

Gelatinase-Associated Phenotypes and Genotypes Among Clinical Isolates of *Enterococcus faecalis* in Poland

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Abstract

Enterococcus faecalis is an important nosocomial pathogen causing serious invasive infections. One of the virulence factors of this pathogen, gelatinase GelE, is a protease whose gene expression is regulated by the Fsr quorum sensing system. In this study, we used a well-characterized collection of 153 clinical *E. faecalis* isolates to investigate the distribution of genes involved in gelatinase expression. Although 140 isolates (91% of the group) harbored the *gelE* gene, only 81 isolates (53%) produced active gelatinase. The gelatinase-negative phenotype was found in several unrelated clones, and appeared to be caused by various genetic events. Isolates of the hospital-adapted clonal complex 2 (CC2) and of CC40 were uniformly gelatinase-positive, while all the CC87 isolates contained the 23.9 kb deletion encompassing most of the *fsr* locus and were gelatinase-negative. No significant differences among isolates of different clinical origin and gelatinase activity or presence of the *fsr* genes were found with the exception of isolates from cerebrospinal fluid, which were more often gelatinase-positive than colonizing isolates.

Key words: *E. faecalis*, clone, *fsr*, gelatinase

Introduction

Enterococci represent important nosocomial pathogens worldwide (Werner *et al.*, 2008), and the major clinically relevant species, *Enterococcus faecalis* causes serious invasive infections, such as endocarditis and bacteremia as well as urinary tract and surgical site infections (Arias *et al.*, 2010). The treatment is complicated by increasing resistance of the species to several antimicrobial agents (Arias *et al.*, 2010), observed also in Poland (Rudy *et al.*, 2005; Kawalec *et al.*, 2007; Piekarska *et al.*, 2008). Several enterococcal virulence factors have been identified and studied extensively (Fisher and Phillips, 2009; Hendrickx *et al.*, 2009). One of them, gelatinase (GelE) of *E. faecalis* is an extracellular zinc metalloendopeptidase (Makinen *et al.*, 1989), whose gene (*gelE*) is co-transcribed with the located directly downstream *sprE* gene encoding serine protease, SprE (Qin *et al.*, 2000; Qin *et al.*, 2001). These proteases provide peptide nutrients to bacteria, as well as their activity causes direct and indirect damage to host tissues (Gilmore, 2002). A number of studies demonstrated that GelE enhances biofilm production (Hancock and Perego, 2004; Kristich *et al.*, 2004; Mohamed *et al.*, 2004), whereas SprE has an oppo-

site effect (Thomas *et al.*, 2008). The interplay of GelE and SprE regulates autolysis of *E. faecalis* cells and the subsequent release of extracellular DNA (eDNA), a critical structural component of biofilm produced by these bacteria (Guiton *et al.* 2010; Thomas *et al.*, 2008). GelE appears also important for cleavage of pheromones responsible for induction of conjugal plasmid transfer and degradation of misfolded bacterial proteins (Waters *et al.*, 2003).

The expression of *gelE* and *sprE* is regulated by the *fsr* locus (Nakayama *et al.*, 2001), a homologue of *agr* of *Staphylococcus aureus* (Balaban and Novick, 1995; Qin *et al.*, 2000). FsrA and FsrC constitute a two-component signal transduction system (TCS), in which FsrA acts as response regulator and FsrC as signal transducer, responding to an increasing extracellular concentration of an 11-amino-acid cyclic peptide, so-called gelatinase biosynthesis-activating pheromone (GBAP). Activated FsrC phosphorylates FsrA which induces transcription of *gelE* and *sprE*, and regulates several other genes, involved in pathogenesis and metabolic pathways (Qin *et al.*, 2000). The GBAP coding sequence was identified within the *fsrD* gene, which is contained in frame in the 3' terminal part of *fsrB*, but independently translated (Nakayama *et al.*, 2006). The *fsrB* product is probably

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involved in the FsrD processing and transport outside the cell (Nakayama *et al.*, 2001; Nakayama *et al.*, 2006). In some isolates, a naturally occurring 23.9-kb chromosomal deletion, encompassing *fsrA*, *fsrB* and a part of *fsrC*, resulting in the gelatinase-negative phenotype was identified (Nakayama *et al.*, 2002). A number of studies using animal models have shown the contribution of GelE, SprE and Fsr to virulence of *E. faecalis*. In a mouse peritonitis model, the gelatinase mutant was attenuated; similarly, *sprE*, *fsrA* and *fsrB* mutants also showed delayed time of killing compared to the wild-type strain (Qin *et al.*, 2000). The virulence of *fsr* mutants was lower than that of a deletion mutant of *gelE* and an insertion mutant in *sprE* (Sifri *et al.*, 2002). Studies using a rabbit endophthalmitis model, an exquisitely sensitive infection system for investigating quorum sensing mechanisms, gave similar results, suggesting possible pleiotropic contribution of the *fsr* locus to the virulence (Engelbert *et al.*, 2004).

Recently, multilocus sequence typing (MLST) has become a method of choice for typing of *E. faecalis*. In the study that introduced the current typing scheme based on seven house-keeping genes, MLST was used for analysis of 110 *E. faecalis* isolates from different sources and geographic locations (Ruiz-Garbajosa *et al.*, 2006). Among four major clonal complexes (CCs) identified, CC2 and CC9 were considered to be hospital-adapted and spread globally. Another study, performed on the collection of *E. faecalis* from hospitals in Poland demonstrated the presence of additional three major endemic clones apart from CC2 and CC9 in this country (Kawalec *et al.*, 2007). We used this well-characterized collection to investigate the distribution of *gelE*, *sprE* and *fsr* genes, and to compare these data with earlier MLST results.

Experimental

Material and Methods

Bacterial isolates. A hundred and fifty-three isolates collected by the National Medicines Institute in Warsaw from 42 medical centers in 28 Polish cities during 1996–2005 were used in this study. These isolates were obtained from various sources: 54 isolates were from invasive infections (blood, peritoneal, cerebrospinal and pleural fluids), 52 isolates were from non-invasive infections (wound, urine, eye), 40 isolates were from carriage (rectum, feces, skin, throat, sputum, vagina, sperm, ear), six isolates were from colonization of catheters and drains, and one isolate was from the hospital environment (Kawalec *et al.*, 2007).

Determination of gelatinase activity. The phenotypic assay of gelatinase activity was performed accord-

ing to the modified method described by Su *et al.* (Su *et al.*, 1991). A 10 µl drop of an overnight culture in Bacto Todd-Hewitt Broth (Becton Dickinson, Sparks, MD) was spotted on a plate with medium containing 3% gelatin (Oxoid, Hampshire, England); 0.5% peptone (Becton Dickinson); 0.3% yeast extract (Becton Dickinson), 1.5% agar (Becton Dickinson); pH 7; after 48 h incubation at 37°C in aerobic conditions, the plate surface was covered with a saturated solution of ammonium sulphate (Sigma-Aldrich Chemie, Steinheim, Germany). Presence of a transparent zone around colonies indicated gelatinase activity.

MLST and detection of *gelE*, *sprE*, *fsrA* and 23.9 kb deletion by PCR. DNA isolation was performed with Genomic Mini kit (A&A Biotechnology, Gdańsk, Poland) according to the manufacturer's instructions. MLST was performed using the standard procedure (Ruiz-Garbajosa *et al.*, 2006) and the database (<http://www.mlst.net/>) to assign alleles and sequence types (STs). Sequences were assembled using the SeqMan program in DNASTAR software package (Lasergene, Madison, WI) and results were analyzed with the eBURST software (Feil *et al.*, 2004) with members of a CC sharing six out of the seven MLST loci (single-locus variants, SLVs). The presence of *gelE*, *sprE* and *fsrA* was determined by PCR with primers described by Lopes *et al.* (Lopes *et al.*, 2006). The amplification was performed in the following conditions: 2 min at 94°C; 30 s at 94°C, 30 s at 55°C, 1 min at 72°C for 35 cycles; 7 min at 72°C. DNA of *E. faecalis* V583 was used as a positive control. For the PCR detection of the 23.9 kb deletion in the *fsr* locus, primers proposed by Roberts *et al.* (Roberts *et al.*, 2004) were used and the elongation time was extended to 2 min. PCR products were analyzed by 1.5% agarose gels electrophoresis (SeaKem LE Agarose, Lonza, Rockland, MN), stained with ethidium bromide (Sigma-Aldrich Chemie).

Dot-blot hybridization assay. For dot-blot hybridization, 50 ng of chromosomal DNA was denaturated with 0.4 M NaOH and 10 mM EDTA solution for 5 min, and vacuum blotted to a Hybond⁺ nylon membrane (GE Healthcare, Buckinghamshire, UK) in a Bio-Dot Microfiltration Apparatus (Bio-Rad, Hercules, CA). DNA was fixed to the membrane by a 0.4 M NaOH wash, followed by neutralization with 2×SSC and exposure to UV radiation for 2 min as recommended by the vacuum-blotter manufacturer. Gene-specific probes for *gelE*, *sprE*, and *fsr* genes were generated by PCR using primers described by Lopes *et al.* (Lopes *et al.*, 2006). Probe labeling and signal detection were carried out according to the ECL Direct Nucleic Labeling and Detection System (GE Healthcare). Membranes were hybridized overnight at 42°C with 100 ng of a probe, washed, incubated with the detection reagents, and exposed on a X-ray film (X-ray Retina Fotochemische

Werke GmbH, Berlin, Germany), which was developed and fixed using Kodak processing chemicals (Sigma-Aldrich Chemie).

Statistical analysis. The chi-squared test was used to evaluate the significance of differences in isolate distributions.

Results

The activity of gelatinase was initially characterized in the previous study (Kawalec *et al.*, 2007) and these data were verified in the current work for reproducibility and a possible loss of phenotype during storage of isolates. These results were entirely congruent with the previous data. Among 153 *E. faecalis* isolates, the gelatinase-positive phenotype (Gel⁺) was found for 81 isolates (53%). In all the Gel⁺ isolates, the *gelE*, *sprE* and *fsrA* genes were detected by both PCR and hybridization, and the presence of *fsrB* and *fsrC* was confirmed by hybridization for a few randomly chosen representatives of this group (the *gelE*⁺, *sprE*⁺ and *fsr*⁺ genotype, Table I). In the group of 72 gelatinase-negative (Gel⁻) isolates, 39 showed the presence of the 23.9-kb deletion by PCR (*gelE*⁺, *sprE*⁺ and Δ *fsr*). As expected, a few checked representatives of this group were negative for *fsrB* but positive for *fsrC* in hybridization. All the remaining 33 isolates were additionally tested for the presence of *fsrB* and *fsrC* by hybridization and showed three different genotypes (Table I). Nine isolates had a complete set of the *gelE*, *sprE* and *fsr* genes; 11 isolates possessed *gelE* and *sprE* but lacked all *fsr* genes and did not show the 23.9-kb deletion by PCR; thirteen isolates lacked all the investigated genes.

The frequency of gelatinase production and the prevalence of *fsr* genes varied depending on the iso-

lation site and these differences were statistically significant ($p=0.009$ and $p=0.008$, respectively, for the whole group). The gelatinase production was common among isolates from the cerebrospinal fluid (75%, *i.e.* 6 Gel⁺ isolates out of 8), urine (71%, 17/24) and vagina (70%, 14/20), less frequent for wound and blood isolates (50%, 14/28 and 44%, 15/34, respectively) and the rarest among isolates from rectum and feces (27%, 3/11) and peritonitis (10%, 1/10).

Gelatinase phenotypes and genotypes were next analyzed in the context of molecular typing data. The previous MLST results (Kawalec *et al.*, 2007) were corrected for four STs, *i.e.* STs 131, 6, 137 and 121 were changed to STs 378 (a new ST), 88, 8 and 220, respectively. In summary, the investigated group of 153 isolates contained 52 STs, grouped into five CCs (21, 40, 87, 88 and 141) and 42 unrelated STs (singletons) by the eBURST analysis. One of the previously defined major epidemic clones, CC2 (the so-called clone A, represented here by 10 isolates of ST6) uniformly produced gelatinase (Table I). All the representatives of the other epidemic clone, CC9 (clone J, 6 isolates of ST9) possessed the full set of analyzed genes; however, one isolate of this ST lost the ability to produce gelatinase. Of the Polish endemic clones, isolates belonging to the previously described clone F (24 isolates of CC87) were all gelatinase-negative due to the presence of the 23.9-kb deletion. In the clone C, consisting of two subclones unrelated in MLST, *i.e.* ST16 and CC40, all CC40 isolates were gelatinase producers, while among representatives of ST16, two out of six isolates lacked the *gelE*, *sprE* and *fsr* genes. Finally, clone I (CC21, 19 isolates) included six Gel⁺ and thirteen Gel⁻ isolates; of the latter, ten possessed *gelE* and *sprE* but lacked the *fsr* genes, two isolates lacked the *gelE*, *sprE* and *fsr* genes and one possessed the full set of analyzed genes.

Table I
Phenotypes and genotypes of *E. faecalis* analyzed in this study in relation to MLST results

Gelatinase phenotype	<i>gelE</i>	<i>sprE</i>	<i>fsrA</i>	<i>fsrB</i>	<i>fsrC</i>	23.9 kb deletion	Number of isolates	Number of invasive isolates	MLST
+	+	+	+	+ ^a	+ ^a	nd	81	23	CCs: <u>21</u> (6), 40 (26), 141 (2) STs: 6 (10), <u>9</u> (5), <u>16</u> (4), 47 (4), 59 (2), 72 (2), 81, 82, 86, 93, 102, 119, 122, 123, 124, 125, 126, 127, 128, 130, 134, 136, 138, 139, 142, 143
-	+	+	-	- ^a	+ ^a	+	39	13	CCs: 87 (24) STs: 8 (3), <u>25</u> (2), 26 (2), 30 (6), 41 (2)
-	+	+	+	+	+	-	9	6	CCs: <u>21</u> , 88 (5) STs: <u>9</u> , <u>25</u> , 120
-	+	+	-	-	-	-	11	5	CCs: <u>21</u> (10) ST: 140
-	-	-	-	-	-	-	13	7	CCs: <u>21</u> (2) STs: <u>16</u> (2), 23, 55 (2), 74, 84, 85, 133, 135, 378

^a detection by dot-blot performed for representatives; nd, not determined.

The numbers of isolates, if higher than one, are given in the brackets; CCs and STs present in more than one group are underlined.

The majority of sporadic isolates produced gelatinase and had the *gelE*⁺, *sprE*⁺ and *fsr*⁺ genotype.

Discussion

The isolates used in this study were typed previously by pulsed-field electrophoresis (PFGE) and MLST, and a number of major clones were described (Kawalec *et al.*, 2007). The aim of the present research was to investigate the distribution of the *gelE* and *sprE* genes, and genes involved in the regulation of gelatinase expression in this well-characterized collection.

The gelatinase coding gene was present in 91% of isolates tested (140/153), whereas the gelatinase activity was found in only 53% (81/153) of them. In other studies, involving large groups of isolates from various clinical materials, it was shown that 56% and 59% of isolates produced gelatinase, while 88% and 92% were *gelE*-positive, respectively (Roberts *et al.*, 2004; Mohamed and Murray, 2005). Among 31 endodontic *E. faecalis*, *gelE* was detected in all tested isolates, whereas the gelatinase activity was found in 74% of them (Sedgley *et al.*, 2005). Certain studies, however, showed a lower prevalence of *gelE*⁺ and GelE⁺ isolates, accounting for 45–74% (Bittencourt de Marques and Suzart, 2004; Piekarska and Jagielski, 2007) and 34% of analyzed collections of clinical isolates (Piekarska and Jagielski, 2007), respectively. The most common cause for a loss of gelatinase production is the 23.9 kb deletion encompassing most of the *fsr* locus which was present *e.g.* in 26 of 33 Gel⁻ isolates from patients with urinary tract infections (Nakayama *et al.*, 2002). Another likely cause for the gelatinase-negative phenotype is a point mutation in the *gelE* gene (Nakayama *et al.*, 2002) or loss of the gene as observed *e.g.* for the genome of the probiotic strain Symbioflor (Domann *et al.*, 2007).

In our study, the frequency of gelatinase activity and the presence of the *fsr* genes varied depending on the site of isolation, being the highest for the isolates from the cerebrospinal fluid. The observation is consistent with the significantly increased prevalence of the *fsr* locus found for endocarditis isolates compared to fecal isolates (Pillai *et al.*, 2002) as well as the fact that GelE plays an important role during infection in a rabbit endocarditis model (Thurlow *et al.*, 2010) which altogether might indicate the importance of GelE and Fsr TCS for the most severe forms of invasive enterococcal infections. Moreover, gelatinase production was also more often associated with hospital strains of *E. faecalis* than with strains carried in the community (Coque *et al.*, 1995). The same study, however, did not show differences in gelatinase production among enterococci from different forms of hospital infections.

Comparison of the distribution of gelatinase activity and presence of the *fsr* genes with the MLST results showed that these features are typical for hospital-adapted, globally spread CCs, such as CC2 and CC9, as well as for some Polish endemic clones, such as CC40 and most of ST16, while CC87 uniformly showed the presence of the 23.9-kb deletion that excluded the production of gelatinase and activation of other genes dependent from Fsr TCS. We found the deletion also in some other unrelated singleton STs, likely due to a horizontal transfer of the corresponding region of the chromosome (Galloway-Pena *et al.*, 2011). Another endemic clone, CC21 was the most diversified group, containing both Gel⁺ and Gel⁻ isolates, the latter showing various genotypes. In general, CC21 is a heterogeneous, not strictly hospital-adapted group, which encompasses also the isolates of animal and environmental origins (Ruiz-Garbajosa *et al.*, 2006; Kawalec *et al.*, 2007).

Although gelatinase is not essential for *E. faecalis*, it seems to play an important role, both in pathogenesis and in basic metabolic processes of the bacterium. This poses a question, why the Gel⁻ phenotype has emerged in several unrelated clones due to various genetic events, such as deletion of the *fsr* genes, horizontal transfer of the deletion-harboring region or presumable inactivation of the *gelE* gene. These observations suggest some evolutionary benefits associated with the loss of gelatinase production due to *e.g.* a significant cost of this process. It is also hypothesized that, as GelE and SprE are involved in the eDNA release necessary for biofilm formation, Gel⁻ clones may be considered as “quorum sensing cheaters”, that do not respond to the GBAP signal and do not contribute for the rest of the population but rather take advantage of it (Thomas *et al.*, 2008; Thomas *et al.*, 2009).

In conclusion, we used phenotypic and molecular methods for investigation of distribution of gelatinase and the *fsr* locus in a well-characterized collection of clinical isolates of *E. faecalis* and showed association of particular phenotypes and genotypes with enterococcal clones. The loss of gelatinase production appeared independently more than once in *E. faecalis*, however, certain hospital-adapted clones maintain this ability.

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