CRISPR-*CAS*, A PROKARYOTIC ADAPTIVE IMMUNE SYSTEM, IN ENDODONTIC, ORAL AND MULTIDRUG-RESISTANT HOSPITAL-ACQUIRED *ENTEROCOCCUS FAECALIS*

by

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THESIS

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Abstract

Introduction: Microorganisms are vulnerable to invasion by mobile genetic elements such as viruses, plasmids and transposons. The recently discovered CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-associated system (CRISPR-Cas) is an adaptive immunity system found in most archaea and many bacteria that targets and inactivates invading foreign genetic elements. Cells with CRISPR-Cas are more likely to resist the invasion and uptake of foreign DNA such as viruses, plasmids and transposons. Several CRISPR-Cas systems have been identified in prokaryotes, including CRISPR1-*cas* and CRISPR3-*cas* in *E. faecalis* isolates recovered from human, animal, insect and environmental sources. The aims of this study were to: (1) compare the occurrence of CRISPR-*cas* in collections of endodontic (n=34), oral (n=21), and multidrug-resistant hospital-acquired strains of *E. faecalis* (n=23); (2) evaluate the distribution of antibiotic resistance and virulence traits amongst strains without CRISPR-*cas*; and (3) evaluate the co-occurrence of expression of virulence traits and a corresponding gene determinant in multidrug-resistant hospital-acquired *E. faecalis*.

Methods and Materials: *E. faecalis* strains were screened for CRISPR1-*cas* and CRISPR3-*cas* by using PCR, and products were verified by DNA sequencing. Associations were investigated between the occurrence of CRISPR-*cas* and the expression of phenotypic traits (antibiotic resistance, gelatinase activity, bacteriocin production, hemolysin activity, and clumping response to pheromone). Multidrug-resistant hospital-acquired *E. faecalis* strains that express certain virulence traits were screened for the co-occurrence of a corresponding gene. Two-tailed Fisher's exact tests were used to (1) compare the occurrence of CRISPR-*cas* in endodontic, oral and multidrug-resistant hospital-acquired isolates, and (2) evaluate the distribution of antibiotic resistance, gelatinase activity, bacteriocin production, hemolysin activity, and clumping response to pheromone among strains without CRISPR-*cas*. Significance was set at $P<0.05$.

Results: CRISPR-*cas* determinants were present in proportionally more endodontic (25 of 34) and oral (15 of 21) strains than multidrug-resistant hospital-acquired (9 of 23) strains (P=0.01 and 0.04, respectively). Significant associations were found between the absence of CRISPR-*cas* and the presence of antibiotic resistance in strains overall (P=0.04) and bacteriocin activity in endodontic strains (P=0.01). The majority of strains expressing virulence traits also carried the corresponding gene determinants.

Conclusions: The present study is the first to investigate CRISPR-*cas* in isolates recovered from infected root canals. Evidence for the presence of CRISPR-*cas* in the majority of endodontic and oral *E. faecalis* strains raises intriguing questions as to how prokaryotic immune systems might modulate interactions within the polymicrobial endodontic biofilm environment, particularly in response to antimicrobial agents used in root canal treatment. Understanding this process could lead to improved therapeutic strategies in the future.

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Chapter 1: Introduction and Review of the Literature

Enterococcus faecalis are facultative Gram-positive cocci that are widely distributed in nature, animals and humans. *E. faecalis* are frequently recovered from primary root canal infections (1-3), previously treated root canals (3-6) and occasionally from the oral cavity (6-8) from where they might enter an unsealed root canal system (9). In a microbiological profile of unexposed and exposed pulp space of necrotic teeth *E. faecalis* have been identified in both by DNA-DNA hybridization (10). While *E. faecalis* can survive for extended periods in the nutrient-deprived obturated root canal system (11, 12), whether intracanal *E. faecalis* can "cause" periradicular infections is not clear. In an effort related to this question, studies have shown that different *E. faecalis* strains recovered from infected root canals can express different virulence factors, as well as exhibit the presence of homologous virulence determinants (13, 14). In addition, a recent study showed that antibiotic resistance genes can transfer between *E. faecalis* and *Streptococcus gordonii* in root canals ex vivo (15). These data suggest a capacity for the species to modulate virulence expression and antibiotic resistance acquisition under varying environmental conditions in the root canal system (16), and may help to explain why *E. faecalis* are recovered from both symptomatic and asymptomatic cases (4, 17-19).

In contradistinction, the importance of the host's immunity in response to bacterial challenge is well established. For example, small numbers of virulent microorganisms may be sufficient to cause disease in an immunocompromised host (20). In a novel twist, the "health" of a microorganism is also dependent on the presence of its own protective "immune" system. Specifically, the recently discovered Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) and genes encoding CRISPR-associated (Cas) proteins, or CRISPR-Cas module, is a prokaryotic immune system widespread among archaea and bacteria that confers resistance to exogenous mobile genetic elements, such as

viruses (phages), plasmids and transposons (21-23). For example, a viral challenge to bacteria with CRISPR-*cas* can integrate new spacers derived from the phage genomic sequence into their own genome (21).

CRISPR-Cas systems are primarily located on the chromosome, but can also be found on plasmids. While considerable diversity exists between the many different CRISPR-Cas systems, they are characterized by a CRISPR locus made up of a varying number of repeating segments (23-25). Each repeating segment has the necessary DNA sequence required to target a different invader.

CRISPR-Cas systems function over three stages: adaptation, expression and interference (23). During the first stage CRISPR-Cas proteins incorporate a small fragment (termed a "spacer") of a foreign genetic element into the repeating CRISPR locus (22). The addition of new spacers in response to phage invasion tends to be polarized to the leader end of the CRISPR locus causing this end to be hypervariable (21). In the second stage an RNA transcript of the CRISPR locus is processed to form small CRISPR RNAs (crRNAs) each of which can identify a specific target for silencing. At the third stage, upon re-exposure to the specific foreign genetic element, the crRNA and CRISPR-Cas proteins target and cleave or silence the invading DNA or RNA (22, 23) (Figure 1). The presence of a CRISPR spacer identical to a phage sequence thus provides resistance to phages containing this particular sequence (21). This allows organisms with the CRISPR system to capture and store fragments of invader sequence and give rise to small RNAs that impart a heritable immunity against invaders (22, 26) (Figure 2).

Although there are some similarities with the eukaryotic systems for adaptive immunity (adaptation, memory) and RNA-based control (small guide RNA), the prokaryotic CRISPR–Cas system is substantially different. There is no sequence homology of the key proteins, and DNA rather than RNA is the prime candidate for target interference. Even now, as many details remain to be discovered, it has become clear that the CRISPR–Cas

system is a specific, dynamic and inheritable protection system in prokaryotes (27).

Several CRISPR-Cas systems have been identified in prokaryotes, including CRISPR1-*cas* and CRISPR3-*cas* in *E. faecalis* isolates recovered from human, animal, insect and environmental sources (28-30). Two CRISPR loci were first identified in the *E. faecalis* OG1RF genome; CRISPR1-*cas* which has the associated *cas* gene, and CRISPR2 which is an orphan locus lacking the functional *cas* genes (28). Subsequently CRISPR3-*cas* was identified in two strains (30), a fruit fly isolate Fly1 (31) and a urine isolate (30). CRISPR-*cas* has since been identified in one-third of 48 *E. faecalis* strains recovered from a variety of sources (e.g. milk, clinical, different animals, endocarditis, blood, fecal, urine), all but one being CRISPR1-*cas* (30), and in 18 of 52 *E. faecalis* strains recovered from (predominantly) clinical, food and environmental sources, all but two being CRISPR1-*cas* (29). Recently, the absence of CRISPR-*cas* in *E. faecalis* was positively correlated with acquired multidrug resistance genes (30), which are often found on mobile genetic elements such as plasmids (8, 32).

The occurrence of CRISPR-*cas* in the endodontic and oral microflora has not been previously evaluated. The first aim of this study was to compare the occurrence of CRISPR-*cas* in collections of endodontic and oral strains and multidrug-resistant hospital-acquired strains of *E. faecalis*; the null hypothesis tested was that there is no difference in occurrence between groups. Because the absence of CRISPR-*cas* could indicate previous uptake of foreign DNA (eg, via horizontal transfer of plasmids encoding virulence and/or antibiotic resistance genes), the second aim was to evaluate the distribution of virulence traits and antibiotic resistance among strains without CRISPR-*cas*. The third aim of the study was to evaluate whether multidrug-resistant hospital-acquired *E. faecalis* that express certain virulence traits also possess a corresponding gene determinant.

Aims of the Study

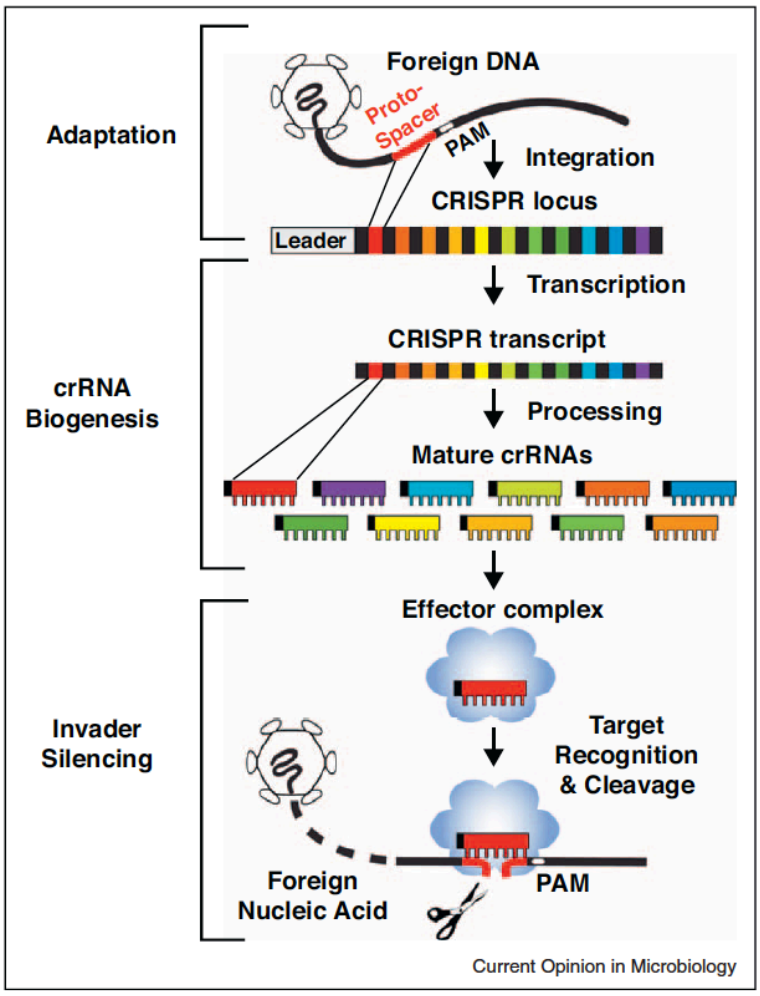
1. The first aim of this study was to compare the occurrence of CRISPR-*cas* in collections of endodontic and oral strains, and multidrug-resistant hospital-acquired strains of *E. faecalis*; the null hypothesis tested was that there is no difference in occurrence between groups.

2. The second aim was to evaluate the distribution of virulence traits and antibiotic resistance among strains without CRISPR-*cas*.

3. The third aim of the study was to evaluate whether multidrug-resistant hospital-acquired *E. faecalis* that express certain virulence traits also possess a corresponding gene determinant.

Figure 1. The three phases of the CRISPR-Cas invader defense pathway.

Reprinted from Current Opinion in Microbiology, Vol. 14, Terns MP and Terns RM, CRISPR-based adaptive immune systems. Pages 321-327, 2011, with permission from Elsevier (22).



Overview of the CRISPR–Cas invader defense pathway. In the adaptation phase, a short fragment of foreign DNA (protospacer) is acquired from the invader and integrated into the host CRISPR locus adjacent to the leader. Protospacer adjacent motifs (PAMs) are found near invader sequences selected for CRISPR integration. The CRISPR locus consists of short direct repeat sequences (black) that separate similarly sized, invader-derived sequences (multiple colors). In the biogenesis phase of the pathway, CRISPR locus transcripts are processed to release individual mature crRNAs (each targeting a different sequence). Mature crRNAs typically retain some of the repeat sequence, which is thought to provide a recognizable signature of the crRNAs. In the silencing phase, crRNA–Cas protein effector complexes recognize foreign DNA or RNA through basepairing of the crRNA. The Cmr and Csn systems affect cleavage of target RNA and DNA, respectively. PAMs provide important auxiliary signals for the recognition of invaders for some DNA-targeting systems.

Figure 2. Overview of the CRISPR-Cas mechanism of action.
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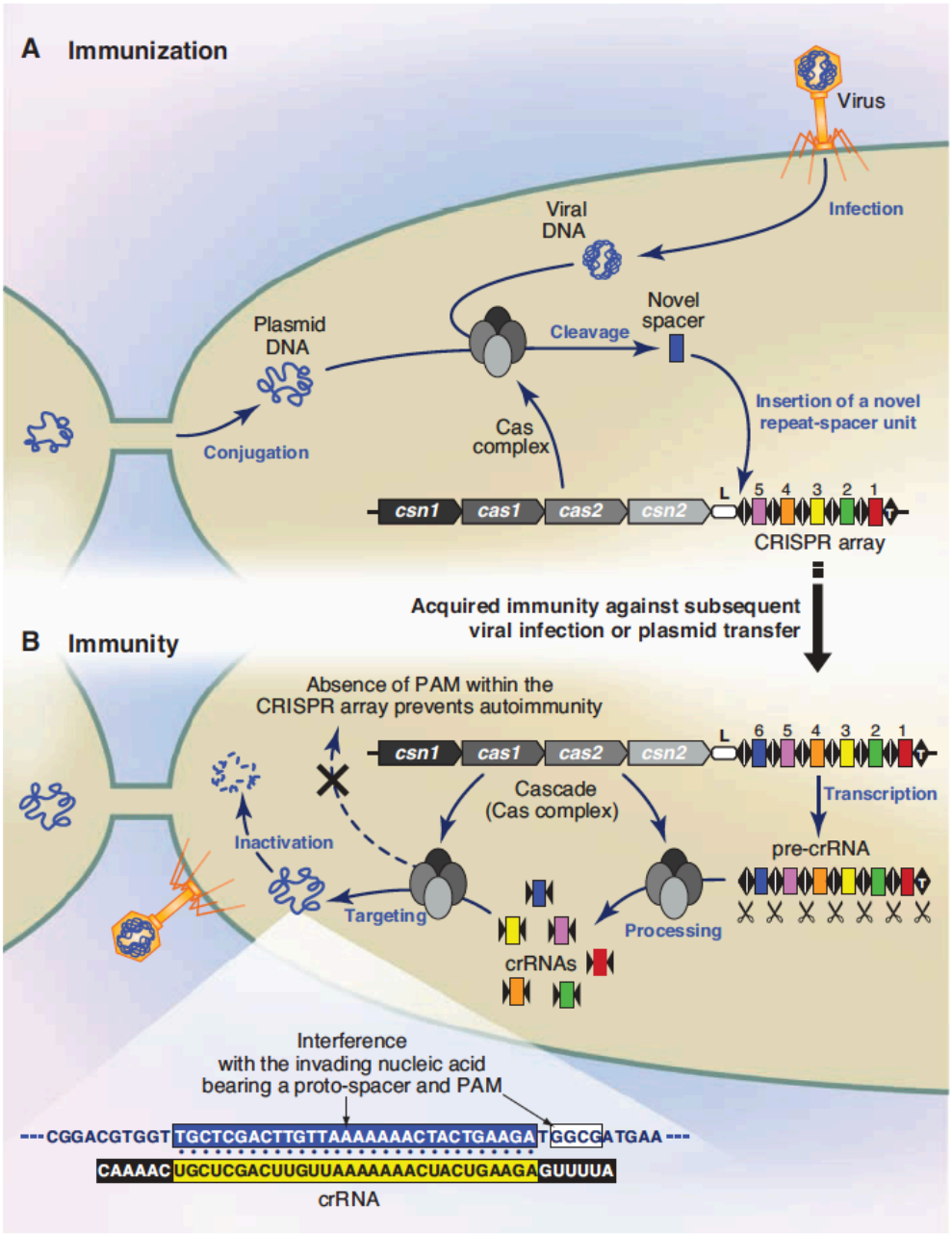


Fig. 2. Overview of the CRISPR/Cas mechanism of action. **(A)** Immunization process: After insertion of exogenous DNA from viruses or plasmids, a Cas complex recognizes foreign DNA and integrates a novel repeat-spacer unit at the leader end of the CRISPR locus. **(B)** Immunity process: The CRISPR repeat-spacer array is transcribed into a pre-crRNA that is processed into mature crRNAs, which are subsequently used as a guide by a Cas complex to interfere with the corresponding invading nucleic acid. Repeats are represented as diamonds, spacers as rectangles, and the CRISPR leader is labeled L.

Chapter 2: Materials and Methods

2.1 Bacterial strains and phenotypic characterization

E. faecalis strains and their sources are listed in Table 1. Strains were taken from minus 80°C stocks and plated onto Todd Hewitt Broth (THB, Becton, Dickinson and Co., Sparks, MD) supplemented with 1.5% agar and incubated aerobically at 37°C for 24 hours. For each strain, expected colony formation, cell morphology, esculin hydrolysis (Figure 3) and Gram stain reaction (Figure 4) were confirmed. 16S rRNA PCR assays were performed for all strains to verify species-specific amplification as previously described (7) (Table 2). Multidrug-resistant hospital-acquired strains (n=23)(33) were screened for antibiotic resistance by using the E-test (bioMérieux, Inc., Durham, NC) (Figure 5), gelatinase activity (Figure 6), bacteriocin production (Figure 7), hemolysin activity (Figure 8), and clumping response to pheromone (Figure 9) using methods previously described (13); endodontic (n=34) and oral (n=21) strains had been previously screened for these traits (7, 8, 13, 34).

Table 1. *Enterococcus faecalis* strains used in this study

Source	Strain name	Reference
Endodontic (n=34)		
Primary treatment	GS3, GS6, GS7, GS8, GS13, GS18, GS19, GS22, GS24, GS27, GS28, GS31, GS32	(13)
Orthograde retreatment	GS1, GS2, GS12, GS16, GS25, GS33	
	ER3/2s, ER5/1	(35)
Endodontic treatment	GS4, GS5, GS9, GS10, GS14, GS15, GS17, GS21, GS23, GS26, GS29, GS30	(13)
	JG2	(36)
Oral (n=21)		
Tongue swab-endodontic patient	AA-T4, AA-T26	(34)
	GS-34	(35)
Oral rinse-dental student	C1	(8)
Oral rinse-endodontic patient	E1, E2, E3, E4, E5, E6, E7, E8, E10, E11	(8)
	OS16, OS25	(7)
	AA-OR3, AA-OR4, AA-OR26, AA-OR34	(34)
	OG1	(37)
Hospital–acquired (n=23)	HNEfs #1 - #20	(33)
	DS16	(32)
	MMH594	(38)
	V583*	(39)
Other	OG1RF*	(40)
	Fly-1*	(31)

*V583 lacks CRISPR1-*cas* and CRISPR3-*cas*; OG1RF encodes CRISPR1-*cas*; #Fly-1 encodes CRISPR3-*cas* (30).

Table 2. Oligoneucleotides used in this study.

Gene		Sequence	Product size (bp)	Reference
E. faecalis 16S rRNA	Ef16SF	5' - CCGAGTGCTTGCACTCAATTGG - 3'		
	Ef16SR	5' - CTCTTATGCCATGCGGCATAAAC - 3'	138	(7)
CRISPR1-*cas*	cas1-F	5'- ATGGGCTGGCGAACGGTAGTGGTTA - 3'	867	(30)
	cas1-R	5'- TCATATCCCAAACTCTGGAACTCCT-3'		
CRISPR1-*cas* loci	neg1cas1F	5'-GCG ATG TTA GCT GAT ACA AC-3'	315	(30)
	neg1cas1 R	5'-CGA ATA TGC CTG TGG TGA AA-3'		
CRISPR3-*cas*	3csnF	5'-GCT GAA TCT GTG AAG TTA CTC-3'	258	(30)
	3csnR	5'-CTG TTT TGT TCA CCG TTG GAT-3'		
CRISPR3-*cas* loci	neg3casF	5´-GAT CAC TAG GTT CAG TTA TTT C-3'	224	(30)
	neg3casR	5'-CAT CGA TTC ATT ATT CCT CCA A-3'		
Gelatinase *gelE*	gelEF	5' - ACCCCGTATCATTGGTTT - 3'	405	(41)
	gelER	5' - ACGCATTGCTTTTCCATC - 3'		
ef1841/fsrC	ef1841F	5' - GATCAAGAAGGGAAGCCACC - 3'	1050	(42)
	fsrC7R	5' - CCAACCGTGCTCTTCTGGA - 3'		
fsrC internal	fsrC6F	5' - ATGATTTTGTCGTTATTAGCTACT - 3'	~1300	(42)
	fsrC7R	5' - CCAACCGTGCTCTTCTGGA - 3'		
Bacteriocin/Hemo -lysin cylA	cylAF	5' - GACTCGGGGATTGATAGGC - 3'	688	(43)
	cylAR	5' - GCTGCTAAAGCTGCGCTTAC - 3'		
Pheromone response *asa*	asaF	5' - CCAGCCAACTATGGCGGAATC - 3'	529	(43)
	asaR	5' - CCTGTCGCAAGATCGACTGTA - 3'		
Adherence factor *esp*	espF	5' - TTGCTAATGCTAGTCCACGACC - 3'	932	(38)
	espR	5' - GCGTCAACACTTGCATTGCCGA - 3'		

Figure 3. Example of Enterococcocel agar test to confirm expected colony formation, cell morphology and esculin hydrolysis.

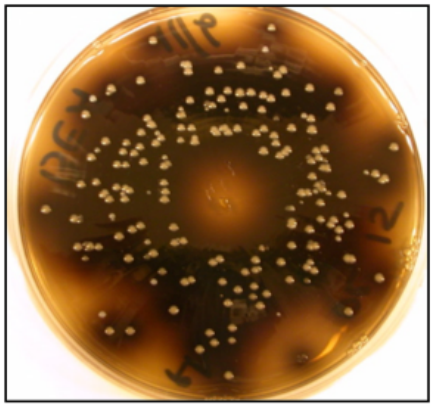


Figure 4. Example of Gram staining of Gram positive cocci *E. faecalis*.

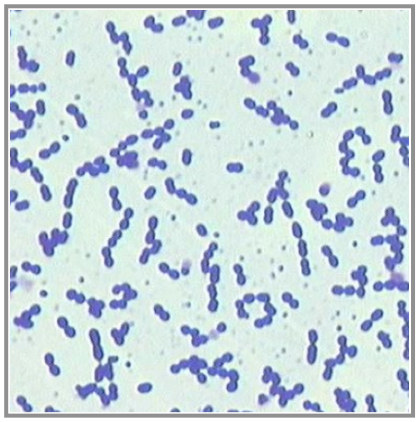


Figure 5. Example of E-tests demonstrating control strains, *E. faecalis* V583 and OG1RF, as vancomycin resistant and susceptible, respectively.

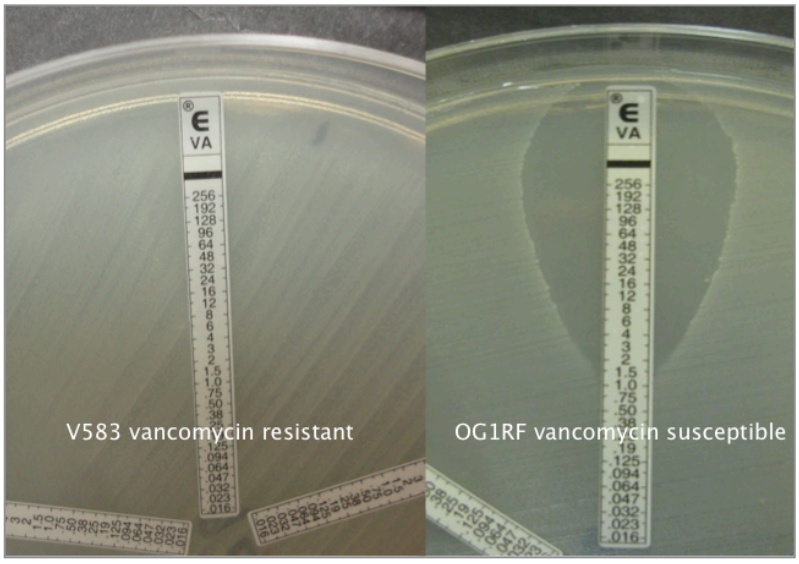


Figure 6. Example of gelatinase activity assay.

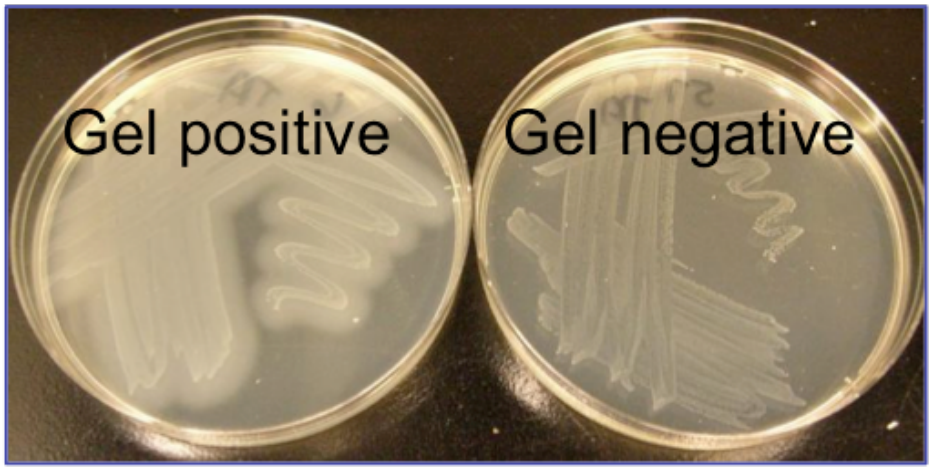


Figure 7. Example of growth inhibition of lawn strain by bacteriocin producer strain (center stab colony).

Figure 8. Example of hemolysin activity assay.

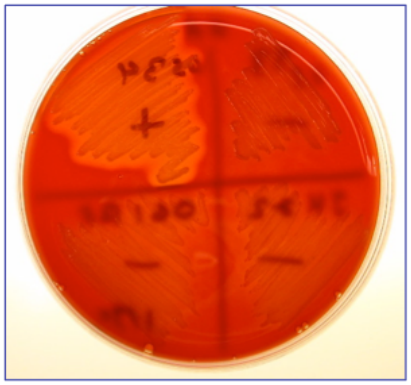
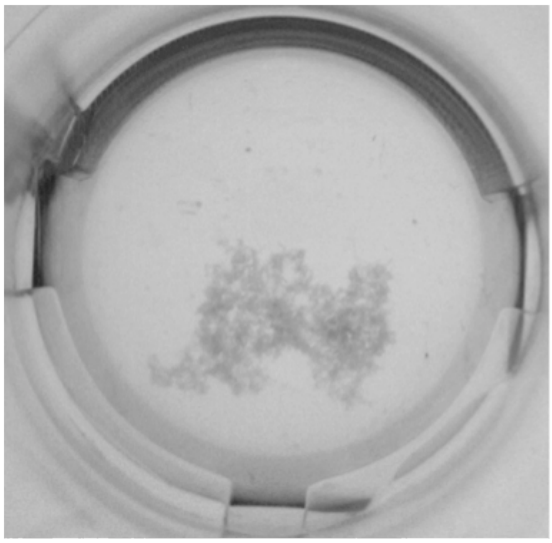


Figure 9. Example of positive clumping response in the pheromone response assay.



2.2 Total DNA extraction, PCR amplification and sequencing

Table 2 lists oligonucleotides used in this study. Total DNA was isolated as previously described (7), and re-suspended in sterile nuclease-free water to a final stock concentration of 50 ng/μL. Primer sequences for E. faecalis CRISPR1-cas cas1 were: For-5'-ATG GGC TGG CGA ACG GTA GTG GTT A-3', Rev-5'-TCA TAT CCC AAA CTC TGG AAC TCC T-3' for an expected 867 base-pair product. Primer sequences for CRISPR3-cas csn1 were: For-5'-GCT GAA TCT GTG AAG TTA CTC-3', Rev-5'-CTG TTT TGT TCA CCG TTG GAT-3' for an expected 258 base-pair product (30). To verify the absence of false negative PCR results, those strains without CRISPR1-cas PCR products were screened using primers that anneal outside the CRISPR1-cas loci: For-5'-GCG ATG TTA GCT GAT ACA AC-3', Rev-5'-CGA ATA TGC CTG TGG TGA AA-3' for an expected 315 base-pair product; those strains without CRISPR3-cas PCR products were screened using primers that flank the CRISPR3-cas loci: For-5'-GAT CAC TAG GTT CAG TTA TTT C-3', Rev-5'-CAT CGA TTC ATT ATT CCT CCA A-3' for an expected product size of 224 bp (30). Control strains used for PCR screening were E. faecalis OG1RF (CRISPR1-cas present), Fly-1 (CRISPR3-cas present) and V583 (CRISPR1-cas and CRISPR3-cas absent) (30).

Primer sequences for *E. faecalis* virulence gene gelatinase (*gelE*) were: For-5'-ACC CCG TAT CAT TGG TTT-3', Rev-5'-ACG CAT TGC TTT TCC ATC - 3' for an expected 405 base-pair product (41). If a particular strain did not demonstrate gelatinase activity but did contain the gene those strains were further screened to determine if the existing gene may be defective by using primer sequences for *E. faecalis* ef1841F: For-5'-GAT CAA GAA GGG AAG CCA CC-3' and fsrC7R: Rev-5'-CCA ACC GTG CTC TTC TGG A-3' for an expected 1050 base-pair product size (42). The strains that were not positive for ef1841F/fsrC7R were further screened for *fsrC* internal using primer sequences for *E. faecalis* fsrC6F: For-5'-ATG ATT TTG TCG TTA TTA GCT ACT-3', and fsrC7R: Rev-5'-

CCA ACC GTG CTC TTC TGG A-3' for an expected approximately 1300 base-pair product size (42). Primer sequences for *E. faecalis* virulence gene hemolysin (*cylA*) were cylAF: For-5'-GAC TCG GGG ATT GAT AGG C-3' and cylAR: Rev-5'-GCT GCT AAA GCT GCG CTT AC-3' for an expected 688 base-pair product size (43). Primer sequences for *E. faecalis* for pheromone response (asa) were: asaF: For-5'-CCA GCC AAC TAT GGC GGA ATC-3' and asaR: Rev-5'-CCT GTC GCA AGA TCG ACT GTA-3' for an expected 529 base-pair product size (43). Primer sequences for *E. faecalis* for adherence factor (*esp*) were espF: For-5'-TTG CTA ATG CTA GTC CAC GAC C-3' and espR: Rev-5'-GCG TCA ACA CTT GCA TTG CCG A-3' for an expected 932 base-pair product size (38).

PCR amplifications used an Eppendorf Mastercycler (Brinkmann Instruments, Inc., Westbury, NY). 100 ng total DNA template were prepared for 50 μL PCR amplifications as follows: 45 μL Platinum PCR SuperMix (High Fidelity) (Invitrogen) with primers (10 μM final concentration) and template DNA to a final volume of 50 μL. The PCR amplification conditions were an initial step of 95°C for 60 seconds, followed by 35 consecutive cycles at 94°C for 20 seconds, 60°C for 30 seconds and 72°C for 30 seconds. PCR products were stained with SYBR Green and analyzed by electrophoresis using 1.5% - 3% agarose gels in TBE buffer, and visualized by fluorescence under UV light (Figure 10). Amplified PCR products were correlated with a 100 base-pair ladder (Invitrogen) to confirm conjunction with their expected PCR amplicon size. PCR products were purified with a QIAquick PCR purification column (Qiagen) and submitted for sequencing to the Oregon Health and Science University MMI DNA Services Core (Portland, OR). Nucleotide sequence alignments were compared to the NCBI GenBank database for final verification of amplification by using the online ClustalW2 multiple sequence alignment tool http://www.ebi.ac.uk/Tools/msa/clustalw2/ (44) and visualized using 4Peaks software (4Peaks Version 1.7.2 by A. Griekspoor and Tom Groothuis, mekentosj.com) (Figure 11).

2.3 Genotypic characterization

PCR was utilized to screen for the presence of virulence genes for gelatinase activity (*gelE*), hemolysin/bacteriocin activity (*cyl*), and clumping response to pheromone (*asa*) in clinical isolates of multidrug-resistant hospital-acquired *E. faecalis* (n = 23) (Table 2). Selected PCR products were purified and underwent DNA sequencing for verification of amplification as described previously.

2.4 Statistical analysis

Two-tailed Fisher's exact tests were used to compare the occurrence of CRISPR-*cas* in endodontic, oral and multidrug-resistant hospital-acquired isolates. Two-tailed Fisher's exact tests were used to evaluate the distribution of antibiotic resistance, gelatinase activity, bacteriocin production, hemolysin activity, and clumping response to pheromone among strains without CRISPR-*cas*. Significance was set at $P<0.05$.

Figure 10. Representative image showing gel analysis of PCR amplification products with *E. faecalis*-derived 16S rRNA gene primers.

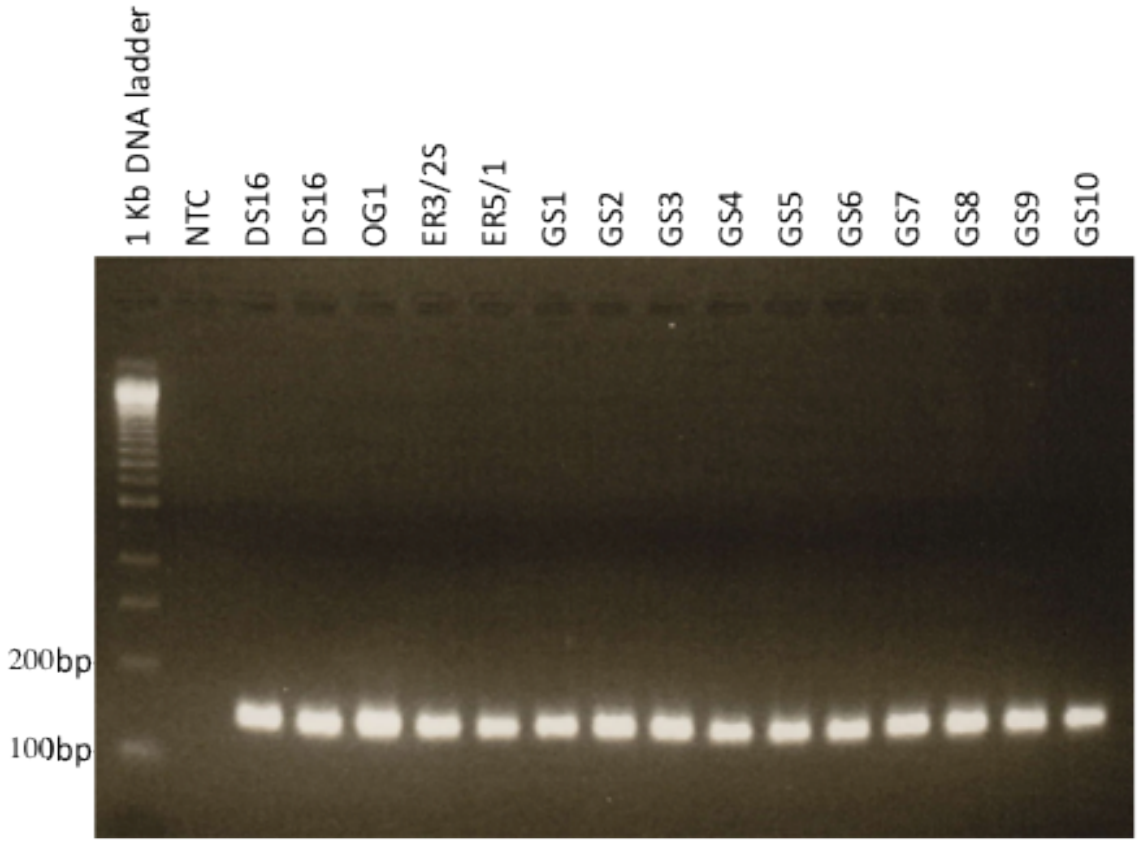
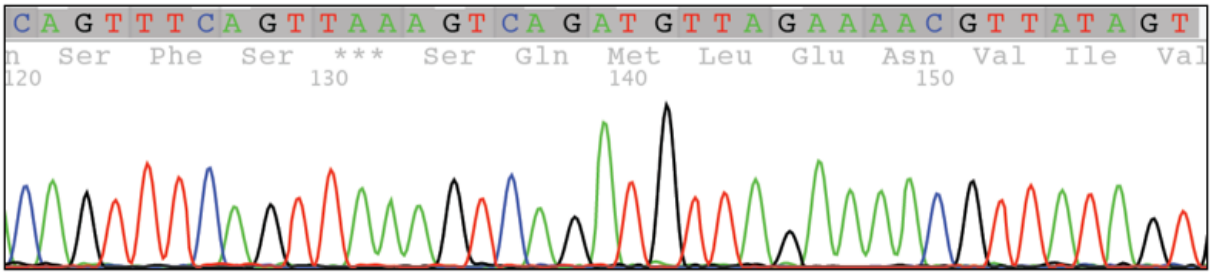


Figure 11. Example of DNA sequencing as viewed with 4Peaks software.



Chapter 3: Results

3.1 Occurrence of CRISPR-*cas*

PCR products were available for all strains. The overall occurrence of CRISPR-*cas* in *E. faecalis* isolates from different sources is shown in Tables 3 and 4. Overall, CRISPR1-*cas* or CRISPR3-*cas* determinants were present in proportionally fewer multidrug-resistant hospital-acquired strains (9 of 23) than endodontic (25 of 34) (P=0.01) and oral (15 of 21) strains (P=0.04). The null hypothesis was rejected. The CRISPR1-*cas* determinant was present in proportionally fewer endodontic (8 of 34) and multidrug-resistant hospital-acquired strains (3 of 23) than oral strains (15 of 21) (P=0.0007 and P=0.0002, respectively), with no difference between endodontic and multidrug-resistant hospital-acquired strains. The CRISPR3-*cas* determinant was present in proportionally more endodontic strains (20 of 34) compared to multidrug-resistant hospital-acquired strains (6 of 23) (P=0.03), and was not identified in any oral strains. Three endodontic strains had both CRISPR1-*cas* and CRISPR3-*cas*. The remaining endodontic strains were significantly more likely to harbor CRISPR3-*cas* than CRISPR1-*cas* (P=0.006) (Figure 12), while the reverse was true for oral strains (P<0.0001) (Figure 13). There was no difference in the occurrence of CRISPR1-*cas* versus CRISPR3-*cas* multidrug-resistant hospital-acquired strains (Figure 14).

The sequenced CRISPR1-*cas* and CRISPR3-*cas* PCR products were 97-100% identical to the corresponding *E. faecalis* OG1RF and Fly-1 sequences. The sequenced products verifying absence of false negative PCR results were 94-100% identical to the corresponding *E. faecalis* V583 sequence (Appendices 1-6).

3.2 Virulence traits and antibiotic resistance

Phenotypic characteristics of multidrug-resistant hospital-acquired strains are shown in Table 5; phenotypic characteristics of endodontic and oral strains are reported elsewhere (7, 8, 13, 34). Overall, a significant association was found between the absence of either

CRISPR1-*cas* or CRISPR3-*cas* and the presence of antibiotic resistance (*P*=0.04) (Table 6). Other significant associations were found between the absence of CRISPR1-*cas* and the absence of hemolysin activity (*P*=0.01), and between the absence of CRISPR3-*cas* and presence of gelatinase activity (*P*=0.005), bacteriocin activity (*P*=0.02), and antibiotic resistance (*P*=0.0009) (Table 6)

In endodontic strains, significant associations were found between the absence of both CRISPR-*cas* determinants and the presence of bacteriocin activity (*P*=0.02) (Table 7), between the absence of CRISPR1-*cas* and the presence of a clumping response to pheromone (*P*=0.03) (Table 7), and between the absence of CRISPR3-*cas* and the presence of bacteriocin activity (*P*=0.03) (Table 7). Within oral and multidrug-resistant hospital-acquired subgroups, no significant associations were found (Table 7).

3.3 Genotypic characterization of multidrug-resistant hospital acquired *E. faecalis*

Genotypic characteristics and phenotypic characteristics of multidrug-resistant hospital-acquired strains are shown in Table 8. The co-occurrence of genotypic and phenotypic virulence traits are shown in Figure 15. The majority of strains expressing each virulence trait also possessed the corresponding gene determinant. Seventeen of the 18 (94%) strains that expressed the gelatinase phenotype possessed the corresponding *gelE* gene determinant. Eight of the 12 (66%) strains that expressed hemolysin/bacteriocin also possessed the *cyl* gene determinant. Eight of the nine strains (88%) that exhibited a clumping response to pheromone also possessed the *asa* gene determinant (Table 8).

Table 3. Occurrence of CRISPR-*cas* in *E. faecalis*

Source	CRISPR1-*cas* OR CRISPR3-*cas*[b]		CRISPR1-*cas*[c]		CRISPR3-*cas*[d]		CRISPR1-*cas* AND CRISPR3-*cas*	
	Present	Absent	Present	Absent	Present	Absent	Present	Absent
Endodontic (n=34)	25	9	8	26	20	14	3	9
Oral (n=21)	15	6	15	6	0	21	0	6
Hospital-acquired multi- drug resistant (n=23)	9	14	3	20	6	17	0	14

[a] CRISPR1-*cas* is present in strains GS1, GS9, GS10, GS16, GS19, GS25, GS30, GS31, E1, E4, E8, E11, E12, OS16, OS25, AA-OR3, AA-OR4, AA-OR26, AA-OR34, AA-T4, AA-T26, GS34, OG1, HNEf2, HNEf3 and DS16. CRISPR3-*cas* is present in strains GS3, GS4, GS5, GS6, GS7, GS8, GS9, GS10, GS12, GS13, GS14, GS15, GS16, GS17, GS21, GS22, GS24, GS26, GS27, GS32 and HNEf7, HNEf14, HNEf15, HNEf17, HNEf18, HNEf19. Both CRISPR1-*cas* AND CRISPR3-*cas* are present in strains GS9, GS10 and GS16.

[b] Absent in more hospital-acquired multi-drug resistant than endodontic (P=0.0136) and oral (P=0.0396) strains

[c] Absent in more endodontic and hospital-acquired multi-drug resistant than oral strains (P=0.0007 and P=0.0002, respectively)

[d] Absent in more oral than endodontic and hospital-acquired multi-drug resistant (P<0.0001 and P=0.0002, respectively) with a significant difference between endodontic and hospital-acquired multi-drug resistant (P=0.0291)

Table 4. CRISPR1-*cas* and/or CRISPR3-*cas* in endodontic, oral and multidrug-resistant hospital-acquired strains of *E. faecalis*

Strain	CRISPR1-*cas*	CRISPR3-*cas*	n
Endodontic (n=34)			
GS2, GS18, GS23, GS28, GS29, GS33, JG2, ER3/2s, ER5/1	-	-	9
GS1, GS19, GS25, GS30, GS31	+	-	5
GS3-GS8, GS12-GS15, GS17, GS21, GS22, GS24, GS26, GS27, GS32	-	+	17
GS9, GS10, GS16	+	+	3
Oral (n=21)			
E2, E3, E5-E7, E10	-	-	6
E1, E4, E8, E11, E12, OS16, OS25, AA-OR3, AA-OR4, AA-OR26, AA-OR34, AA-T4, AA-T26, GS34, OG1	+	-	15
Hospital-acquired multi-drug resistant (n=23)			
HNEf1, HNEf4-HNEf6, HNEf8-HNEf13, HNEf16, HNEf20, V583, MMH594	-	-	14
HNEf2, HNEf3, DS16	+	-	3
HNEf7, HNEf14, HNEf15, HNEf17- HNEf19	-	+	6
Other (n=2)			
OG1RF	(+)	-	
Fly 1		(+)	

Figure 12. Occurrence of CRISPR1-*cas* versus CRISPR3-*cas* in endodontic strains of *E. faecalis*

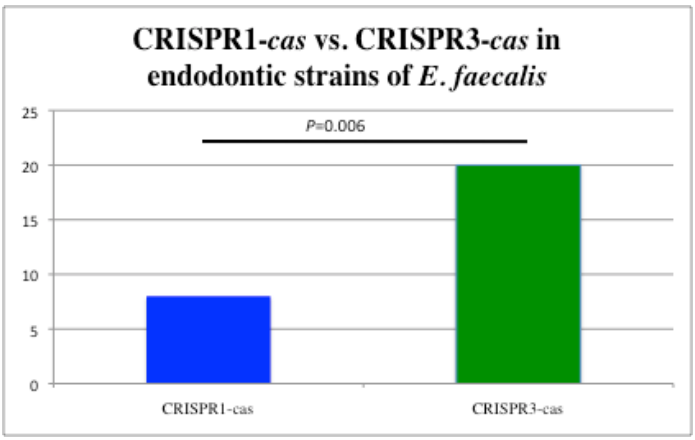


Figure 13. Occurrence of CRISPR1-*cas* versus CRISPR3-*cas* in oral strains of *E. faecalis*

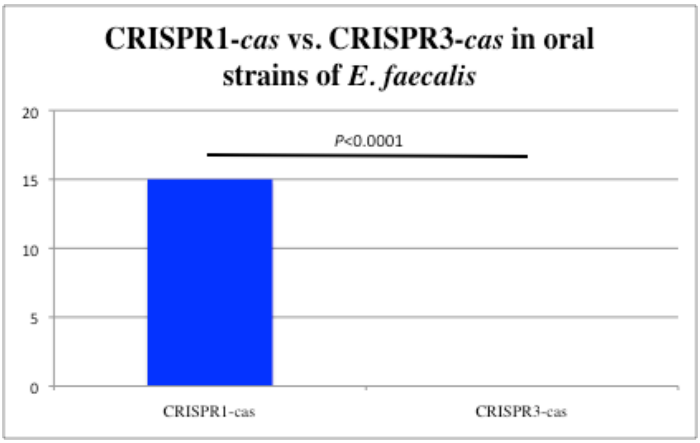


Figure 14. Occurrence of CRISPR1-*cas* versus CRISPR3-*cas* in multidrug-resistant hospital-acquired oral strains of *E. faecalis*

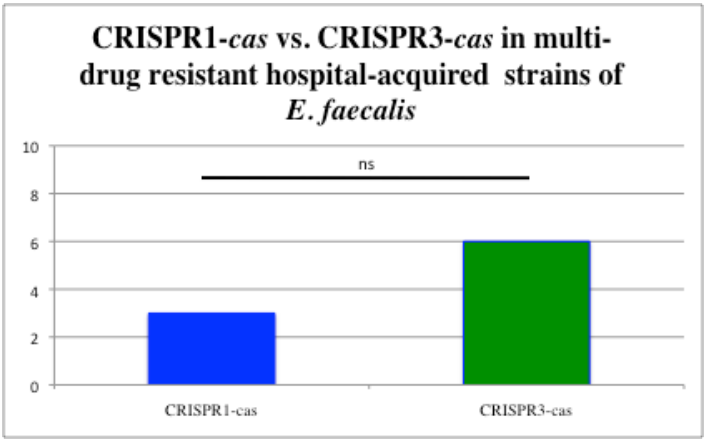


Table 5. Phenotypic characteristics of multidrug-resistant hospital-acquired *E. faecalis*

Strain#	**Phenotype**				
	Gelatatinase	Bacteriocin	Hemolysin	Pheromone response^	Antibiotic resistance~
HNEf1	+	+	-	-	Va, Fu, Tc, Em, Km, Cm, Gm
HNEf2	+	-	-	+	Va, Fu, Tc,Sm, Em, Km, Cm, Gm
HNEf3	-	-	-	-	Va, Fu, Sm, Em, Km, Cm, AM, Gm, Rf, Pg
HNEf4	-	-	-	-	Va, Fu, Em, Km, Cm, Gm
HNEf5	+	+	-	-	Va, Fu, Em, Km, Cm, Gm, Pg
HNEf6	+	-	-	-	Va, Fu, Tc, Em, Km, Cm, Gm
HNEf7	+	+	-	-	Va, Fu, Sm, Em, Km, Cm, Gm
HNEf8	+	+	-	-	Va, Fu, Tc, Sm, Em, Km, Cm, Gm
HNEf9	-	+	-	-	Va, Fu, Em, Km, Cm, Gm
HNEf10	+	-	-	-	Va, Fu, Sm, Em, Km, Cm, Gm
HNEf11	-	-	-	-	Va, Fu, Sm, Em, Km, Cm, Gm
HNEf12	+	-	-	-	Va, Fu, Sm, Em, Km, Cm, Gm, Pg
HNEf13	+	+	-	+	Va, Fu, Sm, Em, Km, Cm, Gm
HNEf14	-	-	+	-	Va, Fu, Sm, Em, Km, Cm, AM, Gm, Pg
HNEf15	+	-	-	-	Va, Fu, Sm, Em, Km, Cm, Gm
HNEf16	+	+	-	+	Va, Fu, Sm, Em, Km, Cm, Gm
HNEf17	+	-	-	+	Va, Fu, Sm, Em, Km, Cl, Cm, Gm
HNEf18	+	+	-	+	Va, Fu, Sm, Em, Km, Cl, Cm, Gm, Rf
HNEf19	+	+	-	+	Va, Fu, Sm, Em, Km, Cl, Cm, Gm
HNEf20	+	-	-	+	Va, Fu, Sm, Em, Km, Cm, Gm
DS16	+	+	+	+	Fu, Tc, Sm, Em, Km, Cm
MMH594	+	+	+	+	Fu, Em, Km, Cl, Cm, Gm, Pg
V583	+	-	-	-	Va, Fu, Em, Km, Cm, Gm

CRISPR1-*cas* is present in HNEf2, HNEf3 and DS16. CRISPR3-*cas* is present in HNEf7, HNEf15, and HNEf17-HNEf19.

^ Positive control *E. faecalis* strains for pheromone tests were OG1X/pAM373, OG1SS/pCF10, 39-5 (harboring pPD1) and OG1X/pPAD1.

~ MICs determined using E-test (AB Biodisk, Solna, Sweden). Antibiotics tested were: ampicillin (Am), benzylpenicillin (Pg), chloramphenicol (Cm), clindamycin (Cl), erythromycin (Em), fusidic acid (Fu), gentamicin (Gm), rifampin (Rf), streptomycin (Sm), tetracycline (Tc), and vancomycin (Va).

Table 6. Associations between phenotypic characteristics and the occurrence of CRISPR-*cas* in *E. faecalis* (n=78)

	CRISPR1-*cas* or CRISPR3-*cas*			CRISPR1-*cas*			CRISPR3-*cas*		
	Present	Absent	*P*	Present	Absent	*P*	Present	Absent	*P*
Antibiotic resistance									
Present	**20**	**25**	**0.04**	**17**	**28**	**ns**	**6**	**39**	**0.0009**
Absent	**23**	**10**		**9**	**24**		**16**	**17**	
Gelatinase activity									
Present	**27**	**21**	**ns**	**13**	**36**	**ns**	**19**	**29**	**0.005**
Absent	**16**	**14**		**13**	**16**		**3**	**27**	
Bacteriocin activity									
Present	**16**	**18**	**ns**	**12**	**22**	**ns**	**5**	**29**	**0.02**
Absent	**27**	**17**		**14**	**30**		**17**	**27**	
Pheromone response									
Present	**20**	**10**	**ns**	**8**	**22**	**ns**	**10**	**22**	**ns**
Absent	**28**	**10**		**18**	**30**		**12**	**34**	
Hemolysin activity									
Present	**7**	**3**	**ns**	**7**	**3**	**0.01**	**0**	**10**	**ns**
Absent	**36**	**32**		**19**	**49**		**22**	**46**	

Two-tailed Fishers' Exact Test http://www.graphpad.com/quickcalcs/index.cfm
ns = not significant

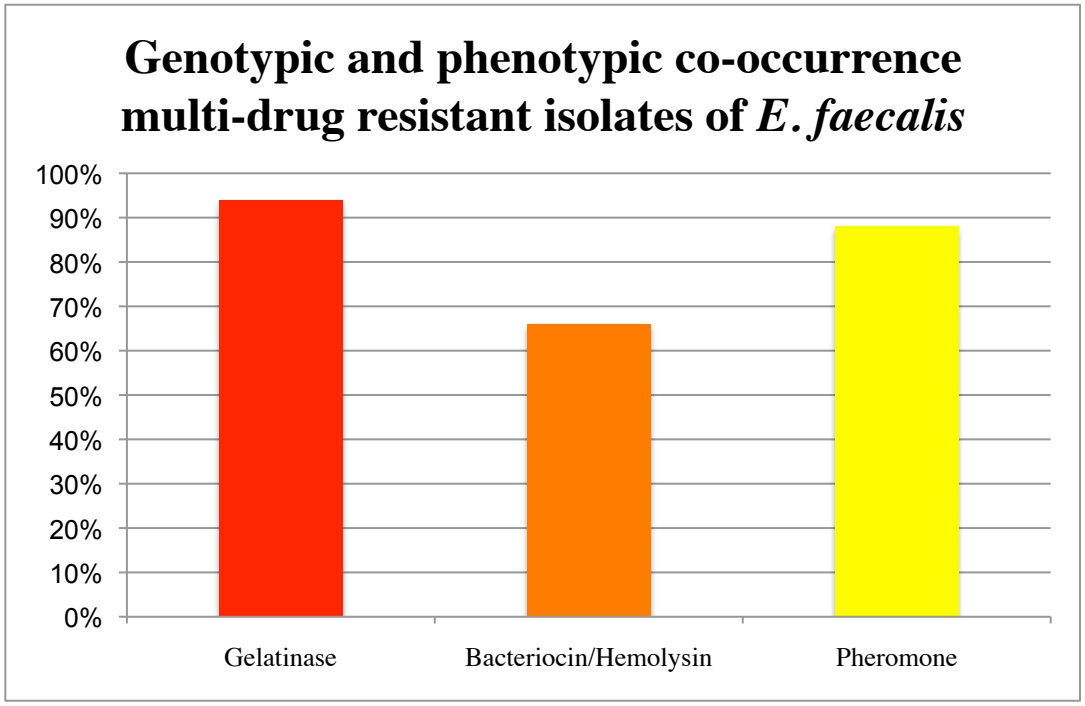
Table 7. Associations between phenotypic characteristics and CRISPR1-*cas*, CRISPR3-*cas*, or either CRISPR1-*cas* or CRISPR3-*cas* in *E. faecalis* from different sources

	CRISPR1-*cas*	CRISPR1-*cas*	*P*	CRISPR3-*cas*	CRISPR3-*cas*	*P*	Either CRISPR1 or 3-*cas*		*P*
	Present	Absent		Present	Absent		Present	Absent	
	n	*n*		*n*	*n*		*n*	*n*	
Oral (n=21)									
Gelatinase activity									
Present	5	1	ns	0	5	ns	4	1	ns
Absent	10	5		0	16		11	5	
Bacteriocin activity									
Present	8	2	ns	0	10	ns	8	2	ns
Absent	7	4		0	11		7	4	
Pheromone response									
Present	5	0	ns	0	5	ns	5	0	ns
Absent	10	6		0	16		10	6	
Hemolysin activity									
Present	6	1	ns	0	7	ns	6	1	ns
Absent	9	5		0	14		9	5	
Antibiotic resistance									
Present	11	3	ns	0	14	ns	11	3	ns
Absent	4	3		0	7		4	3	
Endodontic (n=34)									
Gelatinase activity									
Present	6	19	ns	16	9	ns	20	5	ns
Absent	2	7		3	6		4	5	
Bacteriocin activity									
Present	3	10	ns	4	9	0.02	6	7	0.02
Absent	5	16		15	6		18	3	
Pheromone response									
Present	1	15	0.03	9	7	ns	10	6	ns
Absent	7	11		10	8		14	4	
Hemolysin activity									
Present	0	0	ns	0	0	ns	0	0	ns
Absent	8	26		19	15		24	10	
Antibiotic resistance									
Present	3	5	ns	3	5	ns	5	3	ns
Absent	5	21		16	10		19	7	
Hospital (n=23)									
Gelatinase activity									
Present	2	16	ns	3	15	ns	3	15	ns
Absent	1	4		0	5		1	4	
Bacteriocin activity									
Present	1	10	ns	1	10	ns	2	9	ns
Absent	2	10		2	10		2	10	
Pheromone response									
Present	2	7	ns	1	10	ns	5	4	ns
Absent	1	13		2	10		4	10	
Hemolysin activity									
Present	1	2	ns	0	3	ns	1	2	ns
Absent	2	18		3	17		3	17	
Antibiotic resistance									
Present	3	20	ns	3	20	ns	4	19	ns
Absent	0	0		0	0		0	0	

Table 8. Phenotypic characteristics and presence of virulence gene determinants of multidrug-resistant hospital-acquired isolates of *E. faecalis* (n=23)

	Phenotype	Genotype			Phenotype		Genotype	Phenotype	Genotype	
								Pheromone		Adherence Factor
Strain	Gelatinase	*gelE* F/R	*ef184*F/*fsr*R	*fsrC* F/R	Bacteriocin	Hemolysin	*cylA* F/R	Response	*asa* F/R	*esp* F/R
HNEf1	+	+			+	-	-	-	+	-
HNEf2	+	-			-	-	-	+	+	-
HNEf3	-	-			-	-	-	-	+	+
HNEf4	-	+	-	+	-	-	-	-	+	-
HNEf5	+	+			+	-	+	-	+	-
HNEf6	+	+			-	-	+	-	+	-
HNEf7	+	+			+	-	+	-	+	-
HNEf8	+	+			+	-	-	-	+	+
HNEf9	-	+	+		+	-	-	-	+	+
HNEf10	+	+			-	-	-	-	+	-
HNEf11	-	+	-	+	-	-	+	-	+	+
HNEf12	+	+			-	-	+	-	+	-
HNEf13	+	+			+	-	+	+	+	-
HNEf14	-	-			-	+	-	-	+	-
HNEf15	+	+			-	-	+	-	+	+
HNEf16	+	+			+	-	+	+	-	-
HNEf17	+	+			-	-	+	+	+	-
HNEf18	+	+			+	-	+	+	+	-
HNEf19	+	+			+	-	+	+	+	-
HNEf20	+	+			-	-	+	+	+	-
V583	+	+	(-)		-	-	-	-	+	-
DS16	+	+			+	(+)	+	(+)	+	+
MMH594	+	+			+	+	+	+	+	-
Other/Misc										
OG1RF (lab strain)	(+)	(+)				-		-		
AA-OR26		(-)								
FA2-2			(+)				(-)		(-)	(-)
E1							(+)		(+)	
MMH594										(+)
( )=control strain										

Figure 15. Genotypic and phenotypic co-occurrence of virulence traits in multidrug-resistant hospital-acquired *E. faecalis*



Chapter 4: Discussion

Microorganisms are vulnerable to invasion by mobile genetic elements such as viruses, plasmids and transposons. CRISPR-Cas systems are highly adaptive immune systems present in most archaea and many bacteria that provide intracellular protection against these invading genetic elements (23, 45). In this study endodontic and oral *E. faecalis* strains were significantly more likely to possess CRISPR-*cas* determinants than multidrug-resistant hospital-acquired strains. Interestingly, and for reasons that are not clear, the majority of endodontic strains were more likely to harbor CRISPR3-*cas* than CRISPR1-*cas*, and no CRISPR3-*cas* were detected in oral strains. Most of the endodontic strains were obtained from dental patients in Sweden in 1994-1995, whereas all the other strains came from patients in the United States in 2002-2004.

Genes relating to antibiotic resistance, as well as virulence traits, can be found on plasmids that respond to pheromones (46). Possession of virulence traits that can be encoded on mobile elements capable of horizontal gene transfer might provide a selective advantage over other species in the infected root canal (11, 15). Virulence factors with the potential to promote adaptation and survival in different environments have been identified in enterococci recovered from infected root canals (13, 14). Of 31 *E. faecalis* strains from infected root canals, potential virulence traits expressed included production of gelatinase by 23 strains, and production of aggregation substance by 16 strains in response to pheromones in *E. faecalis* culture filtrate (13). A clumping response to pheromone indicates the production of aggregation substance and the potential for conjugative horizontal gene transfer (8). *E. faecalis* can harbor transferable high level antibiotic resistance and virulence determinants carried on plasmids (8, 47). Pheromone-initiated conjugative transfer of plasmids occurs at a higher frequency with clumping inducing agents. In an early study, examination of 100 clinical isolates of *Streptococcus faecalis* showed a significant correlation between drug-resistance and the ability to produce and respond to clumping

inducing agents (48). Therefore it was of interest to determine whether associations exist between the absence of CRISPR-*cas* and the presence of virulence traits in endodontic strains. In this study 15 of those isolates that exhibited a clumping response to pheromone also lacked the CRISPR1-*cas* determinant (P=0.03), but nine of those strains possessed CRISPR3-*cas*, suggesting no association.

There was a significant association between the absence of CRISPR-*cas* and the presence of antibiotic resistance (P=0.04). Similarly, Palmer and Gilmore reported that a lack of CRISPR-*cas* was positively associated with multidrug antibiotic resistance and suggested that antibiotic therapy inadvertently selects for enterococci with compromised genome defense (30). In the present study 21 of the 23 multidrug-resistant hospital-acquired strains were vancomycin resistant. In contrast, in the endodontic and oral strains, tetracycline resistance was the most common (n=19 strains), with multiple antibiotic resistance present in only one endodontic and six oral strains (7, 8, 13, 34). Tetracycline resistance encoded on conjugative transposons is highly transferrable in *E. faecalis* (49). Our data showing that 14 of the 19 tetracycline resistant endodontic and oral strains possessed either CRISPR1-*cas* or CRISPR3-*cas* supports previous speculation that conjugative transposons may evade the CRISPR-*cas* system (30).

Bacteriocins are antimicrobial proteins or peptides produced by many strains of Gram-positive and Gram-negative bacteria that can be bactericidal to other members of the same species (narrow spectrum) or across genera (broad spectrum). The production of a bacteriocin can provide the producer strain with a selective advantage over other strains, especially those closely related to the bacteriocin-producing strain (50). Lindenstrauss et al. found a significant association between the absence of CRISPR-*cas* and the presence of the cytolysin operon (which encodes hemolysin and bacteriocin activity) in *E. faecalis* strains recovered from predominantly clinical sources (29). Similarly, in this study endodontic strains lacking CRISPR-cas were more likely to demonstrate bacteriocin activity.

Surprisingly, a significant association was found between the absence of CRISPR1-*cas* and the absence of hemolysin activity (P=0.01); the reasons for this are unknown.

Genotypic evidence of potential virulence traits were identified in multidrug-resistant hospital-acquired strains of *E. faecalis* known to express the virulence traits gelatinase and hemolysin/bacteriocin activity (*cyl*), and clumping response to pheromone. Seventeen of the 18 (94%) strains that expressed the gelatinase phenotype also possessed the corresponding *gelE* gene determinant. The one strain that expressed the trait but did not have the gene was HNEf 2. There were three strains (HNEf4, 9, 11) that did not express the gelatinase trait, but did have the *gelE* gene (Table 8). Since expression of gelatinase is regulated by a quorum sensing system encoded by the *fsr* gene cluster these strains were further screened for defects in the gene cluster, known to be associated with the gelatinase-negative phenotype (42). In one of the isolates possessing the gelatinase-negative phenotype (HNEf9), the 1kb 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 23.9kb deletion sequence of the fsr gene cluster upstream to gelE (39); in the remaining two strains (HNEf4 and HNEf11) the internal fsrC product was detected indicating a defective fsr gene cluster (42).

While the majority of strains expressing each virulence trait also possessed the corresponding gene determinant (Figure 15), it should be noted that six strains possessing the *cyl* gene determinant did not express hemolysin/bacteriocin activity, and eight strains possessing the *asa* gene determinant did not clump in response to pheromone (Table 8). The reasons for non-functionality of genes was not explored further but may be attributable to a variety of regulatory factors (51-53). Similarly, there were five endodontic and four multidrug-resistant hospital-acquired strains that contained CRISPR3-*cas* determinants but also contained the determinants confirming their absence. These results were confirmed by DNA sequencing. The reason for this redundancy is not clear at this time.

Details are rapidly emerging about the acquisition, mechanisms, and dynamic evolution of the various CRISPR-Cas immune systems and their associated genes (22, 23, 54). From a clinical perspective the absence of a CRISPR-Cas immunity system might facilitate cell survival under certain conditions, e.g. by allowing uptake of antibiotic resistance genes in an antibiotic environment, but could also render the cell more vulnerable to attack by other selfish genetic elements (e.g. phages). Conversely, possession of a functional CRISPR-Cas system might facilitate survival by way of stabilizing the genome while allowing the cell to acquire information about the external environment via foreign DNA, integrate this information into the genome, and subsequently pass it on to progeny (54). No information directly pertaining to the root canal or oral environment, or specifically to *E. faecalis*, could be found about selective forces for the acquisition of CRISPR-*cas*. However, an analyses of 370 other prokaryotic genomes concluded that there was strong evidence for the propagation of CRISPR-*cas* genes to occur via horizontal gene transfer (55). Clinically, to what extent microbial cells residing in a root canal or oral biofilm participate in horizontal gene transfer of CRISPR-*cas* genes remains to be established.

Chapter 5: Summary and Conclusions

Microorganisms are vulnerable to invasion by mobile genetic elements such as viruses, plasmids and transposons. The recently discovered CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-associated system (CRISPR-Cas) is an adaptive immunity system found in most archaea and many bacteria that targets and inactivates invading foreign genetic elements. Cells with CRISPR-*cas* are more likely to resist the invasion and uptake of foreign DNA such as viruses, plasmids and transposons.

The aims of this study were to: (1) compare the occurrence of CRISPR-*cas* in collections of endodontic (n=34), oral (n=21), and multidrug-resistant hospital-acquired strains of *E. faecalis* (n=23); (2) evaluate the distribution of antibiotic resistance and virulence traits amongst strains without CRISPR-*cas*; and (3) evaluate whether multidrug-resistant hospital-acquired *E. faecalis* that express certain virulence traits also possess a corresponding gene determinant.

To accomplish these aims, *E. faecalis* strains were screened for CRISPR1-*cas* and CRISPR3-*cas* by using PCR, and products were verified by DNA sequencing. Associations were investigated between the occurrence of CRISPR-*cas* and the expression of phenotypic traits (antibiotic resistance, gelatinase activity, bacteriocin production, hemolysin activity, and clumping response to pheromone). Whether multidrug-resistant hospital-acquired *E. faecalis* strains that express certain virulence traits also possess a corresponding gene determinant was also evaluated.

It was found that CRISPR-*cas* determinants were present in proportionally more endodontic (25 of 34) and oral (15 of 21) strains than multidrug-resistant hospital-acquired (9 of 23) strains (P=0.01 and 0.04, respectively). Significant associations were found between the absence of CRISPR-*cas* and the presence of antibiotic resistance in strains overall (P=0.04) and bacteriocin activity in endodontic strains (P=0.01). As expected, genotype and

phenotype correlated for all tested conditions. The findings from this study have been published in the Journal of Endodontics (56).

Finally, the present study is the first to investigate CRISPR-*cas* in isolates recovered from infected root canals. Evidence for the presence of CRISPR-*cas* in the majority of endodontic and oral *E. faecalis* strains raises intriguing questions as to how prokaryotic immune systems might modulate interactions within the polymicrobial endodontic biofilm environment. As further details emerge regarding the occurrence and function of the CRISPR-Cas systems, their role in this process may become more clear.

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Appendices

Appendix 1. Results of Clustal W2 sequence alignments for CRISPR1-*cas* in oral strains

	(OG1RF)	(OG1RF)	(V583)	(V583)	Final Determination after DSEQ	
Strain	cas1F	cas1R	neg1cas1F	neg1cas1R	CRISPR1-cas	
Oral (n=21)						Oral (n=21)
E1	98	98			Yes	Yes=15
E2			96	98	No	No=6
E3			96	98	No	
E4	100	100			Yes	
E5			97	99	No	
E6			97	99	No	
E7			98	100	No	
E8	100	100			Yes	
E10			97	98	No	
E11	100	100			Yes	
E12	99	99			Yes	
OS16	99	100			Yes	
OS25	98	98			Yes	
AA-OR3	99	99			Yes	
AA-OR4	100	99			Yes	
AA-OR26	100	100			Yes	
AA-OR34	100	100			Yes	
AA-T4	100	100			Yes	
AA-T26	100	100			Yes	
GS34	100	100			Yes	
OG1	100	100			Yes	
Values represent percentage of alignment with control strain.						
( ) Control strains were OG1RF and V583						

Appendix 2. Results of Clustal W2 sequence alignments for CRISPR1-cas in endodontic strains

	(OG1RF)	(OG1RF)	(V583)	(V583)	Final Determination after DSEQ	
Strain	cas1F	cas1R	neg1cas1F	neg1cas1R	CRISPR1-cas	
Endodontic (n=34)						Endodontic (n=34)
GS1	100	100			Yes	Yes=8
GS2			98	98	No	No=26
GS3			99	100	No	
GS4			97	100	No	
GS5			99	100	No	
GS6			99	100	No	
GS7			97	100	No	
GS8			93	98	No	
GS9	99	100			Yes	
GS10	100	100			Yes	
GS12			99	100	No	
GS13			99	100	No	
GS14			98	98	No	
GS15			97	100	No	
GS16	100	100			Yes	
GS17			94	94	No	
GS18			98	98	No	
GS19	100	100			Yes	
GS21			98		No	
GS22			98		No	
GS23			97		No	
GS24			98		No	
GS25	99	99			Yes	
GS26			98		No	
GS27			98		No	
GS28			94	96	No	
GS29			98	98	No	
GS30	100	100			Yes	
GS31	100	100			Yes	
GS32			99	100	No	
GS33			96	98	No	
JG2					No	
ER3/2s			97	98	No	
ER5/1			97	98	No	
Values represent percentage of alignment with control strain.						
( ) Control strains were OG1RF and V583						

Appendix 3. Results of Clustal W2 sequence alignments for CRISPR1-cas in multidrug-resistant hospital-acquired strains

	(OG1RF)	(OG1RF)	(V583)	(V583)	Final Determination ater DSEQ	
Strain	cas1F	cas1R	neg1cas1F	neg1cas1R	CRISPR1-cas	
Hospital (n=23)						Hospital (n=23)
HNEf1			99		No	Yes=3
HNEf2	98	98			Yes	No=20
HNEf3	98	98			Yes	
HNEf4			96		No	
HNEf5			98		No	
HNEf6			98		No	
HNEf7			97		No	
HNEf8			97		No	
HNEf9			97		No	
HNEf10			95		No	
HNEf11			96		No	
HNEf12			97		No	
HNEf13			98		No	
HNEf14			100	100	No	
HNEf15			98		No	
HNEf16			98		No	
HNEf17			97		No	
HNEf18			97		No	
HNEf19			97		No	
HNEf20			98		No	
V583					No (control)	
DS16	100	100			Yes	
MMH594					Not available	
Other/Misc						
OG1RF	X	X			Yes (control)	
Fly1						
Values represent percentage of alignment with control strain.						
( ) Control strains were OG1RF and V583						

Appendix 4. Results of Clustal W2 sequence alignments for CRISPR3-*cas* in oral strains

	(Fly1)	(V583)	Final Determination after DSEQ	
Strain	3csnF	neg3casF	CRISPR3-cas	
Oral (n=21)				Oral (n=21)
E1		98	No	Yes=0
E2		97	No	No=21
E3		97	No	
E4		98	No	
E5		98	No	
E6		98	No	
E7		98	No	
E8	97	97	No	
E10		97	No	
E11		97	No	
E12	98	98	No	
OS16		98	No	
OS25		97	No	
AA-OR3		98	No	
AA-OR4		97	No	
AA-OR26		98	No	
AA-OR34		97	No	
AA-T4		97	No	
AA-T26		98	No	
GS34		98	No	
OG1		98	No	
Values represent percentage of alignment with control strain.				
( ) = control strains				

Appendix 5. Results of Clustal W2 sequence alignments for CRISPR3-cas in endodontic strains

Strain	(Fly1) 3csnF	(V583) neg3casF	Final Determination after DSEQ CRISPR3-cas	
Endodontic (n=34)				Endodontic (n=34)
GS1		99	No	Yes=20
GS2		97	No	No=14
GS3	99		Yes	
GS4	99		Yes	
GS5	99		Yes	
GS6	99		Yes	
GS7	99		Yes	
GS8	98	41	Yes	
GS9	100	98	Yes	
GS10	99	97	Yes	
GS12	99		Yes	
GS13	99		Yes	
GS14	99	97	Yes	
GS15	100		Yes	
GS16	99	98	Yes	
GS17	99	98	Yes	
GS18		98	No	
GS19		98	No	
GS21	100		Yes	
GS22	99		Yes	
GS23		98	No	
GS24	99		Yes	
GS25		98	No	
GS26	99		Yes	
GS27	99		Yes	
GS28		98	No	
GS29		97	No	
GS30		98	No	
GS31		98	No	
GS32	98	39	Yes	
GS33		98	No	
JG2		98	No	
ER3/2s		98	No	
ER5/1		98	No	
Values represent percentage of alignment with control strain.				
( ) = control strains				

Appendix 6. Results of Clustal W2 sequence alignments for CRISPR3-cas in multidrug-resistant hospital-acquired strains

Strain				
	(Fly1)	(V583)	Final Determination after DSEQ	
	3csnF	neg3casF	CRISPR3-cas	
Hospital (n=23)				Hospital (n=23)
HNEf1		99	No	Yes=6
HNEf2		98	No	No=17
HNEf3		98	No	
HNEf4		99	No	
HNEf5		99	No	
HNEf6		99	No	
HNEf7	99	41	Yes	
HNEf8		99	No	
HNEf9		97	No	
HNEf10		99	No	
HNEf11		98	No	
HNEf12		99	No	
HNEf13		99	No	
HNEf14	99	82	Yes	
HNEf15	99	100	Yes	
HNEf16		98	No	
HNEf17	99	98	Yes	
HNEf18	99	99	Yes	
HNEf19	99	99	Yes	
HNEf20		98	No	
V583			No (control)	
DS16		98	No	
MMH594		99	No	
Other/Misc				
OG1RF			No	
Fly1			Yes (control)	
Values represent percentage of alignment with control strain.				
( ) = control strains				

Appendix 7. Journal of Endodontics publication

Burley KM, Sedgley CM. CRISPR-Cas, a prokaryotic adaptive immune system, in endodontic, oral, and multidrug-resistant hospital-acquired Enterococcus faecalis. Journal of Endodontics 2012 Nov;38:1511-5.

CRISPR-Cas, a Prokaryotic Adaptive Immune System, in Endodontic, Oral, and Multidrug-resistant Hospital-acquired *Enterococcus faecalis*

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Abstract

Introduction: Microorganisms are vulnerable to invasion by mobile genetic elements such as viruses, plasmids, and transposons. The recently discovered Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)–associated system, or CRISPR-Cas, is an adaptive immunity system found in most archaea and many bacteria that targets and inactivates invading foreign genetic elements. Cells with CRISPR-*cas* are more likely to resist the invasion and uptake of foreign DNA such as viruses, plasmids, and transposons. The aims of this study were to (1) compare the occurrence of CRISPR-*cas* in collections of endodontic (n = 34), oral (n = 21), and multidrug-resistant hospital-acquired strains of *Enterococcus faecalis* (n = 23) and (2) evaluate the distribution of antibiotic resistance and virulence traits among strains without CRISPR-*cas*. **Methods:** *E. faecalis* strains were screened for CRISPR1-*cas* and CRISPR3-*cas* by using polymerase chain reaction, and products were verified by DNA sequencing. Associations were investigated between the occurrence of CRISPR-*cas* and the expression of phenotypic traits (antibiotic resistance, gelatinase activity, bacteriocin production, hemolysin activity, and clumping response to pheromone). **Results:** CRISPR-*cas* determinants were present in proportionally more endodontic (25 of 34) and oral (15 of 21) strains than hospital-acquired (9 of 23) strains (P = .01 and .04, respectively). Significant associations were found between the absence of CRISPR-*cas* and the presence of antibiotic resistance in strains overall (P = .04) and bacteriocin activity in endodontic strains (P = .01). **Conclusions:** Evidence for the presence of CRISPR-*cas* in the majority of endodontic and oral *E. faecalis* strains raises intriguing questions as to how prokaryotic immune systems might modulate interactions within the polymicrobial endodontic biofilm environment. *(J Endod 2012;38:1511–1515)*

Key Words

CRISPR-*cas*, endodontic, *Enterococcus faecalis*, multidrug-resistant hospital-acquired bacteria, oral

Enterococcus faecalis are facultative gram-positive cocci that are widely distributed in nature, animals, and humans. *E. faecalis* are frequently recovered from previously treated root canals (1, 2) and occasionally from the oral flora (3, 4). Although *E. faecalis* can survive for extended periods in the nutrient-deprived obturated root canal system *ex vivo* (5), whether *E. faecalis* can "cause" periradicular infections has not been established. In an effort to address this question, previous studies have shown that *E. faecalis* strains recovered from infected root canals can express different virulence factors (6, 7) and exchange DNA as evidenced by the transfer of antibiotic resistance genes between *E. faecalis* JH2-2 and *Streptococcus gordonii* Challis-Sm in root canals *ex vivo* (8). These data suggest a capacity for the species to modulate virulence expression and antibiotic resistance acquisition under varying environmental conditions in the root canal system and may help to explain the recovery of *E. faecalis* from both symptomatic and asymptomatic cases (9).

In contradistinction, the importance of the host's immunity in response to bacterial challenge is well established. For example, small numbers of virulent microorganisms may be sufficient to cause disease in an immunocompromised host (10). In a novel twist, the "health" of a microorganism is also dependent on the presence of its own protective "immune" system. Specifically, the recently discovered Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) and genes encoding CRISPR-associated (Cas) proteins, or CRISPR-Cas module, are a prokaryotic immune system widespread among archaea and bacteria that confers resistance to exogenous mobile genetic elements such as viruses (phages), plasmids, and transposons (11–13). Although considerable diversity exists between the many different CRISPR-Cas systems, they are characterized by a CRISPR locus made up of a varying number of repeating segments (14). Each repeating segment has the necessary DNA sequence required to target a different invader. CRISPR-Cas systems are primarily located on the chromosome but can also be found on plasmids. They function over 3 stages: adaptation, expression, and interference (13). During the first stage, CRISPR-Cas proteins incorporate a small fragment (termed a spacer) of a foreign genetic element into the repeating CRISPR locus. In the second stage, an RNA transcript of the CRISPR locus is processed to form small CRISPR RNAs (crRNAs), each of which can identify a specific target. At the third stage, on reexposure to the specific foreign genetic element, the crRNA and CRISPR-Cas proteins target and cleave the invading DNA or RNA (12, 13).

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Several CRISPR-*cas* systems have been identified in prokaryotes, including CRISPR1-*cas* and CRISPR3-*cas* in *E. faecalis* isolates recovered from human, animal, insect, and environmental sources (15–17). Two CRISPR loci were first identified in the *E. faecalis* OG1RF genome: CRISPR1-*cas*, which has the associated *cas* gene, and CRISPR2, which is an orphan locus lacking the functional *cas* genes (15). Subsequently, CRISPR3-*cas* was identified in 2 strains (17), a fruit fly isolate Fly1 (18) and a urine isolate (17). CRISPR-*cas* has since been identified in one third of 48 *E. faecalis* strains recovered from a variety of sources, all but 1 being CRISPR1-*cas* (17), and in 18 of 52 *E. faecalis* strains recovered from (predominantly) clinical, food, and environmental sources, all but 2 being CRISPR1-*cas* (16). Recently, the absence of CRISPR-*cas* in *E. faecalis* was positively correlated with acquired multidrug-resistance genes (17), which are often found on mobile genetic elements such as plasmids (19, 20).

The occurrence of the CRISPR-*cas* in the endodontic and oral microflora has not been previously evaluated. The first aim of this study was to compare the occurrence of CRISPR-*cas* in collections of endodontic and oral strains and multidrug-resistant hospital-acquired strains of *E. faecalis*; the null hypothesis tested was that there is no difference in occurrence between groups. Because the absence of CRISPR-*cas* could indicate previous uptake of foreign DNA (eg, via horizontal transfer of plasmids encoding virulence and/or antibiotic resistance genes), the second aim was to evaluate the distribution of virulence traits and antibiotic resistance among strains without CRISPR-*cas*.

Materials and Methods

Bacterial Strains and Phenotypic Characterization

E. faecalis strains and their sources are listed in Table 1. Strains were taken from −80°C stocks and plated onto Todd Hewitt broth (Becton, Dickinson and Co, Sparks, MD) supplemented with 1.5% agar and incubated aerobically at 37°C for 24 hours. For each strain, expected colony formation, cell morphology, esculin hydrolysis, and Gram stain reaction were confirmed. The 16S rRNA polymerase chain reaction (PCR) assays were performed for all strains to verify species-specific amplification as previously described (3). Hospital-acquired strains (n = 23) were screened for antibiotic resistance, gelatinase activity, bacteriocin production, hemolysin activity, and clumping response to pheromone by using methods previously described (6); endodontic (n = 34) and oral (n = 21) strains have been previously screened for these traits (3, 4, 6, 21).

Total DNA Extraction, PCR Amplification, and Sequencing

Total DNA was isolated as previously described (3) and resuspended in sterile nuclease-free water to a final stock concentration of 50 ng/μL. Primer sequences for *E. faecalis* CRISPR1-*cas cas1* were forward: 5′-ATG GGC TGG CGA ACG GTA GTG GTT A-3′, reverse: 5′-TCA TAT CCC AAA CTC TGG AAC TCC T-3′ for an expected 867 base-pair product. Primer sequences for CRISPR3-*cas csn1* were forward: 5′-GCT GAA TCT GTG AAG TTA CTC-3′, reverse: 5′-CTG TTT TGT TCA CCG TTG GAT-3′ for an expected 258 base-pair product (17). To verify the absence of false-negative PCR results, those strains without CRISPR1-*cas* PCR products were screened by using primers that anneal outside the CRISPR1-*cas* loci: forward: 5′-GCG ATG TTA GCT GAT ACA AC-3′, reverse: 5′-CGA ATA TGC CTG TGG TGA AA-3′ for an expected 315 base-pair product; those strains without CRISPR3-*cas* PCR products were screened by using primers that flank the CRISPR3-*cas* loci: forward: 5′-GAT CAC TAG GTT CAG TTA TTT C-3′, reverse: 5′-CAT CGA TTC ATT ATT CCT CCA A-3′ for an expected product size of 224 base pairs (17). Control strains used for PCR screening were *E. faecalis* OG1RF (CRISPR1-*cas* present), Fly-1 (CRISPR3-*cas* present), and V583 (CRISPR1-*cas* and CRISPR3-*cas* absent) (17).

PCR amplifications used an Eppendorf Mastercycler (Brinkmann Instruments, Inc, Westbury, NY). The 100-ng total DNA template was prepared for 50-μL PCR amplifications as follows: 45 μL Platinum PCR SuperMix (High Fidelity; Invitrogen, Carlsbad, CA) with primers (10 μmol/L final concentration) and template DNA to a final volume of 50 μL. The PCR amplification conditions were an initial step of 95°C for 60 seconds, followed by 35 consecutive cycles at 94°C for 20 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. PCR products were stained with SYBR Green (Invitrogen) and analyzed by electrophoresis by using 1.5%–3% agarose gels in tris-borate-ethylenediaminetetraacetic acid buffer and visualized by fluorescence under UV light. Amplified PCR products were correlated with a 100 base-pair ladder (Invitrogen) to confirm conjunction with their expected PCR amplicon size. PCR products were purified with a QIAquick PCR purification column (Qiagen N.V., Venlo, The Netherlands) and submitted for sequencing to the Oregon Health and Science University MMI DNA Services Core (Portland, OR). Nucleotide sequence alignments were compared with the National

TABLE 1. Bacterial Strains

Source	Strain name	Reference
Endodontic (n = 34)		
Primary treatment	GS3, GS6, GS7, GS8, GS13, GS18, GS19, GS22, GS24, GS27, GS28, GS31, GS32	6
Orthograde retreatment	GS1, GS2, GS12, GS16, GS25, GS33, ER3/2s, ER5/1	29
Endodontic treatment	GS4, GS5, GS9, GS10, GS14, GS15, GS17, GS21, GS23, GS26, GS29, GS30	6
	JG2	30
Oral (n = 21)		
Tongue swab, endodontic patient	AA-T4, AA-T26	21
	GS-34	29
Oral rinse, dental student	C1	4
Oral rinse, endodontic patient	E1, E2, E3, E4, E5, E6, E7, E8, E10, E11	4
	OS16, OS25	3
	AA-OR3, AA-OR4, AA-OR26, AA-OR34	21
	OG1	31
Hospital-acquired (n = 23)	HNEfs #1–#20	32
	DS16	20
	MMH594	33
	V583*	34
Other	OG1RF*	35
	Fly-1*	18

*V583 lacks CRISPR1-*cas* and CRISPR3-*cas*; OG1RF encodes CRISPR1-*cas*; #Fly-1 encodes CRISPR3-*cas* (17).

Center for Biotechnology Information GenBank database for final verification of amplification by using the online ClustalW2 multiple sequence alignment tool http://www.ebi.ac.uk/Tools/msa/clustalw2/ (22).

Statistical Analysis

Two-tailed Fisher exact tests were used to compare the occurrence of CRISPR-*cas* in endodontic, oral, and hospital-acquired isolates and to evaluate the distribution of antibiotic resistance, gelatinase activity, bacteriocin production, hemolysin activity, and clumping response to pheromone among strains without CRISPR-*cas*. Significance was set at $P < .05$.

Results

PCR products were available for all strains. The sequenced CRISPR1-*cas* and CRISPR3-*cas* PCR products were 97%–100% identical to the corresponding *E. faecalis* OG1RF and Fly-1 sequences. The sequenced products verifying absence of false-negative PCR results were 94%–100% identical to the corresponding *E. faecalis* V583 sequence.

The occurrence of CRISPR-*cas* in *E. faecalis* isolates is shown in Table 2. Overall, CRISPR1-*cas* or CRISPR3-*cas* determinants were present in proportionally fewer hospital-acquired strains (9 of 23) than endodontic (25 of 34) ($P = .01$) and oral strains (15 of 21) ($P = .04$). The CRISPR1-*cas* determinant was present in proportionally fewer endodontic (8 of 34) and hospital-acquired strains (3 of 23) than oral strains (15 of 21) ($P = .0007$ and $P = .0002$, respectively), with no difference between endodontic and hospital-acquired strains. The CRISPR3-*cas* determinant was present in proportionally more endodontic strains (20 of 34) compared with hospital-acquired strains (6 of 23) ($P = .03$) and was not identified in any oral strains. Three endodontic strains had both CRISPR1-*cas* and CRISPR3-*cas*. The remaining endodontic strains were significantly more likely to harbor CRISPR3-*cas* than CRISPR1-*cas* ($P = .006$), whereas the reverse was true for oral strains ($P < .0001$). The null hypothesis was rejected.

Phenotypic characteristics of multidrug-resistant hospital-acquired strains are shown in Table 3; these characteristics for endodontic and oral strains are reported elsewhere (3, 4, 6, 21). Overall, a significant association was found between the absence of either CRISPR1-*cas* or CRISPR3-*cas* and the presence of antibiotic resistance ($P = .04$), as shown in Table 4. Other significant associations were found between the absence of CRISPR1-*cas* and the absence of hemolysin activity ($P = .01$) and between the absence of CRISPR3-*cas* and presence of gelatinase activity ($P = .005$), bacteriocin activity ($P = .02$), and antibiotic resistance ($P = .0009$). In endodontic strains, significant associations were found between the absence of both CRISPR-*cas* determinants and the presence of bacteriocin activity ($P = .02$), between the absence of CRISPR1-*cas* and the presence of a clumping response to pheromone ($P = .03$), and between the absence of CRISPR3-*cas* and the presence of bacteriocin activity ($P = .03$). Within oral and hospital-acquired subgroups, no significant associations were found (data not shown).

Discussion

Microorganisms are vulnerable to invasion by mobile genetic elements such as viruses, plasmids, and transposons. CRISPR-Cas systems are highly adaptive immune systems present in most archaea and many bacteria that provide intracellular protection against these invading genetic elements (13, 23). In this study endodontic and oral *E. faecalis* strains were significantly more likely to possess CRISPR-*cas* determinants than multidrug-resistant hospital-acquired strains. Interestingly, and for reasons that are not clear, the majority of endodontic strains were more likely to harbor CRISPR3-*cas* than CRISPR1-*cas*, and no CRISPR3-*cas* were detected in oral strains. The majority of the endodontic strains were obtained from dental patients in Sweden in 1994–1995, whereas all other strains came from patients in the United States in 2002–2004.

There was a significant association between the absence of CRISPR-*cas* and the presence of antibiotic resistance (Table 4). Similarly, Palmer and Gilmore (17) reported that a lack of CRISPR-*cas* was positively associated with multidrug antibiotic resistance and suggested that antibiotic therapy inadvertently selects for enterococci with compromised genome defense. In the present study 21 of the 23 multidrug-resistant hospital-acquired strains were vancomycin resistant. In contrast, in the endodontic and oral strains, tetracycline resistance was the most common (n = 19 strains), with multiple antibiotic resistance present in only 1 endodontic and 6 oral strains (3, 4, 6, 21). Tetracycline resistance encoded on conjugative transposons is highly transferrable in *E. faecalis* (24). Our data showing that 14 of the 19 tetracycline-resistant endodontic and oral strains possessed either CRISPR1-*cas* or CRISPR3-*cas* support previous speculation that conjugative transposons may evade the CRISPR-*cas* system (17).

Virulence factors with the potential to promote adaptation and survival in different environments have been identified in enterococci recovered from infected root canals (6, 7). Of 31 *E. faecalis* strains from infected root canals, potential virulence traits expressed included production of gelatinase by 23 strains and production of aggregation substance by 16 strains in response to pheromones in *E. faecalis* culture filtrate (6). A clumping response to pheromone indicates the production of aggregation substance and the potential for conjugative horizontal gene transfer (19). *E. faecalis* can harbor transferable high-level antibiotic resistance and virulence determinants carried on plasmids (19, 25). Therefore, it was of interest to determine

TABLE 2. Occurrence of CRISPR-*cas* in *E. faecalis**

Source	CRISPR1-*cas* or CRISPR3-*cas*†		CRISPR1-*cas*‡		CRISPR3-*cas*§		CRISPR1-*cas* and CRISPR3-*cas*	
	Present	Absent	Present	Absent	Present	Absent	Present	Absent
Endodontic (n = 34)	25	9	8	26	20	14	3	9
Oral (n = 21)	15	6	15	6	0	21	0	6
Hospital-acquired (n = 23)	9	14	3	20	6	17	0	14

*CRISPR1-cas is present in strains GS1, GS9, GS10, GS16, GS19, GS25, GS30, GS31, E1, E4, E8, E11, E12, OS16, OS25, AA-OR3, AA-OR4, AA-OR26, AA-OR34, AA-T4, AA-T26, GS34, OG1, HNEf2, HNEf3, and DS16. CRISPR3-*cas* is present in strains GS3, GS4, GS5, GS6, GS7, GS8, GS9, GS10, GS12, GS13, GS14, GS15, GS16, GS17, GS21, GS22, GS24, GS26, GS27, GS32, and HNEf7, HNEf14, HNEf15, HNEf17, HNEf18, HNEf19. Both CRISPR1-*cas* AND CRISPR3-cas are present in strains GS9, GS10, and GS16.

†Absent in more hospital-acquired than endodontic ($P = .01$) and oral ($P = .04$) strains.

‡Absent in more endodontic and hospital-acquired than oral strains ($P = .0007$ and $P = .0002$, respectively).

§Absent in more oral than endodontic and hospital-acquired strains ($P < .0001$ and $P = .0002$, respectively) with a significant difference between endodontic and hospital-acquired strains ($P = .03$).

TABLE 3. Phenotypic Characteristics of Multidrug-resistant Hospital-acquired *E. faecalis*

	Phenotype				
Strain*	Gelatinase	Bacteriocin	Hemolysin	Pheromone response†	Antibiotic resistance‡
HNEf1	+	+	–	–	Va, Fu, Tc, Em, Km, Cm, Gm
HNEf2	+	–	–	+	Va, Fu, Tc, Sm, Em, Km, Cm, Gm
HNEf3	–	–	–	–	Va, Fu, Sm, Em, Km, Cm, AM, Gm, Rf, Pg
HNEf4	–	–	–	–	Va, Fu, Em, Km, Cm, Gm
HNEf5	+	+	–	–	Va, Fu, Em, Km, Cm, Gm, Pg
HNEf6	+	–	–	–	Va, Fu, Tc, Em, Km, Cm, Gm
HNEf7	+	+	–	–	Va, Fu, Sm, Em, Km, Cm, Gm
HNEf8	+	+	–	–	Va, Fu, Tc, Sm, Em, Km, Cm, Gm
HNEf9	–	+	–	–	Va, Fu, Em, Km, Cm, Gm
HNEfl0	+	–	–	–	Va, Fu, Sm, Em, Km, Cm, Gm
HNEf11	–	–	–	–	Va, Fu, Sm, Em, Km, Cm, Gm
HNEfl2	+	–	–	–	Va, Fu, Sm, Em, Km, Cm, Gm, Pg
HNEfl3	+	+	–	+	Va, Fu, Sm, Em, Km, Cm, Gm
HNEfl4	–	–	+	–	Va, Fu, Sm, Em, Km, Cm, AM, Gm, Pg
HNEfl5	+	–	–	–	Va, Fu, Sm, Em, Km, Cm, Gm
HNEf16	+	+	–	+	Va, Fu, Sm, Em, Km, Cm, Gm
HNEfl7	+	–	–	+	Va, Fu, Sm, Em, Km, Cl, Cm, Gm
HNEfl8	+	+	–	+	Va, Fu, Sm, Em, Km, Cl, Cm, Gm, Rf
HNEfl9	+	+	–	+	Va, Fu, Sm, Em, Km, Cl, Cm, Gm
HNEf20	+	–	–	+	Va, Fu, Sm, Em, Km, Cm, Gm
DS16	+	+	+	+	Fu, Tc, Sm, Em, Km, Cm
MMH594	+	+	+	+	Fu, Em, Km, Cl, Cm, Gm, Pg
V583	+	–	–	–	Va, Fu, Em, Km, Cm, Gm

*CRISPR1-*cas* is present in HNEf2, HNEf3, and DS16. CRISPR3-cas is present in HNEf7, HNEf15, and HNEf17–HNEf19.

†Positive control *E. faecalis* strains for pheromone tests were OG1X/pAM373, OGISS/pCF10, 39-5 (harboring pPD1), and OG1X/pPAD1.

‡Minimum inhibitory concentrations determined by using E-test (AB Biodisk, Solna, Sweden). Antibiotics tested were ampicillin (Am), benzylpenicillin (Pg), chloramphenicol (Cm), clindamycin (Cl), erythromycin (Em), fusidic acid (Fu), gentamicin (Gm), rifampin (Rf), streptomycin (Sm), tetracycline (Tc), and vancomycin (Va).

whether an association exists between the absence of CRISPR-*cas* and the presence of virulence traits in endodontic strains. In this study 15 of those isolates that exhibited a clumping response to pheromone also lacked the CRISPR1-*cas* determinant ($P = .03$), but 9 of those strains possessed CRISPR3-*cas*, suggesting no association.

Bacteriocins are antimicrobial proteins or peptides produced by many strains of gram-positive and gram-negative bacteria and can be bactericidal to other members of the same species (narrow spectrum) or across genera (broad spectrum). The production of a bacteriocin can provide the producer strain with a selective advantage over other strains, especially those closely related to the bacteriocin-producing strain (26). Lindenstrauss et al (16) found a significant association between the absence of CRISPR-*cas* and the presence of the cytolysin operon (which encodes hemolysin and bacteriocin activity) in

TABLE 4. Associations between Phenotypic Characteristics and Occurrence of CRISPR-*cas* in *E. faecalis*

	CRISPR1-*cas*			CRISPR3-*cas*			CRISPR1-*cas* or CRISPR3-*cas*		
	Present	Absent	*P* value	Present	Absent	*P* value	Present	Absent	*P* value
All strains (n = 78)									
Antibiotic resistance									
Present	17	28	NS	6	39	.0009	20	25	.04
Absent	9	24		16	17		23	10	
Gelatinase activity									
Present	13	36	NS	19	29	.005	27	21	NS
Absent	13	16		3	27		16	14	
Bacteriocin activity									
Present	12	22	NS	5	29	.02	16	18	NS
Absent	14	30		17	27		27	17	
Pheromone response									
Present	8	22	NS	10	22	NS	20	10	NS
Absent	18	30		12	34		28	10	
Hemolysin activity									
Present	7	3	.01	0	10	NS	7	3	NS
Absent	19	49		22	46		36	32	
Endodontic strains (n = 34)									
Bacteriocin activity									
Present	3	10	NS	4	9	.03	6	7	.02
Absent	5	16		15	6		18	3	
Pheromone response									
Present	1	15	.03	9	7	NS	10	6	NS
Absent	7	11		10	8		14	4	

NS, not significant.

E. faecalis strains recovered from predominantly clinical sources. Similarly, in this study endodontic strains lacking CRISPR-*cas* were more likely to demonstrate bacteriocin activity.

Details are rapidly emerging about the acquisition, mechanisms, and dynamic evolution of the various CRISPR-Cas immune systems and their associated genes (12, 13, 27). From a clinical perspective the absence of a CRISPR-Cas immunity system might facilitate cell survival under certain conditions, eg, by allowing uptake of antibiotic resistance genes in an antibiotic environment, but could also render the cell more vulnerable to attack by other selfish genetic elements (eg, phages). Conversely, possession of a functional CRISPR-Cas system might facilitate survival by way of stabilizing the genome, while allowing the cell to acquire information about the external environment via foreign DNA, integrate this information into the genome, and subsequently pass it on to progeny (27).

It has been proposed that the possession of virulence traits that can be encoded on mobile elements capable of horizontal gene transfer (HGT) might provide a selective advantage over other species in the infected root canal (8). No information directly pertaining to the root canal or oral environment, or specifically to *E. faecalis*, could be found about selective forces for the acquisition of CRISPR-*cas*. However, an analysis of 370 other prokaryotic genomes concluded that there was strong evidence for the propagation of CRISPR-*cas* genes to occur via HGT (28). Clinically, to what extent microbial cells residing in a root canal or oral biofilm participate in HGT of CRISPR-*cas* genes remains to be established.

In conclusion, the present study is the first to investigate CRISPR-*cas* in isolates recovered from infected root canals. Evidence for the presence of CRISPR-*cas* in the majority of endodontic and oral *E. faecalis* strains raises intriguing questions as to how prokaryotic immune systems might modulate interactions within the polymicrobial endodontic biofilm environment, particularly in response to antimicrobial agents used in root canal treatment. Understanding this process could lead to improved therapeutic strategies in the future.

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The Oregon Health & Science University School of Dentistry

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Brief Summary of Thesis: This study compared the occurrence of CRISPR-*cas* in collections of endodontic, oral strains, and multidrug-resistant hospital-acquired strains of *E. faecalis* and evaluated the distribution of virulence traits and antibiotic resistance among strains without CRISPR-*cas*.