Eur J Clin Microbiol Infect Dis (2012) 31:267–272
DOI 10.1007/s10096-011-1305-z

ARTICLE

Enterococcus faecalis from patients with chronic periodontitis: virulence and antimicrobial resistance traits and determinants

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Received: 30 March 2011 / Accepted: 19 May 2011 / Published online: 10 June 2011

Abstract This study investigated the presence of virulence and resistance traits, as well as their genetic determinants in subgingival *Enterococcus faecalis* from patients with chronic periodontitis. Twenty-four *E. faecalis* strains from a previously multi-locus sequence typing (MLST)-characterized strain collection were examined for virulence-associated phenotypes, antimicrobial susceptibility, and virulence- and antimicrobial-resistant determinants. Gelatinase, hemolysin, and biofilm production were detected in 50, 17, and 100% of the strains, respectively. Genes encoding adherence factors such as *ace*, *efaA*, and *bopD* were detected in all isolates. Other putative virulence determinants, i.e., *EF3314*, *gelE*, *asa*, *esp*, *cylA*, *ef1841*/*fsrC*, and *asa373*, were found in a portion of the strains. Different levels of resistance were observed in these strains, with two strains expressing high-level resistance to erythromycin and gentamicin. The integrase gene and accessory gene(s) of the Tn*916*/Tn*1545* family were detected in ten strains. A direct link was shown between the presence of Tn*916*/Tn*1545*-like elements and resistance to doxycycline and/or erythromycin. The results demonstrated that virulence and antibiotic resistance determinants were prevalent in oral *E. faecalis* strains. It implicates that oral *E. faecalis* might play a role in the pathogenesis of chronic periodontitis and be a potential reservoir for the transferable elements of virulence and antimicrobial resistance.

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Introduction

Enterococci are increasingly associated with nosocomial infections such as bacteremia, infective endocarditis, and urinary tract infections. *Enterococcus faecalis* causes the majority of human enterococcal infections, and *E. faecium* seems to play a relatively more important role in the transfer of antimicrobial resistance [1]. Although the pathogenesis of enterococcal infections is poorly understood, certain virulence factors and antibiotic resistance encoded by mobile genetic elements have been shown to be associated with systemic infections [1]. Whole genome sequencing of *E. faecalis* V583 revealed that over 25% of the genome was made up of mobile and exogenous DNA, including a number of conjugative and composite transposons, a pathogenicity island, integrated plasmid genes and phage regions, and a high number of insertion sequence elements [2]. These genetic determinants confer the traits that provide survival advantages to organisms in unusual environments [3].

Generally, *E. faecalis* has been recovered at a low frequency in the normal oral cavity. In post-treatment apical periodontitis, *E. faecalis* has been recovered at a high rate and is regarded as a causative pathogen [4]. Virulence factors produced by *E. faecalis* are demonstrated to mediate endodontic infections and inflammatory responses in root canals [5]. Moreover, a high proportion of oral *E. faecalis* strains isolated from patients with apical periodontitis express resistance to commonly used antibiotics [6, 7]. *E. faecalis* is recovered at a low frequency from the subgingival area of marginal periodontitis patients. However, antimicrobial

resistance is often detected in the subgingival *E. faecalis* towards several antimicrobial agents [8, 9]. Rams et al. suggested that enterococci might contribute to disease progression in severe subgingival infections and impact the overall effect of periodontal antimicrobial therapy [9]. There is still a big gap in understanding the role of enterococci in chronic periodontitis.

The aims of this study were to investigate the presence of defined virulence and antimicrobial resistance traits and determinants in subgingival *E. faecalis* isolated from patients with chronic periodontitis.

Materials and methods

Bacterial strains

Subgingival dental plaque samples were collected consecutively from the periodontal pockets of over 5 mm of chronic periodontitis patients in 2005–2006. *E. faecalis* strains isolation and characterization by multi-locus sequence typing (MLST) were performed in our previous study [8]. The present study involved the first collection of 24 strains representing 14 MLST sequence types, isolated from 24 periodontitis patients (11 males and 13 females, aged from 36 to 72 years, with average age of 53±9.8 years). Type strain ATCC 29212 and the laboratory strains of DS16, OG1RF, FA2-2, and JH2-2 were used as the reference.

Detection of virulence-associated phenotypes

Hemolysin production

A single-colony inoculum was streaked on Todd–Hewitt agar plates supplemented with 5% defibrinated horse blood and incubated aerobically at 37°C for 48 h. The result was recorded as positive when a clear halo was seen around each colony [10].

Gelatinase production

A single-colony inoculum was streaked on Todd–Hewitt agar plates containing 3% gelatine and incubated aerobically at 37°C for 48 h and subsequently at 4°C for 5 h. A positive result was recorded when a clear halo was seen around each colony [10].

Biofilm formation

Biofilm formation ability was examined as previously described [11]. Briefly, biofilm was formed on a sterile 96-well microtiter plate, dried and stained with 1% crystal violet. An optical density (OD) value derived from the absorption of light at a wavelength of 595 nm when penetrating the biofilm mass by using a VersaMax Tunable Microplate Reader (Molecular Devices Co., Sunnyvale, CA). The OD value was 0.06 for the negative control (*E. faecalis* JH2-2) and 0.28 for the positive control (*E. faecalis* OG1RF). Biofilm formation ability was scored as follows: (−) non-biofilm forming with OD value ≤ 0.06; (±) weak biofilm forming with 0.06 < OD value ≤ 0.30; (+) moderate with 0.30 < OD value ≤ 1; (++) strong with OD value > 1.

Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) was assessed by Etest (AB Biodisk, Solna, Sweden) for six antibiotics, i.e., ampicillin, ciprofloxacin, doxycycline, erythromycin, gentamicin, and vancomycin. The levels of antimicrobial susceptibility were interpreted according to the criteria of the epidemiology cut-off values of the European Committee Antimicrobial Susceptibility Testing (EUCAST).

Detection of virulence genetic determinants

Whole DNA of *E. faecalis* strains was extracted as previously described [12]. Polymerase chain reaction (PCR) assays were applied targeting the virulence determinants of collagen-binding antigen (*ace*), aggregation substance (*asa* and *asa373*), biofilm on plastic surfaces (*bopD*), hemolysin activator (*cylA*), endocarditis antigen (*efaA*), putative surface antigen (*EF 3314*), enterococcal surface protein (*esp*), gelatinase (*gelE*), and the gelatinase-negative phenotype (*ef1841*/*fsrC*) [10, 12].

Detection of antimicrobial resistant genes and mobile genetic elements

Multiplex PCRs were used for detecting the genes of tetracycline resistance, erythromycin resistance, and conjugative transposon Tn*916*/Tn*1545* family [13, 14]. PCR amplicons of *tet*M, *erm*B, and *int* from four strains were confirmed by DNA sequencing with an ABI 3130XL 20 genetic analyzer (Applied Biosystems). The DNA sequences were blasted for sequence similarity to annotated sequences at http://www.ncbi.nlm.nih.gov.

Results

Virulence-associated phenotypes and virulence genetic determinants

The prevalence and distribution of virulence-associated phenotypes and virulence genetic determinants are shown

in Table 1. Moderate or weak biofilm formation was detected in all strains, while gelatinase and hemolysin production were detected in 12 (50%) and four (17%) strains, respectively. The adherence genes of *ace*, *efaA*, and *bopD* were determined in all isolates. Other virulence determinants, i.e., *EF3314*, *gelE*, *asa*, *esp*, *cylA*, *ef1841*/*fsrC*, and *asa373* were determined in 96, 79, 58, 50, 38, 25, and 8% of the strains, respectively.

All 12 gelatinase-positive strains were *gelE* PCR-positive. Seven out of 12 gelatinase-negative strains harbored the *gelE* gene, amongst which six also harbored the *ef1841*/*fsrC* determinant. Four of nine *cylA*-positive strains produced hemolysin. The *esp* gene was determined in 90% of the strains with moderate biofilm formation.

Antimicrobial susceptibility and antimicrobial resistant determinants

The results of the antimicrobial susceptibility assay and resistance determinant detection are shown in Table 2. Different levels of antimicrobial susceptibility were observed in the 24 strains according to the MIC values, i.e., susceptible, intermediate susceptible, resistant, and high-level resistant. All three strains of MLST ST16 expressed resistance or intermediate susceptible to doxycycline, besides two of them, which expressed high-level resistance to gentamicin and/or erythromycin.

Tn*916*-like elements harboring *tet*M and *int* genes were detected in seven strains that expressed resistance or intermediate susceptibility to doxycycline. Tn*1545*-like elements harboring *tet*M, *erm*B, and *int* genes were detected in three strains, including the two strains with high-level resistance to erythromycin and doxycycline. The strains of the predominant MLST STs (ST16, ST21, and ST40) were found often carrying Tn*916*/Tn*1545*-like elements. The sequencing of *tet*M, *erm*B, and *int* amplicons showed 97% sequence identity between *tet*M amplicons and the *tet*M allele of *E. faecalis* strain ET35 (GenBank accession no. FM202723), 96% identity between *erm*B and *erm*B pseudogene from *Enterococcus* species

Table 1 Detection of virulence factors and virulence determinants in 24 subgingival *Enterococcus faecalis* strains

Subgingival strains (ST[a])	Virulence factor			Virulence determinant									
	Gelatinase	Hemolysin	BFA[b]	*ace*	*asa*	*asa373*	*bop*D	*cyl*A	*efa*A	*ef3314*	*esp*	*gelE*	*ef1841*/*fsrC*
P1 (40)	+	−	+	+	+	+	+	+	+	+	+	+	−
P2 (16)	−	+	+	+	−	−	+	+	+	+	+	−	−
P3 (21)	−	−	+	+	+	−	+	−	+	+	+	−	−
P4 (40)	+	−	+	+	+	−	+	−	+	+	+	+	−
P5 (21)	−	−	+	+	+	−	+	−	+	+	+	−	−
P6 (239)	−	−	±	+	−	−	+	−	+	+	−	+	+
P7 (170)	+	−	±	+	+	−	+	+	+	+	−	+	−
P8 (79)	+	−	±	+	+	−	+	−	+	−	−	+	−
P9 (209)	−	−	+	+	−	−	+	−	+	+	+	+	+
P10 (21)	−	−	+	+	+	−	+	−	+	+	+	−	−
P11 (240)	+	−	±	+	+	−	+	−	+	+	−	+	−
P12 (209)	−	−	±	+	−	−	+	−	+	+	+	+	+
P13 (25)	−	−	±	+	−	−	+	−	+	+	+	+	+
P14 (25)	−	−	±	+	−	−	+	−	+	+	+	+	+
P15 (241)	+	−	±	+	+	−	+	+	+	+	−	+	−
P16 (162)	+	−	±	+	+	−	+	+	+	+	−	+	−
P17 (16)	−	+	+	+	−	−	+	+	+	+	+	−	−
P18 (162)	+	−	±	+	+	−	+	−	+	+	−	+	−
P19 (40)	+	−	+	+	+	−	+	−	+	+	+	+	−
P20 (242)	+	−	±	+	+	−	+	−	+	+	−	+	−
P21 (16)	+	−	±	+	−	−	+	+	+	+	−	+	−
P22 (56)	−	+	±	+	−	−	+	+	+	+	−	+	−
P23 (56)	−	+	±	+	−	+	+	+	+	+	−	+	+
P24 (81)	+	−	+	+	+	−	+	−	+	+	−	+	−

[a] Multi-locus sequence typing (MLST) sequence type obtained from our previous study [9]

[b] Biofilm forming ability: (−) no biofilm formation; (±) weak biofilmformation; (+) moderate biofilm formation

Table 2 Antimicrobial susceptibility patterns and resistance determinants in 24 subgingival *E. faecalis* strains

Subgingival strains (ST[a])	Antimicrobial susceptibility						Resistant determinants						
	AM[b]	CI[b]	DC[b]	EM[b]	GM[b]	VA[b]	*tet*K	*tet*L	*tet*M	*tet*O	*tet*S	*erm*B	*int*
P1 (40)	S	S	I	R	S	S	−	−	+	−	−	+	+
P2 (16)	S	S	I	HR	I	S	−	−	+	−	−	+	+
P3 (21)	S	S	I	S	I	S	−	−	+	−	−	−	+
P4 (40)	S	S	S	I	S	S	−	−	−	−	−	−	−
P5 (21)	S	S	I	S	S	S	−	−	+	−	−	−	+
P6 (239)	S	S	S	S	S	S	−	−	−	−	−	−	−
P7 (170)	S	S	I	S	S	S	−	−	+	+	−	−	+
P8 (79)	S	S	S	S	S	S	−	−	−	−	−	−	−
P9 (209)	S	S	S	S	S	S	−	−	−	−	−	−	−
P10 (21)	S	S	I	S	I	S	−	−	+	−	−	−	+
P11 (240)	S	S	S	S	I	S	−	−	−	−	−	−	−
P12 (209)	S	S	S	S	I	S	−	−	−	−	−	−	−
P13 (25)	S	I	S	S	I	S	−	−	−	−	−	−	−
P14 (25)	S	I	I	S	S	S	−	−	+	−	−	−	+
P15 (241)	S	S	S	I	S	S	−	−	−	−	−	−	−
P16 (162)	S	S	S	S	S	S	−	−	−	−	−	−	−
P17 (16)	S	S	R	HR	HR	S	−	−	+	−	−	+	+
P18 (162)	S	S	S	S	S	S	−	−	−	−	−	−	−
P19 (40)	S	S	I	I	S	S	−	−	+	−	−	−	+
P20 (242)	S	S	S	S	S	S	−	−	−	−	−	−	−
P21 (16)	S	S	R	S	S	S	−	−	+	−	−	−	+
P22 (56)	S	I	S	S	S	S	−	−	−	−	−	−	−
P23 (56)	S	S	S	S	S	S	−	−	−	−	−	−	−
P24 (81)	S	S	R	I	S	S	−	+	−	−	−	−	−

S susceptible; *I* intermediate susceptible; *R* resistant; *HR* highly resistant

[a] MLST sequence type obtained from our previous study [9]

[b] *AM* ampicillin; *CI* ciprofloxacin; *DC* doxycycline; *EM* erythromycin;*GM* gentamicin; *VA* vancomycin

clone 191 (GenBank accession no. EU595404), and 98% identity between *int* and the transposase gene of Tn*6085* (GenBank accession no. HM243623).

Discussion

E. faecalis is indigenously capable of acquiring, accumulating, and sharing extrachromosomal elements encoding properties that contribute to pathogenesis and antimicrobial resistance [3]. In this study, biofilm formation and gelatinase and hemolysin production were detected in a substantial proportion of the subgingival *E. faecalis* strains, as well as multiple virulence determinants being detected in each strain.

Gelatinase has been indicated in periapical/periodontal inflammation and bone resorption by the degradation of specific host proteins and impairment of periodontal organic matrix [5, 15]. The current study revealed half of the subgingival strains producing gelatinase, which is consistent with a former study in apical periodontitis [10]. Gelatinase is encoded by the *gelE* gene, whose expression is regulated by a quorum-sensing system encoded by the *fsr* gene cluster [16]. Gelatinase-negative phenotype is suggested in relation to a determinant (*ef1841*/*fsrC*) of a 23.9-kb deletion sequence of the *fsr* gene cluster upstream to *gelE* [17]. This has been verified in the present study. Dental plaque is regarded as a major causative factor in most common dental diseases, e.g., dental caries and periodontal disease. This study showed that all *E. faecalis* strains exhibited biofilm formation ability in vitro. Former investigations also demonstrated that all enterococcal isolates from the bloodstream and urinary tract were capable of producing biofilm [18, 19], but one study reported 59% of clinical *E. faecalis* strains forming biofilm in vitro [20]. A number of factors can influence enterococcal biofilm formation, such as the enterococcal surface protein, gelatinase, *fsr* locus deletions, and poly-

saccharides [21]. The present study indicates an association between biofilm formation and the presence of *bopD* and *esp* determinants. Correspondingly, the *bopD* gene as a sugar-binding transcriptional regulator was suggested to be essential for biofilm production [22], as well as a significant link being observed between the presence of *esp* and the biofilm forming ability on polystyrene [11]. Cytolysin can induces tissue damage through the lysis of erythrocytes and destruction of host cells. In this study, 38% of *E. faecalis* strains were determined to carry the *cylA* gene, but only 17% expressed hemolysin activity. Sedgley et al. determined 36% of the *E. faecalis* endodontitis-associated strains to be capable of producing hemolysin [10]. The genes in the *cyl* operon encode cytolysin, where *cylA* is the only reading frame necessary for the expression of component A, a serine protease.

An increase in antimicrobial resistance has been documented in oral microflora, particularly in bacteria associated with periodontal diseases [23, 24]. The present study recovered a high prevalence of reduced susceptibility to commonly used antibiotics in oral *E. faecalis* strains, which agrees with previous findings [6, 7, 9]. It is believed that the wide use of tetracyclines is conducive to the spread of resistance determinants, such as in the treatment of localized aggressive periodontitis and respiratory tract infections [25, 26]. Two resistance mechanisms are available for tetracycline resistance: ribosomal protection proteins encoded by *tet*M-like genes and efflux pumps encoded by *tet*L-like genes [27]. The *tet*M gene (91%) and *tet*L gene (9%) were both determined in our doxycycline-resistant strains. In comparison to the result of 25% observed in Rams et al.'s study, our study showed erythromycin resistance in 29% of the strains [9]. Erythromycin resistance in *E. faecalis* is typically mediated by rRNA methylase encoded by *erm*B [28], which was detected in all erythromycin-resistant strains in this study. Enterococci are intrinsically resistant to aminoglycosides at low levels, while high-level gentamicin resistance is considered as acquired resistance mediated by composite transposons [29]. In the present study, only one *E. faecalis* isolate expressed high-level gentamicin resistance, as well as co-resistance to tetracycline and erythromycin, and harbored the Tn*1545*-like element. The Tn*916*/Tn*1545* family can spread and increase resistance toward tetracyclines and minocycline and, to a lesser extent, toward erythromycin and kanamycin in a wide variety of gram-positive and gram-negative bacteria [30]. The Tn*916*/Tn*1545* family confers multi-drug resistance encoded by additional antimicrobial resistance determinants integrating into larger conjugative transposons [31]. The present study has revealed a direct link between Tn*916*/Tn*1545*-like elements and the resistance to doxycycline and/or erythromycin. Molecular epidemiological studies demonstrate that ST16-related *E. faecalis* strains are often associated with resistance/co-resistance toward high-level gentamicin, vancomycin, tetracycline, and erythromycin [8, 32, 33]. Combining with our previous findings, the *E. faecalis* in MLST ST16 and ST21 tend to carry the Tn*916*/Tn*1545* family [8]. Inversely, a few different virulence genes and antimicrobial resistance determinants emerge in the strains of one ST clone. Such clonal diversity might be evolved from chromosomal recombination and point mutation in *E. faecalis* [34, 35], which needs to be investigated further.

Overall, the results of the present study implicate that oral *E. faecalis* might play a role in the pathogenesis of chronic periodontitis and be a potential reservoir for the transferable elements of virulence and antimicrobial resistance.

Acknowledgments This study was funded by the University of Tromsø, Norway. We thank Harald M. Eriksen, Johanna E. Sollid, and Bjørn Erik Kristiansen for their kind support in organizing the original collection of strains. We also thank Trine Tesserm for the technical assistance with the Etest.

Declaration of interest Nothing to declare.

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