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Characterization of Collagenase Production by Subspecies of Black Pigment Producing Bacteroides

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CHARACTERIZATION OF COLLAGENASE PRODUCTION BY
SUBSPECIES
of
Black Pigment Producing
Bacteroides

by

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Anthony John Budzinski was born to Lucius and Fernadine Budzinski in Chicago, Illinois on July 25, 1956. He graduated from Saint Patrick High School in June of 1974 and received his Bachelor of Science degree from the Department of Biology at the University of Illinois, Urbana, in June of 1978. He began his Masters of Science research in the Department of Oral Biology at Loyola University School of Dentistry, Maywood, under the guidance of Dr. James C. Hagen in August of 1978. In 1980 he received second place at the American Association of Dental Research Young researchers competition for his abstract; "Characterization of Collagenase Production by Oral Subspecies of Bacteroides melaninogenicus." In September of 1980 he began his D.D.S. studies at Loyola University School of Dentistry, Maywood. He received his D.D.S. degree with dental honors in May of 1984. He is a member of Psi Omega, the Chicago Dental Society and the American Student Dental Association.
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CHAPTER 1

INTRODUCTION

In the past, the most widely recognized and best described anaerobic infections were due to microorganisms acquired exogenously. A classic example of this type of infection is clostridial myonecrosis or "gas gangrene", caused by microorganisms of the genus Clostridium. Infections usually develop following trauma and involve contamination of wounds by Clostridial spores which are widely distributed in nature (67). At the site of injury, the necrotic tissue, blood clots and foreign bodies greatly reduce blood supply to the wound. The anaerobic conditions necessary to germinate spores and initiate the infection (19) are thereby created.

The main pathogenic quality associated with these clostridia is their capability of elaborating large quantities of destructive enzymes, such as alpha toxin. Alpha toxin possesses phospholipase C or lecithinase activity. In addition Clostridium possess collagenase and neuramidase (34), producing infection by causing destruction of both tissue and blood cells (13).

The pathogenic qualities possessed by these organisms, along with their wide distribution in nature, have made them extremely dangerous to man. Improvement of general management of these diseases along with the discovery of effective antimicrobial agents have enabled both the physician and
dentist to control the occurrence of these primary infections in man. Unfortunately, as control of these exogenously acquired anaerobic pathogens became possible, a more complex and less understood class of endogenously acquired anaerobic infections has occurred to take their place.

Obligately anaerobic microorganisms are found endogenously as predominant residents of the skin and mucous membranes (82,91). Although anaerobes are normally found on, or in the body, they may become pathogenic when introduced outside of their normal environment. In fact it has been shown that the majority of anaerobic infections arise from the normal microflora of the body and could be called opportunistic in nature (94,33,2,8,40). Under certain predisposing circumstances, including surgery, malignencies (11), prior antibiotic therapy, diabetes and increased longevity (16), anaerobes from the endogenous microflora have an opportunity to penetrate the normally protective mucosal barrier and begin the infectious process. These infections are caused by the normal microflora which reside at or around the site of infection and are generally polymicrobial with a mixed flora of both obligately and facultatively anaerobic organisms (17-21,62). Infections commonly involves indigenous anaerobes and include brain abscess (37), lung abscess (4,105,106), appendicitis with peritonitis (96), vulvovaginal abscess (22), and oral infections (52,109,55,56,38). The anaerobes most commonly isolated from such infections include **Fusobacteria, Bacteroides melaninogenicus, Peptostreptococcus,**
**Peptococcus** and **Bacteroides fragilis** (2). These nonsporulating anaerobic bacteria are rarely isolated alone, and are usually associated with various other anaerobic and aerobic organisms which exist together in a synergistic relationship (75,86). The fact that each organism must produce the disease in combination when they can not do so alone suggests that their combination is of mutual benefit while it is harmful to the common host. Mixed infections are extremely complex and must be studied in terms of each individual organisms' contribution to the whole as well as the effect of these organisms synergistic relationship on the host (71).

Recent publications from the medical and dental literature have documented an increased recovery rate of the anaerobic microorganism **Bacteroides** from clinical specimens, and have implicated the black pigment-producing oral **Bacteroides** (BPPOB) as playing a major role in the pathogenesis of periodontic and endodontic infections (44,57,79,100,101,113,122,124). While much work has been performed demonstrating the pathogenicity of these organisms in medical infections, there is a paucity of information concerning their actual role in dentally related disease.

The BPPOB (B. asaccharolyticus, B. menlaninogenicus, subsp. intermedius and subsp. melaninogenicus) are thought to be an essential pathogenic component of inflammatory periodontal disease (59,60,109). Due to the prevalence of B. asaccharolyticus in periodontal isolates and the fact that there appears to be a direct correlation between level of
this organism and severity of infection, it has been suggested that *B. asaccharolyticus* is indicative of, and pathologically involved with, periodontitis (71,101,113). The intricacy of these mixed, polymicrobial anaerobic infections has made their study tedious and complex, resulting in a paucity of knowledge concerning their etiology and pathogenesis (58).

One of the most prevalent and poorly understood types of polymicrobial mixed anaerobic infections is Inflammatory Periodontal Disease (IPD) (16,59). Human IPD can be observed as at least three clinically distinct but pathologically interrelated types; acute ulcerative gingivitis, periodontosis and periodontitis. Although all three types of IPD remain clinically distinct, two findings remain consistent. First is the mandatory involvement of the oral microbial flora (41,110,59,16,42,53,100,101,102,118), and second is the destruction of gingival collagen fibers, the major supporting tissue of the periodontium.

First, a direct association can be made between the oral microbial mass and the incidence of IPD (50). The microflora associated with periodontal disease is extremely complex and in many ways reflects the flora of the normal oral cavity (38,16,59,58,10). Bacteria found in the disease state include large numbers of various spirochetes, vibrios, *Bacteroides* species, anaerobic streptococci, anaerobic diptheroids, *Fusobacteria*, *Capnocytophaga*, *Actinobacillus*, and other organisms (93,55). Although the specific microbes involved in each clinical type of IPD may differ, there often
is a dramatic increase in numbers of microorganisms isolated from the disease state. This is an important observation, since the mixed microbiota isolated from IPD possess certain pathogenic qualities, as exemplified by infections attributable to human bites (74,28), typical ulcers, noma, and like conditions.

Perhaps more importantly, shifts in flora composition are observed in IPD. There is a shift from the predominant gram-positive facultative flora supragingivally and in normal pockets to the predominantly anaerobic gram-negative rod flora of the deep periodontal pocket (30). The gram-negative rod most commonly isolated from periodontitis, and implicated as a causative agent of this disease is *Bacteroides melaninogenicus*.

This bacillus has three separate and distinct subspecies. *B. melaninogenicus* subspecies *melaninogenicus intermedius* and *asaccharolyticus*. Each subspecies differs both biochemically and physiologically. It was determined that *B. melaninogenicus* subsp. *asaccharolyticus* was sufficiently different from the other subspecies to be regarded as a separate species designated *Bacteroides asaccharolyticus*.

BPPOB are strictly anaerobic, gram-negative bacillae. It is commonly found as normal flora in the gastrointestinal tract, vagina (33) and oral cavity (108). In the oral cavity, BPPOB are found in concentrations approximating $10^6$ organisms per gram of gingival debris (18,45). This rod increases in
number in plaque, with age (5), gingivitis (104) and chronic periodontitis (103,47,15).

Experimental evidence has shown \textit{B. melaninogenicus} to be required for the pathogenicity of oral flora associated with periodontal disease (59,69,107). As with other infections involving \textit{Bacteroides} the bacterial etiology of periodontal disease involves a polymicrobial mixed flora (111). Oral flora from gingival debris has been shown to cause infections in guinea pigs and rats (109). It was demonstrated that when \textit{B. melaninogenicus} was eliminated from a naturally occurring mixture of human oral flora, such infections could not be induced. Additions of \textit{B. melaninogenicus} back to the inoculum completely restored infectivity (71). MacDonald et al (56) demonstrated that this complex bacterial mixture could be reduced to four organisms and still retain infectivity (59). These include \textit{B. melaninogenicus} two other \textit{Bacteroides} sp., and a facultative diphtheroid.

It has been demonstrated that human oral gram-negative microflora possess endotoxin typical of endotoxin found elsewhere in nature (76) and that these endotoxins could induce inflammatory lesions in the vagina (76), the mucous membranes (88,89) and the gingiva (89). The BPPOB have been shown to possess a physiologically active endotoxin (76). Endotoxin levels were also found to be increased in the affected gingival tissue (14), and even in the cementum of teeth. Although a causative relationship was not established, there was a statistically significant correlation between
amount of endotoxin in human gingival exudate during periodontal disease, and clinical degree of inflammation (including tissue destruction).

Further evidence implicating *B. melaninogenicus* as an essential pathogenic components of inflammatory periodontal disease is its ability to produce an array of substances potentially damaging to the periodontium. These substances include collagenase, fibrinolysin, deoxyribonuclease, chondroiten sulfatase, gleatinase, lipase, hydrogen sulfide, indole, ammonia, organic acids and other cytotoxic substances (25,32,59,60,70,83,95,115,122,112,118,119,127). In spite of the fact that *B. melaninogenicus* has the ability to produce this wide array of potentially destructive substances, and that these toxic factors can cause cell death (12), the actual role of *B. melaninogenicus* in the pathogenesis of such mixed infections as human periodontal disease is unclear. It is not likely that this organism alone initiates the disease process (71). However, once the disease is initiated it is likely that products of *B. melaninogenicus* and other organisms (115) play a role in continuing progression and severity of the disease.

The second consistent finding in IPD is a destruction of collagen fibers. Collagen is the most prevalent protein in man. It is the major constituent of teeth and the supportive tissue which surrounds them. The collagen molecule consists of three helical polypeptide chains which are hydrogen bonded to each other forming a triple helical tropocollagen macro-
molecule. These tropocollagen macromolecules then aggregate in one-quarter staggered arrays to form the collagen fibril. Because of the unusual triple helical structure of collagen, very few enzymes are able to attack it. The only known enzyme able to digest native collagen is collagenase (114). By definition, collagenase are enzymes which are capable of digesting native collagen fibrils under physiological conditions of pH and temperature, and which act by cleaving the helical part of the molecule. This definition excludes proteolytic enzymes such as trypsin and pronase which might attack the non-helical portions of the molecule.

The degradation of collagen by collagenase has been implicated as one of the major forces of gingival destruction in inflammatory periodontal disease. In fact, the degradation of collagen has been shown histochemically to be the earliest observable lesion in IPD (24). Although the exact mechanisms of collagen loss have not been established, two major sources of collagenolytic enzymes exist. First, as can be seen in physiologic remodeling during development, the maintenance of tissue morphology, repair during wound healing, and destruction of collagen associated with "connective tissue disease", collagen in the oral cavity is constantly being destroyed by the body itself (91). The turnover of collagen appears to be controlled by vertebrate tissue collagenase.
The mechanisms of host tissue collagenase on the native collagen molecule is unique. All tissue collagenases cleave the native tropocollagen molecule into two large fragments. The largest of these two fragments include three quarters of the tropocollagen molecule and contains the amino terminal end of these chains. The other fragment which is a quarter portion of the tropocollagen molecule contains the original three carboxy termini.

Most interestingly, it has been suggested that the phagocytic cells involved in inflammation may be an important source of the collagen degrading collagenases (91). In support of this suggestion, Schroeder et al. (99) and Flieder et al. (27) have noted major collagen breakdown in the area of the inflammatory cell infiltrate. Periodontal disease can be described as both an acute and chronic inflammatory pathosis of the periodontium (78). The inflammatory response is activated by bacterial plaque which accumulates at the junction between teeth and gingiva, and promotes a complex series of inflammatory reactions which gradually lead to the destruction of periodontal collagen (50,78). The cellular response consists primarily of polymorphonuclear leukocytes (PMNL) and macrophage infiltration of the gingival tissue. Interestingly, PMNL and macrophages have been shown to be capable of producing collagenase in vitro (91,31).

Both active and latent forms of cellular collagenase have been found in gingival crevicular fluid in both healthy subjects and in subjects with periodontal disease (23,36, 26,92). Most of the collagenase in fluid from healthy
gingiva exists in a latent form. In contrast, fluid from inflamed gingiva contains much collagenase in active form. It has been shown that bacterial plaque is able to activate latent forms of collagenase in gingival fluid of human PMNL (90). It is postulated that bacterial plaque can cause the specific release of collagenase during phagocytosis of plaque substances and non-specific release when leukocytes are destroyed by bacterial toxins.

Bacterial endotoxin from *Escherichia coli* has been shown to stimulate the production of collagenase from macrophages (77,123). Furthermore it was found that the lipid moiety was the portion of the lipopolysaccharide responsible for inducing the collagenase. The biochemical nature of the factors in plaque that activate collagenase remains to be characterized. These factors may vary both quantitatively and qualitatively according to the microbial constituents of plaque.

The second source of enzyme capable of degrading native collagen under physiological conditions is from the oral microbial mass itself. The reported collagenolytic activity of BPPOB and the association of these organisms with clinical disease strongly implicates *Bacteroides* as a major etiologic agent of oral disease. Bacterial collagenase was first discovered in 1922 by H. Henry (39) who observed that cultural filtrates from *Clostridium perfringens* were able to disintegrate fresh muscle tissue, although Henry did not realize that this reaction was potentiated by bacterial
collagenase. In a similar fashion, Weinberg and Randin (120,121) discovered that filtrates from cultures of the anaerobe Cl. histolyticum could slowly digest small pieces of Achilles tendon.

The implications of this finding were ignored or forgotten until 1937 when the German biochemist Maschman (63) found a new enzymatic ability to break down collagen by the organism Cl. perfringens. The enzymes' existence was later confirmed by Oakley et al. in 1946 (84) and clearly identified as collagenase by Bidwell and Van Heyningen in 1948 (9). Many aerobic and anaerobic microorganisms were tested for their ability to produce a collagenase, although Clostridium was the only one found to produce the enzyme (66) the genus Bacteroides was not tested. In 1961, Gibbons and MacDonald were the first investigators to describe the degradation of collagenous substrates by Bacteroides (29). Other investigators then delineated conditions under which the collagenolytic activity was demonstrated (35). Their results suggested that collagenase was a preformed enzyme and did not require the presence of live bacteria. In addition, the pH of the collagen gel did not go below 6.4, consistent with an enzyme capable of hydrolyzing native, insoluble collagen. Robertson et al. found that enzyme activity was enhanced when the microorganisms were grown in peptide-depleted medium (91). It is now known that although Bacteroides collagenase is excreted into the culture supernatant and can be measured in this manner, it is actually cell-associated (91,69) and results obtained by M.
Lantz, R.B. Robertson and S.C. Idolt (1983) J. Dent. Res. 62:289 Abst. No. 1076 indicate that the enzyme is contained within the periplasmic space.

Early studies were not able to differentiate between the different subspecies of Bacteroides. Even today with the distinctions made between the species and subspecies of black-pigmenting oral Bacteroides, there is much controversy as to which of them are able to exhibit collagenolytic activity (91,69,117). Mayrand et al. (71) showed that oral strains of B. melaninogenicus and B. intermedius did not exhibit any collagenase activity. Other workers (Steffen and Hentges 1981, (115) Robertson et al. (1982) (91) using different protocols, assay procedures, and strains found B. assachorolyticus as well as other Bacteroides species to be collagenolytic. Most recently Mayrand et al. (70) using an assay described by Steffen and Hentges (115) found that only one organism, B. gingivalis formly B. asaccharolyticus was able to degrade collagen under their specific set of procedures. In fact, there is little agreement as to which of these microorganisms are actually involved in the pathogenesis of periodontitis.
I. Isolation and Maintenance of Cultures

_Bacteroides melaninogenicus_ subspecies _melaninogenicus_ strain 15930, _B. melaninogenicus_ subsp. _intermedius_ strain 581 and _B. asaccharolyticus_ strain 381 were used throughout this study. Other organisms used were _B. melaninogenicus_ subsp. _intermedius_ strains strains 581B, 587, 379 and _B. asaccharolyticus_ strains 382 and AB-1. Strains 15930 and 25261 were obtained from the American Type Culture Collection (Rockville, Maryland). Strains 381, 382, 379, 581, and 581B were kindly supplied from stock cultures by the Virginia Polytechnic Institute or from deep periodontal pockets by Dr. D.C. Birdsell of the University of Florida (81). Strain AB-1 was isolated from a deep periodontal pocket during a periodontal surgery at Loyola University School of Dentistry. The oral strains of _Bacteroides_ listed above are referred to as black-pigment producing oral _Bacteroides_ (BPPOB). The identity of all _Bacteroides_ used in this study was confirmed by use of the API 20A system (Analytab Products, Plainview New York), which employed a variety of biochemical tests.
Stock cultures of each strain were stored in 1.0 ml defibrinated sheep blood (Ovine Laboratories, Chicago) at -60°C (Model SZB-659, Revco Freezer Co.). For routine work, cultures were maintained at 37°C in enriched Todd-Hewitt Broth or on blood agar (BBL) as described below.

II. Media

A. Liquid media. Several different complex media were used in these studies. Enriched Todd Hewitt Broth (enrTHB), Enriched Trypticase Soy Broth (enrTSB) and Enriched Brain-Heart Infusion Broth (enrBHI) were prepared by adding 0.5% yeast extract (BBL), 0.05% hemin (Eastman Organic Chemical Div., Eastman Kodak Co.), and 0.0005% menadione (Sigma Chem. Co.) to an appropriate amount of THB (BBL), TSB (BBL) or BHI (BBL). All liquid media used in all experiments were allowed to stand at room conditions for 48 hrs. before their inoculation.

B. Solid media. For the routine growth and transfer of BPPOB enriched Todd-Hewitt Blood Agar (enrTHBA) was prepared by the addition on 1.5% agar (Difco labs) and 5% defibrinated sheep blood (Ovine Laboratories) to enrTHB.

C. Selective media. To selectively isolate BPPOB from a variety of microorganisms found in deep periodontal pockets, media was prepared by adding 75 mg/ml kanamycin (51) (Sigma Chem. Co.) and 75 mg/ml L-Cysteine (Nutritional Biochemical Corp.) to enrTHBA. Kanamycin at this concentration will inhibit growth of most other organisms while allowing BPPOB to be isolated.
III. Anaerobic Systems

Routinely, cultures for anaerobic growth were placed in anaerobic jars (BBL) containing a platinum-palladium catalyst. Anaerobic conditions were created by flushing the jar at least three times with a mixture of 5% hydrogen, 10% carbon dioxide and balanced nitrogen (anaerobe grade, Benster Speciality Gas Co.) (43). A methylene blue indicator (BBL) was placed in all jars to monitor anaerobic conditions.

IV. Growth Determinations

In order to determine the characteristics of growth of BPPOB, *B. melaninogenicus* strains 15930, 25261, 581 and 381 were grown in enrTHB under anaerobic conditions at 37°C until visible turbidity could be seen. Each culture was then diluted into an appropriate number of two-day-old tubes of enrTHB to a concentration of $10^5$ cells/ml and then placed in anaerobe jars under anaerobic conditions at 37°C. One tube was removed from anaerobic conditions at 0 time, and then another tube every two to four hours depending on the growth rate of the microorganisms. Serial ten-fold dilution were made from this tube into sterile saline (8.75% NaCl). A 0.1 ml aliquot was spread with a sterile bent glass rod on the surface of duplicate enrTHBA plates giving final dilutions of $10^1$ thru $10^3$. The plates were then placed under anaerobic conditions at 37°C.
for two to four days, removed from incubation, plates containing 30-300 colonies counted, and data recorded.

A. **Dry weight.** To determine the dry weight of cultures grown in broth, duplicate tubes were inoculated. At appropriate time periods, each set of tubes was centrifuged in a IEC HN-SII centrifuge (International Equipment Co., Mass.) at 800 x g for twenty minutes to separate the cellular pellet from the supernatant. The supernatant was discarded and the pellet washed once in saline. These washed cells were centrifuged again and the supernatant discarded. The washed pellet was placed into tubes of saline and resuspended by vortexing (Scientific Industries Inc. New York). The amount of saline added was equal to the original volume of the broth cultures.

Foil laminated bake cups (Maryland Cup Corp, Maryland) were used as weighing cups. These cups were pre-heated in a 70°C oven (National Appliance Corp, Oregon) for two hours and then pre-weighed on a Mettler H10 balance (Mettler Instrument Corp. New Jersey). Within five minutes of preheating, 1.0 ml of the resuspended cells was added to the preweighed cups. A control cup was prepared by adding 1.0 ml of saline to a preweighed cup. These cups were then placed at 70°C overnight, removed from incubation and immediately weighed. Dry weight of cells in mg/ml was determined by subtracting the pre-weighed value of the control cup from the weight of the sample cup.
V. Azocoll Assay

A. Azocoll. Azocoll (Calbiochem., San Diego, CA) is a reddish-purple, insoluble powder prepared by dying hide powder with an azo dye made from tetrazotized binzidine and R-salt (sodium 2-naphthol-3:6 disulphonate). Azocoll is disintegrated when incubated with collagenase,(98) the action of the enzyme being indicated by liberation of a soluble red dye from the insoluble azocoll particles. The amount of color may then be measured spectrophotometrically and amount of enzyme determined by comparing to a standard curve.

B. Amount of azocoll used in assay. In order to determine the proper amount of azocoll which would detect the upper limit of the BPPOB collagenolytic activity, 2 mg, 4 mg, 8 mg, and 18 mg of azocoll were each added to five tubes containing 9.8 ml of enrTHB. A Stock collagenase solution was prepared by adding 5 mg of Clostridiopeptidase A (Sigma Chemical Co.) to 1.0 ml of distilled water and performing serial two-fold dilutions to a final concentration of 0.3 mg/ml. Finally, 0.2 ml of each dilution was added to one of each of the tubes containing the various amounts of azocoll in enrTHB. The tubes were then placed at 37°C and allowed to stand for four hours. At the end of four hours, excess of Azocoll was removed by millipore filtration (Millipore Corp., Mass) through a #1 Whatman filter paper (Whatman Limited, England). The intensity of red dye liberated was determined by use of a Klett-Sommerson Photoelectric Colorimeter (Model 800-3,
Klett Mfg. Co., Inc., New York) with a #54 filter having an appropriate spectral range of 500-570nm. It was found that all levels of enzyme completely degraded 2 mg, 4 mg, and 8 mg samples of Azocoll. However, at concentrations of 18 mg of Azocoll there was an increase in the optical density with increasing concentrations of collagenase, throughout the enzyme range indicating that there was a sufficient amount of Azocoll to be used in our experiments.

C. Standard curve. After the appropriate amount of azocoll to be used has been determined a standard curve of collagenase vs. azocoll was prepared using dilutions of Clostridiopeptidase A as described earlier. Enzyme dilutions ranged from 5 mg/ml to 0.3 mg/ml. Then, 0.2 ml of each dilution was added to 9.8 ml of enrTHB giving final concentrations of enzyme from 0.1 mg/ml to 0.006 mg/ml. Azocoll (18 mg) was then added to each tube and all tubes were incubated at 37°C for four hours. At the end of four hours, excess azocoll was removed by millipore filtration and intensity of red dye liberated was determined photometrically as described above. The optical density readings were then compared to the amount of collagenase present and a standard curve of optical density vs. collagenase concentration made. One unit of activity was defined as the amount of Clostridiopeptidase A necessary to give a Klett reading of 40 after a 4 hour incubation at 37°C in 18 mg of Azocoll. In further experiments this standard curve was used to determine the amount of collagenolytic activity present in BPPOB in units.
D. The assay. To determine the collagenolytic activity of BPPOB, strains were grown in enrTHB under anaerobic conditions at 37°C for two to four days. Cultures were removed from incubation and centrifuged at 800 x g for twenty minutes to separate supernatant from cells. The supernatant was decanted and filtered through a 0.45 millipore filter in a millipore filter unit (Millipore Corporation, Mass) to remove any remaining cells. The cell pellet was washed in TES buffer (0.5M A-tris (hydroxymethyl) methyl 2-aminoethane and 0.05 M CaCl, pH 7.4, Sigma Chem. Co, 3) centrifuged, and the washed pellet suspended in an amount of TES buffer equalling the original volume of the culture. Azocoll (0.018g) was then added to the filtered supernatant and to the washed, resuspended cell pellet. These tubes were then incubated at 37°C for four hours. At the end of four hours, the remaining azocoll was filtered out of both supernatant and cell suspension with a #1 Whatman filter paper. The cell suspension was further filtered with a millipore filter unit containing a 0.45 millipore filter to remove all cells. Color liberated from the insoluble azocoll was read with a Klett-Sommerson Photoelectric Colorimeter and compared to the standard curve to give quantitative amount of collagenolytic activity exhibited by each organism.

VI. Determination of Collagenolytic Activity

In order to determine the collagenolytic activity of the BPPOB, strains 581B, 587, 379, and 581, strains 381,
382, and AB-1 and strain 15930 were grown in tubes of enrTHB under anaerobic conditions at 37°C for nine days (29). Collagenolytic activity was determined using the azocoll assay as described above. In this preliminary screening, only the supernatant was tested for collagenolytic activity (29).

VII. Comparison of Collagenolytic Activity to Bacterial Growth Patterns of B. melaninogenicus Subspecies

A. Growth determinations. BPPOB strains 581, 381, and 15930 were grown in enrTHB under anaerobic conditions at 37°C for 24 hours. Each culture was then diluted into an appropriate number of duplicate tubes of two day old enrTHB to a concentration of $1.0 \times 10^5$ cells per milliliter. Depending on the growth rate of the organism, both of the duplicate tubes were removed from their anaerobic conditions zero and every two to four hours for up to four days. One of these duplicate tubes was used to determine growth patterns by dry weight of organisms as described above.

B. Collagenolytic activity. The other duplicate tube from above, was used to determine the collagenolytic activity of these organisms during their growth. Collagenolytic activity was determined using the azocoll assay described above. Collagenolytic activity was expressed as units per milligram dry weight of organisms.
VIII. Determination of Optimal Conditions for the Collagenolytic Activity of B. melaninogenicus Subspecies

A. Media. B. melaninogenicus subspecies melaninogenicus strain 15930, subspecies intermedius strain 581 and B. asaccharolyticus strain 381 were grown in tubes of enrTHB, enrTSB, enrBHI, and Nutrient Broth (NB) under anaerobic conditions at 37°C for nine days (29). At the end of nine days tubes were removed from incubation, and collagenolytic activity of the three organisms determined using the azocoll assay as described above. Collagenolytic activity was expressed as units per milligram dry weight of organisms.

B. Additives. In order to determine the effect of various additives on the collagenolytic activity of BPPOB, the media described above was supplemented with one of the following; 0.5% yeast extract, 0.05% hemin and 0.0005% menadione or combination of these.

IX. The Effect of Various Parameters on the Collagenolytic Activity of BPPOB

A. pH. In order to determine the effect of pH on the collagenolytic activity of the BPPOB one strain of each was grown in buffered enrTHB. Buffered enrTHB was prepared with McIlvaine buffer solution, (citrate-phosphate buffer) in place of water (72,73). B. melaninogenicus subsp. melaninogenicus strain 15930, B. melaninogenicus subsp. intermedius strain 581 and B. asaccharolyticus strain 381 were grown in 500 ml flasks of buffered enrTHB
buffered to pH 7.2 with a digital ionanalyzer (model 601A, Orion Research, Inc. Massachusetts).

Cultures were grown under anaerobic conditions at 37°C for nine days (29). At the end of nine days cultures were removed from incubation and pH was again determined and then adjusted to pH 7.2. At the same time, collagenolytic activity was determined using the azocoll assay as described above. Collagenolytic activity was expressed as units per milligram dry weight of organisms.

B. Parotid gland secretion (PGS). PGS was obtained through direct cannulation using a Carlson-Crittendon cap placed directly over the Stenson's Duct and sterilized by filtration using a 0.45 millipore filter unit. The effect of PGS on the collagenolytic activity of *B. melaninogenicus* subsp. *melaninogenicus* strain 15930, *B. melaninogenicus* subsp. *intermedius* strain 581 and *B. asaccharolyticus* strain 381 were determined. All organisms were inoculated to envTHB containing 1.0, 5.0 or 10.0% sterile PGS. Duplicate tubes containing 1.0, 5.0 and 10.0% sterile water were also prepared to be used as controls. All tubes were placed under anaerobic conditions at 37°C for nine days (29). At the end of nine days tubes were removed from incubation and collagenolytic activity was determined using the azocoll assay as described above. Collagenolytic activity was expressed in units per milligram dry weight of organism.
X. Electron Microscopy

A. Prefixation. Microorganisms to be visualized were removed from the surface of an enrTHBA plate and placed in 9.0 ml of modified veronal buffer, MVB, pH 6.1 (47). The tube was centrifuged at 800 x g for twenty minutes. Prefixation was completed by placing the cells in a 4% solution of gluteraldehyde in (MVB) with addition of 700 parts per million of the polysaccharide stain, ruthenium red (Sigma Chemical Co., MO) and thoroughly mixed (47). The tube was then centrifuged at 800 x g for twenty minutes and the supernatant discarded.

B. Fixation. Fixation was accomplished by placing the pellet in an OsO₄ solution (MVB, OsO₄, ruthenium red) thoroughly mixing the solution and cells, and letting stand overnight.

C. Dehydration. The tube was again centrifuged at 800 x g for twenty minutes and the supernatant discarded. The specimen was dehydrated sequentially in 70, 90, 95 and three 100% acetone solutions (Mallinkrodt Chemical Co., Kentucky, acetone diluted in distilled water) for one half hour each. The specimen was then centrifuged after each successive dehydration at 800 x g for twenty minutes and the supernatant discarded.

D. Embedding. The specimen was imbedded by placing the cell pellet in 50:50 mixture of Spurr's (Polyscience, Inc. Penn.) and acetone and thoroughly mixed. After one hour the tube was centrifuged at 800 x g for twenty minutes and the supernatant discarded. This procedure was then
duplicated with 100% Spurr's at room temperature for twenty-four hours. Embedding was completed by transferring an appropriate amount of the stained specimen to freshly prepared 100% Spurr's in a BEEM capsule (Polyscience, Inc. Penn.) at 70°C for twenty-four to forty-eight hours.
CHAPTER 3

RESULTS

I. Preliminary Findings

A. Optimal growth media

Various enriched complex media are routinely employed for the growth of anaerobic microorganisms. These media include, enrTHB, enrTSB, enrBHI, and enrNB, and were used because of their practicality, cost and, most importantly, their effectiveness for the growth of fastidious anaerobes. In order to determine a medium in which BPPOB would display their greatest collagenolytic activity, B. asaccharolyticus and B. melaninogenicus subsp. were grown in each media and the supernatant of each tested as described in Materials and Methods. As seen in Table 1, a representative of each subspecies is shown. Greatest collagenolytic activity was grown in enrTHB. Other strains tested gave similar results. Somewhat lower levels of activity could be seen in the enrTSB, while the lowest levels of collagenolytic activity were detected in the enrBHI. Both B. asaccharolyticus and B. melaninogenicus subspecies were unable to grow in enrNB. As determined by dry weight, BPPOB grew in enrTSB, with slightly less growth being observed in both the enrTHB and enrBHI media. (Data not
TABLE 1

Collagenolytic Activity\(^a\) of *Bacteroides melaninogenicus* Subspecies After 9d Growth\(^b\) In Various Media

<table>
<thead>
<tr>
<th>Enriched Growth Media</th>
<th>asaccharolyticus (units/mg dry weight)</th>
<th>intermedius (units/mg dry weight)</th>
<th>melaninogenicus (units/mg dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Todd-Hewitt Broth (BBL)</td>
<td>21.4</td>
<td>6.2</td>
<td>0.15</td>
</tr>
<tr>
<td>Trypticase Soy Broth (BBL)</td>
<td>4.6</td>
<td>2.6</td>
<td>0.45</td>
</tr>
<tr>
<td>Brain Heart Infusion Broth (BBL)</td>
<td>2.3</td>
<td>1.85</td>
<td>0.01</td>
</tr>
<tr>
<td>Nutrient Broth (Difco)</td>
<td>N.G.(^c)</td>
<td>N.G.</td>
<td>N.G.</td>
</tr>
</tbody>
</table>

\(^a\) Release of Azo dye from the collagenous proteolytic substrate Azocoll (Calbiochem) as compared to a Clostridiopeptidase A standard

\(^b\) Cultures were grown at 37°C under anaerobic conditions (modified GasPak system, BBL)

\(^c\) N.G. = no growth
shown). Because of the optimal collagenolytic activity in enrTHB, this medium was employed in all further experiments.

B. Growth characteristics

The characteristic growth patterns of \textit{B. asaccharolyticus} and \textit{B. melaninogenicus} subspecies were determined in order to identify the specific growth phases of each organism. These growth phases represent the immediate metabolic activity of an organism and will be used in later studies.

Typical growth curves of \textit{B. asaccharolyticus}, \textit{B. melaninogenicus} subsp. \textit{melaninogenicus} and \textit{B. melaninogenicus} subsp. \textit{intermedius} are shown in Figure 1, 2 and 3 respectively.

C. Additives

In vivo, many anaerobic organisms, like the BPPOB, require metabolic endproducts which they receive from synergistic relationships with other microorganisms. In vitro, these substances must be supplied to the organisms to ensure optimal growth, although this addition does not truly recreate their normal environment. Traditionally, additives such as yeast extract, hemin and menadione have been used for the growth of BPPOB, although the effect of these additives on collagenolytic activity of oral \textit{Bacteroides} has never been tested.

In this study, yeast extract, hemin, and menadione were tested separately and in combination for their effect on
Figure 1. Representative growth curve of *Bacteroides asaccharolyticus* strain 381 in enrTHB at 37°C under anaerobic conditions. Each point represents the mean viable count of duplicate samples as described in Materials and Methods.
Figure 2. Representative growth curve of *Bacteroides melaninogenicus* subsp. *intermedius* strain 581 in enrTHB at 37°C under anaerobic conditions. Each point represents the mean viable count of duplicate samples as described in Materials and Methods.
Figure 3. Representative growth curve of *Bacteroides melaninogenicus* subsp. *melaninogenicus* strain 15930 in enrTHB at 37°C under anaerobic conditions. Each point represents the mean viable count of duplicate samples as described in Materials and Methods.
collagenolytic activity and described in the Materials and Methods. As shown in Table II, *B. asaccharolyticus* and *B. melaninogenicus* subsp. *intermedius* displayed increased collagenolytic activity, as compared to the THB alone, when grown in media containing hemin and menadione. In contrast, the addition of yeast extract, alone, although stimulating the growth of *B. asaccharolyticus* and *B. melaninogenicus* subsp. *intermedius* depressed these organisms' collagenolytic activity. This potentially result would indicate that the use of yeast extract for in vitro monitoring of collagenolytic activity by these BPPOB could give misleading results. For this reason YE was not used as an additive in any further studies unless otherwise stated. *B. melaninogenicus* subsp. *melaninogenicus*, not shown here, produced an extremely low level of activity in all cases.

II. Collagenolytic Activity of BPPOB

Various strains of *B. melaninogenicus* subspecies were screened for collagenolytic activity as described in the Materials and Methods. As shown in Table III, *B. asaccharolyticus* strain 381 and 382 displayed marked collagenolytic activity as did *B. melaninogenicus* subsp. *intermedius* strains 581 and 581B. *B. melaninogenicus* subsp. *intermedius* strain 379 and 25261 and *B. melaninogenicus* subsp. *melaninogenicus* strain 15930 displayed almost no collagenolytic activity. As a result, it was determined to use only BPPOB strains 381, 581, and 15930 for this study.
<table>
<thead>
<tr>
<th>Addition</th>
<th>Collagenolytic Activity (units/mg dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Subspecies/Strain</td>
</tr>
<tr>
<td></td>
<td>asaccharolyticus/381</td>
</tr>
<tr>
<td>None</td>
<td>1.8</td>
</tr>
<tr>
<td>Yeast Extract (0.5%)</td>
<td>0.8</td>
</tr>
<tr>
<td>Hemin (0.05%) and Menadione (0.0005%)</td>
<td>2.3</td>
</tr>
<tr>
<td>Yeast Extract, Hemin and Menadione</td>
<td>2.25</td>
</tr>
</tbody>
</table>

**a** Release of Azo dye from the collagenous proteolytic substrate, Azocoll (Calbiochem) as compared to a Clostridiopeptidase A standard.

**b** All strains were grown in enriched Brain-Heart Infusion broth (BBL) for 9d at 37°C under anaerobic conditions, (modified GasPak system, BBL).
TABLE 3

Collagenolytic Activity<sup>a</sup> of Bacteroides melaninogenicus Subspecies After 9d Growth<sup>b</sup> in Enriched Todd-Hewitt Broth

<table>
<thead>
<tr>
<th>B. melaninogenicus Subspecies</th>
<th>Strain</th>
<th>Collagenolytic Activity (units/10.0 ml supernatant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>asaccharolyticus</td>
<td>381</td>
<td>20.5</td>
</tr>
<tr>
<td>intermedius</td>
<td>581</td>
<td>17.5</td>
</tr>
<tr>
<td>melaninogenicus</td>
<td>15930</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Release of Azo dye from the collagenous proteolytic substrate Azocoll as compared to a Clostridiopeptidase A standard.

<sup>b</sup> Cultures were grown at 37°C under anaerobic conditions (modified GasPak system, BBL).
Gibbons and MacDonald (29) have compared the collagenolytic activity of underdetermined subspecies of *B. melaninogenicus* during the characteristic growth phases. However, these authors failed to standardize the collagenolytic activity of these organisms with their growth, giving a false representation of these organisms activity over time.

In this study the collagenolytic activity of *B. asaccharolyticus*, *B. melaninogenicus* subsp. *intermedius* and *B. melaninogenicus* subsp. *melaninogenicus*, expressed in units/mg dry weight, were compared to the characteristic growth patterns of each organism as described in Materials and Methods. As seen in Fig. 4 *B. asaccharolyticus* and *B. melaninogenicus* subsp. *intermedius* behave similarly. *B. melaninogenicus* subsp. *melaninogenicus* although displaying a normal growth pattern, exhibit extremely low levels of collagenolytic activity allowing no correlations to be made to its growth pattern.

In both *B. asaccharolyticus* and *B. melaninogenicus* subsp. *intermedius* activity remained extremely low until bacterial dry weight began to increase sharply. Collagenolytic activity peaked at 52 hours for subspecies *intermedius* and at 56 hours for *B. asaccharolyticus*, while bacterial growth reached its peak at 76 hours. Collagenolytic activity could be detected only after logarithmic phase had begun and these organisms had reached a high level of metabolic activity. As the stationary
Figure 4. Collagenolytic activity of BPPOB as related to representative growth curves. Predetermined numbers of 18 h. old microorganisms were inoculated to THB with 0.5% yeast extract and incubated at 37°C under anaerobic conditions. At each time period, a tube was removed and culture supernatant tested for collagenolytic activity as described in Materials and Methods.
Subsp. asaccharolyticus

Subsp. intermedius

Subsp. melaninogenicus

TIME (Hours)

COLLAGENOLYTIC ACTIVITY (Units/mg dry wt)

DRY WEIGHT (mg)
phase of growth was reached, collagenolytic activity began to drop, extracellular collagenolytic enzymes appear to be loosing activity under the growth conditions employed. Once the death phase occured, as indicated by a drop in dry weight, collagenolytic activity again rose, signifying that active enzymes were also released on autolysis of cells. It is quite clear that high levels of collagenolytic activity will be obtained on active growth of cells as well as when they lyse and dye during the continuous culture conditions of the oral cavity.

III. The Effect of Oral Conditions on the Collagenolytic Activity of BPPOB

The oral cavity is an extremely complex and variable environment. Many factors within the oral cavity may effect the collagenolytic activity of one of the BPPOB. In this study, the effect of pH and one of the major components of the saliva, Parotic Gland Secretion (PGS) on the collagenolytic activity of B. asaccharolyticus and B. melaninogenicus subspecies was tested.

A. Determination of the optimal pH for the production of collagen degrading substances

The optimal pH for the collagenolytic activity was found to be over the range of from pH 7.0 - 7.4. More acid or alkaline conditions inhibited collagenolytic activity completely.
B. The effect of PGS on the collagenolytic activity of BPPOB subspecies

As seen in Fig. 5 the effect of PGS on the collagenolytic activity of _B._ *asaccharolyticus* and _B._ *melaninogenicus* subspecies was varied. PGS had a stimulatory effect on the collagenolytic activity of _B._ *asaccharolyticus* and _B._ *melaninogenicus* subsp. *melaninogenicus* while inhibiting this activity in _B._ *melaninogenicus* subspecies *intermedius*. Maximum stimulation of collagenolytic activity was observed for _B._ *asaccharolyticus* and _B._ *melaninogenicus* subsp. *melaninogenicus* when 0.1 ml or 1% PGS was added to the medium. When the PGS was increased to 1.0 ml or 10% of the medium, 95-99% of this stimulation was lost. In contrast, PGS had an inhibitory effect on the collagenolytic activity of _B._ *melaninogenicus* subsp. *intermedius*. Maximum inhibition occurred when 0.5 ml or 5% PGS was added to the medium. When the amount of PGS was decreased to 0.1 ml or 1% of the medium, 99% of this inhibition was lost.

Similarly, when the amount of PGS was increased to 1.0 ml or 10% of the medium, 75% of this inhibition was lost.

The inhibition of collagenolytic activity of _B._ *melaninogenicus* subsp. *intermedius* is an interesting finding. Whether the same substances responsible for the stimulation of collagenolytic activity seen with _B._ *asaccharolyticus* and _B._ *melaninogenicus* subsp. *melaninogenicus* are responsible for the inhibition seen here is unknown.
Figure 5. Effect of parotid gland secretion (PGS) on collagenolytic activity of black pigment-producing oral Bacteroides.
IV. Electron Microscopy

Although the collagenolytic activities of *B. asaccharolyticus* and *B. melaninogenicus* subspecies may play a major role in these organisms pathogenicity, it is also of great importance to know if these bacteria possess the ability to remain at the site of infection and prevent host defense mechanisms. One such way in which bacteria might accomplish this end is to produce some type of extracellular material, such as capsular material, matrix or fibrils. In order to gain some insight as to this ability, a preliminary electron microscopic study on the ultrastructure of *B. asaccharolyticus* and *B. melaninogenicus* subspecies was performed.

Seen in Fig. 6, 7, and 8 is a representative thin sections of (6) *B. asaccharolyticus*, (7) *B. melaninogenicus* subspecies *melaninogenicus* and (8) *B. melaninogenicus* subspecies *intermedius*. As shown, an electron dense material (EM) was consistently observed surrounding cells of *B. asaccharolyticus*.

In contract, *B. melaninogenicus* subsp. *melaninogenicus* possesses distinct fibrils. These type of fibrils classically play a role in an organisms ability to attach. The potential abilities of this organism to stay at the sight of infection, along with its stimulated increase in collagenolytic activity by PGS could greatly enhance its pathogenic potential. *B. melaninogenicus* subsp. *intermedius* does not possess any extracellular matter. Even
Figure 6. Electron microscopy of *B. asaccharolyticus*.

Bacteria were grown in enrTHB at 37°C under anaerobic conditions for 9 days and then fixed and embedded and sectioned as described in Materials and Methods. The 1.5 cm bar represents 1µ.
Figure 7. Electron microscopy of *B. melaninogenicus* subsp. *melaninogenicus*. Bacteria were grown in enrTHB at 37°C under anaerobic conditions for 9 days and then fixed and embedded and sectioned as described in Materials and Methods. The 1.5 cm bar represents 1µ.
Figure 8. Electron microscopy of *B. melaninogenicus* subsp. *intermedius*. Bacteria were grown in enrTHB at 37°C under anaerobic conditions for 9 days and then fixed and embedded and sectioned as described in Materials and Methods. The 1.5 cm bar represents 1µ. 
High cell lines displayed a relatively high level of interferon activity. The overall pathogenicity may be increased due to the occurrence of an acute onset mechanism.
though this organism displayed a relatively high level of collagenolytic activity, its overall pathogenicity may be decreased due to the absence of an attachment mechanism.
CHAPTER 4

DISCUSSION

The species Bacteroides melaninogenicus was first described by Oliver and Wherry in 1921 (85). This species contains strictly anaerobic non-motile, non-sporing, gram-negative coccobacilli which produce dark brown pigmented colonies on media containing blood. In 1973 Moore and Holden (83) were able to divide the dark brown pigmented Bacteroides melaninogenicus into three subspecies Bacteroides melaninogenicus, subsp. melaninogenicus, subsp. intermedius and subsp. asaccharolyticus. Since 1973 much information has accumulated, both biochemical and physiological, (42) amplifying the differences between each subspecies. In addition, it has been observed that of the BPPOB, B. asaccharolyticus is most frequently isolated from clinical infections, including inflammatory periodontal. The distinct biochemical, physiological and clinical differences between the three subspecies provoked the ICSB taxonomic subcommittee on gram-negative anaerobic rods to suggest that B. melaninogenicus subsp. asaccharolyticus was sufficiently different from the other subspecies to be regarded as a separate species designated Bacteroides asaccharolyticus (3).

Prior to 1973, much research was performed implicating B. melaninogenicus as an essential pathologic component
of inflammatory periodontal disease (29,38,59,60,110) Unfortunately, this research was performed without knowing which of the species or subspecies of Bacteroides was involved.

There is currently much controversy as to which microorganisms are isolated from different stages and types of periodontal disease. However, it is clear that B. asaccharolyticus is the most frequently isolated microorganism from advanced periodontitis. In a recent study, White and Mayrand (124) found that, although B. melaninogenicus subsp. intermedius was frequently isolated from pockets of depth 4 to 6 mm, B. asaccharolyticus was still the most frequently isolated from pockets of 6 mm or greater. In addition, they stressed the need to characterize the pathogenic properties of B. asaccharolyticus. The recent accumulation of information illuminating the biochemical, physiological and clinic differences between each organism has made it essential to reevaluate previous information presented on B. melaninogenicus.

In our laboratory it was shown that oral strains of B. asaccharolyticus displayed consistently high collagenolytic activity. B. melaninogenicus subsp. intermedius revealed marked collagenolytic activity in comparison to B. melaninogenicus subsp. melaninogenicus which exhibited extremely low collagenolytic activity. This data coincides with a study (68) which surveyed the collagenolytic activity B. asaccharolyticus
and *B. melaninogenicus* subspecies using radioactively labeled bovine achilles tendon in an assay described by Gislow and McBride (1975).

The potential importance of an enzyme such as this has been described above and elsewhere (91, 69, 115, 121, 53).

Once the collagenolytic activity of *B. asaccharolyticus* and *B. melaninogenicus* subspecies was established it was of considerable interest to determine when this activity was expressed during the characteristic growth patterns of these organisms. An obvious difference in the collagenolytic activities of these organisms might partially explain why *B. asaccharolyticus* is most frequently isolated from clinical infections. When collagenolytic activity was compared to bacterial growth patterns, *B. asaccharolyticus* and *B. melaninogenicus* subsp. *intermedius* behave similarly. *B. melaninogenicus* subsp. *melaninogenicus* although displaying a typical growth pattern, exhibited extremely low amounts of collagenolytic activity.

It was interesting to note that in both *B. asaccharolyticus* and *B. melaninogenicus* subsp. *intermedius* collagenolytic activity remained extremely low until bacterial dry weight began to increase sharply. Only after cells reached a high metabolic activity, during logarithmic phase, could collagenolytic activity be detected. This would indicate that collagenolytic activity is present in actively growing cells.
It is clear that high levels of collagenolytic activity are present while cells are actively growing as well as when they die and lyse in the continuous culture conditions of the oral cavity. This data would indicate that the enzyme produced by *B. asaccharolyticus* and *B. melaninogenicus* subsp. *intermedius* is being released from the cell and is not necessarily dependent upon cell lysis for its entry into the environment.

In contrast, MacDonald and Gibbons, (29) found that the collagenolytic activity of *B. melaninogenicus* could not be seen until stationary phase was reached and culture autolysis began. Similarly, these authors observed that collagenolytic activity increased as autolysis continued. Gibbons and MacDonald (29) suggested that the enzymes responsible remained intracellular until released by autolysis. Unfortunately, these authors failed to standardize collagenolytic activity of *B. melaninogenicus* by equilibrating their values with the quantity of bacteria present. Instead they expressed their collagenolytic determinations in ranges, making definitive interpretation of this data impossible.

In a recent study, Robertson et al. (90) demonstrated that as *Bacteroides* reaches stationary phase, collagenolytic activity is seen in the media, presumably reflecting autolysis. Collagenase has been detected in both culture media and cell sonicates (90,128). It is now generally accepted that although collagenase may be detected in culture supernates, the enzymes is actually cell associated (90,69,
Regardless of the specific time course of production, there is no doubt a large bulk of lysing and dead cells in the subgingival plaque. This material could provide large stores of the collagenolytic enzymes for tissue destruction. The data described above (Fig. 4) clearly indicates that *B. melaninogenicus* subsp. *melaninogenicus* does not produce appreciable levels of collagenase under our experimental conditions. In contrast to this, Robertson et al. found that all species of *Bacteroides* tested, including all *B. melaninogenicus* and *B. fragilis*, did exhibit collagenolytic activity. Mayrand (69) demonstrated that formerly *B. asaccharolyticus* but not *B. melaninogenicus* produced collagenase. Steffen and Hentges (115) showed that all oral *Bacteroides* but not *B. fragilis* produce the enzyme. Most recently, using a slightly different set of assays and procedures, Mayrand et al. (70) have indicated that only *B. gingivalis* possess collagenolytic activity. The discrepancies seen between these studies most likely is due to the variety of strains tested, environmental conditions used, and the sensitivity of the assays. It is important to note that in our study and those of others, the end result of collagen breakdown is due to the combined action of bacterial collagenase and other proteolytic enzymes produced by these microorganisms. In fact, Yamamota (128) demonstrated that *B. gingivalis* produces a somewhat unique proteolytic enzymes not seen in other *Bacteroides* species. Sobhy et al. (107) concluded due to findings from clinically
diseased tissue that both collagenase and proteases were important in the gingival matrix destruction seen in periodontal disease.

The human periodontal pocket, initial site of inflammatory periodontal disease contains fifty or more species of microorganisms (45). The bacteria which inhabit the mouth constitute a ecosystem which has physical, chemical and biological properties that not only dictate the composition of the community, but determines which organisms will dominate the system and which will fail to survive. The biological relations of microorganisms are of the greatest significance in the economy of nature and in the production of disease.

Many factors play a role in the development of clinical infection, yet investigators have concluded that infections caused by several organisms are not microbially specific, but are specific in the biochemical sense (1). The initiation of disease depends not only a specific combination of microbes, but also on the elaboration of a specific combination of require metabolites (69). Research done by MacDonald et al. (59) found that the occurrence of Bacteroides melaninogenicus in guinea pig infections was dependent on the elaboration of Vitamin K like compounds elaborated by other organisms. In this respect, it seemed evident that the elaboration of metabolites by other organisms (along with the natural environment produced by the host in the oral cavity) do effect the pathogenic potentialities of Bacteroides asacharolyticus and other BPPOB. However, the intricacy
and complexity of the nutritional and physiological environment of the periodontal pocket, the site of IPD, can not be reproduced in vitro. The synthetic environment employed in the growth and maintenance of the BPPOB in vitro could alter the metabolic and physiologic patterns of these organisms, ultimately altering their pathogenic qualities.

In this study it was found that the addition of yeast extract, a normal constituent of the "complex media" used to maintain fastidious microorganisms like the BPPOB, although stimulating the growth of \textbf{B. asaccharolyticus} and \textbf{B. melaninogenicus} subspecies, depressed these organisms collagenolytic activity. The specific substance or substances capable of effecting these BPPOB was not established. It is possible that the overall increase in the numbers of peptides or amino acids added to the media through the yeast extract may act as feedback repressors of collagenase synthesis. A similar explanation was given by MacDonald et al. (61) when they discovered that diluting media employed to grow \textbf{B. melaninogenicus} (thus decreasing the overall peptide and amino acid content) decreased growth of organisms while strikingly increasing their collagenolytic activity. More recently, Robertson et al. found that when strains of \textbf{Bacteroides} and \textbf{Actinobacillus} were grown in a peptide depleted medium their enzyme activities were enhanced. No explanation was given (91). This is significant in that a similar explanation could account for the enhancement of the pathogenicity of \textbf{B. asaccharolytics} and \textbf{B. melaninogenicus} subspecies in the presence of other
suitable organisms. If these organisms were found to utilize the endproducts of collagenolytic activity, thus decreasing the peptide and amino acid content of the environment, they could mediate the collagenolytic activity of BPPOB by removing these feedback repressors from the environment.

It is of considerable importance to understand the effect of in vitro conditions which may alter the pathogenic qualities of *B. asaccharolyticus* and *B. melaninogenicus* subspecies. Any in vitro conditions which can alter the collagenolytic activity of these organisms will also alter our understanding of how these BPPOB behave in their natural environment. In order to gain some knowledge of how the collagenolytic activity of these organisms is affected by the intricate and complex nutritional and physiological environment of the periodontal pocket, pH and PGS were tested. By definition, collagenase are proteolytic enzymes capable of degrading native collagen fibrils under physiologic conditions of pH and temperature. Since the physiologic temperature of the oral cavity is normally constant at 37°C, it was necessary to keep this temperature during all experimental studies on the collagenolytic activity of these BPPOB. In contrast, the physiologic pH of the oral cavity is extremely variable. In order to determine if *B. asaccharolyticus* and *B. melaninogenicus* subspecies are active at the pH of the periodontal pocket, pH 7.4, the effect of various pH on the collagenolytic activity of these organisms was tested. In agreement with other authors (29,35) collagen breakdown was greater over a pH of
7.0 to 7.4. More acidic conditions inhibited collagenolytic activity completely. The fact that collagenolytic activity is seen at pH 7.4 in *B. asaccharolyticus* and *B. melaninogenicus* subspecies suggest that it is related to the collagen destruction seen in periodontal disease.

The most obvious environmental factor present in the oral cavity which may have an effect on the collagenolytic activity of *B. asaccharolyticus* and *B. melaninogenicus* is the saliva. Saliva is a mixed secretion more than 90% of which is produced by the parotid, submandibular and sublingual glands. The remainder is contributed by accessory glands present on the soft palate and on the internal surface of the lips and cheeks. In addition, it has been estimated that between 700 and 800 ml of saliva are produced daily. Saliva has many functions of which the most important are protection, digestion, control of water balance and lubrication. The pH of the saliva varies from 5.6 to 8.0 and contains considerable amounts of Ca++. 

One primary means by which such collagenolytic activity might be controlled or altered is by salivary components. Protein (1.8 - 4.2 g/l) and amino acids (40 mg/l) are major components of saliva (45) and may have an effect on collagenolytic activity. Some amino acids have been found to inhibit elaboration of collagenase. Monboisse (80) noted in his study of Acinetobacter that these materials did, in fact, repress synthesis of collagenase. MacDonald et al. (60) reported that when their growth media for Bacteroides was diluted, cultures grew slower, but collagenase activity was
greatly increased. P.B. Robertson et al. (91) found that the collagenolytic activity of stains of *Bacteroides* and *Actinobacillus* were enhanced when grown in a peptide depleated medium. Yeast extract in our study was demonstrated to have essentially the same effect. Proteinaceous components might well be responsible.

Although saliva plays an obvious role in both the environmental and physiological conditions of the oral cavity their has been no information accumulated as to the effect of saliva on the collagenolytic activity of *B. asaccharolyticus* and *B. melaninogenicus* subspecies. One reason which might explain the lack of information on the effect of saliva on the collagenolytic activity of these organisms is the variability of the composition of saliva. Whole (or mixed) saliva, which is usually collected from the mouth during paraffin wax stimulation, not only contains fractions from each gland, but in addition contain food debris, plaque, bacteria, degraded mammalian cells and possibly some gingival sulcular fluid. The extremely variable composition of this fluid would make any explanation of its effect on the BPPOB impossible. For this reason, and because the parotid gland becomes the major contributor to mixed saliva under stimulated conditions (48) it was decided to test the effect of Parotid Gland Secretion, collected with special canniculi directly from the Stenson's Duct. This material is pure and sterile and is truely representative of the secretion of the gland in question. In addition, it has been shown that whole saliva contains vitamin K, which would have a direct effect
on the BPPOB (58). In contrast, the same author discovered that this growth factor was not present in PGS.

The effect of PGS on the collagenolytic activity of \textit{B. asaccharolyticus} and \textit{B. melaninogenicus} subspecies was compared to identical cultures of each species containing equal amounts of H$_2$O. A potentially important observation was that PGS was able to stimulate collagenolytic activity in \textit{B. asaccharolyticus} and \textit{B. melaninogenicus} subsp. \textit{melaninogenicus}. The constituent of the PGS which was able to impose such effects on the collagenolytic activities of these organisms was not determined. However, it is apparent that this factor is in no way related to the amino acid or peptide concentration of PGS, since the effect of PGS was compared to an identically diluted culture with distilled H$_2$O in place of the PGS. Furthermore, the high amino acid and peptide concentration of PGS (58) would be expected to decrease the collagenolytic activity of these organisms as was previously shown by Robertson et al. (91) and would be expected to have the same effect on all three organisms. In this light, it is possible that the PGS contains a substance which acts as an inducer, inactivating the feedback repression of the amino acids and peptides produced through the destruction of collagen. The most important observation which may be drawn from the data is that PGS has a variable effect on the three organisms. The normal study of the collagenolytic activity of these organisms is done in a complex media. Once a singular oral environmental factor is added to this media the organisms behave differently. This
is underlined by the fact that although B. melaninogenicus subsp. melaninogenicus did not exhibit marked collagenolytic activity in culture, once PGS was added its activity increased substantially. In contrast B. melaninogenicus subsp. intermedius which revealed marked collagenolytic in culture, was substantially inhibited by the addition of singular oral environmental factor. It is of great importance in this study of the collagenolytic activity of B. asaccharolyticus and B. melaninogenicus subspecies to realize that oral environmental factors can drastically alter the potential of these organisms to degrade collagen.

Another factor which might play a role in the ability of Bacteroides to initiate and/or participate in periodontal disease would be surface structures such as capsules of fibrils. Dahlen and Nygram (14) demonstrated a uniform polysaccharide lining in all Bacteroides strains yet the surface layer was least expressed in B. melaninogenicus (in about 10% of the cultures examined). Other investigators have also demonstrated a capsule by ruthenium red staining (46, 64, 54, 65, 76, 126). As seen in Fig. (6, 7, and 8) our studies have shown that an electron dense material (EM) was consistently observed surrounding cells of B. asaccharolyticus. In a contrast, B. melaninogenicus subsp. melaninogenicus was shown to possess distinct fibril B. melaninogenicus subsp. intermedius did not possess any extracellular material. Capsuler material or surface structures may play a role in pathogenesis of mixed
infections. Coaggregation between Actinomyces viscosus and Bacteroides gingivalis could not be accounted for only by the fimbriae of Actinomyces. Specific surface structures of Bacteroides gingivalis were proposed as playing a major role in the process. The types of fibrils observed with Bacteroides melaninogenicus subsp. melaninogenicus classically play a role in an organism's ability to attach. The potential abilities to stay at the sight of infection, along with this organism's increased collagenolytic activity in the presence of PGS could greatly enhance its pathogenicity. In contrast, even though the Bacteroides melaninogenicus subsp. intermedius displayed a relatively high level of collagenolytic activity, the absence of any extracellular material may decrease its overall pathogenicity.
CHAPTER V

SUMMARY

Characterization of collagenase production by black-pigment producing oral bacteroides (BPPOB) was investigated. Bacterial cells were grown at 37°C under anaerobic conditions in enriched Todd-Hewitt Broth. In this study, when yeast extract, hemin and menadione were tested separately and in combination for their effect on collagenolytic activity, _B. asaccharolyticus_ and _B. melaninogenicus_ subspecies _intermedius_ displayed increased collagenolytic activity, as compared to Todd-Hewitt Broth alone, when grown in media containing hemin and menadione. In contrast, the addition of yeast extract, alone, though stimulating the growth of _B. asaccharolyticus_ and _B. melaninogenicus_ subsp. _intermedius_ depressed these organisms collagenolytic activity. Collagenase level was determined using the collagenous substrate Azocoll in a colorimetric assay and employing a standard curve of Clostridiopeptidase A.

_B. asaccharolyticus_ and _B. melaninogenicus_ subspecies _intermedius_ displayed marked collagenolytic activity, while activity was not observed in subspecies _melaninogenicus_. When collagenolytic activity was compared to bacterial growth patterns it was found that the highest
level of activity occurred on active growth of cells as well as during cell death and lysis.

When certain oral environmental conditions were tested for their effect on the collagenolytic activity of BPPOB it was found that the optimal pH for production of collagenolytic substances was 7.0 - 7.4. When Parotid Gland Secretion was added to the growth media of the BPPOB both inhibition and stimulation of collagenolytic activity occurred. Parotid Gland Secretion greatly stimulated collagenolytic activity of *B. asaccharolyticus* and *B. melaninogenicus* subsp. *melaninogenicus* (activity not detectable under other conditions) and inhibited collagenolytic activity *B. melaninogenicus* subsp. *intermedius*.

Electron microscopy revealed that *B. asaccharolyticus* and *B. melaninogenicus* subspecies *melaninogenicus* possessed extracellular substances.
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