCR with oligo EF2143_11_BamHI
	EF2143_55_FW	GCAAATGCCAACTCACTATCGTTA	-	To make the deletion by PCR with oligo EF2143_22_XhoI
6	EF2143_66_RV	GCCATAAATACCAACCTCTCCGGA	-	To make the deletion by PCR with oligo EF2143_33_XhoI
7	EF2143_1_BamHI	agcaGGATCCTGTCATTTTTATCCTCCTATCA	BamHI (GGATCC)	To introduce the restriction site in the construction by PCR with oligo EF2143_4_PstI
8	EF2143_2_RV	CTATTTTGCTCTATAATATCGCCGACACTTGTCAAATGATGACCAATTTC	-	Single mutation insertions by PCR with oligo EF2143_5_FW
9	EF2143_3_FW	GAAATTGGTCATCATTTGACAAGTGTCGGCGATATTATAGAGCAAAATAG	-	Single mutation insertions by PCR with oligo EF2143_6_RV
10	EF2143_4_PstI	agcaCTGCAGATTGTTTATTATCTTGGCTACT	PstI (CTGCAG)	To introduce the restriction site in the construction by PCR with oligo EF2143_1_BamHI

11	EF2143_5_FW	CGGTTAATACTGGTATCGC TCATTCCTTT	-	Single mutations insertions by PCR with oligo EF2143_2_RV
12	EF2143_6_RV	TGTTCTAAGGTTGGCTCAT CTGCTAGC	-	Single mutations insertion by PCR with oligo EF2143_3_FW
13	EF2143_7_FW_Verif	CATCATTTGACAAGTGTCG GC	-	To verify the insertion of the single point mutations by PCR with oligo EF2143_4PstI or EF2143_6_RV
14	EF2143_seq_1_FW	GGCGTTGCCAAAACCACAT	-	To verify the sequence of the gene *ef2143*
15	EF2143_seq_2_FW	ACTTATACGGAGTGGGAA ACAAGAAA	-	To verify the sequence of the gene *ef2143*
16	EF2143_seq_3_RV	CGCCTGATTTGACGGTAAT AAGA	-	To verify the sequence of the gene *ef2143*
17	EF2143_seq_4_RV	CAAAATGCGTATACCAAG AATATTGAAA	-	To verify the sequence of the gene *ef2143*
18	EF2144_11_BamHI	agcaGGATCCACATTTCGCG TACTTATT	BamHI (GGATCC)	To introduce the restriction site in the construction by PCR with oligo EF2144_44_PstI
19	EF2144_22_EcoRI	agcaGAATTCGGTTGACTTT TCAGTATC	EcoRI (GGATTC)	To introduce the restriction site to make the deletion by PCR with oligo EF2144_55_FW
20	EF2144_33_EcoRI	agcaGAATTCGCTAATGTGG AAATACAC	EcoRI (GGATTC)	To introduce the restriction site to make the deletion by PCR with oligo EF2144_66_RV
21	EF2144_44_PstI	agcaCTGCAGTATTTATTCA CAGATGAA	PstI (CTGCAG)	To introduce the restriction site in the construction by PCR with oligo EF2144_11_BamHI
22	EF2144_55_FW	CCATTGATTCAATTTATTC ACCCC	-	To make the deletion by PCR with oligo EF2144_22_EcoRI
23	EF2144_66_RV	CGGGCATGTCTAAAACTAT GAGATG	-	To make the deletion by PCR with oligo EF2144_33_EcoRI
24	EF2144_1_BamHI	agcaGGATCCCTGAACAATT AACAGTAGCCGCACAT	BamHI (GGATCC)	To introduce the restriction site in the construction by PCR with oligo EF2144_4_PstI
25	EF2144_2_RV	TTATCATCTAGTTGAGTCA ACACAGCATAATAATCTCC AGGATCAATATC	-	Single mutation insertions by PCR with oligo EF2144_5_FW
26	EF2144_3_FW	GATATTGATCCTGGAGATT ATTATGCTGTGTTGACTCA ACTAGATGATAA	-	Single mutation insertions by PCR with oligo EF2144_6_RV
27	EF2144_4_PstI	agcaCTGCAGATTAGCTAAT TTCTTTGGTTCT	PstI (CTGCAG)	To introduce the restriction site in the construction by PCR with oligo EF2144_1_BamHI
28	EF2144_5_FW	CGAACTCCTGACGCAGAAT TACAAAATAA	-	Single mutations insertions by PCR with oligo EF2144_2_RV
29	EF2144_6_RV	TCTGTTTCAGCATCTCTAC AAGCCTTCTT	-	Single mutations insertion by PCR with oligo EF2144_3_FW

30	EF2144_7_FW_Verif	GGAGATTATTATGCTGTGTTG	-	To verify the insertion of the single point mutations by PCR with oligo EF2144_4PstI or EF2144_6_RV
31	EF2144_seq_1_FW	CTTCAAGACGCAATCAAAAGTTACTCAA	-	To verify the sequence of the gene *ef2144*
32	EF2144_seq_2_FW	TGGCTTGCAAAACCTGGCA	-	To verify the sequence of the gene *ef2144*
33	EF2144_seq_3_RV	GCCAGTTAATCCATTGCTGATATTTG	-	To verify the sequence of the gene *ef2144*
34	EF2144_seq_4_RV	CCCAATTGTACCATCACACCTAAAA	-	To verify the sequence of the gene *ef2144*
35	EF2144_seq_5_FW	CACAGTTAAACAACCGAATTCAAAAGAC	-	To verify the sequence of the gene *ef2144*
36	EF2144_seq_6_RV	CATCAGACTTCGCTTCAGTACTTACTTCT	-	To verify the sequence of the gene *ef2144*
37	pLT06_FW	CAATAATCGCATCCGATTGCAG	-	To check the insert on the plasmid by PCR with oligo pLT06_RV
38	pLT06_RV	CCTATTATACCATATTTTGGAC	-	To check the insert on the plasmid by PCR with oligo pLT06_FW
39	pp5_A_FW	CTTGTGCACGAGATTTGTACGATT	-	To verify pp5 by PCR with oligo pp5_B_RV; To verify pp5-release by PCR with oligo pp5_A_RV
40	pp5_A_RV	GCCGATGGATAAAACTGCCACTTG	-	To verify pp5-release by PCR with oligo pp5_A_FW
41	pp5_B_RV	GTGACCATAGACAGCTAATTCAG	-	To verify pp5 by PCR with oligo pp5_A_FW
42	pp5_C_FW	GCCTCCATTTGCACGTGTAACTCT	-	To verify pp5 by PCR with oligo pp5_C_RV
43	pp5_C_RV	CGCATTACCATTTGATTGGACGAGT	-	To verify pp5 by PCR with oligo pp5_C_FW
44	pp5_attP_FW	CAATGGATTAACTGGCTTGC	-	To verify circularized DNA of pp5 with oligo pp5_attP_RV
45	pp5_attP_RV	ATCCGAAGGAACATTGCTAG	-	To verify circularized DNA of pp5 with oligo pp5_attP_FW

* Bases in lowercase letters are not complementary to the target sequence; Underlined bases correspond to restriction sites.

III.1.4. Plasmids

Table 5 shows the plasmids and their most important characteristics used in this study.

Table 5. Plasmids used in this study.

Plasmid	Characteristics	References
pLT06	Plasmid used to engineer all the isogenic, in-frame deletion mutants used in this study	[64]
pLT06::ΔEF2143	pLT06 carrying the DNA amplifications upstream and downstream of the *ef2143* generating an in-frame deletion of 83,6% of the gene	This study
pLT06::ΔEF2144	pLT06 carrying the DNA amplifications upstream and downstream of the *ef2144* generating an in-frame deletion of 80% of the gene	This study
pLT06::cEF2143	pLT06 carrying the *ef2143* gene with three silent single point mutations to complement the deletion mutant	This study
pLT06::cEF2144	pLT06 carrying the *ef2144* gene with three silent single point mutations to complement the deletion mutant ΔEF2144	This study

III.1.4.1. The pLT06 vector

Thurlow *et al*. combined the pCJK47, pGB354, and pCASPER plasmids to create pLT06, a vector which allows the replication of cloned DNA into *E. coli* or *E. faecalis*[64]. Due to the presence of a temperature-sensitive plasmid replication protein *repA* the pLT06 vector can replicate best at 30°C, while replication does not take place at 42°C[64]. Positive selection of pLT06 is possible due to the presence of an chloramphenicol acetyltransferase gene (pheS)[64]. Additionally, the presence of a β-galactosidase gene (lacZ) in pLT06 allows a blue-white screening of the colonies containing or not containing the plasmid[64].

The pLT06 vector was used for targeted mutagenesis in *E. coli* Top 10 and *E. faecalis* V19 for the two designed mutants by gene deletion *E. faecalis* ΔEF2143 / ΔEF2144, and the complementation of the mutants with three silent single point mutations *E. faecalis* cEF2143 / cEF2144 that were used to be able to track that the reversion was in fact due to a new double-crossover event. Figure 4 illustrates the pLT06 vector and the relative positions of the restriction enzymes BamHI and PstI, which were used to insert the new constructions into the pLT06 vector.

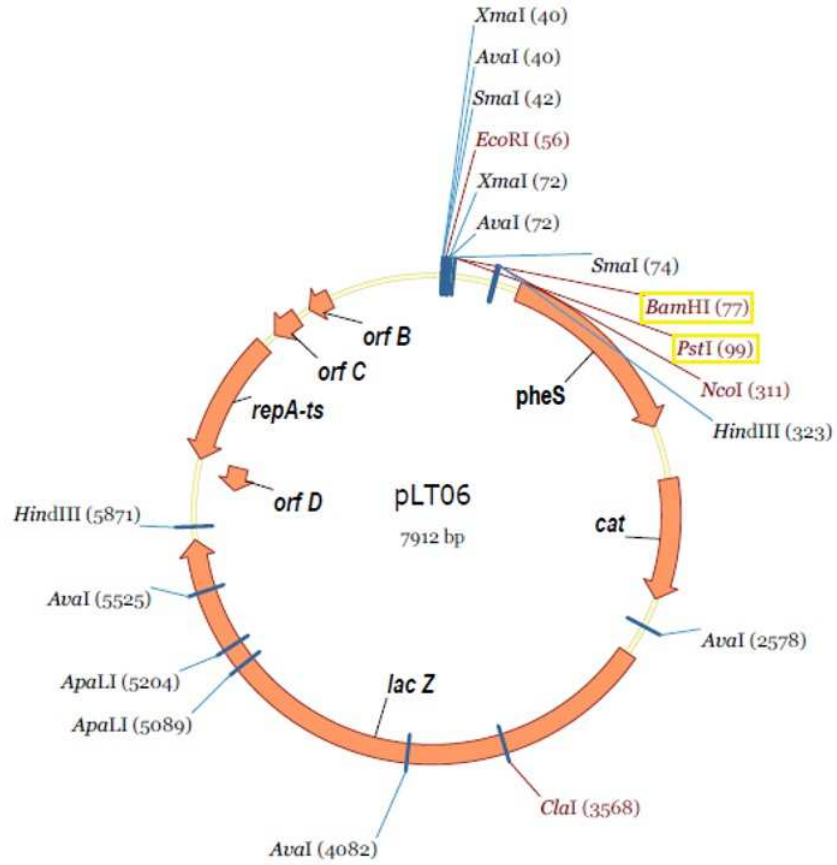

Figure 4. **Sequencing map for the pLT06 vector**[64]. The used restriction enzymes BamHI and PstI are marked in yellow. Unique restriction sites for common restriction enzymes are illustrated in red, multiple restriction sites in black.

III.2. Methods

III.2.1. General culture conditions

III.2.1.1. Culture conditions for *E. coli* strains

E. coli strains were grown under shaking (150-200 rpm) in LB Broth (Luria/Miller; Carl Roth, Germany) at 37 °C. For growing *E. coli* on solid media LB Agar (Carl Roth, Germany) was used. Chloramphenicol 20 µg/mL (Carl Roth, Germany) was added when required to both liquid and solid media. To screen recombinant colonies with a blue-white screening 12

µg/mL of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, AppliChem, Germany) were added to differentiate between the different bacterial clones. *E. coli* strains were kept at 4 °C in between the experiments for a short storage. For long term storage 20 % glycerol (Carl Roth, Germany) was added to the liquid cultures before putting them at -80 °C. The exact compositions of the culture media are listed in table 6 below.

Table 6. Compositions of the culture media used for *E. coli.*

LB Broth	**LB Agar**
Tryptone 10 g/L	Tryptone 10 g/L
Yeast extract 5 g/L	Yeast extract 5 g/L
Sodium chloride 10 g/L	Sodium chloride 10 g/L
	Agar 15 g/L
pH 7.0 ± 0.2	pH 7.0 ± 0.2

III.2.1.2. Culture conditions for *E. faecalis* strains

E. faecalis strains were grown in liquid media without agitation at 37 °C in either BHI (Brain-Heart-Infusion Broth; Carl Roth, Germany), in TSB (Tryptic soy broth; Carl Roth, Germany) or in GM17 (M17 supplemented with glucose 0.5 %, Sigma-Aldrich, USA). To grow them on solid media BHIA plates (Brain-Heart-Infusion agar; Carl Roth, Germany) or TSA plates (Tryptic soy agar; Carl Roth, Germany) were used. If necessary, Chloramphenicol (20 µg/mL) were added to the media and / or 40 µg/mL of X-Gal to screen recombinant colonies with a blue-white screening. *E. faecalis* strains were kept at 4 °C in between experiments for short storage. For long term storage 20 % glycerol was added to the liquid culture before putting them at -80 °C. The exact compositions of the culture media are listed in table 7 below.

Table 7. Compositions of the culture media used for *E. faecalis.*

BHI	**BHIA**
Calf Brain Infusion 7.5 g/L	Calf Brain Infusion 7.5 g/L
Beef Heart Infusion 10 g/L	Beef Heart Infusion 10 g/L
Peptone 10 g/L	Peptone 10 g/L
Glucose 2 g/L	Glucose 2 g/L
Sodium Chloride 5 g/L	Sodium Chloride 5 g/L
Disodium Phosphate 2.5 g/L	Disodium Phosphate 2.5 g/L
	Agar 15 g/L
pH 7.4 ± 0.2	pH 7.4 ± 0.2
TSB	**TSA**
Caseine peptone (pancreatic digest.) 17 g/L	Caseine peptone (pancreatic digest.) 15 g/L
Soya peptone (papain digest.) 3 g/L	Soya peptone (papain digest.) 5 g/L
Dipotassium hydrogen phosphate 2.5 g/L	Sodium chloride 5 g/L
Sodium chloride 5 g/L	Agar 15 g/L
Glucose 2.5 g/L	
pH 7.3 ± 0.2	pH 7.3 ± 0.2
M17	
Ascorbic acid 0.5 g/L	Sodium glycerophosphate 19 g/L
Lactose 5 g/L	Soya peptone 5 g/L
Magnesium sulfate 0.25 g/L	Tryptone 2.5 g/L
Meat extract 5 g/L	Yeast extract 2.5 g/L
Meat peptone 2.5 g/L	pH 7.0 ± 0.2 (25 °C)

III.2.2. Extraction of plasmid DNA

Plasmid DNA from *E. coli* were extracted by using the PureYield™ Plasmid Miniprep System (Promega, Germany) by following the manufacturer´s instructions.

III.2.3. Polymerase chain reaction (PCR)

Two different enzymes were used to perform the different PCR experiments. Either the Q5 High-Fidelity PCR Kit (New England BioLabs, UK) or the GoTaq G2 Hot Start Polymerase (Promega, Germany) following the manufacturer´s instructions. For both kits 12.5 µL of the

enzyme, 9 µL of nuclease free water, 1.25 µL of each primer and 1 µL of the corresponding template were mixed for each reaction.

The Q5 PCR kit was used for reactions that required further DNA manipulation like enzymatic digestion, DNA ligation or DNA sequencing. For all routine PCR reactions without further DNA manipulation i.e. gene identification or colony screening the GoTaq was used. The temperature programs for the different PCRs are listed in the table 8 below.

Table 8. Conditions for PCR experiments with GoTaq and Q5.

	GoTaq			Q5		
Cycle step	**Temperature**	**Time**	**Cycle**	**Temperature**	**Time**	**Cycle**
Initial denaturation	95 °C	2 min	1	98 °C	30 sec	1
Denaturation	95 °C	30 sec	34	98 °C	8 sec	40
Annealing	*X	30 sec	34	*X	20 sec	40
Extension	73 °C	1 min/ 1 kb	34	72 °C	25 sec/ 1 kb	40
Final extension	73 °C	5 min	1	72 °C	2 min	1

*The used annealing temperature (X) was depending on the used primers.

III.2.4. Agarose gel electrophoresis

Agarose gel electrophoresis was performed according to Green *et al.* as follows[65]. A 1 % agarose gel was made with solid agarose (Biozym Scientific, Germany) and TBE buffer (Tris-Borate-EDTA with 10.8 g Tris base (Carl Roth, Germany), 5.5 g boric acid (Carl Roth, Germany) and 0.7 g EDTA (VWR International, USA) per liter). After, the mixture was boiled until it was homogenous. Then 1.0 µL of Midori Green Advance DNA Stain (NIPPON Genetics Europe, Germany) per 100 mL of gel was added to make the DNA visible in UV-light. Once the gel was solidified it was covered with TBE Buffer in a Mini-Sub cell GT (Bio Rad, USA) chamber. The PowerPac Basic (Bio Rad, USA) was running 35 minutes at 120 V and 400 mA. To analyze the size of the DNA fragments either the GeneRuler 1 kb DNA ladder or the GeneRuler 1 kb Plus DNA ladder (both Thermo Fisher Scientific, USA) was added in one well of the gel. The ChemiDoc™ MP Imaging System (Bio Rad, USA) was used to evaluate the gel.

III.2.5. DNA purification

If necessary, DNA was purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Germany) following the manufacturer´s instructions.

III.2.6. DNA digestion

All restriction enzymes were purchased from New England BioLabs (UK). In general, 1 µL of the corresponding restriction enzyme was added to 1 µg of DNA and digested following the manufacturer´s instructions. All reaction conditions i.e. incubation and heat inactivation of the respective restriction enzyme were adjusted according to the manufacturer´s instructions. After digestion, the DNA was purified as mentioned above.

III.2.7. DNA ligation

After purification, the ligation of the digested DNA was performed using the T4 DNA ligase (New England BioLabs, UK). Usually 1 µL of the T4 DNA Ligase was added to a mix of 2 µL of T4 DNA Ligase Reaction Buffer (New England Bio Labs, UK), 3-8 µL of the purified respective vector and 7-15 µL of purified DNA insert (vector insert ratio of 1:5) and adjusted to a final volume of 20 µL with nuclease free water (Promega, Germany). The mixture was incubated then at 37 °C for 30 minutes and the enzyme was heat inactivated at 65 °C for 10 minutes.

III.2.8. Transformation

III.2.8.1. Preparation of *E. coli* electrocompetent cells

E. coli electrocompetent cells were prepared according to the protocol of Dower *et al.* as follows [66]. The corresponding *E. coli* strain was inoculated from a frozen stock into 10 mL of LB broth and incubated at 37 °C overnight with shaking. After 18 hours, 10 mL of the

ON bacterial culture were added to 500 mL of fresh LB broth and incubated at 37 °C with shaking until an OD 600 nm of about 0,8 (approximately 2,5 hours). The bacterial suspension was then centrifuged at 4000 rpm for 30 minutes at 4 °C. After discarding the supernatant, the cells were washed two times with decreasing volumes of ice-cold ultra-pure water (500 mL, 250 mL) and two more times with decreasing volume of ice-cold 10 % glycerol (100 mL, 50 mL) centrifuging the bacterial suspension in between each washing step at 4000 rpm for 30 minutes at 4 °C. After the last centrifugation step the cell pellet was resuspended in 1 mL of ice-cold 10 % glycerol and aliquoted in 100 µL portions before storage at -80 °C.

III.2.8.2 Transformation of plasmids into *E. coli*

Electroporation was performed using the Electro Cell Manipulator (BTX, USA) to transform plasmids into *E. coli,* following the settings of Dower *et al.*[66]. Usually 1-2 µL (10 ng) of the ligated DNA products were mixed with 100 µL of *E. coli* electrocompetent cells in a sterile 2 mm Gene Pulser cuvette (Bio Rad, USA). After electroporation at 2500 V, 200 Ω and 25 µF, the cells were rapidly resuspended in 1 mL of LB broth and transferred to a 1.5 mL micro centrifuge tube (Sarstedt, Germany). The bacteria were grown at 37 °C for 45 minutes before streaking them on LB agar plates with the corresponding antibiotic and X-Gal when needed. Finally, the plates were incubated at 37 °C overnight.

III.2.8.3. Preparation of *E. faecalis* electrocompetent cells

E. faecalis electrocompetent cells were prepared according to the protocol of Ladjouzi *et al.* as follows[67]. 10 mL bacterial culture were inoculated with the appropriate *E. faecalis* strain from a frozen stock and incubated overnight at 37 °C without agitation in TSB media. The ON culture was diluted 1:10 with fresh media and incubated at 37 °C until an OD 600 nm between 0.5-1.0 (approximately 3 hours). Then, the bacterial culture was chilled on ice for 20 minutes before centrifuging it at 4000 rpm for 15 minutes at 4 °C. After resuspending the cell pellet in 3 mL of ice-cold 10 % glycerol, the suspension was split in two 2 mL tubes (Sarstedt, Germany) and centrifuged at 13000 rpm for 1 minute. The cells were resuspended

in 500 µL of a lysozyme solution (for full composition of the solution see table 9 below) containing freshly added lysozyme at 25 µg/mL (Sigma-Aldrich, USA) and mutanolysin at 2 µg/mL (Sigma-Aldrich, USA). The bacterial suspensions were incubated for 30 minutes at 37 °C and later centrifuged at 13000 rpm for 1minute at 4 °C. Bacterial cells were then washed three times with 1 mL of ice-cold electroporation buffer (EB, for full composition of the buffer see table 9 below), centrifuging the bacterial suspension in between each washing step at 13000 rpm for 1 minute at 4 °C. After the washing steps the cell pellets were resuspended in 300 µL of EB. For storage aliquots of 70 µL were frozen at -80 °C.

Table 9. Compositions of the lysozyme solution and the electroporation buffer.

Lysozyme Solution 50 mL		**Electroporation Buffer (EB) 50 mL**	
Tris	10 mM	Sucrose	0.5 M
Sucrose	20 %	Glycerol	10 %
EDTA	10 mM		
NaCl	50 mM		
pH	8.0		

III.2.8.4. Transformation of plasmids into *E. faecalis*

The transformations were performed by electroporation using the Electro Cell Manipulator. Briefly, 1-2 µL (10 ng) of the purified plasmid were mixed with 70 µL of the prepared electrocompetent cells into a sterile 1 mm electroporation cuvette (Carl Roth, Germany). After the electroporation was performed at 1600 V, 200 Ω and 25 µF, the cells were rapidly resuspended in 400 µL of STSB (TSB supplemented with 0.5 M sucrose) and transferred to a 1.5 mL tube (Sarstedt, Germany). The bacteria suspensions were incubated at 37 °C without agitation for 2 hours before plating them on BHI agar containing the corresponding antibiotic and X-Gal at 40 µg/mL when needed. Finally, plates were incubated at 37 °C for 24 to 48 hours.

III.2.9. Blue-white screening

To differentiate between colonies that contain or not the pLT06 plasmid, blue-white screening was performed according to the method described by Sambrook *et al*.[65]. As follows, 20 µg/mL X-Gal were added to the corresponding agar when screening for *E. coli* colonies and 40 µg/mL X-Gal were added when screening for *E. faecalis* colonies. Cells containing the plasmid were detected by its blue color that is due to the production of ß-galactosidase and with this changing X-Gal into a blue pigment. In contrast cells without the plasmid will appear white.

III.2.10. DNA sequencing and analyses

To sequence DNA, either purified PCR products or plasmids were sent to Eurofins (Germany) following the company instructions. The sequenced fragments were analyzed using Vector NTI 11.0 (Invitrogen, USA) software.

III.2.11. Plasmid construction and targeted mutagenesis

III.2.11.1. Construction of the plasmid pLT06 carrying the mutants by gene deletion ΔEF2143 and ΔEF2144 of *E. faecalis* V19

Different PCRs were performed to delete over 80% of the bases pairs (bp) of genes *ef2143* and *ef2144* from *E. faecalis* V19 as it is shown in figure 5 in the results section. Restriction sites were added in the primers and used to introduce a complementary sequence when needed (see table 4).

To generate the in-frame deletion of the genes two DNA fragments were obtained. The first fragments of about 1.1 kb upstream of the region to be deleted, were amplified with primer-pairs EF2143_11_BamHI / EF2143_22_XhoI and EF2144_11_BamHI / EF2144_22_EcoRI for *ef2143* and *ef2144* respectively. For the downstream fragments of about 1.1 kb of the region to be deleted, amplifications were done with the primer-pairs EF2143_33_XhoI / EF2143_44_PstI and EF2144_33_EcoRI / EF2144_44_PstI for *ef2143* and *ef2144*

respectively. The resulting PCR products were purified and digested with XhoI for gene *ef2143*, and EcoRI for gene *ef2144*. After ligations were performed as described in chapter III.2.7., the ligated fragments were used as DNA template for a PCR amplification using the primers EF2143_11_BamHI / EF2143_44_Pstl and EF2144_11_BamHI / EF2144_44_Pstl for *ef2143* and *ef2144* respectively. This PCR products were purified, digested using the BamHI and Pstl restriction enzymes and then ligated to the pLT06 vector previously digested with the same endonucleases.

The above described constructions of the pLT06 vector harboring the ΔEF2143 / ΔEF2144 fragments were transformed by electroporation into *E. coli* Top 10 as described in chapter III.2.8.2. To verify that the bacteria contain the pLT06 vector with the desire construction, blue grown colonies were screened by PCR using the primers pLT06_FW and pLT06_RV. After confirmation of the constructions by PCR, the plasmids with inserts of a correct size were extracted using the PureYield™ Plasmid Miniprep System and send to sequencing in order to verify the DNA sequence of the inserts.

III.2.11.2. Targeted mutagenesis of the mutants by gene deletion ΔEF2143 / ΔEF2144 into *E. faecalis* V19 by homologous recombination

After DNA sequencing confirmation, the different constructions were transformed into *E. faecalis* V19 as described in chapter III.2.8.4. The presence of the desire pLT06 construct in blue grown colonies was again verified by PCRs using primer-pairs pLT06_FW and pLT06_RV.

To achieve the desire mutations by double cross over, pLT06 positive blue grown colonies were incubated overnight at 42 °C in BHI supplemented with chloramphenicol at 20 µg/mL. For the next seven days, 10 µL of the ON-culture from the day before were inoculated in 10 mL of fresh BHI pre-warmed at 42 °C containing chloramphenicol at 20 µg/mL, and incubated again overnight at 42 °C.

Starting on day eight with the second cross over with the goal to lose the pLT06 vector, 10 µL of the cultures were re-inoculated in 10 mL of fresh BHI pre-warmed at 30 °C without

antibiotics. Then, they were incubated at 30 °C for 4 hours, followed by incubation at 42 °C overnight. This step was repeated for five to six days.

After this, serial dilutions until 10^{-6} of the cultures were prepared and plated on BHI agar containing X-Gal 40 µg/mL. After incubating the plates overnight at 42 °C, white grown colonies were streak out on BHI agar containing X-Gal 40 µg/mL, as well as on BHI agar containing X-Gal 40 µg/mL and chloramphenicol 20 µg/mL. The plates were again incubated overnight at 42 °C.

The white colonies that grew only in the plates without antibiotic were tested for the presence of the desire mutations and absence of the plasmid. For this propose, primer-pairs EF2143_seq_1_FW / EF2143_seq_3_RV and EF2144_seq_1_FW / EF2144_seq_3_RV were used for PCRs.

III.2.11.3. Construction of the plasmid pLT06 carrying the mutants by single point mutations cEF2143 and cEF2144 of *E. faecalis* V19

For the mutants by single point mutations cEF2143 / cEF2144 of *E. faecalis* V19 different PCRs were performed to change three single nucleotides without changing the encoded amino acid sequence as it is shown in figure 10 in the results section.

Primer-pairs EF2143_5_FW / EF2143_2_RV, EF2144_5_FW / EF2144_2_RV, EF2143_3_FW / EF2143_6_RV, and EF2144_3_FW / EF2144_6_RV were used to amplify fragments of about 1 kb. To induce the three single point mutations in each new construction, primers EF2143_2_RV / EF2144_2_RV and EF2143_3_FW / EF2144_3_FW were ordered already containing the nucleotide changes.

After purification, the PCR products were mixed in 1:1 ratio and diluted 1:10 in nuclease free water. Then, this DNA mix was used as the new DNA template for PCR reactions using the primer-pairs EF2143_1_BamHI / EF2143_4_Pstl and EF2144_1_BamHI / EF2144_4_Pstl. The reactions generated fragments of about 1.7 kb that included the three single point mutations and the restriction sites BamHI and Pstl. The PCR products were

purified, digested using the two restriction enzymes mentioned before, and then ligated to the pLT06 vector previously digested with the same endonucleases.

The above-described constructions of the pLT06 vector harboring the cEF2143 / cEF2144 inserts were transformed by electroporation into *E. coli* Top 10 as described in chapter III.2.8.2. To verify that the bacteria contain the pLT06 vector with the desire construction, blue grown colonies were screened by PCR using the primers pLT06_FW and pLT06_RV. After confirmation of the constructions by PCR, the plasmids with inserts of a correct size were extracted using the PureYield™ Plasmid Miniprep System and send to sequencing in order to verify the correct nucleotide sequence of the cloned DNA.

III.2.11.4. Targeted mutagenesis of the mutants by single point mutations cEF2143 into *E. faecalis* ΔEF2143, and cEF2144 into *E. faecalis* ΔEF2144 by homologous recombination

After DNA sequencing confirmation, the construction of the mutant by single point mutation cEF2143 was transformed into *E. faecalis* ΔEF2143, and the construction cEF2144 was transformed into *E. faecalis* ΔEF2144 as described in chapter III.2.8.4.

The same protocol as described for the targeted mutagenesis of the mutants by gene deletion ΔEF2143 / ΔEF2144 into *E. faecalis* V19 in chapter III.2.11.2. was used with the following modifications:

To verify the first cross over after seven days of inoculating the bacterial cultures every day, PCRs with primer-pairs pLT06_FW with EF2143_6_RV / EF2144_6_RV, as well as pLT06_RV with EF2143_6_RV / EF2144_6_RV were performed. A PCR product in one of these primer-constellations proved the single integration after the first cross over.

Moreover, to confirm the mutants by single point mutation cEF2143 and cEF2144 after the second cross over, primer-pairs EF2143_6_RV / EF2143_7_FW_verif and EF2144_6_RV / EF2144_7_FW_verif were used for PCRs. Additionally, the mutations were confirmed by DNA sequencing.

III.2.12. Growth behavior of *E. faecalis* V19 and the mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144

The following protocol was performed to test the growth characteristics of the wild type (WT) *E. faecalis* V19 and the designed mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144. BHI agar plates were inoculated with 10 µL of the respective bacterial culture and grown overnight at 37 °C. On the next day, a single colony of the bacterial strain to be tested was inoculated in 5 mL TSB and grown at 37 °C. After 18 hours, 2 mL of the culture were centrifuged at 4 °C and 8000 rpm for 4 minutes. The supernatant was discarded, the bacteria were resuspended in 2 mL of PBS (Dulbecco's Phosphate Buffered Saline, Biochrom, Germany), and the bacterial suspension was then inoculated in 7 mL of fresh TSB until an OD 600 nm of 0.05 was reached. The exact compositions of the PBS used are listed in table 10. Growth of the bacteria was measured with the GENESYS™ 20 Vis-Spectrophotometer (Thermo Fisher Scientific, USA) at 600 nm every hour for 8 hours. The experiment was performed three times.

Table 10. Exact composition of the Phosphate Buffered Saline.

Dulbecco´s Phosphate Buffered Saline (Biochrom, Germany)	
NaCl	8000 mg/l
KCl	200 mg/l
Na_2HPO_4	1150 mg/l
KH_2PO_4	200 mg/l
$MgCl_2$ $6H_2O$	100 mg/l
$CaCl_2$	100 mg/l

III.2.13. Release of pp5 from *E. faecalis* V19, *E. faecalis* ΔEF2143 and *E. faecalis* ΔEF2144

The following protocol from Matos *et al*. was performed to test if the mutants *E. faecalis* ΔEF2143 / ΔEF2144 were still able to release the pp5 after the mutations[32]. The *E. faecalis* V19 was used as a positive control.

Briefly, 7 µL of the frozen stock of the bacterial strain to be tested were inoculated in 7 mL of BHI and incubated overnight at 37 °C. The next day, 500 µL of the overnight cultures were reinoculated in 8 mL of fresh BHI supplemented with 1 mM $MgSO_4$ (Sigma-Aldrich, USA) and 1 mM $CaCl_2$ (Sigma-Aldrich, USA) and incubated for 1.5 hours at 37 °C until an OD 600 nm of 0.17 – 0.23. Then, Mitomycin C (in the following MMC, Carl Roth, Germany) to a final concentration of 4 µg/mL was added to each sample and the cultures were again incubated at 28 °C for 4 hours. The bacterial cultures were then centrifuged for 5 min at 4000 rpm and the supernatants were filtered with a 0.22 µL filter (Carl Roth, Germany) and checked for the pp5 release by different PCRs.

Primer pair pp5_C_FW / pp5_C_RV was used to check for the pp5 in the bacterial supernatants, while primer-pair pp5_A_FW / pp5_A_RV was used to verify the release of pp5 from *E. faecalis* V19 / ΔEF2143 / ΔEF2144. For both primer-pairs used, PCRs products of about 500 bp were expected. Additional PCRs with primers pp5_attP_FW and pp5_attP_RV with location at the attP sites were made to verify the presence of circularized DNA of pp5 in the bacterial cultures. PCR products of about 1400 bp were expected.

III.2.14 Transduction of pp5 into *E. faecalis* V19pp- and *E. faecalis* V19pp3+

To test if transduction of pp5 into *E. faecalis* V19 cured from all seven phages (*E. faecalis* pp-) and *E. faecalis* V19 containing only phage 3 (*E. faecalis* pp3+) was possible, different protocols with some changes following Yasmin *et al.* and Rossmann *et al.* were performed[11,50]. The WT or the respective mutants by gene deletion *E. faecalis* ΔEF2143 and ΔEF2144 were used as phage-donor strains. *E. faecalis* pp- and *E. faecalis* pp3+ were used as phage-acceptor strains.

The first steps are similar to the experiment for the release of pp5 and can be found in chapter III.2.13. In contrast to the steps described in chapter III.2.13., the phage-donor strains were grown for 2 hours at 37 °C until an OD 600 nm of 0.17 – 0.2 prior induction with MMC. BHI soft agar (0.32 % agar supplemented with 10 mM $MgSO_4$ and 10 mM $CaCl_2$) was prepared and prewarmed at 42 °C.

Different dilutions of the supernatants from the respective phage-donor strain with phage buffer (for full composition of the buffer see table 11 below) ranging from 10^{-1} to 10^{-3} were prepared. The phage-acceptor stains were currently grown until an OD 600 nm of 0.17 – 0.23. After, 50 µL of the respective phage-acceptor strain and 100 µL of the phage-donor stain were pipetted into 5,5 mL of the prewarmed soft agar, plated on BHI plates and rest at room temperature for 1.5 hours prior incubation overnight at 37 °C. To check for the favored transduction, 14 plaques were randomly selected for each condition using a 10 µL pipette tip to take the plaque resuspending it on 30 µL of phage buffer. Later, 1µL of the phage solution was used as PCR template using primer-pair pp5_A_FW and pp5_B_RV. A PCR amplification with and an expected size of about 550 bp indicates a positive transduction of pp5 into the different acceptor strains.

Table 11. Exact composition of the phage buffer.

Phage buffer	
NaCl	150 mM
$MgSO_4$	10 mM
Tris	40 mM
pH	7.4

III.2.15 Biofilm formation of *E. faecalis* V19 and the mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144

To test the biofilm formation of the *E. faecalis* V19 and its corresponding mutants and complementation's *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144, the strain to be tested was grown on TSA-S blood plates at 37 °C overnight. On the next day, a bacterial suspension was prepared in PBS from the colonies in the agar plates to prepare a dilution at an OD 660 nm of 0.01 in TSBg (TSB + glucose 1.0 %). Then, 100 µL of the bacterial suspension were added per well on 96-well-plates (Sarstedt, Germany). AI-2 was added if intended to each sample up to a concentration of 300 µM - 500 µM. The plates were sealed before incubation overnight at 37 °C.

On the next day, the media was removed, and the wells were washed two times with 195 µL PBS. Then, 170 µL of Chrystal violet (Sigma-Aldrich, USA) 0.2 % were added to each well and incubated for 15 min at 37 °C before washing again two times with 195 µL PBS. After, 170 µL of ethanol 96 % (Sigma-Aldrich, USA) were added, the plates were sealed and incubated at room temperature for 60 min. The biofilm formation was measured by ELISA at an OD 595 nm with the Synergy H1 Hybrid Multi-Mode Reader (BioTek, USA). TSB + glucose 1.0 % was used as a negative control.

III.2.16. Production of cytokine TNF-α by *E. faecalis* V19 and the mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144

The following protocol was performed to determine whether different genes in the pp5 from *E. faecalis* V19, specifically the genes *ef2143* and *ef2144*, are involved in virulence and inflammatory response. The supernatants of the different *E. faecalis* mutants ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144 were evaluated for cytokine TNF-α secretion after incubation with freshly isolated human Peripheral Blood Mononuclear Cells (PBMCs).

The different *E. faecalis* strains were grown overnight at 37 °C without agitation in TSB media. On the next day, 500 µL of the overnight culture were diluted in 6,5 mL fresh TSB and grown 2.5 hours at 37 °C until an OD 600 nm of 0.18-0.23 was reached. MMC was added at a final concentration of 4 µg/mL prior incubation for 2 hours at 37 °C. After, the cultures were centrifuged for 15 min at 4000 rpm and 4 °C. The supernatants were filtered with a 0.22 µl filter and frozen at -20 °C.

For isolation of PBMCs, 13 mL of peripheral blood from a healthy human donor were collected in Sodium-Heparin tubes (Sarstedt, Germany) and mixed with one volume of PBS. 15 mL of ficoll (Sucrose-epichlorohydrin-copolymer, GE Healthcare, Sweden) were laid in a 50mL falcon tube (Sarstedt, Germany) and 26 mL of the mixture blood/PBS were carefully added on top without disturbing the ficoll phase. The mixture was centrifuged for 30 min at 693 xg and 20 °C without a break. After, the white cell interface was carefully collected and poured into a new tube, avoiding the collection of blood plasma and the ficoll. Collected

cells were washed twice with 50mL of PBS by centrifuging for 10 min at 20 °C with decreasing centrifugal force (first time at 443 xg and second time at 249 xg). The cell pellet was resuspended in 1 mL Dulbecco´s Modified Eagle Medium with GlutaMAX (DMEM Media - GlutaMAX™-I, Gibco, USA), supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Gibco, USA) and Penicillin-Streptomycin (Gibco, USA) up to a final concentration of 50 U/mL. The total amount of PBMCs isolated were enumerated in a Neubauer chamber. For stimulation, 800 µL of cells at $9.47x10^5$ cells/mL were seeded per well on a 24-well plate (Sarsetdt, Germany). The bacterial supernatants (200 µL) were added to each well and incubated for 24 hours at 37 °C in a humidified 5 % CO_2 atmosphere. On the next day, the plate was centrifuged at 500 xg for 5 min and 800 µL of the supernatants were collected. The supernatants stimulated PBMCs were analyzed by commercial human TNF-α ELISA (TNF alpha Human ELISA Kit, Invitrogen, USA) following the manufacturer´s instructions. LPS (Biosearch Technologies, UK) at a final concentration of 50 ng/mL was used as a positive control.

III.2.17. Influence of *E. faecalis* V19 and the mutants *E. faecalis* ΔEF2143 / ΔEF2144 on the bacterial virulence using the animal model *Galleria mellonella*

The following protocol according to Reffuveille *et al.* was used to investigate the virulence of the designed deletion mutants *E. faecalis* ΔEF2143 / ΔEF2144[68]. The larvae of the great wax moth *Galleria mellonella* were used as a virulence model, since they are known as a good model system for the investigation of potential virulence factors[69]. Wildtype *E. faecalis* V19 was used as control.

The different *E. faecalis* strains were grown overnight at 37 °C without agitation in GM17 media (Exact compositions are listed in table 7). On the next day, larvae (about 0.3 g and 3 cm in length) of *Galleria mellonella* were infected subcutaneously with the washed *E. faecalis* strain using a syringe pump (KD Scientific, USA). In the process, 1.7 - 2.3 x 10^6 CFU in 10 µl of sterile saline buffer were injected per larvae prior incubation at 37 °C. 30 larvae per tested bacterial mutant were infected. Finally, the larvae mortality rate was monitored each hour from 17 to 24 hours post infection.

III.2.18 Statistical evaluations

The program PRISM version 5.00 (GraphPad Software, USA) was used for the statistical evaluations. The growth of biofilm formation in dependence of AI-2 were investigated with the non-parametric Man-Whitney-U-Test. For the TNF-α detection the non-parametric Kruskal-Wallis-Test followed by non-parametric Dunn´s post-test was used. The non-parametric Log-Rank-Test was used for the statistical evaluation to investigate the bacterial virulence using *Galleria mellonella*. For all experiments p-values of ≤ 0.05 have been defined as statistically significant.

IV. Results

IV.1. Construction and targeted mutagenesis of the mutants by gene deletion *E. faecalis* ΔEF2143 and *E. faecalis* ΔEF2144

IV.1.1. Construction of the pLT06::ΔEF2143 vector carrying the insert for the *ef2143* gene deletion

To generate an in-frame deletion mutant of the gene *ef2143* from *E. faecalis* V19, 393 bp out of 468 bp of the gene (83,97 %) were deleted. For this, different PCRs were preformed to create a plasmid that contained the mutated gene as described in the materials and methods section.

Figure 5 illustrates the procedure for the construction of the mutants by gene deletion ΔEF2143 and ΔEF2144 as well as the relative position of the primers used.

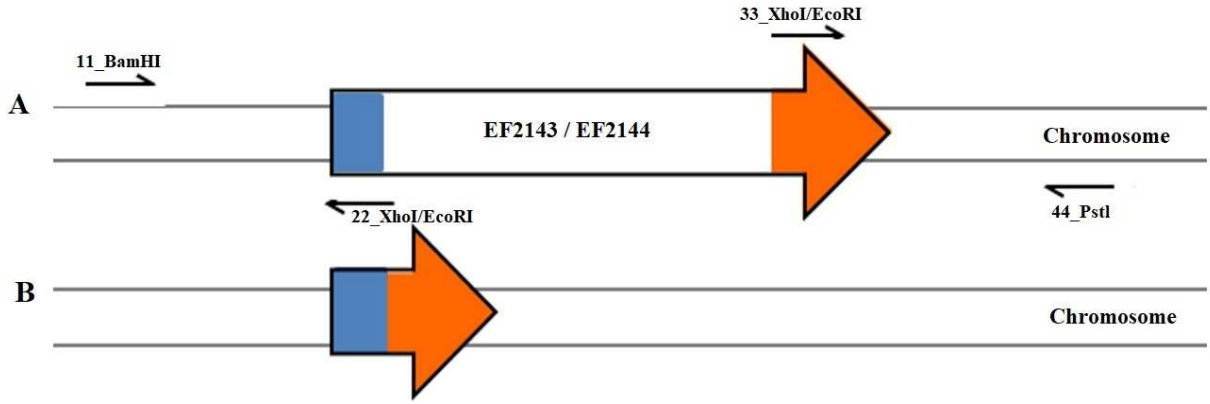

Figure 5. Mutagenesis strategy for the construction of the mutants by gene deletion ΔEF2143 and ΔEF2144 from *E. faecalis* V19. (A) Scheme of gene *ef2143* / *ef2144* from *E. faecalis* V19 before the mutation by gene deletion. The area to be deleted is shown as white box between the orange and blue areas. The relative positions of the used primers are illustrated. **(B)** Scheme of genes ΔEF2143 / ΔEF2144 after deleting about 80% of the gene.

A DNA fragment of 1111 bp upstream of the region to be deleted was created by PCRs with primers EF2143_11_BamHI and EF2143_22_XhoI, using as DNA template the genomic DNA of *E. faecalis* V19. The downstream fragment of 1003 bp was created with primers

EF2143_33_XhoI and EF2143_44_PstI using the same genomic DNA as template. The resulting DNA amplifications are shown in figure 6 (A).

The two fragments were purified, digested, ligated into pLT06, and electroporated into *E. coli* Top 10 as described in the materials and methods section. After transformation, the correct size of the DNA template inserted into the pLT06 vector was verified using primer-pair pLT06_FW and pLT06_RV, and as DNA template a suspension of the colonies that grew in presence of chloramphenicol. A PCR product of 2507 bp was obtained as it is shown in figure 6 (B), confirming the correct incorporation of the insert in the plasmid. The correct sequence of the fragment was also confirmed by DNA sequencing (data not shown).

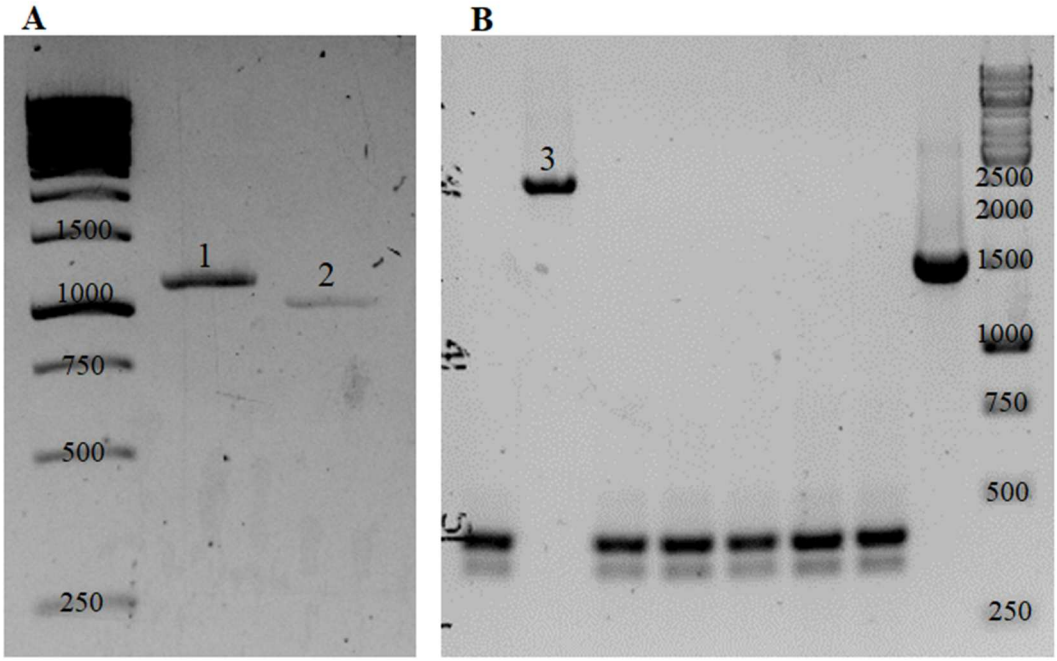

Figure 6. **Different electrophoresis agarose gels for the construction of the pLT06 vector carrying the insert ΔEF2143.** The band sizes from the GeneRuler 1 kb DNA ladder are marked. **(A)** PCR amplifications of the upstream and downstream fragments surrounding the area to be deleted. Band **1** with the expected size of 1111 bp made with using the primers EF2143_11_BamHI and EF2143_22_XhoI. Band **2** was made with primers EF2143_33_XhoI and EF2143_44_PstI with an expected size of 1003 bp. **(B)** PCR amplifications to verify the size of the desire construction after transformation into *E. coli* Top 10. Band **3** shows the expected size of 2507 bp and was amplified using the primers pLT06_FW and pLT06_RV.

IV.1.2. Construction of the pLT06::ΔEF2144 vector carrying the insert for the *ef2144* gene deletion

682 bp out of 861 bp (79,21 %) of the gene *ef2144* from *E. faecalis* V19 were deleted to generate an in-frame deletion mutant. To construct a plasmid that contains the mutated gene, different PCRs were performed as described in the text above and shown in figure 5.

A DNA fragment of 1068 bp upstream of the region to be deleted was created by PCRs with primers EF2144_11_BamHI and EF2144_22_EcoRI, using as DNA template the genomic DNA of *E. faecalis* V19. The downstream fragment of 1171 bp was created with primers EF2144_33_EcoRI and EF2144_44_PstI using the same genomic DNA as template. The resulting DNA amplifications are shown in figure 7 (A).

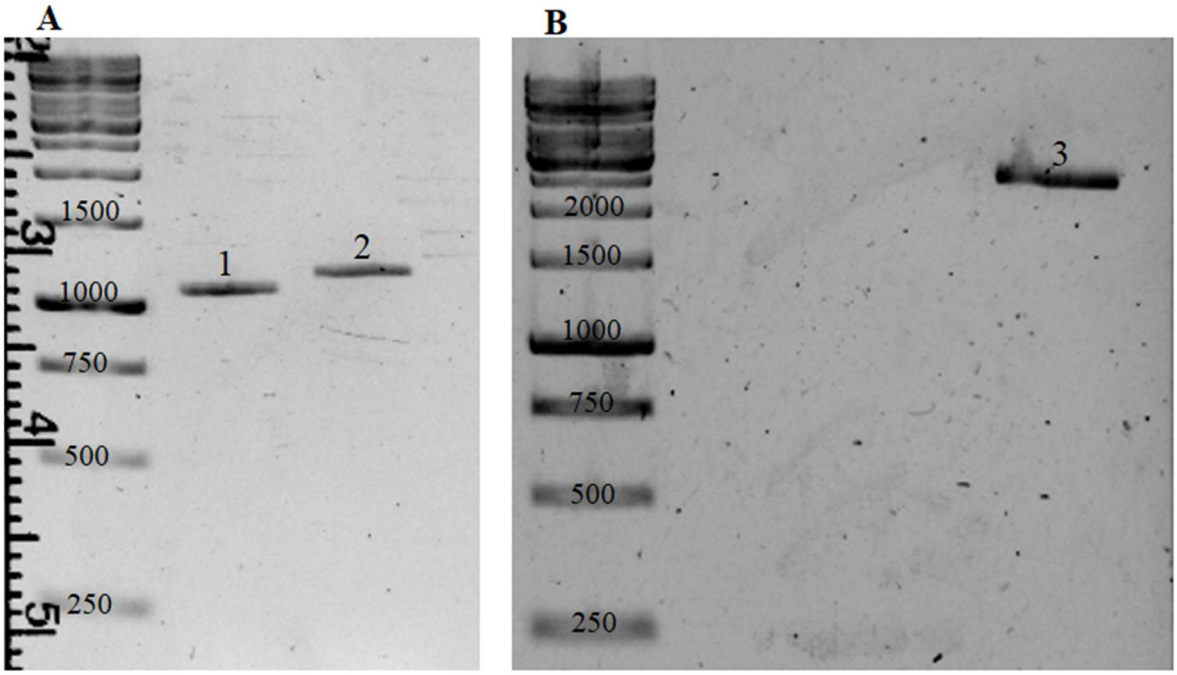

Figure 7. Different electrophoresis agarose gels for the construction of the pLT06 vector carrying the insert ΔEF2144. The band sizes from the GeneRuler 1 kb DNA ladder are marked. **(A)** PCR amplifications of the upstream and downstream fragments surrounding the area to be deleted. Band **1** with the expected size of 1068 bp made with using the primers EF2144_11_BamHI and EF2144_22_EcoRI. Band **2** was made with primers EF2144_33_EcoRI and EF2144_44_PstI with an expected size of 1171 bp. **(B)** PCR amplifications to verify the size of the desire construction after transformation into *E. coli*

Top 10. Band **3** shows the expected size of 2239 bp and was amplified using the primers pLT06_FW and pLT06_RV.

The following steps were performed as described in the text above: The two fragments were purified, digested, ligated into pLT06 and electroporated into *E. coli* Top 10. The correct size of the inserted DNA was verified using primer-pair pLT06_FW and pLT06_RV using as DNA template a suspension of the colonies that grew in presence of chloramphenicol. A PCR product of 2239 bp was obtained as it is shown in figure 7 (B). The correct sequence of the fragment was also confirmed by DNA sequencing (data not shown).

IV.1.3. Targeted mutagenesis of the mutants by gene deletion ΔEF2143 / ΔEF2144 into *E. faecalis* V19 by homologous recombination

To generate in-frame deletion mutants, the pLT06 vectors carrying the constructions ΔEF2143 or ΔEF2144 were transformed into *E. faecalis* V19. Figure 8 shows the schematic of the targeted mutagenesis into *E. faecalis* V19 by double cross over, using the pLT06 vector as plasmid.

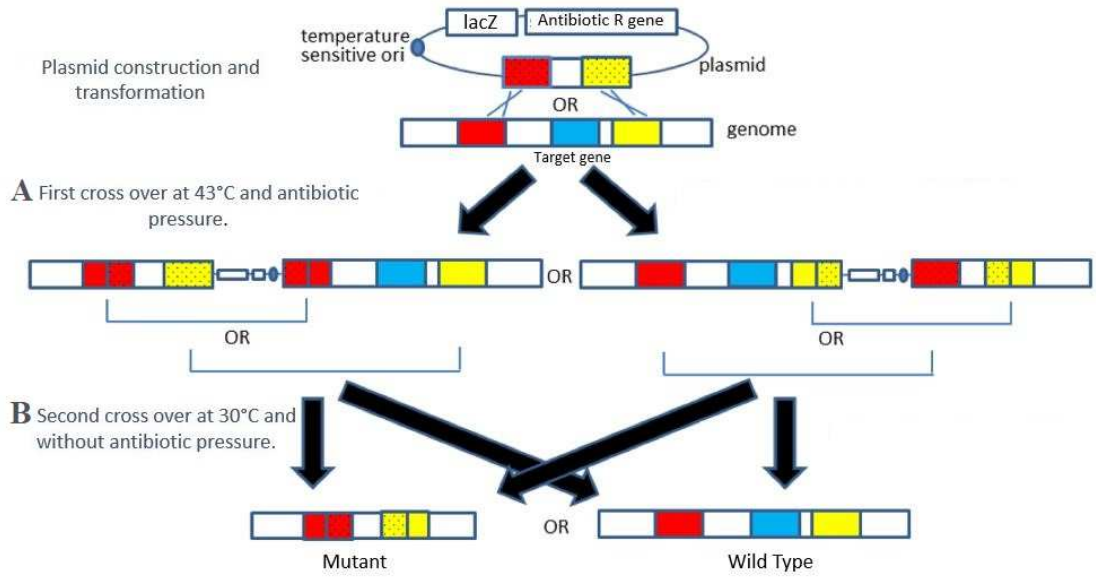

Figure 8. Scheme of the targeted mutagenesis by homologous recombination [70]. The blue boxes are the regions to be deleted. The red and yellow boxes are the open reading

frames upstream and downstream of the regions to be deleted. **(A)** Forcing chromosomal integration of the plasmid at the first cross over by growing the bacteria at high temperature and under antibiotic pressure. **(B)** The second cross over is performed by growing the bacteria at lower temperature without antibiotic pressure, promoting the excision of the plasmid from the chromosome and leaving either the mutated or WT allele.

The first cross over was performed to promote the integration of the pLT06 vector carrying the desired constructs ΔEF2143 / ΔEF2144 into the bacteria's chromosome by homologous recombination. For this, incubation at 42 °C and antibiotic selection with chloramphenicol was used, as only bacteria with the plasmid integrated in their chromosome were able to survive antibiotic selection. To get rid of the plasmid, the second cross over was performed by growing bacteria at 30 °C without antibiotics. To confirm that the targeted mutagenesis took place and bacteria were cured of plasmid pLT06, several dilutions from overnight cultures of the second cross-over were examined by blue-white-screening as described in the materials and methods section.

Once white colonies were obtained that were not able to grow in presence of the chloramphenicol, the expected mutants by gene deletion or the WT strains were confirmed by PCR.

To verify the mutant *E. faecalis* ΔEF2143, PCRs using primers EF2143_seq_1_FW and EF2143_seq_3_RV were performed. The expected mutant presented a band size of 651 bp, while the bacterial WT without deletion of a part of gene *ef2143* had a band size of 1044 bp, as it is shown in figure 9 (A).

The mutant *E. faecalis* ΔEF2144 was verified by PCRs using primers EF2144_seq_1_FW and EF2144_seq_3_RV as shown in figure 9 (B). The expected mutant showed a band size of 850bp, while the WT had a band size of 1532 bp.

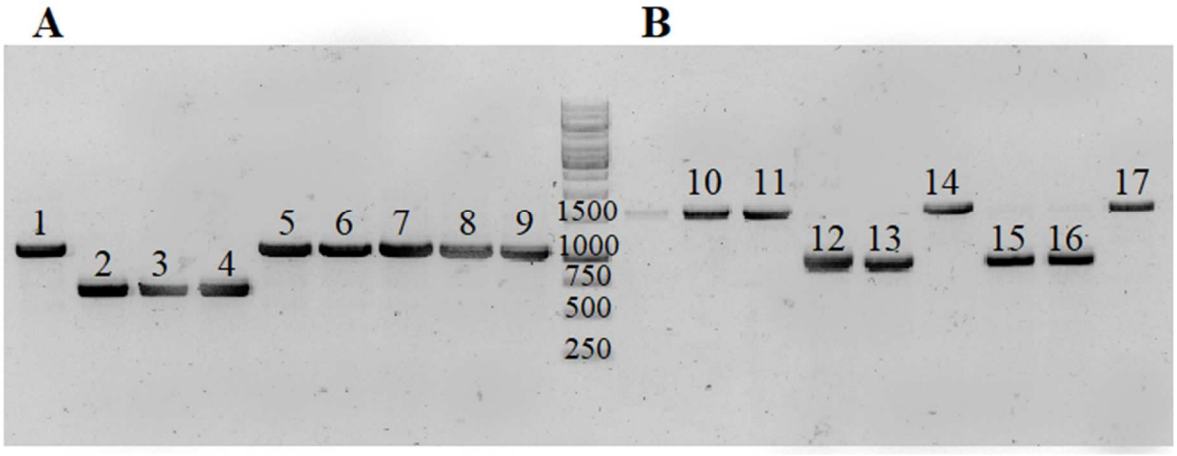

Figure 9. Different electrophoresis agarose gels of the mutants by gene deletion *E. faecalis* ΔEF2143 and *E. faecalis* ΔEF2144. The band sizes from the GeneRuler 1 kb DNA ladder are marked. **(A)** PCRs to verify the mutant *E. faecalis* ΔEF2143 with primers EF2143_seq_1_FW and EF2143_seq_3_RV. Bands **2, 3, 4** with an expected size of 651 bp correspond to the ΔEF2143 mutant. Bands **1, 5, 6, 7, 8, 9** with an expected size of 1044 bp are still the *E. faecalis* V19 WT. **(B)** PCRs to verify the mutant *E. faecalis* ΔEF2144 with primers EF2144_seq_1_FW and EF2144_seq_3_RV. Bands **12, 13, 15, 16** with an expected size of 850 bp correspond to the ΔEF2144 mutant. Bands **10, 11, 14, 17** with an expected size of 1532 bp are the *E. faecalis* V19 WT.

IV.2. Construction and targeted mutagenesis of the mutants by single point mutations *E. faecalis* cEF2143 and *E. faecalis* cEF2144

IV.2.1. Construction of the pLT06::cEF2143 vector carrying the insert for the mutant by single point mutation cEF2143

The pLT06 vector was used to construct the mutant by single point mutations cEF2143 of *E. faecalis* V19. Three single base changes were introduced by PCR without changing the corresponding amino acids encoded by the gene *ef2143*. Figure 10 shows the procedure used for the construction of the mutants by single point mutations *E. faecalis* cEF2143 and *E. faecalis* cEF2144, as well as the relative positions of the primers used.

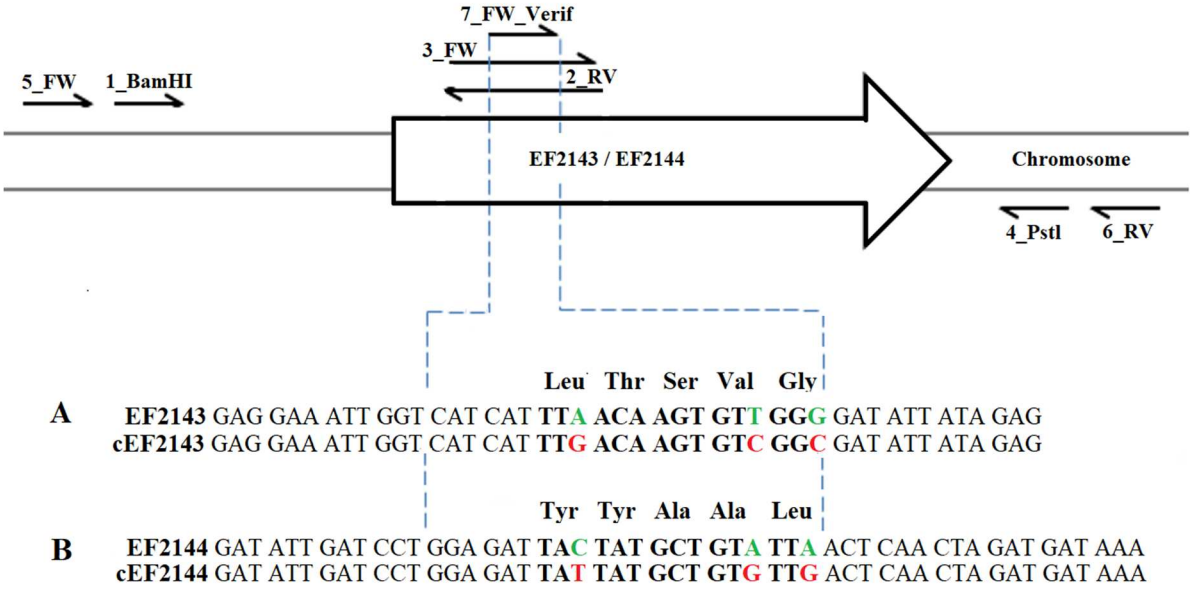

Figure 10. Mutagenesis strategy for the construction of the mutants by single point mutations *E. faecalis* cEF2143 and *E. faecalis* cEF2144. The relative positions of the used primers are marked. The sequence of primer EF2143_7_FW_Verif / EF2144_7_FW_Verif is illustrated with a blue line. The three original bases to be changed per gene are marked in green, the three single base changes after mutation without changing the corresponding amino acid sequence are illustrated in red. **(A)** Extract from gene *ef2143* and mutant cEF2143. **(B)** Extract from gene *ef2144* and mutant cEF2144.

A DNA fragment of 1063 bp including the focused area of the three single base changes and the upstream area of this region was created by PCRs with primers EF2143_5_FW and EF2143_2_RV, using as DNA template the genomic DNA of *E. faecalis* V19. The fragment of 955 bp including the area of the three single base changes and the downstream area of this region was made with primers EF2143_3_FW and EF2143_6_RV using the same genomic DNA as template. Both PCR results are shown in figure 11 (A). To insert the single base changes in the PCR products, primers EF2143_2_RV and EF2143_3_FW were used with these three base changes.

The two fragments were purified, mixed in 1:1 ratio and used as DNA template for PCRs with primers EF2143_1_BamHI and EF2143_4_PstI to insert the restriction enzymes BamHI and PstI. This PCRs resulted with a fragment of 1740 bp as it is shown in figure 11

(B). After purification, digestion and ligation with the pLT06 vector, the construction was electroporated into *E. coli* Top 10.

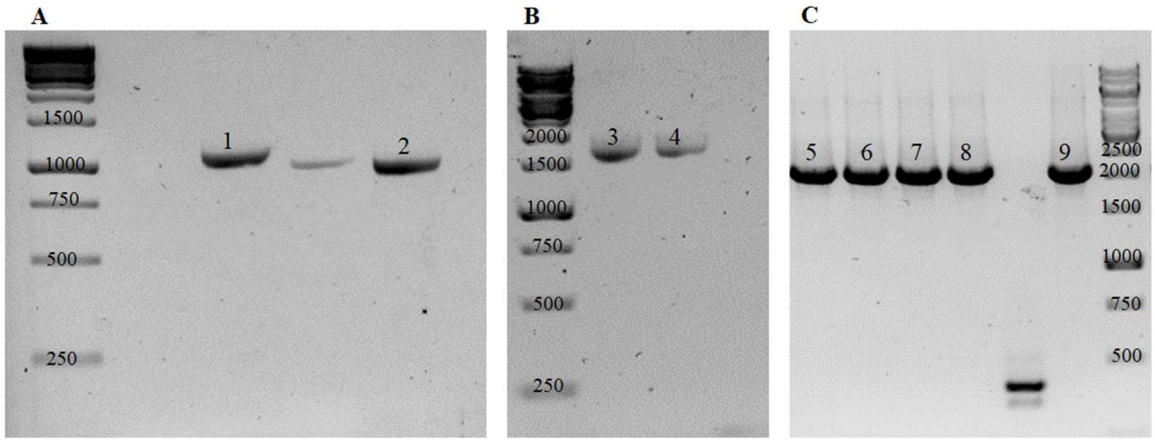

Figure 11. Different electrophoresis agarose gels for the construction of the pLT06 vector carrying the insert cEF2143. The band sizes from the GeneRuler 1 kb DNA ladder are marked. **(A)** PCR amplifications of the upstream and downstream fragments, each including the three single base changes. Band **1** with the expected size of 1063 bp made with using the primers EF2143_5_FW and EF2143_2_RV. Band **2** was made with primers EF2143_3_FW and EF2143_6_RV with an expected size of 955 bp. **(B)** PCR amplifications to insert the restriction enzymes BamHI and PstI. Band **3** and **4** show the expected size of 1740 bp and were amplified using the primers EF2143_1_BamHI and EF2143_4_PstI. **(C)** PCR amplifications to verify the construction of the pLT06 vector carrying the insert for the mutant by single point mutations cEF2143 in *E. coli* Top 10. Band **5-9** with the expected size of 2113 bp made with using the primers pLT06_FW and pLT06_RV.

After transformation, confirmation of the correct incorporation of the DNA inserted into pLT06 was verified using primers pLT06_FW and pLT06_RV. A suspension of the colonies that grew in presence of chloramphenicol were used as DNA template, and a PCR product of 2113 bp was obtained as it is shown in figure 11 (C). The correct sequence of the three single point mutations in gene *ef2143* from *E. faecalis* V19 was also confirmed by DNA sequencing at it is shown in figure 12.

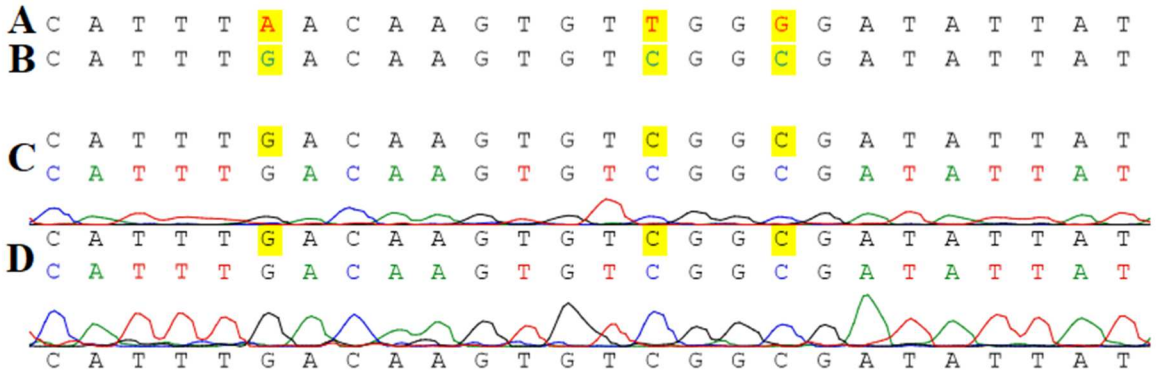

Figure 12. DNA sequencing to verify the mutant cEF2143. Three single base changes illustrated in yellow/black were made without changing the corresponding amino acid encoded. **(A)** DNA sequence extract from gene *ef2143* of *E. faecalis* V19. The three single bases before mutation are marked in yellow/red. **(B)** Expected DNA sequence extract from mutant cEF2143 with the three base changes marked in yellow/green. **(C/D)** DNA sequence extract from mutant cEF2143 sequencing results to verify the three single base changes which are marked in yellow/black.

IV.2.2. Construction of the pLT06::cEF2144 vector carrying the insert for the mutant by single point mutation cEF2144

A schematic of the mutant cEF2144 with three single point mutations without changing the corresponding amino acids encoded by the gene *ef2144* is shown in figure 10. The construction of the pLT06 vector carrying the insert for mutant cEF2144 was achieved as described for mutant cEF2143 the text above with the following changes:

A DNA fragment of 968 bp including the area with the three single base changes and the upstream area of this region was created by PCRs with primers EF2144_5_FW and EF2144_2_RV, using the genomic DNA of *E. faecalis* V19 as DNA template. The fragment of 1003 bp including the focused area of the three single base changes and the downstream area of this region was made with primers EF2144_3_FW and EF2144_6_RV. Both PCR products are shown in figure 13 (A). To insert the three base changes in the PCR products, primers EF2144_2_RV and EF2144_3_FW were used with these three base changes.

The two fragments were purified, mixed in 1:1 ratio and used as DNA template for PCRs with primers EF2144_1_BamHI and EF2144_4_Pstl to insert the restriction enzymes BamHI and Pstl. This PCRs resulted with a fragment of 1680 bp as it is shown in figure 13 (B). After purification, digestion and ligation with the pLT06 vector, the construct was electroporated into *E. coli* Top 10.

After transformation, confirmation of the correct incorporation of the DNA inserted into pLT06 was verified using primers pLT06_FW and pLT06_RV. A suspension of the colonies that grew in presence of chloramphenicol were used as DNA template, and a PCR product of 2073 bp was obtained as it is shown in figure 13 (C).

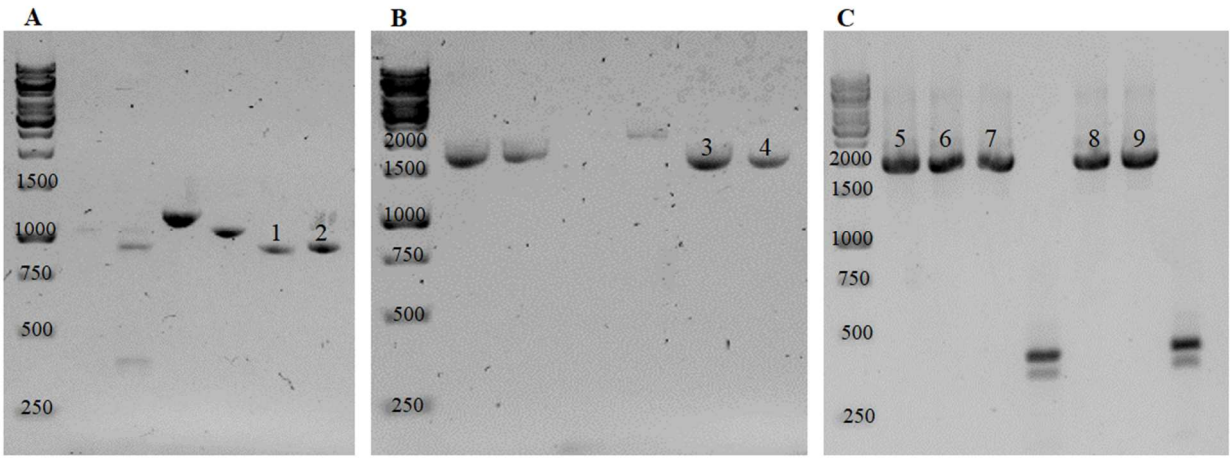

Figure 13. Different electrophoresis agarose gels for the construction of the pLT06 vector carrying the insert cEF2144. The band sizes from the GeneRuler 1 kb DNA ladder are marked. **(A)** PCR amplifications of the upstream and downstream fragments, each including the three single base changes. Band **1** with the expected size of 968 bp made with using the primers EF2144_5_FW and EF2144_2_RV. Band **2** was made with primers EF2144_3_FW and EF2144_6_RV with an expected size of 1003 bp. **(B)** PCR amplifications to insert the restriction enzymes BamHI and Pstl. Band **3** and **4** show the expected size of 1680 bp and were amplified using the primers EF2144_1_BamHI and EF2144_4_Pstl. **(C)** PCR amplifications to verify the construction of the pLT06 vector carrying the insert for the mutant by single point mutations cEF2144 in *E. coli* Top 10. Band **5-9** with the expected size of 2073 bp made with using the primers pLT06_FW and pLT06_RV.

The correct sequence of the three single point mutations in gene *ef2144* from *E. faecalis* V19 was also confirmed by DNA sequencing at it is illustrated in figure 14.

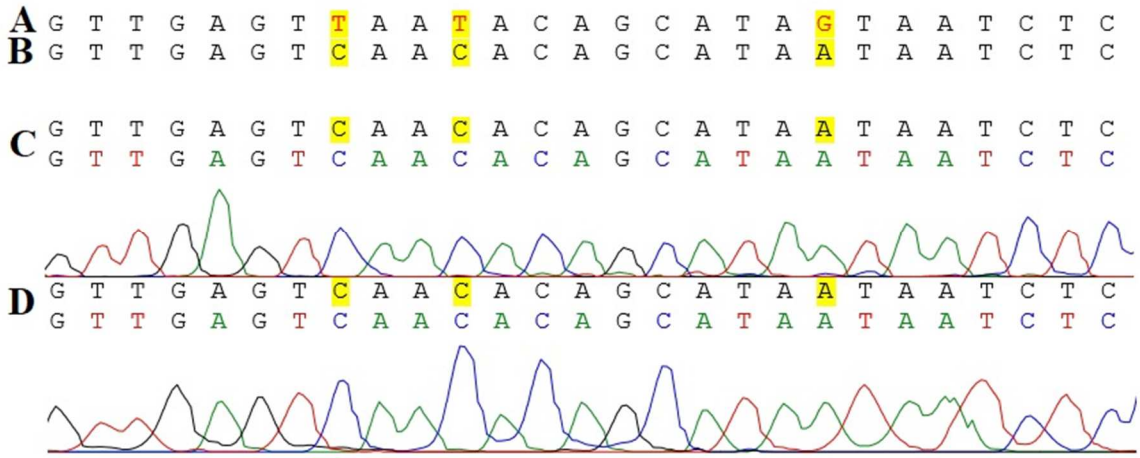

Figure 14. DNA sequencing to verify the mutant cEF2144. Three single base changes illustrated in yellow/black were made without changing the corresponding amino acid encoded. **(A)** DNA sequence extract from gene *ef2144* of *E. faecalis* V19. The three single bases before mutation are marked in yellow/red. **(B)** Expected DNA sequence extract from mutant cEF2144. **(C/D)** DNA sequence extract from mutant cEF2144 sequencing results to verify the three single base changes.

IV.2.3. Targeted mutagenesis of the mutants by single point mutations cEF2143 into *E. faecalis* ΔEF2143, and cEF2144 into *E. faecalis* ΔEF2144 by homologous recombination

After DNA sequencing confirmation, pLT06 carrying the construct cEF2143 was transformed into *E. faecalis* ΔEF2143, and the pLT06 vector carrying the construct cEF2144 was transformed into *E. faecalis* ΔEF2144.

The first cross over was performed at high temperature (42 °C) and under antibiotic selection with chloramphenicol. As the schematic of the targeted mutagenesis in figure 8 illustrates, the integration of the pLT06 vector into the bacteria's chromosome can take place in two different directions, both were tested by different PCRs. For the pLT06 vector carrying the construct of mutant cEF2143, primer-pairs pLT06_FW with EF2143_6_RV, and pLT06_RV with EF2143_6_RV were used. For the pLT06 vector carrying the construct of the mutant cEF2144, primer-pairs pLT06_FW with EF2144_6_RV, and pLT06_RV with

EF2144_6_RV were used. A PCR product in one of these primer-constellations confirmed the single integration after the first cross over, as shown in figure 15 (A, B).

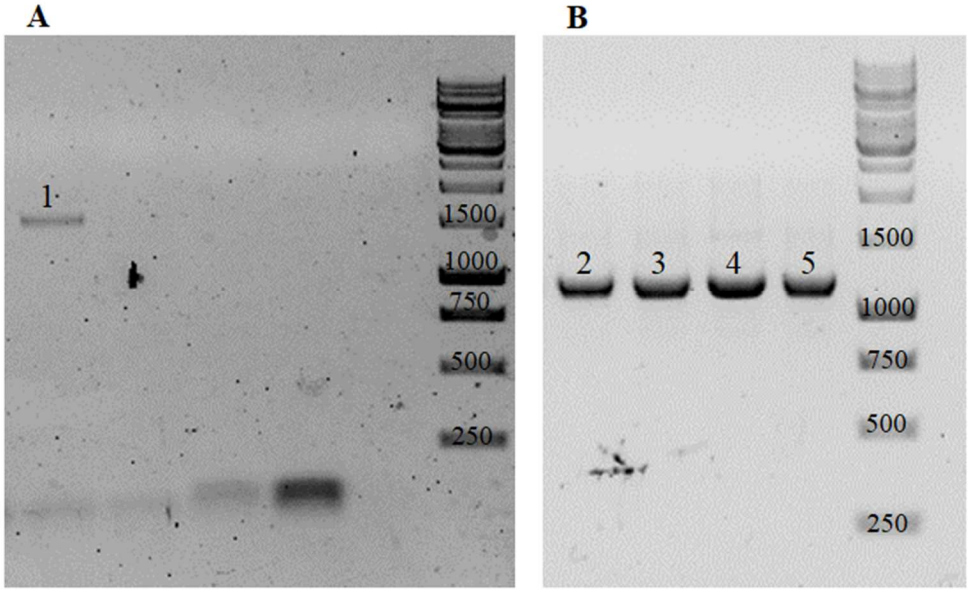

Figure 15. Different electrophoresis agarose gels to verify the single integration of the pLT06 vector carrying cEF2143 / cEF2144 after the first cross over of targeted mutagenesis by homologous recombination. The band sizes from the GeneRuler 1 kb DNA ladder are marked. **(A)** PCR amplification of the integration from the pLT06 vector carrying the construction for mutant cEF2143. Band **1** made with using primers pLT06_FW and EF2143_6_RV. **(B)** PCR amplification of the integration from the pLT06 vector carrying the construction for mutant cEF2144. Bands **2-5** made with using primers pLT06_FW and EF2144_6_RV.

To remove the plasmid, the second cross over was performed by incubation at lower temperature (30 °C) and without antibiotics. To test if the targeted mutagenesis was successful, cultures of the second cross-over were examined by blue-white-screening. Once white colonies that were not able to grow in presence of the chloramphenicol were obtained, several PCRs were done to verify if they were the expected mutants cEF2143 / cEF2144 to complement the deletion mutants.

To verify the mutant *E. faecalis* cEF2143, PCRs using primers EF2143_seq_1_FW and EF2143_seq_3_RV were performed, as shown in figure 16 (A). A PCR product of 1044 bp was obtained for the expected mutant cEF2143, while the mutant by gene deletion ΔEF2143 without the desire construction presented with a band size of 651 bp. The mutant *E. faecalis*

cEF2144 was verified by PCRs using primers EF2144_seq_1_FW and EF2144_seq_3_RV, as shown in figure 16 (B). A PCR product of 1532 bp was obtained for the expected mutant cEF2144, while the mutant by gene deletion ΔEF2144 without the desired construct demonstrated a band of 850 bp. The two mutants with single point mutations were also confirmed by DNA sequencing (data not shown).

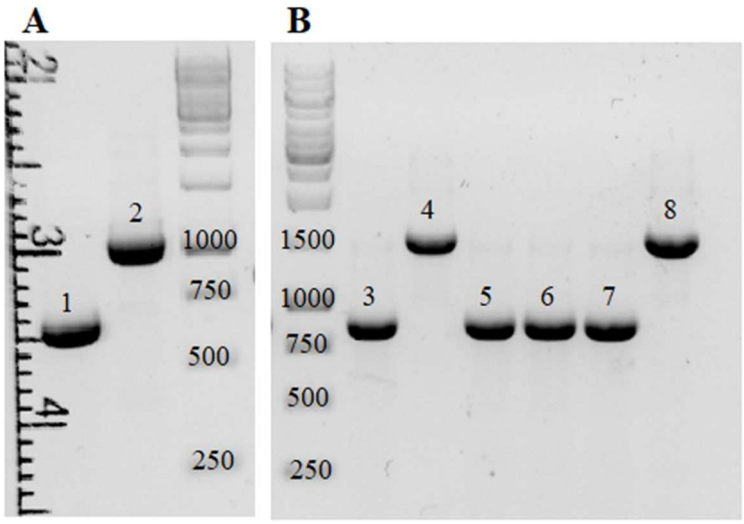

Figure 16. Different electrophoresis agarose gels to verify the complementation mutants by three single point mutations cEF2143 and cEF2144. The band sizes from the GeneRuler 1 kb DNA ladder are marked. **(A)** PCR amplifications to verify the mutant *E. faecalis* cEF2143 with using the primers EF2143_seq_1_FW and EF2143_seq_3_RV. Band **1** without the desire construction and the size from the mutant by gene deletion ΔEF2143 with a product of 651 bp and band **2** with the expected size of 1044 bp corresponding to the cEF2143 mutant. **(B)** PCR amplifications to verify the mutant *E. faecalis* cEF2144 with using the primers EF2144_seq_1_FW and EF2144_seq_3_RV. Bands **4** and **8** with the expected size of 1532 bp for the complementation mutant cEF2144, Bands **3, 5, 6** and **7** without the desire construction and the size of 850 bp corresponding to the ΔEF2144 mutant.

IV.3. Growth of *E. faecalis* V19 and mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144

To test the growth behavior of *E. faecalis* V19 and the mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144, a single colony of the respective bacterial strain was inoculated in TSB and incubated for 18 hours at 37 °C. Afterwards, the culture was

centrifuged, the bacteria were resuspended in PBS and inoculated in fresh TSB until an OD 600 nm of 0.05. Growth of the bacteria at 37 °C was measured every hour for 8 hours. The experiment was performed in triplicate.

Figure 17 shows the growth behavior of the tested bacterial strains showing no significant difference in growth between the WT and the mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144.

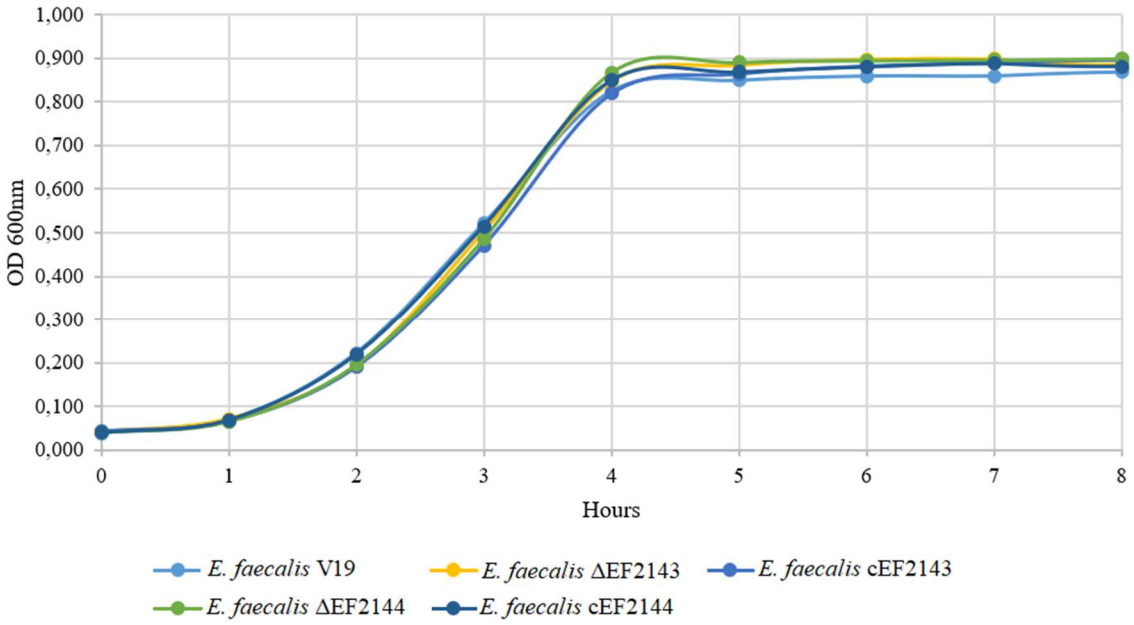

Figure 17. Growth behaviour of *E. faecalis* V19 and the designed mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144. The growth in OD 600 nm is shown as a function of time**.**

IV.4. Release of pp5 from *E. faecalis* V19, *E. faecalis* ΔEF2143 and *E. faecalis* ΔEF2144

To test if *E. faecalis* ΔEF2143 and *E. faecalis* ΔEF2144 mutants were able to release pp5 after deletion of 80 % of the respective genes, phage release was induced with MMC as

described in the materials and methods section. The *E. faecalis* V19 wildtype was used as a control. Different primer-pairs were used to verify the pp5 as shown in figure 18.

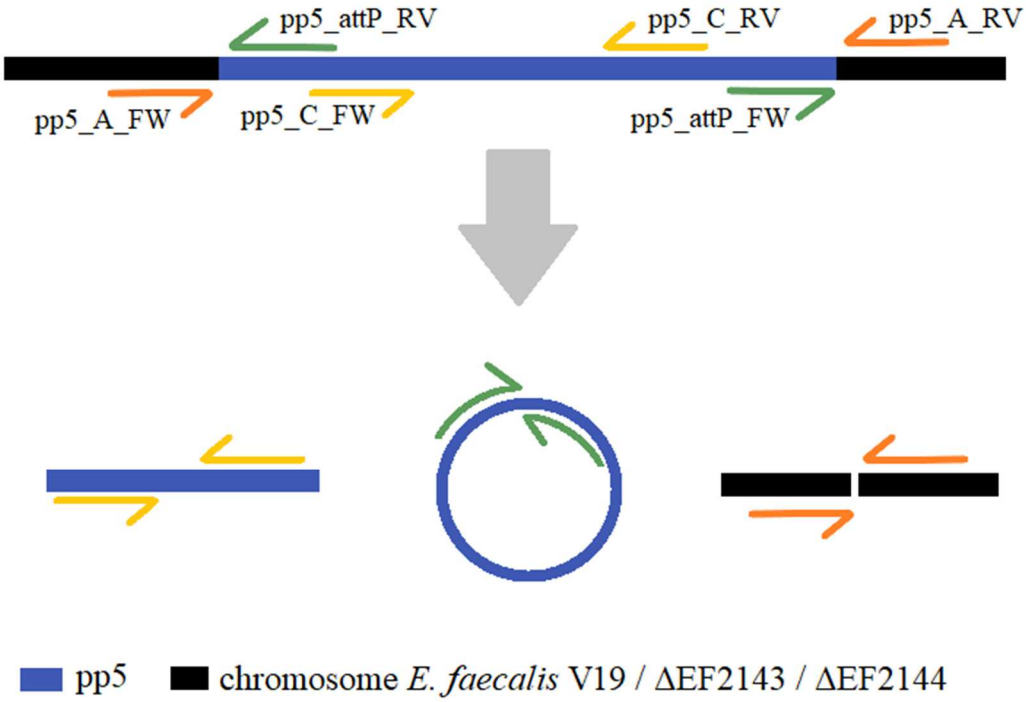

Figure 18. Scheme of the primer-pairs used to verify the pp5 release from *E. faecalis* V19 and the mutants *E. faecalis* ΔEF2143 / ΔEF2144. The figure was modified after Matos *et al*[32]. Primers pp5_A_FW and pp5_A_RV with location in the chromosome of *E. faecalis* were used to verify the release of pp5 and are shown in orange. Primers pp5_C_FW and pp5_C_RV are binding a unique gene of pp5 to verify the presence of pp5 and are shown in yellow. Primers pp5_attP_FW and pp5_attP_RV with location at the attP sites were used to verify circularized DNA of pp5 and are shown in green.

The bacterial supernatants containing the expected released phages were used for the following different PCRs: The presence of pp5 in the bacterial supernatants was verified using primers pp5_C_FW and pp5_C_RV, which are binding a unique gene of the pp5. A PCR fragment of 508 bp was expected as it is shown in figure 19. To verify the release of pp5, primers pp5_A_FW and pp5_A_RV with location within the chromosome of *E. faecalis* V19 / ΔEF2143 / ΔEF2144 were used. A DNA fragment of 475 bp was expected if the pp5 was released. Figure 19 shows both, PCR products of 475 bp made with primer-pair

pp5_A_FW / pp5_A_RV and products of 508 bp made with using primers pp5_C_FW / pp5_C_RV.

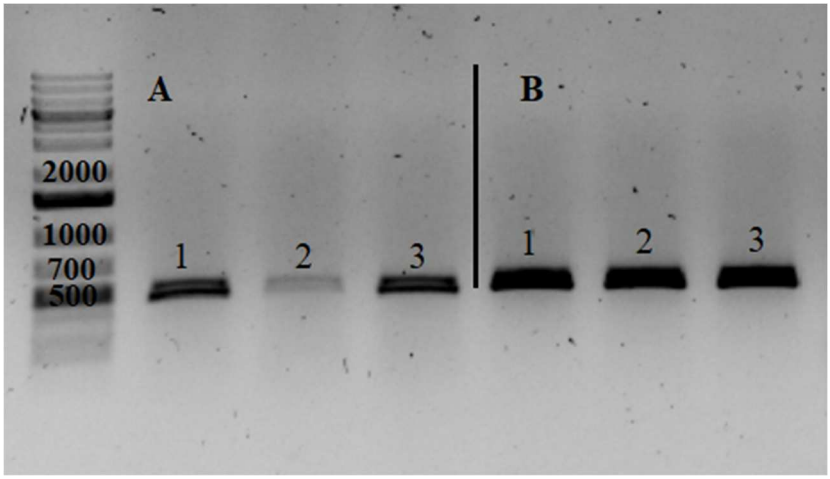

Figure 19. Electrophoresis agarose gel to verify the pp5 release from *E. faecalis* V19 and *E. faecalis* ΔEF2143 / ΔEF2144. The band sizes from the GeneRuler 1 kb Plus DNA ladder are marked. All PCRs amplifications made with using as DNA template the supernatants from the bacterial cultures induced for the release of pp5 with MMC. Bands **1** are PCR products from *E. faecalis* V19, Bands **2** from *E. faecalis* ΔEF2143, Bands **3** from *E. faecalis* ΔEF2144. **(A)** PCRs amplifications with the expected size of 475 bp made with using the primers pp5_A_FW and pp5_A_RV. **(B)** PCRs amplifications with the expected size of 508 bp made with using the primers pp5_C_FW and pp5_C_RV.

To further investigate the pp5 release from *E. faecalis* V19 and the mutants ΔEF2143 / ΔEF2144, primers pp5_attP_FW and pp5_attP_RV that will show the circularized DNA of the pp5 were used to confirm the presence of infective virions in the induced supernatants. PCRs were performed in triplicate samples and products of 1379 bp were obtained as it is shown in figure 20.

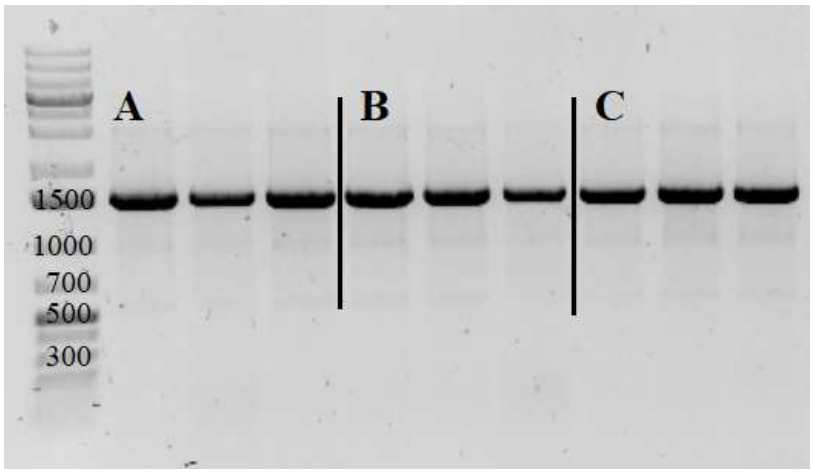

Figure 20. Electrophoresis agarose gel to verify circularized DNA from the pp5 in *E. faecalis* V19 and *E. faecalis* ΔEF2143 / ΔEF2144. The band sizes from the GeneRuler 1 kb Plus DNA ladder are marked. All PCR amplifications made with using primers pp5_attP_FW and pp5_attP_RV and an expected size of 1379 bp. **(A)** PCR products from *E. faecalis* V19. **(B)** PCR products from *E. faecalis* ΔEF2143. **(C)** PCR products from *E. faecalis* ΔEF2144.

IV.5. Transduction of pp5 into *E. faecalis* V19pp- and *E. faecalis* V19pp3+

We investigated whether the pp5 can be transduced into *E. faecalis* pp- and *E. faecalis* pp3+ after deletion of about 80% of the *ef2143* / *ef2144* genes. For this, the *E. faecalis* V19 and the mutants *E. faecalis* ΔEF2143 / ΔEF2144 were used as phage-donor strains, while *E. faecalis* pp- / pp3+ were used as phage-acceptor strains.

The obtained bacterial supernatants after the release of pp5 from the phage-donor strains were then mixed with BHI soft agar and the phage-acceptor strains prior to plating the mixture on BHI agar. Fourteen plaques were randomly picked up on the next day and checked by PCRs for the transduction of pp5 into *E. faecalis* pp- / pp3+. Figure 21 shows a schematic of the primers used.

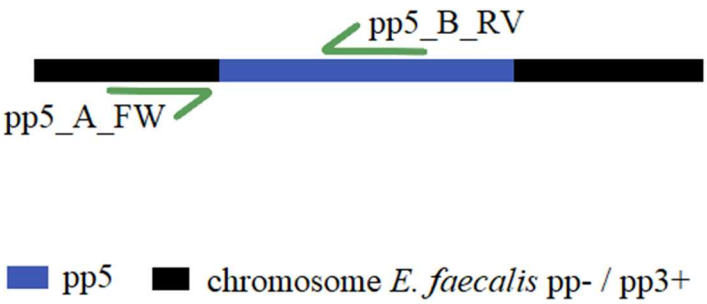

Figure 21. Scheme of the primers used to screen for the transduction of pp5 into *E. faecalis* pp- / pp3+. Primers pp5_A_FW and pp5_B_RV are shown in green and were used to check for the transduction of pp5 into *E. faecalis* pp- / pp3+. While primer pp5_A_FW is located within the chromosome of the respective phage-acceptor strain, primer pp5_A_RV is located within the pp5. For a successful transduction of pp5 a PCR product of 543 bp was obtained.

Primer pp_5_A_FW with location within the chromosome of the phage-acceptor strains *E. faecalis* pp- / pp3+ and primer pp5_B_RV with location within the pp5 were used to check the transduction. PCR products of 543 bp were obtained if the transductions were successful. Figure 22 shows the transduction of pp5 from *E. faecalis* V19 / ΔEF2143 / ΔEF2144 into *E. faecalis* pp-, while figure 23 shows the transduction into *E. faecalis* pp3+. In both transduction experiments, the mutant phages seem to be less infective than the wild type resulting in fewer PCR products with more faint bands.

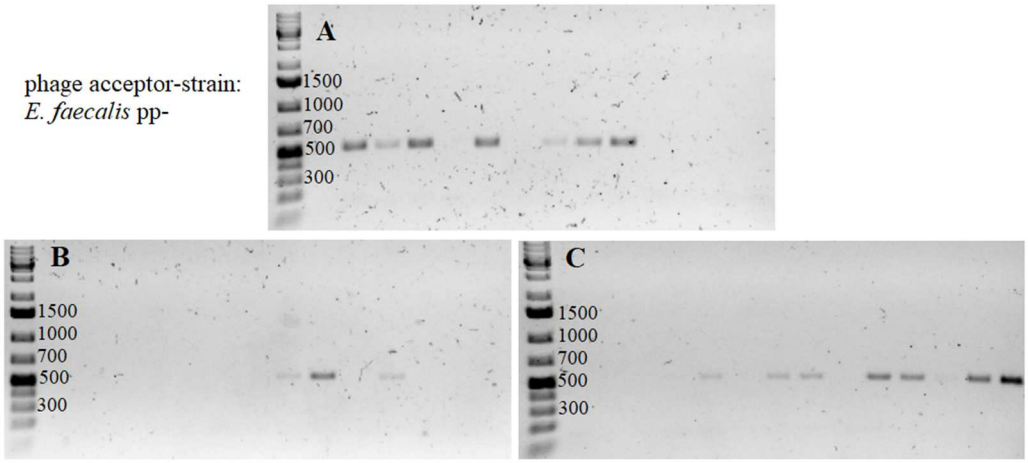

Figure 22. Electrophoresis agarose gels to verify the transduction of pp5 into *E. faecalis* pp-. The band sizes from the GeneRuler 1 kb Plus DNA ladder are marked. The PCR

products with a band size of 543 bp were obtained using primers pp5_A_FW and pp5_B_RV. **(A)** *E. faecalis* V19 as phage-donor strain. **(B)** *E. faecalis* ΔEF2143 as phage-donor strain. **(C)** *E. faecalis* ΔEF2144 as phage-donor strain.

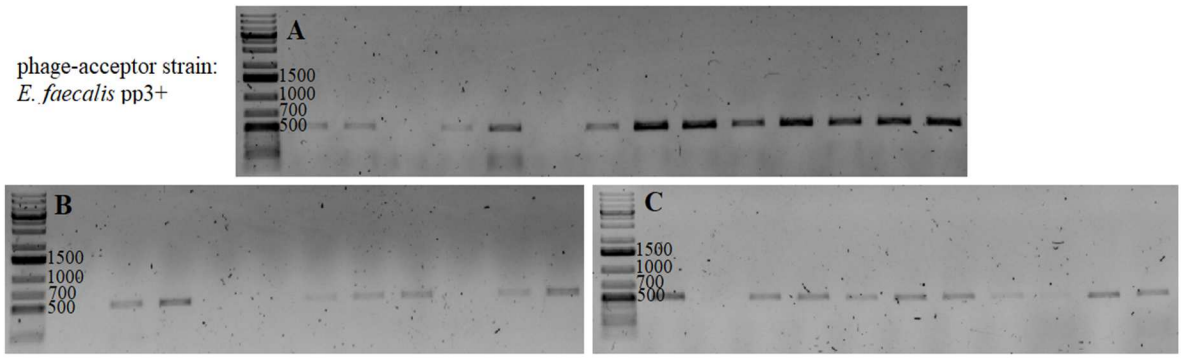

Figure 23. Electrophoresis agarose gels to verify the transduction of pp5 into *E. faecalis* pp3+. The band sizes from the GeneRuler 1 kb Plus DNA ladder are marked. The PCR products with a band size of 543 bp were obtained using primers pp5_A_FW and pp5_B_RV. **(A)** *E. faecalis* V19 as phage-donor strain. **(B)** *E. faecalis* ΔEF2143 as phage-donor strain. **(C)** *E. faecalis* ΔEF2144 as phage-donor strain.

IV.6. Biofilm formation of *E. faecalis* V19 and the mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144

To test the biofilm formation of *E. faecalis* V19 and its mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144, single bacterial colonies were grown on TSA-S blood plates. After, they were inoculated in PBS, diluted with TSBg and grown until an OD 600 nm of 0.01. AI-2 was added if intended in different concentrations before incubating the plates overnight. The biofilm growth was measured in an ELISA reader as described above after the plates were washed several times with PBS, Chrystal violet and ethanol.

Figure 24 shows a 24 hours of biofilm growth with AI-2 added at different concentrations. The WT showed a significant reduction of biofilm formation with 300 µM AI-2 added (from 0.871 to 0.777 at OD 595 nm). The addition of 500 µM AI-2 showed approximately the same amount of biofilm formation for the *E. faecalis* V19 as the sample without AI-2 added. A comparison of the WT with the designed showed a significant higher biofilm formation

for mutant ΔEF2144 (0.997 at OD 595 nm) without the supplementation of AI-2 in contrast to the WT (0.87 at OD 595 nm). The complementation mutant EF2144, on the other hand, did not showed significantly higher biofilm formation in comparison with the WT.

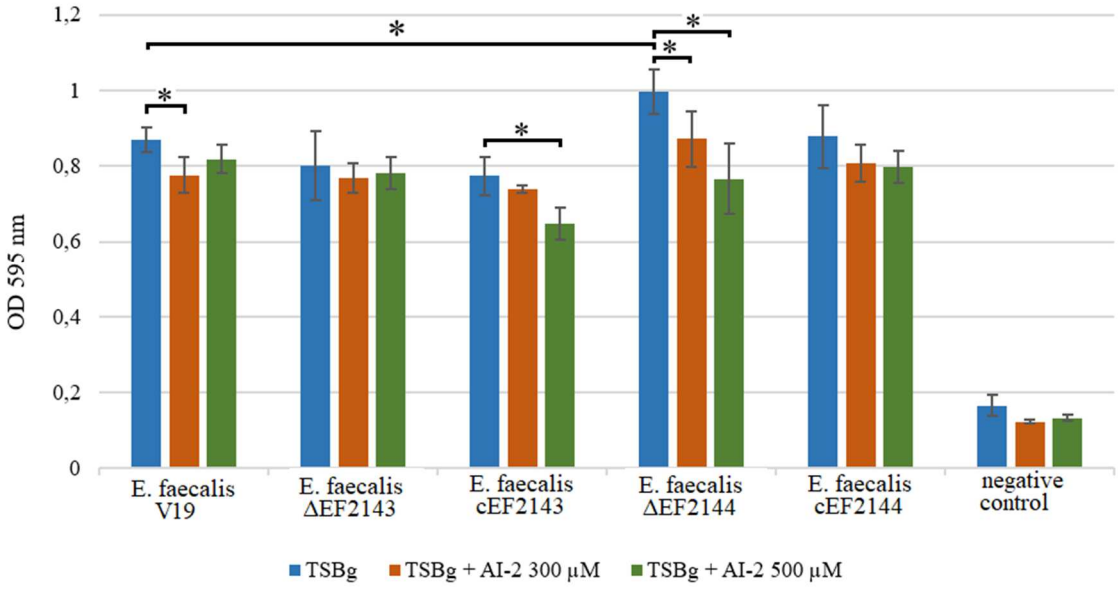

Figure 24. Biofilm formation of *E. faecalis* V19 and the mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144 with AI-2 added in different concentrations. The biofilm growth is shown at the OD of 595 nm. The blue bars show the biofilm growth without induction with AI-2. For the biofilm growth of the red bars 300 µM AI-2, and for the green bars 500 µM of AI-2 were added. The data were tested with a non-parametric Man-Whitney-U-Test. A p-value of ≤0.05 was consider statistically significant and denoted with an asterisk. The bars represent the mean value of the data and the error bars represent the standard error of the mean value.

For the deletion mutant of gene *ef2143* no differences in biofilm were measured no matter if or how much AI-2 was added. In contrast, the complementation mutant of gene e*f2143, E. faecalis* cEF2143 showed a significantly decreased biofilm formation when 500 µM AI-2 were added (from 0.774 to 0.649 at OD 595 nm).

For the deletion mutant, as well as for the complementation mutant of gene *ef2144* a reduced biofilm formation was measured with addition of AI-2 in different concentrations. This reduction was significant in the deletion mutant ΔEF2144 for 300 µM AI-2 (from 0.997 to 0.872 at OD 595 nm) as well as for 500 µM AI-2 (from 0.997 to 0.767 at OD 595 nm).

IV.7. Production of cytokine TNF-α by *E. faecalis* V19 and the mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144

To test weather different genes in the pp5 from *E. faecalis* V19 are involved in virulence and inflammatory response, human PBMCs were stimulated with bacterial supernatants of the WT and the mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144 as described in the materials and methods section. The inflammatory response was measured by human cytokine TNF-α ELISA.

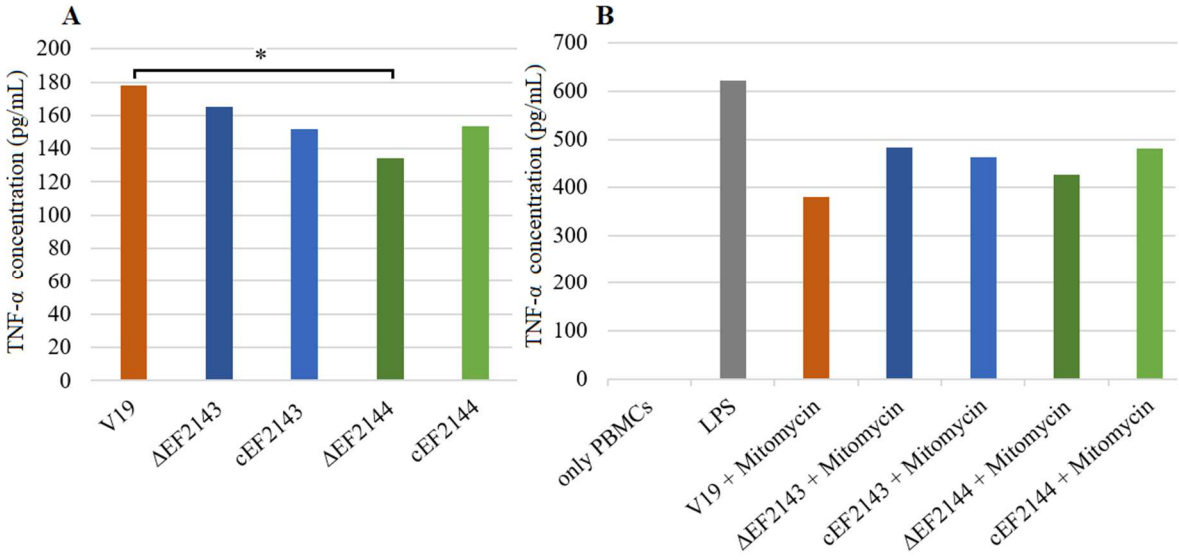

Figure 25. TNF-α detection after stimulation of PBMCs with bacterial supernatants from *E. faecalis* V19 and the mutants *E. faecalis* ΔEF2143 / ΔEF2144 / cEF2143 / cEF2144. The TNF-α detection is shown in pg/mL measured by ELISA **(A)** without or **(B)** with induction with 4 µg/mL MMC. Only PBMCs were used as negative control, LPS was used as positive control. Statistical significance for the inflammatory response was investigated with the non-parametric Kruskal-Wallis-Test followed by the non-parametric Dunn´s post-test. A p-value of ≤0.05 was consider statistically significant and is denoted with an asterisk.

As it is shown in figure 25 (A, B), the TNF-α detection was measured without or after induction with MMC at a final concentration of 4 µg/mL. In general, all samples without induction with MMC showed a much lower cytokine TNF-α response (between 134.4 – 178.2 pg/mL) than samples after induction with MMC (between 379.5 – 482.1 pg/mL).

Compared to the *E. faecalis* V19 (178.2 pg/mL) a significant reduction of the inflammatory response was measured in the mutant *E. faecalis* ΔEF2144 (134.4 pg/mL) without induction with MMC. No further significant differences could be measured.

IV.8. Influence of *E. faecalis* V19 and the mutants *E. faecalis* ΔEF2143 / ΔEF2144 on bacterial virulence using the animal model *Galleria mellonella*

The larvae of the great wax moth *Galleria mellonella* were used as virulence model to investigate how the deletion mutants *E. faecalis* ΔEF2143 / ΔEF2144 are affecting the bacterial virulence in an in vivo model. As control *E. faecalis* V19 was used. After injection of the bacterial culture into *Galleria mellonella*, the survival of the larvae was observed over 24 hours as described in the materials and methods section and shown in figure 26.

After the experiment had been repeated with 30 larvae per investigated bacterial strain, 12 from 30 larvae (40 %) infected with the *E. faecalis* V19 were alive after 24 hours of observation. In *Galleria mellonella* infected with the investigated deletion mutants 11 from 30 larvae (36,7 %) were alive for mutant *E. faecalis* ΔEF2143, and 10 from 30 larvae (33,3 %) were alive for mutant *E. faecalis* ΔEF2144.

As it is shown in figure 26, no significant differences with p-values of 0.602 – 1 between the survival of *Galleria mellonella* infected with *E. faecalis* V19 or the mutants were observed after 24 hours. Interestingly, 22 hours after the bacterial challenge statistically significant differences with a p-value of 0.0469 between the moths infected with the *E. faecalis* V19 and the mutant *E. faecalis* ΔEF2144 were observed.

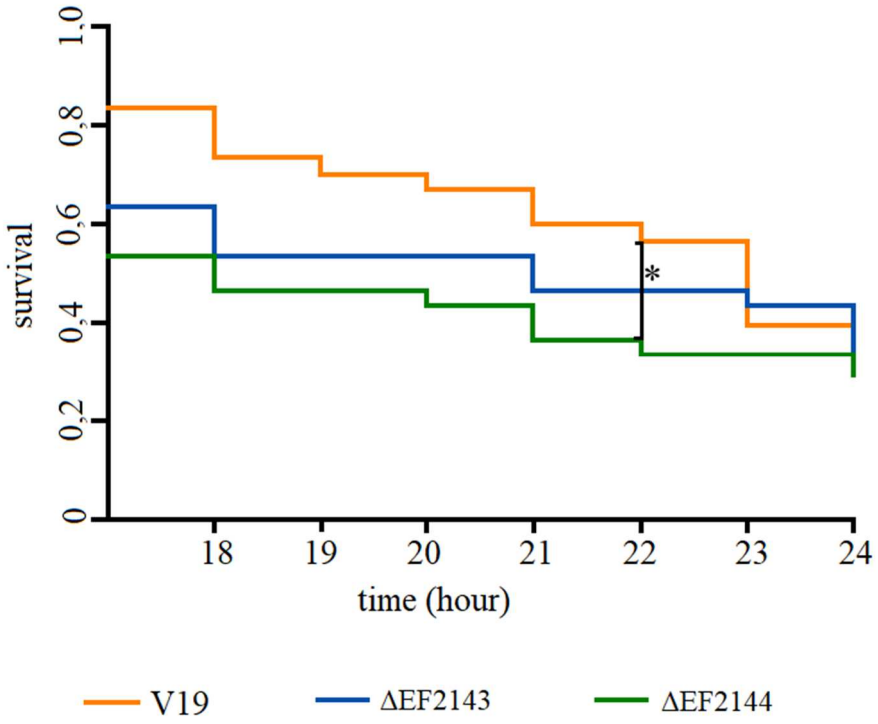

Figure 26. Survival of *Galleria mellonella* infected with *E. faecalis* V19 or the mutants *E. faecalis* ΔEF2143 / ΔEF2144. The survival is expressed in hours as a function of time. The statistical evaluations were made using the non-parametric Log-Rank-Test. A p-value of ≤ 0.05 was consider statistically significant and is denoted with an asterisk. No significant differences between the survival of *Galleria mellonella* infected with the *E. faecalis* V19 compared to *Galleria mellonella* infected with one of the mutants *E. faecalis* ΔEF2143 / ΔEF2144 could be measured after 24 hours of observation. 22 hours after injection of the *Galleria mellonella*, significant differences in the survival of the moths infected with the WT and the mutant *E. faecalis* ΔEF2144 were observed.

V. Discussion

Enterococci are responsible for up to 14 % of all healthcare-associated infections in many clinical settings[10]. In particular, *E. faecalis* plays an important role and causes sometimes life-threatening diseases[10,11]. Phages are viruses that can integrate into the bacterial genome and thus influence the properties of the bacteria[25]. They have become a focus in research as alternative therapies for antibiotics during the recent years of increasing bacterial resistances to antibiotics[43].

In the present work we hypothesized that the *ef2143* and *ef2144* genes of *E. faecalis* V583ΔABC (V19) are, as bacteriophage-derived genes, involved in the virulence of *Enterococcus faecalis*. Experiments from our research group showed an involvement of prophage 5 in the virulence of *E. faecalis* V19, making genes *ef2083 – ef2145* from pp5 of *E. faecalis* V19 interesting to study[11]. It is known from the literature that the *ef2144* gene is one of 90 lipoprotein-encoding genes of *E. faecalis* V19[56]. As only five of these, including *ef2144*, are present in a bacteriophage-related region, this gene became one focus of our interest in investigations[55,56]. Comparing the *ef2143* gene by BLAST with other gram-positive bacteria, this gene became also interesting as it could be a putative toxin in a bacteriophage related region[54]. However, a recent review of our previous results comparing EF2143 by BLAST to other previously unavailable gram-positive bacteria DNA sequences suggest that the *ef2143* gene encodes for a metallo-endopeptidase, more precisely a ImmA / IrrE metallo-endopeptidase[54]. Taking these new findings into account, the results of this work should be discussed, before the *ef2143* gene is re-evaluated as putative toxin.

To determine the role of the investigated genes in pp5 and their contributions to virulence of *E. faecalis*, two in-frame deletion mutants were generated. The genetic material of the *ef2143* and *ef2144* genes was modified by performing various PCRs to delete 83.97 % and 79.21 % of the respective gene obtaining the *E. faecalis* ΔEF2143 and ΔEF2144 mutants. To study the selected genes in detail, the two deletion mutants were complemented by reconstituting the removed genes inserting back the deleted DNA including three single point silent mutations resulting on the complementation mutants *E. faecalis* cEF2143 and cEF2144. The siltent point mutations were included to be able to assess that the reconstitution had taken

place and distinguish the reconstituted mutants from the wild type. After the successful construction of all four desired mutants, we analyzed whether the mutations generated were affecting the bacterial growth. We were able to show that the *ef2143* and the *ef2144* genes are not affecting the bacterial growth of *E. faecalis,* which was expected based on our literature research[71]. These results are indicating that the *ef2143* and the *ef2144* genes and the designed mutants are not lacking or affecting any genetic information responsible for growth compared to the WT. The publication of Mehmeti *et al.* supported our assumption, as they investigated up- and downregulation of gene transcripts during different growth rates of *E. faecalis*[71]. Among 223 gene transcripts affected by different growth rates of *E. faecalis*, both genes investigated in this study, *ef2143* and *ef2144*, were not differentially expressed under the conditions evaluated[71].

It is known that biofilm formation plays an important role in the virulence of bacteria, and that most enterococci and especially different *E. faecalis* strains (among them *E. faecalis* V19) are strong biofilm producers[13,57,61]. The results from Rossmann *et al*. supported our decision to investigate biofilm formation of the mutants *E. faecalis* ΔEF2143 and ΔEF2144[11]. First, Rossmann *et al.* were able to prove an increased detachment of subfractions from biofilms of *E. faecalis* V19 under high AI-2 concentrations (i.e. 100 µM)[11]. In addition, a designed mutant of *E. faecalis* V19 with deletion of the whole pp5 showed decreased biofilm formation in contrast to *E. faecalis* V19 containing pp5[11]. In fact, we were able to show that the mutants are at least slightly affecting the biofilm formations of *E. faecalis*. After the addition of 300 µM or 500 µM of AI-2, all mutants except the *E. faecalis* ΔEF2143 mutant showed a tendency to lower biofilm formation. These reductions were significant for the *E. faecalis* cEF2143 mutant and for the *E. faecalis* ΔEF2144 mutant. It can be assumed, that the addition of AI-2 leads to detachments of subfractions of formed biofilms to release phages and subsequently spread the infection, as it was also shown by Rossmann *et al*[11]. In contrast to Rossmann *et al.* we were able to see differences in the biofilm formation only with higher concentrations of AI-2 added. These differences in concentrations (100 µM by Rossmann *et al.*, 300 – 500 µM in this study) might be explained by different techniques and reagents used for the testing of biofilm formation and the instability of AI-2 as reagent [11]. While in both studies microtiter plates for biofilm formation

were used, which is the most common method, we dissolved the adherent cells with ethanol before quantification while Rossmann *et al.* measured the adherent cells itself[11,72]. Without the addition of AI-2, the *E. faecalis* ΔEF2144 mutant showed a significant higher production of biofilm when compared to the WT. The deletion of the *ef2144* gene thus seems to be associated with a lower dispersal of subfractions of already established biofilms if no AI-2 was added to stimulate the biofilm distribution. It therefore seems that the presence of the *ef2144* gene somehow interferes with biofilm formation and can thus be part of the genes important for the contribution to virulence of pp5. However, further research is needed to understand the exact mechanism.

As described above, gene *ef2143* is described to encode for a metallo-endopeptidase of *E. faecalis* V19 according to recent findings by comparison with BLAST to other gram-positive bacteria[54]. However, the Imma/Irre metallo-endopeptidases family has been insufficiently investigated and described in research so far. Some publications on other metallo-endopeptidases and proteases are indicating that they are influencing biofilm formation of bacteria[73]. For example, Kumar *et al.* were able to show that the matrix metalloprotease-1 prevents and destroys biofilm formations in *E. faecalis*[73]. Considering these findings and the assumption that metallo-endopeptidases of the Imma/Irre family could possibly also prevent and destroy biofilm formation, the results from this study may be explained from a different point of view. Both, the WT and the mutant for complementation of gene *ef2143*, *E. faecalis* cEF2143, showed a tendency to lower biofilm formation under induction with AI-2. This could indicate the disruption of the biofilm formation when the Imma/Irre metallo-endopeptidase is present in the bacterial genome. With the deletion of the *ef2143* gene in mutant *E. faecalis* ΔEF2143, the biofilm formation may be more stable, and therefore no disruption of the biofilm was shown. Through these observations, an influence of the metallo-endopeptidase on the biofilm formation seems possible. Nevertheless, further experiments are required to understand the exact impact of the *ef2143* gene on the properties of *E. faecalis* and on the temperate phage. The fact that the deletion of the *ef2143* gene is having an influence on the pp5 itself is discussed below.

For more than 30 years now, the measurement of cytokine TNF-α after stimulation of PBMCs plays an important role correlating with a potential inflammatory response against the bacteria investigated[74,75]. Rossmann *et al*. showed that a mutant of *E. faecalis* V19 lacking pp5 produces less TNF-α after induction with AI-2 than the *E. faecalis* V19 containing pp5, indicating that pp5 may play an important role in the virulence of *E. faecalis*[11]. Therefore, we decided to test the mutants created in this study for their TNF-α response to see if *ef2143* or *ef2144* are affecting the inflammatory response of *E. faecalis* V19. Without the induction with MMC, mutant *E. faecalis* ΔEF2144 showed a significant reduction of the TNF-α production compared to the WT, and a reconstitution of the phenotype in the *E. faecalis* cEF2144 mutant. This indicates that the deletion of *ef2144* may play a role in the inflammatory process of *E. faecalis* V19 and is supported by the literature since many bacterial proteins have been described to play a proinflammatory role[41,55,56]. This may be important in several diseases, such as inflammatory bowel disease, but also fasting and stunting[56,76–78]. By the induction with MMC, both, the WT and all mutants, showed a TNF-α response at least a twice as high. Surprisingly, the mutants with deletion of *ef2143* or *ef2144* showed a slightly increased TNF-α production that was not statistically significant. A theoretical possibility is that induction with MMC damaged the bacterial DNA, led to an instable genome and might have influenced the results of the experiment. There are many publications on the fact that MMC can damage human DNA and leads to diseases based on this DNA damages, so that damages by MMC to bacterial DNA is not unlikely[79,80]. However, since in contrast to Rossman *et al.* MMC and not AI-2 was used for induction, the results should be evaluated with caution and more experiments should be performed to confirm our findings.

The different and in the first moment seemingly contradictory results for the biofilm- and the cytokine TNF-α assay may be explained through different environmental and population conditions of the bacteria during which the experiments were performed. Thus, planktonic bacteria were used for the cytokine measurement, for which generally a different behaviour is known than for bacteria encompassed in a biofilm and usually used for the biofilm-assay[81]. For example, Mittal *et al.* showed different TNF-α responses of the gram-negative bacteria *Pseudomonas aeruginosa* in urinary tract infections depending on whether the bacteria were

in a planktonic state or encompassed in a biofilm[82]. Also, it is well known that different genes from bacteria are expressed depending on the environmental circumstances which can lead to different properties of the same bacteria[81]. To compare the results better with each other, further studies need to examine the mutants under preferably similar environmental conditions.

It is known that the greater wax moth *Galleria mellonella* is a simple and commonly used animal model to study virulence factors of bacterial pathogens, and that *E. faecalis* V19 is, in contrast to some other enterococci, virulent enough to kill the moths[83,84]. To further investigate the properties of the *ef2143* and *ef2144* genes, we decided to inject both, the WT and the mutants ΔEF2143 / ΔEF2144 into *Galleria mellonella*. The survival of the moths was observed after the injection. After 24 hours of monitoring no significant differences between the survival of *Galleria mellonella* injected with the WT or one of the mutants *E. faecalis* ΔEF2143/ ΔEF2144 were observed. This indicates that the mutations do not affect the virulence of *E. faecalis* to such an extent that they lead to a decreased or increased killing of the moths. When studying the survival curves, we noticed that the *Galleria mellonella* injected with the *E. faecalis* ΔEF2143 / ΔEF2144 mutants died faster within the first 22 hours than the moths injected with the *E. faecalis* V19. At that time point, the differences in killed moths were significant between the *E. faecalis* ΔEF2144 mutant and the WT. With the knowledge on the cytokine TNF-α and the results from the TNF-α assay, these findings can be placed into context. Toll-like receptors detect specific molecular particles present for example in bacterial products[85]. This pathway then leads to the release of cytokines like TNF-α, which is an important factor to activate the signaling cascade of the immune system[42]. The decreased TNF-α response of the *E. faecalis* ΔEF2143 / ΔEF2144 mutants without induction with MMC in the TNF-α assay may lead to a slower and decreased response of the immune system. Therefore, we hypothesize that this behavior could result in a higher killing-rate of the *Galleria mellonella* injected with the deletion mutants within the first hours of the experiment. However, more experiments must be carried out in order to investigate the exact mechanism of virulence through the deletion of *ef2143* and *ef2144* from *E. faecalis* V19. For instance, a double mutant of *E. faecalis* V19 with simultaneous deletion

of both genes *ef2143* and *ef2144* could be helpful to further understand the contributions to virulence that these genes have.

Matos *et. al.* published that *E. faecalis* V19 can release six of seven phages under different conditions by induction with Ciprofloxacin and MMC, and Rossmann *et. al*. showed that also AI-2 can induce the phage release[11,32]. We wanted to examine whether the mutants *E. faecalis* ΔEF2143 and *E. faecalis* ΔEF2144 were still able to release pp5 and can subsequently infect other bacteria. Having success in the pp5 release, we were able to prove that the *ef2143* and *ef2144* genes are not encoding any information needed for pp5 to excise from the chromosome of *E. faecalis*. Knowing from not yet published data of our research group, that the pp3 of *E. faecalis* V19 seems to be the dominant phage of *E. faecalis* V19, we decided to use *E. faecalis* containing already this dominant pp3, *E. faecalis* pp3+, as phage-acceptor stain for the pp5 transduction. Also, we used an *E. faecalis* strain without any phages in its genome (*E. faecalis* pp-) as another phage-acceptor stain. The WT of pp5, pp5 with deletion of *ef2143* (pp5ΔEF2143) and pp5 with deletion of *ef2144* (pp5ΔEF2144) were used to test the possible transduction of pp5. We were able to demonstrate, that the *ef2143* and *ef2144* genes are not encoding information needed to infect other bacteria. Nevertheless, we were able to see differences in the transductions between the *E. faecalis* V19 and the mutants *E. faecalis* ΔEF2143 / ΔEF2144. Thus, we detected more and stronger bands in the PCRs for the transduction with the WT into *E. faecalis* pp3+ / pp-. Accordingly, the PCRs bands for the transductions of the mutants *E. faecalis* ΔEF2143 / ΔEF2144 were fewer and lighter, indicating that the deletion of *ef2143* / *ef2144* are somehow affecting pp5 itself. This impairment of pp5 trough the deletion of *ef2143* and *ef2144* could be another reason for the differences we were able to observe in the other experiments like the TNF-α detection, biofilm formation or survival of the in vivo model *Galleria mellonella*. Since we were not able to assess the full impact of gene deletions, further experiments would be necessary to completely characterize these phage-related genes. For example, the transduction into probiotic strains of *E. faecalis*, e.g. *E. faecalis* Symbioflor, could be helpful to further investigate the possible transduction of pp5 after the genetic modifications.

V.1. Conclusion and perspectives

In this study we investigated the bacteriophage-related genes *ef2143* and *ef2144* from *E. faecalis* V19 to understand their role as possible transferrable virulence factors. In summary, we were able to show that both genes, *ef2144* more than *ef2143*, are affecting the virulence of *E. faecalis* V19, but to a smaller extend than expected. This was demonstrated with the help of mutants by gene deletion of the *ef2143* / *ef2144* genes, and by complementation of these mutants. We were able to show differences in the dispersal of biofilm formation under high concentrations of AI-2 to spread the infection, and reduction of the cytokine TNF-α response after stimulation of PBMCs. No differences in the survival rate of the in vivo model *Galleria mellonella* injected with the WT and the deletion mutants *E. faecalis* ΔEF2143 / ΔEF2144 were observed. However, we were able to see differences in the killing of the moths. Thus, moths injected with one of the deletion mutants died faster within the first 22 hours post infection than moths injected with the WT, and these differences were statistically significant for *E. faecalis* ΔEF2144.

However, considering that Rossmann *et. al*. were able to show a much higher contribution to virulence of pp5, the contribution to virulence of the two genes investigated in this study was lower than expected[11]. This indicates that maybe other genes or the combination of the ones we studied are involved in the contribution to the virulence that pp5 has on *E. faecalis* V19. Therefore, more genes from pp5 could be interesting targets to study in the future to elucidate the complete contribution to virulence of pp5. Moreover, a mutant with simultaneous deletion of both investigated genes could be helpful to understand the contribution to virulence of *ef2143* and *ef2144* in its entirety.

VI. Summary

In this work we investigated bacteriophage-related genes and their contribution on virulence of the gram-positive bacteria *Enterococcus faecalis*. Enterococci and especially *E. faecalis* are responsible for many, sometimes life-threatening, infections in many clinical settings[10,11]. As it is becoming more important to find alternative therapies in times of increasing resistances to antibiotics, bacteriophages became one important focus of research[20,25]. The fact that these viruses can integrate into the genome of bacteria and thus influence their properties led to studies to use these phages for phage therapy[29]. Even though the E. *faecalis* V583 has seven prophages, phage 5 raised our interest after Rossmann *et al.* showed that this phage contributed to the virulence of *E. faecalis*[11,32]. We were then interested to find out which of the pp5-genes are responsible for its contribution to virulence and decided to study the following genes: *ef2143*, which turned out to encode for a metallo-endopeptidase and *ef2144* that encodes a lipoprotein[54,56]. We created four mutants to investigate the *ef2143* and *ef2144* genes: The *E. faecalis* ΔEF2143 / ΔEF2144 mutants with deletion of approximately 80 % of the respective gene, and the *E. faecalis* cEF2143 / cEF2144 mutants with three silent single point mutations for complementation of the deletion mutants.

After the phenotypic characterization, we were able to observe a reduced biofilm formation with supplemented AI-2 in high concentrations for the *E. faecalis* ΔEF2144 mutant. Despite our expectations, the *E. faecalis* ΔEF2143 / ΔEF2144 mutants produced a little bit more cytokine TNF-α after the induction with MMC. This may be explained by DNA damages by MMC. Without the induction with MMC, the mutants showed the expected reduction of the TNF-α response, which was statistically significant for the *E. faecalis* ΔEF2144 mutant. With help of the in vivo model *Galleria mellonella*, we were able to show that the deletion of the *ef2143* / *ef2144* genes are not affecting the total virulence of *E. faecalis* to such an extend resulting in decreased killing of the moths. Nevertheless, the speed at which the moths died was different between the WT and the deletion mutants. In addition, we were able to show the ability of the *E. faecalis* ΔEF2143 / ΔEF2144 mutants to release pp5 from the chromosome. Also, the possible transduction of pp5ΔEF2143 / pp5ΔEF2144 into other

bacteria was shown, whereby weaker and fewer PCR bands indicated an influence of the mutations on the characteristics of pp5. Overall, we were able to show that the investigated *ef2143* and *ef2144* contribute to the virulence of the pp5. However, the contribution to virulence was less than expected, indicating that probably more genes are involved in the virulence of the pp5 and are interesting to study in future.

VI.1. Zusammenfassung

In dieser Arbeit wurden Gene von Bakteriophagen und ihr Beitrag an der Virulenz von grampositiven Bakterien wie *Enterococcus faecalis* untersucht. Enterokokken, und unter ihnen *E. faecalis*, sind für viele, manchmal sogar lebensbedrohliche Infektionen in Krankenhäusern verantwortlich[10,11]. Da es in Zeiten zunehmender Antibiotikaresistenzen immer wichtiger wird, alternative Therapien zu finden, wurden Bakteriophagen zu einem wesentlichen Schwerpunkt der Forschung[20,25]. Der Aspekt, dass diese Viren sich in das Genom von Bakterien integrieren können und dadurch Eigenschaften ihres Wirtes beeinflussen können, wird als Ansatz für die Phagentherapie genutzt[29]. Obwohl *E. faecalis* V583 insgesamt sieben Phagen besitzt, weckte besonders Phage 5 unser Interesse zur weiteren Untersuchung, nachdem Rossmann *et. al.* einen starken Beitrag dieser Phage hinsichtlich der Virulenz von *E. faecalis* zeigen konnten[11,32]. Unserem Interesse folgend wollten wir herausfinden, welche der pp5-Gene für den Beitrag an der Virulenz verantwortlich sind und die folgenden Gene wurden daraufhin genauer untersucht: *ef2143*, welches eine Metallo-Endopeptidase kodiert und *ef2144*, welches ein Lipoprotein kodiert[54,56]. Es wurden vier verschiedene Mutanten hergestellt, um diese für die Untersuchung der Gene *ef2143* und *ef2144* zu verwenden: Die Mutanten *E. faecalis* ΔEF2143 / ΔEF2144 mit Deletion von ca. 80 % des jeweiligen Gens, und die Mutanten *E. faecalis* cEF2143 / cEF2144 mit drei stillen Punktmutationen zur ergänzenden Untersuchung der Deletionsmutanten.

Nach der Charakterisierung des Phänotyps konnte eine reduzierte Biofilmbildung, mit in hohen Konzentrationen supplementiertem AI-2, für die *E. faecalis* ΔEF2144 Mutante festgestellt werden. Entgegen vorherigen Annahmen produzierten die *E. faecalis* ΔEF2143 / ΔEF2144 Mutanten nach der Induktion mit MMC etwas mehr Zytokin TNF-α als erwartet. Dies kann durch mögliche DNA-Schäden durch MMC erklärt werden. Ohne die Induktion mit MMC zeigten die Mutanten die erwartete Reduktion der TNF-α Produktion, welche sich für die *E. faecalis* ΔEF2144 Mutante statistisch signifikant zeigte. Mit Hilfe des in vivo Models *Galleria mellonella* konnte gezeigt werden, dass die Deletion der Gene *ef2143* / *ef2144* die Virulenz von *E. faecalis* nicht in einem solchen Umfang beeinträchtigte, dass

weniger Motten starben. Nichtsdestotrotz zeigte sich die Zeitspanne, in der die Motten starben, zwischen dem Wildtyp und den Deletionsmutanten unterschiedlich. Darüber hinaus konnte gezeigt werden, dass die *E. faecalis* ΔEF2143 / ΔEF2144 Mutanten die Fähigkeit besitzen pp5 aus ihrem Chromosom freizusetzen. Auch die Fähigkeit der Transduktion von pp5ΔEF2143 / pp5ΔEF2144 in andere Bakterien konnte gezeigt werden, wobei wenigere und schwächere PCR-Banden auf einen Einfluss der Mutationen auf die Eigenschaften von pp5 hindeuteten. Insgesamt konnte gezeigt werden, dass die untersuchten Gene *ef2143* und *ef2144* zur Virulenz der Phage 5 beitragen. Dieser Beitrag zur Virulenz zeigte sich geringer als zunächst angenommen und deutet darauf hin, dass weitere Gene mit möglichem Beitrag zur Virulenz von pp5 interessante Kandidaten für weitere Untersuchungen sind.

VII. References

1. Schleifer, K. H. & Kilpper-Balz, R. Transfer of Streptococcus faecalis and Streptococcus faecium to the Genus Enterococcus nom. rev. as Enterococcus faecalis comb. nov. and Enterococcus faecium comb. nov. *Int. J. Syst. Bacteriol.* **34**, 31–34 (1984).

2. Sherman, J. M. THE STREPTOCOCCI. *Bacteriol. Rev.* **1(1) 3-97**, (1937).

3. Franz, C. M. A. P., Huch, M., Abriouel, H., Holzapfel, W. & Gálvez, A. Enterococci as probiotics and their implications in food safety. *Int. J. Food Microbiol.* **151**, 125–140 (2011).

4. Lebreton, F., Willems, R. J. L. & Gilmore, M. S. *Enterococcus Diversity, Origins in Nature, and Gut Colonization*. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection* (Massachusetts Eye and Ear Infirmary, 2014).

5. Hardie, J. M. & Whiley, R. A. Classification and overview of the genera Streptococcus and Enterococcus. *Soc. Appl. Bacteriol. Symp. Ser.* **26**, 1S-11S (1997).

6. Fisher, K. & Phillips, C. The ecology, epidemiology and virulence of Enterococcus. *Microbiology* **155**, 1749–1757 (2009).

7. Kayser, F. H. Safety aspects of enterococci from the medical point of view. *Int. J. Food Microbiol.* **88**, 255–262 (2003).

8. Byappanahalli, M. N., Nevers, M. B., Korajkic, A., Staley, Z. R. & Harwood, V. J. Enterococci in the environment. *Microbiol. Mol. Biol. Rev.* **76**, 685–706 (2012).

9. Gawryszewska, I. *et al.* Invasive enterococcal infections in Poland: the current epidemiological situation. *Eur. J. Clin. Microbiol. Infect. Dis.* **35**, 847–856 (2016).

10. Sievert, D. M. *et al.* Antimicrobial-Resistant Pathogens Associated with Healthcare-Associated Infections Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009–2010. *Infect. Control Hosp. Epidemiol.* **34**, 1–14 (2013).

11. Rossmann, F. S. *et al.* Phage-mediated Dispersal of Biofilm and Distribution of Bacterial Virulence Genes Is Induced by Quorum Sensing. *PLOS Pathog.* **11**, e1004653 (2015).

12. Arias, C. A. & Murray, B. E. Emergence and management of drug-resistant enterococcal infections. *Expert Rev. Anti. Infect. Ther.* **6**, 637–655 (2008).

13. Kristich, C. J., Li, Y.-H., Cvitkovitch, D. G. & Dunny, G. M. Esp-Independent Biofilm Formation by Enterococcus faecalis. *J. Bacteriol.* **186**, 154–163 (2004).

14. Arias, C. A. & Murray, B. E. The rise of the Enterococcus: beyond vancomycin resistance. *Nat. Rev. Microbiol.* **10**, 266–278 (2012).

15. Guidelines for the Evaluation of Probiotics in Food, Food and Agriculture Organization of the United Nations World Health Organization. (2002).

16. Fritzenwanker, M. *et al.* Complete Genome Sequence of the Probiotic Enterococcus faecalis Symbioflor 1 Clone DSM 16431. *Genome Announc.* **1**, e00165-12-e00165-12 (2013).

17. Fijan, S. Microorganisms with Claimed Probiotic Properties: An Overview of Recent Literature. *Int. J. Environ. Res. Public Heal.* **11**, 4745–4767 (2014).

18. Habermann, W., Zimmermann, K., Skarabis, H., Kunze, R. & Rusch, V. Einfluß eines bakteriellen Immunstimulans (humane Enterococcus faecalis-Bakterien) auf die Rezidivhäufigkeit bei Patienten mit chronischer Bronchitis. *Arzneimittelforschung* **51**, 931–937 (2011).

19. Lobanovska, M. & Pilla, G. Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? *Yale J. Biol. Med.* **90**, 135–145 (2017).

20. Hollenbeck, B. L. & Rice, L. B. Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence* **3**, 421–569 (2012).

21. Sahm, D. F. *et al.* In vitro susceptibility studies of vancomycin-resistant Enterococcus faecalis. *Antimicrob. Agents Chemother.* **33**, 1588–1591 (1989).

22. Paulsen, I. T. *et al.* Role of Mobile DNA in the Evolution of Vancomycin-Resistant Enterococcus faecalis. *Science (80-.).* **299**, 2071–2074 (2003).

23. Arias, C. A., Contreras, G. A. & Murray, B. E. Management of Multi-Drug Resistant Enterococcal Infections. *Clin. Microbiol. Infect.* **16**, 555–62 (2010).

24. Rigottier-Gois, L. *et al.* Large-Scale Screening of a Targeted Enterococcus faecalis Mutant Library Identifies Envelope Fitness Factors. *PLoS One* **6**, e29023 (2011).

25. Casjens, S. Prophages and bacterial genomics: what have we learned so far? *Mol. Microbiol.* **49**, 277–300 (2003).

26. Pelzek, A. J., Schuch, R., Schmitz, J. E. & Fischetti, V. A. Isolation, Culture, and Characterization of Bacteriophages. *Curr. Protoc. Essent. Lab. Tech.* **7**, 4.4.1-4.4.33 (2013).

27. Weeks, C. R. & Ferretti, J. J. The gene for type A streptococcal exotoxin (erythrogenic toxin) is located in bacteriophage T12. *Infect. Immun.* **46**, 531–536 (1984).

28. Chibani-Chennoufi, S., Bruttin, A., Dillmann, M.-L. & Brussow, H. Phage-Host Interaction: an Ecological Perspective. *J. Bacteriol.* **186**, 3677–3686 (2004).

29. Brüssow, H. & Hendrix, R. W. Phage Genomics. *Cell* **108**, 13–16 (2002).

30. Leiman, P. G., Kanamaru, S., Mesyanzhinov, V. V., Arisaka, F. & Rossmann, M. G. Structure and morphogenesis of bacteriophage T4. *Cell. Mol. Life Sci.* **60**, 2356–2370 (2003).

31. Gandon, S. *et al.* Why Be Temperate: Lessons from Bacteriophage λ. *Trends Microbiol.* **24**, 356–365 (2016).

32. Matos, R. C. *et al.* Enterococcus faecalis Prophage Dynamics and Contributions to Pathogenic Traits. *PLoS Genet.* **9**, e1003539 (2013).

33. Wagner, P. L. & Waldor, M. K. Bacteriophage Control of Bacterial Virulence. *Infect. Immun.* **70**, 3985–3993 (2002).

34. Brussow, H., Canchaya, C. & Hardt, W.-D. Phages and the Evolution of Bacterial

Pathogens: from Genomic Rearrangements to Lysogenic Conversion. *Microbiol. Mol. Biol. Rev.* **68**, 560–602 (2004).

35. Arber, W. Horizontal Gene Transfer among Bacteria and its Role in Biological Evolution. *Life* **4**, 217–224 (2014).

36. Hargreaves, K. R., Kropinski, A. M. & Clokie, M. R. J. What Does the Talking?: Quorum Sensing Signalling Genes Discovered in a Bacteriophage Genome. *PLoS One* **9**, e85131 (2014).

37. Fuqua, W. C., Winans, S. C. & Greenberg, E. P. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* **176**, 269–75 (1994).

38. Jiang, Q., Chen, J., Yang, C., Yin, Y. & Yao, K. Quorum Sensing: A Prospective Therapeutic Target for Bacterial Diseases. *Biomed Res. Int.* **2019**, 1–15 (2019).

39. Haque, S. *et al.* Quorum sensing pathways in Gram-positive and -negative bacteria: potential of their interruption in abating drug resistance. *J. Chemother.* **31**, 161–187 (2019).

40. Ramachandran, G. Gram-positive and gram-negative bacterial toxins in sepsis. *Virulence* **5**, 213–218 (2014).

41. Romero-Saavedra, F. *et al.* Characterization of Two Metal Binding Lipoproteins as Vaccine Candidates for Enterococcal Infections. *PLoS One* **10**, e0136625 (2015).

42. Balkwill, F. TNF-α in promotion and progression of cancer. *Cancer Metastasis Rev.* **25**, 409–416 (2006).

43. Abedon, S. T., Kuhl, S. J., Blasdel, B. G. & Kutter, E. M. Phage treatment of human infections. *Bacteriophage* **1**, 66–85 (2011).

44. Cisek, A. A., Dąbrowska, I., Gregorczyk, K. P. & Wyżewski, Z. Phage Therapy in Bacterial Infections Treatment: One Hundred Years After the Discovery of Bacteriophages. *Curr. Microbiol.* **74**, 277–283 (2017).

45. Neill, J. O. '. Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations The Review on Antimicrobial Resistance Chaired. (2014).

46. Verbeken, G. *et al.* European regulatory conundrum of phage therapy. *Future Microbiol.* **2**, 485–491 (2007).

47. Viertel, T. M., Ritter, K. & Horz, H.-P. Viruses versus bacteria--novel approaches to phage therapy as a tool against multidrug-resistant pathogens. *J. Antimicrob. Chemother.* **69**, 2326–2336 (2014).

48. Kutateladze, M. & Adamia, R. Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends Biotechnol.* **28**, 591–5 (2010).

49. Schooley, R. T. *et al.* Development and Use of Personalized Bacteriophage-Based Therapeutic Cocktails To Treat a Patient with a Disseminated Resistant Acinetobacter baumannii Infection. *Antimicrob. Agents Chemother.* **61**, (2017).

50. Yasmin, A. *et al.* Comparative Genomics and Transduction Potential of Enterococcus faecalis Temperate Bacteriophages. *J. Bacteriol.* **192**, 1122–1130 (2010).

51. Duerkop, B. A., Clements, C. V., Rollins, D., Rodrigues, J. L. M. & Hooper, L. V. A composite bacteriophage alters colonization by an intestinal commensal bacterium. *Proc. Natl. Acad. Sci.* **109**, 17621–17626 (2012).

52. Brown, L., Wolf, J. M., Prados-Rosales, R. & Casadevall, A. Through the wall: Extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nat. Rev. Microbiol.* **13**, 620–630 (2015).

53. Abedon, S. T. & Lejeune, J. T. Why bacteriophage encode exotoxins and other virulence factors. *Evol. Bioinform. Online* **1**, 97–110 (2007).

54. BLAST: Basic Local Alignment Search Tool. Available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi. (Accessed: 5th September 2019)

55. Kovacs-Simon, A., Titball, R. W. & Michell, S. L. Lipoproteins of Bacterial Pathogens. *Infect. Immun.* **79**, 548–561 (2011).

56. Reffuveille, F., Leneveu, C., Chevalier, S., Auffray, Y. & Rincé, A. Lipoproteins of Enterococcus faecalis: Bioinformatic identification, expression analysis and relation to virulence. *Microbiology* **157**, 3001–3013 (2011).

57. Donlan, R. M. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clin. Infect. Dis.* **33**, 1387–1392 (2001).

58. Mohamed, J. A. & Huang, D. B. Biofilm formation by enterococci. *J. Med. Microbiol.* **56**, 1581–1588 (2007).

59. Donlan, R. M. & Costerton, J. W. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **15**, 167–93 (2002).

60. Costerton, J. W., Geesey, G. G. & Cheng, K.-J. How Bacteria Stick. *Scientific American* **238**, 86–95 (1978).

61. Sandoe, J. A. T., Witherden, I. R., Cove, J. H., Heritage, J. & Wilcox, M. H. Correlation between enterococcal biofilm formation in vitro and medical-device-related infection potential in vivo. *J. Med. Microbiol.* **52**, 547–550 (2003).

62. Ch'ng, J.-H., Chong, K. K. L., Lam, L. N., Wong, J. J. & Kline, K. A. Biofilm-associated infection by enterococci. *Nat. Rev. Microbiol.* **17**, 82–94 (2019).

63. Taga, M. E. & Xavier, K. B. Methods for Analysis of Bacterial Autoinducer-2 Production. in *Current Protocols in Microbiology* 1C.1.1-1C.1.15 (John Wiley & Sons, Inc., 2011). doi:10.1002/9780471729259.mc01c01s23

64. Thurlow, L. R., Thomas, V. C. & Hancock, L. E. Capsular Polysaccharide Production in Enterococcus faecalis and Contribution of CpsF to Capsule Serospecificity. *J. Bacteriol.* **191**, 6203–6210 (2009).

65. Green, M. R. & Sambrook, J. Molecular Cloning. (2012).

66. Dower, W. J., Miller, J. F. & Ragsdale, C. W. High efficiency transformation of E. coli by high voltage electroporation. *Nucleic Acids Res.* **16**, 6127–45 (1988).

67. Ladjouzi, R. *et al.* Analysis of the tolerance of pathogenic enterococci and

Staphylococcus aureus to cell wall active antibiotics. *J. Antimicrob. Chemother.* **68**, 2083–91 (2013).

68. Reffuveille, F. *et al.* Involvement of peptidylprolyl cis/trans isomerases in Enterococcus faecalis virulence. *Infect. Immun.* **80**, 1728–35 (2012).

69. Park, S. Y., Kim, K. M., Lee, J. H., Seo, S. J. & Lee, I. H. Extracellular gelatinase of Enterococcus faecalis destroys a defense system in insect hemolymph and human serum. *Infect. Immun.* **75**, 1861–9 (2007).

70. Nakashima, N. & Miyazaki, K. Bacterial Cellular Engineering by Genome Editing and Gene Silencing. *Int. J. Mol. Sci.* **15**, 2773–2793 (2014).

71. Mehmeti, I. *et al.* Growth rate-dependent control in Enterococcus faecalis: effects on the transcriptome and proteome, and strong regulation of lactate dehydrogenase. *Appl. Environ. Microbiol.* **78**, 170–6 (2012).

72. Azeredo, J. *et al.* Critical review on biofilm methods. *Crit. Rev. Microbiol.* **43**, 313–351 (2017).

73. Kumar, L., Cox, C. R. & Sarkar, S. K. Matrix metalloprotease-1 inhibits and disrupts Enterococcus faecalis biofilms. *PLoS One* **14**, (2019).

74. Oshiro, Y. & Morris, D. L. TNF-α release from human peripheral blood mononuclear cells to predict the proinflammatory activity of cytokines and growth factors. *J. Pharmacol. Toxicol. Methods* **37**, 55–59 (1997).

75. Kornbluth, R. S., Gregory, S. A. & Edgington, T. S. Initial characterization of a lymphokine pathway for the immunologic induction of tumor necrosis factor-alpha release from human peripheral blood mononuclear cells. *J. Immunol.* **141**, 2006–15 (1988).

76. Golińska, E. Virulence factors of Enterococcus strains isolated from patients with inflammatory bowel disease. *World J. Gastroenterol.* **19**, 3562 (2013).

77. Steck, N. *et al.* Enterococcus faecalis Metalloprotease Compromises Epithelial

Barrier and Contributes to Intestinal Inflammation. *Gastroenterology* **141**, 959–971 (2011).

78. Ocvirk, S. *et al.* Surface-Associated Lipoproteins Link Enterococcus faecalis Virulence to Colitogenic Activity in IL-10-Deficient Mice Independent of Their Expression Levels. *PLOS Pathog.* **11**, e1004911 (2015).

79. Lee, Y.-J., Park, S.-J., Ciccone, S. L. M., Kim, C.-R. & Lee, S.-H. An in vivo analysis of MMC-induced DNA damage and its repair. *Carcinogenesis* **27**, 446–453 (2006).

80. Bargonetti, J., Champeil, E. & Tomasz, M. Differential toxicity of DNA adducts of mitomycin C. *Journal of Nucleic Acids* **2010**, (2010).

81. Stoodley, P., Sauer, K., Davies, D. G. & Costerton, J. W. Biofilms as complex differentiated communities. *Annu. Rev. Microbiol.* **56**, 187–209 (2002).

82. Mittal, R., Sharma, S., Chhibber, S. & Harjai, K. Evaluation of tumour necrosis factor-alpha and interleukin-1beta in an experimental pyelonephritis model induced with planktonic and biofilms cells of Pseudomonas aeruginosa. *Can. J. Infect. Dis. Med. Microbiol.* **20**, e35 (2009).

83. Hanin, A. *et al.* Screening of in vivo activated genes in Enterococcus faecalis during insect and mouse infections and growth in urine. *PLoS One* **5**, (2010).

84. Ramarao, N., Nielsen-Leroux, C. & Lereclus, D. The insect Galleria mellonella as a powerful infection model to investigate bacterial pathogenesis. *J. Vis. Exp.* e4392 (2012). doi:10.3791/4392

85. Akira, S. & Takeda, K. Toll-like receptor signalling. *Nature Reviews Immunology* **4**, 499–511 (2004).

VIII. Appendix

VIII.1. Acknowledgements

First, I would like to thank Prof. Johannes Hübner for entrusting me with this very interesting and important topic. Thank you for guiding me to an experimental thesis. Thank you for your help and support throughout the whole project and beyond during medical school.

Many thanks to Dr. Luis Felipe Romero Saavedra! Felipe, I probably would have never put this work on paper without your help. Thank you for your patience and calmness, for the always new motivation, and your positive thinking even if sometimes not everything worked out as we imagined.

I am grateful for all the colleagues from the AG Hübner and the Gencenter. I felt very welcome from day one and it was such a great time with all of you! A very special thanks to Diana. You held the group together not only in the lab but also beyond and you were always willing to help. Thank you so much!

A very special thanks to Dr. Eliette Bisson and Isabelle Rince from the research group of Prof. Axel Hartke, UR Risques micobiens U2RM, University of Caen in France, and to Dr. Fernanda Paganelli from the research group of Prof. Rob Willems, University of Utrecht in Netherlands. Thank you very much for actively supporting us in some of the experiments. I would also like to take the chance to thank the research group of Prof. Christoph Klein for all the positive input and for sharing the lab with us.

Last, but not least I would like to thank my family and my friends. You are all always there for me and supporting me in everything I put into my head.

It really was an honor, thank you all!

Eva Holzapfel

VIII.2. Curriculum vitae

VIII.3. Publikationsliste

Knoflach K, **Holzapfel E**, Roser T, Rudolph L, Paolini M, Muenchhoff M, Osterman A, Griese M, Kappler M, von Both U. Case Report: Unilateral Sixth Cranial Nerve Palsy Associated With COVID-19 in a 2-year-old Child. Front Pediatr. 2021 Dec 17;9:756014. doi: 10.3389/fped.2021.756014. PMID: 34976891; PMCID: PMC8718702.