Oral Microbiology Immunology 2008: 23: 449–454
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

Possible role of the adhesin ace and collagen adherence in conveying resistance to disinfectants on *Enterococcus faecalis*

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Kayaoglu G, Erten H, Ørstavik D. Possible role of the adhesin ace and collagen adherence in conveying resistance to disinfectants on Enterococcus faecalis. *Oral Microbiol Immunol 2008: 23: 449–454*. © 2008 The Authors. Journal compilation. © 2008 Blackwell Munksgaard.

Introduction: This study aimed to evaluate whether the presence of the *ace* gene and Ace-mediated binding to collagen confers on *Enterococcus faecalis* resistance against common endodontic disinfectants.
Methods: Isogenic strains of *E. faecalis*: OG1RF (wild-type) and TX5256 (*ace* insertion mutant of OG1RF) were grown in brain–heart infusion broth at 46°C overnight. Standardized bacterial suspensions were pretreated for 1 h either with acid-soluble collagen or acidified phosphate-buffered saline (ac-PBS). Bacteria were challenged with chlorhexidine digluconate (CHX), iodine potassium-iodide (IKI), sodium hypochlorite (NaOCl), and calcium hydroxide [$Ca(OH)_2$]. Samples were removed at 1, 3, and 6 h, and cultured on Todd–Hewitt agar plates. Colonies were counted, the absolute values were log transformed, and the data were statistically analyzed using Fisher's least significant differences test and *t*-test.
Results: OG1RF was more resistant than TX5256 to IKI, NaOCl, and $Ca(OH)_2$ ($P < 0.05$). Collagen-exposed OG1RF was more resistant than the ac-PBS-pretreated OG1RF against CHX at 3 h and against IKI at 1 h ($P < 0.05$); no significant difference was found against NaOCl. As expected, the *ace* mutant strain, TX5256, pretreated with collagen or ac-PBS did not differ significantly in viability when challenged with CHX, IKI, and NaOCl. An unexpected result was found for $Ca(OH)_2$: collagen-pretreated OG1RF and TX5256 were both more susceptible than ac-PBS-pretreated OG1RF and TX5256, respectively ($P < 0.05$).
Conclusion: The presence of the *ace* gene confers resistance against IKI, NaOCl, and $Ca(OH)_2$ on *E. faecalis*. Exposure to collagen makes the wild-type bacterium more resistant against CHX and IKI; however, exposure to collagen apparently decreases resistance to $Ca(OH)_2$.

Key words: adhesin; antimicrobial; disinfection; extracellular matrix protein; susceptibility

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Accepted for publication February 24, 2008

Enterococcus faecalis, a common inhabitant of the intestinal tract in humans, can cause serious clinical diseases including endocarditis, bacteremia, and urinary tract infections, and is among the major nosocomial pathogens (11, 25). *E. faecalis* is also found frequently in cultures of persistent root canal infections that are refractory to endodontic treatment, commonly as the sole species (17, 18).

Adhesion to and colonization of the host by microorganisms are the first steps in the

establishment of most infectious diseases. In endodontic infection, dentinal tubule colonization by *E. faecalis* has been shown to occur through adhesion of the bacterium to collagen, the main organic component of the dentine (9). Ace is a proteinaceous adhesin of *E. faecalis* that allows the bacterium to adhere to collagen, its function and structure closely resembles the collagen adhesin Cna of *Staphylococcus aureus*, and it possesses a trench-shaped binding site that accommodates the triple-helical collagen molecule (13, 19). Ace is an important factor in the binding of *E. faecalis* to dentine (8). It is produced by the bacterium under stressful growth conditions, such as during growth at 46°C (19). Ace can also be produced by the bacterium under physiological conditions, not just at 46°C (14), and its production can be induced by host factors such as collagen and serum (15).

In laboratory experimental conditions, *E. faecalis* can be eliminated rapidly using disinfectants (2, 3). However, when dentine is included in the experimental design, it cannot be eliminated easily (4, 16). This can at least partly be attributed to interactions between dentine and the disinfectant (4), and it is also possible that an interaction between dentine and *E. faecalis* might render the bacterium resistant. However, there is scarce knowledge as to how *E. faecalis* survives disinfectants and predominates in persistent endodontic infections.

Adhesins produced on the bacterial cell surface have been found to exert protective effects against antibiotics and antimicrobial polypeptides (5, 23). The adherence phenomenon for some microorganisms is also associated with resistance against antimicrobials and adverse conditions (1, 10). Accordingly, it can be speculated also for *E. faecalis* that its adhesins and/or adherence confers on the bacterium resistance to antimicrobials.

This study sought to assess whether the presence of the *ace* gene and Ace-mediated binding to collagen conferred on *E. faecalis* resistance to common endodontic disinfectants.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this experiment were *E. faecalis* OG1RF (TX4002), which is the wild-type, Ace-producing strain (12, 13), and TX5256, an *ace* disruption mutant derivative of OG1RF that does not produce Ace (13). The strains were kindly provided by Dr B.E. Murray. The bacteria were maintained on brain–heart infusion agar plates at 4°C. The agar plate for the TX5256 strain also included kanamycin (2000 μg/ml). For the experiments, a few colonies from the plates were inoculated and grown in brain–heart infusion broth at 46°C for 12 h. The cells were washed twice (8000 ***g***, 5 min) and resuspended in phosphate-buffered saline (PBS, pH 7.4). The bacterial density was standardized to yield comparable baseline bacterial numbers [10^7 colony-forming units (CFU)/ml].

Disinfectants

The disinfectants tested in this experiment were: calcium hydroxide [$Ca(OH)_2$, Merck, Darmstadt, Germany] solution, chlorhexidine digluconate (CHX; 20% solution; Sigma Chemical Co., St Louis, MO), sodium hypochlorite (NaOCl; 1% solution) and iodine-potassium iodide (IKI; 2% solution). NaOCl and IKI were obtained from a pharmacist on prescription. The $Ca(OH)_2$ solution was prepared by dissolving an excess of $Ca(OH)_2$ powder in distilled water overnight, and filtering the saturated solution through a 0.22-μm pore size filter (Millipore, Bedford, MA).

Experimental groups and pretreatment conditions

Acid-soluble collagen type I (Sigma) was dissolved in 0.2 N acetic acid (50 mg/50 ml). This solution was further diluted in PBS (1 : 4, volume/volume). To group 1 (OG1RF) and group 2 (TX5256) (each at a 100 μl volume of a 10^7 CFU/ml bacterial concentration) was added 25 μl of the collagen solution. The resultant concentration of collagen in the mixture was 50 μg/ml and the pH of the mixture was 4.9. To group 3 (OG1RF) and group 4 (TX5256) (each at a 100 μl volume of a 10^7 CFU/ml bacterial concentration) was added 25 μl PBS, the pH of which was adjusted using acetic acid to make it similar to the pH of the collagen solution (the PBS with acetic acid added is cited hereafter as ac-PBS). Pretreatment was carried out for 1 h at room temperature.

Challenge conditions and testing of disinfectant susceptibility

At the end of pretreatment, 25 μl ac-PBS was added to groups 1 and 2, and 25 μl collagen was added to groups 3 and 4, with the purpose of making the collagen concentration identical in all groups. Fifty microliters was immediately removed by pipette from each group to measure the baseline bacterial count (it was found to be 1×10^7 CFU/ml in all groups). Then, 100 μl disinfectant was added to each group. The tubes were incubated at room temperature. Fifty microliters was removed from each tube after 1, 3, and 6 h, serially diluted, and plated on Todd–Hewitt agar plates. The agar plates contained no kanamycin. The CFUs were counted after incubation at 37°C for 2 days. Absolute bacterial numbers were converted to $\log_{10}$ values and the percentage reduction according to the baseline viable count was calculated for each sample. The experiment was performed with three parallels and in triplicate on different days. Using $\alpha = 0.05$ as the level for statistical significance, the data obtained were analyzed using Levene's test for the equality of variances and *t*-test for differences between the reduction rates of the groups. Group 1 was compared with group 3, and group 2 was compared with group 4 to assess the effect of binding/exposure to collagen on disinfectant susceptibility. For the assessment of the effect of the presence of Ace for disinfectant susceptibility (OG1RF versus TX5256), data from groups 1 and 3 (OG1RF cells), and groups 2 and 4 (TX5256 cells) were pooled and killing curves were prepared. The slopes were compared statistically using Fisher's least significant difference test at $P = 0.05$.

Disinfectant dosage

The dosage of each disinfectant that reduced the CFU of the bacteria at 3 h by 90–99% (with reference to absolute baseline bacterial numbers) was used in the experiments. This dosage was determined before the experiment in a pilot experiment with group 3 bacteria, according to the protocol described above. Ten- and twofold serial dilutions (in distilled water) of each disinfectant were tested to find the appropriate dosage. According to the pilot experiments, the appropriate dosage for each disinfectant was: CHX, 0.005%; IKI, 0.0001%; NaOCl, 0.0002%; and $Ca(OH)_2$, full strength.

Adherence assay

The function of Ace on the cell surface of the wild-type bacterial strain under the growth conditions used here was confirmed by adherence assay. The assay was performed as described previously but with minor modifications (6). Briefly,

96-well microtiter plates (Greiner, Frickenhausen, Germany) were coated with collagen type I (50 μg/ml PBS) overnight at 4°C. Wells were blocked with bovine serum albumin (Sigma; 100 μg/ml PBS). Bacteria (OG1RF and TX5256) were grown at 46°C, centrifuged twice, washed with PBS, and standardized to a concentration of 10^7 CFU/ml. The bacteria were pretreated for 1 h with either collagen or ac-PBS at the same concentrations to which they were exposed during the main experiment. The bacterial suspensions at a volume of 200 μl were then added to the collagen-coated wells and incubated for 2 h. Non-adherent bacteria were removed by washing them three times with PBS. The remaining bacteria were stained with crystal violet (1%). The stain captured by the cells was solubilized by addition of ethanol : acetone (4 : 1). The intensity of the stain was measured spectrophotometrically as the optical density at 560 nm. The bacterial adherence was quantified according to known standards.

The effects on OG1RF of pretreatment with anti-Ace A immunoglobulin Gs (IgGs) and preimmune IgGs were also tested as described above. Sera were provided by Dr B.E. Murray; their preparation has been described elsewhere in detail (13). Briefly, a 1008-base-pair DNA fragment coding for the complete Ace A domain was amplified from OG1RF and cloned into a pBAD/HisA vector followed by electroporation into *Escherichia coli*. The recombinant Ace A domain was overexpressed in Luria–Bertani broth and the recombinant protein was eluted and purified. After verifying a single reacting band of His-tagged recombinant Ace A on a Western blot with anti-His antibodies, this protein was used to raise polyclonal antibodies by immunization of rabbits (13). IgGs from the serum of immune or preimmune rabbits were purified by chromatography using Protein A–Sepharose (Amersham Biosciences, Piscataway, NJ). The final concentration of IgGs in each group was 3 μg/ml. The assay was performed with at least two parallels and in triplicate.

Results

Adherence assay

Wild-type OG1RF pretreated with ac-PBS adhered at relatively high numbers to collagen type I. The results of the adherence assay are given in Fig. 1. Pretreatment of OG1RF with either collagen or anti-Ace IgGs significantly reduced binding to collagen-coated surfaces. Consistent with an earlier study (13), pretreatment with preimmune IgGs had no effect on the adherence of OG1RF to collagen. For TX5256, binding to collagen surfaces was similar and at relatively low levels whether the cells were pretreated with ac-PBS or collagen. These results confirmed that OG1RF, but not TX5256, specifically produced the collagen adhesin Ace and that the solubilized collagen in the pretreatment medium was bound by the bacterial adhesin at a significant rate.

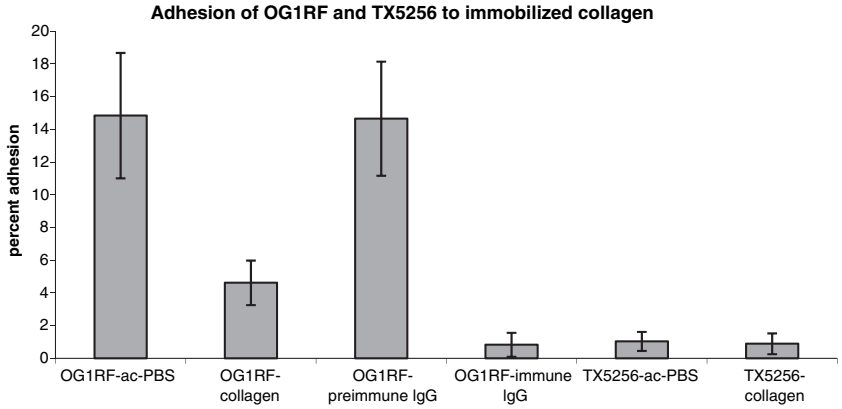

Fig. 1. Results of the adherence assay with OG1RF and TX5256. Mean values of bacterial adhesion to collagen-coated wells after pretreatment with acidified (ac)-PBS, collagen, preimmune or immune (anti-Ace A) IgGs. Bars represent standard deviation for three experiments. At least two wells were used for each group per experiment.

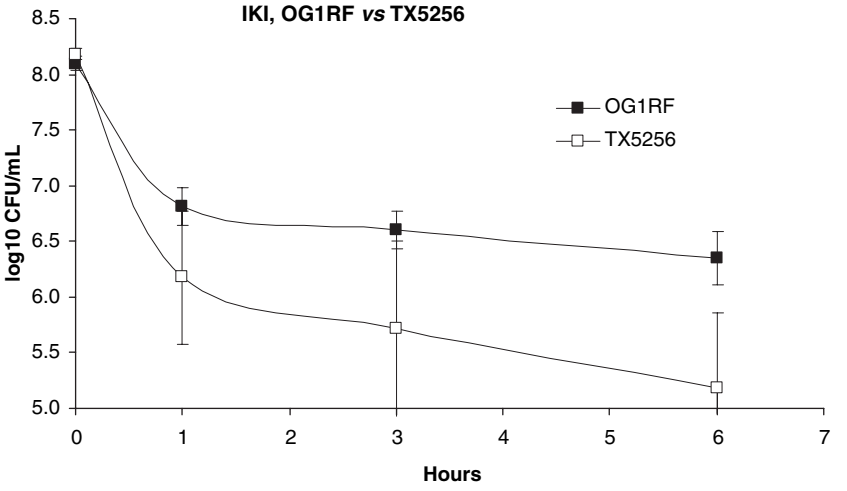

Fig. 2. Killing curves for the Ace+ (OG1RF) and the Ace− (TX5256) strains after challenge with IKI. Bars represent standard deviation for three experiments ($n = 6$ for each value).

Effect of the *ace* gene on disinfectant susceptibility

The Ace-producing strain (OG1RF) was more resistant than the Ace-deficient strain (TX5256) against IKI, NaOCl, and $Ca(OH)_2$ (Figs 2–4, $P < 0.05$). No significant difference was found between the strains against CHX (Fig. 5, $P > 0.05$).

Effect of cell–collagen binding on disinfectant susceptibility

Collagen-bound OG1RF was more resistant than the ac-PBS-pretreated OG1RF against CHX at 3 h, and against IKI at 1 h ($P < 0.05$) as shown in Table 1. No significant difference was found between the collagen-pretreated and ac-PBS-pretreated OG1RF against CHX and IKI for other observation periods. No significant difference was found between the collagen-pretreated and ac-PBS-pretreated OG1RF against NaOCl. As expected, TX5256 pretreated with collagen or ac-PBS did not differ significantly in viability when challenged against CHX, IKI, or NaOCl. Unexpected results were found for $Ca(OH)_2$: collagen-pretreated OG1RF and TX5256 were both more susceptible than ac-PBS-pretreated OG1RF and TX5256, respectively, at all observation periods ($P < 0.05$).

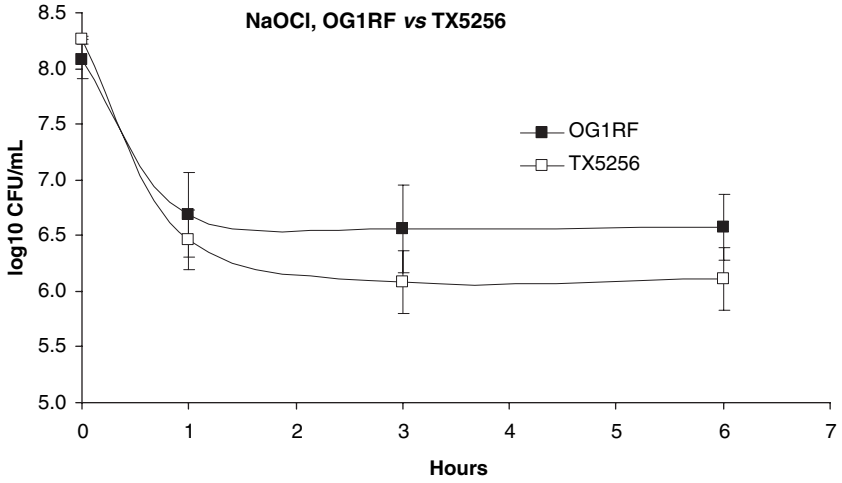

Fig. 3. Killing curves for the Ace+ (OG1RF) and the Ace− (TX5256) strains after challenge with NaOCl. Bars represent standard deviation for three experiments (n = 6 for each value).

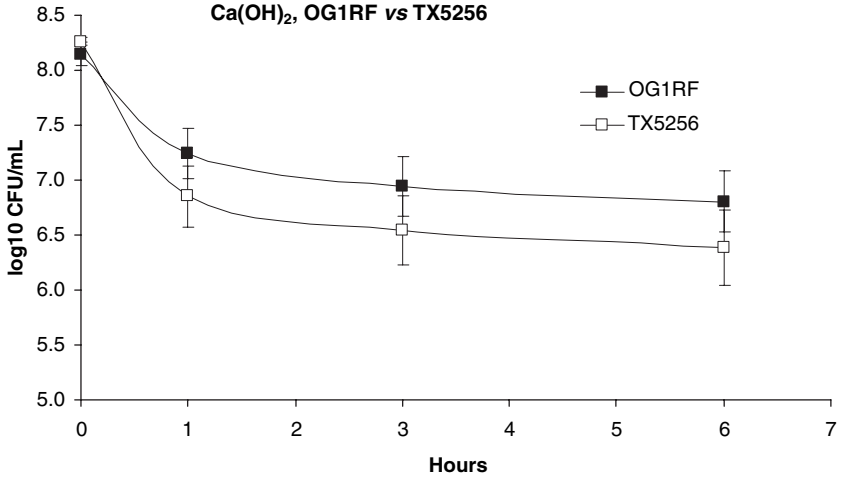

Fig. 4. Killing curves for the Ace+ (OG1RF) and the Ace− (TX5256) strains after challenge with $Ca(OH)_2$. Bars represent standard deviation for three experiments (n = 6 for each value).

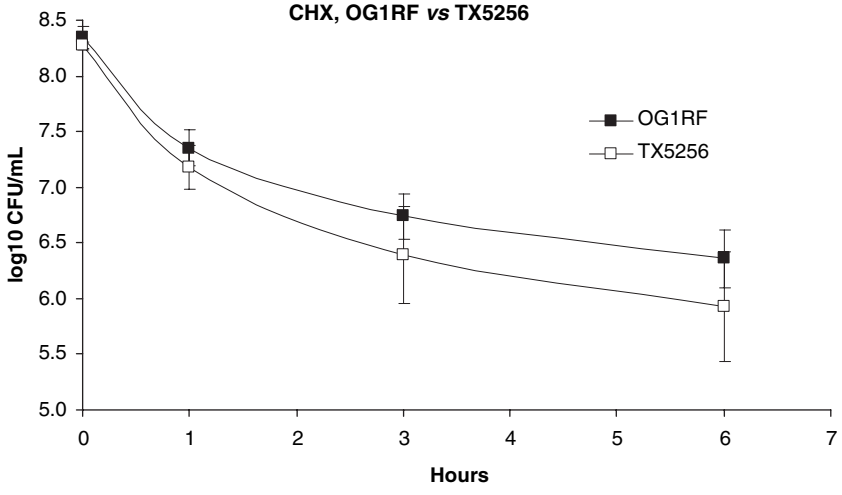

Fig. 5. Killing curves for the Ace+ (OG1RF) and the Ace− (TX5256) strains after challenge with CHX. Bars represent standard deviation for three experiments (n = 6 for each value).

Discussion

The main finding in this study was that the presence of the *ace* gene and also the Ace-mediated binding to collagen conferred on *E. faecalis* resistance to some of the endodontic disinfectants. Unexpectedly, and in contrast with the general pattern of the experimental results, collagen-exposed OG1RF and TX5256 were both more susceptible to $Ca(OH)_2$.

Previous studies have suggested that the presence of an adhesin on the bacterial surface could relate to resistance against antimicrobials. These studies found, for example, that the *Yersinia enterocolitica* adhesin YadA conferred on the bacterium resistance against granulocyte granule extracts (23) and the *E. coli* adhesin Dr conferred resistance against ampicillin (5). In line with these findings, the presence of the *ace* gene, which is responsible for the production of the adhesin Ace, conferred on *E. faecalis* resistance against IKI, NaOCl, and $Ca(OH)_2$. There was a tendency for resistance also against CHX; however, this was not statistically significant. It is possible that the proteinaceous Ace material in OG1RF could have limited the diffusion of the disinfectants into the cytoplasm or buffered the disinfectants and rendered them less effective. Change in the bacterial cell surface's hydrophobicity or electric charge after adhesin expression could also have affected the interaction between the bacterium and the disinfectant to the favor of bacterial survival. However, it should be noted that the mutant strain TX5256 was generated by a chromosomal insertion and the possibility that the inserted plasmid may have had a polar effect on downstream genes that are related to bacterial resistance cannot be disregarded.

This study found that *E. faecalis* that adhered to collagen was more resistant against IKI and CHX at different periods. One study that has investigated the effect of adhesion by *Pseudomonas aeruginosa* to collagen membrane also found significant resistance of the adhering bacteria against an aminoglycoside (22). However, the authors believed that this effect was the result of biofilm formation on the collagen membrane surface, a well-recognized mechanism of antimicrobial resistance. Another study showed that cells of *Candida albicans* gained immediate resistance against an antifungal agent upon adherence to silicone surfaces (10). This resistance did not require biofilm formation, but was associated with upregulation of efflux pump genes that function to reduce the

Table 1. Percent reduction (vs. time = 0) of the log10 *CFU/ml* values of collagen-pretreated and ac-PBS-pretreated strains after challenge with the disinfectants.

	Group 1 OG1RF + collagen	Group 3 OG1RF + ac-PBS	Group 2 TX5256 + collagen	Group 4 TX5256 + ac-PBS
CHX				
1 h	**10,64** (1,34)	**12,99** (2,06)	**12,65** (1,21)	**13,98** (3,06)
3 h	**16,56** (1,14)	**21,82** (2,18)*	**20,63** (2,15)	**24,95** (6,92)
6 h	**21,73** (2,04)	**25,80** (3,71)	**25,97** (3,28)	**30,97** (7,40)
IKI				
1 h	**13,90** (0,71)	**17,83** (2,22)*	**23,16** (6,80)	**26,02** (8,05)
3 h	**17,62** (1,55)	**19,27** (2,53)	**28,35** (8,13)	**32,15** (11,70)
6 h	**21,11** (3,03)	**22,09** (2,99)	**32,45** (3,42)	**41,05** (9,67)
NaOCl				
1 h	**17,11** (3,15)	**17,71** (3,81)	**21,30** (3,35)	**22,15** (3,86)
3 h	**19,57** (3,51)	**18,23** (3,29)	**27,41** (3,24)	**25,32** (3,76)
6 h	**19,82** (2,72)	**17,62** (0,98)	**26,80** (4,19)	**25,29** (2,86)
$Ca(OH)_2$				
1 h	**12,66** (1,73)	**9,60** (0,54)*	**19,87** (0,56)	**14,32** (1,87)*
3 h	**16,79** (1,24)	**12,81** (1,08)*	**23,82** (1,80)	**17,86** (1,78)*
6 h	**18,56** (0,16)	**14,46** (1,77)*	**25,94** (1,65)	**19,38** (2,14)*

Mean and standard deviation (numbers in parentheses) for three experiments (n = 3 for each value). Group 1 to be compared with Group 3; Group 2 to be compared with Group 4. Statistically significant difference has been indicated with*.

intracellular drug accumulation (10). In another study, *E. coli* adhering to red blood cells produced stress proteins, such as RpoE, GroEL, and GroES, that enable the bacteria to survive under adverse conditions (1). It was discussed that contact between bacteria and the eukaryotic cell membrane induces specific changes in bacterial gene expression that would be beneficial for its survival in the new environment (1). This may apply also to *E. faecalis*: cells of *E. faecalis* encountering extracellular matrix proteins (e.g. collagen, laminin, fibrinogen) as the elements to be exploited for the colonization of the host may modify its gene expression for protection and adaptation to the stressful condition. Woody et al. (24) argued that it would take a longer period to cause rupture of the bacterial cytoplasmic membrane in cases where the bacterium is associated with collagen; they found, however, no significant difference in the rate of destruction of *E. coli* on collagen-coated glass coverslips compared with *E. coli* placed on uncoated glass coverslips when challenged against high pH (24).

Surprisingly, the collagen-exposed OG1RF and TX5256 were more susceptible to $Ca(OH)_2$. The isoelectric point (pI) of type I collagen is about 5.5 (26) and at a pH below the pI, proteins carry a net positive charge. Therefore, the net charge of type I collagen in the pretreatment medium of pH 4.9 is assumed to be positive. Once $Ca(OH)_2$ was added, it is possible that under these conditions the collagen-associated bacteria could have electrically attracted the hydroxyl ions (OH^-) dissociating from $Ca(OH)_2$ and been eliminated more readily. The antimicrobial effect of $Ca(OH)_2$ is considered to be mainly the result of the OH^- ions. Hydroxyl ions are highly oxidant free radicals that show extreme reactivity, reacting with several biomolecules. The lethal effects of OH^- ions are probably through damage to the bacterial cytoplasmic membrane by a mechanism called lipid peroxidation, damage to the DNA, and protein denaturation (20). Besides OH^- ions, Ca^{2+} ions could also have some effect. It was shown that *E. faecalis* could calcify in a calcium-enriched medium, forming intra- and extracellular mineral deposits (21). Another study showed that soluble type I collagen deposited on a mesenchymal cell layer induced calcium accumulation of the cultured cells and further participated in the mineralizing matrix (7). In the study presented here, collagen-associated *E. faecalis* similarly could have calcified in the presence of Ca^{2+} ions and probably lost the ability to grow in culture. The OG1RF strain fits both explanations (elimination via OH^- or Ca^{2+} ions) because it specifically binds to collagen through Ace. However, for TX5256, it might be that the bacterial surfaces were covered by collagen, but without specific adhesin–ligand binding. Then, both strains gave similar results when challenged against $Ca(OH)_2$.

This study suggests that the presence of the *ace* gene confers resistance to *E. faecalis* against IKI, NaOCl, and $Ca(OH)_2$. Adherence to collagen makes the bacterium more resistant against CHX and IKI; however, exposure to collagen apparently decreases the resistance to $Ca(OH)_2$. These findings are important in explaining the persistence of *E. faecalis* despite endodontic treatment where these disinfectants are commonly used. More studies are needed, particularly on the effect of collagen-adherence by *E. faecalis* on resistance against $Ca(OH)_2$.

Acknowledgments

This study was supported in part by the Research Council of Norway. The authors would like to thank Dr Murat Atan for performing the statistical analysis.

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