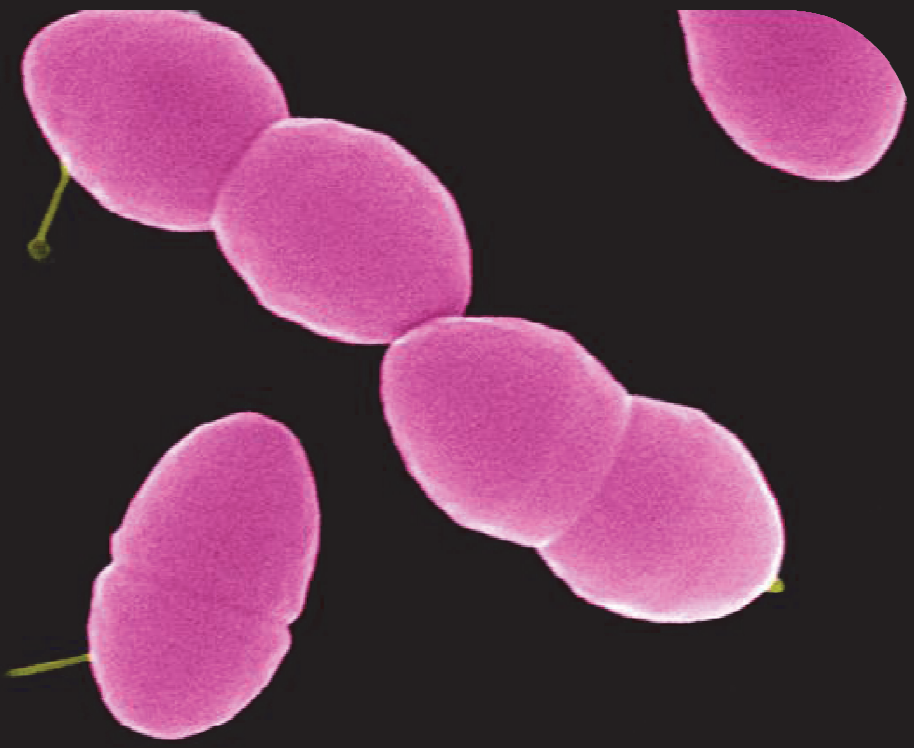
Enterococcus faecalis V583 prophages: Dynamic interactions and contribution to bacterial pathogenic traits

Renata Filipa Cruz de Matos



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
July, 2013

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To my parents Gisela e Fernando.

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"And in this crazy life, and through these crazy times
It's you, it's you, You make me sing.
You're every line, you're every word,
YOU'RE MY EVERYTHING".
(Michael Bublé, Everything)

Thank you all for being part of my life,
Renata

Abstract

Enterococcus faecalis is a firmicute of the human gastrointestinal tract (GIT) core-microbiome. This commensal bacterium is one of the first to colonize the GIT of humans after birth and remains associated with the adult human gut microbiota at sub-dominant levels. Although harmless, certain strains can become pathogenic in immune-compromised and elderly patients causing urinary tract infections, bacteremia and infective endocarditis. This bacterial species has been recognized as an opportunistic pathogen for several decades, and now ranks as a major cause of hospital-acquired infections worldwide. Some *E. faecalis* isolates are particularly adapted to the hospital environment, and this adaptation was recently linked with enrichment in mobile genetic elements, including plasmids and temperate bacteriophages.

Lysogeny is frequently considered as an adaptive evolutionary process in which a temperate bacteriophage is maintained in the prophage state, as it confers additional properties to the bacterial strains. Temperate phages can contribute to bacterial fitness or virulence in at least three ways: lysogenic conversion, gene disruption and lysis-mediated competitiveness.

E. faecalis phages studies are mostly dedicated to the applications of lytic phages and, in spite of the temperate bacteriophage status as key players in the evolution of pathogenic strains, studies on *E. faecalis* prophages are scarce. Therefore, using the polylysogenic *E. faecalis* strain V583 as a model, two main goals were established for this thesis: to study the ability of V583 prophages to engage on a lytic cycle and produce active phage particles; and to determine their impact on *E. faecalis* pathogenicity.

To accomplish the first goal we have established which of the seven V583 prophages (pp1 to pp7) were able to excise from the bacterial

chromosome in the wild-type strain. We next constructed a set of isogenic strains lacking one to all of the six excisable prophages. Polylysogenic and monolysogenic strains were characterized regarding prophage activity at four levels: excision from bacterial chromosome, replication, DNA encapsidation and virion infectivity. From the six active prophages only pp1, pp3, pp5 and pp7 produce active phage particles. Intricate interactions were unraveled between V583 prophages: i) pp1 inhibits excision of pp4 at 37°C, ii) pp3 and pp5 inhibit excision of pp6, and iii) pp7 was identified as the first enterococcal phage-related chromosomal island (PRCI) and was named EfCIV583 for *Enterococcus faecalis* chromosomal island of V583. This PRCI is involved in a molecular piracy phenomenon that culminates with the hijacking of P1 structural proteins. The *E. faecalis* EfCIV583/P1 system resembles that of *Staphylococcus aureus* SaPIs. Though PRCIs are suspected to be widespread in Gram-positive bacteria, such molecular piracy phenomenon has never been reported or demonstrated in gram-positive species other than *S. aureus*. Moreover, we determined that certain environmental cues, such as antibiotics, increase prophage induction as well as virion production and would thus contribute to horizontal gene spreading, especially in the hospital setting.

In order to investigate prophage contribution to bacterial pathogenic traits, the wild-type (polylysogenic) and the phage-deleted strains were compared under various conditions such as, sensitivity to chemical compounds, including antibiotics, biocides and oxidative stress inducing compounds, biofilm formation and adhesion to human platelets. While prophages were not shown to impact on bacterial resistance to chemical compounds or on biofilm-forming abilities, they did contribute to bacterial adhesion to human platelets. Opportunistic pathogens, including *E. faecalis*, can occasionally gain entry into the human circulatory system, induce a transient bacteremia and develop an infected thrombus on the surface of the heart valve. Adhesion to platelets is thought to be a key

step towards this infective endocarditis. *E. faecalis* is known to bind and aggregate human platelets but the underlying molecular mechanisms have yet to be discovered. Interestingly, pp1, pp4 and pp6, involved in platelet binding, encode predicted phage tail proteins homologous to the platelet binding proteins PblA and/or PblB of *Streptococcus mitis* phage SM1. This suggests that these proteins are likely to mediate *E. faecalis* binding to platelets. This work provides for the first time a direct correlation between prophages and bacterial adhesion to human platelets, suggesting a role of *E. faecalis* prophages in the development of nosocomial infective endocarditis.

This thesis reports the first thorough genetic study of *E. faecalis* prophages in a polylysogenic strain. It sheds light on complex prophage interactions including molecular piracy by a phage-related chromosomal island, which resembles the SaPIs of *Staphylococcus aureus*. It also unravelled a direct correlation between prophages and *E. faecalis* adhesion to human platelets. The data generated by this thesis work constitute solid foundations for future research on both functional and ecological impact of *E. faecalis* temperate phages.

Resumo

Enterococcus faecalis é uma das espécies bacterianas constituintes da microbiota natural do trato gastrointestinal humano. Esta espécie bacteriana comensal coloniza o trato gastrointestinal logo após o nascimento e permanece associada à microbiota do indivíduo adulto, em níveis subdominantes. Apesar de inofensiva para pessoas saudáveis, pode tornar-se patogénica em pacientes imunocomprometidos e idosos, causando infeções urinárias, bacterémias e endocardites. Esta espécie bacteriana é reconhecida como patogénica oportunista sendo uma das mais frequentes causas de infeções nosocomiais no mundo. Algumas estirpes de *E. faecalis* estão particularmente bem adaptadas ao ambiente hospitalar, tendo sido recentemente reconhecido que esta adaptação está associada a um enriquecimento dos seus genomas em elementos genéticos móveis, nomeadamente plasmídeos e bacteriófagos temperados.

A lisogenia é considerado um processo evolutivo no qual um bacteriófago temperado permanece no cromossoma bacteriano como profago, conferindo propriedades adicionais ao hospedeiro bacteriano. A existência de bacteriófagos temperados no genoma bacteriano pode contribuir de diferentes formas para a adaptabilidade da bactéria ao meio e/ou virulência, nomeadamente pela conversão lisogénica, interrupção de genes e aumento da competitividade devido à lise bacteriana.

Apesar do seu carácter importante na evolução de estirpes patogénicas, estudos de bacteriófagos temperados em *E. faecalis* são praticamente inexistentes. De modo a colmatar a falta de conhecimento deste tipo de bacteriófagos em *E. faecalis*, foi escolhida como modelo neste estudo a estirpe *E. faecalis* V583 e foram estabelecidos dois grandes objectivos para esta tese: estudar a capacidade dos bacteriófagos da estirpe V583 de efetuarem um ciclo lítico e produzirem

partículas fágicas funcionais; e determinar o contributo destes bacteriófagos na patogénese de *E. faecalis*.

Para atingir o primeiro objectivo começou por se determinar quais dos 7 profagos (pp1-pp7) de V583 são capazes de se excisar do cromossoma bacteriano na estirpe selvagem. Seguidamente foi construído um conjunto de estirpes isogénicas, que variando na sua composição em genomas fágicos, permitiram caracterizar a atividade dos profagos a quatro níveis: excisão do cromossoma bacteriano, replicação, encapsidação do DNA e infetividade das partículas fágicas. Dos 6 profagos ativos, só pp1, pp3, pp5 e pp7 são capazes de produzir partículas fágicas funcionais. Foram ainda identificadas interações complexas entre os profagos de V583: i) pp1 inibe a excisão de pp4 a 37°C, ii) pp3 e pp5 inibem a excisão de pp6, e iii) pp7 foi identificado como a primeira '*phage-related chromosomal island*' (PRCI) em enterococos e foi renomeada EfCIV583 para '*Enterococcus faecalis* chromosomal island of V583'.

Esta PRCI está envolvida num mecanismo de "pirataria" molecular que culmina no sequestro das proteínas estruturais do fago P1. O sistema EfCIV583/P1 de *E. faecalis* é semelhante ao sistema das SaPIs de *Staphylococcus aureus*. As PRCIs são conhecidas sobretudo em *S. aureus*, e embora exista a noção de que estarão disseminadas entre as bactérias Gram positivas, não existem evidências experimentais da sua existência noutras espécies bacterianas. O presente estudo revelou também que determinadas condições ambientais, tais como a presença de antibióticos, aumentam a indução dos profagos e consequentemente a produção de partículas fágicas, podendo deste modo contribuir para a disseminação de genes por transferência horizontal, particularmente em ambiente hospitalar.

De modo a avaliar a contribuição dos profagos de *E. faecalis* para o seu poder patogénico, uma estirpe selvagem (WT) e um conjunto de

estirpes com diferentes composições em genomas fágicos foram comparadas, em diferentes condições: sensibilidade a compostos químicos, como antibióticos, biocidas e compostos que induzem o stress oxidativo, formação de biofilmes e adesão às plaquetas humanas. Não foi evidenciada qualquer associação entre a presença de profagos e a resistência aos compostos químicos testados ou a formação de biofilmes. No entanto os profagos pp1, pp4 e pp6 contribuem para a adesão dos enterococos às plaquetas humanas. Devido ao seu carácter oportunista, *E. faecalis* acede à corrente sanguínea, onde pode induzir uma bacterémia e consequentemente originar um trombo na superfície de uma válvula cardíaca. A adesão de bactérias às plaquetas humanas é considerada uma etapa importante no desenvolvimento de endocardite. Apesar dos enterococos poderem aderir, assim como agregar plaquetas, os mecanismos moleculares envolvidos nestes processos ainda não foram descobertos. Os profagos envolvidos na adesão de *E. faecalis* V583 às plaquetas humanas (pp1, pp4 e pp6), contêm nos seus genomas proteínas estruturais homólogas às proteínas do fago SM1 de *Streptococcus mitis* PblA e/ou PblB, envolvidas na adesão destas bactérias às plaquetas humanas. Isto sugere que as proteínas de *E. faecalis* possam ter uma função semelhante. Este trabalho evidencia pela primeira vez uma correlação direta entre profagos e a adesão de *E. faecalis* às plaquetas humanas, indicando uma contribuição dos profagos para o desenvolvimento de endocardites em meio hospitalar.

A tese aqui apresentada constitui o primeiro estudo genético sistemático dos profagos de uma estirpe poli-lisogénica de *E. faecalis,* um pré-requisito que estabeleceu a atividade dos profagos e gerou dados sólidos que facilitarão os estudos futuros em bacteriófagos temperados de enterococos. Adicionalmente, este trabalho revelou interações complexas entre os profagos de *E. faecalis*, entre as quais um fenómeno

de “pirataria” molecular envolvendo EfCIV583 e o fago P1, semelhante à SaPI de *Staphylococcus aureus*. Por último evidenciou também uma relação direta entre determinados profagos e a adesão de *E. faecalis* às plaquetas humanas.

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Thesis outline

Polylysogeny is frequently considered as the result of an adaptive evolutionary process in which temperate bacteriophages shape bacterial genomes by the acquisition of new genes, thus making them important for evolution of both bacterial populations and infectious agents. Although considered a harmless commensal of the gastrointestinal tract of humans, *Enterococcus faecalis* ranks among the leading causes of hospital acquired bacterial infections. It is therefore recognized as an opportunistic pathogen. Though important in other bacterial species and well studied regarding their impact on bacterial evolution, fitness, and pathogenicity, bacteriophages have been scarcely studied in *E. faecalis*. Recent data on the impact of temperate bacteriophages for the diversity of *E. faecalis* genomes and the potential use of phages against antibiotic resistant strains have renewed the interest on *E. faecalis* bacteriophages. Thus, the poor knowledge on *E. faecalis* temperate bacteriophages motivated the work of this thesis.

Chapter 1 gathers updated knowledge on the three key players of this thesis: *E. faecalis*, bacteriophages and phage-associated genetic elements. The dual lifestyle of *E. faecalis* is discussed in correlation with its rise as major cause of nosocomial infections. Bacteriophage biology and general characteristic are reviewed and their relevance for community species composition in complex microbial ecosystems is emphasized. Finally, the molecular piracy mechanisms used by phage-associated genetic elements to hijack phage structural proteins are described in detail: starting from the best studied case, satellite phage P4 of *Escherichia coli*, for which most of the molecular interplay is known, to a more recent described family of phage-related chromosomal islands (PRCIs), from which the best studied are the *Staphylococcus aureus* pathogenicity islands (SaPIs).

Chapter 2 is focused on the study of *E. faecalis* V583 prophages activity. We report the conditions that induce prophages into the lytic cycle together with a sophisticated interplay between prophages that dictates the functionality of some of them. Furthermore we identify the first enterococcal phage-related chromosomal island and its helper phage.

Chapter 3 reports a series of experiments performed in order to identify conditions in which prophages contribute to *E. faecalis* biological traits. By establishing the importance of prophages carrying platelet-like binding proteins for *E. faecalis* adhesion to human platelets we provided a link between enterococcal bacteriophages and pathogenesis.

The general discussion of Chapter 4 summarizes the main achievements and future directions of the work developed during this thesis in regards with the current knowledge on prophage-prophage interactions and their contribution to host biological traits. The importance of *E. faecalis* prophages in natural ecosystems such as human associated microbial communities is brought up.

Abbreviations

Abi	Abortive system
ADP	Adenosine diphosphate
BEA	Bile Esculin Agar
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
CC	Clonal Complex
CFU	Colony Forming Units
CGH	Comparative Genome Hybridization
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DCO	Double-Crossing Over
DNA	Deoxyribonucleic Acid
dsDNA	double stranded DNA
ECM	Extracellular Matrix
eDNA	extracellular DNA
EfCI	*Enterococcus faecalis* Chromosomal Island
FIGE	Field-Inverted Gel Electrophoresis
GFP	Green Fluorescent Protein
GIT	Gastrointestinal Tract
GRAS	Generally Recognized As Safe
GTA	Gene Transfer Agents
HiRECC	High-Risk Enterococcal Clonal Complex
ICE	Integrative Conjugative Elements
kb	kilo base
LAB	Lactic Acid Bacteria
LB	Luria-Bertani medium
M17G	M17 Glucose

CDM	Chemically Defined Medium
$MgSO_4$	Magnesium Sulfate
MLST	Multi Locus Sequence Type
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
OD	Optical Density
PAI	Pathogenicity Island
Pbl	Platelet binding protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PFGE	Pulse-Field Gel Electrophoresis
PFU	Plaque Forming Unit
PM	Phenotype Microarray
pp	prophage
PRCI	Phage-Related Chromosomal Island
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Specie
SaPI	*Staphylococcus aureus* Pathogenicity Island
SE	Enterotoxins
SEM	Scanning Electron Microscopy
SNP	Single Nucleotide Polymorphism
SpyCI	*Streptococcus pyogenes* Chromosomal Island
ssDNA	single stranded DNA
ST	Sequence Type
TEM	Transmission Electron Microscopy
Tet	Tetracycline
TSST	Toxic Shock Syndrome Toxins
UV	Ultra-Violet
WT	Wild-Type

Chapter 1

General Introduction

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I. *ENTEROCOCCUS*

1. General description of the genus

Enterococci are robust Gram-positive, catalase-negative, facultative anaerobic bacteria. They are ovoid in shape and grow in short chains, pairs or single cells and belong to the phylum *Firmicutes*, class *Bacilli* and family *Enterococcaceae*. Initially classified as group D streptococci, enterococci were proven to be different from the streptococci on the basis of DNA hybridization experiments [1]. Sequentially, *Enterococcus* was given formal genus status in 1984 [1]. Enterococci have the capacity to grow between 10°C and 45°C, in 6.5% NaCl and at pH 9.6, to survive upon heating at 60°C for 30 min, and to hydrolyze esculin into esculitin [2]. They produce lactic acid from glucose through the homofermentative pathway, and thereby belong to the group of lactic acid bacteria (LAB). Given their intrinsic robustness, they are ubiquitous in several ecological niches: they colonize the gastrointestinal and genito-urinary tracts and the oral cavity of humans and animals; they are also found in soil, sand, water, food products and plants and their detection in water is considered as an indicator of faecal contamination [3-6]. Contrary to other LAB, enterococci are not considered “Generally Recognized As Safe” (GRAS) [7], nevertheless, they have been used in food processing due to their proteolytic and esterolytic activities, which contribute to the ripening and aroma development of traditional cheeses such as Cheddar, Feta and Mozzarella [8]. They are also considered as potential probiotics for human and animals, to treat or prevent diarrhea and irritable bowel syndrome, and for health improvement such as reducing cholesterol in the plasma or immune regulation [9-11]. Indeed, *E. faecalis* Symbioflor 1 is used for immune regulation to combat recurrent chronic sinusitis or bronchitis [12,13]. However, despite their potential beneficial roles, their ability to produce biogenic amines in cheese and fermented sausages as well as

their propensity for genetic exchange constitute negative aspects for their utilization as probiotics [14,15].

Nowadays, the genus *Enterococcus* is composed of 44 species [16] from which *Enterococcus faecalis* is currently the most studied due to its prominence in nosocomial infections. However, the interest on *Enterococcus faecium* has risen since it became also an important cause of nosocomial infections over the years [17]. Of the two species, *E. faecalis* harbors the greater innate capacity to cause disease whereas *E. faecium* relies mostly on its multiple antibiotic resistances to drugs of last resort to become infectious [18]. As an example of their propensity to acquire antibiotic resistance, from 1993 to 2002, *E. faecium* isolates resistant to vancomycin raised from 28.9% to 72.4% [19].

Genetic diversity of *E. faecalis* has been reported using various molecular typing methods, comparative genome hybridization (CGH) or analysis of full genome sequences. The most informative molecular typing method for enterococci epidemiology is the multi locus sequence typing (MLST). This method is based on nucleotide sequences of seven housekeeping genes and allows to compare genetic relatedness between strains. It generates an allelic profile that is compared to those within a database to identify the sequence type (ST) or define a new one, if the allelic profile is new. The use of MLST to study *E. faecalis* population unraveled a non-clonal structure for which recombination plays an important role driving genetic variation of this species [20]. At present, a total of 517 STs exist in the database (http://efaecalis.mlst.net). The diversity is so remarkable among *E. faecalis* isolates that the MLST analysis of 110 strains of different sources and geographical locations were resolved into 55 STs [20]. A similar study, with 386 strains generated 105 STs [21]. MLST analyses group the majority of multi-drug resistant isolates of *E. faecalis* into seven major clonal complexes: CC2, CC9, CC16, CC21, CC30, CC40 and CC87, designated as High-Risk

Enterococcal Clonal Complexes (HiRECC), of which CC2 and CC87 include almost exclusively hospital isolates [20-23]. CGH studies of *E. faecalis* isolates revealed considerable differences in the genomic content, as already suspected by MLST-generated data. This diversity was mostly related to the presence and absence of genetic mobile elements such as phages and conjugative transposable elements [24-27]. The first complete genome sequence of *E. faecalis* was released in 2003 and belongs to strain V583 [28]. This isolate is a member of the HiRECC-2, and was the first vancomycin resistant clinical isolate reported in the United States [28]. Since then, the analysis of sixteen *E. faecalis* genomes, recovered from strains isolated over a period of 80 years, confirmed the association of the genomic content differences among *E. faecalis* strains with the presence or absence of genetic mobile elements [29,30]. Interestingly, genome size was inversely correlated with the presence of a CRISPR/Cas functional system, defined as a bacterial system directed against invading DNA (see below on section II.1.2.3). Actually, strains with greater average genome size lacked CRISPR/Cas, like V583. Palmer and collaborators suggest a model in which increased genome size is the result of mobile element accretion due to compromised genome defenses [30,31]. They also correlated the loss of a functional CRISPR/Cas system in the modern *E. faecalis* hospital-adapted lineages with the influx of acquired antibiotic resistance genes [29-31]. A large variety of conjugative plasmids, transposons and integrative and conjugative elements (ICE) have been involved in genetic transfer of resistance and virulence determinants, thus contributing to the success of *E. faecalis* as a nosocomial pathogen [32-34]. Even if CC2 isolates are enriched in mobile genetic elements and surface protein encoding genes [35], the *E. faecalis* opportunism factors characterized so far are widespread among isolates independently of their origin, and none is essential for enterococcal infections [36-38].

In all, the high genetic diversity contributes to the difficulty to predict *E. faecalis* pathogenic potential from the gene content.

2. *E. faecalis*: commensal & opportunistic pathogen

E. faecalis is a commensal bacterium of the gastrointestinal tract (GIT) of humans and other animals as well as insects [39,40]. It colonizes the GIT of humans just after birth and remains associated with the adult human gut microbiota as part of the sub-dominant species [41]. Despite its status as primary colonizing bacteria in the human gut and although harmless in healthy individuals, it has emerged, for the last 30 years, as an important cause of nosocomial infections. It is responsible for 70% of enterococcal-induced infections in hospital setting [42]. This opportunistic pathogen causes wound-, bloodstream- and urinary tract infections and endocarditis, mainly in hospitalized patients [4,43,44].

The mechanisms by which inoffensive commensal *E. faecalis* may become major hospital-acquired pathogens are still not understood but this seems to be a multiparametric phenomenon. Both host and bacterial factors are implicated: equilibrium of the intestinal microbiota and immune system on the host side, as well as bacterial opportunistic traits [45]. A key step in this process concerns *E. faecalis* entry into the bloodstream, which can be achieved through two pathways. The first one involves direct entry by rupture of physical barriers. The second is related to the colonization of the GIT associated with an imbalance of the microbiota. In this case the bacteria need to cross the epithelial barrier to reach the bloodstream and the lymphatic system [46]. Bacterial opportunistic traits are defined as genetic elements that increase the capacity of a microorganism to induce disease [45]. Three main features contribute to the success of *E. faecalis* in hospital environments and to its emergence and prevalence as a nosocomial pathogen: i) its inherent capacity to withstand environmental stresses; ii) a wide range of opportunism factors [47]; and, iii) the

accumulation of different antimicrobial resistance mechanisms, both innate and acquired [48,49].

E. faecalis is a very robust bacterium with an outstanding ability to cope with relatively high concentrations of host-produced inhibitory compounds and nutrient limitations as well as antibiotics used for therapy [50-52]. In addition, it owns a plethora of opportunism factors, effectors and regulators, that allows it to thrive in the hospital setting and participate in the four main stages of the infectious process (Table 1): persistence on abiotic surfaces, entry into the bloodstream, colonization of infection site and tissue damage [53-55]. The best way to survive in hospital environment is through biofilm growth, which protects bacteria against the action of antibiotics and biocides [56]. Indeed, *E. faecalis* counts on several proteins that contribute to biofilm formation such as Esp [57], Ebp [58], Bee [59] and StrA [60,61]. To enter the bloodstream and further colonize the host, *E. faecalis* uses factors such as adhesins, like aggregation substance (AS) [62], enterococcal surface protein (Ebp) [63] and Ace [64] that promote adherence to host tissues. Moreover, factors that possess immune evasion properties are essential to cell survival and persistence, such as the exopolysaccharides (Epa and Cps) [65,66] or GelE [67]. The final stage is the clinical manifestation of infection that leads to cell and tissue damage (like heart valves) from the activity of proteins that include cytolysin, gelatinase and serine protease [68-70].

Antibiotics interrupt cellular functions through different modes of action: attack of the cell wall and cell membrane integrity or interference with DNA and protein synthesis. *E. faecalis* exhibits a broad range of intrinsic tolerance to low concentrations of several classes of antibiotics including aminoglycosides, β-lactams and quinolones [5,71,72]. In addition, single or multiple antibiotic resistance may arise by point mutations in the drug binding site, like for quinolones [73] and ampicillin, or by acquisition of resistance genes, as observed for the β-lactams [74],

aminoglycosides, macrolides, chloramphenicol, tetracycline and glycopeptides, of which vancomycin resistance is the most clinically relevant [5,71-73,75] (Figure 1).

Table 1. *E. faecalis* opportunism factors [49,53].

Gene or locus	Virulence factor	Putative role	References
Cell surface determinants			
AS proteins	Aggregation substance	Adhesion, tissue colonization, endocarditis	[62,76,77]
Esp	Surface protein	Biofilm formation	[57]
Ace	Adhesion to collagen	Adhesion to ECM, endocarditis	[64,78]
Bee	Biofilm enhancer	Biofilm formation	[59]
Ebp	Endocarditis and biofilm associated pili	Biofilm formation and adhesion to human platelets	[58,79]
ElrA	Surface protein	Role in experimental peritonitis, resistance to host defenses	[80]
StrA	Sortase	Biofilm formation, role in catheter-associated UTIs	[60,61]
Exopolysaccharides			
cps cluster	Capsular polysaccharides	Resistance to host defenses	[66]
epa cluster	Enterococcal polysaccharide antigen	Resistance to host defenses	[81]
Secreted factors			
GelE	Gelatinase	Tissue damage, formation of biofilms, immune evasion	[67,70,82]
SprE	Serine protease	Tissue damage	[69]
CylA-M	Cytolysin	Tissue damage	[68]
Regulators			
FsrA-D	-	*gelE*, *sprE* and ace regulation	-
CylR1-R2	-	Cytolysin regulation	-

From a clinical point of view, multidrug-resistant *E. faecalis* isolates are difficult to treat and constitute a major medical challenge due to limited therapeutic options. A recent study with strains from various human sources in Europe supports the concept that *E. faecalis* is globally highly

resistant to clinically used antibiotics, and higher levels of resistance are associated with hospital environment [21,83].

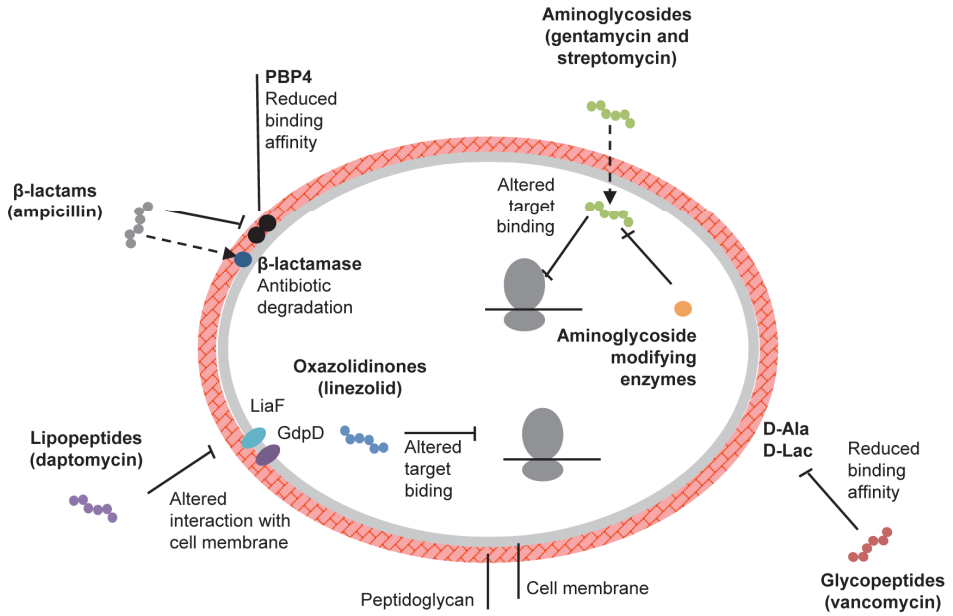


Figure 1. **Mechanism of *E. faecalis* antibiotic resistance**. In addition to their intrinsic resistance to several antibiotics, *E. faecalis* can acquire new antibiotic resistances as well as high-level antibiotic resistance either by point mutations and/or acquisition of genetic mobile elements. High-level resistance to aminoglycosides can be acquired by point mutation on ribosomal subunits, resulting in altered target binding, or by capture of genes for aminoglycoside–modifying enzymes. Resistance to vancomycin involves two pathways: replacement of the terminal D-Ala of peptidoglycan precursors by D-Lac or a D-ser, resulting in high or low-level resistance, respectively. Resistance to linezolid involves mutations in genes encoding domain V of 23S rRNA. Resistance to the lipopeptide daptomycin involves altered interactions with the cell membrane by the action of membrane proteins LiaF and GdpD. Resistance to β-lactams can be acquired through mutations on *pbp4* gene or acquisition of β-lactamases. Adapted from [53].

Severe *E. faecalis* infections can be treated by the synergistic action of ampicillin and aminoglycosides (gentamycin or streptomycin). When a strain is resistant to vancomycin, new antibiotics such as quinupristin-dalfopristin, linezolid or daptomycin can be administered. However, strains resistant to those antibiotics have already been identified (Figure 1) [84]. Furthermore, since antibiotic resistance is frequently linked to genetic mobile elements, their dissemination to pathogenic bacteria in hospitals, such as *Staphylococcus aureus*, represents a major health concern. Actually, *S. aureus* isolates resistant to vancomycin were found to contain the enterococcal transposon Tn*1546* harboring the *vanA* resistance operon [85,86]

One of the most serious complications of *E. faecalis* bacteremia is the development of infective endocarditis, characterized by the formation of an infected thrombus on the surface of a heart valve. Enterococci, staphylococci and streptococci are responsible for the majority of nosocomial infective endocarditis that has a case-fatality rate of more than 50% [87,88]. This infection is very difficult to treat due to low antibiotic penetration in the thrombus and to multidrug resistance. Host tissue damage factors, such as cytolysin [89], pili [58], Asc10 [77], Ace [78] and gelatinase [90,91] described above, are important to the development of infective endocarditis. In addition, recent studies suggest that *E. faecalis* pili and platelet-binding proteins can also be involved in endocarditis development due to their ability to bind and aggregate platelets. This is considered as a first step in the development of infective endocarditis [79,92]. The existence of different factors causing the same disease, together with the multidrug-resistance illustrates the multifactorial nature of *E. faecalis* pathogenesis.

II. BACTERIOPHAGES

1. Biology

Bacteriophages are viruses that specifically infect bacteria and represent the most abundant ecological entities on earth with an estimation of population size of more than 10^{30} viral particles [93]. After their independent discovery in 1915 by Frederick Twort and in 1917 by Felix d'Herelle for their antibacterial activity, bacteriophages were mainly explored in the western countries for their applications in molecular biology and genomics. While empirically used for phage therapy in Soviet Georgia, phages have gained recent attention for their potential use as therapeutic and biocontrol agents of pathogens in the era of multidrug-resistant bacteria (Figure 2) [94].

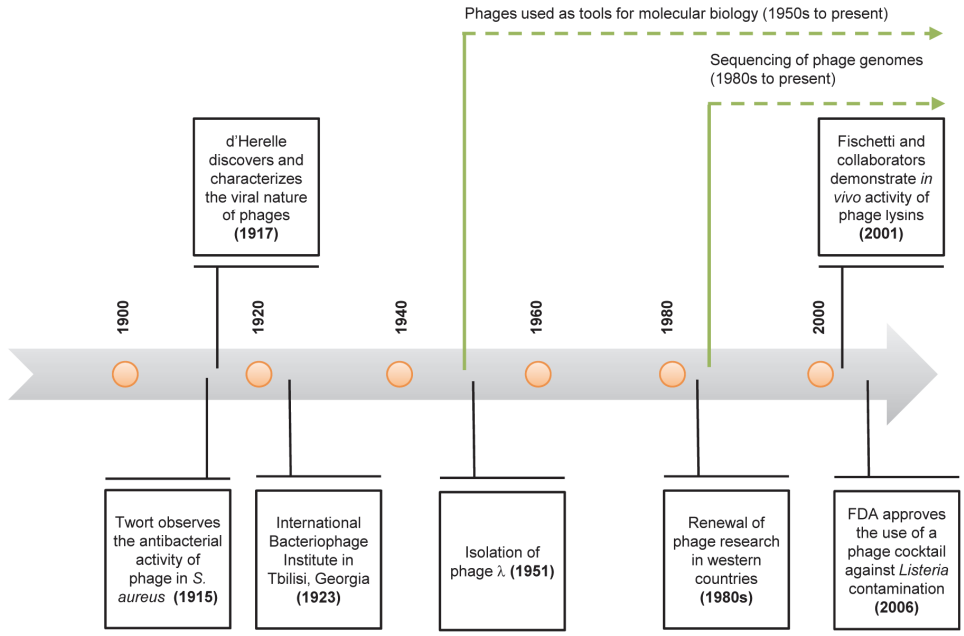


Figure 2. Timeline of major milestones in phage history. Adapted from [94].

Phages exist as extracellular entities made of proteins that protect the genetic material, double- or single-stranded DNA or RNA. Regarding morphology, they are divided in four morphotypes: tailed, polyhedral, filamentous and pleomorphic (Table 2). Tailed phages account for 96% of all prokaryotic viruses, and are grouped in the order *Caudovirales* [95]. The *Siphoviridae* family with long non-contractile tails represent 61% of them [96]. They infect mostly enterobacteria and members of the *Streptomyces*, *Mycobacterium*, *Bacillus*, *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Pseudomonas* and *Vibrio* genus [95].

Table 2. Overview of prokaryotic virus families [95].

Shape	Nucleic acid	Family	Characteristics	Members
Tailed	dsDNA (L)	*Myoviridae*	Tail contractile	1320
		Siphoviridae	Tail long, non-contractile	3229
		Podoviridae	Tail short	771
Polyhedral	ssDNA (C)	*Microviridae*	Conspicuous capsomers	40
	dsDNA (C, S)	*Corticoviridae*	Complex capsid, lipids	3
	dsDNA (L)	*Tectiviridae*	Inner lipid vesicle, pseudotail	19
	dsDNA (L)	SHI group*	Inner lipid vesicle	1
	dsDNA (C)	STV1 group*	Turret-shaped protrusions	1
	ssRNA (L)	*Leviviradae*	Poliovirus-like	39
	dsRNA (L, M)	*Cystoviridae*	Envelope, lipids	3
Filamentous	ssDNA (C)	*Inoviridae*	Long filaments, short rods	67
	dsDNA (L)	*Lipothrixviridae*	Envelope, lipids	7
	dsDNA (L)	*Rudiviridae*	Stiff-rods, TMV-like	3
Pleomorphic	dsDNA (C, S)	*Plasmaviridae*	Envelope, lipids, no capsid	5
	dsDNA (C, S)	*Fuselloviridae*	Lemon-shape, envelope	11
	dsDNA (L, S)	*Salterprovirus*	Lemon-shape, envelope	1
	dsDNA (C, S)	*Guttaviridae*	Droplet-shape	1
	dsDNA (L)	*Ampullaviridae*	Bottle-shape, helical NC	1
	dsDNA (C)	*Bicaudaviridae*	Two-tailed, helical NC	1
	dsDNA (L)	*Globulaviridae*	Envelope, spherical, lipids, helical NC	1

C, circular; L, linear; M, multipartite; NC, nucleocapsid; S, supercoiled; *, waiting classification.
Members indicate number of phages examined by electron microscopy.

1.1 Lytic *vs* temperate bacteriophages

Typically, phage genomes are organized into large operons of functionally related genes that are temporally and sequentially expressed [97]. They present a conserved overall gene order reflecting their modular

organization: left attachment site (*attL*), lysogeny control, DNA replication, transcriptional regulation, DNA packaging, head morphogenesis, tail morphogenesis, lysis module and the right attachment site (*attR*). Lysogenic conversion genes, defined as prophage genes that improve host fitness, are normally located between the lysis module and the *attR*, and have a different GC content when compared with the rest of the phage genome. Tailed bacteriophages use mostly two strategies to propagate: the lytic and the lysogenic cycles. During the lytic cycle, phages produce progeny right after infection and cause bacterial lysis. The lysogenic cycle is performed by temperate phages, and is characterized by the establishment of a prophage state in which the phage genome is integrated into the bacterial chromosome by reciprocal recombination. It is thus, replicated and segregated as part of the bacterial chromosome until induction of the lytic cycle (Figure 3). Both cycles begin by phage recognition of specific receptors on the surface of the bacterial host. Sequentially, DNA is injected into the bacterial cell and circularizes by annealing of the cohesive *cos* sites then, one of the cycles is initiated depending on phage nature. In the lysogenic cycle, recombination, coordinated by the phage integrase, occurs between unique attachment sites on both phage DNA (*attP*) and bacterial chromosome (*attB*) promoting phage integration. A recent survey of prophages found in the sequenced bacterial genomes showed that prophage integrates into tRNA, intergenic regions and open reading frames for genes at similar frequency [98]. In order to maintain the prophage state, lytic genes are turned-off by a phage-encoded repressor. This repressor is also responsible for immunity against incoming phages of the same type. The prophage state is usually very stable, however prophages can be induced to enter the lytic cycle through different genetic switches the most common being the SOS response (Figure 3) [99]. The SOS response is a conserved pathway that controls a set of genes involved in DNA repair

through the action of two proteins, LexA and RecA. Upon DNA damage, RecA forms a complex with ssDNA and becomes activated (RecA*). This complex works as a co-protease that promotes LexA autocleavage. The resulting decrease of LexA cellular pool leads to the activation of the SOS regulon [100]. Prophages are repressed by a phage-encoded repressor that, like LexA, undergoes autocleavage in presence of RecA*. This will induce the prophage into the lytic cycle [101].

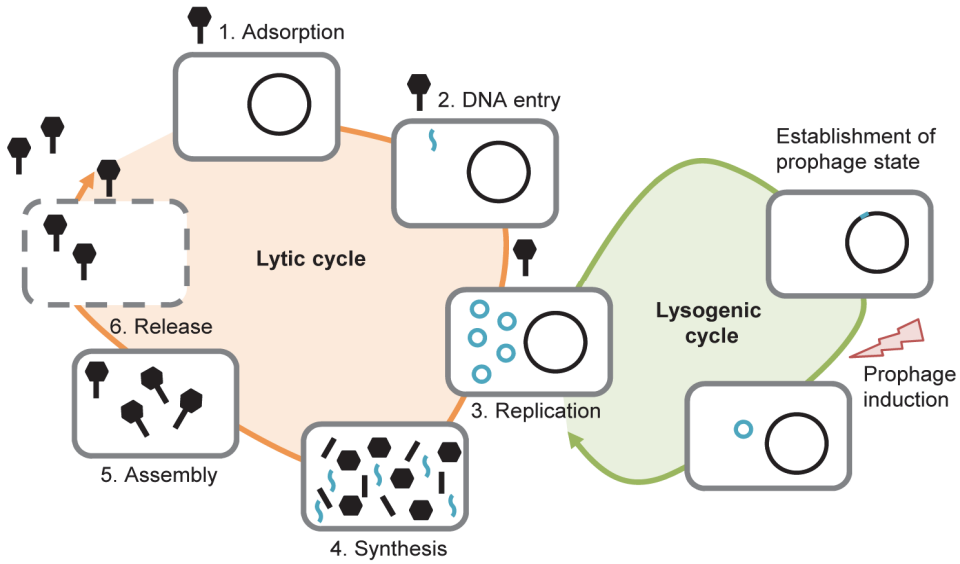


Figure 3. Lytic *vs* lysogenic cycle. In the lytic cycle, the phage adsorbs to the surface of the cell and injects its genome into the host cytoplasm followed by circularization. Phages hijack the host cell machinery in order to replicate its genome and produce its proteins. Phage proteins are assembled into mature phage particles and released by a cell-lysis event coordinated by the action of two phage-encoded proteins, holin and endolysin. In the lysogenic cycle, after DNA injection and circularization, the phage integrates its genome at a specific attachment site (*attB*) into the host chromosome establishing a prophage state. The phage DNA replicates along with the bacterial host chromosome. Prophages are stably maintained in the host chromosome until environmental stimuli (i.e. pressure, temperature and nutritional deficiency) or cellular damage induces the prophage into the lytic cycle and release progeny.

Upon prophage excision, a process mediated by both integrase and excisionase, temperate phages engage the lytic pathway, a set of coordinated events that they share in common with the cycle of lytic phages. Bacterial genome is rapidly degraded, and the phage genome is replicated. Late genes, encoding proteins involved in virion assembly and cell lysis, are transcribed. Once produced and accumulated at suitable levels, head and tail structural proteins are assembled independently and then joined to form a functional virion. Head assembly begins with the production of a procapside (an empty coat protein shell) followed by phage genome uptake into the procapside through the portal protein. Phage genome is recognized and packaged by the terminase complex. During genome packaging the procapsid shell undergoes expansion and becomes more angular in overall appearance [102]. Next, generally through the action of two phage proteins, holin and lysine, bacterial cell lysis occurs, releasing the phage progeny that can search for a new host and begin a new cycle [103].

1.2 Bacterial immunity to phage infection

Bacteriophages have co-evolved with their bacterial host for more than 35 billion years resulting in an everlasting arms race that leads to continuous variation and selection towards adaptations of the host, and counter-adaptations of the phage [104]. Most steps of phage lytic cycle can be targeted by an anti-phage mechanism and counteracted by a phage adaptation. The understanding of the phage-host dynamics is particularly relevant in two circumstances: in the dairy industry, for which phages can impair drastically fermentation processes and thus the overall production, and also in phage therapy to avoid mutual resistances.

1.2.1 Preventing phage adsorption

In order to infect host cells, bacteriophages must recognize a specific receptor on the surface of the bacteria. In addition to the diversity of receptors, achieved by receptor direct alteration through mutagenesis, down-regulation or loss, bacteria have evolved a number of ways to prevent phage adsorption such as production of extracellular matrix or competitive inhibitors [105]. Phages have counteracted by changing their specificity for another receptor, producing carbohydrate-degrading enzymes such as lyases or hydrolases or by recognizing lipopolysaccharides as receptors. In a very elegant experiment of coevolution between phage λ and *E. coli*, Meyer and collaborators demonstrated that upon downregulation of phage λ natural receptor LamB by the host strain, λ has acquired several point mutations in its receptor binding protein that allows it to bind to a new receptor, OmpF [106].

1.2.2 Preventing phage DNA entry

Superinfection exclusion systems block phage DNA entry into the host cell conferring immunity to a specific phage. They are normally coded in prophage genomes and their principal role is to avoid an infection by a second phage. Two distinct superinfection exclusion mechanisms that block DNA injection of a particular subset of 936-phage group were identified in *Lactococcus lactis* strains MG1363 and IL1402 [107]. Each of the systems allows normal phage adsorption but affects phage DNA replication [107]. Gene *ltp* from *Streptococcus thermophilus* temperate phage TP-J34, codes for a lipoprotein involved in superinfection exclusion against *S. thermophilus* phages and also phage P008 from *L. lactis* [108].

1.2.3 Degradation of foreign DNA

Two different mechanisms for degradation of invading DNA have been described: restriction-modification and CRISPR/Cas systems.

Restriction-modification systems are composed of two sequence-specific recognition activities: a restriction endonuclease (REase) that digests incoming foreign genetic material and a methyltransferase (MTase) that protects host genetic material from degradation through methylation of specific bases [109]. Thus, all non-methylated DNA is recognized as foreign and digested. Phages overcome this system by stimulating host MTase to methylate phage DNA, by inhibiting the REase or incorporating unusual bases in their DNA to evade the REase. This was observed for *Bacillus subtilis* phages that replace thymine by 5-hydroxymethyluracil [110].

CRISPR interference is the only adaptive immunity system in prokaryotes. CRISPR loci consist of an array of short direct repeats separated by highly variable spacer sequences of precise length that correspond to previously captured foreign DNA from bacteriophages or plasmids [111,112]. CRISPR associated (*cas*) genes are located immediately downstream of the repetitions and encode the protein machinery in charge of the CRISPR activity. In the adaptation phase CRISPR loci incorporates additional spacer sequences to enlarge their activity against invader foreign DNA (Figure 4). Thus, the spacer content reflects the many different elements that have been encountered by the bacterial host. Once a spacer is established it can be used for protection through the interference phase of the CRISPR pathway. This phase involves the transcription of the CRISPR loci into a single RNA transcript that is cleaved by the Cas proteins in order to generate CRISPR RNA (crRNA) units, each containing one targeting spacer. These units will then interfere with the incoming foreign nucleic acid through hybridization, leading to its degradation by the bacterial cell degradation machinery (Figure 4) [112]. Despite CRISPR/Cas adaptive character, bacteriophages have already stroke back by finding a way to circumvent CRISPR immunity.

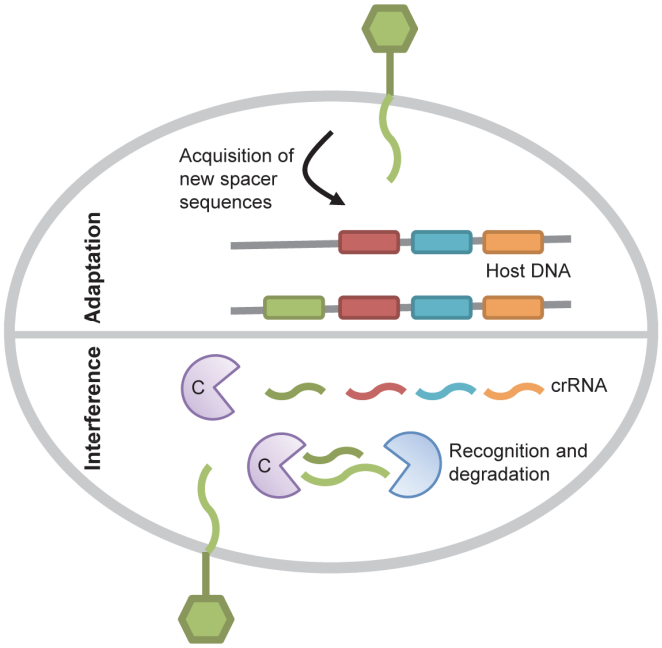


Figure 4. CRISPR/Cas immunity mechanism. In the adaptation phase of CRISPR immunity, new spacers from the invading DNA are incorporated into CRISPR loci. During the interference phase, repeats and spacers are transcribed into a long precursor that is processed by a complex, made of Cas proteins, to generate CRISPR RNAs (crRNAs). This complex together with the correspondent crRNA detects the foreign incoming DNA, targeting the phage genome for degradation. Adapted from [113].

Bondy-Denomy and collaborators described recently several anti-CRISPR phage-encoded genes mediating phage evasion to CRISPR/Cas system in *Pseudomonas aeruginosa* by a yet unknown mechanism [114]. Seed and collaborators report a *Vibrio cholerae* phage, ICP1, which uses its own CRISPR/Cas system to evade a phage immunity system coded on a host chromosomal island, PLE. Interestingly, and in relation with section III.2 of this chapter, PLE has been recognized as a phage-related chromosomal island (PRCI) due to its length, base composition and genome organization [115]. Upon ICP1 infection, PLE on the bacterial host circularizes and inhibits ICP1 lytic infection by an unknown mechanism. In order to progress on the lytic cycle, ICP1 uses its

CRISPR/Cas to target PLE for destruction. If ICP1 has not a complete CRISPR/Cas system, lytic infection is abolished due to the lack of complementarity between ICP1-CRISPR spacers and the target chromosomal island [115].

1.2.4 Abortive infection (Abi)

Abortive infection (Abi) system acts later in the phage infectious process and targets phage replication, transcription, translation or genome packaging [116]. Abi leads to the premature death of the phage-infected bacteria, which limits phage replication and favours the surrounding bacterial population. Although widespread in different bacteria such as *E. coli* and *B. subtilis*, most of Abi systems have been identified in *L. lactis* [116]. Abi systems are generally coded by plasmids. Similarly in *E. coli* and *L. lactis*, Lit and AbiD1, respectively, code for an enzyme activated upon phage infection that promotes cleavage of essential and conserved components of the bacteria translational apparatus [117,118]. Recently, the mechanism underlying *L. lactis* AbiQ abortive infection was unravelled. AbiQ belongs to the type III toxin-antitoxin systems, which involve an antitoxic RNA molecule interacting with its cognate toxic protein [119]. This system resembles to another type III toxin-antitoxin system also involved in an abortive infection mechanism described in *Erwinia carotovora* [120]. ABIQ protein has an endoribonuclease activity. However, the exact mechanism through which it kills phage-infected bacteria is still unknown [119].

2. Ecological impact

Bacteriophages have a major impact on the ecological balance and dynamics of microbial life. They are found where bacteria thrive, whether it is on sediments, aquatic systems or the human body. In nature, phages face several constraints imposed by the different environmental conditions

they can encounter. The most important conditions are the host availability and its nutritional and metabolic states that dictate the outcome of viral infections and proliferation [121]. Regardless of the difficulties, bacteriophage influence many biogeochemical and ecological processes, determining biological production and community species composition, as well as patterns of matter and energy transfer [122]. Although most of the bacteriophage ecological studies have been performed in the aquatic environment, the dynamic relationship phage-host can be extended to other complex microbial ecosystems such as human associated microbial communities [123]. For example, in the gut, phages are suspected to shape the functionality and diversity of the human intestinal microbiome [124]. One of the models that explain how phages control community species composition is called 'killing-the-winner'. It stipulates that viruses control the most abundant or fastest growing population, enabling less-competitive or slower-growing populations to co-exist with the dominant, fast-growing hosts. The reduction in size of the dominant host population gives the opportunity for new hosts to become abundant thus maintaining the high diversity in the population [125]. Metagenomic studies have recently revealed the bacteriophage diversity and abundance within the human gastrointestinal tract [126,127]. Imbalanced composition of the intestinal microbiota or dysbiosis is associated with many intestinal diseases, such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and obesity [128,129]. Given the beneficial or harmful effects of phages on a bacterial population, phages are suspected to influence the balance between intestinal symbionts and pathobionts under certain circumstances [124,130].

2.1 Prophage induction & impact on host fitness

It is largely accepted that temperate phages have a major impact on the development of bacterial pathogenesis and they are key players in the

evolution of bacteria by shaping their genomes through horizontal gene transfer [131]. Lysogeny is a common trait among bacteria, suggesting that prophages provide advantages to their host whether it is a pathogen, commensal or free living organism. This would compensate the potential negative aspects of carrying extra DNA, such as metabolic burden from extra DNA replication and cell lysis after prophage induction. Thus, to benefit bacterial fitness, evolution would select lysogenic strains with mutations that inactivate the prophage ability to perform the lytic cycle. Next, large-scale deletion of prophage genes would decrease the metabolic burden, while genes that increase host fitness would be maintained [132]. In support of this theory, it is common to find multiple prophages in various stages of functionality in the same bacterial chromosome, as is the case of *Streptococcus pyogenes*, *Lactobacillus plantarum* and different strains of *E. coli* [133-136]. Indeed, in addition to fully functional prophages that can be induced to start a lytic cycle and release phage progeny, bacterial genomes, also harbor non-functional prophage-like entities: i) defective prophages, that are prophages in a state of mutational decay. This is the case of the nine *E. coli* K-12 prophages, CP4-6, DLP12, e14, rac, Qin, CP4-44, CPS-53, CPZ-55 and CP4-57 [136]; ii) prophage satellites, that are elements whose genome has evolved to hijack structural proteins from a helper phage. The best characterized example of such an interaction occurs between satellite phage P4 and phage P2 (see section III.1 of this Chapter for details) [137]; and iii) gene transfer agents (GTAs), that are tailed phage like particles that package random fragments of the bacterial genome. These virion-like particles can transduce bacterial DNA into another host, in which they can recombine with the chromosome and be vertically inherited. The best characterized GTA is encoded on the *Rhodobacter capsulatus* chromosome [138].

When bacterial strains harbor more than one prophage they are called polylysogenic. Polylysogeny is frequent among pathogens, such as *Escherichia coli* O157:H7 strain Sakai (18 prophages) and *Streptococcus pyogenes* M3 (6 prophages) (Figure 5) [133,134], but also among opportunistic pathogens and dairy strains such as *E. faecalis* V583 (7 prophages) and *Lactococcus lactis* IL1403 (6 prophages), respectively [28,139].

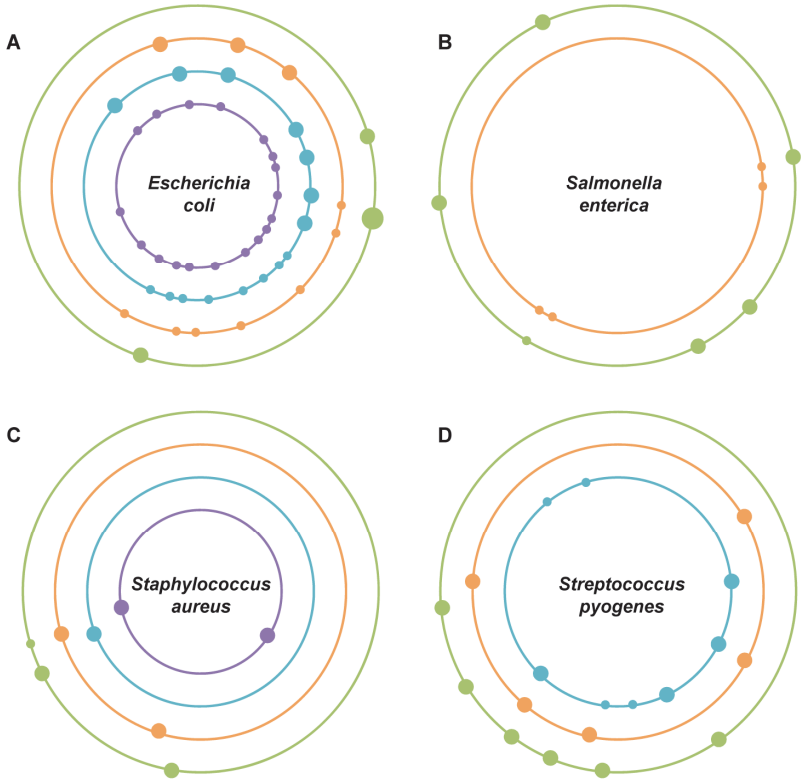


Figure 5. Polylysogeny in human bacterial pathogens. Examples of human pathogens for which the accumulation of prophages has a major impact for pathogenesis. From the center to periphery: (A) *Escherichia coli* genomes from O157:H7 Sakai, O157:H7 EDL933, K12 and CFT073; (B) *Salmonella enterica* genomes from serovar Thyphimurium LT2 and serovar Tiphy CT18; (C) *Staphylococcus aureus* genomes from Mu50, N315, MW2 and 8325; (D) *Streptococcus pyogenes* genomes from M1, M3 and M18. Prophages are indicated by dots, which size if proportional to phage genome length. Circles represent bacterial chromosomes, but are not at scale. Adapted from [140].

A well studied case of impact of polylysogeny on pathogenesis and emergence of hypervirulent clones is illustrated by *Streptococcus pyogenes*. Serotype M3 strains isolated in the 1920s contains φ315.5 (encoding the SpeA1 exotoxin) while M3 strains causing disease in the 1940s have an additional prophage, φ315.2, encoding the streptococcal superantigen (SSA). At the same time, a nonsynonymous mutation in SpeA1 on φ315.5 gave rise to SpeA3, a significantly more mitogenic variant. Contemporary serotype M3 strains have one more prophage, φ315.4, which encodes exotoxin SpeK and streptococcal phospholipase A_2, Sla (Figure 6) [141]. Thus, subclone M3 has accumulated prophages over time resulting in the emergence of a superbug built from a unique combination of phage-encoded virulence factors [134].

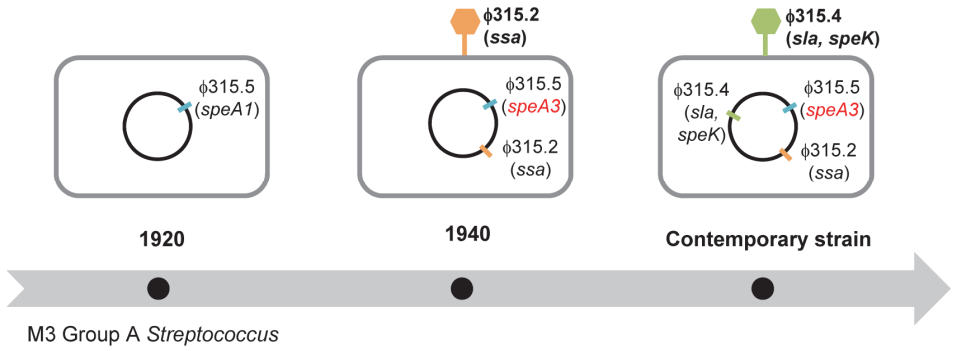


Figure 6. **Hypothesis for the emergency of M3 hypervirulent clones**. Early in the 20th century, an ancestral strain, harboring phage 315.5, acquired phage 315.2, harboring SSA. Subsequently, a single nucleotide mutation resulting in a single amino acid transformed SpeA1 in SpeA3 variant encoded by phage 315.5. Later, this strain gained phage 315.4 that encodes both Sla and SpeK toxins and disseminated widely in the mid late 1980s. Adapted from [141].

Prophages can impact bacterial fitness in several ways: introduction of new fitness factors through lysogenic conversion, gene disruption and lysis-mediated competitiveness upon prophage induction and [142]. They

initiate lytic cycle upon induction, frequently due to SOS-response activation. Antibiotics targeting DNA replication, such as ciprofloxacin and trimethoprim, can induce the SOS response and consequently prophages. Frequently, they also enhance the expression of phage-encoded virulence factors [143]. Some β-lactams, like ampicillin, penicillin, cloxacillin and ceftriaxone, are also able to induce an SOS-response [144]. Antibiotic-induced SOS response and consequent prophage and prophage-related elements induction, promotes horizontal gene transfer of virulence genes [145]. β-lactams induced SOS response has been attributed to the formation of reactive oxygen species (ROS) [146,147]. However recent reports claim that these antibiotics do not induce the production of ROS, and that their effect is probably due to direct inhibition of cell-wall assembly, protein synthesis and DNA replication [148,149].

2.1.1 Lysogenic conversion

Lysogenic conversion was first reported in *Corynebacterium diphtheriae*, the etiological agent of diphtheria. Freeman reported that non-lysogenic strains become toxicogenic after infection with a *tox*$^{+}$ corynebacteriophage β [150]. This is the typical situation in which bacteriophages encoding virulence factors can convert their bacterial host from a non-pathogenic strain into a strain with increased virulence. The *tox* gene, which codes for diphtheria toxin (DT), is activated by low iron concentration. After production, DT is secreted and attached to the cell surface of respiratory epithelium cells where it promotes cell death [151]. Since, the list of lysogenic conversion genes and their mechanisms of action in many other bacterial pathogens has grown and includes both Gram-positive and Gram-negative bacteria such as *S. aureus*, *S. pyogenes*, *E. coli*, *S. enterica*, *Pseudomonas aeruginosa* and *Vibrio cholerae* (Table 3) [142]. The most important factors for the success of a bacterium in hospital setting are those that contribute to host colonization,

acquisition of nutrients and evasion to the innate and adaptive immune systems. In many of these circumstances, prophage-encoded factors can provide an advantage to the bacterial host (Table 3) [142,152]. For example, different phage-encoded proteins promote bacterial evasion from the immune system through the modification (glucosylation or acetylation) of the O-antigen of different bacteria, such as *S. enterica* [153,154], *P. aeruginosa* [155,156], *Shigella flexneri* [153,157] and *Neisseria meningitidis* [158]. Other phage-encoded proteins contribute to bacterial survival to eukaryotic cell environment such as oxidative stress. Phages *Gifsy*-2 and *Fels*-1 from *S. enterica* code for superoxide dismutases protecting the bacteria against host produced ROS [159]. Finally, prophages carry an arsenal of extracellular toxins used by their bacterial host to induce damage in eukaryotic cells. They perform functions such as cytotoxic (as CTX from *P. aeruginosa*) [160], cardiotoxic (as TOX from *C. diphtheriae*) [150] or neurotoxic (as C1 from *C. botulinum*) [161]. Interestingly, two prophages of the *E. coli* O157:H7 strain and other Shiga-toxins producing *E. coli* (STEC) encode Shiga-toxins, Stx1 and Stx2, which cause severe hemorrhagic colitis and hemolytic uremic syndrome [162]. Antibiotic treatments of these infections are deleterious for the patients as they induce prophages, and consequently increase toxin production [163,164].

2.1.2 Gene disruption

Lysogenization of *S. aureus* by φ13 results in loss of β-toxin expression due to prophage integration into the 5' end of β-toxin gene (*hlb*) [165]. In *Listeria*, φ10403S insertion interrupts *comK* gene, which corresponds to the master regulator of DNA uptake competence system in *B. subtilis*. Prophage excision restores ComK that can activate its target genes, involved in *Listeria* pathogenesis [166].

Table 3. Phage encoded bacterial virulence factors [142,152].

Protein	Gene	Phage	Bacterial host	References
Adhesion for bacterial host attachment				
Vir	*vir*	MAV1	*Mycoplasma arthritidis*	[167]
PblA and PblB	*pblA*, *pblB*	SM1	*Streptococcus mitis*	[168]
Effector proteins involved in invasion				
Effector protein	*sopE*	SopEφ	*S. enterica*	[169]
Effector protein	*sseI* (*gtgB*)	*Gifsy*-2	*S. enterica*	[159]
Effector protein	*sspH1*	*Gifsy*-3	*S. enterica*	[159]
Proteins altering antigenicity				
O-antigen acetylase	*lap, oac, wzyβ*	D3	*P. aeruginosa*	[155,156]
Membrane protein	Pmn1	Mu-like	*Neisseria meningitidis*	[158]
Glucosylation	*rfb*	ε^{34}	*S. enterica*	[154]
Glucosylation	*gtr*	P22	*S. enterica*	[153]
O-antigen acetylase	*oac*	Sf6	*S. flexneri*	[157]
Glucosyl transferase	*gtrII*	SfII, SfV, SfX	*S. flexneri*	[153]
Enzymes				
Superoxide dismutase	*sodC*	Sp4, 10	*E. coli* O157	[170]
Superoxide dismutase	*sodC-I*	*Gifsy*-2	*S. enterica*	[159]
Superoxide dismutase	*sodC-III*	*Fels-1*	*S. enterica*	[159]
Neuraminidase	*nanH*	*Fels-1*	*S. enterica*	[159]
Hyaluronidase	*hylP*	H4489A	*S. pyogenes*	[171]
Staphylokinase	*sak*	φ13	*S. aureus*	[172]
Phospholipase	*sla*	315.2	*S. pyogenes*	[141]
DNase/streptodornase	*sdn, sda*	315.6, 8232.5	*S. pyogenes*	[141]
Extracellular toxins				
Diphtheria toxin	*tox*	β-phage	*C. diphtheriae*	[150]
Neurotoxin	*C1*	Phage C1	*C. botulinum*	[161]
Shiga toxins	*stx1, stx2*	H-19B	*E. coli*	[173]
Enterohaemolysin	*hly2*	φFC3208	*E. coli*	[174]
Cytotoxin	*ctx*	φCTX	*P. aeruginosa*	[160]
Enterotoxin	*see, sel*	NA	*S. aureus*	[175]
Enterotoxin P	*sep*	φN315	*S. aureus*	[176]
Enterotoxin A	*entA*	φ13	*S. aureus*	[172]
Enterotoxin A	*sea*	φMu50A	*S. aureus*	[176]
Exfoliative toxin A	*eta*	φETA	*S. aureus*	[177]
Toxin type A	*speA*	T12	*S. pyogenes*	[178]
Toxin type C	*speC*	CS112	*S. pyogenes*	[179]
Cholera toxin	*ctxAB*	CTXφ	*V. cholerae*	[180]
Leukocidin	*pvl*	φPVL	*S. aureus*	[181]
Superantigens	*speA1-A3, speC, I, H, M, L, K, ssa*	8232.1	*S. pyogenes*	[142]

Since competence is not known for *Listeria*, the DNA uptake system is believed to act as a secretion system of proteins involved in phagosomal escape [166].

2.1.3 Lysis-mediated competitiveness

Being a lysogen can be advantageous for the host since they can perform phage-mediated killing of sensitive competitors [182] or deleterious as for *S. aureus* lysogenic strains whose prophages are induced through the activation of the SOS-system in response to H_2O_2 produced by a niche competitor such as *Streptococcus pneumoniae* in the nasopharynx [183]. In a different scenario, prophage-induced lysis allows the release of the platelet binding proteins, PblA and PblB, from *Streptococcus mitis* φSM1 and their interaction with other *S. mitis* cells promotes their fixation to human platelets [168,184,185]. Finally, phage-induced lysis is important to the dispersion of biofilm cells, considered a key step in biofilm development. The dead cells release enzymes that help to break down the biofilm matrix and provide nutrients for the bacteria to found a new biofilm structure [186].

3. Technological applications

Bacteriophages are nowadays recognized for their technological applications in the modern biotechnology industries. Although phage therapy is their main application, many other applications have been developed such as: i) phage display, in which heterologous peptides or proteins are displayed on the surface of M13 and related filamentous phages through fusion with a coat protein [187,188]; ii) development of phage delivered vaccines by phage-display vaccination, in which phages are designed to present a specific antigenic peptide [189] and, iii) as deliver vehicles for gene therapy. Phages can be targeted to specific eukaryotic cell types and deliver gene cassettes [190].

If phage therapy or the use of bacteriophages as anti-bacterial agents started early in the 20th century, it was abandoned in the Western Europe and United States due to a lack of understanding of phage biology and to the appearance and generalization of antibiotic therapy. However, it continued to be developed in some Eastern countries and in the former Soviet Union, mainly at the Eliava Institute in Georgia where they constructed a considerable collection of lytic phages targeting bacterial pathogens (Figure 2) [191,192]. The renewed interest on phage therapy is mostly due to the emergence of multidrug resistant pathogenic bacteria.

Nowadays, phage therapy follows two main approaches: the development of phage-encoded lytic enzymes (lysins) or phages as direct antimicrobial agents [94,193]. Lytic enzymes participate in the lysis of the host cell by degrading one of the 5 major bounds within the peptidoglycan [194]. Lysins of Gram-positive bacteria have 2 domains: N-terminal that has at least one catalytic activity and the C-terminal that binds specifically to a substrate in the cell wall of the host bacteria [193]. Studies of different laboratories have shown that lysine domains can be swapped to obtain lytic enzymes with multiple lytic activities and/or multiple binding domains. [195,196]. This capacity allows the creation of new enzymes with high specificity and cleavage potential. For example, Garcia and collaborators created a new lysine harboring the same binding domain for pneumococci but able to cleave different peptidoglycan bounds which increases its therapeutic potential [197]. Lysins present a number of therapeutic advantages comparing with phages, as they have not yet presented adverse reactions during *in vivo* trials or bacterial resistance [198-200]. They were tested successfully in animal models to treat infections caused by gram-positive pathogens such as *Bacillus anthracis* [200], *S. pneumoniae* [198] and *S. aureus* [201].

Phage therapy has known a great development and successful tests have been done in animal models of infection with antibiotic-resistant

bacteria such as *E. coli* [202], *P. aeruginosa* [203,204] and *S. aureus* [205]. Recently, several phage-based products have reached clinical trials in order to: control nasal carriage of *S. aureus* [206], treat chronic otitis induced by antibiotic resistant *P. aeruginosa* [207] and treat young children with *E. coli* induced diarrhea (clinical trial identifier NCT00937274). Bacteriophages have also been successfully used as biocontrol agents against biofilms and for food protection [208]. Growing interest in such application is facilitated by less stringent regulations than for medical use. For example, LISTEX^{TM}, a phage product against *Listeria monocytogenes* in meat and cheese products, has been readily approved by the FDA and USDA and commercialized in Europe [209]. In conclusion, phage-based products are getting public acceptance due to improved biomedical technologies together with a better understanding of the biological properties and the mechanisms of phage-bacteria interactions.

4. Bacteriophages of *E. faecalis*

Within Gram-positive bacteria, temperate bacteriophages are key vectors of horizontal gene transfer of virulence genes and evolution. Despite bacteriophage impact in the emergence of pathogenesis, and their role in *E. faecalis* genome diversity, the interest on these mobile genetic elements is scarce [24-26]. However the potential use of enterococcal phage for therapeutic applications has been renewed in the era of multidrug resistance. Studies on *E. faecalis* bacteriophages are mainly focused on the development of lytic phages and/or their lysins as therapeutic options against *E. faecalis*, including vancomycin resistant strains. *E. faecalis* phages have been isolated from diverse types of environments such as sewage, effluent and stool specimens [210], human saliva [211] and teeth root canals [212]. Typically, they are tailed dsDNA bacteriophages and belong to the *Siphoviridae* and *Myoviridae* families. To a lesser extent, non-tailed filamentous (*Inoviridae*), polyhedral

(*Leviviridae*) and pleomorphic (*Guttaviridae* and *Fuselloviridae*) bacteriophages have also been identified [210].

So far, four *E. faecalis* lytic phages and their respective lysins have been reported as suitable for phage therapy *in vitro* but only φEF24C was successfully tested *in vivo* in a sepsis mice model [213-218]. Generally, *E. faecalis* phages and their lysins are specific for their host and cell wall targets however, in two cases, PlyV12 and EFAL-1, were found to be also active against other Gram-positive pathogens such as staphylococci and streptococci [214,218]. In a different scenario *E. faecalis* lytic phages have been also developed as microbial source tracking tools to detect human faecal pollution in aquatic environments [219,220].

As they are not considered suitable for phage therapy due to their intrinsic ability to recombine, studies on temperate bacteriophages have been regarded as secondary. Three publications have started to tackle the impact of temperate phages in *E. faecalis* strains [212,217,221]. Those studies report that *E. faecalis* strains are frequently lysogenic and polylysogenic: strain V583 harbors 7 prophages, and from a collection of 47 *E. faecalis* isolates, 5 were lysogenic and 12 polylysogenic [28,217]. Among these temperate phages several potential fitness factors were predicted such as platelet-binding proteins suggesting a potential role of prophages in *E. faecalis* pathogenesis [217]. Furthermore, two studies report that *E. faecalis* temperate phages are able to mediate transduction of antibiotic resistance traits emphasizing the need for a better knowledge on these elements [217,221].

III. PHAGE-ASSOCIATED GENETIC ELEMENTS

Genomes of bacterial species can evolve through mutation, rearrangements or horizontal gene transfer. The acquisition of external DNA is a fundamental process in the diversification of the majority of Bacteria and Archaea, and has particular importance for plant and human pathogens [222].

Genomic islands are large chromosomal regions that differ in base composition from the core genome. They can confer selective advantages to host bacterium under different environmental conditions or niches [223]. Therefore they have been grouped, accordingly with their functions in: i) metabolic islands (utilization of new carbon and nitrogen sources), ii) degradation islands (degradation of novel compounds), iii) resistance islands (resistance to heavy metals and antibiotics) and iv) pathogenicity islands (virulence determinants). Their mobility may involve the help of other mobile elements such as plasmids, integrative conjugative elements (ICEs) or phages. Since 1963 chromosomal islands are known to use helper phages for transduction [224]. They were discovered in *Escherichia coli* and involve phage satellite P4 and helper phage P2. Since then, the mechanisms of interaction between these two elements, and other P2-related phages, have been thoroughly studied and reviewed [137,225]. Later in 1998, phage-related chromosomal islands (PRCIs) were identified in *Staphylococcus aureus*. Members of this family are called SaPIs, for *Staphylococcus aureus* pathogenicity islands. PRCIs are a family of genetic elements that share genome organization and uses helper phages to package their genomes and get disseminated. These elements are suspected to be widespread in Gram-positive bacteria, such as *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Lactococcus lactis* and *Enterococcus faecalis* [226,227]. A similar system has also been

described in *Sulfolobus* and it involves nonconjugative plasmids that use fuselloviruses for packaging and dissemination [228,229].

B. Lindqvist used the words 'molecular piracy' to describe the relationship between P4/P2. G. Christie has extended these words to the description of PRCI/helper phage system. In both cases, a genetic element uses another genetic element (helper phage) to obtain structural proteins and get transduced, compromising helper phage propagation. The following section will detail the bacterial molecular piracy mechanisms of P4/P2 and SaPI/helper phage systems and to a lesser extent the knowledge on the pSSVx/i archaeal system.

1. Satellite bacteriophage P4

P4 is a natural phasmid (phage-plasmid) that, as a prophage lysogenizes their hosts and expresses superinfection immunity, or replicates autonomously and maintains itself as a multicopy plasmid, in the absence of a helper phage [230]. P4 is also able to perform a lytic cycle, but requires a P2-related phage [230]. P2 is an *E. coli* temperate phage of the *Myoviridae* family [231]. The helper phage provides the proteins needed for assembly of phage particles, packaging of P4 DNA and lysis of the host cell. The resulting P4 particles are made of P2 proteins. The small P4 genome (11.6 kb) is packaged in a capsid of 1/3 the size (45 nm) of those of P2 capsids (60 nm) [232]. P2 is a prototype of non-inducible class of temperate phages and is only induced in the presence of P4 [233]. Even if most of the interplay mechanism has been established between P4 and P2, other P2-like phages function as helpers for P4 and are disseminated in 30% of *E. coli* strains as well as other *Enterobacteriaceae* [234].

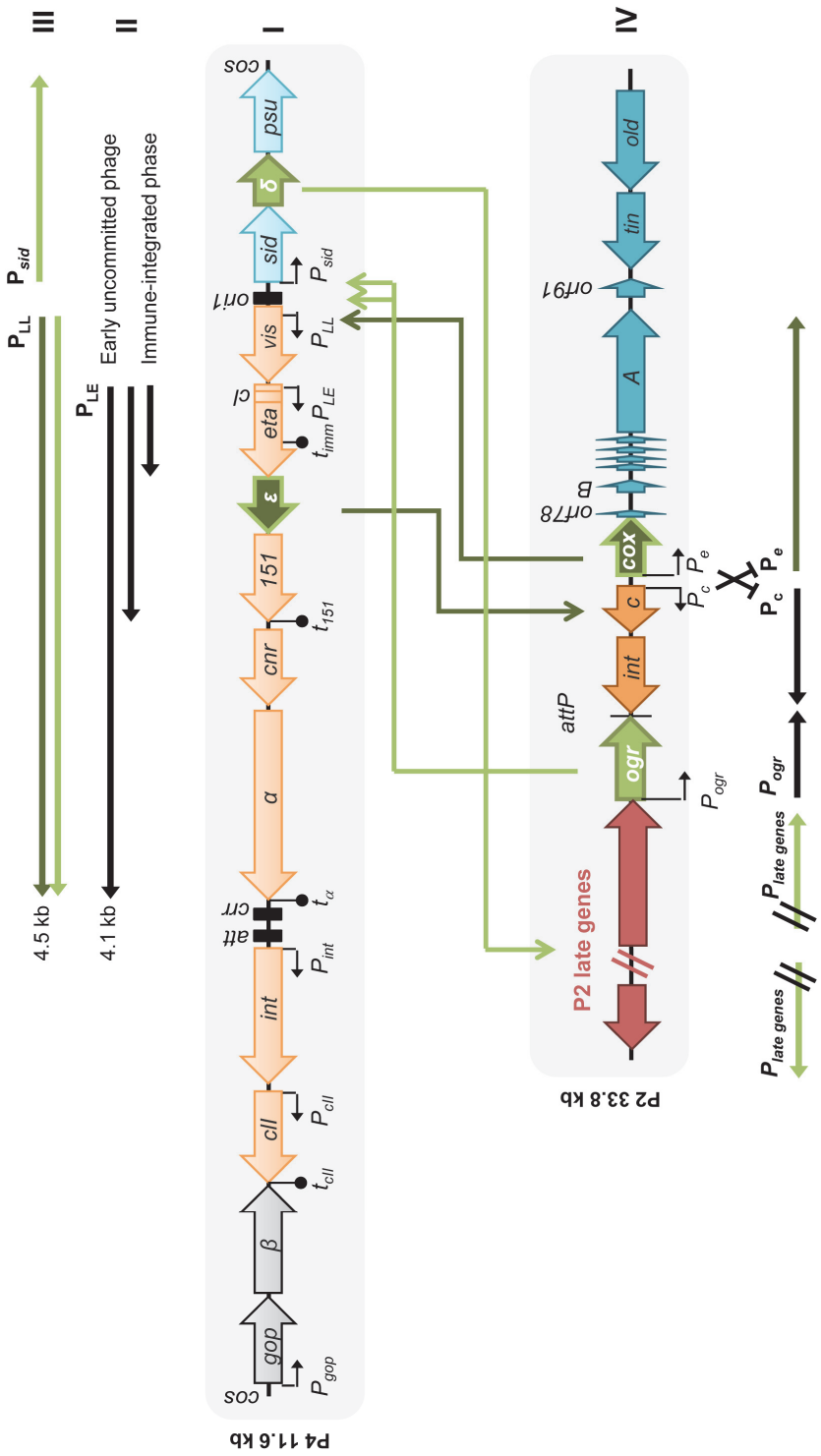
1.1 P4 genome organization

P4 linear map and its transcripts are depicted in Figure 7. All functions required for lysogenic, lytic and plasmid growth comprises 80% of the genome. P4 genome harbors 3 main promoters: P_{LE} and P_{LL}, which control leftward genes, and P_{sid} that controls rightward genes, transcribed divergently from the origin of replication, *ori1* [235] (Figure 7). The leftward genes comprise functions required for the lysogenic, lytic and plasmid propagation such as the integration *att* site, the integrase gene (*int*) and the α operon, that encodes functions required for P4 lytic and plasmid propagation (replication: α and *cnr*; helper prophage derepression: ε) and the immunity determinant, required to prevent P4 replication [236]. The rightward genes belong to the *sid* operon and encode regulatory and morphogenetic proteins involved in the plasmid and/or lytic development [237]: the positive regulator gpδ, and the two head morphogenesis proteins Sid and Psu. Sid determines the small size of the P4 capsid [238] and Psu helps to stabilize the viral particle (Figure 7) [239].

Sequence homology between P4 and genome of the helper phage P2 is less than 1%. It is restricted to the 55 bp *cos* sites and to the positive regulators gpδ (P4) and Ogr (P2). The *cos* sites determine DNA maturation and packaging specificity for P4 and P2 DNA by P2 head proteins [240] and the positive regulators activate transcription from both P4 and P2 late promoters [241].

1.2 P4 lifestyle

After infection of a host cell, P4 can adopt different life styles depending on the presence/absence of the helper phage (Figure 8). If a helper phage is present, the outcome of the infection is usually lytic growth where P4 is dependent on all morphological and lytic functions of the helper phage. In the absence of a helper phage the lytic cycle does not proceed, and P4 infection may lead to either an immune-integrated


III
II
I
IV
Early uncommitted phage
Immune-integrated phase
P4 11.6 kb
P2 33.8 kb
4.5 kb
4.1 kb
cos
psu
δ
sid
ori1
vis
cI
eta
ε
151
cnr
α
att
crr
int
cII
β
gop
old
tin
ori91
A
B
ori78
cox
c
int
attP
ogr
P2 late genes
P late genes

phase, analogous to the lysogenic state, or the establishment of the multicopy plasmid mode of maintenance [230].

1.3 The regulatory network

A reciprocal intricate network that involves the interaction between P2 and P4 regulators maintains the different developmental stages of P4 (uncommitted phase, immune integrated, multicopy plasmid and the lytic states). The uncommitted replication phase starts immediately after P4 infection of a bacterial strain. It is characterized by an early transcriptional burst of the α operon from the P_{LE} promoter, followed by the activation of a negative control system, the immunity control. The establishment of immunity is irreversible and marks the end of the uncommitted phase upon which P4 can integrate or follow the lytic-plasmid state. Generally, the immune-integrated state is preferred at 99% in the absence of P2, whereas the lytic cycle is favored at 50 to 70% in the presence of P2 (Figure 8) [242]. In the immune-integrated state, P4 gene expression is limited to the P_{LE} immunity region and the P4 genome is passively replicated by the bacterial chromosome. The lytic and multicopy plasmid states are characterized by the activation of late promoters P_{LL} and P_{sid}.

Figure 7. P4 genome and P2-P4 regulatory network. The arrows above and below the maps indicate early and late transcripts from P2 and P4 main operons. Transcription starts and terminators are represented by arrows and hanging circles, respectively. **I.** P4 genes and sites; **II.** P4 early transcripts; **III.** P4 late transcripts, activated during the multicopy plasmid state or lytic growth; **IV.** Partial P2 map, early and late transcripts. P2-P4 mutual derepression (dark green lines): Cox protein coded by P2 genome derepress P4 P_{LL} promoter leading to activation of replication functions as well as P4 excision. Epsilon (ε) on P4 genome binds directly to P2 repressor inducing transcription of early P2 genes from P_e. P2-P4 reciprocal transactivation (light green lines): P2 and P4 transactivate late gene operons through the action of the activators Ogr and δ. Adapted from [230].

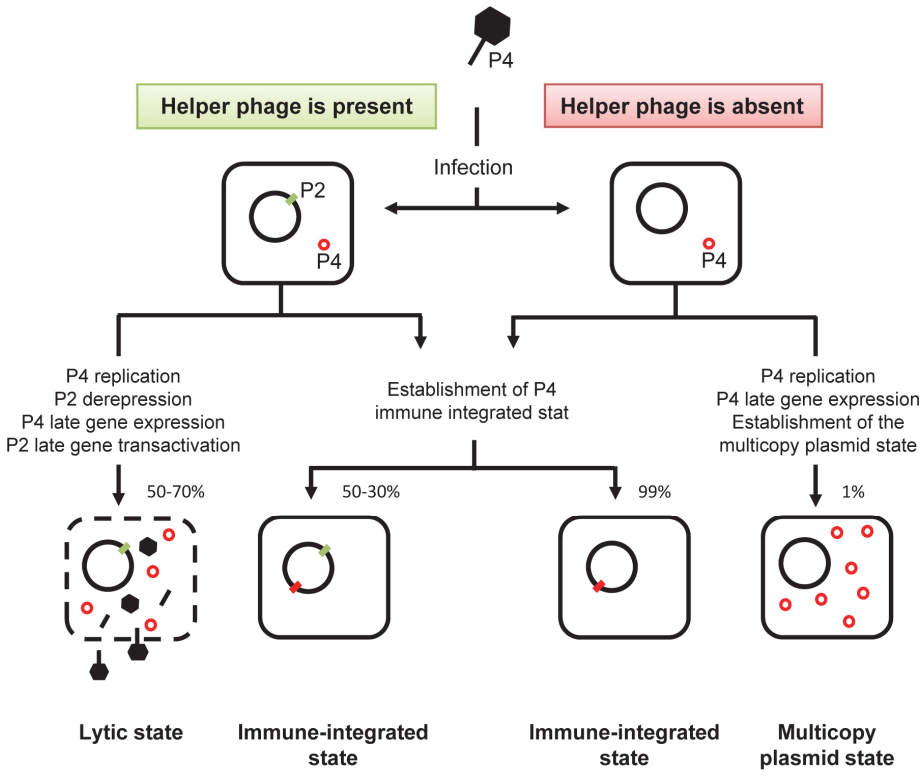


Figure 8. P4 life style scenarios. The presence of P2 helper phage dictates the outcome of P4 infection. P4 lytic cycle is preferentially used when P2 is present, whereas P4 immune state is preferred in the absence of P2. Adapted from [137].

1.3.1 P4 uncommitted phase

P4 replication occurs right after infection, producing a large number of P4 DNA molecules [243]. Replication requires the multifunctional P4 α protein that has primase and helicase activities and specifically binds to DNA sequences at P4 replication origin, composed of *ori1* and *crr* sites [244]. During the uncommitted phase, α operon is transcribed from the strong constitutive promoter P_{LE}. Transcription from P_{LE} yields RNA molecules of different length (Figure 7): (i) full-length mRNA 4.1 kb long, which covers the entire operon; (ii) 1.3-kb mRNA, which stops at t_{151}, a

terminator upstream of the replication genes *cnr* and α, and (iii) a family of transcripts less than 0.5 kb long, which stops at t_{imm} [245-247]. Therefore different portions of the α operon are differentially expressed. The long transcripts are no longer detected beyond 15 minutes after P4 infection, meaning that transcription from P_{LE} allows the expression of the replication genes for a restricted time, after which expression is limited to the 5' untranslated portion of the operon encoding the immunity functions [248]. The immune mode of transcription from P_{LE} is then irreversibly established. The change on transcriptional patterns, from expression of the entire α operon, to the immune mode correlates with the appearance of the mature CI RNA, encoded by the *cI* gene. The CI RNA is produced by processing of transcripts that cover the immunity region. CI RNA is a short stable RNA and functions as P4 immunity factor [245-249].

1.3.2 P4 immune-integrated phase

This phase requires establishment of the immune condition, which prevents expression of the replication genes, and leads to P4 integration into the bacterial chromosome. P4 integration occurs at the preferential site *attB* that corresponds to the 3' end of *leuX* gene, coding for $tRNA^{Leu}$ by the action of P4 integrase, which acts also as an excisionase [250-252]. Excision requires a second P4 protein, Vis. It occurs spontaneously at low frequency and, at much higher efficiency, upon infection of a P4 lysogen by the helper P2 [253]. Vis is also a regulatory protein that controls transcription of late promoters P_{LL} and P_{sid} (see III.1.3.3).

1.3.3 P4 multicopy plasmid state

Maintenance of the plasmid state requires a balanced expression of α and *cnr* (copy number regulation) genes that activate replication and negatively controls P4 copy number, respectively [254]. Control of P_{LL} is essential for expression of the replication functions when P4 is in the

plasmid state. P_{LL} is controlled by the opposing actions of the δ gene product, expressed from P_{sid}, and Vis, encoded by the first gene transcribed from P_{LL}. These two proteins activate and repress P_{LL}, respectively. P_{LL} is located 400 nucleotides upstream of P_{LE} [236] and the transcription termination barrier imposed by the immunity system is bypassed through the activation of termination-insensitive transcription from an upstream promoter, P_{LL} (Figure 7) [236,255]. Indeed, translation of two small ORFs from P_{LL} prevents CI RNA-mediated transcription termination [255].

1.3.4 P4 lytic cycle

P4 lytic cycle requires P2 morphogenetic and lytic functions. P4 takes over P2 regulatory network through mutual derepression in order to lift the immunity mechanisms and reciprocal transactivation of the late operons. P4 lytic cycle may occur within different frameworks: i) P4 infecting a P2 lysogen, ii) P4 and P2 co-infecting a nonlysogenic host, iii) P4 lysogen being infected by P2 and, iv) P4 plasmid carrier being infected by P2. In each case, P4 senses the presence of the helper phage and responds through activation of its own functions in order to take over helper phage gene expression [137].

P2 early operons are controlled by a pair of divergent promoters that regulate lysogeny functions (P_c and P_e) (Figure 7). P2 immunity repressor C is part of the leftward promoter. It regulates itself, and represses Cox that reciprocally blocks C expression [256]. Cox is also responsible for P2 excision from the host chromosome [257]. Derepression of P2 prophage by P4 is controlled by ε gene product that binds directly to P2 immunity repressor [233,258]. On the other hand, P2 may derepress P4 prophage via activation of P_{LL} late promoter by Cox [253]. P4 and P2 late operons, code for morphogenic functions and can be reciprocally transactivated by Ogr and gpδ, coded in P2 and P4 genomes, respectively. They activate

transcription of both the four P2 late operons as well as the two P4 late operons, which are controlled by P_{LL} and P_{sid} (Figure 7) [259]. Ogr and gpδ recognize the same promoters on both genomes with distinct binding efficiencies: gpδ has higher affinity for P2 promoters and Ogr for P4 [137]. This differential affinity allows P4 to control the production of the P2 structural proteins independently of P2 regulators.

The outcome of the satellite-helper interaction depends on the infection conditions. When a P2 lysogen is infected by P4, the helper prophage immunity is lifted by P4 gpε, which leads to P2 early-gene expression and P2 DNA replication *in situ* without excision of the integrated prophage genome [260]. P2 late expression may occur either by normal P2 activation mechanism, that requires P2 DNA replication and Ogr transcriptional activation, or directly through P4 transactivation of P2 late genes by gpδ [259], which activates P2 transcription from the same promoters used by Ogr. The low excision efficiencies of P2 together with the redirection of its capsids explain the low P2 titers in this situation [261]. When P4 and P2 co-infect a host, progeny of both prophages is produced at similar proportions and gpε is dispensable for P4 lytic cycle [261]. On the contrary, when a P2 infects a P4 lysogen, P4 lytic cycle is activated, but the production of P4 particles is very low. P2 Cox activates P4 late promoter P_{LL} and consequently the expression of the α operon. Later on, P4 P_{sid} may be efficiently activated by Ogr [260]. Finally, when P2 infects a P4-plasmid carrier, P4 lytic cycle is already activated and thus Cox is no longer needed. In this case P4 strongly interferes with P2 growth [242,243].

1.4 P4 morphogenesis

In order to get its DNA packaged, P4 needs to interfere with P2 packaging. P2 capsids (60 nm) are assembled as precursors called procapsids from gpN, several copies of a gpO scaffolding protein and a

portal, gpQ, that forms a vertex through which the DNA is packaged (Figure 9) [262]. During the assembly pathway these proteins are processed yielding mature cleaved products called N*, Q* and O* [262,263]. The protein O is process in O*, which corresponds to the N-terminal proteolytic domain [262]. P4 gets packaged into small phage particles (45 nm) hijacking helper phage structural proteins [232]. This molecular piracy phenomenon depends on a P4-encoded size determination protein (Sid) that forms an external scaffold surrounding the P4 procapsids [264,265]. P4 capsid assembly is completed through the maturation of gpN, gpO and gpQ, as for P2, removal of Sid and by the addition of the Psu decoration protein, which stabilizes P4 capsids against environmental stress [239,266]. P2 and P4 share the same *cos* site sequence, which determines that they will be packaged through the same pathway [240].

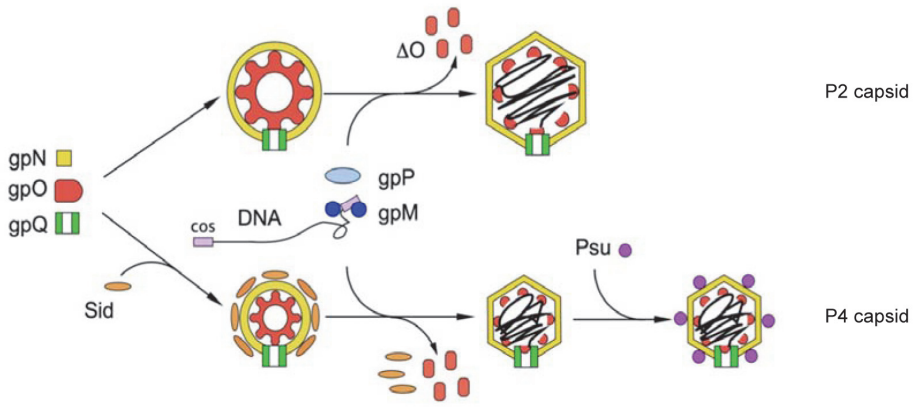


Figure 9. P2 and P4 capsid assembly and DNA encapsidation [226]. Phage procapsids are assembled from the major capsid protein (gpN), scaffolding protein (gpO) and portal protein (gpQ). The presence of P4 Sid protein leads to the formation of an external scaffold and is sufficient for small phage particle formation. DNA is packaged into procapsids by terminase complexes coded by P2 (*gpP* and *gpM*) that recognize the same *cos* sequences on both P2 and P4 genomes. P4-encoded Psu is added to P4 capsids as decoration.

Their genomic DNAs are packaged into the empty large and small procapsids, respectively, through the action of P2 terminase proteins gpM

and gpP [267].Next, the capsids undergo major structural transitions that lead to more hexagonal mature capsids [262,268]. Capsid size determination seems to be the only mechanism of interference that P4 uses against P2 [226].

As the first example of molecular piracy in which a genetic element uses a helper phage to get packaged and transduced at high rates, the molecular partners and the regulatory networks of the P2/P4 system has served as a foundation work to study of SaPI/helper phage system in *S. aureus*.

2. Phage-related chromosomal islands

The first phage-related chromosomal islands (PRCIs) were identified as highly mobile, superantigen-encoding genetic elements known as the *Staphylococcus aureus* pathogenicity islands (SaPIs). *S. aureus* is a serious nosocomial pathogen that relies on genetic mobile elements, such as chromosomal islands, to acquire and disseminate virulence and antibiotic resistance genes. SaPIs transport genes of toxic shock syndrome toxins (TSST) and enterotoxins (SE) [269]. The first pathogenicity island identified in *S. aureus* was SaPI1 that encodes TSST1 and SEK toxins [270]. The interaction between SaPI1 and its helper phage, 80α, led to the first model of SaPI molecular piracy [271]. SaPI1 was described as a chromosomal island that specifically needs phage 80α to excise from the host chromosome and to get its genome packaged. SaPI1 DNA is packaged in small phage-like particles compatible with its genome size that are released upon phage-induced lysis [271]. The authors also noticed that SaPI1 induction interferes with phage 80α, multiplication, blocking plaque formation and phage burst-size [271]. Since then, 16 more SaPIs have been identified, from which 9 are inducible by a helper phage (Figure 10).

SaPI
Virulence factors
Helper phages
Genome organization
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 kb
int
stl str xis
pri
rep
ori pif
Packaging genes
terS
Unknown
SaPI4 - EP
SaPI1028 - EP
SaPIbov1 tst, sec, sel φ11 and 80α
SaPIbov2 bap 80α
SaPImw2 sec -
SaPIm4 fhuD EP
ShPI2 - -
SaPI1 tst, ear, sek, seq 80α and φ13
SaPI3 seb, ear, sec, sel -
SaPI5 ear, seq -
SaPIm1/n1 tst, sec 80α
SaPI2 tst, eta 80 and 80α
SaPI122 mrd EP
SaPI6D actA -
SaPI6Da actA -
SsPI5305 aad, fosB -
SaRIfusB fusB SaRIfusB

As described below, subsequent work from Novick-Penades-Christie groups refined the initial model, and unraveled the molecular players involved in the molecular piracy phenomenon.

2.1 SaPI genome organization

SaPIs share a core of genes that controls major steps of their life cycle and a set of variable accessory genes, such as superantigen-, antibiotic resistance- or toxin-encoding genes. SaPIs carry their own integrase gene (*int*), required for self-integration into the host chromosome. Two divergently oriented promoters located downstream of the *int* gene regulate two major transcript units: *stl* and *str*. Stl is the master repressor of *str* transcript, that controls SaPI excision to packaging cycle, and is inactivated during phage induction [227,272] (Figure 10).

Figure 10. SaPI genomes, virulence factors, helper phages and genome organization. Genes are coloured according to their sequence and function: *int* and *xis* (excisionase) are yellow; transcription regulators are dark blue; replication genes (including the primase gene (*pri*) and the replication initiator gene (*rep*)) are purple; the replication origin (*ori*) is red; encapsidation genes are green, with the terminase small subunit gene (*terS*) in light green; superantigen and other accessory genes are pink; and *pif* is light blue. Genes encoding hypothetical proteins are orange. Helper phages correspond to SaPI inducing phages. *tst*: toxic shock syndrome toxin 1; *seb*: enterotoxin B; *sec*: enterotoxin C, *ear*: penicilin-biding protein; *sek*: enterotoxin K; *sel*: enterotoxin L; *seq*: enterotoxin Q; *eta*: exfoliatin A; *bap*: biofilm-associated protein (BAP); *fhuD*: ferrichrome ABC transporter; *mdr*: multidrug resistance; *aad*: aminoglycoside adenyl transferase (aminoglycoside resistance); *fosB*: glutathione thionyl phosphatase (fosfomycin resistance); *fusB*: FusB (fusidic acid resistance); *ermA*: ribosome methylase (macrolide-lincosamide-streptogamin B resistance); *actA*: GNAT family homologue (acetyl transferase protein); EP: endogenous phage; ˉ not known. Adapted from [227].

2.2 SaPI lifestyle

SaPIs are stably maintained in the chromosome until a resident helper phage, induced by the SOS-response, or a superinfecting helper phage, deactivates the *stl*-mediated integration state of the SaPI and induces SaPI life cycle (Figure 11). Helper phages are temperate phages found in *S. aureus* that belong to the *Siphoviridae* family, with dsDNA genomes ranging from 39.6 to 45.9 kb [273]. They are induced by antibiotics largely used in therapy, such as fluoroquinolones and β-lactams. Thus, antibiotic use indirectly promotes dissemination of the SaPIs and their toxins in the hospital environment [144,145].

2.2.1 SaPI integration

SaPIs integrate into the host chromosome at specific sites, called *attC*, in a reaction catalyzed by their integrase. The known SaPIs occupy six different *attC* sites in the *S. aureus* chromosome determined by their integrase specificity (Figure 12) [274] and each SaPI has a corresponding *attS* insertion site sequence. The six different *attC* sites occur only once in all *S. aureus* genomes sequenced so far, and when a strain carries more than one SaPI, they occupy different *attC* sites. SaPIs integrate in secondary *attC* sites if their preferential site is already occupied [227].

Figure 11. SaPI life style scenarios [227]. (A) SaPI induction by an SOS-induced helper prophage. After induction and excision of the helper phage, phage dUTPase relieves the Stl-mediated repression of the SaPI, allowing production of SaPI proteins. SaPI excisionase (Xis) subsequently promotes the excision of the SaPI, restoring *attC* and *attS* sites. Subsequent SaPI replication generates concatenated linear copies of SaPI, which are cleaved by the terminase complex into individual copies during packaging into small phage-like particles entirely made of phage proteins. (B) SaPI infection. SaPI DNA circularizes upon entry into the bacterial cell. The circular DNA is integrated at the chromosomal *attC* site by cross-over with the SaPI *attS* site. This process requires the SaPI integrase. Stl silences the expression of SaPI genes, keeping the element integrated.

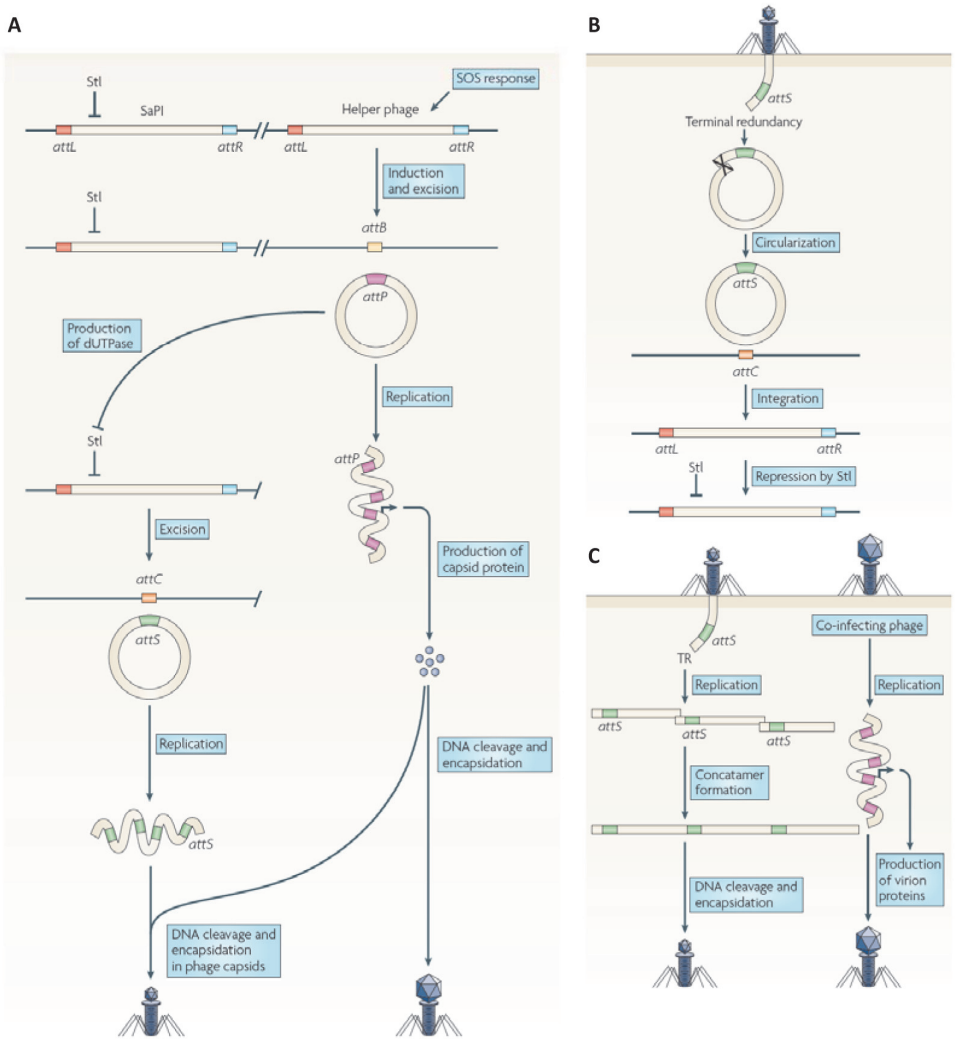


Figure 11. SaPI life style scenarios [227] (*cont.*). For A and B see page 54. (C) Co-infection of a SaPI and a helper phage. In this case, SaPI DNA is replicated prior to integration. Phage proteins are synthetized leading to the production of phage capsids, which after size adaptation are also used to package the replicated SaPI DNA. *attP* and *attS* are the prophage and SaPI core attachment sequences, respectively; *attB* and *attC* are the prophage and SaPI core chromosomal attachment sequences, respectively.

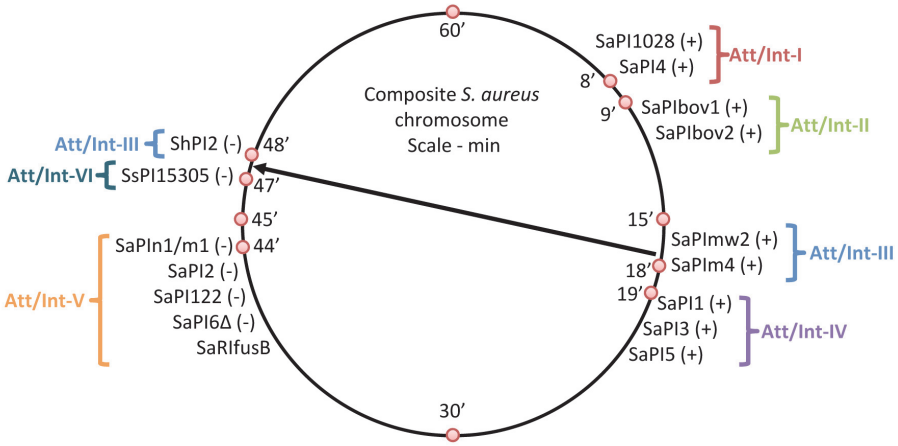


Figure 12. Chromosomal locations of the SaPI *attC* sites. The circle represents a composite staphylococcal chromosome with scales in minutes. All elements at a given site have the same integrase, thus the sites are designated “att/int” and are numbered in clockwise order. Adapted from [274].

2.2.2 SaPI derepression

As mentioned before, SaPIs are stably inserted in the chromosome until a helper phage relieves Stl-dependent repression on *str* promoter. Helper phage genes that conduct this action are different depending on the SaPI [275]. Phage 80α induces 3 different SaPIs using 3 different phage non-essential proteins, which act as antirepressors by direct binding to the Stl proteins: SaPI1 is induced by *sri*, SaPIbov1 by *dut* and SaPIbov2 by *orf15* (Figure 13). The specific interaction between different SaPI repressors and helper-phage-encoded antirepressors constitutes a primary determinant of the SaPI-helper phage specificity [276]. Dut is a dUTP phosphatase and controls SaPIbov1 derepression by switching between the active dUTP-bond form and the inactive (apo-state) conformation. This conversion is catalyzed by its intrinsic dUTPase activity

[277]. Once *str* transcription is activated, excision, catalyzed by SaPI *int* and *xis* (excisionase) proteins, and replication can occur [278]. SaPI replication is initiated at the SaPI-specific replication origin using SaPI-coded initiator and primase [279,280]. SaPI DNA replicates as linear concatemers using the host cell replication machinery, and is packaged by a headful mechanism, like helper phage DNA [271]. In some cases, like for SaPIbov1 and SaPIbov2, SaPIs carry a Sip protein (staphylococcal integration protein) that allow them to excise and integrate at the *attC* site independently of the presence of a helper phage [281].

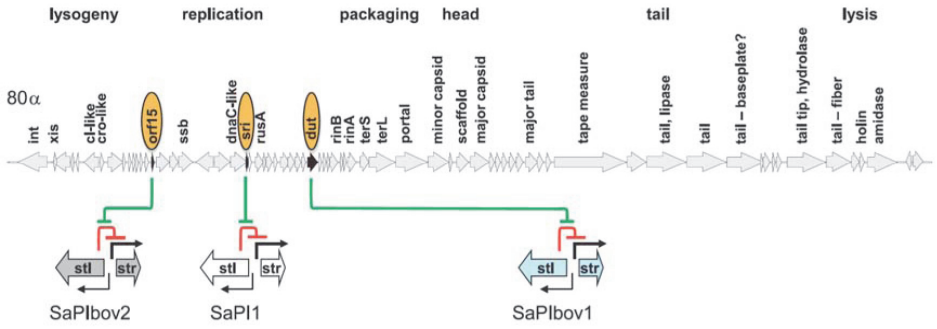


Figure 13. SaPI derepression by multiple helper phage genes [226]. The known derepression functions are highlighted in yellow circles on the 80α genome map. The immunity regions from three different SaPIs are shown below. Transcriptional activation and repression are represented by green and red lines, respectively.

2.2.3 SaPI capsid size redirection and DNA packaging

SaPI DNA is packaged mostly in small particles composed of phage proteins [282,283]. In order to produce smaller capsids, SaPIs must redirect the helper phage assembly pathway to form capsids that are 1/3 smaller (45 nm) than those of the helper phage (60 nm) [271,284].

SaPI1 and its helper phage 80α is the most well studied system regarding capsid size redirection and DNA packaging. Capsid assembly of phage 80α , as in other dsDNA bacteriophages, starts with the formation of a precursor procapsid made of major capsid protein (gp47) and a scaffolding protein (gp46) that acts as a chaperone for the assembly process [226]. The portal protein (gp42) and a few copies of a minor protein of unknown function (gp44) are also incorporated into the procapsid [282,285]. SaPI1 proteins involved in size determination are called capsid morphogenesis proteins and have been named CpmB (gp6) and CpmA (gp7) (corresponding to SaPIbov1 gp8 and gp9, respectively) (Figure 14). CpmB act as an internal scaffold protein while CpmA function is less clear [284,285].

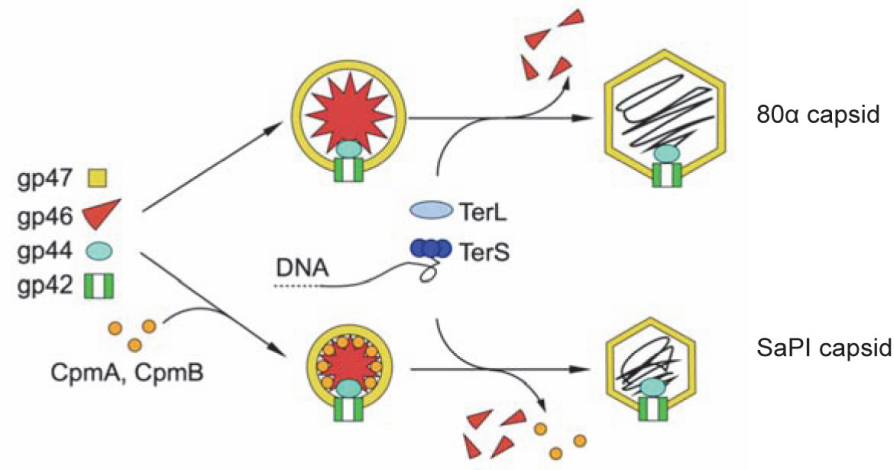


Figure 14. SaPI and phage 80α capsid assembly pathways [226]. Phage procapsids are assembled from the major capsid protein (gp47), scaffolding protein (gp46), portal protein (gp42) and a minor capsid protein (gp44). The presence of CpmAB of SapI leads to the formation of small capsids. CpmB forms an internal scaffold. DNA is packaged into procapsids by terminase complexes together with the removal of the scaffold and expansion of the capsids.

CpmA and CpmB are conserved and always appear together in 13 out of 16 SaPIs sequenced to date [227]. SaPIbov2 and SaPIbov5, two exceptions that do not present *cpmAB* genes in their genomes, are packaged in large capsids [275,286]. Moreover, in the case of SaPIbov2, the large size of its genome makes it impossible to package into small capsids [275]. Although essential for small capsid formation, CpmAB are not essential for SaPI packaging and propagation, since that in their absence, SaPIs are transduced at high frequencies but in large capsids [287].

Phage 80α DNA is packaged through a classical mechanism that requires a portal protein and the terminase complex, consisting of the small (TerS) and large (TerL) terminase subunits (Figure 15). TerL is responsible for prohead binding, DNA translocation and cleavage whereas TerS recognizes and binds to DNA [102]. When a SaPI is present, SaPI encoded TerS binds to a phage encoded TerL and both direct the specific cleavage and packaging of SaPI DNA through the binding to SaPI-specific *pac* sequence [226]. SaPI TerS binding to phage TerL is promoted by SaPI Ppi protein, which interferes with packaging of phage DNA by direct binding to phage TerS (see below) [286]. The majority of SaPI particles are packaged into small capsids, however in some cases they are packaged into large capsids as multimers with full transducing capacity. Fragments of phage DNA can also be packaged into small capsids during mobilization by SaPI1 but not with SaPIbov1. Since the DNA will represent only a fragment of the genome, the resulting virions will not be viable [288]. The ability of SaPI TerS to form a functional complex with the helper phage TerL illustrates another level of specificity between the SaPIs and their helper phages.

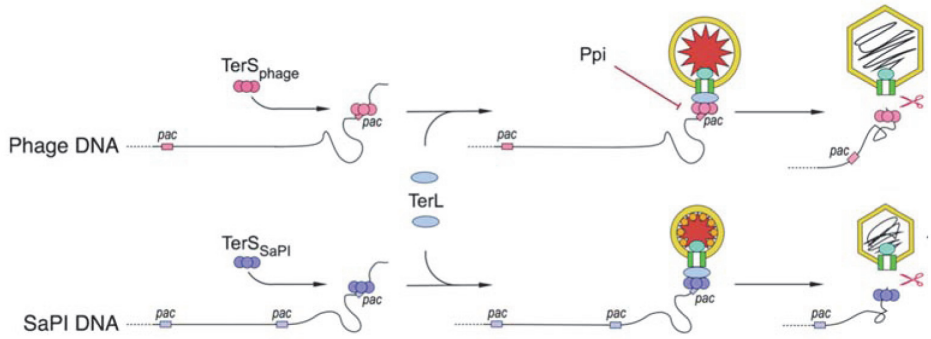


Figure 15. Model for SaPI packaging redirection [226]. *pac* sites on the concatemeric phage DNA are recognized by the phage encoded TerS and packaged into procapsids through the action of phage TerL. The *pac* sites of SaPI DNA are specifically recognized by the SaPI encoded TerS that also uses the phage TerL for DNA packaging.

2.2.4 Interference

SaPIs interfere with the multiplication of their helper phage by blocking plaque formation and reducing burst-size [270,286]. So far, three mechanisms of SaPI interference with the production of their helper phages have been described [286].

CpmAB-mediated diversion of capsomer proteins to produce small sized capsids is a very efficient interference mechanism as reported for SaPI1, a SaPI *cpmAB*-encoding gene. SaPIbov2 that doesn't produce small sized particles nor harbors *cpmAB* genes, uses another mechanism: Ppi-mediated interference with packaging. Ppi is a SaPI-coded protein dedicated to phage interference against packaging of the helper phages. Ppi interferes with the interaction between phage TerL and TerS subunits, blocking the cleavage and packaging of phage DNA [286]. SaPIbov2 efficiently blocks phage 80α multiplication by this mechanism. All SaPIs sequence have a *ppi* gene indicating that they can use it for phage interference alone or in addition to other mechanisms depending on the target helper phage. Different allelic variants of both *cpmAB* and *ppi* determine different levels of interference that in some cases are additive

or redundant. For example, SaPIbov1 *cpmAB* and *ppi* are redundant against phage 80α since they can both interfere at the same level with phage multiplication. On the other hand SaPI2 *cpmAB* and *ppi* produce additive interference against the helper phage [286]. A third interference mechanism was identified between SaPI2 and phage 80 involving SaPI2 ORF17 by a yet unknown mechanism [286].

2.3 PRCIs in other bacteria

SaPIs are extremely well adapted to their pirate life using at least three different mechanisms (Cpm, Ppi and ORF17) to interfere with their helper phages life cycle and ultimately hijack their structural proteins and get transduced. Particularly prone for dissemination of their toxin genes to staphylococci [275], and to other species such as *Listeria monocytogenes* [289], phage-like particles of SaPIs represent a major human health concern. PRCIs are a family of genetic elements that share genome organization and uses helper phages to package their genomes and get disseminated. If their functionality has been established only in *S. aureus*, PRCI-like elements are dispersed in gram-positive bacteria and were recently predicted in the Gram-negative bacterium *Vibrio cholerae* [115]. However, packaging and transfer of these predicted elements (e.g. LICI1 and LICI2 in *L. lactis*, EfCI538 and EfCI18508 in *E. faecalis*, SsuC1 in *Streptococcus suis*, SpyCIM1 in *S. pyogenes*, and PLE in *V. cholerae*) have yet to be demonstrated [115,227]. Based on gene organization, and despite evidence of molecular piracy a *S. pyogenes* defective prophage SF370.4 was renamed SpyCIM1 for *S. pyogenes* chromosomal island M1 [227,290]. Interestingly, SpyCIM1 acts as a genetic switch to control DNA mismatch repair (MMR) in strain M1 by dynamic excision and reintegration into the 5' end of *mutL* in response to cell growth or upon DNA damage. During exponential growth or in response to DNA damage, SpyCIM1 excises from the chromosome and replicates as an episome allowing

mutL to be transcribed and restores DNA mismatch repair (MMR). As cell density rises, SpyCIM1 reintegrates into the chromosome restoring the mutator phenotype [290,291]. This example illustrates how the integrated or episomal state of a predicted PRCI may impact on a bacterial population.

3. Satellite plasmids: pSSVx and pSSVi

Sulfolobus solfataricus and *Sulfolobus acidocaldarius* of the order *Sulfolobales* are tractable species used as model organisms for molecular biology studies among hyperthermophiles and extremophiles Archaea [228]. The genome sequence of *Sulfolobus* revealed a high number of extrachromosomal genetic elements from which fusellovirus is the most studied [292]. *Sulfolobus* REY15/4 strain harbors a fusellovirus, SSV2, and small plasmid molecule (pSSVx) disseminated in small phage-like particles made of SSV2 components. It is believed that pSSVx replicates in the host cell as a plasmid and belongs to the pRN family of *Sulfolobus* plasmids [228]. Upon superinfection with the viruses SSV2, infectious virus particles of two different sizes are released. The smaller virus particles probably contain the smaller genome of pSSVx whereas the larger particles contain the viral genome of SSV2. The genome of pSSVx contains two conserved open reading frames (ORFs) present only in plasmids that use a helper phage for transduction. Therefore, they were suspected to be involved in the hijacking of the fusellovirus packaging system [228]. Another *Sulfolobus* non-conjugating plasmid, pSSVi, was identified in *S. solfataricus* P2 that is able to integrate into the host chromosome and disseminate in phage-like particles like pSSVx. pSSVi did not harbor the two ORFs predicted to be involved in phage components hijacking, however, pSSVi and pSSVx share another ORF that is proposed to be involved in viral packaging and spreading. Differently from PRCIs and P4 that impair their helper phages

propagation, pSSVi seems to have a positive impact on its helper phage by improving SSV2 replication [229].

4. Concluding remarks

Temperate bacteriophages are multifaceted genetic elements. They are traditionally involved in bacterial evolution through lysogenic conversion; they also have the ability to perform horizontal gene transfer of their own, or other bacterial genes. Molecular piracy in which a genetic element, such as a chromosomal island, a defective prophage or a plasmid, uses a temperate phage to get its genome packaged is a widespread phenomenon in both Bacteria and Archaea. Despite the diversity of molecular mechanisms, all of the pirate elements manipulate their helper phages in order to gain control of their structural proteins. The evolutionary origins of the parasite element are still unknown but they seem different as P4 would have evolved from a plasmid and SaPIs from a prophage [226,227]. Since the identification of the first SaPI in *S. aureus*, the phage-related chromosomal islands are suspected to be disseminated among Gram-positive bacteria. Experimental evidence of molecular piracy in Gram positive, other than staphylococci was provided during this thesis with the identification of the first enterococcal phage-related chromosomal island, EfCIV583 (Chapter 2).

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Chapter 2

Enterococcus faecalis prophage dynamic interactions

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SUMMARY

Polylysogeny is frequently considered to be the result of an adaptive evolutionary process in which prophages confer fitness and/or virulence factors, thus making them important for evolution of both bacterial populations and infectious diseases. The *Enterococcus faecalis* V583 isolate belongs to the high-risk clonal complex 2 that is particularly well adapted to hospital environment. Its genome carries 7 prophage-like elements (V583-pp1 to -pp7), one of which is ubiquitous in the species. In this study, we investigated the induction and activity of the V583 prophages. We systematically analyzed the ability of each prophage to excise from the bacterial chromosome, to replicate and to package its DNA. We also created a set of *E. faecalis* isogenic strains that lack from one to all six non-ubiquitous prophages by mimicking natural excision. Our work reveals that prophages of *E. faecalis* V583 excise from the bacterial chromosome and are able to produce active phage progeny. Intricate interactions between V583 prophages were also unveiled: pp7, coined EfCIV583 for *E. faecalis* chromosomal island of V583, hijacks capsids from helper P1 leading to the formation of distinct virions and pp1, pp3 and pp5 inhibit excision of prophages pp4 and pp6. The hijacking exerted by EfCIV583 on helper P1 capsids is the first example of molecular piracy in Gram positive, other than staphylococci. Finally, we have shown that fluoroquinolone increases prophage activity and can thus contribute to horizontal gene spreading.

INTRODUCTION

Acquisition of external DNA by horizontal gene transfer and gene loss are major driving-forces of bacterial genome evolution. Temperate bacteriophages contribute actively to such evolution as they integrate into and excise from the bacterial chromosome [1]. They also mediate horizontal gene transfer by transduction within and across bacterial species [2,3]. By doing so, the integration of a temperate phage into the bacterial genome can provide new genetic properties to the bacterial host, and under some circumstances lead to the emergence of new pathogens within species, as shown for *Corynebacterium diphtheriae*, *Escherichia coli* and *Vibrio cholerae* [4-6].

Frequently, prophages are found in various stages of functionality in a bacterial chromosome. In addition to fully functional prophages that can be induced to start a lytic cycle and release phage progeny, bacterial genomes harbor also prophage-like entities that are defective as they fail to give progeny alone. Nevertheless, they can still harbor functional genes that contribute to their DNA mobilization and/or to bacterial host fitness by providing active genes such as *S. aureus* toxins or *E. coli* effectors [7-9]. This is the case of the Phage-Related Chromosomal Islands (PRCIs) of some Gram-positive bacteria that are mobile genetic elements, initially described as *S. aureus* pathogenicity islands (SaPIs). They encode mobilization functions as well as toxic shock toxins, other virulence and antibiotic resistance genes [7,10]. They can also modulate host gene activity by dynamic excision and reintegration like the PRCI SpyCI of *Streptococcus pyogenes* [11]. PRCIs are mobilized by hijacking structural proteins of a helper phage to form specific-virions [12]. The genomic organization and the current knowledge of the molecular mechanisms of these pirate elements have been reviewed recently [13,14]. Excision of SaPIs from the bacterial chromosome is induced upon infection by a

helper phage or by induction of an endogenous prophage [15]. Following excision, DNA of SaPIs self replicates as concatemers and is packaged as monomers and multimers within small and large capsids, respectively, made of helper phage proteins [16,17]. Redirection of helper phage proteins by SaPIs has been associated with interference mechanisms, which differ between SaPI elements [18,19]. While PRCIs have been recently predicted in other gram-positive bacteria *in silico* [13], demonstration of their activity is still pending.

Beyond the understanding of the involvement of individual prophages in bacterial strain phenotypes and ecology, a few studies have started to tackle the more complex question of the impact of polylysogeny on bacterial physiology. For example, cryptic prophages of *E. coli* improve growth, contribute to protection against antibiotics or stress and increase virulence or biofilm formation [20,21], while temperate phages contribute to virulence of *S. enterica* [22] and *S. aureus* [23] and confer competitive fitness to *S. enterica* [24]. Polylysogeny often leads to intricate phenomenon of prophage interferences that are likely to influence behavior of the bacterial host as shown in *E. coli* and *S. enterica* [20-22].

Enterococcus faecalis is a low-GC Gram-positive bacterium whose primary habitat is the gastrointestinal tract of a wide range of animals and humans. This member of the core human microbiota [25] exhibits different lifestyles. It is commonly found in diverse environments including food, water, soil and plants but it is also associated with life threatening infections. *E. faecalis* ranks among the leading causes of hospital acquired bacterial infections, and causes mostly urinary tract and intra-abdominal infections, infective endocarditis and bacteremia [26]. Epidemiological studies have revealed few enriched clonal complexes (CCs) of multi-drug resistant colonizing and/or invasive isolates among hospital-associated strains [27,28]. Of these high-risk enterococcal clonal

complexes, CC2 isolates are particularly well adapted to hospital environment and associated with invasive disease [29].

The strain V583 belongs to CC2 and was the first vancomycin resistant isolate found in the United States [30]. The chromosome of V583 harbors seven prophage-like elements (V583-pp1 to V583-pp7, named hereafter pp1 to pp7), one of which (pp2) is found in all *E. faecalis* isolates and is considered to be part of the core genome [31,32]. Interestingly, CC2-isolates are enriched in prophage-genes, supporting the idea that these mobile genetic elements may contribute to increased survival of CC2 isolates in the host [33]. Noticeably, *E. faecalis* polylysogeny has been reported recently in a collection of clinical isolates, which carried up to 5 distinct inducible phages [34], indicating that polylysogeny is not specific to the V583 isolate. Even though several phage-encoded potential fitness factors have been pointed out [31,34,35], the contribution of these prophages to the lifestyle of *E. faecalis* and to its biological traits remains largely unknown.

The aim of this study was to establish if the *E. faecalis* V583 prophages are biologically active and determine the conditions that allow their induction and dissemination. Out of the seven prophages predicted, we show that six are inducible and four form infectious virions through a sophisticated regulatory network, which revealed the first enterococcal phage-related chromosomal island. Moreover, our findings demonstrate a correlation between the use of antibiotics, such as fluoroquinolones, and the induction and release of phage particles, which can promote bacterial fitness, and the dissemination of virulence and antibiotic traits.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Strains used in this study are listed in Table S1. *E. coli* strains were grown at 37°C in LB medium with shaking. *E. faecalis* strains were grown in static conditions in appropriate media, either BHI or M17 supplemented with 0.5% glucose (M17G) at 37ºC, unless differently stated. Growth was monitored by measuring optical density at 600 nm (OD_{600}). Antibiotics were used at the following concentrations: erythromycin, 10 µg/ml for *E. faecalis* and 150 µg/ml for *E. coli*; and ampicillin 100 µg/ml.

Prophage induction, total DNA extraction and semi-quantitative PCR

E. faecalis strains were grown at 37°C in M17G up to OD_{600} = 0.2, and prophages were induced by adding mitomycin C, ciprofloxacin, trimethoprim or ampicillin at a final concentration of 4 µg/ml, 2 µg/ml, 0.04 µg/ml and 2 µg/ml, respectively. Cultures were grown for 2 hours at 28, 37 or 42°C, depending on the experimental assay. Cells were collected by centrifugation at 4°C and total DNA was extracted as previously [36]. The resulting DNA samples were screened for circular form of phage DNA (*attP* region) and for the excision site left on the chromosome (*attB* region) after prophage induction, by PCR using primer pairs (Table S2): ef303f / ef0355f and ef0302f / ef0357f (pp1), OEF591 / OEF592 (pp2), OEF531 / OEF532 and OEF653/ OEF654 (pp3), OEF546 / OEF547 and OEF551 / OEF640 (pp4), OEF533 / OEF534 and OEF655 / OEF656 (pp5), OEF548 / OEF549 and OEF557 / OEF624 (pp6) and OEF560 / OEF561 and OEF585 / OEF657 (pp7). Control PCR was performed with primers targeting the chromosomal gene *ef3155* using ef3155f and ef3155r primers, listed in Table S2. PCR amplifications were carried out in a Mastercycler gradient apparatus (Eppendorf, Courtaboeuf, France) using Taq DNA polymerase (Qbiogene, Illkirch, France). Analysis of PCR

products was monitored by agarose gel electrophoresis. Semi-quantitative PCR was performed on serial dilutions of quantified DNA recovered from both induced and non-induced cultures. For each DNA sample, both *attB* and *attP* regions were amplified using from 20 to 32 cycles. Excision *versus* replication was evaluated by comparing the amount of *attB* and *attP* PCR products, respectively, after gel electrophoresis. For each prophage, attachment site sequence was determined by sequencing the PCR products corresponding to the junction of both the circular form and the excision site. Sequencing was performed by GATC Biotech, France.

Construction of prophage deleted strains

Independent markerless deletions on different phage combinations were constructed through double-crossing over as described previously [37]. The 5'- and 3'-terminal regions of each phage were PCR-amplified from V583 chromosomal DNA and fused by PCR in such a way that the attachment site (*att*B) was reconstructed allowing for re-infection by the cognate phage. PCR amplifications were made with the following primers: OEF634 / OEF635 and OEF636 / OEF637 (pp1), OEF470 / OEF471 and OEF472/ OEF473 (pp3), OEF626 / OEF627 and OEF628 / OEF629 (pp4), OEF476 / OEF477 and OEF478 / OEF479 (pp5), OEF618 / OEF619 and OEF620 / OEF621 (pp6), OEF641 / OEF642 and OEF643 / OEF644 (pp7), listed in Table S2. All the plasmids obtained during this work are listed in Table S1. The strain deleted for all studied prophages (strain pp^{-}) was obtained by removing prophages 3, 5, 4, 6, 7 and 1 sequentially. Sequencing of the deletion site and PFGE confirmed the deletion and the absence of other major genome rearrangements.

Phage DNA extraction

V583 prophages were induced by addition of ciprofloxacin at 2 µg/ml to 100 ml of an exponential-phase culture (OD 600 = 0.2) further cultivated

for 4 h at 28, 37 and 42°C. The induced culture was centrifuged at 6 500 g for 20 minutes at 4 °C. The supernatant was collected and filtered through a 0.22 µm filter. Filtrate was supplemented with PEG 6000 (10% final conc., v/v) and NaCl 1 M and incubated overnight at 4°C. Phage particles were then pelleted by centrifugation at 7 600 g for 1 hour at 4°C. Supernatant was discarded and the phage pellet was soaked. The pellet was resuspended in 100 µl of SM buffer [38]. PEG was removed by chloroform extraction before treating the phage particles with 4 units of DNase I (Sigma) for 1 hour at 37°C to remove contaminating bacterial chromosomal DNA. Next, phage particles were disrupted at 80°C for 10 minutes in the presence of SDS 1%, proteins were removed by phenol/chloroform extraction, DNA was precipitated with ethanol and finally resuspended in 20 µl of TE containing 20 µg/ml of DNAse-free RNaseA (Sigma).

Field-inverted gel electrophoresis (FIGE) and Southern-blot

DNA from phage particles was analyzed by field inversion gel electrophoresis (FIGE, BioRad) on 1% agarose gel in TBE for 22h at 11°C. Migration conditions were the following: forward voltage 6 V/cm, reverse voltage 4 V/cm, switch time 0.2 - 1.0 sec, linear ramp. The gel was stained with ethidium bromide and monitored on a UV transillumination table, before transferring DNA onto a Nylon membrane (QBiogene) by Southern-blot [36]. Individual phage genomes were identified by hybridization with phage-specific probes amplified by PCR on genomic DNA with the following primers (Table S2): OEF573/OEF574 (pp1); OEF575/OEF576 (pp3); OEF577/OEF578 (pp4); OEF488/OEF489 (pp5); OEF579/OEF580 (pp6); OEF581/OEF582 (pp7). Probe labelling and hybridization detection was performed with DIG DNA labeling and detection kit (Roche) according to manufacturer's instructions.

Phage lysis plaque assay

Two ml of ciprofloxacin phage-induced cultures were collected after centrifugation for 20 min at 6000 g at 4 °C. Supernatants were collected and filtered on 0.22 µm filters. Filtrates were tested on indicator strain for plaque formation. Briefly, 50 µl of indicator strain grown in BHI up to OD_{600} = 0.2 was mixed with 4 ml of BHI containing 0.2% agarose (Lonza, LE) and 10 mM $MgSO_4$ and plated to form a lawn. 10 µl of each filtered-supernatant sample were spotted on the indicator bacterial lawn. Plates were incubated overnight at 28°C, 37°C or 42°C. Plaque formation was visually detected. When needed, plaques were identified by PCR in two independent experiments. Briefly, twenty plaques formed on each indicator strains were probed systematically for by both pp1 and EfCIV583 with specific primers (Table S2).

Scanning Electron Microscopy (SEM)

Bacterial suspensions immersed in a fixative solution (2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4) were deposited on sterile cover-glass discs (Marienfeld, VWR, France) and kept 1 hour at room temperature before overnight storage at 4°C. The fixative was removed, and samples were rinsed three times for 10 min in the sodium cacodylate solution (pH 7.4). The samples underwent progressive dehydration by soaking in a graded series of ethanol (50 to 100%) before critical-point drying under CO_2. Samples were mounted on aluminum stubs (10 mm diameter) with conductive silver paint and sputter coated with gold-palladium (Polaron SC7640; Elexience, France) for 200 s at 10 mA. Samples were visualized by field emission gun scanning electron microscopy. They were viewed as secondary electron images (2 kV) with a Hitachi S4500 instrument (Elexience, France). Scanning Electron Microscopy analyses were performed at the Microscopy and Imaging

Platform MIMA2 (Micalis, B2HM, Massy, France) of the INRA research center of Jouy-en-Josas (France).

Transmission Electron Microscopy (TEM)

Lysates of strains *pp1*$^{+}$ and *pp1*$^{+}$ *pp7*$^{+}$ were recovered 6h after ciprofloxacin-induction at 2 µl/ml. P1 was propagated on *pp*$^{-}$ strain and EfCIV583 on *pp1*$^{+}$ as described previously for phage plaque assay. Phages were recovered from the top agarose by addition of water and diffusion at 4°C during 4 hours. Next, samples were centrifuged for 15 minutes at 7 000 g and supernatant was filtrated through 0.22 µm pore filters. Phages samples were concentrated by ultra filtration through a Centricon YM-100 filter unit (Millipore, Molsheim, France). Bacteriophage solutions were applied on carbon-coated grids and subsequently stained with uranyl acetate (2% in water). Observations were performed with a JEOL 1200 EXII electron microscope.

RESULTS

Prediction of phage functions coded by the V583 prophages

Generally, temperate phage genomes are organized in modules of genes corresponding to important functions for their life cycle, which facilitates temporal order of gene transcription. Six modules are classically recognized: lysogeny, replication, transcriptional regulation, head and tail morphogenesis, DNA packaging and lysis [39,40]. The chromosome of *E. faecalis* V583 harbors seven prophage-like elements [31]. Table 1 summarizes the presence and absence of functions relevant to the identifiable modules on V583 prophages. Five of the seven prophages (pp1, pp3, pp4, pp5 and pp6) contain genes for all modules suggestive of genome completeness. All five contain an integrase, but only pp1 bears a recognizable excisionase function, which enables prophages to excise from the bacterial chromosome. However, it cannot be excluded that integrases use alternative accessory proteins such as recombination directionality factors allowing them to mediate prophage integration and excision [41]. Thus, prophages 1, 3, 4, 5 and 6 seem to have all necessary functions to undertake a complete lytic cycle (Figure 1). Among them, pp3 and pp5 have similar gene organization. Genes encoding potential fitness factors, namely homologs of *S. mitis* platelet binding proteins PblA and PblB (pp1, pp4 and pp6), a ferrochelatase (pp4) and a more recently identified toxin ADP-ribosyltransferase (pp1) have been predicted [31,35]. The genomes of pp2 and pp7 are particularly small in comparison with the five other prophages (~12 Kb versus >36 Kb). According to recent reports, pp2 belongs to the *E. faecalis* core genome [32,33,42] and the lack of an integrase gene suggests that pp2 is a remnant phage. Prophage 7 encodes an integrase and a replication related protein, however it lacks the head and tail morphogenesis modules essential for capsid formation as well as genes involved in DNA packaging

Table 1. Summary of predicted essential phage functions in V583 prophages.

V583 prophage	Prophage size (kb)	Prophage localization	Lysogeny			Replication	Morphogenesis			DNA packaging	Lysis
			Repressor (CI) / anti-repressor (Cro)	Integrase	Excisionase		Portal	Head	Tail		
pp1	38.2	*ef0303-ef0355*	+	+	+	+	+	+	+	+	+
pp2	14.6	*ef1276-ef1293*	+	-	-	+	-	-	+	-	+
pp3	47.3	*ef1417-ef1489*	+	+	-	+	-	+	+	+	+
pp4	39.0	*ef1988-ef2043*	+	+	-	+	+	+	+	+	+
pp5	43.0	*ef2084-ef2145*	+	+	-	+	-	+	+	+	+
pp6	36.0	*ef2798-ef2855*	+	+	-	+	+	+	+	+	+
pp7[a]	12.0	*ef2936-ef2955*	+	+	-	+	-	-	-	-	-

[a] Renamed EfCIV583 in this work

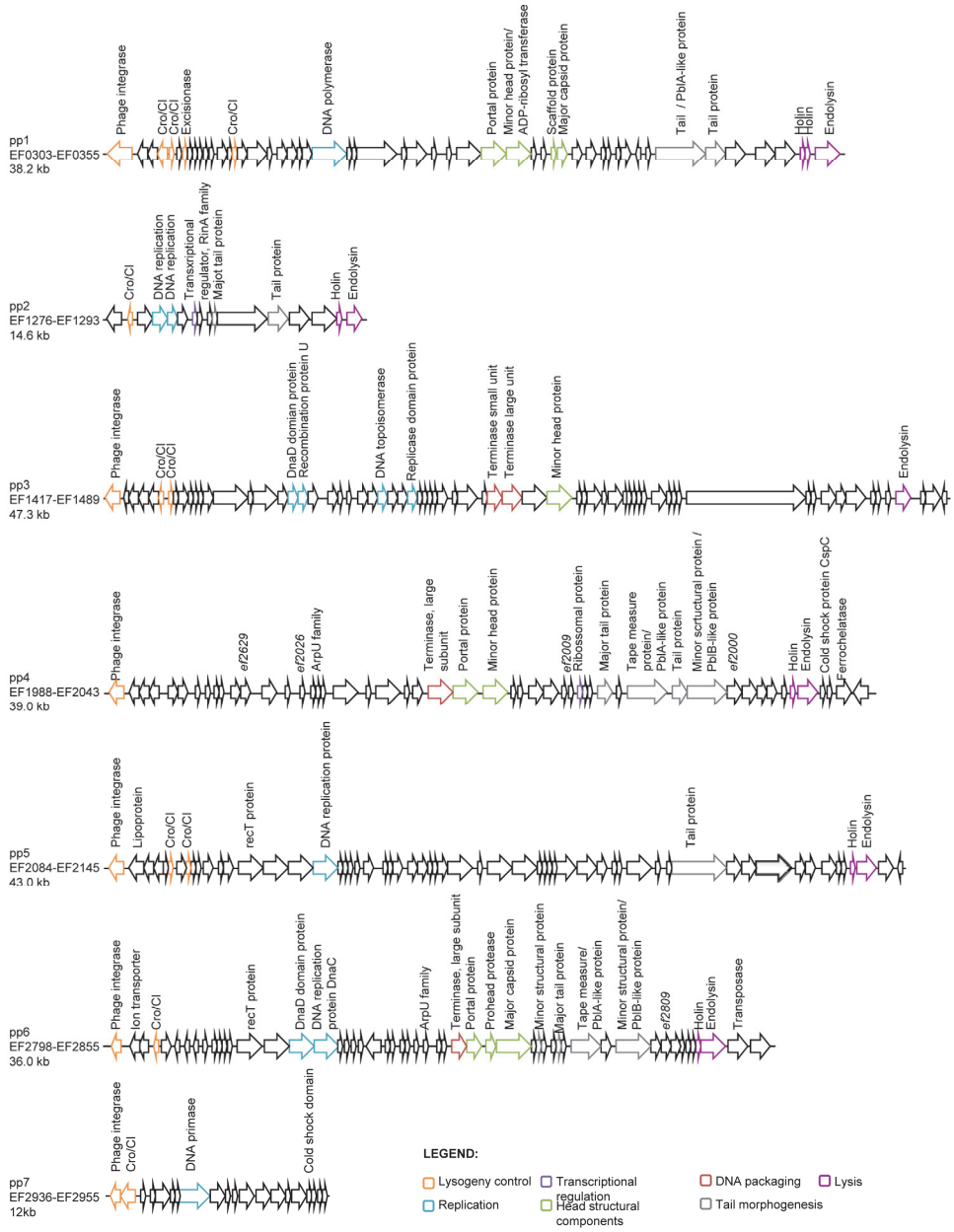


Figure 1. Genomic organization of *E. faecalis* V583 prophages. Open-reading frames are indicated by arrows. Only genes encoding predicted function are annotated. Colors correspond to the seven functional modules of temperate phages as depicted at the bottom right corner.

and lysis. These predictions suggest that pp7 is either defective or belongs to the family of the phage-related chromosomal islands (PRCIs) predicted in Gram-positive bacteria, including *E. faecalis* [13]. Altogether, five of the V583 prophages are predicted to form active particles autonomously.

Prophages 1, 3, 4, 5 and 7 excise from the chromosome

Prophages excise from the bacterial chromosome by inactivation of their repressor triggered either spontaneously or by a signal, which frequently depends on the induction of the SOS response [43]. To confirm our predictions on prophage activity, we tested the ability of the V583 prophages, under various environmental stresses, to accomplish the four major steps of temperate phage life cycle: excision, replication, DNA packaging and production of infectious particles.

We studied the activity of V583 prophages in the strain VE14089, which is a V583 derivative cured of its plasmids, and referred to as WT hereafter, a genetically tractable strain compared to the original V583 [36]. To determine whether prophages were able to excise from the chromosome, bacteria were challenged either with chemical compounds known to trigger prophage induction through SOS response and/or formation of reactive oxygen species (mitomycin C, ciprofloxacin, thrimetoprim, ampicillin), or with varying temperatures (28, 37 and 42°C) for 2 hours. Total DNA was recovered and analyzed by PCR to search for expected products of chromosomal excision and prophage circularization, referred as *attB* and *attP* region, respectively (Figure 2A). Results of amplification of the *attB* region resulting from prophage excision obtained with or without mitomycin C and ciprofloxacin are presented in Figure 2B. The excision of pp1, pp3, pp5 and pp7 was already detected under non-inducing conditions, indicating a basal natural excision in laboratory growth conditions.

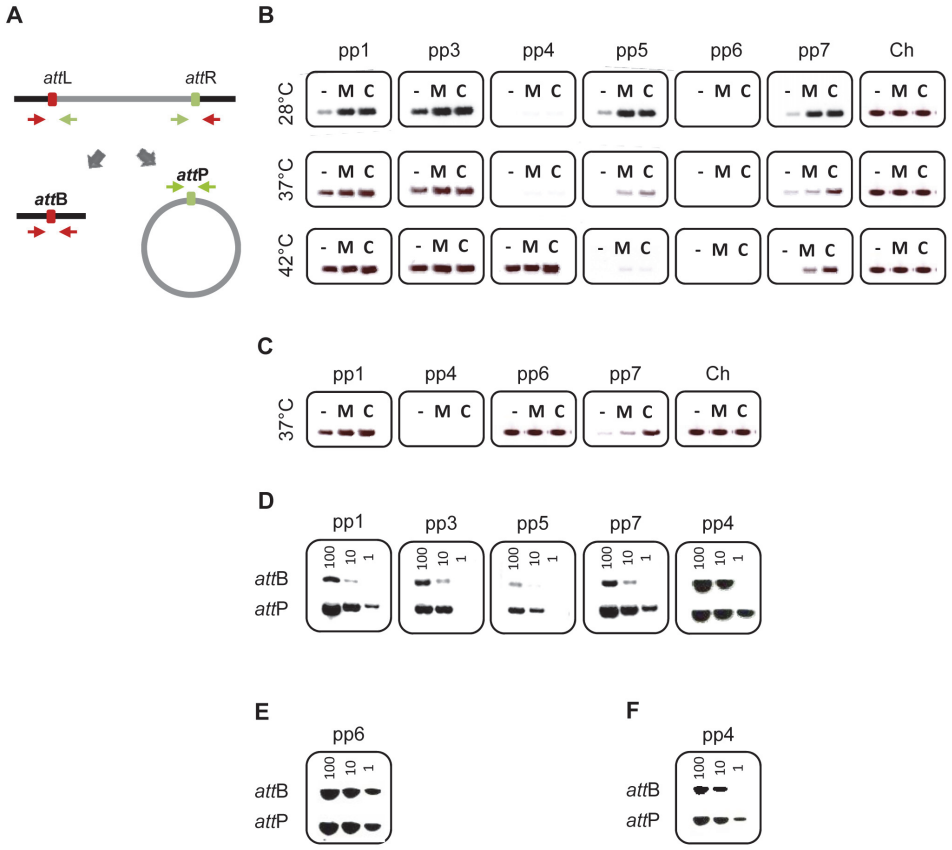


Figure 2. Prophage excision and replication. Agarose gel analysis of prophage excision and circularization products corresponding to *attB* and *attP* regions, respectively, probed by PCR. (A) Experimental approach: two sets of primers were used to detect prophage excision from the chromosome. The first set in red targets the excision site on the chromosome (*attB)* and the second set in green targets prophage circular forms (*attP*). (B) Prophage excision products corresponding to *attB* region were probed by PCR in WT cultures induced with 2 µg/ml of mitomycin C (M), or ciprofloxacin (C) or uninduced (-) at 28, 37 and 42°C. Ch corresponds to amplification of a strain specific chromosomal gene. (C) Prophage excision products in strain *pp3*$^{-}$ *pp5*$^{-}$ at 37°C. (D-F) Excision and circularization products probed by semi-quantitative PCR on 100, 10 and 1 pg of total bacterial DNA prepared from cultures of WT (D) and strains *pp3*$^{-}$*pp5*$^{-}$ (E) *pp4*$^{+}$ (F) induced for 2 h with 2 µg/ml of ciprofloxacin at 37°C except for detection of pp4 products in the WT strain that were obtained from DNA prepared after induction at 42°C. Twenty and 32 PCR cycles were used to amplify products of pp1 and pp7 and products of pp3, pp4 and pp5, respectively. These results are representative of three independent experiments.

Yet, prophages responded differently to environmental challenges. While pp3 was equally induced at 28, 37 and 42°C, natural induction of pp1 increased with temperature but those of pp5 and pp7 decreased with temperature. Strikingly, excision of pp4 was detected only at high temperature (42°C) (Figure 2B). Both mitomycin C and ciprofloxacin increased or triggered pp1, pp3, pp4, pp5 and pp7 excision from the bacterial chromosome at all tested temperatures. Trimethoprim challenge induced prophages similarly to ciprofloxacin, while ampicillin had no effect on prophages induction (data not shown). No excision of pp2 and pp6 was detected in any of the tested conditions. The PCR amplification fragments of the excision and integration sites were sequenced and confirmed the *in silico* predictions from V583 genomes of the attachment (*att*) core sequences (Table 2). This information was further used to reconstitute the prophage integration sites upon deletion by homologous recombination (see below). Note that excision of pp4 restores an open reading frame in an operon encoding competence-like genes [44] and that pp7 integrates in the promoter region of a putative xanthine/uracil permease gene. Together, these results revealed that pp1, pp3, pp4, pp5 and pp7 excise from the chromosome and that all prophages, but pp3 show different responses to environmental cues such as temperature and antibiotics, suggesting potential population heterogeneity of WT strain depending on each growth condition. However, in all conditions tested pp2 and more surprisingly pp6, were not excised.

Prophages 3 and 5 inhibit excision of pp6

Because complex mechanisms of interference between prophages have been reported [18,45], we wondered whether elimination of some prophages would facilitate excision of pp2 and pp6. For this purpose, we deleted successively prophages pp3 and pp5 (see materials and methods) and then checked for pp2 and pp6 excision. Interestingly, pp6

was excised in strain *pp3*$^{-}$ *pp5*$^{-}$ deleted for pp3 and pp5 (Figure 2C), whereas it was not excised in strains deleted either for pp3 or pp5 alone (data not shown). Although excisable in the absence of pp3 and pp5, pp6 does not replicate (Figure 2E). While pp6 basal level of excision was not increased upon temperature or chemical challenge, this finding allowed us to determine the pp6 *att* core sequence (Table 2). Finally, prophage deletions were performed to generate strain *pp*$^{-}$ deleted for all prophages but pp2. Again, no excision of pp2 was detected validating that pp2 is a phage remnant. We conclude that pp6 excision is repressed by both pp3 and pp5. Thus we demonstrated that prophages carried by the V583 *E. faecalis* chromosome, with the exception of pp2, can excise and thereby may form phage progeny.

Table 2. Prophage *att* core sequence predicted and confirmed experimentally from V583 genome[a].

Prophage	Genes	Integration site	Sequence 5'-3'
pp1	*ef0303-ef0355*	3' end of *ef0302*	CCTTGGGATCCAATGGG
pp3	*ef1417-ef1489*	3' end of *ef1416*	ACAAACGCAACATGTTCGCTTTA TTAGGTAAACCAGG
pp4	*ef1988-ef2043*	Within *cglD*-like[b] gene	CCACTCCCCATCTGAAATT
pp5	*ef2084-ef2145*	3' end of *tRNA-Thr2*	GGCAGGTGGCT
pp6	*ef2798-ef2855*	Downstream of 3' end of *ef2856*	TAAATTATTTAGTTTCACGGTGT AA
pp7[c]	*ef2936-ef2955*	Upstream of 5' end of *ef2935*	TATTAATGAAACAACGTG

[a] Genome accession number: AE016830

[b] *cglD* stands for for comG-like [44]

[c] Renamed EfCIV583 in this work

Prophages 1, 3, 5 and 7 form infectious virions

We then established which V583 prophages were able to replicate their genome after excision. Levels of both prophage circular forms and chromosomal excision regions from non-induced and ciprofloxacin-

induced cultures were compared using semi-quantitative PCR. Circular forms of pp4 at 42°C, and pp1, pp3, pp5 and pp7 at 37°C were at least 10-fold more abundant than the corresponding chromosomal excision regions, respectively (Figure 2D). In contrast, no replication activity was detected for pp6 in a *pp3*$^{-}$ *pp5*$^{-}$ strain (Figure 2E). To further investigate whether DNA of prophages could be packaged into phage particles, we precipitated phage particles from ciprofloxacin-induced cultures of wild-type strain at 28ºC, 37ºC and 42°C and strain *pp3*$^{-}$ *pp5*$^{-}$ at 37°C and extracted packaged DNA. Samples of phage DNA were analyzed by FIGE followed by Southern-blot hybridization with prophage-specific probes. In all the tested conditions, packaged DNA of pp1 (38.2 kb), pp5 (43.0 kb) and pp7 (12 kb) were detected, showing that pp1, pp5 and pp7 DNAs were encapsidated whereas DNA of pp3, pp4, and pp6 was not detected (Figure 3).

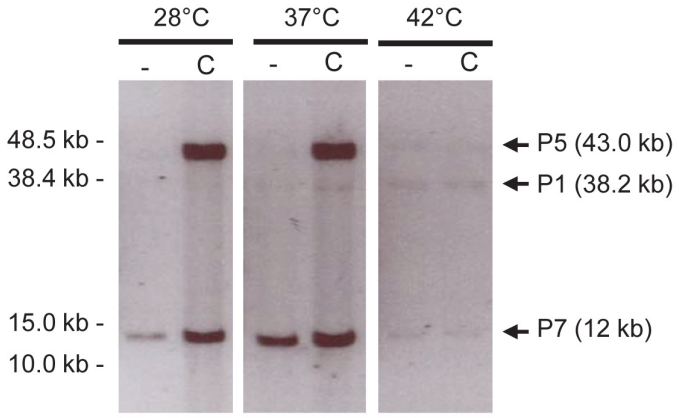


Figure 3. Detection of prophage packaged DNA. Encapsidated prophage DNA recovered from supernatant of WT cultures obtained at different temperatures 2h after ciprofloxacin treatment at 2 µg/ml and detected by southern-blot hybridization with prophage specific probes. Non-treated and ciprofloxacin treated cultures correspond to lanes (-) and (C), respectively. DNA of pp1, pp5 and pp7 is encapsidated at all temperatures under inducing conditions.

As pp7 does not encode its own capsid proteins (Table 1), it might need a helper phage to form particles like PRCIs (see below). Packaged DNA of pp5 and pp7 was less abundant at 42°C, as expected (see Figure 2B). While the absence of pp6 DNA-containing particles correlates with the lack of pp6 replication, non-detection of particles of pp3 and pp4 DNA could be explained either because their DNA was not packaged or the techniques used were not appropriate to isolate the cognate phage particles.

Finally, to determine which *E. faecalis* prophages have kept full viral activity, we examined their ability to form infectious virions. As a way to recognize the different virions generated by the WT strain, we constructed a set of isogenic strains deleted for individual prophages, namely strains *pp1*$^{-}$, *pp3*$^{-}$, *pp4*$^{-}$, *pp5*$^{-}$, *pp6*$^{-}$ and *pp7*$^{-}$, which harbored the natural *att*B integration site of the deleted prophage previously determined (see materials and methods) (Table S1). In a naïve scheme, such strains should be immune to superinfection by all phages except the one that no longer stands in the bacterial genome. Phage-deleted strains were infected with supernatants of ciprofloxacin-induced cultures from WT and *pp3*$^{-}$ *pp5*$^{-}$ strains (Table S3). Plaques were detected on strains *pp3*$^{-}$, *pp5*$^{-}$, and *pp7*$^{-}$, suggesting that particles containing pp3, pp5 or pp7 DNA are infectious. Interestingly, despite encapsidation of pp1 DNA (Figure 3), lytic activity of pp1 DNA-containing particles was not detected on strain *pp1*$^{-}$. This result suggested that either pp1 DNA-containing particles were non-infectious, or that strain *pp1*$^{-}$ was still immune to P1 (see below). No plaque formation was observed on indicator strains *pp4*$^{-}$ and *pp6*$^{-}$, indicating that although pp4 and pp6 were excised and pp4 replicated, these prophages are deficient for the formation of infectious particles.

Since phage interactions or interference could occur during particle or plaque formation we constructed monolysogen strains for each prophage, named *pp1*$^{+}$ to *pp7*$^{+}$, and tested the ability of their ciprofloxacin-induced

supernatants to form infectious particles on a *pp*$^{-}$ strain deleted for all prophages (Table S3). The results confirmed that pp3 and pp5 produced infective virions and that pp4 and pp6 did not. As expected, we confirmed that pp6 circular forms were detected in strain *pp6*$^{+}$ in non-induced conditions (data not shown). Prophage 4, which excision depends on a high temperature (42°C) in strain WT, excises readily and replicates at 37°C in strain *pp4*$^{+}$, deleted of all prophages but pp4 (Figure 2F). This observation suggests that some of the other V583 prophages could interfere with pp4 excision at 37°C in the WT strain. Interestingly, supernatant of the pp1 monolysogen strain (*pp1*$^{+}$) formed plaques on strain *pp*$^{-}$, indicating that pp1 DNA containing particles were infectious in the absence of other prophages. In contrast, the pp7 monolysogen strain (*pp7*$^{+}$) failed to produce infectious particles; further supporting that pp7 requires a helper phage.

Despite the absence of visible lysis upon prophage-inducing treatments, we evaluated the effect of prophage induction on bacterial population by assessing the growth of the strains WT, *pp*$^{-}$, *pp1*$^{+}$ and *pp3*$^{+}$ *pp5*$^{+}$ 6 h after ciprofloxacin-mediated induction. Ciprofloxacin treatment of wild-type strain lowered the growth of approximately 10 % compared to the untreated culture while similar treatment had no effect on strain *pp*$^{-}$ deleted for all prophages (Figure S1). Moreover, the strains *pp1*$^{+}$ and *pp3*$^{+}$ *pp5*$^{+}$ showed significantly decreased biomass when treated with ciprofloxacin. These observations suggest that V583 prophages are induced or perform full lytic cycle in a fraction of the bacterial population only, thereby leading to a mixed population with different combination of excised prophages.

In sum, these results demonstrate that pp1, pp3, pp5 and pp7 produce infective virions in specific conditions. Despite their excision, pp4 and pp6 are unable to produce infectious particles. Noticeably, pp4 and pp6 genomes contain several pseudogenes located in the morphogenesis

module (*ef2000*, *ef2009, ef2026*, *ef2029* for pp4 and *ef2809* for pp6) that could explain that these phages are defective in capsid assembly. While phages 1 (P1), 3 (P3) and 5 (P5) are autonomous, pp7 requires a helper phage to form infectious particles. P3 and P5 provide self-immunity to their bacterial host, and P1 shows cross-immunity with at least one of the other prophages.

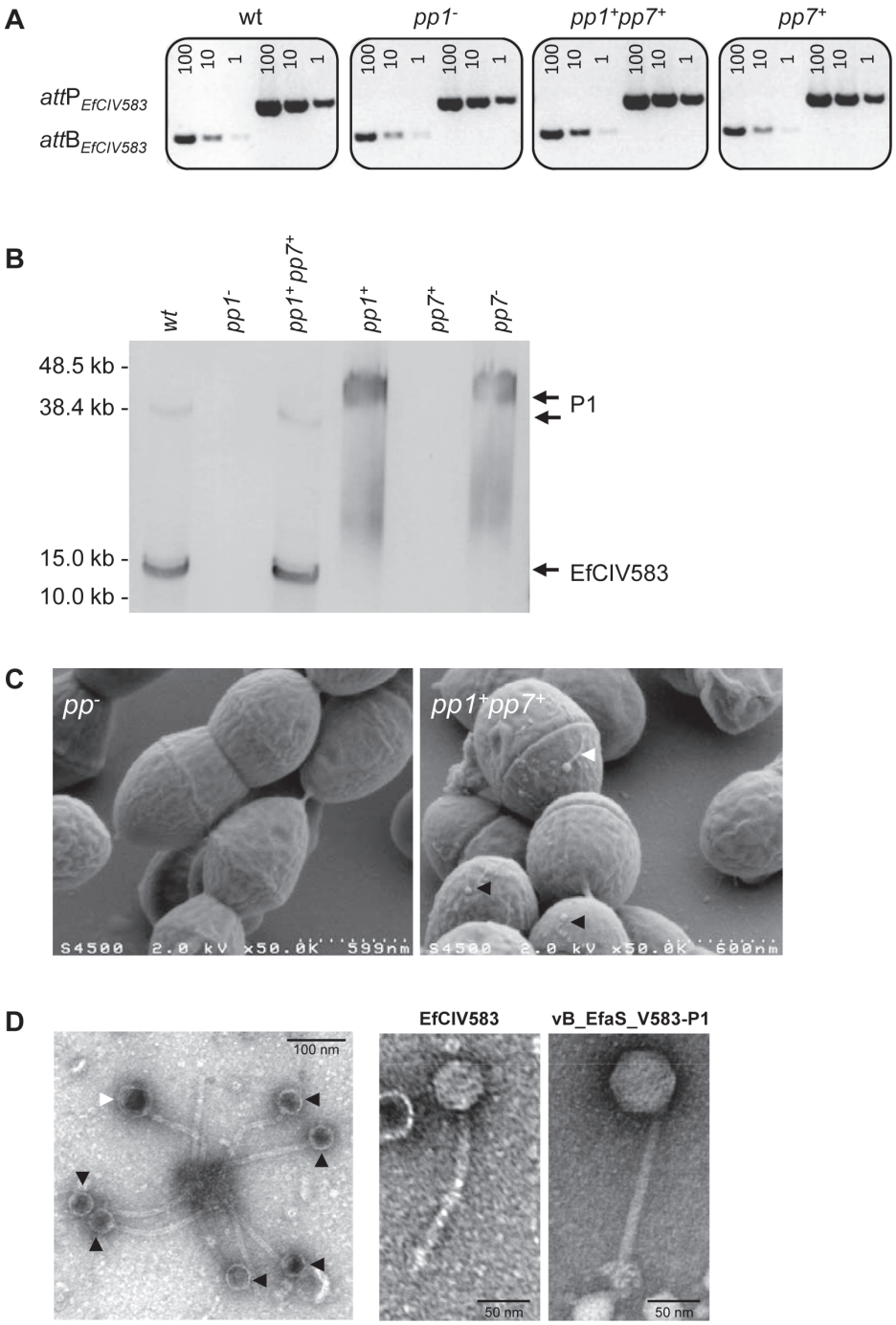
Prophage 7 requires P1 as a helper phage for encapsidation

Subordination of pp7 to a helper phage to form infectious particles was correlated with comparative genomic hybridization data (Akary and Serror, *unp. data*) and sequence analysis of available genomes, which indicate that pp7 is present only in few CC2 isolates, which also carry pp1 whereas pp1 is sometimes present alone [33,46]. Thus, we hypothesized that P1 acts as a helper phage of pp7. To test our hypothesis, we constructed strain *pp1*$^{+}$ *pp7*$^{+}$, which contains pp1 and pp7 only (Table S1). Supernatants of ciprofloxacin-treated isogenic strains *pp1*$^{-}$ and *pp1*$^{+}$ *pp7*$^{+}$ were tested for plaque formation on the indicator strain *pp7*$^{-}$. While the dilysogen strain *pp1*$^{+}$ *pp7*$^{+}$ gave plaques, deletion of pp1 abrogated plaque formation, demonstrating that pp1 is necessary and sufficient for production of P7 virions. We conclude that the presently described pp7 corresponds to the phage-related *E. faecalis* chromosomal island, predicted by Novick and collaborators [13], and rename pp7 as EfCIV583 for *E. faecalis* chromosomal island V583.

To identify the step at which pp1 was required for production of EfCIV583 virions, we analyzed both the excision and replication of EfCIV583 and the packaging of EfCIV583 DNA in WT and the isogenic strains *pp1*$^{-}$, *pp1*$^{+}$ *pp7*$^{+}$, *pp7*$^{+}$ and *pp1*$^{+}$ by semi-quantitive PCR. Excision (*attB* region) and replication (*attP* region) products of EfCIV583 were detected in *pp1*$^{-}$ and *pp7*$^{+}$ strains at the same level as strains wild type and *pp1*$^{+}$ *pp7*$^{+}$ (Figure 4A), showing that pp1 is not required for EfCIV583

excision and replication. Next, DNA from phage particles produced by ciprofloxacin-treated WT and the isogenic strains *pp1*$^{-}$, *pp1*$^{+}$ *pp7*$^{+}$, *pp7*$^{+}$ and *pp1*$^{+}$ was recovered and analyzed as described above. Particles containing EfCIV583 DNA were recovered from WT and *pp1*$^{+}$ *pp7*$^{+}$ strains, while EfCIV583 DNA was no longer packaged in the absence of pp1 (strain *pp1*$^{-}$) or when present as a single element (strain *pp7*$^{+}$) (Figure 4B), indicating that pp1 is required for packaging of EfCIV583 DNA. Independent hybridizations revealed that EfCIV583 DNA is encapsidated as monomers only since no signal was detected at high molecular weight (data not shown). Noticeably, while the amount of the EfCIV583 DNA was similar between strains, the amount of pp1 DNA increased significantly when EfCIV583 was deleted (strain *pp1*$^{+}$), suggesting that EfCIV583 DNA hijacks P1 proteins at the expense of P1 particles production.

Figure 4. **Interaction between *E. faecalis* pp1 and pp7 (EfCIV583).** (A) Semi-quantitative PCR detection of EfCIV583 circular forms (*attP*) and excision sites (*attB*) in wild-type (WT) and strains *pp1*$^{-}$, *pp1*$^{+}$ *pp7*$^{+}$ and *pp7*$^{+}$. Excision and circularization products probed by semi-quantitative PCR on 100, 10 and 1 pg of total bacterial DNA prepared from cultures of WT and strains *pp1*$^{-}$, *pp1*$^{+}$ *pp7*$^{+}$ and *pp7*$^{+}$ induced for 2 h with 2 µg/ml of ciprofloxacin at 37°C. Twenty cycles were used to amplify products of pp1 and EfCIV583. These results are representative of two independent experiments. (B) Prophage DNA extracted from precipitated phage particles obtained from lysates of WT and strains *pp1*$^{-}$, *pp1*$^{+}$ *pp7*$^{+}$ and *pp7*$^{+}$ was separated by FIGE and analyzed by Southern-blot and hybridized sequentially using specific probes for pp1 and EfCIV583 genomes. The approximately 38.2 kb and 12 kb band corresponds to P1 and EfCIV583 genome, respectively. As ascertained by pp1-specific hybridization, migration of P1 DNA was delayed in lane *pp1*$^{+}$ and *pp7*$^{-}$ compared to lanes WT and *pp1*$^{+}$*pp7*$^{+}$. Lambda DNA mono-cut mix (NEB) was run next to the samples to validate band sizes. **(**C) Scanning electron microscopy images of bacterial cells from strains *pp*$^{-}$ and *pp1*$^{+}$*pp7*$^{+}$ after ciprofloxacin treatment. (D) Transmission electron microscopy images of phages produced by strain *pp1*$^{+}$*pp7*$^{+}$ after ciprofloxacin treatment. White and black arrows indicate big and small sized particles attributed to P1 and EfCIV583, respectively. Enlarged images of EfCIV583 and P1 (renamed vB_EfaS_V583-P1) are shown on the right.


A
wt
pp1-
pp1+pp7+
pp7+
100
10
1
attP EfCIV583
attB EfCIV583
B
wt
pp1-
pp1+pp7+
pp1+
pp7+
pp7-
48.5 kb -
38.4 kb -
15.0 kb -
10.0 kb -
P1
EfCIV583
C
pp-
pp1+pp7+
D
100 nm
EfCIV583
vB_EfaS_V583-P1
50 nm
50 nm

The above molecular evidences for EfCIV583 pirating P1 proteins correlate with respective phage titers (Table 3). First, EfCIV583 titer was 10-fold higher than the titer of P1 in lysates from strain *pp1*$^{+}$ *pp7*$^{+}$, supporting that when present, EfCIV583 outnumbers P1 particles. Secondly, P1 titer of lysates from strain *pp1*$^{+}$ was 100-fold higher than in lysates from strain *pp1*$^{+}$ *pp7*$^{+}$, indicating that EfCIV583 impairs the production of P1 particles. Interestingly, P1 particles are infectious on strains *pp1*$^{-}$ *pp7*$^{-}$ and *pp*$^{-}$, but not on strains *pp1*$^{-}$ nor *pp7*$^{+}$ (Tables 3 and S3), further supporting that EfCIV583 interferes with P1 growth.

Table 3. Production of infectious P1 and EfCIV583 virions.

Indicator strain	Lysate (pfu/ml)		
	WT	*pp1*$^{+}$ *pp7*$^{+}$	*pp1*$^{+}$
pp^{-}	3.0 x 10^{2}	1.6 x 10^{3}	1.1 x 10^{5}
pp1^{-} ***pp7***$^{-}$	1.5 x 10^{2}	4.0 x 10^{3}	1.5 x 10^{5}
pp1^{-}	-	-	-
pp7^{-}	2.0 x 10^{3}	1.8 x 10^{4}	-

SaPI are usually encapsidated into small-headed phage particles, distinguishable from their helper phage particles [16]. Indeed here, as pp1 and EfCIV583 genomes differ in size, P1 and EfCIV583 particles were expected to be distinguishable in size. Scanning electron microscopy observation of a ciprofloxacin treated culture from the *pp1*$^{+}$ *pp7*$^{+}$ dilysogen revealed the existence of two phage size particles (Figure 4C), which were further confirmed by transmission electron microscopy (Figure 4D). Measurement of the capsids grouped the particles into small and large-size groups of ~ 46 nm and ~ 62 nm of width, respectively (Figure 5). Both particles harbored similar size tail of ~165 nm in length. As a control, P1 particles obtained from strain *pp1*$^{+}$ were also analyzed. Their size corresponds to the size of the large-size capsids produced by strain *pp1*$^{+}$ *pp7*$^{+}$, strongly indicating that large and small capsids belong to P1 and

EfCIV583 virions, respectively. In addition, we confirmed that P1 belongs to the *Siphoviridae* family with a non-contractile tail (Figure 4D). Accordingly to Kropinsky's nomenclature proposal for bacterial virus [47], we propose to rename phage 1 "vB_EfaS_V583-P1".

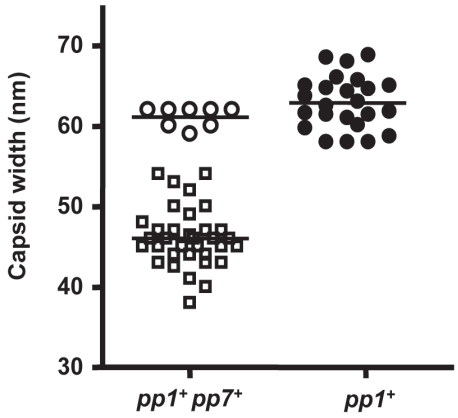
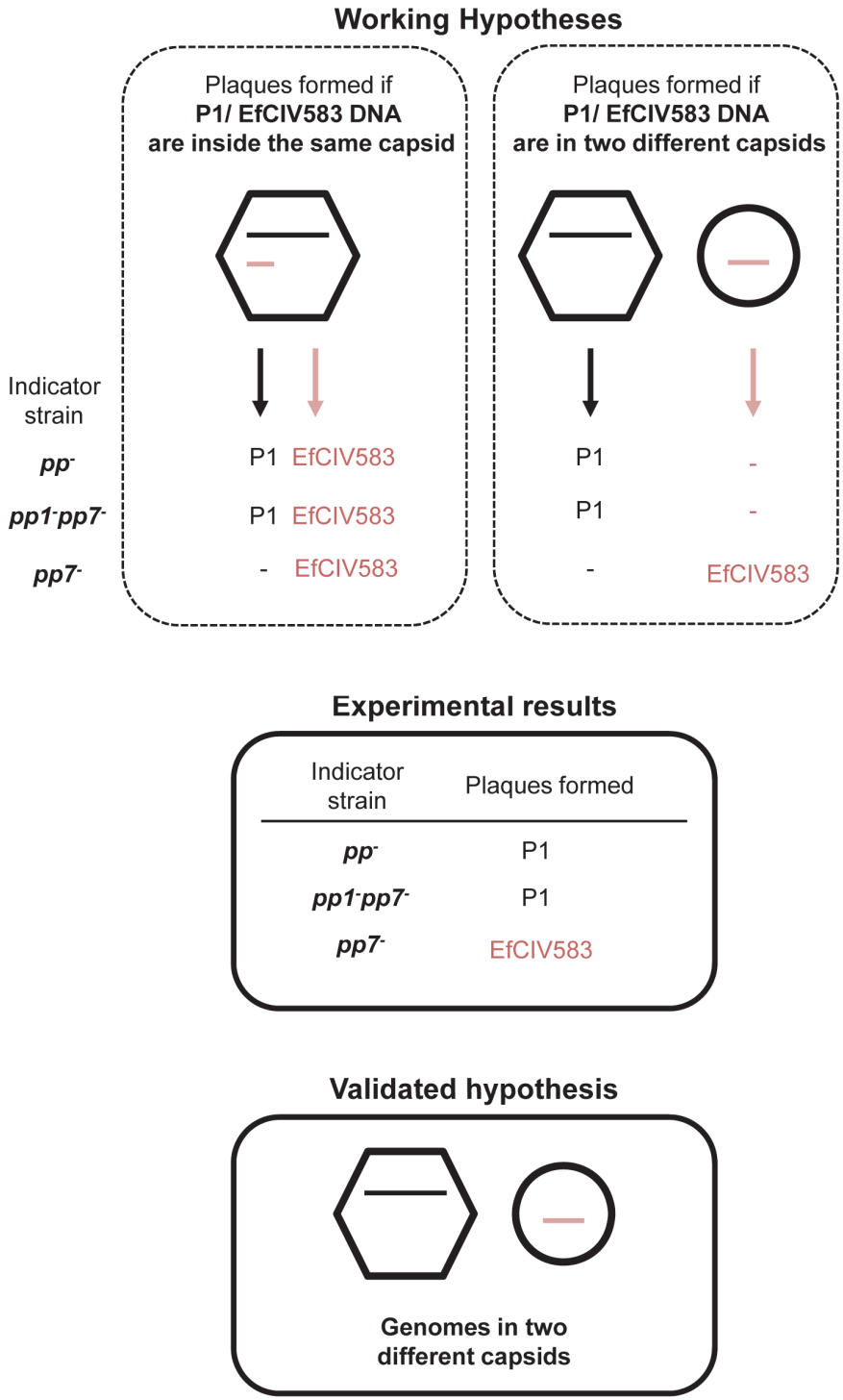


Figure 5. Capsid size distribution of virions produced by strains *pp1*$^{+}$ *pp7*$^{+}$ and *pp1*$^{+}$. Scatter plot of the capsid width (nm) measured particles for strains *pp1*$^{+}$ *pp7*$^{+}$ (n=42) and *pp1*$^{+}$ (n=24). Strain *pp1*$^{+}$ *pp7*$^{+}$ produced two groups of different capsid size, small and large with a mean width of 46.1 ± 3.7 nm and 61.1 ± 1.3 nm, respectively. Strain *pp1*$^{+}$ produced homogenous capsid size with a mean width of 62.9 ± 3.3 nm.

During completion of this manuscript, Duerkop et al, proposed that P1 and EfCIV583 were encapsidated together in a composite phage [48]. This hypothesis does not fit with the results reported here, and to completely exclude this possibility, we investigated particles infectivity on different indicator strains and identified the resulting plaques by phage-specific PCR (Figure 6). A mixed lysate of P1 and EfCIV583 was propagated on strains devoid of both pp1 and EfCIV583 (e. g. strains *pp*$^{-}$ and *pp1*$^{-}$ *pp7*$^{-}$). Under such circumstance, EfCIV583 should not form plaques, unless its DNA is indeed always encapsidated with that of its helper phage (Figure 6). Plaques were screened for the presence of EfCIV583 DNA, and none were found positive. As a control, the same lysate grown on a pp1 positive

lawn gave EfCIV583 positive plaques, as expected. Thus, we conclude that EfCIV583 DNA is encapsidated separatly into small size particles, and does not travel along with its helper phage. We can nevertheless explain how Duerkop et al. came to their inappropriate conclusion (see discussion). According to our results and in keeping with SaPIs elements, pp1 and EfCIV583 DNA are packaged in distinct particles and we propose that large and small phages correspond to packaging of pp1 and EfCIV583 DNA, respectively. Altogether, our results demonstrate that EfCIV583 is a self-excisable and -replicative phage-related element, using P1 as a helper phage.

Figure 6. DNA of pp1 and EfCIV583 are packaged in separated capsids. Presentation of two working hypotheses for pp1 and EFCIV583 DNA packaging in a dilysogen strain and experimental results corroborating one of them. On the left, DNAs are packaged inside the same capsid. The resulting virions are predicted to deliver both DNA during infection and to form plaques containing both P1 and EFCIV583 virions since pp1 is required for formation of EfCIV583 virions on indicator strains *pp*$^-$ and *pp1*$^-$ *pp7*$^-$, both deleted for pp1 and EfCIV583. On the right, pp1 and EfCIV583 DNAs are packaged separately in two different capsids. The resulting virions would deliver either pp1 or EfCIV583 DNA during infection of strains *pp*$^-$ and *pp1*$^-$ *pp7*$^-$, and would form only P1 plaques since pp1 is required for formation of EfCIV583 virions and co-infection by two particles is highly improbable. However, EfCIV583 virions would be detected on the indicator strain *pp7*$^-$ (harboring pp1). Lysates of strain *pp1*$^+$ *pp7*$^+$ were tested on indicator strains *pp*$^-$, *pp1*$^-$ *pp7*$^-$ and *pp7*$^-$ and the resulting plaques were identified by pp1 and EfCIV583-specific PCRs. Our results strongly support that P1 and EfCIV583 genomes are packaged in two different capsids since plaques formed by *pp1*$^+$ *pp7*$^+$ lysates on indicator strains *pp*$^-$ and *pp1*$^-$ *pp7*$^-$ were identified as P1 plaques only, while EfCIV583 virions were detected on indicator strain *pp7*$^-$.


Working Hypotheses
Plaques formed if
P1/ EfCIV583 DNA
are inside the same capsid
Plaques formed if
P1/ EfCIV583 DNA
are in two different capsids
Indicator strain
pp-
pp1-pp7-
pp7-
P1 EfCIV583
P1 EfCIV583
- EfCIV583
P1 -
P1 -
- EfCIV583
Experimental results
Indicator strain
Plaques formed
pp-
P1
pp1-pp7-
P1
pp7-
EfCIV583
Validated hypothesis
Genomes in two different capsids

Prophage 1 interferes with pp4 excision

Having observed that pp4 excises spontaneously at 37°C in a monolysogen strain, we analyzed the presence of pp4 circular forms in a panel of strains containing various prophages to understand which one(s) was interfering with its excision (Figure S2). Interestingly, the presence of pp4 circular form at 37°C was strictly correlated with the absence of pp1 prophage, indicating that pp4 excision is blocked at 37°C when pp1 is present.Spontaneous excision of pp4 at 42°C in WT strain suggests that the inhibitory effect of pp1 is thermosensitive. Indeed, P1 titer of supernatants of a monolysogen strain increased 10-fold, from 10^4 pfu/ml to 10^5 pfu/ml when grown at 42°C, further supporting that P1 repressor is thermosensitive. This result reveals another level of *E. faecalis* prophage interactions, in which prophage pp1 interferes with excision of pp4.

Potential plasmid-prophage interactions

We next investigated whether phages were produced as readily in the V583 parental strain as in the plasmid-cured strain used hitherto. For this, supernatants of V583 cultures treated or not with ciprofloxacin were plated on the same set of indicator strains *pp1*$^-$, *pp3*$^-$, *pp4*$^-$, *pp5*$^-$, *pp6*$^-$, *pp7*$^-$ as above. PRCI EfCIV583, but not P3 nor P5 was found to form plaques on the corresponding deleted strains. These results show that strain V583 exhibits a lower efficiency to produce phages compared to its plasmid-cured derivative, and suggest that plasmid-curing has somehow caused an increase of the basal level of prophage induction, indicating a possible interference of plasmids with prophages. If plasmid pCM194 can increase phage production of a SPO2 lysogen *Bacillus subtilis* strain [49], it is also possible that plasmids interfere negatively with phage production and contribute to prophage accumulation leading to polylysogenic strains.

DISCUSSION

In the present study, we characterized the biological activity (excision, replication and virion production) of six *E. faecalis* predicted prophages of a plasmid-cured derivative of the polylysogenic V583 isolate, a representative of the hospital-adapted clade CC2. We show that all of the predicted prophages, except V583-pp2, are able to start a lytic cycle, with four of them (V583-pp1, V583-pp3, V583-pp5 and V583-pp7) leading to the production of phage progeny, which is exacerbated by clinically relevant antibiotics. Besides showing that phages P1, P3 and P5 are autonomous and confer self immunity to their bacterial host, we identified three levels of prophage interactions: i) the herein demonstrated phage-related chromosomal island EfCIV583 (V583-pp7) hijacks P1 capsids and interferes with P1 infectivity, ii) pp1 exerts a temperature-dependent inhibition of pp4 excision, and iii) pp3 and pp5 block excision of pp6. Altogether, the interplay between these prophages potentiates their mobility and biological activities.

Polylysogeny is found in a variety of bacterial species, including *E. faecalis* [34] and it is frequently considered as the result of an adaptive evolution process in which prophages are maintained as they confer advantageous properties to the bacterial strains [23,50-54]. As a way to maintain and propagate themselves, prophages interfere with each other through a variety of mechanisms in different bacterial species [18,52,55]. We demonstrate that EfCIV583 is a phage-related chromosomal island that excises and replicates autonomously as an episome, but specifically requires P1 structural proteins for production of infectious virions. Correlating the genome length of each prophage with the electron microscopic observations and virion infectivity, we propose that P1 and EfCIV583 virions encapsidate into large and small size particles, respectively. Moreover, as packaging of EfCIV583 DNA mobilizes P1

structural proteins, EfCIV583 outcompetes with the formation of P1 particles and interferes with P1 plaque forming ability. Our conclusions on P1 and EfCIV583 DNA packaging and autonomy of the helper phage P1 differ from those recently reported by Duerkop et al., 2012 [48]. Their data can be fully explained by the chromosomal island-helper phage interaction that we have described between EfCIV583 and P1, except for the apparent absence of P1 particles in the supernatant of a V583 strain mutated for EfCIV583 (their Fig. 2D), which leads the authors to suggest that P1 depends on EfCIV583 for its growth. However, the indicator strains used in this experiment are not appropriate to count P1 plaques as they are lysogen for P1 and therefore immune to P1 (WTs of their Fig. S6). According to our data and as depicted in Figure 7, the interaction between *E. faecalis* P1 and the phage-related chromosomal island EfCIV583 is a case of molecular piracy, which involves hijacking of P1 structural proteins by EfCIV583 DNA to be disseminated into small capsids. This is to our knowledge the first example of Gram positive, other than staphylococci, in which such molecular piracy phenomenon has been described. In spite of the resemblance with the well studied system of the SaPIs and their helper phages [13,14], EfCIV583/P1 system is different in several ways. First, with the exception of SaPIbov1 and SaPIbov2 [56], SaPIs are generally stably maintained into the bacterial genome [13] through the action of a SaPI-encoded master repressor [15], which is inactivated by helper-phage specific antirepressors [57,58]. Here, spontaneous excision of EfCIV583 in a monolysogen strain suggests that the activity of its predicted repressor (EF2954) is controlled by a helper phage-independent mechanism. Furthermore as EfCIV583 excision is increased by ciprofloxacin, this repressor is likely under the control of the SOS response. Similarly, SpyCIM1 of *S. pyogenes* responds to SOS system, however the implication of a helper phage for its induction remains to be investigated. Excision and reintegration of SpyCIM1 adjust

the adaptation capacity of the host strain by modulating expression of the gene *mutL* [11,59].

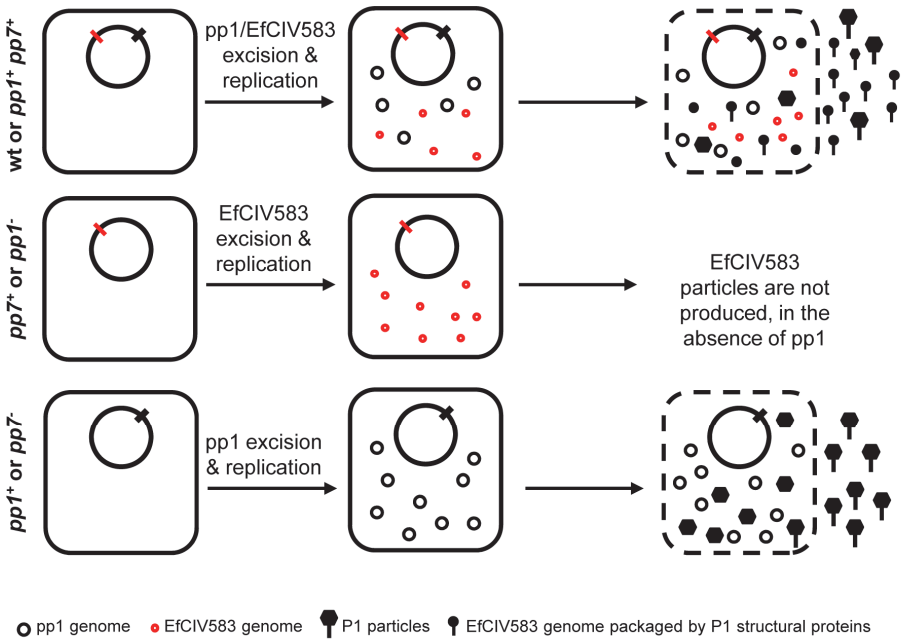


Figure 7. Model of P1 / EfCIV583 interplay. Infectious P1 and EfCIV583 particles are produced by strains WT and *pp1*$^{+}$*pp7*$^{+}$ whereas no particles are produced in the absence of pp1 (strains *pp1*$^{-}$ and *pp7*$^{+}$), showing that pp1 is required to form EfCIV583 virions. This hijacking phenomenon impairs the production of P1 particles in favor to EfCIV583. As observed by SEM and TEM, strain *pp1*$^{+}$*pp7*$^{+}$ produces two different sizes of phage particles: the biggest package most probably P1 and the smallest EfCIV583. In the absence of EfCIV583 (strains *pp7*$^{-}$ and *pp1*$^{+}$), P1 virions are produced at higher titer.

Given that EfCIV583 integrates into the promoter region of a putative xanthine/uracil permease gene, it may dynamically modulate xanthine or uracil utilization. Secondly, while the best studied SaPIs, SaPI1 and SaPIbov, form mostly small capsids, their DNA can also be packaged as

multimers into large capsids [60,61]. In the case of EfCIV583, the packaging specificity seems to be tightly controlled since EfCIV583 DNA is packaged exclusively as the monomeric form. Lastly, different interference mechanisms used by the SaPIs to counteract capsid formation and DNA packaging of the helper phage have been recently deciphered [18,19]. They rely on capsid morphogenesis (*cpm*) and phage packaging interference (*ppi*) genes of which no close homolog can be identified in EfCIV583 (R. Guerois, Pers. Comm.), suggesting that EfCIV583 may use other mechanisms to interfere with P1. Thus, besides expanding and strengthening the concept of molecular piracy within bacteria, the enterococcal EfCIV583/P1 system exhibits specific molecular mechanisms that deserve to be further investigated.

Remarkably, pp4 and pp6 are kept silent by other prophages. Prophage pp1 negatively interferes with pp4 excision at 37°C but not at 42°C. As excision of pp1 is increased at 42°C, a simple explanation may be that the pp1 repressor is itself thermosensitive and controls pp4. We also found that pp6 excises only when pp3 and pp5 are deleted from the wild type strain. Since single deletion of pp3 or pp5 had no effect on pp6 induction, it is conceivable that pp3 and pp5 exert redundant repression of pp6 induction. Noticeably, pp3 and pp5 share the highest homology compared to the other V583 prophages, suggesting a potential crosstalk. A recent study from Lemire *et al*., 2011 described a mechanism of antirepressor-mediated control of prophage induction involving recognition of both cognate and non-cognate repressors of Gifsy prophages in *Salmonella* [45]. Interestingly, their work suggests coordinate induction of lytic cycle of prophages in polylysogenic strains. Prophage interferences and low efficiency of lysis on V583 upon induction may contribute to maintain diversity within the bacterial population and ensure survival. Further genetic and molecular studies will be required to characterize the

crosstalk mechanisms between *E. faecalis* prophages, including prophage-related chromosomal islands.

Gene dissemination is an important biological aspect through which temperate phages impact on bacterial species [17,62,63]. Prophage mediated gene transduction has been recently reported between *E. faecalis* strains and between enterococcal species [34,64]. We demonstrated that V583 pp1, pp3, pp5 and EfCIV583 form infectious particles suggesting that they are capable of mediating horizontal gene transfer. Their excision is enhanced by SOS-triggering agents, including mitomycin C, trimethoprim and the fluoroquinolone antibiotic ciprofloxacin. Fluoroquinolones inhibit DNA gyrase and topoisomerase IV and cause DNA double-strand breaks, as such, they are among the most efficient phage-inducing antibiotics [65,66]. Fluoroquinolones promote release of Shiga toxins encoded by prophages from *Escherichia coli* [67], potentiate the spread of virulence traits in *Staphylococcus aureus* [68] and eventually reduce strain competitiveness [69]. Though the impact of the *E. faecalis* prophages in promoting both strain fitness and horizontal gene spreading has yet to be studied, phage-inducing antibiotics may contribute to the emergence of *E. faecalis* polylysogenic strains, such as V583. Treatment with fluoroquinolones was identified as a risk factor for infection or colonization by vancomycin-resistant enterococci in the U.S, where the CC2 isolates have emerged [70].

Given the complexity of the interplay between V583 prophages, we anticipate that mixed *E. faecalis* subpopulations may be formed upon prophage induction and could favor survival of one or several of them as described for different bacterial species especially in biofilms [20,71-73]. In all, temperate phages are likely to potentiate *E. faecalis* genetic and physiological flexibility for optimal adaptation during colonization or infection.

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SUPPLEMENTARY INFORMATION

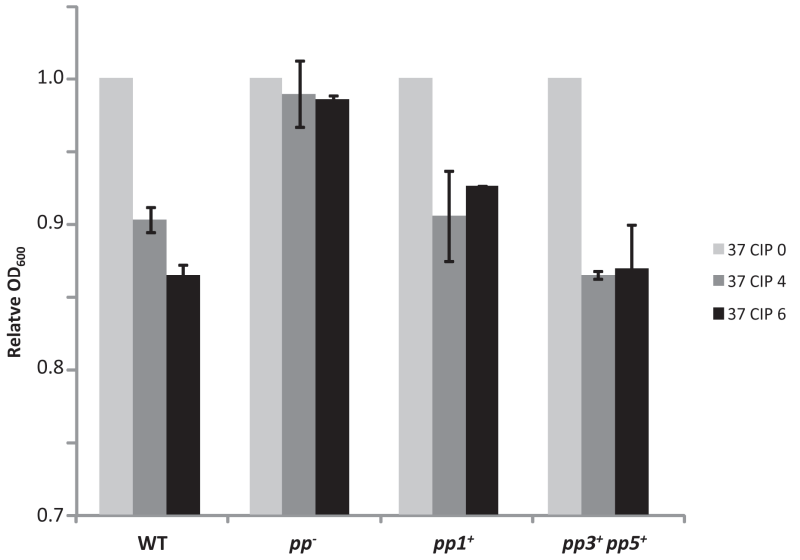

Figure S1. Growth of ciprofloxacin-treated strains. The WT and isogenic strains *pp*$^{-}$, *pp1*$^{+}$ and *pp3*$^{+}$ *pp5*$^{+}$ were grown at early exponential growth phase (OD_{600} ~ 0.2) before treatment with ciprofloxacin at 4 or 6 µg/ml. Relative optical density (OD_{600}) was calculated for each strain as the ratio of OD_{600} of the ciprofloxacin-induced cultures (Cip 4 and Cip 6) with the non-induced culture (Cip 0) 6 h after addition of ciprofloxacin later. The mean and the standard error of the mean (SEM) obtained on two independent cultures for each strain is shown.

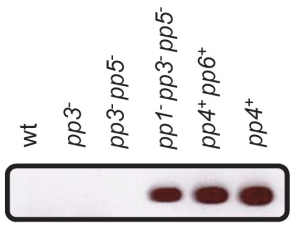

Figure S2. pp1 interference with pp4 excision. PCR detection of pp4 circular forms in different isogenic strains (see Figure 2A): WT, *pp3*$^{-}$, *pp3*$^{-}$ *pp5*$^{-}$, *pp1*$^{-}$ *pp3*$^{-}$ *pp5*$^{-}$, *pp4*$^{+}$ *pp6*$^{+}$ and *pp4*$^{+}$ (see Table S1). Circular forms of pp4 are detected only in the absence of pp1.

Table S1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Short name	Relevant characteristics	Reference or source
Strain			
E. coli			
TG1		*supE hsdD5 thi* (Δ*lac-proAB*) F' (*traD36 proAB-lacZΔM15*)	[74]
GM1674		*dam*$^-$*dcm*$^-$ *repA*$^+$	[75]
E. faecalis			
VE14002	V583	V583 vancomycin resistant clinical isolate	[30]
VE14089	WT	V583 vancomycin resistant clinical isolate cured of its plasmids	[36]
VE14492	V583pTEF1	V583 vancomycin resistant clinical isolate cured of its plasmids transconjugated with pTEF1	This study
VE14279	*pp3*$^-$	pp3 deletion in *E. faecalis* VE14089	This study
VE18306	*pp6*$^-$	pp6 deletion in *E. faecalis* VE14089	This study
VE18310	*pp4*$^-$	pp4 deletion in *E. faecalis* VE14089	This study
VE18284	*pp7*$^-$	pp7 deletion in *E. faecalis* VE14089	This study
VE14285	*pp5*$^-$	pp5 deletion in *E. faecalis* VE14089	This study
VE18299	*pp3*$^-$*pp5*$^-$	pp3 and 5 deletion in *E. faecalis* VE14089	This study
VE18313	*pp1*$^-$	pp1 deletion in *E. faecalis* VE14089	This study
VE18316	*pp1*$^+$*pp7*$^+$	pp3, pp4, pp5 and pp6 deletion in *E. faecalis* VE14089	This study
VE18566	*pp1*$^-$*pp7*$^-$	pp1 and pp7 deletion in *E. faecalis* VE14089	This study
VE18576	*pp4*$^+$*pp6*$^+$	pp1, pp3, pp5 and pp7 deletion in *E. faecalis* VE14089	This study
VE18589	*pp7*$^+$	pp1, pp3, pp4, pp5 and pp6 deletion in *E. faecalis* VE14089	This study
VE18590	*pp*$^-$	pp1, pp3, pp4, pp5, pp6 and pp7 deletion in *E. faecalis* VE14089	This study
VE18559	*pp1*$^-$*pp3*$^-$*pp5*$^-$	pp1, pp3 and pp5 deletion in *E. faecalis* VE14089	This study
VE18562	*pp1*$^+$	pp3, pp4, pp5, pp6 and pp7 deletion in *E. faecalis* VE14089	This study
VE18581	*pp6*$^+$	pp 1, pp3, pp4, pp5 and pp7 deletion in *E. faecalis* VE14089	This study
VE18582	*pp4*$^+$	pp1, pp3, pp5, pp6 and pp7 deletion in *E. faecalis* VE14089	This study
VE18583	*pp3*$^+$*pp5*$^+$	pp1, pp4, pp6 and pp7 deletion in *E. faecalis* VE14089	This study
Plasmids			
pGEM-T		Amp^r, linearized with 3' T overhangs, ori ColE1	Promega
pG+host9		Erm^r, repATs	[76]
pVE14354		Amp^r, pGEM-T with *ef1417-ef1489* deletion	This study
pVE14357		Amp^r, pGEM-T with *ef2085-ef2145* deletion	This study
pVE14362		Ery^r, pG+host with *ef1417-ef1489* deletion	This study
pVE14364		Ery^r, pG+host with *ef2085-ef2145* deletion	This study
pVE14367		Amp^r, pGEM-T with *ef2798-ef2855* deletion	This study
pVE14368		Ery^r, pG+host with *ef2798-ef2855* deletion	This study
pVE14369		Amp^r, pGEM-T with *ef1988-ef2043* deletion	This study
pVE14370		Ery^r, pG+host with *ef1988-ef2043* deletion	This study
pVE14371		Amp^r, pGEM-T with *ef0303-ef0355* deletion	This study
pVE14372		Ery^r, pG+host with *ef0303-ef0355* deletion	This study
pVE13473		Amp^r, pGEM-T with *ef2936-ef2955* deletion	This study
pVE13474		Ery^r, pG+host with *ef2936-ef2955* deletion	This study

Table S2. Primers used in this study.

Primer name	Sequence (5'→3')*	Position of the primer 5' end†	Reference or source
ef0302f	CGTGGATGGACGAATACAC	289 146	This study
ef0303f	GCAGTACAGATTATAAAA	289 672	This study
ef0355f	GATCGGCAACAAGTAATGTC	326 034	This study
ef0357f	AGAACATCAGTACATTTTACC	326 635	This study
ef3155f	ACAGCACCAGACCCGACAG	3 026 754	This study
ef3155r	ACGACGAGGTTCCATGTGATG	3 026 256	This study
OEF470	AGACTAGAGTTACTACCCC	2 003 732	This study
OEF471	AGCCACCTGCCGGGCTTG	2 004 735	This study
OEF472	CAAGCCCGGCAGGTGGCTTTTTGGTAAAATCCCAACTC	2 048 272	This study
OEF473	GCATCACCTGCGATATTTC	2 049 399	This study
OEF474	TTGTGTTTAACACTCGGAGC	2 003 577	This study
OEF475	AATTGGGTAGTTTCGACTTG	2 049 708	This study
OEF476	GAAAGCGCCACAAATCTTC	1 396 960	This study
OEF477	CTATAGGCGTGCATTTAAATC	1 397 966	This study
OEF478	GATTTAAATGCACGCCTATAGTTTGTAAATAACAGAAGAAAA	1 446 359	This study
OEF479	AATAGAGATACGCTCAGCC	1 447 403	This study
OEF480	CTGGCTGGATTGATTTACC	1 396 770	This study
OEF481	CAACAGCTTCTTCACTCTC	1 447 606	This study
OEF483	CATATTTTCACCTCCTATCC	1 402 370	This study
OEF485	TCTCTAGGATGTTGTTGAGG	1 401 513	This study
OEF488	GCCGCACATATAGATGATG	2 045 368	This study
OEF489	CTAATTTCTTTGGTTCTGCC	2 047 006	This study
OEF490	CAGAGTTCCTAGGAGTAAC	2 045 792	This study
OEF491	GTTGTTCTAAGGTTGGCTC	2 046 561	This study
OEF531	GACGTAGCAATGGTACTGG	1 399 238	This study
OEF532	GATCGTTCAGGTAACATTAG	1 445 898	This study
OEF533	ATCCGAAGGAACATTGCTAG	2 005 292	This study
OEF534	CAATGGATTAACTGGCTTGC	2 047 356	This study
OEF546	CAGTTCGAGTCCTGTATGG	1 923 230	This study
OEF547	AGAACGGCTTTTCAGAGAAG	1 962 161	This study
OEF548	ACCTAGCATTCGTAGAACC	2 701 530	This study
OEF549	TACTATGCCGACTTAGACTG	2 736 242	This study
OEF551	AGGCTACATTACTAAAGAGC	1 962 985	This study
OEF552	TCCGTATTGATAAGCAATGC	1 926 694	This study
OEF553	GTTCAATCGACGGATGTAA	1 927 314	This study
OEF554	TAGCCTGCATTTGCTGAAG	291 436	This study
OEF555	CTTTTGCGGTACCATTAACG	291 919	This study
OEF557	GCAACAGATGCTAATGGAG	2 737 353	This study
OEF558	GCCAATCCATTTAACGAAGC	2 703 739	This study
OEF559	GTATCCTATCGATGGTGTG	2 704 251	This study
OEF560	TCGAAGGTTCCTGATGAAC	2 817 577	This study
OEF561	ACTGCTCTCACCAAATGTAG	2829302	This study
OEF564	AGCCAATTGGTAACGTCCAC	2 817 997	This study

Primer name	Sequence (5'→3')*	Position of the primer 5' end†	Reference or source
OEF565	TTCAGCTCCTAATCTAGTAG	2 818 606	This study
OEF573	ATGCAGACTACCAAGTCATG	323 573	This study
OEF574	ATCATGTGCATAGCCAAAGC	325 002	This study
OEF575	AGATTGTTGTGAAGCGAACG	1 443 399	This study
OEF576	TGAGCAACTTAAAGGAGGTG	1 441 968	This study
OEF577	TCAGCACGTTCATTTAATCC	1 958 890	This study
OEF578	ATTCTTCGCAAATTTGACGG	1 960 329	This study
OEF579	GATCAGAATGGGTAGCTAAG	2 732 677	This study
OEF580	ATCGTCAGATGGTTTAGCAC	2 734 199	This study
OEF581	ACATCCGTCATTGACTTACG	2 825 960	This study
OEF582	CTATTAAGAGTGGAACGTGG	2 827 442	This study
OEF585	GTACGGTTGGATTAACGAAC	2 816 552	This study
OEF591	CGGAAGCAAGAGTTGAAAGC	1 255 569	This study
OEF592	TTGCCAATCGGACCAAACG	1 259 906	This study
OEF618	TAGCCATATGAGACGAAACG	2 699 418	This study
OEF619	GTTAGATAGAGCCTAGAATC	2 700 416	This study
OEF620	GATTCTAGGCTCTATCTAACTAAATTATTTAGTTTCACGGTG	2 736 907	This study
OEF621	AACCATGCAATTAACTGCG	2 737 969	This study
OEF622	AAACGATTGATAGTGAACCG	2 699 195	This study
OEF623	TGGAGAAGTCACACCTAATC	2 738 142	This study
OEF624	ACGATGTTACTCGCCTAAC	2 700 282	This study
OEF626	AATAACGTACCCGTCTTTTC	288 080	This study
OEF627	TGCCAAAACAGTTGGCGC	289 226	This study
OEF628	GCGCCAACTGTTTTGGCACCTTGGGATCCAATGGGCGC	326 495	This study
OEF629	TTGATTGATGCTGAAGGTAG	327 598	This study
OEF630	CGTAAAATGAAAGGACGATG	287 920	This study
OEF631	TGACAATCAACGTTACCAAC	327 779	This study
OEF634	TATGTATAATCGAGGGTCAC	1 921 627	This study
OEF635	CAAATATACGAAGAAAATTAAC	1 922 596	This study
OEF636	GTTAATTTTCTTCGTATATTTGCCACTCCCCATCTGAAATTG	1 962 541	This study
OEF637	ATTTGATGCGCCATACAACC	1 963 549	This study
OEF638	TGGGAACAAATTAGCACCTC	1 921 400	This study
OEF639	GTCCATACATTCTGGTTACC	1 963 762	This study
OEF640	GAATATCCCTGCTATCACAC	1 922 523	This study
OEF641	GGTTGTAATAGCTGTGATTCC	2 815 695	This study
OEF642	CACGTTGTTTCATTAATAAAT	2 816 749	This study
OEF643	ATTTATTAATGAAACAACGTGTTTAATCATATAATAAACCAA	2 829 750	This study
OEF644	AGTGTCAATCATCCGGAACTG	2 830 756	This study
OEF645	GAAATGCTGTATGTCAATGGC	2 815 501	This study
OEF646	ATCTTCTTGCCACGATTATCC	2 830 927	This study
OEF653	AAGTGCCAACAATGGATGC	1 397 790	This study
OEF656	TCATCATTGTACTCCACTCC	2 048 416	This study
OEF657	ATCAGTGAAATGGTTGTTCG	2 829 802	This study

* Sequences added for fusion PCR are underlined.
† Position of the primer 5' end in the V583 genome.

Table S3 V583 phages infection and immunity.

Indicator strain	Lysate								
	$WT_{37°C}$	$WT_{42°C}$	*pp3⁻pp5⁻*	*pp1⁺*	*pp3⁺pp5⁺*	*pp4⁺*	*pp6⁺*	*pp7⁺*	*pp1⁺pp7⁺*
pp⁻	+	+	+	+	+	-	-	-	+
pp1⁻	-	-	-	-	-	-	-	-	-
pp3⁻	+	+	-	-	+	-	-	-	-
pp4⁻	-	-	-	-	-	-	-	-	-
pp5⁻	+	+	-	-	+	-	-	-	-
pp6⁻	-	-	-	-	-	-	-	-	-
pp7⁻	+	+	+	-	-	-	-	-	+
pp1⁻pp7⁻	+	+	+	+	-	-	-	-	+
pp3⁻pp5⁻	+	+	-	-	+	-	-	-	-
pp1⁺	+	+	+	-	+	-	-	-	+
pp3⁺pp5⁺	+	+	+	+	-	-	-	-	+
pp4⁺	+	+	+	+	+	-	-	-	+
pp6⁺	+	+	+	+	+	-	-	-	+
pp7⁺	+	+	+	-	+	-	-	-	-

+ Plaque formation detection

- No plaque formation detection

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Chapter 3

Phage contribution to *Enterococcus faecalis* biological traits

This chapter contains data of the manuscripts in preparation:
Matos, RC, Rigottier-Gois, L, Aleksandrzak-Piekarczyk, T, Kennedy, SP, Deschamps, J, Briandet, R, Lopes, MF, Serror, P. Phage contributions to *Enterococcus faecalis* V583 biological traits. *In preparation*.

Rigottier-Gois, L, Madec, C, Navickas, A, Matos, RC, Akary-Lepage, E, Kennedy, SP, Mistou, MY and Serror, P. Three *Enterococcus faecalis* key genes in the adaptation to the mammalian gut. *In preparation*.

Platelet-binding data was published in:
Matos, RC, Lapaque, N, Rigottier-Gois, L, Debarbieux, L, Meylheuc, T, Gonzalez-Zorn, B, Repoila, F, Lopes, MF, Serror, P. *Enterococcus faecalis* prophage dynamics and contributions to pathogenic traits. PLOS Genetics *in press*. 10.1371/journal.pgen.1003539.

Author contributions:
The author of this thesis was involved in the majority of the experiments. Experimental design, data analysis and manuscript preparation were done by the author of this thesis and the supervisors Pascale Serror and Maria de Fatima Lopes. Nicolas Lapaque and Lionel Rigottier-Gois supervised the platelet binding assays and the mice experiments, respectively. Sean Kennedy performed the genome sequencing and Tamara Aleksandrzak-Piekarczyk performed the phenotypic microarray tests.

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SUMMARY

Temperate bacteriophages impact on the fitness of their bacterial host in a number of different ways that depend either on cell lysis-mediated competitiveness, gene disruption or the acquisition of fitness factors that are able to convert their bacterial host, in a process known as lysogenic conversion, from a non-pathogenic strain into a strain with increased virulence. *Enterococcus faecalis* has a dual nature: commensal of the gastro-intestinal tract of humans and opportunistic pathogen in fragilized or immunocompromised patients. *E. faecalis* pathogenicity remains poorly understood, but it is multifactorial, involving both bacterial and host contributions. Studies on enterococcal temperate phages are scarce and their role on strain fitness and pathogenicity has yet to be addressed. Enrichment of prophage genes in *E. faecalis* isolates of the high-risk enterococcal clonal complex 2 (HiRECC-2), that gathers hospital-adapted strains, supports that *E. faecalis* prophages may be important for bacterial adaptation to hospital setting. In this work, we addressed the impact of enterococcal temperate bacteriophages in the lifestyle of V583, a polylysogenic CC2 isolate. For this purpose, we compared the behaviour of the WT strain and its phage-deleted derivatives on sensitivity to chemical compounds, intestinal colonization, adhesion to human platelets, mice infectivity and biofilm formation. While prophages seem to have no major impact on sensitivity to chemical compounds, intestinal colonization, mice infectivity and biofilm formation in the tested conditions, prophages encoding platelet-like binding proteins promote adhesion to human platelets. Adhesion to human platelets is a key step towards the development of infective endocarditis, thus involving prophages in *E. faecalis* V583 pathogenicity.

INTRODUCTION

Bacteriophages are the most abundant entities on earth with an estimation of population size of more than 10^{30} viral particles [1]. They have a major impact on the ecological balance and dynamics of microbial life, and are also recognized as key players in the evolution of bacteria by shaping their genomes through horizontal gene transfer [2]. Lysogeny, which involves the integration of a temperate phage into the bacterial chromosome is more frequent than initially thought, and affects bacteria regardless of their lifestyles [3]. Thus, increasing evidence indicates that prophages can possibly confer advantages to their hosts whether they are pathogens, commensals or free-living organisms [4-6].

Temperate phages contribute to bacterial fitness or virulence in several ways: introduction of fitness factors, gene disruption, protection from lytic infection and lysis of competing strains [3]. Import of fitness factors is also referred to as lysogenic conversion, which confers new traits to the host bacterium by providing genes that are not essential for the phage life cycle. Over the last few years, a plethora of prophage-associated genes that contribute to various aspects of bacterial pathogenesis has been identified [7,8]. They encode functions such as ADP-ribosyltransferase toxins in *Pseudomonas aeruginosa* and *Vibrio cholerae* [9,10], detoxifying enzymes, e.g. *sod*C in *Escherichia coli* O157 [11], type III effector proteins such as *sop*E, *sse*I, *ssp*H1 in *Salmonella enterica* [12-14], among many others (for review see [3]). Integration of a prophage may modify bacterial virulence or adaptability by disrupting bacterial genes or operons such as the *Staphylococcus aureus* β-toxin-encoding gene inactivated upon integration of bacteriophage φ13 [15]. Prophage excision in a fraction of the bacterial population can also impact bacterial fitness. This excision, followed in most cases by induction of the phage lytic cycle can be beneficial for the surviving population. For

example, excision of a *Listeria monocytogenes* prophage is sufficient to restore a functional transcriptional regulator promoting escape from the phagosome and thus intracellular growth [16]. Likewise, excision of the prophage-like element $Skin^{Cd}$ restores *sigK* gene in *Clostridium difficile* that is an essential step for proper activation of σ^k, required for efficient sporulation [17]. In other cases, a complete lytic cycle is required for the expression and release of the Shiga toxins in *E. coli* [18] or for promoting adhesion to human platelets via PblA and PblB, two platelet-binding proteins that are integral part of the phage SM1 tail from *Streptococcus mitis* [19]. Indeed, PblA and PblB code the tail tape measure protein and the tail fiber protein, respectively [20]. These proteins are released in the medium upon phage-induced bacterial lysis. Then, they interact with choline residues on the surface of *Streptococcus mitis* intact cells, and promote their adhesion to α2-8-linked sialic residues on platelet membrane gangliosides [20,21]. Adhesion to platelets is considered as a key step towards the development of infective endocarditis generated by *S. mitis* [20]. Phage-mediated lysis is also important for biofilm formation and differentiation since it facilitates dispersion of the surviving cells in order to form a new biofilm, as seen for *Pseudomonas aeruginosa* [22] or due to the release of extracellular DNA (eDNA) which enhances biofilm development in *Streptococcus pneumoniae* [23].

Enterococci are commensal bacteria of the gastrointestinal tract (GIT) of humans and other animals as well as insects. They also colonize the genito-urinary tract and the oral cavity, and are detected in several environmental niches such as soil, sand, water, food products and plants, [24-27]. Given their intrinsic robustness, capacity to withstand environmental stresses, including high concentrations of antibiotics and biocides, and propensity to exchange genetic material, they have emerged as a major cause of nosocomial infections worldwide [28-31]. *Enterococcus faecalis* ability to cause infection and persistence in the

hospital settings has been associated with strain ability to form biofilms known to promote bacterial survival in harsh conditions such as desiccation and antibiotic-rich environments [32-36]. *E. faecalis* V583 autolysis and subsequent release of eDNA is an important factor for biofilm development [37,38]. While autolysins have been implicated in *E. faecalis* V583 ability to form biofilms due to bacterial lysis the impact of phage-induced lysis has not yet been addressed.

The *E. faecalis* V583 was isolated in the beginning of the 80s and belongs to the HiRECC-2, that gathers strains adapted to hospital setting harboring several antibiotic resistances and virulence factors [39]. The genome of this strain contains 6 prophage-like elements (V583-pp1 to V583-pp6, named hereafter pp1 to pp6) and a phage-related chromosomal island (EfCIV583) that we have characterized regarding induction and activity [40]. Interestingly, CC2-isolates are enriched in prophage-genes, supporting that these mobile genetic elements may have a role in increased survival of CC2 isolates in the host and hospital environment [41]. Even though several phage-encoded potential fitness factors have been predicted: platelet binding-like proteins (in pp1, pp4 and pp6), a ferrochelatase (in pp4) and more recently, a toxin ADP-ribosyltransferase (in pp1) [39,42,43], their contribution to *E. faecalis* lifestyle remains to be explored.

In this study we evaluated the importance of 6 non-ubiquitous prophages for *E. faecalis* lifestyle by mimicking conditions that may be faced by these bacteria: chemical stress, intestinal colonization in presence of a competitive microbiota, adhesion to human platelets, mice infectivity and biofilm formation. For this purpose, we used a polylysogenic WT and a set of isogenic phage-deleted strains. Capacity to withstand chemical stress, to colonize the intestine and infect the organs of mice, and to form biofilms was similar between the strains. However, several phage-deleted strains bound differentially to human platelets. Adhesion to

platelets correlates with prophages that encode platelet binding-like proteins and with their ability to perform lytic cycle. With this work we demonstrate the contribution of pp1, pp4 and pp6 to human platelet adhesion, suggesting a role of *E. faecalis* prophages in the development of nosocomial infective endocarditis.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Strains used in this study are listed in Table 1. *E. faecalis* was grown in static conditions in appropriate media, either M17 supplemented with 0.5% glucose (M17G), chemically defined medium (CDM) [44] or bile-esculin agar (BEA) at 37ºC. Growth was monitored by measuring optical density at 600 nm (OD_{600}). When needed, strains were transformed with plasmid pMV158-gfp [45], as described previously [46]. Tetracycline was used at 4 µg/ml for *E. faecalis* when required.

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmid	Short name	Relevant characteristics	Reference
Strains			
VE14002	V583	V583 vancomycin resistant clinical isolate	[47]
VE14089	WT	V583 vancomycin resistant clinical isolate cured of its plasmids	[46]
VE18239	WT-GFP$^+$	V583 vancomycin resistant clinical isolate cured of its plasmids transformed with pMV158-gfp	This study
VE18590	*pp*$^-$	VE14089 deleted for pp1, pp3, pp4, pp5, pp6 and EfCIV583	[40]
VE18595	*pp*$^-$-GFP$^+$	VE18590 transformed with pMV158-gfp	This study
VE18562	*pp1*$^+$	VE14089 deleted for pp3, pp4, pp5, pp6 and EfCIV583	[40]
VE18583	*pp3*$^+$ *pp5*$^+$	VE14089 deleted for pp1, pp4, pp6 and EfCIV583	[40]
VE18582	*pp4*$^+$	VE14089 deleted for pp1, pp3, pp5, pp6 and EfCIV583	[40]
VE18581	*pp6*$^+$	VE14089 deleted for pp1, pp3, pp4, pp5 and EfCIV583	[40]
VE18589	*pp7*$^+$	VE14089 deleted for pp1, pp3, pp4, pp5 and pp6	[40]
Plasmids			
pMV158-gfp		Tet^R, GFP	[45]

Genome re-sequencing and identification of polymorphisms

VE14089 (WT) and VE18590 (*pp*$^-$) (Table 1) genomes were sequenced on a Life Technologies 5500XL NGS system at the MetaQuant platform, INRA Jouy-en-Josas (www.mgps.eu). Mapping of the sequencing reads to the *E. faecalis* VE14089 reference sequence along with SNP analysis was performed using the Lifescope v2.0 software (Life Technologies). To identify any insertion, deletions, or duplication, mapping was compared to the reference V583 genome, by analysis of BAM alignment files in the Integrated Genomic Viewer (IGV, Broad Institute). Polymorphisms between V583 reference sequence, deposited in NCBI under the accession number NC_004668 [39], VE14089 and VE18590 genomes were confirmed by PCR with primers listed on Table 2 followed by sequencing at GATC Biotech, France.

Phenotype microarrays for microbial cells

To access phenotypic differences between the polylysogenic strain (WT) and the phage deleted strain (*pp*$^-$), pre-configured phenotypic sensitivity plates from BIOLOG were used: PM9, 10, 11C, 12B, 13B, 14A, 15B, 16A, 17A, 18C, 19 and 20B. Detailed plaque configurations are available at the provider website: http://www.biolog.com. Briefly, PM9 contains test for osmotic and ion effects, PM10 tests for pH effect and PM11C-20B contain chemicals that target stress pathways in the cell. Incubation and monitoring of optical density were performed using the OmniLog-PM system. Data were analyzed using the Omnilog-PM software. These experiments were performed at the Institute of Biochemistry and Biophysics (Warsaw, Poland), in a laboratory equipped with BIOLOG technology and according to the manufacturer's instructions, which includes undisclosed growth media. To study differences of growth obtained with BIOLOG test, the growth of the two strains was tested in CDM in presence of copper chloride (9, 18, 36 µg/ml), carbenicillin (2, 4, 8

µg/ml), trimethoprim (0.05, 0.1 and 0.2 µg/ml) and potassium tellurite (0.07, 0.15, 0.3 µg/ml). Growth was followed by monitoring optical density at 600 nm at 30 minutes intervals for 15 hours in a TECAN microplate reader.

Table 2. Primers used in this study.

Primer name	Target ORF	Sequence (5'→3')	Reference or source
OEF723	EF0151	AGGTACAGAATTGACGAAAG	This study
OEF724	EF0151	CCATTTTTACGATACCGACG	This study
OEF701	EF0172	AATGCAAAAGGAGACGTTGG	This study
OEF702	EF0172	AATCGTCCACTATCTCGTG	This study
OEF703	EF0295	ACAAAGAAATGGTAGAGGAG	This study
OEF704	EF0295	CAAATCAAAGCCAGCATTAC	This study
OEF707	EF0573	CCACAAATTACTAGTGCAG	This study
OEF708	EF0573	TCTCCTTTAGCATCTACTTG	This study
OEF709	EF1007	AGTCTGTGTATGAAGTACCC	This study
OEF710	EF1007	TAAAGCGCTAGGAGTTCTTC	This study
OEF711	EF1021	TTTTAATCAGGAACCCACGG	This study
OEF712	EF1021	TTCTGCTTTCCCTTCTGACG	This study
OEF727	EF1725	CTTCTGTTACGACATAATCC	This study
OEF728	EF1725	TCGATTAATCCAACACCAGC	This study
OEF721	EF1976	TAGAACTGTTTCGCCACCAC	This study
OEF722	EF1976	AGAGGTGAGAACATGAAGTG	This study
OEF713	EF2060	TCTGCATAATTTCGAGCACG	This study
OEF714	EF2060	TTGGCGAATGAGTACACTAC	This study
OEF729	EF2461	TCATAGGTTTCAGGAGTTGC	This study
OEF730	EF2461	CCGATGTGATTCGAATGAAC	This study
OEF717	EF2678	CCTGCCATTAATTGTGCTTG	This study
OEF718	EF2678	TTCGCGAAAAGGAGTGATGC	This study
OEF731	EF2688	CCTAATAATTGGCTGGTATG	This study
OEF732	EF2688	GTGAGTCGATGAAATGGAG	This study
OEF719	EF2914	GTCATAGATTGCGAAAGGAC	This study
OEF720	EF2914	GAACTACGTATCGTTAAGCG	This study
OEF733	EF2933	CCTGTCAATTCTAATACGTG	This study
OEF734	EF2933	AACGCATTAAATCACGAGAG	This study

Mouse intestinal colonization model

Six- to 8-weeks old male CF-1 mice (Harlan, USA) were used for intestinal colonization experiments as described previously [48]. Briefly, mice received a daily dose of 1.4 mg of subcutaneous clindamycin for three days. On the fourth day, mice were administered with 10^{10} CFU of *E. faecalis* strains prepared as dried frozen pellets, as described previously [46], by orogastric inoculation using a feeding tube (Ecimed) (Rigottier-Gois et al., in preparation). Fecal samples were collected at baseline and at 1 and 4 days after orogastric inoculation of the strains. Endogenous enterococci were evaluated by plating serial dilutions of fecal samples from control mice on BEA. Inoculated *E. faecalis* strains were monitored by plating on BEA supplemented with vancomycin at 6 µg/. All animals were handled in strict accordance with good animal practice as defined by the local animal welfare bodies (Unit IERP, INRA Jouy-en-Josas, France) and all animal work was carried out under the authority of license issued by the Direction des Service Vétérinaires (accreditation number A78-187 to LR-G).

Platelet binding assay

The ability of *E. faecalis* to bind to human platelets was assessed as previously described [49]. Briefly, platelet-enriched cells were obtained from human blood by adding Lymphoprep (Axis-shield, Norway) followed by centrifugation at 800 g for 25 minutes, at room temperature. After formation of the different phases, the second cloudy phase from the top, containing the platelets, was carefully removed, resuspended in PBS and centrifuged at 300 g for 10 minutes, at room-temperature. Supernatant rich in platelets was kept and washed five times in PBS at 500 g for 10 minutes, at room temperature as described by [50]. After, platelets were fixed in paraformaldehyde 3.2% and immobilized on poly-L-lysine for 1h at 37°C. Unbounded platelets were washed with PBS prior to saturation with

a 1% casein solution for 1h at 37°C. After removal of the saturating solution, platelets were incubated for 1h at 37°C with the indicated bacteria at a MOI of 1. After six washes, the bound bacteria were counted by plating serial dilutions on M17G. Binding was expressed as a percentage of the inoculum followed by normalization to wild-type strain adhesion. Platelet binding assays were performed three times in triplicate using platelets prepared from buffy coats of three, healthy and anonymous volunteers obtained through the Etablissement Français du Sang (EFS, Ile de France, Le Chesnay, France). As required for blood donation, written informed consents were obtained by EFS from all donors.

Distribution analysis of pp1, pp4 and pp6 genes in *E. faecalis* draft genomes

Nucleotide sequences of pp1, pp4 and pp6 annotated genes on *E. faecalis* V583 genome were aligned against the publicly available *E. faecalis* draft genomes (http://www.ncbi.nlm.nih.gov) using BLASTN, and their presence or absence was predicted as described by Solheim et al., 2011 [41]. Briefly, predictions were made based on a score calculated as the number of identical nucleotides divided by the query gene length. Genes with a score higher than 0.75 were considered similar. Strains positive for pp1, pp4 or pp6 genes are listed in Table S2 along with their geographical origin, year of isolation, source, sequence type (ST) and presence of CRISPR1 and CRISPR3 *cas* genes. STs of strains TX4244, ATCC29212, TX1467 and ERV were deduced *in silico* accordingly with the MLST scheme developed by Ruiz-Garbajosa et al., 2006 [51]. Sequences for the seven housekeeping genes *ghd* (ef1004), *gyd* (ef1964), *pstS* (ef1705), *gki* (ef2788), *aroE* (ef1561), *xpt* (ef2365) and *ygiL* (ef1364) were recovered from NCBI web site and STs determined using the *E. faecalis* MLST database (http://efaecalis.mlst.net). Presence of *cas*

genes from CRISPR1 and CRISPR3 [52,53] was also predicted by BLAST search.

Mouse infectivity models

Groups of 10 six-week-old male C57BL6 mice were infected intraperitoneally with a sub-lethal dose of $1x10^8$ CFU of *E. faecalis* strains prepared as dried frozen pellets [46]. Mice were sacrificed 12 hours post-infection, and organs (hearts, kidneys, livers and spleens) were aseptically removed, weighed, homogenized, and serially diluted in saline solution for colony counts on BEA medium supplemented with vancomycin at 6 µg/ml. *E. faecalis* burden were expressed as CFU/g of organ. Statistical analyses were performed using GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA). Bacterial counts were compared using the unpaired *t* test, and P values of < 0.05 were considered statistically significant.

In the neutropenia model, six-week-old male C57BL6 mice were immunocompromised by intravenous administration of vinblastine (Velbe®) at 5 mg/kg. Seventy-two hours later, mice were injected intraperitoneally with a sub-lethal dose of $1x10^8$ CFU of *E. faecalis* strains. Mice were sacrificed 24 hours post-infection, and organs were aseptically removed and treated as above.

Biofilm formation assays

Biofilm formation assays and data analysis were performed accordingly to Bridier et al. [54]. Briefly, 250 µl of green fluorescent protein (GFP) labeled strains cultured overnight in M17G in presence of tetracycline were diluted to approximately 10^7 CFU/ml, and seeded in wells of a polystyrene 96-well microtiter plate (Greiner Bio-one, France). After 1 h adhesion at 37°C, wells were rinsed with 150 mM NaCl in order to eliminate non-adherent bacteria. Wells were refilled with 250 µl M17G and microplate was incubated for 24 and 48 hours at 37°C. At each time

point, wells were rinsed with 150 mM NaCl and refilled with M17G. The microtiter plate was mounted on the motorized stage of a Leica SP2 AOBS confocal laser-scanning microscope (LEICA Microsystems, France) at the MIMA2 microscopy platform (http://voxel.jouy.inra.fr/mima2). Emitted fluorescence was recorded within the range 500–600 nm in order to visualize GFP fluorescence. Three stacks of horizontal plane images (512Å~512 pixels corresponding to 119 Å~119 µm) with a z-step of 1 µm were acquired for each biofilm at different areas in the well. Two independent experiments were performed in triplicate for each strain. Three-dimensional projection of biofilm structure was reconstructed using the Easy 3D function of the IMARIS 7.0 software (Bitplane, Switzerland). Quantitative structural parameters of the biofilms were calculated using PHLIP [55].

RESULTS

pp$^-$ genome sequence analysis

The *E. faecalis* strain VE14089, referred as WT in this study, is a plasmid-cured derivative of V583 (VE14002) obtained after chemical treatments (Figure 1A) [46]. To study, the potential role of the six non-ubiquitous prophages or phage-related elements pp1, pp3, pp4, pp5, pp6 and EfCIV583 in *E. faecalis* lifestyle, we used a set of isogenic strains deleted for individual or combinations of phage-elements in strain VE14089 [40]. Strain VE18590 (*pp*$^-$*)* is deleted for the six non-ubiquitous prophages of V583, representing a 215 kb loss (~ 6.6 % in chromosome size) compared with VE14089. It was obtained by sequential deletion of pp3, pp5, pp4, pp6, EfCIV583 and pp1 (Figure 1B). In preliminary experiments, we compared the growth of strain *pp*$^-$ with the wild-type parental strain in M17G and CDM, at 37°C (Figure 1C). No major change in the growth on rich and minimal media was observed, supporting the accessory nature of these elements and suggesting that no major underlying sequence changes resulted from the serial genetic recombination events. Previously, we excluded major chromosomal rearrangements in *pp*$^-$ strain, due to the sequential deletion events, by pulse-field gel electrophoresis [40]. As a prerequisite to study prophage impact on bacterial phenotypes, we sequenced and compared the whole genome of both *pp*$^-$ and WT strains, which were also compared with the V583 reference genome sequence [39].

Along with the expected prophage deletions, comparison of the whole genome sequence of WT and VE18590 (*pp*$^-$) uncovered only 6 single nucleotide polymorphisms (SNPs), which we confirmed after amplification and re-sequencing using WT and *pp*$^-$ as templates. The SNPs are located within six open reading frames (ORFs) not localized in the close vicinity of the deletion region (Table 3).

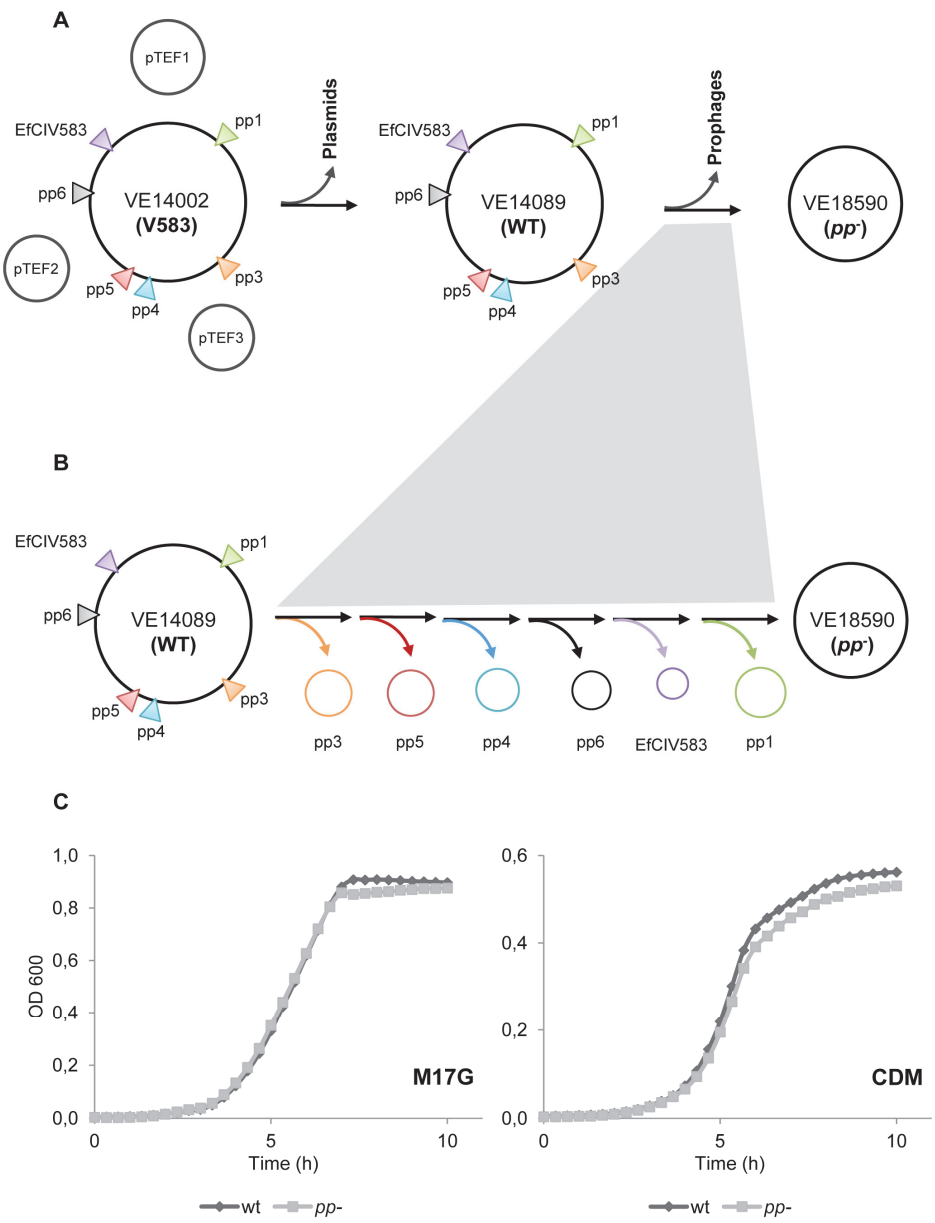

Figure 1. V583 derivatives used in the genome sequencing project. (A) VE14089 was obtained from VE14002 (V583) by plasmid curing (pTEF1, pTEF2 and pTEF3) through chemical treatments [46]. VE18590 was obtained from the plasmid-cured V583 by sequential deletion of pp3, pp5, pp4, pp6, EfCIV583 and pp1 through double crossing-over. (B) Prophage sequential deletions. (C) WT and *pp*$^{-}$ growth curves at 37°C in M17G and CDM.

Table 3. SNPs in VE18590 (*pp*$^-$) compared to VE14089 (WT).

Reference position[a]	Variation type	Variation	Annotation	Predicted function	Amino acid change	Side chain polarity and charge change	SNAP prediction[b]
151768	SNP	C → G	EF0151	Hypothetical protein	T98R	Polar → Basic polar Neutral → Positive	Non-neutral
1590374	SNP	C → T	EF1725 (*fhs*)	Formate-tetrahydrofolate ligase	A166T	Nonpolar → Polar	Neutral
2213130	SNP	C → A	EF2461	Inositol monophosphatase	G200V	NA	Neutral
2634343	SNP	C → T	EF2688	Snf2 family protein	E120K	Acidic polar → Basic polar Negative → Positive	Neutral
2555129	SNP	G → A	EF2882	Malonyl CoA-acyl carrier protein	Silent	NA	NA
2607835	SNP	C → A	EF2933	Redox sensing transcriptional repressor Rex	A171S	Nonpolar → polar	Neutral

[a]Position in *E. faecalis* V583 reference sequence *pp*$^-$; NA: not applicable.
[b]http://www.rostlab.org/services/snap/submit. Neutral: no effect on the protein function; Non-neutral: effect on the protein function.

One gene has a silent mutation, and the others have missense mutations resulting in an amino acid substitution in the encoded protein, however prediction of the functional consequences of SNPs remains challenging [56]. Of the predicted functions, EF1725 a formate-tetrahydrofolate ligase and EF2461 an inositol monophosphatase exert two important metabolic activities, suggesting that the substitution has no major impact on strain growth. EF2933 is annotated as oxidative stress response regulator of the Rex family, however recent experimental evidences indicate that contribution of EF2933 to redox homeostasis is minor [57].

Strain WT is a derivative of V583. In addition to the 20.5 kb insertion from *efa0063* to *efa0006* of pTEF1 plasmid between genes *ef3209* and *ef3210* [46], a total of 43 differences, including deletions, insertions and SNPs, were detected between WT and V583 genome sequences [39]. From these, 34 differences have been recently reported by Palmer et al., [58], leading us to consider them as real differences, which may be due to sequencing errors and/or reflect the evolution of independent isolates of strain V583 (Table S1). The other 9 differences were confirmed after PCR amplification and sequencing of the corresponding regions using WT and VE14002, our V583 isolate as templates (Table 4). They are all located within ORFs. One deletion and 2 missense SNPs are in both VE14002 and WT strains, being specific of our laboratory strains (Table 4). Remarkably, the deletion in *ef1007* generates a predicted protein of 243 residues annotated as regulator of sugar fermentation. Noticeably, this protein is predicted in all available *E. faecalis* genomes, but V583, strongly indicating an assembly error in the reference genome. The remaining six SNPs were specific to VE14089 and are probably due to the chemical agents used for plasmid curing (Table 4). The nonsense SNP generates a premature stop codon in *ef0295* predicted to encode subunit J of a V-type ATP synthase acting as a primary ion pump transporting Na^+ or K^+ ions.

Table 4. SNPs in VE14089 compared to reference sequence V583 [39].

Reference position[a]	Variation type	Variation	Annotation	Predicted function	Amino acid change	Side chain polarity and charge change	SNAP prediction[b]
Differences on VE14002 and WT							
966197	Deletion	CATGT → -	EF1007	Sugar fermentation stimulation protein	Frameshift	NA	NA
979343	SNP	G → A	EF1021	N-acetyltransferase	V156I	NA	Neutral
1914901	SNP	G → A	EF1976 (*prmA*)	Ribosomal protein L11 methyltransferase	I74F	NA	Neutral
Differences specific of VE14089							
169833	SNP	C → A	EF0172	Sugar-binding transcriptional regulator, LacI family	T34N	NA	Non-neutral
280276	SNP	C → T	EF0295	V-type ATPase, subunit J	Q45Stop (make protein of 44 aa)	NA	NA
536621	SNP	C → A	EF0573	Hypothetical protein	Silent	NA	NA
1982291	SNP	G → A	EF2060 (*cydB*)	Cytochrome d ubiquinol oxidase, subunit II	Silent	NA	NA
2589844	SNP	T → A	EF2678	Regulatory protein SpxA	N31I	Polar → nonpolar	Non-neutral
2792148	SNP	C → T	EF2914 (*greA*)	Transcription elongation factor	G12E	Nonpolar → Acidic polar Neutral → Negative	Non-neutral

[a]Position in *E. faecalis* V583 reference sequence in NCBI. NA: not applicable

[b]http://www.rostlab.org/services/snap/submit. Neutral: no effect on the protein function; Non-neutral: effect on the protein function.

Such mutation is likely to impair ion transport and affect osmotic adjustment impacting the overall fitness of VE14089 compared with VE14002 [46]. Moreover, the three other substitutions predicted as non-neutral are in annotated transcription factors. The substitution in the DNA-binding domain of the predicted sugar-binding transcriptional regulator EF0172 may affect regulatory efficiency of its unknown targets. The *E. faecalis* SpxA regulator EF2678 is a global transcriptional regulator, which modulates RNA polymerase specificity in response to oxidative stress in aerobic condition. It promotes colonization of the peritoneum and dissemination in the blood in mice [59]. The transcription elongation factor GreA acts on the fidelity and processivity of RNA polymerase [60]. However, the substitution effect on these two regulators is difficult to predict.

Overall, the plasmid-cured VE14089 strain harbors 1 truncated gene (*ef0295*) and 5 missense mutated ORFs relative to our V583 strain. Six additional mutations (5 missense and 1 silent) were accumulated in VE14089-derivative strain *pp*$^-$ during sequential prophage deletions.

WT and *pp*$^-$ growth in presence of chemical compounds

We previously showed that VE14089 prophages are induced by antibiotics and SOS-inducing agents. To examine the impact of deleting the six excisable prophage elements on *E. faecalis* physiology, we compared the growth of WT and *pp*$^-$ strains in the presence of 288 different compounds, at 4 different concentrations each, using BIOLOG phenotypic array plates PM9-20. We found 16 compounds in the presence of which *pp*$^-$ reached higher OD than WT, and 5 for which WT growth was favored. Each compound effect was observed at a specific concentration (Table 5).

Table 5. BIOLOG results.

Molecule	Target / mode of action	Functional family	Concentration (µg/mL)
Compounds for which WT was fittest			
Lincomycin	Protein synthesis	Antibiotic	60
Erythromycin	Protein synthesis 50S ribosomal subunit	Antibiotic	6.6
Copper chloride	Transport, toxic cation	Biocide	18
Phenylarsine oxide	Tyrosine phosphatase	Biocide	3.7
Antimony (III) chloride	Toxic cation	Biocide	50
Compounds for which *pp*$^-$ was fittest			
Democlocycline	Protein synthesis 30S ribosomal subunit	Antibiotic	0.3
Rolitetracyclin	Protein synthesis	Antibiotic	1.6
Carbenicillin	Cell wall	Antibiotic	4
Trimethoprim	Folate antagonist	Antibiotic	0.1
Blasticidin	Protein synthesis	Antibiotic	50
5-fluorouracil	Thymidylate synthetase	Antimetabolite	0.8
Amitriptyline	Membrane, transport	Antimicrobial effect	210
Protamine sulfate	Membrane, transport	Antimicrobial peptide	52
Acriflavin	DNA intercalator	Biocide	24
Sodium dichromate	Toxic anion, SO_4 analog	Biocide	6.5
1,10-Phenanthroline	Chelator, Fe^{++}, Zn^{++}, divalent metal ions	Biocide	34
Chlorohexidine	Membrane, biguanide, electron transport	Biocide	0.5
8-hydroxyquinoline	Chelator, lipophilic	Biocide	315
Menadione	Respiration, uncoupler	Oxidation	300
1-chloro-2,4-dinitrobenzene	Glutathione oxidation	Oxidation	1077
Potassium tellurite	Transport, toxic anion	Oxidation	0.15

The implicated compounds belong to three main functional classes: biocides, antibiotics and oxidative stress inducing agents. Out of the 21 compounds, we selected copper chloride, potassium tellurite, carbenicillin and trimethoprim as representative chemical agents to challenge WT and *pp*$^-$ strain. The biocides copper chloride and potassium tellurite cause

damage to the bacterial cell through the formation of reactive oxygen species [61,62]. Carbenicillin and trimethoprim are two antibiotics used in hospitals to treat bacterial infections. BIOLOG protocol uses a chemical defined medium of undisclosed composition to grow *E. faecalis*. Thus, we attempted to approach BIOLOG growth conditions, using a chemically defined medium [44] to grow bacteria in the presence of the selected compounds, tested at three different concentrations (see Materials and Methods). None of the differences observed with the PM plates were confirmed: WT and *pp*$^-$ growth was similar in the presence of trimetoprim and carbenecillin, and was the opposite of the BIOLOG results for copper chloride and potassium tellurite (Figure 2). Absence of reproducibility between the experimental settings may be related to the difference of growth medium composition and monitoring conditions.

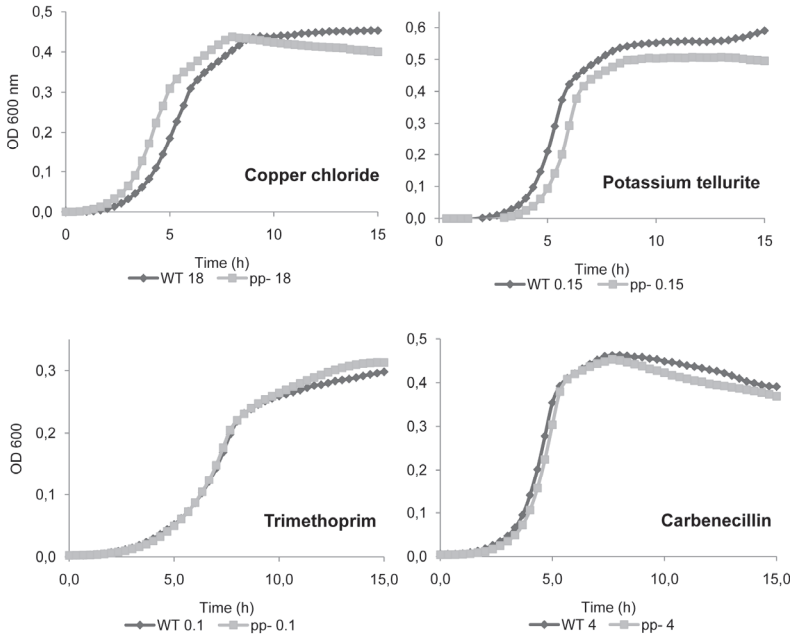

Figure 2. WT and *pp*$^-$ growth curves in presence of selected compounds. Copper chloride, potassium tellurite, trimethoprim and carbenicillin were used at 18 µg/mL, 0.15 µg/mL, 0.1 µg/mL and 4 µg/mL, respectively. Early stationary phase cultures were diluted 1/500 in 200 µL of CDM and incubated at 37°C for 15h in a TECAN microplate reader. Data presented are representative of two independent experiments.

Prophages do not modify intestinal colonization potential in conventional mice

To evaluate prophages impact on bacterial ability to colonize the gastro-intestinal tract (GIT), we used a model based on the orogastric inoculation of *E. faecalis* in mice harboring their complex microbiota imbalanced by a treatment with clindamycin [63]. Clindamycin is a lincosamide with broad spectrum against firmicutes and obligate anaerobic bacteria, which favors the emergence of enterococci by causing microbiota dysbiosis, and facilitates GIT colonization by inoculated *E. faecalis* strains [63,64]. To analyze the effect of different prophages and limit the number of animals used, we tested colonization ability of strains WT, *pp1*$^{-}$ and *pp7*$^{+}$, that differ in prophage content and phage production *in vitro* [40]. Strain WT harbors all prophages and produces P1, P3, P5 and EfCIV583 particles; strain *pp1*$^{-}$ carries all but pp1 prophage and produces only P3 and P5 while strain *pp7*$^{+}$ harbors only EfCIV583 and does not produce phage particles. GIT colonization by these strains was monitored for 96h (Figure 3A and B). WT strain transiently colonized the mouse gut at ~10^{9} CFU/g one day after inoculation, decreasing to between 10^{5} and 10^{4} CFU/g four days after arrest of antibiotic treatment. No significant difference in the efficiency of the GIT colonization was observed between the WT and the *pp1*$^{-}$ strains, indicating that the presence of pp1 or the production of P1 and EfCIV583 virions is dispensable for GIT colonization by *E. faecalis* V583 in a complex ecosystem of intestinal microbiota. Similarly, no significant difference of GIT colonization was observed between WT and *pp7*$^{+}$ or between *pp1*$^{-}$ and *pp7*$^{+}$. Altogether, these results indicate that prophages and virions do not confer a detectable advantage to WT in the gut microbiota of mice.

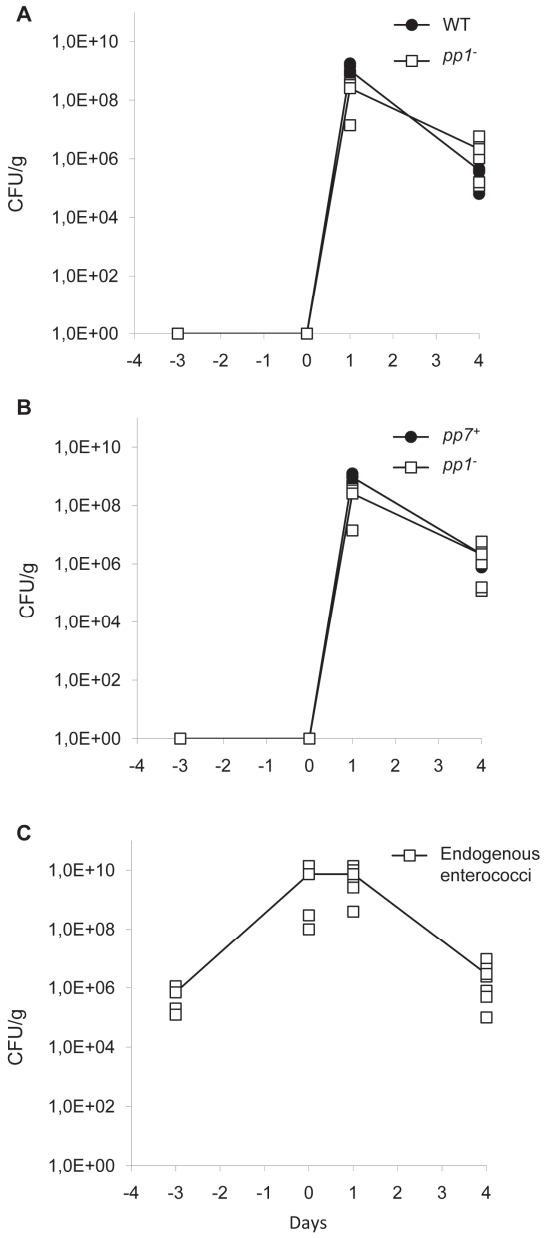

Figure 3. Mice gastro-intestinal tract colonization by *E. faecalis* strains WT, *pp1*$^-$ and *pp7*$^+$. After three days of subcutaneous administration of clindamycin, 1x10^{10} CFUs of each strain were force-fed independently into five mice. *E. faecalis* burden in stools was monitored 24h and 96h after oral gavage. (A) WT *vs pp1*$^-$. (B) *pp7*$^+$ *vs pp1*$^-$. (C) Endogenous enterococci levels before and after clindamycin treatment. No significant differences in the efficiency of colonization between strains were observed. Data presented are representative of two independent experiments.

In addition to the strains under study, we also monitored the endogenous enterococci of the mice gut microbiota and we observed a transient colonization that reached the highest level after the 3 days of clindamycin treatment (Figure 3C). A decrease of endogenous enterococci 4 days after arrest of clindamycin treatment was observed as for the inoculated strain. Flourishing endogenous enterococci were identified at the species level, using partial sequencing of the 16S rRNA genes (Rigottier-Gois et al., in preparation). Interestingly, *E. faecalis* was identified as a member of the clindamycin-treated gut microbiota, forcing inoculated *E. faecalis* to compete with endogenous isolates for the same niche.

Prophages promote bacterial binding to human platelets

PblA and PblB of *S. mitis* phage SM1 are multifunctional proteins that are both important for platelet adhesion and for normal phage morphogenesis: PblA is the phage tape measure protein and PblB is the tail fiber [20]. Prediction of pp1-, pp4- and pp6-encoded platelet-binding factors prompted us to investigate *E. faecalis* V583 prophages impact on binding to human platelets [39] (Figure 4).

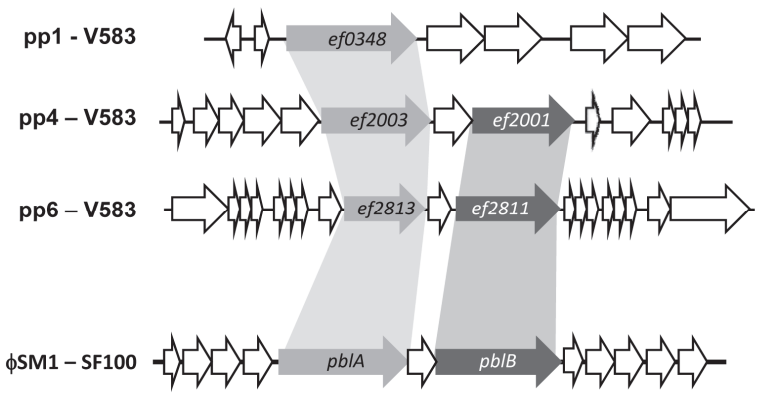

Figure 4. *E. faecalis* platelet-like binding proteins identified in V583 prophages. pp1, pp4 and pp6 carry homologous genes to *pblA* and *pblB*, both code for platelet-binding proteins identified in phage SM1 from *Streptococcus mitis*.

Since neither pp3 nor pp5 encode predicted PbI, strain *pp3*$^{+}$ *pp5*$^{+}$ that harbors pp3 and pp5 only, was constructed as a negative control and verified for the production of P3 and P5 particles (Table 1). We tested the ability of strains WT, *pp*$^{-}$, *pp1*$^{+}$, *pp4*$^{+}$, *pp6*$^{+}$, *pp7*$^{+}$ and *pp3*$^{+}$ *pp5*$^{+}$ to bind human platelets (Figure 5).

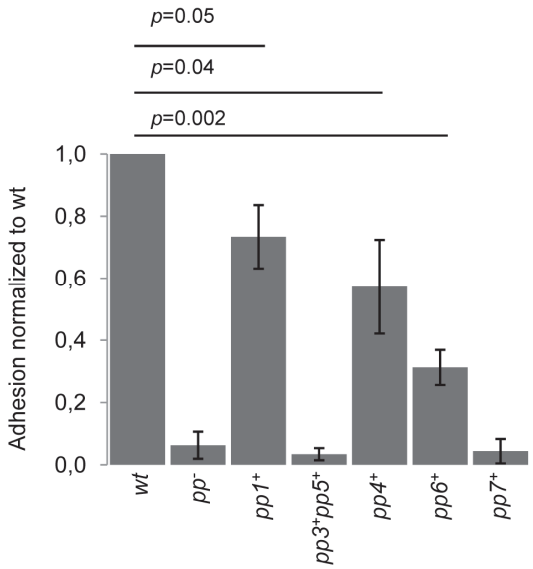

Figure 5. Impact of prophages on *E. faecalis* adhesion to human platelets. The values shown are normalized to the percentage of adhesion to platelets of the WT strain. Data are expressed as mean ± SD. Platelet binding assays were performed in platelets from three different donors. P value is indicated.

Removal of six V583 prophages (*pp*$^{-}$) reduced by ~8-fold the adhesion ability of *E. faecalis*, revealing that prophages contribute to the interaction with human platelets. Similarly to *pp*$^{-}$ strain, strains that have pp3 and pp5 or EfCIV583 bound poorly to platelets, indicating that pp3, pp5 and EfCIV583 are not involved in platelet adhesion. In contrast, strains carrying prophage-encoding platelet-binding factors, i.e. *pp1*$^{+}$, *pp4*$^{+}$ and *pp6*$^{+}$, bound significantly more than *pp*$^{-}$ strain, with a significant higher platelet adhesion for strains *pp1*$^{+}$ and *pp4*$^{+}$. These results show that *E.*

faecalis platelet-binding ability correlates with prophage-encoding platelet-binding factors. Given the importance of bacterial-platelet binding in the development of infective endocarditis it is tempting to speculate that these phages might contribute to enrich the repertoire of virulence traits of the species.

In addition to V583 genome, prophage-encoded platelet-binding proteins have also been identified in other *E. faecalis* isolates and all of them harbor tail proteins with sequence identity to either PblA or PblB [42]. BLAST search homology for the 160 genes of pp1, pp4 and pp6 were performed against the ~80 *E. faecalis* draft genomes publically available. While, prophage gene orthologs were detected in 63 genomes (Table S2) full-length genomes of pp1, pp4 and pp6 were detected in only few *E. faecalis* strains (Figure 6-8). Interestingly, pp1 orthologs are less disseminated than pp4 and pp6 ones. Moreover, despite the low representation of each CC, pp1, pp4 and pp6 orthologs seem to be enriched in CC2 and CC9 strains. While, *pblA*-like *ef0348* is mostly associated with pp1-orthologs, *pbl*-like orthologs of pp4 and pp6 (*ef2001*, *ef2003*, *ef2811* and *ef2813*) are not systematically detected with pp4- and pp6-orthologs. Among *E. faecalis* genomes, three CRISPR loci were identified: CRISPR1, CRISPR2 and CRISPR3. CRISPR1 and 3 are linked with *cas* genes and exhibit the typical organization of Nmeni CRISPR/Cas subtype [65], whereas CRISPR2 is an orphan locus and is not associated with *cas* genes [52,53]. CRISPR-Cas has been correlated with the absence of genetic mobile elements, such as prophages [52], and antibiotic resistance [53]. Interestingly, presence of CRISPR1 and CRISPR3 do not inversely correlate with pp1, pp4 and pp6 genes (Table S2).

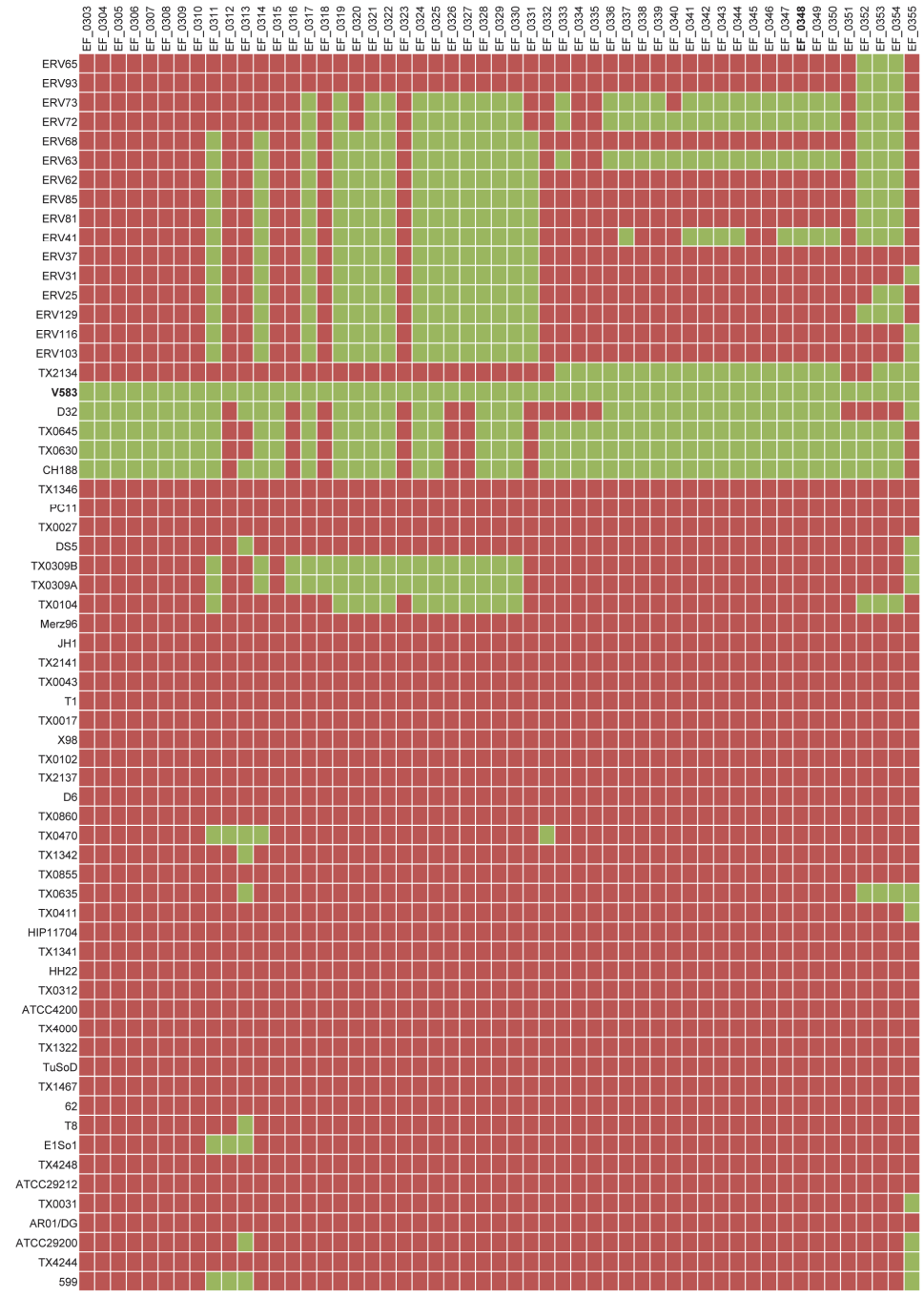

Figure 6. Distribution of pp1 gene orthologs in *E. faecalis* sequenced genomes. *pbl* genes are highlighted in bold.

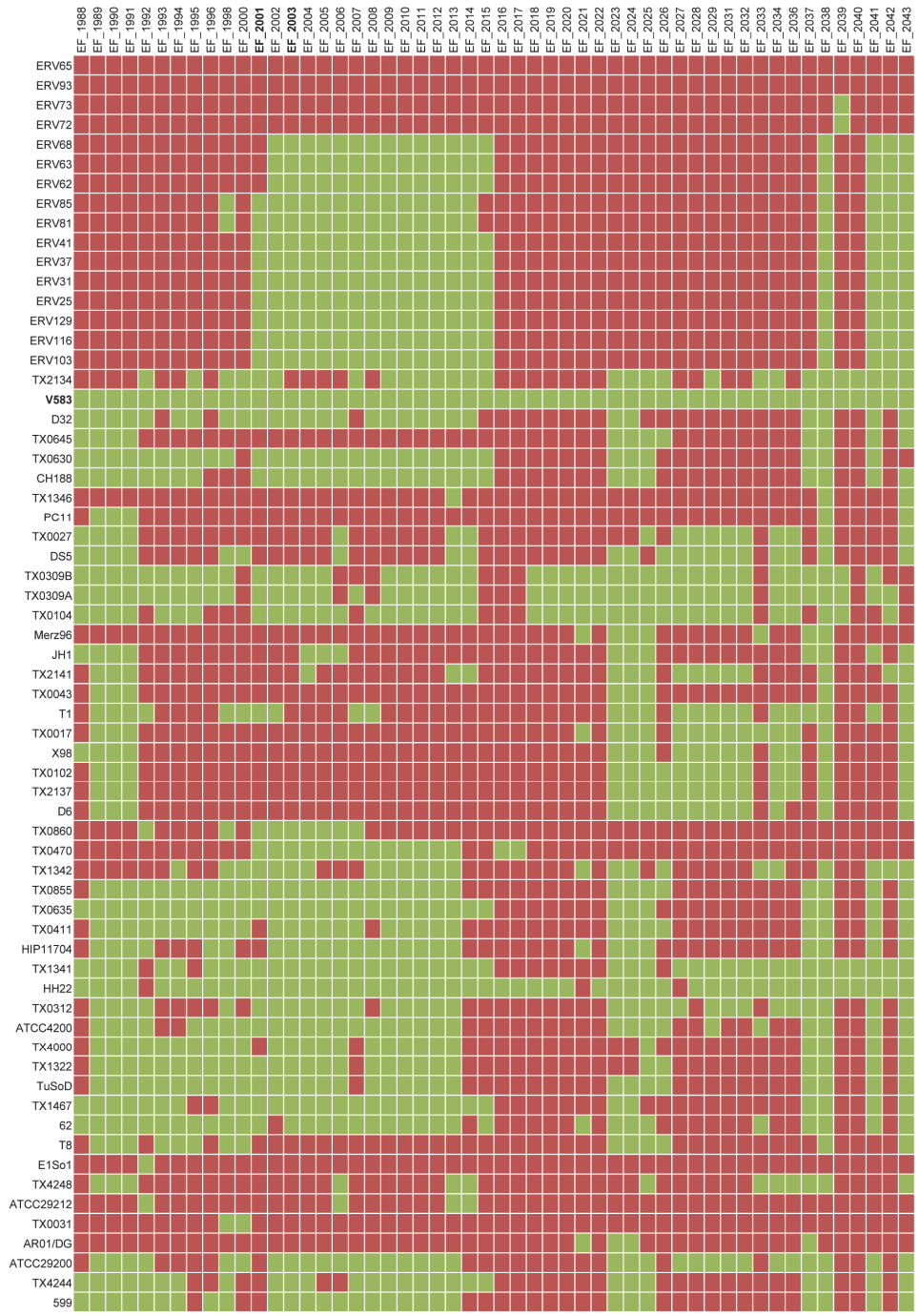

Figure 7. Distribution of pp4 gene orthologs in *E. faecalis* sequenced genomes. *pbl* genes are highlighted in bold.

Figure 8. Distribution of pp6 gene orthologs in *E. faecalis* sequenced genomes. *pbl* genes are highlighted in bold.

Prophages do not significantly impact bacterial infectivity in mice

To evaluate the impact of prophages carrying *pbl*-like genes *in vivo*, we compared the ability of the most adherent strain (*pp1*$^+$) with strain *pp*$^-$ to infect healthy mice. Both strains were injected intraperitoneally at 1x10^8 CFU. Mice were sacrificed 12 hours post-infection and bacterial burdens on heart, kidneys, liver and spleen were evaluated. In average, *pp1*$^+$ and *pp*$^-$ presented similar counts on heart (~1.4x10^4 CFU/g), kidneys (~7x10^4 CFU/g), liver (~4x10^5 CFU/g) and spleen (~9x10^5 CFU/g) (Figure 9), indicating that *pp1*$^+$ has no detectable effect on bacterial infectivity in mice.

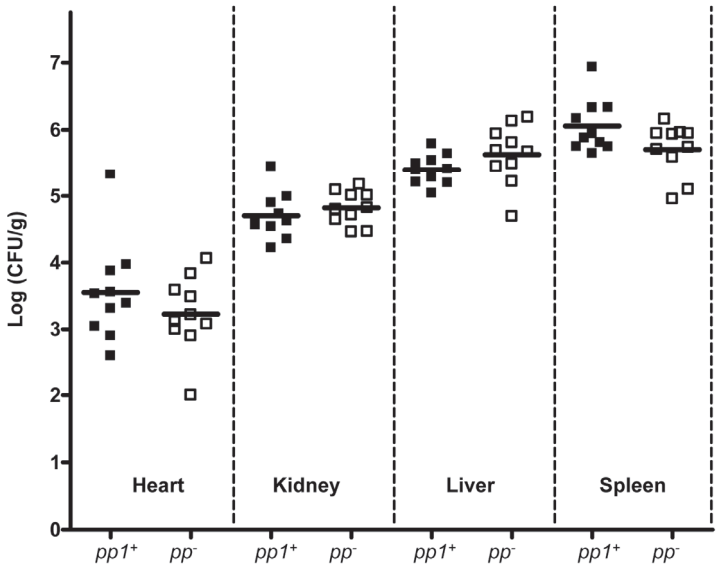

Figure 9. Mice infectivity by *pp1*$^+$ and *pp*$^-$ strains. Mice were injected intraperitoneally with 1x10^8 CFUs of each strain. *E. faecalis* bacterial counts in the heart, kidneys, liver and spleen were monitored 12 hours after injection. No significant differences in the efficiency of organ colonization between strains were observed.

To evaluate the impact of all prophages on bacterial infectivity in conditions that mimic immunocompromised hosts, we tested WT and *pp*$^-$ strains in a neutropenic mice model adapted from Balloy et al., [66]. Overall, our data show that mice response upon bacterial infection is

highly variable which is illustrated by the variation on bacterial counts reached in each organ, 24 hours after injection (Figure 10). Despite the high variability, bacterial burdens detected on heart, kidneys, liver and spleen are globally similar between WT and *pp*$^-$ strains (Figure 10). In average, bacteria were detected in the heart at ~5x10^4 CFU/g, in the kidneys at ~8.2x10^4 CFU/g, in liver at ~9x10^5 CFU/g and in the spleen at ~1.2x10^6 CFU/g. Analysis of blood samples recovered from mice after induction of neutropenia revealed a high variability in leukocytes, neutrophils, lymphocytes and monocytes counts, reflecting heterogeneous restoration of cellular immunity. In regards of such variability, bacterial infectivity of strains WT and *pp7*$^+$ deserves to be assessed either in healthy mice, for a shorter period of time, or in a refined neutropenic model.

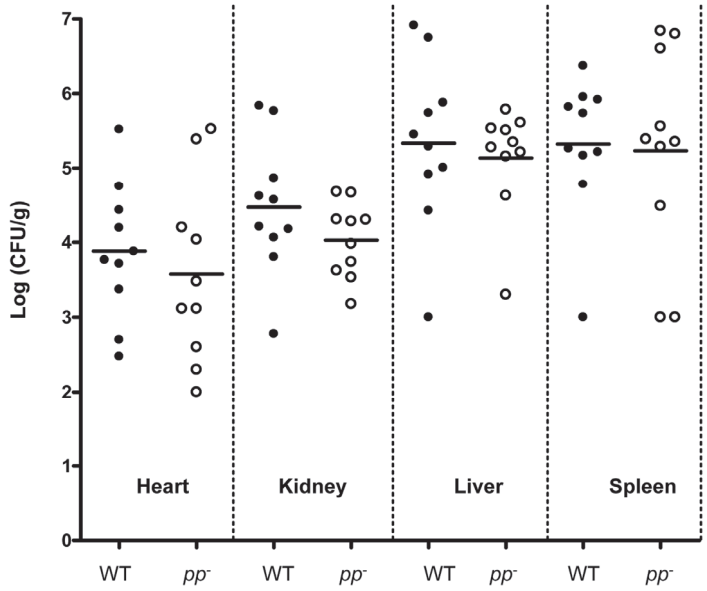

Figure 10. Mice infectivity by WT and *pp*$^-$. Immunocompromised mice were injected intraperitoneally with 1x10^8 CFUs of each strain. *E. faecalis* bacterial counts in the heart, kidney, liver and spleen were monitored 24 hours after injection. No significant differences in the efficiency of organ colonization between strains were observed.

Prophages have no significant impact on biofilm formation

Given that biofilm-forming ability enhances *E. faecalis* persistence in the hospital setting, we compared biofilm-forming ability of strains WT and *pp*$^{-}$ in static conditions at 24h and 48h. Three quantitative structural parameters of the biofilms were calculated: i) biovolume, which represents the overall volume of cells (μm^3), ii) roughness, which provides a measure of variations in biofilm thickness, and is an indicator of the superficial biofilm interface heterogeneity, and iii) maximum thickness (μm) of biofilm (Figure 11). Overall, the two strains formed biofilms with identical values for each measured parameter, indicating that prophages do not impact on biofilm formation in the tested conditions. A recent study by Bridier et al., evaluated the ability of several *E. faecalis* isolates to form biofilm in TSB using the same approach [54]. The *E. faecalis* strains tested, including V583 strain (ATCC 700802), form biofilms that have 10 times more biovolume and are two times thicker than the biofilms formed by WT and *pp*$^{-}$ strains [54]. We cannot exclude that WT strain forms less biofilm than V583 as a result of plasmid curing. Hence, differential biofilm formation between WT and *pp*$^{-}$ strains might be difficult to detect.

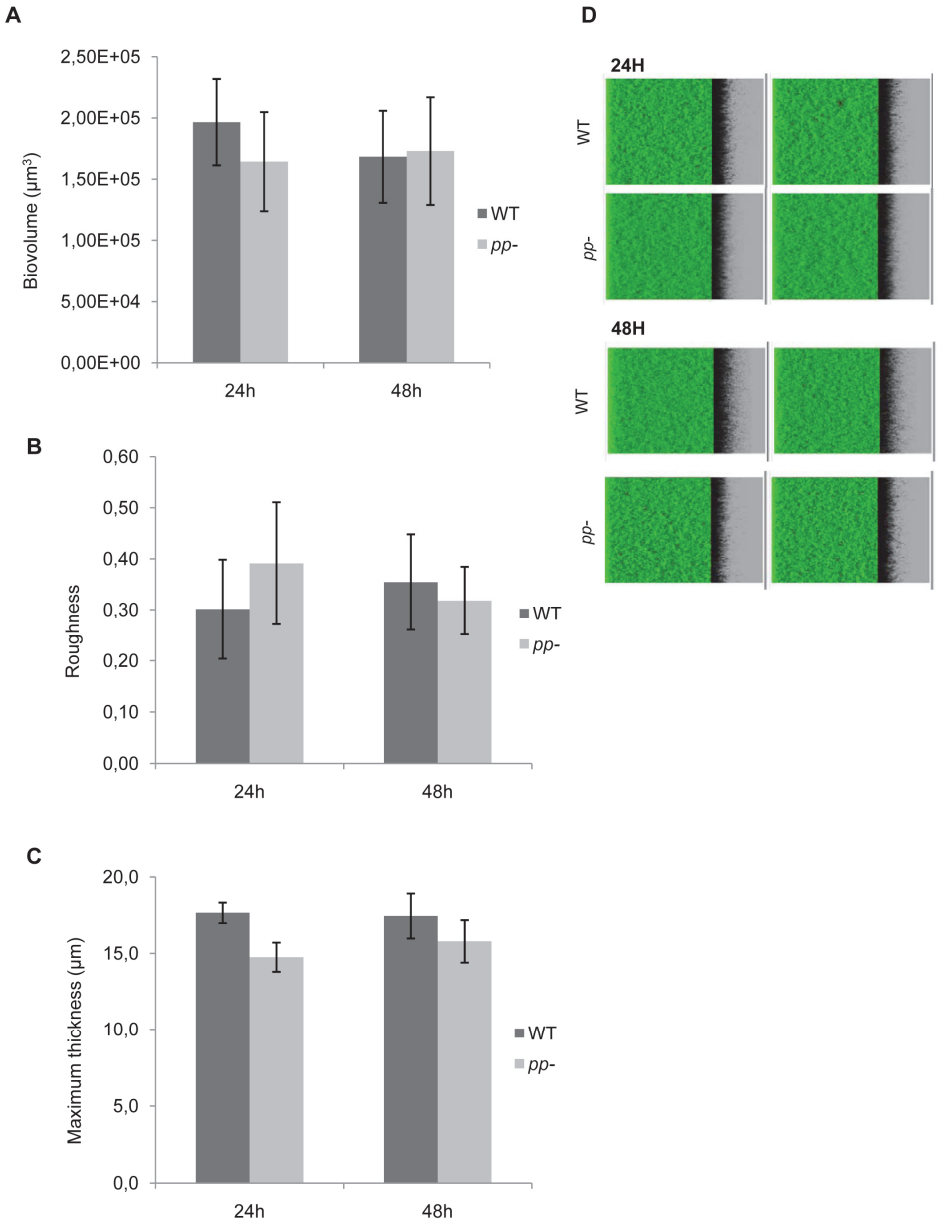

Figure 11. Biofilm quantitative structural parameters. (A) Biovolume. (B) Roughness. (C) Maximum thickness. Data are expressed as mean ± SD and are the result of two independent experiments performed in triplicates. (D) Three-dimensional projections of biofilms structure reconstructed with the Easy 3D function of the IMARIS 7.0 software for WT and *pp*$^{-}$ strain at 24 and 48h. These images represent an aerial view of biofilm structures with the shadow projection on the right.

DISCUSSION

Upon DNA injection and establishment of the prophage state, temperate bacteriophages can impact bacterial fitness in several ways. Either they carry useful genes for the bacteria, and are maintained in the prophage state or they help to eliminate niche competitors through induction of the lytic cycle and release of phage progeny. In the present study, we provide for the first time direct evidence that prophages carrying *pbl*-like genes are important for *E. faecalis* adhesion to human platelets. pp1, pp4 and pp6 encode predicted phage tail proteins homologous to platelet binding proteins, PblA and/or PblB, of *S. mitis* φSM1 [39,42]. Thus is likely that these proteins (EF0348, EF2003, EF2001, EF2811, EF2813) mediate *E. faecalis* binding to platelets. Platelet binding activity of *S. mitis* is linked to lysis induced by φSM1, implying that lytic activity of this prophage is required [19,20]. Upon prophage induction, platelet-binding proteins PblA and PblB, coded in φSM1, exert a dual function. They are part of φSM1 capsids as a tape measure and side tail fiber respectively, and they bind as free proteins to the cell wall of non-induced bacteria, promoting bacterial adhesion to platelets [20]. Interestingly, *E. faecalis* platelet-binding capacity varies from strong for the *pp1*$^+$ strain, to intermediate for the *pp4*$^+$ strain and to low for the *pp6*$^+$ strain. It is possible that the various platelet-binding efficiencies are the consequence of Pbls distinct binding capacity. However, such variation correlates with the efficiency of pp1, pp4 and pp6 to perform their lytic cycle [40]. Indeed, adhesion is minimal for the strain that harbors pp6, which only excises, maximal for strain that forms pp1 progeny, and intermediate with pp4 that excises and replicates. Even if pp4 and pp6 do not form infectious virions, they excise from the chromosome suggesting that their genes are expressed. We propose that this correlation may reflect different levels of expression and/or accessibility resulting from prophage activity. *E. faecalis*

adhesion to platelets has been reported before with strain OG1RF and clinical *E. faecalis* strains isolated from infective endocarditis [67,68]. Inactivation of the pathogenicity island-encoded transcriptional regulator PerA in *E. faecalis* E99 represses orthologs of the putative pp4-*pblA*, pp4-*pblB* and pp4-lysin genes, and increases the ability of E99 to bind to human platelets [49]. This apparent contradiction may result from the concomitant induction of the enterococcal biofilm-associated pilus *epb* locus that also promotes adhesion to human platelets [68]. PerA is present in several *E. faecalis* strains but its expression is probably dependent on the orientation of an IS element inserted upstream [69]. V583 has a *perA* gene and the IS orientated in the same direction, suggesting that *perA* is transcribed. It is possible that V583 PerA contributes to the platelet-adhesion phenotype mediated by pp4. Infectivity of $pp1^{+}$ strain, the most adherent to human platelets, did not differ from pp^{-} strain in healthy and immunocompromised mice models. This result indicates that either pp1 does not promote infectivity or that mice are not suitable for analyzing *E. faecalis* interaction with platelets. Given that pp1 mediates adhesion to human platelets *in vitro*, it is possible that the bacterium interacts with human-specific receptors. Since adhesion to platelets can lead to platelet activation, which promotes infective endocarditis, the role of prophage-encoded Pbl-like proteins in *E. faecalis* pathogenesis deserves further investigation.

GIT colonization and persistence are key steps for *E. faecalis* pathogenesis. Infectious virions produced by lysogenic strains are likely to form progeny on phage sensitive strains and thus be an asset for the bacteria in competitive environments like the complex microbial ecosystem of the GIT [70-72]. We investigated V583 prophages impact on intestinal colonization using strains that harbors different combinations of phage genomes. Our results show that P1, P3, P5 and EfCIV583 genomes and/or particles do not represent an advantage to their host in a

complex ecosystem where other enterococci, including *E. faecalis*, are overrepresented. Endogenous *E. faecalis* present in the mice microbiota may not be sensitive to V583 phage infection, thus competitive advantage of the prophage-producing strains WT and *pp1*$^{-}$, if any, would not be detected. Duerkop and collaborators recently reported that pp1 and EfCIV583 confer a subtle competitive fitness against phage sensitive strains in the intestine of gnotobiotic mice devoid of endogenous microbiota [73]. However, their experiments are difficult to interpret as the CH188 competitor strain was lysogen for P1 [40]. Competition between isogenic lysogenic and susceptible strains in a complex GIT ecosystem deserves to be further investigated.

E. faecalis ability to thrive in a broad range of ecological niches derives from its capacity to cope with a broad range of chemical compounds, including antibiotics and biocides. Comparison of WT and *pp*$^{-}$ growth using phenotypic microarray plates revealed growth differences in the presence of 21 compounds, mainly antibiotics, biocides and ROS inducing compounds. For the majority of compounds WT growth reached lower cell density when compared with *pp*$^{-}$ strain possibly due to phage induction followed by WT cell lysis. Consistently, we have previously demonstrated that trimethoprim induces *E. faecalis* V583 prophages [40]. Conversely, *pp*$^{-}$ growth reached lower cell density with a few number of compounds. In this case, prophages may carry additional genes that confer advantages for the bacteria as reported for the prophage pool of *E. coli* K12 [74]. Unfortunately, the effects of the identified compounds could not be reproduced in a medium different from the one used for microarray phenotyping. However, this technical problem needs to be further worked out to verify whether prophage impact on strain fitness towards the identified chemical compounds, particularly with potassium tellurite and copper chloride, which cause slight growth difference in both tested conditions.

Biofilm growth confers higher resistance to hostile environments encountered in the hospital setting and during pathogenesis [35,75]. Recent studies reported the importance of phages and phage remnants in *E. coli* and *P. aeruginosa* for biofilm formation, especially for the dispersal phase [76,77]. To withstand harsh conditions and shield against biocides, antimicrobials and predators, *E. faecalis* can grow in biofilm [24-26,78]. V583 prophages do not influence biofilm formation by its host, at least during the first 48h, since both strains produce biofilms with similar quantitative structural parameters (biovolume, roughness and maximum thickness). Nevertheless, we cannot exclude that over time, phage production, in WT strain, could enhance cell lysis and release of eDNA and thus promote the formation of a more robust biofilm. Although biofilms formed by WT and *pp*$^-$ present the same characteristics, it would be interesting to determine their resistance to antibiotics and biocides.

Overall, the six consecutive deletion events performed to generate *pp*$^-$ produced only 6 SNPs compared with WT. Given a genome of ~3.23 Mbp and ~1200 cell generations separating the two sequenced strains, the spontaneous mutation rate is estimated at $\sim 1.5\times10^{-9}$ SNPs per base pair per generation. This rate is 10-fold higher than the rate estimated for *E. coli* [79]. Such a difference may result from the use of antibiotics during *pp*$^-$ construction, since their usage is known to accelerate mutation rate [80]. Nevertheless, even if these mutations may not be silent in a future study, the absence of striking phenotypic differences between WT and *pp*$^-$ indicates that the mutations were not detrimental to *pp*$^-$.

In this work, we studied V583 prophages contribution to resistance to chemical compounds, intestinal colonization, adhesion to platelets, infectivity and biofilm formation. Under the tested conditions, prophage presence did not significantly impact on biofilm formation, intestinal colonization and infectivity of the host strain. Their role to sustain growth in the presence of chemical compounds needs to be further investigated.

pp1, pp4 and pp6 promote *E. faecalis* adhesion to human-platelets, probably through the release of PblA/B-like proteins upon prophage induction. Establishing a direct link between Pbls and endocarditis is required to open new perspectives towards diagnosis and eventually prevention of enterococcal endocarditis.

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SUPPLEMENTARY INFORMATION

Table S1. Differences detected in VE14089 common with Palmer et al., [58].

Reference position[a]	Variation type	Variation	Annotation	Predicted function
132143	Insertion	- → G	IR *ef0126-0127*	-
179987	Deletion	A → -	EF0183	Hypothetical protein
191992	SNP	T → A	EF0199	Ribosomal protein S7
229297	Deletion	C → -	IR *ef0252-0253*	-
611688	SNP	G → T	EF0660	MATE efflux family protein
872056	SNP	C → A	EF0906	Hypothetical protein
921232	SNP	A → G	EF0958	PTS system, IIABC components
931162	SNP	G → T	IR *ef0967-0968*	-
998111	SNP	C → T	IR *ef1036-1037*	-
1170952	Insertion	- → T	EF1205	Transcriptional regulator
1303060	Deletion	A → -	IR *ef1331-1332*	-
1474120	SNP	C → A	EF1519	Cation-transporting ATPase, E1-E2 family
1534568	Deletion	A → -	EF1581	Transketolase
1571355	SNP	C → T	EF1614	DNA topoisomerase IV, A subunit
1651141	SNP	T → C	EF1704	Sensory box histidine kinase
1651146	SNP	T → C	EF1704	Sensory box histidine kinase
1917084	SNP	T → A	EF1979	ATPase, AAA family
2059477	Insertion	-- → AA	EF2155	Phosphoglucomutase family protein
2117617	Insertion	- → C	EF2204	Aminopeptidase
2232992	Deletion	T → -	EF2308	Hypothetical protein
2234179	Insertion	- → G	IR *ef2311-2312*	-
2234474	SNP	C → G	EF2312	DNA topoisomerase III
2309182	SNP	C → A	IR *ef2380-2381*	-
2323031	Deletion	C → -	EF2399	Acetyltransferase
2325873	Insertion	- → G	EF2405	Hypothetical protein
2452948	Insertion	- → C	IR *ef2528-2529*	-
2452957	Insertion	- → C	IR *ef2528-2529*	-
2570780	SNP	T → C	IR *ef2658-2659*	-
2578163	SNP	C → G	IR *ef2665-2667*	-
2677895	Deletion	A → -	EF2772	Drug resistance transporter
3000492	Deletion	- → C	EF3124	Polypeptide deformylase
3015992	SNP	T → A	IR *ef3142-3244*	-
3100859	Deletion	G → -	EF3231	Ribosomal protein L13
3125849	Insertion	- → C	EF3251	Hypothetical protein

Table S2. Strains used to study prophage genes distribution and *cas* genes presence.

Strain	Geographical origin	Year of isolation	Source	CC	ST	C1[1]
599	-	-	-	16	16	+
TX4244	Netherlands	1998	Fecal	27	27	+
ATCC29200	Canada	<1974	Urogenital	21	21	+
AR01/DG	New Zealand	2001	Dog	108	108	-
TX0031	USA	<1993	Endocarditis	21	21	+
ATCC29212	USA	-	Urine	30	30	-
TX4248	Netherlands	2002	Seal	40	40	+
E1Sol	Salomon Islands	1960s	Fecal	93	93	+
T8	Japan	<1992	Urine	8	8	-
62	Norway	2002	Feces	66	66	-
TX1467	-	-	Feces	40	40	+
TuSoD	USA	<2009	Root canal	364	364	-
TX1322	USA	1994	Feces	8	64	-
TX4000	-	<1974	-	8	8	-
ATCC4200	-	1926	Blood	105	105	+
TX0312	USA	<1998	Urine	21	21	-
HH22	USA	1981	Urine	2	6	-
TX1341	USA	1994	Fecal	287	287	-
HIP11704	USA	2002	Clinical	4	4	-
TX0411	USA	<1954	Clinical	8	90	-
TX0855	Thailand	1980	Urine	4	4	-
TX0635	USA	1986	Urine	9	9	-
TX1342*	USA	1994	Feces	134	134	-
TX0470	USA	1963	Clinical	110	110	+
TX0860	Thailand	1980	IV catheter	28	11	-
D6	Denmark	-	Pig	16	16	+
TX2137	Spain	2001	Fecal	16	16	+
TX0102	USA	<2001	Endocarditis	21	21	+
X98	-	1934	Fecal	19	19	+
TX0017	USA	1992	Endocarditis	144	144	-
T1	Japan	<1950	-	21	21	-
TX0043	USA	1983	Endocarditis	19	19	+
TX2141	Spain	2001	Blood	25	25	-
JH1	UK	<1974	Clinical	40	40	-
Merz96	USA	2002	Blood	103	103	-
TX0104	USA	<2002	Endocarditis	2	2	-
TX0309A	USA	<1996	Clinical	2	6	-
TX0309B	USA	<1996	Clinical	2	6	-
DS5	USA	<1974	-	55	55	+
TX0027	USA	1975	Endocarditis	55	55	+
PC1.1	Australia	-	Feces	40	40	-
TX1346	-	-	-	192	192	-
CH188	USA	1980s	Liver	9	9	-
TX0630	Argentina	1989	Blood	9	9	-
TX0645	Lebanon	1989	Blood	23	10	-

Strain	Geographical origin	Year of isolation	Source	CC	ST	C1[1]
D32	Denmark	2001	Pig	40	40	+
V583	USA	1987	Blood	2	6	-
TX2134	Netherlands	1998	Fecal	30	30	-
ERV103	-	-	-	238	238	-
ERV116	-	-	-	238	238	-
ERV129	-	-	-	238	238	-
ERV25	-	-	-	238	238	-
ERV31	-	-	-	238	238	-
ERV37	-	-	-	238	238	-
ERV41	-	-	-	238	238	-
ERV81	-	-	-	238	238	-
ERV85	-	-	-	238	238	-
ERV62	-	-	-	238	238	-
ERV63	-	-	-	238	238	-
ERV68	-	-	-	238	238	-
ERV72	-	-	-	238	238	-
ERV73	-	-	-	238	238	-
ERV93	-	-	-	238	238	-
ERV65	-	-	-	238	238	-

[1]CRISPR1.[+] Present; [-] Absent. *TX1342 harbors a CRISPR3 locus.

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Chapter 4

General Discussion and Perspectives

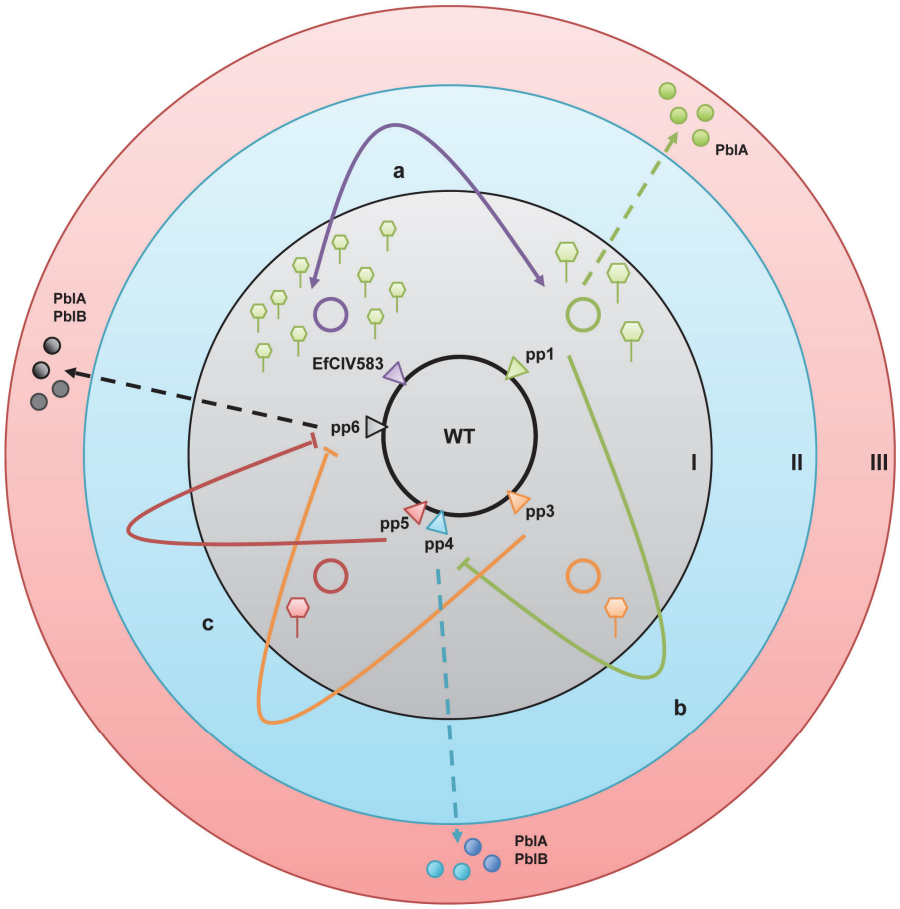

Figure 1. *E. faecalis* V583 prophages. I (grey circle). Prophage induction and particle production. pp1, pp3, pp4, pp5, pp6 and EfCIV583 induction occurs spontaneously and can be enhanced by environmental cues such as antibiotics. pp1, pp3, pp5 and EfCIV583 active particles are produced at different titers. **II** (blue circle). V583 prophage-prophage interactions: a) pp1 and EfCIV583 are involved in a phenomenon of molecular piracy in which EfCIV583 hijacks P1 structural proteins (Chapter 2); b) pp1 blocks pp4 excision at 37°C; c) pp3 and pp5 block pp6 excision. **III** (red circle). Prophage contribution to bacterial biological traits. pp1, pp4 and pp6 contribute for V583 adhesion to human platelets, probably through the action of *pbl* genes, in a process that correlates with prophage's ability to accomplish a full lytic cycle (Chapter 3).

Polylysogeny and prophage interaction networks

The advent of whole genome sequencing revealed the extensive dynamic of bacterial genomes that can suffer accretion or reductive evolution depending on bacterial lifestyles. Accretions in genome size rely on the duplication or acquisition of exogenous genetic material, whereas reductive evolution relies on drift and deletions that remove nonfunctional DNA [1]. The larger genome size of free-living bacteria reflects frequent acquisition of new genes and greater need for metabolic versatility. On the contrary, the transition to intracellular lifestyle probably reduces the opportunity for gene uptake and imposes the need to retain the most essential functions [1]. Most of the bacterial species harbor prophage elements, and polylysogeny is frequently encountered among bacteria. The most extreme cases of polylysogeny are illustrated by *Escherichia coli* O157:H7 strains Sakai and K12 in which prophage genome elements represent 16% and 18% of the genome content, respectively [2,3]. Furthermore, the acquisition of multiple prophages plays a fundamental role in the emergence of pathogenic strains, as observed in *Streptococcus pyogenes*, for which the content of prophages accounts for up to 12% of the bacterial genome and correlates with the severity of the virulence [4].

Enterococcus faecalis prophages are probably important for the evolution of the species as they account for an important part of the intraspecies variability [5,6]. In the V583 strain, seven prophage-related elements (pp1-pp7) are present, representing ~6.4 % of its total genome. pp2 is ubiquitous among *E. faecalis* strains; pp1, pp3, pp4, pp5 and pp6 are excisable and participate in unanticipated prophage-prophage interactions; and pp7 was re-classified, based on our experimental evidences, as phage-related chromosomal island (PRCI) and renamed EfCIV583 [7]. Four phage particles are released and belong to P1, P3, P5 and EfCIV583 (Figure 1.I). EfCIV583 is not only the first PRCI to be

identified in *Enterococcus* genus but it is also the first for which experimental evidences of molecular piracy have been provided in species other than *Staphylococcus aureus*. EfCIV583 hijacks P1 structural proteins in order to get its own genome packaged and probably disseminated (Figure 1.II). Although the resemblance with the molecular piracy system of *S. aureus* SaPIs and their helper phages are numerous [8,9], EfCIV583/P1 system is different in two main aspects: i) EfCIV583 excises spontaneously through a helper phage-independent mechanism, whereas SaPIs are generally stably maintained into the bacterial genome through the action of a SaPI-encoded master repressor, which is inactivated by helper-phage specific antirepressors [10,11]; ii) EfCIV583 DNA is packaged exclusively in the monomeric form while some SaPIs can have their DNA packaged in the monomeric form, into small capsids, or as multimers, into large capsids [12,13]. As SaPIs, EfCIV583 controls helper phage structural proteins and interferes with its helper phage titer. P1 titer is thus 10-fold lower than EfCIV583. Interestingly, although the proportion is maintained, in the context of the WT polylysogenic strain P1 and EfCIV583 particles are released at lower amounts than by the dilysogen strain *pp1*$^{+}$*pp7*$^{+}$. This difference could be due to prophage within-host competition in the WT, which was showed to impair phage productivity in a polylysogenic strain of *E. coli* [14]. Interestingly pp4 and pp6 are kept silent through unknown mechanisms by pp1 and pp3/pp5, respectively (Figure 1.II). Prophages in a polylysogenic strain develop mechanisms to interact with each other in order to guarantee their vertical propagation. The fastest prophage to accomplish the lytic cycle dictates the time of lysis for all other prophages [15]. As none of the single deletions (pp3 or pp5) had effect on pp6 excision and pp3 and pp5 share the highest homology among V583 prophages, their ability to repress pp6 excision might be redundant. Recently, a study described a mechanism of antirepressor-mediated control of prophage induction based on the

recognition of cognate and non-cognate repressors of Gifsy prophages in *Salmonella* [16]. This interaction could lead to the coordination of lytic cycles in a polylysogenic strain in order to give time to 'slower' phages to finish their cycles [16]. Prophage interference can influence strain's behavior and impact on its fitness. Various types of prophage-prophage interactions occur within the prophage pool of *E. coli* O157:H7 strain Sakai, and these interactions promote the activity of strain defective prophages [2]. Furthermore, prophages can interact with other mobile genetic elements such as plasmid pCM194, which increases phage production of a SPO2 lysogen *Bacillus subtilis* strain [17]. It is the case of strain V583, which exhibits a lower efficiency of phage production compared to its plasmid-cured derivative. Moreover, prophages regulate host gene expression and improve the fitness of the lysogen under certain conditions, such as acid stress [18]. Actually, a prophage-encoded AraC-like regulator, PatE, activates transcription of several acid resistance pathways in the enterohemorrhagic *E. coli* strain EDL933 [18]. Although this aspect of prophages interaction with their host was not addressed in this thesis, it might be relevant to determined V583 transcriptomes in the presence and absence of prophages.

Within the polylysogenic strain V583, prophages interact with each other in order to get excised and produce phage active particles. Those interactions deserve further investigation that will allow determining the molecular mechanism underlying them. The identification of the enterococcal phage-related chromosomal island, EfCIV583, constitutes for us a major point of interest. Given PRCIs prevalence and impact on *S. aureus* virulence, it would be interesting to study PRCI distribution in enterococci and determine their impact for bacterial fitness. Regarding the molecular piracy phenomenon, several questions remain to be investigated namely the molecular partners, in both pp1 and EfCIV583,

coordinating the interference mechanism and the re-direction of the capsid size.

Environmental cues involved in prophage induction

Once the prophage state is established, prophages are stably maintained in the host cell by the expression of a repressor that blocks phage lytic cycle and provides immunity to the lysogenic strain against phage superinfection. pp1, pp3, pp5 and EfCIV583 of *E. faecalis* V583 provide homo-immunity to their lysogenic host, which can constitute an advantage for the host in complex ecosystems. Prophages can switch irreversibly into the lytic development and release phage progeny under certain conditions, which generally trigger the SOS response [19]. This process allows prophages to sense host physiological state and escape bacterial cell death. Although the classical induction pathway involves RecA and the SOS response, in other cases it can occur independently [20]. Triggering signals for the SOS response are diverse and include exposure to DNA-damaging agents such as UV light, mitomycin C and certain antibiotics, reactive oxygen species, pressure and chelating agents [21-25]. Lambda and other lambdoid phages can be induced independently of RecA through the action of RcsABC system, which is involved in capsular polysaccharide synthesis [26]. RcsB and RcsC are members of a two-component system whereas RcsA is an additional transcriptional activator in. RcsA pathway is activated under high cell density mediated by a quorum-sensing effector (acyl-homoserine lactone) that upon binding to its receptor induces the switch into the lytic growth for *E. coli* λ prophage [27]. RcsA role in prophage induction may involve the regulation of an alternative RecA co-protease [20].

Opportunistic bacteria are likely to face different stressful conditions imposed by their lifestyles: commensal and pathogen. In addition to the

stresses encountered in the human host, *E. faecalis* copes with several other environmental conditions that may induce its prophages. *E. faecalis* V583 prophages are induced spontaneously and their excision is globally enhanced by sub-inhibitory concentrations of ciprofloxacin, mitomycin C and trimethoprim, while temperature has different effects depending on the prophage. Fluoroquinolones and β-lactams are the most efficient phage-inducing antibiotics [28]. Ciprofloxacin is a fluoroquinolone antibacterial agent that traps DNA gyrase on DNA and blocks the replication fork movement, inducing the SOS-response [22]. Actually, increase of *rec*A transcription was concomitant with phage induction in *E. faecalis* in presence of ciprofloxacin (data not shown), suggesting that this antibiotic induces V583 prophages through activation of the SOS-system. On the contrary, ampicillin had no inducing effect on V583 prophages or on *recA* transcription. Antibiotic-mediated induction of the SOS-response promotes the spread of virulence traits by generalized or specialized transduction in *S. aureus* [22] and *E. coli* [29]. Therefore, the use of SOS-inducing antibiotics in hospital setting and for animal growth promotion has consequences both at the resistance level and for the dissemination of virulence traits [30,31]. Despite not being recognized as typical SOS-system inducing condition, temperature can impact phage induction and activity. High temperature has the strongest negative effect on phage DNA packaging as illustrated for *Escherichia coli* (STEC) prophages [32]. Indeed, high temperature decreases excision efficiency of pp5 and EfCIV583 but provokes the release of pp4 from pp1 inhibitory effect, possibly due to thermosensitivity of pp1 repressor.

Although poorly studied, spontaneous excision of prophages maintains a small but constant background of prophage particles and induces lysis on a fraction of the population. It also provides a competitive advantage for the lysogens by killing phage-sensitive strains [33,34]. Induction of prophages not only releases phage particles but can also

increase toxin production, which worsens the disease symptoms like Shiga toxin-encoding prophages [35,36]. V583 harbors three prophages, pp1, pp4 and pp6, carrying platelet-binding proteins (Pbl) involved in the adhesion to human platelets (Figure 1.III) and an ADP ribosyl-transferase (EFV toxin on pp1) [7,37]. EFV toxin expression in yeast induces cell death through its ADP ribosyl-transferase activity [37]. This family of toxins, which includes cholera and diphtheria toxins, damage host cells by mono-ADP-ribosylation of intercellular targets that are usually key regulatory proteins in the target cell [38]. Both *pbls* and *efv* genes are annotated as phage structural proteins and therefore they seem to have a dual role. As for Pbls, EFV toxin is non-classically secreted (without signal peptide) and it is likely released upon phage-induced cell lysis. Spontaneous induction of V583 prophages can release these genes into the different environments where the strain thrives and promote horizontal gene transfer. The work of this thesis reports a direct correlation between prophages harboring platelet-binding proteins and bacterial adhesion to human platelets. To improve our understanding of this phenomenon, it should be established whether PblA and PblB are involved in the phenotype and how pp4 and pp6, that are unable to complete a full lytic cycle, release their Pbls and thus contribute to platelet adhesion. Given the contribution of other virulence factors for *E. faecalis* adhesion to platelets, such as Ebp [39], the relative contribution of Pbls and those other factors should be determined. As adhesion to platelets is considered the first step towards development of infective endocarditis, monolysogenic strains for pp1, pp4 and pp6 should be tested in an infective endocarditis model [40]. However, the outcome of such test may depend on the specificity of the platelet receptors.

In addition to the spreading of virulence factors, prophage excision can restore gene function or modify their expression [41-43]. In the case of *E. faecalis* V583 prophages, excision of pp4 restores an open reading

frame of an operon that encodes orthologs of a DNA uptake machinery [44]. Interestingly, Rabinovitch and colleagues recently reported that excision of the *L. monocytogenes* prophage φ10403S induces the expression of a DNA uptake machinery needed for intracellular growth [41]. Although, probably not functional in strain V583, due to a premature stop codon in another gene of the same operon [44], such a complex is likely to be expressed in strains devoid of mutation. Resemblance of the DNA uptake machinery with type IV secretion systems suggests that its expression may confer new biological traits.

Bacteriophages in human associated microbial communities

Understand lysogeny is extremely important due to prophages impact on host physiology, community composition in complex ecosystems and gene transfer. Recent metagenomic studies of different environmental niches as diverse as soil, ocean and human oral and intestinal microbiota have highlighted the importance of bacterial virome impact on microbial communities [45-48]. Interestingly, the microbial metabolic activities encoded by the viromes were broad and enriched in nucleic acid metabolism and virulence genes [45].

E. faecalis is a member of several human and animal associated microbial communities such the oral cavity and the gastrointestinal tract (GIT) [49,50], and *E. faecalis* phages have been found in root canals [51], saliva [52] and intestine [53]. Lysogeny state is favored under poor growing conditions due to nutrient limitations, and also lack of suitable hosts such as in human related microbial ecosystems [54]. As a member of the oral cavity microbiota and as a major cause of post-treatment endodontic infections, *E. faecalis*, similarly to *S. mitis*, has the potential to gain access to the bloodstream through the root canals [55,56]. Metagenome analysis of the oral cavity microbiota demonstrated that *pblA*

and *pblB* sequences are enriched in the oropharyngeal and saliva samples [48]. Prophages can also be induced with some ingested substances and represent a risk factor in the development of endocarditis [48]. Accordingly with these data, it is reasonable to think that the presence in the oral cavity of *E. faecalis* strains that harbor prophages carrying *pbl* genes could also represent a risk of infection.

Prophages of commensal bacteria have the potential to shape the gut microbial communities and the resulting metabolic activities, as well as promote horizontal gene transfer. V583 prophages present a basal level of spontaneous excision, which is increased by environmental stress including antibiotics. It was reported recently that P1 and EfCIV583 may represent a competitive advantage to their hosts in the gut [57], however the phage-susceptible competitors they have used did not seem appropriate. Antibiotic pressure is an important driving force in bacterial evolution, and the extensive use of antibiotics that induce prophages can stimulate their dissemination and favor host fitness [30]. While we have shown that antibiotics and temperature influence prophage induction, other stress found in the GIT (e. g. physiochemical defenses of the host, including low pH of the stomach and elevated osmolarity) may also induce enterococcal prophages. Prophage induction can be thus a factor contributing to dysbiosis in the GIT due to their potential impact on the ratio between symbionts and pathobionts.

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