Scholar Commons
USF University of South Florida

University of South Florida
Scholar Commons

Graduate Theses and Dissertations | Graduate School

2004

Detection, cloning, and analysis of a u32 collagenase in streptococcus mutans gs-5

Marios Ioannides
University of South Florida

Follow this and additional works at: http://scholarcommons.usf.edu/etd

 Part of the American Studies Commons

Scholar Commons Citation

Ioannides, Marios, "Detection, cloning, and analysis of a u32 collagenase in streptococcus mutans gs-5" (2004). *Graduate Theses and Dissertations.*
http://scholarcommons.usf.edu/etd/1090

This Thesis is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.

Detection, Cloning, and Analysis of a U32 Collagenase in *Streptococcus mutans* GS-5

by

Marios Ioannides

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Biology
College of Arts and Sciences
University of South Florida

Major Professor: My Lien Dao, Ph.D.
Daniel Lim, Ph.D.
Andrew Cannons, Ph.D.

Date of Approval:
July 2, 2004

Keywords: gelatinase, metalloproteinase, collagen, U32 peptidase, dental root decay

© Copyright 2004, Marios Ioannides

AKNOWLEDGMENTS

I would like to express my gratitude to my advisor, Dr. My Lien Dao, for her encouragement, enthusiastic spirit, and guidance during my graduate studies, especially for helping me discover my strength and develop my research skills. I would also like to thank the members of my committee, Dr. Daniel Lim and Dr. Andrew Cannons, for their valuable advice and words of encouragement.

I would like to thank my colleagues, Thomas Han, Ross Myers, Valerie Carson, and Justin Federico for their help, positive interaction, and especially for making the laboratory a friendly, energetic, and comfortable environment for productive research.

I would like to thank my grandparents and especially my parents for their love, confidence in me, and for their enormous emotional and financial sacrifice in the last seven years in order to send me abroad and offer me a better education. My gratitude is endless and I am blessed to have them in my life.

Last but not least, I would like to thank my girlfriend, Juliana Saavedra, for her friendship, encouragement, and understanding during the last four years of my studies. She has been a source of inspiration to me, and with her everything seems to be so much easier and within reach.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Detection, Cloning, and Analysis of a U32 Collagenase in *Streptococcus mutans* GS-5

Marios Ioannides

ABSTRACT

Streptococcus mutans is a recognized principal etiologic agent in coronal caries. Although *S. mutans* has the ability to bind collagen and degrade FALGPA, a synthetic peptide mimicking collagen substrate, its role in dental root caries has not yet been fully elucidated. Degradation of collagen fibrils in dentin was attributed to *S. mutans*, but a collagenase enzyme has not yet been isolated from this organism. Considering the increased incidence of dental root decay among the elderly, an understanding of the role of the pathogenic factors is necessary to the development of preventive measures. The present study has focused on the cloning and analysis of *S. mutans* collagenase enzyme. Toward this goal, a putative collagenase gene was identified in *S. mutans* UA159 by genomic analysis and a primer set was designed and used to amplify the corresponding gene in *S. mutans* GS-5 used as a model organism. The PCR product was cloned into the vector pCR 2.1 TOPO-TA, and the gene sequenced and analyzed. Alignment of the *S. mutans* GS-5 and UA159 putative collagenase genes showed 99% homology. The gene was next cloned in frame into the inducible expression vector pET100/D TOPO. Induction and expression of recombinant protein in *E. coli* were confirmed by SDS-PAGE and Western

immunoblotting, while biochemical analysis indicated that it was a calcium-dependent metalloproteinase. Enzyme analysis of the recombinant enzyme showed both gelatinolytic and collagenolytic activity. Further analysis of the GS-5 gene using databases such as ExPASy, Pfam, and SMART indicated that it was highly homologous to the U32 peptidase family, which includes the PrtC collagenase of *Porphyromonas gingivalis,* a bacterium causing periodontitis. The present study was the first to unequivocally demonstrate the existence of a collagenase gene in *S. mutans*, and to identify it as a member of the U32 peptidase family. The obtaining of the *S. mutans* collagenase gene should help in further investigation of the role of this enzyme in dental root decay and its potential use as a dental root caries vaccine.

INTRODUCTION

Dental root decay is an infectious disease, with high prevalence in older individuals worldwide (19). It has been shown that nearly two thirds of tested elderly people experienced root caries, with 23% having untreated root caries (48). Nowadays, with the high technological advances, the excellent health care, and dental health awareness, there is a need for the populations to retain their teeth much longer than ever before (38, 46). Thus, an adequate understanding of the pathology of oral diseases is essential in order to devise preventive measures and to insure the quality of life in aging populations.

Dental root caries is initiated on root surfaces that have been exposed to the oral environment due to gum recession (19). The etiology of the gingival recession varies from aging as the leading factor, to trauma. It has been shown that gingival recession is a frequent phenomenon in people aged 30 years and older with a 2.8- fold increase in root caries in old individuals (8,37). Other factors associated with gum recession include anatomical factors or deficiencies, such as loss of alveolar bone, tooth shape, and abnormal tooth position. Furthermore, trauma resulting from vigorous toothbrushing and operating procedures could often be related to gum recession (37). The now exposed root is susceptible to microbial attack (42), which, if not taken care by professionals, can result in the infection of root canal and ultimately in the potential loss of the tooth (48). The factors involving the etiology and process of dental root decay,

even though similar to coronal caries, seems to be more complicated because of higher susceptibility of the exposed dentin, the main structural component of tooth root (14). Dentin, which lacks the highly mineralized enamel present in the crown, is composed of an inorganic material (hydroxyapatite) (14) and an organic matrix 90% of which is type I collagen and 10% non-collagenous proteins (14,33). Furthermore, the susceptible root caries involve two additional factors: the presence of bacteria and fermentable carbohydrates (8).

In accordance to a number of studies, the microorganisms isolated from root caries lesions include predominately *Streptococcus* spp., *Actinomyces* spp., *Lactobacillus* spp. and yeast (14, 42). While both root and coronal caries involve demineralization of the dentin and enamel respectively, the rate of this process differs substantially. In root caries loss of dentin minerals occurs twice as rapidly as compared to the enamel demineralization at higher pH, since the crown enamel has almost twice as much minerals (8). As mentioned before, due to gingival recession, the dentin is exposed to the oral microbial environment. Dung *et al.* (14), in their review about the molecular pathogenesis of root caries, proposed a possible mechanism of the disease process. Production of acid, after fermentation of certain carbohydrates by oral bacteria, such as sucrose, results in the demineralization of the exposed dentin. This is followed by another oral bacterial invasion involving proteolytic degradation of dentin's collagen, mainly by collagenases. Furthermore, according to the same author, collagenolytic activity of acid-demineralized dentin is enhanced by the addition of trypsin. It was also suggested that acid pretreatment may cause a degradation

of collagen by trypsin and other non-specific proteases. Another *in vitro* investigation (29) involving a pH cycling model, which closely mimics a demineralization/remineriläztion process in the oral environment, suggested that the organic matrix is attacked during remineralization and predominately after demineralization of the dentin. Studies by Dung *et al.* (15) and van Strijp *et al. (*47), showed that proteases, such as collagenases or gelatinases present in saliva, tissue, and dentin may be activated either by the presence of bacteria or pH fluctuation in the oral environment. These host derived proteases may also play an important role in root dentin collagen degradation. Even though all studies are in agreement that demineralization of dentin and degradation of its organic matrix is a pre requisite for the pathogenesis of root decay, the exact mechanism of this multifunctional disease is yet to be investigated.

Collagen is the major fibrous component of animal extracellular connective tissues: skin, tendon, blood vessels, bone, and dentin. To date at least 19 types of collagen have been characterized (21). Dentin collagen -type I- consists of two identical α1 and a different α2 chain (49), with the repeating sequence Gly-X-Y present in all three, with X and Y often being proline and hydroxyproline respectively. These 100kDa fibrous proteins are laterally aggregated and twist around a common axis to form the triple helical structure of collagen. Stability of this structure is conferred to the “steric repulsion of the proline and hydroxyproline residues” (43) as well as the covalent cross-linkages, mineral coating, and interaction of collagen with non-collagen proteins (14). Disruption of inter- and intra- molecular linkages of collagen molecule results in the dispersal

of the polypeptide chains and in the production of gelatin (21). In this state, the strands are vulnerable to cleavage by a wide variety of nonspecific proteases.

Collagen degradation is a result of a group of enzymes called collagenases. These enzymes digest the peptide bonds of fibrous collagen in the triple helix region, under physiological conditions and pH (21). Mammalian collagenases produced by tissue resident cells, macrophages, or neutrophils, aid in tissue regeneration and remodeling as well as in immune responses against invasion of host tissue pathogens (12,40). As they are responsible for degradation of extracellular matrix, they belong to the family of matrix metalloproteinases (MMP). Activity and stability of these proteases depend on the presence of Zn^{2+} and Ca^{2+} (9, 12). Moreover, invading microorganisms may also have the ability to produce collagenases. Unlike mammalian collagenases that attack their substrate at specific regions producing two distinct fragments (21), bacterial collagenases possess broad substrate specificities and can degrade both native and denatured collagens. Over the last few years many microorganisms have been reported to produce enzymes that degrade collagen. Among them are some that are associated with human diseases, thus identifying the presence of the enzyme as a possible virulence factor.

Much of the knowledge of bacterial collagenases has come from studies involving the enzyme from *Clostridium histolyticum.* This Gram-positive, spore forming, anaerobic organism has been associated with progression of myonecrosis (54). It has been shown to produce up to seven different collagenases/gelatinases, ranging from 68kDa to 130kDa (34). Investigations

though, have been concentrated mainly on the 116kDa collagenase (ColH). Initially, cloning and expression of the *colH* gene revealed that the 116kDa collagenase successfully degraded Pz-peptide (54), a synthetic peptide analog to collagen. Interestingly, zymography of the partially purified collagenase revealed the presence of two gelatinases (116kDa, 98kDa). Sequence analysis of the two indicated that the 98kDa gelatinase is a result of the C-terminal cleavage of ColH, finding which suggests that the C terminal of the enzyme is responsible for binding or accessing the native collagen (54). In accordance of many research groups (27, 54), ColH was found to be a zinc metalloproteinase containing the consensus sequence HEXXH that corresponds to the zinc-binding site and the catalytic site (27). Mutations of the codons at the catalytic site (histidine residues) resulted in the abolishment of zinc binding as well as catalytic activity of the enzyme, result that confirmed that zinc is required for enzyme activity (27). Matsushita *et al.* (35), in an effort to characterize a 78kDa gelatinase in *C. histolyticum,* showed that there is another gene, *colG,* which also confers collagenolytic activity. The 116kDa enzyme, initially produced as an inactive pro-enzyme, was isolated from the culture supernatant and able to hydrolyze the Pz-peptide, insoluble collagen, as well as gelatin (34, 35). Results from hybridization experiments and sequence analyses of the two genes suggested that *colH* and *colG* are a single copy genes; thus, the presence of the seven different collagenases/gelatinases are due the proteolytic cleavage of the C- terminus of either one the genes resulting in the multiplicity of collagenases (35).

Clostridium perfringens is another tissue invasive pathogen, able to degrade collagen (2, 36). The 120kDa enzyme (ColA) was able to degrade the Pz-peptide, as well as type I fibrils. Analysis of the deduced aminoacid sequence of the *colA* gene revealed great similarities with the products of *colH* and *colG* genes found in *C. histolyticum*, having a greater degree of resemblance with the *colG* (35,36,54). A Pz-peptide sequence as well as the zinc-binding motif were also identified, with the former probably being a signal sequence for self processing of the inactive pro-enzyme (36). Notable is the fact that all three collagenases ColA, ColH, and ColG, showed to have high homology with the *Vibrio alginolyticus* collagenase (34,35,36,54). Alignment of the aminoacid sequences indicated the presence of the HEXXH motif. Also, similarities of the proposed segmental enzyme structures suggested that the three organisms have a common ancestral collagenase gene, which was then diverged and duplicated in each descendant (34, 35).

The PrtC collagenase, produced by the periodontal pathogen *Porphyromonas gingivalis*, is another well studied proteolytic enzyme. Unlike the previously mentioned homologues, this enzyme does not possess the zinc-binding motif. Instead, it is a member of U32-peptidase family, with a conserved catalytic domain yet to be characterized. Kato *et al*. (28) successfully cloned and characterized the *prtC* gene. SDS-PAGE showed a 35kDa enzyme, but in the active form the enzyme acts as a dimer as shown by gel filtration assays. According to the same research group, the enzyme has no significant similarities with its clostridial counterparts. It neither hydrolyzed gelatin nor the Pz-peptide,

while it degraded azocoll, heat denatured type I collagen, as well as fibrillar reconstituted type I collagen (28). The presence of Zn^{2+} and Fe^{3+} inhibited the enzyme's activity. The metal chelator EDTA also inhibited collagen degradation, but there was no effect on the activity when the enzyme was incubated with PMSF (28). On the other hand, Ca^{2+} stimulated the activity of the purified protein. Furthermore, Houle *et al.* (23) used three different assays using fluorescein-labeled Type I collagen and two cysteine proteinases mutants (Arg- and Lys-gingipapains) as substrates, in order to test for the collagenolytic activity of *P. gingivalis*. Their results demonstrated that the ability of the organism to hydrolyze native collagen resides mainly in the presence of Arg- gingipapain. One possible reason for the contradictory results of Kato and Houle is the heterogeneity of the *prtC* gene. Indeed, PCR and sequence analysis performed on six clinical isolates by Wittstock *et al.* (51) showed that none of the sequences agreed with that of the *prtC* gene reported by Kato, and each one had distinct differences as compared to each other. Further clarification regarding the function and role of *prtC* gene is necessary.

Vibrio parahemolyticus is a human pathogen that produces collagenase (PrtV). Cloning and expression of the gene showed activity against type I, II, III, and IV (55, 56) collagen. The 62 kDa protein is highly hydrophobic and active at temperature as high as 40^{o} C. EDTA and 1,10-phenanthroline inhibit the activity of the enzyme (55). This is also a Zn-metalloproteinase, but double immunodiffusion with antibodies against PrtV showed no similarities between the other two zinc-metalloproteinases of *Vibrio alginolyticus* and ColH (56).

Collagenolytic activity was also detected in Group B streptococci. This group of organisms has been associated with neonatal diseases and premature rupture of the amniochorionic membrane, probably due to the presence of collagenases (25). Jackson *et al.* showed that the cell-associated enzyme was able to degrade the synthetic peptide analogue to collagen, FALGPA, as well as to migrate through the placental tissue *in vitro*. Inhibition assays also revealed that EDTA and 1,10-phenanthroline inhibited the enzyme activity suggesting that GBS collagenase also needs a metal co-factor for its activity (25). Additionally, cross reactivity with anti-clostridial antibodies as well as the presence of gelatinolytic activity of the enzyme further supported the notion that GBS collagenase is closely related to ColH (25).

Streptococcus mutans, has been established by many research groups as an oral pathogen, especially in relation to dental caries (5, 29). In particular, *S. mutans* serotype c strains are most frequently isolated from the human oral cavity (29). This acidouric organism has the ability to produce lactic acid while attached tightly on tooth surfaces (29, 53). A number of proteins and enzymes , including glucosyltranferases, glucan binding proteins, and wall associated proteins, have been extensively studied and their role in the colonization of the tooth and the induction of oral diseases has been established. Directed mutagenesis by Qian and Dao (39) demonstrated that the wall-associated protein A (WapA) is important in sucrose depended aggregation and binding of the organism to smooth surfaces. Another important factor that promotes the accumulation of *S. mutans* on the tooth surface is the production of glucan from

sucrose via glucosyltranferases (GTFs) (29, 45). There are three different types of GTFs based on their ability to produce water-soluble or water insoluble glucans (29, 45). GTFs, besides producing glucans, also have the ability to bind these glucose polymers (45), and consequently promote colonization of the tooth and built up of dental plaque. In the same line, *S. mutans* produces three non-enzymatic glucan binding proteins, GBPA, GBPB, and GBPC (29). Once the organism establishes itself on the tooth surface, the tooth enamel and the exposed dentin in the root are demineralized by acid production. This gives the organism even more opportunities to colonize and to further invade the tooth, through binding and degradation of collagen. It is noteworthy that immunization with a single antigen only provides a decrease in caries score, while inactivation of a single gene in *S. mutans* can only cause a decrease in virulence. These results indicate that dental caries involves multiple virulence factors.

Furthermore, *S. mutans* is considered to be one of the major pathogenic microorganisms isolated from root caries (8, 14, 29), but its role in the pathogenesis of the disease is not yet clear. Studies on the microbiology of root caries by Shen *et* al. (42) demonstrated that *S. mutans* was isolated in high numbers, implying the importance of this pathogen to the disease. Another research group suggested that the high prevalence of *S. mutans* from patients who previously received periodontal treatment may be of importance in the development of root caries in these patients (5). Several reports in the literature (5, 32) have proved that the cell surface antigen I/II is necessary for the initial adherence of the organism on collagen type I and dentin tubules. Isogenic

mutants showed reduction in binding of cells, suggesting that antigen I/II may be important in the process of dental or root caries. In favor of this, two *S. mutans* isolates were shown to have significantly high specific affinity ratio to type I collagen (33). This was in support with the finding of Switalski *et al*. (44) who demonstrated that *S. mutans* isolated from adult patients had the ability to bind collagen type I independently of the environmental conditions and changes in pH. In addition to collagen-binding properties *S. mutans* also has the ability to invade host tissues (31). Yet, *S. mutans* collagenolytic activity is still in the early stages of investigation. Initially, human isolates of the bacterium have been shown to cause extensive loss of bone and the breakdown of periodontal ligament in gnotobiotic rats (21). Harrington *et al*. (22) also demonstrated that the organism possesses collagenolytic activity by describing two extracellular proteases (50 and 52kDa) that are capable of hydrolyzing PZ-PLGPA and azocoll. Later, Jackson *et al*. (24) discovered that *S. mutans* also expressed a cell-associated collagenolytic enzyme that degraded the synthetic collagen peptide FALGPA, and that the organism could migrate through placental tissue *in vitro*. In the same work it was demonstrated that the collagenolytic activity of *S. mutans* was inhibited by EDTA, and 1,10-phenanthroline. Gelatin zymogram analysis showed the ability of the organism to hydrolyze gelatin (22,24), while immunoblot analysis of *S. mutans* proteins indicated that it cross reacted with polyclonal rabbit anti-*C. histolyticum* collagenase antiserum.

Previously in Dr. Dao's laboratory, a 4020bp contig sequence was obtained from the Oklahoma Genome Center *S. mutans* sequencing project

database. BLAST search showed sequence similarities with other putative collagenases. Submission of the contig sequence to the Open Reading Frame Finder (OPR Finder) at the NCBI, revealed an open reading frame of 1287 bp that encoded for 428 amino acid protein. PCR amplification, using a set of primers that flanked the ORF of the gene, produced a 1.5 kb fragment but failed to show expression and activity of the enzyme when cloned into pCR2.1-TOPO. Additionally, immunoscreening of *S. mutans* GS-5 library with a rabbit anti *C. histolyticum* collagenase gave false positive results due to the cross reaction of antibodies with epitopes present on unrelated proteins (Seijo, Biology M.S. Thesis, USF, Tampa, FL).

In the present study, the ORF of *S. mutans* GS-5 collagenase (*smcol*) was amplified by PCR and the gene was first cloned into pCR2.1-TOPO for sequence analysis. Primers were then designed to amplify *smcol* from the *S. mutans* genomic DNA directly in order to clone the gene in-frame into the inducible pET100/D-TOPO expression vector. Expression of collagenase and gelatinase activity in the recombinant *E. coli* was demonstrated.

MATERIALS AND METHODS

Chemicals and Reagents

Restriction endonucleases and PCR reagents were purchased from Promega Inc. (Madison, WI) and used according to the manufacturer's protocol. Cloning vectors pCR2.1-TOPO TA and pET100/D TOPO were from Invitrogen (Carlsbad, CA). PCR primers were obtained from Qiagen (Valencia, CA). All other chemicals and reagents were purchased from Sigma Aldrich Chemical Company (St. Louis, MO) or Fisher Scientific (Pittsburg, PA) unless otherwise specified.

Bacterial Strains and Growth Conditions

S. mutans GS-5 serotype c, originally obtained from J. J. Ferretti (University of Oklahoma Health Sciences Center, Oklahoma City, OK), was cultured in brain heart infusion broth (BHI obtained from Difco, Detroit, MI) at 37^{o}C with 5% CO_2. Competent *E. coli* Top 10 was obtained from Invitrogen and used for the cloning of recombinant plasmids. Competent *E. coli* BL21 Star (DE3) (Invitrogen) was used for the cloning and expression of the recombinant *S. mutans* collagenase (*smcol*) gene. LB medium containing Ampicillin added at 100 μg per ml (LB-amp) was used for the selection of transformants and culture of recombinant *E. coli*. Plates were prepared by adding agar at 1.5%.

Plasmid Isolation

Plasmid isolation was performed by the alkaline lysis procedure using the QIAprep Miniprep™ kit and protocol from Qiagen Inc (Valencia, CA). Briefly, 1ml of an overnight culture was centrifuged at 10,000 X g and the pellet was resuspended in 250µl of 50mM Tris.Cl (pH 8) buffer containing 10 mM EDTA and 0.1mg/ml of RNase A. Following a sequential addition of 250µl of 200mM NaOH containing 1% SDS solution and 350µl of 3M Potassium Acetate, the sample was mixed gently and centrifuged for 10 minutes. The supernatant was carefully applied to the QIAprep column (Qiagen) and centrifuged briefly. The column was washed with 0.5ml of Guanidine and isopropanol solution followed by 0.75ml of 80% ethanol. Finally, the plasmid DNA was eluted by the addition of 50µl of 10mM Tris.Cl (pH 8.5), and centrifuged for 1 minute at maximum speed in a microcentrifuge. The plasmid DNA obtained was measured in a SmartSpec Plus spectrophotometer (Bio-Rad, Richmond, CA) in order to determine concentration and purity ($OD_{260/280}$ > 1.7), and analyzed by electrophoresis in a 1% agarose gel containing ethidium bromide.

Isolation of Genomic DNA from *S. mutans* GS-5

Genomic DNA isolation was performed using the Wizard™ Genomic Isolation Kit obtained from Promega and according to the manufacturer's protocol. Briefly, a 5 ml overnight culture of *S. mutans* GS-5 was aliquoted in 1.5 ml centrifuge tubes and centrifuged at 13,000 X g for 2 minutes. The supernatant was discarded and the pellet was resuspended in 480µl of EDTA

and 100 μl of 10mg/ml lysozyme. The sample was centrifuged for 2 minutes at 13,000 X g. The pellet was resuspended in 600μl of in Nuclei Lysis Solution and incubated for 60 minutes at 80°C. Three μl of RNase solution were added and the sample was mixed and incubated for 15 minute at 37°C. Next, the sample was treated with 200μl of Protein Precipitation Solution and vortex-mixed at maximum speed in a microcentrifuge for 20 seconds. After incubation on ice for 5 minutes and subsequent centrifugation for 3 minutes the supernatant was transferred to a new microcentrifuge tube containing 600μl of room temperature isopropanol. The sample was then mixed and centrifuged for 2 minutes at 13,000 X g. The pellet was washed with 70% ethanol and centrifuged again for 2 minutes. Finally, the pellet was air-dried and resuspended in 100μl of autoclave-sterilized distilled water. Concentration of DNA was determined by measurement in the SmartSpec Plus spectrophotometer (Bio Rad) and the $OD_{260/280}$ of 1.7 or higher was indicative of purity.

PCR Amplification of Genomic DNA

Direct PCR amplification was performed on *S. mutans* genomic DNA for subsequent cloning and expression of the *smcol* gene. For TOPO TA cloning, 2X PCR Master Mix (Promega) was used. *Taq* polymerase and dNTPs were used at a final concentration of 1.25 U and 200μM respectively. The 1.2kb fragment (*smcol*) was amplified using 0.2μM of the primers F1: ATGACAAAACAATTAAAACG and R1: TAAGTTCTAACAGTAAGGC under the following amplification profile: Initial denaturation at 95°C for 1 minute, 30 cycles

each consisting of a denaturation step at 95 ^{o}C for 1 minute, an annealing step at 50 ^{o}C for 1 minute, and extension step at 72 ^{o}C for 1 minute and a final extension at 72 ^{o}C for 7 minutes. The PCR product flanked by dA overhangs was cloned into the TOPO TA cloning vector and used to transform *E. coli* Top 10 as described below. The plasmid containing the *smcol* gene inserted into the TOPO TA vector was isolated from the recombinant *E. coli* and designated *smcol*-TA.

The PCR reaction mixture for amplification of the *smcol* gene for cloning into the pET100/D-TOPO vector (*smcol*-pET), as described below, consisted of the following: 2.5 U of *Pfu* Turbo Cx hotstart DNA polymerase (Stratagene, La Jolla, CA), 25ng of DNA template, 0.2μM of each of the following primers : F2:CACCATGACAAAACAATTAAAACG and R1:TTAAGTTCTAACAGTAAGGC 200μM dNTPs, and 1X reaction buffer (Stratagene). An initial denaturation step was performed at 95^{o}C for 2 minutes. Next, the sample was subjected to 30 cycles of the following : Denaturation at 95^{o}C for 30 seconds-Annealing at 50^{o}C for 30 seconds-Extension at 72^{o}C for 30 seconds. After the last cycle the products were extended for another 10 minutes at 72^{o}C. The final PCR products were analyzed by electrophoresis in 1% agarose gel, and DNA was stained with ethidium bromide for observation under UV light.

Cloning of the 1.2 Kb PCR Product into pCR 2.1 -TOPO TA Vector

The *smcol* fragment obtained by PCR amplification was cloned into the pCR2.1-TOPO vector. Briefly, 3μl of fresh PCR products were mixed with 1μl of TOPO vector and 2μl of sterile distilled water. 2μl of the above cloning reaction

were used to transform 250µl of *E. coli* Top 10 cells, using the heat shock method. The cells were mixed with the insert, incubated on ice for 30 minutes and then heat-shocked for 30 seconds at 42^{o}C. Then, 250µl of room temperature of SOC medium (Invitrogen) was added to the tube and incubated at 37^{o}C with shaking for an additional 1 hour. Finally, 25µl and 50µl of the transformed cells were spread on pre-warmed LB-amp agar plates. Due to the presence of the *Lac*Z*a* fragment in the vector for easy blue/white colony screening, the identification of recombinant *E. coli* was performed by adding 40µl of 40mg/ml X-gal on the LB plate and white colonies were selected. Recombinant plasmid was isolated and analyzed by PCR amplification (using forward M13 primer (Invitrogen) and reverse R1 primer), and restriction digestion by *Eco*RI. Results were analyzed by agarose gel electrophoresis. The gene was also sequenced and the resulting sequence was used as a query for investigation using databases on the Internet.

Subcloning of the *smcol* Gene Into the Expression Vector pET100/D-TOPO

The *smcol* fragment obtained by PCR was cloned into pET100/D-TOPO vector and used to transform competent *E. coli* BL21 (DE3) by the heat shock method as described previously with some modifications: the fresh PCR product was first diluted 1:50 and 4µl of the diluted PCR product were added to 1µl of the vector and 1µl of distilled water. Transformation of *E. coli* was performed a described below. Plasmid was isolated from recombinant *E. coli*, analyzed by agarose gel electrophoresis, and amplified by PCR using a combination of the

following primers: T7 forward and reverse with F2 and R1 primers. Gene sequencing was also performed to make sure that the *smcol* gene was ligated in frame with the vector and in the correct orientation.

Expression of *smcol*-pET

Recombinant *smcol*-pET plasmid (10ng in a 5µl volume) was used to transform 250µl of competent *E. coli* BL21 Star (DE3) by the heat shock method. After 1 hour of incubation at 37^{o}C the transformation reaction was added to 10ml of LB-amp and grown for 16 hours at 37 ^{o}C with shaking. Then, 500 µl or 2.5ml of the overnight culture was added to 10ml or 50ml of LB-amp medium and grown for another 2 hours or until OD_{600} reached 0.5. The culture was then divided into 2 tubes, one of which was induced by the addition of 1mM isopropyl β-D thiogalactoside (IPTG), while the other was used as a non-induced control. Competent cells transformed with empty vector were used as a negative control. From each culture 500µl were removed every hour for 4 hours and analyzed by SDS PAGE.

SDS-PAGE

Samples to be analyzed (Recombinant *E. coli* cells, cell extract or pure enzyme) were separated on a 12% SDS polyacrylamide gel (0.75mm) using a Mini-PROTEAN II electrophoresis cell (Bio-Rad). Separation was performed at 150V for 1h and the gel was stained with Coomassie brilliant blue R-100

overnight. The stain was then removed by incubating the gels for 30 minutes in a destaining solution (Methanol: Acetic acid: H_2O at a ratio of 4:1:5 respectively).

Purification of Recombinant *S. mutans* Collagenase

Smcol was cloned into the pET vector in order to obtain a fusion protein with 6XHis-tag at the N- terminal in order to facilitate its purification by affinity column chromatography using ProBond^{TM} (Invitrogen) resin precharged with Ni^{2+}. Purification was performed under native and hybrid conditions as described by the manufacturer. The pellet from an overnight 50ml culture was harvested and resuspended in 8ml a buffer containing 50mM $NaPO_4$-0.5M NaCl (pH 8). The solution was sonicated on ice using six 10-second bursts at high intensity with a 10-second cooling period between each burst. After 15-minute centrifugation at 3000 X g the supernatant was removed and purified under native conditions. In order to recover the insoluble proteins the pellet was purified under hybrid conditions. Briefly, the pellet was resuspended in 8ml of Guanidinium Lysis buffer (6M Guanidine HCl, 20mM NaPO4, 500mM NaCl, pH 7.8), sonicated on ice with three 5-second pulses at high intensity and centrifuged for 15 minutes. The supernatant was then used for column purification.

Columns were prepared according to the respective sample (soluble or insoluble). Initially, the resin was washed with sterile water followed by a Native Binding Buffer wash (50mM $NaPO_4$, 0.5M NaCl, 10mM imidazole, pH 8) or Denaturing Binding Buffer wash (8M Urea, 20mM NaPO4, 500mM NaCl, pH 7.8). The supernatant containing the soluble proteins was applied onto the column and

allowed to bind for 1 hour on a rotating wheel. The supernatant was then aspirated and the resin was washed three times with Native Binding Buffer (50mM $NaPO_4$, 0.5M NaCl) and used to pack a column. The soluble protein was collected in 1ml fractions after the addition of 8ml of Native Elution Buffer.

Sample containing insoluble proteins was added to the column and allowed to bind for 30 minutes. Unbound proteins were removed and the resin was washed sequentially with a Denaturing Binding Buffer (pH 7.8) and twice with a Denaturing Wash Buffer (pH 6). The resin was then resuspended four times in Native Wash Buffer and used to pack a column. Elution and collection of the protein was as above. Purified lysate of *E. coli* BL21 transformed with an empty vector served as the negative control. The samples obtained were analyzed by SDS-PAGE.

Western Blot Analysis

Western immunoblot was performed on cell lysate samples containing collagenase and on the purified recombinant protein obtained under hybrid conditions. Proteins were separated by SDS-PAGE as previously described. Transfer of protein bands to nitrocellulose was carried out overnight at 30V in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membrane was blocked for 1 hour in 10ml blocking buffer (10% Dry milk in PBST) and washed twice with PBST (0.05% Tween-20 in PBS). The membrane was then incubated for 1 hour with anti-HisG (Invitrogen) diluted 1: 5,000 in blocking buffer and washed twice with PBST at room temperature. Finally, the membrane was

incubated for 1 hour with rabbit anti-mouse IgG (Sigma) diluted 1:30,000 in blocking buffer at room temperature. Excess conjugate was removed by two washes with PBST. The band development was performed according to Dao's method (11) by incubating the membrane 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.

Sample Preparation for Collagenase Assay

Cell lysates used for collagen assay were prepared as followed: A pellet from centrifugation (8000 X g for 10 min) of 10ml culture was resuspended in lysis buffer and freeze-thawed 4 times on dry ice and 42^{o}C water bath respectively. The lysates were then centrifuged at 8000 X g for 20 minutes to remove intact cells, cellular debris, and cell membranes. 100µl of the lysate was used for collagenase assay. To determine collagenase activity in whole recombinant *E. coli* cells, 10ml culture were centrifuged at 800 X g for 10 minutes and the pellet obtained was resuspended in 2ml collagen assay reaction buffer. 100µl of the sample was used for the assay. Before readings were taken, the reaction mixture was centrifuged briefly for 10 seconds.

Collagenase Assay

Collagenase activity was measured using the EnzChek^{TM} Collagenase Assay Kit (Molecular Probes Inc., Eugene, OR) as described by the manufacturer's protocol. Each 200µl-reaction mixture contained 80µl of 1X

Reaction Buffer (0.05M Tris-HCl, 0.15M NaCl, 5mM $CaCl_2$, 0.2mM NaN, pH 7.6) and 20µl of DQ collagen in a final concentration of 100µg/ml. Various concentrations of the purified recombinant enzyme were used for the assay (10, 20, 30, or 40 µg of the recombinant enzyme diluted in 1X Reaction Buffer) as well as cell lysates and whole cells. *Clostridium histolyticum* collagenase IV (0.4 U/ml, Molecular Probes Inc.) was used as a positive control , 100µl of reaction buffer were used as a negative blank, and whole cells and cell lysate from *E. coli* BL21 transformed with empty vector were used as negative controls. 10µg of trypsin were used to assess the specificity of the assay. The samples were incubated at room 37^{o}C for 24h and the fluorescence from the digestion products was measured using a fluorometer at excitation and emission wavelengths of 490 and 520nm respectively. Then, the highest concentration of the purified enzyme was incubated for additional 24h in order to see the rate of collagen degradation as compared to the positive control. All experiments were performed in triplicate.

Gelatin Hydrolysis

Determination of gelatinolytic activity was performed initially by gelatin zymography. Samples were incubated at room temperature for 2 hours with 2X Sample Buffer (1.25M Tris-HCl, pH 6.8, Glycerol, 2.5% SDS, 0.005% Bromophenol Blue), without heating, and under non-reducing condition (without the addition of β-mercaptoethanol) and then applied in ready made 10% polyacrylamide gels containing 0.1% gelatin (Bio-Rad). The electrophoresis was performed at 125V for 1 hour and the gels were incubated with 2.5% Triton X-

100 (Renaturing Buffer) for 30 minutes at 25°C, and then overnight (16h) at 37°C with a buffer containing 50mM Tris-HCl, 0.2M NaCl, 5mM $CaCl_2$, and 0.02% Brij 35. Finally, the gels were stained with 0.5% Coomassie Blue R-250 and destained with the destaining solution. Areas of gelatinase activity appeared as clear bands against a blue background. The samples tested included purified recombinant collagenase, IPTG induced *E. coli* BL21 whole cells containing the *smcol*-pET, *C. histolyticum* collagenase as a positive control, and *E. coli* BL21 transformed with empty vector as a negative control. Additionally, 40µg of the purified enzyme was incubated for 24h at 37°C using 20µl/ml DQ gelatin. The same controls used in the collagen assay were also tested in parallel. All experiments were performed in triplicate.

Collagenase Inhibition Assay

Collagenase activity was assayed as described above but in the presence of the following known protease inhibitors: 10mM of EDTA, 10mM of 1,10-phenanthroline, or 2mM PMSF. Incubation was at 37°C for 48h. Release of fluorescence was recorded and compared to that obtained in the absence of protease inhibitor. All experiments were performed in triplicate**.**

Protein Concentration

Protein concentration was determined by the method of Bradford (6). A serial dilution of BSA was prepared and assayed in parallel with the test samples. After addition of the Dye Reagent Concentrate (Bio Rad) to the samples at the

concentration specified by the manufacturer the absorption at OD_{595} was recorded. Values obtained with the BSA were used to establish a standard curve from which the protein concentration of the test samples was derived.

DNA Sequencing and Sequence Analysis

DNA sequencing of recombinant plasmids was performed by the Molecular Biology Core Facility at the H. Lee Moffitt Cancer Center & Research Institute (Tampa, FL). The sequence of *smcol*-TA and *smcol*-pET were used as a query for various databases on the internet. The sequence obtained from cloning of the gene in pCR2.1-TOPO was initially compared to the *S. mutans* UA 159, (Oklahoma Genome Center) using BLAST2seq database (1). Additionally, other databases such as SWISSPROT, PROTOMAP, DAS (10,16,17) gave information about conserved sequences of the deduced aminoacid sequence**,** expected molecular weight and potential transmembrane regions.

RESULTS

PCR Amplification of *S. mutans* Collagenase Gene and Cloning into pCR 2.1 TOPO-TA Vector

Following genomic isolation of *S. mutan*s GS-5, the gene of interest (*smcol*) was amplified by PCR. Custom primers were designed based on the DNA sequence of putative *S. mutans* UA159 collagenase (Oklahoma Genome Center). The aim was to amplify the gene from the start codon (ATG) through the stop codon (TAA). Once the 1287bp amplicon was obtained and confirmed by agarose gel electrophoresis (Fig. 1), the gene was cloned into pCR2.1-TOPO TA vector for sequence analysis. TOPO TA vectors (Invitrogen) employ an easy and efficient way to clone PCR products. The single deoxyribose adenosine (dA) overhangs added by *Taq* polymerase to the 3' end of PCR products, are complementary to the 3' deoxyribose thymidine (dT) overhangs of the linearized vector, thus binding spontaneously the PCR product and the vector. Additionally, the vector is conjugated with Topoisomerase I, an enzyme that cleaves the phosphodiester bonds at specific sites on the vector backbone. This creates a high energy covalent bond between the phosphate residue of the cleaved DNA and the enzyme (tyrosine molecule). This energy is released when the newly inserted DNA attacks the bond, thus reversing the reaction by ligating the inserted gene into the vector and releasing the Topoisomerase.

Positive clones were selected on the basis of blue/white screening. White color colonies were an indication of positive clones, because of interruption of the *LacZ* gene by the inserted gene. On the other hand, blue colonies suggested that the gene was not present in the specific clone. 10 white colonies were selected and analyzed for the presence of the collagenase gene. The cloning efficiency was as high as 80%. Only one positive clone was used for further analyses.

After plasmid isolation, the recombinant plasmid (*smcol*-TA) was subjected to restriction enzyme digestion by *Eco*RI and PCR analysis (Fig. 2). As anticipated, restriction enzyme digestion gave two distinct bands around 3.9 kb and 1.2 kb (Fig. 2A) corresponding to the linearized vector and the *smcol* insert, respectively. PCR amplification using 20 ng of the *smcol*-TA as a template and M13-complementary forward primer (vector) and c*ol*-complementary R1 reverse primer confirmed these results as a single discrete band of approximately 1.3 kb was obtained (Fig. 2B)

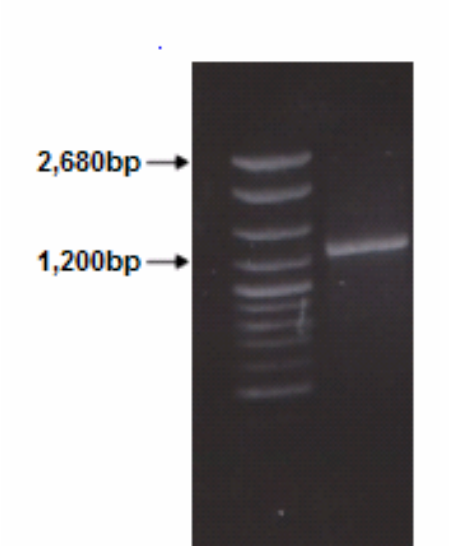


Figure 1. PCR amplification of the putative collagenase gene of *S. mutans* GS-5. Agarose gel electrophoresis of the PCR product showed a DNA band of approximately 1.2 kb.

Sequence Analysis of *smcol*-TA

The sequence of *smcol*-TA was obtained and analyzed. Alignment of *smcol* sequence with that of the corresponding gene in UA 159 strain showed 99% homology (Fig. 3). There were a total of 4 nucleotide variations that resulted in three amino acid differences and one silent mutation. Furthermore, the deduced amino acid sequence of the gene was used as a query for sequence analyses. Alignment using the TBLASTX database, showed high similarity of the protein with other proteases, predominately collagenases, including the PrtC collagenase from the periodontopathogen *P. gingivalis*. These preliminary results open the way for further investigation of the *S. mutans* GS-5 collagenase gene.

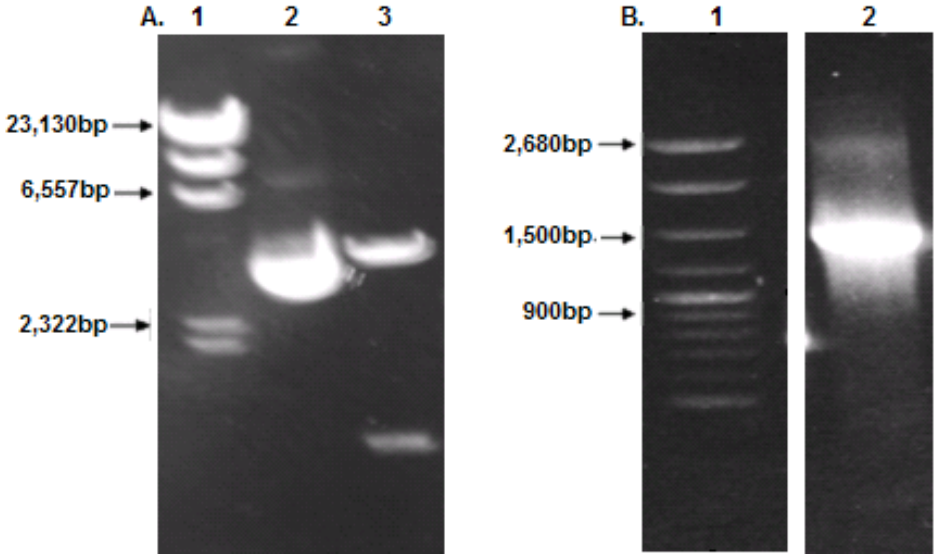


Figure 2. Insertion of PCR product into pCR 2.1-TOPO was confirmed by restriction enzyme digestion (A) and PCR amplification (B).

A: Lane 1: λ-Hind III MW marker
Lane 2: Supercoiled recombinant plasmid
Lane 3: *Eco*RI digest of the recombinant vector produced 2 distinct bands corresponding to the linearized vector (3.9Kb) and the *smcol* insert (1.2kb)

B: Lane 1: 100bp ladder.
Lane 2: PCR amplification of the inserted gene produced the expected size product.

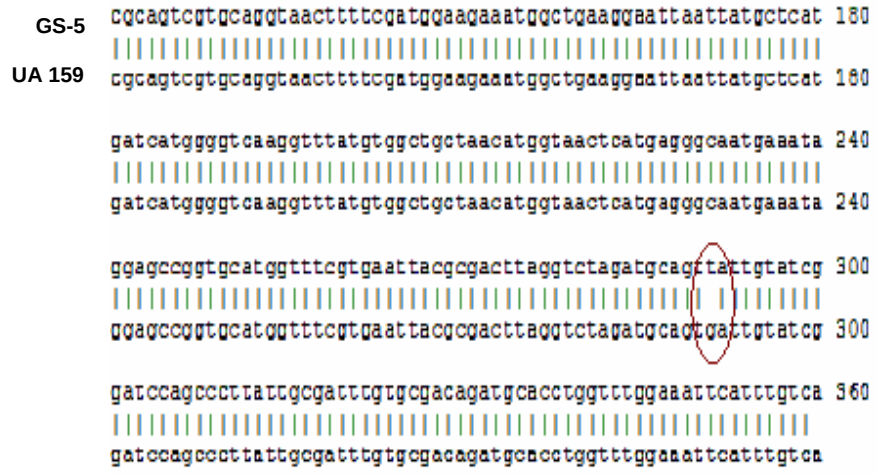


Figure 3. Alignment of the *smcol* sequence of strain GS-5 with the corresponding sequence of UA 159 strain. Alignment (BLAST2seq) revealed 99% similarity between the two genes. The red circle represents 1 of the 4 nucleotides where the two sequences showed discrepancy.

Cloning of the *smcol-p*ET Gene

To clone and express *S. mutans* collagenase the pET100/D-TOPO vector (Invitrogen) was used as it offered many advantages over previous TOPO TA cloning vectors which require the use of *Taq* polymerase to generate PCR product with dA overhangs. Like TOPO TA vectors the pET100/D-TOPO vector was constructed with topoisomerase properties, but instead of *Taq* polymerase it requires the use of proofreading polymerase such as *Pfu* hotstart polymerase that removes the overhangs. Hence, the gene of interest can be cloned in frame with the genes encoded by the vector. Another requirement for in-frame as well as directional ligation is the presence of 5'-CACC-3' upstream of the desired gene to be cloned. This ensures efficient ligation of the gene due to the presence of 3' complementary sequence on the linearized vector. Thus, a forward primer incorporating the 4 additional nucleotides was used (F2 primer). As an expression vector, pET-100 contains an IPTG-inducible promoter, as well as a

binding site for lac repressor to reduce basal expression of the protein. It also consists of an N-terminal histidine fusion tag that allows purification and detection of the recombinant protein.

The 1.2 kb PCR product (Fig. 4) obtained was diluted 1:50 and used for subcloning into the pET100/D-TOPO. Since no blue/white screening was possible, 10 randomly selected colonies were chosen for analysis of positives clones. The cloning efficiency was only 40%. Only one positive clone was used in subsequent studies. As previously observed, restriction enzyme analysis of the plasmid isolated from the recombinant clone showed two discrete bands corresponding to the linearized vector (5.7 kb) and the *smcol* insert (1.2 kb) (Fig. 6A). Confirmation of the cloned gene came from PCR amplification using *smcol*-pET as a template and combinations of 2 sets of primers (Table 1, Figure 5): T7 forward and reverse primers hybridizing to vector sequence and F2 and R1 primers hybridizing to the *smcol* gene. As shown in Figure 6B different sizes of PCR products ranging from 1.2 – 1.5 kb were obtained. The 1.2 kb reflects the actual size of the *smcol* gene (Lane 3, F2 and R1 primers), while the rest (Lanes 2 and 4, T7 Forward and R1, F2 and T7 reverse respectively) indicate that the 5' and 3' of the collagenase gene was successfully ligated. The larger 1.5 kb (Lane 5, T7 forward and reverse) confirmed that there was no deletion in the vector in the proximity of the insertion site.

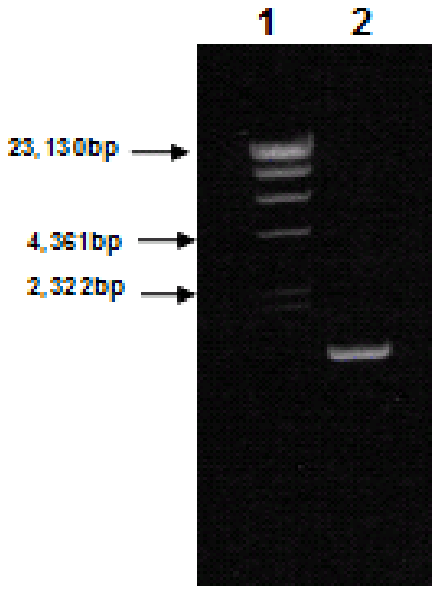


Figure 4. PCR product obtained from genomic DNA
Lane 1: λ-Hind III MW marker
Lane 2: 1287bp PCR product

Sequence Analysis of *smcol*-pET

The cloned *smcol*-pET was subjected to sequence analysis in order to detect any possible mutations that had potentially taken place during cloning or PCR amplification as well as to confirm that the gene was ligated in-frame with the histidine residues (Figure 5). BLAST2 alignment (1) of the *smcol*-pET gave 100% similarity with the respective sequence of the *S. mutans* UA 159. A more extensive analysis of the deduced amino acid sequence was also performed using various databases to examine important motifs and conserved regions of the suspected collagenase. Databases, such as ExPASy, Pfam, and SMART (3,16,41), gave more information about the protein in search. The results indicated high homology with U32 family peptidases. Even though these enzymes are of unknown catalytic origin, they are represented by well

characterized species, *P. gingivalis*. The consensus sequence present in all U32 peptidases is E-x-F-x(2)-G-[SA]-[LIVM]-C-x(4)-G-x-C-x-[LIVM]-S and is used as a signature pattern for these families. Furthermore, the sequence was run as a query using the ProtoMap database. ProtoMap (17) classifies all proteins in SWISSPROT and TrEMBL databases into groups of related proteins. The query was identified as a member of cluster #1872. There are 24 members that belong to this cluster, 13 of which belong to the U32 family peptidases. Notable is the fact at 50% of the members are collagenases, including *P. gingivalis* prtC, while the rest are putative bacterial proteases. In agreement with the results mentioned above, PSI-BLAST (1) placed the consensus sequence in U32 Peptidase family as well. The European Molecular Biology Laboratory (EMBL) database also placed the enzyme into E.C. 3.4, a designated broad family of hydrolases that act on peptide bonds; members of this family include collagenases such as the well characterized zinc-metalloproteinases of *C. histolyticum* and *V. alginolyticus*. In order to get a more conclusive picture of the protein, another annotation was employed. Investigations regarding the presence of any phylogenetic pattern between our sequence and the Cluster of Orthologous Group of Protein (COG) database (1) were also performed. This program consists of carefully annotated sets of likely orthologs and assigns new proteins to COGs by comparing them to the sequences in the COG database. When three or more genome-specific best hits (BeTS) are detected then the query sequence is considered a likely new COG member (4). Submission of our query gave BeTS to 6 clades of COG0826 designated as collagenase and related proteases.

Another important information was also obtained, which added to our knowledge of the enzyme in search. Dense Alignment Surface (DAS) method uses a single sequence as a query and predicts possible transmembrane segments in prokaryotic membrane proteins (10). Using the deduced aminoacid sequence as a query it was suggested that the enzyme potentially spans the membrane at position 96 through 108.

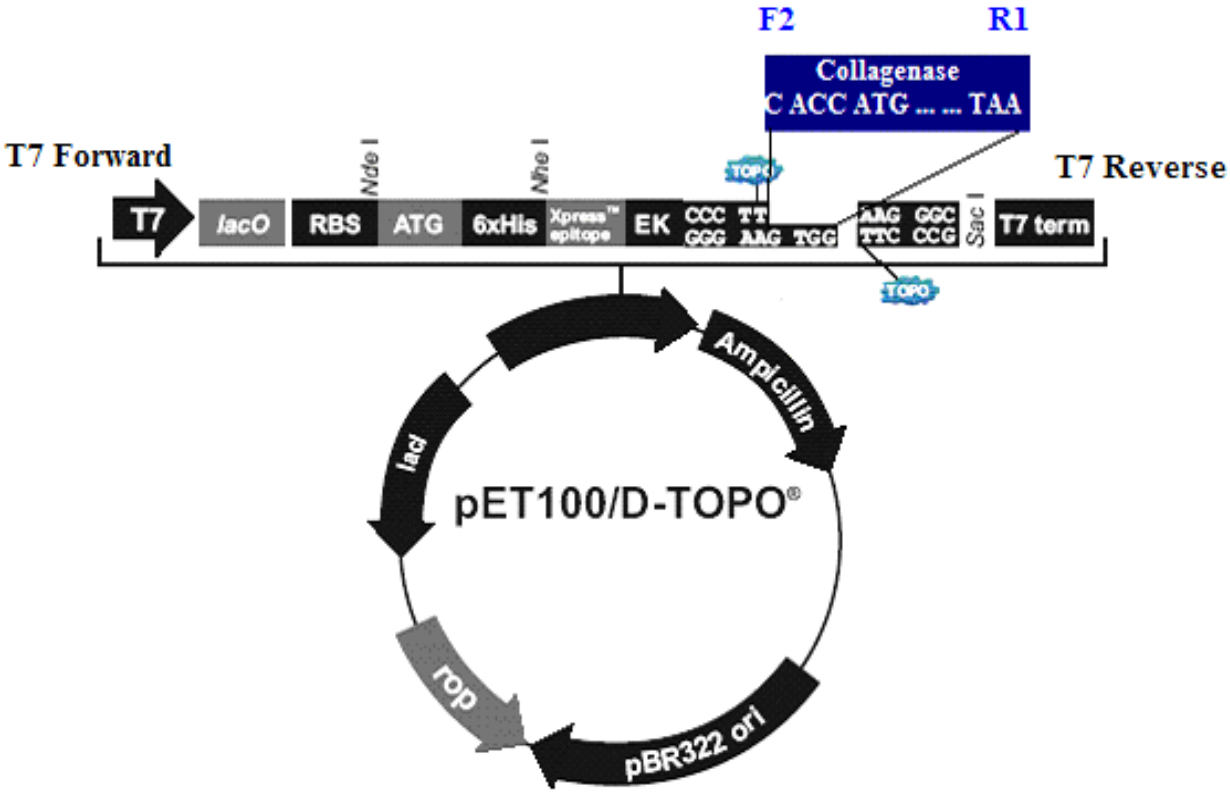


Figure 5. Map of pET 100/D-TOPO with collagenase insert (blue). The 5'-CACC-3' in the 5' end of the insert provides directional and in-frame cloning of the gene with the 6x His fusion tag. Ampicillin resistant positive clones were selected and the recombinant plasmid was subjected to sequence analysis.T7 promoter was IPTG induced for high expression of the gene.

Table 1. PCR primer pairs used for confirmation of successful ligation of *smcol* in pET100

Forward Primer	Reverse Primer	DNA Size (bp)	Lane (Figure 6)
T7 Forward	R1	1489	2
F2	R1	1291	3
F2	T7 Reverse	1373	4
T7 Forward	T7 Reverse	1563	5

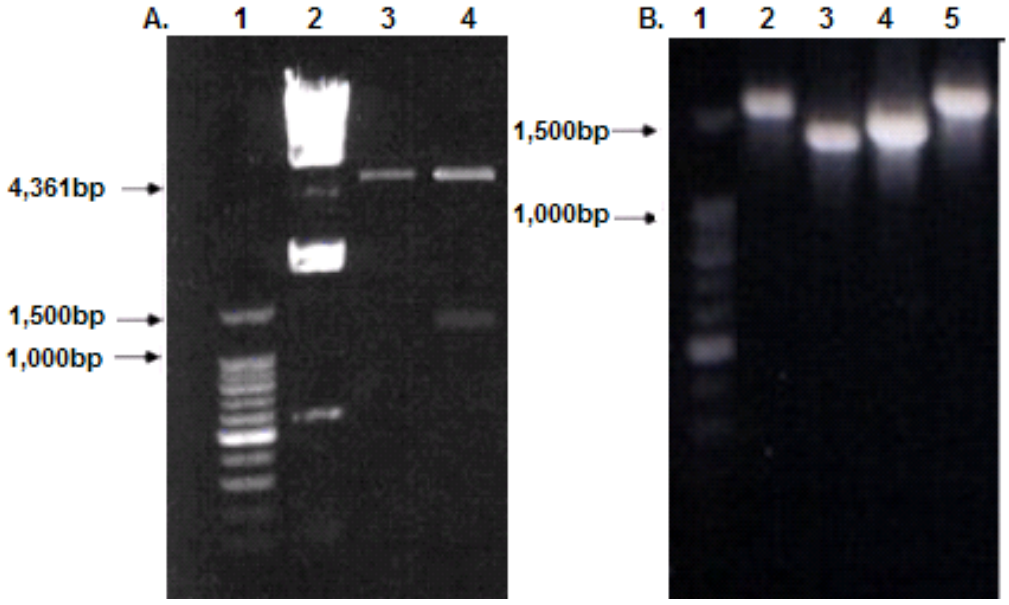


Figure 6. Confirmation of successful insertion of the collagenase gene into pET 100/D-TOPO.

A. Lanes 1 and 2: Molecular weight standard
Lane 3: Undigested recombinant plasmid
Lane 4: Recombinant plasmid digested with *Eco*RI:Two bands were obtained corresponding to linearized vector (5.7 kb) and the *smcol* insert (1.2kb)

B.PCR products generated by different primer sets (Table 1) confirmed successful ligation and correct orientation of the insert.

SDS PAGE of the IPTG Induced *smcol*-pET

1mM of IPTG was used to induce *E. coli* BL21 harboring the *smcol*-pET recombinant plasmid. The induction was performed over a 4-hour period with samples taken every hour after the addition of IPTG. Figure 7 shows the results obtained when samples were analyzed by SDS PAGE. Induction was already apparent at 1 hour post-induction as compared to at time 0, and the expression continued to be induced over the 4 hour period. One strong unique band of 50kDa was observed to increase in size with time. The size of the induced band was larger than the anticipated collagenase size of 47kDa to account for the presence of the 3 kDa His-tag.

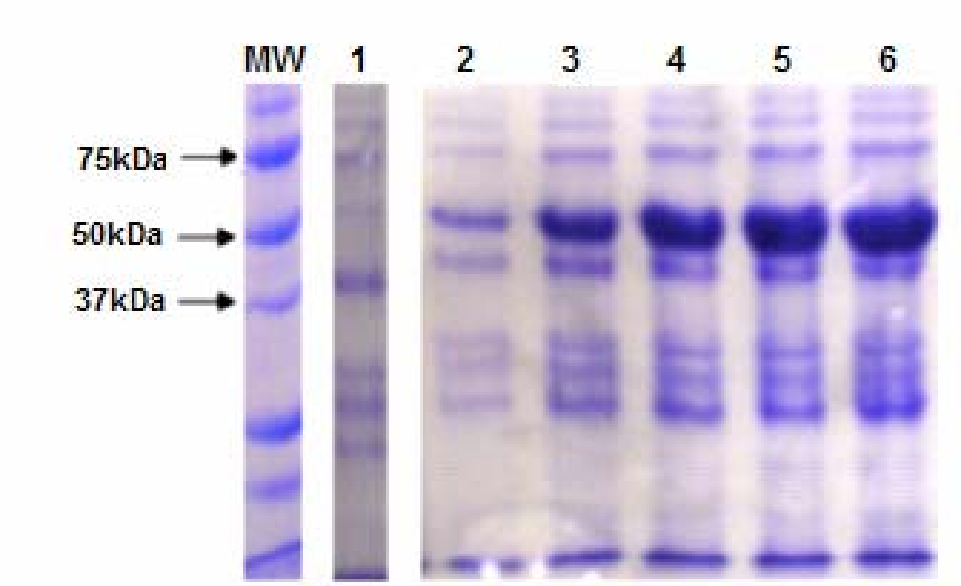


Figure 7. SDS analysis of the induced *E. coli* BL 21 cells transformed with the recombinant plasmid *smcol*-pET. Equal volumes of cell lysates were loaded into each lane. Induction by IPTG was followed over 4–hour time period and samples were collected at hourly intervals. Lane 1: *E. coli* BL 21 transformed with empty vector (negative control); Lanes 2-6: Expression of a 50kDa band was evident at 1 hour post-induction and increased thereafter. MW: SDS molecular weight marker

SDS and Western Blot analysis of Purified Recombinant Protein

High affinity purification column was used in order to purify the recombinant protein that was tagged with six tandem histidine residues at the N-terminal end. The samples applied onto the column consisted of protein purified under native and hybrid conditions. Although there was no protein eluted from the soluble supernatant under native conditions, the insoluble pellet that was recovered after lysis, showed one strong 50kDa band after SDS analysis (Fig. 8). This sample, in parallel with a non-purified sample as well as a negative control, was subjected to western blot analysis in order to confirm that the 50kDa was the anticipated fused protein. As shown in Figure 9A, the non-purified sample (Lanes 2, 3) showed many bands with higher intensity being the 50kDa fused protein. In agreement with this, the purified protein also showed high intensity band at the same area confirming that the enzyme was successfully expressed and purified (Fig. 9B). Smaller bands with lower size and intensity were also present in the fraction tubes.

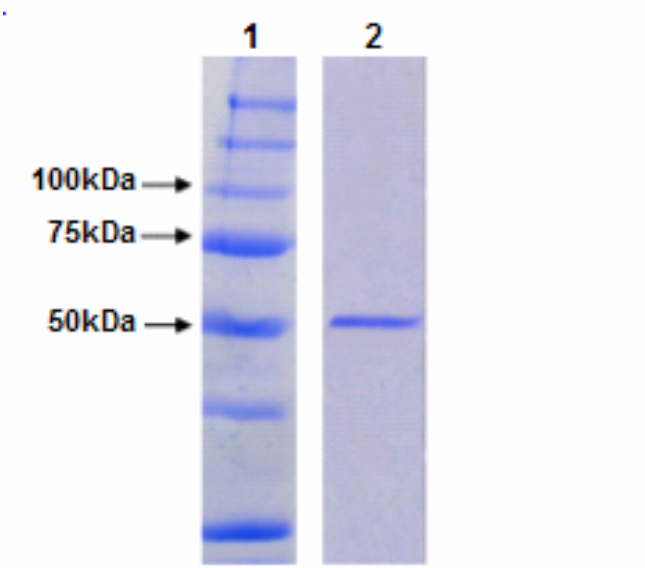


Figure 8. SDS-PAGE of purified recombinant protein. Purity was confirmed by the obtaining of a single 50kDa band corresponding to the expression of a fusion protein formed by the 47kDa *S. mutans* collagenase and the 3kDa his-tag. Lane 1: SDS MW marker; Lane 2: Purified recombinant fusion protein.

Gelatin Hydrolysis

Gelatin zymogram was performed in order to investigate the ability of the enzyme to degrade gelatin, a denatured form of collagen. After 16 hours of incubation at 37^{o}C with collagenase sample buffer, *E. coli* whole cells indicated the presence of gelatinolytic activity as a clear band of approximately 90-100 kDa over a blue background (Fig. 10). No activity was observed with the negative control or with the pure fusion enzyme protein. In order to see if a longer incubation time would have any effect on the gelatinolytic activity of the enzyme, the purified collagenase-his tag fusion protein was incubated for 24h at 37^{o}C with fluorescein conjugated gelatin. Increase in fluorescence would be an indication of gelatin hydrolysis. As in the gelatin zymography experiment, no significant fluorescent change was observed at 16h incubation, but at 24 hours a significant

increase in the fluorescence was observed. The result suggested that the purified fusion protein had weak activity. The specificity of the assay was demonstrated by the strong activity observed with *C. histolyticum* collagenase, whereas the negative control had no effect on the substrate.

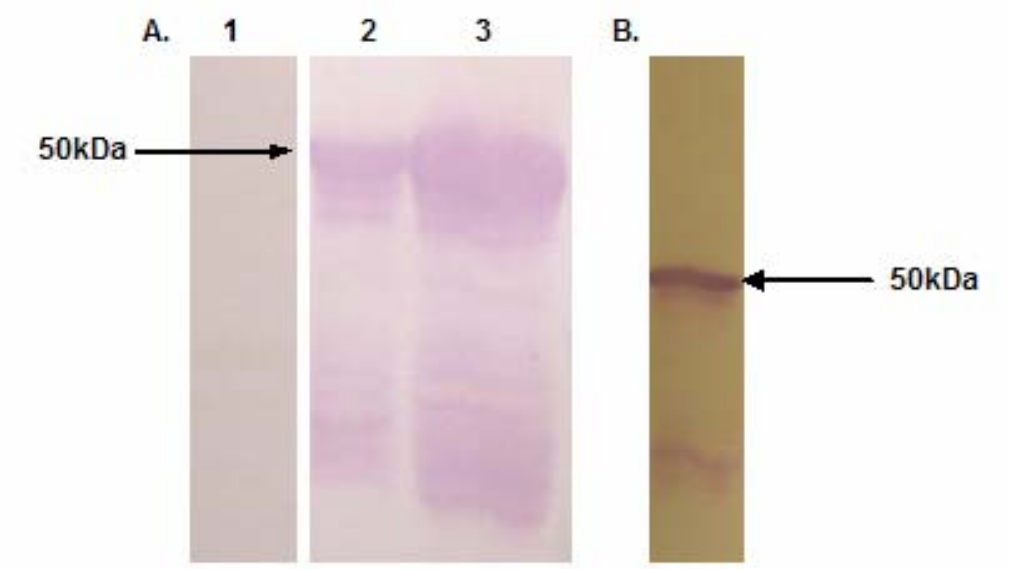


Figure 9. Western immunoblots of *E. coli* cell lysates containing the His-Collagenase fusion protein (A) and the purified collagenase (B). Expression of the fusion protein was tested by western blot analysis using a mouse specific antibody to his-tag as a primary antibody and an alkaline phosphatase conjugated rabbit as a secondary antibody. A purple coloration was indicative of a positive reaction
A. Lane 1: *E. coli* transformed with empty vector(-)
Lane 2: *E. coli* transformed with recombinant vector 1h after induction
Lane 3: *E. coli* transformed with recombinant vector 4h after induction
B. Purified recombinant protein.

Collagen Assay

True collagenases are capable of hydrolyzing collagen in its native form. The Enzchek collagenase assay kit (Molecular Probes) provides a sensitive and rapid way to assay for collagen hydrolysis. Fluorescein-conjugated Type I collagen was used as a substrate, and any significant increase in fluorescence

would be an indication of collagen hydrolysis. Initially, as showed in figure 11, only the 30 and 40µg of the purified enzyme demonstrated significant collagenolytic activity 24 hours after incubation at 37^{o}C (Fig. 11A). A sample containing 40µg of purified fusion protein was next incubated for 48h before measurement of collagenolytic activity (Fig. 11B). Collagen degradation was comparable to the levels observed with that of the positive *C. histolyticum* collagenase (positive control). As anticipated, no activity was observed with the negative control. It is noteworthy that whole recombinant *E. coli* cells degraded collagen while the cell lysate supernatant did not, suggesting that the enzyme may be expressed as bound to the cell membrane (Fig. 11A). The specificity of the assay was confirmed by the failure of trypsin, a non-specific protease, to degrade native collagen. A small background of fluorescence was observed and attributable due to the degradation of non-helical components of collagen or to the presence of small amounts of denatured collagen spontaneously generated during the commercial preparation of the substrate.

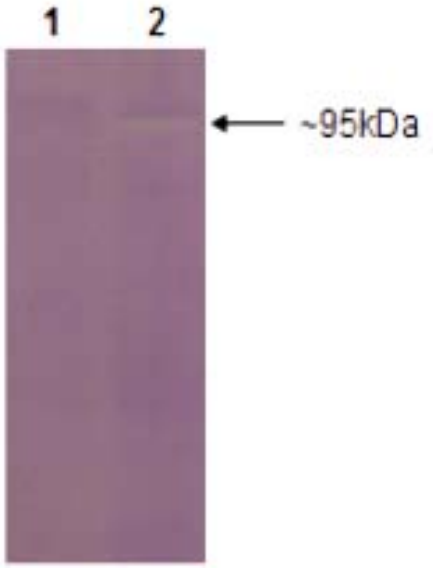
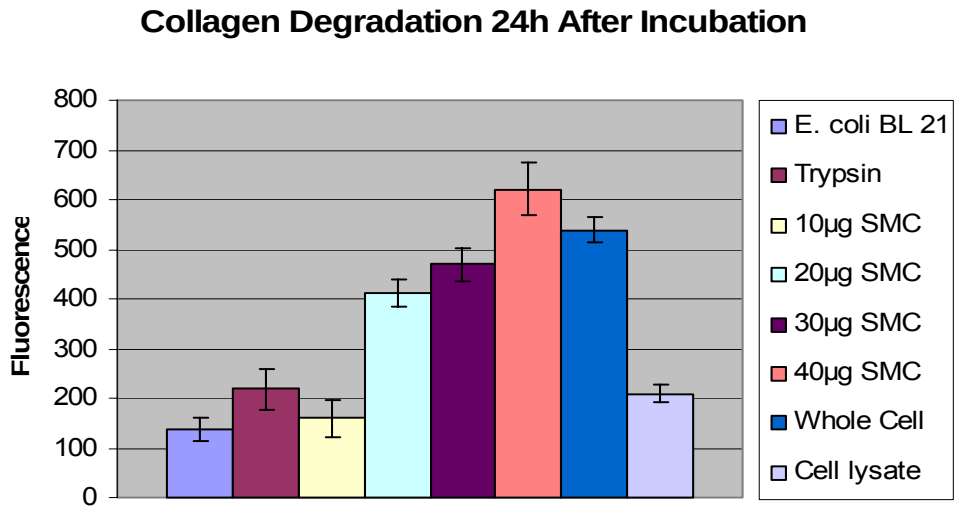


Figure10. Gelatin zymogram of recombinant *E. coli* cells and purified recombinant protein. The samples were separated in a gelatin-containing gel under non-reducing conditions, and incubated for 16h at 37^{o}C. A clear band in the gel indicated gelatin degradation.
Lane 1: Purified recombinant fusion protein
Lane 2: Recombinant *E. coli* whole cells expressing the recombinant protein as a 95kDa band corresponding to a dimer of the 50kDa observed in SDS-PAGE under reducing conditions

Inhibition Assay

Three different inhibitors were used to further characterize the properties of the *S. mutans* GS-5 collagenase. As shown in Table 2, only EDTA reduced the enzyme activity. No inhibition took place with either the metalloproteinase inhibitor 1,10-phenathroline or the serine protease inhibitor PMSF.

A.



B.

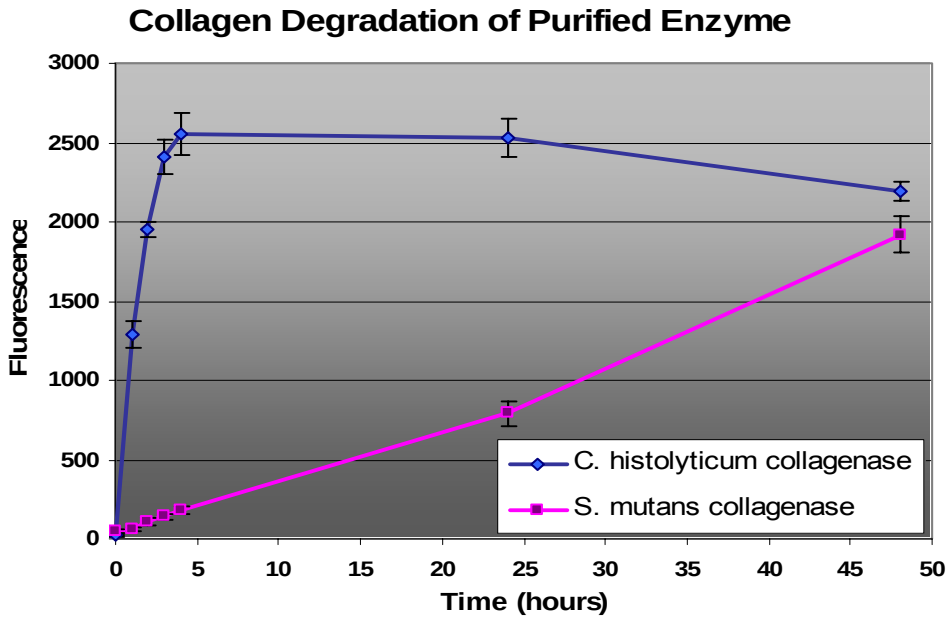


Figure 11. Degradation of fluorescein-conjugated Type I collagen by purified fusion protein. Increase in fluorescence indicated collagen degradation. A. *E. coli* cells transformed with empty vector and trypsin were used as negative controls. *E. coli* cell lysate did not show significant degradation after 24h incubation, while whole cells degraded a significant amount of collagen. Different amounts of purified enzyme were tested (10-40µg) to show dose-dependent enzyme activity. SMC: *Streptococcus mutans* collagenase. B. Considerable amounts of collagen degradation was evident 48h after incubation. Activity of 0.4U/ml of *C. histolyticum* collagenase was tested a positive control for the assay.

Table2. Inhibition assays for collagenase activity

Organism	Fluorescence w/o Inhibitor	Fluorescence w/t inhibitor		
		EDTA 10mM	1,10-PHEN 10mM	PMSF 2mM
C. histolyticum (0.4U/ml)	2524.88	452.15	393.2	3349.87
S. mutans GS-5 (40µg)	2618.2	1040.15	2276.55	2542.45

Three common protease inhibitors were used to further characterize the *S. mutans* collagenase enzyme (EDTA, 1,10- phenanthroline, PMSF). Activity of *S. mutans* collagenase was reduced by the presence of EDTA, but not by 1,10-phenanthroline nor PMSF, whereas *C. histolyticum* collagenase was drastically inhibited by EDTA and 1,10 phenanthroline. These results were consistent with the difference in the nature of the two enzymes.

DISCUSSION

The present study aimed at the cloning of a collagenase gene of *S. mutans* and its expression as an active enzyme in recombinant *E. coli.* Although *S. mutans* has been implicated in dental root decay and found in dental root in association with collagen fibril degradation, there has been no proof of the production of collagenase enzyme by *S. mutans*.

A two step cloning approach was taken. First, the pCR2.1-TOPO was used in the cloning of PCR amplified *smcol* gene for sequence analysis. Once the sequence was established a strategy was devised in order to clone the *smcol* gene into the expression vector pET100/D-TOPO in frame and directionally in order to insure its expression. Primers were designed directly on the start and stop codon so that only the open reading frame was PCR amplified. Sequence analysis of the initial cloned gene in the pCR2.1-TOPO TA vector showed 99% similarity (Fig. 3) with *S. mutans* UA 159, but 100% homology was observed with the gene cloned in the pET100/D-TOPO. This difference in results was due to the error prone nature of *Taq* polymerase as compared to the proof reading *Pfu* hotstart polymerase used in the *smcol* gene amplification for cloning into pET100/D-TOPO. This enzyme is characterized by a 3'-5' exonuclease activity that minimizes the possibility of inserting mutations during PCR amplification.

The cloned gene was successfully expressed as a 50 kDa protein at high levels after induction with IPTG (Fig. 7). The recombinant protein appeared to be

associated with the insoluble membrane fraction of the recombinant *E. coli* and consequently was difficult to recover from the insoluble pellet. Nevertheless, western blot analysis of the purified protein confirmed that the expressed, purified protein was the desired enzyme. As depicted in Figure 9, there was a major 50kDa recombinant protein band corresponding to the 47kDa *S. mutans* collagenase fused with a 3kDa His-Tag. The presence of some smaller bands in the western blot was probably attributable to proteolytic degradation, a phenomenon commonly observed with the expression of *S. mutans* antigens in *E. coli* as reported by Yoder *et al.* for the *S. mutans* wall-associate protein (53).

Jackson *et al.* (24) showed that the *S. mutans* cell-associated enzyme was responsible for the collagenolytic activity of the organism. In the present study it is suggested that the collagenase enzyme spans the membrane from aminoacid 96 through 108 (10). This was confirmed by the observed collagenase activity in recombinant *E. coli* whole cells (Fig. 11A) and lack of activity in the soluble cell extract.

Inhibition of *S. mutans* collagenase by EDTA was in agreement with prior works (22, 24), thus the enzyme can be considered a metalloproteinase. Unlike bacterial zinc metalloproteinases that are inhibited by both EDTA and 1,10-phenanthroline, the purified *S. mutans* fusion enzyme activity was reduced only by the addition of EDTA, and not by 1,10 phenanthroline. These data are in partial agreement with Jackson *et al.* (24) that showed inhibition of *S. mutans* cell-associated collagenolytic activity by both EDTA and 1, 10- phenanthroline. This apparent discrepancy may be attributed to either a difference in assay

conditions, testing of whole *S. mutans* GS-5 cells in the study by Jackson *et al.* (1997) versus a purified *S. mutans* collagenase-fusion protein in the present study, or to the possibility of more than one collagenase enzyme being produced by *S. mutans*. The possibility of such an occurrence was supported by the presence of two antigen bands in previous western immunoblot of *S. mutans* (24). Further purification of the *S. mutans* enzyme without the His-tag and activity testing will determine whether or not there was any interference caused by the extra His-tag region.

Sequence analysis of the deduced amino acid sequence gave high similarities with other bacterial proteases, mainly collagenases. Specifically, *S. mutans* collagenase belongs to the U32 family peptidase, a broad family of enzymes with unknown catalytic domain. A shared characteristic is the presence of two cysteine residues within the consensus sequence E-x-F-x(2)-G-[SA]-[LIVM]-C-x(4)-G-x-C-x-[LIVM]-S. Surprisingly, PrtC is one of the well characterized members of the U32 peptidases. Alignment of the *S. mutans* collagenase with the PrtC of *P. gingivalis* deduced amino acid sequences only showed 48% homology. This reflected the heterogeneity in the U32 enzyme family. Indeed, heterogeneity of the *prt*C gene was also observed in different clinical isolates (51). Nevertheless, the two enzymes shared two important characteristics. Firstly, Kato *et al.* showed by gel filtration that PrtC was a dimer in its active form. Similarly, in the present work, gelatin zymogram analysis showed an active band of 95kDa under non-reducing conditions. Secondly, both

enzymes, PrtC and *S. mutans* collagenase were inhibited by EDTA suggesting a requirement for Ca^{2+}, but not by 1, 10-phenanthroline.

Dental root decay is a disease implicating many organisms and different virulence factors, making it hard to investigate a single factor individually. Despite this difficulty, we successfully cloned and expressed *S. mutans* collagenase in *E. coli* and demonstrated that it had both collagenase and gelatinase activity. Experimental results (Fig. 11B) indicated that the activity was relatively weaker than that of the *C. histolyticum* collagenase. This observation was, however, in agreement with the slow progression of dental root decay in infected individuals, and the *in vitro* studies of *S. mutans* collagenolytic activity (24).

Undoubtedly, more work is needed to fully elucidate the nature and function of *S. mutans* collagenase in dental root decay. From the data presented above, it is already clear that *S. mutans* collagenase is a novel enzyme, sharing some characteristics with *P. gingivalis* PrtC but not all. For instance, PrtC did not have gelatinolytic activity. The present study has provided the tool necessary to further our understanding of *S. mutans* collagenase. Not only have we conclusively demonstrated for the first time the existence of a collagenase gene in *S. mutans* GS-5, but with the obtaining of a recombinant *E. coli* clone expressing the *S. mutans* enzyme, further analysis will be greatly facilitated.

REFERENCES

1. Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman.1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." Nucleic Acids Res. 25:3389-3402.

2. Awad,M.M.,D.M. Ellemor, A.E. Bryant, O. Matsushita, R.L. Boyd, D.L.Stevens, J.J. Emmins, and J.I Rood. 2000. Construction and virulence testing of a collagenase mutant of *Clostridium perfringens*. Microb. Pathog. 28, 107-17.

3. Bateman A, E. Birney, L. Cerruti , R. Durbin, L. Etwiller, S.R. Eddy, S. Griffiths-Jones, K.L. Howe, M. Marshall, E.L Sonnhammer. The Pfam Protein Families Database. 2002. Nucleic Acids Research 30(1):276-280

4. Baxevanis, A.D. and B.F.F. Ouellette. Bioinformatics. A practical guide to the analysis of genes and proteins. 2nd Ed. Wiley Interscience. New York. 2001.

5. Beg, A.M, M.N. Jones, T. M. Torbert, and R.G. Holt. 2002. Binding of *Streptococcus mutans* to extracellular matrix molecules and fibrinogen. Bioch. Biophys. Res. Communic. 298: 75-79.

6. Bradford M.M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. 1976. Anal. Biochem. 72: 248-54.

7. Brady, J.J., L. Marloes, A. Tilburg, C.E. Alford, and W.P. McArthur. 2000. Monoclonal antibody-mediated modulation of the humoral immune response against mucosally applied *Streptococcus mutans*. Infec. Immun. 68:1796-1805.

8. Burgess, O.B., J.R. Gallo. 2002. Treating Root Surface Caries. Dent. Clin. N. Am. 46: 385-404.

9. Chang, Y.C., S.F. Yang, J.Y. Liu, and Y.S. Hsieb. 2002. Regulation of matrix metalloprotease production by cytokines, pharmacological agents, and periodontal agents in human periodontal ligament and fibroblast cultures. J. Period. Res. 37: 129-203.

10. Cserzo, M., E. Wallin, I. Simon, G. von Heijne and A. Elofsson.1997. Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the Dense Alignment Surface method. Prot. Eng.10(6):673-76.

11. Dao, M.L. 1985. An improved method of antigen detection in nitrocellulose: In situ staining of alkaline phosphatase conjugated antibody. J. Immunol. Methods. 82: 225-231.

12. De Souza, A.P., S.R.P. Line. 2002. The biology of matrix metalloproteases. Rev. FOB. 10(1): 1-6.

13. Dumas J, N . Hurion, R. Weill, and B.Keil. 1991. Collagenase in mineralized tissues of human teeth. Arch Oral Biol.36(4):299-304

14. Dung, S.Z., A. H. Liu. 1999. Molecular pathogenesis of root dentin caries. Review. Oral Diseases. 5: 92-99.

15. Dung, S.Z., R.L Grecory, and G.K Stookey. Effect of lactic acid and proteolytic enzymes on the release of organic matrix components from human root dentine. 1995. Caries Res. 26 (6): 483-89.

16. Gasteiger E., A. Gattiker, C. Hoogland, I. Ivanyi, R.D. Appel, and A. Bairoch. ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 31:3784-3788.

17. Golan, Y., Linial, N., and Linial M. 2000. ProtoMap: Automatic classification of protein sequences and hierarchy of protein families. Nucleic Acids Research, 28: 49-55.

18. Hahn, C.L., A.M. Best, and J.G. Tew. 2000. Cytokine induction by *Streptococcus mutans* and pulpal pathogenesis. Infec. Immun. 68(12):6785-89.

19. Hahn, P., D. Reinhardt, H.G. Schaller, and E. Hellwig. 1999. Root lesions in a group of 50–60 year-old Germans related to clinical and social factors. Clin. Oral Invest. 3:168–74

20. Han, T.K., S. Yoder, C. Cao, K.E. Ugen, and M.L. Dao. 2001. Expression of *Streptococcus mutans* wall-associated protein A gene in Chinese hamster ovary cells: Prospect for dental caries DNA vaccine. DNA and Cell. 20(9): 595-601.

21. Harrington, D.J. 1996.Bacterial collagenases and collagen-degrading enzymes and their potential role in human disease. Infec. Immun. 64(6): 1885-91.

22. Harrington, D.J and R.R.B. Russel. Identification and characterization of two extracellular proteases of *S. mutans*. 1994. FEMS Microb. Letters. 121: 237-42.

23. Houle, M.A., D. Grenier, P. Plamondon, and K. Mikayama. 2003. The collagenolytic activity of *Porphyromonas gingivalis* is due to Arg-gingipain. FEMS Letters. 181-85.

24. Jackson, R.J., D. V. Lim, and M.L. Dao. 1997. Identification and analysis of a collagenolytic activity in *Streptococcus mutans*. Cur. Microb. 34:49-54.

25. Jackson, R.J., M.L. Dao, and D.V. Lim. 1994. Cell-associated collagenolytic activity by group B streptococci. Infec. Immun. 62(12): 5647-51.

26. Juarez, Z.E., and M.W. Stinson. 1999. An extracellular protease of *Streptococcus gordonii* hydrolyzes Type IV collagen and collagen analogues. Infec Immun. 67:271-278.

27. Jung, C.M., O. Matsushita, S. Katagama, J. Minami, J. Sakurai, and A. O. Kabe. 1999. Identification of metal ligands in *Clostridium histolyticum* collagenase. J. Bact. 181(9): 2816-22.

28. Kato T., N. Takahashi, and H. K. Kuramitsu. 1992. Sequence analysis and characterization of the *Porphyromonas gingivalis prtC* gene, which expresses a novel collagenase activity. J. Bact. 174(12):3889-3895

29. Kawasaki, K. and J.D.B. Featherstone. Effects of collagenase on root demineralization. 1997. 76(1): 588-95.

30. Koga T, Oho T, Shimazaki Y, Nakano Y. 2002. Immunization against dental caries. Vaccine 1-18.

31. Love R.M. 2002. The effect of tissue molecules on bacterial invasion of dentin. Oral Microb. Immun. 17:32-37.

32. Love, R.M., M. McMillan, and I.F. Jenkinson. 1997. Invasion of dentinal tubules by oral streptococci is associated with collagen mediated by Antigen I/II family peptides. Infec. Immun. 65(12): 5157-64.

33. Makihira, S., H. Nikawa, M. Tamgami, T. Hamda, H. Nishimura, K. Ishida, and H. Yamashiro. 2002. Bacterial and *Candida* adhesion to intact and denatured collagen *in vitro*. Mycoses. 45: 389-92.

34. Matsushita, O. and A. Okabe. 2001. Clostridial hydrolytic enzymes degrading extracellular components. Toxicon. 39: 1769-80.

35. Matsushita, O., C.M. Jung, S. Katayama, J. Minami, Y. Takahashi, and A. Okabe. 1999. Gene dublication and multiplicity of collagenases in *Clostridium histolyticum*. J. Bact. 181(3):923-933

36. Matsushita, O., K. Yoshihara, S. Katayama, J. Minami, and A. Okabe. 1994. Purification and characterization of a *Clostridium perfringens* 120-kilodalton collagenase and nucleotide sequence of the corresponding gene. J. Bact. 176(1): 149-156.

37. Moawia, M.K. and R.E. Cohen. 2003. The etiology and prevalence of gingival recession. JADA. 134: 220-25.

38. Oral Health 2000: Facts and Figures. U.S. Department of Health and Human Services.May2000. http://www.cdc.gov/OralHealth/factsheets/sgr2000-fs1.htm.

39. Qian H and M.L. Dao. 1993. Inactivation of the *Streptococcus mutans* wall-associated protein A gene (wapA) results in a decrease in sucrose-dependent adherence and aggregation. Infec. Immun. 61(12):5021-8.

40. Romanelli, R., S. Mancini, C. Laschinger, C.M. Overall, J. Sodek, and C.A.G. McCulloh. 1999. Activation of neutrophils collagenase in periodontidis. Infec. Immun. 67(5): 2319-26.

41. Schultz J., F. Milpetz, Bork P, and C.P. Ponting. 1998. SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl Acad. Sci U S A. 95(11):5857-64

42. Shen, S., L.P. Samaranayake, H.K. Yip, and J.E. Dyson. 2002. Bacterial and yeast flora of root surface caries in elderly, ethnic Chinese. Oral Microbiol. 8: 207-217.

43. Stryer, L.1995. Biochemistry .4th Edition, W. H. Freeman and Company, NewYork.

44. Switalski, L.M., W.G. Buthcer, P.C. Caufield, and M. S. Lantz. 1993. Collagen mediates adhesion of S*treptococcus mutans* to human dentin. Infec. Immun. 61:4119-4125.

45. Taubman, M.A., D.J. Smith, C.J. Holberg, and J.W. Eastcott. 2000. Coimmunization with complementary glycosyltransferases peptides results in enhances immunogenicity and protection against dental caries. Infec. Immun. 68:2698-2703.

46. Van der Reijden,W. A., N. Dellemijn-Kippuw , A. M. Stijne-van Nes , J. J. de Soet and J. van Winkelhoff. 2001. Mutans streptococci in subgingival plaque of treated and untreated patients with periodontitis. J Clini Periodontol. 28: 686-91.

47. Van Strijp, A.J.P., D.C. Jansen, J. DeGroot, J.M. ten Cate, and V. Everts.2003. Host-derived proteinases and degradation of dentine collagen in situ. Caries Res. 37: 58-65.

48. Vargas, M. C, E.A. Kramarow, and J.A. Yellowitz. 2001.The Oral Health of Older American. CDC.

49. Vitagliano, L, G. Zagari, and H.A. Scheraga. 1995. Structure of type I collagen. Molecule based on conformational energy. Computating the triple stranded helix and the N terminal telopeptide. J. Mol. Biol. 247: 68-80.

50. Warren, J.J., H.J. Cowen, C.M. Watkins, and H.S. Hand. 2000. Dental caries prevalence and dental utilization among very old. J Am Dent Assoc. 131(11)1571-19.

51. Wittstock M., T.F. Flemming, H. Schmidt, and H. Karch. 2000. Heterogeneity of *prtC* gene of *Porphyromonas gingivalis*. Oral Microbiol. Immun. 15: 33-39.

52. Wittstock M., H. Schmidt, T.F. Flemming, R. Mutters, and H. Karch. 1996. Serodiagnosis of *Porphyromonas gingivalis* infection by immunoblot analysis with recombinant collagenase. J. Clin. Microb. 34(10): 2411-13.

53. Yoder, S., C. Cao, K.E. Ugen, and M.L. Dao. 2000. High-level expression of truncated wall associated protein A from the dental cariogenic *Streptococcus mutans*. DNA and Cell. 19:401-408.

54. Yoshihara, K., O. Matsushita, J. Minami, and A. Okabe. 1994. Cloning and nucleotide analysis of the *colH* gene from *Clostridium histolyticum* encoding a collagenase and a gelatinase. J. Bact. 176(21): 6489-649.

55. Yu, M.S. and C.Y. Lee. 1999. Expression and characterization of the *prtV* gene encoding a collagenase from *Vibrio parahemolyticus* in *Escherichia coli*. Microbiology. 145: 143-150.

56. Yu, M.S., M.N. Yap, and C. Y. Lee. 2000. Metal content and biochemical analyses of a recombinant collagenase prtV from *Vibrio parahemolyticus.* Micriobiol. Immunol. 44(10): 805-13.