

Relevance of the clustered regularly interspaced short palindromic repeats of *Enterococcus faecalis* strains isolated from retreatment root canals on periapical lesions, resistance to irrigants and biofilms

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Abstract. A high prevalence of *Enterococcus faecalis* (*E. faecalis*) is observed in teeth with root canal treatment failures. Clustered regularly interspaced short palindromic repeats (CRISPR) are widely distributed in prokaryotes that have adaptive immune systems against mobile elements, including pathogenic genes. The present study investigated the relevance of the CRISPR in *E. faecalis* strains isolated from retreated root canals on biofilms, periapical lesions and drug resistance. A total of 20 *E. faecalis* strains were extracted from the root canals of teeth referred for root canal retreatment. CRISPR-Cas loci were identified by two pairs of relevant primers and polymerase chain reaction. The susceptibility of the 20 isolated strains to intracanal irrigants was evaluated by 1- and 5-minute challenges with a mixture of a tetracycline isomer, an acid and a detergent (MTAD), 2% chlorhexidine (CHX) and 5.25% sodium hypochlorite (NaOCl). The microtiter plate assay and crystal violet staining were used to compare the biofilm formation of the *E. faecalis* isolate strains. Out of the 20 *E. faecalis* isolate strains, 5 strains that lacked CRISPR-cas determinants exhibited significant periapical lesions. Among the 15 strains containing CRISPR-cas determinants, 8 were isolated from root canals with inadequate fillings and 7 were isolated from root canals without any fillings. The five strains lacking CRISPR-cas loci were observed to be more resistant to

MTAD and 2% CHX than the 15 strains that had CRISPR-cas loci. All of the strains exhibited the same susceptibility to 5.25% NaOCl. Furthermore, the 5 strains lacking CRISPR-cas determinants generated more biofilm than the other 15 strains. Thus, the results of the present study suggested that *E. faecalis* root canal isolates lacking CRISPR-cas exhibit higher resistance to intracanal irrigants, stronger biofilm formation and generate significant periapical lesions.

Introduction

Post-treatment apical periodontitis is typically caused by diverse pathogens, including Gram-positive and Gram-negative bacteria, and is characterized by the appearance and persistence of apical periodontic lesions (1,2). Microbiological culturing techniques and molecular biological methods have been employed to investigate the microbiota from post-treatment root canal infections and have demonstrated that *Enterococcus faecalis* (*E. faecalis*) was highly prevalent in teeth that had undergone unsuccessful root canal therapies (3,4). *E. faecalis* possesses numerous virulence factors, including cytolysin, lytic enzymes, pheromones, aggregation substances and lipoteichoic acid, and transmits these virulence traits into other species, further contributing to apical periodontitis (5,6). Furthermore, *E. faecalis* may overcome the challenges of starvation and intracanal medication and persist as a pathogenic bacterium in the root canal system (7). When an adequate nutrition supply becomes available, the starved *E. faecalis* recover, resulting in increased pathogenicity (8). Therefore, *E. faecalis* may serve an important role in contributing to endodontic treatment failure.

Bacteria-like eukaryotes usually have an adaptive immune system that facilitates sequence-specific immunity against exogenous mobile genetic elements. Clustered regularly interspaced short palindromic repeats (CRISPR) loci encode this system (9,10). *E. faecalis* is a common nosocomial pathogen that generates pathogenicity and resistance to antibiotics by acquiring mobile genetic elements, including antibiotic resistance genes (11). It has been demonstrated that among strains of *E. faecalis*, CRISPR1-cas and CRISPR3-cas loci are variable, and an inverse correlation between the presence of

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CRISPR-cas loci and acquired antibiotic resistance has been observed in clinically isolated strains of *E. faecalis* (11,12). CRISPR-cas supplies *E. faecalis* with a specific sequence and provides a defense against plasmids, phages, pathogenicity islands and other elements (12,13). Multidrug-resistant *enterococci* usually lack complete CRISPR loci and maybe invaded by mobile genetic elements (13). The complete genome of *Enterococcus faecalis* V583, a vancomycin-resistant clinical isolate, indicated that up to 25% of its genome consists of mobile elements (14).

Burley and Sedgley (15) investigated CRISPR-cas determinants that existed in an endodontic and oral *E. faecalis* isolate and observed that the occurrence of CRISPR-cas determinants was significantly higher in endodontic and oral *E. faecalis* than in hospital-acquired strains. It has been determined that the absence of CRISPR-cas is associated with the presence of antibiotic resistance (12,15). CRISPR-cas impedes mobile genetic elements, which may include pathogenic genes. As a result, the presence and absence of CRISPR-cas determinants might be associated with the pathogenicity of a *E. faecalis* root canal isolate strain, further contributing to periapical lesions (16,17). The aim of the present study was to investigate the possible relevance of a CRISPR-cas determinant of the *E. faecalis* root canal isolate strain to post-treatment periapical lesions. In addition, the CRISPR-cas determinant associated with biofilm formation and the resistance of the *E. faecalis* root canal isolate strain were assessed.

Materials and methods

Strain isolation. A total of 20 *E. faecalis* strains were isolated from the root canals of 20 patients who visited the Department of Operative Dentistry and Endodontics, Guanghua School of Stomatology, Sun Yat-sen University (Guangzhou, China) from January 2013 to January 2015, and these patients underwent endodontic treatment for at least one year. The teeth had a complete coronal filling according to a previous study (18). The appearance of an apical zone in these 20 teeth was revealed by radiographs and periapical lesion zones were measured using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). *E. faecalis* strains were sampled from the root canals as follows: Following rubber dam isolation, the operative field was disinfected with 2.5% NaOCl. Samples were harvested using sterile paper points and then transferred into 2xYT medium (Qingdao-Hope Bio-Technology Co., Ltd., Qingdao, China). *E. faecalis* were isolated by inoculation in KF streptococcal agar medium (Qingdao-Hope Bio-Technology Co., Ltd.) and streaking on Bile Esculin Azide agar (BEA; cat. no. 212205; BD Biosciences, Franklin Lakes, NJ, USA). Isolated strains were identified by their bile resistance, colony morphology, Gram staining characteristics and oxygen tolerance, as well as by 16s ribosomal DNA according to polymerase chain reaction (PCR) analysis. Among the 20 *E. faecalis* isolates, 12 were from teeth with imperfect root canal fillings and 8 were from teeth without a previous root canal filling. The present study was approved by the Ethics Review Committee of Institute of Stomatological Research, Sun Yat-Sen University (Guangzhou, China). All patients signed a consent form to take part in the study.

CRISPR loci identification. CRISPR1-cas and CRISPR3-cas are two common CRISPR loci of *E. faecalis* (12,15). To examine CRISPR loci, two pairs of relevant primers (Table I) were devised to detect CRISPR1 or CRISPR3 loci according to a procedure by Burley and Sedgley (15). Total DNA from the 20 *E. faecalis* isolates was extracted using an E.Z.N.A.[®] Bacterial DNA Kit (D3350; Omega Bio-Tek, Inc., Norcross, GA, USA). PCR amplification was performed using 50 μ l of each sample and Taq DNA Polymerase (ET101; Tiangen Biotech Co., Ltd., Beijing, China) according to a procedure by Burley and Sedgley (15). PCR products (20 μ l) were analyzed using agarose gel electrophoresis, purified with an Agarose Gel DNA Extraction kit (product no. 11696505001; Roche, Basel, Switzerland) and submitted for sequencing at BGI-Tech Solutions, Co., Ltd. (Shenzhen, China). The nucleotide sequences were exported by Chromas software (v2.6.1; Technelysium Pty Ltd., Brisbane, Queensland, Australia) and the online ClustalW2 multiple sequence alignment tool (www.ebi.ac.uk/Tools/msa/clustalw2) was used to perform nucleotide sequence alignments according to the National Center for Biotechnology Information GenBank database (www.ncbi.nlm.nih.gov).

Assay of antibacterial activity. A microplate dilution method was used to examine the susceptibility of the 20 *E. faecalis* isolates to three routine intracanal irrigants: 5.25% NaOCl, 2% chlorhexidine (CHX) and mixture of tetracycline isomer acid and detergent (MTAD) consisting of 3% doxycycline, 4.25% citric acid and 0.5% polysorbate 80 detergent. *E. faecalis* OG1RF was used as a control due to its intrinsic CRISPR loci. *E. faecalis* isolates and OG1RF were grown to the exponential phase at a bacterial concentration of $\sim 10^9$ colony-forming units/ml. Aliquots (1 ml) of *E. faecalis* were centrifuged at 7,610 x g at 4°C for 5 min. The supernatant was discarded and pellets were subsequently washed twice with phosphate-buffered saline (PBS) and challenged by resuspension in 5.25% NaOCl, 2% CHX or MTAD for 1 or 5 min. The survival rates were examined by a 10-fold gradient dilution and plate counts. The antibacterial assay of each strain was repeatedly performed three times on different days.

Evaluation of biofilm formation. A microtiter plate assay and crystal violet staining were used to evaluate and compare the biofilm formation of *E. faecalis* isolates. The bacteria were grown in brain heart infusion broth, to the exponential phase and were diluted 1:200 in tryptic soy broth (TSB; BD Biosciences) with 1% glucose. Subsequently, 200 μ l diluted bacterial culture was added to 96-well microtiter plates and incubated at 37°C for 24 h to allow for biofilm formation. *E. faecalis* biofilms on the bottom of the wells were evaluated by crystal violet staining following the protocol described in a previous study (19). In brief, following washing with PBS, the biofilms on the bottom of the wells were stained with 0.1% crystal violet for 15 min and subsequently washed with sterile distilled water. The bound dye was released by addition of 33% acetic acid and measured at 570 nm using a microplate reader. Each test was repeated four times in three independent experiments.

Statistical analyses. SPSS v18.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses. In the biofilm

Table I. CRISPR-Cas primers used for determining the CRISPR-cas determinant in *Enterococcus faecalis* root canal isolate strains.

CRISPR loci	Primer sequence (forward)	Primer sequence(reverse)	Amplicon length (bp)
CRISPR1-cas	ATGGGCTGGCGAACGGTAGTGGTT	TCATATCCCAAACCTCTGGAACCTCCT	867
CRISPR1-cas	GCGATGTTAGCTGATACAAC	CGAATATGCCTGTGGTGAAA	315
CRISPR3-cas	GCTGAATCTGTGAAGTTACTC	CTGTTTTGTTACCGTTGGAT	258
CRISPR3-cas	GATCACTAGGTTCAAGTTATT	CATCGATTCAATTCTCTCAA	224

CRISPR, clustered regularly interspaced short palindromic repeats.

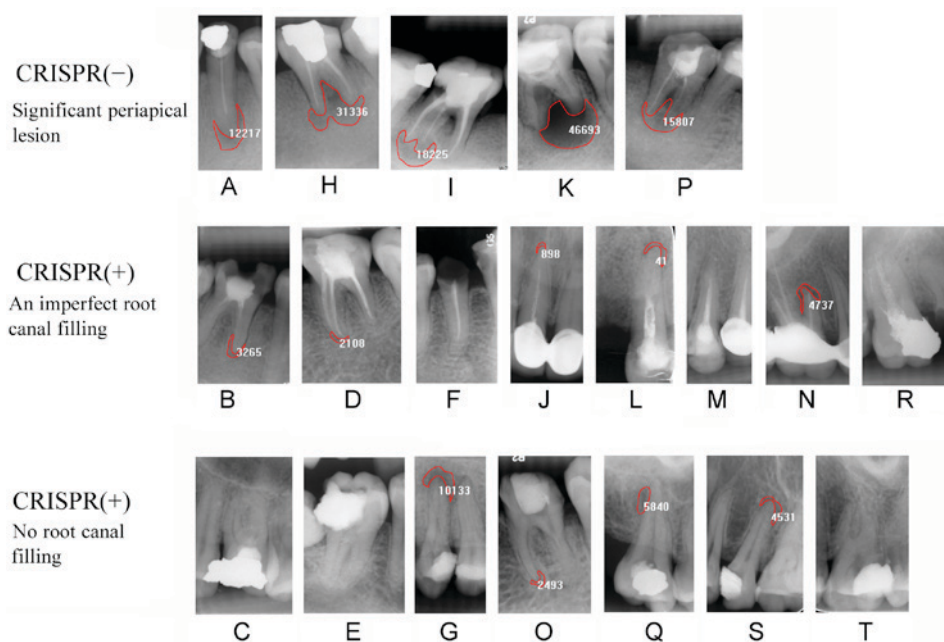


Figure 1. X-ray of periapical lesions in 20 teeth referred for root canal retreatment. The A, H, I, K, and P strains without a CRISPR-cas determinant were from teeth with significant periapical lesions, the B, D, F, J, L, M, N, and R strains in the presence of a CRISPR-cas determinant were from root canals with an inadequate filling, and the C, E, G, O, Q, S, and T strains with the presence of CRISPR-cas determinants were from root canals without a filling. CRISPR, clustered regularly interspaced short palindromic repeats.

assay, one-way analysis of variance and Tukey's honestly significant difference test were employed to compare the optical density (OD) values at 570 nm of the biofilm formation of 20 *E. faecalis* isolates and OG1RF. All values were expressed as the mean \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

X-rays of 20 teeth with endodontic treatment. Out of the 20 *E. faecalis* isolate strains, the A, H, I, K, and P strains did not contain a CRISPR-cas determinant. X-rays of the corresponding teeth revealed significant periapical lesions and the area value of periapical lesions measured using Image-Pro Plus software was $>10,000$ IOD (integrated optical density). However, the other 15 *E. faecalis* isolate strains did contain CRISPR-cas determinants. Among these 15 strains, 8 strains were isolated from teeth with an imperfect root canal filling and 7 strains were isolated from teeth without a root canal filling. A majority of the 15 teeth exhibited a slight or moderate

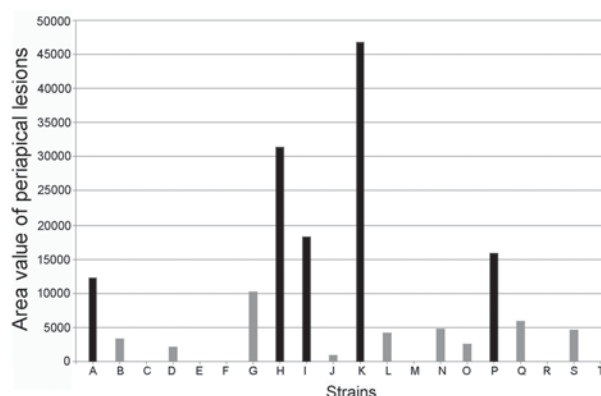


Figure 2. Periapical lesion area of 20 teeth referred for root canal retreatment. The appearance of an apical periodontitis lesion in the 20 teeth was revealed by radiographs and periapical lesion areas were measured using Image-Pro Plus software. The A, H, I, K, and P isolate strains lacking a CRISPR-cas determinant were from teeth with larger periapical lesion areas (black columns), while the other 15 isolate strains with the presence of a CRISPR-cas determinant were from teeth with smaller periapical lesions areas (gray columns). CRISPR, clustered regularly interspaced short palindromic repeats.

Table II. Survival rates (%) of 20 *E. faecalis* root canal isolate strains with or without CRISPR-cas determinants following 1- and 5-min challenges with routine intracanal irrigants.

<i>E. faecalis</i>	CRISPR	MTAD		2% CHX		5.25% NaOCl	
		1 min	5 min	1 min	5 min	1 min	5 min
A	(-)	7.20	1.00	5.20	0.32	0.012	0
B	(+)	2.50	0.12	0.80	0.05	0.0023	0
C	(+)	2.80	0.11	0.42	0.071	0.0037	0
D	(+)	0.50	0.11	0.24	0.04	0	0
E	(+)	1.20	0.21	2.60	0.12	0.0028	0
F	(+)	1.20	0.15	0.86	0.04	0.0085	0
G	(+)	1.80	0.09	0.58	0.02	0.0025	0
H	(-)	5.2	0.5	2.50	0.48	0.0008	0
I	(-)	8.0	1.1	4.20	0.36	0.0019	0
J	(+)	0.68	0.09	0.05	0.001	0.0013	0
K	(-)	6.10	0.98	4.20	0.80	0.0037	0
L	(+)	4.60	0.082	0.35	0.024	0	0
M	(+)	0.91	0.25	0.40	0.08	0.0005	0
N	(+)	3.50	0.34	1.50	0.034	0.0037	0
O	(+)	0.52	0.25	0.04	0.005	0.0025	0
P	(-)	2.90	1.15	4.80	0.52	0.0038	0
Q	(+)	0.084	0.008	2.40	0.057	0	0
R	(+)	1.50	0.16	1.20	0.15	0.0022	0
S	(+)	1.80	0.20	0.58	0.06	0.0064	0
T	(+)	0.95	0.04	0.38	0.10	0.0019	0
OG1RF	(+)	1.50	0.24	1.20	0.15	0.0028	0

CHX, chlorhexidine; MTAD, mixture of 3% doxycycline, 4.25% citric acid and 0.5% polysorbate 80 detergent; *E. faecalis*, *Enterococcus faecalis*; CRISPR, clustered regularly interspaced short palindromic repeats.

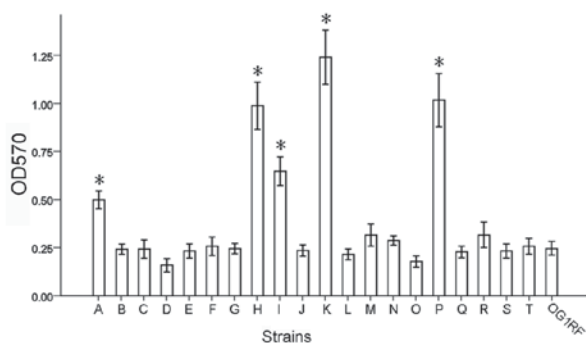


Figure 3. Biomass assay of biofilms in 20 different *E. faecalis* root canal isolate strains by crystal violet staining. The strain OG1RF was referred to as the control. * $P < 0.05$, biofilm biomass of the *E. faecalis* isolate strains vs. the control OG1RF. OD570, optical density at 570 nm.

periapical lesion with an area of $<10,000$ IOD, with the exception of strain G (Figs. 1 and 2).

Susceptibility to antibacterial irrigants. Overall, the assay of antibacterial activity indicated that the 5 strains that did not contain CRISPR-cas loci were more resistant to MTAD or 2% CHX than the 15 strains that had CRISPR-cas loci; neither group exhibited significant differences in the 5.25% NaOCl

susceptibility assay. However, among the 15 strains with CRISPR-cas loci, a few exceptional cases were encountered; for instance, the survival rates of strains L and N were 4.60 and 3.50% following a 1-min challenge with MTAD. The survival rates of strains E and Q were 2.60 and 2.40%, respectively, following a 1-min challenge with 2% CHX (Table II).

Biofilm formation ability. In the biofilm assay, the five strains (A, H, I, K and P) without CRISPR-cas loci had significantly higher OD values compared with the control OG1RF strain ($P < 0.05$; Fig. 3). Furthermore, the OD values of these strains were markedly higher compared with that of the other 15 isolate strains with CRISPR-cas determinants (Fig. 3).

Discussion

The CRISPR-cas module is a defense system against invading mobile genetic elements and is present in ~40% of bacteria and ~90% of archaea (20). The present study determined that among 20 test *E. faecalis* root canal isolates, 15 isolates presented with CRISPR-cas determinants, which was in accordance with results published by Burley and Sedgley (15) who demonstrated that 25 out of 34 endodontic isolates presented with CRISPR-cas determinants; however, these *E. faecalis* isolates were from teeth referred for primary, orthograde and

endodontic treatment. In the present study, the CRISPR-cas determinant of *E. faecalis* isolates from teeth that were referred for root canal retreatment was investigated and it was observed that teeth infested with *E. faecalis* without CRISPR-cas determinants exhibited significant periapical lesions. The absence of CRISPR-cas determinants was associated with bacteriocin activity and a clumping response to pheromones in *E. faecalis* endodontic isolates (15). A clumping response to pheromones induces the generation of an aggregation substance and may lead to conjugative horizontal gene transfer, which causes transferable high-level antibiotic resistance and exposure to virulence determinants that are carried on plasmids (21,22). Enterococci have been frequently identified in root canals with persistent periapical lesions and in teeth with insufficient root fillings (23,24). Although enterococci have generally been observed in filled root canals without radiographic lesions, they are able to cause periapical lesions (25). The present study suggested that there may be a correlation between the CRISPR-cas determinant of *E. faecalis* and the degree of periapical lesions, which, however, may be caused by a number of factors.

CRISPR-cas systems mediate immunity to invading exogenous mobile genetic elements in three distinct stages: Adaptation, expression and interference (20). CRISPR-cas systems capture invading nucleic acids from exogenous genetic elements into their CRISPR arrays and generate a memory of past invaders. When re-exposed to the specific foreign genetic element, CRISPR-cas systems target and specifically cleave homologous invasive nucleic acids in the same manner that CRISPR RNAs (crRNAs) guide Cas proteins (20,26,27). Numerous pathogenic genes of enterococci originate from exogenously acquired genes and CRISPR-cas systems limit the invasion of mobile genetic elements (12). Therefore, the five isolates with an absence of CRISPR-cas determinants identified in the present study are vulnerable to invasion by foreign pathogenic genes and may contribute to the generation of significant periapical lesions.

Palmer and Gilmore (12) and Burley and Sedgley (15) have indicated that the lack of CRISPR-cas is associated with resistance to multiple antibiotics. The present study attempted to evaluate the relevance of CRISPR-cas determinants in resistance to root canal irrigants. The antibacterial activity of MTAD depends on doxycycline, a tetracycline (28). The tetracycline resistance gene belongs to a conjugative transposon and is highly transferrable in *E. faecalis*. In the present study, the majority of the *E. faecalis* exhibited resistance to MTAD, with a 1-minute survival rate of >1%. *E. faecalis* lacking CRISPR-cas exhibited a higher resistance to MTAD. Similarly, *E. faecalis* isolates that lacked CRISPR-cas exhibited a higher resistance to a synthetic cationic disinfectant, CHX, compared with *E. faecalis* isolates that contained CRISPR-cas. Although CHX is a disinfectant, its antibacterial mechanism acts on microbial cell membranes in a similar fashion to certain antibiotics (29). However, *E. faecalis* isolates were susceptible to NaOCl regardless of whether they contained CRISPR-cas determinants. NaOCl is a bacteriolytic and antibacterial agent and the antibacterial mechanism of NaOCl is based on its high pH and the irreversible oxidation of the sulfhydryl (SH) groups of essential bacterial enzymes (30). For any enzyme, this irreversible oxidation of

SH groups may occur in bacteria and is not influenced by the CRISPR-cas determinant.

E. faecalis often grow by biofilms mode on the root canal post-treatment (31,32). In the present study, *E. faecalis* isolates with a lack of a CRISPR-cas determinant demonstrated higher biofilm formation than those containing a CRISPR-cas determinant. The CRISPR-cas determinant in bacteria defends against foreign DNA (9). *E. faecalis* without CRISPR-cas determinants may be invaded by mobile elements, including plasmids, prophages and pathogenicity islands (PAI) (12). The *E. faecalis* PAI is extensively distributed among the different strains, contains a highly variable gene, and encodes a number of pathogenicity factors, including cytolysin, aggregation substance and enterococcal surface protein, which are involved in bacterial biofilm and colonization (33). Thus, *E. faecalis* from a root canal infection may adhere to the bottom of the plates and generate a great deal of biofilm, possibly due to the presence of adhesive proteins. The *E. faecalis* PAI originates from an exogenous horizontal gene transfer and contains ≥ 100 additional genes with unknown roles that are possibly associated with the biofilm formation capacity, virulence and drugresistance of *E. faecalis* (34). Therefore, the CRISPR-cas system is able to prevent mobile gene transfers and *E. faecalis* gains more virulence factors in the absence of a CRISPR-cas determinant.

E. faecalis, a common nosocomial pathogen, has been widely studied for antibiotic selection, gastrointestinal colonization, endocarditis and nosocomial infection (11,35-37). CRISPR-Cas is an RNA-mediated adaptive immune system that is widespread in archaea and bacteria (38). However, the role of the CRISPR-cas of bacteria in periapical periodontitis has remained elusive. The results of the aforementioned studies suggested that a CRISPR-cas determinant may regulate the biofilm formation and intracanal irrigant resistance of *E. faecalis* root canal isolates, and further influence the degree of periapical lesion formation. However, it must be considered that the degree of the periapical lesion depends on multiple factors and the CRISPR-cas of an *E. faecalis* pathogenic bacterium might only be partly responsible for the periapical lesion. *E. faecalis* is a predominant pathogen in post-treatment root canal infections. A further important consideration is whether CRISPR-cas regulates horizontal pathogenic gene transfer between *E. faecalis* and other root canal pathogens, which may determine the amount of virulence factor dissemination between the root canal pathogens and further influence the development of periapical lesions.

In conclusion, CRISPR-cas may serve a role in *E. faecalis* pathogenicity and periapical periodontitis. *E. faecalis* root canal isolates lacking CRISPR-cas determinants may exhibit greater resistance to intracanal irrigants, undergo stronger biofilm formation and generate significant periapical lesions. However, further studies on additional root canal isolate strains of *E. faecalis* are required to determine the relevance of *E. faecalis* CRISPR-cas in drug-resistance, biofilms and periapical lesions.

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