Occurrence, Population Structure, and Antimicrobial Resistance of Enterococci in Marginal and Apical Periodontitis[▽]

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Subgingival plaque samples and root canal samples were collected from 2,839 marginal periodontitis (MP) patients and 21 apical periodontitis (AP) patients. *Enterococcus* species were identified by a series of phenotypic and genotypic tests. Antimicrobial susceptibility assays were performed by an agar disk diffusion test. Multilocus sequence typing (MLST), eBURST, and minimum spanning tree were used for enterococcal genetic clustering and population analysis. *Enterococcus faecalis* was recovered from 3.7% MP patients and 9.5% AP patients, and *Enterococcus faecium* was recovered from 0.04% MP patients. Enterococci were detected more often in older male patients. *E. faecalis* isolates of MP were found resistant to tetracycline (49.1%), erythromycin (8.5%), trimethoprim (2.8%), and gentamicin (1.9%), while one AP isolate was resistant to tetracycline. A total of 40 sequence types (STs) were resolved in 108 *E. faecalis* isolates. Comparison with *E. faecalis* international MLST database revealed that 27 STs were previously found, 13 STs were novel, and several major clonal complexes in the database were also found in MP isolates. The tetracycline-resistant isolates distributed mainly in the major clonal complexes and singletons, whereas the erythromycin-resistant isolates were more dispersed. Although the rate of occurrence of enterococci recovered in the MP and AP samples was low, 50% of these isolates are resistant to at least one antimicrobial agent, which is most often tetracycline. This implies that subgingival *E. faecalis* might represent a reservoir of resistance to tetracycline and erythromycin. The subgingival *E. faecalis* isolates show high genetic diversity but are grouped, in general, with the known isolates from the international database.

Enterococci are commensal organisms well adapted to survive in human and animal intestinal tracts (15). Occasionally they become opportunistic pathogens causing urinary tract infections, septicemia, and endocarditis (16). In the United States, *Enterococcus faecalis* has been found to account for approximately 60% of health care-associated enterococcal infections and *Enterococcus faecium* for most of the remaining part (13).

Enterococci display intrinsic resistance to a number of antimicrobial agents and have a propensity for acquiring resistance to currently prescribed antimicrobials, which poses serious problems in the treatment of invasive enterococcal infections (3, 7, 18). Molecular typing of *E. faecalis* and *E. faecium*, e.g., multilocus sequence typing (MLST), has shown the emergence of specific genetic lineages of enterococci, i.e., hospital-adapted clones of antimicrobial-resistant *E. faecalis* and *E. faecium* (11, 40). *E. faecalis* clonal complexes CC2, CC9, and CC87 seem to be widely distributed in hospital patients in Europe and the United States (17, 31).

Enterococci have been isolated at low rates and in small numbers from the oral cavities of healthy individuals (10, 28,

33). However, in patients with posttreatment apical periodontitis (AP), enterococci are predominant bacteria, with reported prevalence ranging from 29% to 77% (37). Moreover, enterococcal biofilms have been observed in root canals of patients with AP after treatment for AP (8, 39). Enterococci recovered in root canals of AP patients often express resistance toward commonly used antimicrobials (6, 26).

Only a few studies have reported on the occurrence of enterococci in marginal periodontitis (MP) patients, but with a variation from 1% to 47.8% (29, 36). There is a considerable gap in our knowledge concerning the prevalence of enterococci in MP, their genetic population structure, antimicrobial susceptibility profile, and their role in pathogenesis.

The aims of the present study were as follows: (i) to investigate the occurrence and species distribution of enterococci in subgingival and root canal samples from MP and AP patients, respectively; (ii) to examine their antimicrobial susceptibility profile; (iii) to analyze the genetic relatedness of oral *E. faecalis* isolates and compare their population structure with *E. faecalis* international MLST database; and (iv) to assess the association, if any, between sequence type (ST) and specific antimicrobial-resistant phenotypes.

MATERIALS AND METHODS

Bacterial sampling. The bacterial samples were collected from the subgingival areas of 2,839 MP patients throughout Norway in 2005 to 2006 according to a standard protocol at www.unilabs.no/Fagomrader/Medisinsk-mikrobiologi/-Informasjon-til-tannleger (Telelab, Skien, Norway). Briefly, three sterile paper

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TABLE 1. Occurrence of *E. faecalis* and *E. faecium* and age and gender of MP and AP patients

Patient	Enterococcus	No. of patients	% of patients	Gender (%)		Age range (yr) (mean $\pm$ SD)
				Male	Female	
MP	Total	2,839		43.3	56.7	15–96 (53 $\pm$ 11.3)
	<i>E. faecalis</i>	106	3.7	57.5	42.5	31–85 (56 $\pm$ 11.1)
	<i>E. faecium</i>	1	0.04	100		49
AP	Total	21		57.1	42.9	26–75 (50 $\pm$ 12.0)
	<i>E. faecalis</i>	2	9.5	50	50	48, 66

points were inserted into each periodontal pocket for 15 s and transported in Stuarts transport medium (SSI 28733, Denmark). In addition, 47 bacterial samples were collected from the root canals of 21 AP patients by an endodontist, Anne Kjaereng, in Tromsø, Norway. Three sterile paper points were inserted into each root canal of an AP tooth for 15 s and transported as described above. *E. faecalis* ATCC 29212, *E. faecium* ATCC 19434, and *Enterococcus gallinarum* ATCC 49608 were used as reference strains in species identification. *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 51299 were used as quality controls in antimicrobial susceptibility tests.

Isolation of enterococci and identification of enterococcal species. A pilot study covering all 47 AP samples and 101 MP samples revealed the same efficiency in isolation of enterococci by using enterococcal broth enrichment (14) and cephalixin (cefalexin) aztreonam arabinose (CAA) enterococcal selective agar (9) in parallel. Therefore, direct inoculation of samples on CAA agar and reculturing on human blood agar were applied in this study. Enterococcal species identification was performed by a series of phenotypic tests including the Oxoid biochemical identification system PYA (Oxoid Ltd., Hampshire, England), Prolex streptococcal grouping latex kit (Pro-Lab Inc., Richmond Hill, Ontario, Canada), and Tellur diagnostic tablet (ROSCO Diagnostica A/S, Taastrup, Denmark). All enterococcal isolates were confirmed to the species level by molecular identification.

Molecular identification of *E. faecalis* and *E. faecium* by 16S rRNA and D-alanine-D-alanine ligase (DDL) gene PCR. Rapid isolation of total bacterial DNA was performed as previously described (5). Molecular identification of *E. faecalis* and *E. faecium* was carried out by PCR with species-specific primers targeting the inner fragments of *E. faecalis* 16S rRNA and the *E. faecium* *ddl* gene (2, 34). PCR products were defined by size determination after agarose gel electrophoresis.

Antimicrobial susceptibility assays. Antimicrobial susceptibility test of enterococci was performed by an agar disk diffusion method, using a semiconfluent inoculum on ISA agar (Oxoid, Basingstoke, Hampshire, United Kingdom). The following antimicrobials were tested: ampicillin (10 μ g), erythromycin (ERY) (15 μ g), gentamicin (GEN) (30 μ g), tetracycline (TET) (30 μ g), linezolid (10 μ g), and trimethoprim (TMP) (5 μ g) (Oxoid). The results were interpreted according to the Swedish Reference Group for Antimicrobials. Susceptibility to vancomycin was examined by an agar dilution method (38). *E. faecalis* ATCC 51299 (*vanB*) and *E. faecalis* ATCC 29212 served as control strains.

MLST. Multilocus sequence typing (MLST) of *E. faecalis* isolates was performed as described previously (31). Briefly, the internal fragments of seven housekeeping genes, *gdh*, *gyl*, *pstS*, *gki*, *aroE*, *xpt*, and *yqjL*, were amplified, purified, and bidirectionally sequenced by ABI Prism BigDye3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI Prism 377 automated DNA sequencer (Applied Biosystems). The sequences were analyzed by using DNASTAR version 5 software (DNASTAR, Inc. Madison, WI). Allele numbers were assigned by comparing the sequence at each locus to all known corresponding alleles available at the *E. faecalis* MLST website (<http://efaecalis.mlst.net>). The ST was determined by the combination of the seven alleles, which was derived from the same website. The new alleles and STs identified in this study were deposited in the database. The isolates from MP and AP patients were clustered on an eBURST diagram and a minimum spanning tree based on the STs.

Statistical analysis. SPSS 15.0 for Windows was used for statistical analysis of the age (*t* test) and gender (chi-square test) of the patients. A *P* value of less than 0.05 was considered to be statistically significant. eBURST software 3.0 (<http://efaecalis.mlst.net/eburst/>) was used to generate a diagram based on STs, with single-locus variants (SLVs) and double-locus variants (DLVs) within a clonal complex. BioNumerics software (version 4.0; Applied Maths, Sint-Martens-Latem, Belgium) was used to generate a minimum spanning tree under the categorical coefficient of similarity and the priority rule of the highest number of SLVs: the ST type that had the highest number of SLVs would be linked first.

TABLE 2. Antimicrobial susceptibility of *E. faecalis* and *E. faecium* isolates from MP and AP patients

Antimicrobial	No. of isolates showing resistance or susceptibility ^a					
	MP patients				<i>E. faecalis</i> (<i>n</i> = 2) from AP patients	
	<i>E. faecalis</i> (<i>n</i> = 106)		<i>E. faecium</i> (<i>n</i> = 1)			
	S	R	S	R		
Ampicillin	106	0	1	0	2	0
Erythromycin	98	9	1	0	2	0
Gentamicin	105	2	1	0	2	0
Tetracycline	54	52	1	0	1	1
Linezolid	106	0	1	0	2	0
Trimethoprim	104	3	1	0	2	0
Vancomycin	106	0	1	0	2	0

^a Resistance (R) or susceptibility (S) according to the Swedish Reference Group for Antimicrobials.

RESULTS

Occurrence of enterococci from MP and AP patients. A total of 109 enterococcal isolates were isolated from 107 (3.8%) MP patients and two (9.5%) AP patients. All but one isolate (*n* = 108) were identified as *E. faecalis*, and the one isolate was *E. faecium* that was recovered from an MP patient. The occurrence of enterococci and distribution in patients are shown in Table 1. Statistical analyses indicated that enterococci occurred more frequently in males than in females (*P* = 0.002) and that the patients carrying subgingival enterococci were significantly older than those without subgingival enterococci (*P* = 0.003).

Antimicrobial susceptibility. Antimicrobial susceptibility of the enterococci in MP and AP patients are summarized in Table 2. Generally 50% *E. faecalis* isolates (53 in MP patients and one in an AP patient) were resistant to at least one antimicrobial agent. *E. faecalis* isolates in MP expressed resistance to TET (49.1%), ERY (8.5%), TMP (2.8%), and GEN (1.9%). One of the two AP isolates was resistant to TET. The only *E. faecium* isolate was susceptible to all the tested antimicrobials. Vancomycin resistance was not detected. Multiresistance was detected in MP *E. faecalis* isolates, with 3.8% isolates resistant to three antimicrobials (TET, ERY, and GEN or TMP), and 4.7% isolates resistant to two (TET and ERY or TMP). Monoresistance (TET or ERY) was detected in 41.5% isolates.

Genetic clustering of *E. faecalis*. A total of 106 *E. faecalis* MP isolates and two AP isolates were resolved into 40 STs, of which 23 STs comprised only one isolate. The most frequently found types were ST21 (*n* = 19 [17.6%]), ST40 (*n* = 11 [10.2%]), ST30 (*n* = 7 [6.5%]), and ST56 (*n* = 6 [5.6%]). The two AP isolates were resolved into ST40 and ST56. Figure 1A illustrates the eBURST cluster analysis of 108 *E. faecalis* isolates from MP and AP patients based on the group stringency (six/seven shared alleles).

Figure 1B shows the assignment of the 108 isolates to the international *E. faecalis* MLST database. Twenty-seven STs comprising 93 (86.1%) *E. faecalis* isolates existed already in the database, while 12 singletons (*n* = 14 [13.0%]) were found for the first time in this study, i.e., ST236 and ST241 (*n* = 2 for each ST) and ST226, ST237, ST238, ST239, ST240, ST242, ST244, ST245, ST246, and ST247 (*n* = 1 for each ST). The

FIG. 1. eBURST diagram of *E. faecalis* sequence types. (A) The diagram includes the 106 *E. faecalis* isolates from MP patients and two isolates from AP samples that are classified into 40 MLST STs in the present study. Each ST is presented as a node together with a number (the ST number). The size of the node reflects the number of isolates. Pink lines connect single-locus variants, and blue lines connect double-locus variants. (B) The diagram is drawn with 460 *E. faecalis* isolates classified into 167 MLST STs from the database and the present study. Each ST is presented as a node together with a number (the ST number). The size of the node reflects the number of isolates. Blue nodes represent the predicted founder. Black numbers represent the STs from the database. Green numbers represent the new STs from the present study. Pink numbers represent the STs from both the present study and database. Pink or black lines connect SLVs, and blue lines connect DLVs.

major clonal complexes like CC21, CC25, CC30, CC40, CC44, and CC72 in the database were also observed in MP isolates.

Clonal distribution of antimicrobial susceptibility. The minimum spanning tree illustrates the distribution of antimicrobial resistance among the 40 STs of the 108 *E. faecalis* isolates from MP and AP patients (Fig. 2). TET resistance was observed in 17 STs ($n = 53$) but mainly in major STs, i.e., ST21, ST56, ST40, and ST16 (Fig. 2A). ERY resistance was observed in seven STs ($n = 9$) belonging to CC40 and six singletons (Fig. 2B). Figure 2C shows the distribution of complex antimicrobial resistance among different STs. ST21 and ST56 comprised the isolates mostly resistant to TET, 89.5% and 100%, respectively. ST40 and ST30 comprised isolates either susceptible or resistant to two or one antimicrobial agent. ST16 displayed the most complex antimicrobial susceptibility profile, with the isolates resistant to three, two, and one antimicrobial agent or susceptible. Three of the 12 new singletons, ST246, ST244, and ST241, contained antimicrobial-resistant isolates. ST246 with one isolate expressed multiresistance to three antimicrobials (TET, ERY, and GEN). CC72 comprised the isolates susceptible to all tested antimicrobials.

DISCUSSION

This study was undertaken to examine the occurrence, antimicrobial susceptibility profile, and genetic characteristics of enterococci in subgingival and root canal samples from MP and AP patients. Enterococcal isolates were identified to the species level, characterized by MLST, and examined for their susceptibility to antimicrobial agents used in the treatment of enterococcal infections.

The present study reports the first isolation of *E. faecium* from a subgingival sample of an MP patient. *E. faecium* has rarely been detected in the oral cavity of a healthy individual, but it has been detected from infected root canals and periapical areas in endodontic patients after treatment (6, 27). These observations suggest that *E. faecium* might not be an indigenous organism in the oral cavity. *E. faecalis* was identified in 3.7% (106/2,839) MP patients. The frequency of *E. faecalis* in MP patients is consistent with 5.1% reported in a previous study using comparable cultivation techniques (29). In contrast, a recent study showed a high prevalence of 48.1% MP patients carrying *E. faecalis* in subgingival samples and 17.1% periodontally healthy subjects carrying *E. faecalis* in sulcus samples by using a PCR method (36). The high prevalence could be explained by the detection of noncultivable enterococci and a higher sensitivity of PCR-based techniques, which needs to be verified in other studies. An early study revealed the carriage rates of enterococci in dental plaque samples among different groups by culturing, i.e., 6.0%, 5.4%, 13.0%, and 19.2% among a healthy group of university students and staff, a group of patients with toothaches, hemodialysis patients, and the dialysis unit staff, and most of the enterococci were *E. faecalis* (35). Moreover, a recent review article concluded that *E. faecalis* appears in a low number of oral samples in healthy individuals (28). Overall, these studies suggest that *E. faecalis* is a part of the human oral flora but occurs at a low prevalence or transiently.

E. faecalis was identified in 9.5% (2/21) AP patients, one before and one after treatment. A recent review has summa-

rized the occurrence of enterococci in AP patients, ranging between 0 and 14% and 29 and 77% in patients before and after treatment, respectively (37). *E. faecalis* is claimed to play a pathogenic role in posttreatment AP that is associated with biofilm formation and acquired antimicrobial resistance (37, 39). A number of putative virulence factors have been detected in AP-associated *E. faecalis* isolates, i.e., virulence genes, such as *ace*, *gelE*, *esp*, *efaA*, and *cylA*, and substances, such as hemolysin, gelatinase, and aggregation substance (32, 34). Alternatively, the occurrence of enterococci in root canals could also be interpreted as a result of ecological selection rather than a putative role in pathogenesis (25).

Our statistical analysis showed a tendency of higher frequency of *E. faecalis* in older male MP patients. This might be relevant to the previous findings of a preponderance of enterococcal endocarditis in older males (21). Other enterococcal infections are known to occur more often in elderly people, e.g., infections of the urinary tract, endocarditis, and bacteremia (12, 20, 22). However, a few studies demonstrate that the prevalence of *E. faecalis* in the oral cavities of endodontic patients is not affected by age and gender (33, 35).

The antimicrobial susceptibility assay revealed that 50% of MP *E. faecalis* isolates expressed resistance to at least one antimicrobial agent, mostly TET (49.1%). The high frequency of TET resistance is comparable to a previous finding of 58% MP *E. faecalis* resistant to TET in the United States (29). On the other hand, the low frequencies of resistance to ERY (8.5%) and GEN (1.9%) contrast with the high prevalence of corresponding resistance (25% to ERY, 50% to GEN) found in the same study (29). A total of 8.5% of *E. faecalis* isolates in MP patients were resistant to more than one antimicrobial agent, with multiresistance to three antimicrobials (TET, ERY, and GEN or TMP) and two antimicrobials (TET and ERY). The high prevalence of TET resistance and the occurrence of multiresistance against TET and ERY likely are associated with the presence of the Tn916-Tn1545 family, which are conjugative transposons carrying *tet(M)* and/or *erm(B)*. Tn916-Tn1545 was originally isolated from *E. faecalis* and has been shown to be present in many bacterial species within the oral microbiota (4).

Antimicrobial usage is considered a driving force behind the selection and spread of antimicrobial resistance. A recent national study of antimicrobial prescriptions demonstrates that Norwegian dentists prescribe only 1.2% and 2.8% of the national consumption of TET and macrolides/lincosamides, respectively (1). There is no clear correlation between the low prescription of TET in Norwegian dentistry and the high prevalence of TET resistance in our collection of oral *E. faecalis* isolates. However, TET has been a widely used antimicrobial agent in the treatment of respiratory tract infections in Norway (23). In addition, previous studies have shown a high prevalence of TET resistance and *tet(M)* determinants in bacterial isolates from periodontal pockets (24). The high prevalence of TET resistance in oral *E. faecalis* could be the result of the promiscuous spread of *tet(M)* containing Tn916-related elements within oral microbiota, while our data do not allow further speculations on this. To sum up, our observations are consistent with the general recognition of oral microflora as an important reservoir for antimicrobial resistance (30, 42, 43).

To our knowledge, the present study is the first to use MLST

A

B

C

FIG. 2. Minimum spanning tree for clonal distribution of antimicrobial susceptibility. Colors indicate resistance to TET (A), ERY (B), and four antimicrobial agents (C). Every circle represents a ST (the ST number is shown in the circle), and the size of the circle represents the number of isolates. Short thick lines connect SLVs, thin lines connect DLVs, and broken lines connect triple-locus variants (black) or STs with more than three alleles different (gray). Pie charts indicate ST distribution. Clonal complexes are indicated by colored backgrounds. In panel A, red indicates TET resistance. In panel B, red indicates ERY resistance. In panel C, red indicates resistance against three antibiotics (TET, ERY, and GEN or TMP); blue indicates resistance against two antibiotics (TET and ERY or TMP), yellow indicates resistance against one antibiotic (TET or ERY), and green indicates susceptibility.

to establish the population structure of oral enterococci. The eBURST snapshots illustrate a high genetic diversity of oral *E. faecalis* isolates that were assigned to 40 different STs, five CCs and 29 singletons. A number of studies demonstrate a high genetic diversity of *E. faecalis* isolated from hospitalized patients, surveillance samples, animals, and food (17, 18, 31). Based on the hypothesis that all the STs within one clonal complex represent ancestry and evolutionary descent (41), the 108 isolates are supposed to be the descendants of 33 ancestries from the whole *E. faecalis* MLST database.

Besides the genetic diversity, common genetic origins and a similar population structure are shared by *E. faecalis* isolates in this study and those in the database, e.g., 86.1% *E. faecalis* isolates and 27 of 40 STs presenting in the MLST database and the major ST21, ST40, and ST30 also predominating in the database. The database documents that most strains in ST21 (80%), CC40 (81.6%), and CC30 (77.8%) are from hospitalized patients mostly from Poland (17, 31); the remaining strains are from human community and animal reservoirs in Spain, Denmark, and The Netherlands (17, 31). The presence of corresponding STs and CCs in both our study and the database indicates that oral *E. faecalis* strains can be cross

transmitted from other sources, such as hospitalized patients, healthy individuals, and animals in different countries. Moreover, these genetic lineages are well adapted to several ecological niches. Additionally, the present study has identified 12 new singletons comprising 13.2% isolates in MP patients. It is unclear whether these novel singletons are unique for subgingival conditions where selective pressure may exist, e.g., unusual pH, nutrients, redox potential, oxygen tension, temperature, and complex microbiota (19).

Minimum spanning tree analysis revealed a wide clonal distribution of the *E. faecalis* isolates expressing antimicrobial resistance. TET-resistant isolates are found mainly in major CCs and singleton clones, while ERY-resistant isolates are found more widely. The highly diverse genetic background of TET and ERY resistance isolates is typical for resistance determinants residing on mobile genetic elements that can be transferred horizontally. In the present study, CC21, CC30, CC40, and ST16 were associated with TET resistance, while CC40 and ST16 were associated with ERY resistance. Furthermore, the international MLST database documents that some strains in CC40 are associated with multidrug resistance, and a few strains in CC21 are associated with glycopeptide resis-

tance. These pieces of evidence imply that there is somehow an association between CC40, CC21, and antimicrobial resistance. Interestingly, the high-risk global clonal complexes of *E. faecalis* (CC2, CC9, and CC87), which have emerged in hospital outbreaks and enterococcal infections in the United States and some European countries, have not been identified in our isolates (17, 18, 31). On the whole, oral *E. faecalis* isolates may be associated with antimicrobial-resistance-related clones CC40 and CC21, but not to hospital-adapted clonal complexes. The isolates in ST16, CC40, and CC30 displaying multidrug resistance indicate that one genetic group may contain the *E. faecalis* isolates with different antimicrobial susceptibility levels.

In conclusion, the present study shows a low rate of *E. faecalis* isolated from MP and AP patients. Only one *E. faecium* was recovered from an MP patient. A total of 50% of the *E. faecalis* isolates are resistant to at least one antimicrobial agent, which is most often TET. MLST analysis revealed a high genetic diversity among *E. faecalis* isolates. The population structure of oral isolates coincides with that of the international MLST database, although new STs were observed. There is somehow an association between specific CCs/STs and antimicrobial resistance, and subgingival *E. faecalis* could be regarded as a reservoir for resistance to TET and ERY.

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