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 colony-forming units (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 < .0001). Genetic complementation of the cbdA-deficient strain restored the collagen-binding phenotype. By day 12, significantly fewer (P = .03) cbdA-deficient than parental CFUs were recovered from instrumented canals. Conclusions: 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 the persistence of oral streptococci in instrumented root canals. (J Endod 2013;39:39–43)

Key Words
Bacteria, collagen, endodontics, microbiology, Streptococcus gordonii

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 (2–4). 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 is found in primary and persistent endodontic infections (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, the 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. Therefore, the possibility was 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 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

Bacterial Strains and Growth Media

The S. 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 SGD_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. *Escherichia coli* strain DH5α (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 μg/mL for *E. coli* and 250 μg/mL for *S. gordonii*, and erythromycin, 5 μg/mL.

**Molecular Methods**

DNA cloning, polymerase chain reaction (PCR) amplification, plasmid purification, and Southern blotting were performed using standard molecular methods (15). 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) were used for constructing strains and plasmids and the verification of 16S ribosomal RNA (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 National Center for Biotechnology Information BLAST algorithm (15) to identify proteins similar to *E. faecalis* Ace. Online protein structural prediction programs ([http://www.ebi.ac.uk/swissprot/](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 the strain DL1 chromosomal template were sequentially cloned upstream and downstream of *aad9*. Primers *XboI*386usF and *EcoRI*386usR (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 *HindIII*386dsF and *BamHI*386dsR 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 nucleotide numbers 514 to 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 *XhoI* and *BamHI* and electrophoresed through agarose gel, and purified with Qiaex II beads (Qia gen, 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 1,027 nucleotides of *cbda* were replaced by the 1158-bp *aad9* gene; this isogenic mutant derivative of strain DL1 was designated BN1386.

**Complementation of the *cbda* Mutation**

A 1,910-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 *cbdaSalF* and *cbdaBamHI* (Table 1). The amplicon was cloned into the *SalI* and *BamHI* 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 precoated with type I collagen (Thermo Fisher Scientific, Waltham, MA) were blocked with phosphate-buffered saline (PBS, pH = 7.4) containing 0.2% w/v bovine serum albumin and washed three times with PBS. Late–log phase bacterial cultures grown in BHI were collected by centrifugation (1,000g for 10 minutes), washed twice in PBS, and resuspended in PBS-Tween 80 (Bio-Rad Laboratories, Hercules, CA) (0.1% v/v) bovine serum albumin (0.1% w/v) to an optical density at 600 nm (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 minutes and air dried. Bound cells were stained with a crystal violet solution (0.01% w/v crystal violet, 0.5% w/v isopropanol, and 0.5% v/v ethanol) for 15 minutes at room temperature. Wells were then washed twice with PBS and destained with ethanol-acetone solution (4:1). The retained crystal violet as an indicator of bound cells was determined by the measurement of absorbance at 570 nm in a microtiter plate reader with internal statistical software (Model AD340; Beckman Coulter, Brea, CA). Assays were performed in triplicate, and experiments were repeated at least twice. Values were expressed as the average absorbance plus or minus standard deviation. The statistical significance (*P* < .05) of differences between strains was compared using the Student’s *t* test.

**Table 1. Oligonucleotide Primers for PCR**

<table>
<thead>
<tr>
<th>Use</th>
<th>Primer name</th>
<th>Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Construction of strain BN1386</td>
<td><em>XhoI</em>386usF</td>
<td>5’TACCTGAGATGTCATTACTGTGATGGATAC3’</td>
</tr>
<tr>
<td></td>
<td><em>EcoRI</em>386usR</td>
<td>5’TGAATTCATGAAAGTACTTTCTACTCTGGT3’</td>
</tr>
<tr>
<td></td>
<td><em>HindIII</em>386dsF</td>
<td>5’TAAAGGTGTCGCTAGCTAGCAG3’</td>
</tr>
<tr>
<td></td>
<td><em>BamHI</em>386dsR</td>
<td>5’TGATGATGTCGCTAGCTAGCAG3’</td>
</tr>
<tr>
<td>Complementation</td>
<td><em>cbdASalF</em></td>
<td>5’ACCGGTGACACAAAGCTGTTTCTAGACAGATGG3’</td>
</tr>
<tr>
<td>Verification of 16S rRNA genes</td>
<td><em>cbdABamHI</em></td>
<td>5’ACGGGTGACACAAAGCTGTTTCTAGACAGATGG3’</td>
</tr>
<tr>
<td></td>
<td>8F</td>
<td>5’ACGGGTGACACAAAGCTGTTTCTAGACAGATGG3’</td>
</tr>
<tr>
<td></td>
<td>1391R</td>
<td>5’ACGGGTGACACAAAGCTGTTTCTAGACAGATGG3’</td>
</tr>
</tbody>
</table>

*Engineered restriction sites for cloning are underlined.
**Ex Vivo Model for Bacterial Survival**

The University Human Subjects Institutional Review Board 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 deidentified patients under the age of 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 ProTaper 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, Schaan, Liechtenstein), copiously washed with sterile distilled water, and reautoclaved. Preliminary experiments had confirmed that direct contact with the composite resin had no effect on the 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 μL (≈2 × 10^9 colony-forming units [CFUs]) were inoculated into the instrumented canals. Access openings were closed with sterile cotton, and teeth were placed apices downward 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 of incubation, CFUs were counted and expressed as average plus or minus standard deviations. Groups were compared for statistical significance using the Student’s t test with SPSS software (SPSS Inc, 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 performed 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 with 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_1650, a 2,085-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 CBD 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 to 310 and is similar to the conserved region found in proteins comprising Pfam PF05737. A conserved stalk region (amino acids 340-410) is similar to the Pfam PF05738 domain that is thought to function by holding the CBD in a functional conformation on the bacterial cell surface. Because sequence similarity does not ensure 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**

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, Fig. 1). Strain BN1386 showed significantly less ability to bind to the plates than control strain UB1360 (P = .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 = .097).

**Figure 1.** Stained streptococcal cells bound to immobilized collagen type 1. Error bars indicate ± standard deviation. *Differs from parental strain, P < .0001; differ from each other, P = .003.
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 (~2 × 10^9 cells) of parental and mutant cells of strains DL1 and BN1386 were present in the preparative cultures and inoculated into teeth (Fig. 2). On day 3, similar bacterial numbers were recovered from teeth inoculated with either strain DL1 or BN1386 (1.6 × 10^5 ± 8.5 × 10^4 DL1 CFU/tooth compared with 1.9 × 10^5 ± 6.4 × 10^4 BN1386 CFU/tooth, P = .35). At each subsequent time point, the number of recovered strain BN1386 CFUs decreased consistently compared with strain DL1 CFUs (Fig. 2). By day 12, approximately 10-fold fewer bacteria were recovered from teeth inoculated with strain BN1386 than from strain DL1-inoculated controls (1.0 × 10^4 ± 1.0 × 10^3 CFUs compared with 1.1 × 10^5 ± 2.4 × 10^4 CFUs, respectively; P < .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 similar to the *E. faecalis* collagen-binding protein Ace (2). Ace has been implicated as an enterococcal virulence factor in endodontic infections because of 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 a region of *cbdA* was 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. Because type 1 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). Therefore, we 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 the 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 multistep 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 that might affect the bacterial metabolism or adhesive processes provided a focused insight into the role of a specific bacterial factor in the attachment and survival processes. After 12 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 performed with the complemented strain BN1386/pUB1000:cbdA because 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 collagen-bridged adhesion-receptor interactions that facilitate the formation of micropeloid 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 the 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.

Acknowledgments

The authors deny any conflicts of interest related to this study.

References