

A simple model host for identifying Gram-positive virulence factors

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We demonstrate the use of the nematode *Caenorhabditis elegans* as a facile and inexpensive model host for several Gram-positive human bacterial pathogens. *Enterococcus faecalis*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*, but not *Bacillus subtilis*, *Enterococcus faecium*, or *Streptococcus pyogenes*, kill adult *C. elegans*. Focusing our studies on the enterococcal species, we found that both *E. faecalis* and *E. faecium* kill *C. elegans* eggs and hatchlings, although only *E. faecalis* kills the adults. In the case of adults, a low inoculum of *E. faecalis* grows to a high titer in the *C. elegans* intestine, resulting in a persistent infection that cannot be eradicated by prolonged feeding on *E. faecium*. Interestingly, a high titer of *E. faecium* also accumulates in the nematode gut, but does not affect the longevity of the worms. Two *E. faecalis* virulence-related factors that play an important role in mammalian models of infection, *fsr*, a putative quorum-sensing system, and cytolsin, are also important for nematode killing. We exploit the apparent parallels between Gram-positive infection in simple and more complex organisms by using the nematode to identify an *E. faecalis* virulence factor, *ScrB*, which is relevant to mammalian pathogenesis.

Gram-positive pathogens in the genera *Streptococcus*, *Staphylococcus*, and *Enterococcus* are leading causes of human infections, causing diseases such as pneumonia, meningitis, bacteremia, endocarditis, and necrotizing fasciitis. The genus *Enterococcus*, the main focus of this article, is particularly problematic because of multidrug resistance, including resistance to vancomycin, often the antibiotic of last resort. Nevertheless, only a limited number of enterococcal virulence-related factors have been described, including cytolsin (Cyl), a factor called aggregation substance (AS), a zinc metalloprotease (gelatinase), and *fsr*, a putative quorum-sensing system thought to be involved in gelatinase and/or serine protease regulation (1–5). As the development of antibiotic resistance continues to erode one of the greatest advances in modern health care, it is crucial to identify bacterial targets that can form the basis of novel anti-infective therapies.

One reason that relatively little is known about enterococcal virulence factors is that the mammalian models used to study enterococcal infections are cumbersome and expensive. Using a mammalian host to screen enterococcal mutant libraries for avirulent mutants, for example, would be prohibitively time consuming and expensive because of the large number of animals involved. Therefore, we have sought to develop alternative nonvertebrate hosts for *Enterococcus* and other Gram-positive human pathogens.

Previously, our laboratory and others have shown not only that the Gram-negative human pathogens *Pseudomonas aeruginosa* and *Salmonella enterica* kill the nematode *Caenorhabditis elegans*, but also that *P. aeruginosa* and *S. enterica* virulence factors required for mammalian pathogenesis also are required for efficient killing of *C. elegans* (6–10). In this article, we demonstrate the suitability of using *C. elegans* as a model host for Gram-positive infection. We show that clinical isolates of *En-*

terococcus faecalis, *Streptococcus pneumoniae*, and *Staphylococcus aureus* kill adult *C. elegans*, and that several features of the *E. faecalis*–*C. elegans* model that we have studied suggest that some aspects of human Gram-positive pathogenesis can be modeled successfully in a simple nonvertebrate host.

Materials and Methods

Assay of *C. elegans* Killing by Bacteria. Bacterial strains were grown on brain heart infusion (BHI) agar medium (Difco) in 35-mm tissue-culture plates (Falcon). Appropriate antibiotics were added to the medium to selectively prevent growth of *Escherichia coli*. Tetracycline (12.5 μ g/ml) was used for *E. faecalis* strains E001, E002, E006, and E009 and for *Enterococcus faecium* strains E003 and E007. Gentamicin (25–50 μ g/ml) was used for *E. faecalis* strains V583, OG1RF, and OG1X, and 5 μ g/ml polymyxin B (American Research Products, Belmont, MA) was used for the experiment shown in Fig. 1A with *S. aureus* A003, *E. faecium* E007, *E. faecalis* E002, *S. pneumoniae* SP003, *Streptococcus pyogenes* GAS002, and *Bacillus subtilis* PY79. Lawns of bacteria were grown as follows: 2 ml of BHI was inoculated with a single colony of the appropriate strain and grown at 37°C for 4–5 hr, and 10 μ l of the culture was spread on each plate. The plates were incubated at 37°C overnight. *Streptococcus* plates were grown in a 5% CO₂/95% air atmosphere.

C. elegans strain N2 was maintained and propagated on *Escherichia coli* strain OP50 with standard techniques (11). Between 20 and 30 *C. elegans* L4 or young adult hermaphrodites were transferred from a lawn of *Escherichia coli* OP50 to a lawn of the bacterium to be tested, incubated at 25°C, and examined at \approx 24-hr intervals with a dissecting microscope for viability. Worms were considered dead when they did not respond to touch with a platinum wire pick. Each experimental condition was tested in duplicate or triplicate. Nematode survival was plotted with the Kaplan–Meier method using STATA 6 statistical software (Stata, College Station, TX). The same software was used for testing equality of survival (log-rank and Wilcoxon tests). *P* values of <0.05 were considered statistically significant.

Measuring the Number of Bacteria Within the *C. elegans* Digestive Tract.

Five *C. elegans* were picked at the times indicated in Fig. 2, and the surface bacteria were removed by washing the worms twice in 4- μ l drops of M9 medium on a BHI agar plate containing 25 μ g/ml gentamicin. The nematodes were placed in a 1.5-ml Eppendorf tube containing 20 μ l of M9 medium with 1% Triton X-100 and were mechanically disrupted by using a pestle. The volume was adjusted to 50 μ l with M9 medium containing 1% Triton X-100

Abbreviations: cfu, colony-forming units; Cyl, cytolsin; AS, aggregation substance; *ScrB*, sucrose-6-phosphate hydrolases.

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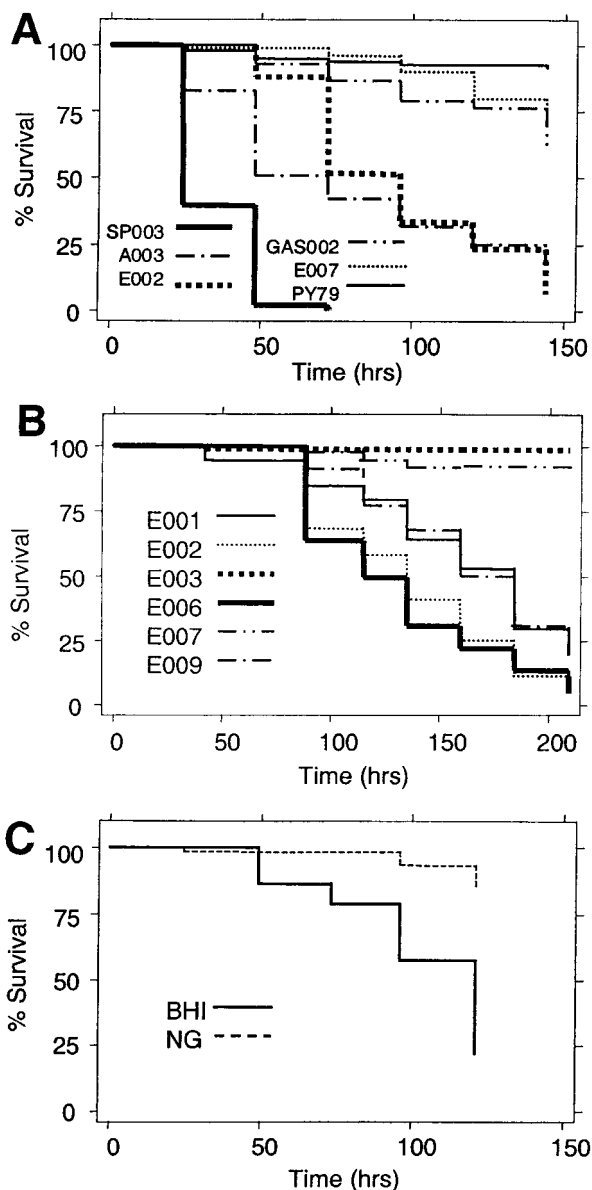


Fig. 1. *C. elegans* killing by Gram-positive pathogens. (A) Survival of *C. elegans* fed on clinical isolates of *S. pneumoniae* (SP003), *S. aureus* (A003), *E. faecalis* (E002), *S. pyogenes* (GAS002), *E. faecium* (E007), and nonpathogenic *B. subtilis* (PY79). (B) Survival of *C. elegans* fed on lawns of *E. faecalis* (E001, E002, E006, and E009) and *E. faecium* (E003 and E007) clinical isolates. (C) Survival of *C. elegans* fed on lawns of *Escherichia coli* strain OP50 grown on BHI medium or nematode growth (NG) medium.

which was diluted and plated on BHI agar containing 15 $\mu\text{g/ml}$ vancomycin, 50 $\mu\text{g/ml}$ ampicillin, or 12.5 $\mu\text{g/ml}$ tetracycline to select for strains V583, E007, or E003, respectively. Each data point represents the mean cfu from triplicate samples, and the error bars represent the standard deviation.

Microscopic Pictures of *C. elegans*. Nematodes were exposed for 3 days to bacteria on BHI plates and then placed on a pad of 2% agarose in a 5- μl drop of 30 mM NaN_3 in M9 medium. The worms were examined at 100 \times magnification with an Axioplan2 microscope (Zeiss) with Nomarski optics.

Screening for *E. faecalis* Tn917 Mutants Attenuated in Nematode Killing. A library consisting of $\approx 1,000$ OG1RF Tn917 insertion mutants was constructed as described (12). The mutants were

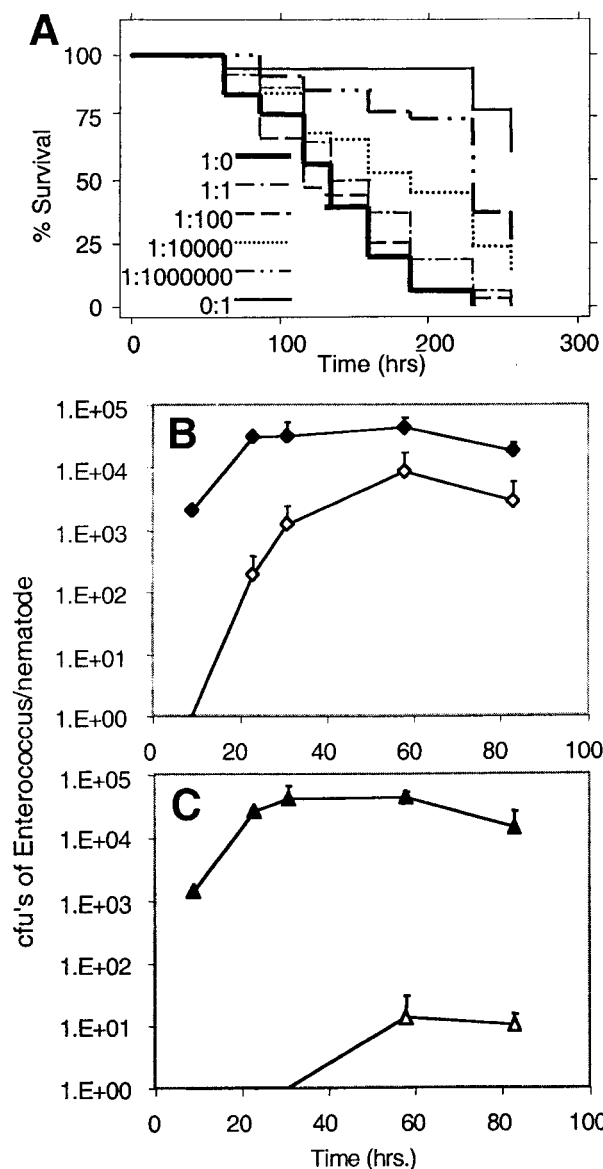


Fig. 2. *E. faecalis* proliferates in the nematode gut. (A) Various ratios of *E. faecalis* V583 to *E. faecium* E007 were plated to form bacterial lawns. The survival of *C. elegans* fed on these lawns was measured. (B) *C. elegans* were placed on a lawn of *E. faecalis* V583 and *E. faecium* E007 in the ratio of 1:1,000. After 12 hr, the *C. elegans* were washed and moved to a plate of E007 (time 0). At the times indicated, nematodes were washed and ground, and dilutions of the resulting suspension were plated on selective media. The number of colony-forming units (cfu) of V583 (\diamond) and E007 (\blacklozenge) per worm was calculated. (C) The same protocol as described for B was followed except that two *E. faecium* strains with different drug sensitivities were used. *C. elegans* were initially plated on a lawn of E003 (\triangle) and E007 (\blacktriangle) in a ratio of 1:1,000. Note that the error bars correspond to the standard deviations calculated from three experiments and are present on only the upper side of the curves because of the logarithmic scale.

assayed for their ability to kill *C. elegans* as described above, except that only eight worms were placed on each lawn, and the number of live worms was determined only on days 2 and 6. Mutants that caused less than average killing were tested twice more with the standard assay to confirm the attenuated phenotype.

Mouse Peritonitis Model. For the experiment shown in Fig. 4C, inoculum preparation, colony counts, and use of animals were

carried out as described (4), except that each inoculum was diluted a final 1:10 in 35% sterile rat fecal extract (SRFE; ref. 13). In the experiment shown in Fig. 4D, the cells were resuspended in saline to an optical density of 1.7 to 2.0 at 600 nm and diluted 1:10 in 40% SRFE. ICR-CD1 mice (Charles River Breeding Laboratories) were used. Survival curves were plotted and analyzed as described above for nematode killing. Guidelines of the Animal Welfare and the Animal Care Committees of the University of Texas Health Science Center and the Institutional Animal Care and Use Committee of Massachusetts General Hospital, respectively, were followed throughout the course of the animal experiments.

Results

***E. faecalis*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* Kill Adult *C. elegans*.** We tested whether Gram-positive clinical isolates obtained from the Massachusetts General Hospital Clinical Laboratory could kill *C. elegans* as described in *Materials and Methods*. All strains of *E. faecalis*, *S. aureus*, and *S. pneumoniae* tested killed *C. elegans*. Representative data are shown in Fig. 1 A and B. The most virulent *S. pneumoniae* strains killed quickly, with an LT₅₀ (time for half of the worms to die) of ≈1 day. The most virulent *S. aureus* and *E. faecalis* strains had LT₅₀s of ≈2 days and 4 days, respectively (Fig. 1 A and B). In contrast, *B. subtilis* [strain PY79 (14)] and several clinical isolates of *Streptococcus pyogenes* and *E. faecium* did not cause significant killing (Fig. 1 A and B). Interestingly, *Escherichia coli* strain OP50, the food source used by most laboratories studying *C. elegans* (11), caused significant killing when grown on BHI (LT₅₀ = 6 days; Fig. 1C). Perhaps the rich BHI medium induces the expression of *Escherichia coli* factors that are lethal for *C. elegans* but are not expressed on the commonly used NG medium.

We studied the *E. faecalis*–*C. elegans* model in detail to investigate further the feasibility of using *C. elegans* as a model host for Gram-positive pathogens. We chose *E. faecalis*, in part, because we observed that both *E. faecalis* and *E. faecium* killed *C. elegans*' eggs and hatchlings in a manner that seems to be mechanistically distinct from the killing of the adult nematodes (data not shown). It was technically convenient that *C. elegans* failed to produce a brood on enterococcal lawns, as it was easier to keep track of the original adult nematodes. We used the previously studied *E. faecalis* isolates V583 (15) (recently sequenced and available at www.tigr.org) and OG1RF (16) for most of the following experiments because they killed *C. elegans* at approximately the same rate as the most virulent new isolates that were tested.

A Small Initial Inoculum of *E. faecalis* Can Proliferate in the Nematode Gut. To determine whether a relatively small number of *E. faecalis* cells could kill *C. elegans*, we took advantage of the fact that *E. faecium* did not kill *C. elegans* under the conditions tested. L4 *C. elegans* were placed on mixed lawns of *E. faecalis* and *E. faecium* in ratios of 1:1, 1:10², 1:10⁴, and 1:10⁶. As shown in Fig. 2A, even at a ratio of 1:10⁶ *E. faecalis* to *E. faecium* cells, a significant amount of killing of *C. elegans* was observed compared with feeding on 100% *E. faecium*. Indeed, there was no diminution in killing until the *E. faecalis* to *E. faecium* ratio was less than 1:10². In general, as the percentage of *E. faecalis* decreased, we observed an increasingly longer lag time before killing eventually commenced. We hypothesized that the lag in killing corresponded to the time required for a small initial inoculum of *E. faecalis* that the nematodes had ingested to proliferate in the *C. elegans* intestine. To test this hypothesis, *C. elegans* were fed on a mixed lawn of *E. faecalis* and *E. faecium* in the ratio of 1:10³. After 12 hr, the worms were transferred to a lawn consisting only of *E. faecium*. At various times, the nematodes were washed to remove bacteria from their surfaces and then disrupted to recover bacteria from inside their digestive

tracts. By plating on appropriate selective media, *E. faecalis* and *E. faecium* cfu could be distinguished readily. As shown in Fig. 2B, the number of *E. faecalis* cfu within the nematode gut increased over the course of a couple of days from undetectable levels to about 10⁴ bacteria per worm. This latter result indicates that *E. faecalis* not only proliferates in the *C. elegans* intestine but also can cause a persistent infection.

Surprisingly, as shown in Fig. 2B, we found that the worms also had a stable population of about 10⁵ *E. faecium* cells in their intestines during the course of the experiment, although *E. faecium* does not cause death. In contrast, when *C. elegans* feeds on *Escherichia coli*, essentially no live bacteria can be recovered from the intestine (6, 10). Thus, in the experiment shown in Fig. 2B, whereas the number of *E. faecium* cells per worm remained approximately constant, the ratio of *E. faecalis* to *E. faecium* increased from less than 1:10³ to 1:10. In other similar experiments, the ratio reached 1:1. The increase of *E. faecalis* within the gut is not simply a reflection of *E. faecalis* overgrowing the bacterial lawn on which the *C. elegans* were feeding. Random sampling of the lawns showed that the ratio of *E. faecalis* to *E. faecium* never exceeded 1:10³ (data not shown).

To investigate the accumulation of *E. faecium* in the *C. elegans* intestine further, we carried out a transfer experiment with the *E. faecium* strains E003 and E007, which can be distinguished by their antibiotic resistance phenotypes, plated at an initial ratio of 1:10³ E003 to E007 cells. When the worms on these plates were transferred to a lawn of only E007 cells, the ratio of E003 to E007 in the digestive tract never exceeded the initial ratio of 1:10³ (Fig. 2C). We also experimented with moving nematodes from a lawn of pure E003 to a lawn of pure E007. After 24 hr, most of the E003 cells had been cleared from the nematode's gut, suggesting that the high titer of *E. faecium* observed in the gut is not primarily a consequence of a persistent colonization (data not shown).

To confirm the observation of high titers of bacteria in the nematodes' intestines in the above experiments, we examined microscopically worms fed on *Escherichia coli*, *B. subtilis*, *E. faecalis*, and *E. faecium* lawns. As observed (6, 10), worms fed on *Escherichia coli* had no visible intact bacteria present in their intestinal lumen, and the lumen was slender in appearance (Fig. 3 A and B). In marked contrast, the lumens of worms fed on *E. faecalis* (Fig. 3 E and F) and *E. faecium* (Fig. 3 C and D) were full of intact bacteria, and the intestines were grossly distended. It is unlikely that the nematodes fail to grind *E. faecalis* and *E. faecium* efficiently because of their thick Gram-positive cell walls. Worms feeding on *B. subtilis* (strain PY79) contained no visible bacteria and had slender intestinal lumens (Fig. 3 G and H).

Cyl Causes Faster Killing of *C. elegans*. *E. faecalis* has many naturally occurring conjugative plasmids, some of which encode a Cyl that lyses both eukaryotic and prokaryotic cells (17) and functions as a virulence factor in mouse models of endophthalmitis (5, 18) and peritonitis (2, 4). These plasmids also produce a cell surface protein called AS that promotes plasmid transfer (17) and also is thought to play a role in pathogenesis by mediating *E. faecalis* binding to host tissues (19). There is a significant increase in mortality in a rabbit model of endocarditis when both AS and Cyl are expressed (1).

To determine whether Cyl or AS plays a role in *C. elegans* killing, we tested isogenic strains of *E. faecalis* OG1X (20) carrying mutant derivatives of pAD1 that encode both AS and Cyl. Plasmid pAM944 has a Tn917 disruption of the AS gene and is therefore Cyl⁺ AS⁻, whereas Tn917 disrupts the Cyl operon in pAM9058 (Cyl⁻ AS⁺). pAM714 has a nondisruptive Tn917 insertion (Cyl⁺ AS⁺) (1) which was used as a control. As shown in Fig. 4A, OG1X strains containing plasmid pAM714 (Cyl⁺ AS⁺) or pAM944 (Cyl⁺ AS⁻) killed *C. elegans* faster than OG1X ($P < 0.001$). There was no significant difference between OG1X

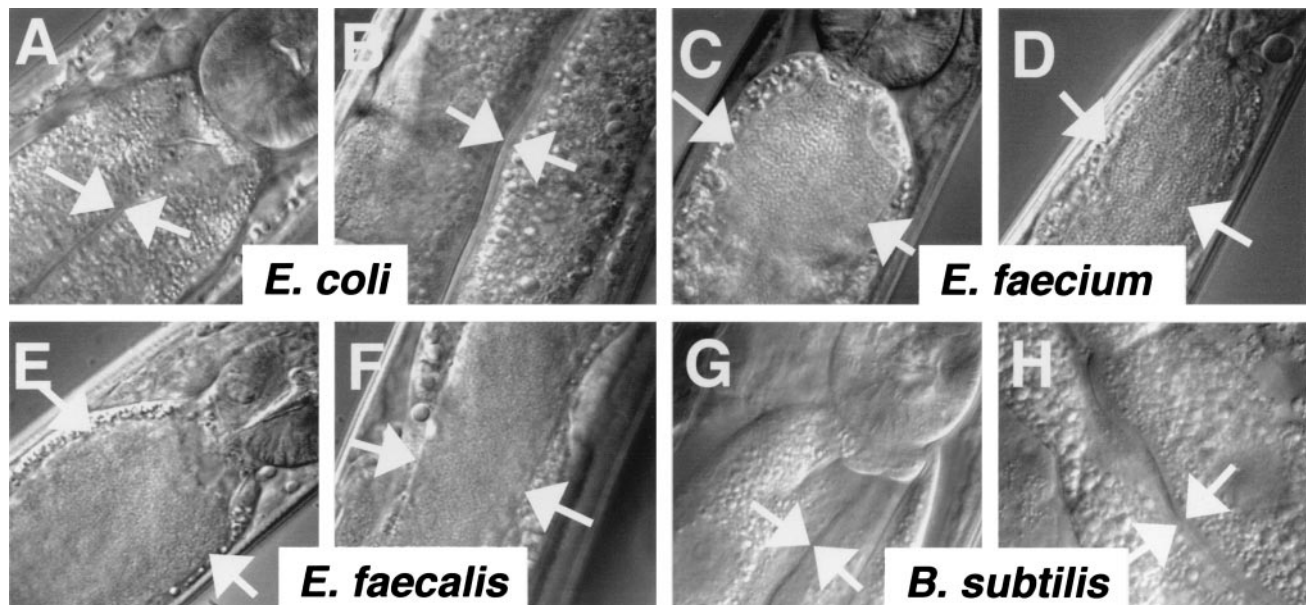


Fig. 3. Intact bacteria and gut distension visualized in *C. elegans* feeding on *E. faecalis* and *E. faecium* but not *Escherichia coli* or *B. subtilis*. Micrographs were taken of worms fed on lawns of bacteria on BHI plates for 3 days. Arrows point to the borders of the intestinal lumen. (A, E, C, and G) Beginning of intestinal tracts of worms fed on *Escherichia coli*, *E. faecalis*, *E. faecium*, and *B. subtilis*, respectively. In the upper right-hand corner of each image, a round structure is visible that corresponds to the grinder organ, which functions to disrupt ingested bacteria. (B, F, D, and H) Middle of the intestinal tracts.

containing no plasmids and OG1X containing pAM9058 (Cyl⁻AS⁺). These data suggest that Cyl is a virulence factor that affects *C. elegans* killing independently of the presence or absence of AS.

A Deletion in *fsrB* Causes Attenuation in the Mouse and the Nematode.

Previous work has shown that a disruption of *E. faecalis gelE*, which encodes gelatinase, causes attenuation of *E. faecalis* strain OG1RF in a mouse peritonitis model (4). Three genes located directly upstream of *gelE*—*fsrA*, *fsrB*, and *fsrC*—seem to be involved in *gelE* regulation. FsrA and FsrB are homologous to two component response regulators and sensor kinases (3), respectively, and a nonpolar *fsrB* deletion blocks the production of gelatinase (21). Based on the homology of FsrA, FsrB, and FsrC to the *S. aureus* quorum-sensing system encoded by *agrA*, *agrB*, and *agrC*, *fsrB* may encode a processor of a putative *E. faecalis* signal peptide (3).

Fig. 4B shows that *C. elegans* died much more slowly when placed on lawns of the *fsrBΔ* gelatinase regulatory mutant (strain TX5266) than when placed on lawns of the isogenic parental strain OG1RF ($P < 0.001$). *E. faecalis* OG1RF mutants containing disruptions in *fsrA*, *fsrB*, and *gelE* also caused prolonged survival of *C. elegans*, which was nearly identical to that observed with *fsrB* (data not shown). As shown in Fig. 4C, the *fsrBΔ* mutant is also less virulent in the mouse i.p. injection model ($P < 0.0009$).

An Avirulent *E. faecalis* Mutant Identified by Using *C. elegans* Is Also Attenuated in the Mouse.

In an attempt to identify previously unknown enterococcal virulence factors, we screened an *E. faecalis* transposon Tn917 library for mutants that were attenuated in killing nematodes. About 20 such mutants were identified, one of which, designated IC-C9, was studied in further detail. When IC-C9 was tested in the mouse i.p. infection model described above, the mice exhibited prolonged survival relative to the mice injected with parental strain OG1RF ($P < 0.0104$; Fig. 4D). DNA blot hybridization with a Tn917-specific probe indicated that mutant IC-C9 contains a single transposon insertion (data not shown). By using primers specific to the transpo-

son in combination with arbitrary primers, a DNA fragment flanking the transposon insertion was amplified (22) and sequenced, thereby identifying the gene in which Tn917 had been inserted. The translated sequence was highly homologous to sucrose-6-phosphate hydrolases (ScrB) of other bacterial species (48% identity and 64% similarity with ScrB of *Streptococcus sobrinus*).

Discussion

Several Gram-negative human pathogens, including *P. aeruginosa* and *S. enterica*, have been shown to kill *C. elegans* when presented to the nematodes as a source of food (6–8, 10). In this article, we demonstrate that Gram-positive human pathogens including *E. faecalis*, *S. aureus*, and *S. pneumoniae* also kill *C. elegans*, and that *E. faecium* and *S. pyogenes* do not exhibit significant killing under the conditions tested. We do not understand why some human pathogens kill *C. elegans* whereas others do not. Initially, we thought that human pathogens able to infect and kill *C. elegans* would tend to be unspecialized, opportunistic species such as *P. aeruginosa* and *E. faecalis*. However, in this article, we show that *S. pneumoniae*, with an ecological niche thought to be limited to the human nasal-pharynx, also kills *C. elegans*. As illustrated in Fig. 1C, one factor that has a critical effect on *C. elegans* killing is the medium used to grow the bacterial lawn. *Escherichia coli* does not kill *C. elegans* when grown on NG medium, but it is an efficient killer when grown on BHI medium. Thus, species such as *E. faecium* and *S. pyogenes*, which did not exhibit killing on BHI medium, may kill *C. elegans* if grown on the appropriate medium. The uses and limitations of *C. elegans* as a model host need to be clarified and defined with further research; we predict that the list of pathogens able to use *C. elegans* as a model host will expand as different conditions are tested.

In the case of *P. aeruginosa*, two mechanistically distinct types of *C. elegans* killing have been identified in our laboratory. “Slow-killing” requires live bacteria and occurs when *C. elegans* are fed *P. aeruginosa* PA14 grown on low-osmolarity minimal medium (10). In contrast, “fast-killing” occurs on high-osmolarity medium, does not require live bacteria, and has been

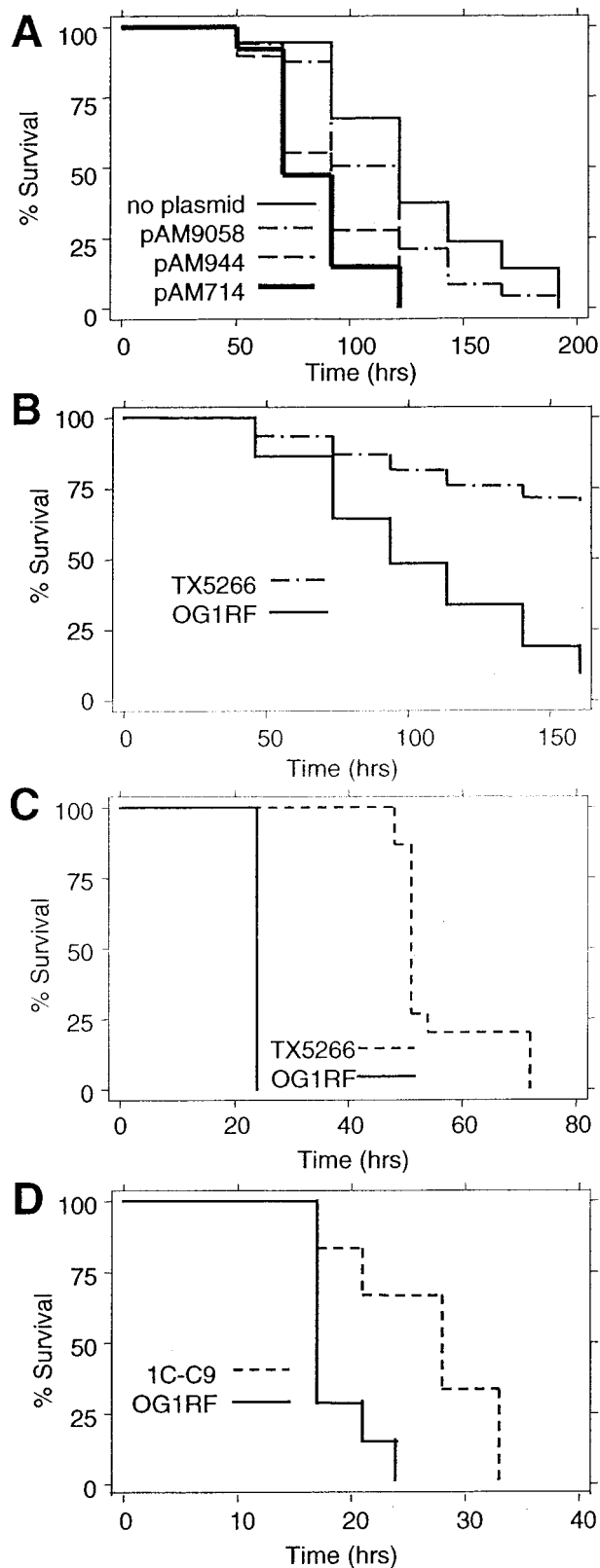


Fig. 4. Mammalian virulence factors enhance *E. faecalis* killing of *C. elegans*. (A) Survival of *C. elegans* placed on lawns of *E. faecalis* OG1X containing no plasmids (Cyl⁻, AS⁻), OG1X + pAM9058 (Cyl⁻ AS⁺), OG1X + pAM944 (Cyl⁺ AS⁻), and OG1X + pAM714 (Cyl⁺ AS⁺). (B) Survival of nematodes feeding on lawns of OG1RF or TX5266 (*fsrBΔ*). (C) Survival of mice infected peritoneally with 1.3×10^8 cfu of OG1RF or 2.1×10^8 cfu of TX5266 (*fsrBΔ*). (D) Survival of mice infected peritoneally with 2.20×10^8 cfu of OG1RF or 2.86×10^8 cfu of 1C-C9.

shown to be mediated by low molecular weight toxins (8). In the case of *E. faecalis*, secreted toxins are probably not the primary cause of killing adults, because antibiotic-treated *E. faecalis* did not kill (data not shown). With the exception of the *P. aeruginosa* toxin-mediated fast killing of worms, the mechanisms by which live *P. aeruginosa*, *S. enterica*, and *E. faecalis* kill worms is not clear. In all three cases, the worms do not appear to become rapidly paralyzed, as is the case in another *P. aeruginosa*-*C. elegans* model system involving *P. aeruginosa* strain PA01 (7).

When *C. elegans* are feeding on *Escherichia coli*, essentially no live bacteria can be recovered from and no intact green fluorescent protein-labeled bacteria can be detected in the digestive system of the nematodes. In contrast, *E. faecalis* not only accumulates in the intestinal lumen, reaching steady-state titers as high as 10^5 cfu per worm (6, 10), but as shown in Fig. 2B, proliferates in the gut, resulting in a long-lasting infection that persists for the entire lifespan of the worms. Similar results were obtained previously with *S. enterica* (6). Overgrowth of *E. faecalis* and *S. enterica* in the intestine of *C. elegans* to the animal's apparent detriment has many characteristics of a true infection. In contrast, although *P. aeruginosa* also accumulates in the *C. elegans* gut, it is not able to establish a persistent infection (10). Interestingly, as shown in Fig. 2B and Figs. 3C and D, *E. faecium* also accumulates to high titers in the intestinal lumen, although it does not kill adult worms and does not form a persistent infection (Fig. 2C).

P. aeruginosa and *S. enterica* containing mutations in well known virulence factors such as *lasR* and *phoPQ*, respectively, are less virulent in *C. elegans*, validating the use of *C. elegans* to identify and study virulence factors relevant for mammalian pathogenesis (6, 10). In this article, we show that two known enterococcal virulence factors, Cyl and FsrB, which have been shown to enhance pathogenesis in various mammalian model systems, also enhance the rate at which *C. elegans* are killed by *E. faecalis*. Cyl disrupts membranes of both eukaryotic and prokaryotic cells (23) and is thought to increase tissue damage in mammalian models of endocarditis (1). *fsrB* putatively encodes a peptide processor necessary for quorum sensing (3). Polar mutations in *fsrB* previously have been shown to increase the survival time for i.p.-infected mice (3), and in this article we show that a nonpolar deletion in *fsrB* increases survival time in both the mouse and the nematode. In the *C. elegans* model, because noncytolytic *E. faecalis* and a *fsrB* deletion mutant are significantly attenuated but remain lethal (Fig. 4), it seems that Cyl and FsrB contribute to, but are not sufficient for, *C. elegans* killing.

In the case of *P. aeruginosa* strain PA14, we used *TnphoA* mutagenesis to identify killing-related factors by simply screening for PA14 mutants that exhibited a decreased rate of killing. This procedure resulted in the discovery of a variety of virulence factors that also are relevant for mouse pathogenesis (8, 9, 24). As shown in Fig. 4D, our preliminary experiments show that *C. elegans* also can be used to identify *E. faecalis* mutants that are attenuated in a mammalian model. Specifically, we have found that a mutant with an insertion in the *scrB* gene is less virulent in both the mouse (Fig. 4D) and the nematode (data not shown) models. Although it is not clear whether there is an appreciable amount of sucrose in the mouse peritoneal cavity, sucrose utilization has been shown to play a role in the formation of dental caries and in endocarditis in the related bacterium *Streptococcus mutans*. Sucrose catabolites are used to synthesize insoluble glucans, exopolymers that may promote adherence to the tooth surface and to the heart valve (25, 26). An alternative explanation for these results is that the Tn917 insertion in *scrB* exerts a polar effect on a downstream virulence factor.

In contrast to Cyl, FsrB, and ScrB, *E. faecalis* AS seems to be a virulence factor only in mammalian infections. Because we have very little information concerning the mechanism by which

E. faecalis (as well as *P. aeruginosa* and *S. enterica*) kill *C. elegans*, we do not understand why some virulence factors important for mammalian infection play a role in *C. elegans* killing, whereas others do not. Another factor that limits our mechanistic understanding of nematode–bacterial interactions is the lack of information concerning the *C. elegans* innate immune response. Although *C. elegans* seem to have homologs of some Toll signaling pathway components important to innate immunity in organisms as diverse as humans and *Drosophila melanogaster*, experiments to date have failed to demonstrate a significant role for the Toll pathway in a *C. elegans* defense response (27). We can hypothesize that shared virulence factors exert their effect by acting on conserved targets shared among diverse organisms. Conversely, virulence factors that have an effect in one species, but not in another, may be targeting factors specific to that species. Therefore, the use of *C. elegans* as a model host for mammalian pathogenesis likely will be limited to studying virulence factors involved in targeting evolutionarily conserved cellular mechanisms.

In conclusion, we have shown that the clinically important Gram-positive pathogens *E. faecalis*, *S. aureus*, and *S. pneu-*

moniae kill adult *C. elegans*. Killing of adult *C. elegans* by *E. faecalis* has characteristics of an infectious process including colonization and proliferation of a small inoculum of bacteria. We show that two previously characterized enterococcal factors necessary for virulence in mammalian models, Cyl and FsrB, a protein that seems to be involved in quorum sensing, increase the rate of *C. elegans* killing. Finally, we provide evidence that *C. elegans* may be a useful model host for identifying novel Gram-positive virulence factors by showing that at least one *E. faecalis* mutation found in an initial screen for attenuation in *C. elegans* killing seems to prolong survival in a mouse model.

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