**IS256 Abolishes gelatinase activity and biofilm formation in
a mutant of the nosocomial pathogen *Enterococcus faecalis*
V583**

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IS256 Abolishes Gelatinase Activity and Biofilm Formation in a Mutant of the Nosocomial Pathogen *Enterococcus faecalis* V583

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Abstract

Enterococcus faecalis is one of the most controversial species of lactic acid bacteria. Some strains are used as probiotic, while others are associated with severe and life threatening nosocomial infections. Their pathogenicity depends on the acquisition of multi drug resistance and virulence factors. Gelatinase, which is required in the first steps of biofilm formation, is an important virulence determinant involved in *E. faecalis* pathogenesis including endocarditis and peritonitis. The gene that codes for gelatinase (*gelE*) is controlled by the Fsr quorum-sensing system, whose encoding genes (*fsrA*, *fsrB*, *fsrC*, and *fsrD*) are located immediately upstream of *gelE*.

The integration of a DNA fragment into the *fsr* locus of a derived mutant of *E. faecalis* V583 suppressed the gelatinase activity and prevented biofilm formation. Sequence analysis indicated the presence of IS256 integrated into the *fsrC* gene at nucleotide position 321. Interestingly, IS256 is also associated with biofilm formation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. This is the first description of an insertion sequence that prevents biofilm formation in *E. faecalis*.

Key words: *Enterococcus faecalis*, insertion sequence, biofilm, gelatinase

Enterococcus faecalis, a Gram-positive bacterium, is generally thought to be a commensal of the mammalian gastrointestinal tract. However, its ability to endure a range of harsh conditions allows it survive in other environments. It is also an opportunistic pathogen that can cause nosocomial infections, including bacteremia and biofilm-based pathogenesises such as endocarditis and urinary tract infections (Paulsen et al. 2003). The problem of hospital-acquired enterococcal infections has been aggravated in recent decades due to the alarming increase in emergent vancomycin-resistant enterococci (VRE) (Pan et al. 2012).

Gelatinase, an extracellular protease, is known to be involved in *E. faecalis* pathogenesis, and is synthesized by approximately 60% of clinical isolates (Galloway-Pena et al. 2011). It is required in the first steps of biofilm formation (Hancock and Perego 2004) and contributes towards virulence by hydrolyzing host substrates such as collagen, fibrinogen, fibrin, endothelin-1, bradykinin, LL-37 and complement components C3 and C3a (Thurlow et al. 2010).

The genes that code for gelatinase (*gelE*) and serine protease (*sprE*) form an operon controlled by the Fsr quorum-sensing system located immediately upstream of *gelE*. The two-component system of enterococcal Fsr includes *fsrA* (a response regulator), *fsrB* (a propeptide processing protein), *fsrC* (histidine kinase), and *fsrD* (a gelatinase biosynthesis activating pheromone [GBAP]) (Nakayama et al. 2006). The Fsr system of *E. faecalis* is required for the production of gelatinase (Hancock and Perego 2004), and appears to be involved in the regulation of other genes important in virulence and metabolism (Bourgogne et al. 2006; Teixeira et al. 2013).

This paper describes the effect of the integration of IS into the *fsrC* gene in a laboratory derivative mutant of *E. faecalis* strain V583.

E. faecalis V583 was isolated in 1987 from a blood culture of a chronically-infected patient. It was the first clinical VRE isolate reported from the United States (Sahm et al. 1989) and Shankar et al., (2002) demonstrated that variations within the structure of the pathogenicity islands modulate the virulence of this strain. During the construction of an *E. faecalis* V583 non-tyramine-producing mutant (Perez et al. 2014) by double-crossover deletion (Jonsson et al. 2009) of the tyrosine decarboxylase (*tdc*) gene cluster (Ladero et al. 2012), it was observed that one of the three independent *E. faecalis* V583 Δtdc mutants obtained did not produce gelatinase. This was confirmed by streaking single colonies on M17 agar plates containing 30 gL⁻¹ of gelatin and incubating overnight at 37°C (Reviriego et al. 2005).

The primers *fsrA1F* (5'-GCAGGAACTACTGAAATCGC-3') and *sprE1R* (5'-CTCGAGATTTCCCGTGATTCTGG-3') were designed based on *E. faecalis* V583 genome sequence (GenBank accession number: AE016830) to PCR- amplify the *fsrABDC-gelE-sprE* locus. The wild type strain and the Δtdc mutants yielded the expected 5767 bp fragment. However, the amplification of the strain unable to produce gelatinase resulted in a 7091 bp amplicon (data not shown). New primers were designed in order to sequence this unexpected fragment: *fsrB1F* (5'-GTGCAATACTTGAAGAGGAGGG-3'), *fsrC1R* (5'-CATATAACAATCCCAACCGTGC-3'), *fsrC1F* (5'-GATAACAAATAGTGTCCAAGCCG-3'), *gelE1R* (5'-CATAAGATTATGCCACTCCTTATCC-3'), *fsrC2R* (5'-TCATCATGTAGGTCCATAAGAACGGC-3') and *fsrC2R* (5'-CGTAAAGCTGCGCTCATAATAGCC-3'). Sequence analysis (performed by Macrogen, Korea) indicated the presence of a 1324 bp DNA fragment integrated into the *fsrC* gene at nucleotide position 321. The insert corresponded to IS256. This was orientated in the same transcriptional direction as the

fsr genes and had a target site duplication of 8 bp in the flanked regions (Figure 1). The nucleotide sequence of the *fsrABCD* locus containing IS256 was deposited in the European Nucleotide Archive under accession number HG794359.

The construction of a *fsrC* mutant strain and their subsequent complementation demonstrated that *fsrC* expression is necessary for *gelE* expression and biofilm production (Hancock and Perego 2004), experiments were performed to determine whether the IS256 in the *fsrC* gene affected the capacity of the strain to form a biofilm on polystyrene microtiter plates (TC Microwell 96U, Thermo Scientific, Denmark). Briefly, *E. faecalis* strains were grown overnight in M17 medium with 14 mM glucose at 37°C. The culture was diluted 1;40 in 200 µl of the same media. The microtiter plates were incubated at 37°C for 24 h in aerobic conditions. The cells were washed, stained with crystal violet and the optical density was determined following a method previously described (Hancock and Perego 2004). The capacity of the gelatinase-negative strain to form biofilms was much reduced compared to the parental strain and Δtdc gelatinase-positive mutants (Figure 2).

These results suggest that the insertion of IS256 into *fsrC* leads to a truncated histidine kinase. This would lead to impaired GBAP signalling, therefore preventing the expression of *gelE* and biofilm formation.

It has been indicated the high plasticity of the *E. faecalis* genome in the area of the Fsr system (Galloway-Pena et al. 2011). The gelatinase-negative phenotype has been reported for both natural and laboratory *E. faecalis* strains (Teixeira et al. 2012). It has a number of genetic causes, mostly involving the *fsr* locus (Shankar et al. 2012). However, this is the first report of an IS causing *gelE* not to be expressed.

IS256, which encodes a transposase, is widespread in the genomes of multi-resistant staphylococci and enterococci. The sequence appears as multiple free copies as well as forming the ends of the composite aminoglycoside resistance-mediating transposon Tn4001 (Hennig and Ziebuhr 2010). *E. faecalis* V583 has multiple copies of IS256 in its genome, 6 in the chromosome and 4 spread across 3 plasmids (Paulsen et al. 2003).

IS256 has been identified as a marker of multidrug resistance and biofilm-formation in clinical isolates of staphylococci (Hennig and Ziebuhr 2010). It is associated with biofilm formation via its reversible transposition into the *ica* operon in *S. epidermidis* (Ziebuhr et al. 1999) and *S. aureus* (Valle et al. 2007), and into the *agr* operon in *S. aureus* (Cafiso et al. 2007). Biofilm-associated genes and regulators seem to be an important hot-spot for IS256 integration in staphylococci. As shown by the present results, IS256 is also associated with biofilm-forming genes in enterococci.

It has been shown that a temperature of 30°C favors the transposition of IS256 in *S. aureus* (Valle et al. 2007). The present production of *E. faecalis* mutants by homologous recombination (Jonsson et al. 2009) involved growth at 28°C for approximately 75 generations, which may have similarly favored the transposition process.

It is difficult to speculate upon the survival/evolutionary benefit that silencing the Fsr system would bring. In any event, minority subpopulations of *E. faecalis* GBAP quorum non-responders - perhaps arising from accumulation of mutations in the *fsr* locus - have been reported (Thomas et al. 2009). Thus, the transposition of IS256 might provide a mechanism for the generation of GBAP non-responders, which might have an evolutionary advantage under certain conditions. It has been suggested that, in

staphylococci, the shutdown of biofilm formation by IS256 may help dissemination into novel habitats (Ziebuhr et al. 1999).

Given the clinical importance of biofilm formation, and of gelatinase as a promising target for therapeutic intervention against multidrug-resistant and virulent *E. faecalis* strains, further investigations should be performed to unravel the complex regulation of the Fsr system. The genetic causes of phenotypic change in biofilm-forming ability should be determined, taking into account the importance of mobile elements in genome flexibility, adaptation and evolution.

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References

- Bourgogne, A., Hilsenbeck, S.G., Dunny, G.M., and Murray, B.E. 2006. Comparison of OG1RF and an isogenic *fsrB* deletion mutant by transcriptional analysis: the Fsr system of *Enterococcus faecalis* is more than the activator of gelatinase and serine protease. *J Bacteriol* 188(8): 2875-2884.
- Cafiso, V., Bertuccio, T., Santagati, M., Demelio, V., Spina, D., Nicoletti, G., and Stefani, S. 2007. *agr*-Genotyping and transcriptional analysis of biofilm-producing *Staphylococcus aureus*. *FEMS Immunol Med Microbiol* 51(1): 220-227.

- Galloway-Pena, J.R., Bourgogne, A., Qin, X., and Murray, B.E. 2011. Diversity of the *fsr-gelE* region of the *Enterococcus faecalis* genome but conservation in strains with partial deletions of the *fsr* operon. *Appl Environ Microbiol* 77(2): 442-451.
- Hancock, L.E., and Perego, M. 2004. The *Enterococcus faecalis* *fsr* two-component system controls biofilm development through production of gelatinase. *J Bacteriol* 186(17): 5629-5639.
- Hennig, S., and Ziebuhr, W. 2010. Characterization of the transposase encoded by IS256, the prototype of a major family of bacterial insertion sequence elements. *J Bacteriol* 192(16): 4153-4163.
- Jonsson, M., Saleihan, Z., Nes, I.F., and Holo, H. 2009. Construction and characterization of three lactate dehydrogenase-negative *Enterococcus faecalis* V583 mutants. *Appl Environ Microbiol* 75(14): 4901-4903.
- Ladero, V., Fernandez, M., Calles-Enriquez, M., Sanchez-Llana, E., Canedo, E., Martin, M.C., and Alvarez, M.A. 2012. Is the production of the biogenic amines tyramine and putrescine a species-level trait in enterococci? *Food Microbiol* 30(1): 132-138.
- Nakayama, J., Chen, S., Oyama, N., Nishiguchi, K., Azab, E.A., Tanaka, E., et al. 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(23): 8321-8326.
- Pan, S.C., Wang, J.T., Chen, Y.C., Chang, Y.Y., Chen, M.L., and Chang, S.C. 2012. Incidence of and risk factors for infection or colonization of vancomycin-resistant enterococci in patients in the intensive care unit. *PLoS One* 7(10): e47297.

Paulsen, I.T., Banerjee, L., Myers, G.S., Nelson, K.E., Seshadri, R., Read, T.D., et al. 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science* 299(5615): 2071-2074.

Perez, M., Calles-Enríquez, M., Ingolf, N., Martin, M.C., Fernandez, M., Ladero, V., and Alvarez, M.A. 2014. Tyramine biosynthesis is transcriptionally induced at low pH and improves the fitness of *Enterococcus faecalis* in acidic environments. *Applied Microbiology and Biotechnology*.

Reviriego, C., Eaton, T., Martin, R., Jimenez, E., Fernandez, L., Gasson, M.J., and Rodriguez, J.M. 2005. Screening of virulence determinants in *Enterococcus faecium* strains isolated from breast milk. *J Hum Lact* 21(2): 131-137.

Sahm, D.F., Kissinger, J., Gilmore, M.S., Murray, P.R., Mulder, R., Solliday, J., and Clarke, B. 1989. In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. *Antimicrob Agents Chemother* 33(9): 1588-1591.

Shankar, N., Baghdayan, A.S., and Gilmore, M.S. 2002. Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis*. *Nature*. 13(6890):746-750

Shankar, J., Walker, R.G., Ward, D., and Horsburgh, M.J. 2012. The *Enterococcus faecalis* exoproteome: identification and temporal regulation by Fsr. *PLoS One* 7(3): e33450.

Teixeira, N., Santos, S., Marujo, P., Yokohata, R., Iyer, V.S., Nakayama, J., et al. 2012. 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. *Microbiology* 158(Pt 2): 519-528.

Teixeira, N., Varahan, S., Gorman, M.J., Palmer, K.L., Zaidman-Remy, A., Yokohata, R., et al. 2013. *Drosophila* host model reveals new *Enterococcus faecalis* quorum-sensing associated virulence factors. *PLoS One* 8(5): 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(4): 1022-1036.

Thurlow, L.R., Thomas, V.C., Narayanan, S., Olson, S., Fleming, S.D., and Hancock, L.E. 2010. Gelatinase contributes to the pathogenesis of endocarditis caused by *Enterococcus faecalis*. *Infect Immun* 78(11): 4936-4943.

Valle, J., Vergara-Irigaray, M., Merino, N., Penades, J.R., and Lasa, I. 2007. sigmaB regulates IS256-mediated *Staphylococcus aureus* biofilm phenotypic variation. *J Bacteriol* 189(7): 2886-2896.

Ziebuhr, W., Krimmer, V., Rachid, S., Lossner, I., Gotz, F., and Hacker, J. 1999. A novel mechanism of phase variation of virulence in *Staphylococcus epidermidis*: evidence for control of the polysaccharide intercellular adhesin synthesis by alternating insertion and excision of the insertion sequence element IS256. *Mol Microbiol* 32(2): 345-356.

Legend of figures

Figure 1.

Diagram showing the IS256 insertion. IS256 was inserted into the *fsr* locus of the *E. faecalis* V583 Δ *tdc* gelatinase-negative strain.

Figure 2.

Biofilm formation on polystyrene microtiter plates. 1. *E. faecalis* V583 parental strain; 2. *E. faecalis* V583 Δ *tdc* strain; 3. *E. faecalis* V583 Δ *tdc* gelatinase-negative strain. The asterisk indicates statistically significant difference ($P \leq 0.001$; Student's *t*-test) in comparison to the other conditions.

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Fig. 1. Diagram showing the *IS256* insertion site in the *fsr* locus of the *E. faecalis* V583 Δ *tdc* gelatinase-negative strain.

Figure 2