Journal of Microbiology (2014) Vol. 52, No. 3, pp. 200–210
DOI 10.1007/s12275-014-4011-6

REVIEW

Enterococcus Infection Biology: Lessons from Invertebrate Host Models

Grace J. Yuen[1,2] and Frederick M. Ausubel[1,3]*

[1]*Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, USA*
[2]*Program in Immunology,* [3]*Department of Genetics, Harvard Medical School, Boston, MA 02115, USA*

(Received Jan 7, 2014 / Accepted Jan 21, 2014)

The enterococci are commensals of the gastrointestinal tract of many metazoans, from insects to humans. While they normally do not cause disease in the intestine, they can become pathogenic when they infect sites outside of the gut. Recently, the enterococci have become important nosocomial pathogens, with the majority of human enterococcal infections caused by two species, *Enterococcus faecalis* and *Enterococcus faecium*. Studies using invertebrate infection models have revealed insights into the biology of enterococcal infections, as well as general principles underlying host innate immune defense. This review highlights recent findings on *Enterococcus* infection biology from two invertebrate infection models, the greater wax moth *Galleria mellonella* and the free-living bacteriovorous nematode *Caenorhabditis elegans*.

Keywords: *Enterococcus*, innate immunity, *Caenorhabditis elegans*, *Galleria mellonella*

Introduction

The enterococci are a widely dispersed group of bacteria and more than 40 species have been described (Murray *et al.*, 2013). Two enterococcal species, *E. faecium* and *E. faecalis,* are commonly found among the commensal microflora in the human gut and typically do not cause disease. However, they can be important agents of human disease, especially when they infect extra-intestinal sites. The first disease associated with *Enterococcus* was a case of infective endocarditis in 1899 (MacCallum and Hastings, 1899). Enterococci are currently the third most common nosocomial pathogen (12% of all hospital infections) causing urinary tract and wound infections, infective endocarditis, endophthalmitis, and peritonitis, which are often complicated by antibiotic drug resistance (Fischetti and American Society for Microbiology, 2006). The majority of human enterococcal infections are caused by *E. faecalis* (80–90%), with *E. faecium* comprising most of the remainder (10–15%) (Jett *et al.*, 1994; Johnson, 1994). It is important to understand the biology of enterococcal infections to combat the rising rates of nosocomial infections. Within the past 25 years, many important insights have been gained into enterococcal infection biology from the viewpoint of host-pathogen interactions. In this review, we will focus on the insights gleaned from invertebrate infection models that have revealed how enterococcal species have become such successful opportunistic, nosocomial pathogens, as well as the mechanisms by which the host innate immune system defends itself against enterococcal infection.

*For correspondence. E-mail: ausubel@molbio.mgh.harvard.edu; Tel.: +1-617-726-5969; Fax: +1-617-726-5949

Rationale for studying host-pathogen interactions in invertebrate models

Understanding the relationship between enterococcal virulence mechanisms and the host response has helped form an integrated view of enterococcal pathogenesis. Given that multiple interconnected factors with complex inter-relationships between both the host and the microbe are at play (Table 1), the use of a genetically tractable host and pathogen resulting in a dual genetic model allows one to deconstruct host-pathogen interactions in greater detail than is possible in vertebrate models.

Invertebrate infection models have revealed important insights into host-pathogen interactions, including guiding principles underlying both the infectious process and the host immune response that are relevant to mammalian infections (Casadevall, 2005; Lemaitre *et al.*, 2012; Vogel, 2012). The ability to use simple invertebrate hosts to study innate immunity and host-pathogen interactions is enabled by the evolutionary conservation of both ancient innate host defenses and bacterial virulence mechanisms. In general, the shared use of virulence factors by a pathogen infecting different hosts suggests that the mechanisms of pathogenesis are often host-independent (Waterfield *et al.*, 2004; Irazoqui *et al.*, 2010). Similarly, if a particular pathogen activates similar defense responses in both invertebrate and vertebrate hosts, it suggests that the underlying mechanisms of innate immunity against the pathogen may be conserved across metazoans. Thus, while the specific immune responses activated in a model host are likely to be most representative of phylogenetically related species, the categories of responses and the signaling pathways involved may be more highly conserved and may reflect common features of metazoan infection biology (Irazoqui *et al.*, 2010).

Two invertebrate organisms have been used most successfully as model hosts for the study of enterococcal patho-

Table 1. Facets of host immunity and pathogen virulence

Host	Pathogen

• Innate immunity
  ○ Sensors
  ▪ Pattern recognition
  ▪ Altered host/damage recognition
  ○ Effectors
  ▪ Antimicrobial peptides
  ▪ Lysozyme
  ○ Phagocytosis
  ○ Sequestration of pathogen
	• Quorum sensing
	• Toxic mediators
	  ○ Matrix-degrading enzymes
	  ○ Cytolysin
	  ○ Reactive oxygen species
	• Surface adhesion molecules
	• Biofilm
	• Persistence in environment and host

• Adaptive immunity
  ○ Humoral immunity (antibody-mediated)
  ○ Cellular immunity	

genesis and will be discussed at length in this review: the greater wax moth *Galleria mellonella* and the free-living bacteriovorous nematode *Caenorhabditis elegans* (Fig. 1). Compared to *C. elegans*, *G. mellonella* has a relatively complex innate immune system that may correlate better with observations in mammalian hosts. On the other hand, *C. elegans* is genetically tractable with many genetic and genomic tools are available, as well as an extensive worldwide community of scientists focused on all aspects of *C. elegans* biology. This facilitates the discovery of novel features of the host-pathogen interaction. The advantages and disadvantages of these two infection models will be discussed at length in this review. While a few preliminary studies of enterococcal infection have also been carried out using *Drosophila melanogaster* as a model host (Cox and Gilmore, 2007; Teixeira *et al.*, 2013), there has been very limited follow-up on these experiments.

Invertebrate model systems of enterococcal infection

Galleria mellonella as a model host

Galleria mellonella (hereafter referred to as *Galleria*) larvae have been used to identify and characterize bacterial virulence factors that also cause disease in mammals, including humans, in part because of a good correlation between the role of bacterial virulence factors in the *Galleria* infection model and mice (Jander *et al.*, 2000). Studies using *Galleria* have identified several novel enterococcal virulence factors. While *Galleria* lacks a fully sequenced genome and genetic tractability, the *Galleria* infection model has been successfully used to biochemically identify enterococcal virulence factors in the insect hemolymph, as well as for studying novel protective responses in the host. The *Galleria* model of *Enterococcus* infection is also biologically relevant. The predominant organism colonizing the intestines of wild-caught *Galleria* larvae is the enterococcal species *E. hirae*, which is thought to have the potential to escape from the intestinal lumen to the body cavity during the larval to pupal transition, thereby inducing the host defenses of *Galleria* (Bucher and Williams, 1967; Dunn *et al.*, 1994).

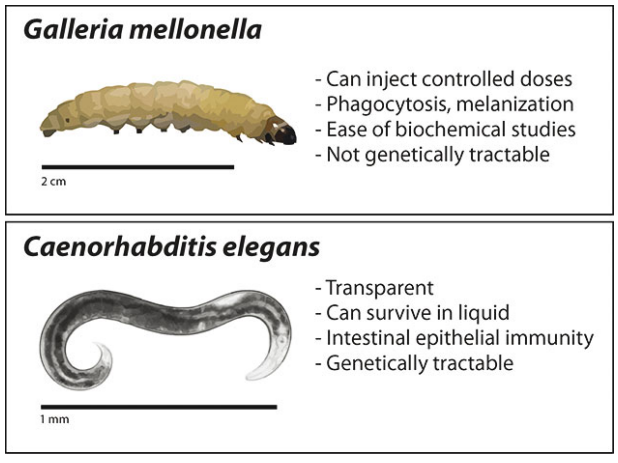

Fig. 1. Key features of two invertebrate models.

There are three main ways in which *Galleria* combats bacterial infections. First, circulating phagocytic hemocytes patrol the hemolymph, primed to recognize, engulf and sequester invading microbes. Second, upon detection of the pathogen within the inner cavity of the insect, proteolytic cascades can be quickly triggered, activating the melanization response (the synthesis and deposition of melanin to sequester pathogens at a wound site), followed by hemolymph coagulation, and opsonization (Tang, 2009). The melanization response of insects is analogous to the formation of an abscess in mammals following an infection, in which neutrophils accumulate within a tissue to prevent the spreading of the pathogen. Third, *Galleria* is able to induce a menagerie of antimicrobial immune effectors such as lysozymes, as well as antimicrobial peptides, such as cecropins and moricins, which can be rapidly synthesized by the fat body (Goldsmith and Marec, 2010). The insect larval midgut is the functional equivalent of the mammalian intestine. It is not only a major part of the digestive tract, but also regulates metabolism and the immune response (Apidianakis and Rahme, 2011).

An added benefit of using *Galleria* for pathogenesis studies is that infections can be carried out at 37°C or higher, as *Galleria* tolerates relatively high temperatures, unlike both *D. melanogaster* and *C. elegans* (maximum 25°C) (Glavis-Bloom *et al.*, 2012). This allows *Enterococcus* to grow optimally, and at human body temperature, for experimental studies. The larger size of the *Galleria* larva, compared to other invertebrate models, also allows it to be infected with larger, more controlled doses of the pathogen. In contrast,

Table 2. Enterococcal virulence factors

Virulence factor	Description
Secreted toxic mediators	
GelE	Gelatinase; matrix metalloproteinase of broad proteolytic activity
SprE	Serine protease; homology to *S. aureus*
Cytolysin	Member of lantibiotic class of bacteriocins; able to lyse prokaryotic and eukaryotic cells
Surface adhesion proteins	
Esp	Enterococcal surface protein; adhesion to epithelium, biofilm formation and conjugation
Ace	Adhesin to collagen
Fss1-3	Adhesin to fibrinogen
Ebp	Adhesin to fibrinogen
Acm	Adhesin to collagen
Aggregation substance	Group of proteins encoded on pheromone-responsive, conjugative plasmids; role in transfer of antibiotic resistance genes and extracellular matrix proteins
Other toxic mediators	
Extracellular ROS	Superoxide and hydrogen peroxide; can induce oxidative stress in nearby host cells

infection of *C. elegans* is carried out by allowing the animals to feed *ad libitum* on the pathogen, which does not control for dosage.

C. elegans as a model host

C. elegans is an excellent, although relatively new, model for studying pathogenesis and immunity. In the wild, *C. elegans* inhabits decaying vegetation and fruit and thus encounters a diverse microcommunity, rich in microbes (Felix and Braendle, 2010). As their environment naturally contains noxious bacteria, *C. elegans* must recognize the presence of pathogenic bacteria in their food and environment in order to mount effective immune and detoxification responses.

The simplicity of the small roundworm *C. elegans* has allowed it to become an attractive system for the study of innate immunity. The adult *C. elegans* worm has 20 non-renewing intestinal epithelial cells (IECs) that share many common morphological features with mammalian IECs, including a "brush border" of microvilli, anchored into a cellular structure called a terminal web (Irazoqui *et al.*, 2010). As the worm has no motile immune cells, it is dependent on these IECs to mediate intestinal immunity. The worm innate immune system can be easily activated by replacing non-pathogenic *E. coli*, the normal laboratory food source for *C. elegans*, with a pathogenic microbe. Its genetic tractability easily allows for thorough analysis of genetic pathways, especially using whole-genome RNAi screens (Kamath *et al.*, 2001; Kamath and Ahringer, 2003).

The small size of *C. elegans* and its ability to survive in liquid culture makes it amenable to cost-effective and rapid scaling-up for biochemical analysis (Walhout and Boulton, 2006), as well as for high-throughput screening of chemical libraries in whole-animals – a platform that would be impractical to implement in the case of *Galleria* larva (or even *Drosophila*), let alone vertebrates (Moy *et al.*, 2006, 2009). Additionally, testing the effect of small volumes of rare natural compounds on infection biology is only currently possible using the worm infection model.

Mechanisms of enterococcal virulence

There are a number of factors that contribute to the success of *E. faecalis* in establishing nosocomial infections (Table 2). In addition to its ability to readily acquire antibiotic resistance and persist in harsh environments, *Enterococci* also have a substantial toolkit of virulence factors (Kayaoglu and Orstavik, 2004; Fisher and Phillips, 2009; Sava *et al.*, 2010; Arias and Murray, 2012). In the sections below, we discuss the virulence factors and virulence-related processes that *Enterococcus* uses to establish an infection and the role that these factors play in pathogenesis in *Galleria* and *C. elegans* (Fig. 2).

Quorum sensing

Quorum sensing is the means by which bacteria are able to sense a species- or population-specific cell density via the production and secretion of small signal molecules. Quorum sensing can ultimately activate signaling transduction and thus modulate physiological behavior within the bacteria (Podbielski and Kreikemeyer, 2004). In *Enterococci*, quorum-sensing regulation is driven by the Fsr regulon, which is homologous to the Agr system of staphylococci, perhaps the most well-described cyclic peptide-mediated quorum-sensing system among Gram-positive bacteria. The Agr quorum sensing system (Lyon and Novick, 2004; Kong *et al.*, 2006; Novick and Geisinger, 2008; Gospodarek *et al.*, 2009) and, to some extent, the Fsr system (Carniol and Gilmore, 2004; Cook and Federle, 2013), have been discussed in several reviews. Briefly, the *fsr* quorum sensing system is comprised of the four genes *fsrA* (a response regulator), *fsrB* (a processing enzyme), *fsrC* (a sensor histidine kinase), and *fsrD* (a pro-peptide of the autoinducer) (Nakayama *et al.*, 2001). Following its production, the pro-peptide FsrD is processed and cyclized by FsrB to yield gelatinase biosynthesis activating pheromone (GBAP), an 11-amino acid cyclic peptide containing a lactone linkage (Nakayama *et al.*, 2006). GBAP then interacts with sensor kinase FsrC, which leads to the phosphorylation of regulator FsrA, thereby inducing the transcription of not only the *fsrBC* operon, but also two nearby genes encoding secreted products, the serine protease

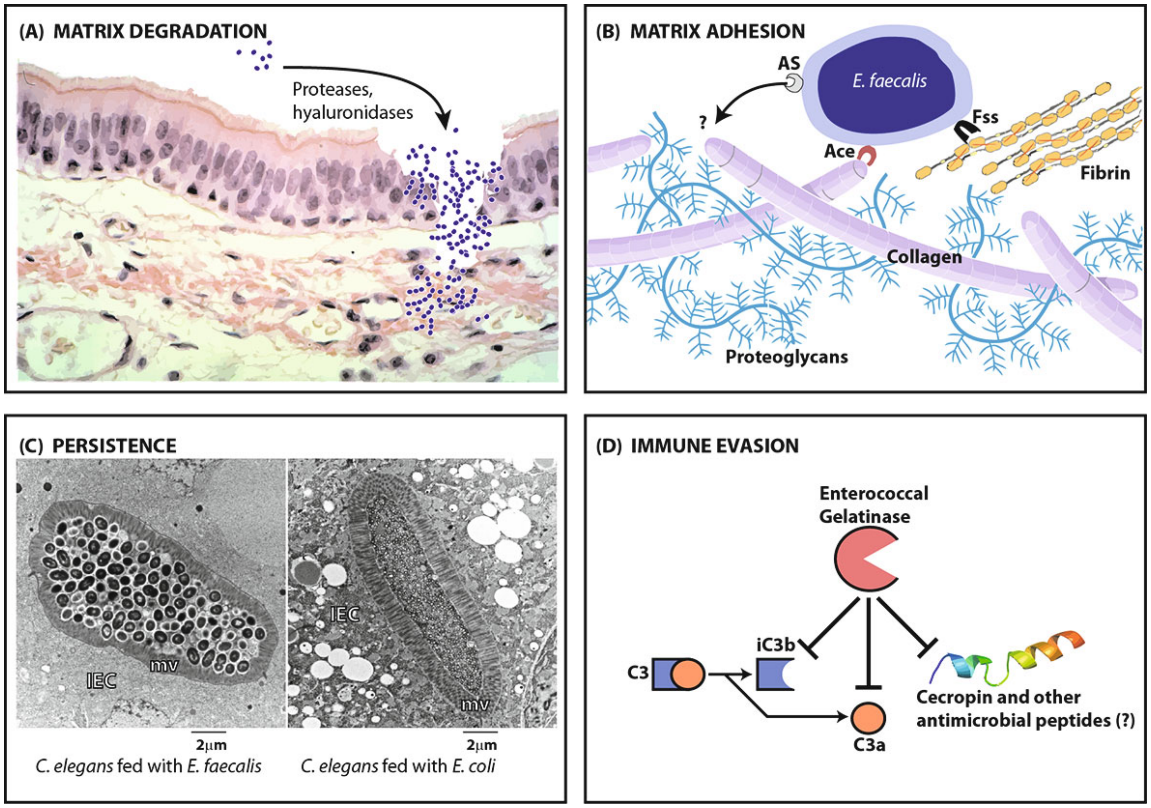

Fig. 2. Mechanisms of enterococcal virulence. (A) *Enterococci* produce a number of enzymes (e.g., hyaluronidase, gelatinase, and serine proteases) that degrade the host extracellular matrix, which allow the bacteria to invade host tissues. (B) *E. faecalis* expresses several adhesins that allow it to bind the extracellular matrix and cell surface of the host. Aggregation substance (AS) facilitates the adherence of *E. faecalis* to extracellular matrix proteins. Ace and Fss are bacterial adhesins that bind collagen and fibrin, respectively. (C) Cross-sections of *C. elegans* animals showing the intestinal lumen. After infection with *E. faecalis*, the *C. elegans* gut becomes distended with live enterococcal cells, forming a persistent infection in the worm (left). Most enterococcal cells are intact, with some even undergoing fission. In contrast, nearly all *E. coli* are degraded by the *C. elegans* pharyngeal grinder and intestine (right). Microvilli (mv) and intestinal epithelial cell (IEC) are labeled. (D) *Enterococcal gelatinase* GelE aids in evading the immune system of both *G. mellonella* and humans. GelE degrades cecropin, an insect antimicrobial peptide that is induced early in infection. Additionally, GelE is able to hydrolyze human C3a and degrade C3b, two complement proteins generated by C3 activation. This inhibits opsonization, as well as the formation of the membrane attack complex, two important roles of the complement cascade.

SprE and the zinc-metalloprotease gelatinase GelE. The Fsr two-component regulatory system is a major regulator of virulence in *E. faecalis*, and disruption of the *fsr* operon impairs the transcription of the two downstream proteases (SprE and GelE), as well as the development of biofilms (Hancock and Perego, 2004; Pillai *et al.*, 2004). *E. faecalis fsrB* mutants are attenuated in virulence in both the *C. elegans* and *Galleria* infection models, as well as in mammalian infection models (mouse peritonitis and rabbit endophthalmitis) (Singh *et al.*, 2005; Gaspar *et al.*, 2009).

Does the host interfere with quorum sensing, and if so, how does the host counteract its effects? Because quorum sensing is a feature of live and active bacteria, It has been proposed that quorum sensing may be targeted by the host immune system, which may try to recognize and inhibit the quorum sensing molecules (Vance *et al.*, 2009). In one study, a specific acyl homoserine lactone produced by *P. aeruginosa* (3OC12-HSL), but not its chemically-related counterparts, was found to induce the chemotaxis of human polymorphonuclear neutrophils (PMNs) via a host receptor on the PMNs that directly binds 3OC12-HSL (Zimmermann *et al.*, 2006). It is possible that the immune system has been selected to recognize these quorum-sensing auto-inducers as they are released from bacteria during the initial phases of biofilm formation at a time in the infection process when it may still be early enough for innate immune cells to stave off the infection. It has also been reported that *C. elegans* recognizes *P. aeruginosa* quorum sensing molecules, which results in aversive feeding behavior (Beale *et al.*, 2006). There may be a similar, corresponding interaction between enterococcal quorum sensing molecules and the host immune system. While there are no reports that this has been observed in *Galleria* or *C. elegans*, this interaction may be worthwhile to explore in the future.

Toxic mediators

Matrix-degrading enzymes: Proteases secreted by bacteria that degrade host tissues serve to provide peptide nutrients for the bacteria. In the case of bacterial pathogens, the secretion of proteases is often concomitant with pathogenesis. Bacterial proteases damage the host through multiple mechanisms: direct destruction of both cells and the extracellular matrix in the host tissue, indirect damage by proteolytic ac-

tivation of bacterial matrix metalloproteinases or host proteolytic cascades (Sorsa *et al.*, 1992), the processing of pathogenic virulence factors, and inhibition of host immunity (Lantz, 1997).

Gelatinase (GelE) is a secreted zinc metalloproteinase that allows *Enterococcus* to degrade and invade host tissues by degrading the collagenous matrix and that can interfere with host inflammatory processes by hydrolyzing a broad range of substrates including fibrinogen, fibrin, endothelin-1, bradykinin, human cathelicidin (LL-37), and complement components C3 and C3a (Makinen *et al.*, 1989; Makinen and Makinen, 1994; Schmidtchen *et al.*, 2002; Waters *et al.*, 2003; Park *et al.*, 2007). GelE is also critical for biofilm development in *E. faecalis*, as it activates a peptidoglycan-degrading enzyme called autolysin, which is responsible for the release of extracellular DNA and the formation of biofilm (Thomas *et al.*, 2009). The formation of biofilm allows *Enterococcus* to adhere to and survive on urethral catheters, urethral stents, and cardiac valves, and enhances its survival in an antibiotic-rich environment (Reid *et al.*, 1992; Donlan and Costerton, 2002; Sabbuba *et al.*, 2002; Tunney and Gorman, 2002; Carniol and Gilmore, 2004). Injection of purified GelE was found to be toxic to *Galleria* in a dose-dependent manner. *E. faecalis gelE* mutants are also attenuated in virulence in *Galleria.* Although the insect does not contain any of the canonical targets of GelE such as collagen, GelE acts on other targets in *Galleria*. For example, GelE was shown to directly hydrolyze a cecropin-like antimicrobial peptide in *Galleria*, analogous to its ability to degrade immune mediators in the human serum such as the complement component C3a. As C3a and C3a-derived peptides have broad antibacterial properties, the degradation of C3a by GelE may serve as an efficient way to cripple immune responses downstream of C3a. GelE is thus capable of subverting and inhibiting the immune system in both an insect and a mammalian infection model (Park *et al.*, 2007). In contrast, although injection of purified enterococcal extracellular serine protease SprE does not cause any obvious damage to *Galleria,* SprE was shown to be necessary for the full virulence of *E. faecalis* in killing in *C. elegans*, as a *sprE* mutant was attenuated in virulence in the *C. elegans* infection model (Sifri *et al.*, 2002).

Like gelatinase, hyaluronidase is also a degradative enzyme; it hydrolyzes hyaluronic acid, contributing to host tissue damage and invasiveness of the infection. It is thought that disruption of host tissue may also allow other secreted bacterial factors to permeate the tissue, magnifying the damage (Takao *et al.*, 1997). The breakdown of host tissue products by hyaluronidase may also allow *Enterococcus* to access and metabolize the nutrients of its host (Toto *et al.*, 1968; Hynes and Walton, 2000).

Cytolysin: The enterococcal cytolysin, a secreted two-peptide lytic toxin, is related to the extensive group of lanthionine-containing bacteriocins produced by Gram-positive bacteria and is capable of lysing both prokaryotic and eukaryotic cells in response to quorum sensing signals (Coburn and Gilmore, 2003). The molecular properties of enterococcal cytolysin and its regulation have been well studied (Coburn and Gilmore, 2003; Cox *et al.*, 2005; Van Tyne *et al.*, 2013). One retrospective study found that of nearly 200 clinical *E. faecalis* isolates, 45% expressed cytolysin, which is encoded on a pheromone-responsive plasmid on a pathogenicity island (Huycke *et al.*, 1991). The presence of cytolysin in a human infection has been reported to render the infection five times more acutely lethal (Huycke *et al.*, 1991). In the wild, enterococci have been isolated from *Galleria* larvae, and some of these isolates have been observed to exhibit lytic activity (Jarosz, 1975, 1979). In the *C. elegans* infection model (Garsin *et al.*, 2001), as well as in a *Drosophila* oral ingestion model (Cox and Gilmore, 2007), noncytolytic *E. faecalis* strains were attenuated in virulence, although they still remained lethal to *C. elegans*.

Surface adhesion proteins

Enterococcal surface protein (Esp) is a surface adhesin that controls adherence to host tissues, some of which have been implicated in endocarditis. Following host heart tissue damage, *Enterococcus* uses surface adhesins to adhere to the exposed extracellular matrix of injured tissue (Heikens *et al.*, 2011). The *esp* gene encodes a large LPxTG-anchored surface protein, which contributes to biofilm formation on abiotic surfaces. One study identified a putative surface antigen structurally related to Esp in vancomycin-resistant *E. faecalis* strain V583 called EF3314. Mutations in EF3314 resulted in attenuated virulence in the *C. elegans* infection model, suggesting that this surface antigen may have targets in both mammals and nematodes (Creti *et al.*, 2009).

Another potential *E. faecalis* adhesion is the protein Ace, a collagen- and laminin-binding microbial surface component recognizing adhesive matrix molecules (MSCRAMM). Ace-specific antibodies have been found in the sera of patients with enterococcal infections, and in particular, those patients with *E. faecalis* endocarditis, implying that the environment is an efficient trigger of Ace expression and that the expression of Ace is quite common (Nallapareddy *et al.*, 2000). Several *in vivo* environmental triggers, including high temperatures and the presence of collagen or bile salts, can trigger transcription of *ace*. However, the first indication that Ace has a role in addition to its binding the extracellular matrix, came from *Galleria* infection studies, where *ace* mutants were found to be attenuated in virulence (Lebreton *et al.*, 2009). As collagen and laminin are both absent in insects, it is possible that Ace has other targets in *Galleria*, and perhaps in mammals as well.

Enterococcal aggregation substance (AS) is a pheromone-responsive, surface-bound, plasmid-encoded protein that promotes clumping of bacterial cells to facilitate plasmid exchange, as well as adherence to intestinal epithelial cells by specifically binding extracellular matrix proteins. Additionally, AS allows *E. faecalis* to directly bind human neutrophils through complement receptor type 3 (CR3) binding (Vanek *et al.*, 1999). Despite the close proximity of the bacteria to the neutrophils, AS-expressing *E. faecalis* strains are resistant to neutrophil killing (Rakita *et al.*, 1999). Indeed, the oxidative burst (superoxide production) from the neutrophil may be responsible for causing collateral damage to neighboring tissues, without seriously damaging the adherent *E. faecalis* cells. Interestingly, *E. faecalis* AS is not a virulence factor in *C. elegans* killing (Garsin *et al.*, 2001), suggesting that the host target of AS may be absent.

Novel concepts in host-pathogen interactions revealed by invertebrate studies

Stress and its relation to virulence

Bacterial virulence is closely related to the ability of pathogens to adapt to different stresses. As bacteria traverse between the outside environment and the host during the course of the free-living and infection stages of their life cycles, they encounter myriad stressors, such as changing pH, temperature, and osmolarity, as well as the presence of reactive oxygen species. To establish a successful infection, a pathogen must be able to rapidly adapt to a changing environment, which is accompanied by many changes in gene expression levels of regulators and effectors of the stress response. These stress response genes are often required for bacterial virulence and have been explored in the *Galleria* model. Mutants corresponding to several stress-response genes were found to be attenuated in virulence, including the *clpB* gene, which encodes a Clp ATPase that is critical for promoting protein folding, as well as the assembly and degradation of proteins (de Oliveira *et al.*, 2011). Mutations in a second stress response gene that encodes methionine sulfoxide reductase A also results in attenuated *Galleria* killing, suggesting the importance of antioxidant repair enzymes in virulence (Zhao *et al.*, 2010). Surprisingly, however, a deletion mutation in the *E. faecalis slyA* gene, which encodes a member of the MarR/SlyA family of bacterial transcription factors that is predicted to be a regulator of stress responses, was shown to enhance virulence in *Galleria*, an unexpected result for a transcriptional activator (Michaux *et al.*, 2011). The enhanced virulence of the Δ*slyA* mutant in *Galleria* is consistent with increased persistence in macrophage assays and *in vivo* mouse infections. Given these data, it is possible that a small subset of genes that are overexpressed in the Δ*slyA* mutant may be responsible for the enhanced virulence phenotype.

The "stringent response" is a bacterial response to nutritional stress (e.g., amino acid starvation) and is an important component of enterococcal virulence. During the bacterial stringent response, two modified guanine nucleotides, guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), together known as (p)ppGpp, accumulate in the bacterial cell and act as "alarmones" or chemical messengers indicating stress, shutting down global transcription and selectively activating the transcription of genes regulating amino acid biosynthesis and stress-related survival. In *Enterococcus*, (p)ppGpp synthesis is catalyzed by two enzymes, RelA (the bifunctional synthetase/hydrolase of the alarmone) and RelQ (the monofunctional synthetase responsible for basal levels of alarmone during homeostasis). The alarmone (p)ppGpp was found to accumulate during vancomycin treatment and amino acid starvation. While the *E. faecalis relA* and *relQ* single mutants do not show attenuated virulence in the *C. elegans* infection model, a *relAQ* double mutant, in which (p)ppGpp production is completed abrogated, was highly attenuated in its virulence (Abranches *et al.*, 2009). A similar result was obtained in *Galleria* (Gaca *et al.*, 2012), and *relAQ* deficient strains also exhibited lower survival rates in macrophages *in vitro* than either the *relA* or *relQ* singly-deficient strains. These data support the model that basal levels of (p)ppGpp are required for full *E. faecalis* virulence in both *C. elegans* and *G. mellonella* as well as in mammalian cells. It is possible that the host innate immune system may recognize (p)ppGpp: the immune system is able to recognize the bacterial secondary messenger cyclic di-GMP as a MAMP (Karaolis *et al.*, 2007; McWhirter *et al.*, 2009; Yin *et al.*, 2012), which, like (p)ppGpp, is also derived from the modification of a nucleotide.

Environmental persistence and biofilm production

The ability of *Enterococcus* to survive in a multitude of environments, from the human gut to abiotic surfaces in hospitals, reflects its hardiness as a pathogen and capacity for environmental persistence. In addition to being thermotolerant (growing at 10-45°C), pH-tolerant (pH 4.8–9.6) and salt-resistant (up to 28% NaCl), *Enterococcus* is also able to survive extended desiccation (Gilmore, 2002), which is unusual for a bacterium that is incapable of sporulation. This may explain how *Enterococcus* is transferred from person to person in the hospital. *Enterococcus* may be able to enter a viable but non-culturable state when faced with nutrient-poor environments and may exist in this form on inanimate objects, such as bedrails and medical station keyboards (Lleo *et al.*, 1998; Giard *et al.*, 2001; Heim *et al.*, 2002). These intrinsic properties allow *Enterococcus* to flourish in a variety of environments, and may have been evolutionarily selected for by its need to survive in harsh environments, such as the intestinal tracts of animals.

The ability of *Enterococcus* to form a biofilm also allows *Enterococcus* to persist on abiotic surfaces as well as within the host. Biofilm protects pathogenic bacteria from the host immune response, as well as antibiotics and antimicrobials, which leads to chronic enterococcal infections that are difficult to treat (Lewis, 2001). Two potential *E. faecalis* regulators of biofilms may also control virulence in the *C. elegans* infection model. Whole-organism screening using the *C. elegans* infection model has helped identify novel regulators of enterococcal biofilm. In screens for *E. faecalis* transposon mutants that were attenuated in killing *C. elegans,* two related, potential virulence factors were identified: the transcriptional repressor ScrR, and a gene that is highly homologous to sucrose-6-phosphate hydrolases (ScrB) of other bacterial species and likely regulated by ScrR. This was intriguing, as sucrose utilization has been demonstrated to have a role in the formation of biofilms in dental caries and endocarditis by *Streptococcus mutans*, in which the sucrose catabolites are used to synthesize insoluble glucans to promote adherence to the tooth surface and heart valves (Garsin *et al.*, 2001; Maadani *et al.*, 2007).

The oral *E. faecalis* isolate, OG1RF, is devoid of many genes related to pathogenicity and is comparatively avirulent in humans. V583 is a virulent strain and was also the first sequenced vancomycin-resistant *E. faecalis* strain. The genome of V583 contains 25% mobile elements, including a pathogenicity-associated island (Shankar *et al.*, 2002, 2006; Paulsen *et al.*, 2003). However, both OG1RF and V583 have comparable virulence in the *C. elegans* infection model, even though OG1RF is believed to lack many genes thought to be required for virulence in humans (Garsin *et al.*, 2001; Bourgogne *et al.*, 2008). One possibility is that the genes re-

quired for virulence in humans are not necessary to render *Enterococcus* pathogenic in *C. elegans*, whereas *E. faecalis* genes that promote commensal enterococcal colonization of the human gut in healthy individuals (seen in both OG1RF and V583) are sufficient to cause virulence in *C. elegans* infection. This latter conclusion is supported by studies of enterococcal metabolism. *Enterococcus* has an impressive flexibility to catabolize a smorgasbord of carbon sources (Deibel, 1964a; Farrow *et al.*, 1983; Devriese *et al.*, 1993). *Enterococcus* is also able to use certain amino acids as energy and carbon sources (Deibel, 1964b; Roon and Barker, 1972). Concomitantly, and almost paradoxically, *Enterococcus* also has a number of auxotrophies, requiring a number of amino acids (e.g., valine, leucine, histidine, and tryptophan), as well as vitamins (e.g., biotin, riboflavin and nicotinic acid) (Murray *et al.*, 1993; Gilmore, 2002). These features of enterococcal metabolism are conserved across both virulent and avirulent *E. faecalis* strains and may have been selected for by competition as a commensal organism in the mammalian GI tract (Vebo *et al.*, 2010).

Why are *E. faecalis* infections more common than *E. faecium* infections? One reason may be that *E. faecalis* is simply more abundant in the gastrointestinal tract than *E. faecium*; indeed, one study found that *E. faecalis* was on average 100 times more prevalent than *E. faecium* in the gut (Noble, 1978). An interesting observation from enterococcal infection of *C. elegans* may hint at a reason for dominance of *E. faecalis*. Infection with *E. faecalis or E. faecium* rapidly distends the *C. elegans* intestine, which fills up with live enterococcal cells. However, only *E. faecalis* results in a persistent and lethal infection in the worm, even with a small inoculum of *E. faecalis* bacterial cells. This may suggest that *E. faecalis* has an inherent ability to establish a persistent infection, perhaps contributing to its virulence, but the unique characteristics of *E. faecalis* that allow it to form a persistent infection remain unknown. There does not appear to be a single virulence factor that contributes to the bulk of *E. faecalis* pathogenicity, as *E. faecalis* transposon mutant library screens have not picked up any single mutant that renders *E. faecalis* completely attenuated. Although the mechanism for *E. faecalis* persistence in *C. elegans* is unknown, we speculate that *E. faecalis* may be better at competing for and establishing a protected niche for itself in the *C. elegans* intestine.

Innate immune perception of enterococcal infection

The mammalian innate immune response involves the perception of a bacterial infection by pattern recognition receptors (PRRs), which recognize conserved microbial structures (e.g., flagellin, lipopolysaccharide, unmethylated CpG motifs) as pathogen- or microbe-associated molecular patterns (M/PAMPs). The detection of bacterial M/PAMPs by host PRRs activates downstream signaling pathways that culminate in the production of immune effectors that inhibit the infection (Medzhitov, 2001). However, the localized context of infection is likely to be just as important for innate immune recognition of a pathogen, since M/PAMPs are present on non-pathogenic bacteria as well as pathogenic bacteria. Additionally, M/PAMPs are often thought to be invariant bacterial features, but pathogens are often able to vary their M/PAMPs, such as in the case of the many variants of LPS among different bacteria. It has been proposed that the immune system may respond to M/PAMPs in the context of other signals, derived from either the host or the pathogen, that are only generated during a pathogenic infection, called patterns of pathogenesis (Vance *et al.*, 2009).

These principles also apply to the innate immune system of invertebrates. In the case of *C. elegans*, several immune signaling pathways have been identified, although no specific M/PAMPs that activate *C. elegans* immunity have been discovered. Chemical genetics may be one approach to identifying novel and potentially conserved immune signaling pathways in *C. elegans*, which may ultimately lead to the identification of the most upstream innate immune receptors in *C. elegans*, and potentially, the identification of specific bacterial M/PAMPs recognized by such receptors. One chemical genetics study employed a liquid-based, high-throughput screen of small molecules and extracts to identify compounds that promoted the survival of *E. faecalis*-infected worms (Moy *et al.*, 2006, 2009). One compound selectively acted upon the *C. elegans* host via the activation of the well-studied and conserved p38 MAP kinase, PMK-1 (Pukkila-Worley *et al.*, 2012). However, the precise biological target of this molecule is unknown. It is possible that this activating compound is modified in the host to generate a molecule resembling a pattern of pathogenesis or may activate signals that are also transduced by receptors that recognize patterns of pathogenesis. In addition to identifying novel immune activators, this chemical genetics approach can also be used to identify antimicrobials (which inhibit the growth of or kill bacteria) and inhibitors of bacterial virulence (which may not have an effect on bacterial survival, but may modulate bacterial physiologic behavior).

The DAF-2/DAF-16 (homologous to IGF-1 receptor and FOXO, respectively) insulin signaling pathway is also involved in the *C. elegans* defense against *Enterococcus*. When the DAF-2 receptor is prevented from inhibiting DAF-16, genes involved in regulating lifespan, dauer formation, and pathogen and stress resistance are activated, leading to enhanced resistance to *E. faecalis* and other pathogens (Garsin *et al.*, 2003; Murphy *et al.*, 2003). A number of DAF-16-regulated genes are necessary for the neutralization and detoxification of ROS, such as superoxide dismutases (which reduce superoxide to hydrogen peroxide) and catalases (which convert hydrogen peroxide to water and oxygen) (Chavez *et al.*, 2007). This suggested that countering ROS is crucial component of an effective immune response; however, it was unknown whether the ROS was primarily generated by *E. faecalis,* or the host in response to pathogenic infection. It was later shown that *C. elegans* host intestinal cells, following infection by *E. faecalis*, generate extracellular ROS via the NADPH oxidase BLI-3, the *C. elegans* homolog of mammalian dual oxidase Duox1, which contributed to pathogen resistance (Chavez *et al.*, 2007). The production of ROS, in tandem with the infection, activates a p38 MAPK PMK-1 cascade to induce the Nrf-family transcription factor SKN-1, which in turn upregulates stress and antioxidant responses during infection to protect against the collateral damage inflicted by endogenous ROS production (Hoeven *et al.*, 2011; Papp *et al.*, 2012). This response may serve as

an ancient form of immunity, much like the oxidative burst observed in macrophages and neutrophils. It remains unknown, however, by what mechanism the dual oxidase is activated to eventually activate the immune response.

How is *Enterococcus* infection perceived by the *C. elegans* host immune system? Is it through recognition of conserved structural motifs specific to pathogens, or by the recognition of perturbed cellular processes in the host? There is increasing evidence for the latter (Dunbar *et al.*, 2012; McEwan *et al.*, 2012; Melo and Ruvkun, 2012). Host innate immune defense appears to be closely related to surveillance pathways that monitor core cellular activities (e.g., organelle function, protein translation, etc.), which may also allow the host to perceive the presence of pathogens that produce virulence factors that subvert fundamental host functions.

E. faecium and its opportunistic traits

In comparison with other enterococcal species, strains of *E. durans* and *E. faecium* are either avirulent or weakly virulent in *G. mellonella* compared to *E. faecalis*. This intriguing interspecies difference in pathogenesis has also been observed in the *C. elegans* infection model, and may imply the presence of *E. faecalis*-specific virulence factors.

It is not always the case, however, that *E. faecium* is avirulent. In studies with *C. elegans*, *E. faecium* that is grown anaerobically and then exposed to aerobic conditions, produces hydrogen peroxide, which is able to kill *C. elegans* rapidly without a few hours. The explanation behind this phenomenon is related to *E. faecium* being a facultative anaerobe relying on glycolysis and fermentation to generate energy. In *E. faecium*, lactic acid fermentation is favored under anaerobic conditions. However, under aerobic conditions, mixed acid fermentation takes place, where NADH oxidases use molecular oxygen as an electron acceptor to regenerate NAD^+ from NADH. In turn, in these aerobic conditions, molecular oxygen is reduced to hydrogen peroxide. The NADH peroxidase, which scavenges the hydrogen peroxide, is only expressed under aerobic conditions. Thus, the immediate exposure of anaerobically grown *E. faecium* to aerobic conditions allows for the production and accumulation of hydrogen peroxide, with levels of NADH peroxidase that are too low to scavenge efficiently (Moy *et al.*, 2004). It was also found that hydrogen peroxide production increased as aerobically-grown *E. faecium* entered the stationary phase. Taken together, the production of hydrogen peroxide may be one mechanism by which *E. faecium* is able to impede or kill its fellow competing bacteria, as well as damage host tissue or immune cells via oxidative stress.

Oxidative stress may be a key signal used by *E. faecium* to promote opportunistic traits. The *E. faecium* AsrR protein was identified as an oxidative stress sensor that uses cysteine oxidation to sense hydrogen peroxide. AsrR and its putative homologs are present in *E. faecium*, *E. gallinarum*, and *E. casseliflavus*, but absent in *E. faecalis*. AsrR was found to regulate 181 genes related to many features of pathogenesis aside from oxidative stress, including resistance to antibiotics and antimicrobial peptides (Lebreton *et al.*, 2012). Two important adhesion-encoding genes, *acm* and *ecbA*, were also found to be upregulated, and contributed to the increased persistence and colonization observed in *Galleria* infection, which was supported further by the observed increased adhesion to human intestinal cells and biofilm formation in two different *in vitro* assays.

Conclusions

There remain several unanswered questions in *Enterococcus* infection biology, and many of these questions are especially tractable using invertebrate model systems, which often allow for the genetic dissection on both the host and pathogen fronts. The use of invertebrate infection models will prove especially useful in recapitulating facets of the *Enterococcus* infection that involve the interaction between *Enterococcus* and host tissues, rather than the interaction between *Enterococcus* and individual cells (e.g., macrophages), which can be recapitulated in cell culture. By virtue of their small size, the *C. elegans* allows high throughput genetic and chemical screens in whole-animal infections for novel virulence factors as well as anti-infective compounds. In addition, *C. elegans* is especially genetically tractable and thus can be used for forward- and reverse-genetic screens to identify host factors required for defense against *Enterococcus*. However, genetic tools are rapidly improving, and it will not be long before *Galleria* can also be manipulated genetically, perhaps through the use of the CRISPR-Cas system (Ran *et al.*, 2013a, 2013b) and other genome editing tools. The use of bacterial genetics tools such as Tn-seq (van Opijnen and Camilli, 2010), which utilizes next-generation sequencing for identifying transposon insertion mutants of interest, will also help elucidate the unique features of *Enterococcus* virulence such as its ability to induce a persistent infection.

References

Abranches, J., Martinez, A.R., Kajfasz, J.K., Chavez, V., Garsin, D.A., and Lemos, J.A. 2009. The molecular alarmone (p)ppGpp mediates stress responses, vancomycin tolerance, and virulence in *Enterococcus faecalis*. *J. Bacteriol.* **191**, 2248–2256.

Apidianakis, Y. and Rahme, L.G. 2011. *Drosophila melanogaster* as a model for human intestinal infection and pathology. *Dis. Model Mech.* **4**, 21–30.

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

Beale, E., Li, G., Tan, M.W., and Rumbaugh, K.P. 2006. *Caenorhabditis elegans* senses bacterial autoinducers. *Appl. Environ. Microbiol.* **72**, 5135–5137.

Bourgogne, A., Garsin, D.A., Qin, X., Singh, K.V., Sillanpaa, J., Yerrapragada, S., Ding, Y., Dugan-Rocha, S., Buhay, C., Shen, H., and *et al.* 2008. Large scale variation in *Enterococcus faecalis* illustrated by the genome analysis of strain OG1RF. *Genome Biol.* **9**, R110.

Bucher, G.E. and Williams, R. 1967. The microbial flora of laboratory culture of the greater wax moth and its effects on rearing parasites. *J. Invert. Pathol.* **9**, 467–473.

Carniol, K. and Gilmore, M.S. 2004. Signal transduction, quorum-sensing, and extracellular protease activity in *Enterococcus faecalis* biofilm formation. *J. Bacteriol.* **186**, 8161–8163.

Casadevall, A. 2005. Host as the variable: model hosts approach the immunological asymptote. *Infect. Immun.* **73**, 3829–3832.

Chavez, V., Mohri-Shiomi, A., Maadani, A., Vega, L.A., and Garsin,

D.A. 2007. Oxidative stress enzymes are required for DAF-16-ediated immunity due to generation of reactive oxygen species by *Caenorhabditis elegans*. *Genetics* **176**, 1567–1577.

Coburn, P.S. and Gilmore, M.S. 2003. The *Enterococcus faecalis* cytolysin: a novel toxin active against eukaryotic and prokaryotic cells. *Cell. Microbiol.* **5**, 661–669.

Cook, L.C. and Federle, M.J. 2013. Peptide pheromone signaling in *Streptococcus* and *Enterococcus*. *FEMS Microbiol. Rev.* doi: 10.1111/1574-6976.12046.

Cox, C.R., Coburn, P.S., and Gilmore, M.S. 2005. Enterococcal cytolysin: a novel two component peptide system that serves as a bacterial defense against eukaryotic and prokaryotic cells. *Curr. Protein Pept. Sci.* **6**, 77–84.

Cox, C.R. and Gilmore, M.S. 2007. Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis. *Infect. Immun.* **75**, 1565–1576.

Creti, R., Fabretti, F., Koch, S., Huebner, J., Garsin, D.A., Baldassarri, L., Montanaro, L., and Arciola, C.R. 2009. Surface protein EF3314 contributes to virulence properties of *Enterococcus faecalis*. *Int. J. Artif. Organs* **32**, 611–620.

de Oliveira, N.E., Abranches, J., Gaca, A.O., Laport, M.S., Damaso, C.R., Bastos Mdo, C., Lemos, J.A., and Giambiagi-deMarval, M. 2011. *clpB*, a class III heat-shock gene regulated by CtsR, is involved in thermotolerance and virulence of *Enterococcus faecalis*. *Microbiology* **157**, 656–665.

Deibel, R.H. 1964a. The Group D Streptococci. *Bacteriol. Reviews* **28**, 330–366.

Deibel, R.H. 1964b. Utilization of arginine as an energy source for the growth of *Streptococcus faecalis*. *J. Bacteriol.* **87**, 988–992.

Devriese, L.A., Pot, B., and Collins, M.D. 1993. Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. *J. Appl. Bacteriol.* **75**, 399–408.

Donlan, R.M. and Costerton, J.W. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **15**, 167–193.

Dunbar, T.L., Yan, Z., Balla, K.M., Smelkinson, M.G., and Troemel, E.R. 2012. *C. elegans* detects pathogen-induced translational inhibition to activate immune signaling. *Cell Host Microbe* **11**, 375–386.

Dunn, P.E., Bohnert, T.J., and Russell, V. 1994. Midgut antibacterial defenses of Manduca sexta following infection and during metamorphosis. *In* Hoffmann, J.A., Janeway, C.A.Jr., and Natori, S. (eds.), Phylogenetic Perspectives in Immunity: The Insect Host Defense, pp. 105–113. Landes, Austin, Texas, USA.

Farrow, J.A., Jones, D., Phillips, B.A., and Collins, M.D. 1983. Taxonomic studies on some group D Streptococci. *J. Gen. Microbiol.* **129**, 1423–1432.

Felix, M.A. and Braendle, C. 2010. The natural history of *Caenorhabditis elegans*. *Curr. Biol.* **20**, R965–969.

Fischetti, V.A. and American Society for Microbiology. 2006. Gram-positive pathogens. ASM Press, Washington, D.C., USA.

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

Gaca, A.O., Abranches, J., Kajfasz, J.K., and Lemos, J.A. 2012. Global transcriptional analysis of the stringent response in *Enterococcus faecalis*. *Microbiology* **158**, 1994–2004.

Garsin, D.A., Sifri, C.D., Mylonakis, E., Qin, X., Singh, K.V., Murray, B.E., Calderwood, S.B., and Ausubel, F.M. 2001. A simple model host for identifying Gram-positive virulence factors. *Proc. Natl. Acad. Sci. USA* **98**, 10892–10897.

Garsin, D.A., Villanueva, J.M., Begun, J., Kim, D.H., Sifri, C.D., Calderwood, S.B., Ruvkun, G., and Ausubel, F.M. 2003. Long-lived *C. elegans daf-2* mutants are resistant to bacterial pathogens. *Science* **300**, 1921.

Gaspar, F., Teixeira, N., Rigottier-Gois, L., Marujo, P., Nielsen-LeRoux, C., Crespo, M.T., Lopes Mde, F., and Serror, P. 2009. Virulence of *Enterococcus faecalis* dairy strains in an insect model: the role of *fsrB* and *gelE*. *Microbiology* **155**, 3564–3571.

Giard, J.C., Laplace, J.M., Rince, A., Pichereau, V., Benachour, A., Leboeuf, C., Flahaut, S., Auffray, Y., and Hartke, A. 2001. The stress proteome of *Enterococcus faecalis*. *Electrophoresis* **22**, 2947–2954.

Gilmore, M.S. 2002. The enterococci : pathogenesis, molecular biology, and antibiotic resistance. ASM Press, Washington, D.C., USA.

Glavis-Bloom, J., Muhammed, M., and Mylonakis, E. 2012. Of model hosts and man: using *Caenorhabditis elegans*, *Drosophila melanogaster* and *Galleria mellonella* as model hosts for infectious disease research. *Adv. Exp. Med. Biol.* **710**, 11–17.

Goldsmith, M.R. and Marec, F. 2010. Molecular biology and genetics of the Lepidoptera. CRC Press/Taylor & Francis, Boca Raton, FL, USA.

Gospodarek, E., Bogiel, T., and Zalas-Wiecek, P. 2009. Communication between microorganisms as a basis for production of virulence factors. *Pol. J. Microbiol.* **58**, 191–198.

Hancock, L.E. and Perego, M. 2004. The *Enterococcus faecalis fsr* two-component system controls biofilm development through production of gelatinase. *J. Bacteriol.* **186**, 5629–5639.

Heikens, E., Singh, K.V., Jacques-Palaz, K.D., van Luit-Asbroek, M., Oostdijk, E.A., Bonten, M.J., Murray, B.E., and Willems, R.J. 2011. Contribution of the enterococcal surface protein Esp to pathogenesis of *Enterococcus faecium* endocarditis. *Microbes Infect.* **13**, 1185–1190.

Heim, S., Lleo, M., Bonato, B., Guzman, C.A., and Canepari, P. 2002. The viable but nonculturable state and starvation are different stress responses of *Enterococcus faecalis*, as determined by proteome analysis. *J. Bacteriol.* **184**, 6739–6745.

Hoeven, R., McCallum, K.C., Cruz, M.R., and Garsin, D.A. 2011. Ce-Duox1/BLI-3 generated reactive oxygen species trigger protective SKN-1 activity via p38 MAPK signaling during infection in *C. elegans*. *PLoS Pathog.* **7**, e1002453.

Huycke, M.M., Spiegel, C.A., and Gilmore, M.S. 1991. Bacteremia caused by hemolytic, high-level gentamicin-resistant *Enterococcus faecalis*. *Antimicrob. Agents Chemother.* **35**, 1626–1634.

Hynes, W.L. and Walton, S.L. 2000. Hyaluronidases of Gram-positive bacteria. *FEMS Microbiol. Lett.* **183**, 201–207.

Irazoqui, J.E., Urbach, J.M., and Ausubel, F.M. 2010. Evolution of host innate defence: insights from *Caenorhabditis elegans* and primitive invertebrates. *Nat. Rev. Immunol.* **10**, 47–58.

Jander, G., Rahme, L.G., and Ausubel, F.M. 2000. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *J. Bacteriol.* **182**, 3843–3845.

Jarosz, J. 1975. Lysozyme-like lytic enzyme of *Streptococcus faecalis* and its role in larval development of wax moth, *Galleria mellonella*. *J. Invert. Pathol.* **26**, 275–281.

Jarosz, J. 1979. Gut flora of *Galleria mellonella* suppressing ingested bacteria. *J. Invert. Pathol.* **34**, 192–198.

Jett, B.D., Huycke, M.M., and Gilmore, M.S. 1994. Virulence of enterococci. *Clin. Microbiol. Rev.* **7**, 462–478.

Johnson, A.P. 1994. The pathogenicity of enterococci. *J. Antimicrob. Chemother.* **33**, 1083–1089.

Kamath, R.S. and Ahringer, J. 2003. Genome-wide RNAi screening in *Caenorhabditis elegans*. *Methods* **30**, 313–321.

Kamath, R.S., Martinez-Campos, M., Zipperlen, P., Fraser, A.G., and Ahringer, J. 2001. Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol.* **2**, RESEARCH0002.

Karaolis, D.K., Means, T.K., Yang, D., Takahashi, M., Yoshimura, T., Muraille, E., Philpott, D., Schroeder, J.T., Hyodo, M., Hayakawa, Y., and *et al.* 2007. Bacterial c-di-GMP is an immunostimulatory molecule. *J. Immunol.* **178**, 2171–2181.

Kayaoglu, G. and Orstavik, D. 2004. Virulence factors of *Enterococcus faecalis*: relationship to endodontic disease. *Crit. Rev.*

Oral. Biol. Med. **15**, 308–320.

Kong, K.F., Vuong, C., and Otto, M. 2006. *Staphylococcus* quorum sensing in biofilm formation and infection. *Int. J. Med. Microbiol.* **296**, 133–139.

Lantz, M.S. 1997. Are bacterial proteases important virulence factors? *J. Periodontal Res.* **32**, 126–132.

Lebreton, F., Riboulet-Bisson, E., Serror, P., Sanguinetti, M., Posteraro, B., Torelli, R., Hartke, A., Auffray, Y., and Giard, J.C. 2009. *ace*, which encodes an adhesin in *Enterococcus faecalis*, is regulated by Ers and is involved in virulence. *Infect. Immun.* **77**, 2832–2839.

Lebreton, F., van Schaik, W., Sanguinetti, M., Posteraro, B., Torelli, R., Le Bras, F., Verneuil, N., Zhang, X., Giard, J.C., Dhalluin, A., and *et al.* 2012. AsrR is an oxidative stress sensing regulator modulating *Enterococcus faecium* opportunistic traits, antimicrobial resistance, and pathogenicity. *PLoS Pathog.* **8**, e1002834.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. 2012. Pillars article: the dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell. 1996. 86: 973-983. *J. Immunol.* **188**, 5210–5220.

Lewis, K. 2001. Riddle of biofilm resistance. *Antimicrob. Agents Chemother.* **45**, 999–1007.

Lleo, M.M., Tafi, M.C., and Canepari, P. 1998. Nonculturable *Enterococcus faecalis* cells are metabolically active and capable of resuming active growth. *System. Appl. Microbiol.* **21**, 333–339.

Lyon, G.J. and Novick, R.P. 2004. Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria. *Peptides* **25**, 1389–1403.

Maadani, A., Fox, K.A., Mylonakis, E., and Garsin, D.A. 2007. *Enterococcus faecalis* mutations affecting virulence in the *Caenorhabditis elegans* model host. *Infect. Immun.* **75**, 2634–2637.

MacCallum, W.G. and Hastings, T.W. 1899. A case of acute endocarditis caused by *Micrococcus zymogenes* (nov. spec.), with a description of the microorganism. *J. Exp. Med.* **4**, 521–534.

Makinen, P.L., Clewell, D.B., An, F., and Makinen, K.K. 1989. Purification and substrate specificity of a strongly hydrophobic extracellular metalloendopeptidase ("gelatinase") from *Streptococcus faecalis* (strain 0G1-10). *J. Biol. Chem.* **264**, 3325–3334.

Makinen, P.L. and Makinen, K.K. 1994. The *Enterococcus faecalis* extracellular metalloendopeptidase (EC 3.4.24.30; coccolysin) inactivates human endothelin at bonds involving hydrophobic amino acid residues. *Biochem. Biophys. Res. Commun.* **200**, 981–985.

McEwan, D.L., Kirienko, N.V., and Ausubel, F.M. 2012. Host translational inhibition by *Pseudomonas aeruginosa* exotoxin A triggers an immune response in *Caenorhabditis elegans*. *Cell Host Microbe* **11**, 364–374.

McWhirter, S.M., Barbalat, R., Monroe, K.M., Fontana, M.F., Hyodo, M., Joncker, N.T., Ishii, K.J., Akira, S., Colonna, M., Chen, Z.J., and *et al.* 2009. A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP. *J. Exp. Med.* **206**, 1899–1911.

Medzhitov, R. 2001. Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* **1**, 135–145.

Melo, J.A. and Ruvkun, G. 2012. Inactivation of conserved *C. elegans* genes engages pathogen- and xenobiotic-associated defenses. *Cell* **149**, 452–466.

Michaux, C., Sanguinetti, M., Reffuveille, F., Auffray, Y., Posteraro, B., Gilmore, M.S., Hartke, A., and Giard, J.C. 2011. SlyA is a transcriptional regulator involved in the virulence of *Enterococcus faecalis*. *Infect. Immun.* **79**, 2638–2645.

Moy, T.I., Ball, A.R., Anklesaria, Z., Casadei, G., Lewis, K., and Ausubel, F.M. 2006. Identification of novel antimicrobials using a live-animal infection model. *Proc. Natl. Acad. Sci. USA* **103**, 10414–10419.

Moy, T.I., Conery, A.L., Larkins-Ford, J., Wu, G., Mazitschek, R., Casadei, G., Lewis, K., Carpenter, A.E., and Ausubel, F.M. 2009. High-throughput screen for novel antimicrobials using a whole animal infection model. *ACS Chem. Biol.* **4**, 527–533.

Moy, T.I., Mylonakis, E., Calderwood, S.B., and Ausubel, F.M. 2004. Cytotoxicity of hydrogen peroxide produced by *Enterococcus faecium*. *Infect. Immun.* **72**, 4512–4520.

Murphy, C.T., McCarroll, S.A., Bargmann, C.I., Fraser, A., Kamath, R.S., Ahringer, J., Li, H., and Kenyon, C. 2003. Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. *Nature* **424**, 277–283.

Murray, B.E., Singh, K.V., Ross, R.P., Heath, J.D., Dunny, G.M., and Weinstock, G.M. 1993. Generation of restriction map of *Enterococcus faecalis* OG1 and investigation of growth requirements and regions encoding biosynthetic function. *J. Bacteriol.* **175**, 5216–5223.

Murray, P.R., Rosenthal, K.S., and Pfaller, M.A. 2013. Medical microbiology. Elsevier/Saunders, Philadelphia, USA.

Nakayama, J., Cao, Y., Horii, T., Sakuda, S., Akkermans, A.D., de Vos, W.M., and Nagasawa, H. 2001. Gelatinase biosynthesis-activating pheromone: a peptide lactone that mediates a quorum sensing in *Enterococcus faecalis*. *Mol. Microbiol.* **41**, 145–154.

Nakayama, J., Chen, S., Oyama, N., Nishiguchi, K., Azab, E.A., Tanaka, E., Kariyama, R., and Sonomoto, K. 2006. Revised model for *Enterococcus faecalis fsr* quorum-sensing system: the small open reading frame *fsrD* encodes the gelatinase biosynthesis-activating pheromone propeptide corresponding to Staphylococcal AgrD. *J. Bacteriol.* **188**, 8321–8326.

Nallapareddy, S.R., Singh, K.V., Duh, R.W., Weinstock, G.M., and Murray, B.E. 2000. Diversity of *ace*, a gene encoding a microbial surface component recognizing adhesive matrix molecules, from different strains of *Enterococcus faecalis* and evidence for production of Ace during human infections. *Infect. Immun.* **68**, 5210–5217.

Noble, C.J. 1978. Carriage of group D streptococci in the human bowel. *J. Clin. Pathol.* **31**, 1182–1186.

Novick, R.P. and Geisinger, E. 2008. Quorum sensing in staphylococci. *Ann. Review Genet.* **42**, 541–564.

Papp, D., Csermely, P., and Soti, C. 2012. A role for SKN-1/Nrf in pathogen resistance and immunosenescence in *Caenorhabditis elegans*. *PLoS Pathog.* **8**, e1002673.

Park, S.Y., Kim, K.M., Lee, J.H., Seo, S.J., and Lee, I.H. 2007. Extracellular gelatinase of *Enterococcus faecalis* destroys a defense system in insect hemolymph and human serum. *Infect. Immun.* **75**, 1861–1869.

Paulsen, I.T., Banerjei, L., Myers, G.S., Nelson, K.E., Seshadri, R., Read, T.D., Fouts, D.E., Eisen, J.A., Gill, S.R., Heidelberg, J.F., and *et al.* 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science* **299**, 2071–2074.

Pillai, S.K., Sakoulas, G., Eliopoulos, G.M., Moellering, R.C. Jr., Murray, B.E., and Inouye, R.T. 2004. Effects of glucose on fsr-mediated biofilm formation in *Enterococcus faecalis*. *J. Infect. Dis.* **190**, 967–970.

Podbielski, A. and Kreikemeyer, B. 2004. Cell density–dependent regulation: basic principles and effects on the virulence of Gram-positive cocci. *Int. J. Infect. Dis.* **8**, 81–95.

Pukkila-Worley, R., Feinbaum, R., Kirienko, N.V., Larkins-Ford, J., Conery, A.L., and Ausubel, F.M. 2012. Stimulation of host immune defenses by a small molecule protects *C. elegans* from bacterial infection. *PLoS Genet.* **8**, e1002733.

Rakita, R.M., Vanek, N.N., Jacques-Palaz, K., Mee, M., Mariscalco, M.M., Dunny, G.M., Snuggs, M., Van Winkle, W.B., and Simon, S.I. 1999. *Enterococcus faecalis* bearing aggregation substance is resistant to killing by human neutrophils despite phagocytosis and neutrophil activation. *Infect. Immun.* **67**, 6067–6075.

Ran, F.A., Hsu, P.D., Lin, C.Y., Gootenberg, J.S., Konermann, S., Trevino, A.E., Scott, D.A., Inoue, A., Matoba, S., Zhang, Y., and

Zhang, F. 2013a. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* **154**, 1380–1389.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., and Zhang, F. 2013b. Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308.

Reid, G., Denstedt, J.D., Kang, Y.S., Lam, D., and Nause, C. 1992. Microbial adhesion and biofilm formation on ureteral stents *in vitro* and *in vivo*. *J. Urol.* **148**, 1592–1594.

Roon, R.J. and Barker, H.A. 1972. Fermentation of agmatine in *Streptococcus faecalis*: occurrence of putrescine transcarbamoylase. *J. Bacteriol.* **109**, 44–50.

Sabbuba, N., Hughes, G., and Stickler, D.J. 2002. The migration of *Proteus mirabilis* and other urinary tract pathogens over Foley catheters. *BJU Int.* **89**, 55–60.

Sava, I.G., Heikens, E., and Huebner, J. 2010. Pathogenesis and immunity in enterococcal infections. *Clin. Microbiol. Infect.* **16**, 533–540.

Schmidtchen, A., Frick, I.M., Andersson, E., Tapper, H., and Bjorck, L. 2002. Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol. Microbiol.* **46**, 157–168.

Shankar, N., Baghdayan, A.S., and Gilmore, M.S. 2002. Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis*. *Nature* **417**, 746–750.

Shankar, N., Baghdayan, A.S., Willems, R., Hammerum, A.M., and Jensen, L.B. 2006. Presence of pathogenicity island genes in *Enterococcus faecalis* isolates from pigs in Denmark. *J. Clin. Microbiol.* **44**, 4200–4203.

Sifri, C.D., Mylonakis, E., Singh, K.V., Qin, X., Garsin, D.A., Murray, B.E., Ausubel, F.M., and Calderwood, S.B. 2002. Virulence effect of *Enterococcus faecalis* protease genes and the quorum-sensing locus *fsr* in *Caenorhabditis elegans* and mice. *Infect. Immun.***70**, 5647–5650.

Singh, K.V., Nallapareddy, S.R., Nannini, E.C., and Murray, B.E. 2005. Fsr-independent production of protease(s) may explain the lack of attenuation of an *Enterococcus faecalis fsr* mutant versus a *gelE-sprE* mutant in induction of endocarditis. *Infect. Immun.* **73**, 4888–4894.

Sorsa, T., Ingman, T., Suomalainen, K., Haapasalo, M., Konttinen, Y.T., Lindy, O., Saari, H., and Uitto, V.J. 1992. Identification of proteases from periodontopathogenic bacteria as activators of latent human neutrophil and fibroblast-type interstitial collagenases. *Infect. Immun.* **60**, 4491–4495.

Takao, A., Nagashima, H., Usui, H., Sasaki, F., Maeda, N., Ishibashi, K., and Fujita, H. 1997. Hyaluronidase activity in human pus from which *Streptococcus intermedius* was isolated. *Microb. Immunol.* **41**, 795–798.

Tang, H. 2009. Regulation and function of the melanization reaction in Drosophila. *Fly* **3**, 105–111.

Teixeira, N., Varahan, S., Gorman, M.J., Palmer, K.L., Zaidman-Remy, A., Yokohata, R., Nakayama, J., Hancock, L.E., Jacinto, A., Gilmore, M.S., and de Fatima Silva Lopes, M. 2013. *Drosophila* host model reveals new *Enterococcus faecalis* quorum-sensing associated virulence factors. *PloS One* **8**, e64740.

Thomas, V.C., Hiromasa, Y., Harms, N., Thurlow, L., Tomich, J., and Hancock, L.E. 2009. A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of *Enterococcus faecalis*. *Mol. Microbiol.* **72**, 1022–1036.

Toto, P.D., Santangelo, M.V., and Madonia, J.V. 1968. Use of hyaluronic acid and chondroitin sulfate by bacterial isolates from carious dentin. *J. Dental Res.* **47**, 1056–1061.

Tunney, M.M. and Gorman, S.P. 2002. Evaluation of a poly(vinyl pyrollidone)-coated biomaterial for urological use. *Biomaterials* **23**, 4601–4608.

van Opijnen, T. and Camilli, A. 2010. Genome-wide fitness and genetic interactions determined by Tn-seq, a high-throughput massively parallel sequencing method for microorganisms. *Curr. Protoc. Microbiol.* **Chapter 1**, Unit1E 3.

Van Tyne, D., Martin, M.J., and Gilmore, M.S. 2013. Structure, function, and biology of the *Enterococcus faecalis* cytolysin. *Toxins* **5**, 895–911.

Vance, R.E., Isberg, R.R., and Portnoy, D.A. 2009. Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. *Cell Host Microbe* **6**, 10–21.

Vanek, N.N., Simon, S.I., Jacques-Palaz, K., Mariscalco, M.M., Dunny, G.M., and Rakita, R.M. 1999. *Enterococcus faecalis* aggregation substance promotes opsonin-independent binding to human neutrophils via a complement receptor type 3-mediated mechanism. *FEMS Immunol. Med. Microbiol.* **26**, 49–60.

Vebo, H.C., Solheim, M., Snipen, L., Nes, I.F., and Brede, D.A. 2010. Comparative genomic analysis of pathogenic and probiotic *Enterococcus faecalis* isolates, and their transcriptional responses to growth in human urine. *PLoS One* **5**, e12489.

Vogel, S.N. 2012. How discovery of Toll-mediated innate immunity in *Drosophila* impacted our understanding of TLR signaling (and vice versa). *J. Immunol.* **188**, 5207–5209.

Walhout, A.J.M. and Boulton, S.J. 2006. Biochemistry and molecular biology, The online review of *C. elegans* biology. *In* Walhout, A.J.M. and Boulton, S.J. (eds.), WormBook.

Waterfield, N.R., Wren, B.W., and Ffrench-Constant, R.H. 2004. Invertebrates as a source of emerging human pathogens. *Nat. Rev. Microbiol.* **2**, 833–841.

Waters, C.M., Antiporta, M.H., Murray, B.E., and Dunny, G.M. 2003. Role of the *Enterococcus faecalis* GelE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. *J. Bacteriol.* **185**, 3613–3623.

Yin, Q., Tian, Y., Kabaleeswaran, V., Jiang, X., Tu, D., Eck, M.J., Chen, Z.J., and Wu, H. 2012. Cyclic di-GMP sensing via the innate immune signaling protein STING. *Mol. Cell* **46**, 735–745.

Zhao, C., Hartke, A., La Sorda, M., Posteraro, B., Laplace, J.M., Auffray, Y., and Sanguinetti, M. 2010. Role of methionine sulfoxide reductases A and B of *Enterococcus faecalis* in oxidative stress and virulence. *Infect. Immun.* **78**, 3889–3897.

Zimmermann, S., Wagner, C., Muller, W., Brenner-Weiss, G., Hug, F., Prior, B., Obst, U., and Hansch, G.M. 2006. Induction of neutrophil chemotaxis by the quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone. *Infect. Immun.* **74**, 5687–5692.