Oral Microbiology Immunology 2005: 20: 10–19
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Oral Microbiology and Immunology

Virulence, phenotype and genotype characteristics of endodontic *Enterococcus* spp.

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Sedgley CM, Molander A, Flannagan SE, Nagel AC, Appelbe OK, Clewell DB, Dahlén G. Virulence, phenotype and genotype characteristics of endodontic Enterococcus *spp. Oral Microbiol Immunol 2005: 20: 10–19.* © Blackwell Munksgaard, 2005.

Background/aims: Enterococci have been implicated in persistent root canal infections but their role in the infection process remains unclear. This study investigated the virulence, phenotype and genotype of 33 endodontic enterococcal isolates.
Methods: Phenotypic tests were conducted for antibiotic resistance, clumping response to pheromone, and production of gelatinase, hemolysin and bacteriocin. Genotype analysis involved polymerase chain reaction amplification of virulence determinants encoding aggregation substances *asa* and *asa373*, cytolysin activator *cylA*, gelatinase *gelE*, gelatinase-negative phenotype *ef1841/fsrC*, adherence factors *esp* and *ace*, and endocarditis antigen *efaA*. Physical DNA characterization involved pulsed-field gel electrophoresis of genomic DNA, and plasmid analysis.
Results: Potential virulence traits expressed included production of gelatinase by *Enterococcus faecalis* (n = 23), and response to pheromones in *E. faecalis* culture filtrate (n = 16). Fourteen strains produced bacteriocin. Five strains were resistant to tetracycline and one to gentamicin, whereas all were susceptible to ampicillin, benzylpenicillin, chloramphenicol, erythromycin, fusidic acid, kanamycin, rifampin, streptomycin and vancomycin. Polymerase chain reaction products encoding *efaA*, *ace*, and *asa* were detected in all isolates; *esp* was detected in 20 isolates, *cylA* in six isolates, but *asa373* was never detected. The gelatinase gene (*gelE*) was detected in all isolates of *E. faecalis* (n = 31) but not in *Enterococcus faecium* (n = 2); a 23.9 kb deletion sequence corresponding to the gelatinase-negative phenotype was detected in six of the eight *E. faecalis* isolates that did not produce gelatinase. Pulsed-field gel electrophoresis and plasmid analyses revealed genetic polymorphism with clonal types evident. Plasmid DNA was detected in 25 strains, with up to four plasmids per strain and a similar (5.1 kb) plasmid occurring in 16 isolates.
Conclusions: Phenotypic and genotypic evidence of potential virulence factors were identified in endodontic *Enterococcus* spp., specifically production of gelatinase and response to pheromones.

Key words: endodontic; *Enterococcus* spp.; genotype; phenotype; virulence

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Accepted for publication July 29, 2004

Enterococci are gram-positive cocci that form part of the normal gastrointestinal tract flora in animals and humans (22). Clinical studies have shown that enterococci make up a small proportion of the flora in untreated root canal infections (5, 53). However, enterococci have long been implicated in persistent root canal infections (4, 20, 50). Along with streptococci and gram-positive rods, enterococci have been identified as the organisms most commonly recovered from the root canals of teeth with failed endodontic treatment (6, 37, 52).

The role of enterococci in root canal infections remains unclear. Fabricius et al. (21) showed that *Enterococcus faecalis*, as monocultures, survived for 6 months in all

of nine inoculated root canals in monkeys, although there was only a weak inflammatory periapical response. It has been suggested that enterococci may be selected in root canals undergoing standard endodontic treatment because of low sensitivity to antimicrobial agents (13, 38, 42). Further, Love (33) postulated that the proficiency with which *E. faecalis* can invade dentinal tubules facilitates protection from chemomechanical root canal preparation and intracanal dressing techniques. Subsequently, when the opportunity arises, *E. faecalis* could be released from the tubules into the root canal space and act as a source of reinfection. If this is true then investigation of potential virulence factors would be useful in understanding their role, if any, in endodontic infections. Potential virulence factors of interest would include those that promote adaptation and survival in different environments, such as *Enterococcus* surface protein (Esp) and aggregation substance (AS), and factors that enable secretion of toxins (e.g. cytolysin) and proteases (e.g. gelatinase).

In this study the phenotypic and genotypic characteristics of enterococci isolated from the root canals of patients receiving endodontic treatment in Sweden in 1994/1995 were investigated.

Material and methods

Microorganisms

All bacterial strains used in these investigations are listed in Table 1.

Thirty-three enterococci which were recovered from the root canals of patients attending the Department of Endodontology/Oral Diagnosis, Göteborg University, Sweden in 1994/1995 were investigated. Root canal sample collection, processing and identification methods followed those described previously (13). Information on endodontic treatment and root canal sample growth were available for 23 patients (Table 1).

Freeze-dried samples of the strains were transferred to the University of Michigan. Strains were rehydrated in Todd Hewitt Broth (THB, Difco; Becton Dickinson and Company, Sparks, MD) and a 100 µl aliquot was plated on THB agar supplemented with 4% defibrinated horse blood (Colorado Serum Co., Denver, CO) and incubated aerobically at 37°C for 24–48 h. One colony was then transferred to bile esculin azide agar (Difco) and incubated aerobically at 37°C for 24–48 h. All strains were presumptively confirmed to be enterococci based on growth in the presence of bile and azide, and esculin hydrolysis. Conventional biochemical reactions were used to further characterize the organisms (23). All isolates were characterized as gram-positive cocci, catalase-negative, nonmotile, pyruvate-positive, capable of growth in THB supplemented with 6.5% NaCl at 42°C and identified using API 20 Strep identification kits (Analytical Profile Index; Bio Mérieux SA, Marcy-Etoile, France), with type strain *E. faecalis* ATCC 47077 serving as a positive control. The identity of *Enterococcus faecium* strains was independently confirmed at the Clinical Microbiology/Virology Laboratory, University Hospital, University of Michigan, Ann Arbor, MI.

Strains were stored in 50% sterile glycerol at − 80°C and, when required, were transferred to THB supplemented with 1.5% agar and incubated aerobically at 37°C for 24–48 h followed by a second transfer to THB agar and aerobic incubation for 24 h at 37°C.

Identification of *E. faecalis* by 16S rRNA analysis

Total DNA was extracted according to the method of Molander et al. (36) with minor modifications. Briefly, 1.5 ml of an overnight culture was pelleted and resuspended in 50 µl sterile nuclease-free water (Invitrogen, Carlsbad, CA), heated at 100°C for 5 min and then cooled to room temperature. To each bacterial suspension were added 1 mg lysozyme, 150 µg achromopeptidase, and 15 µg mutanolysin to facilitate bacterial cell wall degradation. After incubation for 1 h at 37°C, genomic DNA extractions were performed using the Wizard DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's suggested protocol but scaled down to accommodate a 100 µl sample. Total DNA template was resuspended in sterile nuclease-free water, quantified using a multichannel Beckman-Coulter DU® 604 spectrophotometer (Foster City, CA) and adjusted to a final stock concentration of approximately 100 ng/µl.

Molecular identification of *E. faecalis* was determined by polymerase chain reaction (PCR) utilizing sequence-specific primers derived from the full-length *E. faecalis* 16S rRNA gene sequence (Accession # Y18293; National Center for Biotechnology Information, NCBI) (Table 2). PCR amplifications were prepared in a 30 µl final reaction volume with the following: 20 ng total DNA template; 6 pmol of each primer; 100 µM dNTPs (Invitrogen, Carlsbad, CA); 2 U HotStarTaq DNA polymerase (Qiagen, Valencia, CA); 3 µl 10X PCR buffer (Qiagen); 4 µl of 25 mM $MgCl_2$ to give a final Mg^{2+} concentration of 2.5 mM. PCR conditions were as follows: 15 min initial enzyme activation/DNA denaturation step at 95°C followed by 35 consecutive cycles at 94°C for 20 s; 68°C for 45 s; 72°C for 15 s. PCR products were analyzed by electrophoresis using 1.5% agarose gels (containing ethidium bromide) in TBE buffer, and visualized by UV fluorescence using an AlphaImager™ 2200 Documentation and Analysis System (Alpha Innotech Corporation, San Leandro, CA).

Hemolysin and gelatinase production

Strains were assessed for hemolytic activity by streaking a single-colony inoculum onto THB agar supplemented with 4% defibrinated horse blood (Colorado Serum Co.), sheep, rabbit or bovine blood (Hemostat Laboratories, Dixon, CA) followed by incubation at 37°C for 24 h. Blood hemolysin activity resulted in a clear halo around each colony. *E. faecalis* DS16 served as a positive control and *E. faecalis* FA2-2 as a negative control.

Gelatinase activity was assessed using samples from isolated colonies to inoculate agar containing 3% gelatin which was then incubated at 37°C for 24–48 h in aerobic and anaerobic (10% hydrogen, 5% carbon dioxide, 85% nitrogen) conditions. Gelatinase activity was evident as a clear halo around each colony. *E. faecalis* OG1RF was used as a positive control and *E. faecalis* OG1-X and *E. faecalis* FA2-2 were negative controls.

Bacteriocin production

E. faecalis strains were tested for production of bacteriocin activity essentially as previously described (28) along with positive and negative control bacteriocin producer strains *E. faecalis* DS16 and *E. faecalis* FA2-2, respectively. Indicator strains were *E. faecalis* FA2-2, *E. faecalis* DS16, *E. faecium* 409 and *Staphylococcus aureus* ATCC 6538 grown in THB, and *Escherichia coli* DH5α and *E. coli* ATCC 29417 grown in LB broth (Difco) to stationary phase. Each indicator strain (10 µl) was added to 3 ml of liquefied THB soft agar (0.75%) and poured onto a THB agar plate. When this had solidified, samples from single colonies of potential

Table 1. Bacteria used in this study

Strains	Description						Reference
	Patient information						
	Treatment type	Age	Gender	Tooth	Treatment Provider Type	Clinical sample growth	
Endodontic strains*							
E. faecalis							
GS1	Retreatment	69	Female	11	Specialist Endodontist A	5 +	This study
GS2	Retreatment	40	Male	37	Specialist Endodontist A	5 +	This study
GS3	Primary treatment	37	Female	46	Specialist Endodontist B	4 +	This study
GS4	N/A						This study
GS5	N/A						This study
GS6	Primary treatment	59	Male	32	General practitioner C	1 +	This study
GS7	Primary treatment	57	Female	33	Specialist Endodontist	5 +	This study
GS8	Primary treatment	81	Female	22	General practitioner D	5 +	This study
GS9	N/A						This study
GS10	N/A						This study
GS12	Retreatment	50	Male	23	General practitioner E	4 +	This study
GS13	Primary treatment	51	Male	27	General practitioner F	4 +	This study
GS14	N/A						This study
GS15	N/A						This study
GS16	Retreatment	43	Female	46	General practitioner G	5 +	This study
GS17	N/A	69	Female	11	General practitioner H	5 +	This study
GS18	Primary treatment	75	Male	43	General practitioner I	5 +	This study
GS19	Primary treatment	42	Female	37	General practitioner J	5 +	This study
GS21	N/A						This study
GS22	Primary treatment	29	Female	46	General practitioner K	4 +	This study
GS23	Primary treatment	45	Female	16	Specialist Endodontist B	1 +	This study
GS24	Primary treatment	51	Female	47	Specialist Endodontist	5 +	This study
GS25	Retreatment	59	Male	36	Specialist Endodontist	5 +	This study
GS26	N/A						This study
GS27	Primary treatment	62	Female	35	Specialist Endodontist	4 +	This study
GS28	Primary treatment	54	Female	13	General practitioner C	5 +	This study
GS29	N/A						This study
GS30	N/A						This study
GS31	Primary treatment	53	Male	26	General practitioner I	5 +	This study
GS32	Primary treatment	45	Female	46	Specialist Endodontist B	1 +	This study
GS33	Retreatment	41	Male	27	Specialist Endodontist L	5 +	This study
E. faecium							
GS11	Retreatment	57	Female	37	Undergraduate	5 +	This study
GS20	Retreatment	64	Male	16	Undergraduate	1 +	This study

	Relevant characteristics	
Nonendodontic strains		
E. faecalis		
FA2-2	Resistant to rifampin and fusidic acid	(9)
JH2-2	Resistant to rifampin and fusidic acid	(30)
OG1RF	Resistant to rifampin and fusidic acid. Produces gelatinase	(17)
OG1X	Resistant to spectinomycin. Does not produce gelatinase	(29)
V583	Clinical isolate (resistant to vancomycin, gentamicin, erythromycin and tetracycline)	(44)
MMH594	Clinical isolate (resistant to vancomycin, gentamicin)	(47)
39–5	Carries pPD1, responds to cPD1 pheromone	(62)
5952	Carries pOB1, responds to cOB1 pheromone	(40)
OG1X/pAD1	pAD1 encodes hemolysin/bacteriocin, responds to cAD1 pheromone	(9)
DS16	Carries pAD1, responds to cAD1 pheromone	(9)
OG1X/pAM373	Responds to cAM373 pheromone	(14)
FA378	FA2-2 carrying pAM378 (pAM373::Tn*918*), responds to cAM373 pheromone	(7)
OG1SS/pCF10	Responds to cCF10 pheromone	(16)
OG1X/pPD1	pPD1 encodes bacteriocin, responds to cPD1 pheromone	(62)
ATCC 47077		ATCC
E. faecium 409	Clinical isolate (resistant to vancomycin)	§
E. faecium BM4105-RF	Resistant to rifampin and fusidic acid	(58)
S. aureus ATCC 6538		ATCC
E. coli ATCC 29417		ATCC
E. coli DH5alpha		Invitrogen

N/A, not available. ATCC, American Type Culture Collection.
§University of Michigan Hospital.
*****Patient and treatment information is not available for GS4, 5, 9, 10, 14, 15, 21, 26, 29, 30.
Growth of clinical sample on Brucella agar: 5+, very heavy; 4+, heavy; 3+, moderate; 2+, sparse; 1+, very sparse (Göteborg University).
Teeth are identified using FDI ISO-3950 notation.
Specific treatment providers are designated by letter, where known.

Table 2. Oligonucleotides used in the study

Gene		Sequence	Product size (bp)	Reference
E. faecalis 16S rRNA	Ef16SF	5' - CCGAGTGCTTGCACTCAATTGG - 3'	138	This study
	Ef16SR	5' - CTCTTATGCCATGCGGCATAAAC - 3'		
ace	aceF	5' - GGAATGACCGAGAACGATGGC - 3'	616	(11)
	aceR	5' - GCTTGATGTTGGCCTGCTTCCG - 3'		
efaA	efaAF	5' - GCCAATTGGGACAGACCCTC - 3'	688	(11)
	efaAR	5' - CGCCTTCTGTTCCTTCTTTGGC - 3'		
asa	asaF	5' - CCAGCCAACTATGGCGGAATC - 3'	529	(11)
	asaR	5' - CCTGTCGCAAGATCGACTGTA - 3'		
asa373	asa373F	5' - GGACGCACGTACACAAAGCTAC - 3'	619	(11)
	asa373R	5' - CTGGGTGTGATTCCGCTGTTA - 3'		
cylA	cylAF	5' - GACTCGGGGATTGATAGGC - 3'	688	(11)
	cylAR	5' - GCTGCTAAAGCTGCGCTTAC - 3'		
esp	espF	5' - TTGCTAATGCTAGTCCACGACC - 3'	932	(47)
	espR	5' - GCGTCAACACTTGCATTGCCGA - 3'		
gelE	gelEF	5' - ACCCCGTATCATTGGTTT - 3'	405	(19)
	gelER	5' - ACGCATTGCTTTTCCATC - 3'		
ef1841/fsrC	ef1841F	5' - GATCAAGAAGGGAAGCCACC - 3'	1050	(39)
	fsrC7R	5' - CCAACCGTGCTCTTCTGGA - 3'		
fsrC internal	fsrC6F	5' - ATGATTTTGTCGTTATTAGCTACT - 3'	ca. 1300	(39)
	fsrC7R	5' - CCAACCGTGCTCTTCTGGA - 3'		

producer strains were stabbed into the agar. After aerobic incubation overnight at 37°C, clear zones were visible around the stabs of bacteriocinogenic strains.

Aggregation substance production

Production of aggregation substance in response to pheromones was determined using microtiter dilution assays as previously described (17). Culture filtrates containing naturally occurring pheromone activities were prepared from plasmid-free *E. faecalis* JH2-2 as previously described (17). *E. faecalis* DS16 (harboring pAD1), *E. faecalis* OG1X/pAM373, *E. faecalis* OG1SS/pCF10, *E. faecalis* 39–5 (harboring pPD1) and *E. faecalis* OG1X/pAD1, each carrying a pheromone-responsive plasmid, were used as positive controls for pheromone response (8). Assays were conducted using microtiter plates. Culture filtrate (100 µl) was placed in the first well of the assigned row and serially diluted (two-fold) into 50 µl THB. Responder strains (50 µl of a bacterial suspension, after overnight growth in THB with fresh THB in a 1 in 10 dilution) were added to each well in the assigned row. Plates were incubated for 4 h at 37°C on an orbital shaker. Pheromone response was observed as visual clumping (aggregate formation) of the responder cells during growth in one or more of the wells.

Antimicrobial susceptibility

Minimal inhibitory concentrations (MICs) were determined using the *E*-test (AB Biodisk, Solna, Sweden) (2). Antibiotics tested were ampicillin, benzylpenicillin, chloramphenicol, clindamycin, erythromycin, fusidic acid, gentamicin, kanamycin, rifampin, streptomycin, tetracycline and vancomycin. Bacterial suspensions in 0.85% phosphate-buffered saline adjusted to equal the turbidity of a 0.5 McFarland standard were spread onto Mueller–Hinton agar (Difco). Following application of *E*-test strips, plates were incubated aerobically at 35°C for 16–20 h and MICs read from the interpretive scale at the point at which the ellipse of inhibition intersected the strip.

Virulence gene detection by PCR

Primers targeting segments of the enterococcal virulence determinants, aggregation substance (*asa* and *asa373*), surface adhesin (*esp*), cytolysin activator (*cylA*), gelatinase (*gelE*) and the gelatinase-negative phenotype determinant (*ef1841/fsrC*), endocarditis antigen (*efaA*), and collagen binding antigen (*ace*) have been previously described (11, 19, 39, 47). Strains used as positive or negative amplification controls or also investigated are listed in Table 1.

PCR conditions were modified to accommodate a common annealing temperature profile of 58°C for all PCR reactions, based on initial temperature-gradient PCR amplifications of each target. Briefly, 100–200 ng total DNA template was prepared for 30 µl PCR amplifications with the following: 6 pmol of each respective primer; 100 µM dNTPs (Invitrogen); 2 U HotStarTaq DNA polymerase (Qiagen); 3 µl 10X PCR buffer (Qiagen). The PCR conditions were as follows: 15 min initial enzyme activation/DNA denaturation step at 95°C followed by 35 consecutive cycles at 94°C for 20 s; 58°C for 45 s; 72°C for 60 s.

Those *E. faecalis* strains positive for the *gelE* genotype but not producing gelatinase activity were examined for the presence or absence of a 23.9 kb deletion segment corresponding to the *fsr* gene cluster upstream of *gelE* by PCR amplification utilizing the proposed primer set according to Nakayama et al. (39). The PCR conditions for a 30 µl final reaction volume based on initial temperature-gradient PCRs were as follows: 15 min initial enzyme activation/DNA denaturation step at 95°C followed by 35 consecutive cycles at 94°C for 20 s; 56°C for 45 s; 72°C for 60 s.

PCR products were analyzed by electrophoresis using 1.5% agarose gels (containing ethidium bromide) in TBE buffer. Gels were analyzed under ultraviolet (UV) fluorescence as previously described. All resulting amplified virulence gene PCR products were correlated with a 1 Kb-Plus ladder (Invitrogen) in conjunction with their expected PCR amplicon size. The PCR product from four randomly sampled reactions for each gene was purified with a QIAquick PCR purification column (Qiagen) and submitted to the University of Michigan DNA Sequencing Core Facility (Ann Arbor, MI) for sequencing. Each resulting nucleotide sequence was compared to the NCBI GenBank database for final verification of amplification.

Pulsed-field gel electrophoresis (PFGE) and plasmid DNA preparation

Genetic profiles of total cellular DNA were obtained using pulsed-field gel electrophoresis (PFGE). DNA isolation and digestion for PFGE was essentially as previously described (15). Ethidium bromide-CsCl gradient-prepared plasmid DNA was isolated as previously described (61). Restriction enzymes were obtained from Invitrogen (Carlsbad, CA) and New England Biolabs (Beverly, MA) and used as recommended by the manufacturer. Genomic DNA restriction fragments were separated on 0.8% agarose gels in TBE buffer. Plasmid DNA restriction fragments were separated on 0.7% agarose gels in TBE buffer. The gels were stained with ethidium bromide for 30 min and the bands were visualized by fluorescence under UV light.

Results

The characteristics of 33 endodontic enterococcal strains are presented in Table 3.

Phenotype

Thirty-one isolates were identified as *E. faecalis*. Two isolates were identified as *E. faecium* (GS11 and GS20), both recovered from endodontic retreatment cases. Twenty-three strains (70%) expressed gelatinase activity under both aerobic and anaerobic growth conditions. Bacteriocin production was evident in 14 strains (42%) using indicator strains *E. faecalis* FA2-2, *E. faecalis* DS16 and *E. faecium* 409. *S. aureus* ATCC 6538, *E. coli* DH5α and *E. coli* ATCC 29417 showed no sensitivity. None of the isolates expressed hemolysin activity on horse, rabbit, sheep or bovine blood agar. All were susceptible to ampicillin, benzylpenicillin, chloramphenicol, erythromycin, fusidic acid, kanamycin, rifampin, streptomycin, and vancomycin. MICs for clindamycin, to which enterococci are intrinsically resistant (48), ranged from 0.5 to 64 μg/ml. *E. faecium* GS11 was resistant to gentamicin (MIC >1024 μg/ml) and five strains (*E. faecalis* GS8, GS9, GS24, GS30, GS31) were resistant to tetracycline (MIC >16–32 μg/ml). Sixteen strains (48%) exhibited a clumping ('pheromone') response when exposed to *E. faecalis* JH2-2 culture filtrate.

Virulence genes

PCR products consistent with genes encoding adherence factors EfaA and Ace were found in all isolates. An *esp* gene was present in 20 isolates (61%), including both *E. faecium* isolates. A *cylA* gene was present in six isolates (18%). An aggregation substance gene, *asa*, was present in 100% of the isolates. [We note that the primers used to generate the product representing aggregation substance (Table 2), do not discriminate between several known aggregation substance genes. Indeed the *asaF* and *asaR* primers correspond to identical sequences in pAD1, pPD1 and pCF10, whereas *asa373* is unique.] An *asa373* gene was not detected in any strain. The *gelE* gene was detected in all *E. faecalis* isolates but not in the *E. faecium* strains. However, in six of the eight *E. faecalis* isolates possessing the gelatinase-negative phenotype, the 1 kb PCR product corresponding to the 3′ end of *ef1841* and the 3′ end of the *fsrC* product for gelatinase-negative phenotype was detected, indicating a 23.9 kb deletion sequence of the *fsr* gene cluster upstream to *gelE* (39). In the remaining two strains (GS17 and GS18) the internal *fsrC* product was not detected, indicating a defective *fsr* gene cluster.

DNA analysis

PFGE evaluation of genomic DNA showed genetic polymorphism with evidence of genetically related *E. faecalis* isolates (clonal groups) (Fig. 1). Prior to plasmid analyses, clonal groups provisionally identified by PFGE were: Group 1. GS3, GS4, GS5, GS6, GS7, GS12 and GS21; Group 2. GS23 and GS29; Group 3. GS13 and GS26; and Group 4. GS32 and GS33. All other strains had unique PFGE patterns.

Plasmid DNA preparations showed that at least 25 strains contained plasmid DNA, with one to four plasmids per strain (Fig. 2). Based on size and restriction pattern, a similar small (5.1 kb) plasmid occurred in 16 of the isolates. Clonal groups identified by PFGE described above were further differentiated by plasmid analyses (Fig. 2): Group 1 (i) GS4 and GS5, (ii) GS3, GS12, and GS21; Group 2, GS23 and GS29; Group 3, GS13 and GS26; and Group 4, GS32 is plasmid free while GS33 contains two plasmids, one of which is similar to one found in GS23 and GS29.

Discussion

Of clinical significance is whether endodontic enterococci found in previously treated root canals carry potential virulence factors. Phenotypic tests showed that 23 strains (70%) produced gelatinase. Interestingly, where clinical data were available, gelatinase activity was expressed in 13/14 (93%) of strains recovered from primary endodontic infections compared to 2/8 (25%) from retreatment cases (Table 3). Gelatinases are extracellular zinc endopeptidases capable of hydrolyzing gelatin, collagen and other proteins/peptides (35, 51). They are produced by a large proportion of *E. faecalis* isolated from hospitalized patients and patients with endocarditis (10) and may contribute to increased severity of endocarditis in animal models (25). Expression of *gelE* contributed to the increased dissemination of *E. faecalis* in high-density environments (60) and was associated with increased adhesion of *E. faecalis* to dentin *in vitro* (26). Previously, *E. faecalis* 2 SaR (also known as OG1), a strain recovered from a human oral cavity and found to exhibit gelatinase activity, was capable of inducing caries in germ-free rats (24). In view of a recent report that expression of GelE enhanced biofilm formation of *E. faecalis* in microtiter plates (31), investigations are indicated into the role of gelatinase production by *E. faecalis* in root canal biofilms.

In the present study, phenotypic testing revealed apparent 'silent' genes where the presence of the determinant using PCR did not correlate with its phenotypic expression, an observation that has been previously reported (1, 11, 18, 19). For example, gelatinase activity was not detected in eight *E. faecalis* isolates where *gelE* was detected (Table 1). Expression of gelatinase is regulated by a quorum sensing system encoded by the *fsr* gene cluster. The data show that the gelatinase-negative phenotype observed in the eight *E. faecalis* isolates can be attributed to defects in the *fsr* gene cluster, known to be associated with the gelatinase-negative phenotype (39).

A potential explanation for the persistence of *E. faecalis* in the root canal might be an association with expression of adherence factors. Genes for the adherence factors EfaA protein, a homolog of cell surface adhesins found on streptococcal species, and Ace (adhesin of collagen from enterococci) protein were present in all endodontic strains, similar to reports for 'medical' strains (11, 19). Ace may aid binding of *E. faecalis* to dentin (26). The *esp* gene was found in 61% of endodontic isolates, in contrast to a reported occurrence of 100% of endocarditis and bacteremia isolates (1). The Esp protein is associated with colonization and persistence of *E. faecalis* in urinary tract infections in mice (46). Another adherence factor, aggregation substance, is a pheromone-inducible surface protein of *E. faecalis.* Expression of aggregation substance can increase adhesion to collagen type 1, but not type IV (43). A PCR product representative of aggregation substance, Asa, was found in all endodontic strains. Reports of the incidence of determinants for aggregation substance in clinical isolates have been contradictory; some indicate a high prevalence in clinical isolates compared to strains from healthy individuals (10, 59), whereas others have reported a similar prevalence in both clinically associated and commensal strains (1, 27). The determinants for a number of aggregation substance genes are known to exhibit strong homology (e.g. those related to pAD1, pPD1 and pCF10), thus it was

Table 3. Characteristics of *Enterococcus* spp

		Phenotypic tests~					Virulence genes								Plasmids^				
																Size			
Strain#	Treatment	Gelatinase	Bacteriocin	Hemolysin	Antibiotic resistance	Clumping (“pheromone” response	*gelE*	*Ef1841/ fsr*	*esp*	*asa*	*asa373*	*ace*	*cylA*	*efaA*	Number	L	M	S	5.1kb
Endodontic strains																			
GS3	Primary	+	–	–	–	+	+	–	–	+	–	+	–	+	2	1		1	+
GS6	Primary	+	–	–	–	+	+	–	–	+	–	+	–	+	4	1	1	2	+
GS7	Primary	+	–	–	–	–	+	–	–	+	–	+	–	+	2			2	+
GS8	Primary	+	–	–	TC	–	+	–	–	+	–	+	+	+	–				
GS13	Primary	+	–	–	–	–	+	–	+	+	–	+	–	+	2			2	
GS18	Primary	–	–	–	–	+	+	–	+	+	–	+	–	+	2	1	1		
GS19	Primary	+	–	–	–	+	+	–	–	+	–	+	–	+	2	1		1	+
GS22	Primary	+	+	–	–	+	+	–	+	+	–	+	–	+	4	1		3	+
GS23	Primary	+	+	–	–	+	+	–	+	+	–	+	–	+	2	1		1	+
GS24	Primary	+	–	–	TC	–	+	–	–	+	–	+	–	+	1	1			
GS27	Primary	+	+	–	–	+	+	–	+	+	–	+	–	+	3	1		2	+
GS28	Primary	+	+	–	–	–	+	–	+	+	–	+	+	+	–				
GS31	Primary	+	+	–	TC	–	+	–	+	+	–	+	–	+	–				
GS32	Primary	+	+	–	–	–	+	–	+	+	–	+	+	+	–				
GS1	Retreatment	+	–	–	–	–	+	–	–	+	–	+	–	+	1	1			
GS2	Retreatment	–	+	–	–	+	+	+	–	+	–	+	–	+	2	1		1	+
GS11	Retreatment	–	+	–	GM	–	–	–	+	+	–	+	–	+	–				
GS12	Retreatment	–	–	–	–	+	+	+	+	+	–	+	–	+	2	1		1	+
GS16	Retreatment	+	–	–	–	–	+	–	+	+	–	+	–	+	–				
GS20	Retreatment	–	+	–	–	–	–	–	+	+	–	+	–	+	2	1		1	
GS25	Retreatment	–	–	–	–	–	+	+	–	+	–	+	+	+	1			1	+
GS33	Retreatment	–	+	–	–	+	+	+	–	+	–	+	+	+	2	1		1	+
GS4	Unknown	+	–	–	–	+	+	–	–	+	–	+	–	+	3	1	1	1	+
GS5	Unknown	+	–	–	–	+	+	–	+	+	–	+	–	+	3	1	1	1	+
GS9	Unknown	+	–	–	TC	–	+	–	+	+	–	+	–	+	–				
GS10	Unknown	–	+	–	–	–	+	+	+	+	–	+	–	+	1		1		
GS14	Unknown	+	+	–	–	+	+	–	+	+	–	+	–	+	3	1	1	1	+
GS15	Unknown	+	–	–	–	+	+	–	–	+	–	+	–	+	1	1			
GS17	Unknown	–	–	–	–	–	+	–	–	+	–	+	–	+	–				
GS21	Unknown	+	–	–	–	+	+	–	+	+	–	+	–	+	2	1		1	+
GS26	Unknown	+	–	–	–	–	+	–	+	+	–	+	+	+	2			2	
GS29	Unknown	–	+	–	–	+	+	+	+	+	–	+	–	+	2	1		1	+
GS30	Unknown	+	+	–	TC	–	+	–	+	+	–	+	–	+	1	1			
Non–endodontic strains																			
FA2–2		(–)*	(–)	(–)		(–)	+	(+)	(–)	(–)	(–)	+	(–)	+					
OG1X		(–)					+		–	–	–	+	–	+					
OG1RF		(+)*					(+)												
DS16			(+)	(+)		(+)													
V583							+	(–)	–	+	–	+	–	(+)					
MHH 594							+	–	(+)	+	–	(+)	+	+					
OG1X/pAD1						(+)	+		–	(+)	–	+	(+)	+					
FA378						(+)	+		–	–	(+)	+	–	+					
OG1SS/pCF10						(+)	+		–	+	–	+	–	+					
OG1X/pPD1						(+)	+		–	+	–	+	+	+					

Table 3. Continued.

Strain#	Treatment	Phenotypic tests~					Virulence genes							Plasmids^			
		Gelatinase	Bacteriocin	Hemolysin	Antibiotic resistance	Clumping ("pheromone" response	*gelE*	*Ef1841 fsr*	*esp*	*asa*	*asa373*	*ace*	*cylA*	*efaA*	Number	Size L	Size M	Size S
5952						(+)	+		–	+	–	+	+	+				5.1kb
BM4150-RF							(–)					(–)		(–)				

#All strains are *E. faecalis* except GS11, GS20 and BM4105–RF, which are *E. faecium*. Identification of strains was based on phenotypic tests, API 20 Strep and 16S rRNA analysis.
*Indicates positive (+) or negative (–) findings in strains used as controls in phenotypic tests and analysis of PCR products. Shaded areas indicate not applicable or not tested.
~Antibiotic resistance was determined using the E-test. Antibiotics: gentamicin (GM), tetracycline (TC). All endodontic isolates were sensitive to ampicillin, benzylpenicillin, chloramphenicol, erythromycin, fusidic acid, kanamycin, rifampin, streptomycin, and vancomycin.
^PFGE and plasmid DNA analyses identified clonal groups: Group 1. (i) GS4, GS5, (ii) GS3, GS12, GS21; Group 2. GS23, GS29; Group 3. GS13, GS26.
Plasmid size estimates are: large (L), >40 kb; mid–size (M), 15–40 kb; or small (S), <15 kb. A similar 5.1kb plasmid was found in 16 strains.

Fig. 1. Pulsed field gel electrophoresis of *Sma*I-digested genomic DNA from endodontic *E. faecalis* strains. A) *E. faecalis* GS1, GS2, GS8-GS10, GS14-GS19. B) *E. faecalis* GS3- GS7, GS12, GS13, GS21, GS22; *E. faecium* GS11, GS20. C) *E. faecalis* GS23–GS33. Note similarities between GS3-GS7, GS12 and GS21 ('Group 1'); GS23 and GS29 ('Group 2'); GS13 and GS26 ('Group 3′); GS32 and GS33 ('Group 4'). Reference standard: lambda phage DNA.

not surprising that all the endodontic strains that exhibited a clumping ('pheromone') response also gave rise to a representative PCR product. Interestingly, strains not exhibiting a clumping response also gave rise to such a PCR product, suggesting the presence of inactive or defective genes for aggregation substance. One type of aggregation substance, that encoded by pAM373, which bears little homology with the others, was not evident in the clinical isolates.

Pulsed-field gel electrophoresis has been utilized in the molecular epidemiologic typing of nosocomial enterococci (15, 56). In the present study, PFGE of endodontic isolates demonstrated genotypic polymorphism with evidence of several clonal groups, defined as genetically related isolates (55). The existence of groups of genetically similar strains from different patients treated by different providers cannot be explained using the retrospective clinical data available, but it is apparent that in certain strains, while chromosomal DNA sequences appeared similar by PFGE, plasmid DNA differed. Plasmids are autonomous, covalently closed circu-

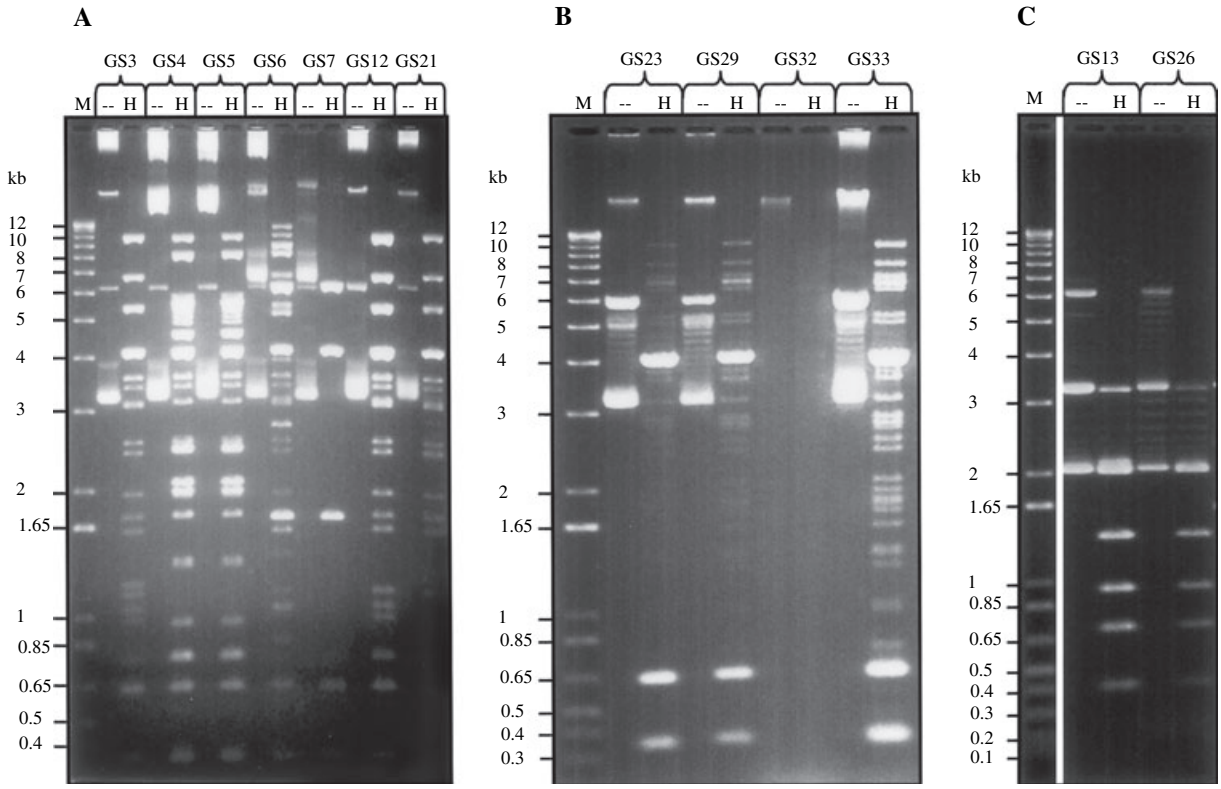

Fig. 2. Plasmid analysis of selected *E. faecalis* isolates. Lanes M, molecular size marker (1 Kb Plus DNA Ladder, Invitrogen); –, undigested; H, digested with *Hin*dIII. Strain designations are shown above the lane designations. In some cases, determination of plasmid number and size involved additional gel analyses (data not shown). A) Isolates classified based on PFGE pattern as clonal Group 1. GS3, GS12, and GS21 are similar in plasmid content (each has a 5.1 kb plasmid along with a nearly identical large plasmid). GS4 and GS5 appear to be alike in plasmid content (having similar 5.1 kb, mid-size, and large plasmids). GS6 and GS7 each contain two similar small plasmids (one being a 5.1 kb plasmid); however, GS6 has two additional plasmids. B) Isolates of clonal Groups 2 (GS23 and GS29) and 4 (GS32 and GS33). GS23 and GS29 each contain a 5.1 kb plasmid, as well as a similar large plasmid comparable to the one found in GS33. GS32 appears to be plasmid-free, whereas GS33 contains a 5.1 kb plasmid along with a large plasmid. C) Isolates of clonal Group 3. GS13 and GS26 are alike in plasmid content.

lar, double-stranded, supercoiled DNA elements. They range in size from ~1 kb to >200 kb, in contrast to the chromosomal DNA size of about 3.2 mb (41). Plasmids frequently confer traits that facilitate growth/survival under atypical conditions, for example, resistance to antibiotics and metal ions. *E. faecalis* can participate in plasmid-mediated horizontal transfer of virulence determinants, emphasizing the importance of these organisms in nosocomial infections (8). In the present study, plasmid DNA was recovered from 25 of the 33 endodontic isolates. Apart from the five strains resistant to tetracycline and one resistant to gentamicin, the strains were highly susceptible to the antibiotics studied.

Sixteen strains responded ('clumped') to a culture filtrate known to contain numerous pheromones, suggesting the potential to transfer plasmid DNA between strains. The pheromones are small linear peptides secreted by *E. faecalis* strains lacking the corresponding plasmid. One strain may secrete multiple pheromones specific for inducing transfer of different families of plasmids. There is evidence that aggregation substance itself can contribute to virulence (8). A 'pheromone' (clumping) response by almost half of the strains demonstrates the potential for transfer of genetic elements. The detection of a similar plasmid (5.1 kb) recovered from 14 strains exhibiting a clumping response is noteworthy, and investigations on the plasmid content of these endodontic enterococci are ongoing.

Interactions between species found in root canal infections would be expected to range from antagonistic to synergistic. The production of bacteriocins, evident in 14 endodontic isolates, may provide the producer strain with a selective advantage over other strains, especially those closely related to the bacteriocin-producing strain (57). Important to this simple phenotypic test, however, is the provision of appropriate growth conditions for both producer and responder, thereby limiting the sets of interactions that can be assessed *in vitro*. Further, while *in vitro* experiments showed that enterococci inhibited the growth of anaerobes and *Streptococcus milleri* recovered from a monkey root canal, this inhibitory activity was not reproduced when the same combinations of strains were deposited subcutaneously in rabbits (12). In contrast, synergistic interactions between species are known to occur readily in dental plaque, and combinations of nonenterococcal bacterial species found in endodontic infections have been associated with increased pathogenicity in animal models (3, 21, 49, 54). On a molecular level, specific interbacterial coaggregation may directly influence the etiology of endodontic diseases: for example, the ability of *Porphyromonas gingivalis* to invade dentinal tubules with *Streptococcus gordonii* (but not with *Streptocccus mutans*) (32, 34). Evaluation of the occurrence of coaggregation between endodontic enterococci and other endodontic infection species would seem worthwhile.

The present data were compared to those from a recent study on enterococci

recovered from the oral cavity (oral rinse) of endodontic patients attending the University of Michigan Dental School (45). Despite differences in geographic location (Sweden vs. United States), sampling years (1994/1995 vs. 2002/2003) and sample size and processing, oral and endodontic enterococci demonstrated susceptiblility to most antibiotics. However, differences included a greater incidence of gelatinase activity and pheromone response in endodontic strains from Sweden (76% and 42%, respectively) compared to oral rinse strains from the United States (36% and 9%, respectively).

Acknowledgments

This study was supported by NIH Grant GM33956, Vetenskapsradet Medicin 255004612 and the Endodontic Research Fund, University of Michigan. We thank Susan Donabedian for assistance with pulsed field gel electrophoresis.

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