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Streptococcus gordonii* collagen-binding domain protein CbdA may enhance bacterial survival in instrumented root canals *ex vivo

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Abstract

Introduction—The surface-associated collagen-binding protein Ace of *Enterococcus faecalis* has been implicated as a virulence factor that contributes to bacterial persistence in endodontic infections. The purpose of this study was to determine if proteins with amino acid sequence similarity to Ace found in more abundant oral streptococci could play a similar role in potentially enhancing endodontic infections.

Methods—A *Streptococcus gordonii* gene similar to *ace* was identified by genome sequence searches *in silico*. An isogenic derivative of strain DL1 with a disruption in the identified gene was constructed by allelic replacement. Parent and mutant strains were characterized for their ability to bind immobilized collagen type-1 in a microtiter plate binding assay. Survival of the strains in a human tooth *ex vivo* instrumented root canal model was compared by inoculating canals with parental or mutant bacteria and determining the CFUs recovered at various time points over a 12-day period.

Results—The *S. gordonii* gene, encoding a protein with a conserved collagen-binding domain similar to that of Ace, was designated *cbdA*. The *cbdA*-deficient cells were less able to bind collagen type-1 than parental cells ($P < 0.0001$). Genetic complementation of the *cbdA*-deficient strain restored the collagen-binding phenotype. By day 12 significantly fewer ($P = 0.03$) *cbdA*-deficient than parental CFUs were recovered from instrumented canals.

Conclusion—A gene encoding a putative collagen-binding protein was identified in *S. gordonii*. Fewer *S. gordonii* *cbdA*-deficient cells survived *ex vivo* compared with parental cells, suggesting that collagen-binding proteins may contribute to persistence of oral streptococci in instrumented root canals.

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Introduction

Studies of virulence factors that contribute to the persistence of bacteria within endodontic infections have often focused on *Enterococcus faecalis*, with less emphasis on the more abundant oral streptococci. These closely-related gram positive cocci may share pathogenic determinants that enhance their survival and persistence in endodontic infections (1). The *E. faecalis* surface protein designated adhesin of collagen from enterococci (Ace) has been implicated as a virulence factor in endodontic disease because it promotes the ability of the bacteria to bind to collagen and dentin (2, 3, 4). Collagen binding proteins similar to Ace in structure and function are found in many gram positive cocci including staphylococci and pyogenic streptococci (5, 6) and share a conserved collagen binding domain (CBD) held on the bacterial surface in a functional conformation by a stem domain (7).

The human oral commensal microorganism *Streptococcus gordonii* is a significant component of dental plaque (8) and found in primary and persistent endodontic infections (reviewed in 9). Proposed endodontic virulence factors in this species include surface proteins SspA and SspB, which have been shown to bind to collagen and have been implicated in bacterial survival in dentin tubules (10). However, disruption of *sspA* and *sspB* does not completely abrogate the ability of *S. gordonii* cells to bind collagen (11), nor do the SspA and SspB proteins share the conserved CBD and stem domains found in Ace and similar proteins. The possibility was therefore investigated that collagen-binding surface proteins similar to Ace may exist in *S. gordonii* and may share similar potential pathogenic properties. An *in silico* approach was used to search the genome sequence of *S. gordonii* and a gene encoding a protein with an amino acid sequence similarity to Ace was identified. Data supporting a role for this streptococcal determinant in mediating collagen binding and bacterial survival in instrumented root canals *ex vivo* are presented.

Materials and Methods

Bacteria strains and growth medium

The *Streptococcus gordonii* parental strain DL1 (Challis) and its isogenic derivatives strain UB1360 (deletion of DNA encoding both SspA and SspB; 12) and strain BN1386 (deletion of DNA within *S. gordonii* genome locus SGO_1650, designated the collagen binding domain gene, *cbdA*; this study) were grown in Brain Heart Infusion (BHI) or Todd Hewitt (TH) (Becton Dickinson and Co. Sparks, MD) medium. *E. coli* strain DH5 α (Life Technologies, Carlsbad, CA), used for cloning plasmids carrying DNA used for *S. gordonii* allelic exchange, was grown in Luria-Bertani medium incubated with aeration at 37°C. Antibiotics were added to the medium when needed for selection at the following concentrations: spectinomycin (50 μ g/ml for *E. coli* and 250 μ g/ml for *S. gordonii*) and erythromycin (5 μ g/ml).

Molecular methods

DNA cloning, PCR amplification, plasmid purification, and Southern blotting were performed using standard molecular methods (13). Modifications for *S. gordonii* included using cells made competent with heat-inactivated serum for transformation (14) and using mutanolysin and lysozyme to facilitate bacterial cell lysis for DNA extractions. Oligonucleotide primers (Life Technologies) used for constructing strains, plasmids and verification of 16S rRNA sequences of recovered bacteria are shown in Table 1. DNA sequencing reactions were determined by cycle sequencing using BigDye Terminator primers on an ABI 3730xl sequencer (Life Technologies). Sequences were edited and assembled using AssemblyLIGN MacVector V7 software (MacVector Inc. Cary, NC).

Computer analysis to identify *S. gordonii* CBD proteins

An *in silico* examination of the *S. gordonii* genome sequence (GenBank accession number CP000725) used the NCBI Blast algorithm (15) to identify proteins similar to *E. faecalis* Ace. Online protein structural prediction programs (<http://www.ebi.ac.uk/swissprot/>) were used to identify conserved domains within identified proteins including the CBD (pFam05737) and CnaB-type stalk (pFam05738) (16) found in the collagen binding family of proteins, and potential signal and gram-positive anchor sequences that would be predictive of cell surface proteins.

Construction of *S. gordonii* strain DL1 derivative with a deletion in a CBD protein with Ace similarity

A DNA fragment was constructed in *E. coli* using the cloning plasmid pGem7 that carries the *aad9* gene for spectinomycin resistance (17). PCR amplicons from strain DL1 chromosomal template were sequentially cloned upstream and downstream of *aad9*. Primers *Xho*I1386usF and *Eco*RI1386usR (Table 1) were used to amplify the region upstream of the *cbdA* nucleotides encoding the CBD; the amplicon was cloned upstream of *aad9*. Similarly, primers *Hind*III1386dsF and *Bam*HI1386dsR were used to amplify the region downstream of the *cbdA* nucleotides that encode the stalk domain; this amplicon was cloned downstream of *aad9*. This resulted in a cloned 1.9-kb fragment in which nucleotides number 514–1542 of *cbdA*, which encoded the conserved CBD and CnaB-type stalk domains, were replaced with a 1.1-kb gene encoding spectinomycin resistance. The cloned fragment was released from the plasmid by digestion with *Xho*I and *Bam*HI, electrophoresed through agarose gel, and purified with Qiaex II beads (Qiagen, Valencia, CA) according to the manufacturer's directions. The linear fragment was transformed into serum-competent strain DL1 cells and transformants were selected on agar plates containing spectinomycin. After incubation at 36 °C for 48 hours to allow allelic exchange, isolates were picked and their chromosomal DNA was prepared for Southern hybridization analysis (15) and direct nucleotide sequencing of chromosomal amplicons. The transformant strain selected for study had the desired chromosomal sequence in which 1027 nucleotides of *cbdA* was replaced by the 1158-bp *aad9* gene; this isogenic mutant derivative of strain DL1 was designated BN1386.

Complementation of the *cbdA* mutation

A 1910-bp fragment encoding an internal fragment of the *cbdA* gene that excluded the encoded CbdA signal sequence and cell wall anchor was amplified by PCR using DL1 chromosomal DNA and the primers *cbdA*SalIF and *cbdA*BamHIR (Table 1). The amplicon was cloned into the *Sal*I and *Bam*HI sites of the replicative expression plasmid pUB1000 (12). The nucleotide sequence fidelity of the resulting plasmid, which carried an in-frame chimeric *cbdA* gene, was confirmed to ensure correct DNA coding. The purified plasmid was designated pUB1000:cbdA and transformed into strains DL1 and BN1386.

Binding of *S. gordonii* strains to immobilized collagen

Commercially available microtiter plates pre-coated with type-1 collagen (Thermo Fisher Scientific, Waltham MA) were blocked with phosphate buffered saline (PBS; pH 7.4) containing 0.2% v/v Bovine Serum Albumin (BSA) and washed three times with PBS. Late-log phase bacterial cultures grown in BHI were collected by centrifugation ($1000 \times g$ for 10 min), washed twice in PBS and resuspended in PBS-Tween 80 (0.1% v/v)-BSA (0.1% w/v) to an OD₆₀₀ of 0.5. Two hundred microliter aliquots of the cell suspension were added to each well and incubated at room temperature for 2 hours with gentle shaking at 50 rpm. Unbound cells were removed by washing twice with PBS. The remaining attached cells were fixed with methanol for 30 min, air dried and bound cells were stained with crystal violet solution (0.01% w/v crystal violet, 0.5% v/v isopropanol, 0.5% v/v ethanol) for 15

min at room temperature. Wells were then washed twice with PBS, destained with ethanol-acetone solution (4:1), and the retained crystal violet as an indicator of bound cells was determined by measurement of absorbance at 570 nm in a microtiter plate reader with internal statistical software (Beckman Coulter Model AD340, Brea CA). Assays were done in triplicate and experiments were repeated at least twice. Values were expressed as average absorbance plus or minus standard deviation. Statistical significance ($P < 0.05$) of differences between strains was compared using a Student's *t*-test.

Ex vivo model for bacterial survival

The University Human Subjects Institutional Review Board (HSIRB) approved this study and the use of the extracted teeth in a modified *ex vivo* model (18). Twenty noncarious, intact single-rooted teeth were extracted from de-identified patients under age 25. Teeth were rinsed with PBS, placed in a solution of 50% glycerine/ 50% ethanol (v/v) autoclaved and stored in 100% humidity. Teeth were aseptically decoronated and instrumented to Pro Taper size F3 apical file (Tulsa Dentsply, Tulsa OK) using 6% NaOCl irrigant followed by 17% EDTA to remove the smear layer. Canals were then washed with sterile distilled water, sealed apically with tetric evoceram composite resin (Ivoclar Vivadent AG, FL-9494 Schaan/Liechtenstein), copiously washed with sterile distilled water and re-autoclaved. Preliminary experiments had confirmed that direct contact with the composite resin had no effect on growth of any of the *S. gordonii* strains. Mid-log phase bacterial cells of strain DL1 or BN1386 grown in TH broth medium without antibiotic were concentrated by centrifugation and resuspended in PBS. Aliquots of 15 μ l ($\sim 2 \times 10^9$ CFUs) were inoculated into the instrumented canals. Access openings were closed with sterile cotton and teeth were placed apices downwards in a 100% humidity foil-sealed container and incubated aerobically at 36 °C for up to 12 days. At each time point, teeth were removed, aseptically crushed with sterile metal forceps into fine particles, resuspended in TH medium, sonicated to release and disrupt bacterial chains, and the recovered viable bacterial CFUs were determined by dilution and plating on agar medium. After 48 hours incubation, viable CFUs were counted and expressed as average plus or minus standard deviations. Groups were compared for statistical significance using a Student's *t*-test with SPSS software (IBM, New York, NY). Recovered colonies were examined for uniform morphology and alpha hemolysis on Columbia blood agar (Becton Dickinson and Co). To verify the bacteria identities at the molecular level, chromosomal DNA was prepared from randomly selected isolates ($n=10$ for each group) and PCR was done with a 56 °C annealing temperature and primers 8F and 1391R (Table 1; 19), designed to amplify species-specific regions of the 16S rRNA genes. DNA sequences of the resulting amplicons were compared to that of the inoculant strains.

Results

***In silico* analysis of the *S. gordonii* *cbdA* locus**

Examination of the *S. gordonii* genome sequence identified locus SGO_1386, a 2085-bp open reading frame encoding a protein of 694 amino acids with a predicted processed molecular mass of 76.5 kDa and a 56% overall similarity to the Ace protein of *E. faecalis*. The gene was designated *cbdA* because it encodes a protein with a collagen binding domain sequence. The CbdA protein has a predicted signal sequence at the amino terminus to facilitate protein export and an LPXTG motif followed by a hydrophobic domain at the carboxyl terminus suggesting that CbdA is anchored to the *S. gordonii* cell surface. The predicted CBD spans amino acids 189–310 and is similar to the conserved region found in proteins comprising pFam PF50737 and a conserved stalk region (amino acids 340–410) is similar to the pFam PF0578 domain that is thought to function by holding the CBD in a functional conformation on the bacterial cell surface. Since sequence similarity does not

assure functional activity, the phenotype conferred by the newly-identified protein was examined.

CbdA-deficient *S. gordonii* cells are less able to bind to immobilized type I collagen

Both strain BN1386, and the previously characterized, negative control strain UB1360, in which the tandem genes encoding collagen-binding proteins SspA and SspB are deleted, showed significantly less ability to bind to collagen coated microtiter plates than parental strain DL1 cells ($P < 0.0001$ for both strains; Figure 1). Strain BN1386 showed significantly less ability to bind to the plates than control strain UB1360 ($P = 0.003$). The complementation of the chromosomal mutation in strain BN1386 by a plasmid borne copy of *cbdA* increased the ability of BN1386/pUB1000:*cbdA* to bind to collagen to near parental levels ($P = 0.097$).

Strain BN1386 is less able than the parental strain to survive in instrumented canals *ex vivo*

Strains DL1 and BN1386 had similar doubling times when grown in TH medium at 36 °C indicating the strains had comparable planktonic growth curves under rich medium conditions (data not shown). Viable counts confirmed that similar numbers ($\sim 2 \times 10^9$ cells) of parental and mutant cells of strains DL1 and BN1386 were present in the preparative cultures and inoculated into teeth (Figure 2). On day 3, similar bacterial numbers were recovered from teeth inoculated with either strain DL1 or BN1386 ($1.6 \times 10^5 \pm 8.5 \times 10^4$ DL1 CFU/tooth compared with $1.9 \times 10^5 \pm 6.4 \times 10^3$ BN1386 CFU/tooth, $P = 0.35$). At each subsequent time point, the number of recovered strain BN1386 CFUs decreased consistently compared to strain DL1 CFUs (Figure 2). By day 12, ca. 10-fold fewer bacteria were recovered from teeth inoculated with strain BN1386 than from strain DL1-inoculated controls ($1.0 \times 10^4 \pm 1.0 \times 10^4$ CFUs compared with $1.1 \times 10^5 \pm 2.4 \times 10^4$ CFUs, respectively; $P < 0.03$ Student's *t*-test). All recovered bacteria had uniform colony morphologies on agar plates. Nucleotide sequences of all amplicons from recovered strains were 100% identical to the expected *S. gordonii* 16S rRNA gene sequence.

Discussion

S. gordonii is among the most commonly recovered oral streptococci from endodontic infections (1). Computer-assisted genome searches identified a gene in *S. gordonii* that encodes a putative cell surface collagen binding protein, CbdA with amino acid sequence similarity to the *E. faecalis* collagen binding protein, Ace (2). Ace has been implicated as an enterococcal virulence factor in endodontic infections due to its ability to bind collagen and dentin (3, 4). The role of the newly-identified related streptococcal protein was investigated using a genetic approach. An isogenic mutant derivative of the parental *S. gordonii* strain DL1 was constructed in which regions of *cbdA* were deleted so that the cells could not express a functional CbdA protein. The resulting derivative strain, BN1386, was less able to bind to collagen type I in an *in vitro* assay than was strain DL1, indicating that the loss of collagen binding ability was due to the *cbdA* mutation. When DNA encoding the deleted domains of *cbdA* carried on a streptococcal expression plasmid was transformed back into the mutant strain, the ability of the genetically complemented bacterial cells to bind to collagen was restored. These results suggest that CbdA confers an ability to bind type I collagen to the streptococcal cells.

The ability of a bacterium to attach to a surface is a first step in colonization. Since type I collagen is the primary component of human dentin (20), the ability to bind to collagen has been suggested to allow binding to dentin and promote the establishment of microcolonies within the tubules and/or the formation of biofilms on canal walls (10). We therefore

hypothesized that the ability to bind to collagen might confer a survival advantage to bacterial cells within the environment of an instrumented root canal. Accordingly, an *ex vivo* tooth model (18) was modified and used to compare the abilities of strain DL1 and its *cbdA*-deficient derivative, strain BN1386, to survive in instrumented human root canals. Although it has some limitations, this model has been used to elucidate roles of specific bacterial virulence factors using isogenic mutants of *E. faecalis* by measuring their survival within this limited nutrient environment. Model systems such as this are used for initial characterization of putative virulence factors so that individual variables can be selectively controlled and interpreted in order to dissect the roles of individual components in the multi-step infectious process; this preliminary characterization is generally done before proceeding to the use of animal models. In this study the *ex vivo* tooth model was used to begin to clarify the role of a putative virulence determinant by examining isogenic strains of *S. gordonii*. This species was used as a representative of the mitis group of oral streptococci which are generally more abundant in the oral cavity and in endodontic infections than enterococci (8, 9). The use of a single species inoculum in the absence of confounding variables such as serum or necrotic tissue which might affect the bacterial metabolism or adhesive processes provided a focused insight into the role a specific bacterial factor in the attachment and survival processes. After twelve days of incubation within an instrumented canal, significantly fewer *cbdA*-negative cells survived, suggesting that the absence of CbDA impaired the ability of the cells to interact with dentin components and survive in the *ex vivo* environment. Survival assays were not done with the complemented strain BN1386/pUB1000:cbdA since the maintenance of the plasmid within the cell requires growth in the presence of erythromycin. The addition of antibiotic pressure would add an additional selective factor that would confound interpretation of the results.

Although the streptococcal survival experiment was not conducted over a long time period, the trend was apparent. In a clinical setting, the situation is, of course, much more complex. Leakage products or tissue remnants may enhance nutrition availability and the presence of multiple bacterial species can influence metabolic interactions of the microbial community present in the canals. It is possible that the ability to bind collagen could allow for the formation of adhesin-receptor interactions that facilitate formation of microcolony aggregates or biofilms (21). Indeed, oral streptococci are routinely recovered from recalcitrant cases (9), and clinical studies indicate that *S. gordonii* are recovered associated with root ends rather than within the periradicular tissues of persistent periradicular lesions (22), suggesting that this tooth structure-association may provide a preferred niche for these bacteria in the endodontic infection process. It is possible that the ability to bind collagen has conferred an advantage to bacteria that can colonize dentin surfaces. It has also been suggested that the ability of enterococci to bind to collagen via Ace may protect the bacteria from effects of antiseptics commonly used during endodontic treatment (23, 24); it is possible that similar collagen binding proteins, which are being detected by sequence similarity in the genomes of many streptococcal species as more genome sequence data become available, could play a similar role. Additional studies examining the survival of CBD-deficient cells in the presence of host factors such as serum, which might affect binding (25) or provide nutrition (21, 26) in an *ex vivo* model, as well as extension of these studies to include multispecies bacterial communities in more complex model systems, would further clarify a potentially pathogenic role of these commonly occurring (27, 28) streptococcal collagen binding proteins. Understanding roles of bacterial adhesins and colonization factors, particularly those that are shared by many species of oral bacteria, may provide insights into a long-term goal of designing more effective treatment strategies for endodontic infections.

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References

1. Chávez de Paz LE, Svensäter G, Dahlen G, Bergenholtz G. Streptococci from root canals in teeth with apical periodontitis receiving endodontic treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2005; 100:232–41. [PubMed: 16037782]
2. Rich RL, Kreikemeyer B, Owens RT, LaBrenz S, Narayana SVL, Weinstock GM, Murray BE, Höök M. Ace is a collagen binding MSCRAMM from *Enterococcus faecalis*. *J Biol Chem.* 1999; 274:26939–45. [PubMed: 10480905]
3. Hubble TS, Hatton JF, Nallapareddy SR, Murray BE, Gillespie MJ. Influence of *Enterococcus faecalis* proteases and the collagen binding protein Ace, on adhesion to dentin. *Oral Microbiol. Immunol.* 2003; 18:121–6.
4. Kowalski WJ, Kasper EL, Hatton JF, Murray BE, Nallapareddy SR, Gillespie MJ. *Enterococcus faecalis* adhesin, Ace, mediates attachment to particulate dentin. *J Endod.* 2006; 32:634–7. [PubMed: 16793469]
5. Xu Y, Rivas JM, Brown EL, Liang X, Hook M. Virulence potential of the staphylococcal adhesin CNA in experimental arthritis is determined by its affinity for collagen. *J Infect Dis.* 2004; 189:2323–33. [PubMed: 15181582]
6. Kreikemeyer B, Nakata M, Oehmcke S, Gschwendtner C, Normann J, Podbielski A. *Streptococcus pyogenes* collagen type I-binding Cpa surface protein. *J Biol Chem.* 2005; 280:33228–39. [PubMed: 16040603]
7. Symersky J, Patti JM, Carson M, House-Pompeo K, Teale M, Moore D, Jin L, Schneider A, DeLucas LJ, Hook M, Narayana SV. Structure of the collagen-binding domain from a *Staphylococcus aureus* adhesin. *Nat Biol.* 1997; 4:833–8.
8. Frandsen EVG, Pedrazzoli V, Kilian M. Ecology of viridans streptococci in the oral cavity and pharynx. *Oral Microbiol Immunol.* 1991; 6:129–33. [PubMed: 1945494]
9. Siqueira JF Jr, Rôças IN. Diversity of endodontic microbiota revisited. *J Dent Res.* 2009; 88:969–81. [PubMed: 19828883]
10. Love RM, Jenkinson HF. Invasion of dentinal tubules by oral bacteria. *Crit Rev Oral Biol Med.* 2002; 13:171–83. [PubMed: 12097359]
11. Love RM, McMillan MD, Jenkinson HF. Invasion of dentinal tubules by oral streptococci is associated with collagen recognition mediated by the antigen I/II family of polypeptides. *Infect Immun.* 1997; 65:5157–64. [PubMed: 9393810]
12. Heddle C, Nobbs AH, Jakubovics NS, Gal M, Mansell JP, Dymock D, Jenkinson HF. Host collagen signal induces antigen I/II adhesin and invasin gene expression in oral *Streptococcus gordonii*. *Mol Microbiol.* 2003; 50:597–607. [PubMed: 14617182]
13. Ausubel, FM.; Brent, B.; Kingston, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K. Current protocols in molecular biology. New York: John Wiley & Sons, Inc; 1987.
14. Lawson J, Gooder H. Growth and development of competence in the group H streptococci. *J Bacteriol.* 1970; 102:820–5. [PubMed: 5464272]
15. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol.* 1990; 215:403–10. [PubMed: 2231712]
16. Bateman A, Birney E, Durbin R, Eddy SR, Howe KL, Sonnhammer EL. The pFam protein families database. *Nucleic Acids Research.* 2000; 28:263–6. [PubMed: 10592242]
17. Leblanc DJ, Lee LN, Inamine JM. Cloning and nucleotide base sequence analysis of a spectinomycin adenylyltransferase *aad9* determinant from *Enterococcus faecalis*. *Antimicrob Agents Chemother.* 1991; 35:1804–10. [PubMed: 1659306]
18. Sedgley CM, Lennan SL, Appelbe OK. Survival of *Enterococcus faecalis* in root canals *ex vivo*. *Int Endod J.* 2005; 38:735–42. [PubMed: 16164688]

19. Baker GC, Smith JJ, Cowan DA. Review and re-analysis of domain-specific 16S primers. *J Microbiol Methods*. 2003; 55:541–55. [PubMed: 14607398]
20. Mjor IA. Human coronal dentine: structure and reactions. *Oral Surg Oral Med Oral Pathol Oral Radio Endod*. 1972; 33:810–23.
21. Svensater G, Bergenholtz G. Biofilms in endodontic infections. *Endodontic Topics*. 2004; 9:27–36.
22. Subramanian K, Mickel AK. Molecular analysis of persistent periradicular lesions and root ends reveals a diverse microbial profile. *J Endod*. 2009; 35:950–7. [PubMed: 19567313]
23. 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; 23:449–54. [PubMed: 18954349]
24. Kayaoglu G, Erten H, Bodrumlu E, Ørstavik D. The resistance of collagen-associated, planktonic cells of *Enterococcus faecalis* to calcium hydroxide. *J Endod*. 2009; 35:46–9. [PubMed: 19084123]
25. Chávez de Paz LE, Bergenholtz G, Svensäter G. The effects of antimicrobials on endodontic biofilm bacteria. *J Endod*. 2010; 36:70–7. [PubMed: 20003938]
26. Chávez de Paz LE. Redefining the Persistent Infection in Root Canals: Possible role of biofilm communities. *J Endod*. 2007; 33:652–62. [PubMed: 17509401]
27. Xu P, Alves JM, Kitten T, Brown A, Chen Z, Ozaki LS, Manque P, Ge X, Serrano MG, Puiu D, Hendricks S, Wang Y, Chaplin MD, Akan D, Paik S, Peterson DL, Macrina FL, Buck GA. Genome of the opportunistic pathogen *Streptococcus sanguinis*. *J Bacteriol*. 2007; 189:3166–75. [PubMed: 17277061]
28. Ajdic D, McShan WM, McLaughlin RE, Savic G, Chang J, Carson MB, Primeaux C, Tian R, Kenton S, Jia H, Lin S, Qian Y, Li S, Zhu H, Najjar F, Lai H, White J, Roe BA, Ferretti JJ. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proc Natl Acad Sci USA*. 2002; 99:14434–9. [PubMed: 12397186]

Figure 1.

Stained streptococcal cells bound to immobilized collagen type 1. Error bars indicate $\pm$ S.D.

*Differs from parental strain $P < 0.0001$; differ from each other $P = 0.003$.

Figure 2.

Average CFUs of each strain recovered from inoculated teeth at each time point.

*Differs from parental strain $P < 0.03$.

TABLE 1

Oligonucleotide Primers for PCR.

Use	Primer name	Sequence ^a
Construction of strain SG1386	XhoI1386usF	5' T <u>ACTCGAG</u> ATGTCATTACTCGGATGTATC3'
	EcoRI1386usR	5' TAGA <u>ATTCC</u> AGTGAAGTTAATCTTACCTGCTG3'
	HindIII1386dsF	5' ATA <u>AAGCTT</u> GGTGGCTAAAGTTAGCTATGAC3'
	BamHI1386dsR	5' AT <u>GGA</u> TCCCTGGTAACTGTTGGTAAAGCTATC3'
Complementation	cbdAFSall	5' ACGCGT <u>CGACA</u> ACAAGGTCTTTGCTACAGAACATG3'
	cbdAR BamHI	5' ACGCGGATCCTTCTTGCTGTTCTTGCTTGATGGAG3'
Verification of 16S rRNA genes	8F	5' AGAGTTTGATCATGGCTCAGGACGA3'
	1391R	5' ACGGGCGGTGTGTACA3'

^aEngineered restriction sites for cloning are underlined