Streptococcus gordonii Collagen-binding Domain Protein CbdA May Enhance Bacterial Survival in Instrumented Root Canals *Ex Vivo*

Peter J. Moses, DDS, Daniel A. Power, PhD,*† Amy M. Jesionowski, BS,*† Howard F. Jenkinson, PhD,‡ Eugene A. Pantera, Jr, DDS, MS,* and M. Margaret Vickerman, DMD, PhD*†*

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 SGO_1650, designated the collagen-binding domain gene, *cbdA* [this study]) were grown in brain-heart infusion

*Departments of Periodontics and Endodontics and †Oral Biology, School of Dental Medicine, University at Buffalo, State University of New York, Buffalo, New York; and ‡School of Oral and Dental Sciences, University of Bristol, Lower Maudlin Street, Bristol, United Kingdom.

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Address requests for reprints to Dr M. Margaret Vickerman, University at Buffalo School of Dental, Medicine, 223 Foster Hall, Buffalo, New York, NY 14214. E-mail address: mmv4@buffalo.edu

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(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 (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 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/) 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 *Xho*I1386usF and *EcoR*I1386usR (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 *Hin*dIII1386dsF 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 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 *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 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 *cbdASal*IF and *cbdABam*HIR (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 precoated with type 1 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 (1,000*g* 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 (OD_{600}) 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% v/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

Use	Primer name	Sequence*
Construction of strain BN1386	*Xho*I1386usF	5′TACTCGAGATGTCATTACTCGGATGTATC3′
	*EcoR*11386usR	5′TAGAATTCCAGTGAAGTTAATCTTACCTGCTG3′
	*Hin*dIII1386dsF	5′ATAAGCTTGGTGGCTAAAGTTAGCTATGAC3′
	*Bam*HI1386dsR	5′ATGGATCCTGGTAACTGTTGGTAAAGCTATC3′
Complementation	*cbdASal*IF	5′ACGCGTCGACAACAAGGTCTTTGCTACAGAACATG3′
	*cbdABam*HIR	5′ACGCGGATCCTTCTTGCTGTTCTTGCTTGATGGAG3′
Verification of 16S rRNA genes	8F	5′AGAGTTTGATCATGGCTCAGGACGA3′
	1391R	5′ACGGGCGGTGTGTACA3′

*Engineered restriction sites for cloning are underlined.