Virulence of *Enterococcus faecalis* dairy strains in an insect model: the role of *fsrB* and *gelE*

Frédéric Gaspar,¹ Neuza Teixeira,¹ Lionel Rigottier-Gois,² Paulo Marujo,¹ Christina Nielsen-LeRoux,³ Maria Teresa Barreto Crespo,¹ Maria de Fátima Silva Lopes¹ and Pascale Serror²

Correspondence

Pascale Serror
pascale.serror@jouy.inra.fr

¹IBET and ITQB-UNL, Quinta do marquês, Av da República, 2781-901 Oeiras, Portugal

²INRA-U888-UBLO, Domaine de Vilvert, 78352 Jouy en Josas cedex, France

³INRA-U1249-GME, Domaine de la Minière, 78285 Guyancourt, France

Despite the existence of various virulence factors in the *Enterococcus* genus, enterococcal virulence is still a debated issue. A main consideration is the detection of the same virulence genes in strains isolated from nosocomial or community-acquired infections, and from food products. The goal of this study was to evaluate the roles of two well-characterized enterococcal virulence factors, Fsr and gelatinase, in the potential virulence of *Enterococcus faecalis* food strains. Virulence of unrelated *Enterococcus* isolates, including dairy strains carrying *fsr* and *gelE* operons, was compared in the *Galleria mellonella* insect model. *E. faecalis* dairy strains were able to kill larvae and were as virulent as strain OG1RF, one of the most widely used for virulence studies. In contrast, *Enterococcus durans* and *Enterococcus faecium* strains were avirulent or poorly virulent for *G. mellonella*. To evaluate the role of *fsrB* and *gelE* in virulence of *E. faecalis* dairy strains, both genes were deleted independently in two strains. The $\Delta fsrB$ and $\Delta gelE$ deletion mutants both produced a gelatinase-negative phenotype. Although both mutations significantly attenuated virulence in *G. mellonella*, the $\Delta fsrB$ strains were more strongly attenuated. These results agree with previous findings suggesting the involvement of *fsrB* in the control of other cell functions relevant to virulence. Our work demonstrates that the presence of functional *fsrB*, and to a lesser extent *gelE*, in dairy enterococci should be considered with caution.

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INTRODUCTION

Enterococcus is a peculiar and controversial genus of Gram-positive lactic acid bacteria. It includes commensal species that inhabit the gastrointestinal tracts of humans and animals, which can be used as starter cultures in food fermentations, as animal health supplements and/or as probiotics. However, they are also capable of causing opportunistic infections, including bacteraemia, endocarditis, meningitis, and wound, urinary tract and nosocomial bloodstream infections. Owing to their robustness, these bacteria are able to contaminate and maintain viability in diverse environments such as soil, sand, water, plants and food (Mundt, 1986). In the particular case of fermented food products, enterococci belong to the non-starter flora, although they have been proposed as starters for the production of certain cheeses and other fermented milk products (Aarestrup *et al.*, 2002). They are of particular relevance for traditionally made cheeses in south European countries, where they play an important role in the

ripening process, through proteolysis, lipolysis and citrate metabolism, therefore contributing to the organoleptic characteristics of the product.

Enterococcus faecalis is the predominant species in human/animal-associated environments, and therefore the most studied species of this genus (Tannock & Cook, 2002). *E. faecalis*, together with *Enterococcus faecium*, are also the most frequently found species in dairy food products, with varying prevalence as a function of the country and cheese type (Aarestrup *et al.*, 2002). *E. faecalis* is also responsible for up to 80 % of enterococcal-associated nosocomial infections, followed by *E. faecium*. One explanation for the over-representation of *E. faecalis* among clinical isolates may relate to its natural abundance, or to the presence of virulence factors (Hancock & Gilmore, 2000). Although harmless in healthy individuals, enterococcal clinical isolates become pathogenic in patients in intensive care units and in hospitalized patients with impaired immune systems. This has been associated with a variety of virulence factors carried by *E. faecalis* (Ogier & Serror, 2008).

One of the most studied *E. faecalis* virulence factors is gelatinase, a metalloprotease able to degrade several

The GenBank/EMBL/DBJ accession number for the *fsr*–*gelE* region sequence of *E. faecium* QS32 is FJ858146.

substrates, such as gelatin, casein, haemoglobin and other bioactive peptides, including *E. faecalis* sex pheromones (Kayaoglu & Orstavik, 2004). Gelatinase is encoded by *gelE*, which is in an operon with *sprE*, which encodes a serine protease (Qin *et al.*, 2000). Its phenotypic expression requires the regulatory system encoded by the *fsrABC* operon (Lopes *et al.*, 2006; Qin *et al.*, 2000), recently renamed *fsrABDC* (Nakayama *et al.*, 2006). *fsr* is homologous to the *agr* quorum sensing system of *Staphylococcus aureus*, and has also been implicated in virulence of *E. faecalis* independently of *gelE* (Bourgogne *et al.*, 2006). The role of *gelE* and *fsr* loci in *E. faecalis* virulence has been demonstrated in different mammalian infection models (Mohamed & Murray, 2006), in the *Caenorhabditis elegans* nematode model (Sifri *et al.*, 2002) and in the *Arabidopsis thaliana* plant model (Jha *et al.*, 2005). More recently, gelatinase has been implicated in evasion of the immune system of the insect *Galleria mellonella*, suggesting that gelatinase may participate in virulence in this system (Park *et al.*, 2007). Screening of both *gelE* and *fsr* in *E. faecalis* isolates from different origins revealed their dissemination in fermented milk products (Lepage *et al.*, 2006; Lopes *et al.*, 2006; Semedo *et al.*, 2003). However, the roles for *Fsr* and gelatinase in the potential virulence of *E. faecalis* food strains have never been established. In the present study, two gelatinase-positive *E. faecalis* dairy strains carrying *gelE* and the complete *fsr* operon, and their respective isogenic *gelE* and *fsrB* deletion mutants, were tested for virulence in the *G. mellonella* infection model.

METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains used in this study are listed in Table 1. Enterococci were grown in M17 broth supplemented with 0.5% (w/v) glucose (M17BGlucose) or M17 agar supplemented with 0.5% glucose (M17AGlucose) at 37 °C without aeration. *Escherichia coli* strains were grown aerobically in

Luria–Bertani broth or on LB agar at 37 °C. Antibiotics were used at the following concentrations: erythromycin, 30 µg ml⁻¹ for *Enterococcus faecalis* and 150 µg ml⁻¹ for *Escherichia coli*; ampicillin, 80 µg ml⁻¹.

General DNA techniques. General molecular biology techniques were performed by standard methods (Sambrook *et al.*, 1989). Restriction enzymes, polymerases and T4 DNA ligase were used according to manufacturers' instructions. PCR amplification was performed using a Biometra or Eppendorf thermocycler. When necessary, PCR products and DNA restriction fragments were purified with QIAquick purification kits (Qiagen) or Montage Life Science kits (Millipore). Plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen). Electrotransformation of *Escherichia coli* and *Enterococcus faecalis* was carried out as described by Dower *et al.* (1988) and Dunny *et al.* (1991), using a Gene Pulser apparatus (Bio-Rad). Plasmid inserts were sequenced at Baseclear.

Construction of in-frame *gelE* and *fsrB* deletion mutants of strains QA29B and LSE4a Markerless *gelE* and *fsrB* deletion mutants of *E. faecalis* were constructed essentially as described by Brinster *et al.* (2007). Briefly, 5' and 3' flanking regions of *fsrB* and *gelE* were amplified from chromosomal DNA of each strain by PCR with primers OEF-232, OEF-233, OEF-234 and OEF-235, and OEF-236, OEF-237, OEF-238 and OEF-239, respectively (Table 2). The two cognate PCR fragments were fused by PCR using the external primers OEF232 and OEF235, and OEF-236 and OEF-239 for *fsrB* and *gelE*, respectively, and the resulting product was cloned into pGEM-T (Promega). The inserted PCR fragment was removed from its cloning vector by restriction enzymes and subsequently cloned into pG+host9 plasmid (Maguin *et al.*, 1996), which was then electroporated into *E. faecalis*. The *fsrB* and *gelE* single- and double-crossover mutants were selected as described by Brinster *et al.* (2007). Successful targeted mutations of *fsrB* and *gelE* in strains LSE4a and QA29B were first identified by PCR screening and were confirmed by Southern blot analysis.

Gelatinase activity assay. The phenotypic assay of gelatinase activity was performed as described by Lopes *et al.* (2006). Briefly, *Enterococcus* strains were grown on agar plates containing 3% (w/v) gelatin (Oxoid) and flooded with a saturated solution of ammonium sulfate (Merck). A transparent halo around cells indicated gelatinase activity.

Table 1. Strains used in this study

Strains	Relevant characteristics	Reference or source
<i>Escherichia coli</i> DH5α	F ⁻ φ80dlacZΔM15 Δ(lacZYA-argF)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44 λ⁻ thi-1 gyrA96 relA1</i>	Grant <i>et al.</i> (1990)
<i>Escherichia coli</i> TG1 <i>repA</i>	<i>supE hsdD5 thi</i> (Δ <i>lac-proAB</i>) F ⁻ (<i>traD36 proAB-lacZ</i> ΔM15) <i>repA</i>	Law <i>et al.</i> (1995)
OG1RF	Fus ^r Rif ^r , plasmid free wild-type strain, Gel ⁺	Dunny <i>et al.</i> (1978)
QA29B	Wild-type <i>E. faecalis</i> ; isolated from cheese, Gel ⁺	This study
LSE4a	Wild-type <i>E. faecalis</i> ; isolated from milk, Gel ⁺	This study
LN68	Wild-type <i>E. faecalis</i> ; isolated from milk, Gel ⁺	Lopes <i>et al.</i> (2006)
QSE32	Wild-type <i>E. faecium</i> ; isolated from cheese, Gel ⁺	Alves <i>et al.</i> (2004)
QN1	Wild-type <i>E. durans</i> ; isolated from milk, Gel ⁻	Ribeiro <i>et al.</i> (2007)
TX5264	<i>gelE</i> in-frame deletion in OG1RF, Gel ⁻	Sifri <i>et al.</i> (2002)
TX5266	<i>fsrB</i> in-frame deletion in OG1RF, Gel ⁻	Qin <i>et al.</i> (2001)
QA29BΔ <i>fsrB</i>	<i>fsrB</i> in-frame deletion in QA29B, Gel ⁻	This study
QA29BΔ <i>gelE</i>	<i>gelE</i> in-frame deletion in QA29B, Gel ⁻	This study
LSE4aΔ <i>fsrB</i>	<i>fsrB</i> in-frame deletion in LSE4a, Gel ⁻	This study
LSE4aΔ <i>gelE</i>	<i>gelE</i> in-frame deletion in LSE4a, Gel ⁻	This study

Table 2. Primers used in this study

Primer name	Sequence (5'–3')*	Position of the primer 5' end	Reference
OEF-232	GAAAGGGATGAGTGAACAAATG	bp 839 upstream of the <i>fsrB</i> start codon	Sifri <i>et al.</i> (2002)
OEF-233	CTTTGTCCATTGTGTTTTCTCTG	bp 78 in <i>fsrB</i>	Sifri <i>et al.</i> (2002)
OEF-234	<u>CAGGAAAAACACAATGGACAAAGTGGATGGGACAAA-</u> <u>CTGAAAAACC</u>	bp 54 before the <i>fsrB</i> stop codon	This study
OEF-235	TAGCCAACAAACGAATCACAACC	bp 1020 after the <i>fsrB</i> stop codon	Sifri <i>et al.</i> (2002)
OEF-236	CTAAAAGTGATTGTTGATGTGC	bp 639 upstream of the <i>gelE</i> start codon	Qin <i>et al.</i> (2001)
OEF-237	ATCAACAGTAACGCCTTCC	bp 258 in <i>gelE</i>	Qin <i>et al.</i> (2001)
OEF-238	<u>GGAAGGCGTTACTGTTGATATT</u> CAGGTAAACC- AACCAAGTG	bp 45 upstream of the <i>gelE</i> stop codon	This study
OEF-239	GATTATTTGCCTTCTTTTCAGC	bp 1047 after <i>gelE</i> stop codon	Qin <i>et al.</i> (2001)
sprE_2	TCAAACAAACGAAACTGGC	1 761 697†	This study
sprE3'	TGCCTTCTTTTCAGC	1 761 921	
sprE_3	CATTCTTAAACTTTTCAGCCAC	1 762 591	
gelE3'	CAAGCTAAAACCGGC	1 762 832	
msprE	TAACCTTGATCGCCGG	1 762 858	
gelE	TCATTCAATGACCAG	1 762 961	
sprE5'	TGAATCTGTTCTGGTC	1 762 986	
msprE_3	AAAGTTTGAACTTTCAGATACCC	1 763 120	
msprE_2	AAAGTTTGAACTTTCAGATACCC	1 763 120	
gelE_3	CAACACTCTGAGTATCCGCACC	1 763 362	
mgelE_2	AACGGATAACACAGGGG	1 763 829	
gelE_2	GTGTAAAGCAATTCCCG	1 763 831	
mgelE_3	TCTTCGCCAACTGGTGACC	1 764 008	
fsrC3'	TTGCTTTATCCTCCC	1 764 545	
mgelE	AATATTTACGCAGGG	1 764 605	
gelE5'	TTGAGTTATGAGGGG	1 764 700	
fsrC_3	TTTCTTTTACATATAACAATCCCC	1 764 840	
mfsrC_2	TGAAGAAACGATTGCACCAACC	1 765 263	
fsrC_2	TTTATAATCATGACGAAACATCGC	1 765 328	
mfsrC_3	TTTTTGTTGTTTTGATTTTCGCC	1 765 573	
fsrB3'	AACATTAATGCCGC	1 765 917	
mfsrC	TTTGTTTCGTTTGCGGC	1 765 942	
fsrB	GTCCAAATATATTGGGC	1 766 115	
fsrC5'	GACAATGGATGGGAC	1 766 118	
fsrA3I	TTCGCTTAACGTCCC	1 766 635	
mfsrB	TTGAAGAGGAGGGCG	1 766 814	
fsrB5'	TTACTTAGGGAGGG	1 766 893	
fsrA_2	CTAGGAAAAAGATATTAGTTGGGC	1 767 442	
mfsrA	ATGAGTGAACAAATGGC	1 767 638	
fsrA5'	GTTTTTGTTCAGG	1 767 788	
mfsrA_2	CAAGAACAGTTGGCGGTTG	1 767 908	

*Sequences added for fusion PCR are underlined.

†Position of the primer 5' end in the V583 genome.

G. mellonella mortality assay. *G. mellonella* eggs were hatched at 25 °C, and the larvae were reared on bee's wax and pollen (Naturalim) until the last instars which were used for the infection experiments. *Enterococcus* strains were grown in M17BGl and collected by centrifugation 1 h after they had reached stationary phase. Bacterial cells were washed with 0.9 % saline solution and stored as a dry, frozen pellet at –80 °C. Before inoculation, the frozen bacterial pellet was suspended in 1 ml saline solution and serial dilutions were plated on M17AGlu plates in order to determine the bacterial count of the pellet.

Groups of 20–30 *G. mellonella* larvae, starved for 24 h and weighing about 200 mg, were injected at the base of the last proleg with 10 µl

each bacterial inoculum ($\sim 2 \times 10^8$ cells ml⁻¹) using a microinjector (KDS 100; KD Scientific) with a 1 ml syringe and 0.45 × 12 mm needles (Terumo). A control group of larvae received saline solution only. The size of the inoculum was confirmed by determining the number of c.f.u. on M17AGlu. Five infected larvae were kept per Petri dish, without food, at 37 °C and survival was monitored every 24 h for 2–5 days, depending on the experiment. Experiments were repeated at least three times. The mortality rate was compared using a two-tailed unpaired *t*-test at the 95 % confidence interval. Survival curves were constructed by the Kaplan–Meier method and compared by log-rank analysis (GraphPad Prism, version 4.0; GraphPad Software). *P*-values of <0.05 were considered statistically significant.

Sequence analysis of the *fsr*–*gelE* region of *E. faecium* QSE32.

PCR amplification of overlapping fragments of the *fsr*–*gelE* region was carried out using Expand High Fidelity DNA polymerase (Roche) and the primers indicated in Table 2. Sequencing was performed at Baseclear. The sequence was analysed using Vector NTI 10.3.0 (Invitrogen) and the final DNA sequence, and the deduced protein sequence were analysed using Vector NTI 10.3.0 (Invitrogen) and BLAST from the NCBI website (<http://blast.ncbi.nlm.nih.gov/>).

RESULTS**Killing of *G. mellonella* by isolates of *E. faecalis* and other enterococcal strains**

Increasing interest in using *G. mellonella* as a surrogate model to study virulence of various micro-organisms led us to compare virulence of enterococcal strains. We determined whether strains of enterococci other than *E. faecalis* GM could kill *G. mellonella* (Park *et al.*, 2007). Mortality rates at 48 h post-infection are presented in Fig. 1. All *E. faecalis* strains tested (LN68, V583, LSE4a, OG1RF and QA29B) were able to kill between 60 and 98 % of *G. mellonella* larvae with inocula of about 2×10^6 c.f.u. The most virulent *E. faecalis* strains for *G. mellonella* were the clinical strain V583 and milk isolates LN68 and LSE4a. Maximum killing by LN68 was reached within 24 h (data not shown), indicating that this isolate was more rapidly lethal for *G. mellonella*. The clinical strain OG1RF and the

Fig. 1. Killing of *G. mellonella* larvae by various enterococci isolates. Percentage mortality of *G. mellonella* larvae at 48 h post-infection with *E. faecalis* strains LN68 ($2.07 \times 10^6 \pm 0.5 \times 10^6$ c.f.u.), V583 ($1.67 \times 10^6 \pm 0.06 \times 10^6$ c.f.u.), LSE4a ($2.45 \times 10^6 \pm 0.13 \times 10^6$ c.f.u.), OG1RF ($2.0 \times 10^6 \pm 0.2 \times 10^6$ c.f.u.) and QA29B ($2.07 \times 10^6 \pm 0.11 \times 10^6$ c.f.u.), *E. durans* strain QN1 ($1.94 \times 10^6 \pm 0.19 \times 10^6$ c.f.u.) and *E. faecium* strain QSE32 ($1.8 \times 10^6 \pm 0.1 \times 10^6$ c.f.u.). Larvae were infected with $\sim 2 \times 10^6$ bacteria as indicated in parentheses. Data were obtained from three independent experiments and are expressed as the mean values $\pm$ SD. Asterisks indicate a significant difference (** $P < 0.005$, *** $P < 0.0005$) relative to the LN68 strain.

cheese isolate QA29B were significantly less virulent with ~ 72 and ~ 60 % killing after 48 h, respectively. Interestingly, *E. durans* strain QN1 and *E. faecium* strain QSE32 did not kill *G. mellonella* larvae significantly. These data show that *E. faecalis* strains from both clinical and food origin have the ability to kill *G. mellonella* larvae, whereas *E. durans* and *E. faecium* species seem to be avirulent or of low virulence for *G. mellonella* under the conditions used.

Gelatinase and Fsr affect *E. faecalis* virulence in *G. mellonella*

We asked whether the *G. mellonella* model could discriminate between the roles of *gelE* and *fsrB* in virulence, using OG1RF and isogenic strains TX5264 and TX5266 (kindly provided by B. Murray) deleted for *gelE* and *fsrB*, respectively. Larvae were infected with $\sim 2 \times 10^6$ c.f.u. OG1RF, TX5264 and TX5266. As shown in Fig. 2, the three strains have similar killing rates of *G. mellonella* at 48 h. However, killing was significantly delayed for larvae injected with the Δ *gelE* strain (TX5264); 50 % of the larvae were dead after 24 h, compared to 80 % of those injected with OG1RF or TX5266 ($P < 0.05$). This result indicates that *gelE*, but not *fsrB*, contributes to OG1RF virulence in the *G. mellonella* infection model.

To study the role of *gelE* and *fsrB* in the potential virulence of *E. faecalis* food isolates, we collected strains QA29B and LSE4a in two distant areas of cheese production in Portugal (Semedo *et al.*, 2003; Lopes *et al.*, 2006). According to PFGE and MLST analysis, these two isolates are not genetically related (data not shown). Besides the *fsrABDC* and *gelE*–*sprE* operons, both strains carried the virulence

Fig. 2. Role of *E. faecalis* gelatinase in killing of *G. mellonella* larvae. Survival of *G. mellonella* after injection of *E. faecalis* strains OG1RF (2.1×10^6 c.f.u. per larva), Δ *gelE* (TX5264, 1.89×10^6 c.f.u. per larva) and Δ *fsrB* (TX5266, 1.71×10^6 c.f.u. per larva). Larva infection doses are indicated in parentheses. ♦, OG1RF; ○, Δ *gelE*; □, Δ *fsrB*. One representative experiment of three independent experiments is shown.

genes *agg*, *esp* and *efaAfs*, none of which was haemolytic (results not shown). We successfully generated independent in-frame *gelE* and *fsrB* deletion mutants by allelic exchange in *E. faecalis* QA29B and LSE4a, as described by B. Murray and co-workers (Qin *et al.*, 2001; Sifri *et al.*, 2002). As expected, the resulting $\Delta fsrB$ and $\Delta gelE$ deletion mutants failed to produce detectable gelatinase in a standard plate assay (data not shown). Next, their virulence was examined using the *G. mellonella* infection model. As for OG1RF, inactivation of the *gelE* gene in the two strains significantly decreased killing of *G. mellonella* larvae; 65 and 32 % of larvae infected with the *gelE* mutants were dead after 48 h, compared to 88 and 60 % of those infected with LSE4a and QA29B, respectively ($P < 0.05$). Moreover, inactivation of *fsrB* in LSE4a and QA29B clearly reduced virulence compared to the isogenic WT strains (Fig. 3). After 48 h post-infection, the QA29B $\Delta fsrB$ mutant killed 15 % of the *G. mellonella* larvae, versus 60 % killing by the isogenic WT strain. Thus, in contrast to results in OG1RF, *fsrB* appears to be implicated in infection by certain *E. faecalis* isolates.

Sequence analysis of the *E. faecium* QSE32 *fsr-gelE* region

To check whether the low virulence of the *E. faecium* QS32 strain in *G. mellonella* could result from the absence of a functional Fsr system, we sequenced the entire *fsr-gelE* region of this strain. The gene organization was identical to

E. faecalis V583, and the sequence shared 98 % identity at both the nucleotide and amino acid sequence level with *gelE* of *E. faecalis* V583 (data not shown). Therefore, we concluded that the Fsr system of *E. faecium* QS32 is most probably functional and that *gelE* conferred gelatinase activity. The low virulence of *E. faecium* QS32 seems to be independent of the functionality of the *fsr-gelE* region.

DISCUSSION

Simple invertebrates, the nematode *C. elegans* and insects *Drosophila melanogaster* and *G. mellonella* have recently attracted interest as models for screening of virulence factors of pathogenic microbes, including *E. faecalis*, or for elucidating their effects in the host (Maadani *et al.*, 2007; Park *et al.*, 2007; Schneider *et al.*, 2007). Although adaptive immunity is unique to vertebrates, the innate immune response seems to be well conserved between vertebrates and invertebrates. In contrast to the nematode, which lacks the cellular immune response involved in phagocytosis of bacteria (Fares & Greenwald, 2001), insects have both cellular and humoral responses, making them attractive models to study bacteria–host interactions (Vallet-Gely *et al.*, 2008). Although the *G. mellonella* infection model has been efficiently used to identify virulence factors in Gram-negative bacteria and in fungi (Schell *et al.*, 2008; Mylonakis *et al.*, 2005; Brennan *et al.*, 2002; Choi *et al.*, 2002; Cotter *et al.*, 2000; Xu *et al.*, 1991), this model has been mainly used to characterize virulence factors in *Bacillus cereus* and *Bacillus thuringiensis*. These bacteria are virulent to *G. mellonella* by oral infection (Salamitou *et al.*, 2000; Fedhila *et al.*, 2006) and by injection into the haemocoel (Bouillaut *et al.*, 2005). A recent study demonstrated killing of *G. mellonella* larvae by at least one *E. faecalis* strain isolated from larval cadavers (Park *et al.*, 2007). In the present study, we used the *G. mellonella* model to compare virulence of several *Enterococcus* strains from food and clinical origins. Using this simple infection model, we demonstrated the efficacy of the *G. mellonella* model, and establish for the first time that gelatinase and Fsr virulence factors of *E. faecalis* contribute to the virulence of food isolates.

E. durans and *E. faecium* strains were relatively avirulent or of low virulence, killing only 0–3 % of larvae, whereas *E. faecalis* strains had killing rates of 60–98 %. Although we used different strains of *E. faecalis* and *E. faecium* in this study, our results with *G. mellonella* correlate well with the *C. elegans* model, for which *E. faecium* strains were less virulent than *E. faecalis* strains (Garsin *et al.*, 2001; A. Alberti, personal communication).

Attenuated virulence of *gelE*-deficient *E. faecalis* strains in the *G. mellonella* infection model is consistent with results in mammal and nematode models (Engelbert *et al.*, 2004; Sifri *et al.*, 2002; Qin *et al.*, 2000). This result correlates with the recent finding that gelatinase degrades cecropin, an antimicrobial peptide important for the immune system

Fig. 3. Effects of *gelE* and *fsrB* inactivation on killing of *G. mellonella*. Percentage mortality of *G. mellonella* larvae at 48 h post-infection with *E. faecalis* strains LSE4a ($2.45 \times 10^6 \pm 0.14 \times 10^6$ c.f.u.), LSE4aΔ*gelE* ($1.91 \times 10^6 \pm 0.02 \times 10^6$ c.f.u.) and LSE4aΔ*fsrB* ($1.94 \times 10^6 \pm 0.49 \times 10^6$ c.f.u.), QA29B ($2.07 \times 10^6 \pm 0.11 \times 10^6$ c.f.u.), QA29BΔ*gelE* ($2.32 \times 10^6 \pm 0.42 \times 10^6$ c.f.u.) and QA29BΔ*fsrB* ($2.32 \times 10^6 \pm 0.29 \times 10^6$ c.f.u.). Larvae were infected with $\sim 2 \times 10^6$ bacteria as indicated in parentheses. Data were obtained from three independent experiments and are expressed as mean values $\pm$ SD. Asterisks indicate a significant difference (* $P < 0.05$, ** $P < 0.005$) relative to the wild-type strain.

in the haemolymph of larvae of *G. mellonella* (Park *et al.*, 2007).

Since inactivation of *gelE* does not abolish *E. faecalis* virulence, other factors might be involved in *G. mellonella* virulence. Although SprE protease is still expressed in *gelE*-inactivated strains, it does not seem to have insecticidal activity (Park *et al.*, 2007). Based on the high genome diversity between *E. faecalis* isolates (Bourgogne *et al.*, 2008; Lepage *et al.*, 2006), it is likely that the strains used in this study carry other factors involved in virulence in *G. mellonella* or differentially express them. Interestingly, the OG1RF genome encodes a putative extracellular protease (OG1RF_0194), which remains to be characterized (Bourgogne *et al.*, 2008).

The major regulatory system of gelatinase, the *fsr* operon, is among the most probable candidates to be involved in virulence in *G. mellonella*. While no significant difference in virulence was observed between larvae inoculated with the OG1RF and the *fsrB* isogenic mutant, we found that *fsrB* contributes to virulence in *G. mellonella* in the two food isolates studied. The result with OG1RFΔ*fsrB* is in agreement with a previous study in which *fsrB* inactivation in OG1RF did not modify virulence in a rat endocarditis model (Singh *et al.*, 2005). The authors proposed that residual production of gelatinase was sufficient to allow induction of endocarditis. In this case, however, residual gelatinase production would have had to be strongly induced *in vivo*, especially as virulence was evaluated at just 24 h post-infection. As proposed above, *fsrB* may directly or indirectly regulate other factors important for virulence in OG1RF. Since inactivation of *fsrB* in LSE4a and QA29B food isolates appears to be more relevant for virulence, we propose that OG1RF has additional factors that contribute to its pathogenic potential, as suggested by its intrinsic virulence in a mouse peritonitis model (Bourgogne *et al.*, 2008), or that these food isolates lack factors important for virulence in such a model. Taken together, present results and previous work suggest that the genetic background of the strain may be as important as the infection or host model used to assess virulence. Singh *et al.* (2005) used an endocarditis model to demonstrate that a *gelE sprE* double mutant significantly decreased the evolution of endocarditis compared to infection by WT OG1RF; the *fsrB* mutant was not significantly attenuated compared to wild-type OG1RF. Using different models, i.e. rabbit endophthalmitis, mouse peritonitis, *C. elegans* and *G. mellonella* (this study), the *fsrB* mutant strain of OG1RF was more attenuated than the double protease mutant (Engelbert *et al.*, 2004; Sifri *et al.*, 2002). An important conclusion of this work is that the role of virulence genes studied in one model cannot always be extrapolated to other models.

Transfer of virulence or antibiotic resistance genes has been observed *in vitro* (Eaton & Gasson, 2001) and *in vivo* (Mater *et al.*, 2005; Lester *et al.*, 2006). Although our finding does not preclude that enterococcal food isolates will be virulent in other infection or animal models or

pathogenic for humans, it indicates the *in vivo* activity of the gelatinase and Fsr present in food isolates, and calls for surveillance in monitoring virulence traits in food strains which represent a potential reservoir for virulence genes.

In summary, we used the *G. mellonella* infection model as a surrogate virulence model to compare virulence of three *Enterococcus* species, and *E. faecalis* isolates and isogenic deletion mutants. Our data show that *fsrB* and to a lesser extent *gelE* significantly contribute to the virulence of *E. faecalis* food isolates. This simple animal model may provide insights for risk assessment of food isolates.

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