

Investigating the determinants of

***Enterococcus faecalis* virulence**

in *Drosophila melanogaster*

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Statement of originality

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Abstract

Enterococcus faecalis is a Gram-positive bacterium found in the normal gut microbiota of diverse species, including vertebrates and invertebrates, as well as being common in the environment. It is also an opportunistic pathogen with a broad host range. One of the hosts *E. faecalis* can infect is the fruit fly, *Drosophila melanogaster*. The *Drosophila* immune response is distinct from that of humans and interacts with *E. faecalis* differently. To study this interaction we carried out experimental evolution via serial passage of *E. faecalis* in *Drosophila*. We generated *E. faecalis* strains with much-enhanced ability to survive and proliferate within this host. Strains selected in this way are specifically resistant to the Toll-induced Bomanin family of effector peptides, resulting not only in higher *E. faecalis* numbers but also in a significant increase in pathogenicity. Many of these *Drosophila*-selected strains also exhibit marked increases or decreases in antimicrobial resistance. Whole genome sequencing showed that most selected strains carried single mutations and that many of these mutations were in genes encoding proteins known to be involved in bacterial surface characteristics and antimicrobial resistance (*mprF_2*, *liaF*, *yxdM*, *croS*, *bgsA*). To test if *Drosophila* antimicrobial peptides kill *E. faecalis* using mechanisms similar to antibiotics we generated *E. faecalis* strains that were resistant to daptomycin. Some of these daptomycin-adapted strains also acquired resistance to the *Drosophila* immune response. Daptomycin-adapted *E. faecalis* strains have mutations in the same genes or the same regulatory systems as were observed in *Drosophila*-adapted strains. As common genetic mechanisms underlie the resistance of *E. faecalis* to daptomycin and the *Drosophila* immune response, these results indicate these two systems target the same conserved bacterial properties in *E. faecalis*. They also demonstrate that the selection and emergence of antibiotic resistance *in vivo* does not require antibiotic exposure.

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Abbreviations

AA	Amino acid
Ala	Alanine
Bbd	Bombardier
BDSC	Bloomington <i>Drosophila</i> Stock Center
BHA	Brain heart agar
BHI	Brain heart infusion
BLUD	Bacterial load upon death
BomS2	Bomanin short-form 2
cDNA	Complementary DNA
CFU	Colony forming units
CroRS	Ceftriaxone resistance response regulator and sensor kinase
DAG	Diacylglycerol
Dap	Daptomycin
DAP	Diaminopimelic acid
Def	Defensin
DGlcDAG	Diglucosyldiacylglycerol
Dif	Dorsal-related immunity factor
Dipt	Diptericin
DNA	Deoxyribonucleic acid
Dro	Drosocin
Drs	Drosomycin
EDTA	Ethylenediamine tetra-acetic acid
EE	Experimental evolution

EPA	Enterococcal polysaccharide antigen
FITC-PLL	Fluorescein isothiocyanate-labelled poly-L-Lysine
GalNAc	N-acetylgalactoseamine
GFP	Green fluorescent protein
GlcNAc	N-acetylglucosamine
GNBP	Gram-negative binding protein
GP	Generalised polarisation
HA	Human influenza haemagglutinin
HK	Histidine kinase
IKK	I κ B Kinase
IM-HK	Intra-membrane sensing histidine kinase
Imd	Immune deficiency
IS	Insertion element
I κ B	Inhibitor of NF- κ B
JNK	C-jun N-terminal kinase
Key	Kenny
LB	Lysogeny broth
LiaRS	Lipid II interacting antibiotics response regulator and sensor
LTA	Lipoteichoic acid
Lys-PG	Lysyl-phosphatidylglycerol
MGlcDAG	Monoglucosyldiacylglycerol
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MOPS	3-(N-morpholino)propanesulfonic acid
MprF	Multiple peptide resistance factor

mRNA	Messenger RNA
MS	Mitis Salivarius
Mtk	Metchnikowin
Myd88	Myeloid differentiation primary response 88
M17G	M17 media supplemented with Glucose to a final concentration of 0.5%
NEB	New England BioLabs Inc.
NF- κ B	Nuclear factor κ -light chain enhancer of activated B cells
O/N	Overnight
OD ₆₀₀	Optical density at 600nm
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PASTA kinase	Penicillin binding protein and serine-threonine kinase associated kinase
PBS	Phosphate buffered saline
PC	Plate control
PCR	Polymerase chain reaction
PG	Phosphatidylglycerol
PGRP	Peptidoglycan Recognition Protein
PI	Post infection
PO	Phenol oxidase
PPO	Prophenol oxidase
PRR	Pattern recognition receptors
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RpL4	Ribosomal protein L4
rpm	Revolutions per minute
RR	Response regulator
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SDS	Sodium dodecyl sulphate
SGM17	M17G supplemented with sucrose and glycine to a final concentration of 0.5M and 2%
SNP	Single nucleotide polymorphism
SPE	Spätzle processing enzyme
Spz	Spätzle
Suc-Glc	0.5M sucrose, 10% glycerol suspension
Tak1	TGF- β activated kinase 1
TBS	Tris buffered saline
TBS-T	TBS with 0.1% Tween 20
TCS	Two-component system
TE	Tris buffered saline with 1mM EDTA
TGF- β	Transforming growth factor β
UAS	Upstream activating sequence
Van	Vancomycin
w/v	Ratio of weight-to-volume
WT	Wild type
WTA	Wall teichoic acid

Chapter 1: Introduction

1.1 *Enterococcus faecalis*

1.1.1 General characteristics of Enterococci

Enterococci are Gram-positive, facultatively anaerobic, catalase-negative, non-spore forming cocci that may appear as short chains, pairs, or single cells. Members of this genus have the ability to grow in 6.5% NaCl, in a wide range of temperatures (from 10 to 45°C) and are tolerant of high concentrations of bile (Murray, 1990; Sherman, 1938). Currently, 62 species form part of this genus, with the latest described in 2022 (Chung et al., 2022). Enterococci are ubiquitous and have been isolated from a wide range of environmental sources (soil, water), they are found in association with plants, in dairy and fermented food products, and are common members of the microbiota of vertebrates and invertebrates (Martin & Mundt, 1972; Mundt, 1963).

While Enterococci are a core component of animal microbiomes, they can also be pathogenic. The first description of the pathogenic nature of Enterococci was by Thiercelin in 1899. He described a Gram-positive diplococcus of intestinal origin that could translocate to the bloodstream and cause infection (Thiercelin, 1899; Thiercelin & Jouhaud, 1899). Before the late 20th century, pathogenic infections with Enterococci were rare. However, since the 1980s, Enterococci have emerged as a leading cause of nosocomial infections (Arias & Murray, 2012; Murray, 1990). Enterococci can cause a variety of infections and are common causes of bacteraemia, urinary tract infections, endocarditis, and infections associated with catheters and other implanted medical devices (Agudelo Higueta & Huycke, 2014). In humans, *E. faecalis* and *E. faecium* are the most common cause of pathogen infections (Murray, 1990). *E. faecalis* is more pathogenic than *E. faecium* but infections caused by *E. faecium* are harder

to treat due to the high prevalence of vancomycin resistance in this species (Kao & Kline, 2019). Thus, the severity of the infection caused by these two species is a product of their pathogenicity as well as the ease of treatment.

1.1.2 Antimicrobial resistance in Enterococci

The emergence of Enterococci as a leading cause of nosocomial infections is aided in part by their intrinsic and acquired resistance to several antibiotics. Enterococci are intrinsically resistant to cephalosporins, trimethoprim-sulfamethoxazole, and most β -lactams excluding only a limited number of penicillins and have low levels of resistance to aminoglycosides and clindamycin (Murray, 1990). In addition to this, Enterococci have acquired resistance to antibiotics such as vancomycin, chloramphenicol and tetracyclines (Hummel et al., 2007; Miller et al., 2014; Schwarz et al., 2001). High-level ampicillin resistance has been found in clinical isolates of *Enterococcus faecium* (Kristich et al., 2014).

Due to their presence in the gastrointestinal tract of humans and other organisms, Enterococci are in a good position to not only acquire but also spread antibiotic resistance genes (Palmer et al., 2010; Sommer et al., 2009). For example, high-level vancomycin resistance in *Staphylococcus aureus* is due to the interspecies transfer of a plasmid containing a vancomycin-resistance gene cluster from *E. faecalis* to *Staphylococcus aureus* (Weigel et al., 2003).

1.1.3 *E. faecalis* is a generalist pathogen

Some enterococcal species are known to associate with specific hosts, while some are found in association with multiple hosts (Lebreton et al., 2014). Of the different enterococcal species, *Enterococcus faecalis* is considered to be the most ubiquitous and most pathogenic (Eaton & Gasson, 2001). Bacteria that infect multiple different hosts are expected to differentiate into distinct lineages with each lineage having an advantage in a particular environment (Lebreton et al., 2013; Palmer et al., 2012; Richardson et al., 2018). For example, *E. faecium* is broadly divided into two clades, A and B; strains from clade A are commonly isolated from hospital infections and animals, whereas clade B strains associate with healthy humans (Pontinen et al., 2021; Zhong et al., 2019). Similarly, *Staphylococcus aureus* causes infections in humans and a diverse range of animals. The isolates responsible for these infections often preferentially associate with a single host species. Thus, *S. aureus* strains are considered to be host specialists (Guinane et al., 2010; Matuszewska et al., 2020). However, *E. faecalis* strains from diverse origins do not show any signs of host specialization. This suggests that *E. faecalis* is a generalist pathogen.

A property of *E. faecalis* that highlights its generalist nature is the observation that the *E. faecalis* pangenome consists of a large core genome that is stable between *E. faecalis* strains isolated at different times and from different origins. Clustering of strains based on contents of either core or accessory genomes in *E. faecalis* is not observed, and the size of the core and accessory genome also do not vary considerably between *E. faecalis* strains. This is in contrast to sister species *E. faecium*, which has a small core genome and shows extensive clustering based on accessory gene contents. Additionally, there is a difference in the size of the accessory genomes of *E. faecium* strains of hospital and animal origin. Human-associated *E. faecium* strains show an increase in genome size, indicating their ability to take up new genes (Zhong et al., 2019).

1.1.4 Environment plays an important role in *E. faecalis* biology

Given its generalist nature, the biology of *E. faecalis* is shaped by its interactions with its environment, and the properties that make it a successful pathogen may have been evolutionarily selected for by the stressful environments it resides in. For example, while there is no doubt that the increase in the incidence of antimicrobial resistance in Enterococci is due to the inappropriate use of antimicrobials, there is evidence that interactions with non-human hosts and environments play a considerable role in the acquisition of antibiotic resistance traits by *E. faecalis* and Enterococci. Some antibiotic resistance operons in Enterococci bear resemblance to those seen in antibiotic-producing soil organisms such as *Actinomycetes* (Li et al., 2004; Marshall et al., 1997). Furthermore, enterococcal species such as *E. casseliflavus* and *E. gallinarum* that are not commonly associated with humans are resistant to vancomycin (Palmer et al., 2012). These strains are more commonly isolated from the gut of fowl and other animals and are not a common cause of pathogenic infections. Antimicrobial resistance traits have also been identified in *E. faecalis* isolates from non-hospital settings and non-human origin (Pontinen et al., 2021).

Interactions with the environment can also play a role in the development of traits that are important for host colonization and success. A recent study found that adaptive traits such as effective colonization and resistance to environmental stressors, that enable *E. faecalis* to succeed in hospital environments, may have originated during or before the paleozoic age (Lebreton et al., 2017). Following this, Pontinen *et al.* have described the emergence of hospital-associated clusters of *E. faecalis* during the mid-19th century, before the wide-spread introduction of antibiotics (Pontinen et al., 2021). In addition to this, the presence of important virulence factors of Enterococci such as cytolysin is also not limited to clinical strains and has been found in strains of environmental and animal origin (La Rosa et al., 2015). Finally, genes

that are responsive to commonly encountered stressors such as heat and salt stress can also function as important virulence factors in Enterococci (de Oliveira et al., 2011).

1.2 *E. faecalis* cell envelope

The bacterial cell envelope provides protection from the external environment and confers strength and rigidity to the cell. The *E. faecalis* cell envelope is similar to that found in other Gram-positive bacteria. The core structure of the *E. faecalis* cell envelope consists of a thick peptidoglycan cell wall surrounding a single phospholipid bilayer that forms the cell membrane (**Figure 1.1**) (Hancock et al., 2014). Embedded in the cell wall and membrane are anionic cell surface polymers called teichoic acids (Hancock et al., 2014). There are two types of teichoic acids, lipoteichoic acids (LTA) which are anchored to the phospholipid membrane, and wall teichoic acids (WTA), which are covalently linked to the peptidoglycan. The LTA in *E. faecalis* is composed of a glycerol phosphate polymer with kojibiose substitutions (a disaccharide of 1,2-linked glucose) and is attached to the cell membrane by a glycolipid anchor. WTA has a more complex structure and consists of glucose, galactose, N-acetyl galactosamine, N-acetyl glucosamine, and ribitol phosphate units. Both LTA and WTA are decorated with D-alanine (Neuhaus & Baddiley, 2003). *E. faecalis* also expresses a rhamnopolysaccharide on its surface, called the Enterococcal Polysaccharide Antigen (EPA). The EPA is made up of a rhamnan backbone to which teichoic acids are covalently linked (Guerardel et al., 2020).

Other components of the *E. faecalis* cell envelope not directly relevant to this thesis include cell surface-associated proteins, lipoproteins, microbial surface components recognizing adhesive matrix molecules, and the enterococcal capsular polysaccharides (Hancock et al., 2014).

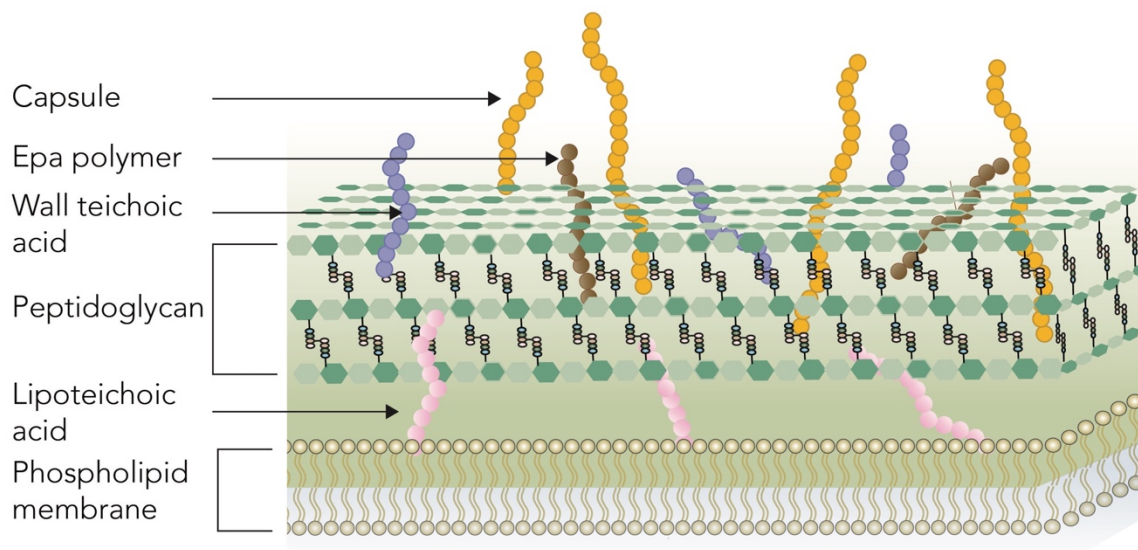


Figure 1.1 The enterococcal cell envelope

The enterococcal cell envelope is made up of a thick layer of peptidoglycan. Bound to the peptidoglycan are wall teichoic acids, the enterococcal polysaccharide antigen and the capsule. Underneath the peptidoglycan is the phospholipid bilayer to which lipoteichoic acids are bound.

1.2.1 Cell envelope stress response in *E. faecalis*

Because *E. faecalis* lives in many different environments, this bacterium needs to be responsive to a wide range of stresses both abiotic and host-derived. The bacterial cell envelope is the first line of defense against these stresses; monitoring changes in cell envelope integrity, and adapting in response to them, is vital for bacterial survival. The ability of bacteria to sense and adapt in response to changes in the environment is often mediated by signal transduction systems such as two-component systems (TCS) (Stock et al., 2000).

Two component signalling systems are ubiquitous in bacteria (Hoch, 2000). They consist of a sensor histidine kinase (HK) that senses changes in the external environment and a cytoplasmic response regulator (RR) that modulates gene expression (Hoch, 2000; Kellogg & Kristich, 2018; Zschiedrich et al., 2016). Upon sensing a change in environmental stimuli, the

sensor kinase auto phosphorylates on a conserved histidine residue. This phosphoryl group is then transferred to a conserved aspartate residue on the response regulator. The phosphorylated regulator can now bind to the promoters of target genes and activate or repress their transcription. Sensor kinases can also act as a phosphatase and dephosphorylate their cognate response regulators (Hoch, 2000; Kellogg & Kristich, 2016). The balance between kinase and phosphatase activities of the sensor kinase determines the outcome of the TCS. Thus, if there more kinase activity of the sensor kinase there is greater downstream activation of the target genes whereas, the opposite is true if there is greater phosphatase activity of the kinase.

In *E. faecalis*, 17 sensor kinase-response regulator pairs have been identified based on sequence homology to TCSs in *B. subtilis* and *E. coli* (Hancock & Perego, 2002). Of these, the *croRS*, *yxdJK*, and the *liaFSR* regulatory systems play an important role in regulating the cell envelope stress response of *E. faecalis*. These systems were initially characterized to be important in mediating the response to cell surface targeting antibiotics (Hancock & Perego, 2004; Le Breton et al., 2003; F. Teng et al., 2002). However, it is now known that these TCS regulate bacterial response to a wide range of cell envelope stresses including the action of the host immune response (Cardona et al., 2018; Khan et al., 2019; Muller et al., 2008; Rincon et al., 2019; Werth et al., 2014). In addition to these three systems, the *vanRS* regulatory system that is responsible for high-level vancomycin resistance may also form a part of the cell envelope stress response in *E. faecalis* (Arthur & Courvalin, 1993). However, the *E. faecalis* strain used in our study lacks this system and thus, we will not be discussing its role in cell envelope stress response.

1.2.2 YxdJK and the bacitracin resistance network

The *yxdJK* two-component signalling system is homologous to the *bceRS* TCS found in *Bacillus subtilis* (Gebhard et al., 2014; Mascher, 2006). Based on the specific architecture of the YxdK and BceS histidine kinases, they are included among the intra-membrane sensing HKs (IM-HK). IM-HKs have a short N-terminal domain with a small extracellular loop, <25 amino acids, between two transmembrane helices (Mascher, 2006; Mascher et al., 2003). Because this loop is so short, it is believed that they cannot sense specific external stimuli. In Firmicutes, IM-HK containing TCS can often be found in association with a neighbouring ABC transporter whose activity is regulated by the TCS (Dintner et al., 2011; Mascher et al., 2003). In these systems, the extracellular domain of the permease component of the ABC transporter is important for sensing the signal, which is then relayed to the histidine kinase. Activation of the sensor histidine kinase in turn results in the transcription of the operon encoding the ABC transporter (Bernard et al., 2007).

BceRS systems are often involved in the efflux of peptide antimicrobial compounds out of the cell. The ABC transporters associated with these TCS are believed to be involved in both the sensing of stimuli and the detoxification of these peptides (Dintner et al., 2011; Mascher et al., 2003; Rietkotter et al., 2008; Yoshida et al., 2011). In *E. faecalis*, YxdJK has been found to interact with more than one ABC transporter: a BceAB-type ABC transporter, and a second ABC transporter, YxdLM (Gebhard et al., 2014). These three components (YxdJK, BceAB and YxdLM) form the bacitracin regulatory network in *E. faecalis* (**Figure 1.3**). The organization of this network differs from that observed in *B. subtilis* in two ways. First, YxdJK interacts with two different ABC transporters, and secondly, neither of these transporters are genetically linked to it (Gebhard et al., 2014). This lack of genetic linkage is not unique to *E. faecalis* and the presence of regulatory networks where TCSs interact with ABC transporters encoded at a different location on the chromosome has been observed in both *Staphylococcus*

aureus and *Listeria monocytogenes* (Hiron et al., 2011; Jiang et al., 2019; Yoshida et al., 2011). When BceRS-type TCSs regulate the expression of more than one ABC transporter, it is believed that the signalling and detoxification roles are split between the two ABC transporters (Hiron et al., 2011). In *E. faecalis*, it has been experimentally shown that BceAB is important for the sensing of stimuli, whereas YxdLM is essential for the resistance to bacitracin (Gebhard et al., 2014). However, it is unclear how YxdLM, the ABC transporter responsible for bacitracin resistance exerts its effect.

Although termed the bacitracin regulatory network, the expression of *yxdJK* is upregulated in response to a number of different cell wall damaging stressors (Gebhard et al., 2014). Given the non-specific nature of the substrates activating this system and its localisation in the cytoplasmic membrane, there is a possibility that in addition to being responsive to bacitracin, this TCS also senses and responds to membrane damage. As well as its two ABC transporters, YxdJK also regulates the expression of the *dlt* operon and *mprF* (Morris et al., 2022; Prater et al., 2019).

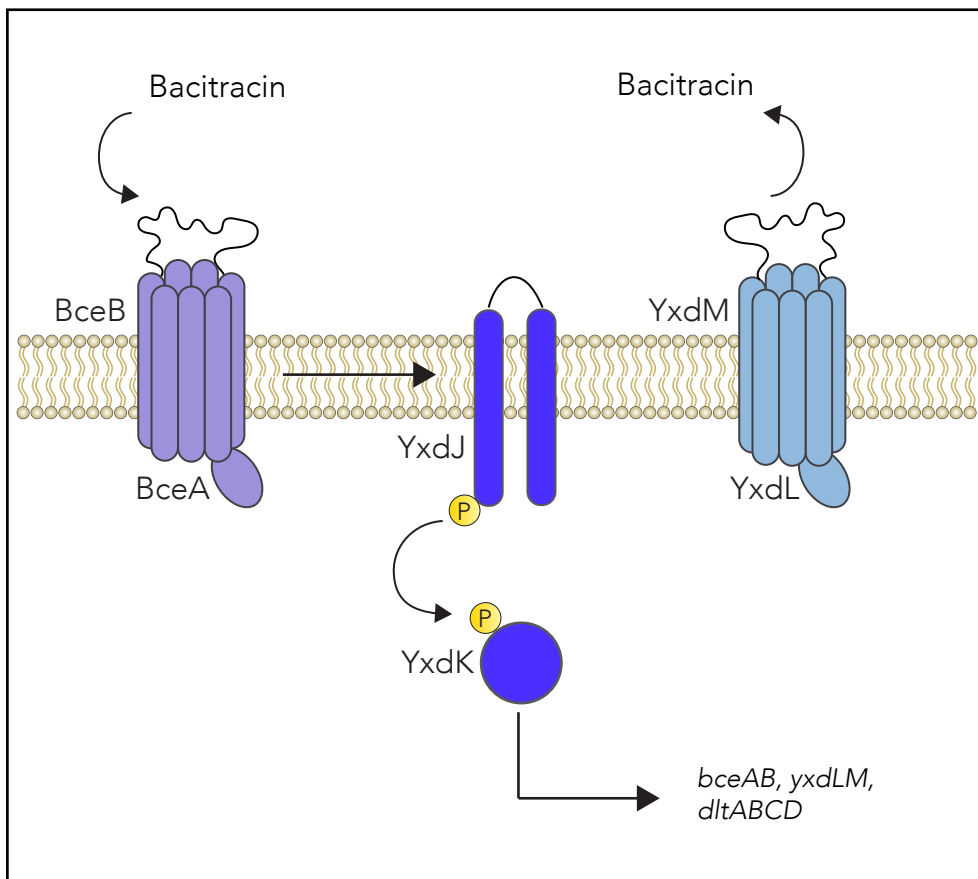


Figure 1.2 The bacitracin regulatory network in *E. faecalis*

Bacitracin is sensed by the ABC transporter permease protein BceB and this signal is relayed to the YxdJK two-component system resulting in its activation. Activation of this system results in the upregulation of the ABC transporters *bceAB* and *yxdLM* as well as the *dltABCD* operon. YxdLM is believed to be involved in the efflux of bacitracin to the cytoplasm. In addition to the action of bacitracin, the YxdJK two-component system is also upregulated in response to cell membrane stress. However, the activating signal for this is unclear as it is not believed to be mediated through BceAB.

1.2.3 The CroRS two-component system

CroRS stands for ceftriaxone resistance response regulator and sensor kinase, and as the name suggests, this two-component system plays an important role in the intrinsic resistance of *E. faecalis* to certain β -lactams (Comenge et al., 2003; Djoric & Kristich, 2015; Hancock & Perego, 2004). Disruption of the CroRS TCS leads to a 4000-fold increase in sensitivity to ceftriaxone. In response to the other β -lactams, *croRS* mutants show increased sensitivity to second and third-generation cephalosporins but not to first-generation cephalosporins or ampicillin (Comenge et al., 2003). This system is also involved in the response to other cell envelope targeting antibiotics such as teixobactin, bacitracin and vancomycin (Darnell et al., 2019). However, it is not clear whether CroRS is important for tolerance or resistance in this context, as conflicting roles have been described (Darnell et al., 2019; Hancock & Perego, 2004). CroRS is important for bacterial virulence and mutants for this TCS show attenuated virulence and impaired macrophage survival (Muller et al., 2018; Muller et al., 2008). CroRS is also involved in cell wall biosynthesis, as changes in the expression of this system can impact cell morphology and lead to growth impairments (Le Breton et al., 2003).

The expression of the CroRS TCS is upregulated in response to a wide range of cell envelope damaging agents including bile, sodium dodecyl sulphate, oxidative stress and cell surface targeting antibiotics (Darnell et al., 2019; Djoric & Kristich, 2015; Muller et al., 2018; Solheim et al., 2007). CroRS can also be cross-regulated by other sensor kinases; VanRS and CisRS have been shown to interact with and regulate the expression of CroRS (Jiang et al., 2022; Kellogg & Kristich, 2016). However, not all strains of *E. faecalis* contain the CisRS and VanRS systems and as a result, this cross-regulation could be the result of specialisation to a particular environment. In addition to this, penicillin binding protein and serine-threonine kinase associated (PASTA) kinases, a type of eukaryotic like serine-threonine kinase can also regulate the expression of this system. Increased phosphorylation of CroS by the PASTA

kinase Irek, has been observed under conditions of cell-wall stress (**Figure 1.3**)(Kellogg & Kristich, 2018). However, while the external stimuli that activate the expression of this system are known, there is limited knowledge regarding the CroRS regulon. In addition to regulating its own expression, only three other genes have been experimentally confirmed to be regulated by the CroRS TCS; *salB*, a secreted protease; *glnQ*, the first gene of a predicated glutamine transport system and *pbp5*, a low-affinity penicillin binding protein (Comenge et al., 2003; Kellogg & Kristich, 2016; Muller et al., 2006; Muller et al., 2018). SalB is a secreted protein that is important for cell wall biosynthesis and cell morphology, but it does not contribute to CroRS-associated cephalosporin resistance, whereas, pbp4 has been recently described to contribute to cephalosporin resistance in *E. faecalis* (Breton et al., 2002; Le Breton et al., 2003; Muller et al., 2006). However, pbp5 is not responsible for the complete resistance phenotype observed in *croRS* mutants (Timmler et al., 2022).

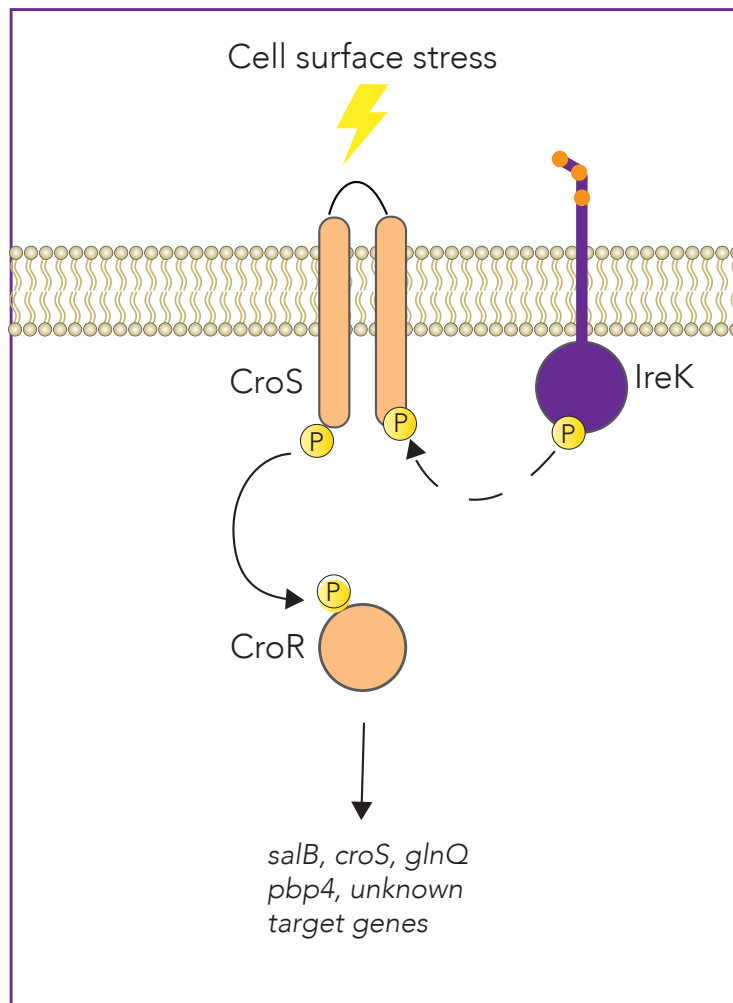


Figure 1.3 The CroRS two-component system

The CroRS two-component system is activated in response cell surface stress and results in the expression of downstream target genes. CroR-mediated gene expression can also be mediated via the PASTA kinase IreK which phosphorylates a conserved threonine on CroS in response to cell wall stress.

1.2.4 The LiaFSR three-component system

The third regulatory system that plays an important role in the cell envelope stress response in *E. faecalis* is the LiaFSR three-component system. This system consists of the TCS LiaRS, in which LiaS, an intra-membrane sensing HK, and LiaR, its cognate response regulator, are in association with LiaF, a membrane-embedded accessory protein (Jordan et al., 2008). LiaF represses the activity of the TCS by inhibiting LiaS in the absence of membrane stress (**Figure 1.4**). LiaF is conserved in all Firmicutes that have a LiaRS TCS, and this system has been well characterized in *B. subtilis* (Eldholm et al., 2010; Jordan et al., 2006; Suntharalingam et al., 2009). In *E. faecalis*, *liaFSR* regulates its own expression as well as that of a separate operon, *liaXYZ* (Khan et al., 2019; Ota et al., 2021). Recently, it has been found that LiaX negatively regulates the expression of the LiaFSR system and this may be dependent on LiaYZ (Khan et al., 2019). However, LiaX can only exert its effect in the presence of a functional LiaFSR system, highlighting the complex regulation of this cell envelope stress response system.

The expression of the *liaFSR* system is upregulated in response to a wide range of cell envelope damaging agents including antibiotics and antimicrobial peptides. It is weakly activated in response to alkaline stress, surfactants, ethanol and detergents (Jordan et al., 2008; Jordan et al., 2006; Mascher et al., 2004). This system is important for resistance to a wide range of antibiotics and antimicrobial peptides. *E. faecalis* strains that lack a functional LiaFSR system show increased sensitivity to a number of antimicrobials including daptomycin, nisin, gallindermin, merscadin, friulimicin, bacitracin, and telavancin (a semi-synthetic derivative of vancomycin), as well as the human antimicrobial peptides, LL-37 and defensin B (Reyes et al., 2015). Due to its importance in response to lipid II cycle inhibitors such as bacitracin, nisin and vancomycin, this system is called the lipid II interacting antibiotic response regulator and sensor (LiaRS) (Jordan et al., 2006).

The LiaFSR system plays a particularly important role in daptomycin resistance and mutations in this system have been associated with clinical resistance to daptomycin (Arias et al., 2011; Ota et al., 2021). As a result, the physiological changes driven by this system in response to cell membrane stress have been well characterized. Mutations in this system affect the binding of daptomycin to the cell surface (Tran et al., 2013). Under normal conditions, daptomycin concentrates at the septum of *E. faecalis* cells; however, in strains that have a constitutively active LiaFSR system, binding is distributed over the cell surface. This non-specific binding and reduced efficacy of daptomycin is due to the redistribution of the cardiolipin domains away from the cell division machinery (Reyes et al., 2015; Tran et al., 2013). However, this is not the only mechanism through which this system promotes resistance to daptomycin. LiaX, whose expression is regulated by LiaFSR, has been shown to directly bind daptomycin forming a daptomycin-LiaX complex (Khan et al., 2019). The formation of this complex results in sequestration of the antibiotic, thereby protecting the cell membrane from its action. LiaFSR can also upregulate the expression of the *dlt* operon via the YxdJK TCS in response to the action of daptomycin (Morris et al., 2022).

While most studies of involving the LiaFSR system have focused on the mechanisms of daptomycin resistance, as this system is responsive to several cell envelope stresses, it is plausible that similar mechanisms are used to respond to stresses such as host immune responses. Supporting this idea, reduced binding of mammalian cationic antimicrobial peptide LL-37 has been observed in strains with a constitutively active LiaFSR system (Werth et al., 2014). Additionally, LiaX has also been shown to bind to LL-37 and the formation of this complex leads to cell membrane remodelling (Khan et al., 2019). Strains that have a disrupted LiaFSR system are also attenuated in their virulence in a nematode model of infection and are more susceptible to neutrophil-mediated killing (Reyes et al., 2015; Rincon et al., 2019).

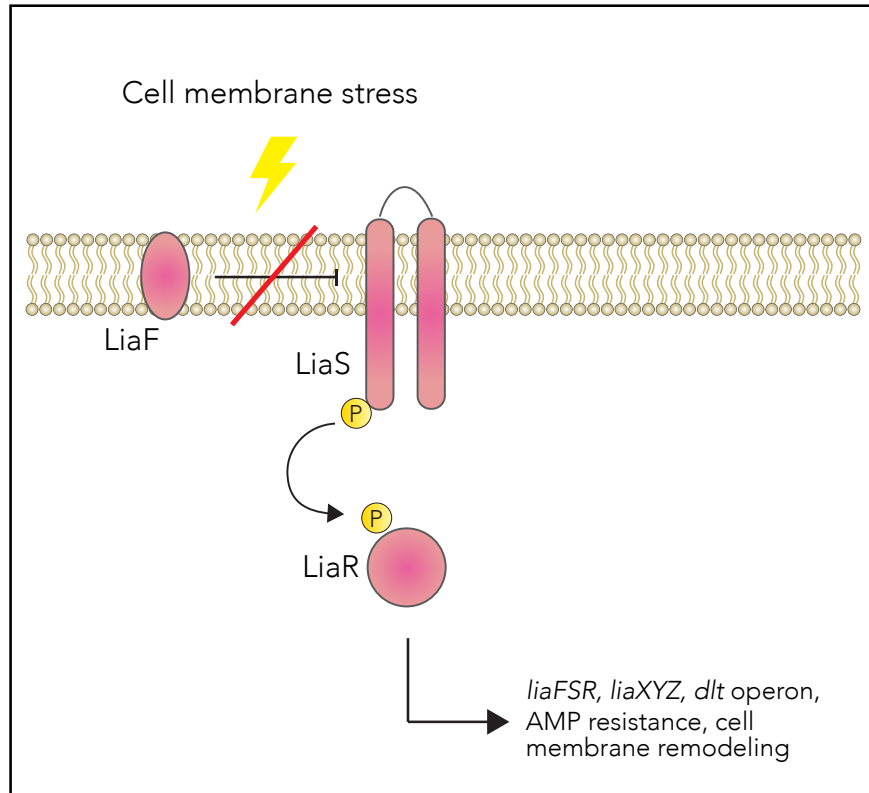


Figure 1.4 The LiaFSR three-component system

In the absence of cell membrane stress, the three-component signalling system, LiaFSR, is in the 'OFF' state due to the inhibition of LiaS by LiaF. Under conditions of cell membrane stress, LiaF no longer inhibits LiaS. LiaS responds to membrane stress by phosphorylating its cognate response-regulator, LiaR which regulates downstream expression of genes.

1.2.4.1 The cell surface as a determinant for bacterial virulence and antimicrobial resistance

The bacterial surface is the target of host and antimicrobial action and changes in bacterial surface properties can lead to reduced killing by the host immune response and altered susceptibility to antimicrobials. As a result, several genes that encode for bacterial surface properties have been characterised as virulence factors and antimicrobial determinants. These include genes coding for proteins that regulate surface charge, membrane fluidity and those involved in the biosynthesis of lipoteichoic acid and the EPA.

Bacteria can alter the electrostatic properties of their cell surface in response to cell envelope perturbations, typically reducing the negative charge of the cell surface to reduce interaction with cationic antimicrobials or host effectors. In Gram-positive bacteria, two distinct mechanisms that reduce the negative charge of the cell surface have been identified. The first is through modification of the negatively charged membrane lipid phosphatidylglycerol (PG) with positively or neutrally charged amino acids (Peschel et al., 2001). The addition of amino acids such as lysine to PG to form lysyl-phosphatidylglycerol (Lys-PG) results in the reduction of the net negative surface charge of bacterial membranes (Peschel et al., 2001; Roy & Ibba, 2008). This process is mediated by the protein MprF (multiple peptide resistance factor), which is a bifunctional protein with a synthase and flippase domain (Ernst et al., 2015). The synthase domain of this protein mediates the transfer of amino acids from aminoacyl-tRNA to PG, and the flippase translocates aminoacyl-PG from the inner leaflet to the outer leaflet of the cytoplasmic membrane. Due to its role in reducing the net negative charge of the membrane, MprF is important for bacterial resistance to cationic antimicrobial peptides of diverse origins. By promoting evasion of killing by host cationic antimicrobial peptides, MprF can function as a virulence factor during infection (Ernst & Peschel, 2011; Nowak et al., 2021; Peschel et al., 2001; Thedieck et al., 2006).

Enterococcus faecalis has two predicted MprF proteins, MprF 1 and MprF 2 (Bao et al., 2012). Of these, MprF 2 is functionally similar to the MprF protein found in *S. aureus*, and has been found to regulate the aminoacylation of PG (Bao et al., 2012). Mutations in MprF2 are associated with increased sensitivity to cationic antimicrobial peptides, enhanced neutrophil-mediated killing, increased biofilm formation, and increased opsonophagocytic killing (Bao et al., 2012; Kandaswamy et al., 2013; Rigottier-Gois et al., 2011). However, it is unclear whether MprF2 functions as a virulence factor; Bao *et al.* have shown that mice infected with *mprF2* mutants show similar bacterial burdens to those infected with the control strain (Bao et al., 2012). However, Rigottier-Gois *et al.* have observed that in a *Galleria* model of infection, strains lacking a functional MprF2 protein have attenuated virulence (Rigottier-Gois et al., 2011). This difference could be the result of differences in strain background between the two studies. In *E. faecium*, the activity of MprF2 is regulated by the TCS YxdJK which is activated in response to cell envelope stress (Prater et al., 2019). However, it is unclear if the same is true in *E. faecalis*.

The second mechanism used by Gram-positive bacteria to alter their electrostatic properties involves the D-alanylation of teichoic acids by proteins encoded by the *dlt* operon. The addition of positively charged D-alanine esters on the negatively charged teichoic acids confers a net-positive charge on the teichoic acids (Neuhaus & Baddiley, 2003). The *dlt* operon encodes four to five proteins (Dlt(X)ABCD); DltABCD are conserved in all low-GC, Gram-positive bacteria (Kovacs et al., 2006; Reichmann et al., 2013). Similar to MprF, bacterial strains mutant for any component of this operon show defects in virulence, increased sensitivity to cationic antimicrobial peptides, enhanced killing by neutrophils and decreased adherence to host cells (Coupri et al., 2019; Fabretti et al., 2006; Kamar et al., 2017; Kovacs et al., 2006; Kristian et al., 2005; Peschel et al., 1999). In Enterococci, the activity of this operon is regulated by the LiaFSR and YxdJK TCS (Gilmore et al., 2020; Prater et al., 2019; Zeng et al., 2022).

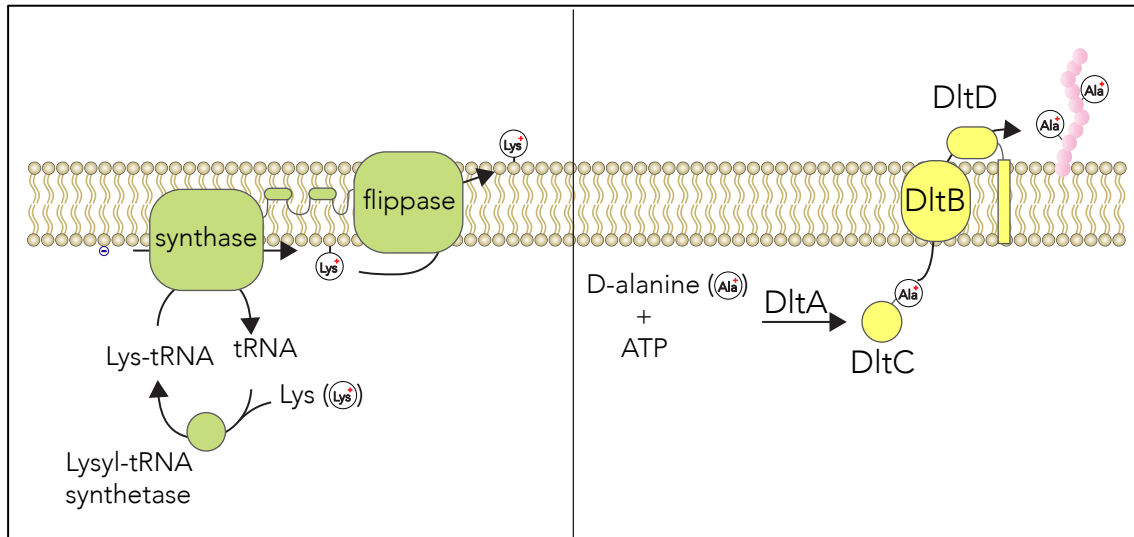


Figure 1.5 Mechanisms of *E. faecalis* surface charge regulation

E. faecalis can alter its surface charge using two distinct mechanisms. The first involves the MprF protein (left panel). MprF is involved in the addition of positively charged amino groups such as lysine to the bacterial phosphatidylglycerol in the inner leaflet of the cytoplasmic membrane and in the flipping of this positively charged phosphatidylglycerol to the outer leaflet of the cytoplasmic membrane. The second mechanism used by *E. faecalis* to alter its surface charge is through D-alanylation of teichoic acids by the *dltABCD* operon (right panel). DltA catalyses the ligation of D-alanine to the carrier protein DltC in the presence of ATP. From DltC, the D-alanine is believed to be transferred to an intermediate and translocated across the cytoplasmic membrane with the help of DltB. DltD aids in the the final transfer of D-alanine to the LTA.

In addition to D-alanylation, other modifications of the LTA can also have an impact on bacterial virulence and resistance to antimicrobials (Fedtke et al., 2007; Grundling & Schneewind, 2007; Hesser et al., 2020; Mello et al., 2020). In *E. faecalis*, the glycolipid moiety that anchors the LTA to the cell membrane is synthesized via a two-step process (Theilacker et al., 2009; Theilacker et al., 2011). The first step involves the glycosylation of diacylglycerol (DAG) by the enzyme BgsB to form monoglucosyldiacylglycerol (MGlcDAG). The second enzyme, BgsA then adds an additional glucose moiety to MGlcDAG to form diglucosyldiacylglycerol (DGlcDAG) which is the glycolipid anchor for LTA in *E. faecalis*.

Disruption of *bgsA* results in increased accumulation of the DGlcDAG precursor molecule, MGlcDAG (Theilacker et al., 2009). Disruption of *bgsB*, on the other hand, results in strains having no glycolipids or glycolipid derivatives (Theilacker et al., 2011). Deletion mutants of *bgsA* and *bgsB* display impaired adherence to host cells, poor biofilm formation, and longer LTA chains (Theilacker et al., 2009; Theilacker et al., 2011). The changes in the composition of the glycolipid anchor also have an impact on *E. faecalis* virulence. Increased LTA chain length results in a stronger host immune response. As a result, these *bgsA* and *bgsB* mutants show enhanced clearance from hosts. However, this strong immune response has also been associated with worse host outcomes due to uncontrolled immune activation (Diederich et al., 2014; Theilacker et al., 2015).

Changes in the enterococcal polysaccharide antigen (EPA) can also result in altered virulence and antimicrobial resistance in *E. faecalis*. EPA is a cell wall polymer made up of primarily glucose, rhamnose with small quantities of galactose, N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) (Guerardel et al., 2020; Korir et al., 2019). The synthesis of this polymer is directed by the *epa* locus, made up of a core region (*epaA-R*) that is conserved between *E. faecalis* strains and a variable region (Palmer et al., 2012; Xu et al., 1998; Xu et al., 2000). The core region encodes the rhamnan backbone of this polymer, whereas, the

variable region codes for the decorations on the EPA. These decorations are surface exposed while the rhamnan backbone is buried in the cell wall. It has been recently identified that these EPA decorations are teichoic acids that are cross-linked to the rhamnan backbone (Guerardel et al., 2020).

Mutations in core genes of the *epa* operon result in changes in the polysaccharide content of these polymers. For example, mutations in *epaQ* result in an extensive reduction of rhamnose from EPA (Korir et al., 2019; Teng et al., 2009). As EPA is hypothesized to be involved in stabilizing the cell envelope, EPA deletion mutants have altered cell shape (Dale et al., 2015; Korir et al., 2019). Several studies have implicated EPA as an important virulence determinant in *E. faecalis*. Disruption of genes in the *epa* operon result in attenuation of *E. faecalis* virulence in different models of infection, enhanced phagocytosis and killing by neutrophils, reduced translocation of bacteria across epithelial cells, defects in biofilm formation and architecture, and attachment defects (Ramos et al., 2019; Rigottier-Gois et al., 2014; Singh et al., 2009; Fang Teng et al., 2002; Teng et al., 2009; Xu et al., 1998; Xu et al., 2000; Zeng et al., 2004). Disruption of this operon is also linked to an increase in resistance to cephalosporins. This is believed to be due to increased signalling through the croRS TCS, but no evidence for this has been reported (Korir et al., 2019; Smith et al., 2019).

Finally, changes in bacterial membrane fluidity have also been associated with altered resistance to the antibiotic daptomycin. Daptomycin-resistant strains of *E. faecalis* have a more rigid cell membrane (Arias et al., 2011; Mishra et al., 2012). These strains have altered composition of the cell membrane phospholipids, with a decrease in the abundance of PG (Mishra et al., 2012; Tran et al., 2013). Some daptomycin-resistant strains also show diversion of cardiolipin enriched domains away from the cell division septum (Tran et al., 2013). Changes in the localisation of these lipid microdomains results in the diversion of daptomycin away from the cell division machinery, leading to reduced bacterial killing (Tran et al., 2013).

1.3 Enterococci and insects

Due to the high level of conservation of the innate immune response between vertebrates and invertebrates, insects have been widely used to study host-pathogen interactions. These studies have primarily made use of insect hosts such as the wax moth and the fruit fly to identify new virulence factors of Enterococci (Cox & Gilmore, 2007; Gaspar et al., 2009; Holt et al., 2015; Martino et al., 2018; Park et al., 2007; Rigottier-Gois et al., 2011; Teixeira et al., 2013). However, in addition to being used as a tool to identify bacterial virulence factors, insects can also be used to study natural interactions between Enterococci and non-human hosts. Enterococci have been found to interact with a wide range of insects. In 1972, Martin and Mundt, described the association of Enterococci with 213 different insects that had been isolated during a 14-month period, including *Diptera*, *Lepidoptera*, *Coleoptera*, and *Hymenoptera* (Martin & Mundt, 1972). Of the different enterococcal species identified, *E. casseliflavus* was found to be the most prevalent, while *E. faecalis* was the second most common. Further highlighting the close association between *E. faecalis* and insects, a study looking at *E. faecalis* colonisation of the fruit fly by Cox & Gilmore, identified that irrespective of the origin of *Drosophila*, wild or laboratory reared, *E. faecalis* was found to colonize almost every fly stock tested (Cox & Gilmore, 2007). *E. faecalis* is not only a gut commensal in insects, but is also capable of causing systemic infection. *E. faecalis* has been isolated from the hemolymph, the equivalent of blood, of wild *Drosophila*, and has been cultured from mites that feed on hemolymph of honeybees (Lazzaro et al., 2006; Pakwan et al., 2017). Additionally, gut dysbiosis in insects can also result in the translocation of *E. faecalis* from the gut of insects into circulation, similar to what is observed in humans (Mason et al., 2011).

Since *E. faecalis* is a generalist that shows little host specificity, its interactions with insect hosts can influence its biology in ways that are relevant during human infection. This influence is not necessarily limited to the selection of virulence factors or evasion strategies that work

in the same way in different hosts. For example, in response to systemic infection, the insect immune response exerts its action via antimicrobial effector peptides that interact with and kill bacteria directly (Eleftherianos et al., 2021). While the mechanism used by most of these antimicrobial effector molecules to kill bacteria are not fully elucidated, it is known that some share common mechanisms of action with mammalian antimicrobial peptides and clinically used antimicrobials. This includes insect cecropins, which function like mammalian defensins and colistin (Eleftherianos et al., 2021; Ledger et al., 2022). These molecules bind and disrupt bacterial membranes through electrostatic interaction. Thus, there is a possibility that interactions with insect immune systems can also result in altered susceptibility to clinically used antimicrobials. However, until the work reported in this thesis, this had not been demonstrated.

Work in this thesis is focused on understanding the pressures encountered by *E. faecalis* in an insect host, the fruit fly *Drosophila melanogaster*, as a way of understanding the mechanisms of insect immunity and the mechanisms by which bacteria might escape immune killing. This would provide greater understanding of the ways insect immune responses have shaped the biology of this clinically-important microbe.

1.3.1 The *Drosophila melanogaster* immune response to *E. faecalis* infection

The fruit fly *Drosophila melanogaster* (*D. melanogaster*) is a natural host of the bacterium *E. faecalis* (Cox & Gilmore, 2007; Lazzaro et al., 2006). As a model organism, the immune response of *Drosophila* has been extensively studied. In response to *E. faecalis* systemic infection, three arms of the *Drosophila* immune response are now known to play a role: humoral responses, cellular responses, and the melanisation cascade.

1.3.1.1 Role of the Toll pathway during *E. faecalis* infection

The humoral immune response in *Drosophila* is mediated by the fat body and involves the activation of NF- κ B transcription factors by the Immunodeficiency (Imd) and Toll pathways (Lemaitre et al., 1995). The *Drosophila* humoral immune response to *E. faecalis* is mediated via the Toll pathway (Rutschmann et al., 2002). This pathway is activated upon detection of Lys-type peptidoglycan, predominantly found in Gram-positive bacteria, or β -1,3-glucan, a component of fungal cell walls, by the pattern recognition proteins peptidoglycan recognition protein (PGRP)-SA and gram-negative binding protein-1 (GNBP-1) (**Figure 1.6**) (Gobert et al., 2003; Michel et al., 2001). The Toll pathway can also be activated in response to microbial proteases (El Chamy et al., 2008; Issa et al., 2018). Detection of microbial cell wall components and proteases results in an extracellular proteolytic cascade which culminates in the activation of the Spätzle Processing Enzyme (SPE). The activated SPE subsequently cleaves Spätzle (spz) which can now bind to the Toll receptor (Weber et al., 2003). Activation of the Toll receptor results in the assembly of a multimeric complex, which includes the adaptor proteins MyD88 and Tube, and the serine-threonine kinase, Pelle (Lemaitre et al., 1996; Towb et al., 1998). Formation of this complex induces phosphorylation and subsequent degradation of the I κ B-like Cactus. The NF- κ B-like transcription factor, Dorsal-related Immunity Factor (Dif), is then released from inhibition and can translocate from the cytoplasm to the nucleus and regulate the expression of immune responsive genes such as antimicrobial peptides and other immune effectors (Ip et al., 1993; Manfrulli et al., 1999). Of the different immune effectors secreted as a result of Toll pathway activation, the Bomanins are known to be the most important for the immune response against *E. faecalis* (Clemmons et al., 2015).

1.3.1.2 Bomanins are the primary immune effectors during *E. faecalis* infection

The Bomanins are a family of twelve secreted peptides that are essential for the immune response against *E. faecalis* and fungi (Clemmons et al., 2015). Several members of this family were first described in a screen for peptides induced as result of immune response and were termed immune-induced molecules (Uttenweiler-Joseph et al., 1998). These immune-induced molecules were finally characterized in the year 2015 by Clemmons and colleagues, who termed these peptides as the ‘Bomanins’ after Hans Boman, who carried out ground-breaking work in the field of insect immunity (Boman et al., 1972; Clemmons et al., 2015). They showed that this family of peptides is specific to the *Drosophila* genus, and thus represents a relatively young family of effectors. Given the specificity with which they are needed for *E. faecalis* infection, there is a possibility that this family of peptides may have arisen due to repeated interactions with this bacterium.

The Bomanins lack similarity to other *Drosophila* antimicrobial peptides and are instead defined by a conserved CXXC motif. Three types of Bomanins exist, each type carries 1-2 copies of a 16-AA long motif that consists of the CXXC region surrounded by a region of high amino acid (AA) conservation. The first are short-form Bomanins, whose mature peptide length is 16 to 17 AA. There are six short-form Bomanins; they exhibit very high levels of sequence conservation, having only two to three variable amino acids in the CXXC region. The second type of Bomanins are tailed Bomanins. These peptides consist of the Bomanin motif followed by a C terminal extension that is 15 to 82 AA long. The *Drosophila* genome encodes three tailed Bomanins. The third type is the bicipital Bomanins. They are made up of two Bomanin motifs at each end joined by a linker region that is 43 to 103 AA long. Bicipital Bomanins exhibit lower sequence conservation in the Bomanin motif compared to the short-form Bomanins, and the linker shows no obvious conservation. Ten of the twelve Bomanins are located on the second chromosome at cytogenic position 55 in the Bom55C cluster. Flies

lacking this cluster (*Bom*^{55CA}) are unable to mount an effective immune response against *E. faecalis* infection. The survival outcomes of *Bom*^{55CA} flies when challenged with *E. faecalis* are identical to those observed with Toll pathway mutants, indicating that the loss of these peptides is equivalent to the loss of the entire pathway (Clemmons et al., 2015). It was also recently shown that no other characterized *Drosophila* antimicrobial peptide is important for the immune response against *E. faecalis* (Hanson et al., 2019).

Bombardier (*bbd*) is another gene strongly expressed upon Toll pathway activation. Similar to the Bomanins, it has orthologs only in the *Drosophila* genus (Lin et al., 2019). Analysis of hemolymph from flies deficient for *bbd* (*bbd*Δ) show that these flies do not produce any short-form Bomanins. However, transcript levels of Bomanins are the same in these flies compared to wild-type (WT) flies. Thus, *bbd* is believed to be essential for the post-transcriptional processing of short-form Bomanins, but its exact role in this process is unclear. *bbd*Δ flies infected with *E. faecalis* show defects in both resistance and tolerance of infection, some flies die with a lower bacterial burden than that observed in wild-type (WT) flies, while some die with the same bacterial burden (Lin et al., 2019). The observed defect in resistance to *E. faecalis* infection is likely to be due to the absence of short-form Bomanins in the hemolymph, whereas, the defects in tolerance may be the consequence of increased accumulation of short-form Bomanins in the fat body. Supporting this idea, infection of *bbd*Δ flies with heat-killed *E. faecalis* also results in poor survival outcomes and this survival defect is rescued when both *bbd* and the *Bomanins* are absent from the flies.

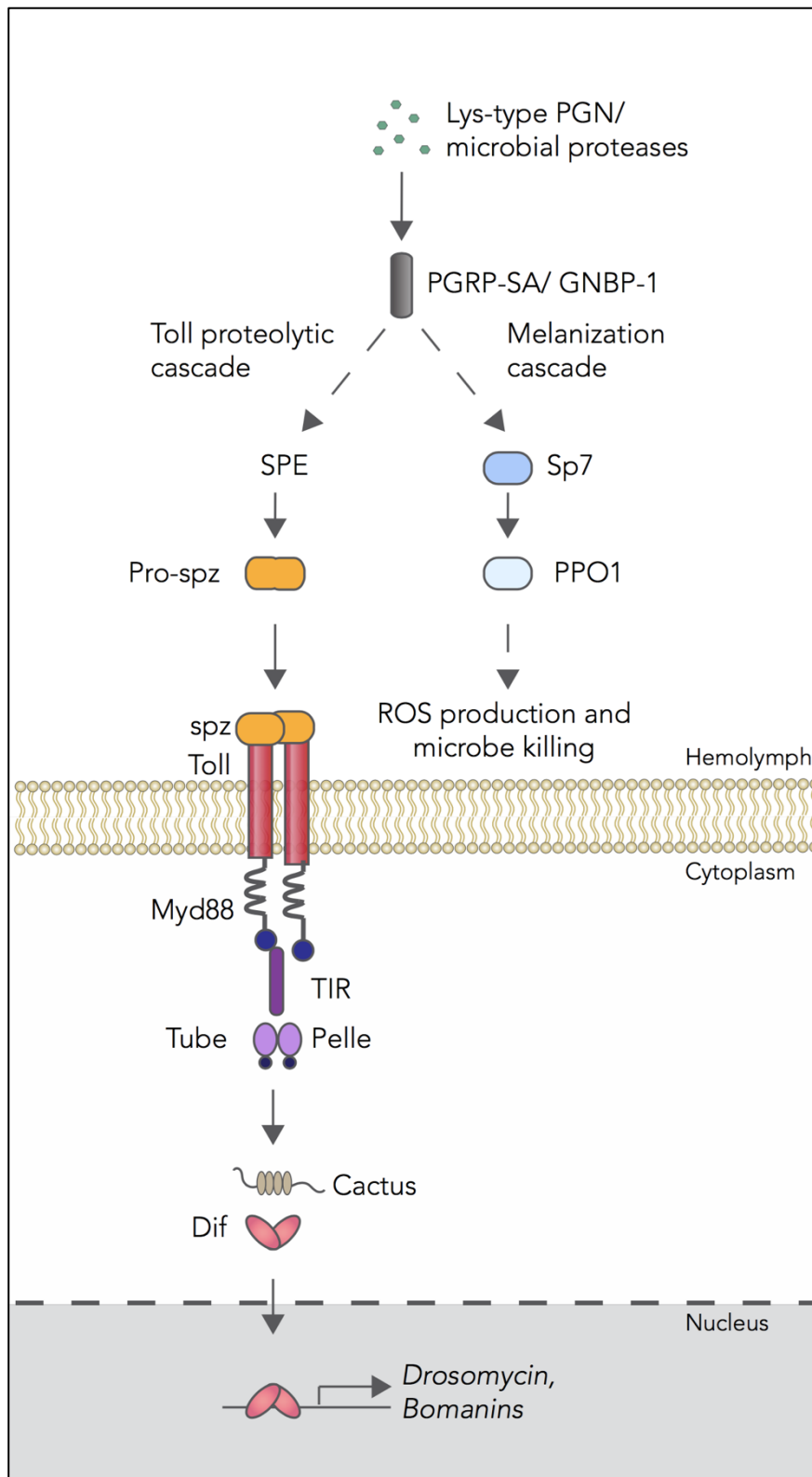


Figure 1.6 The Toll pathway and the melanization cascade

Recognition of Lys-type peptidoglycan or microbial proteases by pattern recognition proteins, PGRP-SA and GNBP-1 results in the initiation of two extracellular proteolytic cascades; one results in the activation of the spätzle processing enzyme (SPE) and the other results in the activation of the melanization cascade. Activated SPE cleaves spätzle (spz) which then binds to the transmembrane Toll receptor. This binding

initiates an intracellular signalling cascade that results in translocation of NF- κ B-like transcription factor Dif to the nucleus and the transcription of immune-responsive genes such as the antimicrobial peptide *Drosomycin* and the *Bomanin* family of peptides. Activation of the melanization cascade culminates in the production of phenol oxidase (PO) which catalyses the oxidation of phenols, resulting in the polymerization of melanin and the production of reactive oxygen species.

1.3.1.3 Role of the imd pathway during *E. faecalis* infection

The Imd pathway bears resemblance to the mammalian tumour necrosis factor receptor pathway (Leulier et al., 2000). This pathway is activated in response to the detection of diaminopimelic acid (DAP)-type peptidoglycan (which is generally associated with Gram-negative bacteria), by PGRP-LC at the cell surface or by PGRP-LE intracellularly. The Imd pathway is also activated in response to wounding. Briefly, injury or detection of bacterial peptidoglycan by PGRP-LC or PGRP-LE results in the recruitment and activation of the imd protein. This leads to the activation of two distinct signalling processes that converge on the NF- κ B like Rel family transcription factor Relish, which mediates gene expression of immune-responsive genes in this pathway. The first process involves the recruitment and activation of the adaptor protein FADD, and the caspase Dredd, while the second process involves activation of TAK1 and the IKK complex that results in Relish phosphorylation (Ferrandon et al., 2007; Georgel et al., 2001; Leulier et al., 2000; Leulier et al., 2002; Naitza et al., 2002). Unphosphorylated Relish is present in the cytoplasm due to its inhibitory I κ B domain. Upon phosphorylation of Relish, this inhibitory domain is cleaved by Dredd (Stoven et al., 2003). This enables Relish to translocate to the nucleus and activate expression of antimicrobial peptides and other immune-responsive genes (**Figure 1.7**) (Buchon et al., 2014).

While the Imd pathway is primarily activated in response to infection with Gram-negative bacteria, it has also been shown to contribute to the immune response against certain Gram-positive pathogens such as *Streptococcus pneumoniae* (Pham et al., 2007). In recent years, it has also become clear that significant cross-talk between Toll and Imd pathways, and

pathway activation by sterile injury, results in complex interactions between infection sensing and target gene specificity. For example, during *E. coli* infection, induction of *Drosomycin* (*Drs*) can be mediated by Toll or the Imd pathway, whereas during *Micrococcus luteus* or *Aspergillus fumigatus* infection, *Drs* expression is mediated only by the Toll pathway (De Gregorio et al., 2002). Expression of *Diptericin*, which is an antimicrobial peptide whose expression is normally thought to be regulated specifically by the Imd pathway, has also been found to be upregulated in uninfected flies expressing a constitutively active allele of Toll (Mulinari et al., 2006). Finally, *Rel/spz* double-mutants die faster during *E. faecalis* infection compared to *spz* mutants suggesting that the Imd pathway may play a direct role in the immune response during *E. faecalis* infection, but this has not been directly tested (De Gregorio et al., 2002).

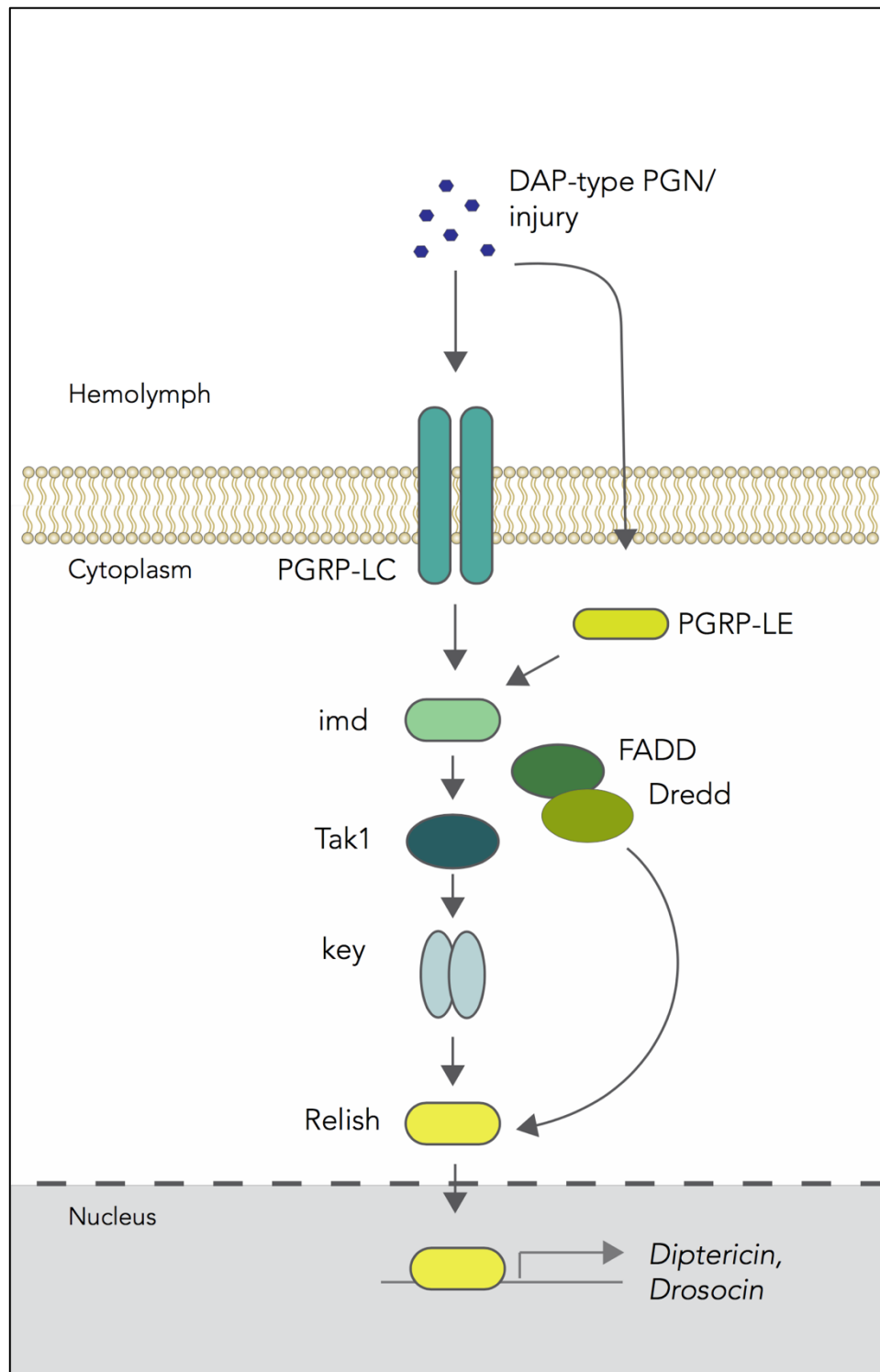


Figure 1.7 The Imd signalling pathway

The Imd signalling pathway is activated upon injury or the detection of DAP-type peptidoglycan by the pattern recognition receptors, PGRP-LC and PGRP-LE. This activation initiates a signal transduction pathway that results in the cleavage of NF- κ B-like transcription factor Relish, which translocates to the nucleus and drives the expression of Imd target genes such as the antimicrobial peptides *Drosocin* and *Diptericin*.

1.3.1.4 Contributions of the melanization cascade to the immune response during *E. faecalis* infection

Melanization plays an important role in the antimicrobial immune response in arthropods and is activated in response to infection or injury (Nam et al., 2012). The melanization cascade in *Drosophila* is activated upon the detection of microbial components by PGRP-SA and GGBP-1 (Dudzic et al., 2019) (**Figure 1.6**). This detection event results in a serine protease cascade which culminates in the cleavage of the zymogen prophenol oxidase (PPO) to produce phenol oxidase (PO). Phenol oxidase is a key enzyme for melanin formation and catalyzes the oxygenation of monophenols to o-diphenols and further oxidation to o-quinones. These quinones can be directly toxic to bacteria or polymerize to form melanin which can encapsulate bacteria. The deposition of melanin at wound sites can accelerate wound healing and the production of reactive oxygen species by this biosynthetic pathway can be directly harmful to the pathogens (Ferrandon et al., 2007). Flies encode three different PPOs: PPO1, PPO2, and PPO3. PPO1 and PPO2 contribute to hemolymph melanization in larvae and adult flies. In larvae, PPO1 and PPO2 are found in crystal cells, and in adult flies, they are found in the hemolymph (Binggeli et al., 2014; Dudzic et al., 2015). PPO3 is present in lamellocytes. As lamellocytes are not present in adult flies, PPO3 is not expected to play a role in the wounding or immune response in these flies.

Activation of the melanization cascade does not lead to the same outcome with every infection: it is beneficial for some infections, whereas for some others it is harmful (Ayres & Schneider, 2008). For example, PPO activity is essential for the immune response against the Gram-positive bacterium *S. aureus*; infection of flies deficient in PPO activity with *Streptococcus pneumoniae*, on the other hand, leads to improved outcomes after infection (Ayres & Schneider, 2008). Studies of the *Drosophila* immune response to *E. faecalis* infection describe conflicting roles for this pathway. Inactivation of the serine proteases that are involved in the cleavage of PPO1 has been shown to result in increased resistance to *E. faecalis* infection

(Ayres & Schneider, 2008; Leclerc et al., 2006). This enhanced resistance to infection did not lead to changes in survival outcomes but resulted in reduced bacterial load 48 hours after infection (Ayres & Schneider, 2008). However, another study found that deletion of PPOs results in increased susceptibility to *E. faecalis* infection (Binggeli et al., 2014). The Toll pathway and the melanization cascade share common upstream activation mechanisms (serine proteases, PGRP-SA, GNBPs) and mutations in the serine proteases specific to the melanization cascade result in enhanced expression of Toll-regulated antimicrobial peptides (Dudzic et al., 2019). This might explain the increase in resistance to infection observed in the absence of certain serine proteases.

1.3.1.5 Contributions of the cellular immune response to *E. faecalis* infection

Cellular responses in *Drosophila* are performed by hemocytes, which are analogous to mammalian blood cells. Flies have different types of hemocytes: macrophage-like plasmatocytes, crystal cells, and lamellocytes. In adult flies, the cellular immune response is believed to be specifically performed by plasmatocytes (Lemaitre & Hoffmann, 2007). The cellular immune response is known to contribute to the immune response against *E. faecalis*. Ablation of hemocytes in adult flies by expression of pro-apoptotic genes, or phagocytosis blockade using latex beads, results in increased susceptibility to *E. faecalis* infection (Defaye et al., 2009; Nehme et al., 2011).

In larvae and adult flies, plasmatocytes have been shown to aid the immune response during infection in two ways: first, through phagocytosis of invading microbes, and second, by regulating the expression of antimicrobial peptides (Shia et al., 2009). Larval hemocytes express the Toll agonist *spz*, which then drives Toll activation and antimicrobial peptide transcription in the fat body. Ablation of hemocytes by expression of proapoptotic genes

results in reduced antimicrobial peptide transcript levels in the larval fat body (Charroux & Royet, 2009; Shia et al., 2009). In adult flies, the mechanisms behind hemocyte-mediated antimicrobial peptide regulation differ from larval hemocytes. For example, TGF- β -like signals from the hemocytes can repress the transcription of antimicrobial peptides in response to wounding or infection (Clark et al., 2011).

1.3.2 Mechanistic underpinnings of *Drosophila* defense against *Enterococcus faecalis*

As summarized in **Sections 1.3.1.1-5**, our knowledge about the host factors important for the *Drosophila* immune response during *E. faecalis* infection has increased immensely in the past few years. We now know that Bomanins are the primary immune effectors important for the immune response during *E. faecalis* infection (Binggeli et al., 2014; Clemmons et al., 2015; Hanson et al., 2019). We also know that the cellular immune response and the melanization cascade might contribute to this immune response (Ayres & Schneider, 2008; Binggeli et al., 2014; Defaye et al., 2009; Elrod-Erickson et al., 2000; Nehme et al., 2011). However, despite these discoveries, relatively little is known about mechanisms used by the *Drosophila* immune response and the Bomanins in particular to kill *E. faecalis*. While previous studies have established that the Bomanins are not cationic, they do not function by modulating the host immune response and they require some host factors for their activity, these studies have not been informative about their mechanisms (Lindsay et al., 2018). We can gain novel insights into the *Drosophila* immune response by characterising bacteria that are resistant to the action of the *Drosophila* immune response and its immune effectors. To generate these immune-resistant bacteria we have made use of experimental evolution.

1.4 Experimental evolution

Experimental evolution involves the laboratory-based propagation of a population of organisms over a time period during which it is expected that the population will exhibit phenotypic change (either neutral change or adaptation to an applied selective pressure). This approach is widely used to study adaptive mechanisms in response to stress (Elena & Lenski, 2003; Kawecki et al., 2012). Experimental evolution is a popular tool to study microbial adaptation (Rodriguez-Pastor et al., 2022; Van den Bergh et al., 2018). Many microbes' small genome and quick generation time, and the possibility of working with large populations, enable evolutionary changes to be observed on a short time scale. Early microbial evolution studies were often designed to test the evolutionary theories of adaptation (Lenski, 2017). The most well-known example of an experiment of this kind is the long-term evolution experiment by Richard Lenski (Lenski, 2017; Lenski et al., 1991). In this experiment, twelve populations of *E. coli* were propagated in glucose-limiting conditions in 1988. These cultures were then serially subcultured in fresh media daily. This experiment is still ongoing. Originally set up to study the repeatability of evolution, this study has enabled direct observation and analysis of many key evolutionary concepts, including adaptation by natural selection, parallelism, fitness landscapes, clonal interference, epistasis, and pleiotropy (Bohannan et al., 1999; Clune et al., 2008; Cooper et al., 2003; Crozat et al., 2005; de Visser & Lenski, 2002; Elena & Lenski, 1997, 2003; Jagdish & Nguyen Ba, 2022; Maddamsetti et al., 2015; Ostrowski et al., 2005; Remold & Lenski, 2004; Rozen & Lenski, 2000; Woods et al., 2006). In recent years, the field of experimental evolution has undergone a shift. The availability of low-cost genome-scale sequencing has made it possible to identify the molecular genetic changes underpinning evolutionary phenotypic change (Van den Bergh et al., 2018). Thus, experimental evolution can now be used to study the molecular basis of adaptation to specific pressures.

Experimental evolution coupled with whole-genome sequencing can provide molecular insights into the targets of selection. As a result, this approach has been widely adopted to study the evolution of antimicrobial resistance in bacteria (Jansen et al., 2013; Kapel et al., 2022; MacLean, 2020; Miller et al., 2013; Papkou et al., 2020; Prater et al., 2019; Santos-Lopez et al., 2022). In these studies, *in vitro* evolution of bacteria is often performed through serial transfer or continuous culture in the presence of antimicrobial compounds. At the end of the evolution experiment, bacteria that have become resistant to the antimicrobial compound of interest are assayed to identify the phenotypic and genetic changes associated with this evolved resistance. Given the simple nature of this set-up, it is not surprising that a similar approach is also used to study the action of host immune components such as antimicrobial peptides or macrophages on infecting pathogens (Dobson et al., 2014; Perron et al., 2006; Ramiro et al., 2016). However, while this strategy is useful to study the stress imposed by important components of the host immune response under controlled environmental conditions, it is not representative of the stress exerted by an intact immune response. This was emphasized in a recent study: *S. aureus* strains were experimentally evolved to become resistant to Tenecins, a family of antimicrobial peptides from the mealworm beetle, *Tenebrio molitor*. The Tenecin-resistant strains had mutations in one of two genes; the first encoded an efflux pump, and the second a TCS homologous to the BceRS system. These mutations were expected to result in increased virulence of *S. aureus* during infection (Makarova et al., 2018). However, upon testing these strains for their virulence *in vivo*, it was observed that these strains exhibited reduced virulence compared to the wild type strain, suggesting that, while these mutations were beneficial in a one-on-one interaction with Tenecins, they have no such benefit when challenged with an intact immune response (El Shazely et al., 2019; Johnston et al., 2016).

Thus, in order to use experimental evolution to understand the interaction with the intact immune response, selection must take place in an intact host or hosts (Rodriguez-Pastor et

al., 2022; Van den Bergh et al., 2018). This approach provides a more complete view of the stress faced by an invading pathogen. At the end of the evolution experiment, the fitness of the passaged populations is compared to the ancestor to determine whether the microbes have adapted. Genetic and phenotypic analyses of these adapted populations enable the identification of the changes that have allowed adaptation, thus providing an insight into the mechanisms of immunity as experienced by the microbial target.

A major advantage of *in vivo* experimental evolution over *in vitro* evolution is that *in vivo* evolution is a more accurate reflection of the complexity of the selective pressures encountered by microbes in the host. However, there are many challenges in setting up a successful *in vivo* evolution experiment (Rodriguez-Pastor et al., 2022; Van den Bergh et al., 2018). *In vivo* experimental evolution is more cost and labour intensive compared to *in vitro* experiments due to the need to rear hosts for serial transfer. As a result, these experiments are often constrained by small sample sizes. These experiments also typically take a longer period of time to complete—or, conversely, evolution is monitored over a short timescale compared to *in vitro* evolution experiments. Nonetheless, *in vivo* experimental evolution has been used extensively to study interactions between pathogens and their hosts (Allen et al., 2021; Cooper et al., 2020; Huo et al., 2022; Rodriguez-Pastor et al., 2022; Salgado et al., 2022). It can be used to gain insight into factors that are important for organ-specific colonization. For example, a recent study explored the bacterial properties important for *in vivo* nasopharyngeal colonization of *S. pneumoniae* (Cooper et al., 2020). They serially passaged *S. pneumoniae* in nasopharynx of mice and observed that at the end of the experiment, serially passaged strains showed greater adherence to respiratory cells compared to the ancestral strain. This increased adherence was associated with mutations in the gene, *dltB*. However, this enhanced ability to colonise the nasopharynx correlated with a fitness disadvantage during pulmonary infection. This was because the properties that

enabled bacteria to become more adherent to the cells also made them more sensitive to the action of host antimicrobial peptides.

In vivo experimental evolution has also been used to study the contribution of the host immune response in the evolution of antimicrobial resistance during infection (Huo et al., 2022). In this study, the evolution of fluoroquinolone resistance in *Acinetobacter baumannii* was monitored in the lungs of mice replete with or depleted of neutrophils. They observed that the rise of variants that showed altered drug resistance was quick in mice that were immunodeficient, whereas the presence of neutrophils in immunocompetent mice restricted the appearance of these variants. They also observed that the immunological state of the host determined the types of alleles that were acquired during selection, as rare alleles of resistance were observed in strains isolated from immunodeficient hosts. These alleles were responsible for bacterial persistence in the presence of the antibiotic and resulted in antibiotic-treatment failure.

However, these studies were expensive, had a small number of replicate populations, and were slow as they were performed in mammalian hosts. Performing experimental evolution of bacteria in flies would be quicker, cheaper and higher-throughput compared to mammalian hosts. This would be especially useful for studying the interactions between broad-host human pathogens like *E. faecalis* and flies, as insects are a natural host of this bacterium.

Work in this thesis is focused on understanding the mechanisms of insect immunity and the mechanisms by which bacteria might escape immune killing. To study this I propose to experimentally evolve *E. faecalis* in response to the systemic immune response of *Drosophila* by serial passage. In addition to this, as *E. faecalis* is a broad-host pathogen, studying its interaction with the immune response of *Drosophila*, one of its natural hosts, can also provide insight into how these interactions can influence the biology of *E. faecalis*.

1.5 Aims

Enterococcus faecalis is a generalist bacterium with a wide host range. Insects are a natural host of this bacterium and it is believed that this interaction has influenced the biology of this *E. faecalis*. However, not much is known about the mechanisms through which *E. faecalis* is killed by the *Drosophila* immune response and the host components involved in this response. As a result, the first **aim** of this project is to understand how the *Drosophila* immune response interacts with and kills *E. faecalis*. As interaction with this immune response can influence the biology of this broad-host pathogen, the second **aim** of this project is to understand the consequences of the interaction between *E. faecalis* and the *Drosophila* immune response, specifically in the context of bacterial traits that are relevant for human health and disease.

Chapter 2: Materials and Methods

2.1 Bacterial strains, plasmids and growth conditions

2.1.1 Bacterial strains and plasmids

The bacterial strains used in this study are shown in **Table 2.1** and the plasmids used are shown in **Table 2.2**.

2.1.2 Growth conditions

E. faecalis Tissier strain (ATCC 19433) was used for all serial passage experiments. *E. faecalis* was grown in brain heart infusion (BHI) broth (Oxoid), brain heart agar (BHA, Merck), Mueller Hinton broth (MHB, Sigma), M17 broth (Difco) or on *Mitis Salivarius* (MS) agar (Sigma-Aldrich). MS agar is a selective medium for *Streptococci* and *Enterococci* spp. It inhibits most Gram-negative bacilli and Gram-positive bacteria except *Streptococci* and *Enterococci*. MS agar is supplemented with potassium tellurite which acts a differentiating agent amongst *Streptococci* and is reduced by *Enterococcus* spp to form black colonies. Unless explicitly stated, with the exception of *Drosophila* serial passage experiments, all other infection experiments were performed with *E. faecalis* grown in sterile filtered BHI.

For experiments involving plasmid selection, *E. faecalis* was grown in sterile filtered BHI with 20 µg/ml of erythromycin (Sigma-Aldrich) or 5 µg/ml tetracycline (Sigma-Aldrich) added. *Staphylococcus aureus* was grown in tryptic soy broth (TSB, Sigma-Aldrich) with the addition of 5 µg/ml tetracycline. *Escherichia coli* was grown in lysogeny broth (LB, Sigma-Aldrich) with the addition of 200 µg/ml erythromycin. M17 broth was used in the preparation of electrocompetent *E. faecalis*. For experiments involving daptomycin, media were supplemented with 0.5mM CaCl₂.

Table 2.1 Bacterial strains used in this study

Strain	Description	Unique ID	Source
	<i>Enterococcus faecalis</i>		
ATCC 19433/NCTC 775 (Tissier)	Ancestral strain (<i>E. faecalis</i> type strain)	Ancestor	NCTC
EE1S1C1	Fly passage experiment 1, Population 1, Colony 1		This study
EE1S1C2	Fly passage experiment 1, Population 1, Colony 2		This study
EE1S1C3	Fly passage experiment 1, Population 1, Colony 3		This study
EE1S1C4	Fly passage experiment 1, Population 1, Colony 4		This study
EE1S2C1	Fly passage experiment 1, Population 2, Colony 1	Strain A	This study
EE1S2C2	Fly passage experiment 1, Population 2, Colony 2		This study
EE1S2C3	Fly passage experiment 1, Population 2, Colony 3		This study
EE1S2C4	Fly passage experiment 1, Population 2, Colony 4		This study
EE1S3C1	Fly passage experiment 1, Population 3, Colony 1		This study
EE1S3C2	Fly passage experiment 1, Population 3, Colony 2		This study
EE1S3C3	Fly passage experiment 1, Population 3, Colony 3		This study

EE1S3C4	Fly passage experiment 1, Population 3, Colony 4		This study
EE2S1C1	Fly passage experiment 2, Population 1, Colony 1		This study
EE2S1C2	Fly passage experiment 2, Population 1, Colony 2	Strain B	This study
EE2S1C3	Fly passage experiment 2, Population 1, Colony 3		This study
EE2S1C4	Fly passage experiment 2, Population 1, Colony 4		This study
EE2S1C5	Fly passage experiment 2, Population 1, Colony 5		This study
EE2S2C1	Fly passage experiment 2, Population 2, Colony 1		This study
EE2S2C2	Fly passage experiment 2, Population 2, Colony 2		This study
EE2S2C3	Fly passage experiment 2, Population 2, Colony 3	Strain C	This study
EE2S2C4	Fly passage experiment 2, Population 2, Colony 4		This study
EE2S2C5	Fly passage experiment 2, Population 2, Colony 5		This study
EE2S3C1	Fly passage experiment 2, Population 3, Colony 1		This study
EE2S3C2	Fly passage experiment 2, Population 3, Colony 2		This study
EE2S3C3	Fly passage experiment 2, Population 3, Colony 3		This study
EE2S3C4	Fly passage experiment 2, Population 3, Colony 4		This study
EE2S3C5	Fly passage experiment 2, Population 3, Colony 5		This study

EE2S4C1	Fly passage experiment 2, Population 4, Colony 1		This study
EE2S4C2	Fly passage experiment 2, Population 4, Colony 2		This study
EE2S4C3	Fly passage experiment 2, Population 4, Colony 3		This study
EE2S4C4	Fly passage experiment 2, Population 4, Colony 4		This study
EE2S4C5	Fly passage experiment 2, Population 4, Colony 5		This study
EE2S5C1	Fly passage experiment 2, Population 5, Colony 1		This study
EE2S5C2	Fly passage experiment 2, Population 5, Colony 2		This study
EE2S5C3	Fly passage experiment 2, Population 5, Colony 3		This study
EE2S5C4	Fly passage experiment 2, Population 5, Colony 4		This study
EE2S5C5	Fly passage experiment 2, Population 5, Colony 5		This study
EE2S6C1	Fly passage experiment 2, Population 5, Colony 1		This study
EE2S6C2	Fly passage experiment 2, Population 5, Colony 2		This study
EE2S6C3	Fly passage experiment 2, Population 5, Colony 3		This study
EE2S6C4	Fly passage experiment 2, Population 5, Colony 4		This study
EE2S6C5	Fly passage experiment 2, Population 5, Colony 5	Strain D	This study
EE3S1C1	Fly passage experiment 3, Population 1, Colony 1		This study

EE3S1C2	Fly passage experiment 3, Population 1, Colony 2		This study
EE3S1C3	Fly passage experiment 3, Population 1, Colony 3		This study
EE3S2C1	Fly passage experiment 3, Population 2, Colony 1		This study
EE3S2C2	Fly passage experiment 3, Population 2, Colony 2		This study
EE3S2C3	Fly passage experiment 3, Population 2, Colony 3		This study
EE3S3C1	Fly passage experiment 3, Population 3, Colony 1	Strain E	This study
EE3S3C2	Fly passage experiment 3, Population 3, Colony 2		This study
EE3S3C3	Fly passage experiment 3, Population 3, Colony 3		This study
EE3S4C1	Fly passage experiment 3, Population 4, Colony 1		This study
EE3S4C2	Fly passage experiment 3, Population 4, Colony 2		This study
EE3S4C3	Fly passage experiment 3, Population 4, Colony 3		This study
EE3S5C1	Fly passage experiment 3, Population 5, Colony 1		This study
EE3S5C2	Fly passage experiment 3, Population 5, Colony 2		This study
EE3S5C3	Fly passage experiment 3, Population 5, Colony 3		This study
EE3S6C1	Fly passage experiment 3, Population 6, Colony 1		This study
EE3S6C2	Fly passage experiment 3, Population 6, Colony 2		This study

EE3S6C3	Fly passage experiment 3, Population 6, Colony 3		This study
EE3S7C1	Fly passage experiment 3, Population 7, Colony 1		This study
EE3S7C2	Fly passage experiment 3, Population 7, Colony 2		This study
EE3S7C3	Fly passage experiment 3, Population 7, Colony 3		This study
EE3S8C1	Fly passage experiment 3, Population 8, Colony 1		This study
EE3S8C2	Fly passage experiment 3, Population 8, Colony 2		This study
EE3S8C3	Fly passage experiment 3, Population 8, Colony 3		This study
EE3S9C1	Fly passage experiment 3, Population 9, Colony 1		This study
EE3S9C2	Fly passage experiment 3, Population 9, Colony 2		This study
EE3S9C3	Fly passage experiment 3, Population 9, Colony 3		This study
EE3S10C1	Fly passage experiment 3, Population 10, Colony 1		This study
EE3S10C2	Fly passage experiment 3, Population 10, Colony 2		This study
EE3S10C3	Fly passage experiment 3, Population 10, Colony 3	Strain F	This study
PC1	Plate control, Colony 1	PC1	This study
PC2	Plate control, Colony 2	PC2	This study
PC5	Plate control, Colony 5	PC5	This study

PC6	Plate control, Colony 6	PC6	This study
PC7	Plate control, Colony 7	PC7	This study
Daptomycin passaged population 1 Colony 1	Daptomycin passage experiment, Population 1, Colony 1	Dap Strain 1	This study
Daptomycin passaged population 1 Colony 2	Daptomycin passage experiment, Population 1, Colony 2		This study
Daptomycin passaged population 1 Colony 3	Daptomycin passage experiment, Population 1, Colony 3		This study
Daptomycin passaged population 2 Colony 1	Daptomycin passage experiment, Population 2, Colony 1	Dap Strain 2	This study
Daptomycin passaged population 2 Colony 2	Daptomycin passage experiment, Population 2, Colony 2		This study
Daptomycin passaged population 2 Colony 3	Daptomycin passage experiment, Population 2, Colony 3		This study
Daptomycin passaged population 3 Colony 1	Daptomycin passage experiment, Population 3, Colony 1	Dap Strain 3	This study

Daptomycin passaged population 3 Colony 2	Daptomycin passage experiment, Population 3, Colony 2		This study
Daptomycin passaged population 3 Colony 3	Daptomycin passage experiment, Population 3, Colony 3		This study
Daptomycin passaged population 4 Colony 1	Daptomycin passage experiment, Population 4, Colony 1		This study
Daptomycin passaged population 4 Colony 2	Daptomycin passage experiment, Population 4, Colony 2		This study
Daptomycin passaged population 4 Colony 3	Daptomycin passage experiment, Population 4, Colony 3	Dap Strain 4	This study
Daptomycin passaged population 5 Colony 1	Daptomycin passage experiment, Population 5, Colony 1	Dap Strain 5	This study
Daptomycin passaged population 5 Colony 2	Daptomycin passage experiment, Population 5, Colony 2		This study
Daptomycin passaged population 5 Colony 3	Daptomycin passage experiment, Population 5, Colony 3		This study

Media Strain 1	Media passage experiment, Population 1, Strain 1		
Media Strain 2	Media passage experiment, Population 2, Strain 1		
Media Strain 3	Media passage experiment, Population 3, Strain 1		
Media Strain 4	Media passage experiment, Population 4, Strain 1		
Media Strain 5	Media passage experiment, Population 5, Strain 1		
	<i>Staphylococcus aureus</i>		
SH1000- pMV158-mCherry	Strain containing plasmid with constitutive expression of mCherry		S. Mesnage
SH1000- pMV158-GFP	Strain containing plasmid with constitutive expression of GFP		S. Mesnage
	<i>Escherichia coli</i>		
DH5α	Cloning strain		NEB

Table 2.2 Bacterial plasmids used in this study

Plasmid	Description	Source
pTet2op-dltABC	<i>E. coli</i> / <i>E. faecalis</i> shuttle vector expressing dltABC operon under Tet control. Erm ²⁰⁰ in <i>E. coli</i> , Erm ³⁰ in <i>E. faecalis</i>	S. Mesnage
pMV158-GFP	Plasmid for constitutive expression of GFP	S. Mesnage
pMV158-mCherry	Plasmid for constitutive expression of mCherry	S. Mesnage
pTet2op-yxdM	Plasmid expressing <i>yxdM</i> under Tet control	This study
pTet2op-dcuR	Plasmid expressing <i>dcuR</i> under Tet control	This study
pTet2op-epaQ	Plasmid expressing <i>epaQ</i> under Tet control	This study

2.2 *Drosophila* experiments

2.2.1 Fly crosses

Adult *Drosophila* were anesthetized using CO₂ and placed on a porous gas pad. Male and female flies were sorted under a light microscope and were separated based on their anatomical differences. Male flies are smaller and have darker abdomen and genitalia than females. Male flies also have sex combs, thick black hair-like structures which can be used to grasp the female during mating.

For collection of virgin female flies, fly vials containing hatching pupae were cleared of any adults and left for new flies to eclose. Female flies that had eclosed up to 8 hours post vial emptying were collected as during this period they will have not mated. Additionally, virgin females can also be visually identified through their light colouring and the presence of a dark spot in the abdomen, called the meconium, which consists of waste products from pupation. Collected female flies were kept at room temperature for 2-3 days before setting up crosses to ensure the collected females were virgins. Flies from any vial found to have eggs or larvae were discarded, because this indicated that they were not virgins. Each experimental cross consisted of 4 males and 4 females in one vial. Flies were kept in the same vial for 2-3 days before being transferred to new vials. Each cross was transferred to fresh food 2 times before flies were discarded.

For crosses utilizing the Gal4-UAS system, virgin female flies were used. The Gal4-upstream activating sequence (UAS) system, is a transcription activation system commonly used in *Drosophila* to achieve tissue-specific expression of genes (Brand & Perrimon, 1993). The system has two parts, the first being the Gal4 gene, which encodes the yeast transcription activator protein Gal4, which is under the control of a tissue-specific enhancer. The second half of this system is the UAS sequence which is linked to the gene of interest. Gal4 under the

control of a tissue specific driver specifically binds to the UAS sequence and facilitates the transcription of the target gene leading to tissue specific expression.

5-7 day old male flies were used for all experiments. Flies were kept on a diet composed of 10% yeast (w/v), 8% fructose (w/v), 2% polenta (w/v), 0.8% w/v agar (w/v), 0.075% methylparaben (w/v) and 0.0825% propionic acid (v/v). All experimental crosses included in this study were housed at 25°C. The only exception to this was when rearing flies at 25°C could have an effect on fly development. For instance, flies expressing *Toll^{10b}* were kept at room temperature until the flies eclosed, after which the males were sorted and moved to 25°C for 5-7 days. The fly lines used in this project are described in **Table 2.3**.

Table 2.3 Fly lines used in this study

Line	Genotype	Description	Source
w ¹¹¹⁸	w ¹¹¹⁸	Isogenic wild-type control	Bloomington <i>Drosophila</i> Stock Center (BDSC)
outbred	Outbred wild-type	Recently wild-derived fly population	E. Sucena (Faria & Sucena, 2017)
Dif	w ¹¹¹⁸ ; <i>Difcnbw</i>	Line carrying loss of function allele of <i>Dif</i>	D. Ferrandon, Strasbourg (Rutschmann, Jung, Zhou, et al., 2000)
Bom Δ 55c	w ¹¹¹⁸ ; <i>Bom</i> ^{Δ55c}	Fly line lacking 10/12 Bomanin effector peptides	S. Wasserman, UCSD (Clemmons et al., 2015)
w;; Δ AMP	w ¹¹¹⁸ ; Δ AMP; Δ AMP	Fly line lacking 10/14 <i>Drosophila</i> AMPS	M. Hanson, B. Lemaitre, EPFL (Hanson et al., 2019)
w;; Δ Drs	w ¹¹¹⁸ ; Δ Drs	Line carrying loss of function allele of <i>Drs</i>	M. Hanson, B. Lemaitre, EPFL (Hanson et al., 2019)
c564-Gal4	w ¹¹¹⁸ ; c564-Gal4	Fat-body driver line. It also expresses in the gut and hemocytes.	BDSC

UAS-Toll ^{10B}	<i>w¹¹¹⁸(UAS-Toll^{10B})/(FM7h)</i>	UAS line expressing a constitutively active Toll receptor	BDSC
imd	<i>w¹¹¹⁸; imd</i>	Line carrying loss of function allele of <i>imd</i>	BDSC
key	<i>w¹¹¹⁸; key^{c02831}/(SM6a)</i>	Line carrying loss of function allele of <i>key</i>	BDSC
Tak1	<i>+(Tak1²⁵²⁷)</i>	Line carrying loss of function allele of <i>Tak1</i>	BDSC (Polaski et al., 2006)
++;+	<i>++;+</i>	Background control for Tak1 flies	BDSC
PPO ^{1Δ} , PPO ^{2Δ}	<i>w¹¹¹⁸; PPO^{1Δ}, PPO^{2Δ}</i>	Line carrying loss of function alleles of <i>PPO1</i> and <i>PPO2</i>	M. Hanson, B. Lemaitre, EPFL (Binggeli et al., 2014)

2.2.2 Drosophila injections

Fly injections were carried out with microinjection needles produced from borosilicate glass capillaries (World Precision Instruments) and a needle puller (Model PC-10, Narishige). Injections were performed using a Picospritzer III system (Parker Hannifin), and the injection volume was calibrated by expelling a drop of liquid from the needle into a pot of mineral oil and halocarbon oil (both Sigma). The expelled drop was measured using the microscope graticule to obtain a final injection volume of 50 nanolitres (nl). Flies were anesthetized using CO₂ and injected with either sterile PBS (Fisher) or 50 nl of bacterial suspension in sterile PBS.

The bacterial suspensions for injections were prepared by centrifuging 1 ml of an overnight culture at 4000x g for 5 min. The supernatant was then discarded and the pellet was resuspended in sterile PBS. Finally, the suspension was adjusted to the required OD₆₀₀ reading using sterile PBS.

2.2.3 Experimental evolution

Serial passage of *E. faecalis* in *Drosophila* was performed on three separate occasions, each including multiple replicate populations. For the initial round of serial passage, three replicate sets for selection were set up. Each set was treated exactly the same way – for example, injected on the same day, using the same initial bacterial suspension. The infection was allowed to proceed for 72 hours at 29°C after which eight live flies from each set were homogenized in 100 µl sterile PBS and plated on *MS* agar. Bacteria were allowed to grow overnight, then scraped off the plate and resuspended in sterile PBS. Each bacterial suspension was adjusted to OD₆₀₀ = 1 and injected into 20 male flies. The infection was again allowed to proceed for 72 hours. This cycle was repeated six times. For the second round of

serial passage, six replicate selections were set up, infection was allowed to proceed for 24 hours instead of 72 hours, and ten cycles of serial passage were carried out. For the third round of serial passage, ten replicate selections were set up, flies were infected with *E. faecalis* for 24 hours before plating, and ten cycles of serial passage were carried out.

To control for effects due to selection on *MS* agar (as opposed to selection by growth in the fly), plate controls were performed. An overnight culture of *E. faecalis* streaked onto *MS* agar plate was resuspended in sterile PBS, adjusted to $OD_{600} = 1$ and then re-plated onto a new *MS* agar plate. This process was repeated for ten cycles and ten replicate sets for selection were set up. These plate controls exhibited no changes in ability to grow in the fly as a result of this selection process; two plate controls (PC2 and PC7) were subjected to enhanced whole genome sequencing to generate complete, closed genome sequences for variant-calling, as no such sequence was previously available for the *E. faecalis* type strain (Section 2.3).

For all evolution experiments, bacteria were grown only on solid media i.e. *MS* agar. This was done to reduce interbacterial competition during *in vitro* culture.

2.2.4 Single colony isolations

To remove any effect of multiple populations in our experiments, I wanted to isolate single colonies of bacteria. I streaked out the passaged bacterial populations out onto *MS* Agar plates. Single colonies were then selected at random and grown overnight to make glycerol stocks. Survival analysis and bacterial quantifications of flies infected with these single colonies were then performed to determine whether these single colonies recapitulated the behaviour of the bacterial population they were isolated from. For single colonies isolated from the first two rounds of serial passage, an $OD_{600} = 1$ bacterial inoculum was used for infection experiments. For single colonies isolated from the third round of serial passage, $OD_{600} = 0.3$

of bacterial inoculum was used. I proceeded with analysis of at least one representative single colony-derived strain from most populations, including all that exhibited increased proliferation *in vivo* relative to the ancestor (12/19 populations).

2.2.5 Survival experiments

All experiments were performed on 5-7 day old, age-matched male flies. Flies were grouped into uninjected controls, sterile PBS-injected controls, and flies infected with *E. faecalis*. Each group consisted of 15-30 flies. Survival experiments were conducted at 29°C and vials were checked each day for fly deaths. Vials were placed horizontally to minimize possibility of fly death from getting stuck to the food, and flies were transferred to fresh food every 3-4 days. Unless explicitly stated, survival experiments were carried out using $OD_{600} = 0.3$ inoculum of bacteria. At this concentration, a significant difference in the survival outcomes of flies infected with ancestral strain compared to the *Drosophila*-adapted strains was observed. This difference was less clear if flies were infected with $OD_{600} = 1$ concentration of bacteria.

2.2.6 Bacterial load quantification

Unless explicitly stated, bacterial quantification experiments were carried out using $OD_{600} = 0.3$ inoculum of bacteria. For each experiment, at least 16 individual flies were injected with *E. faecalis*. Eight of those flies were incubated for 8 hours at 29°C while the remaining 8 were collected for a zero hour time point immediately after injections. Individual flies were then homogenized in 100 µl of sterile PBS, serially diluted and plated onto *MS* agar plates. The plates were incubated overnight at 37°C, and colonies were counted to determine the number of bacterial colony forming units (CFUs) per fly.

2.2.7 *Drosophila* gut competition assay

Flies were fasted on 1% agar supplemented with 2% PBS overnight at 25°C. 10 µl of a monoculture of bacteria or mixed culture (1:1) of GFP-tagged ancestral and *Drosophila*-adapted strains were added to fly food. Fasted flies were transferred onto inoculated food for 3 hours and then transferred to clean food. Flies were collected 3 hour post infection (transfer), 3 hours post-transfer and 24 hours post-transfer. Single flies were homogenized in 100 µl of PBS and plated on BHI for bacterial load quantification. Plates were incubated overnight at 37°C.

Bacterial load from flies fed a single culture of bacteria was quantified as described in. For flies fed a mixed culture of bacteria, colonies was counted under a light and fluorescent microscope. This was done to distinguish between fluorescently tagged ancestral strain and untagged *Drosophila*-adapted strain. As fluorescent colonies directly corresponded to the *Drosophila*-adapted strains to quantify the ancestral strain in each sample I used the following formula:

$$\begin{aligned} & \text{Total no. of colonies per sample} - \text{Fluorescent colonies per sample} \\ & = \text{No. of ancestral colonies per sample} \end{aligned}$$

To calculate the relative proportions of the ancestral and *Drosophila*-adapted strains I made use of the following formula:

$$\frac{\text{No. of fluorescent or ancestral colonies}}{\text{Total no. of colonies}} \times 100$$

2.3 Whole genome sequencing and genome assembly

Genomic DNA was extracted using the Wizard genomic DNA purification kit and sequenced at MicrobesNG (University of Birmingham, UK). PC2 and PC7 which are the two sub-populations of the ancestral strain were sequenced using a combination of short and long read sequencing to generate complete, closed genome sequences with no gaps; these served as references against which the sequences of other isolates were compared because there was previously no fully-assembled sequence for *E. faecalis* Tissier. All other strains were sequenced using short-read sequencing at coverage of at least 30x. For long-read sequencing the Oxford Nanopore platform was used and for short-read sequencing, Illumina MiSeq or HiSeq 2500 technology platforms with 2x250-bp paired-end reads was used. Sequences were assembled by MicrobesNG. In brief, Trimmomatic (Bolger et al., 2014) was used to trim raw reads which were mapped to the closest available reference genome identified by Kraken (Wood & Salzberg, 2014). The reads were mapped using BWA-MEM (Li & Durbin, 2009) to assess the quality of data. Additionally, *de novo* assembly of the reads using SPAdes (Bankevich et al., 2012) was carried out and the reads were mapped onto the resultant contigs using BWA-MEM.

2.4 Variant calling

Variant calling was performed using Snippy rapid haploid variant calling on Galaxy web platform using the public servers at <https://usegalaxy.eu/> and <https://usegalaxy.org.au/> with default parameters (Jalili et al., 2020; Seemann, 2015). To evaluate if there were complex mutations in the assembled sequences the trimmed reads were mapped to the ancestral genome using Bowtie2 with the default parameters for paired-end sequencing. Read alignments were visualized with IGV version 2.4.9 (Thorvaldsdottir et al., 2013) and manually assessed for deletions, insertions, and copy-number variation. Variants identified during sequencing were validated by PCR amplification and Sanger sequencing. Sequences of primers used for PCR amplification are detailed in **Table 2.10**.

2.5 Molecular Biology techniques

2.5.1 RT-qPCR

In this thesis, RT-qPCR was used to analyze gene expression in both *Drosophila* as well as bacteria. Sample preparation differs between these two sample types and is described below.

Sample preparation:

For *Drosophila* gene expression:

Three flies per sample were homogenized in 100µl TRI reagent (Sigma T9424) in a 1.5ml microcentrifuge tube using a pestle. Each timepoint consisted of 4 samples per condition. Samples were stored at -20°C prior to nucleic acid extraction.

For bacterial gene expression:

3 ml of stationary or mid-exponential phase bacteria ($OD_{600} = 0.5$) were centrifuged for 5 min at 4000x g. The supernatant was discarded and the pellet was resuspended in 100 µl of TRI reagent (Sigma T9424) and 1 µl of Glycerol (ThermoScientific). Samples were then transferred to Lysing Matrix B tubes (2 ml, MP Biomedicals) and homogenized using a FastPrep 24 (MP Biomedicals) (6 m/s, 2 x 40 s, 300 s rest time). Samples were centrifuged at 13,000x g for 5 min and 100 µl of the supernatant was transferred to a new microcentrifuge tube.

RNA isolation and reverse transcription

From this step onwards, all samples (*Drosophila* and bacterial) were treated the same. Nucleic extraction was carried out by adding 20 µl of chloroform to each sample after which the samples were shaken thoroughly and incubated for 3 min at room temperature. Samples were centrifuged at 12,000x g for 5 min at 4°C and the upper aqueous layer of each sample was then added to new microcentrifuge tubes containing 50 ul of isopropanol. Samples were

vortexed and after 10 min at room temperature, centrifuged at 12,000x g for 10 min. The supernatant was then discarded and 200 µl of 75% ethanol was added to the pellet. If needed, the samples were stored at -20°C before continuing.

For RNA extraction, samples stored in 75% ethanol were centrifuged at max speed for 5 min and the ethanol was discarded. A second quick round of centrifugation was carried out to eliminate any ethanol. Next, a reaction mixture containing: 34 µl ddH₂O, 4 µl 10x DNase buffer (Thermo Scientific), 2 µl RNase-free DNase I (1 U/µl, Thermo Scientific) was added to the samples and the samples were incubated at 37°C for 30 min. The reaction was stopped by the addition of 4 µl 25 mM EDTA (Thermo Scientific) followed by incubation at 65°C for 10 min.

For reverse transcription, 1 µl of random DNA hexamers (200 µg/ml, Thermo Scientific) was added to 10 µl of each DNase treated sample. The samples were incubated at 70°C for 5 min. A reaction mix consisting of the components described in **Table 2.4** was prepared and samples were incubated at room temperature for 10 min followed by 1-2 hours of incubation at 37°C. The reaction was stopped by incubation at 70°C for 10 min.

10 µl of each prepared complementary DNA (cDNA) sample were added to a single microcentrifuge tube to make a pooled sample. This pooled sample was diluted 1 in 5 eight times in TE (10mM Tris, 1mM EDTA) and this was used for generating standard curves. The remaining 10 µl of each sample was diluted 1 in 40 in TE (10mM Tris, 1mM EDTA). The diluted samples and pooled dilution series were stored at - 20°C.

PCR reactions were set up on ice, a reaction mix containing 5 µl of SensiMix SYBR Green (Bioline QT650) and 0.2 µl primer mix (10 µM) was added to 4.8 µl of cDNA samples or pooled sample dilutions. Amplification was carried out using an using an Corbett RG-6000 or Qiagen Rotor-Gene Q thermocycler using conditions described in **Table 2.5**. Primers were synthesized by Invitrogen and their sequences are listed in **Table 2.6**.

The pooled sample dilution series were used to generate a standard curve for each gene during a run. Gene expression of the samples was calculated by making use of this standard curve. The expression of the samples was then normalised to the expression of the Rpl4 for *Drosophila* and 23S RNA for bacterial gene expression.

Table 2.4 Components for reverse transcription of mRNA

Component	Volume (μl)
Reverse transcriptase buffer (5x, Thermo Scientific)	4
dNTPs (10mM, Thermo Scientific)	2
RiboLock RNAse inhibitor (Thermo Scientific)	1
RevertAid M-MuLV reverse transcriptase (200U/μl, ThermoFisher EP0441)	0.1
DNase treated RNA	11
Water	1.9
Total	20

Table 2.5 RT-qPCR cycle conditions

Reaction stage	Temperature	Time	No. of cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	57	30 s	40
Extension	72	30 s	40

Table 2.6 List of RT-qPCR primers used in this study

Primers used for gene expression analysis of *Drosophila*

Gene	Forward	Reverse
<i>Rpl4</i>	TCCACCTTGAAGAAGGGCTA	TTGCGGATCTCCTCAGACTT
<i>IM2</i>	ACCGTCTTTGTGTTCCGGTCT	GATTACCACATTTCTGGATCG
<i>Drs</i>	GTACTTGTTCCGCCCTCTTCG	CTTGACACACGACGACAG
<i>Dro</i>	CCATCGAGGATCACCTGACT	CTTTAGGCGGGCAGAATG
<i>Mtk</i>	TCTTGGAGCGATTTTTCTGG	TCTGCCAGCACTGATGTAGC
Primers used for gene expression analysis of <i>E. faecalis</i>		
Gene	Forward	Reverse
<i>23S</i>	CCTATCGGCCTCGGCTTAG	AGCGAAAGACAGGTGAGAATCC
<i>yxdM</i>	AGTCGCCGCTTCTTCTTCAT	CGTTGCTCGGGCAATGATTC
<i>yxdJ</i>	AACCGACCGAAAATGGGTCA	TTAGGACCGTAATCGTGCCG
<i>nucF</i>	CGGCGAGGCTGGTTTAATTG	AACGCCCCTTTTTGAATGGC
<i>liaF</i>	GGCGTTTAAACCGTTTGCCA	ATAGCTGAGCGAGCACCTTG
<i>liaS</i>	GGGGTTTCTGTTTCGTTGTGC	GCGGGAGTTGCATGATTGAG
<i>mprF_2</i>	CGGGTACATAGCCGAAACGA	AATGCGTTGTGATGGGCAAC
<i>croS</i>	CCGCTTACGTCAAAGCGAAA	CGGTACACTTGGTTGACGGA
<i>croR</i>	CCGCTCTTGAGTTTGGTATCCT	GTTGCCACACACGCTCAAAA
<i>yihY</i>	TTCCGATTAGGAACGACCGC	TCCAAGCGTTAAAATGGCCG
<i>salB</i>	AAGCACCAGTTGCCTCTTCA	TGCGTGCTTGATTGAGTTGC

2.5.2 Polymerase chain reaction

PCR amplification was carried out to validate variants identified during sequencing, for amplification of genes used in cloning and for validation of positive clones after transformations. Reactions were set up as described in **Table 2.7** and amplification was carried out using an Applied Biosciences 2720 or a G-storm gradient thermocycler using conditions described in Table 2.2.

In instances when purified bacterial DNA was not used for amplification, colony PCR was performed. Reactions were set up in 200 µl PCR tubes and a single colony was added to the reaction mix as described in **Table 2.7**. Amplification was carried out using conditions described in **Table 2.8**. Primers were synthesized by Invitrogen and their sequences are detailed in **Table 2.10**. The PCR products were then analysed by gel electrophoresis (**2.5.3**).

Table 2.7 Components of the PCR reaction

Component	Volume (µl)
DreamTaq buffer (10x)	5
dNTPs (10mM)	1
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
DreamTaq polymerase	0.25
Template DNA	2 µl or direct inoculation from single colony
Water	Volume required to make final volume 50
Total	50

Table 2.8 PCR cycle conditions for amplification of purified DNA

Reaction stage	Temperature	Time	No. of cycles
Initial denaturation	95	30 s	1
Denaturation	95	30 s	30
Annealing	55	30 s	30
Extension	68	1 min	30
Final extension	68	5 min	1

Table 2.9 Colony PCR cycle conditions

Reaction stage	Temperature	Time	No. of cycles
Initial denaturation	95	4 min	1
Denaturation	94	1 min	35
Annealing	56	1	35
Extension	72	1 min	35
Final extension	72	4 min	1

Table 2.10 List of PCR primers used in this study

Primers for validation of mutations		
Gene	Forward	Reverse
<i>dcuR</i>	AATAAACGAGATGAAGATACGC	GCTGTATTTGCGCCACTTTTC
<i>yxdM</i> - 1*	CGTATTCTCACTTTGAATGC	CCTTTAACGGCGTGAATGAG
<i>yxdM</i> - 2*	CTGGGTTGGTCTGAATCAGAC	GATGAAATTGTCCAAAATGG
<i>liaF</i>	CGAGTAAAATAAATGACAGAGGG	GATTTCAATTATATGCGTCCC
<i>mprF</i> _2.1*	GGGTCATTTATTTGCCAAAAAC	CCAAAAGTTGGATACTCATT
<i>mprF</i> _2.2*	GATGTACCGATTCTTTAATTG	CTTATATCCCAAAGAAGAACCG
<i>mprF</i> _2.3*	CAACTTATGGCGGGAATATTG	GTATAAAAAGCTTGACAACCTC
<i>nucF</i>	GGCTTTACGATTCGTCAACG	GTGCAGGTTTCAGTTGTGACG
<i>epaQ</i> _1*	CAGAACGGGGCATATATTTATCG	CAGTATCCGATTCCGCACATAAG
<i>epaQ</i> _2*	GTTTGGTTTTGTCTGGAGTAG	CATAATGTCAGCGGACAAGAAAG
<i>croS</i>	CAAACAGTGTGGGGAGTTGG	CTCTTTTCTATAGAATACCG
<i>croR</i>	GGATTTTGTCTTTTGTAGCA	CTGCTAATAACTCGCTGATTTT
<i>bgsA</i>	TAGTTAGTAAAGCAGAAAAGG	GACTTCTTTTAACTCGTAAC
<i>yyiH</i>	GTCACAAGGCGAAGAATTGAC	GTTCTGGCAACGGTTTAGAC

<i>DUF domain protein</i>	CATTGCTGTTTTACGAGGTGA	GTGGTGCCAGAACTTTTTTAAAT
<i>ygaE</i>	CTAGAGAAAAGTGAAGAATT	CCTCCGTTTAAAAAGGTTTC
<i>bceAB_1*</i>	ATGCAAGCAACGATTGGTGG	AACTAAAAAGTCCACGGGGC
<i>bceAB_2*</i>	AGTATGCACACGGTCAACAG	AACTGTAAAAACCGCTTCAC
<i>fmt</i>	GAAGCGTATATGGAGGAAC	TTCTTGGGGATTTTTTCAGCC
<i>pspC</i>	GTAAAATTTAGTAGACGAGG	GTGTATTAACAACCAATCGCT
<i>yxdJ</i>	TATATGGTGCCCTAGATAGG	ATCACCTCTTTAATTTGCTG
Primers for construction of complementation plasmids by Gibson assembly :		
Gene	Forward	Reverse
<i>dcuR</i>	CTTTAGTGATGATGGTGATGGTGATGGTGGGATCC AATTCTCCGATACGATTGATAGGG	TCATTGATAGAGTGAGCTCAAGGAGGAGACTGACCAT GAACCTATTGATTATTGAAGACGATC
<i>yxdM</i>	CTTTAGTGATGATGGTGATGGTGATGGTGGGATCC ATCTTTCACCTAAGTGATAAACTTTTTTGAAG	TCATTGATAGAGTGAGCTCAAGGAGGAGACTGACCAT GAATTTTAATCAGTTTGTTATAAGAAACACG
<i>liaF</i>	CTTTAGTGATGATGGTGATGGTGATGGTGGGATCC TACACGGATCACCTCAACATC	TCATTGATAGAGTGAGCTCAAGGAGGAGACTGACCAT GAATAACCCTTGGCGCTTT

<i>mprF_2</i>	CTTTAGTGATGATGGTGATGGTGATGGTGGGATCC GTCAATATTTTTTTGATCGACAATGAG	TCATTGATAGAGTGAGCTCAAGGAGGAGACTGACCAT GAAACAAATTATTCATTGGATCAAAACG
<i>nucF</i>	TCATTGATAGAGTGAGCTCAAGGAGGAGACTGAC CGTGAAAAAAGACTATCAAGG	CTTTAGTGATGATGGTGATGGTGATGGTGGGATCCTT CCTTAGATGAATGGACTTTT
<i>epaQ</i>	CTTTAGTGATGATGGTGATGGTGATGGTGGGATCC TCTGCGATGTCTTTGGACAC	TCATTGATAGAGTGAGCTCAAGGAGGAGACTGACCAT GGGAAAAGTATTGAATAGAATCGG
Primers for validation of constructs generated by Gibson assembly :		
Gene	Forward	Reverse
<i>dcuR</i>	CGTAGCGTTAACAGATCTACTC	CTCGTTGGCAGCCGTAATCAGG
<i>yxdM</i>	CGTAGCGTTAACAGATCTACTC	CACGGCTAAGAAAGATTTAAC
<i>liaF</i>	CGTAGCGTTAACAGATCTACTC	CCAGCGGCGTTTAAATCGTTTGC
<i>mprf_2</i>	CGTAGCGTTAACAGATCTACTC	GGGAAATAAAGACCGCCACC
<i>nucF</i>	CGTAGCGTTAACAGATCTACTC	CCCACGACAACATAATCAGG
<i>epaQ</i>	CGTAGCGTTAACAGATCTACTC	GGAATCAACTGTGGCAACTCC

*Genes greater than 1000 bp in length were amplified using sets of overlapping primers

2.5.3 Agarose gel electrophoresis

To analyse DNA fragments after amplification or restriction digestion, gel electrophoresis was performed. 1% agarose gels were made by dissolving agarose powder (Fisher) in 1x Tris-acetate-EDTA (TAE) buffer by heating. Once the agarose gel mixture was cooled, SYBR safe DNA gel stain (Strattech) at 1:10000 dilution of was added. DNA samples were mixed with 6x loading dye and 10 µl was loaded onto the gel along with DNA ladder (NEB). Gels were run in TAE buffer at 100 mV for 30 mins after which the bands were visualized and images were captured using Syngene ingenius gel imaging system.

2.5.4 Gel extraction and PCR purification

After amplification and gel electrophoresis, PCR products were purified. If a single fragment was visualized after gel electrophoresis, DNA was purified from the reaction tube using the Qiagen PCR purification kit (Qiagen). If more than one fragment was visualized, gel extraction was performed using the Qiagen gel extraction kit (Qiagen). DNA was visualized and bands of interest were cut out using a scalpel and purified. The purified DNA was quantified using a Nanodrop and stored at -20°C in EB buffer (Qiagen).

2.5.5 Sanger sequencing

For Sanger sequencing, the samples were diluted to concentration of 10 ng/µl and primers were diluted to concentration of 3.2 pmol/µl. Sanger sequencing was performed by Source biosciences and sequencing data were analysed using Ape (Jorgensen, 2022).

2.5.6 Plasmid purification and digestion

Plasmid DNA from *E. coli* and *S. aureus* was purified for transformation into *E. faecalis*. DNA was extracted from a 5 ml of overnight culture using the QIAprep Miniprep kit (Qiagen). The concentration of the plasmid DNA was determined using a Nanodrop and the DNA was stored in EB buffer at -20°C.

For Gibson assembly, restriction digestion of pTet2op-dltABC was carried out with NcoI (NEB) and BamHI (NEB) to generate a linearized plasmid. Each reaction consisted of 1 µl of Cutsmart buffer (NEB), 1 µg of DNA, 1 µl of restriction enzyme (NEB) and MilliQ water up to a total volume of 10 µl. Reactions were incubated at 37°C for 1-2 hours. The digested products were then analysed by gel electrophoresis and gel extracted.

2.5.7 Gibson Assembly

Plasmids containing genes of interest were constructed using Gibson assembly. The primers used for Gibson assembly were designed using NEBuilder Assembly Tool and are described in **Table 2.10**. The PCR fragments used for Gibson assembly were generated and purified as described in **Section 2.5.4**. Plasmids were assembled using the Gibson Assembly Cloning kit (NEB). Each reaction consisted of 50 ng vector DNA, a two-fold molar excess of insert DNA, 10 µl 2X Gibson assembly master mix and water in a final volume of 10 µl. Reactions were incubated at 50°C in a heat block for 30 min. Following incubation, samples were stored at -20°C and subsequently used for transformation in *E. coli*.

2.5.8 Transformation of competent *E. coli*

Gibson assembly ligation reactions were transformed into *E. coli* DH5α cells (NEB). Briefly, 5 µl of ligation reaction was added to 50 µl of *E. coli* DH5α cells. The mixture was incubated on ice for 30 min, heat-shocked for 1 min at 42°C, and then incubated on ice again for 2 min. SOC medium (NEB) was added to the cells and they were incubated for 1 h at 37°C, shaking at 180 rpm. The cells were then spread on LB agar plates containing 200 µg/ml of erythromycin and grown overnight at 37°C.

2.5.9 Generation of electrocompetent *E. faecalis*

Electrocompetent *E. faecalis* was generated as follows. *E. faecalis* was grown overnight at 37°C in M17 media supplemented with glucose to a final concentration of 0.5% (M17G) without shaking. In parallel, 100 ml of M17G supplemented with sucrose and glycine to a final concentration of 0.5 M and 2% (v/v) respectively (SGM17) were added to a sterile 250 ml flask and warmed overnight at 37°C. 1 ml of overnight culture was subcultured into 100 ml of pre-warmed SGM17 media and grown at 37°C without shaking until it reached OD₆₀₀ = 0.5. The culture was centrifuged and washed 3 times in 1 L ice-cold suspension of (0.5 M sucrose, 10% glycerol). The cells were resuspended in 1 ml Suc-Gly, 50 µl aliquots prepared, and aliquots frozen with liquid N₂.

2.5.10 Transformation of Electrocompetent *E. faecalis*

Electroporation was performed as follows. First, electroporation cuvettes, microcentrifuge tubes and DNA were cooled and electrocompetent bacteria were thawed on ice. DNA (4 µl, 100-1000 ng) was added to 50 µl of electrocompetent cells and transferred to a 0.2 cm

electroporation cuvette (Bio-Rad). Bacteria were electroporated using a Bio-Rad Gene Pulser (25 μ F, 200 Ω , 2.4 kV). After electroporation 1 ml of cold M17G media supplemented with 0.5 M sucrose, 10 mM CaCl_2 , 10 mM MgCl_2 was added to the cells and the cells were kept on ice for 5 min. Cells were then incubated at 37°C for 1-3 hours without shaking and plated on BHA supplemented with 5 μ g/ml tetracycline or 30 μ g/ml erythromycin.

To generate fluorescently labelled *E. faecalis*, pVM158-GFP, a plasmid constitutively expressing GFP was used. To generate rescue constructs for *yxdM*, *dcuR*, and *epaQ*; pTet2op-dltABC, containing the gene of interest was purified from *E. coli* DH5 α and used. Protein expression for rescue constructs was induced using anhydrotetracycline at 10 ng/ml.

2.6 Bacterial growth and survival assays

2.6.1 Growth curves

Growth curves were set up in sterile 96-well plates (Grenier Bio-One, 655161). 200 μ l of milliQ distilled water was added to all the peripheral wells to prevent absorption of media. Overnight cultures were added to wells containing MHB supplemented with 22.153 mM glucose to get a final concentration of 5×10^5 CFUml⁻¹ in a total volume of 200 μ l. For negative controls, three wells were filled with media but not bacteria. The plates were then placed in a TECAN Infinite M200 PRO plate reader. The plates were incubated for 18h at 37°C (180 rpm) and absorbance at 600 nm was measured every 30 min. Each strain was measured in triplicate.

2.6.2 Determination of Antibiotic Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) of antibiotics were determined using a 2-fold serial broth dilution protocol. Briefly, overnight cultures were incubated in a sterile 96-well plate with a range of antibiotic concentrations to get a final concentration of 5×10^5 CFU ml⁻¹ in MHB. The range of antibiotic concentrations was generated by 2-fold serial dilutions in media starting from the highest concentration of antibiotic and resulting in final volume of 200 μ l. After static incubation at 37°C for 18 hours, the MIC was defined as the lowest concentration for which there was no visible bacterial growth. For negative control, three wells were filled with media but not bacteria. **Table 2.11** describes the concentration of antibiotics used in this thesis.

Table 2.11 Antibiotic concentrations used for MIC's

Antibiotic	Highest concentration (μ g/ml)	Lowest concentration (μ g/ml)
Ampicillin (Sigma-Aldrich)	4	0.0625
Bacitracin (BioVision)	512	8
Ceftriaxone (Sigma-Aldrich)	8	0.125
Daptomycin (Tocris)	32	0.5
Gentamicin (Sigma-Aldrich)	512	8
Linezolid (LKT labs)	8	0.125
Nisin (Sigma- Aldrich)	512	8
Polymyxin-B (Abcam)	1024	16
Teicoplanin (Enzo Life Sciences)	8	0.125

Vancomycin (Sigma-Aldrich)	8	0.125

2.6.3 *E. faecalis* survival in human blood

Overnight cultures were washed twice in sterile PBS and adjusted to 10^6 CFU/ml. 10 μ l of this bacterial suspension was added to the wells of a sterile 96-well flat-bottomed plate (Grenier Bio) and incubated with 90 μ l of freshly-donated human blood (collected in EDTA-treated tubes; BD Biosciences). Blood from each of four individual volunteers was assayed twice. For the 0 hour timepoint, bacteria were immediately serially diluted in sterile PBS and plated onto BHA. For the 6 hour timepoint, plates were sealed with Parafilm and incubated for 6 hours at 37 °C with shaking at 180 rpm. Bacteria were enumerated by serial dilution and plating onto BHA.

2.6.4 Bile killing assay

Bile tolerance was assayed using a protocol modified from Zhou *et al.* (Zhou et al., 2019). The ancestral *E. faecalis* strain was challenged with four different concentrations of bile: 5%, 10%, 15% and 20% in BHI. A clear difference in the CFU's between the bile and no bile control was observed when *E. faecalis* was challenged with a 10% bile suspension. As a result, all further experiments were carried out using a 10% bile suspension in BHI.

To assay bile tolerance, 100 μ l of overnight culture was added to 3 ml of 10% bile suspension in BHI. The samples were incubated for 1 hour at 37°C with shaking at 180 rpm. Control suspensions without bile were also set up in parallel. After incubation, 1 ml of this suspension

was centrifuged for 5 mins at 4000x g. The supernatant was discarded, and the pellet was resuspended in 1 ml of sterile PBS. Bacteria were serially diluted and plated onto BHA.

2.6.5 Antibiotic adaptation of bacterial strains

Antibiotic adaptation of *E. faecalis* was carried out by repeated exposure to increasing concentrations of antibiotic. 100 µl of an overnight culture was exposed to 1,2,4,6, or 8X MIC of a particular antibiotic in 3 ml of BHI. The cultures were incubated for ~16 hours at 37°C with shaking at 180 rpm. The following day, 100 µl was sub-cultured from the culture with the highest antibiotic concentration in which visible bacterial growth was seen into two fresh cultures, one with the same concentration of antibiotic and the other with 2x this concentration. This process was repeated for 10 cycles of passage with 5 parallel replicate populations. Glycerol stocks were made at each cycle from the culture showing visible growth at the highest antibiotic concentration for each replicate population. Controls without antibiotic were run in parallel to account for media-effects. At the end of the experiment, bacteria from glycerol stocks were cultured in the absence of antibiotic, single colonies selected, and then MIC of these clonal populations were tested to confirm that resistance was fixed. These clonal drug-resistant strains were then used for downstream analysis.

2.7 Analysis of bacterial surface properties

2.7.1 Surface charge measurements

Bacterial surface charge was measured using a fluorometric method that quantified binding of FITC-PLL to bacterial surface. FITC-PLL is a positively charged fluorescent dye that binds to negatively charged bacterial surfaces. For measurements of bacterial surface charge, 200 µl

aliquots of overnight cultures were incubated with 80 µg/ml FITC-PLL (MW 15,000 – 30,000; Sigma) for 10 min at room temperature in the dark. Samples were washed three times in sterile PBS and transferred to a black-walled 96-well plate (Nunc MicroWell 96-Well Optical-Bottom Plates, Thermo Scientific 165305). Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. Measurements were made using a TECAN Infinite M200 PRO plate reader.

2.7.2 Membrane fluidity measurements

Membrane fluidity was measured using laurdan, a fluorescent dye sensitive to membrane packing. The emission spectrum for laurdan when lipids are disordered is 490 nm which shifts towards 440 nm when the lipids are packed (Sanchez et al., 2012). To measure membrane fluidity, 200 µl aliquots of overnight cultures were incubated with 100 µM laurdan for 5 min in the dark at room temperature. Samples were washed three times in sterile PBS and transferred to a black-walled 96-well plate (Nunc MicroWell 96-Well Optical-Bottom Plates, Thermo Scientific 165305). Membrane fluidity was measured by using an excitation wavelength of 330 nm and measuring fluorescence at emission wavelengths of 460 nm and 500 nm. Generalised polarisation (GP) was then calculated using the formula $GP = (I_{460} - I_{500}) / (I_{460} + I_{500})$. I_{460} and I_{500} are the fluorescence measurements at 460 and 500 nm respectively. Measurements were made using a TECAN Infinite M200 PRO plate reader.

2.7.3 Western Blot for lipoteichoic acid

Western blot was used to analyze changes in lipoteichoic acid composition of the different *Drosophila*-adapted strains. 6 ml of OD₆₀₀ = 1 culture was centrifuged and resuspended in 1 ml of sterile PBS. This suspension was transferred to Lysing Matrix B tubes (2 ml, MP

Biomedicals) and homogenization (2 x 40 s, 300 s rest time) at 6 m/s was performed (FastPrep 24, MP Biomedicals). Samples were centrifuged at 200x g for 1 min to settle the beads and 500 µl of supernatant was transferred to new microcentrifuge tubes. Samples were then centrifuged for 15 min at 17,000x g, the supernatant discarded and the pellet resuspended in 100 µl of 2X Laemmli buffer. Following this, samples were boiled for 20 min, centrifuged for 4 mins at 16,000x g and 10 µl of the sample and protein ladder (NEB P7706) were loaded onto pre-cast 12% Poly-acrylamide gel (NuPAGE 12% Bis-Tris gel, Invitrogen NPO343BOX). Electrophoresis was performed using 3-(N-morpholino) propanesulfonic acid (MOPS) buffer and gels were run at 300mA, 200V, 50 Watts for 50 min (Novex Life Technologies). Composition of buffers used for Western Blot are described in **Table 2.12**.

Following electrophoresis, LTA was transferred via electroblotting onto polyvinylidene fluoride (PVDF) Immobilon Transfer membrane (Millipore). The PVDF membrane was incubated in methanol prior to transfer. The transfer apparatus consisted of two sheets of Whatmann paper and two sponges that had been previously soaked in ice-cold transfer buffer in addition to the gel and PVDF membrane. The transfer was performed at 400mA, 250V, 50W for 1 hour using a Life Technologies wet transfer apparatus. The transfer was carried out at 4°C and the contents of the chamber (Biorad) were stirred continuously using a magnetic stirrer.

Following transfer, the membrane was blocked at room temperature for 1 hour in TBS-T (TBS, 0.1% Tween-20) supplemented with 5% milk (Cell Signalling) on a rolling platform. After blocking, the membrane was washed 3 x 5 min in TBS-T to remove excess blocking solution. Incubation with primary antibody, monoclonal LTA antibody (Hycult HM2048 mAb 55 monoclonal LTA antibody) in a 1:5000 dilution in 5% bovine serum albumin (BSA, Serva) in TBS-T was done overnight at 4°C on a rolling platform. The membrane was washed three times with TBS-T and incubation with secondary antibody in a 1:5000 dilution (anti-mouse HRP, Cell Signalling), was performed in 5% milk in TBS-T for 2 hours at room temperature.

Following incubation with secondary antibody, the membrane was washed again with TBS-T, 3 x 5 min. The antibody-stained membrane was then incubated for 5 min with chemiluminescent HRP-substrate solution (SuperSignal West Pico PLUS, Thermo Scientific 34580) and blots were imaged using a Bio-Rad Chemidoc XRS+ imaging system. Images were analyzed using ImageJ (Schneider et al., 2012) and the bands corresponding to LTA were identified by comparison to bands of known mass in the protein ladder.

Table 2.12 Compositions for buffers for Western blots

Buffer	Composition
2x Laemmli buffer	4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue in 0.125 M Tris HCl (pH 6.8)
MOPS running buffer (20x stock solution, 500 ml)	104.6 g MOPS, 60.6 g Tris-Base, 10 g SDS 3 g EDTA in ddH ₂ O (made upto 500 ml)
Transfer buffer (2L)	6.06 g Tris Base, 28.8 g Glycine, 400 ml MeOH, 1600 ml ultra-pure H ₂ O
TBS 10x stock solution	24.2 g Tris-base, 80 g NaCl, in ddH ₂ O (made upto 1L)
TBS-T	100 ml 10X TBS stock solution, 10ml 10% Tween20 (Sigma-Aldrich P1379) in ddH ₂ O (made upto 1 L)

2.8 Statistical analysis and data visualization

Data visualisation was performed using Graphpad Prism version 9 and R Studio with R version 4.0.4. All statistical analyses were performed using R Studio. In all boxplots, the heavy line indicates the median, the extremes of the box indicate the first and third quartiles, and the whiskers a further 1.5x the interquartile range or to the most extreme value, whichever is closer to the median. Growth curves were analysed and plotted using the 'growthcurver' package in R Studio (Sprouffske & Wagner, 2016). Survival data were initially analysed using the Cox proportional hazards models, with ANOVA for pair-wise comparisons. Survival curves were plotted using the Kaplan-Meier method. For all other assays, I tested for normality using a Shapiro-Wilk test before selecting the appropriate statistical tests. For normally distributed data, comparisons were made using ANOVA followed by Tukey's test for multiple comparisons. For non-normally distributed data, Mann-Whitney test was used for comparisons between two conditions and Kruskal-Wallis test was performed for comparisons involving more than two conditions. Corrections for multiple comparisons were made using Bonferroni's adjustment.

Chapter 3: Development of a system to study the *in vivo* interaction of *Enterococcus faecalis* with the *Drosophila* immune response

3.1 Introduction

Experimental evolution is a technique that is commonly used to study the mechanisms by which organisms adapt in response to pre-determined selective pressures (Elena & Lenski, 2003). Early studies utilizing this technique were often limited to studying the phenotypic consequences of adaptation to a given stress (Lenski et al., 1991; Lenski & Travisano, 1994). However, in recent years, aided by the introduction of next generation sequencing technologies, identification of the genetic basis for adaptation at a low cost is now possible (Brockhurst et al., 2011). As a result, a direct link between stress exerted by a particular environment and genetic and phenotypic changes that are an outcome of this selection can be established.

When utilized to understand interactions between insect immune responses and pathogens, experimental evolution has been primarily used to study the mechanisms of host-adaptation to pathogens and the consequences of this adaptation in terms of cross-resistance to other infections (Leitao et al., 2020; Martins et al., 2014). Other studies have used this experimental evolution to identify the targets of insect antimicrobial peptides (Dobson et al., 2014; El Shazely et al., 2019; Johnston et al., 2016). However, to my knowledge, no study has used

experimental evolution to identify the mechanisms through which the insect immune response exerts its action *in vivo*. The use of experimental evolution in this context allows for the recapitulation of the different stresses that a pathogen might encounter during host infection. In an *in vivo* setting this is particularly advantageous to study the mechanisms of immune components that require unknown host factors to exert their effect, such as the Bomanin family of effector peptides (Cooper et al., 2020; Huo et al., 2022; Lindsay et al., 2018).

3.2 Aims

The **aim** of this chapter was to generate *E. faecalis* strains that have adapted to the action of the *Drosophila* immune response. To do this I designed and optimized a protocol for experimental evolution of *E. faecalis* in *Drosophila* during systemic infection. Bacterial populations generated as a result of this serial passage were screened to identify *E. faecalis* strains that had adapted to the *Drosophila* immune response. Genetic and phenotypic characterization of these strains can provide us with an insight into the mechanistic nature of the *Drosophila* immune response against this bacterium as well as inform us about the consequences of this interaction on bacterial phenotypes relevant for human disease.

3.3 Experimental evolution of *E. faecalis* in *Drosophila melanogaster*

I performed experimental evolution by the serial passaging *E. faecalis* in *Drosophila*. I took into consideration several different factors while designing my experimental evolution protocol. Some of these factors are commonly considered when designing experiments studying host-pathogen interactions such as the bacterial inoculum and the end-point of infection (or period of infection). Some other factors are more relevant in terms of the evolutionary process such as the number of replicate populations that are propagated and the number of cycles of passage that need to be carried out. These different parameters can influence the outcome of selection and need to be optimized based on the trait being selected. In this project, the aim was to select bacteria that had adapted in response to the *Drosophila* immune response. This meant I wanted to generate bacteria that grew to higher numbers in comparison to the control strain when in the presence of an active and strong fly immune response. To generate these strains I performed three serial passage experiments and over the course of these experiments, I optimized parameters that I identified as problematic. Some choices I made during the first round of serial passage were arbitrary and served to help me identify issues and optimize the experimental setup.

The first parameter I focused on was bacterial inoculum. An initial low dose of bacteria can not only lead to quick bacterial clearance by the host but also create a bottleneck during adaptation. This can influence the outcome of the experiment as chance expansion and fixation of a particular allele may play a bigger role than selection (Cooper et al., 2020; Elena & Lenski, 2003; Vogwill et al., 2016). Having too high an initial bacterial dose is not optimal either and can lead to rapid bacterial proliferation by overwhelming the ability of the immune response to control infection. In this instance, the presence of an immune response may only

correspond to a weak or ineffective selective pressure as pathogen proliferation occurs at high rates even when adaptive traits are absent. These two scenarios are not suitable for studying bacterial adaptation to the immune response. An ideal experiment to study this process involves having an initial inoculum that is large enough to avoid bottlenecks whilst at the same time small enough to allow the immune response to function as an effective selective pressure.

To identify a bacterial inoculum that would be suitable for the experimental evolution setup, I performed survival experiments by infecting flies with 50 nl of *E. faecalis* at two optical densities (OD); $OD_{600} = 0.1$ and $OD_{600} = 1.0$ (**Figure 3.1A, B**). I observed a bacterial dose-dependent effect on host lifespan. Flies infected with an inoculum of $OD_{600} = 0.1$ show no differences in survival compared to uninjected control flies, indicating the possibility of bacterial clearance. In flies that were infected with $OD_{600} = 1.0$ of *E. faecalis*, I observed a reduction in host survival compared to the uninjected controls. $OD_{600} = 1.0$ corresponds to approximately 8,000-10,000 colony forming units (CFUs) (**Figure 3.2**) and represents a large initial concentration of bacteria. Infection of flies with this bacterial dose does not appear to result in rapid clearance by the immune response nor does it overwhelm the immune response and kill all the infected flies. As this matches the criteria I set out above, I chose this concentration of bacteria ($OD_{600} = 1.0$) for my evolution experiments.

Another important factor that I considered was the period of time the bacteria are allowed to evolve in the host. It is important that bacteria are given enough time to adapt in response to the *Drosophila* immune response. Based on the median survival of flies infected with $OD_{600} = 1.0$ (seven days), I decided that the infection should proceed for 72 hours after which bacteria are recovered from flies, grown on selective media, and used for the next passage. For the first evolution experiment, I carried out six cycles of serial passage.

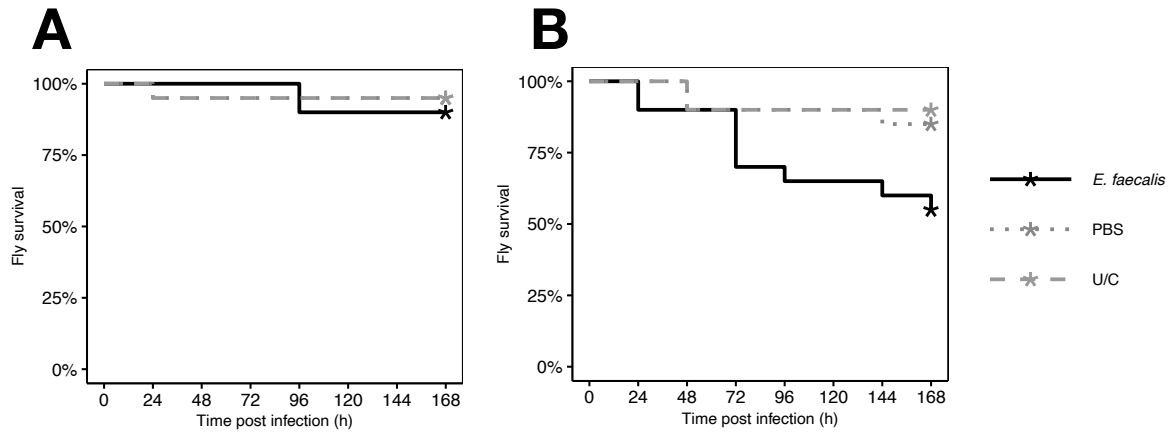


Figure 3.1 *Drosophila* survival after infection with *E. faecalis* at two concentrations

Infection with a low inoculum of *E. faecalis* ($OD_{600} = 0.1$), leads to little to no mortality (**A**), whereas infection with a higher inoculum of *E. faecalis* ($OD_{600} = 1.0$), leads to an increase in host mortality compared to uninjected and mock-infected controls (**B**). In **A** and **B**, $n = 20$ per condition.

While carrying out the first evolution experiment, I noticed that after a few rounds of serial passage, there was an increase in the virulence of *E. faecalis* in one of the replicates, and this was evident within 24 hours of infection. Beyond this time point, no more host mortality was observed. As a result, I was concerned about the possibility that bacteria could be under a selective pressure by 72 hours which is not specific to the *Drosophila* immune response. Thus, to gain a better insight into *E. faecalis* growth dynamics in *Drosophila*, I decided to quantify bacterial numbers over the first 24-hours of infection using $OD_{600} = 1.0$ inoculum of bacteria (**Figure 3.2**). I observed that between 2 to 6 hours post-infection, bacterial growth in the fly was exponential, however, at 8 and 24 hours post-infection there was a bimodal distribution in bacterial numbers with the strongest reduction in numbers occurring at 24 hours post-infection. This indicated that in some flies bacterial growth was being controlled by the action of the *Drosophila* immune response by 24 hours. Therefore, for the next two evolution experiments, I selected a 24-hour time point for serial passage, i.e. infection was now allowed to proceed for 24 hours rather than 72 hours. To ensure that bacteria were in the fly for a

comparable time to the first evolution experiment, I increased the number of cycles of serial passage from six to ten for these experiments.

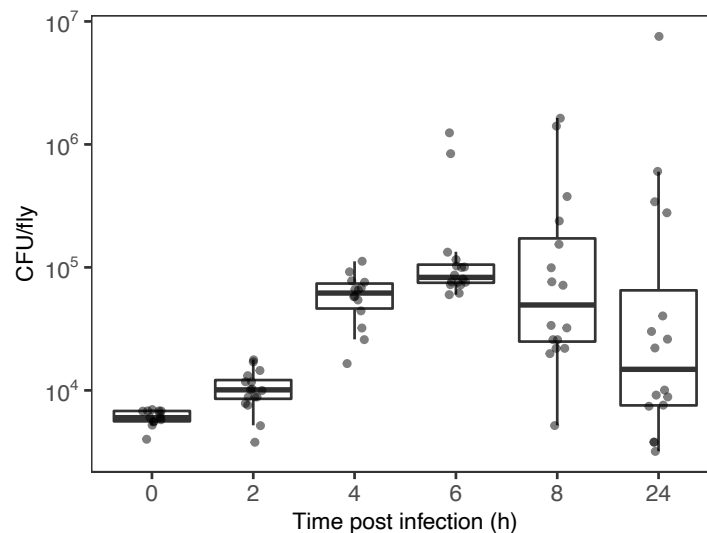


Figure 3.2 *In vivo* growth dynamics of *E. faecalis* in *Drosophila melanogaster*

Growth of *E. faecalis* over a period of 24-hours after systemic infection in *Drosophila melanogaster*. These experiments were performed using $OD_{600} = 1.0$ inoculum of bacteria.

The final factor I took into consideration while designing the evolution experiment is the number of replicate populations. Replicate populations can provide insight into the reproducibility of evolutionary processes at both phenotypic and genetic levels. They can also help identify the relative contributions of the selective pressure and stochastic processes such as genetic drift on the final phenotype. For the first evolution experiment, I decided to propagate three replicate populations. However, after characterizing the bacteria generated from this experiment, I could not observe any convergence in bacterial phenotypes among these three replicate populations (**Figure 3.4A-F**). As a result, I decided to increase the number of replicate populations to six for the second serial passage experiment and to ten replicate populations for the third serial passage experiment.

I wanted to ensure that the bacteria generated at the end of the serial experiment were the result of competition in the host and not due to competition in media. As a result, solid media was used throughout the serial passage experiment as this allowed for the co-existence of multiple bacterial clone which would not be possible in liquid media.

Finally, to take into consideration changes in bacterial behaviour that could be the result of growth on selective media, I carried out plate control experiments. Here, *E. faecalis* was passed through the *Mitis salivarius* agar (MS agar) plate ten times. Plate control experiments were run in parallel to the second evolution experiment. A simplified schematic describing the experimental evolution set-up is shown in **Figure 3.3**.

When the serial passage experiment was being performed I believed that the ancestral strain was clonal. However, it became clear as I performed more experiments that this was not the case and the ancestral strain was heterogenous and made up of two recently diverged subpopulations of bacteria. More detailed information about this is in **Chapter 5**.

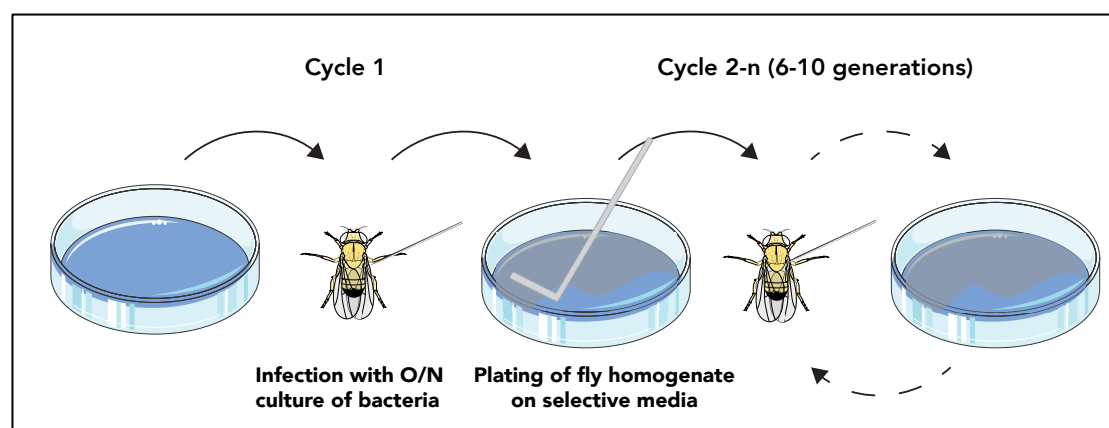


Figure 3.3 Experimental evolution schematic

Simplified schematic describing the set-up used to perform serial passage of *E. faecalis* in *Drosophila melanogaster*. A detailed description of the experimental evolution protocol can be found in **Materials and Methods** section of the thesis. 'O/N' stands for overnight.

3.4 Screening of *Drosophila*-passaged *E. faecalis*

By the end of the three serial passage experiments, I had generated 19 populations of *Drosophila*-passaged *E. faecalis*. The next step involved screening these populations to identify populations that had adapted in response to the *Drosophila* immune response. In the context of my experimental set-up, I considered strains to be adapted if they gained the ability to grow to higher numbers in the fly compared to the control strain. As the bacterial populations I generated could be mixed or clonal, I isolated and performed experiments with at least three single colonies from each passaged population for the screen. If different single colonies isolated from the same population showed similar behaviour in flies I would consider this population to be potentially clonal in nature. To carry out my screen, I used two different assays; in the first assay I quantified bacterial load 8 hours post-infection and in the second assay I monitored changes in host survival after infection. To eliminate the possibility that any changes in bacterial numbers 8 hours post-infection could be due to different input bacterial doses, I performed bacterial quantifications immediately after infection as well (0-hour timepoint).

For populations generated during the first serial passage, I carried out the screen using $OD_{600} = 1.0$ inoculum of bacteria that were grown on solid media. At this inoculum, I previously observed that infection of flies with the ancestral strain resulted in approximately 50% host mortality in a week (**Figure 3.1B**) and also that median bacterial load at 8 hours post-infection was lower than the initial inoculum (**Figure 3.2**). Focusing first on the identification of bacterial strains which had adapted in response to the *Drosophila* immune response, I observed that colonies from all three populations showed an increase in bacterial numbers eight hours post-infection compared to the initial inoculum (**Figure 3.4A, C, and E**). This enhanced bacterial growth *in vivo* was not accompanied by an increase in host mortality when flies were infected with colonies from populations 1 and 3 (**Figure 3.4B, F**). Infection with two out of four colonies

from population 2 led to $\geq 50\%$ host mortality 24 hours post-infection (**Figure 3.4D**). Flies infected with colonies from population 2 had substantially higher bacterial loads compared to flies infected with bacteria from the two other passaged populations. There was also no bimodal distribution of bacterial numbers, rather the distribution was more or less clustered. Bimodal distribution of bacterial numbers is a characteristic feature of *E. faecalis* infection in *Drosophila* (Duneau et al., 2017). The differences in bacterial numbers in flies that have been infected with the same inoculum of bacteria are believed to be due to differential activation of the immune response between these different hosts. Thus, flies that have initiated a strong immune response early in infection can control bacterial growth, whereas higher bacterial numbers are often associated with flies whose immune response has been unable to control bacterial growth. Flies infected with colonies from population 2 appear to be unable to control their infection suggesting that these bacteria have adapted in response to the *Drosophila* immune response and become resistant to its action. As for the clonal nature of these populations, I observed that single colonies from populations 1 and 3 had similar phenotypes when assaying for host survival, but the bacterial quantifications were harder to interpret here due to the bimodal distribution of bacteria. Colonies from population 2 showed similar behaviour when assayed for bacterial numbers and three out of four colonies showed similar levels of host mortality in the survival assay.

Screening for *Drosophila*-adapted *E. faecalis* from bacteria generated from the first round of experimental evolution did not yield straightforward results. It was not very clear at this stage whether the increase in bacterial numbers I observed in flies infected with bacteria from populations 1 and 3 was due to adaptation or was random. Similarly, any changes in host mortality could not be attributed to changes in virulence as I did not have the ancestral *E. faecalis* strain for comparison. In my hands, the behaviour of *E. faecalis* was extremely variable between experiments and thus more conclusions could not be drawn at this stage.

From this round of serial passage, I sequenced one representative colony from each of populations 2 and 3 (**More information in Chapter 5**).

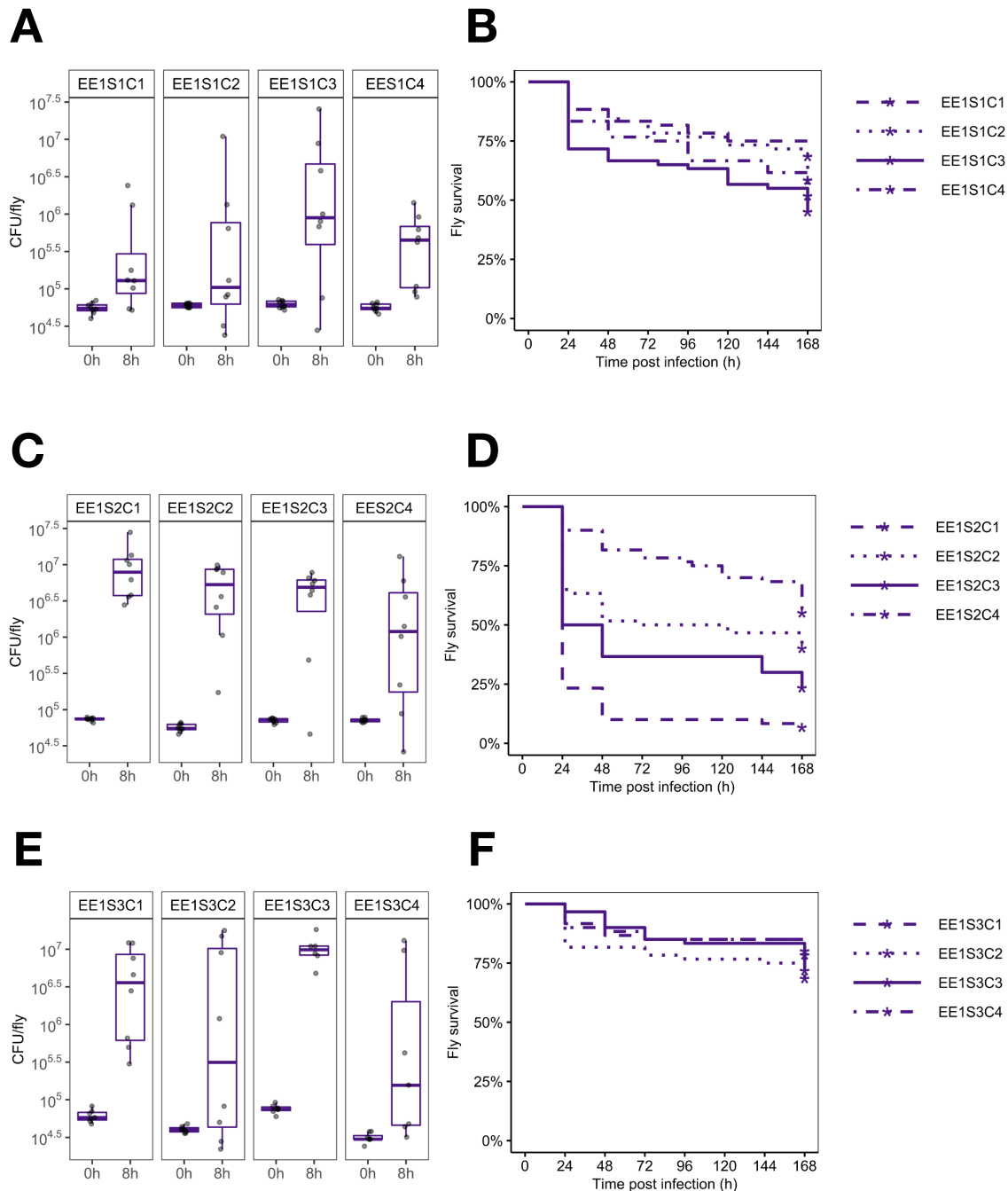


Figure 3.4 Analysis of the clonality and virulence of bacterial populations generated during the first round of serial passage

Survival analysis and bacterial quantifications of four single colonies from each passed population was carried out to identify whether the bacterial populations were mixed or clonal in nature and to identify changes in bacterial virulence (**A-F**). For **A**, **C** and **E**, $n = 8$ per condition per time-point, all data points are

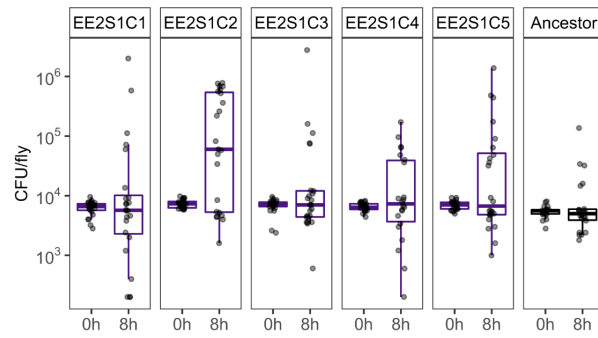
plotted on the graph. For **B**, **D** and **F**, $n = 15/20$ per condition, with data pooled from three independent experiments. These experiments were carried out using $OD_{600} = 1.0$ inoculum of bacteria.

Screening for *Drosophila*-adapted strains from bacteria generated in the second serial passage experiment was performed using the same conditions as those used for the previous round. However, this time I decided to include the ancestral *E. faecalis* strain as a control. An internal ancestral control allows us to assay for both changes in bacterial virulence as well as the clonal nature of colonies isolated from the same population. Infection with colonies from populations 1, 3, 4, and 5 did not diverge much from the control strain in terms of both bacterial numbers and host survival post-infection (**Figure 3.5A, B, E, F, G, H, I, J**). Infection with colonies from populations 2 and 6 on the other hand resulted in significantly higher bacterial loads and increased host mortality (**Figure 3.5C, D, K, L**). **Statistical analysis for all experiments in this thesis can be found in the appendix.**

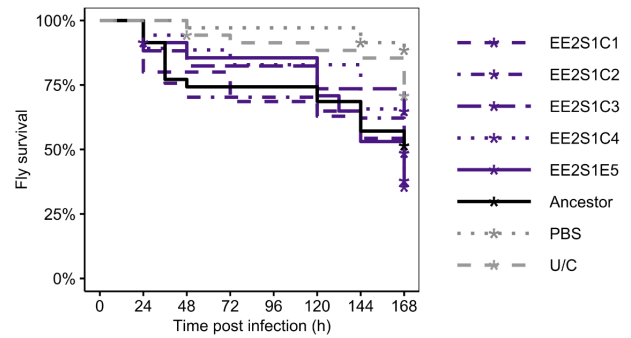
I also observed that during this screen, the ancestral strain was extremely variable in its behaviour between experiments, sometimes resulting in 10% host mortality and sometimes in 80% host mortality within 24 hours of infection. As a result, it was unclear whether the increase in virulence in some strains was due to an increase in overall virulence in *E. faecalis* due to a non-host effect or whether it was the result of adaptation to the immune response. Similar to what I observed with colonies from populations generated during the first round of evolution, colonies from the same populations generated during the second serial passage experiment also showed similar fitness phenotypes when assayed for host mortality after infection. Bacterial colonies from populations 1, 3, 4, and 5 showed a bimodal distribution of numbers and again it was hard to interpret from this whether the populations were clonal. However, colonies from populations 2 and 6 showed clustered distribution and similar bacterial numbers 8 hours post-infection, suggesting that these populations were clonal. Finally, given the

extremely variable nature of the ancestral strain during this round of screening, I sequenced at least one representative colony from all six populations generated in this experiment.

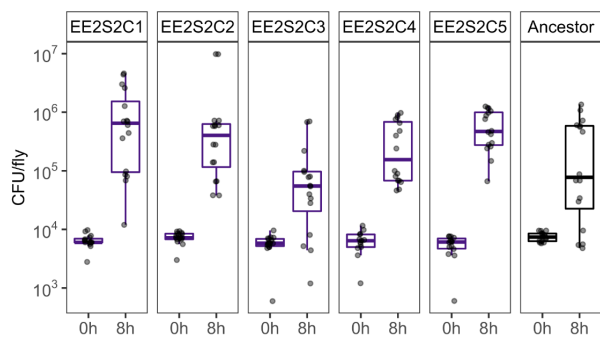
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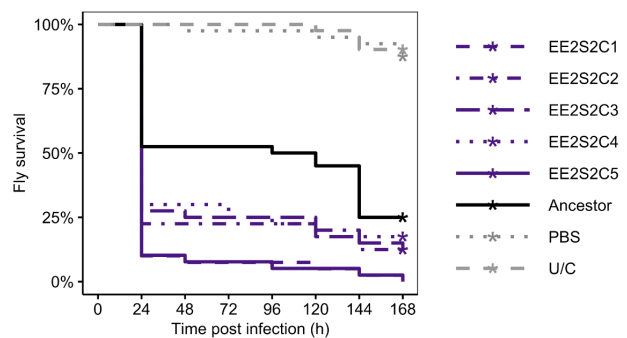
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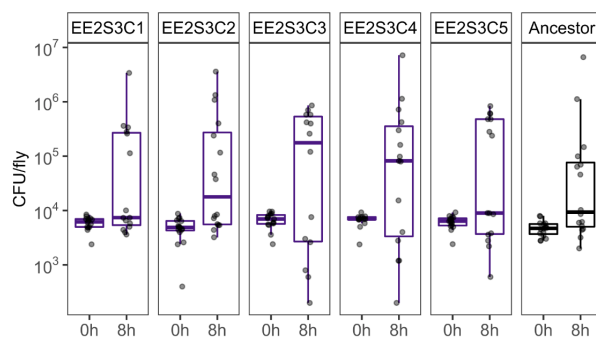
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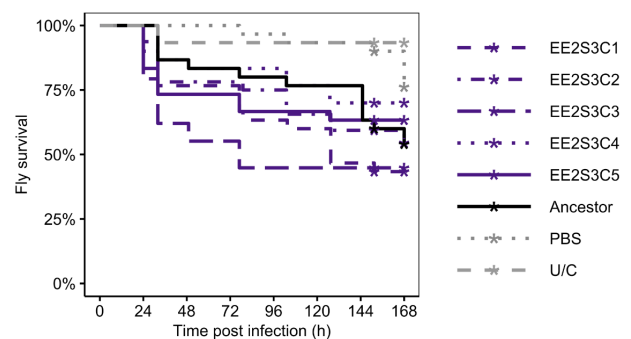
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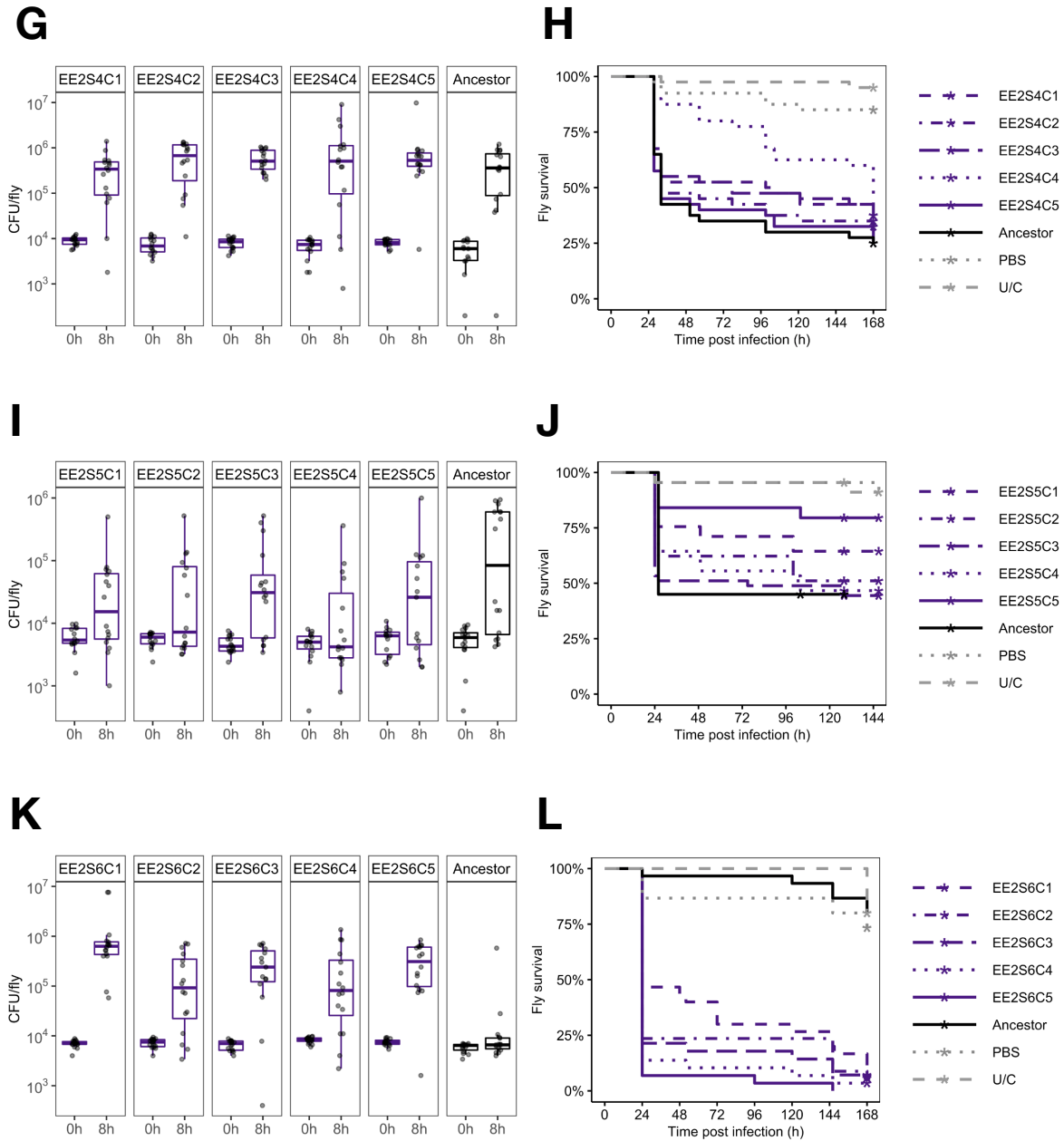
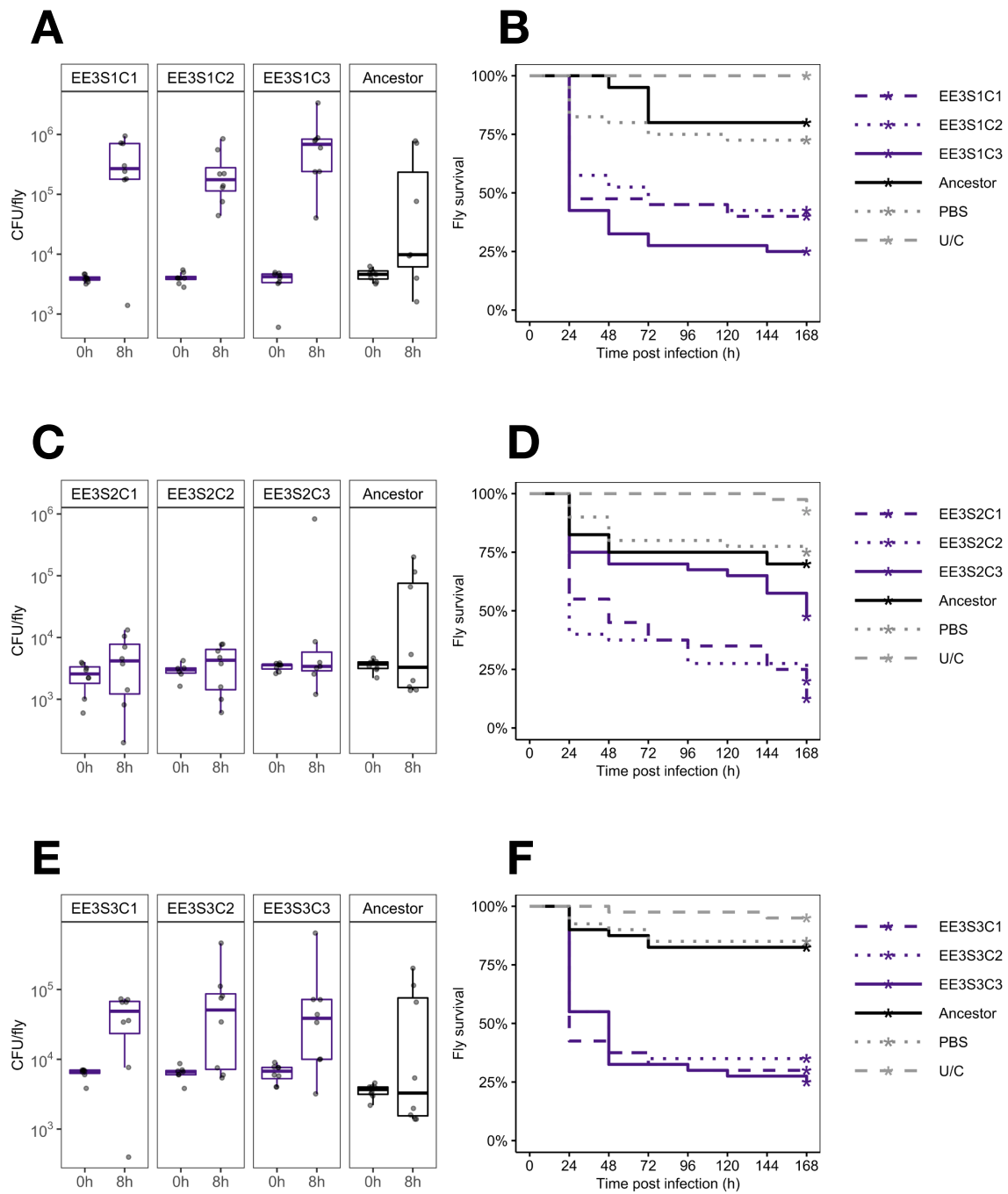


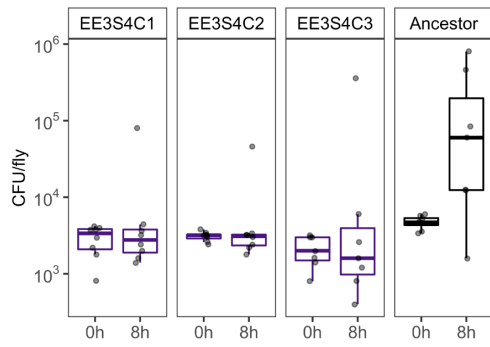
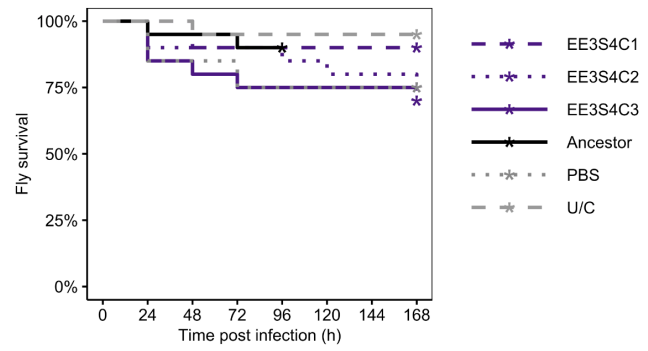
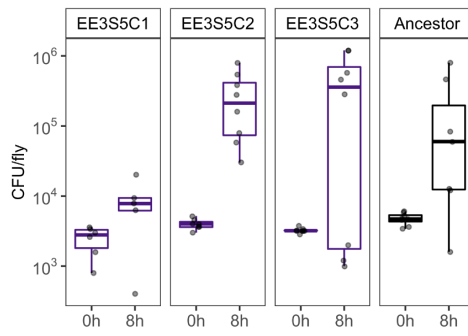
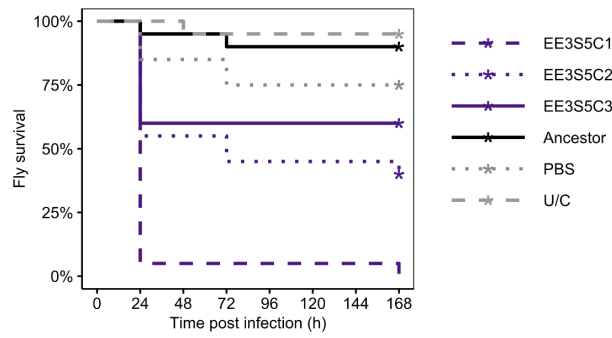
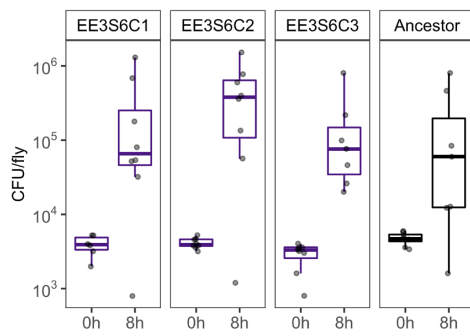
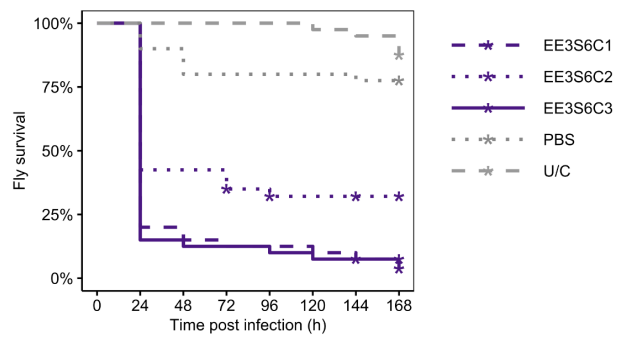
Figure 3.5 Analysis of the clonality and virulence of bacterial populations generated during the second round of serial passage

Survival analysis and bacterial quantifications of five single colonies from each passaged population was carried out to identify whether bacterial populations were mixed or clonal in nature. Changes in bacterial virulence were also quantified by comparison with behaviour of ancestor strain (A-L). For bacterial quantification experiments $n = 8$ per condition per time-point, with data pooled from two independent experiments. For survival experiments, $n = 15-20$ per condition, with data pooled from two independent experiments. These experiments were carried out using $OD_{600} = 1.0$ inoculum of bacteria.

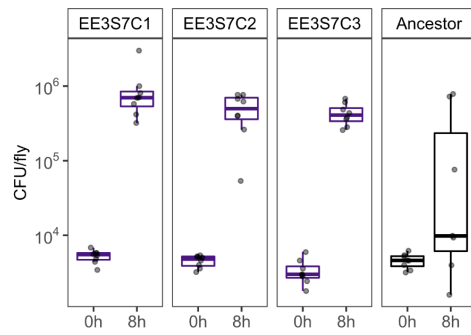
Screening for *Drosophila*-adapted strains from the third serial passage experiment was carried out using $OD_{600} = 0.3$ of inoculum. I observed that at $OD_{600} = 0.3$ of inoculum, the ancestral strain was less variable between experiments. For this screen, I grew the single bacterial colonies in sterile filtered brain heart infusion (BHI) broth instead of *MS* agar as I identified that autoclaving of media was the source of variation that I observed during the previous screens (Data not shown). I also made use of an ancestral control for almost every experiment here except for the survival assays for populations 6 and 10.

Colonies from populations 2 and 4 showed no changes in virulence (bacterial numbers and host mortality) compared to the ancestral control (**Figure 3.6C, D, G, H**). Colonies from population 10 did not show any increase in bacterial numbers compared to the ancestral control but led to approximately 90% host mortality within 24 hours of infection (**Figure 3.6S, T**). Infection of flies with colonies from all other populations resulted in an increase in both bacterial numbers and host mortality compared to the control strain (**Figure 3.6A, B, E, F, I-R**). For most populations, I did not see any divergence in phenotypes between colonies from the same population indicating again that the populations might be clonal. Single representative colonies from all populations except populations 2 and 4 were sequenced (**More information in Chapter 5**).

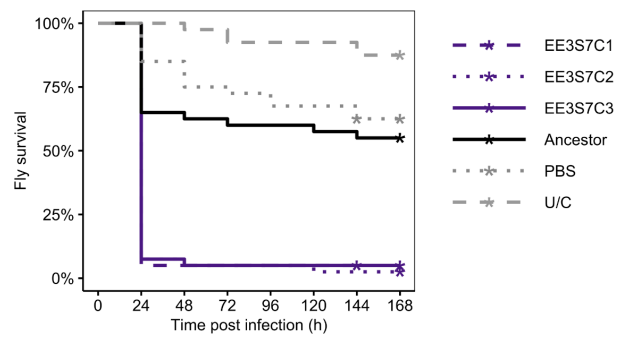


G**H****I****J****K****L**

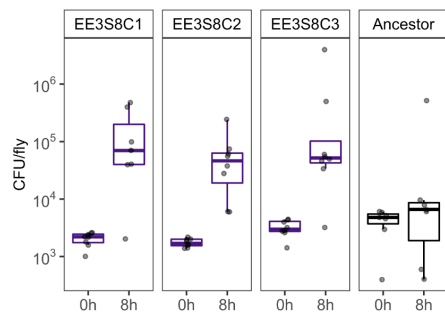
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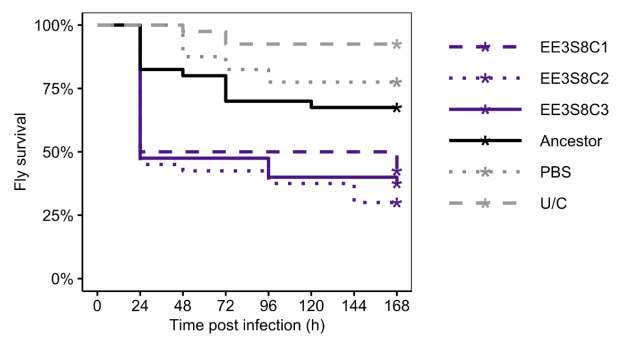
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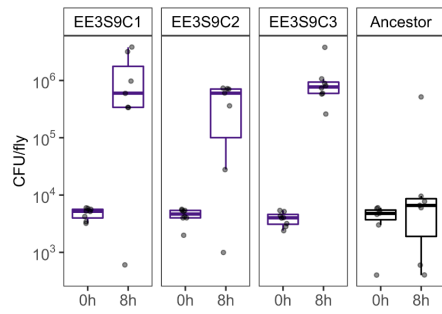
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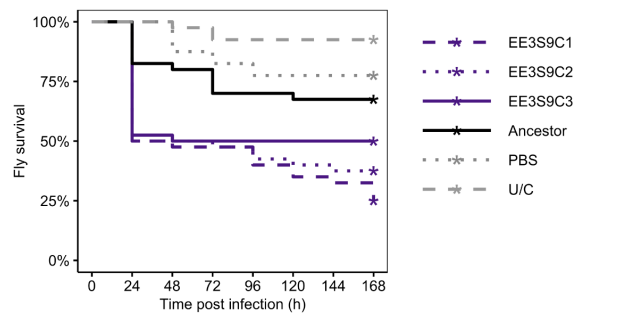
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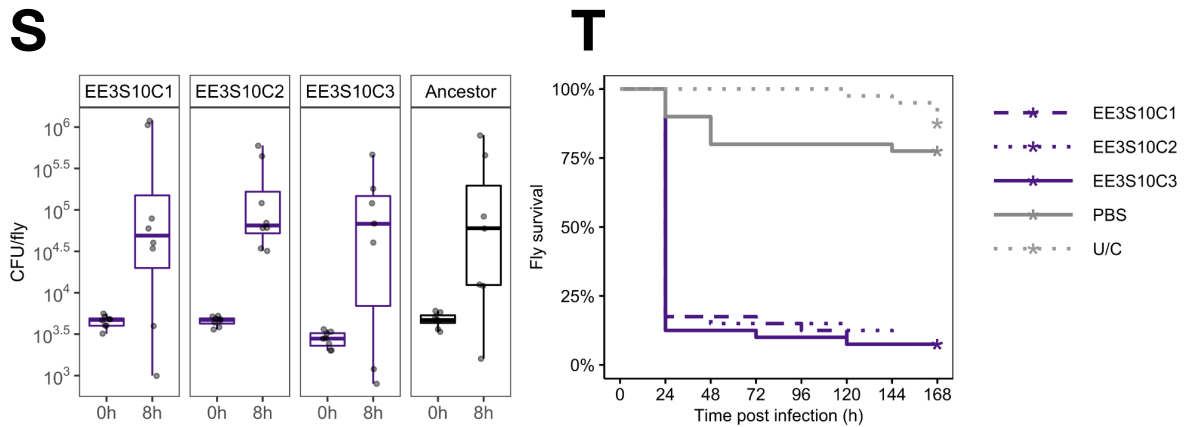


Figure 3.6 Analysis of the clonality and virulence of bacterial populations generated during the third round of serial passage

Survival analysis and bacterial quantifications of three single colonies from each passaged population was carried out to identify whether bacterial populations were mixed or clonal in nature. Changes in bacterial virulence were also quantified by comparison with behaviour of ancestor strain (A-T). For bacterial quantification experiments $n = 8$ per condition per time-point, with data pooled from two independent experiments. For survival experiments, $n = 15-20$ per condition, with data pooled from two independent experiments. Some experimental controls are shared between different populations as experiments were performed at the same time. Experimental evolution and screening of these strains was performed with the help of **Dr. Carolina J Simoes da Silva Pereira**. These experiments were carried out using $OD_{600} = 0.3$ inoculum of bacteria.

As mentioned in **Section 3.1.1**, I also carried out the serial passage of *E. faecalis* on *MS* agar to take into consideration the contributions of the media during the evolutionary process. I isolated single colonies from these populations and carried out fly infections. This passage and screen were performed in parallel to the second evolution experiment and as result, the same conditions were used for infections. I identified that colonies from media passaged bacterial populations (plate controls) did not deviate significantly from the control strain both in host mortality and bacterial numbers (**Figure 3.7A, B**). The only exception to this was the strain PC7, which showed an increase in host mortality compared to the control strain. However, this increase fell within the realms of variability that was observed in *E. faecalis* experiments during this round of serial passage with $OD_{600} = 1.0$ of inoculum. As a result, I

can conclude that the media does not seem to contribute to the increased virulence observed in some of the *Drosophila*-passaged bacterial strains.

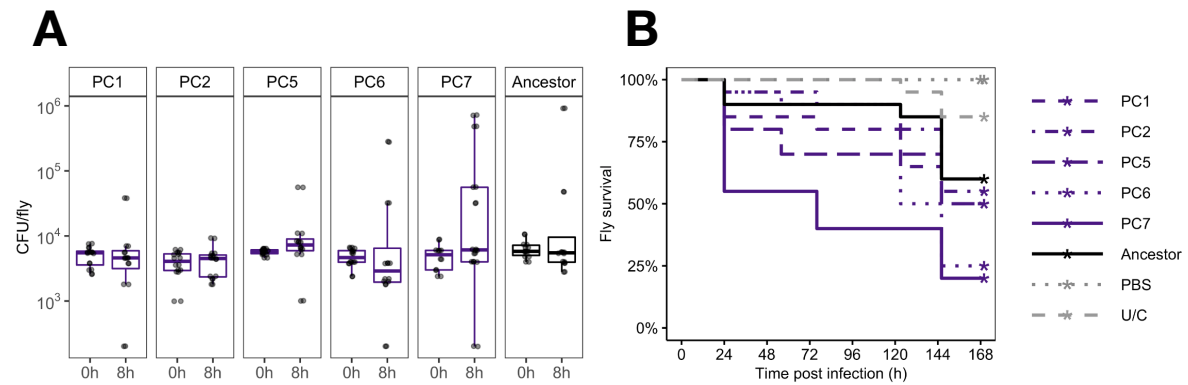


Figure 3.7 Media does not contribute to increase in virulence observed in some *Drosophila*-passaged *E. faecalis* strains

Survival analysis and bacterial quantifications of five single colonies from bacterial populations that have been serially passaged on *MS* agar ten times. Changes in bacterial virulence of these bacteria were quantified by comparison with behaviour of ancestral strain after infection (**A,B**). For bacterial quantification experiments $n = 8$ per condition per time-point, with data pooled from two independent experiments. For survival experiments, $n = 15-20$ per condition. These experiments were performed using $OD_{600} = 1.0$ inoculum of bacteria.

3.5 Characterization of single-colonies of *Drosophila*-passaged bacteria

Due to the variable virulence of the ancestral strain between experiments, it was hard to interpret the results from the strains generated in the first and second evolution experiments. However, by changing the growth medium for *E. faecalis* and by lowering the concentration of inoculum used for infection, I managed to resolve this issue. Accordingly, for strains generated during the third round of serial passage, I could clearly identify the bacterial populations that exhibited increased virulence compared to the ancestral strain. As a result, I decided to carry out infection experiments again, this time making use of single representative colonies from all bacterial populations which appeared to exhibit increased virulence during the initial screen. This included a representative colony from population 2 from the first round of passage, representative colonies from populations 1, 2, and 6 from the second round of passage; and representative colonies from all populations except 2 and 4 from the third round of passage. All infections were performed using $OD_{600} = 0.3$ of bacteria as the inoculum which was grown in sterile filtered BHI. I previously identified that this was a good dose to observe differences in virulence between the *Drosophila*-passaged and the ancestral strain, as with an $OD_{600} = 1.0$ of inoculum, these differences were less obvious.

On infection of flies with the above mentioned *Drosophila*-passaged single colonies, I observed a 10-100 fold increase in bacterial number 8 hours post-infection compared to the ancestral strain (**Figure 3.8A-C**). This increase in bacterial load correlated with a significant increase in host mortality compared to infections with the ancestral strain within 24 hours of infection (**Figure 3.8B, D**). These phenotypes were previously observed during the population level screen for most of these strains, but by making use of a lower bacterial inoculum I could now observe how much more virulent these strains were compared to the ancestral strain. I

termed these strains as *Drosophila*-adapted strains. I generated twelve *Drosophila*-adapted strains over the course of three serial passage experiments. However, for the rest of the thesis, I will focus mainly on six of these (**explained in Chapter 5**). These six strains are EE1S2C1 (**Strain A**), EE2S1C2 (**Strain B**), EE2S2C3 (**Strain C**), EE2S6C5 (**Strain D**), EE3S3C1 (**Strain E**), EE3S10C1 (**Strain F**).

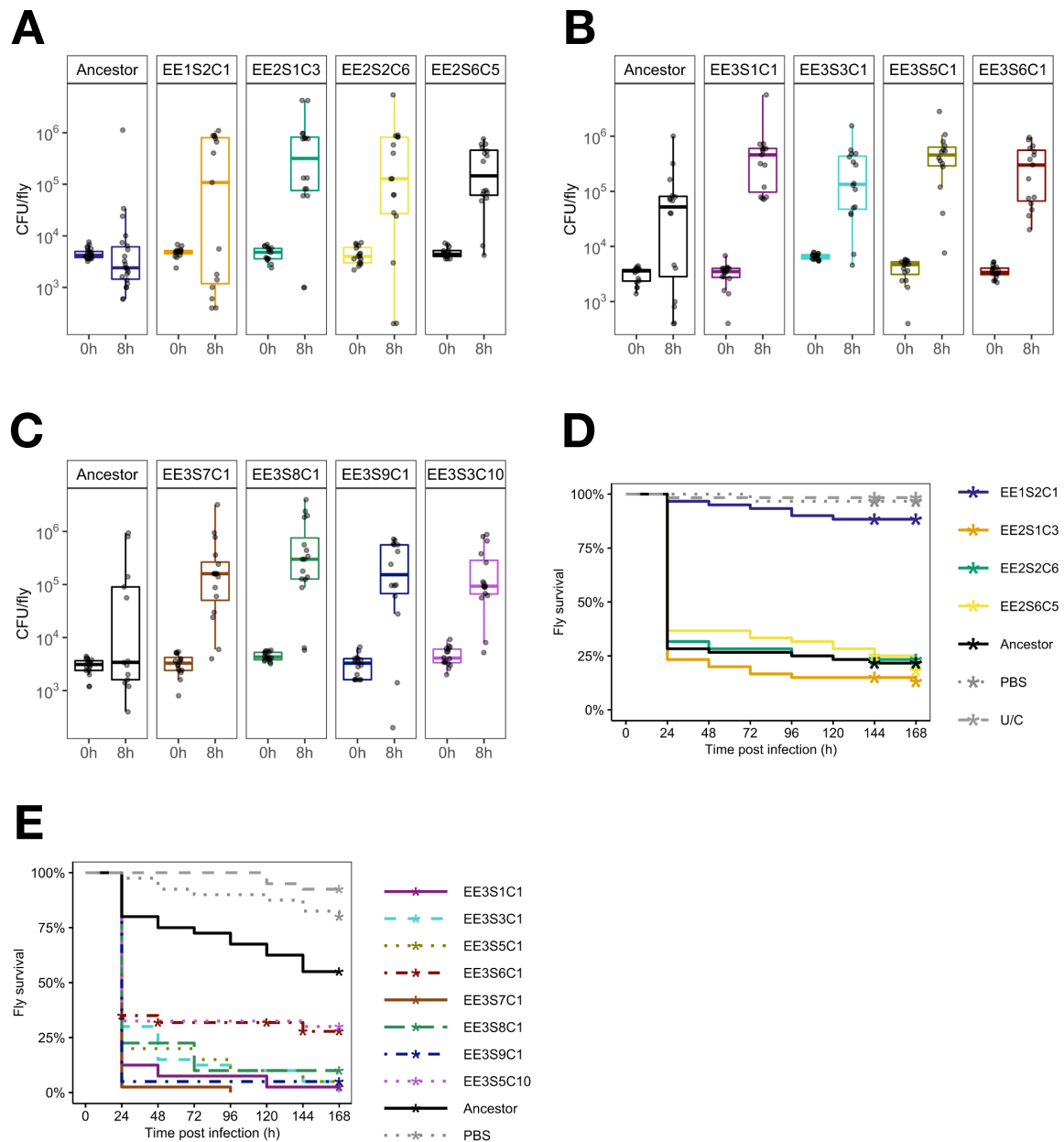


Figure 3.8 Analysis of virulence of *Drosophila*-passaged single colonies

Survival analysis and bacterial quantifications of representative single colonies from passaged population was carried out using the same infection conditions to identify changes in virulence that could be directly comparable to ancestral strain. The single colonies tested here, show increased proliferation in *Drosophila* 8-hours post-infection (**A-C**) and cause significantly greater mortality (**D, E**) compared to the ancestor strain. For bacterial quantification experiments $n = 8$ per condition per time-point, with data pooled from two independent experiments. For survival experiments, $n = 15-20$ per condition, with data pooled from two independent experiments. These experiments were carried out using $OD_{600} = 0.3$ inoculum of bacteria.

3.6 *E. faecalis* adaptation is not specific to laboratory reared *Drosophila melanogaster*

All the experiments carried out until this point were in inbred w^{1118} flies, used as wild type in our laboratory. However, because these flies are inbred, they do not reflect the diversity in immune responses observed in wild flies (Lazzaro et al., 2004). To test that my strains had adapted in response to a core element of the *D. melanogaster* immune response and not something more specific to w^{1118} flies and the laboratory environment I carried out survival experiments with a recently-collected outbred population of *D. melanogaster* (Faria & Sucena, 2017). I observed an increase in mortality in these flies similar to what was observed in w^{1118} flies after infection with the *Drosophila*-adapted *E. faecalis* (Figure 3.9).

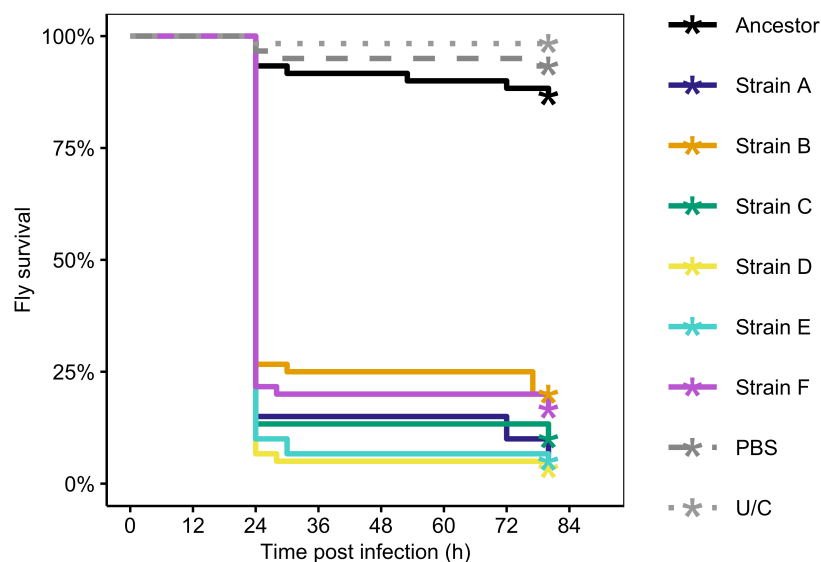


Figure 3.9 Survival outcomes of outbred *D. melanogaster* upon infection with w^{1118} -adapted *E. faecalis*
Infection of outbred populations of *D. melanogaster* with w^{1118} -adapted *E. faecalis* results in high host mortality 24-hours post-infection compared to infection with control strain. n=20 per experiment, with data pooled from three independent experiments. These experiments were carried out using OD₆₀₀ = 0.3 inoculum of bacteria and were performed by **Dr. Catarina Nunes**.

3.7 Discussion

3.7.1 Optimization of conditions for experimental evolution and screening

In this chapter, I have described the process of generating *E. faecalis* strains that have adapted in response to the immune action of *Drosophila melanogaster*. The first step in generating these strains was designing an experimental evolution protocol for the serial passage of *E. faecalis* in *Drosophila*. While designing this protocol, I identified different parameters that could influence the outcome of the selection process. Over the course of three serial passage experiments, I optimized these parameters to make this setup more robust. For example, I changed the period of time bacteria spent in the fly from 72 hours to 24 hours to ensure that the immune response was the strongest selective pressure encountered by the bacteria. I also ensured that any changes made to these parameters during optimization were comparable to conditions used in the earlier versions of the protocol so that bacteria generated from all three experiments could be used for further analysis.

One recurring problem I had during the evolution experiment was the high degree of variability in the behaviour of the ancestral strain between experiments. I hypothesize that this variation is due to the altered availability of amino acids and sugar in the media due to heat sterilization. It has been described that the caramelization process of sugars in media can result in the depletion of essential amino acids such as cysteine and the formation of toxic intermediate products that can inhibit the growth of lactic acid bacteria (Finkelstein & Lankford, 1957; Lankford et al., 1957). In data not shown in this thesis, I identified that by changing autoclave conditions of certain media I could deplete the availability of some essential amino acids, and *E. faecalis* showed reduced growth in these conditions *in vitro*. This growth impairment was no longer present if filter sterilization of media was performed. As it is not possible to filter sterilize *MS* agar, I decided to switch to sterile filtered BHI as the growth media for screening of strains from the third serial passage experiment.

3.7.2 Outcomes of serial passage of *E. faecalis* in *Drosophila*

Once the serial passage experiment was completed, the next step involved screening the different passaged populations to identify *E. faecalis* that had adapted in response to the action of the *Drosophila* immune response. From the screen, I identified that 12/19 populations of *E. faecalis* that were passaged in *Drosophila* showed enhanced proliferation in the host. The higher bacterial burden in flies infected with these strains correlated with an increase in host mortality indicating that the increase in virulence observed in these strains is directly linked to their enhanced ability to proliferate in the fly. Considering that the bacteria recovered from flies during serial passage was always grown on solid media, there was the possibility that the final passaged populations might be made up of different bacterial colonies. To understand if these populations were mixed or clonal, I performed the screen with at least three single colonies from each passaged population of *E. faecalis*. While results from the first serial passage were not very clear due to issues with media, it became clearer later on that the bacterial populations were likely to be clonal in nature. This observation was later confirmed using whole genome sequencing (**Chapter 5**). Once I identified that the adaptive populations were clonal, I confirmed the *in vivo* growth advantage and enhanced virulence using single representative colonies of the populations using standardized conditions. I also identified that the adaptation in these strains was not specific to laboratory reared in-bred flies as I observed that infection of a wild-derived outbred population of flies with these strains resulted in a similar increase in virulence.

Not every population of *E. faecalis* that was passaged in *Drosophila* ended up with the ability to grow better in this host. Some passaged populations remained unchanged from the ancestral strain in their behaviour at the end of the experiment. This result was unexpected as I anticipated that upon facing the strong selective pressure exerted by the *Drosophila* immune response, all passaged populations would adapt. However, my selection experiments were

performed over a short period and there is a chance that the adaptive allele may have not appeared or become fixed in these populations by the time I ended my selection. Thus, if I continued the evolution experiment for a longer period, there is a possibility that all passaged populations would have reached the common path of adaptive evolution.

Chapter 4: Host factors involved in the selection of the *Drosophila*-adapted strains

4.1 Introduction

In the previous chapter, I generated *E. faecalis* strains that had adapted in response to the *Drosophila* immune response. As a result of this adaptation, these strains acquired the ability to rapidly proliferate in the hemolymph of wild type *Drosophila*. They also exhibited increased virulence, leading to significantly greater host mortality. My next step was to identify which host component these strains had adapted in response against. Multiple components of the *Drosophila* immune response have been suggested to play a role during *E. faecalis* infection. These include the Toll-mediated immune response via the Bomanins, the cellular immune response, and the melanization cascade (Ayres & Schneider, 2008; Binggeli et al., 2014; Clemmons et al., 2015; Defaye et al., 2009; Nehme et al., 2011). While the Bomanins are known to be essential for the immune response against *E. faecalis*, the contributions of hemocytes and the melanization cascade are not very clear. In addition to this, the role played by the Imd pathway, the other pattern recognition signalling pathway in *Drosophila*, has not been explored during *E. faecalis* infection. As a result, it is unknown whether the stress encountered by *E. faecalis* during systemic infection of *Drosophila* is solely mediated by the Toll pathway or if other systems are involved.

Once I have identified the host factors important for the selection of resistance of *E. faecalis* to the systemic immune response of *Drosophila*, I can also look at trade-offs associated with this adaptation. In *Drosophila*, *E. faecalis* can cause both gut and systemic infection (Cox &

Gilmore, 2007; Lazzaro et al., 2006). Thus, there is the possibility that enhanced resistance to the systemic immune response can render *E. faecalis* more susceptible or resistant to the gut immune response. I can test this by performing oral infections of flies with the *Drosophila*-adapted strains and comparing their fitness to the ancestral strain in this environment.

4.2 Aims

The first **aim** of this chapter was to identify the selective pressure (host component) that the systemically-adapted strains had adapted in response to. To do this, I assayed bacterial fitness in flies with defects in immune components suggested to be involved during *E. faecalis* infection. In the absence of the selective pressure, the *Drosophila*-adapted strains and the ancestral strain should have similar fitness, allowing for the identification of host components involved in the selection process. This could also tell us if the strains had adapted in response to a single strong selective pressure or if different immune components were at play during this interaction.

The second **aim** of this chapter was to identify whether adaptation to the systemic immune impacted the ability of *E. faecalis* to colonize the fly gut. To understand this, I assayed the fitness of the systemically-adapted strains in the *Drosophila* gut in the absence of or in competition with the ancestral strain.

4.3 Toll pathway-mediated Bomanins peptides are involved in the selection for immune resistance

The Toll signalling pathway is essential for the humoral immune response against Gram-positive bacteria such as *E. faecalis* (Nehme et al., 2011; Tauszig-Delamasure et al., 2002). This pathway is activated upon the detection of Lys-type peptidoglycan by the PRRs; GGBP-1 and PGRP-SA, this detection results in the activation of a proteolytic cascade. The final step of this signalling pathway involves the translocation of the transcription factor *Dif* to the nucleus, where it drives transcriptional activation of immune-responsive genes including a variety of antimicrobial effector peptides. As *E. faecalis* carries Lys-type peptidoglycan, looking at whether this pathway was involved in the selection for immune resistance in the *Drosophila*-adapted *E. faecalis* strains was my first step. To that end, I assayed bacterial fitness in flies that were mutant for *Dif*. I made use of *Dif^l* flies (hereafter, *Dif*), carrying a strong loss of function allele of the gene (Rutschmann, Jung, Hetru, et al., 2000).

Similar to experiments performed in **Chapter 3**, I assayed bacterial fitness by monitoring changes in host survival and bacterial numbers post-infection in flies infected with the *Drosophila*-adapted strains compared to those infected with the ancestral strain. I observed that *Dif* flies showed rapid host mortality when infected with either the ancestral or *Drosophila*-adapted strains (**Figure 4.1A**). At 8-hours post-infection, *Dif* flies had similar bacterial loads, in the range of 10^6 to 10^7 CFU/fly, irrespective of the strain they were infected with (**Figure 4.1B**). These results suggest that the *Dif*-mediated immune response is involved in the selection of immune resistance in these strains as infection of flies with loss of function mutation in *Dif* results in no observable differences between the virulence of the ancestral and *Drosophila*-adapted strains.

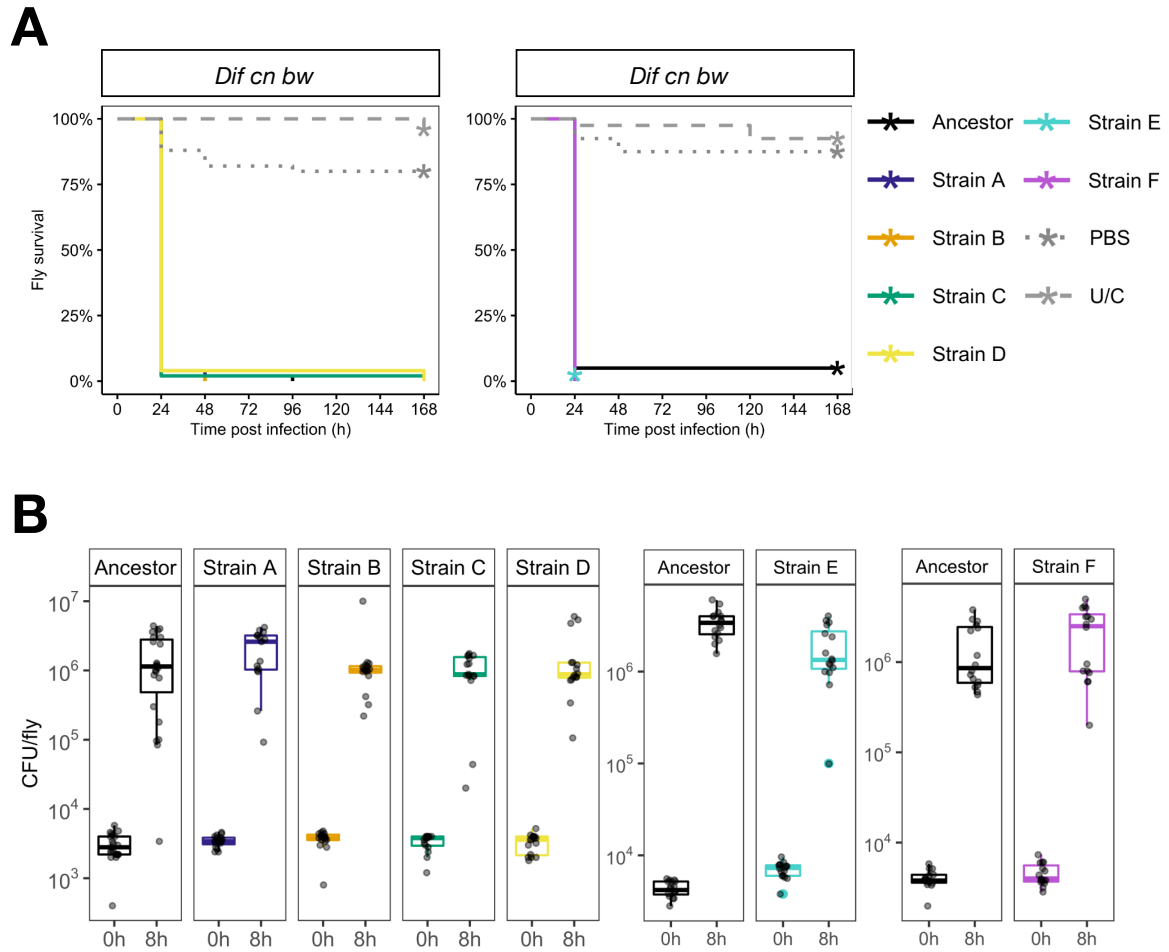


Figure 4.1 Ancestral *E. faecalis* and *Drosophila*-adapted strains show similar virulence in *Dif* mutants

Flies mutant for *Dif* were infected with ancestral and *Drosophila*-adapted *E. faecalis* strains. Infection with either the ancestral or *Drosophila*-adapted *E. faecalis* strains resulted in rapid host mortality 24-hours post-infection (A). No difference in median bacterial load was observed 8-hours post-infection between *Dif* flies infected with the ancestral or *Drosophila*-adapted strains (B). In A, $n = 15-20$ per condition, with data pooled from two independent experiments. In B, $n = 8$ per condition per time-point, with data pooled from two independent experiments.

Having established that the *Dif*-mediated immune response played a role in the selection of immune resistance, I wanted to test if these strains had adapted in response to the action of a particular Toll-responsive immune effector. The primary Toll-specific antibacterial effectors are the antimicrobial peptide Drosomycin and the Bomanin family of peptides. To test if the Bomanins were involved in the selection for immune resistance, I assayed bacterial fitness in *Bom*^{Δ55C} flies. These flies have a 9 kb deletion that eliminates 10/12 Bomanin peptides (Clemmons et al., 2015).

To make direct comparisons between bacterial fitness in wild-type flies encoding all the Bomanin peptides and *Bom*^{Δ55C} flies, I carried out infections of *Bom*^{Δ55C} flies alongside their genotype control (*w*¹¹¹⁸). Infections of *w*¹¹¹⁸ flies with *Drosophila*-adapted strains led to rapid host mortality within 24-hours of infection, whereas flies infected with the ancestral strain had low mortality (**Figure 4.2A**). In contrast, infection of *Bom*^{Δ55C} flies with either the ancestral or *Drosophila*-adapted strains resulted in rapid host mortality within 24-hours of infection (**Figure 4.2A**). All infected *Bom*^{Δ55C} flies had bacterial loads in the range of 10⁶ to 10⁷ CFU/fly 8 hours post-infection, and no differences in proliferation between the ancestral control strain and the *Drosophila*-adapted strains were observed (**Figure 4.2B**). The response to *E. faecalis* infection in *Bom*^{Δ55C} flies is similar to what I previously observed in *Dif* flies (**Figure 4.1**) indicating that this family of effector peptides is likely a critical factor in the selection of immune resistance in the *Drosophila*-adapted strains.

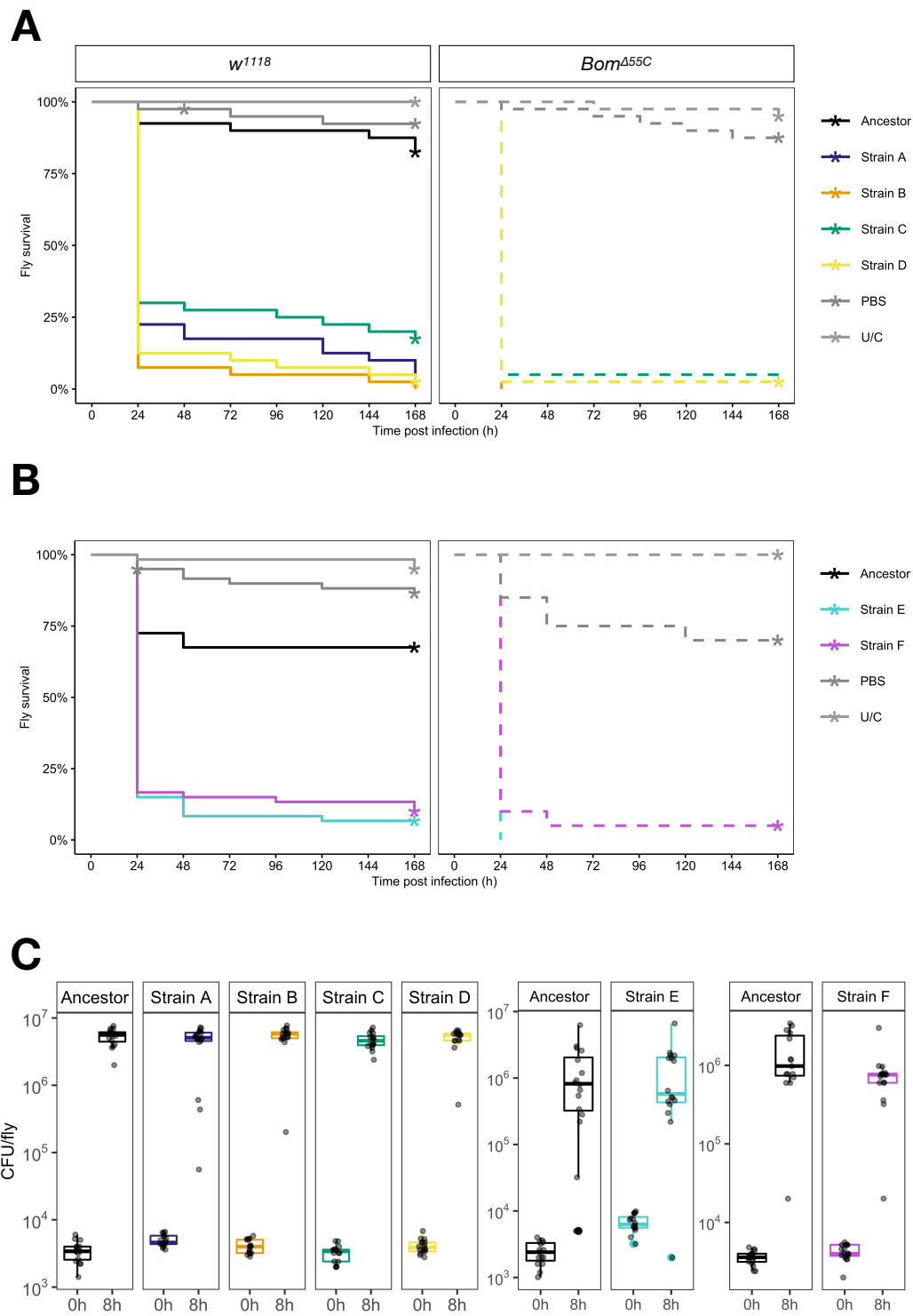


Figure 4.2 Ancestral and *Drosophila*-adapted strains show similar virulence in flies lacking most of the Bomanin peptides

A, B: *Bom*^{Δ55C} flies which are deficient for 10/12 Bomanin peptides and genotype control *w*¹¹¹⁸ were infected with the ancestral and *Drosophila*-adapted *E. faecalis* strains. In *w*¹¹¹⁸ flies, only infections with the

Drosophila-adapted strains resulted in rapid host mortality while the infection with the ancestral control strain resulted in little mortality compared to uninfected and mock-infected controls. However, infection with either the ancestral or *Drosophila*-adapted strains resulted in high host mortality 24-hours post-infection in *Bomanin*-deficient flies. **C:** *Bom^{ΔSG}* flies infected with ancestral and *Drosophila*-adapted *E. faecalis* strains show no difference in median bacterial load 8-hours post-infection. In **A** and **B**, n = 15-20 per condition, with data pooled from two independent experiments. In **C**, n = 8 per condition per time-point, with data pooled from two independent experiments.

As mentioned earlier, activation of the Toll signalling pathway also results in the transcription of antimicrobial peptides such as *Drosomycin*. Until the recent description of the Bomanins, *Drosomycin* was thought to be the main antibacterial effector of this pathway. To test whether *Drosomycin* is involved in the immune response to and the subsequent selection of immune-resistance in the *Drosophila*-adapted strains, I made use of flies lacking *Drosomycin* (hereafter, *Drs*) (Hanson et al., 2019). Interestingly, I observed that the both the ancestral and *Drosophila*-adapted strains exhibited reduced virulence in *Drs* flies compared to their *w¹¹¹⁸* controls (**Figure 4.3A**). Finally, to exclude the possibility that any other antimicrobial peptide was involved in the selection of my strains, I also infected flies that lacked all known antimicrobial peptides but retained the Bomanins (*AMPΔ14*). *AMPΔ14* flies showed an increase in mortality upon infection with all *E. faecalis* strains, including the ancestral strain, compared to the *w¹¹¹⁸* genotype controls (**Figure 4.3B**). However, while a greater proportion of *AMPΔ14* flies died after infection compared to *w¹¹¹⁸* flies, the difference in virulence between ancestral control and *Drosophila*-adapted strains was retained between the two fly genotypes. Thus, the increase in mortality observed in *AMPΔ14* flies appears to be the result of an overall increase in susceptibility of these flies to *E. faecalis* infection. These results suggest that antimicrobial peptide-mediated immune response is not actively involved in the selection of my strains. However, it is not clear why *Drs* flies survive better after infection with the *Drosophila*-adapted strains.

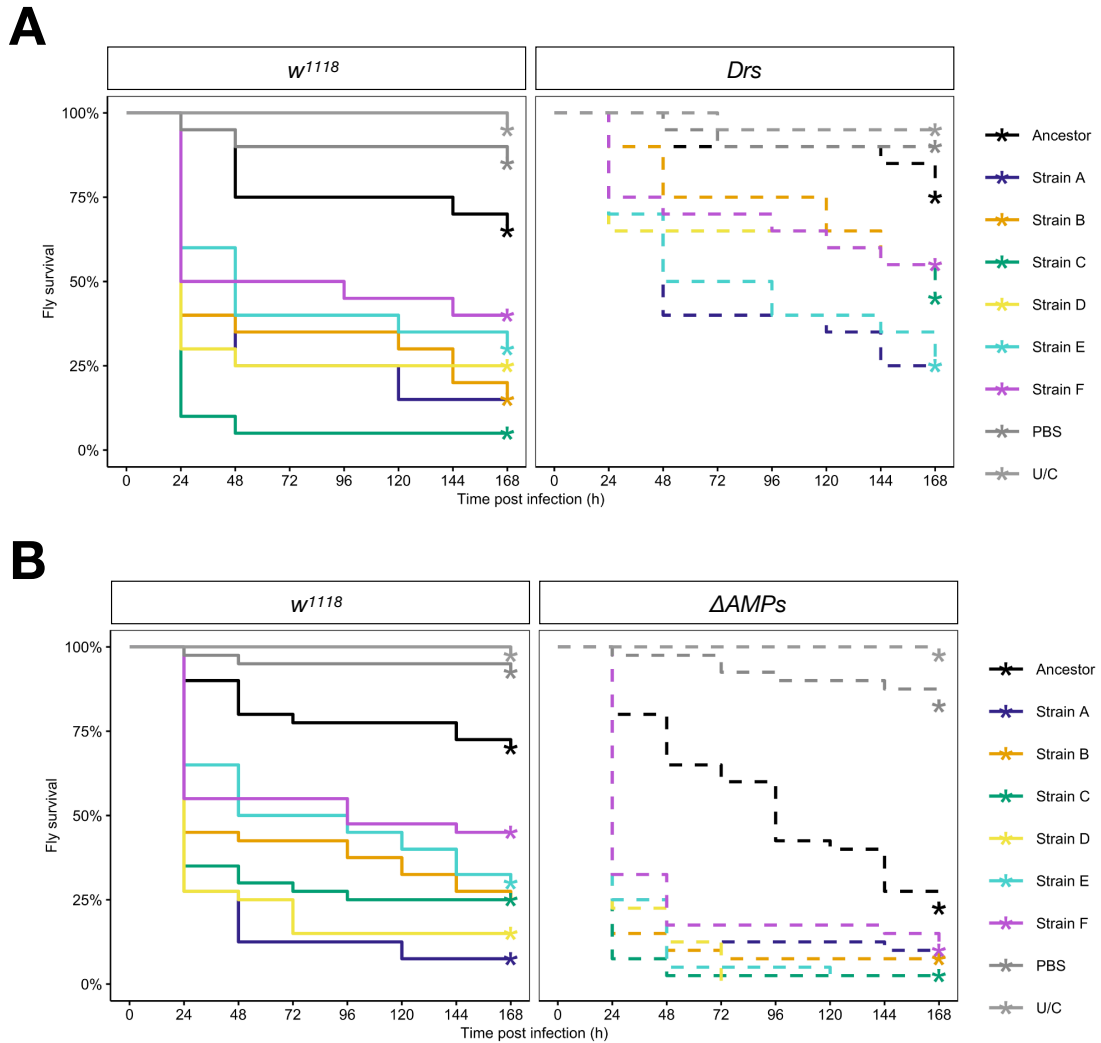


Figure 4.3 *Drosophila* antimicrobial peptides are not involved in the selection of immune-resistant strains

A: Flies deficient for the antimicrobial peptide *Drosomycin* (*Drs*) and the control genotype (*w¹¹¹⁸*) were infected with the ancestral and *Drosophila*-adapted *E. faecalis* strains. As observed previously, *w¹¹¹⁸* flies infected with *Drosophila*-adapted *E. faecalis* strains exhibited >50% host mortality 24-hours post-infection while those infected with the ancestral *E. faecalis* strain did not. *Drs* flies had better outcomes compared to the *w¹¹¹⁸* and 24-hours post-infection, <50% host mortality was observed in all conditions. **B:** Flies lacking all known antimicrobial peptides (Δ AMP) and the control genotype (*w¹¹¹⁸*) were infected with the ancestral and *Drosophila*-adapted strains. Infection with the *Drosophila*-adapted strains resulted in high host mortality 24-hours post-infection in both the control genotype and antimicrobial peptide-deficient flies. Infection with the ancestral *E. faecalis* strain also resulted in a slight increase in mortality of Δ AMP flies compared to *w¹¹¹⁸* flies. However, the difference in virulence between the *Drosophila*-adapted strains and the ancestral *E. faecalis* strains was maintained between the two genotypes. In **A** and **B**, n = 15-20 per condition, with data pooled from two independent experiments.

Results from this section of the chapter indicate that the Bomanins induced by Toll pathway activity are likely to be the primary selective pressure encountered by *E. faecalis* during *Drosophila* infection. However, it was unclear whether these bacteria would exhibit similar resistance to higher than normal levels of Toll-pathway effectors. To answer this question, I next assayed bacterial fitness in flies that expressed a constitutively active allele of *Toll* in the fat body under the control of the *c564-Gal4* driver (*c564-Gal4>UAS-Toll^{10b}*) alongside their control genotypes (*c564-Gal4/+* and *0>UAS-Toll^{10b}*). *c564-Gal4/+* flies showed similar survival outcomes after infection with the *Drosophila*-adapted strains as previously observed in *w¹¹¹⁸* flies, where infection with the ancestral strain results in low host mortality while infection with the *Drosophila*-adapted strains results in high host mortality within 24-hours of infection (**Figure 4.4**). In contrast to this, three out of five *Drosophila*-adapted strains exhibited reduced virulence in *c564-Gal4>UAS-Toll^{10b}* flies, whereas infection of the other control genotype, *0>UAS-Toll^{10b}*, resulted in an intermediate phenotype. These results suggest that the *Drosophila*-adapted strains are resistant to the inducible Toll pathway, but sufficiently high Toll pathway activation could still protect the host during this infection.

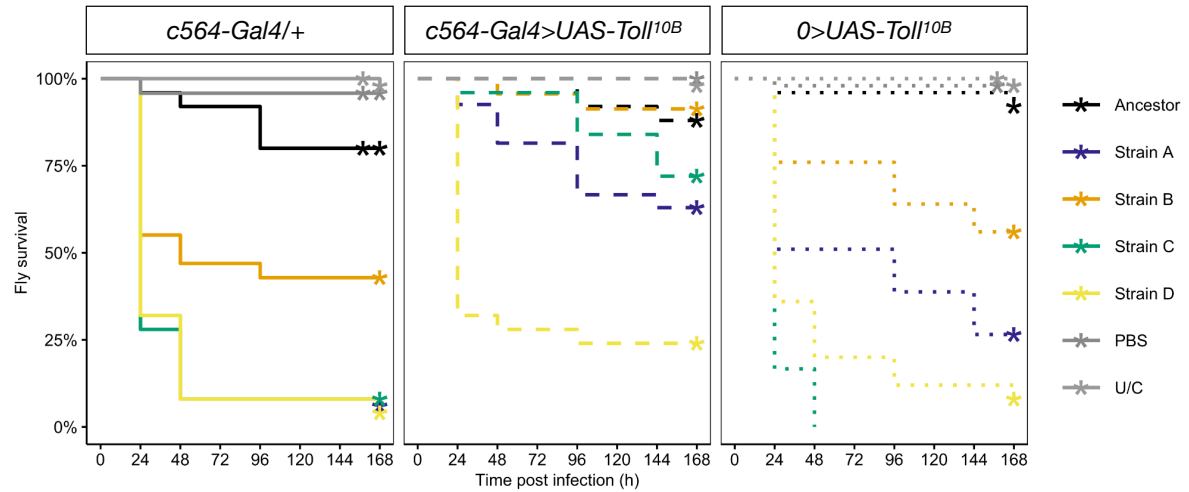
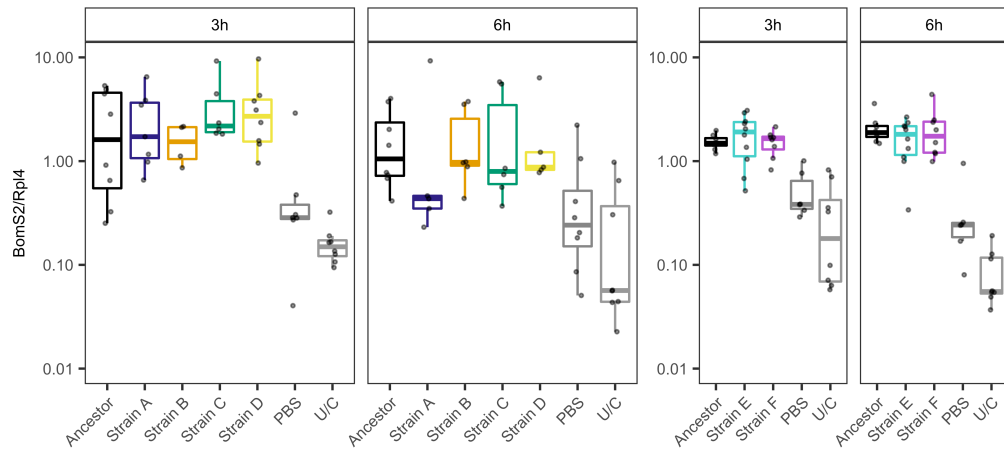
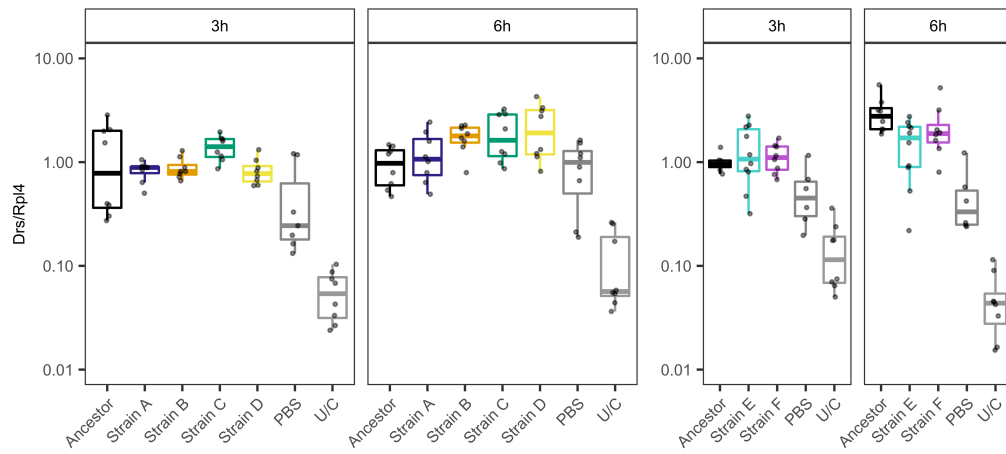
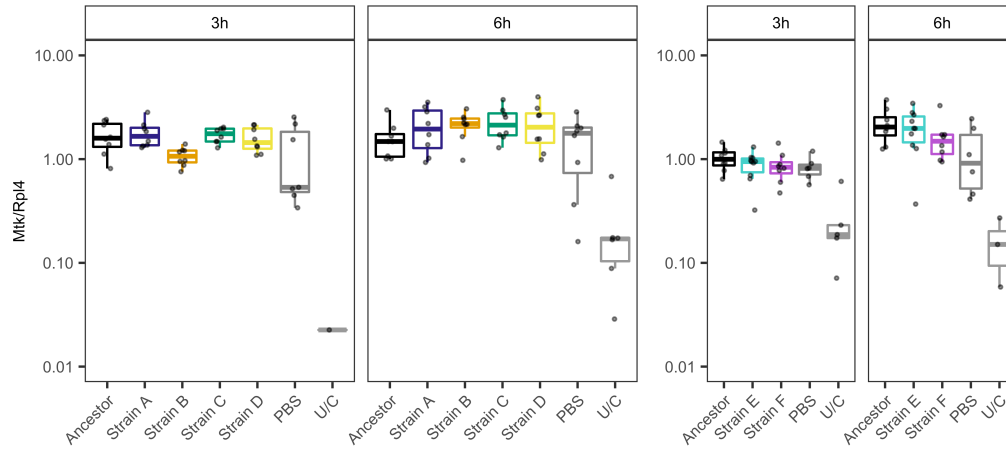


Figure 4.4 Over-expression of the Toll pathway can protect against immune-resistant *E. faecalis* strains

Flies expressing a constitutively active allele of *Toll* (*Toll^{10B}*) in fat body under control of the *c564-Gal4* driver line (*c564-Gal4>UAS-Toll^{10B}*) were infected, alongside their corresponding genotype control lines (*c564-Gal4/+* and *0>UAS-Toll^{10B}*), with the *Drosophila*-adapted and ancestral *E. faecalis* strains. Three out of four *Drosophila*-adapted strains (Strains A-C) exhibited reduced virulence in *c564-Gal4>UAS-Toll^{10B}* flies compared to *c564-Gal4/+* flies, whereas *0>UAS-Toll^{10B}* flies showed an intermediate phenotype. n = 15-20 per condition, with data pooled from two independent experiments.

4.4 *Drosophila*-adapted strains have adapted to resist killing by Bomanins rather than evading detection by the Toll pathway

My results suggest that the Toll pathway and the Bomanin peptides in particular play an important role in the selection of immune resistance in the *Drosophila*-adapted strains. There are two potential routes that the *E. faecalis* strains could take to avoid killing by the Toll-mediated Bomanins; they could have evolved to evade detection by Toll pathway, or they could have evolved to become resistant to killing by the Bomanins. To test which one of these possibilities was responsible for the observed immune resistance, I analyzed transcript levels of some representative immune peptides after infection with the different *E. faecalis* strains (**Figure 4.5A-D**). I observed similar levels of transcripts of the different genes assayed in flies infected with the different *E. faecalis* strains (ancestral or *Drosophila*-adapted) at both 3-hours and 6-hours post-infection. Although *BomS2* transcript levels 6-hours post-infection in flies infected with strain A appeared to be lower than those infected with the ancestral strain, this difference was not statistically significant (**Figure 4.5A**). These results imply that the *Drosophila*-adapted strains are detected normally and have evolved to resist killing by the Bomanins.

A**B****C**

D

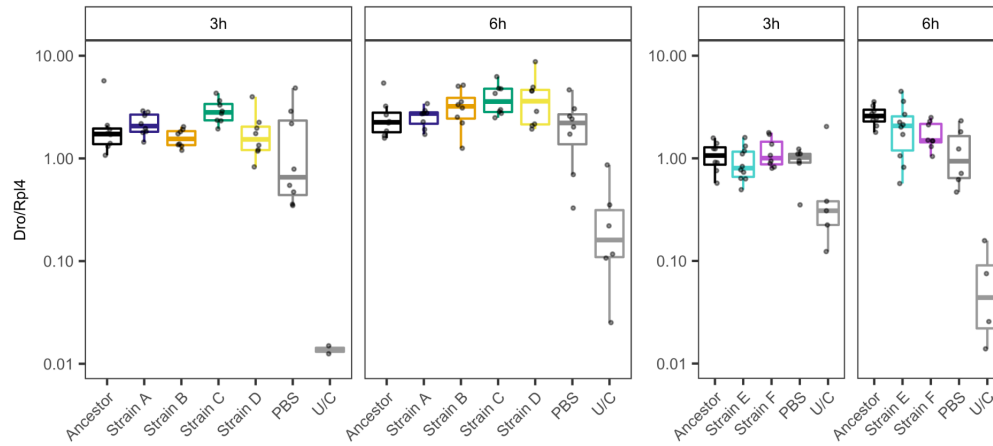


Figure 4.5 Increased virulence of *Drosophila*-adapted strains is not due to reduced activation of the Toll or Imd signalling pathways

w¹¹¹⁸ flies were infected with ancestral and *Drosophila*-adapted *E. faecalis* strains and expression of four immune-responsive genes, *BomS2* (A), *Drs* (B), *Mtk* (C), *Dro* (D) was assayed 3 and 6-hours post-infection. No differences in transcript levels of antimicrobial peptides and *BomS2* were observed in any of the conditions tested with the exception of *BomS2* levels 6-hours post-infection with Strain A, where a reduction in expression was observed. However, this difference is not statistically significant. *n* = 4, per condition per time-point, with data pooled from two independent experiments.

4.5 The Imd pathway may contribute to the immune response against *E. faecalis*

The role of the Toll pathway has been well-defined when it comes to the immune response during Gram-positive infections, but the same cannot be said for the Imd pathway. This pathway is primarily activated in response to infection with Gram-negative bacteria but it can contribute to the immune response against certain Gram-positive pathogens such as *Streptococcus pneumoniae* (Pham et al., 2007). The role of the Imd pathway during *E. faecalis* infection has not yet been explored. As a result, to rule out the involvement of this pathway in the selection of strains, I assayed bacterial fitness in flies that were mutant for the Imd pathway. I made use of *imd*¹⁰¹⁹¹ flies (hereafter, *imd*). This is a loss of function allele that has a 26-nucleotide deletion in the coding region resulting in a frameshift, and these flies are severely immunocompromised against Gram-negative bacterial infections (Chambers et al., 2014; Lazzaro et al., 2006; Pham et al., 2007). Surprisingly, I observed that the *imd* flies infected with a subset of the *Drosophila*-adapted strains (Strains A-D) exhibited better outcomes than those previously observed during infection of *w*¹¹¹⁸ flies (**Figure 3.8A**). Additionally, these strains did not exhibit high levels of proliferation in the *imd* flies 8-hours post-infection, with most flies having only 10³-10⁴ CFU/ml median bacterial load at this time point (**Figure 4.6B**). These experiments were not performed using Strains E and F as they had not yet been generated. Thus, the absence of Imd pathway appears to be protective for the fly during *E. faecalis* infection.

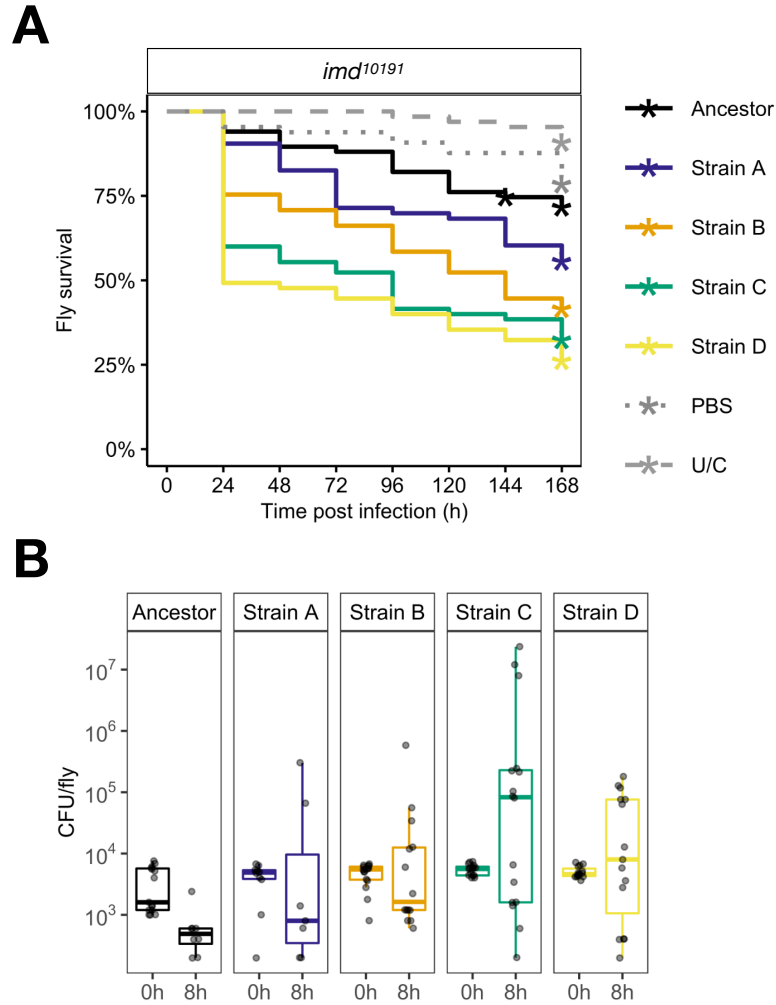


Figure 4.6 *Drosophila*-adapted *E. faecalis* strains exhibit reduced virulence in *imd* flies

Imd flies were infected with the ancestral and *Drosophila*-adapted *E. faecalis* strains and survival outcomes and bacterial numbers 8-hours post-infection were assayed. 24-hours post infection the *imd* flies exhibited <50% mortality under most conditions (A). *Imd* flies also appear to control infection with *Drosophila*-adapted strains better than *w¹¹¹⁸* flies. Most strains show a similar levels of bacterial proliferation 8-hours post-infection to ancestral *E. faecalis* strain. In A, n = 15-20 per condition, with data pooled from two independent experiments. In B, n = 8 per condition per time-point, with data pooled from two independent experiments.

The results from infections of the *imd* flies were surprising. However, I had not performed these experiments alongside *w¹¹¹⁸* flies and there was a possibility that this observed reduction in virulence could be due to problems with media. There was also the possibility that this reduction in virulence was specific to the *imd* allele I used. As a result, I decided to repeat this experiment with a different mutant for the Imd pathway, *kenny* (*key*, hereafter). *Key* is the upstream activator of the NF- κ B transcription factor *Relish*, and the *key* mutants I used, *key^{c02831}*, have a large piggyBac insertion in the coding sequence. These flies are highly susceptible to Gram-negative infection but are believed to function like wild-type in response to Gram-positive infections (Bou Aoun et al., 2011). Infections of *key* flies with the *E. faecalis* strains resulted in an opposite phenotype to what I observed for *imd* mutants (**Figures 4.6A and 4.7A**). *Key* flies showed rapid host mortality within 24-hours of infection, irrespective of the bacterial strain used. These results looked similar to, but weaker than, what I previously observed with the *Dif* and *Bom^{Δ55C}* flies (**Figure 4.1 and 4.2**). However, I was still unclear about the involvement of the Imd pathway in the selection of strains. As a result, I repeated the experiment using a third mutant for the Imd signalling pathway, *Tak1*.

Tak1 is the homolog of the mammalian MAPK kinase kinase, *Tak1* (Transforming growth factor β -activated kinase 1). It activates the downstream Imd as well as the C-jun N-terminal kinase (JNK) signalling pathway. For my experiments, I made use of *Tak1²⁵²⁷* flies (hereafter, *Tak1*) that have a point mutation in the coding sequence resulting in a premature stop codon (Polaski et al., 2006). These flies are not on the *w¹¹¹⁸* background, as a result, infections were performed alongside their background control (+; +; +) instead of *w¹¹¹⁸*. Similar to *key* flies, *Tak1* flies also exhibited rapid host mortality within 24-hours of infection irrespective of the *E. faecalis* strain used for infection (**Figure. 4.7B**). Infection of the control genotype also resulted in similar phenotypes to what I have observed in *w¹¹¹⁸* flies, except strain B which showed reduced virulence in this background. However, as this experiment was only performed once, this observation could be the result of a technical error during fly injections.

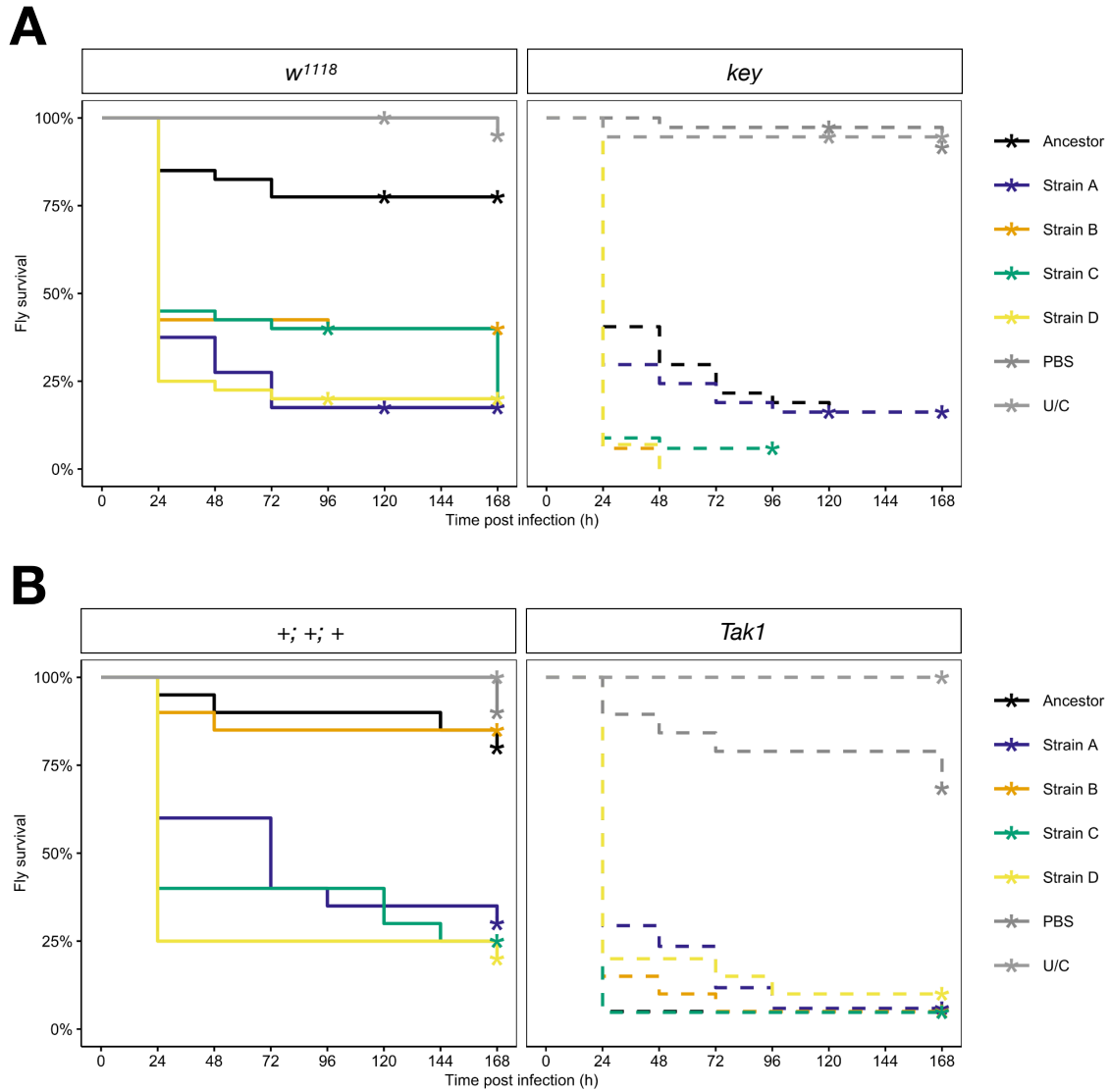
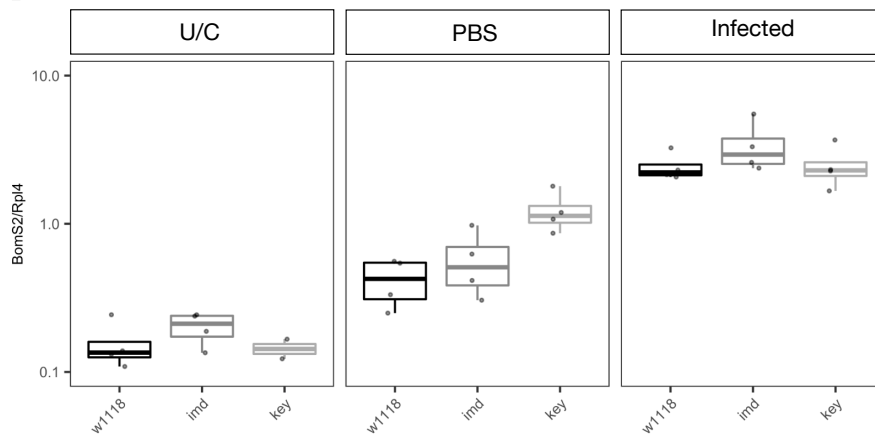
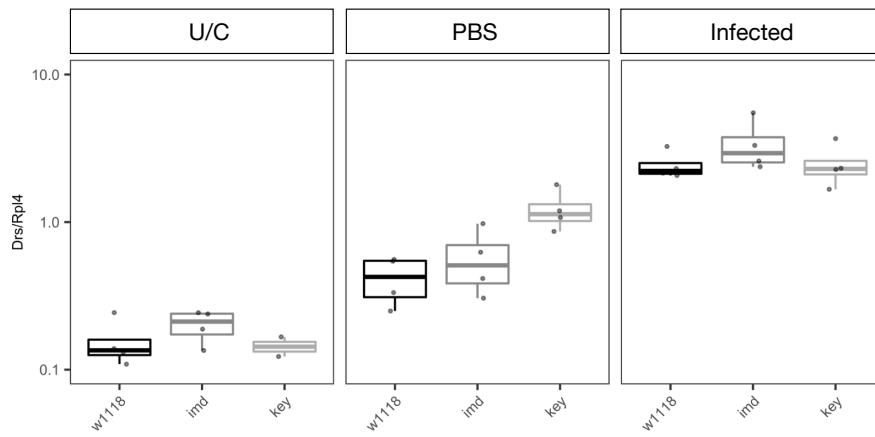
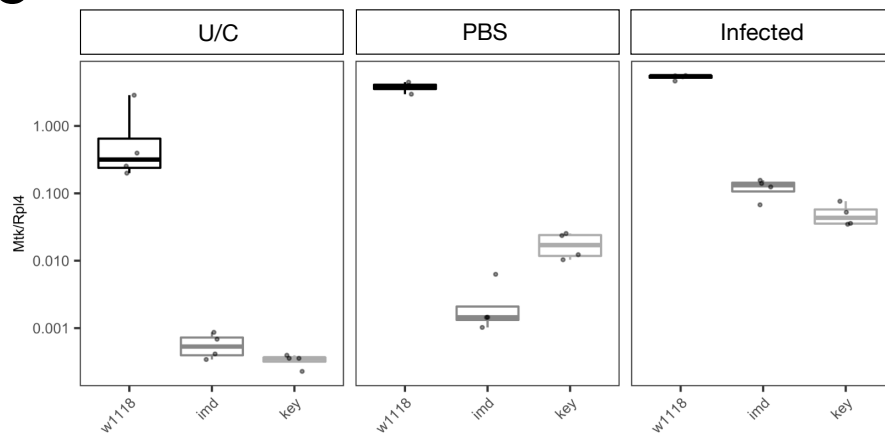


Figure 4.7 *Key* and *Tak1* appear to be important for the immune response against *E. faecalis*

A, B: Flies deficient in *key* and *Tak1*, downstream components of the Imd signalling pathway, as well as their control genotypes, w^{1118} for *key* and $+/+; +/+$ for *Tak1*, were infected with the ancestral and *Drosophila*-adapted strains. Irrespective of the *E. faecalis* strain used, both mutant genotypes (*key* and *Tak1*) showed high host mortality 24-hours post-infection. However, in the control genotypes (w^{1118} and $+/+; +/+$), infection with the ancestral *E. faecalis* strain did not result in high host mortality but infection with most of the *Drosophila*-adapted strains did. In **A**, $n = 15-20$ per condition, with data pooled from two independent experiments. In **B**, $n = 20$ per condition from one experiment.

Results from this section of the chapter show that the absence of some components of the Imd signalling pathway (*key* and *Tak1*) is detrimental during *E. faecalis* infection whereas the absence of other components (*imd*) is beneficial. It is unclear why different mutants of the same signalling pathway would result in these opposite phenotypes. One possibility that could explain these results was that the *imd* mutant that I was working with is not really an Imd pathway mutant, potentially due to contamination of the stock or due to incorrect labelling of the flies. Alternatively, there was the possibility that impairment of the Imd pathway at an early stage could result in the compensatory upregulation of Toll pathway regulated immune genes. To test if either of these was the case, I analyzed transcript levels of representative genes induced by the Toll and the Imd pathways in *w¹¹¹⁸*, *imd* and *key* flies under uninjected, mock-infected and ancestral *E. faecalis* infection conditions (**Figure 4.8**). Gene expression was assayed at 3-hours post-infection and the genes I assayed were *Bomanin short-form 2* (*BomS2*), *Drosomycin* (*Drs*), *Metchnikowin* (*Mtk*), and *Diptericin* (*Dipt*). Of these four, the expression of *BomS2* and *Drs* is regulated by the Toll pathway, the expression of *Mtk* is regulated by both the Toll and Imd pathways whereas the expression of *Dipt* is regulated solely by the Imd pathway.

Under the conditions tested, I did not observe any differences between the three genotypes in the transcript levels of genes whose expression is regulated by the Toll pathway. Transcript levels of antimicrobial peptides, whose expression is either completely or partly regulated by the Imd signalling pathway, was much lower in both *imd* and *key* mutants than in controls, confirming that these lines carried the expected mutations. However, there were clear differences between the two Imd pathway mutants. While there was no difference in the transcript levels of *Mtk* and *Dipt* in uninjected flies, mock-infected *key* flies showed slightly greater induction of *Mtk* whereas *imd* flies showed slightly greater induction of *Dipt*. Finally, I observed that infected *imd* flies showed a slight increase in expression of *Mtk* and *Dipt* compared to infected *key* flies.

A**B****C**

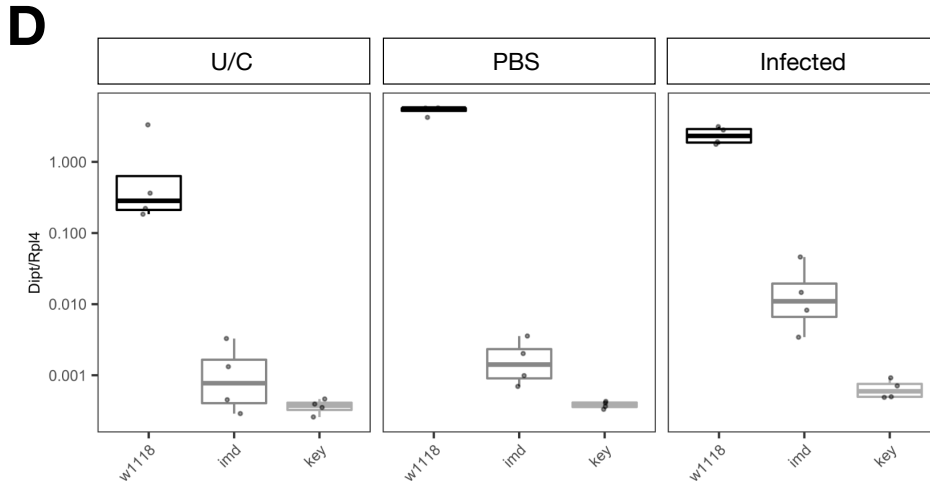


Figure 4.8 Difference in outcome between *key* and *imd* flies is not due differential expression of antimicrobial peptides

w¹¹¹⁸, *imd* and *key* flies were infected with the ancestral *E. faecalis* strain, and changes in the expression of four immune-responsive genes, *BomS2* (A), *Drs* (B), *Mtk* (C), *Dipt* (D) were assayed 3-hours post-infection. Induction of immune genes regulated by the Toll pathway; *BomS2* and *Drs*, appeared to be of similar magnitude between the three genotypes under uninfected, mock-infected, and infected conditions (A, B).. However, statistical analyses were not performed to confirm if there were any statistically significant differences due to the small sample size. *Imd* and *key* flies showed reduced expression of genes regulated partly or completely by the Imd pathway, *Mtk* and *Dipt* compared to *w¹¹¹⁸* flies in all conditions (C, D). n = 4 per genotype per condition, all data points are plotted on the graph.

4.6 The melanization cascade does not play a role in the immune response against *E. faecalis*

Two differing views on the role of melanization in the immune response against *E. faecalis* have been described. Binggeli *et al* described that flies lacking the terminal enzymes of the melanization cascade, the prophenol oxidase's (PPO's), are more susceptible to *E. faecalis* infection (Binggeli et al., 2014). On the other hand, two studies have suggested that PPO's are not important for the immune response during *E. faecalis* infection (Ayres & Schneider, 2008; Leclerc et al., 2006). These studies made use of flies that had a loss of function allele

of *Sp7*, which is a serine protease that functions directly upstream of *PPO1*. Additionally, Ayres *et al.* observed that flies with an inactive melanization pathway had reduced bacterial load after *E. faecalis* infection compared to the control genotype.

To test whether this arm of the *Drosophila* immune response was involved in the selection of immune resistance, I carried out infections in *PPO1^Δ*, *PPO2^Δ* flies. Similar to infections carried out with the *Imd* flies, infections were performed with a subset of *Drosophila*-adapted strains Strains A-D as Strains E and F had not been generated at the time this experiment was performed. *PPO1^Δ*, *PPO2^Δ* flies lack both prophenol oxidases involved in the melanization response in adult *Drosophila*. Similar to what I observed in *Drs* and *imd* flies; *PPO1^Δ*, *PPO2^Δ* flies also exhibited better outcomes after infection with *Drosophila*-adapted strains compared to the wild-type controls, *w¹¹¹⁸* (**Figure 4.9A**) (Binggeli et al., 2014). This was interesting, as these flies are more susceptible to wounding and are not as healthy as the *w¹¹¹⁸* flies, as observed during the later stages of infection. There was no longer a difference in killing between the *Drosophila*-adapted strains and the ancestral strain in these double mutants. I also quantified bacterial loads in *w¹¹¹⁸* and *PPO1^Δ*, *PPO2^Δ* double mutant flies after infection with the ancestral strain and a representative *Drosophila*-adapted strain, strain D (**Figure 4.9B, C**). *PPO1^Δ*, *PPO2^Δ* flies had lower bacterial load 8-hours post-infection compared to *w¹¹¹⁸* flies after infection with either strain. Similarly, at 24-hours post-infection, *PPO1^Δ*, *PPO2^Δ* flies infected with the ancestral strain had lower bacterial numbers compared to the *w¹¹¹⁸* flies whereas, in experiments where strain D was used for infections, no *w¹¹¹⁸* were alive 24-hours post-infection to test for bacterial load while *PPO1^Δ*, *PPO2^Δ* flies showed further clearance of bacteria (**Figure 4.9B, C**). Taken together, results suggest that the melanization cascade does not contribute to the immune response during *E. faecalis* infection.

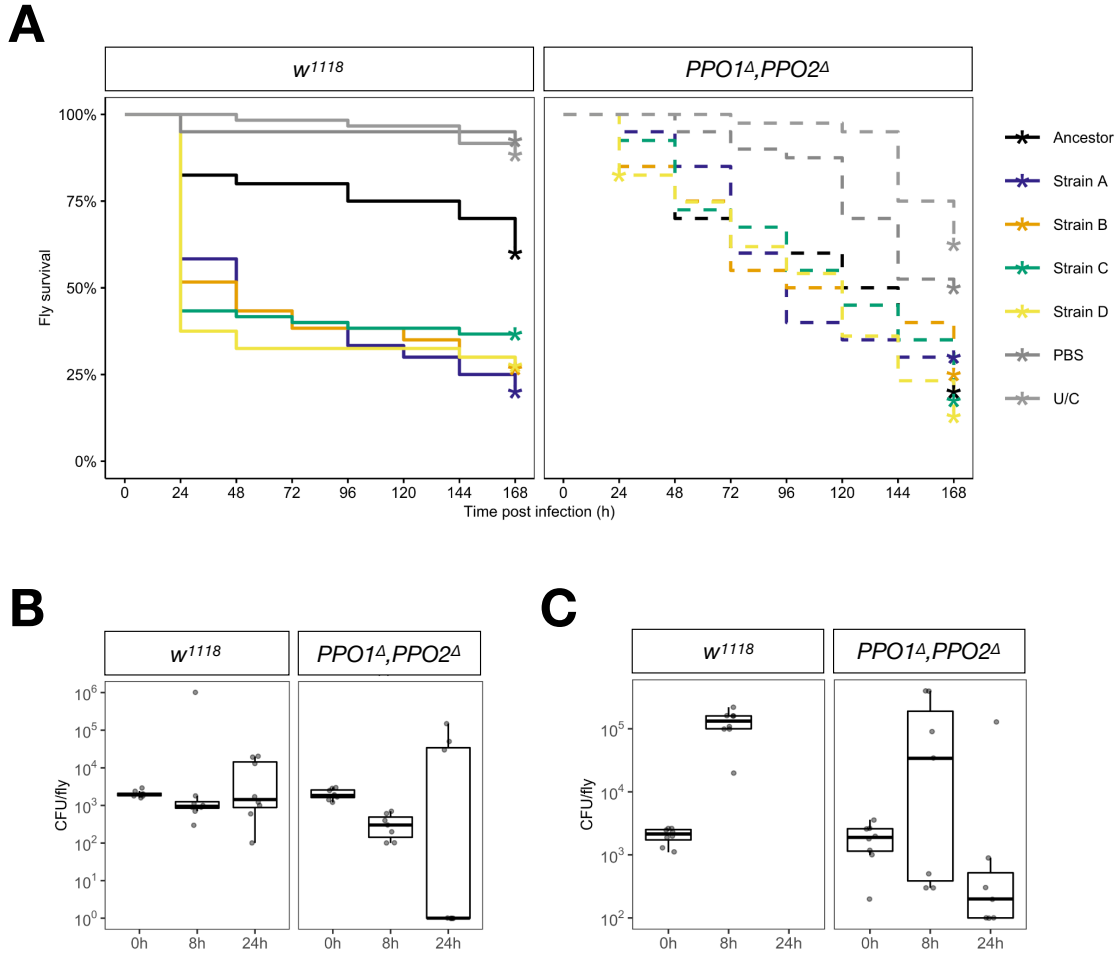


Figure 4.9 *PPO* double mutants show improved outcomes after infection with *Drosophila*-adapted strains

A: *PPO1^Δ,PPO2^Δ* flies alongside *w¹¹¹⁸* flies were infected with the ancestral and *Drosophila*-adapted strains and survival outcomes were assayed. The *Drosophila*-adapted strains exhibited reduced virulence in *PPO1^Δ,PPO2^Δ* flies compared to the *w¹¹¹⁸* flies. **B and C:** Bacterial load quantification of *PPO1^Δ,PPO2^Δ* and *w¹¹¹⁸* flies was performed after infection with either ancestral *E. faecalis* strain (**B**) or Strain D (**C**). Compared to *w¹¹¹⁸* flies, *PPO1^Δ,PPO2^Δ* flies had lower bacterial loads 8-hours post-infection with both the ancestral strain and Strain D. This trend was also observed at 24-hours in flies infected with the ancestral *E. faecalis* strain, but this comparison was not possible for flies infected with Strain D, as by 24-hours post-infection, there were no live *w¹¹¹⁸* flies to carry out quantifications. However, *PPO1^Δ,PPO2^Δ* flies infected with Strain D have lower bacterial loads at 24-hours compared to 0-hours and 8-hours. In **A**, n= 15-20 per genotype per condition, with data pooled from two independent experiments. In **B**, n= 8 per condition per time-point, all data points are plotted on the graph.

4.7 *Drosophila*-adapted strains do not have trade-offs in other environments

The strains I generated exhibited a strong growth advantage in the hemolymph during systemic infection. However, it was not clear whether adaptation to this specific environment would have an impact the ability of these strains to grow in a different environment in *Drosophila*. To test this, I assayed the growth of these strains in the *Drosophila* gut, in competition with and in absence of the ancestral strain. Briefly, flies were fasted overnight on agar supplemented with PBS, then transferred to food inoculated with either a monoculture or mixed culture of bacteria (1:1 ancestral and *Drosophila*-adapted strain). After 3 hours, flies were tipped onto clean food; this time point is termed as ‘Transfer’. Flies were collected at Transfer and 3 and 24-hours post-transfer for bacterial load quantification (**Figure 4.10**).

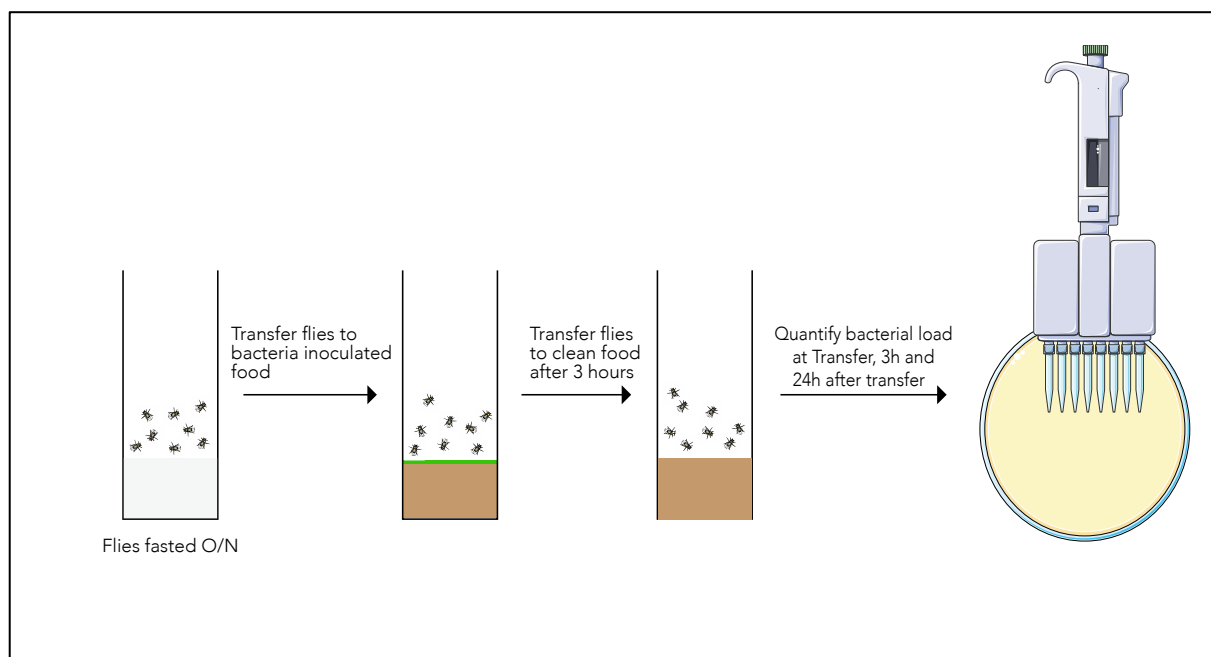
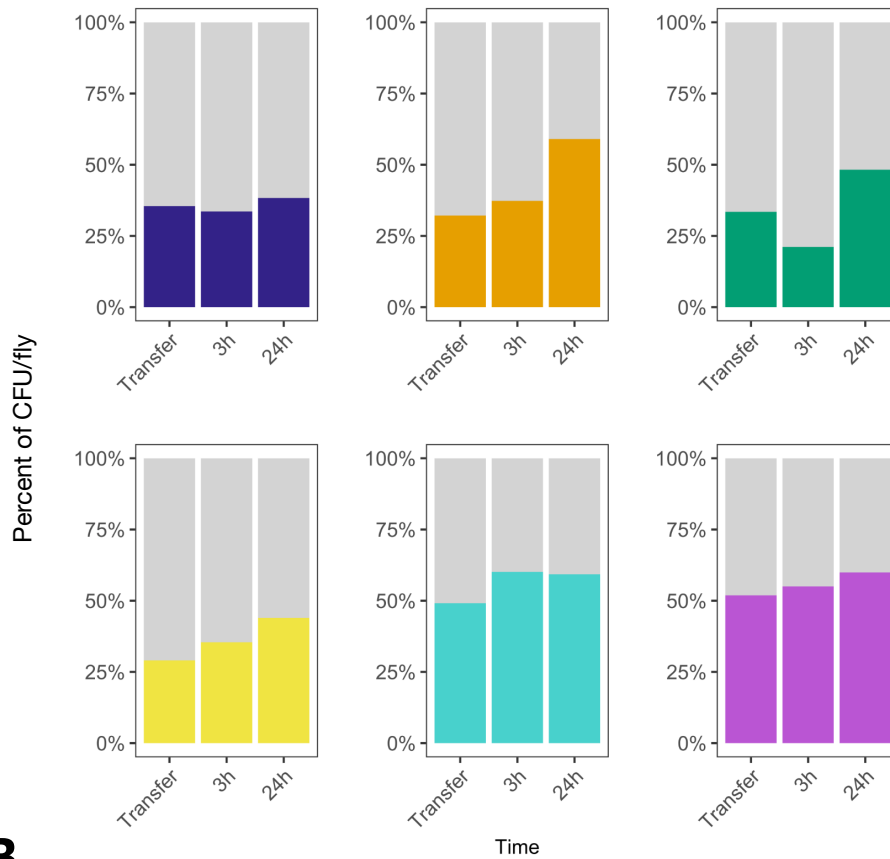


Figure 4.10 Set-up of the *Drosophila* feeding experiment

For these experiments, I made use of a GFP-labelled ancestral strain. As a result, in conditions where flies were fed with a mixed culture of bacteria, I could distinguish between the non-fluorescent *Drosophila*-adapted strains and the fluorescent ancestral strain. As shown in **Figure 4.11**, the relative abundance of each strain during competition with the ancestral strain was quantified and appeared to be stable over the course of infection. Similarly, I did not observe any specific trends when flies were fed monocultures of most of the strains, except for strain B. This strain displayed a tendency grow to higher numbers in the *Drosophila* gut (**Figure 4.11**).

A



B

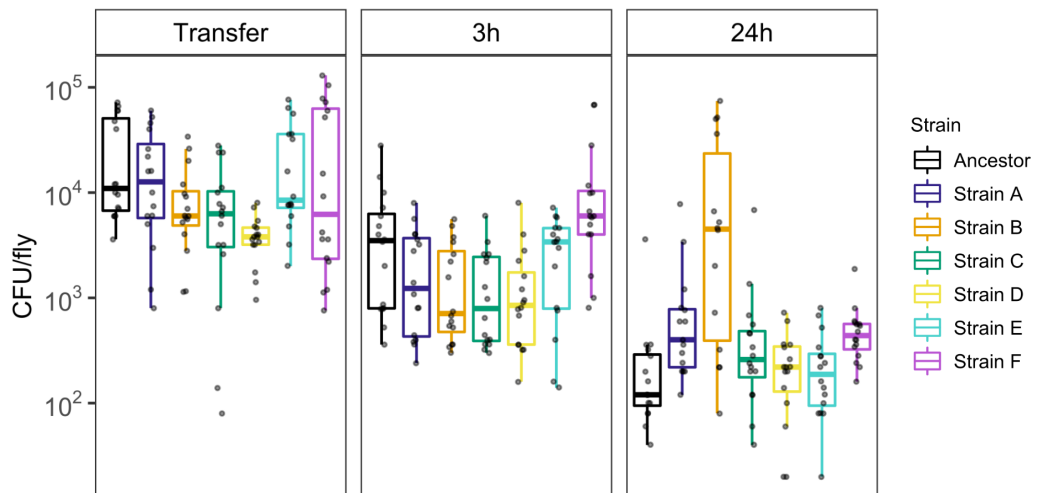


Figure 4.11 Adaptation to the systemic immune response does not result in a trade-off in the *Drosophila*-gut

Flies were orally infected with either a mixed culture of *E. faecalis* consisting of 1:1 concentrations of GFP labelled ancestral strain and a *Drosophila*-adapted strain (**A**) or with a single *E. faecalis* strain (**B**). Bacterial numbers were quantified at three time points after infection; Transfer, 3-hours post-transfer, and 24-hours

post-transfer (A detailed description of this setup is described in **Materials and Methods**). **A:** Relative abundance of *Drosophila*-adapted strains in competition with the ancestral strain, where the abundance of the ancestral strain is indicated by grey. **B:** Colony forming units of bacteria present in the fly gut during monoculture infection. For **A** and **B**, $n = 8$ per condition per time point, with data pooled from two experiments. These experiments were performed with the help of **Dr. Marc Dionne**.

4.8 Discussion

4.8.1 *Drosophila*-adapted strains have adapted in response to Bomanin-mediated selective pressure

To identify the host components involved in the selection of the *Drosophila*-adapted strains, I performed experiments with *Drosophila* mutants that had defects in their immune response. Infection of flies having a loss of function mutation in *Dif*, the NF- κ B transcription factor of the Toll pathway with the *Drosophila*-adapted strains resulted in no differences in virulence between these strains and the ancestral strain (**Figure 4.1**). Supporting the role of the Toll pathway in the selection of these strains, I observed that constitutive expression of the Toll pathway was protective after infection with the *Drosophila*-adapted strains, in comparison to control genotypes where infection with these strains resulted in high levels of host mortality (**Figure 4.4**). The Toll signalling pathway regulates the expression of a number of immune effector peptides. To identify which of these were involved in the selection of the strains, I next carried out experiments in flies that lacked different immune effectors. I did not observe any differences in virulence between the *Drosophila*-adapted strains and the ancestral strain in flies that lacked 10/12 of the Bomanins, whereas the *Drosophila*-adapted strains exhibited increased virulence compared to the ancestral strain after infection of flies lacking any other antimicrobial peptides (**Figure 4.2, 4.3A**). These results suggest that interaction with the Toll-mediated Bomanins was likely to be the primary stress encountered by *E. faecalis* during host infection.

One way the *Drosophila*-adapted strains could become resistant to the Bomanin-mediated immune response is by evading detection by the Toll pathway and it has previously been described that changes in bacterial peptidoglycan and cell envelope components such as wall teichoic acids affect the activation of the Toll signalling pathway (Atilano et al., 2011; Vaz et al., 2019; Wang et al., 2006). Alternatively, bacteria can evolve to resist killing by the Bomanins. However, as these peptides do not have any activity *in vitro*, it is difficult to test this possibility. As a result, I performed RT-qPCR of representative immune genes including *BomS2* after infection with the different *Drosophila*-adapted strains to identify changes in the induction of the Toll and Imd signalling pathways (**Figure 4.9**). I did not observe differences in the transcript levels of the genes tested in flies infected with the different strains. These results indicate that the *Drosophila*-adapted strains have evolved to resist killing by the Bomanin peptides and not to evade detection by pattern recognition receptors.

An interesting observation I had during these experiments was that the *Drs* flies showed improved outcomes after infection with the *Drosophila*-adapted strains in comparison to *AMPΔ14* and *w¹¹¹⁸* flies (**Figure 4.3B**). A similar phenotype has previously been described by Hanson *et al.* where flies lacking *Mtk*, *Drs*, and *Def* exhibited better survival outcomes compared to flies lacking *Mtk* and *Drs* after infection with the fungi, *Candida albicans* (Hanson et al., 2019). These observations suggest that—at least for infections where the immune response is mediated via the Toll pathway—depending on the infection, the absence of particular antimicrobial peptides can be beneficial to the host. However, why this would be the case is not clear. One explanation could be that in the absence of *Drosomycin*, more resources are focused on the production of the Bomanins, resulting in a stronger immune response after infection. An alternative mechanism could involve compensatory upregulation of other immune genes by the Toll pathway prior to infection. As observed in *c564-Gal4>UAS-Toll^{10b}* flies, increased levels of *Bomanins* and other Toll-regulated antimicrobial peptides prior to infection can be protective against the *Drosophila*-adapted strains. Thus, by analyzing the transcript

levels of Toll-regulated genes in uninjected and infected *Drs* flies I can identify why these flies have better outcomes after this infection.

4.8.2 The role of the Imd pathway in the immune response during *E. faecalis* infection

Infection of flies mutant for the Imd pathway with the *E. faecalis* strains resulted in confusing survival outcomes (**Figure 4.6**). The *Drosophila*-adapted strains showed reduced virulence in *imd* flies; whereas, all *E. faecalis* strains, including the ancestral strain, showed increased virulence in *key* and *Tak1* flies (**Figure 4.7**). This difference in the outcomes of *imd* flies compared to *key* and *Tak1* flies after infection with *E. faecalis* was unexpected. I initially wondered whether the *imd* mutant was mislabelled or contaminated, as a result, I analyzed the transcript levels of representative genes regulated by Toll and imd pathways in *w¹¹¹⁸*, *imd*, and *key* flies after *E. faecalis* infection (**Figure 4.8**). I identified that all three genotypes had similar expression profiles for genes induced by the Toll pathway. The expression of Imd-regulated *Mtk* and *Dipt* was reduced in both the *imd* and *key* flies compared to the *w¹¹¹⁸* flies, therefore, it appears that both genotypes are mutants for the Imd signalling pathway. However, it also appears that the *imd* mutant I are working with is a hypomorph and thus some of the observed differences between the *imd*, *key* and *Tak1* flies could be the result of this. Experiments using other alleles of *imd* will need to be performed to help confirm if the observed phenotype is specific to the *imd¹⁰¹⁹¹* allele or if it is conserved for all *imd* mutants.

The results with *key* and *Tak1* flies suggested that the signalling components downstream of *imd* are important for the immune response against *E. faecalis*. It is known that *Relish*-dependent gene expression is not important for Toll pathway functionality. In addition to this, the increased susceptibility to *E. faecalis* observed in these flies is not due to the absence of Imd-regulated antimicrobial peptides as the ancestral *E. faecalis* strain shows similar levels of

virulence in *AMPΔ14* and *w¹¹¹⁸* flies. Work in the Dionne Lab has previously identified that during an infection, *Drosophila* degrades endogenous host proteins to fuel its immune response (Pearson, 2021). This purine catabolism is dependent on the Imd pathway but is believed to be independent of *Relish* activation and translocation during Gram-positive bacterial infection. Amino acids liberated as a result of this catabolism are hypothesized to be used for the synthesis of immune genes such as Bomanins. Work in this thesis has mainly focused on looking at changes in transcript levels of different immune genes. Changes in amino acid availability and subsequent effects on protein translation are not reflected when I look at gene expression. To confirm the role of the Imd pathway in the synthesis of antimicrobial peptides such as Bomanins, it is important to look at peptide levels rather than transcript levels. This can be achieved by making use of fly lines where antimicrobial peptides are tagged with epitopes such as FLAG or HA (hemagglutinin) allowing for the detection and quantification of the tagged peptides using commercially available anti-FLAG and anti-HA antibodies.

4.8.3 Role of melanization in the immune response during *E. faecalis* infection

My results suggest that the absence of the melanization cascade is beneficial for flies during *E. faecalis* infection. These results are consistent with observations made by Ayres *et al.*, where they identified that inactivation of this pathway is protective during infection with non-melanizing Gram-positive bacteria such as *E. faecalis* and *S. pneumoniae* (Ayres & Schneider, 2008). In this study, the protective effect was observed only in terms of bacterial numbers after infection with *E. faecalis* while survival outcomes were the same between mutant and wild-type flies. However, in my experiments, I observed that *PPO*-deficient flies showed reduced bacterial numbers and better survival compared to *w¹¹¹⁸* flies after infection with the *E. faecalis*

strains (**Figure 4.9**). This could be because V583, the *E. faecalis* strain used by Ayres *et al.*, is different from the one I used for my experiments. V583 produces more virulence factors such as proteases compared to my control strain and these virulence factors have been identified to be important in determining the survival outcomes during infection of *PPO*-deficient flies (Teixeira *et al.*, 2018).

Even though I use the same fly lines for my experiments, my results are not identical to those observed by Binggeli *et al.* (Binggeli *et al.*, 2014). However, infection experiments in this thesis involved injection of bacteria suspended in PBS into the abdomen, whereas Binggeli *et al.* infected flies by pricking with a solid needle coated with bacteria in the thorax. It has previously been shown that the site of injection can influence the outcome of infection (Chambers *et al.*, 2014). For example, injection of *Sp7* flies in the thorax with *E. faecalis* results in a clear increase in mortality compared to wild-type flies injected in the thorax. However, no such increase is observed when flies are injected in the abdomen (Chambers *et al.*, 2014). Thus, while injection in the thorax results in an impaired immune response, this is not dependent on the presence of the melanization cascade.

The melanization cascade and the Toll pathway share upstream signalling components including PGRP-SA. As a result, there is the possibility that the absence of *PPO*'s might result in a more productive immune response similar to what I think is happening in the *Drs* flies. This hypothesis can be easily tested by looking at transcripts of Toll-induced genes to see if *PPO1^Δ*, *PPO2^Δ* flies have greater levels of Bomanins in uninfected conditions or if they produce higher levels of Bomanins after infection. I also know that reactive oxygen species (ROS) are a byproduct of the melanization cascade and can result in damage to both the host and pathogen. Thus, there is also the possibility that by eliminating the production of ROS I may be limiting host damage and this could contribute to the observed improved outcomes after infection.

4.8.4 Adaptation of *E. faecalis* to the systemic immune response does not result in a trade-off during gut infection

Adaptation to one stress is often accompanied by a corresponding increase in susceptibility to a different stress. Such trade-offs have been observed during pathogen specialization to hosts and also during host adaptation in response to a particular pathogen (Martins et al., 2013). While I have mainly focused on the interaction between the systemic immune response of *Drosophila* and *E. faecalis* in this thesis, *E. faecalis* is also commonly isolated from the *Drosophila* gut. The immune response generated as a result of infection of the gut is distinct from that during systemic infection in *Drosophila* (Buchon et al., 2009; Kuraishi et al., 2013). Therefore, there is the possibility that adaptation to the systemic immune response could result in changes in bacterial susceptibility to these components of the gut immune response. However, compared to the ancestral strain, the *Drosophila*-adapted strains did not show increased or decreased changes in fitness in competition with or in absence of the ancestral strain after oral infection (**Figure 4.11**). However, there is also the possibility that the presence of a GFP-expression plasmid in the ancestral strain could be a burden and lead to reduced growth during competition with the systemically-adapted strains, masking any effects. However, during monoculture infections all strains, including the ancestral strain grew to similar numbers, suggesting that the presence of this plasmid did not affect the growth of the ancestral strain *in vivo*.

The Bomanins are not known to be involved in the immune response during gut infection, and it seems that resistance to the action of these peptides does not influence the behaviour of *E. faecalis* in the gut environment. These results also point towards the idea, that depending on the route and site of infection, *E. faecalis* makes use of different mechanisms to enable successful colonization. Given the generalist nature of this bacterium, this seems to be a likely possibility.

Taken together, the results in this chapter suggest that the Toll pathway and Bomanins specifically are a strong selective pressure encountered by *E. faecalis* during *Drosophila* infection. While the Imd pathway appears to be important for fuelling the immune response, the antimicrobial peptides regulated by this pathway are not important during this infection. Finally, the melanization cascade does not play a role in this immune response. As I have not yet identified the contribution of hemocytes during *E. faecalis* infection, I cannot be confident that the Bomanins are the sole selective pressure encountered by bacterium during *Drosophila* infection. More experiments will need to be performed to understand the contributions of the cellular immune response during *E. faecalis* infection.

Chapter 5: Identification of the genetic and phenotypic changes in *E. faecalis* as a result of *Drosophila* adaptation

5.1 Introduction

In the previous chapter, I identified the host components involved in the selection of immune resistance in my strains. In this chapter, my focus is to identify and characterize the bacterial genes that have been altered as a result of interaction with the *Drosophila* immune response. To identify the genetic changes that are the result of host adaptation, I can carry out whole genome sequencing of these strains. This will provide us with a greater understanding of the targets of the Bomanins and the *Drosophila* immune response. It will also inform us about the diversity of routes available to *E. faecalis* to adapt to this stress. For example, if there is only one route towards adaptation, i.e. all strains have mutations in the same gene, or if mutations in different genes can result in the same phenotypic outcome. Once the genes important for host adaptation have been identified I can carry out *in vitro* characterization of the *Drosophila*-adapted strains to identify the bacterial properties altered as a result of these mutations. In this way, I can identify both the genes and bacterial properties that are important for resistance to the *Drosophila* immune response.

E. faecalis is a generalist pathogen and there is the possibility that adaptation to the *Drosophila* immune response can affect the success of the bacterium during infection of other host species. Additionally, while the mechanisms with which the Bomanins kill *E. faecalis* is unclear, several host effector peptides are believed to kill bacteria using mechanisms similar to cell surface targeting antimicrobials. As a result, mutations that enable

resistance to the *Drosophila*-immune response could influence bacterial virulence in other hosts, as well as susceptibility to antimicrobials. This can be tested by assaying the fitness of the *Drosophila*-adapted strains during these interactions.

5.2 Aims

The first **aim** of this chapter was to identify and validate the mutations that are important for *E. faecalis* adaptation to the *Drosophila* immune response. To do this, I carried out whole genome sequencing of the ancestral and *Drosophila*-adapted strains. I then compared the output sequences of the *Drosophila*-adapted strains to the ancestral strain to identify variants. After the identification of these variants, my next **aim** was to characterize the effects of these mutations on the bacterial phenotype. To do this, I carried out *in vitro* phenotyping of the ancestral and *Drosophila*-adapted strains. Finally, I performed *in vitro* assays to test if adaptation to the *Drosophila* immune response can influence bacterial virulence during infection of other host species and resistance to antimicrobials.

5.3 Identification of variants in the *Drosophila*-adapted strains

To identify the genetic changes enabling resistance to the *Drosophila* immune response, I performed whole genome sequencing of the strains generated during the serial passage experiments (*Drosophila*-passaged and Plate control) and the ancestral strain. Following the sequencing of these strains, I identified variants between the *Drosophila*-passaged strains and the ancestral strain by using the variant calling tool Snippy on the Galaxy platform (Jalili et al., 2020; Seemann, 2015). These analyses were performed alongside **Dr. Marc Dionne**. After the identification of variants, I validated the presence of these mutations by amplifying the genomic region with the mutation in the *Drosophila*-adapted strains and the ancestral strain and Sanger sequencing these amplicons.

Snippy is a useful tool for the identification of indels and SNPs between strains but it cannot tell us whether larger rearrangements have occurred in the genome. I visually inspected read mapping across the genome to search for more complex genome rearrangements. I identified locations within the protein-coding regions where Illumina reads could not be assembled in two of my strains, indicating low-complexity insertions. Through PCR amplification and Sanger sequencing of these regions, I identified that these low-complexity insertions were IS6770 transposon insertions. These findings were later confirmed by **Molly Duffet**, who made use of the tool ISMapper to identify IS6770 insertion sites in my strains (Hawkey et al., 2015). This analysis did not reveal any insertion sites other than the ones I had previously identified.

To eliminate mutations that were the result of growth on media and not due to host selection, I sequenced two plate control strains, PC2, and PC7. Surprisingly, I found that while PC2 was genetically identical to my sequenced ancestral strain, PC7 was not, and contained a few variants (**Table 5.1**). Individual *Drosophila*-adapted strains were on either the PC2 or PC7 genetic background (**Table 5.2**). As mentioned in **Chapter 3**, these results suggest that my

ancestral strain was not clonal and consisted of two recently diverged sub-populations of bacteria. Thus, the genetic background that appeared and took over the population during serial passage may be randomly determined.

Table 5.1 Background mutations identified in plate control strains

Gene	PC2	PC7
<i>glnQ_1</i> / EF1120	X	N163D
<i>Tet_R</i> /EF1326	X	N520D
EF1546	X	T400fs
EF2393	X	S153S
<i>ettA</i> /EF1575	X	H51H
<i>ypdA</i> / EF3197	X	N424H
<i>alr</i> / EF0849	X	H255H
<i>bdhA_2</i> / EF0194	X	N198S
<i>degA_2</i> / EF0172	X	D228G and R187fs

All identified mutations within transcribed regions are shown. Gene names, where not previously used in *E. faecalis*, are derived from homologues in other bacteria. Amino acid locations are relative to the predicted protein in the ancestral strain. “fs” indicates a frameshift in the coding regions of affected genes. “X” indicates the absence of the mutation.

Through whole genome sequencing, I could also confirm that the bacterial populations generated during the serial passage in *Drosophila* were clonal in nature. I identified that different single colonies isolated from the same population had the same mutations to the nucleotide level (**Table 5.2**). Since in every case all single colonies isolated from a single passaged population were genetically identical, I have worked with only one isolate from each population.

Table 5.2 Variants identified in the *Drosophila*-adapted strains

Strain		Background	Gene affected	Gene identifier	Mutation type	Gene function
Old identifier	New identifier					
EE1S2C1	Strain A	PC7	<i>dcuR</i>	EF1210	N175fs	Member of the <i>dcuR</i> / <i>dcuS</i> two-component system that regulates utilization of secondary sources of carbon
			<i>yxdM</i>	EF2049	Q402*	ABC transporter involved in Bacitracin export
			<i>epaQ</i>	EF2178	IS6770	Member of the EPA locus that synthesises enterococcal polysaccharide antigen
EE2S1C1	Strain B	PC7	<i>nucF</i>	EF1188	A97T	Predicted to be involved in cell wall metabolism
EE2S2C1, EE2S2C2, EE3S1C1, EE3S5C1, EE3S6C1, EE3S7C1, EE3S8C1, EE3S9C1	Strain C	PC7	<i>liaF</i>	EF2913	S217I	Member of three-component system that co-ordinates cell envelope response to membrane damage and antimicrobials

EE2S6C1, EE2S6C2, EE2S6C5	Strain D	PC7	<i>mprF_2</i>	EF1027	IS6770	Involved in reducing negative charge of cell membrane
EE3S3C1	Strain E	PC2	<i>bgsA</i>	EF2891	G227E	Involved in the synthesis of the glycolipid anchor that attaches LTA to cell membrane
			<i>croS</i>	EF3290	K19E	Member of the croR/croS two-component system that regulates cell envelope response to cell surface damage and antimicrobials
EE3S10C1	Strain F	PC2	<i>yihY</i>	EF2199	F180L	Brkb family protein

All identified mutations within transcribed regions are shown. Gene names, where not previously used in *E. faecalis*, are derived from homologues in other bacteria. Amino acid locations are relative to the predicted protein in the ancestral strain. “*” indicates an introduced stop codon, “fs” indicates a frameshift, “ins” refers to mutations caused by new insertions of IS6770 elements in the coding regions of affected genes.

After validating the mutations in the different strains, I next performed a literature search to identify the function of these genes. Strain A had acquired mutations in three genes: *yxdM*, *dcuR*, and *epaQ*. *yxdM* encodes an ABC transporter permease protein that is part of the bacitracin regulatory network in *E. faecalis*. This regulatory system is involved in coordinating the bacterial response during conditions of cell envelope stress (Gebhard et al., 2014). *dcuR* encodes the response regulator of the DcuS/DcuR two-component sensor-regulator system. This system senses the presence of C4-dicarboxylic acids and regulates their utilization as a source of energy (Abo-Amer et al., 2004; Janausch et al., 2004; Zientz et al., 1998). The third gene mutated in strain A, *epaQ*, is part of the enterococcal polysaccharide operon and the protein it encodes is important for the incorporation of rhamnose into the EPA backbone (Korir et al., 2019). The *epa* operon is involved in polysaccharide biosynthesis and biofilm formation, and is important for *E. faecalis* virulence. Mutations in this operon have been associated with increased resistance to the antibiotic ceftriaxone (Korir et al., 2019; Sava et al., 2010; Fang Teng et al., 2002; Teng et al., 2009). Strain B had acquired a mutation in the gene encoding a protein homologous to *B. subtilis* NucF. This protein is part of the HAD-type hydrolase superfamily of proteins; members of this family have been described to be involved in cell wall production and cell membrane metabolism, but the exact role of this protein is unclear (Hachmann et al., 2011; Tremblay et al., 2006). Strain C had acquired a mutation in the gene *liaF*. *liaF* is part of the *liaFSR* three component system that is another cell envelope stress response signalling system. Mutations in this system are commonly associated with altered bacterial surface characteristics and daptomycin resistance (Arias et al., 2011; Reyes et al., 2015; Tran et al., 2013). The identified allele, *liaF*^{SS217I}, was present in seven *Drosophila*-adapted strains. One of these was generated during the second serial passage experiment (EE2S2C1), while six others were generated during the third serial passage experiment (EE3S1C1, EE3S5C1, EE3S6C1, EE3S7C1, EE3S8C1, EE3S9C1). This was the only mutation identified in these strains, which were genetically and phenotypically

indistinguishable from each other, I collectively term these strains as strain C. Strain D has a mutation in *mprF_2*, which encodes a bifunctional protein involved in addition of positively charged amino acids to phosphatidylglycerol (Ernst et al., 2015). In addition to regulating bacterial cell surface charge, MprF_2 is also a known virulence factor of *E. faecalis* and is important for resistance to cationic antimicrobial peptides (Bao et al., 2012; Ernst & Peschel, 2011; Kandaswamy et al., 2013). Strain E had acquired mutations in two genes: *croS* and *bgsA*. *croS* encodes the sensor histidine kinase of the CroS/CroR two component system (TCS). Similar to the YxdJK and the LiaFSR system, the CroS/CroR TCS is activated in response to cell envelope stress and mutations in either member of this TCS are linked to enhanced cephalosporin resistance (Comenge et al., 2003; Darnell et al., 2019; Kellogg & Kristich, 2018). The other gene mutated in strain E, *bgsA*, encodes a glycosyltransferase required for synthesis of the glycolipid anchor of the LTA (Diederich et al., 2014; Theilacker et al., 2009; Theilacker et al., 2011). Disruptions in either *bgsA* or *bgsB* result in LTA that is longer in length compared to strains that have a functional copy of these genes. These changes in LTA result in enhanced clearance of *E. faecalis* during infection (Theilacker et al., 2015; Theilacker et al., 2009; Theilacker et al., 2011). Finally, Strain F had a mutation in *yihY*, which codes for an uncharacterized membrane protein. This gene is located adjacent to the *epa operon* but is not co-transcribed with it (Teng et al., 2009).

Most *Drosophila*-adapted strains differed from their ancestral strain by a single mutation. However, strains A and E had acquired mutations in more than one gene. My next step involved identifying which of these mutations contributed to the increased *in vivo* virulence in *Drosophila*. I transformed electrocompetent strain A with plasmids containing the ancestral copy of each gene. Following this, I carried out *Drosophila* survival experiments with four strains: strain A, strain A complemented with WT copy of *epaQ* (*epaQ*), strain A complemented with WT copy of *yxdM* (*yxdM*), and strain A complemented with WT copy of *dcuR* (*dcuR*). Complementing the mutation in *dcuR* did not have any effect on the virulence of the strain. However, both *yxdM* and *epaQ* contributed to Strain A's increased *in vivo* virulence (**Figure 5.1**). This effect was also observed in control conditions where anhydrotetracycline was not added to induce the expression of the genes.

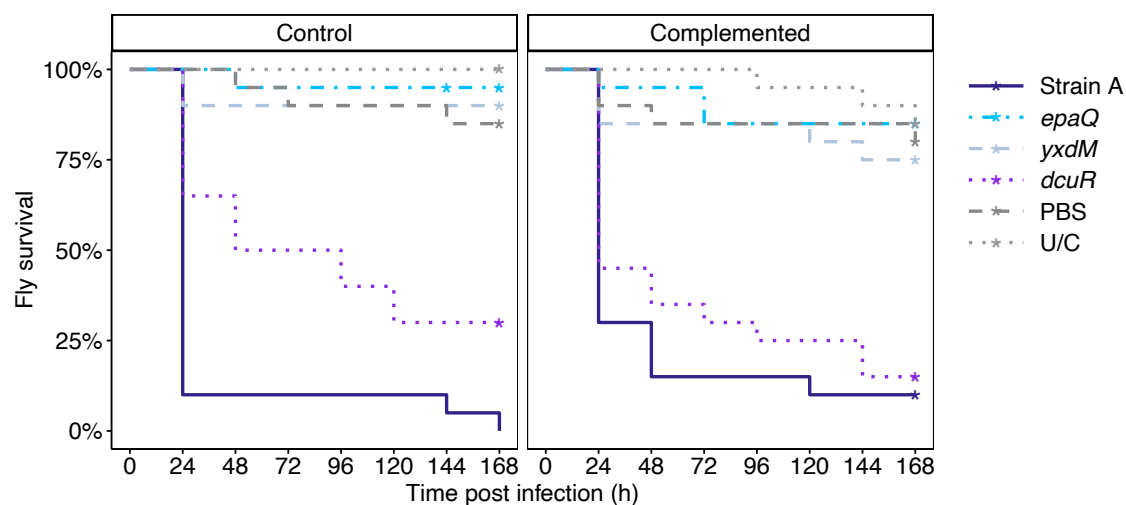


Figure 5.1 Mutations in *epaQ* and *yxdM* contribute to the increased virulence of *E. faecalis* strain A

Survival of *w¹¹¹⁸* flies infected with strain A carrying rescue constructs for *dcuR*, *epaQ*, *yxdM* in absence of (left) or in presence of anhydrotetracycline which drives the expression of the genes in the rescue constructs (right). n = 20 per condition.

I were unable to generate electrocompetent strain E, so I could not make use of the above strategy to identify which of the mutations in strain E contributed to its virulence. Instead, I made use of an alternative strategy where I screened bacteria collected from earlier cycles of selection in the strain E population. Using this approach I was able to isolate a strain carrying only the mutation in *croS* (strain G). Analysis of this strain revealed that both *croS* and *bgsA* contributed to strain E's increased *in vivo* growth as flies infected with strain G showed reduced bacterial load in flies 8-hours post-infection compared to strain E. However, only *croS* seemed to be important for virulence as there was no difference in the survival outcomes of flies infected with strain E or strain G (**Figure 5.2**).

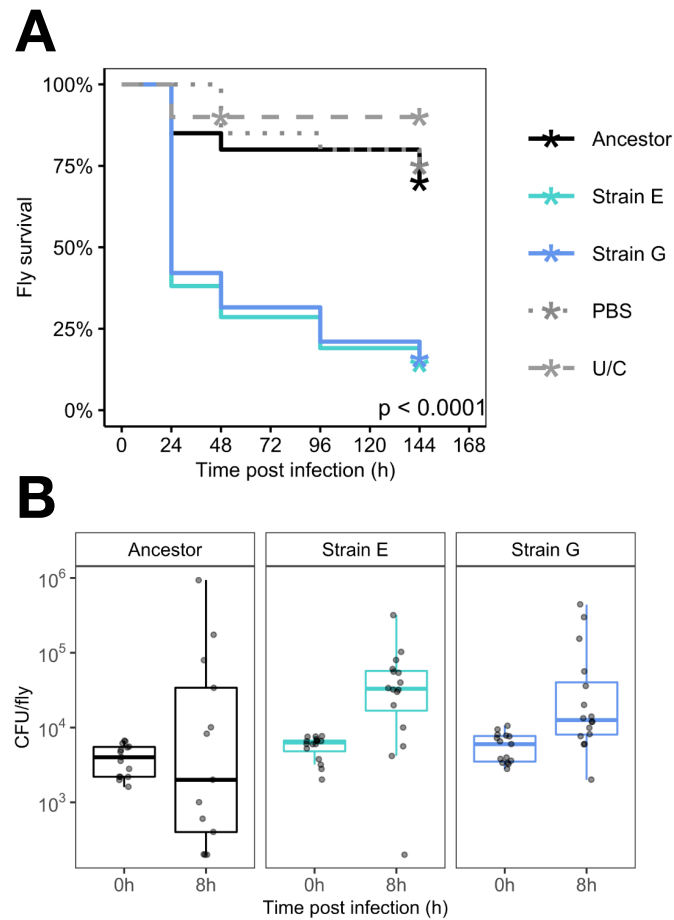
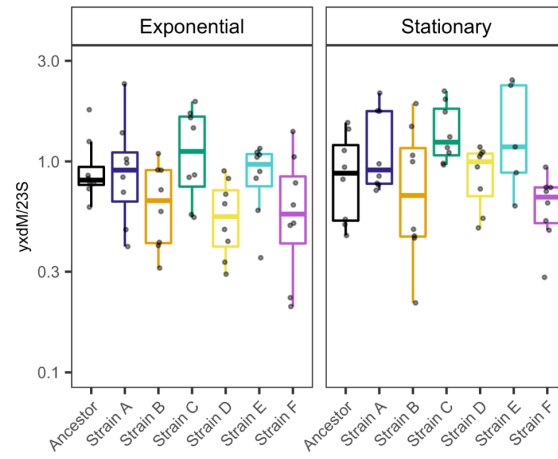
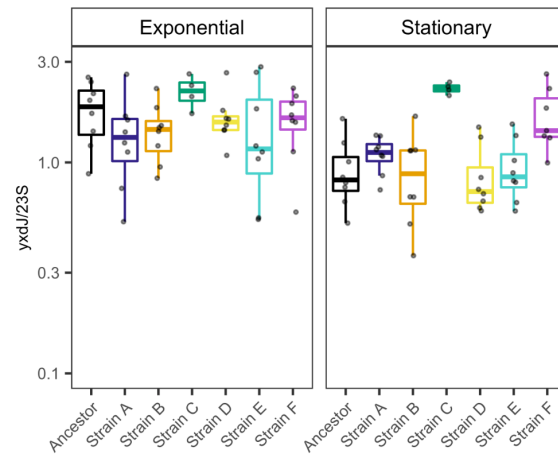
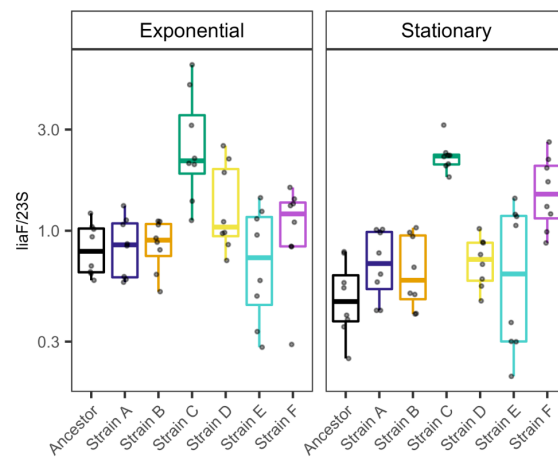


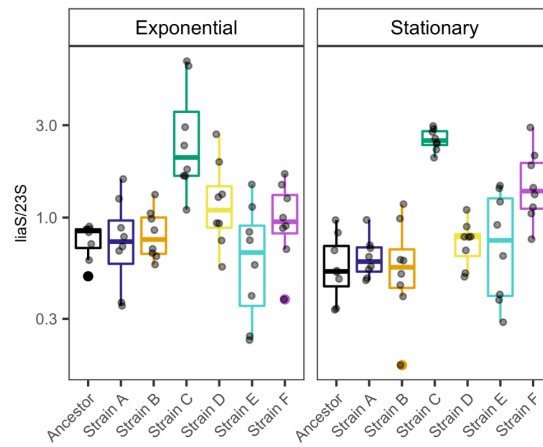
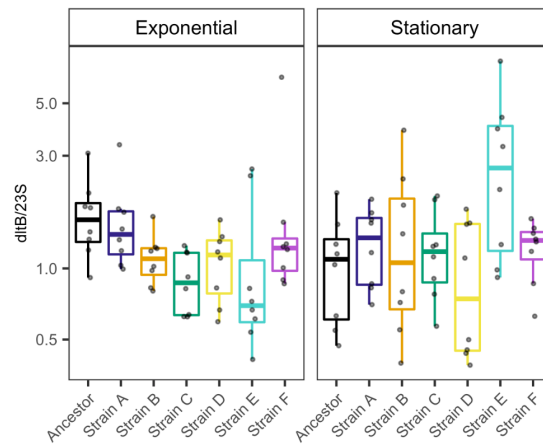
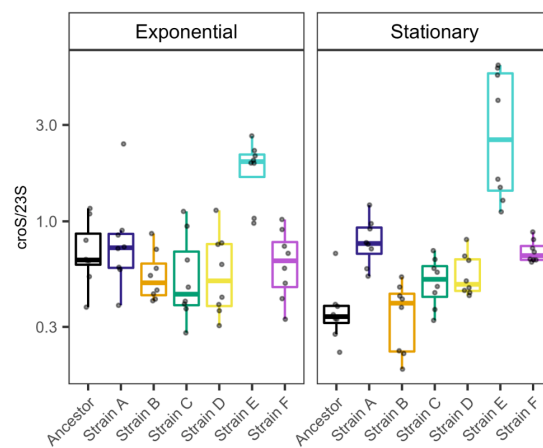
Figure 5.2 Mutations in *croS* and *bgsA* contribute to the increased virulence of *E. faecalis* strain E during *Drosophila* infection

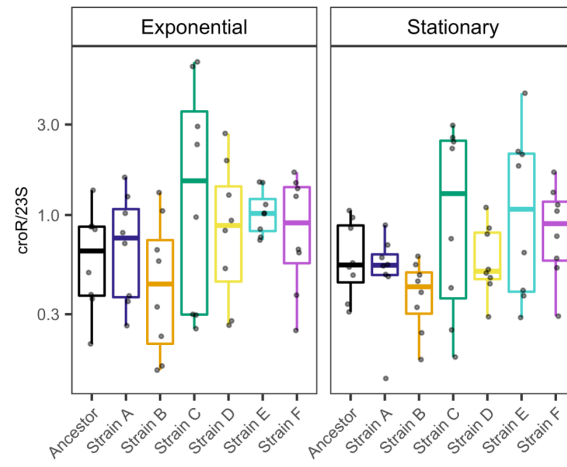
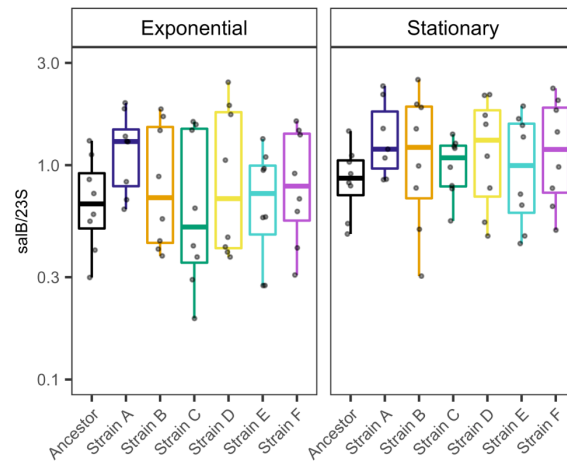
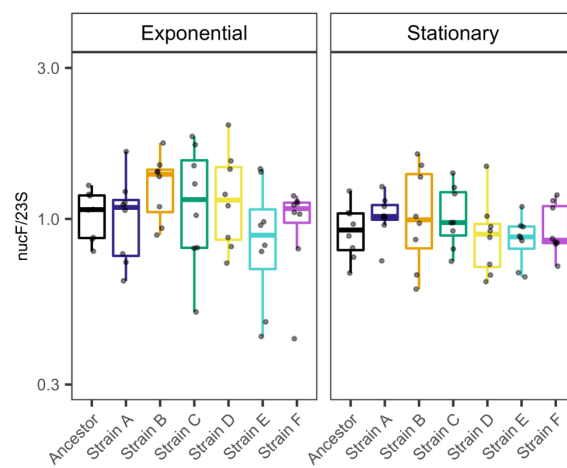
A: Survival of *w¹¹¹⁸* flies infected with ancestor and *Drosophila*-adapted strains E and G. **B:** *E. faecalis* numbers at 0h and 8h after infection of wild-type (*w¹¹¹⁸*) flies with ancestor and *Drosophila*-adapted strains E and G. Values for Strain E and ancestor are the same as those in Figure 3.8 because strain G was performed in parallel as part of the same experiment. In **A**, $n = 20$ per condition. In **B**, $n = 8$ per time point per condition with data pooled from two experiments.

5.4 *Drosophila* adaptation drives changes in cell envelope stress response signalling systems

The *Drosophila*-adapted strains acquired mutations in genes that encode key bacterial surface components, suggesting that the *Drosophila* immune response targets the *E. faecalis* cell surface. Bacterial surfaces are often targeted by host immune effectors and antimicrobials, activating a cell-envelope stress response. *E. faecalis* responds to cell envelope stress via several well-characterized signalling pathways. The *Drosophila*-adapted strains have mutations in three cell envelope stress response systems: *yxdJKLM*, *liaFSR* and, *croRS* (Gilmore et al., 2020). I assayed the activity of these systems by measuring the expression of well-characterized target genes (*yxdJ*, *yxdM*, *liaF*, *liaS*, *croS*, *croR*, *dltB* and *salB*) in *Drosophila*-adapted strains during exponential and stationary phase using RT-qPCR (**Figure 5.3A-H**). No single strain exhibited increased activity of all three pathways, but almost all strains exhibited increased activity of at least one, including strain D and strain F, which did not carry mutations with known direct effects on these signalling mechanisms. Next, I decided to assay the expression of *mprF_2*, *nucF* and, *yihY* to identify if mutations in the signalling systems influenced the expression of these genes (**Figure 5.3I-K**). I did not observe any specific trends in the activity of *nucF*, *mprF_2* among the different strains. However, there is a decrease in the expression of *yihY* in most of the *Drosophila*-adapted strains compared to the ancestral strain in the stationary phase.

A**B****C**

D**E****F**

G**H****I**

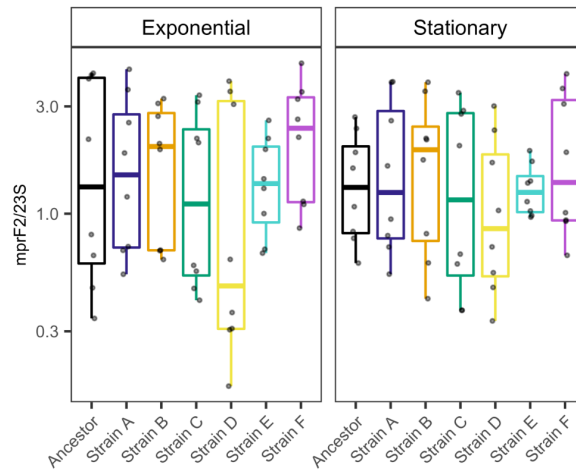
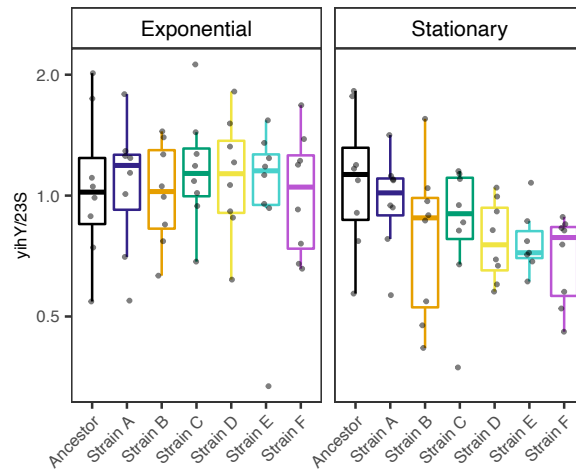
J**K**

Figure 5.3 *Drosophila*-adapted strains show increased activity of cell envelope stress response systems

Gene expression analysis of target genes of the cell envelope stress response systems (*yxdJKLM*, *liaFSR* and *croRS*) in the ancestral and *Drosophila*-adapted strains was measured by RT-qPCR (A-E). Gene expression analysis of other genes identified as mutated was also measured by RT-qPCR (F-J). $n = 4$, with data pooled from two independent experiments.

5.5 *Drosophila*-adapted strains show increased membrane fluidity and surface charge

Several of the *Drosophila*-adapted strains had mutations in genes or systems that were important for regulating bacterial surface properties. As a result, I assayed whether *Drosophila* adaptation resulted in changes to two important bacterial surface properties – membrane fluidity and surface charge. To measure membrane fluidity, I made use of the fluorescent dye laurdan. Laurdan inserts into the phospholipid bilayer and has different emission spectra dependent on lipid packing. When lipids are disordered, the emission peak for laurdan is 490 nm; this shifts to 440 nm when the lipids are packed (Sanchez et al., 2012). Generalized polarisation can be calculated from the fluorescence measurements at these two wavelengths. Higher laurdan generalized polarisation indicates a more tightly packed, thus a more rigid membrane, whereas lower generalized polarisation indicates a more fluid membrane. I observed that all *Drosophila*-adapted strains showed a reduction in generalized polarisation compared to the control strain, indicating greater membrane fluidity (**Figure 5.4**). This trend towards decreased generalized polarisation in the *Drosophila*-adapted strains was observed in both stationary and exponential phase bacteria.

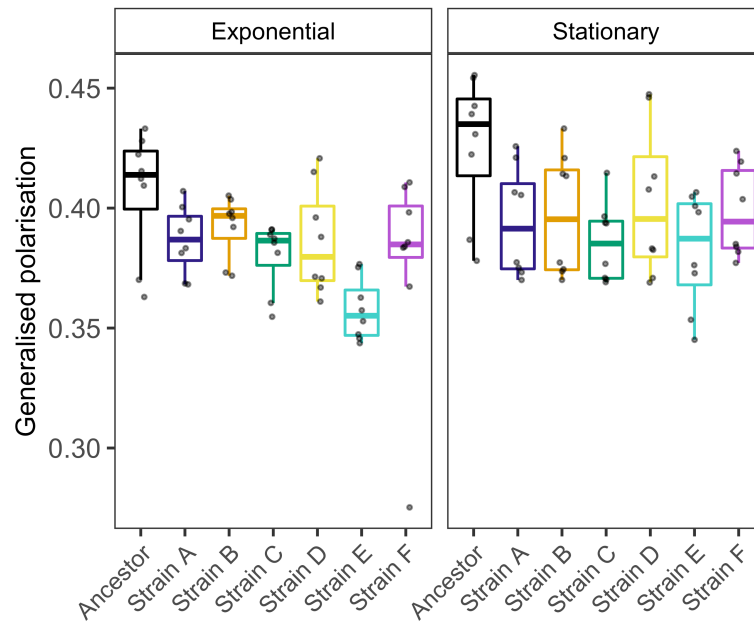


Figure 5.4 The *Drosophila*-adapted strains have greater membrane fluidity

Generalised polarisation of ancestral and *Drosophila*-adapted *E. faecalis* strains as determined by the fluorescent dye laurdan. Reduced generalised polarisation values correlate with increased membrane fluidity. Each strain was measured in duplicate per experiment. The graph represents data pooled from four independent experiments.

To measure bacterial surface charge, I performed a fluorescein isothiocyanate-poly-L-lysine (FITC-PLL) binding assay. Changes in binding of FITC-PLL indicate changes in the surface charge of bacteria as this is a polycationic molecule. Thus, a reduction in fluorescence compared to the control indicates an increase in surface charge whereas an increase in fluorescence suggests a greater negative charge. I observed that all the *Drosophila*-adapted strains showed reduced binding to FITC-PLL during the exponential phase (**Figure 5.5**). This trend was also observed during measurements of FITC-PLL binding to stationary phase bacteria, but the effect was smaller for strains D and F.

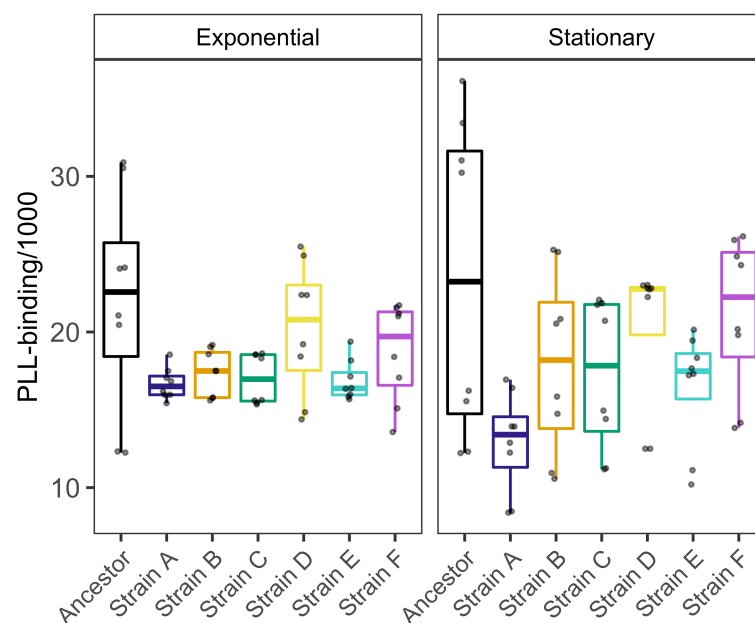


Figure 5.5 Most *Drosophila*-adapted strains have greater surface charge

Surface charge of ancestral and *Drosophila*-adapted *E. faecalis* strains as determined by binding of cationic fluorescent dye FITC-PLL. Reduced binding correlate with increased surface charge. For each experiment, measurements of FITC-PLL binding were carried out in duplicate. The graph represents data pooled from four independent experiments.

5.6 The lipoteichoic acid of most *Drosophila*-adapted strains is not altered as a result of host-adaptation

Two *Drosophila*-adapted strains had mutations in genes that are important during LTA synthesis. These were *bgsA* (Strain E) and *mprF_2* (Strain D). *bgsA*, along with *bgsB*, is involved in the production of diglucosyldiacylglycerol (DGlcDAG), the glycolipid anchor of LTA (Theilacker et al., 2015; Theilacker et al., 2011). In *S. aureus*, it has been described that in absence of these genes, diacylglycerol (DAG) can function as the anchor for this polymer (Fedtke et al., 2007; Grundling & Schneewind, 2007). This change in anchor molecule in turn results in the production of LTA with longer chain lengths. *mprF_2* on the other hand is involved in the synthesis and translocation of aminoacyl-PG. It has recently been described that *B. subtilis* and *S. aureus* strains that have mutations in *mprF* produce LTA that is shorter in length than wild-type strains (Guyet et al., 2022). I carried out SDS-PAGE of bacterial cell extracts followed by Western blot analysis using an LTA-specific antibody to visualize changes in the LTA of the different strains.

Confirming previous observations made by Theilacker *et al.* (Theilacker et al., 2011), the *bgsA* mutant strain, strain E, produced LTA of a different size compared to other strains. This LTA moved slower through the gel indicating a larger size. I also observed that strain E produced more LTA than the other strains, but I did not quantify this (**Figure 5.6**). This increased abundance of LTA has previously been suggested to be due to the increased length of the polymer (Theilacker et al., 2011). It also appeared that strain A might produce slightly more LTA compared to the control strain. However, as the presence of strain E on the Western blot resulted in the saturation of the signal, more experiments will need to be performed without this strain to conclude whether strain A does indeed produce higher amounts of LTA.

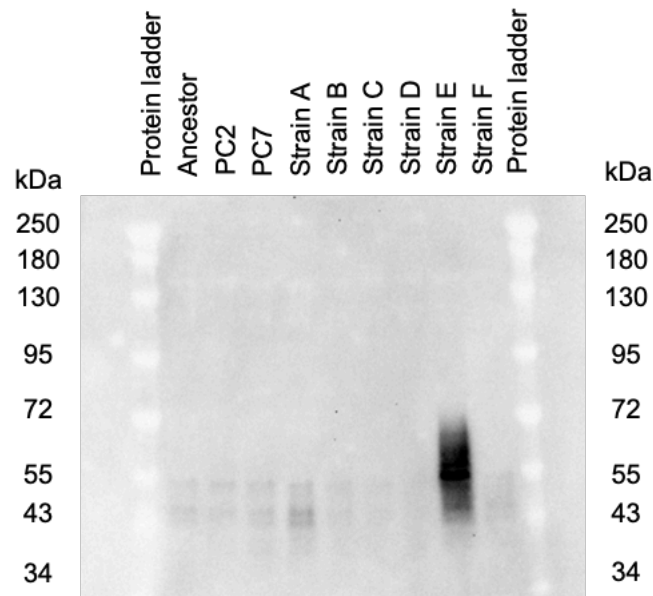


Figure 5.6 Increased virulence of the *Drosophila*-adapted strains is not due to altered LTA

Western blot analysis of LTA of ancestral, plate control and *Drosophila*-adapted strains. The band for LTA is between 43 and 55kDa for most strains with the exception of strain E where it is between 55 and 72kDa. The molecular weights in kDa of the protein markers are indicated. Three independent replicates of western blot analysis were performed and one representative image is shown

5.7 *Drosophila*-adapted strains resist killing by human blood but show no changes in tolerance to bile

The *Drosophila*-adapted strains acquired mutations in genes that are known to be important for bacterial virulence such as *mprF*, *bgsA* and, *epaQ*. Mutations in these genes often result in enhanced clearance during mammalian infection (Nowak et al., 2021; Peschel et al., 2001; Fang Teng et al., 2002; Theilacker et al., 2015). This suggested that the *Drosophila*-adapted strains exhibited altered sensitivity to stresses that would be encountered in mammalian hosts. To test if this was the case, I first assayed the killing of *Drosophila*-adapted strains by whole human blood *ex vivo* (Painter et al., 2017). I observed that while the ancestral strain itself was not killed very efficiently by human blood, all tested *Drosophila*-adapted strains grew significantly more during six hours in human blood than the ancestral strain (**Figure 5.7**).

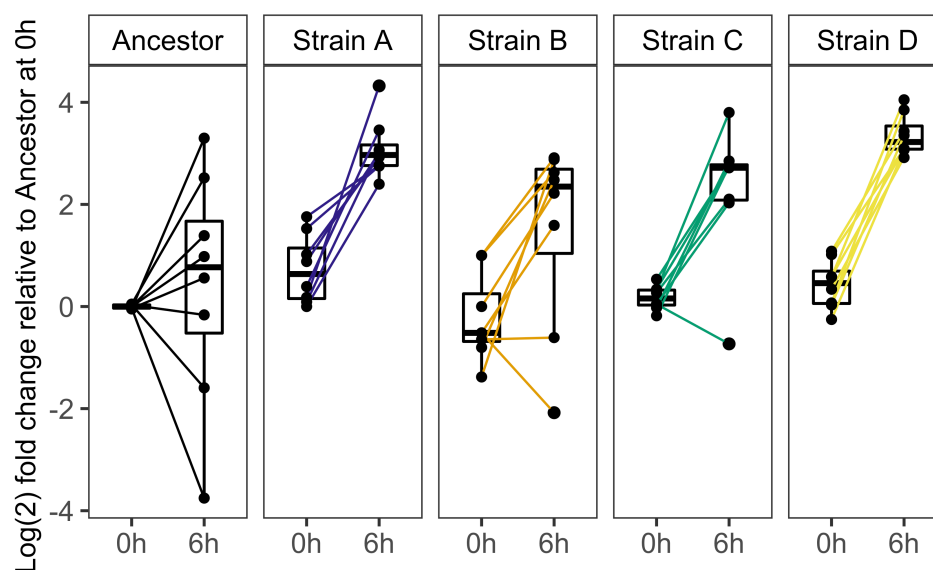


Figure 5.7 *Drosophila*-adapted strains exhibit enhanced resistance to killing by human blood

The ability of ancestor and *Drosophila*-adapted strains A-D to survive and proliferate over 6h in fresh human blood was tested. Bacteria were quantified 0h and 6h after incubation in human blood. In each experiment, each strain was measured in duplicate. The graph represents data pooled from four independent experiments.

Next I decided to assay the ability of the *Drosophila*-adapted strains to tolerate bile, which is a stress commonly encountered by bacteria in the mammalian gut. Bacterial numbers were compared after growth in the presence or absence of 10% bovine bile in BHI for a period of 1 hour (**Figure 5.8**). I observed that strain C exhibited increased tolerance to bile. This result was not unexpected, as mutations in *liaFSR* system have been described to alter bile tolerance in *E. faecium* (Zhou et al., 2019). However, no other tested strain showed any differences in bile tolerance.

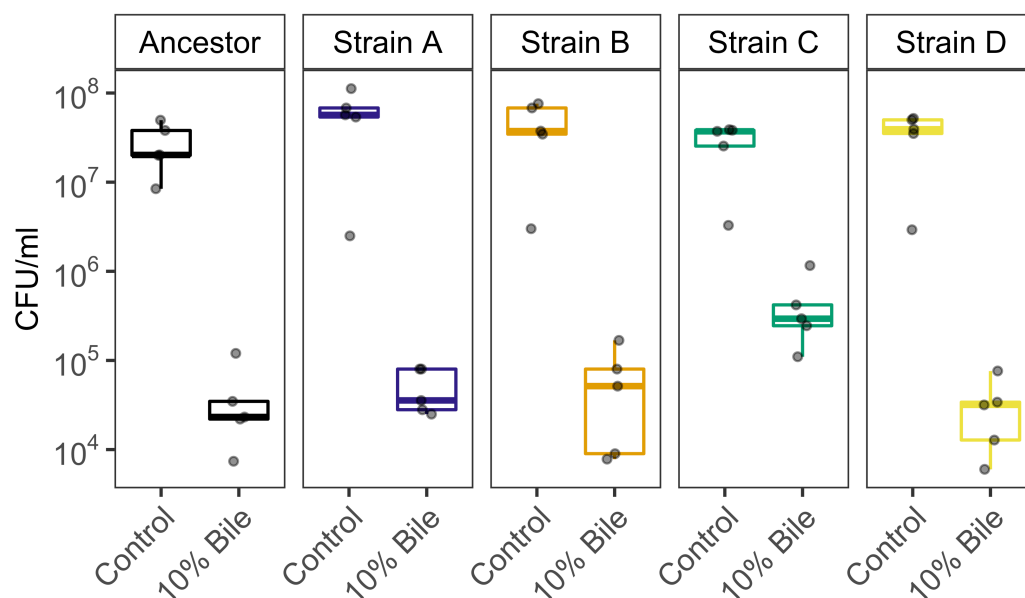


Figure 5.8 Bile tolerance of *Drosophila*-adapted strains

Ability of the ancestral and *Drosophila*-adapted strains (strains A-D) to tolerate 10% bile was tested. Bacteria were subcultured in control media or media containing 10% bovine bile. Bacteria were quantified after 1h of incubation. Data pooled from three independent experiments. For 2/3 experiments, strains were measured in duplicate whereas in the last experiment only single measurements were made.

5.8 *Drosophila*-adapted strains show changes in sensitivity to antimicrobials

Several *Drosophila*-adapted strains had mutations in genes known to be important for antimicrobial resistance or tolerance, including *mprF2*, *epaQ*, *liaF*, *croS* and *yxdM* (Arias et al., 2011; Darnell et al., 2019; Gebhard et al., 2014; Prater et al., 2019). I also observed that these strains had greater membrane fluidity and greater surface charge. Alteration of membrane fluidity is a known mechanism of resistance to the lipopeptide antibiotic daptomycin (Tran et al., 2015). Similarly, bacteria increase their surface charge to reduce electrostatic interaction with cationic antimicrobial peptides (Fedtke et al., 2004). As a result, I tested the minimal inhibitory concentration (MIC) of these strains against a panel of antimicrobials. I also tested the MICs of the plate control strains to exclude the possibility that any changes in antimicrobial susceptibility could be the result of background of the strains.

I observed increases and decreases in the susceptibility of the *Drosophila*-adapted strains to different antimicrobials, with no two *Drosophila*-adapted strains showing the same susceptibility profile (**Figure 5.9**). Most changes were modest; however, confirming previous observations, loss of function mutation in *yxdM* resulted in increased susceptibility of strain A to bacitracin, while the *liaF* mutation in strain C conferred decreased susceptibility to daptomycin (Arias et al., 2011; Gebhard et al., 2014). Finally, strain E showed a 4-fold decrease in susceptibility to both vancomycin and teicoplanin, attributable to its mutation in *croS* (Darnell et al., 2019). All *Drosophila*-adapted strains showed decreased susceptibility to vancomycin, and half showed decreased susceptibility to cationic antimicrobial peptides and daptomycin. Both plate control strains had the same antibiotic susceptibility profile to the ancestral strain, except for a two-fold increase in the susceptibility of PC7 to linezolid.

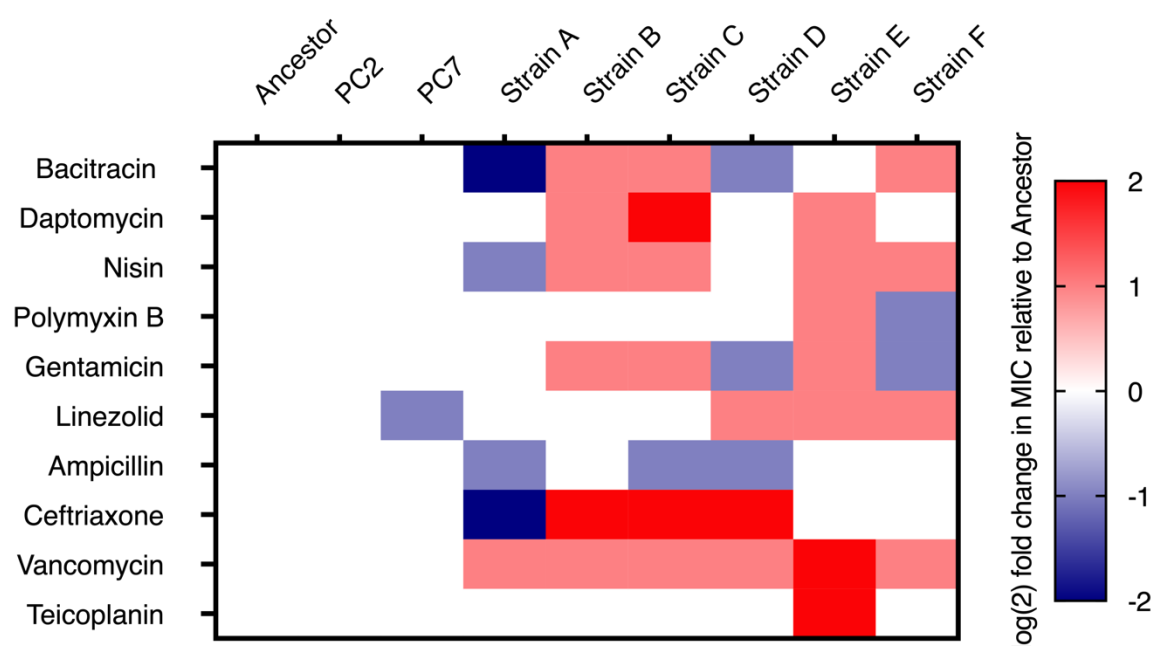


Figure 5.9 Susceptibility of the *Drosophila*-adapted strains to a panel of antibiotics

MIC of the *Drosophila*-adapted strains and the plate control strains relative to the in-experiment ancestral control plotted as a heatmap. Absolute MIC values can be found in **Table 5.3**. Each measurement was made in triplicate in each experiment. Heatmap represents data pooled from three independent experiment.

Table 5.3 Antimicrobial minimal inhibitory concentrations of the *E. faecalis* strains

Antimicrobial	Ancestor	PC2	PC7	Strain A	Strain B	Strain C	Strain D	Strain E	Strain F
Bacitracin	32	32	32	<16	64	64	16	32	64
Daptomycin	4	4	4	4	8	16	4	8	4
Nisin	128	128	128	64	256	256	128	256	256
Polymyxin B	512	512	512	512	512	512	512	1024	256
Gentamicin	64	64	64	64	128	128	32	64	32
Linezolid	2	2	1	2	2	2	4	4	4
Ampicillin	0.5-1	0.5	0.5	0.25	0.5-1	0.5	0.5	0.5	0.5
Ceftriaxone	2-8	2	2	<2	8	8	8	8	8
Vancomycin	1-2	1	1	4	4	4	4	4	2
Teicoplanin	0.5	0.5	0.5	0.5-1	0.5-1	0.5	0.5	2	0.5-1

The full range of measurements on all strains is shown; the heat-map in **Figure 5.5** shows measurements normalized to in-experiment controls to avoid batch-to-batch variation. Measurements are in µg/ml. n=3 per experiment with data pooled from three independent experiment.

5.9 Most *Drosophila*-adapted strains do not have altered growth rate

I assayed growth of the *Drosophila*-adapted strains *in vitro* to confirm that the increased growth in human blood and altered susceptibility to antimicrobials observed in the *Drosophila*-adapted strains was not due to changes in bacterial growth rate (**Figure 5.10**). The average doubling time of the ancestral and most *Drosophila*-adapted was between 27 and 33 minutes. However, strain F showed a longer doubling time of 50 minutes. This difference in doubling time is because one of the technical replicates of strain F always appeared to have different growth behavior compared to the other two technical replicates. This observation was made during all three repeats of the experiment. As a result, I think this difference in growth is due to issues with readings at that well position as the layout of the strains remained the same in the 96-well plate during all three repeats. However, I cannot be confident of this hypothesis until I repeat this experiment changing the position of strain F in the 96-well plate.

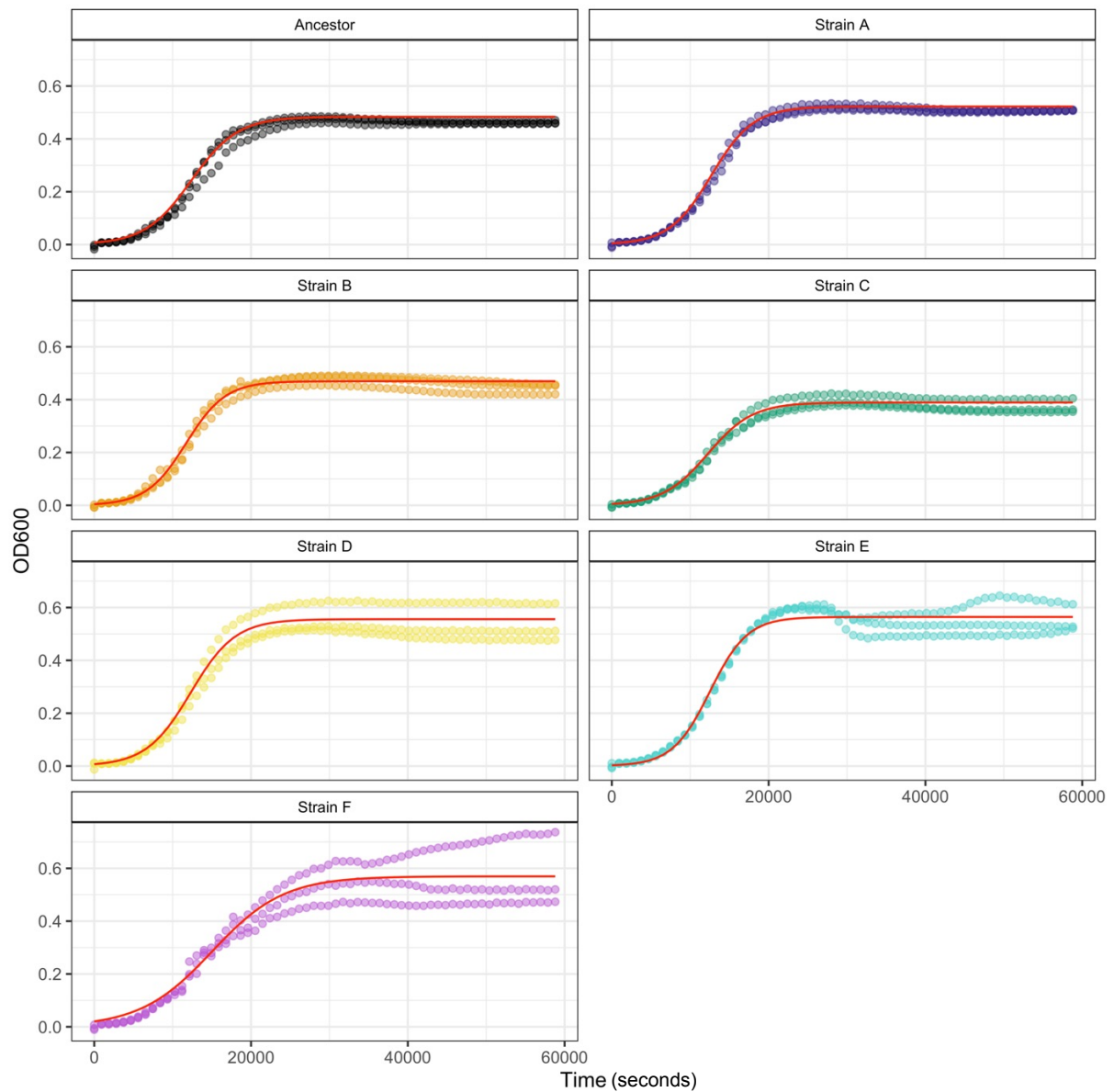


Figure 5.10 *Drosophila*-adapted strains do not show differences in growth rate *in vitro*

Growth of ancestral and *Drosophila*-adapted strains determined by measuring absorbance at OD₆₀₀ over 17h. Three technical replicates were set up per growth experiment and the red line indicates average growth between the three replicates. Three independent growth curve experiments were performed and one representative image is shown.

5.10 Discussion

5.10.1 The genetics of adaptation to the *Drosophila* immune response

To identify the genetic changes in the *Drosophila*-adapted strains that enabled resistance to the *Drosophila* immune response, I sequenced the *Drosophila*-adapted strains, plate control strains and the ancestral strain. I then compared sequences of the *Drosophila*-adapted strains and the ancestral strain to identify adaptive variants in these strains. I also compared the sequences of the plate control strains and the ancestral strain to identify mutations that were acquired as a result of passage on selective media. Through these analyses, I found that PC7 and a subset of the *Drosophila*-adapted strains had common mutations that were absent from the PC2 strain (**Table 5.1**). I also observed that strain A, which was generated during the first serial passage experiment had fewer of these mutations compared to PC7 and the other strains which were generated in the second and third rounds of serial passage. These mutations were conserved at the nucleotide level between these strains, including strain A.

As described in **Chapter 3**, I did not observe any significant differences in the behaviour of the two plate control strains compared to the ancestral strain. Additionally, as I made use of this mixed ancestral strain as a control for all my experiments, I believe that I have managed to include this variation in the bacterial background in my experiments. Finally, for some *in vitro* experiments where I made use of three control strains (ancestral, PC2, PC7), I did not see any contributions of the background mutations to the adaptive phenotypes that the *Drosophila*-adapted strains displayed.

Previously, in **Chapter 3**, I suggested that the *Drosophila*-adapted populations were clonal in nature as different single colonies from the same population showed similar phenotypic behaviour. My sequencing results confirmed this notion as different colonies isolated from the

same population had the same mutations conserved to the nucleotide level (**Table 5.2**). I also identified that six apparently independently-derived *Drosophila*-adapted strains had acquired the same mutation to the allele level. These results suggest that strong parallel evolution has occurred during adaptation to the *Drosophila* immune response. This phenomenon has been frequently described during bacterial adaptation to the host immune response and to antimicrobial stress (Cooper et al., 2020; Miller et al., 2013). However, there is also the possibility that these mutations could be the result of some contamination event during the course of the serial passage experiment. In this case, the repeated isolation of this strain would not represent parallel evolution, but is nonetheless strong evidence that this mutation confers a strong selective advantage on the bacterium. I would need to perform more serial passage experiments to verify if strains containing this mutation are more frequently isolated compared to others to confirm or disprove parallel evolution.

In addition to the acquisition of SNPs and indels by the *Drosophila*-adapted strains, I identified that two of the strains had larger chromosomal rearrangements due to the disruption of coding sequences of genes by an insertion element. The same family of insertion elements (IS), IS6770, was involved in both these insertion events, indicating the possibility that the mobility of this IS is driven by the *Drosophila* immune response. My results also point towards a potential preference for this IS to disrupt genes that encode key bacterial envelope components. This idea is supported by a study that identified that the expression of the *cps* genes in some *E. faecalis* strains is disrupted due to the insertion of an IS6770 element in the promoter region of the *cps* locus (Gaspar et al., 2012).

5.10.2 *E. faecalis* alters its bacterial surface to resist the action of the *Drosophila* immune response

My sequencing results suggested that the bacterial cell surface is the target of the *Drosophila* immune response, as most *Drosophila*-adapted strains acquired mutations in genes that encoded components of the bacterial cell envelope. These included mutations in the cell envelope stress-response systems *yxdJKLM*, *liaFSR*, and *croRS*, which regulate the cellular response to changes in cell envelope integrity. Considering that the mutations in the other *Drosophila*-adapted strains could affect bacterial cell envelope homeostasis, I wondered whether there was increased activation of these cell envelope stress-response systems in my strains. I identified that most strains displayed increased activity of at least one of these systems. This included strains that contained mutations in genes of unknown function. These results also tell us that the mutations in two of the signalling systems, *liaFSR* and *croRS*, lead to increased activation of these systems, as I observe increased expression of their sensor kinases (**Figure 5.3D and 5.3F**). However, mutation in *yxdM* did not result in altered expression of *yxdJ* in strain A (**Figure 5.3B**). This result is not surprising as *yxdM* encodes for the ABC transporter believed to be involved in the efflux of antimicrobial agents such as bacitracin, and is not known to be involved in the signalling aspect of this system (Gebhard et al., 2014). However, more experiments will need to be performed to understand whether mutations in *yxdM* result in increased expression of other genes regulated by *yxdJK*. I also observed that all strains exhibited increased activation of *croS*, during stationary phase; this was unexpected as Le Breton *et al.* previously described that this system is repressed during the stationary phase of growth (LeBreton et al., 2007). Correspondingly, I observed a small increase in the expression of *salB*, a well-defined *croRS* target gene, in all strains during stationary phase (**Figure 5.3H**). I also observed a decrease in the expression of *yihY* in stationary phase *Drosophila*-adapted strains. The function of this gene is unknown and while it is not co-transcribed with the *epa* operon, the increase in expression of the *yxdJK* and

LiaFSR TCS in the strain F suggests that it encodes for some bacterial surface component. However, more experiments will need to be performed to identify what this might be.

I next moved my attention towards identifying if the different mutations resulted in the modification of common bacterial properties. I did not observe any general differences in the size or abundance of LTA produced by these different strains (**Figure 5.6**). However, I discovered that most *Drosophila*-adapted strains had greater membrane fluidity and greater surface charge compared to the control strain (**Figure 5.4 and 5.5**). This included strains B and E, which carried mutations in genes of unknown function. I will need to carry out more experiments to identify the mechanisms behind these changes. As altered membrane fluidity can be the result of changes in the fatty acid composition of the cell membrane, I can test and compare the fatty acid composition of my strains to identify if this was responsible for the increased membrane fluidity of my strains (Klein et al., 1999; Mishra et al., 2012).

Bacteria regulate their surface charge through two different mechanisms. The first mechanism involves the modification of teichoic acids with D-alanine which is mediated by the *dltABCD* operon. The other mechanism used by bacteria to regulate surface charge is through the addition of aminoacyl groups to the membrane lipid phosphatidylglycerol by MprF (Bao et al., 2012; Peschel et al., 2001; Peschel et al., 1999). I did not observe any increases in the activity of either of these systems during my gene expression analysis (**Figure 5.3E and 5.3J**). However, there is a possibility that changes in gene expression of these systems might occur during periods of cell surface stress. While efforts to quantify bacterial gene expression during infection of *Drosophila* have not been successful, I can assay the expression of these genes after challenging the strains with a cell surface stressor such as bacitracin that is known to induce the expression of these systems. I can then compare for differences in the expression of *dltABCD* and *mprF_2* between the ancestral and *Drosophila*-adapted strains. If any of these systems are upregulated to a greater extent I can then quantify the D-alanylation

of LTA's or the abundance of L-PG in the cell membrane to confirm that they are responsible for the phenotype.

Strain D, had a mutation in MprF_2; *mprF* genes encode for bifunctional proteins that can synthesize and add aminoacyl groups to phosphatidylglycerol thereby regulating the bacterial surface charge (Peschel et al., 2001; Roy & Ibba, 2008; Thedieck et al., 2006). As the mutation in strain D is due to the insertion of an IS6770 element early in the coding region of this gene, I had assumed that it was non-functional. Thus, I expected strain D to have more negative surface charge compared to the ancestral strain as this is what has been described previously for *mprF* mutants (Ernst et al., 2009). However, I observed a small increase in surface charge in exponential phase strain D (**Figure 5.5**). This increase in positive charge could be attributed to *dltABCD* mediated D-alanylation of LTA, but I did not observe increased expression of *dltB* in strain D (**Figure 5.3E**).

MprF proteins are made up of two domains, a synthase and flippase domain. It has been previously described that these two domains are separable and mutations in the flippase domain do not affect the function of the synthase domain and vice versa (Ernst et al., 2009). The mutation in strain D was in the flippase domain of MprF_2. Thus, there is a possibility that while the IS6770 element has disrupted the flippase domain of MprF_2, the synthase domain is still functional and can produce aminoacyl groups. In addition to this, while most Firmicutes encode for a single MprF protein, *E. faecalis* encodes for two, MprF_1 (EF0031) and MprF_2 (EF1027). MprF_2 is important for the aminoacylation of PG, but the function of MprF_1 is unclear, although it has recently been described to contribute to PG and L-PG synthesis (Bao et al., 2012; Rashid et al., 2022). As a result, I hypothesize that in strain D, aminoacyl-PG that is synthesized by the synthase domain of MprF_2 is flipped by the flippase domain of MprF_1. One way to validate this hypothesis is by comparing the membrane lipid composition of strain D, a null mutant for MprF_2 and the ancestral strain.

5.10.3 *Drosophila*-adapted strains have a growth advantage in human blood

Several of the *Drosophila*-adapted strains had mutations in genes that were characterized to be important for bacterial virulence or antimicrobial resistance (**Table 5.2**). As a result, I carried out experiments to understand the consequences of *Drosophila* adaptation on these bacterial traits. I identified that adaptation to the *Drosophila* immune response conferred an advantage to *E. faecalis* during incubation with human blood. This result was unexpected as neutrophils are believed to be the dominant mechanism of killing in this experimental setup (Ranganathan et al., 2020). This suggests that there might be some similarities in the mechanisms of killing by the *Drosophila* and human immune response during systemic infection. As strains from the third serial passage experiment were not generated at the time these experiments were performed, it will be interesting to see if they show the same advantage, considering that mutations in *bgsA* result in a virulence defect during bloodstream infections (Theilacker et al., 2009).

5.10.4 *Drosophila*-adapted strains show altered susceptibility to antimicrobials

Antibiotic susceptibility testing of the *Drosophila*-adapted strains identified new determinants for antimicrobial resistance; *nucF* and *yihY*. For some strains, the MIC results validated what has been previously described in the literature. For example, the *liaFSR* system is one of the most important determinants of daptomycin resistance in enterococci. Thus, it is not surprising that strain C, which has a mutation in *liaF* that results in increased activation of this system, shows a four-fold increase in resistance to daptomycin. I also observed that strain A, which

has a mutation in the gene encoding *yxdM*, is more sensitive to bacitracin. *yxdM* encodes the ABC transporter responsible for the efflux of bacitracin out of the cytoplasm; disruption of this transporter results in higher susceptibility to bacitracin (Gebhard et al., 2014). Finally, in agreement with the role of *croRS* in resistance to vancomycin, I observed that strain E shows increased resistance to this antimicrobial (Darnell et al., 2019; Hancock & Perego, 2004). Interestingly, I observed that strain E also shows increased resistance to teicoplanin, another glycopeptide antibiotic. Cross-resistance to vancomycin and teicoplanin has previously been associated with *vanA* type vancomycin resistance in Enterococci (Arthur & Courvalin, 1993).

Not all results from the MIC assays agree with what might be predicted from the literature. Loss of *epaQ* has been shown to result in a 32-fold decrease in susceptibility to ceftriaxone (Korir et al., 2019). However, I observed an eight-fold increase in the susceptibility strain A to ceftriaxone. The *epaQ* gene in strain A is disrupted due to the insertion of an IS6770 element. One reason for this difference could be that Korir *et al.* made use of an engineered null *epaQ* mutant; the insertion of the IS6770 element is probably null for *epaQ* but may also disrupt *epaR*. Second, strain E has a mutation in *croS*, which is the response regulator normally driving cephalosporin resistance in *E. faecalis* (Comenge et al., 2003; Timmler et al., 2022). I also did not observe any changes in the MIC of strain E to ceftriaxone. This could be because the strain E mutation activates, rather than inactivates, *croS*. However, I found the susceptibility of the ancestral strain to ceftriaxone to be highly variable between experiments. Although I performed an in-experiment ancestral control during each experiment, there is a possibility that this variation could be masking a phenotype. As a result, repeating this experiment with a different cephalosporin antibiotic would be beneficial.

Chapter 6: Mechanisms of *E. faecalis* resistance to daptomycin

6.1 Introduction

In **Chapter 5**, I found that adaptation to the *Drosophila* immune response resulted in mutations in genes encoding proteins that regulate the properties of the bacterial surface. This included *mprF_2*, *liaF*, *yxdM* and *croS* that are known to be important for resistance to surface targeting antimicrobials (Arias et al., 2011; Bao et al., 2012; Comenge et al., 2003; Ernst et al., 2018; Gebhard et al., 2014). I found that these genetic changes in the *Drosophila*-adapted strains were accompanied by phenotypic changes such as: increased activation of cell envelope stress response systems, increased cell membrane fluidity, and more positive cell surface charge. These phenotypic changes can also influence bacterial interaction with antimicrobials (Jordan et al., 2008; Kovacs et al., 2006; Mishra et al., 2012). Finally, I found that *Drosophila* adaptation resulted in altered resistance of these strains to vancomycin and other surface-targeting antibiotics. Based on these results I hypothesize that there are similarities between the stress exerted by the *Drosophila* immune response and surface targeting antibiotics, and *E. faecalis* responds to these stresses via similar mechanisms. Therefore, as resistance to the *Drosophila* immune response can result in altered susceptibility to antimicrobials, resistance to surface targeting antimicrobials could result in altered virulence in *Drosophila*.

6.2 Aims

The **aim** of the experiments performed in this chapter, is to identify whether the *Drosophila* immune response and daptomycin target the same conserved bacterial properties in *E. faecalis*. To answer this, I experimentally evolved *E. faecalis* against the antimicrobial daptomycin. If, as suggested by my hypothesis, these two stresses have the same target, then I would expect the daptomycin-adapted strains to acquire mutations in the same genes or systems as those identified in the *Drosophila*-adapted strains. I would also expect that these strains might exhibit greater virulence during *Drosophila* infection.

6.3 Experimental evolution of *E. faecalis* to daptomycin

To investigate whether *E. faecalis* responds to the insect immune response and surface-targeting antimicrobials using the same mechanisms, I needed to generate *E. faecalis* strains that were resistant to the action of one such antimicrobial. I decided to make use of daptomycin instead of vancomycin for these experiments for two reasons. The first reason was that high level vancomycin resistance is not achievable through mutations. Vancomycin resistance in Enterococci is mediated through alterations in peptidoglycan synthesis where, D-Alanine-D-Alanine residues are substituted with either D-Alanine-D-Lactate or D-Alanine-D-Serine residues (Cetinkaya et al., 2000; Gold, 2001; Reynolds, 1998; Reynolds & Courvalin, 2005). This alteration in peptidoglycan synthesis is generally associated with several genes that form part of a gene cluster. Several different vancomycin gene clusters have been identified in Enterococci and they can be present on mobile genetic elements or they can be chromosomally encoded (Chen & Xu, 2015). In *E. faecalis* and *E. faecium*, vancomycin resistance is almost always plasmid borne (Depardieu et al., 2004; Miller et al., 2020). As my ancestral strain lacked these plasmids, high level resistance to vancomycin would not be possible. The second and more important reason for selecting daptomycin for my experiments was that mutations in *mprF*, *yxdJK* and *liaFSR* are associated with clinical levels of resistance to daptomycin (Arias et al., 2011; Miller et al., 2019).

Daptomycin-adapted strains of *E. faecalis* were generated by serial passage of *E. faecalis* to increasing concentrations of daptomycin. Briefly, 100 μ l of an O/N culture was added to 3 ml BHI containing daptomycin at 1,2,4,6, and 8X MIC concentration. The following day, 100 μ l from the culture with visible bacterial growth at the highest antibiotic concentration was sub-cultured into two fresh cultures, one with the same concentration of antibiotic and one with 2x this concentration. Ten cycles of passage were carried out and five replicate populations were

propagated. I also passaged *E. faecalis* in media without daptomycin in parallel to take into account effects of the media.

Over the course of the serial passage, I monitored the evolution of daptomycin resistance in the different populations (**Figure 6.1**). By the end of the 10 cycles of serial passage, all replicate populations were growing in 32X ancestral MIC of daptomycin.

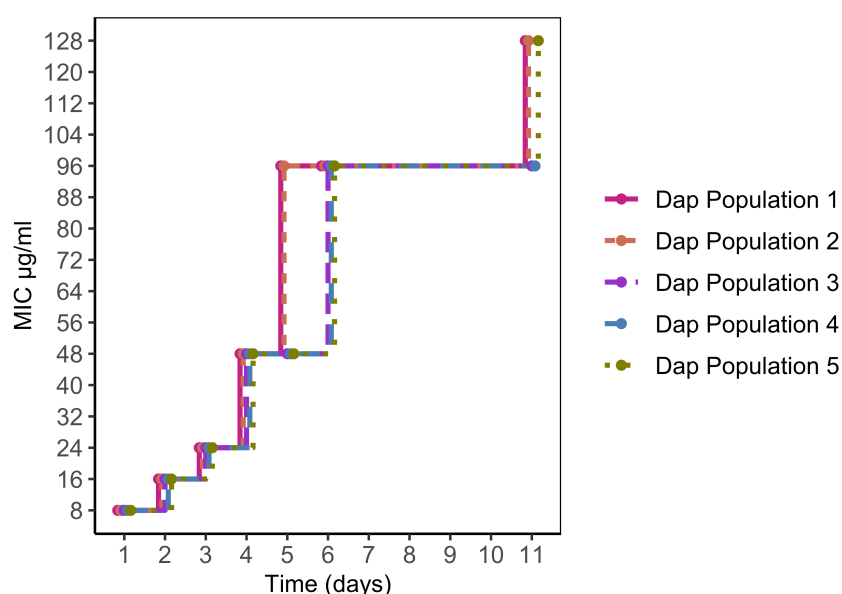


Figure 6.1 *in vitro* evolution of resistance in the daptomycin passaged strains

Progression of daptomycin MIC's through the course of selection, as assayed by the highest concentration of drug allowing visible growth.

Once serial passage was complete, I isolated three single colonies from each daptomycin-adapted population, grew them in BHI in the absence of daptomycin and performed MIC's to confirm that resistance was fixed. I observed that all serially passaged strains showed 16-fold increased daptomycin resistance compared to the ancestral strain.

6.4 Resistance to daptomycin can influence virulence during *Drosophila* infection

After generating daptomycin-adapted strains, I wanted to test whether resistance to daptomycin resulted in changes in bacterial virulence in *Drosophila*. Similar to my approach in Chapter 3, I initially performed experiments with three single colonies from each population to rule out the possibility that the populations were mixed. I observed that colonies from populations 1, 2 and 3 did not show any increase in virulence compared to the control strains (**Figure 6.2A-C**), whereas, colonies from populations 4 and 5 showed an increase in virulence (**Figure 6.2D and E**). The colonies from populations 1, 2, 3 and 5 behaved uniformly during *Drosophila* infection, whereas the colonies from population 4 showed heterogeneous increases in virulence, suggesting that this population may not be clonal. Nonetheless, due to lack of time, I decided to use only one single colony from each population for further analysis.

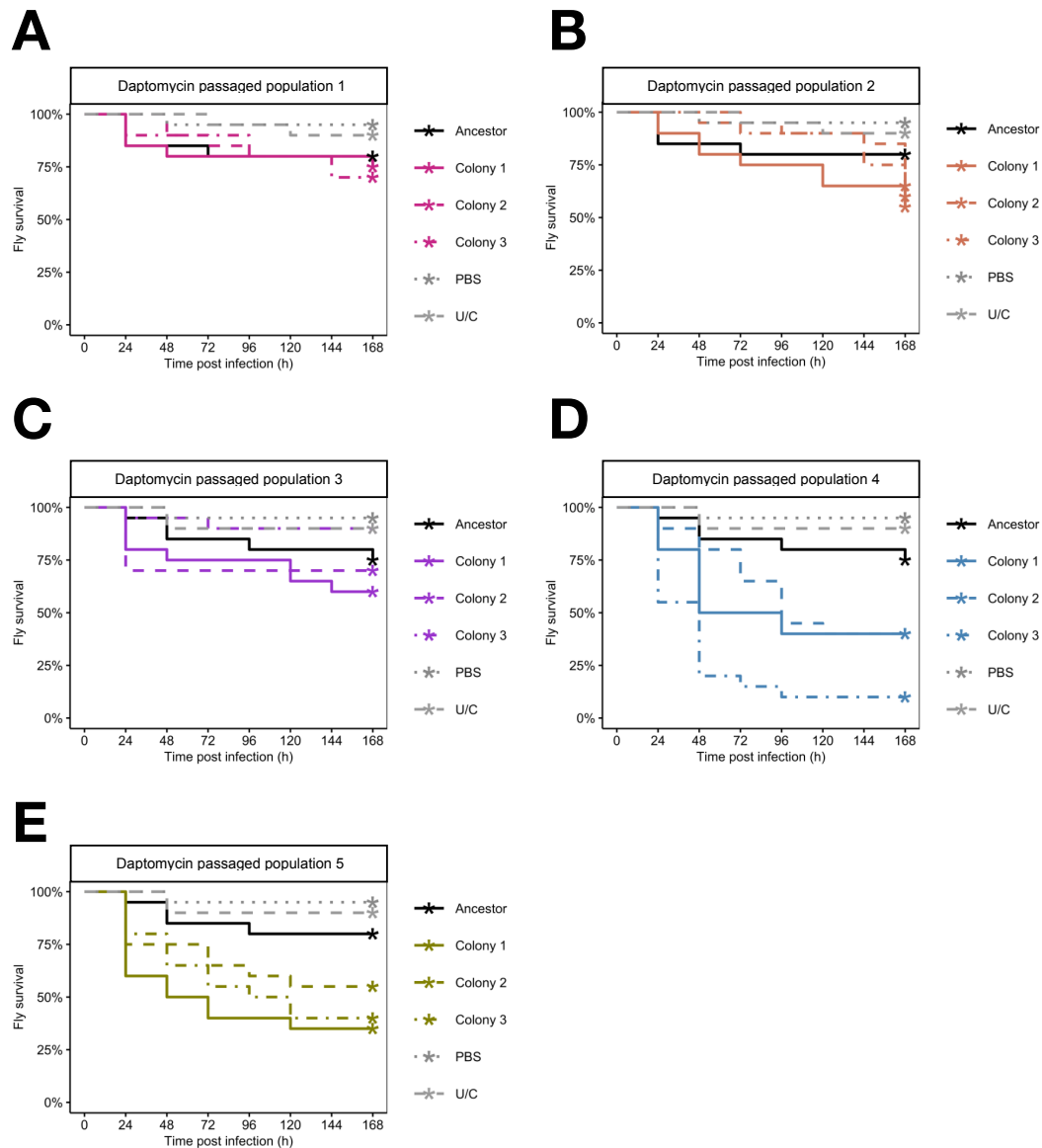


Figure 6.2 Some daptomycin-adapted populations show increased virulence in *Drosophila*

A-E: Survival of wild-type flies infected with ancestor and single colonies from different daptomycin-adapted populations. n = 20 per condition.

I repeated the survival experiments, this time using single representative colonies from each daptomycin-adapted population. These were colony 1 from population 1 (renamed Dap strain 1), colony 2 from population 2 (Dap strain 2), colony 1 from population 3 (Dap strain 3), colony 3 from population 4 (Dap strain 5) and colony 1 from population 5 (Dap strain 5). Similar to what I observed with the population data (**Figure 6.2**), Dap strain 4 and Dap strain 5 exhibited

greater virulence compared to the ancestral and other Dap strains (**Figure 6.3A and B**). This increase in mortality corresponded with increased proliferation of Dap strain 4, but no such increase was observed for Dap strain 5 (**Figure 6.3C**). I also infected flies with media-passaged bacteria (**Figure 6.3D**), but did not observe any significant changes in mortality of flies infected with these compared to flies infected with the ancestral strain.

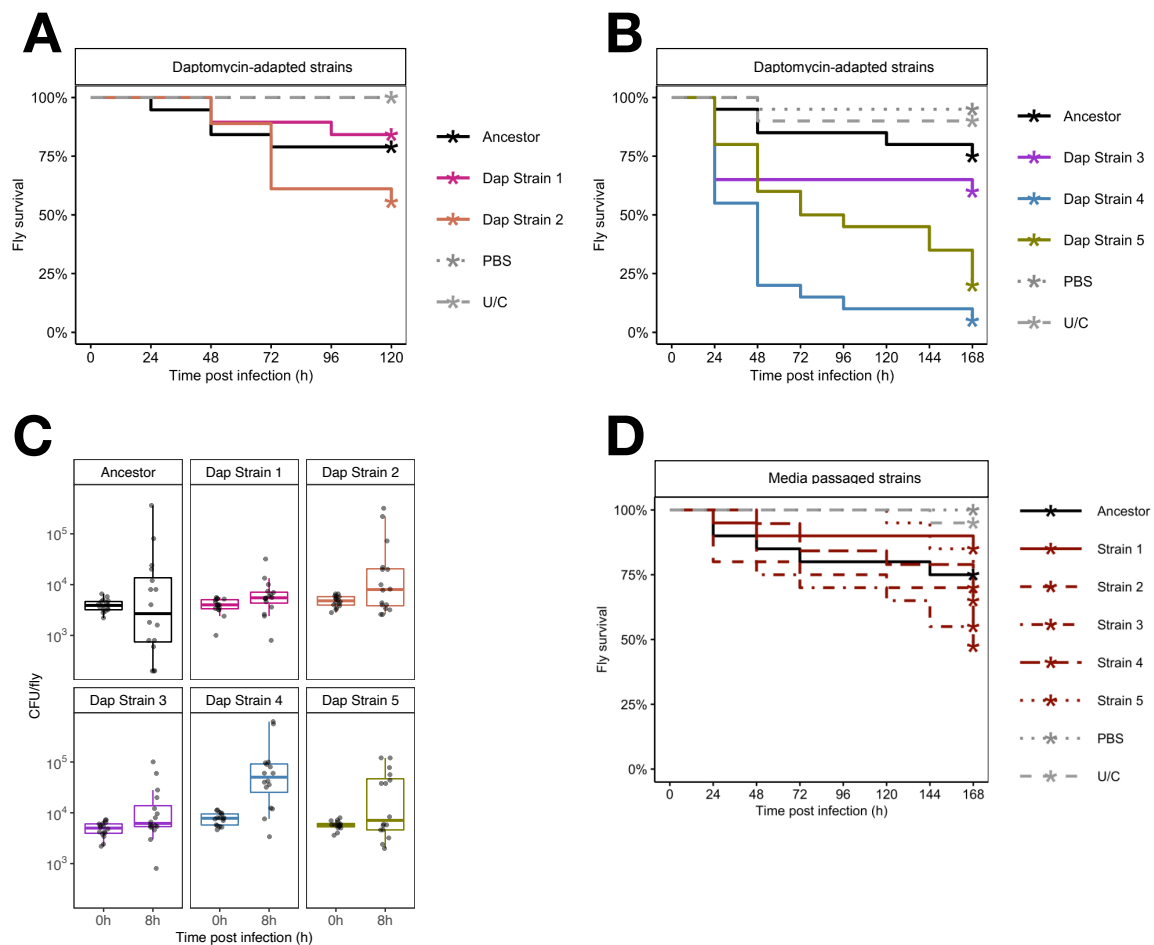


Figure 6.3 Some daptomycin-adapted strains show increased virulence in *Drosophila*

A, B: Survival of wild type flies infected with the ancestral and daptomycin-adapted strains. **C:** *E. faecalis* numbers at 0h (input inoculum) and 8h after infection of wild-type flies with the ancestral and daptomycin-adapted strains. **D:** Survival of wild-type flies infected with the ancestral and media passaged *E. faecalis* strains. For **A, B** and **D**, $n = 20$ per condition. For **C**, $n = 8$ per timepoint per condition, with data pooled from two experiments.

6.5 Identification of allelic variants in the daptomycin-adapted strains

Having identified that daptomycin resistance can result in altered virulence in *Drosophila*, I next sequenced the strains to identify any mutated genes. Bioinformatic analysis to identify variants and experiments to validate these variants were performed as described in **Chapter 5 (Table 5.2)**. I identified that all strains had acquired mutations in genes previously known to be important for daptomycin resistance in Enterococci (*yxdJ*, *bceB* and *liaY*) (Belley et al., 2018; Humphries et al., 2012; Miller et al., 2019; Prater et al., 2019). Dap strain 1 had acquired mutations in three genes: *bceB*, *ygaE* and EF0797. *BceB* encodes the ABC transporter permease that is involved in the activation of the YxdJK TCS; the functions of YgaE and the protein product of EF0797, a DUF domain containing protein, are unknown (Gebhard et al., 2014). Dap strain 2 had acquired two mutations. One of its mutations was also in *bceB* while the second mutation was in a gene encoding a methionyl-tRNA formyltransferase. Dap strain 3 had acquired mutations in two genes: *liaY* and *croS*. *LiaY* encodes for a membrane protein of unknown function and its expression is regulated by *liaFSR* (Khan et al., 2019). Dap strain 4 had mutations in *yxdJ* and *ygaE*. Finally, Dap strain 5 had mutations in *yxdJ*, *bceB*, and *croS*. The mutations in *bceB* and *croS* in this strain were identical with those identified in Dap strains 2 and 3 respectively. It is important to point out that as I did not have time to sequence the media-passaged strain it is not possible to rule out that some of these mutations could be due to growth in media

Table 6.1 Variants identified in the daptomycin-adapted strains

Strain	Background	Gene affected	Gene identifier	Mutation type	Gene function
Dap strain 1	PC2	<i>DUF4097</i>	EF0797	T4fs	Encodes a DUF-domain containing protein
		<i>ygaE</i>	EF1910	M26I	Uncharacterised membrane protein
		<i>bceB</i>	EF2751	M24V	ABC transporter permease involved in signal relay to the yxdJ/yxdK two-component system
Dap strain 2	PC2	<i>fnt</i>	EF3123	G39V	Methionyl-tRNA Formyltransferase
		<i>bceB</i>	EF2751	G112D	ABC transporter permease involved in signal relay to the yxdJ/yxdK two-component system
Dap strain 3	PC2	<i>croS</i>	EF3290	A206S	Member of the croR/croS two-component system that regulates genes involved in autolysis and cell wall metabolism
		<i>liaY</i>	EF1752	I99fs	Part of the liaFSR regulon and important for resistance to daptomycin
Dap strain 4	PC2	<i>ygaE</i>	EF1910	E150*	Uncharacterised membrane protein
		<i>croS</i>	EF3290	A206S	Member of the croR/croS two-component system that regulates genes involved in autolysis and cell wall metabolism

		<i>yxdJ</i>	EF0927	W126C	Sensor kinase of the <i>yxdJ/yxdK</i> two-component system that co-ordinates cell envelope response to bacitracin
Dap strain 5	PC2	<i>bceB</i>	EF2751	G112D	ABC transporter permease involved in signal relay to the <i>yxdJ/yxdK</i> two-component system
		<i>yxdJ</i>	EF0927	W126C	Sensor kinase of the <i>yxdJ/yxdK</i> two-component system that co-ordinates cell envelope response to bacitracin

All identified mutations within transcribed regions are shown. Gene names, where not previously used in *E. faecalis*, are derived from homologues in other bacteria. Amino acid locations are relative to the predicted protein in the ancestral strain. “*” indicates an introduced stop codon, “fs” indicates a frameshift in the coding regions of affected genes.

6.6 Discussion

6.6.1 Mechanisms of *E. faecalis* resistance to daptomycin

I generated *E. faecalis* strains that were resistant to the action of the surface-targeting antimicrobial daptomycin. All the daptomycin-adapted strains acquired mutations in at least one gene that was previously associated with daptomycin resistance. This included mutations in the *yxdJK*-mediated cell envelope response system, and in *liaY*. However, these strains also acquired additional mutations in genes that have not previously been associated with daptomycin resistance, such as the *croRS* two-component system. As it is unclear whether some of these mutations are due to selection in media, I will need to confirm the contributions of the different mutations toward the resistance phenotype. To do this, I can complement these genes in the different strains and quantify daptomycin resistance.

It has previously been described that the path to daptomycin resistance is highly constrained and mutations in the same genes have been identified multiple times in different studies (Arias et al., 2011; Miller et al., 2013). My results are in agreement with this: I observed a high degree of genetic parallelism across the daptomycin-adapted strains, at the level of common alleles, common mutated genes, and common pathways (**Table 6.1**). Work in the Arias & Shamoo labs has described that mutations in the *liaFSR* system are the preferred route for daptomycin resistance in *E. faecalis*, while mutations in *yxdJK* are secondary and occur when the *liaFSR* system is disrupted. (Arias et al., 2011; Miller et al., 2013). However, my results suggest a greater contribution of the *yxdJK*-mediated cell envelope stress response for daptomycin resistance in comparison to the *liaFSR* system. I observed that, except for Dap strain 3, all other strains had acquired mutations either in *yxdJK* or in genes involved in its regulation (*bceB*) and that Dap strain 3 was the only strain that had acquired a mutation in a gene under the control of the *liaFSR* system, *liaY*. However, there were a few differences between the set-up of my experiments and those performed in the Arias & Shamoo labs. For example, my

daptomycin-adapted strains were generated using serial transfer, whereas they used a bioreactor for this purpose. Prater *et al.* have previously identified that in these two environments, *E. faecium* has different evolutionary trajectories for daptomycin resistance, and acquires mutations in different genes (Prater et al., 2019). The founder (ancestral) strain I use for my experiments is also different from the one used in these studies; strain background has also been identified to influence the genotypic evolution of antimicrobial resistance (Card et al., 2021). As a result, there is a possibility that the difference between my results and studies performed by the Arias & Shamoo labs could be due to these differences in experimental conditions.

6.6.2 Daptomycin resistance can influence virulence in *Drosophila*

Two of the daptomycin-adapted strains exhibited increased virulence compared to the ancestral strain during *Drosophila* infection (**Figure 6.3A and B**). This increase in virulence could be attributed to the ability of Dap strain 4 to proliferate to greater numbers in the fly, 8-hours post-infection, but no such increase in proliferation was observed for Dap strain 5 (**Figure 6.3C**). However, the median survival time of flies infected with Dap strain 5 is between 48-72 hours post-infection, whereas, for Dap strain 4, it is 24-48 hours post-infection. As a result, there is a possibility that if bacterial loads were quantified at a later time point during infection I would observe greater bacterial numbers in flies infected with Dap strain 5 compared to those infected with the control strain. Alternatively, I can also perform bacterial load upon death (BLUD) assays for flies infected with Dap strain 5. In this assay, flies are collected and bacterial load quantified within 30 minutes of death, potentially clarifying links between bacterial numbers and overall virulence (Duneau et al., 2017).

Another interesting observation was that infection with Dap strains 1 and 3 no longer resulted in a bimodal distribution of bacteria 8-hours post-infection. This phenotype was especially striking in Dap strain 1 (**Figure 6.3C**). It will be interesting to measure bacterial numbers at a later timepoint in infection to identify if flies infected with these strains continue to display a similar bacterial load or if the strains are cleared. If these bacteria do persist in similar numbers or show small increases over the course of the experiment, it would suggest that these strains have become more tolerant to the action of the *Drosophila* immune response. As all the daptomycin-adapted strains have acquired at least one mutation in a gene involved in the cell envelope stress response system, this is not unlikely.

Experiments in this chapter have mainly focused on identifying whether resistance to daptomycin can also lead to a cross-resistance to the *Drosophila* immune response. However, I previously observed that *Drosophila* adaptation did not always result in an increase in resistance to daptomycin or other surface targeting antimicrobials (**Figure 5.9**). As result, it is important to consider the possibility that resistance to daptomycin could also result in increased sensitivity to the *Drosophila* immune response. To test this, survival experiments can be performed using a high inoculum and differences in killing between the ancestral and daptomycin-adapted strains can be quantified.

6.6.3 *E. faecalis* uses common genetic mechanisms to adapt in response to daptomycin and the *Drosophila* immune response

Sequencing of the daptomycin-adapted strains revealed that all strains had acquired mutations that affected either the same gene or genes that were part of the same regulatory network as those identified in the *Drosophila*-adapted strains (**Table 5.2** and **6.1**). These results confirm that *E. faecalis* makes use of common genetic mechanisms to resist the action of the cell surface targeting antimicrobial daptomycin and the *Drosophila* immune response.

While I have only focused on the mechanisms of *E. faecalis* resistance to daptomycin, the same cell envelope stress response systems are activated in response to *E. faecalis* interaction with other surface targeting antimicrobials such as bacitracin and vancomycin. As a result, I would expect similar genetic outcomes in *E. faecalis* after selection with these antibiotics. Whether these interactions would effect virulence in *Drosophila* remains to be tested. I also found that those strains with mutations in *yxdJ* exhibited the greatest virulence in *Drosophila*. However, as all strains had more than one mutation, more experiments will need to be performed to confirm the role of *yxdJ* in this context. It will also be interesting to characterize these strains further to identify if these mutations result in similar changes to bacterial properties as those observed in the *Drosophila*-adapted strains.

Chapter 7: Discussion

7.1 Background and aim of the project

E. faecalis is a generalist bacterium with a broad host range. It is also an important nosocomial pathogen. It has often been suggested that the properties which make *E. faecalis* such a successful pathogen have been selected for evolutionarily by the different environments in which it resides and the immune responses of the different host species with which it interacts. Insects constitute a natural host of this bacterium, yet little is known about how the insect immune response exerts its action during *E. faecalis* infection. Studies using the model organism *Drosophila melanogaster* have found that a specific family of effector peptides, the Bomanins, is key for the immune response to *E. faecalis*. However, the Bomanins' mechanism of action, and the consequences of this action on the biology of *E. faecalis*, are unclear.

In this project, I sought to further explore the interaction between the insect immune response and *E. faecalis*. To do this, I performed experimental evolution of *E. faecalis* by serial passage in *Drosophila melanogaster* to generate *E. faecalis* strains that were resistant to the action of *Drosophila* immunity. I then characterized these strains to identify the genetic and phenotypic changes that enabled them to become resistant to this immune response. These genetic and phenotypic changes have provided insights into the consequences of the interaction between *E. faecalis* and non-mammalian hosts.

7.2 Summary of the work presented in this thesis

In **Chapter 3**, I designed and optimized a protocol to experimentally evolve *E. faecalis* against the systemic immune response of *Drosophila melanogaster*. Evolution was performed on three occasions consisting of 3-10 parallel replicates. This produced several populations of *Drosophila*-adapted bacteria. Strains were derived from single colonies from these populations and characterized. The *Drosophila*-adapted strains exhibited a significant increase in growth over the first eight hours of infection compared to the ancestral strain. They also exhibited increased virulence, leading to significantly greater host mortality. This evolved resistance was observed was not specific to lab-adapted flies: increased bacterial proliferation and virulence were also observed in a recently wild-derived, outbred population of flies.

In **Chapter 4**, I set out to identify the host factors involved in the selection of this immune response resistance—i.e., host factors contributing to the *Drosophila* immune response during *E. faecalis* infection. To do this, I tested the virulence of the *Drosophila*-adapted strains in flies that were deficient for different components of the immune response. These experiments indicated that the Toll-induced Bomanin peptides played a critical role in the immune response to *E. faecalis* and were the primary selective pressure to which these strains had evolved resistance. The *Drosophila*-adapted strains did not exhibit reduced Toll pathway activation, suggesting that the evolved resistance in these strains was not evasion of detection by the Toll pathway, and thus must be evasion of killing by the Bomanin peptides themselves. I also identified that the melanization cascade was not involved in the immune response to *E. faecalis*, but the Imd pathway contributed to it. However, this contribution was not the result of Imd-mediated antimicrobial peptide production as flies lacking all known antimicrobial peptides showed similar survival outcomes to wild-type flies.

To identify the mechanisms of the interaction between the *Drosophila* immune response and *E. faecalis*, I genetically and phenotypically characterised the ancestral and *Drosophila*-adapted strains in **Chapter 5**. I found that immune resistance in *E. faecalis* could be conferred by a variety of single mutations. Many of the affected genes encoded proteins that regulate bacterial surface properties, including genes known to be important for *E. faecalis* resistance to surface-targeting antimicrobials. As a result of these mutations, most strains displayed phenotypic changes that have been associated with altered resistance to surface targeting antimicrobials such as: increased activation of one or more cell envelope stress response systems, a less negatively-charged cell surface, and an increase in membrane fluidity. Resistance to the *Drosophila* immune response also resulted in greater resistance to killing by human blood and surface-targeting antimicrobials, including vancomycin and daptomycin.

Based on the results from **Chapter 5**, we hypothesized that there were mechanistic similarities between the *Drosophila* immune response and surface targeting antimicrobials in their killing of *E. faecalis*. In **Chapter 6**, I tested this hypothesis. I first generated five populations of daptomycin-adapted *E. faecalis* through *in vitro* experimental evolution. Single colonies isolated from these populations exhibited a 16X increase in daptomycin resistance compared to the control strain. I then infected *Drosophila* with these strains and found that two of these strains showed increased virulence. Sequencing of these strains revealed that all strains had acquired at least one mutation in a gene or system that were also identified in the *Drosophila*-adapted strains (**Table 7.1**) These results confirm our hypothesis as common genetic mechanisms underlie resistance of *E. faecalis* to the action of daptomycin and the *Drosophila* immune response.

Table 7.1 Mutations acquired between experimental evolution experiments

Gene	<i>Drosophila</i> -adapted strain						Daptomycin-adapted strain					System
	A	B	C	D	E	F	1	2	3	4	5	
<i>yxdM</i> EF2049	Q402*											<i>yxdJKLM</i>
<i>yxdJ</i> EF0927										W126C	A136D	
<i>bceB</i> EF2751							M12V	G112D			G112D	
<i>liaF</i> EF2913			S217I									<i>liaFSR</i>
<i>liaY</i> EF1752									I99fs			
<i>croS</i> EF3290					K19E				A206S	A206S		<i>croRS</i>
<i>dcuR</i> EF1210	N175fs											Other
<i>epaQ</i> EF2178	IS6770											
<i>nucF</i> EF1188		A97T										
<i>mprF2</i> EF1027				IS6770								
<i>bgsA</i> EF2891					G227E							
<i>yihY</i> EF2199						F180L						
EF0797							T4fs					
<i>yqjA</i> EF1910							M267I			E150*		
<i>fnt</i> EF3123								G39V				

7.3 Discussion

7.3.1 What is the nature of the stress experiences by *E. faecalis* during *Drosophila* infection?

Insect immune systems are complex, typically deploying a variety of semi redundant effectors against any given infection, so that antimicrobial peptides must act in co-ordination with other host immune components to lead an effective response against pathogens (Lazzaro et al., 2020). Thus, it is assumed that the stress experienced by a pathogen during infection is not necessarily simple. However, our results suggest that this might not be the case, at least during *E. faecalis* infection. Through *in vivo* experimental evolution of *E. faecalis* in *Drosophila*, I generated bacterial strains that were resistant to the action of the intact immune response. The evolution of this resistance was rapid. I then showed that interaction with the Bomanin family of peptides was crucial for the development of this resistance. The ancestral strain and *Drosophila*-evolved strains showed similar proliferation and virulence upon infection of flies that were deficient in this family of peptides, indicating that in this background the *Drosophila*-adapted strains lost their selective advantage (**Figure 4.2**). Taken together, these results suggest that the primary stress experienced by *E. faecalis* during *Drosophila* infection is the action of the Bomanin peptides. If other components of the immune response were involved in the selection of immune resistance we would expect different outcomes. We would expect the strains to acquire multiple mutations to counter the different types of stresses. These mutations would be expected to target genes that influence different bacterial properties. However, I show that in many *Drosophila*-adapted strains, the evolution of this resistance was mediated through a single mutation and these mutations had similar phenotypic consequences (**Table 5.2**). Thus, supporting the idea that no other immune components are involved in the selection of immune resistance in these strains.

While I have established that the Bomanins are the primary stress experienced by *E. faecalis* during *Drosophila* infection, there is a possibility that secondary immune effectors or responses might also be involved. Hemocytes are a likely candidate here, as others have shown that flies deficient in hemocytes show increased susceptibility to *E. faecalis* infection (Defaye et al., 2009; Nehme et al., 2011). There is also the possibility that other antimicrobial peptides could be involved, as I have observed that flies lacking all characterized antimicrobial peptides are more susceptible to infection with the ancestral *E. faecalis* strain compared to wild type flies (**Figure 4.3B**). A third possibility is that secondary immune responses are not related to canonical immune genes or pathways—they might, for example, be mediated by restriction of nutrients over the course of infection. In order to identify the secondary responses that might be contributing to the immune response during *E. faecalis* infection, we could experimentally evolve *E. faecalis* in flies that lack the primary stress, e.g. *Bom*^{Δ55C} flies. These strains can then be characterized to identify the nature of the stress they have adapted in response to and the mechanisms underlying this adaptation.

7.3.2 How do the Bomanins kill *E. faecalis*?

The *Bomanins* encode short secreted peptides (Clemmons et al., 2015; Lindsay et al., 2018). However, little is known about how these peptides exert their action. This is in part due to the fact that, unlike antimicrobial peptides, synthetic Bomanins do not have any antimicrobial activity *in vitro*. One reason for this could be that Bomanins need to undergo some post translational modification to make them active. Antimicrobial peptides are common targets for post translational modifications and these modifications are known to have a direct effect on their activity (Brady et al., 2019; Bulet et al., 1993; Callaway et al., 1993; Hara et al., 1994; Yi et al., 2014). This would explain why synthetic Bomanins have no activity *in vitro*. The Bomanin family of peptides are characterized by a conserved CXXC motif (Clemmons et al., 2015). It

is believed that the two cysteine residues in this motif form a disulphide bridge. However, using predictive bioinformatic tools, I have found that, in short-form Bomanins, these cysteines are also predicted to be good targets for palmitoylation. The addition of acyl groups such as palmitate to short peptides has previously been shown to be important for stability during interaction with bacterial membranes (Greaves et al., 2009; Salaun et al., 2010). The absence of these groups can have a direct impact on the antimicrobial activity of some peptides, and depending on the lipid group added can also increase the positive charge on the peptides (Greaves et al., 2009; Grimsey et al., 2020; Krishnakumari & Nagaraj, 2015; Li et al., 2021; Mathew & Nagaraj, 2015). More experiments will be needed to be performed to confirm whether these peptides are post-translationally modified.

While *in vitro* characterization of the mechanism of Bomanins has not yet been possible, I have generated *E. faecalis* strains that appear to be specifically resistant to the action of the Bomanins. These strains can now be used to gain a better understanding of how the Bomanins exert their action. The *Drosophila*-adapted strains exhibited genetic and phenotypic overlap with strains resistant to the action of the surface targeting antimicrobial daptomycin. This suggests that the Bomanins exert their action using a mechanism similar to these surface-targeting antibiotics—i.e., by disrupting the bacterial cell surface. However, as these peptides do not kill bacteria *in vitro*, we do not have a good assay to measure their activity. An alternative approach to study how these peptides exert their action involves making use of an *in vivo* live imaging approach where we can visualize the interaction between fluorescently labelled-Bomanins and *E. faecalis*. However, the mature peptide length of most short-form Bomanins is 15 amino acids long. As a result, there is the possibility that addition of a fluorescent tag to these peptides might disrupt their activity. Instead, we can label Bombardier, the carrier protein for short-form Bomanins that is known to be important for their secretion in the hemolymph (Lin et al., 2019). Thus, to assay Bomanin killing of *E. faecalis* we propose to visualize the interaction between fluorescently labelled-Bomanins or Bombardier and *E.*

faecalis expressing GCaMP6. GCaMP is synthetic fluorescent protein that fluoresces green when bound to calcium (Nakai et al., 2001). When bacterial cells are intact, green fluorescence may be visible inside the bacterial cell. However, if the Bomanins kill *E. faecalis* by disrupting the bacterial membrane then as a result of influx of calcium due to this disruption, there would a corresponding increase in cytoplasmic GFP fluorescence. The concentration of Ca^{2+} in *Drosophila* hemolymph is approximately 500 μM , while it is present at much lower concentrations in bacterial cytosol (between 2 and 14 μM for *S. pneumoniae* intracellular and around 0.1 μM in *E. coli*). As a result, we expect the corresponding change in green fluorescence due to membrane disruption to be significant (Chapuy-Regaud et al., 2001; Dube et al., 2000; Gangola & Rosen, 1987).

7.3.3 Is a specific type of Bomanin important for the immune response to *E. faecalis*?

The *Bomanin* genes encode for three types of mature peptides: short-form, tailed, and bicipital. All are secreted into the hemolymph during an infection (Clemmons et al., 2015). Previously, it has been shown that a single short-form Bomanin is sufficient for a successful immune response during infection with the yeast *Candida glabrata* (Lindsay et al., 2018). However, it is unclear whether a specific type of Bomanin is important for the killing of *E. faecalis* or whether all three Bomanin types play an important role. To identify whether the *Drosophila* immune response to *E. faecalis* is dependent on a particular type of Bomanin, we could generate fly lines that lack specific types of Bomanins using a systematic gene knockout strategy along the lines used by Hanson *et al* (Hanson et al., 2019). Comparison of the outcome of *E. faecalis* infection of these flies to wild-type flies and flies lacking 10/12 of the Bomanin peptides (*Bom^{Δ55C}*) can help establish the role of these Bomanin types in this immune

response. However, if all three fly lines show a similar phenotype to *Bom*^{455C} flies after *E. faecalis* infection, it will indicate that more than one type of peptide is important.

7.3.4 What are the outcomes of the interaction between *E. faecalis* and the insect immune response?

We found that adaptation to the *Drosophila* immune response was mediated by the acquisition of mutations in genes that encoded for key bacterial surface properties. Some of these genes were characterized to be virulence factors and some others were known determinants for antimicrobial resistance. We also observed that the *Drosophila*-adapted strains exhibited increased resistance to killing by human blood, suggesting that adaptation to the insect immune response can result in a cross-resistance to components of mammalian immune responses (**Figure 5.7**). In fresh human blood *ex vivo*, most killing activity is attributed to neutrophils; it will be interesting to observe whether similar cross-resistance also exists for other components of the mammalian innate immune response, such as the cationic antimicrobial peptides LL-37 and human defensin β .

We also observed that adaptation to the *Drosophila* immune response resulted in changes in antimicrobial resistance of *E. faecalis* to cell surface targeting antimicrobials (**Figure 5.9**). All *Drosophila*-adapted strains showed increased resistance to vancomycin, and half of the strains showed increased resistance to daptomycin and the cationic antimicrobial peptides bacitracin, nisin and polymyxin-B. These results indicate that the *Drosophila* immune response and cell surface-targeting antimicrobials target the same conserved bacterial properties. Consistent with this idea, we observed that *E. faecalis* became resistant to daptomycin by acquiring mutations in the same genes or systems that were previously identified in the *Drosophila*-adapted strains (**Table 7.1**). As these two stresses have the same bacterial target and as resistance to the *Drosophila* immune response could select for antimicrobial

resistance, we hypothesized that resistance to daptomycin may also result in increased virulence in *E. faecalis* during host infection. Supporting this idea we identified that a few of the daptomycin-adapted strains exhibited increased virulence during *Drosophila* infection (Figure 6.3).

Taken together, these results highlight the significant overlap in the mechanisms used by *E. faecalis* to resist the action of the insect immune responses, human immune responses and cell surface targeting antimicrobials. As response to both the *Drosophila* immune response and daptomycin is mediated through cell envelope stress response systems, these results suggest that interactions with non-human hosts could have played an important role in the evolution of genes that critical for the intrinsic antimicrobial resistance in *E. faecalis*. Additionally, we observe that the same bacterial properties are important for resistance to the innate immune response and cell surface targeting antimicrobials. This link between antimicrobial resistance and virulence in *E. faecalis* might have played a critical role in establishing this bacterium as an important nosocomial pathogen, as antimicrobial treatment during host-infection could drive increases in virulence and action of the host-immune response could conversely select for antimicrobial resistance.

7.3.5 Is membrane fluidity the bacterial property important for resistance to the insect immune response?

Resistance to both the *Drosophila* immune response and the action of daptomycin appeared to be mediated by increased activity of the cell envelope stress response systems. In the *Drosophila*-adapted strains, this increase correlated with an increase in membrane fluidity. This suggests that membrane fluidity is the key bacterial property mediating resistance to the Bomanins. We could make use of the daptomycin-adapted strains to test whether this hypothesis is correct. Daptomycin resistance is commonly associated with increases or

decreases in membrane fluidity (Mishra et al., 2012; Nguyen et al., 2022). As only a few of the daptomycin-adapted strains we have generated exhibit increased virulence in flies, the strongest form of this prediction would be that daptomycin-selected strains exhibiting increased virulence exhibit increased membrane fluidity, while those with unchanged virulence should exhibit decreased or unchanged membrane fluidity.

7.3.6 Is the stress exerted by the Bomanins and daptomycin the same?

I observed that the action of the Bomanins and daptomycin resulted in mutations in the same genes or genes that were part of the same regulatory network (**Table 7.1**). This indicated that these two stresses targeted similar bacterial properties. However, this parallelism did not extend to the allele level i.e alleles that were specifically associated with resistance to the action of the Bomanins were not the same as those responsible for resistance to daptomycin and vice versa. This suggests that while the Bomanins and daptomycin might target similar bacterial properties, the alleles selected by these two stresses are specific. Supporting this idea, I observed that even when the same genes were found to carry mutations, the phenotypic outcomes differed. For example, Dap strains containing mutations in *croS* did not always exhibit increased virulence in *Drosophila* (**Figure 6.3**), while the *Drosophila*-adapted strain, Strain E which has a mutation in *croS*, does not exhibit high levels of daptomycin resistance (**Figure 5.9**).

7.3.7 Why is selection in the lab not always reflective of selection in the environment?

Our results suggest that the evolution of resistance of *E. faecalis* to the *Drosophila* immune response can be rapid and acquired through single mutations. Since it is clearly easy to

acquire this resistance, it seems surprising that this is not generally observed in nature. One potential reason for this is because the conditions experienced by *E. faecalis* in nature are different from those it experiences during a laboratory selection experiment. In the lab, *E. faecalis* is under sustained, specific pressure from the *Drosophila* immune response. However, in nature, *E. faecalis* is exposed to a broader range of stresses, such as high and low temperatures, nutrient stress, interaction with other hosts and competition with other bacteria. As a result, there is a possibility that while mutations might appear as a result of interaction with host immune responses, they are counter-selected by a different stress encountered in the environment.

A second possibility is that these mutations do appear and become fixed in a bacterial population. However, this results in the specialization of this population to a particular environment or host. This specialization further enables the acquisition of more host-specific mutations, resulting in the diversification of this bacterial population from *E. faecalis* such that it may no longer be considered to be part of this species. Such diversification is not uncommon in Enterococci. For example, *Enterococcus termitis* and *Enterococcus plantarum* which show close association with termites and plants, respectively, phylogenetically fall in the *Enterococcus faecalis* species group, but they are distinct from *E. faecalis* (Garcia-Solache & Rice, 2019; Svec et al., 2006; Svec et al., 2012). This suggests that these two species are the outcome of specialization to a particular environment from a common ancestor, i.e. *E. faecalis*.

Finally, there is also the possibility that these alleles do exist in nature but either are not identified or are considered to be the result of interactions with antimicrobials rather than interactions with host immune systems.

7.3.8 Why are the alleles associated with daptomycin resistance *in vitro* different from those associated with clinical resistance?

Similar to the alleles we identified in the *Drosophila*-adapted strains are not commonly found in other *E. faecalis* strains, the alleles we generated by *in vitro* adaptation of *E. faecalis* to daptomycin are not often identified in clinically-derived daptomycin resistant *E. faecalis* isolates. We observed that *in vitro* adaptation of *E. faecalis* to daptomycin was mediated by two key cell envelope stress response systems: YxdJK and CroRS. However, clinical resistance of *E. faecalis* is primarily associated with mutations in two distinct sets of genes: first, the *liaFSR* three-component system; second, *cls*, the gene encoding cardiolipin synthase (Arias et al., 2011; Palmer et al., 2011). It has been argued that discrepancies in mutations identified *in vitro* and *in vivo* were the result of differences in strain backgrounds, as different strains have different preferences for genes to be mutated. However, our results suggest that this difference could be because the stresses experienced by the bacterium during *in vitro* and *in vivo* treatment with daptomycin are different. During host infection, *E. faecalis* needs to adapt to the dual pressure exerted by the immune response and the antimicrobial. This is in contrast to *in vitro* evolution of resistance where the action of the antibiotic is the sole stress experienced by the bacterium and adaptation will be specific to this selection. Consistent with this idea, while most of the daptomycin-adapted strains generated by us show high-level resistance to daptomycin, few show a corresponding increase in virulence during *Drosophila* infection (**Figure 6.3**). On the other hand, mutations in the *liaFSR* system result in high-level resistance to both the innate immune response and daptomycin, which explains why mutations in this system are commonly associated with daptomycin resistance *in vivo* (Khan et al., 2019; Rincon et al., 2019). A second factor may be the fact that the concentration of daptomycin *E. faecalis* needs to adapt in response to, in order to be characterized as ‘resistant’ during *in vivo* infection, is significantly lower than what it is exposed to during *in vitro* evolution. As a result,

mutations in *liaFSR* are enough to result in *in vivo* resistance but other alleles are important to provide higher levels of resistance.

7.4 Concluding remarks and future questions

In this project, I have shown that there are similarities in the killing of *E. faecalis* by the *Drosophila* immune response and the antimicrobial daptomycin. Interaction with the *Drosophila* immune response can select for resistance to surface targeting antimicrobials and this resistance is likely due to interaction with the Bomanin family of peptides. Conversely, resistance to daptomycin can result in increased virulence during *Drosophila* infection. However, many questions remain, including:

- We see mechanistic similarities between the immune effectors and antimicrobials. Does this mean that immune interaction primes bacterial antimicrobial resistance during infection?
- Is immune-antimicrobial cross-resistance specific to *E. faecalis* or would it be observed in other generalist pathogens that interact with insects?
- We observed that interaction with the *Drosophila* immune response results in increased resistance to vancomycin. Does this mean that interaction with this host can also influence the maintenance and transmission of plasmids carrying vancomycin resistance?

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Appendix

Statistical data corresponding to Figure 3.5 (Bacterial quantifications)

Comparison	P value
Ancestor - EE2S1C1	1
Ancestor - EE2S1C2	0.007568
Ancestor - EE2S1C3	1
Ancestor - EE2S1C4	1
Ancestor - EE2S1C5	0.589485

Comparison	P value
Ancestor - EE2S2C1	4.59E-07
Ancestor - EE2S2C2	0.166823
Ancestor - EE2S2C3	0.022835
Ancestor - EE2S2C4	0.113052
Ancestor - EE2S2C5	0.001422

Comparison	P value
Ancestor - EE2S3C1	1
Ancestor - EE2S3C2	1
Ancestor - EE2S3C3	1
Ancestor - EE2S3C4	1
Ancestor - EE2S3C5	1

Comparison	P value
Ancestor - EE2S4C1	1
Ancestor - EE2S4C2	1
Ancestor - EE2S4C3	1
Ancestor - EE2S4C4	1
Ancestor - EE2S4C5	1

Comparison	P value
Ancestor - EE2S5C1	1
Ancestor - EE2S5C2	1
Ancestor - EE2S5C3	1
Ancestor - EE2S5C4	0.016572
Ancestor - EE2S5C5	0.7933

Comparison	P value
Ancestor - EE2S6C1	4.59E-07
Ancestor - EE2S6C2	0.166823
Ancestor - EE2S6C3	0.022835
Ancestor - EE2S6C4	0.113052
Ancestor - EE2S6C5	0.001422

Statistical data corresponding to Figure 3.5 (Survivals)

Comparison	P value
Ancestor - EE2S1C1	1
Ancestor - EE2S1C2	1
Ancestor - EE2S1C3	1
Ancestor - EE2S1C4	1
Ancestor - EE2S1C5	1
Ancestor - PBS	1
Ancestor - (U/C)	0.040293

Comparison	P value
Ancestor - EE2S2C1	0.000164
Ancestor - EE2S2C2	0.487
Ancestor - EE2S2C3	1
Ancestor - EE2S2C4	1
Ancestor - EE2S2C5	0.000203
Ancestor - PBS	0.00359

Ancestor - (U/C)	0.00443
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Comparison	P value
Ancestor - EE2S3C1	1
Ancestor - EE2S3C2	1
Ancestor - EE2S3C3	1
Ancestor - EE2S3C4	1
Ancestor - EE2S3C5	1
Ancestor - PBS	1
Ancestor - (U/C)	0.237469

Comparison	P value
Ancestor - EE2S4C1	1
Ancestor - EE2S4C2	1
Ancestor - EE2S4C3	1
Ancestor - EE2S4C4	0.0321
Ancestor - EE2S4C5	1
Ancestor - PBS	9.79E-06
Ancestor - (U/C)	4.49E-07

Comparison	P value
Ancestor - EE2S5C1	0.004016
Ancestor - EE2S5C2	0.287771
Ancestor - EE2S5C3	1
Ancestor - EE2S5C4	0.26388
Ancestor - EE2S5C5	1.6E-05
Ancestor - PBS	3.83E-06
Ancestor - (U/C)	3.86E-06

Comparison	P value
Ancestor - EE2S6C1	3.76E-06
Ancestor - EE2S6C2	6.19E-08
Ancestor - EE2S6C3	4.87E-08

Ancestor - EE2S6C4	1.73E-09
Ancestor - EE2S6C5	7.63E-11
Ancestor - PBS	1
Ancestor - (U/C)	1

Statistical data corresponding to Figure 3.6 (Survivals)

Comparison	P value
Ancestor - EE3S1C1	3.93E-05
Ancestor - EE3S1C2	8.33E-05
Ancestor - EE3S1C3	3.34E-07
Ancestor - PBS	1
Ancestor - (U/C)	1

Comparison	P value
Ancestor - EE3S2C1	2.07E-05
Ancestor - EE3S2C2	6.98E-05
Ancestor - EE3S2C3	0.958381
Ancestor - PBS	1
Ancestor - (U/C)	0.205592

Comparison	P value
Ancestor - EE3S3C1	8.25E-05
Ancestor - EE3S3C2	0.000605
Ancestor - EE3S3C3	6.96E-05
Ancestor - PBS	1
Ancestor - (U/C)	1

Comparison	P value
Ancestor - EE3S4C1	1
Ancestor - EE3S4C2	1
Ancestor - EE3S4C3	1
Ancestor - PBS	1

Ancestor - (U/C)	1
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Comparison	P value
Ancestor - EE3S5C1	9.65E-05
Ancestor - EE3S5C2	0.070433
Ancestor - EE3S5C3	0.506344
Ancestor - PBS	1
Ancestor - (U/C)	1

Comparison	P value
Ancestor - EE3S7C1	3.79E-08
Ancestor - EE3S7C2	3.88E-08
Ancestor - EE3S7C3	8.73E-08
Ancestor - PBS	1
Ancestor - (U/C)	0.022664

Comparison	P value
Ancestor - EE3S8C1	5.77E-06
Ancestor - EE3S8C2	3.33E-07
Ancestor - EE3S8C3	8.97E-07
Ancestor - PBS	1
Ancestor - (U/C)	0.078587

Comparison	P value
Ancestor - EE3S9C1	1.92E-06
Ancestor - EE3S9C2	2.28E-05
Ancestor - EE3S9C3	0.008723
Ancestor - PBS	1
Ancestor - (U/C)	0.087244

Due to small sample size (n = 8), no statistical analysis were performed on bacterial load quantifications between control strain and passaged strains at 8h.

Statistical data corresponding to Figure 3.7 (Survivals)

Comparison	P value
Ancestor - PC1	1
Ancestor - PC2	1
Ancestor - PC5	1
Ancestor - PC6	0.997615
Ancestor - PC7	0.062228
Ancestor - PBS	1
Ancestor - (U/C)	1

Statistical data corresponding to Figure 3.7 (Bacterial quantifications)

Comparison	P value
Ancestor - PC1	1
Ancestor - PC2	1
Ancestor - PC5	1
Ancestor - PC6	0.661815
Ancestor - PC7	1

Statistical data corresponding to Figure 3.8 (Bacterial quantification)

Comparison	P value
Ancestor - EE1S2C1	0.428004
Ancestor - EE2S1C2	0.000385
Ancestor - EE2S2C3	0.013022
Ancestor - EE2S6C5	0.009312

Comparison	P value
Ancestor - EE3S1C1	0.003913
Ancestor - EE3S3C1	0.03469
Ancestor - EE3S5C1	0.0175
Ancestor - EE3S6C1	0.04177
Ancestor - EE3S7C1	0.001846

Ancestor - EE3S8C1	0.000487
Ancestor - EE3S9C1	0.1044
Ancestor - EE3S10C1	0.1044

Statistical data corresponding to Figure 3.8 (Survivals)

Comparison	P value
Ancestor - EE1S2C1	1.01E-10
Ancestor - EE2S1C2	8.74E-09
Ancestor - EE2S2C3	1.17E-08
Ancestor - EE2S6C5	3.61E-09
Ancestor - PBS	1
Ancestor - (U/C)	1

Comparison	P value
Ancestor - EE3S1C1	2.09E-08
Ancestor - EE3S3C1	3.2E-06
Ancestor - EE3S5C1	1.56E-06
Ancestor - EE3S6C1	0.034393
Ancestor - EE3S7C1	8.45E-11
Ancestor - EE3S8C1	1.07E-05
Ancestor - EE3S9C1	3.5E-09
Ancestor - EE3S10C1	0.061057
Ancestor - PBS	1
Ancestor - (U/C)	0.063824

Statistical data corresponding to Figure 3.9 (Survivals)

Comparison	P value
Ancestor – Strain A	6.42E-13
Ancestor – Strain B	1.51E-09
Ancestor – Strain C	1.41E-12
Ancestor – Strain D	1.32E-13

Ancestor – Strain E	2.12E-13
Ancestor – Strain F	1.24E-10
Ancestor – PBS	0.958696
Ancestor – U/C	0.534193

Statistical data corresponding to Figure 4.1 (Survivals)

Comparison	P value
Ancestor - Strain A	1
Ancestor - Strain B	1
Ancestor - Strain C	1
Ancestor - Strain D	1
Ancestor - PBS	1.13796E-16
Ancestor - (U/C)	1

Comparison	P value
Ancestor - Strain E	1
Ancestor - Strain F	1
Ancestor - PBS	5.66359E-11
Ancestor - (U/C)	1.18114E-09

Statistical data corresponding to Figure 4.1 (Bacterial quantification)

Comparison (8h vs 8h)	P value
Ancestor - Strain A	1
Ancestor - Strain B	1
Ancestor - Strain C	1
Ancestor - Strain D	1
Ancestor - Strain E	0.001844
Ancestor - Strain F	0.09686

Statistical data corresponding to Figure 4.2 (Survivals)

Comparison	P value
Ancestor - Strain A	1
Ancestor - Strain B	1
Ancestor - Strain C	1
Ancestor - Strain D	1
Ancestor - PBS	3.18874E-12
Ancestor - (U/C)	1.13289E-08

Comparison	P value
Ancestor - Strain E	1
Ancestor - Strain F	1
Ancestor - PBS	2.29918E-13
Ancestor - (U/C)	1.12391E-06

Statistical data corresponding to Figure 4.2 (Bacterial quantifications)

Comparison (8h vs 8h)	P value
Ancestor - Strain A	1
Ancestor - Strain B	1
Ancestor - Strain C	1
Ancestor - Strain D	1
Ancestor - Strain E	0.91
Ancestor - Strain F	0.1162

Statistical data corresponding to Figure 4.3A

Genotype	Comparison	P value
<i>w1118</i>	Ancestor - Strain A	3.79E-08
<i>w1118</i>	Ancestor - Strain B	0.00215
<i>w1118</i>	Ancestor - Strain C	0.000231
<i>w1118</i>	Ancestor - Strain D	1.41E-06
<i>w1118</i>	Ancestor - Strain E	0.0491

<i>w1118</i>	Ancestor - Strain F	0.682
<i>w1118</i>	Ancestor - PBS	1
<i>w1118</i>	Ancestor - (U/C)	0.911
<i>w₁₁₁₈AMP</i>	Ancestor - Strain A	0.015
<i>w₁₁₁₈AMP</i>	Ancestor - Strain B	0.00079
<i>w₁₁₁₈AMP</i>	Ancestor - Strain C	2.64E-06
<i>w₁₁₁₈AMP</i>	Ancestor - Strain D	0.000177
<i>w₁₁₁₈AMP</i>	Ancestor - Strain E	0.000273
<i>w₁₁₁₈AMP</i>	Ancestor - Strain F	0.189
<i>w₁₁₁₈AMP</i>	Ancestor - PBS	0.00113
<i>w₁₁₁₈AMP</i>	Ancestor - (U/C)	0.0141

Statistical data corresponding to Figure 4.3B

Genotype	Comparison	P value
<i>w1118</i>	Ancestor - Strain A	0.000344
<i>w1118</i>	Ancestor - Strain B	0.055456
<i>w1118</i>	Ancestor - Strain C	6.81E-05
<i>w1118</i>	Ancestor - Strain D	0.040177
<i>w1118</i>	Ancestor - Strain E	0.017291
<i>w1118</i>	Ancestor - Strain F	1
<i>w1118</i>	Ancestor - PBS	1
<i>w1118</i>	Ancestor - (U/C)	0.974841
<i>Drs</i>	Ancestor - Strain A	0.000344
<i>Drs</i>	Ancestor - Strain B	0.055456
<i>Drs</i>	Ancestor - Strain C	6.81E-05
<i>Drs</i>	Ancestor - Strain D	0.040177
<i>Drs</i>	Ancestor - Strain E	0.017291
<i>Drs</i>	Ancestor - Strain F	1
<i>Drs</i>	Ancestor - PBS	1
<i>Drs</i>	Ancestor - (U/C)	0.974841

Statistical data corresponding to Figure 4.4

Genotype	Comparison	P value
c564/+	Ancestor - Strain A	5.7E-19
c564/+	Ancestor - Strain B	4.58E-05
c564/+	Ancestor - Strain C	7.62E-25
c564/+	Ancestor - Strain D	1.2E-29
c564/+	Ancestor - PBS	0.177926
c564/+	Ancestor - (U/C)	0.133997
c564>UAS-Toll10B	Ancestor - Strain A	5.7E-19
c564>UAS-Toll10B	Ancestor - Strain B	4.58E-05
c564>UAS-Toll10B	Ancestor - Strain C	7.62E-25
c564>UAS-Toll10B	Ancestor - Strain D	1.2E-29
c564>UAS-Toll10B	Ancestor - PBS	0.177926
c564>UAS-Toll10B	Ancestor - (U/C)	0.133997
UAS-Toll10B/+	Ancestor - Strain A	5.7E-19
UAS-Toll10B/+	Ancestor - Strain B	4.58E-05
UAS-Toll10B/+	Ancestor - Strain C	7.62E-25
UAS-Toll10B/+	Ancestor - Strain D	1.2E-29
UAS-Toll10B/+	Ancestor - PBS	0.177926
UAS-Toll10B/+	Ancestor - (U/C)	0.133997

Statistical data corresponding to Figure 4.5

<i>BomS2</i>			
3h			
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.3823	Ancestor vs Strain E	0.8785
Ancestor vs Strain B	0.1304	Ancestor vs Strain F	0.5726
Ancestor vs Strain C	0.1949	Ancestor vs PBS	0.000666
Ancestor vs Strain D	0.5054	Ancestor vs U/C	0.000155
Ancestor vs PBS	0.1949		
Ancestor vs U/C	0.000311		
6h			

Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.8785	Ancestor vs Strain E	0.7209
Ancestor vs Strain B	0.3282	Ancestor vs Strain F	0.5726
Ancestor vs Strain C	0.4418	Ancestor vs PBS	0.000666
Ancestor vs Strain D	0.1304	Ancestor vs U/C	0.000155
Ancestor vs PBS	0.02067		
Ancestor vs U/C	0.002953		

<i>Dro</i>			
3h			
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.1304	Ancestor vs Strain E	0.7209
Ancestor vs Strain B	0.7209	Ancestor vs Strain F	0.5726
Ancestor vs Strain C	0.01476	Ancestor vs PBS	0.4908
Ancestor vs Strain D	0.7984	Ancestor vs U/C	0.01041
Ancestor vs PBS	0.2786		
Ancestor vs U/C	0.000155		
6h			
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.4418	Ancestor vs Strain E	0.01041
Ancestor vs Strain B	0.2786	Ancestor vs Strain F	0.2031
Ancestor vs Strain C	0.03792	Ancestor vs PBS	0.004662
Ancestor vs Strain D	0.2345	Ancestor vs U/C	0.000155
Ancestor vs PBS	0.7209		
Ancestor vs U/C	0.000155		

<i>Drs</i>			
3h			
Comparison	P value	Comparison	P value
Ancestor vs Strain A	1	Ancestor vs Strain E	0.4418
Ancestor vs Strain B	1	Ancestor vs Strain F	0.2031
Ancestor vs Strain C	0.7209	Ancestor vs PBS	0.0293

Ancestor vs Strain D	1	Ancestor vs U/C	0.000155
Ancestor vs PBS	0.02067		
Ancestor vs U/C	0.000155		
6h			
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.5054	Ancestor vs Strain E	0.08298
Ancestor vs Strain B	0.004662	Ancestor vs Strain F	0.02052
Ancestor vs Strain C	0.06496	Ancestor vs PBS	0.000666
Ancestor vs Strain D	0.04988	Ancestor vs U/C	0.000155
Ancestor vs PBS	1		
Ancestor vs U/C	0.000155		

<i>Mtk</i>			
3h			
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.7055	Ancestor vs Strain E	0.2911
Ancestor vs Strain B	0.02206	Ancestor vs Strain F	0.01435
Ancestor vs Strain C	0.8636	Ancestor vs PBS	0.1647
Ancestor vs Strain D	0.7083	Ancestor vs U/C	5.29E-06
Ancestor vs PBS	0.1184		
Ancestor vs U/C	8.34E-05		
6h			
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.2443	Ancestor vs Strain E	0.152
Ancestor vs Strain B	0.09968	Ancestor vs Strain F	0.6126
Ancestor vs Strain C	0.07978	Ancestor vs PBS	0.04861
Ancestor vs Strain D	0.1887	Ancestor vs U/C	0.000163
Ancestor vs PBS	0.8218		
Ancestor vs U/C	0.000336		

Statistical data corresponding to Figure 4.6 (Survivals)

Comparison	P value
Ancestor - Strain A	1
Ancestor - Strain B	0.006355
Ancestor - Strain C	1.11E-05
Ancestor - Strain D	1.75E-07
Ancestor - PBS	1
Ancestor - (U/C)	0.147065

Statistical data corresponding to Figure 4.6 (Bacterial Quantifications)

Comparison	P value
Ancestor - Strain A	1
Ancestor - Strain B	0.033397
Ancestor - Strain C	1.51E-05
Ancestor - Strain D	0.004168

Statistical data corresponding to Figure 4.7A

Genotype	Comparison	P value
<i>w1118</i>	Ancestor - Strain A	6.1E-06
<i>w1118</i>	Ancestor - Strain B	0.00162
<i>w1118</i>	Ancestor - Strain C	0.001062
<i>w1118</i>	Ancestor - Strain D	5.77E-07
<i>w1118</i>	Ancestor - PBS	1
<i>w1118</i>	Ancestor - (U/C)	1
<i>Key</i>	Ancestor - Strain A	1
<i>Key</i>	Ancestor - Strain B	0.036835
<i>Key</i>	Ancestor - Strain C	0.237915
<i>Key</i>	Ancestor - Strain D	0.010284
<i>Key</i>	Ancestor - PBS	0.000136
<i>Key</i>	Ancestor - (U/C)	0.000193

Statistical data corresponding to Figure 4.7B

Genotype	Comparison	P value
Control	Ancestor - Strain A	1
Control	Ancestor - Strain B	0.568695
Control	Ancestor - Strain C	1
Control	Ancestor - Strain D	1
Control	Ancestor - PBS	4.24E-07
Control	Ancestor - (U/C)	1
<i>Tak1</i>	Ancestor - Strain A	1
<i>Tak1</i>	Ancestor - Strain B	0.568695
<i>Tak1</i>	Ancestor - Strain C	1
<i>Tak1</i>	Ancestor - Strain D	1
<i>Tak</i>	Ancestor - PBS	4.24E-07
<i>Tak1</i>	Ancestor - (U/C)	1

Statistical data corresponding to Figure 4.9A

Genotype	Comparison	P value
<i>w1118</i>	Ancestor - Strain A	0.000645
<i>w1118</i>	Ancestor - Strain B	0.004437
<i>w1118</i>	Ancestor - Strain C	0.05353
<i>w1118</i>	Ancestor - Strain D	0.00301
<i>w1118</i>	Ancestor - PBS	0.140647
<i>w1118</i>	Ancestor - (U/C)	0.070884
<i>PPO1Δ,PPO2Δ</i>	Ancestor - Strain A	1
<i>PPO1Δ,PPO2Δ</i>	Ancestor - Strain B	1
<i>PPO1Δ,PPO2Δ</i>	Ancestor - Strain C	1
<i>PPO1Δ,PPO2Δ</i>	Ancestor - Strain D	1
<i>PPO1Δ,PPO2Δ</i>	Ancestor - PBS	0.434171
<i>PPO1Δ,PPO2Δ</i>	Ancestor - (U/C)	0.010259

Statistical data corresponding to Figure 4.11B

Ancestor	P value
24h - 3h	0.001568
24h - Transfer	1.41E-08
3h - Transfer	0.05077
Strain A	P value
24h - 3h	0.174211
24h - Transfer	1.53E-06
3h - Transfer	0.005298
Strain B	P value
24h - 3h	0.705538
24h - Transfer	0.085328
3h - Transfer	0.002188
Strain C	P value
24h - 3h	0.144208
24h - Transfer	5.72E-05
3h - Transfer	0.064541

Strain D	P value
24h - 3h	0.008953
24h - Transfer	4.2E-08
3h - Transfer	0.020554
Strain E	P value
24h - 3h	1.21E-05
24h - Transfer	4.63E-06
3h - Transfer	1
Comparison	P value
24h - 3h	0.008972
24h - Transfer	1.23E-08
3h - Transfer	0.010779

Statistical data corresponding to Figure 5.1

Comparison	P value
Strain A - epaQ	8.46E-09
Strain A - yxdM	2.05E-09
Strain A - malR	1.27E-01
Strain A - PBS	1.14E-09
Strain A - (U/C)	3.47E-08

Statistical data corresponding to Figure 5.2A

Comparisons	P value
Ancestor - Strain E	0.003063
Ancestor - Strain G	0.006466
Ancestor - PBS	1
Ancestor - (U/C)	1

Statistical data corresponding to Figure 5.2B

Comparison (8h vs 8h)	P value
Ancestor - Strain E	0.020544
Ancestor - Strain G	0.109507
Comparison (0h vs 8h)	P value
Ancestor - Ancestor	0.4169
Strain E - Strain E	0.0008339
Strain G - Strain G	0.0006883

Statistical data corresponding to Figure 5.3A

<i>yx_dM</i>			
Stationary phase		Exponential phase	
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.3282	Ancestor vs Strain A	0.9591
Ancestor vs Strain B	0.5737	Ancestor vs Strain B	0.1949
Ancestor vs Strain C	0.04988	Ancestor vs Strain C	0.4418
Ancestor vs Strain D	0.9591	Ancestor vs Strain D	0.03792
Ancestor vs Strain E	0.2345	Ancestor vs Strain E	0.1304
Ancestor vs Strain F	0.02813	Ancestor vs Strain F	0.7984

Statistical data corresponding to Figure 5.3B

<i>yxdJ</i>			
Stationary phase		Exponential phase	
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.1949	Ancestor vs Strain A	0.1949
Ancestor vs Strain B	0.8785	Ancestor vs Strain B	0.2345
Ancestor vs Strain C	0.0001554	Ancestor vs Strain C	0.02067
Ancestor vs Strain D	0.5737	Ancestor vs Strain D	0.7209
Ancestor vs Strain E	0.7984	Ancestor vs Strain E	0.3823
Ancestor vs Strain F	0.004662	Ancestor vs Strain F	0.5054

Statistical data corresponding to Figure 5.3C

<i>liaF</i>			
Stationary phase		Exponential phase	
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.04988	Ancestor vs Strain A	1
Ancestor vs Strain B	0.1605	Ancestor vs Strain B	0.8785
Ancestor vs Strain C	0.0001554	Ancestor vs Strain C	0.0003108
Ancestor vs Strain D	0.04988	Ancestor vs Strain D	0.08298
Ancestor vs Strain E	0.7209	Ancestor vs Strain E	0.7209
Ancestor vs Strain F	0.0001554	Ancestor vs Strain F	0.1304

Statistical data corresponding to Figure 5.3D

<i>liaS</i>			
Stationary phase		Exponential phase	
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.4418	Ancestor vs Strain A	0.8785
Ancestor vs Strain B	0.9591	Ancestor vs Strain B	0.7984
Ancestor vs Strain C	0.0001554	Ancestor vs Strain C	0.0001554
Ancestor vs Strain D	0.1949	Ancestor vs Strain D	0.03792
Ancestor vs Strain E	0.4418	Ancestor vs Strain E	0.4418
Ancestor vs Strain F	0.0006216	Ancestor vs Strain F	0.08298

Statistical data corresponding to Figure 5.3E

<i>dltB</i>			
Stationary phase		Exponential phase	
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.3282	Ancestor vs Strain A	0.5054
Ancestor vs Strain B	0.6454	Ancestor vs Strain B	0.02813
Ancestor vs Strain C	0.7209	Ancestor vs Strain C	0.002953
Ancestor vs Strain D	0.5737	Ancestor vs Strain D	0.02813
Ancestor vs Strain E	0.04988	Ancestor vs Strain E	0.06496
Ancestor vs Strain F	0.3823	Ancestor vs Strain F	0.1949

Statistical data corresponding to Figure 5.3F

<i>croS</i>			
Stationary phase		Exponential phase	
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.0006216	Ancestor vs Strain A	0.7984
Ancestor vs Strain B	0.8785	Ancestor vs Strain B	0.1949
Ancestor vs Strain C	0.06496	Ancestor vs Strain C	0.2345
Ancestor vs Strain D	0.006993	Ancestor vs Strain D	0.2786
Ancestor vs Strain E	0.0001554	Ancestor vs Strain E	0.001865
Ancestor vs Strain F	0.001865	Ancestor vs Strain F	0.5737

Statistical data corresponding to Figure 5.3G

<i>croR</i>			
Stationary phase		Exponential phase	
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.7984	Ancestor vs Strain A	0.8785
Ancestor vs Strain B	0.1304	Ancestor vs Strain B	0.4418
Ancestor vs Strain C	0.5054	Ancestor vs Strain C	0.3282
Ancestor vs Strain D	0.7209	Ancestor vs Strain D	0.4418
Ancestor vs Strain E	0.3823	Ancestor vs Strain E	0.06496
Ancestor vs Strain F	0.2345	Ancestor vs Strain F	0.3282

Statistical data corresponding to Figure 5.3H

<i>salB</i>			
Stationary phase		Exponential phase	
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.02813	Ancestor vs Strain A	0.04988
Ancestor vs Strain B	0.4418	Ancestor vs Strain B	0.5737
Ancestor vs Strain C	0.4418	Ancestor vs Strain C	0.9591
Ancestor vs Strain D	0.3282	Ancestor vs Strain D	0.7209
Ancestor vs Strain E	0.7984	Ancestor vs Strain E	1
Ancestor vs Strain F	0.3823	Ancestor vs Strain F	0.3823

Statistical data corresponding to Figure 5.3I

<i>nucF</i>			
Stationary phase		Exponential phase	
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.02813	Ancestor vs Strain A	0.04988
Ancestor vs Strain B	0.4418	Ancestor vs Strain B	0.5737
Ancestor vs Strain C	0.4418	Ancestor vs Strain C	0.9591
Ancestor vs Strain D	0.3282	Ancestor vs Strain D	0.7209
Ancestor vs Strain E	0.7984	Ancestor vs Strain E	1
Ancestor vs Strain F	0.3823	Ancestor vs Strain F	0.3823

Statistical data corresponding to Figure 5.3J

<i>mprF_2</i>			
Stationary phase		Exponential phase	
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.02813	Ancestor vs Strain A	0.04988
Ancestor vs Strain B	0.4418	Ancestor vs Strain B	0.5737
Ancestor vs Strain C	0.4418	Ancestor vs Strain C	0.9591
Ancestor vs Strain D	0.3282	Ancestor vs Strain D	0.7209
Ancestor vs Strain E	0.7984	Ancestor vs Strain E	1
Ancestor vs Strain F	0.3823	Ancestor vs Strain F	0.3823

Statistical data corresponding to Figure 5.3K

<i>yihY</i>			
Stationary phase		Exponential phase	
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.5054	Ancestor vs Strain A	0.6454
Ancestor vs Strain B	0.1049	Ancestor vs Strain B	1
Ancestor vs Strain C	0.2345	Ancestor vs Strain C	0.5054
Ancestor vs Strain D	0.08298	Ancestor vs Strain D	0.7209
Ancestor vs Strain E	0.02067	Ancestor vs Strain E	0.9591
Ancestor vs Strain F	0.04009	Ancestor vs Strain F	0.7209

Statistical data corresponding to Figure 5.4

Membrane fluidity			
Exponential phase		Stationary phase	
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.06496	Ancestor vs Strain A	0.01476
Ancestor vs Strain B	0.1049	Ancestor vs Strain B	0.02067
Ancestor vs Strain C	0.03792	Ancestor vs Strain C	0.01041
Ancestor vs Strain D	0.1605	Ancestor vs Strain D	0.1605
Ancestor vs Strain E	0.001865	Ancestor vs Strain E	0.01041
Ancestor vs Strain F	0.06496	Ancestor vs Strain F	0.03792

Statistical data corresponding to Figure 5.5

Surface charge			
Exponential phase		Stationary phase	
Comparison	P value	Comparison	P value
Ancestor vs Strain A	0.1049	Ancestor vs Strain A	0.08298
Ancestor vs Strain B	0.1033	Ancestor vs Strain B	0.2786
Ancestor vs Strain C	0.1033	Ancestor vs Strain C	0.2345
Ancestor vs Strain D	0.7209	Ancestor vs Strain D	0.7209
Ancestor vs Strain E	0.1049	Ancestor vs Strain E	0.4418
Ancestor vs Strain F	0.3823	Ancestor vs Strain F	0.7209

Statistical data corresponding to Figure 5.7

Comparison (0h vs 6h)	P value
Ancestor	0.1953
Strain A	0.007813
Strain B	0.02344
Strain C	0.01563
Strain D	0.007813

Statistical data corresponding to Figure 6.2A

Comparison	P value
Ancestor - Colony 1	1
Ancestor - Colony 2	1
Ancestor - Colony 3	1
Ancestor - PBS	1
Ancestor - (U/C)	1

Statistical data corresponding to Figure 6.2B

Comparison	P value
Ancestor - Colony 1	1
Ancestor - Colony 2	1
Ancestor - Colony 3	1
Ancestor - PBS	1
Ancestor - (U/C)	1

Statistical data corresponding to Figure 6.2C

Comparison	P value
Ancestor - Colony 1	1
Ancestor - Colony 2	1
Ancestor - Colony 3	1
Ancestor - PBS	1
Ancestor - (U/C)	1

Statistical data corresponding to Figure 6.2D

Comparison	P value
Ancestor - Colony 1	0.3029
Ancestor - Colony 2	0.7846
Ancestor - Colony 3	0.0002
Ancestor - PBS	1
Ancestor - (U/C)	1

Statistical data corresponding to Figure 6.2E

Comparison	P value
Ancestor - Colony 1	0.0764
Ancestor - Colony 2	1
Ancestor - Colony 3	0.3237
Ancestor - PBS	1
Ancestor - (U/C)	1

Statistical data corresponding to Figure 6.3A and B

Comparison	P value	Comparison	P value
Ancestor - Dap Strain 1	1	Ancestor - Dap Strain 3	1
Ancestor - Dap Strain 2	0.528076	Ancestor - Dap Strain 4	4.6E-08
Ancestor - PBS	0.349591	Ancestor - Dap Strain 5	0.00109
Ancestor - (U/C)	1	Ancestor - PBS	0.419
		Ancestor - (U/C)	1

Statistical data corresponding to Figure 6.3C

Comparison (8h vs 8h)	P value
Ancestor - Dap Strain 1	1
Ancestor - Dap Strain 2	1
Ancestor - Dap Strain 3	1
Ancestor - Dap Strain 4	0.000709
Ancestor - Dap Strain5	0.822603
Comparison (0h vs 8h)	P value

Ancestor - Ancestor	0.7918
Dap Strain 1 – Dap Strain 1	0.02239
Dap Strain 2 – Dap Strain 2	0.1866
Dap Strain 3 – Dap Strain 3	0.03633
Dap Strain 4 – Dap Strain 4	0.0001105
Dap Strain 5 – Dap Strain 5	0.2569

Statistical data corresponding to Figure 6.3D

Comparison	P value
Ancestor – Strain 1	1
Ancestor – Strain 2	1
Ancestor – Strain 3	1
Ancestor – Strain 4	1
Ancestor – Strain 5	1
Ancestor - PBS	1
Ancestor - (U/C)	1