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Cloning and Analysis of Putative Collagenases of the U32 Family in *Streptococcus mutans* and *Streptococcus agalactiae* (Group B Streptococci)

By

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
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Cloning and Analysis of Putative Collagenases of the U32 Family in *Streptococcus mutans* and *Streptococcus agalactiae* (Group B Streptococci)

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ABSTRACT

Analysis of the genomic sequences of *Streptococcus mutans* UA159 and Group B Streptococcus (GBS) strains *Streptococcus agalactiae* NEM316 and *S. agalactiae* 2603V/R indicated the presence of two putative collagenase genes in each organism. *smcol1* from *S. mutans* was previously cloned and analyzed and the results indicated that the enzyme belonged to the U32 family of collagenases/peptidases. This enzyme shares homology with the *prtC* of *Porphyromonas gingivalis,* one of the principal examples of the U32 family of peptidases. Considering the potential role of these enzymes in the pathogenicity of *P. gingivalis* (periodontitis or gum disease), GBS (premature rupture of the amniochorionic membrane) and *S. mutans* (dental root decay), it is necessary to study these enzymes and establish their role in the virulence of these organisms. Toward this goal the present study has focused on cloning collagenase 2 (*smcol2*) from *S. mutans* and cloning collagenase 1 (*gbscol1),* and collagenase 2 (*gbscol2),* from GBS. The information obtained will contribute to a further understanding of the U32 peptidase family.

INTRODUCTION

Collagen is a major structural protein in our bodies, making up a large percentage of human tissue, from the skin to the tendons, eyes, bones, amniotic membrane and teeth. In the mouth, collagen is one of the main components of gingival connective tissue and it is found in the matrix of alveolar bone and cementum, dentin of the tooth and in the basement membrane beneath the gingival epithelium (37). Periodontal tissue is made up primarily of type I collagen, which is made up of three parallel polypeptide chains composed of the sequence Gly-X-Y, with X representing proline and Y representing hydroxyproline. These amino acids give collagen stability and restrict the rotation of the polypeptide backbone (10). Collagen is extremely resistant to degradation because of its tightly coiled triple helix structure that is stabilized by hydrogen bonds and cross-linking and can only be cleaved by collagenases (20).

Until recently it was thought that only a few species of bacteria produced collagenases, namely *Clostridium* and *Vibrio alginolyticus*, but numerous other human pathogens have also been reported to have the ability to break down collagen (17). The specificity of bacterial collagenases is very broad and, unlike vertebrate collagenases that are more specific in their cleavage sites (33), bacterial collagenases are capable of hydrolyzing denatured as well as native collagen. Gelatin is produced when collagen loses its triple helix structure and

becomes denatured. Numerous mammalian proteases are able to hydrolyze gelatin, including pepsin, trypsin and papain. Hydrolysis of collagen may help in bacterial infection and spreading, hence collagenase has been considered as a virulence factor and the focus of numerous studies.

The most widely studied collagenase is a metalloprotease produced by *Clostridium histolyticum* (32) that requires a zinc molecule to retain its hydrolytic activity (47). This zinc-metalloprotease is unique in that it is able to cleave denatured and native collagen (32). Subsequently, *C. histolyticum* has been found to produce different types of collagenases, which are separated into two classes based on their amino acid sequences and peptide substrate requirements (31). *C. histolyticum colH* is a 116 kDa collagenase that co-purifies with a 98 kDa protein, which cleaves denatured collagen, but not native collagen (47). Because they share identical N-terminal sequences and peptide maps, it is believed that the 98 kDa gelatinase is produced by the cleavage of the C-terminal end of the 116 kDa collagenase (32). *C. histolyticum* also produces another collagenase, ColG. It was found that *colG* and *colH* are less than 760 kb apart on *C. histolyticum's* genome. It is hypothesized that the presence of these similar clostridial collagenase genes is due to gene duplication and later divergence (31).

Periodontitis is a collection of diseases involving the destruction of structural proteins in the oral cavity (37) . Periodontal diseases, which are characterized by the destruction of collagen, vary in severity depending on the stage of development, age of the patient and reaction to treatment. Degradation of

gingival connective tissue, the supporting structure of the tooth, including type I collagen, leads to periodontal lesions. Numerous studies have shown that in regions of periodontal tissue degradation, extensive collagenase activity is evident (38). Collagenase activity by bacteria of the oral cavity were first discovered in the 1960's (17). Hence, an understanding of how bacteria interact with collagen of the periodontium is a necessary tool in the understanding of their pathogenecity.

A common bacterium isolated from infected individuals suffering from advanced periodontitis is *Porphyromonas gingivalis* (27). This organism has collagenases, one of which is encoded by *prtC.* The prtC enzyme was found to be able to break down soluble type I collagen (38), as well as fibrillar collagen; however it could not degrade the synthetic collagenase substrate, PZ-PLGPA, gelatin nor denatured type I collagen (27). The ability of *P. gingivalis* to cleave native type I collagen was eliminated by the inactivation of one of the two genes encoding Arg-gingipain A or B (20), suggesting that the collagenase activity of *P. gingivalis* requires the action of both enzymes (27). It was also found that the activity of these two enzymes is dependent on their association with the bacterial cell wall, given that purified enzymes showed no activity (20). Indeed, prtC was classified in the U32 family of peptidases/collagenases based on the presence of a consensus sequence.

One of the most widespread and expensive infectious diseases in the world is dental caries (11), of which *S. mutans* is the primary etiological agent. It has been found that if oral streptococci are inhibited on the root surface, the

development of dental caries is greatly diminished (40). Dental caries results from the acidic end products produced by the metabolism of fermentable carbohydrates in the diet. This drastically decreases the pH in the oral cavity which leads to the dissolution of the tooth enamel and root surface (45). Dental caries is a public health problem world-wide, hence extensive research effort has focused on developing means to prevent this infectious disease. Kassab et al, determined that approximately 24 million people in the United States have tooth surfaces that have 3 millimeters or greater of gingival recession and that the occurrence of gingival recession increased with age and was higher in men compared to women of the same age (26). Fluoridation of water and dental hygiene showed limited success. With current medical research, the average life span has greatly increased, but along with this, the occurrence of dental root decay has also increased.

Dentin, unlike the tooth crown, is made up of organic components and hydroxyapatite, an inorganic material (10). The organic component of dentin is composed of about 90% type I collagen, citrate, lipids and non-collagenous proteins. Root caries begin by exposure of the root surface to the oral environment via recession of the gum and subsequently the exposed dentin becomes vulnerable to microbial infection, which can lead to loss of the tooth (10). Gingival recession, which is defined as the dislocation of the gingival tissue and exposure of the root, can be localized or generalized (26). It has recently been shown that greater than 50% of the general population had one or more locations with gingival recession of 1 millimeter or greater. Many factors are

associated with gingival recession, including age, lack of alveolar bone, the abnormal tooth position, and vigorous tooth brushing (26). The main factors involved in the progression of root caries is the invasion of bacteria into the dentin and fermentable carbohydrates derived from the host's diet (5).

Development of root and coronal caries differs greatly. Root caries involve the decomposition of dentin minerals while coronal caries involves enamel demineralization. Root surface caries seem to be more complex in their treatment and pathology as compared to coronal caries although both types of caries involve acidic demineralization (10). The rate at which coronal and root caries proceeds also differs. The destruction of dentin transpires about twice as quickly as the demineralization of the enamel, due to the fact that the crown is composed of almost double the amount of minerals (5).

One study showed that the microflora that colonized isolated dentin specimens from patients were composed of a diverse community of bacteria (41). Numerous microorganisms have been isolated from root caries lesions including, *Actinomyces* spp, *Streptococcus* spp, and *Lactobacillus* spp (3). It has been found that *Actinomyces* and *Streptococcus* where the dominant species isolated from root surface lesions (41). *S. mutans* is routinely found in root caries plaque samples and has been shown to be one of the major players in root caries disease (3). It has also been found that the inhibition of streptococci at the root surface leads to the decline of root caries (42). *S. mutans* produces a number of proteins that are associated with its cell wall and have been implicated as virulence factors, hence they are the focus of research regarding *S. mutans*

and dental caries (15, 40). Antibodies against these antigens could possibly thwart the development of dental caries by *S. mutans*. The main objective for an anti-dental caries vaccine would be to prevent *S. mutans* from attaching and adhering to oral tissues, which could lead to the prevention of tooth decay on the surface and root of the tooth. With the increasing technology in recombinant DNA techniques, research has focused on identifying and isolating genes involved in the pathogenicity of *S. mutans*. Numerous genes that are involved in coronal caries, such as polymer-forming glucosyltransferases, fructosyl-transferases, and wall associated protein A have been cloned and sequenced (12, 45). In studies where cell wall fractions were exposed to proteases, animals that were immunized with the suspension were not protected against dental caries (13). Indeed, the identification of these cell-surface proteins as potential immunogens against dental caries should be further investigated.

Degradation of collagen in tissues of the dento-epithelial seam leads to the development of a region that has a redox potential level that is lower than that of the surrounding tissue (17). This environment promotes the colonization of anaerobic organisms which can lead to periodontal disease. Infection with *S. mutans* has been shown to degrade the periodontal ligament, instigate the massive loss of bone, while the production of collagenase activity in this organism was substantiated by its ability to hydrolyze collagen fibrils in rat tail tendons (17, 18). *S. mutans* has been found in human root surface carious lesions and is able to bind collagen type I and II (22). *S. mutans* has been shown to possess two extracellular proteases that can hydrolyze PZ-PLGPA and

breakdown type I collagen (18). It is speculated that these enzymes may be a factor in the degradation of collagen of the dentin and cementum of the oral cavity (17). Switalski *et al.* (42) found that *S. mutans* was able to bind collagen in dentin and that it may have a profound effect on the development of root surface caries. It was also demonstrated that *S. mutans* strain GS-5 was capable of breaking down alveolar bone and collagen of the periodontal ligament (18). Jackson *et al*. demonstrated cell-associated collagenase activities in *S. mutans* (22). It was found that *S. mutans* was able to bind collagen and that cell lysate from *S. mutans* cross-reacted with antiserum to collagenase from *C. histolyticum* (22). These characteristics taken together lend to the fact that *S. mutans* plays a considerable role in the pathogenesis of dentinal caries. These virulent factors may aid *S. mutans* in maintaining its environmental niche in the oral cavity and contribute to its ability to cause host tissue damage.

A great deal of focus has been directed on producing a vaccine against coronal caries (16, 24), but this will have no effect on the colonization of dental root by *S. mutans* and subsequent destruction of the root dentine. While coronal caries involves surface adhesins with binding affinity for salivary pellicles and glucan-binding proteins (42), dental root decay is mediated by collagen-binding protein and collagen degrading enzymes. More research is needed in order to identify and characterize the factors involved in dental root caries in order to develop specific prophylactic measures.

Streptococcus agalactiae, also referred to as Group B streptococci (GBS), is the leading cause of severe neonatal bacterial infections, including pneumonia,

sepsis and meningitis. In the United States, about 10,000 instances of GBS infections occur with a 15% mortality ratio (28, 36), being responsible for two to three cases per 1000 live births (14). GBS can be found living asymptomatically in the vaginal epithelium and the lower gastrointestinal tract of healthy adults. GBS has a competitive advantage over other microflora in the vaginal epithelium since it is able to attach to the epithelium and survive in the low pH environment. It is estimated that 10-40% of women who are pregnant are infected with GBS and that 40-70% of these women transmit the bacterium to their child (7). Human newborns contract GBS when they pass through the birth canal or swallow infected amniotic fluid from their mother (30). GBS does not only infect neonates and pregnant women, it also affects people with chronic conditions and the elderly. The incidence of invasive GBS infections has steadily increased in recent years for the immunocompromised and the elderly to numbers similar to the incidence of the newborn population (30). Most newborn that become infected with GBS do not develop disease, but the range of virulence factors attributed to GBS can lead to infection when the infant's immune system fails. These virulence factors include its ability to hinder the newborns defensive system, factors that allow the organism to infect the bloodstream and deep tissue by its ability to invade the epithelial and endothelial barriers, the production of toxins, and mechanisms that allow inflammatory reactions in the host (30). When an infant is born prematurely, the likelihood that the infected newborn will become symptomatic is greatly increased. Generally, 1-3% of infected babies develop early-onset disease (sepsis and meningitis) within the first 24 hours after

birth (7). It has been suggested that vaginal infection or inflammation is linked to preterm rupture of the amniotic membrane and preterm labor (44). Labor before 37 weeks gestation is considered preterm labor (PTL) and delivery (PTD) and is usually preceded by pre-mature rupture of membranes (PPROM) (39). PTL and PTD is generally caused by such factors as: smoking, alcoholism, poor nutrition, health disorders, PROM, multiple gestation, placental abruption and bacterial infection (39). The main cause of PPROM and PTL can be contributed to bacterial infections (39). About 12% of pregnancies in 2001 were caused by preterm birth, with the number of preterm babies being born progressively increasing (39). In premature labors, it has been found that there is a decreased concentration of collagen (44). The fetal membrane is composed of collagen types I, III, and V (2), with type I and type V giving the amniotic membrane its strength (35).

It is hypothesized that GBS may be involved in the premature rupture of the amniotic tissue based on the fact that infection with this organism was associated with the degradation of the amniochorionic membrane and on its ability to degrade the synthetic peptide FALGPA, which mimics collagen (23). However, it was found that GBS was incapable of degrading a film of reconstituted rat tail collagen (30). Lin *et al*. (29) further isolated and tested the suspected collagenase and speculated that it was not a collagenase but an oligopeptidase, belonging to the M3 oligopeptidase family of metallopeptidases. Since the paper has been published, two strains of *S. agalactiae*, 2603V/R (43) and NEM316 (14), have been sequenced and submitted online at www.ncbi.nlm.nih.gov.

Based on sequence analysis of both strains, it was found that *S. agalactiae* does indeed have a gene (*gbs0824* from strain NEM316 and *SAG0805* from strain 2603V/R) similar to *pepF* from *Lactococcus lactis* (66.4% identity, data not shown), which is also an M3 oligopeptidase and displayed many of the same properties as members of this family.

Peptidases are categorized into clans and families where clans represent sets of families that share common ancestry and families are arranged by their catalytic specifications. The peptidase clan U- belongs to MEROPS peptidase family U32 of the clan U- and has an unknown catalytic mechanism. It also contains the consensus pattern: E-x-F-x(2)-G-[SA]-[LIVM]-C-x(4)-G-x-C-x-[LIVM]-S. The most studied peptidase of this family is the prtC collagenase from *Porphyromonas gingivalis*, which is capable of degrading type I collagen and may require a metal cofactor. It is able to degrade soluble type I collagen but not gelatin or synthetic collagenase substrates.

The availability of sequenced genomes online has allowed a more thorough search and analysis of bacterial genomes. And with the convenience of molecular genomics, such as PCR and cloning into expression vectors, it is now possible to easily and efficiently study virulence factors from pathogens. Recently, our lab has isolated and characterized a collagenase gene (*smcol1)* of the U32 peptidase family from *S. mutans* (Ioannides, Biology MS thesis, USF, 2004). *smcol1* was cloned and expressed in *Escherichia coli* and the recombinant protein was purified and studied. The *smcol1* was shown to be identical to the SMU.761 protease in *S. mutans* UA159.

Preliminary analysis of the *S. mutans* UA159 indicated the presence of another putative collagenase (col2) encoded by a gene upstream from *smcol1* (SMU.759). Similar enzymes to *S. mutans* col1 and col2 were also identified in the analysis of GBS NEM316 and 2603V/R genomic sequences (*gbs0762* and *gbs0763* in GBS NEM316, and *SAG0741* and *SAG0742* in strain 2603V/R). The goal of the present study was to clone *smcol2, gbscol1* and *gbscol2* into *E. coli* TOP10 using the arabinose-inducible expression vector system pBAD TOPO® TA (Invitrogen), which allows the expression and purification of soluble recombinant His-tagged protein. Recombinant proteins will be analyzed for collagenase and gelatinase activity. The results obtained in the present studies will add to our understanding of *S. mutans* and GBS role in their respective pathogenicity.

MATERIALS AND METHODS

Chemicals and Reagents

Primers for PCR were produced by Operon Biotechnologies (Huntsville, AL). PCR reagents, restriction enzymes, The Wizard® Genomic DNA Purification Kit and The Wizard® Plus Minipreps Plasmid DNA Purification System were obtained from Promega Inc. (Madison, WI) and used in accordance to the manufacturer's protocols. The expression vector, pBAD TOPO® TA was obtained from Invitrogen Life Technologies (Carlsbad, CA). All other reagents and chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO), Fisher Scientific (Pittsburg, PA), or Bio-Rad Laboratories (Hercules, CA) unless otherwise specified.

Bacterial Strains and Growth Conditions

S. mutans GS-5 serotype c, was initially obtained from J. J. Ferretti (University of Oklahoma Health Sciences Center, Oklahoma City, OK). GBS USF704 (serotype Ib/c, α, β, γ) is a β-hemolytic clinical isolate originally obtained from a septic newborn and was acquired from Dr. Daniel Lim (University of South Florida Department of Biology and Center for Biological Defense, Tampa, FL). Both strains were cultured at 37ºC in BHI broth with 5% CO_2. Chemically competent *E. coli* TOP10 cells were obtained from Invitrogen and used in the

cloning and expression of all recombinant plasmids. Luria-Bertani (LB) medium (Difco, Detroit, MI) containing 100 µg/ml ampicillin (LBA) was used in the selection of transformants and the culturing of recombinant clones expressing the collagenase genes.

Bioinformatical Analysis

The sequences used for the cloning and analysis of the collagenase 2 gene (*smcol2*) from *S. mutans* and the collagenase 1 (*gbscol1),* and collagenase 2 (*gbscol2)* genes from GBS were obtained from the National Center for Biotechnology Information (NCBI) Entrez server. The sequenced genomes of *S. mutans* UA159 (Accession # AE0141) and *S. agalactiae* NEM316 serotype III strain (Accession # AL732656) and *S. agalactiae* 2603V/R serotype V (Accession # AE009948) were used for the genomic analysis of the collagenases. Alignment analysis was performed using the ClustalW WWW Service at the European Bioinformatics Institute (19). Signal peptide analysis was derived from PSIPRED Protein Structure Prediction Server (25, 34).

Isolation of Genomic DNA

The isolation of genomic DNA was accomplished using a genomic DNA purification kit (Promega's Wizard®). The isolation was performed according to the manufacturer's protocol. Briefly, 1 ml from an overnight culture was centrifuged at 16,000 x g for 2 minutes and the supernatant removed. The cell pellet was resuspended in 480 µl of 50 mM EDTA. 120 µl of 10 mg/ml of

lysozyme was added to the cell suspension and incubated at 37°C for 1 hour to weaken the cell wall. The samples were centrifuged at 16,000 x g for 2 minutes. The supernatant was removed and 600 µl of Nuclei Lysis Solution was added to the samples and incubated at 80°C for 5 minutes to lyse the cells. The solution was cooled to room temperature. 3 µl of RNase Solution was added to the cell lysate and incubated at 37°C for 45 minutes. 200 µl of Protein Precipitation Solution was added to the RNase-treated cell lysate and incubated for 5 minutes on ice. The samples were then centrifuged at 16,000 x g for 3 minutes. The supernatant containing the DNA was transferred to a clean 1.5 microcentrifuge tube and 600 µl of room temperature isopropanol was gently mixed with the DNA. The mixture was centrifuged at 16,000 x g for 2 minutes. The supernatant was gently aspirated off and 70% ethanol was added to the pellet. The suspension was again centrifuged at 16,000 x g for 2 minutes and the ethanol aspirated. The pellet was allowed to air dry for 3 hours and 100 µl of DNA Rehydration Solution was added to rehydrate the DNA pellet. The purity and concentration of the DNA was determined by analysis on a 1% agarose gel and measurement on the SmartSpec Plus Spectrophotometer (Bio-Rad Hercules, CA).

PCR Amplification of *S. mutans* and *S. agalactiae* Genomic DNA

Using purified genomic DNA from *S. mutans* GS-5 as a template, PCR was performed to amplify the collagenase 2 gene (*smcol2*). Primers were designed based on the sequenced genome of *S. mutans* UA159 (9). Four primer sets

were developed to amplify the gene with and without the signal peptide. The gene was also amplified with and without the native stop in order to include the V5 epitope and the polyhistidine region of the pBAD vector. PCR was performed using four different primer sets (Table 1) to amplify the *smcol2* gene from *S. mutans* GS-5 genomic DNA under the following conditions: an initial denaturation step at 95ºC for 2 minutes. Then 30 cycles of the following: 95ºC for 1 minute (denaturation step), 1 minute at the corresponding annealing temperature for the specific primer (annealing step), and 72ºC for 1 minute (extension step). Lastly, a final extension step was done at 72ºC for 10 minutes. The PCR mixture used contained the following: 12.5 µl of PCR Master Mix (Promega), 0.2 µM of the forward primer, 0.2 µM of the reverse primer, 25 ng of genomic DNA, and 10.5 µl of H_2O. The PCR products were analyzed by electrophoresis on a 1% agarose gel. The DNA was stained with ethidium bromide and viewed under ultraviolet light.

smcol2 Clones	**Forward Primer**		**Reverse Primer**		**Annealing Temp**
Clone 1	Includes the signal peptide	5'ATGGAAAAAA TTGTTATCACT GCGACTGC	Contains native stop	5'TTACTTAAC TGTTTGCGG ATCAAGC	55.0ºC
Clone 2	Includes the signal peptide	5'ATGGAAAAAA TTGTTATCACT GCGACTGC	Does not contain native stop	5'CTTAACTGT TTGCGGATC AAGC	56.8ºC
Clone 3	Excludes the signal peptide	5'AATATTAAAC CATTTTTAGAA TTAATGAAGGA AATTCAG	Contains native stop	5'TTACTTAAC TGTTTGCGG ATCAAGC	55.4ºC
Clone 4	Excludes the signal peptide	5'AATATTAAAC CATTTTTAGAA TTAATGAAGGA AATTCAG	Does not contain native stop	5'CTTAACTGT TTGCGGATC AAGC	55.4ºC

Table 1. PCR primers used in the amplification of *smcol2*

The sequences for genes *SAG0741, SAG0742, gbs0762* and *gbs0763* of *S.agalactiae* 2603V/R (43) and *S. agalactiae* NEM316 (14), respectively, were used to develop primers for the *gbscol1* and *gbscol2* genes. The reason for this selection is based on the highest homology each one has with the corresponding enzyme in *S. mutans*.

SAG0742 and *gbs0763* were used to design primers for *gbscol1*

Forward = 5' ATGTCTAATGTAAAAAAACGCCCT

Reverse = 5' AGCTCTTACAGTCTTGCTAG

SAG0741 and *gbs0762* were used to design primers for *gbscol2*

Forward = 5' ATGGAAAAAATAATTTTGACAGCGAC

Reverse = 5' TTTTACTGTTGATGGGTCAAAATC

PCR conditions were optimized for the specific primers and the conditions were as described above. The specific annealing temperature for the *gbscol1* primers was 52.7ºC and 51.6ºC for *gbscol2* primers (Operon Biotechnologies).

Cloning of the *smcol2, gbscol1* and *gbscol2* Genes into the pBAD-TOPO® TA Vector

Once the correct size of the PCR products was verified on a 1% agarose gel, the products were cloned into the pBAD TOPO® TA vector (Figure 1). Briefly, for the cloning reaction, 2 µl of fresh PCR product was mixed with 1 µl of pBAD vector, 1 µl of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$) and 1 µl of H_2O and allowed to incubate at 24ºC for 5 minutes. The cloning mixture was then placed

on ice and 2 µl was mixed with 250 µl of One Shot® TOP10 Chemically Competent *E. coli* cells (Invitrogen). The cells were incubated on ice for 15 minutes. Transformation was by heat shock treatment: 30 seconds in a 42ºC water bath followed by immediate cooling on ice. 250 µl of S.O.C. medium (Invitrogen) was added to the cells and they were horizontally incubated at 37ºC for one hour with shaking. Finally, 20 µl and 40 µl samples were cultured on pre-warmed LBA plates. The plates were incubated for 24 hours at 37ºC. After incubation, five ampicillin resistant (Amp^R) clones from each cloning reactions were chosen at random for further screening of each gene.

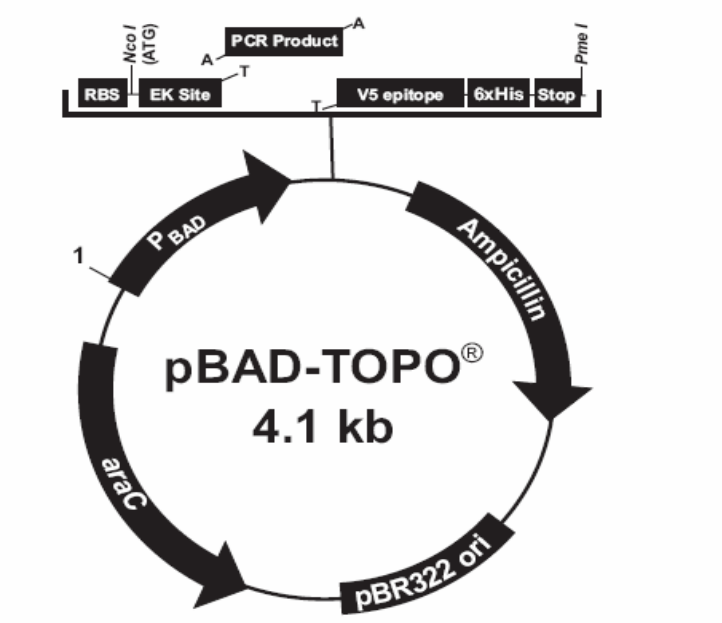

Figure 1. Map of the expression vector pBAD TOPO® TA (Invitrogen)

Analysis of the Recombinant Plasmids

Plasmid DNA was isolated from the clones using the FastPlasmid™ Mini kit from Eppendorf (Westbury, NY) following the manufacturer's protocol. Briefly, the Amp^R transformants containing the recombinant plasmids were grown in LBA for 16 hours at 37ºC with shaking. 1.5 µl of each culture was centrifuged at 16,000 x g for 1 minute and the supernatant was removed. 400 µl of the Lysis Solution was added to the pellet and resuspended with vigorous mixing. The mixture was then incubated for 3 minute at 24°C. The lysate was removed and transferred to a spin column assembly that was then centrifuged for 1 minute at 16,000 x g. The spin column assembly was washed with 400 µl of diluted wash buffer and centrifuged for 1 minute. The solution in the bottom of the tube was decanted and the spin column assembly was centrifuged for an additional minute to remove any excess isopropanol from the assembly. The spin column was then transferred to a clean tube and the plasmid DNA was eluted off by adding 30 µl of elution buffer and centrifuging at 16,000 x g for 1 minute.

The presence of the inserts was verified by PCR using the reverse and forward primers specific for each clone. Once the insert was confirmed to be present, clones that were positive for the insert were analyzed to determine that the PCR product had been inserted in the correct orientation and was in frame with the C-terminal histidine residues. To test for this, the pBAD Forward primer (5' ATGCCATAGCATTTTTATCC) was used with the specific insert's reverse primer. Use of the pBAD Forward primer adds 179 bp to the PCR product. Once

the PCR had been verified by electrophoresis of the PCR product on an agarose gel, one of the clones producing a band of the correct size was chosen and stored frozen in glycerol at -80°C as the laboratory stock strain.

Expression of the Recombinant Proteins

Pilot expression experiments were performed on pBAD/smcol2 clones #2 and #4, pBAD/gbscol1, and pBAD/gbscol2 to determine the optimal concentration of arabinose for induction of the clones and the expression of the recombinant proteins. pBAD/smcol2 clones # 2 and # 4 were chosen for further analysis since they allowed for the production of the polyhistidine (6x) fusion protein tag, with and without the signal peptide, respectively. They were further termed pBAD/smcol2sp, pBAD/smcol2wosp, respectively. pBAD/smcol2 clones #1 and #3 were prepared in prevision of potential problem with enzyme activity being influenced by the polyhistidine fusion protein tag (since this problem did not occur, these two clones were not analyzed further). The expression experiment was done according to the manufacturer's protocol. Briefly, the recombinant clones were inoculated into 2 ml of LBA broth (100 μg/ml ampicillin) and grown for 24 hours at 37ºC with constant shaking. The following day, 0.1 ml of the overnight cultures was added to 10 ml of LBA and allowed to continue incubating at 37ºC with constant shaking. The cultures were allowed to grow to an OD_{600} of 0.5, approximately 2.5 hours. A 1 ml sample of each culture was taken and the cells were sedimented by centrifugation. The supernatant was aspirated and the pellets were frozen for a zero time point sample. For each clone, cultures were

induced at five different concentrations of L-arabinose: 0.2%, 0.02%, 0.002%, 0.0002%, and 0.00002%. Un-induced cultures were also grown that did not have L-arabinose added to it. The cells were then allowed to grow for an additional 4 hours at 37ºC with constant shaking. Each culture was centrifuged to pellet the cells. The supernatant was aspirated and the samples were frozen for further analysis.

SDS-PAGE Analysis of Gene Expression in Recombinant *E. coli*

To determine if the recombinant bacteria produced the protein of interest, the samples obtained above were analyzed by electrophoresis on a 10% SDS-Polyacrylamide gel (1 mm). The cell pellets were thawed, resuspended in 20 µl of 1x SDS-PAGE Sample Buffer (Bio-Rad) and boiled in a water bath for 5 minutes. The samples were then separated by electrophoresis using a Mini-Protean II Electrophoresis Cell (Bio-Rad) at 200V for 1 hour. Once the samples were separated through the gel, the gel was stained with 0.1% Coomassie blue R-250 (in 40% methanol and 10% acetic acid) for one hour, then was destained by incubation in a destaining solution (40% methanol and 10% acetic acid). Numerous washes were used to totally destain the gel and obtain sharp protein bands stained in blue.

Western Blot Analysis of His-Tagged Fusion Protein

A Western immunoblot was performed by separating the samples by SDS-PAGE and then transferring them to a nitrocellulose membrane. The proteins were transferred to the nitrocellulose for one hour at 30V and 100 mA, with an ice pack and constant stirring of the buffer using a Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad). The membrane was then blocked for 16 hours in 10 ml of Western Blot Blocking Solution (5% dry milk in PBS containing 0.05% Tween 20 with) with gentle agitation. Next, the membrane was washed twice with 10 ml of Western Blot Wash Buffer (PBS and 2% Tween 20) for 10 minutes each wash. The primary antibody, murine anti-HisG (Invitrogen), was diluted 1:5000 in Blocking Solution and incubated with the nitrocellulose for one hour at 24ºC with gentle agitation. The antibody was washed away with two washes of Wash Buffer, 10 minutes each wash. Next, the membrane was incubated with the secondary antibody, anti-mouse IgG antibody (Sigma) diluted 1:30,000 in Blocking Buffer, for one hour at 24ºC with gentle agitation. The membrane was washed again with Wash buffer, twice for 10 minutes each wash and then developed. The nitrocellulose was developed via Dao's method (8). Briefly, the membrane is incubated with sodium borate buffer (60mM sodium tetraborate, 10mM magnesium sulfate, pH 9.7) containing 0.025% of O-dianisidine and 0.025% of β -naphthyl acid phosphate. After a 1 hour incubation in the buffer, the membrane was fixed by incubating in Immunoblot Fixing Buffer (methanol: H2O: acetic acid, 4:5:1). The membrane was then rinsed in deionized water and allowed to dry.

Purification of Recombinant Proteins

A fresh culture of the clones from the glycerol frozen stock was streaked onto LBA plates and grown for 24 hours at 37ºC. A culture of competent cells that contained the empty pBAD vector was used as a negative control. The recombinant clones were grown and induced as described above using the optimal concentration of arabinose. The fusion proteins were purified using Qiagen's Ni-NTA Fast Start Superflow Columns. Purification was performed under native conditions and as described by the manufacturer. The cell pellet was resuspended in 10 ml of native lysis buffer, containing lysozyme and benzonase and incubated on ice for 30 min. The lysate was centrifuged at 14,000 x g for 30 minutes at 4°C to pellet the cellular debris. The Fast Start Columns were drained of the shipping buffer and the supernatant containing the soluble fraction of the recombinant protein was applied to the column. The column was washed twice with 4 ml of Native Wash Buffer. The bound 6x His-tagged protein was eluted out with two 1 ml aliquots of Native Elution Buffer. The samples were analyzed by SDS-PAGE and Western Blot as described above.

Protein Concentration

The protein concentration of all samples were determined by the BCA Protein Assay developed by Bradford (4). Briefly, a serial dilution of Bovine Serum Albumin (BSA) was prepared and assayed along with the samples to be tested. After addition of the BCA Working Reagent (Sigma) to the samples, they were incubated for 15 minutes at 60°C and the absorption was read at OD_{595}. The values obtained from the BSA were used to develop a standard curve, which was used to determine the protein concentration of the test samples.

Gelatinase assay

X-ray film coated with gelatin was stained with Coomassie blue R250, and used to assay for gelatinase activity. Ten μl of each purified sample obtained above was dotted onto the stained X-ray film, which was then placed in a humid chamber (box with a piece of wet paper towel placed at the bottom). Incubation was at 37°C for 16 hours, at which time the film was placed under running faucet water. Dots containing digested gelatinase were identified by a cleared zone exposing the shiny film backing.

Collagenase Assay

Controls for the collagenase assay included *C. histolyticum* collagenase as a positive control and trypsin as a negative control. Purified SmCol2 with or without the signal peptide, GBSCol1 with the signal peptide and GBSCol2 with the signal peptide were prepared as follows: The samples were prepared by growing 30 ml cultures for 24 hours at 37°C. The following day, the cells were pelleted and the supernatant removed. The pellet was resuspended in 5 ml of Gelatinase Assay Buffer (GAB: Tris-HCl with $CaCl_2$ at pH 7.4) and freeze-thawed 3 times on dry ice and a 42°C water bath. The cells were then sonicated with short bursts and centrifuged at 8,000 X g for 25 minutes. The supernatant was poured off into clean tubes and the pellet was resuspended in 5 ml of assay Buffer (50mM Tris buffer, 5 mM $CaCl_2$ added at 5mM, pH 7.4). Purified fusion proteins were isolated as described above and assayed for collagenase activity using a specific blue collagenase substrate developed in our laboratory (Dao, unpublished method). Briefly, 100 μl of each sample (each sample was approximately 600 μg/ml) were incubated separately with 1 ml suspension of approximately 15 mg blue collagen type I from bovine tendon in assay buffer. The enzymatic assay using blue collagen was referred to as “blue collagenase assay” (proprietary method). After incubation in an incubator shaker at 37^{o}C, followed by centrifugation, degradation of collagen resulted in the blue coloration of the supernatant, which was quantified by measuring the absorbance of the blue dye at 500nm ($OD_{500nm)}$ using a spectrophotometer.

RESULTS

Comparative Analysis between Putative Collagenase Genes of *S. mutans* and GBS

The completed genomes of *S. mutans* UA159 (9), S*. agalactiae* strain NEM316 serotype III (14) and *S. agalactiae* strain 2603V/R serotype V (43) have been sequenced and, hence were used for this study. Analysis of the genomic sequence of *S. mutans* UA159 revealed two proteases related to collagenase (SMU.759 and SMU.761). These genes were used to find similar genes in Group B streptococci and compare their sequences with other known collagenases from *C. histolyticum* and *P. gingivalis*. A BLink ("BLAST Link") was performed using SMU.759 and SMU761 sequences. It was found that SAG0741 from *S. agalactiae* 2603V/R showed the highest similarities with SMU.759 (78% homology, Figure 3) and SAG0742 from *S. agalactiae* 2603V/R showed the highest similarities with SMU.761 (78% homology, Figure 2). These genes were used for further analysis. SMU.761 was found to be 100% identical to *smcol1*, cloned recently in our lab from GS-5 (NCBI Accession # AY644675) (21). SMU.761 will be termed smcol1 and SMU.759 will be termed smcol2 for the remainder of this study. The most closely related genes were selected for further analysis (Table 2).

Alignment Analysis

Comparative analysis of the deduced amino acid sequence of the genes of interest was done using the alignment program, ClustalW WWW Service at the European Bioinformatics Institute (6) (Table 2). SMU.761 (*smcol1*) showed high homology to SAG0742 from *S. agalactiae* 2604V/R (78%) and gbs0763 from *S. agalactiae* NEM316 (78%) (Figure 2). Minimal similarity was observed between *smcol1* and *colG* (2%) or *colH* (2%). SMU.759 (*smcol2*) was found to have significant similarities with SAG0741 from *S. agalactiae* 2604V/R (77%) and gbs0762 from *S. agalactiae* NEM316 (78%) (Figure 3), Minimal similarity was found to *colG* and *colH*, 7% and 5% respectively. Only 9% homology was observed between *smcol2* and *smcol1*.

Based on the sequence alignment, it was found that *S. agalactiae* 2603V/R and *S. agalactiae* NEM316 presented parallel results with the sequences used (Figure 2 and 3). Hence, SAG0742 and gbs0763 were determined to be the same gene, as SAG0741 and gbs0762. They were termed gbscol1 and gbscol2, respectively.

Organism	Gene	Protein Function
Streptococcus mutans UA159	*SMU.759*	Putative collagenase
Streptococcus mutans UA159	*SMU.761*	Putative collagenase
Streptococcus agalactiae 2603V/R	*SAG0742*	Peptidase, U32
Streptococcus agalactiae NEM316	*gbs0763*	Hypothetical protein
Streptococcus agalactiae 2603V/R	*SAG0741*	Hypothetical protein
Streptococcus agalactiae NEM316	*gbs0762*	Hypothetical protein
Clostridium histolyticum	*colH*	Collagenase
Clostridium histolyticum	*colG*	Collagenase
Porphyromonas gingivalis	*prtC*	Collagenase

Table 2. Genes selected for further bioinformatical analysis.

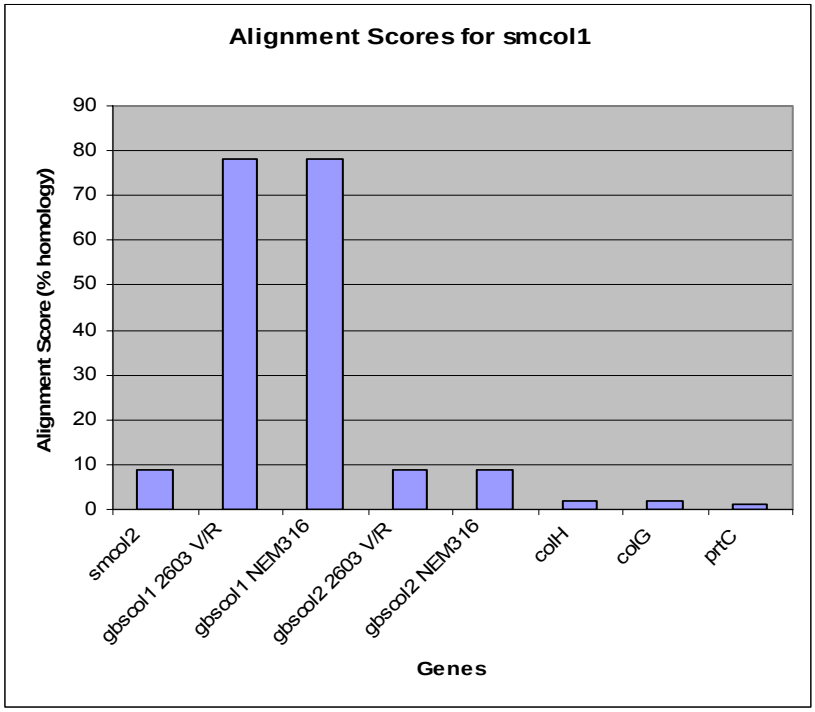

Figure 2. Alignment of homology for *smcol1*

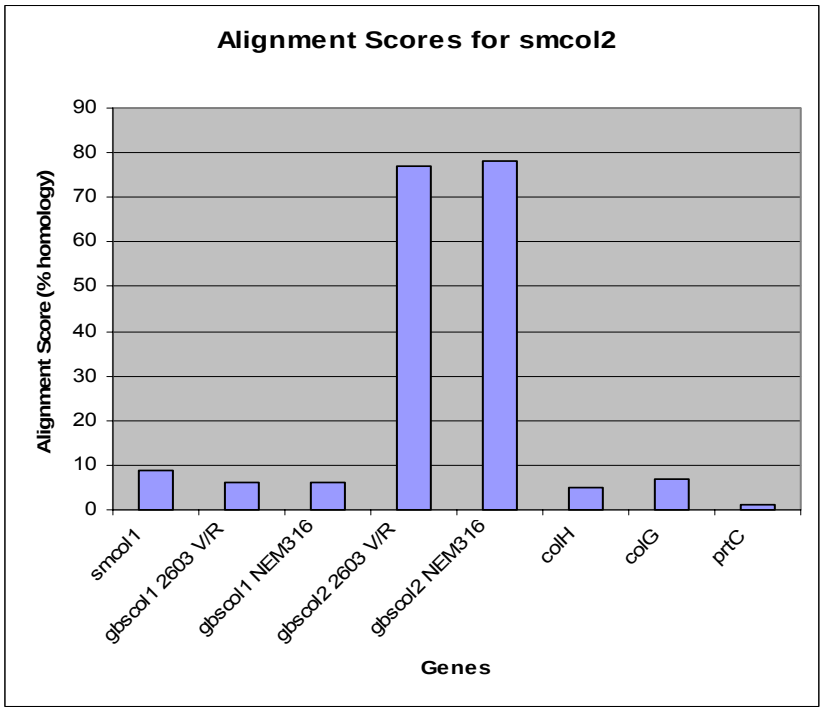

Figure 3. Alignment of homology for *smcol2*

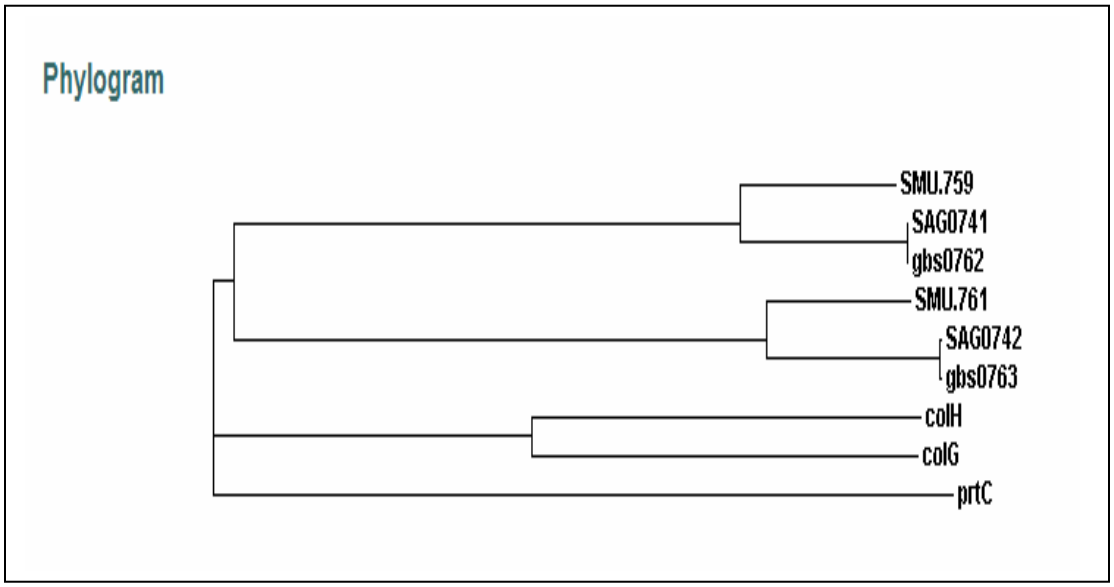

Figure 4. Phylogram. Estimated phylogeny between the selected genes. Branch lengths are proportional to the amount of inferred evolutionary change.

Tables of Homology

The sequence of *smcol2, gbscol1* and *gbscol2* was used as a query to identify homologous genes in other bacteria and to deduce the corresponding amino acid sequence and associated biochemical characteristics. The BLAST analysis indicated high homology with the U32 family of peptidases and collagenases of other related organisms (Tables 4, 5, and 6).

By using the *S. mutans* UA159 *smcol2* sequence as a query, a BLAST search showed 197 hits to 119 unique species (Table 3). Analysis of the deduced amino acid sequence showed notable homology with *Streptococcus suis* collagenase (79%), *Streptococcus pyogenes* putative protease (77%) and *S.*

pyogenes peptidase family U32 (76%). It also showed homology to U32 peptidases of *Enterococcus faecalis* (53%) and *Bacillus anthracis* (31%).

Using the *gbscol1* sequence as a query, a BLAST analysis was done to determine which genes carried the most homology (Table 4). As expected, the collagenases and U32 peptidases of Streptococcal species shared the highest homology to *gbscol1*, ranging from 99%-82%. It was found that *gbscol1* had 77% and 74% homology to *Lactococcus lactis* collagenase and *Enterococcus faecium* U32 peptidase, respectively. It was also found that U32 peptidases from *Listeria monocytogenes* and *Staphylococcus epidermidis* had 63% and 49% homology, respectively.

Using the sequence of *gbscol2* from *S. agalactiae* NEM316, a BLAST search was performed to compare it to other organisms (Table 5). It was found that it shared high homology with other Streptococcal collagenases and peptidases of the U32 family: 79% homology with *S. pyogenes*, 76% homology with *S. suis*, 72% homology with *Streptococcus pneumoniae* and 67% *Streptococcus thermophilus.* It was also found that it shared 56% homology with *L. lactis* and 55% homology with U32 peptidases from *Enterococcus* species. Lastly, it was found that *gbscol2* had 30% homology to U32 peptidases from *Bacillus* species.

Organism	Gene	# of aa/MW	Putative function	% aa Identity	Accession #
Streptococcus mutans GS5	smcol2	309/35477	Collagenase	n/a	n/a
Streptococcus mutans UA159	smu_759	309/35477	Protease	100%	AAN58482
Streptococcus pyogenes MGAS5005	M5005_Spy_0489	122/13653	Peptidase, U32	84%	YP_281852
Streptococcus agalactiae 515	SAL_0796	309/34732	Unknown	83%	ZP_00789279
Streptococcus suis 89/1591	SsuiDRAFT_0788	309/35467	Peptidase, U32	79%	ZP_00875752
Streptococcus pneumoniae R6	spr0710	356/41084	Protein	72%	NP_358304
Streptococcus thermophilus CNRZ1066	str0671	309/35184	Protease	70%	YP_141082
Lactococcus lactis Il1403	L54059	307/34518	Protease	57%	NP_268148
Lactococcus lactis SK11	Llacc01000266	323/36444	Collagenase	57%	ZP_00383784
Enterococcus faecalis V583	EF3280	306/34266	Peptidase, U32	53%	NP_816877
Enterococcus faecium DO	EfaeDRAFT_0731	307/35232	Peptidase, U32	49%	ZP_00604498
Listeria monocytogenes 4b F2365	LMOf2365_0980	302/33585	Peptidase	43%	YP_013581
Bacillus cereus ATCC 10987	BCE4464	309/35461	Peptidase, U32	32%	NP_980757
Bacillus anthracis Sterne	BAS4278	309/35532	Peptidase, U32	31%	YP_030526

Table 3. *smcol2* homologous genes

Organism	Gene	# of aa/MW	Putative function	% aa Identity	Accession #
Streptococcus agalactiae 515	SAL_0797	428/47936	Peptidase, U32	99%	ZP_00789271
Streptococcus pyogenes M49 591	SpyoM01000802	428/47782	Collagenase	90%	ZP_00365935
Streptococcus mutans UA159	SMU.761	428/47906	Collagenase	88%	AAT66437
Streptococcus suis 89/1591	SsuiDRAFT_0787	429/48224	Peptidase, U32	85%	ZP_00875751
Streptococcus thermophilus LMD-9	StheL01000251	434/48605	Collagenase	83%	ZP_00389679
Streptococcus pneumoniae TIGR4	SP1429	428/47762	Peptidase, U32	82%	NP_345886
Lactococcus lactis SK11	Llacc01000267	430/47741	Collagenase	77%	ZP_00383785
Enterococcus faecium DO	EfaeDRAFT_0730	416/46699	Peptidase, U32	74%	ZP_00604497
Enterococcus faecalis V583	EF3279	415/46727	Peptidase, U32	72%	NP_816876
Listeria innocua Clip11262	lin0960	408/45693	Protein	63%	NP_470297
Listeria monocytogenes 4b H7858	LMOh7858_1025	408/45703	Peptidase, U32	63%	ZP_00231410
Bacillus licheniformis ATCC 14580	BLi02862	422/47642	Protease	50%	YP_092425
Bacillus subtilis 168	BSU27340	422/47498	Protein	49%	NP_390612
Staphylococcus epidermidis RP62A	SERP1176	422/47641	Peptidase, U32	49%	YP_188752

Table 4. *gbscol1* homologous genes

Organism	Gene	# of aa/MW	Putative function	% aa Identity	Accession #
Streptococcus agalactiae COH1	SAN_0829	303/34717	Unknown	100%	ZP_00785817
Streptococcus mutans UA159	SMU.759	308/35346	Protease	82%	NP_721176
Streptococcus pyogenes MGAS6180	M28_Spy0470	308/34559	Peptidase, U32	79%	YP_279939
Streptococcus suis 89/1591	SsuiDRAFT_0788	309/35467	Peptidase, U32	76%	ZP_00875752
Streptococcus pneumoniae TIGR4	SpneT_01000657	321/36890	Collagenase	72%	ZP_00404041
Streptococcus thermophilus CNRZ1066	str0671	309/35184	Collagenase	67%	YP_141082
Lactococcus lactis Il1403	L54059	307/34518	Protease	56%	NP_268148
Lactococcus lactis SK11	Llacc01000266	323/36444	Collagenase	55%	ZP_00383784
Enterococcus faecalis V583	EF3280	306/34266	Peptidase, U32	55%	NP_816877
Enterococcus faecium DO	EfaeDRAFT_0731	307/35232	Peptidase, U32	50%	ZP_00604498
Listeria innocua Clip11262	lin0959	302/33722	Protein	44%	NP_470296
Listeria monocytogenes 4b F2365	LMOf2365_0980	302/33585	Peptidase	43%	YP_013581
Bacillus cereus ATCC 14579	BC4377	309/35548	Collagenase	30%	NP_834089
Bacillus thuringiensis 97-27	BT9727_4115	309/35475	Peptidase, U32	30%	YP_038433
Bacillus anthracis Sterne	BAS4278	309/35532	Peptidase, U32	29%	YP_030526

Table 5. *gbscol2* homologous genes

PCR Amplification and Cloning of the *smcol2, gbscol1* and *gbscol2* genes into the pBAD-TOPO Vector

Using genomic DNA from *S. mutans* GS-5 as a template, PCR was used to amplify *smcol2*. The primers were designed based on the sequenced genome of *S. mutans* UA159 (9) and the PCR product obtained was cloned into the pBAD TOPO® TA Cloning vector (Invitrogen). The pBAD vector employs TOPO® Cloning, an easy and efficient method of cloning PCR products. The linearized vector has 3' deoxyribose thymidine (dT) overhangs that are complementary to the deoxyribose adenosine (dA) overhangs added by *Taq* polymerase to the 3' end of PCR products. This allows for the direct incorporation of PCR amplicons by *Taq* polymerase into the linearized plasmid vector. With TOPO® Cloning, Topoisomerase I from Vaccinia virus is bound to the plasmid vector. This enzyme binds duplex DNA at specific sites and cleaves the phosphodiester bonds on the vector backbone. The energy generated from the broken phosphodiester backbone creates a high energy covalent bond between a tyrosyl residue of the enzyme and the phosphate residue of the cleaved DNA. This leads to the release of Topoisomerase I through the attack of the phospho-tyrosyl bond between the enzyme and the DNA by the 5′ hydroxyl of the original cleaved strand. PCR products cloned into pBAD are regulated for expression in *E. coli*. The expression of the PCR product in *E. coli* is determined by the araBAD promoter (pBAD). The pBAD-TOPO® plasmid encodes for the *AraC* gene product, which positively regulates this promoter. The expression of the pBAD vector is controlled through the presence of L-arabinose. When L-arabinose is

not present, transcription from pBAD is extremely low, while expression of pBAD is turned on in the presence of L-arabinose. Protein expression levels can be optimized by varying the concentration of L-arabinose.

Four primer sets were developed to amplify the gene with and without the signal peptide. The gene was also amplified with and without the native stop. In order to include the V5 epitope and the polyhistidine region of the pBAD vector, the native stop must be removed. Once the PCR conditions were optimized for the four primer sets and the PCR product had been verified on agarose gel electrophoresis (Figure 5), it was cloned into the pBAD vector.

The cloning reaction mixture was used to transform *E. coli* TOP10 cells. After an overnight incubation, each primer set produced numerous transformants. Five ampicillin resistant (Amp^R) transformants of each pBAD clone were chosen at random for further screening. Plasmid DNA was isolated from the clones and the presence of the insert was verified through PCR using the above primers. Clones that produced plasmid that was positive for the corresponding PCR product were subjected to further testing.

	Forward Primer	**Reverse Primer**	**MW of PCR Product**
Clone 1	Includes the signal peptide	Contains native stop	927 bp
Clone 2	Includes the signal peptide	Does not contain native stop	924 bp
Clone 3	Excludes the signal peptide	Contains native stop	702 bp
Clone 4	Excludes the signal peptide	Does not contain native stop	699 bp

Table 6. Primers designed for the cloning into the pBAD vector and the anticipated molecular weight of the amplified product

Once the insert had been confirmed to be present, the clones were analyzed to determine that the PCR product had been inserted in the correct orientation and were in frame with the C-terminal His tag. pBAD # 1 only produced one clone that had the insert in the correct orientation, a band at 1106 bp (Figure 6A). This lone clone was used for further investigation. Of the 5 clones screened, pBAD # 2 had two clones in the correct orientation which produced bands at 1103 bp (Figure 6B). Figure 6C shows pBAD # 3 and pBAD # 4 also had two clones, respectively, with the insert in the correct orientation and anticipated size; 881 bp and 878 bp for pBAD # 3 and pBAD # 4, respectively. Based on the PCR results, it was concluded that the *smcol2* had been successfully cloned into the pBAD vector. One clone that produced positive results for the PCR was randomly chosen for further analysis.

The cloning of the *gbscol1* and *gbscol2* was as described for *smcol2*. Primers were designed based on the sequenced genome of *S. agalactiae* 2603V/R and *S. agalactiae* NEM316 for the *gbscol1* and *gbscol2* gene. Once PCR conditions had been optimized, the products were cloned in the pBAD vector (Figure 7).

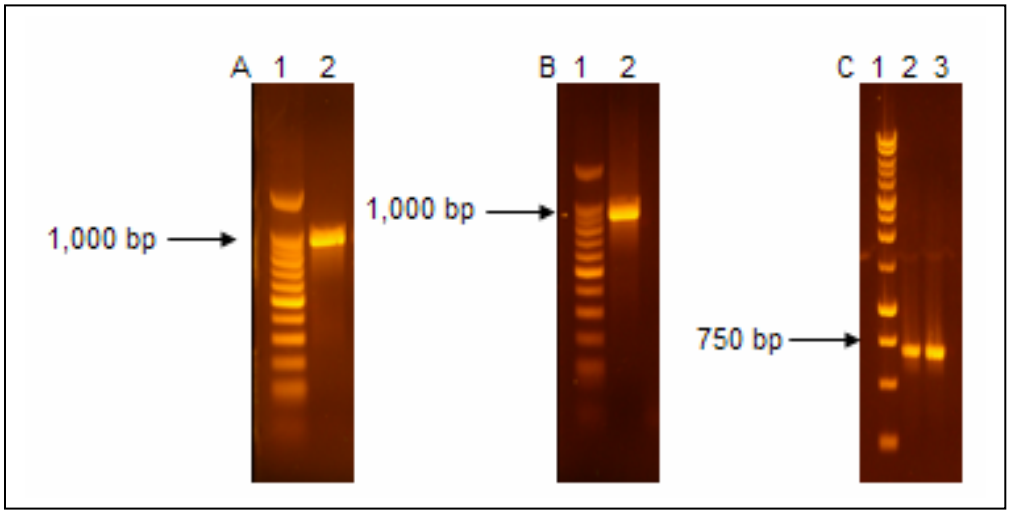

Figure 5. PCR results using *S. mutans* GS-5 genomic DNA and custom designed primers for *smcol2*

A. Lane 1: Molecular weight standard
   Lane 2: PCR product for pBAD clone # 1 = 927 bp
B. Lane 1: Molecular weight standard
   Lane 2: PCR product for pBAD clone # 2 = 924 bp
C. Lane 1: Molecular weight standard
   Lane 2: PCR product for pBAD clone # 3 = 702 bp
   Lane 3: PCR product for pBAD clone # 4 = 699 bp

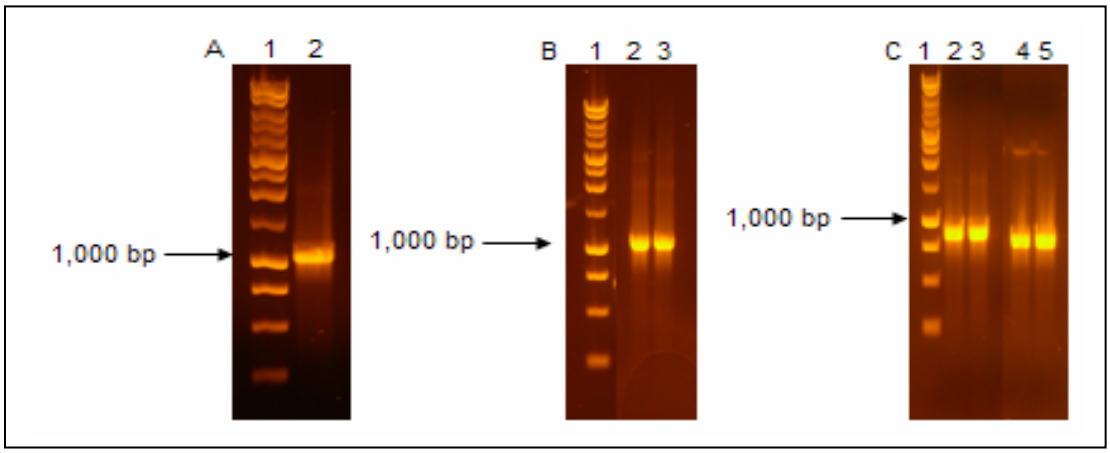

Figure 6. Confirmation of successful insertion and orientation of the *smcol2* PCR products into the pBAD vector using pBAD forward primer and the insert's reverse primer

A. Lane 1: Molecular weight standard
   Lane 2: PCR conformation for pBAD clone # 1 = 1106 bp
B. Lane 1: Molecular weight standard
   Lane 2: PCR confirmation for pBAD clone # 2a = 1103 bp
   Lane 3: PCR confirmation for pBAD clone # 2b = 1103 bp
C. Lane 1: Molecular weight standard
   Lane 2: PCR confirmation for pBAD clone # 3a = 881 bp
   Lane 3: PCR confirmation for pBAD clone # 3b = 881 bp
   Lane 4: PCR confirmation for pBAD clone # 4a = 878 bp
   Lane 5: PCR confirmation for pBAD clone # 4b = 878 bp

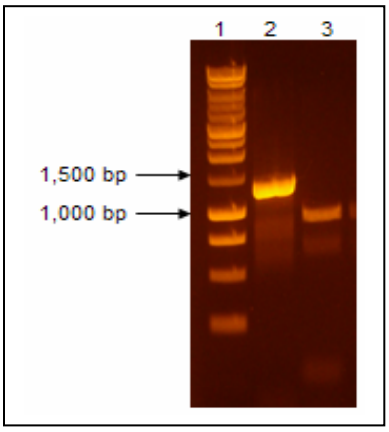

Figure 7. PCR results using *S. agalactiae* USF704 genomic DNA and custom- designed primers for *gbscol1* and *gbscol2*
Lane 1: Molecular weight standard
Lane 2: PCR product for *gbscol1* = 1284 bp
Lane 3: PCR product for *gbscol2* = 924 bp

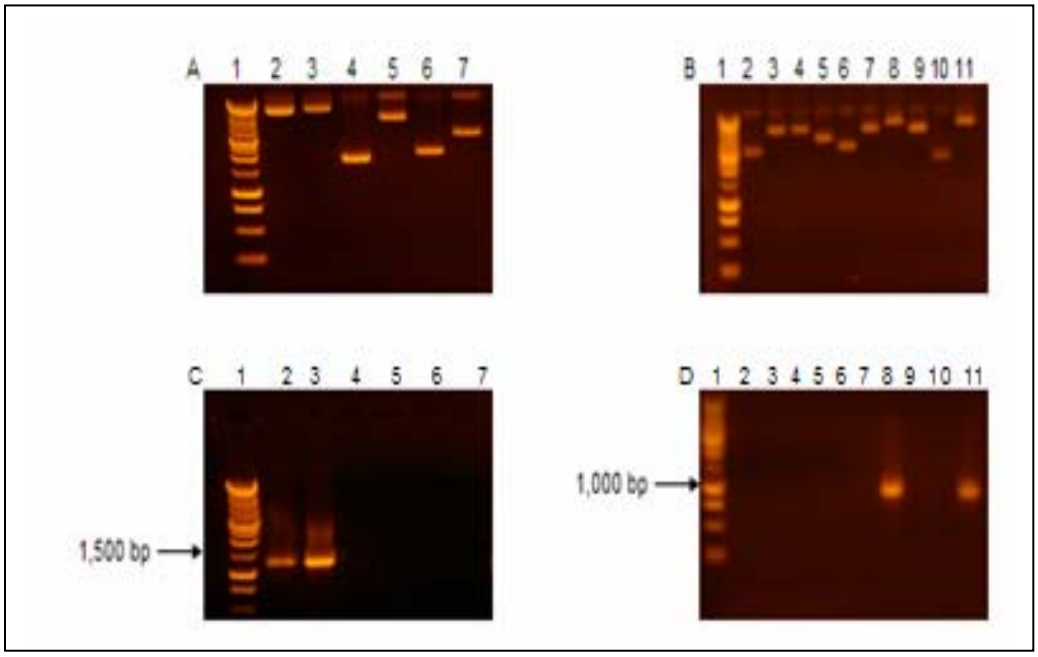

Figure 8: PCR confirmation of successful insertion in the pBAD vector
Figure 8A and 8C: Top lanes show plasmid isolated from pBAD/*gbscol1* transformants. The bottom lanes show PCR using the pBAD forward primer with the above corresponding plasmid. Lanes 2 and 3 show bands at 1463 bp.
Figure 8B and 8D: Top lanes show plasmid isolated from pBAD/*gbscol2* transformants. The bottom lanes show PCR using the pBAD forward primer with the above corresponding plasmid. Lanes 8 and 11 show bands at 1103 bp.

Plasmid DNA was isolated from the clones (Figure 8, top) and the presence of the insert was verified through PCR using the primers specific for the insert. Clones that showed a band at 1284 bp for pBAD/*gbscol1* and 924 bp for pBAD/*gbscol2* were analyzed to determine whether the insert had been inserted in the correct orientation. Gbscol1 and Gbscol2 transformants both showed two clones that had the insert positioned in the correct orientation (Figure 8, bottom). These results show that the *gbscol1* and *gbscol2* had been successfully cloned into the pBAD vector. One of each clone was chosen at random for further studies.

Expression and Detection of pBAD/smcol2 through induction with Arabinose, SDS-PAGE and Western Blot

Pilot expression experiments were performed on pBAD/*smcol2* clone # 2, pBAD/*smcol2* clone # 4, pBAD/*gbscol1* and pBAD/*gbscol2* to determine the optimal concentration of arabinose for induction of the clones and the expression of the recombinant protein. pBAD/*smcol2* clone # 2 will be referred to as smcol2sp (smcol2 with signal peptide) and pBAD/*smcol2* clone # 4 will be referred to as smcol2wosp (smcol2 without signal peptide) throughout the rest of the paper. It was found that the highest production of the recombinant protein in the clones was with 0.2% arabinose. 0.2% arabinose was then used for all subsequent experiments as the inducer concentration.

A Western blot was performed on clones smcol2sp, smcol2wosp, gbscol1 and gbscol2 using an anti-HisG antibody to definitely verify the size and the production of the fusion protein in the corresponding induced recombinant bacteria. As expected, *smcol2sp* clone produced a strong band at 40 kDa and *smcol2wosp* clone produced a smaller band at 31 kDa (Figure 9). The negative control (*E. coli* TOP10 transformed with pBAD-empty vector) showed no reactivity to the anti-HisG antibody. The *gbscol1* clone showed a strong band at 52.5 kDa whereas *gbscol2* showed a band at about 40 kDa. This data confirms that the four clones did produce successful induction of the polyhistidine (6xHis) tagged fusion protein.

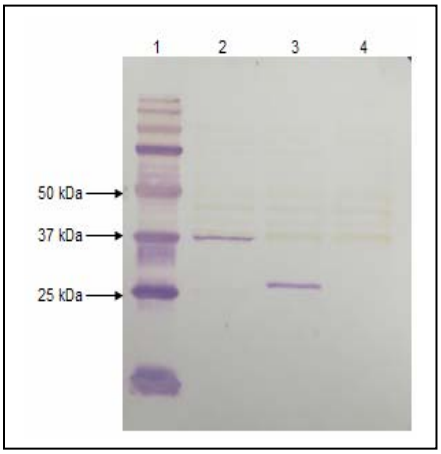

Figure 9: Western Blot of smcol2sp and smcol2wosp
Lane 1: Molecular weight standard
Lane 2: smcol2sp, 40 kDA
Lane 3: smcol2wosp; 31 kDA
Lane 4: Negative control, pBAD empty vector

Purification of the polyhistidine (6xHis) tagged fusion proteins smcolsp, smcolwosp, gbscol1 and gbscol2

A large-scale induction was performed on the four clones using 0.2% arabinose. The presence of the recombinant protein in the induced cells was verified through immunodot analysis using anti-HisG antibody (Figure 10) before isolation. The recombinant protein was then isolated from the cells via Qiagen's NI-NTA Fast Start Columns (Catalog # 30600) using native conditions. The samples were separated by electrophoresis on an SDS-PAGE gel to confirm that the protein of the correct size was isolated (Figure 11). The purified enzymes were then verified to be the correct poly-histamine fusion recombinant protein through Western Blot analysis using anti-HisG antibody. Bands with the anticipated molecular size were observed: 40 kDa smcol2sp, 31 kDa smcol2wosp, 52.5 kDa gbscol1and 40 kDa gbscol2 on both the SDS-PAGE and Western Blot (Figure 12). A Bradford assay was then performed to determine the concentration of the isolated proteins. It was found that the protein concentration of each sample was as follows: smcol2sp, 559.8 ug/ml; smcol2wosp, 580.9 ug/ml; gbscol1, 613.8 ug/ml; gbscol2, 538.9 ug/ml. These samples were analyzed for collagenase activity.

pBAD Clones	Size of protein (kDa)	pI
smcol2sp	40 kDa	5.48
smcol2wosp	31 kDa	5.62
gbscol1	52.5 kDa	5.46
gbscol2	40 kDa	5.28

Table 7. pBAD clones and the anticipated protein size, including the pBAD vector, and pI

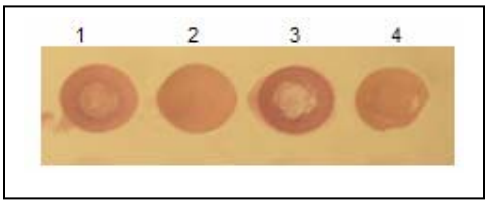

Figure 10: Immunodot of induced clones
Well 1: smcol2sp
Well 2: smcol2wosp
Well 3: gbscol1
Well 4: gbscol2

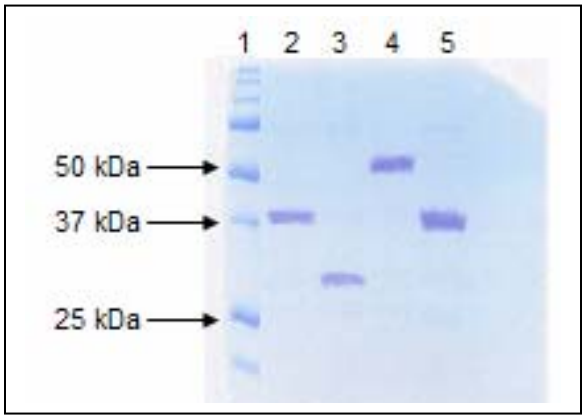

Figure 11: SDS-PAGE of purified recombinant enzymes
Lane 1: Molecular weight standard
Lane 2: Purified smcol2sp, 40 kDa
Lane 3: Purified smcol2wosp, 31 kDa
Lane 4: Purified gbscol1, 52.5 kDa
Lane 5: Purified gbscol2, 40 kDa

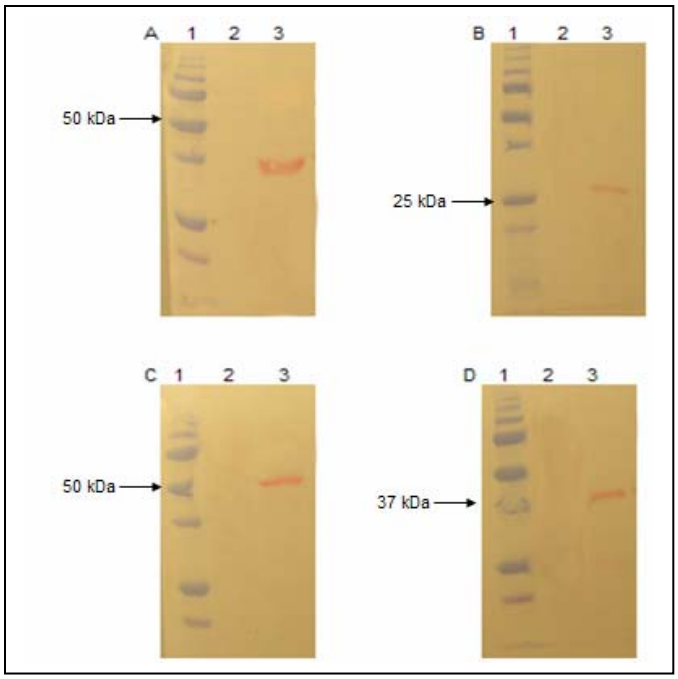

Figure 12: Western Blot of purified enzymes
A. Lane 1: Molecular weight standard
Lane 2: Negative control
Lane 3: Purified smcol2sp, 40 kDa
B. Lane 1: Molecular weight standard
Lane 2: Negative control
Lane 3: Purified smcol2wosp, 31 kDa
C. Lane 1: Molecular weight standard
Lane 2: Negative control
Lane 3: Purified gbscol1, 52.5 kDa
D. Lane 1: Molecular weight standard
Lane 2: Negative control
Lane 3: Purified gbscol2, 40 kDa

Gelatinase Assay

All the samples tested, which included pure recombinant smcol2sp, smcol2wosp, gbscol1 and gbscol2, were positive for gelatinase activity (Figure 13). As anticipated *C. histolyticum* collagenase, and trypsin, used as positive controls, also degraded the gelatin. No gelatinase activity was observed with a BSA dot.

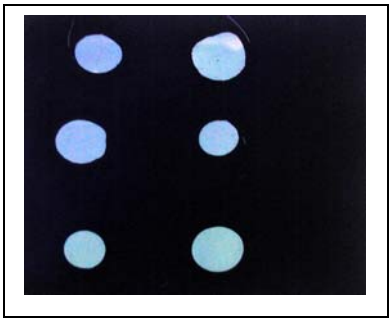

Figure 13. Gelatinase assay using X-Ray film
Top row: smcol2sp; smcol2wosp
Middle row: gbscol1; gbscol2
Bottom row: *C. histolyticum* collagenase; Trypsin
.

Blue Collagenase Assay

The Blue Collagenase Assay was used to determine whether or not the recombinant proteins had true collagenase activity. As anticipated, *C histolyticum* was strongly positive, whereas trypsin was negative (Figure 14). All recombinant enzymes showed the presence of small collagen fragments adhering to the wall of the tube (Figure 14). However, only smcol2sp and gbscol2 (also with the signal peptide) showed some measurable degraded collagen (OD_{500nm}) (Table 8).

Samples	gbscol1	gbscol2	smcol2sp	smcol2wosp	*C. histolyticum*	Trypsin
Abs OD_{500}	0.0472	0.1237	0.1965	0.034	1.0985	0.075
Abs OD_{500}	0.0728	0.2268	0.1467	0.0705	0.913	0.015
Mean	0.060	0.175	0.1716	0.052	1.006	0.045
SD	0.018	0.072	0.035	0.025	0.130	0.042

Table 8. Raw Data from Blue Collagenase Assay

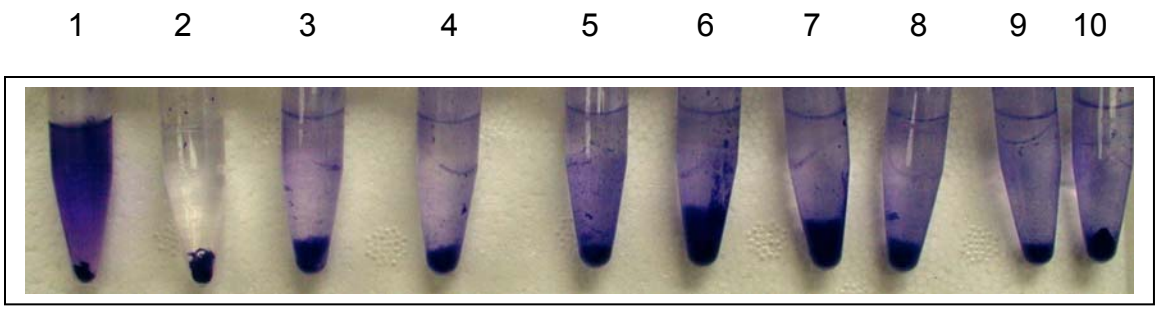

Figure 14. Blue collagenase assay
C. histolyticum collagenase (1) showing digestion of blue collagen
Trypsin (2) showing no degradation of collagen
Partial degradation of collagen into smaller fragments sticking to the tube wall
gbscol1 (3 & 4); gbscol2 (5 & 6); smcol2sp (7 & 8); smcol2wosp (9 & 10)

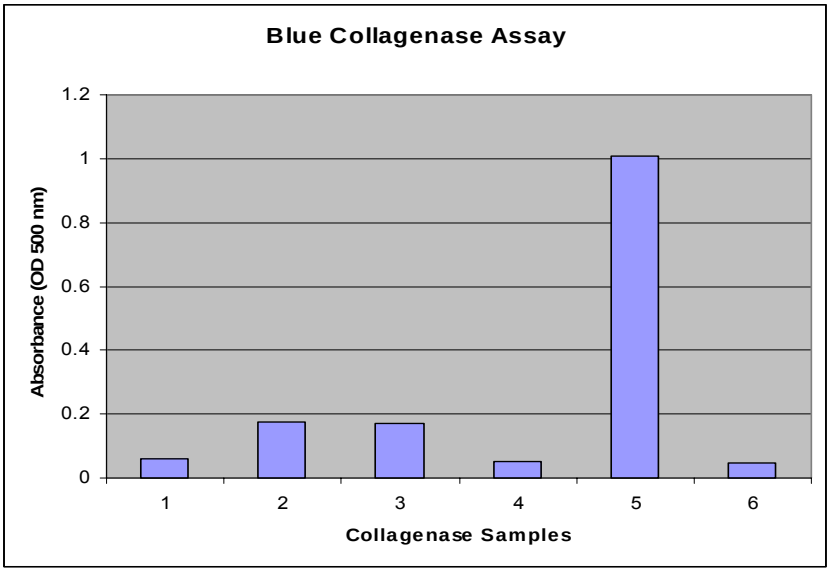

Figure 15: Results of Blue Collagenase Assay
Mean OD_{500nm} of duplicate samples of:
1. Gbscol1
2. Gbscol2
3. Smcol2sp
4. Smcol2wosp
5. *C. histolyticum* collagenase
6. Trypsin

The absorbance observed with samples 1, 4 and 6 is equal to the background level observed with blue collagen incubated in parallel with assay buffer alone.

DISCUSSION

With the recent availability of completely sequenced microbial genomes, analysis of *S. mutans* and GBS genomic sequences allowed the identification of putative collagenase genes in these organisms. Of the putative collagenases identified in *S. mutans* UA159, one identical gene (*smcol1*) was previously cloned from *S. mutans* GS-5 and sequenced (21). Bioinformatical analysis of the second putative collagenase gene, *smcol2*, indicated the possibility of it being also a U32 collagenase. Homologous genes in GBS were identified in two *S. agalactiae* strains NEM316 and 2603V/R. Subsequently, primers were designed for the amplification of these genes using genomic DNA as a template. The PCR products obtained were then cloned into an inducible vector system that allowed for strict regulation of recombinant protein expression.

Recombinant clones harboring the genes of interest in the right orientation were obtained and confirmed by PCR analysis, and the optimal recombinant protein expression was achieved by induction with 0.2% arabinose. Expression of the 6xHis-tagged fusion proteins was confirmed by western immunoblot analysis. SDS-PAGE and Western blot analysis of smcol2sp and smcol2wosp indicated that the presence of the signal peptide did not interfere with protein expression. Hence, subsequent cloning of GBS collagenases was conducted without removal of the signal peptide.

As anticipated from His-tagged fusion protein, the purification of recombinant proteins was much facilitated, and pure proteins were obtained and used in determining their enzymatic activity. Previously, gelatinase activity was observed with *C. histolyticum* collagenase (31), *S. mutans* (22) and GBS (23) but not with *P. gingivalis* prtC collagenase (27). In the present study, gelatinase activity was demonstrated for pure recombinant *S. mutans* and GBS recombinant enzymes, thus indicating that these enzymes were not serine proteases like trypsin as they were not inhibited by PMSF. Since prtC collagenase was reported not to degrade gelatin, the observation of gelatinase activity in *S. mutans* and GBS enzymes denoted that members of the U32 family of peptidases/collagenases were heterogeneous.

It was shown that these three enzymes, *smcol2, gbscol1* and *gbscol2*, had high degree of homology with other bacterial protease, most being U32 peptidases and collagenases (Tables 4, 5 and 6). Based on the ProtoMap database (46) all three enzymes were classified as members of the cluster, 1872. This cluster contains 24 members, with 13 containing the Peptidase family U32 signature It was also found that the European Molecular Biology Laboratory (EMBL) database (1) grouped these proteins into family ENZYME: 3.4.-.- (E.C. 3.4), which are peptide hydrolases, acting on peptide bonds. This family contains other well known collagenases from *Vibrio alginolyticus* and *C. histolyticum*.

Complete degradation of blue collagen by *C. histolyticum* collagenase and the lack of digestion by trypsin demonstrated that the blue collagen substrate prepared in our laboratory was useful in determining true collagenase activity.

Generation of smaller fragments upon incubation with the recombinant proteins indicated the presence of enzymes degrading partially the blue collagen substrate, even when the incubation time was extended to 72 hours. Only the smcol2sp and gbscol2 had the ability to degrade completely some of the collagen. This apparent difference may be due to the fact that the *C. histolyticum* collagenase sample contained a mixture of collagenases and proteases, or that the 6x His-Tag might interfere with collagenase activity. Interestingly, smcol2sp protein showed some complete collagenase activity, but not the smcol2wosp. Considering that collagenase activity was observed with *P. gingivalis* bacteria, but not with purified prtC enzyme, and that collagenase activity involved two enzymes (20), it is hypothesized that perhaps the same holds true for the *S. mutans* and GBS col1 and col2 enzymes.

Prior to the present study, a major obstacle was encountered due to the lack of a true collagenase assay. Synthetic peptide substrate, acid soluble collagen and denatured collagen used in commercially available collagenase assay kits were found not to be specific for collagenase as none contained the typical triple helix of native type I collagen (17). By using the specific blue collagenase assay developed in our laboratory, we were able to observe complete and incomplete collagen degradation activity by col1 and col2 in *S. mutans* and GBS. Since the recombinant enzymes contained the signal peptide and the 6xHis-Tag, it is not possible to extrapolate the data to the native enzymes, which may very well be more active than the recombinant fusion proteins. Nevertheless, the data obtained in the present study already showed that the *S. mutans* and GBS

collagenases, col1 and col2, had both collagenase and gelatinase activity, and that they appeared to be distinct from both *C. histolyticum* collagenase and *P. gingivalis* collagenase. Indeed, genetic analysis indicated that *smcol1*, *smcol2, gbscol1* and *gbscol2* had essentially no homology to *C. histolyticum* collagenases and moderate homology to *P. gingivalis* prtC enzymes. This is in agreement with the distinct differences between bacterial Zn-metalloproteases and U32 peptidases/collagenases, and the heterogeneity among members of the U32 family.

In conclusion, the work presented herein has significantly added to our understanding of *S. mutans* and GBS in dental root decay and premature rupture of the amniochorionic membrane, and provided the direction for future studies. Work is already underway in our laboratory by other members of our research team to inactivate *smcol1, smcol2, gbscol1* and *gbscol2* by allelic exchange in *S. mutans* and GBS to probe whether or not both enzymes are needed for collagenase expression in the respective bacteria, to clone these genes into a vector that will allow the purification of native enzymes for in vitro studies, and to analyze collagenase expression as a function of growth state, planktonic versus biofilms and to identify genes that are similarly regulated by microarray analysis.

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