The incongruent gelatinase genotype and phenotype in *Enterococcus faecalis* are due to shutting off the ability to respond to the gelatinase biosynthesis-activating pheromone (GBAP) quorum-sensing signal

Neuza Teixeira,^{1,2} Sofia Santos,¹ Paulo Marujo,¹ Ryoji Yokohata,³ Vijayalakshmi S. Iyer,⁴ Jiro Nakayama,³ Lynn E. Hancock,⁴ Pascale Serror^{5,6} and Maria de Fátima Silva Lopes^{1,2}

Correspondence

Maria de Fátima Silva Lopes
flopes@itqb.unl.pt

¹Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal

²IBET, Quinta do Marquês, Estação Agronómica Nacional, Apartado 12, 2781-901 Oeiras, Portugal

³Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

⁴Division of Biology, Kansas State University, 19 Ackert Hall, Manhattan, KS 66506, USA

⁵INRA, UMR1319 Micalis, F-78350 Jouy-en-Josas, France

⁶AgroParisTech, UMR Micalis, F-78350 Jouy-en-Josas, France

The concomitant presence of a complete *fsr* quorum-sensing system and *gelE*–*sprE* operons in *Enterococcus faecalis* is known to be essential for the detection of gelatinase activity. However, there are reports of the absence of gelatinase activity despite the presence of complete *fsr* and *gelE* loci. In order to understand this incongruence between genotype and phenotype we sequenced *fsr* and *gelE* loci of the *E. faecalis* LN68 strain, which was previously found to carry both operons but to lack gelatinase activity. Of the 59 nucleotide differences detected compared with the gelatinase-positive V583 strain, we found a nonsense mutation (a premature STOP codon) predicted to truncate the ATPase sensor domain of the FsrC protein, responsible for sensing and transducing the signal from the quorum-sensing molecule. Strain LN68 was highly affected in the expression of the *gelE* and *sprE* genes, further supporting the lack of Fsr-dependent *gelE* induction. When we constructed a V583 mutant with the same premature stop mutation in the *fsrC* gene the resulting strain was no longer able to degrade gelatin. We conclude that the reduced ability to transduce the quorum-sensing signal of the prematurely truncated FsrC protein is sufficient to explain the negative gelatinase phenotype. As the incongruent genotype and phenotype is detected in natural isolates, we believe that the silencing of the quorum-sensing system Fsr may be beneficial for some *E. faecalis* strains.

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INTRODUCTION

Enterococci are natural inhabitants of the oral cavity, intestinal tract and female genital tract of both humans and animals, and are also among the predominant microbiota of traditionally fermented dairy products (Lopes *et al.*,

1999). In contrast to their beneficial role in intestinal homeostasis, these micro-organisms are becoming increasingly important to human health as leading causes of nosocomial infections, namely of the urinary tract, blood-stream, intra-abdominal and pelvic regions, and surgical sites. To do so, they rely on several mechanisms, including the *fsr* operon of *Enterococcus faecalis*, the species most frequently associated with nosocomial infections (Gilmore *et al.*, 2002; Mundt, 1986; Ogier & Serror, 2007). The *fsr* (*Enterococcus faecalis* regulator) two-component system, a homologue of the *agr* system in *Staphylococcus aureus*, is a quorum sensing-dependent regulatory system. The *fsr* operon comprises four

Abbreviation: GBAP, gelatinase biosynthesis-activating pheromone.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of *E. faecalis* strain LN68 is JN246675.

A supplementary figure and a supplementary table are available with the online version of this paper.

genes: *fsrA*, *fsrB*, *fsrC* and *fsrD*. The last encodes an auto-inducing cyclic peptide named gelatinase biosynthesis-activating pheromone (GBAP) that is processed and exported out of the cell by the FsrB protein. Accumulation of GBAP outside cells is sensed by the FsrC histidine kinase, leading to the activation of the response regulator FsrA. Activated FsrA induces expression of the *fsrBDC* genes. These genes are involved in an autoregulatory circuit that results in a boost of GBAP signalling and in induction of the Fsr regulon, among which the *gelE-sprE* operon is the most induced (Bourgogne *et al.*, 2006). This operon encodes gelatinase (GelE), an extracellular zinc metalloprotease (Mäkinen *et al.*, 1989; Su *et al.*, 1991), and SprE, a serine protease (Nakayama *et al.*, 2001a, b; Qin *et al.*, 2000).

Although the secreted protease SprE has been implicated in disease in animal models, the role of SprE is still unknown. GelE is known to contribute to biofilm formation, and contributes also to virulence through degradation of a broad range of host proteinaceous substrates (Hancock & Perego, 2004; Park *et al.*, 2007, 2008; Steck *et al.*, 2011). The role of the *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 (Gaspar *et al.*, 2009; Sifri *et al.*, 2002) and in the *Arabidopsis thaliana* plant model (Jha *et al.*, 2005). Gelatinase has also been implicated in evasion of the immune system of the insect *Galleria mellonella* (Park *et al.*, 2007). Recent transcription studies have also shown that *fsr* and *gelE-sprE* expression is modulated during some stress conditions, namely in blood (Vebo *et al.*, 2009) and urine (Vebo *et al.*, 2010). Despite their evident importance for *E. faecalis* virulence and stress responses inside the host, many reports have shown that both loci are present in enterococcal isolates from different environments. This suggests that both the Fsr system and the GelE and SprE proteins may play a role, not associated with virulence, in the biology of *E. faecalis* (Thomas *et al.*, 2009).

The presence of the *gelE* genetic locus has often been complemented by the search for the phenotype, i.e. detection of gelatinase activity on plates containing gelatin. Qin *et al.* (2000) reported that the gelatinase phenotype requires the concomitant presence of the *fsr* and *gelE* genes. Soon after, Eaton & Gasson (2001) reported the loss by subculturing of the gelatinase phenotype in 20% of the analysed strains. Later, Nakayama *et al.* (2002) suggested that the loss of the gelatinase phenotype after subculturing might be due to a 23.9 kb chromosomal deletion, which was found in urine isolates with silent *gelE* genes. This deletion included the *fsrA*, *fsrB* and partially the *fsrC* gene. However, it has recently been shown that the 23.9 kb chromosomal deletion does not occur spontaneously by subculturing strains in the laboratory, but likely results from horizontal transfer and recombination (Galloway-Peña *et al.*, 2011). We and others have reported the loss of the gelatinase phenotype by subculturing, but also the existence of natural gelatinase-negative strains carrying *gelE* and an apparently complete *fsr* operon (Lopes *et al.*, 2006;

Nakayama *et al.*, 2002). Altogether, these reports indicate that silent *gelE* genes, in the presence of an apparently complete *fsr* operon, occur both in natural and laboratory-subcultured *E. faecalis* isolates. However, no further demonstration of the involvement of mutations in either the *fsr* or the *gelE-sprE* operon in the absence of gelatinase activity has been reported or demonstrated so far.

In the present study we investigated the reason for the incongruence between the *fsr* and *gelE* genotype and the gelatinase phenotype in *E. faecalis* strain LN68 (Lopes *et al.*, 2006). We demonstrate that this incongruence, in our strains with apparently complete *fsr* and *gelE* loci, is correlated with a specific nonsense codon in the FsrC protein. We further demonstrate that this codon change originates a truncated FsrC protein, preventing *E. faecalis* from sensing GBAP.

METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. Enterococcal strains were grown in BHI (brain heart infusion) medium at 37 °C, and *Escherichia coli* strains were grown in LB medium at 37 °C with agitation.

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 thermocycler. When necessary, PCR products and DNA restriction fragments were purified with purification kits (Macherey-Nagel). Plasmids were purified using the Miniprep kit (Macherey-Nagel). Electrotransformation of *Escherichia coli* and *E. faecalis* was carried out as described by Dower *et al.* (1988) and Dunny *et al.* (1991), using a Gene Pulser apparatus (Bio-Rad) (Dower *et al.*, 1988; Dunny *et al.*, 1991). Plasmid inserts were sequenced at BaseClear (The Netherlands).

Sequence analysis of the *fsr* and *gelE-sprE* regions of *E. faecalis* LN68. PCR amplification of overlapping fragments of the *fsr* and *gelE-sprE* regions was carried out using Expand High FidelityPLUS DNA polymerase (Roche) and primers from Gaspar *et al.* (2009). Amplicons were sequenced by BaseClear and DNA sequence analysis was accomplished using the Vector NTI 10.3.0 program (Invitrogen). The results were compared with the V583 genomic DNA sequence available at the J. Craig Venter Institute website (<http://www.jcvi.org/>).

Mutant construction. Markerless *fsrC* mutants of *E. faecalis* LN68 and of a derivative erythromycin-susceptible V583 strain (kindly provided by Axel Hartke, Université de Caen, France) were constructed essentially as described by Brinster *et al.* (2007) (Fig. 1). In this procedure we changed the nucleotide guanine for adenine (position 1764864 in the V583 genome), which leads to the substitution of a tryptophan by a STOP codon (in V583). In LN68, we did the opposite, i.e. we substituted the STOP codon by a tryptophan at position 403. Briefly, flanking regions of *fsrC* were amplified from chromosomal DNA by PCR with primers LN68_fsrC-PGMT5 and LN68_mfsrC_M, LN68_fsrC-PGMT3 and LN68_fsrC_M, for strain LN68; and primers V583_mfsrC_M and LN68_fsrC-PGMT5, LN68_fsrC-PGMT3 and V583_fsrC_M, for strain V583 (Table 1). The two cognate PCR fragments were fused by PCR using the external primers LN68_fsrC-PGMT5 and LN68_fsrC-PGMT3, for both strains, and the resulting products were cloned into pGEM-T (Promega). After being checked by sequencing, the inserted PCR fragment was removed from its cloning vector by restriction enzymes and subsequently cloned into plasmid

Table 1. Strains, plasmids and primers used in this study

Restriction sites are underlined.

Strain, plasmid or primer	Relevant characteristics or sequence	Reference or source
Strains		
<i>Escherichia coli</i> DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (r $\overline{k}$ m $\overline{k}$) <i>phoA</i> <i>supE44</i> λ ⁻ <i>thi-1</i> <i>gyrA96</i> <i>relA1</i>	Grant <i>et al.</i> (1990)
<i>Escherichia coli</i> TG1 RepA	<i>supE</i> <i>hsdD5</i> <i>thi</i> (Δ lac-proAB) F ⁻ (<i>traD36</i> <i>proAB</i> -lacZ Δ M15) <i>glgB::repA</i>	Law <i>et al.</i> (1995)
<i>Escherichia coli</i> EC1000	F ⁻ <i>araD139</i> (<i>ara</i> ABC- <i>leu</i>)7679 <i>gal</i> ⁺ <i>galK</i> <i>lacX74</i> <i>rsp</i> <i>thi</i> <i>repA</i>	Leenhouts <i>et al.</i> (1996)
<i>E. faecalis</i> V583	Clinical isolate, TIGR sequence strain; Van ^R	Sahm <i>et al.</i> (1989)
V583 ErmS	Derivative of <i>E. faecalis</i> V583, susceptible to erythromycin due to deletion of the <i>erm</i> (B)	Yan <i>et al.</i> (2009)
LN68	Wild-type <i>E. faecalis</i> ; isolated from milk, GelE ⁻	Lopes <i>et al.</i> (2006)
LN66	Wild-type <i>E. faecalis</i> ; isolated from milk, GelE ⁻	This study
QSE15	Wild-type <i>E. faecalis</i> ; isolated from cheese, GelE ⁻	This study
QN8	Wild-type <i>E. durans</i> ; isolated from cheese, GelE ⁻	This study
EF_SAVE3	<i>E. faecalis</i> V583 <i>ermS</i> W403STOP, GelE ⁻	This study
EF_SAVE4	<i>E. faecalis</i> LN68 STOP403W, GelE ⁺	This study
EF_SAVE5	<i>E. faecalis</i> LN68 carrying pSAVE7, Ery ^R , GelE ⁺	This study
VT01	<i>E. faecalis</i> V583 Δ gelE, GelE ⁻ , GBAP ⁺	Thomas <i>et al.</i> (2008)
VT03	<i>E. faecalis</i> V583 Δ gelE Δ sprE, GelE ⁻ , SprE ⁻ , GBAP ⁺	Thomas <i>et al.</i> (2008)
VI13	<i>E. faecalis</i> V583 Δ fsrB, GelE ⁻ , GBAP ⁻	This study
Plasmids		
pORI23	<i>Escherichia coli</i> - <i>E. faecalis</i> shuttle plasmid, Ery ^R	Que <i>et al.</i> (2000)
pGEM-T	<i>Escherichia coli</i> replicating plasmid, Amp ^R	Promega
pG + host9	<i>E. faecalis</i> thermosensitive plasmid, Ery ^R	Maguin <i>et al.</i> (1996)
pSAVE3	pGEM-T derivative carrying <i>fsrC</i> _W403STOP from V583	This study
pSAVE4	pGEM-T derivative carrying <i>fsrC</i> _W403STOP from LN68	This study
pSAVE5	pGhost9 derivative carrying <i>fsrC</i> _W403STOP from V583	This study
pSAVE6	pGhost9 derivative carrying <i>fsrC</i> _STOP403W from LN68	This study
pSAVE7	pORI23 derivative carrying a 1595 pb <i>Bam</i> HI/ <i>Pst</i> I <i>fsrC</i> fragment from V583	This study
pLT06	Temperature-sensitive cloning vector	Thurlow <i>et al.</i> (2009)
pVI02	pLT06 containing engineered <i>fsrB</i> deletion	This study
Primers		
sprE_4	TTTCCTGTTTGCTTAATGCCGC	
msrpE_4	GGCTGAAAGTTTAAAGAATGCCAAA	
LN68_fsrC-PGMT5	TTATAAACCAATGATACGGG	
LN68_fsrC-PGMT3	AAAATAAAATTATTATGGATTGCC	
LN68_mfsrC_M	CCCAGAAGAGCACGGTTGGGGATTGTTATATGTAAAAG	
LN68_fsrC_M	CTTTTACATATAACAATCCCCAACCGTGCTCTTCTGGG	
V583_mfsrC_M	CCAGAAGAGCACGGTTGAGGATTGTTATATGTAAAAG	
V583_fsrC_M	CTTTTACAATAACAATCCTCAACCGTGCTCTTCTGG	
fsrC3' <i>Pst</i> I	GAATCCTGCAGTTGCTTTATCCTCCC	
fsrC5' <i>Bam</i> HI	GAATCGGATCCGACAATGGATGGGAC	
RNA23S_fw_V583	AAAGAAATCAACCGAGATTCCCC	
RNA23S_rv_V583	AAACAGTGCTCTACCCCGG	
FsrBP1	GAGAGAATTCCAAGAACAGTTTGGCGGTTG	
FsrBP2	CTCTGGATCCATCGATTAGCATATCGCC	
FsrBP3	GAGAGGATCCGGTCTGATGATACTCCCT	
FsrBP4	CTCTCTGCAGGTTGGTGCAATCGTTTC	
FsrAP1	GGGATTGACGGAATCAAGA	
GelEP2	AAAGATGCCTGTACCTAAAAATG	

pG + host9 (Maguin *et al.*, 1996), which was then electroporated into the respective *E. faecalis* strain. The *fsrC* single- and double-crossover mutants were selected as described by Brinster *et al.* (2007). Successful targeted mutations of *fsrC* in strains LN68 and V583 were first identified by PCR screening and then confirmed by sequencing. The cognate phenotypes were confirmed by gelatinase activity assay.

An isogenic in-frame deletion of *fsrB* in *E. faecalis* V583 was generated using an *Escherichia coli*-enterococcal temperature-sensitive cloning vector, pLT06 (Thurlow *et al.*, 2009). Upstream regions flanking *fsrB* (*efl821*) in the V583 genome were PCR-amplified using the primer pair FsrBP1 and FsrBP2, and the downstream region of *efl821* was amplified using primers FsrBP3 and FsrBP4 (Table 1). The resultant

Fig. 1. Schematic representation of the *fsr* and *gelE* loci in V583 and LN68, and the mutants derived from point mutations, EF_SAVE3 and EF_SAVE4, respectively. The nonsense codon W403STOP is also indicated.

PCR products were digested with *Bam*HI followed by ligation, and the resulting product was reamplified with primers FsrBP1 and FsrBP4. For the construction of the *fsrB* deletion vector pVI02, the amplified product was digested with *Eco*RI and *Pst*I, followed by ligation to similarly digested plasmid vector pLT06. The ligated product was electroporated into *Escherichia coli* EC1000 for propagation, and blue colonies were selected for by culture at 37 °C on LB agar containing chloramphenicol (10 µg ml⁻¹) and X-Gal (80 µg ml⁻¹). Positive clones were identified by PCR using primers OriF and SeqR (Thurlow *et al.*, 2009). Plasmid pVI02 was confirmed by restriction mapping and electroporated into *E. faecalis* V583 cells. To generate *E. faecalis* strain VI13 possessing an in-frame deletion of *fsrB*, a published protocol was used (Thurlow *et al.*, 2009). Primers FsrAP1 and GelEP2 were employed to confirm the presence of the deletion.

***E. faecalis* LN68 complementation with the V583 *fsrC* gene.** To complement strain LN68 with the *fsrC* gene from V583, a 1595 bp *Bam*HI–*Pst*I fragment from V583 was recovered from the PCR amplicon obtained by using primers *fsrC*3' *Pst*I and *fsrC*5' *Bam*HI (Gaspar *et al.*, 2009). The fragment was cloned into the vector pOri23 (Que *et al.*, 2000) cut with *Bam*HI and *Pst*I, resulting in plasmid pOri23–*fsrC*. Primers pOri23_fw and pOri23_rv (Braga *et al.*, 2011) were used to confirm cloning. pOri23–*fsrC* was then introduced into LN68 cells by electroporation. Gelatinase activity was evaluated as described below, on gelatin plates supplemented with 500 µg erythromycin ml⁻¹.

Gelatinase activity. The phenotypic assay for detection of gelatinase activity was performed as described by Lopes *et al.* (2006). Briefly, enterococcal 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. Assays to study the ability of cells to sense and produce GBAP were performed on plates containing 1.5% skimmed milk. A transparent halo around cells detected after 24 h incubation at 37 °C indicated gelatinase production and activity. When indicated, GBAP was added to cells at a final concentration of 10 nM prior to plating 10 µl of culture onto the skimmed milk plates.

RNA extraction and semiquantitative RT-PCR. RNA was extracted from cells grown in BHI broth at 37 °C. Briefly, overnight cultured cells were diluted 1:100 and growth was monitored by following OD₆₀₀. Following exponential, early stationary and late stationary growth phases, cells were collected for RNA isolation. Total RNA was extracted and purified with an RNeasy Mini kit (Qiagen). RNA integrity was checked by electrophoresis on a 1% agarose gel (RNase free), and DNA contamination was checked using primers for 23S (Supplementary Table S1). The cDNA was synthesized using random primers (Roche Diagnostics), 3 µg total RNA and a Transcriptor High Fidelity cDNA Synthesis kit (Roche Diagnostics). Two serial dilutions (1:10 and 1:100) of cDNA were used for PCR in order to amplify cDNA of *fsrA* (primers: *mfsrA*, *fsrA*), *fsrB* (primers: *fsrB*, *mfsrB*), *fsrC* (primers: *fsrC*_2, *mfsrC*), *gelE* (primers: *mgeE*_2, *gelE*) and *sprE* (primers: *sprE*_4, *msrpE*_4) transcripts (Supplementary Table S1). 23S rRNA was used as a control.

RESULTS AND DISCUSSION

The importance of *E. faecalis* gelatinase to virulence and biofilm-forming ability has promoted the detection of the *fsr* and *gelE*–*sprE* operons as a presumption of pathogenicity. However, an incongruent genotype and phenotype may lead to false-positive assumptions on the virulence potential of strains, since the detection of the gene does not necessarily equate to gelatinase activity (Eaton & Gasson, 2001). In a previous study, the *E. faecalis* LN68 dairy strain was found to lack gelatinase activity on plates, despite apparently carrying the entire *fsr* and *gelE*–*sprE* operons, suggesting a defect in the functionality of the *fsr* and *gelE*–*sprE* operons or in their expression (Lopes *et al.*, 2006).

We first sequenced 6106 bp, including the promoter and terminator regions, of both the *fsr* and *gelE*–*sprE* operons of LN68 strain. Comparison of the LN68 sequence with that of *E. faecalis* V583 strain revealed 59 differences. Thirty-one, 18 and one corresponded to silent, missense

and nonsense mutations, respectively. We found also nine differences located in the intergenic regions (Table 2). Even if all of these differences could potentially influence the gelatinase phenotype, either by disturbing the regulation of gene expression through changed transcript stability for silent mutations, or by promoting a change in protein structure for missense mutations, the most drastic effect was predicted to result from the nonsense codon in the *fsrC* gene responsible for the substitution of tryptophan 403 by a STOP codon (Fig. 1). This leads to FsrC₄₀₂, a truncated histidine kinase shortened by the last 45 aa compared with the FsrC of V583 strain (447 aa).

To further investigate how the detected differences in the LN68 sequence would affect the structure of FsrC we used TopPred software (<http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::toppred>). FsrC proteins of both the V583 and the LN68 strain are predicted to have six transmembrane segments and three extracellular loops (Fig. 2). The major difference in LN68 FsrC is a truncated kinase domain due to the nonsense mutation in the C-terminal region. These results support our prediction that substitution of tryptophan 403 by a STOP codon promotes a structural change in the FsrC protein and most likely has an impact on its activity. Noticeably, the GXG triplet highly conserved in the ATPase domain of many histidine kinases is interrupted in LN68 FsrC (Fig. 2). This triplet is important for nucleotide binding and histidine phosphorylation of the cognate response regulators (Parkinson & Kofoed, 1992; Zhu & Inouye, 2002). In response to GBAP, the ATPase domain of FsrC phosphorylates the FsrA response regulator, which activates transcription from P_{fsrB} and P_{gelE} promoters, increasing expression of the *fsrBDC*, *gelE* and *sprE* genes. If phosphorylation of FsrA does not occur, the response regulator will not activate transcription, and expression of the proteases will not increase. Thus, we hypothesize that a truncated ATPase domain of FsrC impairs its ability to transduce GBAP signalling and consequently it cannot induce the expression of the *GelE* and *SprE* proteases. The low transcript levels of the *gelE* and *sprE* genes in LN68 (Supplementary Fig. S1) are in

accordance with the absence of gelatinase activity on gelatin plates.

In order to prove that LN68 has a negative gelatinase phenotype because it is a GBAP non-responder (derived from its functionally impaired FsrC) and not because of any other mutation detected in the *fsrA*, *fsrB* and *gelE* genes (Table 2), we constructed two mutants: EF_SAVE3 in V583, where tryptophan 403 was substituted by a STOP codon, and EF_SAVE4 in LN68, where the 403STOP codon was substituted by a tryptophan (Fig. 1), and we checked for induction of gelatinase activity of LN68, V583 and their derived mutants by exogenous GBAP. The activity of FsrC was indirectly detected by testing gelatinase activity on plates. Compared with the V583 wild-type strain, EF_SAVE3 showed no gelatinase activity, indicating that the nonsense mutation is sufficient to explain loss of FsrC activity (Fig. 3). Unexpectedly, we observed that the *E. faecalis* EF_SAVE4 mutant was unstable, as it reverted easily to the wild-type sequence of the LN68 *fsrC* gene. In fact, if gelatinase activity was tested right after constructing and confirming strain EF_SAVE4, the strain behaved as a gelatinase producer. However, if we repeated the test with EF_SAVE4 subcultures, it behaved as a gelatinase non-producer, and sequencing of *fsrC* showed that the strain reverted to the STOP codon of the wild-type strain. We thus decided to complement strain LN68 with *fsrC* from strain V583, using plasmid pORI23. The resulting strain, *E. faecalis* EF_SAVE5, exhibited gelatinase activity, indicating that expression of *fsrC* from V583 was sufficient to restore gelatinase expression in strain LN68 (Fig. 3).

Then, to investigate whether LN68 and EF_SAVE3 were GBAP non-responders and GBAP producers, we performed two experiments, whose results are presented in Fig. 4. We observed no gelatinase activity when purified GBAP was added to LN68 and EF_SAVE3 cells (Fig. 4a). These results indicate that neither LN68 nor EF_SAVE3 is a GBAP responder, showing that the W403STOP mutation in the FsrC histidine kinase is enough to turn *E. faecalis* into a GBAP non-responder. We then wondered whether these two strains were still producing sufficient amounts of

Table 2. Nucleotide differences identified in *E. faecalis* LN68

Mutation type	Gene/IR*										
	IR	<i>fsrA</i>	IR	<i>fsrB</i>	IR	<i>fsrC</i>	IR	<i>gelE</i>	IR	<i>sprE</i>	IR
Silence	—	2	—	4	—	5	—	12	—	8	—
Missense	—	2	—	3	—	5	—	6	—	3	—
Nonsense	3	—	4	—	—	1	—	—	—	—	1
Deletion	—	—	—	—	—	—	—	—	—	—	—
Insertion	—	—	—	—	—	—	—	—	—	—	—
Other†	3	—	5	—	—	—	—	—	—	—	1

*IR, intergenic region.

†Other: differences found in the intergenic regions.

Fig. 2. Intramembrane structures of FsrC proteins from V583 and LN68, predicted using TopPred software. Mutations found in the *fsrC* gene of LN68 are shown: tyrosine (Y) 54 to cysteine (C); leucine (L) 124 to isoleucine (I); tyrosine 252 to asparagine (N); lysine (K) 385 to arginine (R); tryptophan (W) 403 to STOP. ES, extracellular space; IS, intracellular space; ECL, extracellular loop.

GBAP to induce detectable gelatinase. The results are presented in Fig. 4(b). VI13 is an *fsrB* mutant of V583, which is not able to produce GBAP, but responds to it. In the presence of another GBAP producer strain, it is possible to see a transparent halo around VI13 cells, indicative of gelatinase activity and thus of Fsr induction by GABP. When VI13 was allowed to grow in the proximity of either VT01 or VT03, GBAP producer strains but with a negative gelatinase phenotype, the GBAP produced by VT01 and VT03 was able to induce gelatinase activity in VI13. Similarly, both LN68 and EF_SAVE3 were able to induce gelatinase activity in VI13, although to different extents, and at much lower levels than those seen with VT01 or VT03. This is because both LN68 and EF_SAVE3 have a truncated FsrC, which allows these

strains to produce only basal levels of GBAP to which VI13 responded. Our results thus demonstrate that both LN68 and EF_SAVE3 are GBAP producers.

Recently, Thomas *et al.* (2009) have reported the existence, among planktonic *E. faecalis* cells, of GBAP non-responders, i.e. cheaters, which, at the end of stationary phase, reach approximately 12% of the population. In a population where individuals work cooperatively, cheaters are individual cells that benefit from the cooperation with others, but do not cooperate. In light of our data, it is possible that *E. faecalis* strains accumulate mutations in the *fsr* locus, in particular in *fsrC*, and upon selection, due to still unknown factors, the loss of the ability to transduce the GBAP signal is selected, as if the strains no longer need

Fig. 3. Images from gelatin agar plates inoculated with the V583, EF_SAVE3, LN68 and EF_SAVE5 strains. A transparent halo, indicative of gelatin degradation, is clearly seen around V583 and EF_SAVE5 growth, demonstrating the presence of gelatinase activity in these two strains.

Fig. 4. LN68 and EF_SAVE3 gelatinase activity on skimmed milk plates. (a) Demonstration of the inability of LN68 and EF_SAVE3 to induce gelatinase upon addition of exogenous GBAP. Strain VI13 was used as a proof of concept of this assay. This strain is able to sense GBAP, and induce gelatinase activity upon GBAP addition, but it is not able to produce GBAP. (b) Demonstration of the ability of both LN68 and EF_SAVE3 to produce GBAP. Proof came from the observation of a transparent halo around growth of the VI13 strain, which is not able to produce GBAP and depends on exogenous GBAP to produce visible gelatinase activity.

Fig. 5. Enterococcal strains with an incongruent gelatinase genotype and phenotype. The *fsrC* gene of these strains was sequenced and was found to carry the W403STOP mutation, indicated by a star.

high levels of GelE and SprE for survival, and therefore do not require a fully efficient quorum-sensing Fsr system. As *fsr* has also been suggested to regulate other genes in the genome of *E. faecalis* (Bourgogne *et al.*, 2006), it is also plausible that the advantage of the loss of quorum sensing is selected by other functions in the cell which are shut down. This raises interesting future prospects for further investigating the role of Fsr, GelE and SprE in the biology of *E. faecalis*.

In order to establish whether the GBAP non-responder behaviour in other *E. faecalis* isolates was due to the same W403STOP mutation or to other codon variations, we searched for this nonsense codon in seven genetically unrelated enterococcal isolates from our culture collection, which also carried the apparently complete *fsr* and *gelE* loci, but lacked gelatinase activity (Fig. 5). The same nonsense codon was detected in only two *E. faecalis* tested strains, suggesting that it is likely that other variations in the FsrC sequence lead to the same GBAP non-responder behaviour. The detection of this nonsense codon in *Enterococcus durans* suggests either an ancient event that was disseminated in the genus or a mutation which frequently occurs due to unknown specific conditions. We have also detected other codon variations in the *fsrC* gene of strain LN68. It is possible that these, though not responsible for the loss of FsrC activity, are needed for stabilization of the STOP403W substitution that seems to be deleterious in the LN68 genetic background. It is thus likely that different sets of *fsrC* codon variations will be responsible for negative gelatinase phenotypes in different genomes. In fact, when we searched the *E. faecalis* sequenced genomes (http://www.broadinstitute.org/annotation/genome/enterococcus_faecalis/MultiHome.html), we found different sets of codon variations in *fsrC* among strains with a negative gelatinase phenotype and positive genotype (Galloway-Peña *et al.*, 2011). This fact, together with the inability of LN68 to displace the W403STOP mutation, suggests that each genome may accommodate different sets of codon variations in *fsrC* in order to achieve a gelatinase-negative phenotype.

In summary, previous work suggested that, besides deletion, mutations in any of the *fsr* or *gelE* genes could explain the negative gelatinase phenotype observed in natural *E. faecalis* isolates. However, no proof was available. In the present work we demonstrate that a single nonsense

codon in *fsrC* is enough to explain the incongruence between a *gelE* genotype and a gelatinase-negative phenotype. The substitution of tryptophan 403 by a STOP codon in strain LN68 was shown to generate a truncated FsrC that was most likely impaired in the ATPase activity needed for GBAP signal transduction. By demonstrating the hypothesized cause–effect relationship between codon variations in the *fsr–gelE* loci and the gelatinase-negative phenotype, we came across interesting findings which may prove very important for future research on *E. faecalis* biology and host–pathogen relationships. First, it is clear that the missense mutations that we detected in other *fsr* genes and in *gelE* were not important for the negative gelatinase phenotype, as strain EF_SAVE5, despite still carrying those mutations, was able to produce gelatinase. Therefore, *E. faecalis* achieves the gelatinase-negative phenotype by accumulating mutations in the *fsrC* gene. Accordingly, and despite the fact that each isolate may accumulate different sets of codon variations in *fsrC*, the important fact here is that some *E. faecalis* isolates appear to stabilize a quorum-sensing non-responder behaviour. This is worth studying in future research on *E. faecalis*, as it could shed some light on the role of Fsr and gelatinase in the biology and pathogenicity of this important nosocomial pathogen.

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