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Screening for Protease Genes from
Gene Clone Library of <u>Bacteroides</u> <u>gingivalis</u>

by

Scott C. Palmer, D.D.S.

THESIS

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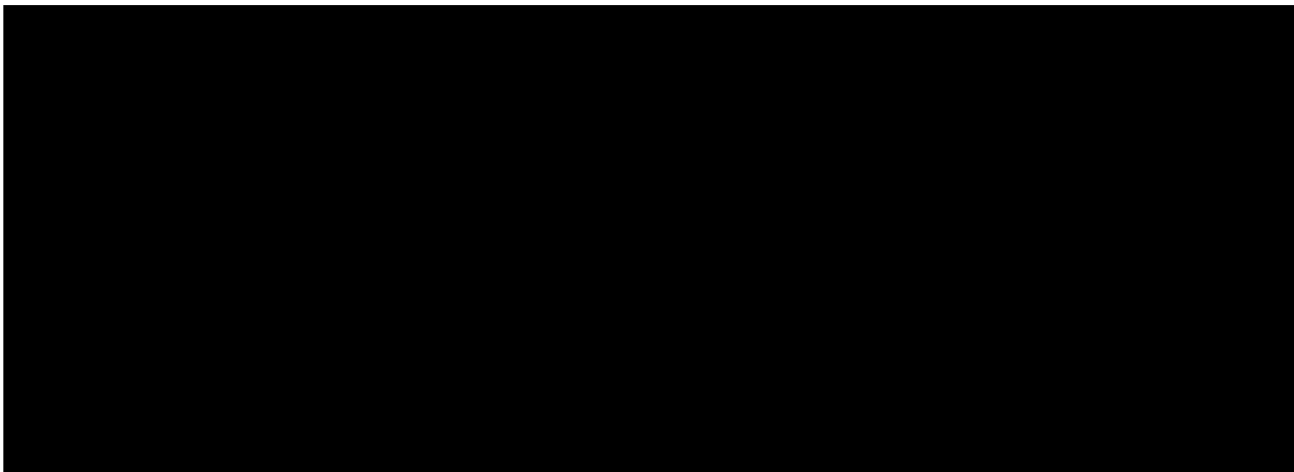
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TABLE OF CONTENTS

LIST OF FIGURES AND ILLUSTRATIONS

INTRODUCTION

Historical Perspective

Periodontal diseases have plagued mankind since the beginning of history. Bone loss indicative of these diseases has been noted in fossil remains of the earliest humans. Written historical records addressing medical topics discuss periodontal diseases and the need for treating them. Some five thousand years ago, the Sumerians fashioned gold decorated toothpicks, presumably with an interest in oral hygiene. Treatment consisted of gingival massage and herbal medications, according to one clay tablet from the Babylonians. Papyrus writings of the Egyptians discuss gingival diseases and treatments, as do ancient Indian and Chinese medical scripts. In one Indian treatise, the Charaka Samhita, oral hygiene is described: "The stick for brushing the teeth should be either astringent or pungent or bitter. One of its ends should be chewed into the form of a brush. It should be used twice a day, taking care that the gums not be injured" (Caranza, 1984).

The Greeks, Romans, and later the Arabs recognized periodontal diseases. Hippocrates of Cos (460-335 B.C.), the father of modern medicine, discussed the function and eruption of teeth. He taught that gingival inflammation was caused by accumulation of pituita or calculus. Roman poets spoke of toothbrushes and gingival massage. Ibn Sina (Avicenna) (980-1037), an Arabic physician, wrote his Canon. Said to be in use over six hundred years, the work included titles such as "Ulcers of the gums", "Bleeding gums", "Recession of the gums", and "Soreness of the gums". Another Arab, Abul-Qasin (936-1013), described the scaling of teeth with special instruments and the removal of hyperplastic gingival tissue (Caranza, 1984).

More modern approaches in the study of dental diseases began in Europe. In 1530,

Ambroise Pare´ (1517-1590) authored the first book entirely devoted to dental practice. The book, Artzney Buchlein, describes "tarter, ... a white, yellow, and black slime that settles on the lower part of the teeth and over the gums". It also presents the concept that systemic as well as local factors may play a role in periodontal diseases, referring to calculus and noxious "humors" which "from the head drop down upon the gums or roots of the teeth and loosen them". Anton van Leeuwenhoek (1632-1723) using the microscope, first described oral bacteria and made drawings of bacilli and spirochetes. Pierre Fauchard (1678-1761), self-educated in dentistry, developed a systematic approach to dentistry. His book, The Surgeon Dentist, transformed the dental profession and educated the next generation of dentists, many of whom relocated to America. Fauchard's writings included the description and usage of five instruments for tartar removal (Caranza, 1984).

Later, in the late nineteenth century, a Russian physician named Znamensky showed a great understanding of the complex nature of periodontal diseases. He observed microscopically the progression of early gingivitis to the more advanced stages of periodontitis, recognizing the infiltration of white blood cells and later the "adsorption of bone". He also described a treatment regimen, which sometimes had a three-month follow-up scraping appointment for areas where his instruments "went too deep into the thickness of the bone - more than a centimetre from the upper front teeth". Znamensky set the stage for later investigations, which sought to explore the importance of the histopathology of this disease (Caranza, 1984).

Since the turn of this century, debate about the etiology of periodontal diseases has centered around two theories, the specific and nonspecific microbial theories. The specific microbial theory proposes that a few specific pathogenic bacterial species are the cause of inflammatory periodontal diseases. Proponents of this theory suggest that the elimination of the specific pathogen is all that would be required to treat the disease. Alternatively, the
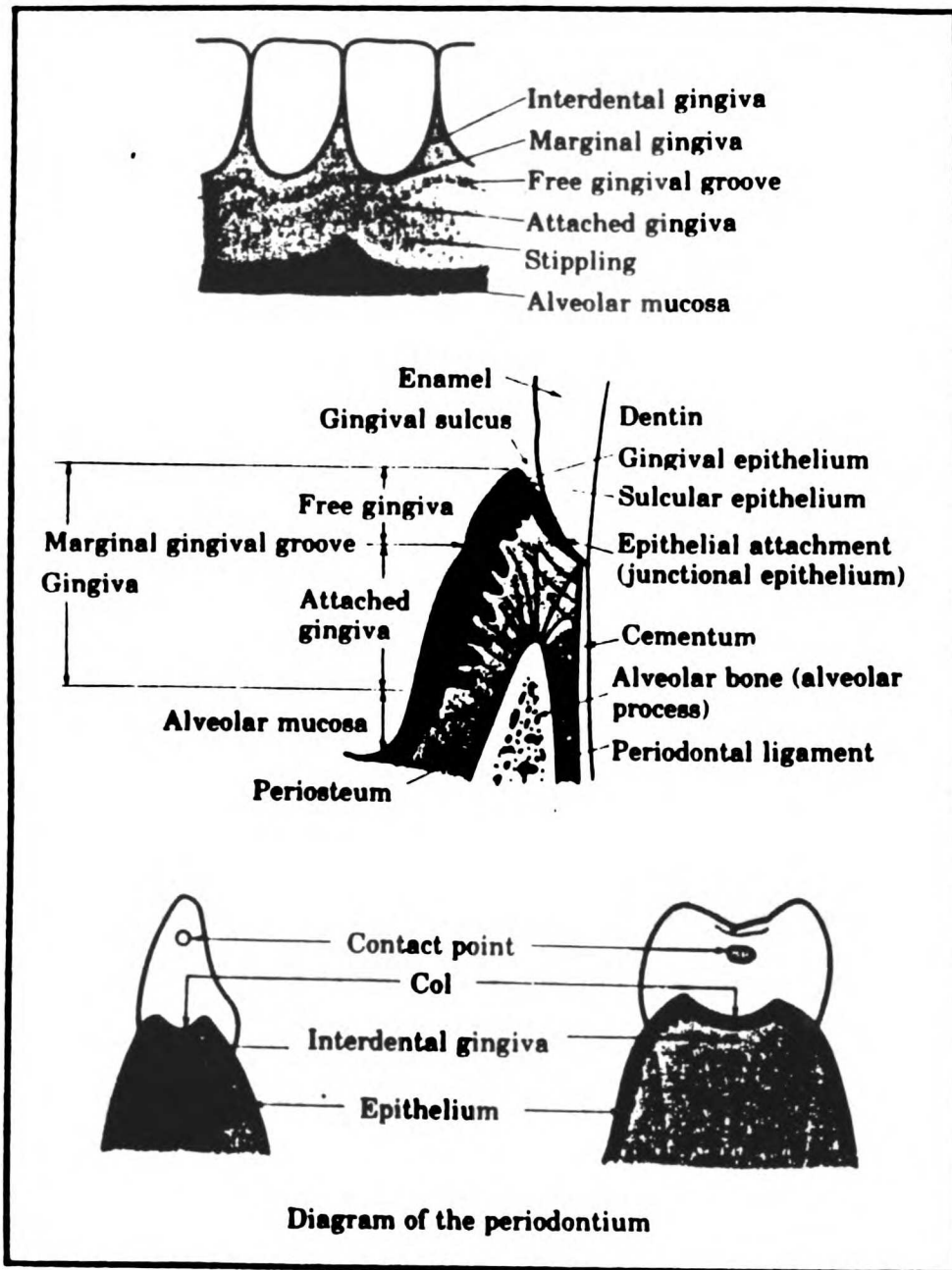
nonspecific microbial theory asserts that a multitude of indigenous oral bacterial species colonize the gingival crevice in the absence of oral hygiene, causing periodontal diseases by triggering host inflammatory hypersensitivity reactions. This theory proposes that many oral bacteria somehow participate in initiating periodontal destruction. The controversy between the specific and nonspecific microbial hypotheses has been mainly academic because treatment is essentially the same in either case. The specific theorists concede that perhaps as many as 6-12 microbial species may be responsible for the various periodontal diseases while the proponents of the non-specific plaque hypothesis admit that some subgingival bacteria are more virulent than others (Theilade, 1986).

Structure of the Periodontium

The periodontium is composed of the two mineralized tissues cementum and alveolar bone connected to each other by gingiva and the periodontal ligament connective tissues. The gingiva surrounding the teeth and bone is composed of dense connective tissue covered by keratinized squamous epithelium. Gingival fibers are arranged in three groups: gingivodental, circular, and transseptal. The healthy periodontium is a tissue of high metabolic activity with turnover rates of the epithelial and connective tissues faster than that of skin. Structural studies reveal that the predominant constituent of the gingiva, cementum, periodontal ligament, and surrounding structures is type I collagen, with type III collagen making up 15-20%. The principal fiber groups of the periodontal ligament, which are collagenous and arranged in bundles, are: transseptal, alveolar crest, horizontal, oblique, and apical (Wooley, 1980).

Figure 1 illustrates the anatomy of the periodontium. The top frontal view outlines the outer appearance of the gingiva and alveolar mucosa. Stippling is the term given to the

3

Figure 1. Anatomy of the Peridontium

- Interdental gingiva
- Marginal gingiva
- Free gingival groove
- Attached gingiva
- Stippling
- Alveolar mucosa

Enamel
Gingival sulcus
Dentin
Gingival epithelium
Sulcular epithelium
Free gingiva
Marginal gingival groove
Gingiva
Epithelial attachment (junctional epithelium)
Attached gingiva
Cementum
Alveolar bone (alveolar process)
Alveolar mucosa
Periodontal ligament
Periosteum

Contact point
Col
Interdental gingiva
Epithelium

Diagram of the periodontium

orange peel-like texture of the gingiva which varies in pattern and extent from one person to the next. In the cross-sectional view in the middle of the figure, the underlying tissues are exposed. Alveolar bone is covered by periosteum which contains the periodontal ligament fibers attaching the cementum of teeth to the underlying bone. The gingival sulcus is lined by sulcular epithelium in the coronal area and junctional epithelium or epithelial attachment at the base of the sulcus. The junctional epithelium undergoes a number of characteristic changes in response to inflammatory processes and is of major interest in the development of periodontal diseases. The lower two illustrations in Figure 1 demonstrate the col, a crater-like depression between facial and lingual gingival papillae. The col may not be present on every tooth, but is likely to be present when two teeth are in contact.

Etiology of Periodontal Diseases

There are two forms of periodontal disease, periodontitis and gingivitis, and there are several types of each. Generalized, chronic gingivitis results in edema, redness, and bleeding upon probing. Acute necrotizing ulcerative gingivitis (ANUG) is another type of gingivitis but, unlike the chronic type, ANUG is painful as a result of ulceration and necrosis mostly between teeth. Periodontitis is a bacterially induced process that ultimately results in connective tissue destruction and loss of support of the teeth. Periodontitis is usually painless, unless accompanied by abscess formation, and is associated with pocket formation, gingival color and texture changes, bone loss, and bleeding upon probing. Twenty to thirty-five percent of all tooth loss can be attributed to periodontitis (Armitage, 1980). Many classification systems for periodontitis have been proposed over the years. One of the more current classifications is that of Page and Schroeder (1982) which includes: prepubertal periodontitis, generalized and local; juvenile periodontitis; rapidly

5

progressing periodontitis; and adult type periodontitis. Adult type periodontitis is the most common form of the disease and is often called chronic adult periodontitis. For the purposes of this thesis the term periodontitis will be a general term for all types and the types will be referred to specifically. While chronic gingivitis and periodontitis are related, the progression of chronic gingivitis to chronic periodontitis is not well understood. As described below, some believe that a multitude of different organisms are involved for each disease.

Periodontitis is caused by the accumulation of dental plaque, which may be accelerated by calculus, faulty restorations, and food impaction (Carranza, 1984). The term plaque is used to describe the association of bacteria to the tooth surface. Plaque is differentiated into two types, supragingival and subgingival. Supragingival plaque starts with the adherence of bacteria to the acquired pellicle of the tooth surface at the gingival one-third of the tooth (Brecx, 1981). Plaque grows by multiplication of bacteria and accumulation of bacterial and host products. Organic products present in plaque include dextran, levan, galactose, and lipids (Carranza et al., 1984). The relationship of supragingival plaque to the progression of periodontal diseases is unclear; however, subgingival and marginal plaque are directly responsible for the initiation and progression of periodontal lesions (Carranza, 1984).

The role of microorganisms in the oral cavity is considerable. The oral cavity becomes infected with facultative organisms by the time an infant is ten hours old. By ten days, most humans have anaerobes present (Socransky and Manganiello, 1971). The bacteria associated with periodontal health are mainly gram-positive coccal and rod-shaped bacteria. Streptococcus sanguis and gram-positive rods have been shown to be the initiators of supragingival plaque (Gibbons and Van Houte, 1978). Once supragingival plaque is started, a bacterial succession occurs in which the populations of bacteria shift

toward a higher proportion of anaerobes. Actinomyces species are most likely to be encountered, although other species may also be cultivated (Socransky et al., 1977). Gingivitis is thought to be initiated by an increase in supragingival organisms, followed by an increase in subgingival plaque. In periodontitis, Bacteroides species increase, as do Hemophilus, spirochetes, and Capnocytophaga (Crawford et al., 1975). In localized juvenile periodontitis (LJP), a disease affecting both males and females from puberty until about age twenty-five, Actinobacillus actinomycetemcomitans has been determined to be the major contributing pathogen (Slots and Genco, 1984).

However, chronic adult periodontitis is a more complex disease than LJP because of the multiplicity of putative pathogens, the variability in microbial flora, and the difficulty in differentiating active from inactive sites at the time of sampling. The subgingival anatomy presents a technically difficult area to sample. The anaerobic environment is not easily duplicated, and since there are more than two hundred bacterial species cultivatable from periodontal pockets, identification is not always easily or readily completed. Because of these difficulties, data may conflict from one study to the next, even when similar sampling, identification, and quantification techniques are used. In addition to cultivation difficulties, an added dimension of complexity has recently been described. Chronic adult periodontitis apparently involves periods of quiescence or inactivity, resulting in less inflammation and little or no bone loss, interspersed with periods of exacerbation during which bone and connective tissues are lost to an active disease process. The time span of these active or inactive states varies (among lesions?, among patients?, randomly for the same lesion?) (Dzink et al., 1988).

The gingival sulcus is an area less likely to be thoroughly cleaned, and these retentive areas provide a relatively stagnant environment in which microorganisms ordinarily not adherent to the tooth may colonize and thrive. As an area of low oxygen concentration, the

gingival sulcus or developing periodontal pocket may harbor anaerobic bacteria. Moreover, spirochetes, Actinobacillus actinomycetemcomitans, and recently Bacteroides gingivalis have been found in the connective tissue proper (Saglie et al., 1982, Slots and Genco, 1984). The presence of micro-organisms in the connective tissue may contribute directly or indirectly to the pathogenic process.

Although most investigators agree that periodontitis is a bacterially induced inflammatory process, it is not clear whether the bacteria themselves cause connective tissue destruction directly, or whether their presence in the gingival sulcus or their invasion into the connective tissue stimulates host factors which actually cause the destruction. Destruction by indirect means may be the result of evasion or inactivation of host defense mechanisms or by triggering of immunopathologic processes mediated by lymphokines. For instance, macrophage activating factor (MAF) is thought to activate macrophages attracted to periodontal sites, causing them to secrete collagenase (Nath et al., 1973). Bacteria may also cause tissue destruction directly by the production of lytic enzymes or toxic products including hyaluronidase-glucuronidase, chondroitin sulfatase, ammonia, hydrogen sulfide, organic acids, endotoxin, and collagenase (Carranza, 1984).

Among the suspected bacterial pathogens associated with periodontitis, the anaerobic, black-pigmented, gram-negative, rod-shaped bacterium Bacteroides gingivalis has been most strongly associated with chronic adult periodontitis (Newman, 1984, Slots, 1982, Laughton et al., 1982, and Mayrand and Holt, 1988). Dzink, Socransky, and Haffajee (1988) concluded that the likelihood of a site being active was increased if B. gingivalis was detected in that site. Slots found that 42-52% of progressing lesions contained B. gingivalis (10-fold higher than nonprogressing sites). Most patients afflicted with chronic adult periodontitis exhibit elevated levels of antibodies to B. gingivalis (Ebersole et al., 1986). The most convincing evidence for the pathogenicity of B. gingivalis came recently

from Holt and coworkers whose successful implantation of a rifampin-resistant strain of B. gingivalis into ligated periodontal sites in rifampin-treated monkeys resulted in periodontitis including attachment loss and and significant rapid bone resorption (Holt et al., 1988).

What substances does B. gingivalis produce that are responsible for its virulence and pathogenicity? Many factors may play a role, either directly or indirectly. B. gingivalis lipopolysaccharide can strongly activate the alternative and classical complement pathways (Okuda and Takazoe; Sundquist and Johansson, 1980). Bacteroides species liberate products that may compete for and block chemotactic receptors on polymorphonuclear leukocytes, reducing their chemotactic ability (Van Dyke et al., 1982). In vitro, B. gingivalis exhibits a greater resistance to phagocytosis than do less-pathogenic species (Sundquist et al., 1982). All Bacteroides species elaborate IgA proteases, and most of them produce IgG proteases as well (Kilian, 1981). B. gingivalis possesses strong fibrinolytic (Wikstrom et al., 1983), as well as gelatinase activity and trypsin-like activities (Van Steenbergen, 1981; Slots, 1981). Extracellular production of phospholipase A is common to all black-pigmented Bacteroides species, as are alkaline and acid phosphatases (Bulkacz et al., 1981; Slots, 1981). DNAse and RNAse, two other enzymes produced by B. gingivalis, also have the capacity to degrade periodontal tissue components (Rudek and Hague, 1976). Lastly, B. gingivalis releases large quantities of volatile sulfides, which may be important cytotoxins in periodontal disease by increasing the permeability of oral mucosa and reducing the rate of collagen synthesis (Tonzetich and McBride, 1981).

Electron microscopic examination of all species of black-pigmented Bacteroides reveals the presence of pili or fimbriae. Pili may aid in the attachment of these bacteria to plaque and other surfaces. Other surface structures of black-pigmented Bacteroides species include long fibers distinct from pili, capsule, lipopolysaccharide, and vesicles associated

with the outer membrane, although the function of these structures remains unclear (Slots and Genco, 1984).

B. gingivalis produces an active, cell-bound collagenase (Robertson et al., 1982). Since B. gingivalis appears to be the major pathogen associated with chronic inflammatory periodontitis, the collagenase it produces is likely to be responsible for at least some of the collagen breakdown characteristic of this disease. Although host collagenases derived from lysed PMNs, macrophages, or osteoclasts are present, the effect of bacterially produced collagenases must not be ignored, since bacterial and vertebrate collagenases have different substrate specificities (Wooley, 1980).

Considering the evidence for the pathogenicity of B. gingivalis in periodontitis, either singularly or in combination with other microorganisms, it is important to attempt molecular biological dissection of this organism in order to understand its role in the disease process. Proteases are likely to play a particularly important role as described earlier. The goal of this research project was to clone the genes encoding putative pathogenically significant proteases from B. gingivalis. After cloning such genes it would be possible to further characterize enzymatic properties, produce quantities of enzyme for further *in vitro* and *in vivo* studies, and continue the characterization of the virulence factors of B. gingivalis. Once proven, the techniques used in this project may easily be applied to other potentially pathogenic oral bacteria.

Overview of Gene Cloning

With the advancement of molecular biological techniques, powerful methods have been developed to study pathogenic mechanisms at a level formerly not possible. Cloning protease genes from B. gingivalis makes it possible to further elucidate their role in the
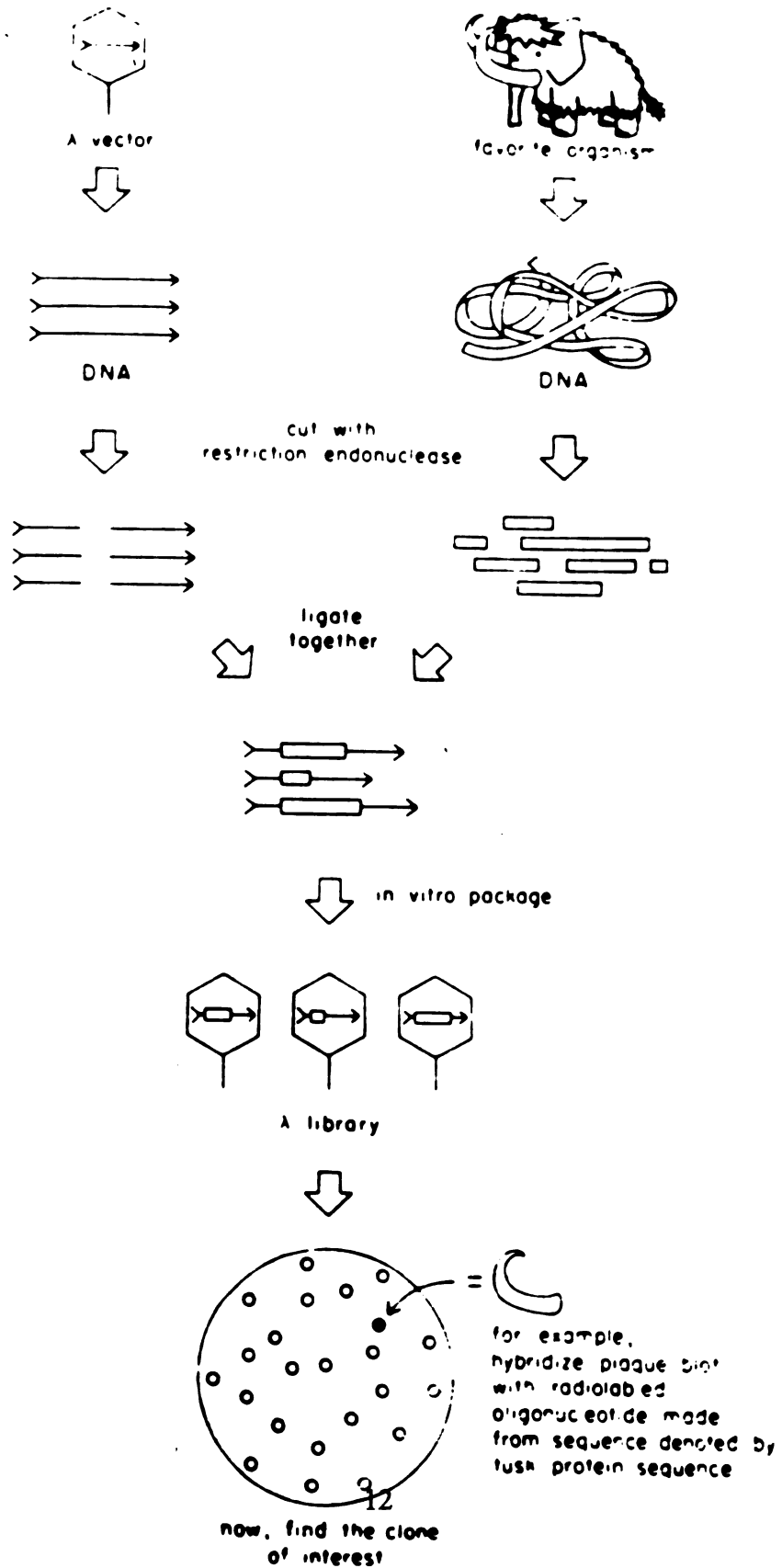
pathogenicity of periodontitis. Biological characterization, purification, and determination of nucleotide and amino acid sequences are each possible once these genes are cloned. Enzymatically inactive mutant forms of these proteases could then be derived, perhaps leading to a vaccine against periodontitis. Indeed, the recombinant DNA techniques now at hand provide powerful biologic tools to develop a better understanding of the molecular mechanisms of many disease processes, including periodontal diseases.

The cloning of genes from any organism first requires the production of a gene clone library. The ideal library would contain overlapping DNA fragments representing an entire genome. Whole genes and their flanking sequences must be contained within the cloned fragments, but the fragments must be small enough to be readily mapped with restriction enzyme techniques. The library should be easily constructed, amplified without losing representative gene clones, screened with proven techniques, and stored without significant loss in titer for years.

In Figure 2, a flow chart of the steps of gene cloning is provided. The DNA from a vector (often a bacteriophage or plasmid) is prepared and cut with a specific restriction endonuclease in order to accept foreign DNA. Also, the DNA from the organism of study is isolated and partially digested in a random fashion (with an restriction endonuclease which yields sticky ends which are compatable with those produced with the vector DNA) into varying size fragments such that all genes will be represented. The foreign DNA is ligated with the vector DNA and the resulting recombinant DNA is packaged *in vitro* into viable phage (in the case of the bacteriophage vector) that may be amplified in bacteria (often E. coli).

Although other vectors exist, the one best fulfilling these characteristics for our purposes is derived from bacteriophage lambda. Bacteriophages are viruses that infect bacteria as their host. Lambda L47.1 is a derivative of the wild type lambda phage (Loenen

Figure 2.   Overview of Gene Cloning

A vector

favorite organism

DNA

DNA

cut with restriction endonuclease

ligate together

in vitro package

A library

now, find the clone of interest

for example, hybridize plaque lift with radiolabeled oligonucleotide made from sequence denoted by tusk protein sequence

12

and Brammar, 1980). In this case the chosen vector, lambda L47.1, will be described in more detail, along with the basic methods/techniques used to make libraries with this vector.

The chromosome of lambda L47.1 is a linear DNA molecule 40.6 kb long. As a result of many manipulations of the lambda genome, this vector has been constructed to accept foreign DNA and retain its infectivity. Cleavage of the vector DNA with BamHI, a restriction endonuclease, creates left and right arms, which together contain the information essential to produce infectious phage particles, as well as a 6.5 kb non-essential (stuffer) fragment. When BamHI is used to cleave λL47.1 DNA, cohesive ends are formed. The sequence recognized is:

<div align="center">
XXXXGGATCCXXXX

XXXXCCTAGGXXXX
</div>

The arrows signify the cleavage points that result in the production of single stranded projections at each end of the arms:

<div align="center">
          GATCCXXXX
XXXXG   GXXXX
XXXXCCTAG
</div>

These ends are compatible with the sticky ends that are created by the partial cleavage of B. gingivalis DNA with the restriction enzyme Sau3A.

When the cleaved vector DNA is mixed with exogenous DNA fragments having the same cohesive ends, and treated with DNA ligase, a proportion of the recombined DNA molecules will contain a foreign (B. gingivalis) DNA fragment flanked by left and right arms of the vector. Ligation of the two arms without the inessential fragment or a foreign fragment of DNA in the center creates a phage genome too small to be packaged. If introduced into a bacterial cell, genomes containing cloned inserts of B. gingivalis DNA

will enter a lytic growth cycle and produce a clone of viral particles. Individual plaques of viral growth, which originate from a singly infected bacterium, are therefore a source of amplified, recombinant phage chromosomes containing one specific sequence from among the population of donor fragments. The infection of the recombined DNA molecules into E. coli may be accomplished either by transfection (as naked DNA) or by in vitro packaging of recombinant genomes into lambda phage particles before infection into the bacteria. We have chosen in vitro packaging, as it is considerably more efficient. The packaging extracts will encapsidate only phage genomes with size ranges of about 38-52 kb and therefore limit the size of clonable inserts to 4-18 kb. The bacterium used for infection by recombinants in this case is a strain of E. coli K-12, which is low in proteolytic activity (Young and Davis, 1983). The B. gingivalis chromosome provides the exogenous source of bacterial DNA for our library production.

Verifying Authenticity and Completeness of Library

Having prepared the library, it is first necessary to confirm that it contains representative cloned fragments of B. gingivalis DNA. There are many ways to accomplish this, one of which involves isolating the DNA from several clones, cleaving the DNA with the restriction endonuclease BamHI, and examining the DNA restriction fragment patterns obtained by agarose gel electrophoresis (Berman et al., 1982). The restriction pattern of a recombinant clone that has lost its nonessential fragment and acquired a cloned fragment of B. gingivalis DNA would be significantly different from the pattern of the original phage lambda L47.1 vector DNA.

Since there are hundreds or thousands of genes in the B. gingivalis genome, and since all of these genes must be represented in the library of B. gingivalis recombinant

clones, it is necessary also to test the library for completeness. This can be done by examining the library for the presence of individual genes, such as those responsible for encoding biosynthetic pathway enzymes. If several of these arbitrarily chosen genes are present in the library, then presumably every other gene is present as well, and the library is complete. This examination may be accomplished selectively by complementation of an auxotroph. The flow chart in Figure 3 outlines this approach and a detailed description follows: An auxotroph is a nutritional mutant of a bacterial strain (Maniatis et al., 1982). Auxotrophic mutants are unable to produce one of the enzymes required for biosynthesis of some essential growth factor, such as an amino acid. The parent strain of such a mutant is termed a prototroph and can produce all its required growth factors. For example, when an amino acid auxotroph is plated on minimal growth medium, either with or without the amino acid it requires for growth, it will grow in the presence of that amino acid but not in its absence. By contrast, a prototroph similarly plated would grow on both plates. An auxotrophic mutant E. coli strain deficient in a particular biosynthetic gene might be complemented by the presence of the corresponding B. gingivalis gene that could encode the missing enzyme. Thus, the complemented auxotrophic mutant would phenotypically resemble a prototroph.
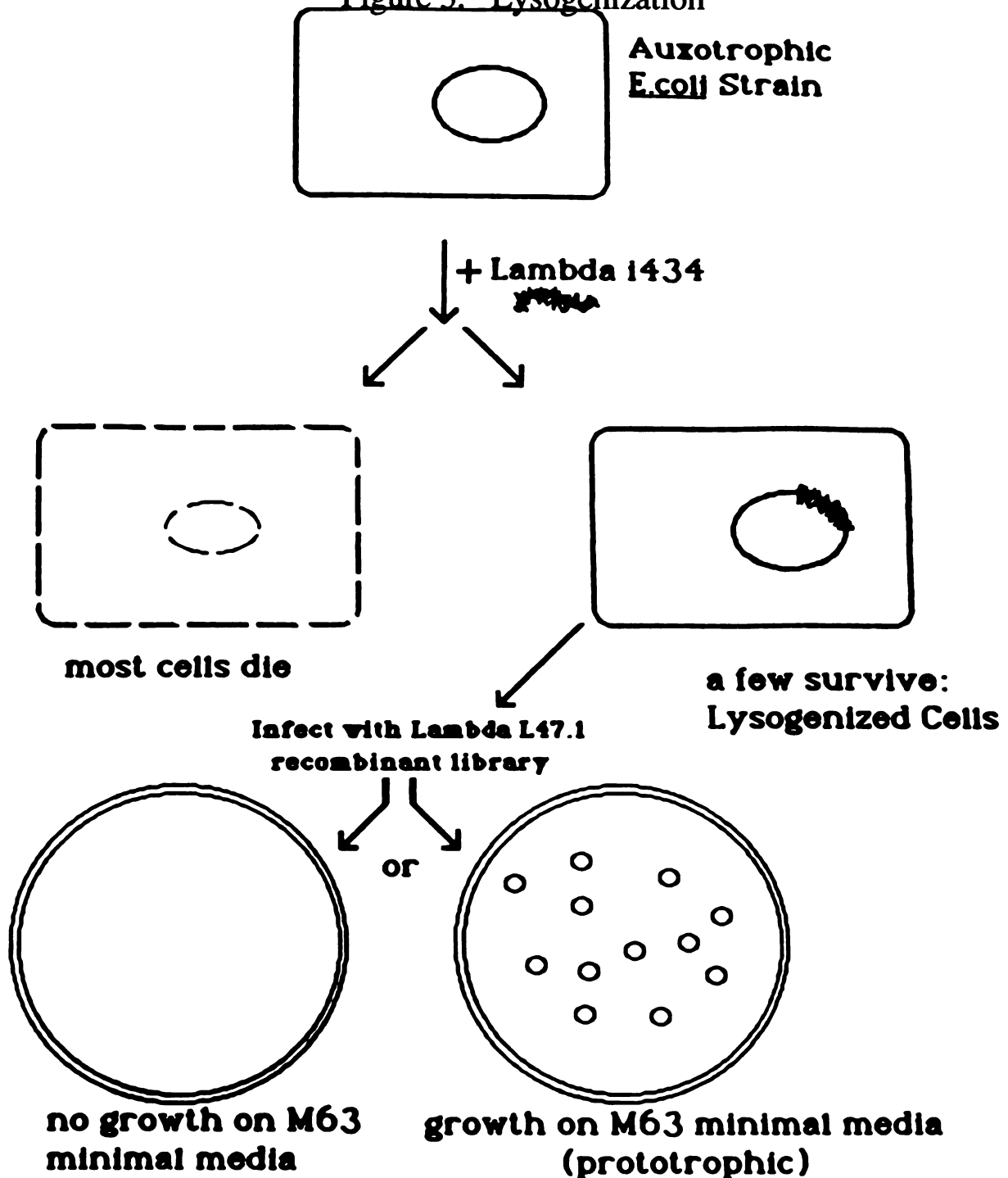
To test the library of recombinant B. gingivalis clones for the Bacteroides counterpart of the defective gene in the auxotrophic E. coli strain, the following steps are performed. First, the E. coli strain is lysogenized with phage λi434. The E. coli strain requires lysogenization to prevent killing upon infection with the recombinant phage library. The gene clone of interest will not be expressed unless the E. coli into which it is infected remains alive. This is done by infecting the λi434 phage into the chosen auxotrophic mutant of E. coli. In a certain percentage of the infected cells, this phage will not kill the cell, but instead will lysogenize it, producing repressor and integrating its DNA into the

bacterial chromosome. The vector phage (λL47.1), used to construct the library, cannot lysogenize by itself but it can be helped to lysogenize by a previously integrated λi434 phage genome. The population of λL47.1 recombinant phage that comprises the library is then infected into the E. coli - λi434 lysogen. These phage will not kill the cells because of the presence of 434 repressor (Maniatis et al., 1982). Recombination can occur between the DNAs of the integrated λi434 and the infecting λL47.1, as there is substantial homology between the chromosomes of these two phages. This recombination causes the λL47.1 genome to be integrated into the λi434 prophage, which resides in the bacterial chromosome. Thus, double lysogens are formed that have cloned fragments of B. gingivalis DNA incorporated into the E. coli chromosome. Since E. coli can express genes cloned from many other gram-negative bacteria (Macrina, 1984), it may be possible to select for clones carrying the gene that encodes the enzyme deficient in the E. coli auxotrophic strain by simply plating the infected E. coli cells on minimal medium lacking the required amino acid. Besides revertants, any other colonies that grow on this medium must have been complemented by the appropriate B. gingivalis gene present in one of the λL47.1 clones, which caused the auxotrophic E. coli lysogen to become prototrophic. Subculturing these E. coli transductants and inducing phage production would generate high frequency transducing lysates, thus confirming the presence of the selected gene within the recombinant clone (see Materials and Methods).

Screening for Proteolytic Clones

With the confirmation of a complete library, it is possible to screen it for the desired clone, one containing a protease gene. Any recombinant library contains many independent clones, and only a small fraction of them will contain the desired gene. The minority of

Figure 3. Lysogenization

Auxotrophic
E.coli Strain

+ Lambda 1434

most cells die

a few survive:
Lysogenized Cells

Infect with Lambda L47.1
recombinant library

or

no growth on M63
minimal media

growth on M63 minimal media
(prototrophic)

Auxotroph converted to prototroph
in one of two ways:
1. reversion of the auxotrophic mutation to prototroph
2. acquisition of DNA necessary for biosynthesis of
   missing growth requirement by transduction of
   cloned B.g. gene

clones that contain the desired gene must then be distinguished from the majority that do not. For this, a screening assay can be utilized to identify clones that contain a protease gene. We have shown that B. gingivalis genes can be expressed in E. coli (see results), and therefore protease-positive plaques were sought. Plaques, small, circular, regions of clearing in a bacterial lawn, are produced by phage infections that kill bacterial cells. The sequence of events is: phage infection, replication, maturation, and finally destruction by lysis of the bacterial cell. After lysis of an infected cell, hundreds of phage particles are released and the surrounding cells in the lawn are infected. This cycle occurs repeatedly until a macroscopically visible zone of clearing is formed. Use of a phage vector that lyses the E. coli cells may be especially beneficial, since the protease produced from the cloned gene may not be secreted by E. coli and lysis of the host cells may be necessary to detect the presence of this enzyme. Even if the protease is not secreted, the formation of plaques will still allow detection of protease-positive clones.

When screening for clones, it helps to eliminate nonrecombinant background phage that may have been produced by reconstitution of the vector during the ligation process. To achieve this, recombinants may first be selected using the Spi⁻ selection (Loenen and Brammar, 1980). This eliminates any vector molecules that have not received an inserted fragment of B. gingivalis DNA, and thus reduces the number of plaques that must be screened for protease production.

Perhaps the best technique available, because of its ease and great sensitivity, is the use of an artificial fluorescent collagenase substrate (collagenase is one of the more likely virulence factors produced by B. gingivalis). For this assay, cellulose acetate membranes impregnated with the substrate are placed on an agar plate containing plaques produced by the recombinant clones. The substrate for this reaction is Z-gly-pro-leu-gly-pro-AFC, where Z is carbobenzoxy (CbZ). AFC (7-amino-4-trifluoromethyl coumarin) when bound

to an amino acid and viewed under ultraviolet light fluoresces blue, but when found free, fluoresces green and has a brighter fluorescence. In this fluorescent substrate assay for collagenase, a two-step reaction occurs. First, the leu-gly bond is cleaved by bacterial collagenase, whose consensus amino acid recognition sequence is R-pro-X-gly-pro, where R is any blocking group, X is an uncharged amino acid, and the cleavage site is between X and gly (Peterkofsky, 1982). Then, a second cleavage occurs, catalyzed by the enzyme dipeptidyl-aminopeptidase IV (DPP-IV), which is provided in the buffer. This enzyme cleaves the pro-AFC bond, liberating free AFC. After the second cleavage (which can occur only following the first cleavage), the substrate will fluoresce green, and collagenase-positive clones can be identified (Smith, 1984). Other nonspecific protease substrates may be used to screen the library, such as casein, gelatin, or colored substrates (e.g. Azocoll) in which red or blue dye is covalently attached to cowhide powder, which is primarily denatured collagen. When collagenase acts, the covalent bonds are cleaved, the dye is solubilized, and the diffusion of dye produces local decolorization in the area of the collagenase-containing phage plaque (Peterkofsky, 1982).

Another powerful method for isolating the collagenase gene is to employ a selective technique based on an auxotroph complementation procedure similar to those described previously. The selection is based upon the ability of an auxotrophic E. coli strain to obtain the amino acid it requires for growth on minimal medium by degrading a peptide collagenase substrate containing that amino acid. The auxotroph can degrade the substrate only if it produces collagenase because of the presence of a cloned B. gingivalis gene. In more general terms, this selection could be used to clone any protease gene. For instance, the collagenase substrate to be used is Z-gly-pro-leu-gly-pro. The premise of this selection is that after infection of a glycine auxotroph of E. coli with the recombinant library, some of the infected E. coli cells will receive the collagenase gene and gain the ability to cleave

the leucine-glycine bond of the substrate, thus producing Z-gly-pro-leu and gly-pro peptides. This particular strain of E. coli was chosen for its ability to utilize gly-pro, but not the intact Z-gly-pro-leu-gly-pro substrate, to satisfy its auxotrophic requirement for glycine. If the original E. coli glycine auxotroph produces enzymes that interfere with this selection (e.g., a peptidase enabling it to use the uncleaved pentapeptide substrate), standard E. coli genetic techniques can be used to generate a suitable mutant derivative having the desired characteristics.

Yet another method for the further screening of protease-positive clones identified by using nonspecific substrates, or as ultimate confirmation of those believed to be collagenase-positive, is the use of undenatured tropocollagen as substrate. For the final identification of collagenase-positive clones, a tropocollagen substrate must be utilized, since other nonspecific proteolytic enzymes might be produced that could cause the scission of denatured collagen, (i.e. gelatin) or of the fluorescent substrate (Harper, 1980). Collagenase cleaves the main-body helical polypeptide chains of the structural protein collagen under normal physiological conditions (Harper, 1980). A method for preparing soluble collagen from tissue involves the use of 0.5 M acetic acid and pepsin. Acid-soluble collagen will form a gel at high concentration (4 mg/ml), neutral pH, and 37°C (Miller and Rhodes, 1982). Thus, a native collagen gel may be applied over solid growth medium containing recombinant phage plaques on an E. coli lawn, and liquefaction of the gel will denote collagenase activity of the screened clones. Alternatively, a more sensitive radioactive assay based on degradation of ^{14}C-acetylated tropocollagen may be employed (Harris and Vater, 1982).

MATERIALS & METHODS

Recombinant DNA Library Production

Cloning Vector:

The vector, bacteriophage lambda L47.1, was obtained from Dr. Jeffrey Felton and propagated as follows: the phage stock was plated for single plaques on L plates (see Appendix) on a lawn of exponential phase E. coli JF50. A pickate was made from a medium-sized plaque and titered. Approximately 1×10^7 JF50 were preadsorbed with 5×10^4 λL47.1 and plated on L plates in a 0.6% soft agar overlay. Twenty such plates were incubated for 6-8 hours at 37ºC until lysis was confluent (complete destruction of bacterial lawn by phage). These plates were harvested using a sterile bent glass rod to scrape the soft agar layer into a centrifuge tube (Miller, 1974). The harvested material was centrifuged in a Sorvall RC2-B (SS-34 rotor) at 12,000 RPM for ten minutes. One milliliter of chloroform was added to the pooled supernatant and the liquid was mixed and stored overnight at 4ºC. The supernatant was decanted from the chloroform with a resulting volume of 51 ml and a titer of 2.5×10^{10} phage per ml.

Next, NaCl and PEG (6000) were added to the phage sample to final concentrations of 2.1% and 10%, respectively and stirred to dissolve at 4ºC for 1 1/2 hours. The stir bar was removed and this solution allowed to sit overnight at 4ºC. The turbid suspension obtained was centrifuged ten minutes at 11,000 x g at 4ºC. The resulting pellet was dissolved in 5 ml of phage 80 buffer (see appendix A). This suspension was clarified by recentrifugation at 11,000 x g at 4ºC for 10 minutes. A cesium chloride block density gradient was made as follows:

21

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CsCl in phage
  80 buffer      refractive index

  2 ml          - 1.372

  1 1/2 ml       - 1.380

  1 ml           - 1.390
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The clarified supernatant was layered onto the top of this block gradient and centrifuged in a Beckman SW 50.1 rotor for 120 minutes at 24,000 RPM. Using a white penlight in a dark room, a bluish band containing the phage was visualized and isolated with a syringe and 20 gauge needle by puncturing through the side of the tube. Approximately 1.5 ml sample was obtained. CsCl solution of refractive index 1.381 was added to bring the volume to 4 ml. This sample was centrifuged for 24 hours to equilibrium at 10°C, 34,000 RPM in a SW50.1 rotor. A one ml sample was collected as described above and dialyzed against phage 80 buffer in a boiled #2 dialysis bag. A total of five changes of buffer in 24 hours were performed. Approximately 0.5 ml was collected at a titer of 1.7×10^{11} phage/ml and stored in a screwcap tube.

Extraction of Phage DNA

One half molar EDTA, pH 8.0, was added to the phage sample to give a final concentration of 20mM. Ten percent SDS was added to a final concentration of 0.5%. Pronase was added to a final concentration of 0.5 mg/ml. This solution was incubated at 37°C for 1 1/2 hours. An equal volume of Tris-buffered phenol (see Appendix A) was added, the solution repeatedly mixed by inverting, and the phases separated by centrifugation in an Eppendorf microcentrifuge for five minutes at room temperature. The phenol phase was removed with a pasteur pipette. This phenol extraction procedure was twice repeated. The phage DNA was then precipitated as follows: 2.5 M sodium acetate

pH 5.2 was added to give a final concentration of 0.25 M followed by two volumes of ice-cold 95% ethanol and this was allowed to sit overnight at -20°C after mixing well. This suspension was centrifuged in an Eppendorf tube at 4°C for 10 minutes. The supernatant was discarded and the tube vacuum desiccated to remove remaining ethanol. Twenty microliters TE (10mM Tris, 1mM EDTA) pH 7.4, was added to dissolve pellet. Approximately 150 μg of DNA was recovered.

BamHI Digestion of λL47.1 DNA

Approximately 20 μg of λL47.1 DNA was added to 5 μl of 10X EcoRI buffer (see Appendix A), 2 μl DTT, and 60 units of BamHI. This mixture was incubated for 1 hour at 37°C, and the reaction was stopped by addition of EDTA to a final concentration of 20 mM. A phenol extraction and ethanol precipitation were performed to repurify the DNA fragments. Products of this digestion were evaluated by electrophoresis through a 0.7% agarose gel (see Figure 4, Appendix C).

The electrophoresis technique employed the following: 0.7% agarose in TBE buffer (see Appendix A) was autoclaved and 5 μg/ml ethidium bromide added. The agarose was poured into a Bio Rad plate which was taped at both ends, and an eight tooth gel comb was utilized to form wells. The samples were usually 10-15 μl and included loading buffer (see Appendix A). The power supply was started at 5-10 volts to run the DNA fragments into the gel and then increased to 40-60 volts. The resulting gels were photographed through a red filter with UV illumination. HindIII fragments of λ DNA of lengths 2.0, 2.3, 4.4, 6.6, 9.4, and 23 kb were normally run in one lane of each gel as size standards.

Bacteroides gingivalis DNA Isolation

Bacteroides gingivalis, ATCC strain #33277, was grown from a large inoculum anaerobically at 37°C in 100 ml trypticase soy broth with menadione, hemin, and DTT for 24 hours (see Appendix A). Cells were harvested by centrifugation at 5000 RPM for 5 minutes. The cell pellet was resuspended in 50 mM Tris, pH 8.0. Lysozyme to 2 mg/ml and SDS to a final concentration of 1% were added to the cell suspension, which was then heated to 65°C. Proteinase K was added to 1 mg/ml and the solution was incubated at 50°C for five minutes. An equal volume of Tris-buffered phenol (pH 7.6) was added, mixed for five minutes, and centrifuged at 3000 RPM for five minutes. The aqueous layer was transferred to a new tube and the phenol extraction repeated twice. One half ml of 3M sodium acetate was added to the 5.5 ml sample and mixed gently. Two volumes of 95% ethanol were added and a visible precipitate formed. This suspension was stored at -20°C for two hours. After centrifugation, the resulting pellet was resuspended in 5 ml TE pH 8.0 (Tris 50 mM, EDTA 1 mM) and 50 μg/ml RNAse and incubated for 30 min at 37°C. Another phenol extraction was performed followed by a chloroform (equal volumes) extraction. The aqueous phase was removed and the DNA was precipitated from it by addition of two volumes of 95% ethanol, and the DNA pellet was redissolved in TE (50/1) and stored at 4°C (Berman, 1982).

Partial Restriction of B. gingivalis DNA with Sau3A

Ten to twenty kb sized fragments of B. gingivalis DNA needed for recombinant library production were generated as follows: after optimal conditions for partial digestion were determined, 500 μg of whole B. gingivalis DNA was added to 100 μl 10X EcoRI buffer and 20 units of Sau3A. The reaction was stopped after incubation for 45 min at

37°C by addition of EDTA to a final concentration of 20 mM and chilling to 0°C. A 38 ml, 10 to 40% linear sucrose density gradient was used to separate the varying size fragments. The reaction mixture (above) was heated to 68°C and cooled to 20°C, layered onto the top of the sucrose gradient, and centrifuged in a Beckman SW28.1 rotor at 25,200 RPM for 24 hours at 20°C. A fractionator was employed to collect 0.5 ml samples of the gradient. A 10 µl aliquot from every third fraction was mixed with loading buffer and analyzed by electrophoresis through a 0.7% agarose gel with HindIII digest of λ DNA standards and ethidium bromide staining (see Figure 5 and 6, Appendix C).

The fractions containing most of the 4-18 kb DNA fragments were then pooled and concentrated 10 fold. The DNA was precipitated by adding NaCl to a final concentration of 0.1M followed by two volumes of 95% ethanol. This mixture was chilled at -20°C for 1 1/2 hours, centrifuged at 10,000 RPM for 5 minutes, and the pellet was dissolved in 100 µl TE (10/1).

Ligation of B.gingivalis DNA and λL47.1 DNA

Approximately 19 µg (26 µl) of Sau3A-digested 4-18 kb fragments of B. gingivalis DNA and 20 µg (70 µl) of BamHI-digested λL47.1 DNA were added to 10 µl of 10X ligation buffer and 2 µl 1 mM ATP. Twelve units of T4 ligase was added, giving a final volume of 118 µl and the mixture incubated at 11°C for 16 hours. The reaction was terminated by the addition of EDTA. The success of the ligation was determined by electrophoresis through a 0.7% agarose gel. Ligation is easily confirmed as right and left arms of λL47.1 DNA are absent and higher molecular weight DNA is seen in the agarose gel.

25

Packaging the Recombinant DNA

The packaging of ligated recombinant DNA into lambda phage particles is complex. In short, the packaging relies on a combination of two lysogenic E. coli strains which are defective in two different steps of phage λ morphogenesis. When a combination of these E. coli strains are lysed in the presence of ATP and λ DNA (provided in the ligated recombinant DNA), infectious viral particles are formed. Packaging extracts purchased from Promega Biotec (Madison, WI) were employed. Extracts were thawed and approximately 2.5 µg ligated DNA was added to the packaging mixture and incubated for two hours at 22°C. Twenty-five µl chloroform was added and the solution gently vortexed. The resulting phage particles were plated on E. coli Q359 grown in L broth plus 0.2% maltose. Strain Q359, a P2 lysogen, only allows plating of phage particles which have received a recombinant stuffer fragment. The library was then harvested and placed in a screw cap tube and stored in 10% glycerol at -70°C.

Screenings

The library of recombinants was plated on E. coli JF50 in a 0.6% soft agar overlay on L plates to give about 500 plaques per plate. Immediately following formation of plaques cellulose acetate membranes called "enzyme overlay membranes" (from Enzyme Systems Product, Livermore, CA) impregnated with the fluorescent substrate for collagenase were wetted with the buffer containing dipeptidyl peptidase IV (DPP-IV) (required for second reaction), laid over the plates, and incubated at 37°C overnight in a humidor. Screening using cellulose acetate membranes containing the fluorescent substrate for trypsin-like activity were similarly performed using the appropriate buffer for wetting.

26

Enzymatic activity against fluorescent substrates was monitored using long wave ultraviolet light. B. gingivalis 33277 cells grown in TSB were used as a positive control for the enzymatic activities.

In a different approach used to isolate single recombinants and amplify their expression, approximately 450 plaques from a plate of recombinants grown on E. coli JF50 were picked and placed in L broth in microtiter dishes. Using a replicator, these individual plaques were amplified by plating on a lawn of E. coli JF50 on L plates. Liquid fluorescent substrates (see Appendix A) specific for gly-pro dipeptidase activity (gly-pro-AFC) and trypsin-like activity (Z-arg-AFC) were sprayed (0.20 ml) onto the plates using an atomizer. The plates were incubated at 37°C and were viewed for activity under long wave UV at varying times. L plates containing 40 µg/ml 5-bromo-4-chloro-3-indolyl phosphate were also used to screen the library for clones expressing alkaline phosphatase activity. This chromogenic substrate turns blue when enzymatically cleaved.

L agar plates containing 1.5% nonfat dry milk were used to screen the B. gingivalis library. The host strain used was E. coli Q359. Putative protease-positive clones were biologically characterized by plating on several bacterial strains. E. coli strain Q359 (a P2 lysogen) plates only Spi⁻ recombinant clones based on the absence of the lambda vector recombinant stuffer fragment. E. coli HB101 (recA⁻) fails to plate recombinants due to absence of lambda vector stuffer fragments. E. coli RVPI will not plate phage P2 which is a possible source of contamination from the P2 lysogen-Q359. E. coli K10, a lysogenic strain with wild type lambda immunity, will not plate wild type lambda. E. coli SM525 lacks lambda cell surface receptor and is therefore resistant to all lambda type phage. E. coli MH4 (λi434) a lysogenic strain with 434 immunity will not plate a recombinant with i434 immunity. The putative protease-positive clones detected on the 1.5% nonfat milk plates were further characterized using the fluorescent substrate techniques previously described.

27

Production of Lysogens of Auxotrophic Strains:

After confirming their auxotrophic requirements by showing lack of growth on M63 glucose minimal medium plates (see Appendix A) and growth on the same plates if the required nutritional factor was supplied at 30 μg/ml, E. coli auxotrophic strains MH2, MH4, C600, MC1066, and BNN24 were grown in L broth to log phase ($OD_{600} \approx 1$) and infected with phage λi434. These were incubated overnight at 37°C. An aliquot was then streaked out for single colony isolation on an L plate and the colonies obtained were likely to be lysogens. To test the putative lysogens, these colonies were purified and further examined for production of phage λi434 . Also, the strains were re-tested to confirm their auxotrophic requirements. Finally, the lysogens were infected with λL47.1 and λC$_I$b$_2$ to confirm their immunity to λL47.1, which carries the immunity region for phage 434.

After these conditions were satisfied, the recombinant library was infected into the lysogenic auxotrophic strains which were then plated on M63 glucose minimal plates. The small number of colonies which grew were suspected to be complemented by genes in the B. gingivalis recombinant library, which encoded the enzyme they lacked. To test that the strains which grew were not simply revertants of the E. coli auxotrophic mutation, the colonies were subcultured in L broth and grown to log phase. The cultures were centrifuged for 5 minutes at 5000 RPM, and chloroform was added to the supernatants. The supernatants were checked for absence of viable bacteria and the phage remaining in the supernatants were again infected into the original lysogenic strains. If the phage present truly represented a complementing recombinant clone, the growth seen on minimal plates, by infecting the auxotrophic E. coli with the phage from the supernatant, would be abundant, because the supernatant would be a high frequency transducing lysate of that phage.

Selection for Collagenase-positive Clones

E. coli AT2457 is a bacterial auxotroph deficient in the ability to produce glycine. This strain was shown to be able to grow on minimal medium (M9) supplemented with either glycine or the dipeptide gly-pro. The strain was also plated on minimal medium supplemented with Z-gly-pro-leu-gly-pro and the strain did not grow. A λi434 lysogenic derivative of AT2457 was constructed as previously described. The recombinant library was then infected into this lysogen as follows: AT2457 (λi434) cells were grown in liquid minimal medium plus 30 μg/ml glycine to log phase, centrifuged for 10 minutes at 5000 RPM in a Sorvall RC2-B (SS-34 rotor), washed with 0.9% saline and resuspended in 0.9% saline after re-centrifugation. Approximately 2×10^5 recombinant phage particles were infected into 10^7 cells of AT2457 (λi434) which were then spread with a sterile glass rod on a minimal medium plate supplemented with the Z-gly-pro-leu-gly-pro pentapeptide at 30 μg/ml. The plate was incubated at 37°C for 5 days. A control plate containing AT2457 (λi434) without the recombinant library was also incubated. After the fifth day, the colonies that appeared on the minimal plate infected with the recombinant library were plated on minimal medium plates with and without the pentapeptide Z-gly-pro-leu-gly-pro to differentiate revertants of the gly mutation from auxotrophs which were being complemented by the recombinant phage.

RESULTS

Library Production

A library of B. gingivalis clones has been made, its authenticity confirmed and a number of screening experiments and selection experiments performed which have yielded one putative protease-positive clone and two biosynthetic pathway enzyme clones.

λL47.1 DNA was isolated and the BamHI digestion of λL47.1 DNA gave the correct profile. In Figure 4 (see Appendix C) lane 2 represents 5 μg BamHI-digested λL47.1 DNA. The expected profile includes the right arm (10kb), left arm (23kb) and the inessential middle fragment (6.5kb).

B. gingivalis DNA was isolated and partially digested with Sau3A. In Figure 5 (see Appendix C), lanes 1 and 8 are lambda HindIII size standards, lane 7 represents uncut B. gingivalis DNA, and lanes 2-6 represent B. gingivalis DNA digested with varying amounts of Sau3A (from lane 6 with 0.015 units to 0.25 units in lane 2).

The results of size selection on a sucrose density gradient are seen in Figure 6 (Appendix C). Lane 1 represents λ HindIII size standards and lane 8 uncut B. gingivalis DNA. Lanes 2-7 show varying size DNA fragment populations with lanes 3 and 4 representing the best samples of 4-18kb B. gingivalis DNA fragments. In Figure 7 (Appendix C), the fraction containing fragments of 4-18kb is confirmed. Lanes 3 and 5 are lambda HindIII size standards and lane 4 represents size-selected B. gingivalis DNA.

After the ligation of Sau3A-digested B. gingivalis DNA with BamHI-cut λL47.1 DNA was performed, packaging the ligated recombinant DNA molecules into viable lambda phage particles was completed, and a recombinant library of cloned genes from B. gingivalis 33277 was thus produced. The titer of the library after a single cycle of

propagation on in E. coli Q359 was 6.5 x 10^6 recombinants per ml. Biological tests confirmed the presence of λL47.1-like phage. The Spi⁻ selection system eliminates the possibility that parental type religated λL47.1 containing the inessential fragment are present once plated on E. coli Q359.

Screenings

The recombinant library was screened for clones expressing a trypsin-like activity using the synthetic fluorogenic substrate carbobenzoxy-L-arginine-7-amino-4-methylcoumarin amide-HCl (trypsin substrate). Similarly, the substrate Gly-Pro-AFC, which detects Gly-Pro peptidase activity, was used to screen the library. Recombinant plaques were also overlaid with cellulose acetate membranes impregnated with the synthetic collagenase substrate. In addition, the library was screened with alkaline phosphate substrate 5-bromo-4-chloro-3-indolyl phosphate. No positive clones were detected using any of the above screenings.

However, when the library was plated on E. coli Q359 using L agar plates containing 1.5% nonfat dried milk, 0.5% of the plaques were surrounded by turbid "haloes", indicating a change in the solubility of the milk protein casein due to the action of some B. gingivalis gene product (see Figures 7 and 8, Appendix C), presumably a protease (see discussion section) (Yanagida, 1986). The putative protease-positive clones were further examined using the previously mentioned fluorescent substrates for known B. gingivalis enzymatic activities. All tests proved negative, while positive controls with B. gingivalis cells confirmed activity against these substrates.

Selections

E. coli auxotrophic mutants MH2 and C600, having nutritional requirements for uracil and threonine respectively, were lysogenized with phage λi434, to prevent killing upon subsequent infection with the library (see introduction). The lysogenic strains were then infected with the phage library at a multiplicity of 10^{-3}, and 10^7 infected cells were plated on minimal medium. Prototrophic transductants were selected on M63 glucose minimal agar plates, and approximately 20 transductants were seen on each plate. High frequency transducing lysates of lambda recombinants containing the B. gingivalis genes encoding enzymes involved in the biosynthesis of uracil (orotidine 5' phosphate decarboxylase) and threonine (enzyme not characterized) have been obtained. These lysates gave confluent growth when infected into the original auxotrophic lysogens and plated on minimal medium. E. coli lysogenic auxotrophic strains MH4, BNN24, and MC1066 did not yield prototrophic transductants, although the experiment was only attempted once.

The selection experiment in which we attempted to obtain clones expressing collagenase by complementation of the auxotrophic E. coli strain AT2457, following infection with the recombinant library, yielded no protease-positive clones.

DISCUSSION

The etiology of periodontitis is not clearly understood, although there is evidence for the ability of Bacteroides gingivalis to cause this disease (Slots, 1982, Mayrand and Holt, 1988). In our investigation we attempted to identify a protease gene among a library of recombinant clones from B. gingivalis. Indeed, a protease-positive clone may have been obtained, and other findings have come from our experimentation. A library of gene clones from B. gingivalis has been generated and the ability of B. gingivalis genes to be expressed in E. coli has been demonstrated. The auxotrophic selection for biosynthetic pathway enzymes revealed important information. First, since the genes for two randomly chosen enzymes have been successfully cloned, the library has been shown to be representative of the B. gingivalis genome. Though DNA analysis of these recombinants is needed, biological characterization following isolation of complemented auxotrophs showed that the auxotrophs had obtained the ability to grow on minimal media due to the expression of a foreign gene and not simply the reversion of the original auxotrophic mutation. While other investigators have found that certain B. gingivalis promoters are inoperative in E. coli, (Dickinson et al., 1988) our results indicate that at least in some cases B. gingivalis promoters are, in fact, active in E. coli. The expression vector λL47.1 was used in our experiments, but the auxotrophic selection experiment utilizes a lysogenized form of the phage in which the vector promotor for expression of cloned genes is repressed. Therefore, to obtain complementation of an auxotroph to a prototroph from the recombinant library, it is likely that the cloned gene is expressed in E. coli with its own (B. gingivalis) promotor. For definitive evidence, careful biochemical analysis would be required. However, our result may indicate that not all B. gingivalis promoters are active

33

in E. coli. Although prototrophic transductants of the E. coli auxotrophs MH2 and C600 were obtained, no such transductants from the other lysogenic auxotrophs (BNN24, MC1066, and MH4) were found. This implies that either B. gingivalis does not possess the genes needed to complement the mutations in these strains, or that the B. gingivalis promoters for these particular genes are not active in E. coli.

A number of proteases from B. gingivalis have been biochemically examined and are suspected of playing a pathogenic role in periodontal tissue destruction. The goal of some investigators is to develop quick clinical tests which determine the presence of destructive B. gingivalis proteases (Slots, 1987). Trypsin-like activity in B. gingivalis species has been found and the enzyme partially characterized. Molecular weight determinations vary from 35 kd to 49 kd (Sorsa et al., 1987 and Ono et al., 1987). The enzyme is inhibited by serine protease inhibitors and activated by reducing agents and human serum. The trypsin-like protease has been found capable of degrading native type IV collagen, but not native type I collagen (Sorsa et al., 1987). Further, the enzyme has an optimal pH of 7.6 with an isoelectric point of 4.9 (Ono et al., 1987).

Gly-pro-peptidase was produced more abundantly by B. gingivalis than by other black-pigmented Bacteroides (Suido et al., 1986). The enzyme has been partially characterized as a 160,000 dalton protein with an isoelectric point of 6.2 (Abiko et al., 1985) and purified by Grenier and McBride. Since the glycyl-prolyl dipeptide occurs repeatedly within the sequence of collagen in the periodontal ligament, the gly-pro peptidase may prove important in destruction of collagen in tissue, once the initial cleavage is made by collagenase.

Screening the library for clones expressing both the gly-pro peptidase and a trypsin-like protease was unsuccessful. The fluorogenic substrates are very sensitive and specific, yet it is possible that the enzymes are expressed in tiny quantities, too small to yield a

34

positive result, or that the enzymes are not expressed in our library at all. One line of investigation not yet attempted is to screen the recombinant library for the presence of these enzymes under anaerobic or reducing conditions. Robertson et al. observed collagenase activity from B. gingivalis without the addition of reducing agents (1982). However, Sundquist, Carlsson and Hänström reported that the addition of 1mM dithiothreitol (DTT) greatly stabilized the collagenolytic activity (1987). Yoshimura et al. (1984) and Sorsa et al. (1987) found that trypsin-like activity from B. gingivalis was considerably enhanced by reducing conditions. On the other hand, Fujimura and Nakamura (1987) partially purified two proteases from cells of B. melaninogenicus which were inactivated by DTT. Furthermore, Grenier and McBride (1988) reported eight proteolytic bands in a 10% SDS polyacrylamide gel, six that required reducing agents for activity and two that were inhibited by reducing conditions. Again, screening the library under reducing conditions may result in a protease-positive clone and should be attempted.

As with the trypsin-like enzyme, collagenase, and gly-pro-peptidase activity shown to exist in B. gingivalis, an active alkaline phosphatase is also present. Again, detection of recombinants expressing this enzyme in our gene clone library using the substrate 5-bromo-4-chloro-3-indolyl phosphate proved unsuccessful.

The most significant result obtained is the turbidity associated with some recombinant plaques when plated in the presence of 1.5% nonfat dried milk. The putative protease-positive clones were purified and biologically examined to exclude the possibility that they may be phage P2, a possible source of contamination from the P2 lysogen E. coli Q359 on which the recombinant library was plated. Also, these turbidity-causing clones were tested to confirm that they are recombinants having characteristics of λL47.1. With these characterizations satisfied, the clones were further tested using the fluorogenic substrates previously described. Grenier and McBride have shown activity of B. gingivalis proteases

35

against casein and had seen precipitation associated with a gly-pro-peptidase which is inhibited by reducing agents (Grenier and McBride, 1987). The fluorogenic substrates did not detect a gly-pro-dipeptidase activity in the plaques of our turbid clones.

When a trypsin solution is spotted on the milk plates, clearing is observed as might be expected. The precipitation or turbidity noted on milk plates is also likely to be an enzymatic process. Rennin, a protease found in the lining of calf stomach, is an enzyme used in the process of cheese making. Rennin cleaves casein in such a way that precipitation of milk or milk curdling occurs, and calf rennin has been expressed in E. coli (Emtage et al., 1983). Serratia marcescens, a gram-negative bacterium, is reported to produce a protease which causes turbid haloes on 1% skim milk plates (Yanigida et al., 1986). We believe that we have cloned a protease from B. gingivalis which causes precipitation of casein due to limited proteolysis. Alternatively, an increased acid concentration associated with the few turbid plaques, but not with the majority of clear plaques, might be responsible for the phenomenon.

When attempts at detecting enzyme activity using the substrates described above with the turbid clones were unsuccessful, a simpler test was attempted. A liquid lysate of the turbid recombinant phage was made and tested for activity against casein. All attempts to show activity on casein were unsuccessful and this result has lent support to the idea that the enzyme which is responsible for this activity is highly labile. The only time this precipitation phenomenon has been seen is during the growth of these clones on plates containing 1.5% dried milk. We believe an enzyme is present but since it is short-lived its characterization is much more difficult than had been first anticipated. The importance of this putative protease in the pathogenesis of periodontal disease is speculative at best, but any B. gingivalis protease may prove to be a factor in the development or progression of periodontal disease.

In an attempt to select for a protease gene using the auxotrophic E. coli strain AT2457, which requires glycine supplementation, by infecting it with the B. gingivalis recombinant gene clone library and plating on minimal media with the pentapeptide Z-gly-pro-leu-gly-pro added, it should be emphasized that the phage promoter on the expression vector was repressed. Although other evidence presented suggests that some B. gingivalis promoters are active in E.coli, it would be desirable to utilize both expression systems when selecting for any gene. Since the E. coli auxotroph is first lysogenized with λi434, the prophage produces a repressor which represses the activity of the vector promoter for expression of cloned genes. Another experiment could be designed in which the same E. coli strain could be used unlysogenized, therefore utilizing the expression system of the vector. The unlysogenized E. coli host would be killed by infection with the recombinant library, so the proposed experiment would have to be designed to allow selection of collagenase-expressing phage independent of survival of the host bacteria.

CONCLUSIONS

We have successfully cloned the gene for orotidine 5' phosphate decarboxylase and a gene in the pathway of threonine biosynthesis from <u>Bacteroides gingivalis</u> 33277. Also, we have isolated a gene clone which displays protease-like activity against casein. We have obtained evidence that at least some <u>B. gingivalis</u> promotors are active in <u>E. coli</u>, and therefore conclude that these applications of molecular biology techniques are useful in the identification and characterization of genes and biological processes in <u>Bacteroides</u> and may be applicable to other putative periodontal pathogenic species, as well.

BIBLIOGRAPHY

Abiko, Y., Hayakawa, M., Murai, S., and Takiguchi, H.: Glycylprolyl dipeptidylaminopeptidase from Bacteroides gingivalis. J. Dent. Res. 64:106-111, 1985.

Armitage, G.C.: Biologic Basis of Periodontal Maintenance Therapy, pp 1-5, Praxis Publishing Co. Berkeley, CA, 1980.

Berman, M.L., Enquist, L.W., and Silhavy, T.J.: Advanced Bacterial Genetics: Cold Spring Harbor, New York 1982.

Brecx, M., Rontrom, A., Theilade, J., and Attstom, R.: Early formation of dental plaque on plastic films. J. Periodontal. Res. 16:213, 1981.

Bulkacz, J., Erbland, J.F., and MacGregor, J.: Phospholipase A activity in supernatants from cultures of Bacteroides melaninogenicus. Biochim. Biophys. Acta. 664:148-155, 1981.

Caranza, F.A.: Glickmans Clinical Periodontology 6th edition, W.B. Saunders Company, Philadelphia, 1984.

Crawford, A., Socransky, S.S., and Bratthall, G.: Predominant cultivable microbiota of advanced periodontitis. J. Dent. Res. 54:209, 1975.

Dickinson, D.P., Kubiniec, M.A., Yoshimura, F., and Genco, R.J.: Molecular cloning and sequencing of the gene encoding the fimbrial subunit protein of Bacteroides gingivalis. Bacteriol. 170:1658-1665, 1988.

Dzink, J.L., Socransky, S.S., and Haffajee, A.D.: The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. J. Clin. Periodontol. 15:316-323,1988.

Ebersole, J.L., Taubman, M.A., Smith, D.J., and Frey, D.E.: Human immune responses to oral microorganisms: Patterns of systemic antibody levels to Bacteroides species. Infect. Immun. 51:507-513, 1986.

Emtage, J.S., Angal, S., Doel, M.T., Harris, T.J.R., Jenkins, B., Lilley, G., and Lowe, P.A.: Synthesis of calf prochymosin (proprennin) in E. coli. Proc. Natl. Acad. Sci. USA 80:3671-3675, 1983.

Fujimury, S., and Nakamura, T.: Isolation and characterization of a protease from Bacteroides gingivalis. Infect. Immun. 55:716-729, 1987.

Gibbons, R.J., and Van Houte, J.: Microbiology of periodontal disease: Textbook of Oral Biology, Philadelphia, W.B. Saunders Company, 1978.

Grenier, G. and McBride, B.C.: Isolation of membrane-associated Bacteroides gingivalis glycylprolyl protease. Infect. Immun. 55:3131-3136, 1987.

Grenier, D., and McBride, B.C.: Characterization and localization of the proteolytic activities of B. gingivalis. J. Dent. Res. (Abstracts) 67:368, 1988.

Harper, E.: Collagenases. Ann. Rev. Biochem. 49:1063-1078, 1980.

Harris, E.D., and Vater, C.A.: Vertebrate Collagenases. Method Enzymol 82:423-452, 1982.

Holt, S.C., Ebersole, J., Felton, J., Brunsvold, M., and Kornman, K.S.: Implantation of Bacteroides gingivalis in nonhuman primates initiates progression of periodontitis. Science 239:55-57, 1988.

Kilian, M.: Degradation of immunoglobulins A1, A2, and G by suspected principal periodontal pathogens. Infect. Immun. 34:757-765, 1981.

Laughon, B.E., Syed, S.A., and Loesche, W.J.: Rapid identification of Bacteroides gingivalis. J. Clin. Microbiol. 15:345-346, 1982.

Loenen, W.A., and Brammar, W.J.: A bacteriophage lambda vector for cloning large DNA fragments made with several restriction enzymes. Gene 10:249, 1980.

Macrina, F.L.: Molecular cloning of bacterial antigens and virulence determinants. Ann. Rev. Microbiol. 38:193-219, 1984.

Maniatis, T., Fritsch, E.F., and Sambrook, J.: Molecular Cloning - A laboratory manual, Cold Spring Harbor, New York, 1982.

Mayrand, D., and Holt, S.C.: Biology of asaccharolytic black-pigmented Bacteroides species. Microbiol. Rev. 52:134-152, 1988.

Miller, E.J., and Rhodes, R.K.: Preparation and characterization of the different types of collagen. Methods Enzymol. 82:33-64,1982.

Miller, J.H.: Experiments in Molecular Genetics: Cold Spring Harbor, New York, 1974.

Nath, I., Poulter, L.W. and Turk, J.L.: Effect of lymphocyte mediators on macrophages in vitro. Clinical Exp. Immunol. 13:455, 1973.

Newman, M.G.: Anaerobic oral and dental infections. Rev. Inf. Dis. 6:5107-5112, 1984.

Okuda, K. and Takazoe, I.: Activation of complement by dental plaque. J. Periodontal. Res. 15:232-239, 1980.

Ono, M., Okuda, K., Takazoe, I.: Purification and characterization of a thiol protease from B.gingivalis 381. Oral Microbiol. Immunol. 2:77-81, 1987.

Page, R.C. and Schroeder, H.E.: Periodontics in Man and Other Animals. Basel, S. Karger, 1982.

Peterkofsky, B.: Bacterial collagenase. Methods Enzymol. 82:453-476, 1982.

Robertson, P.B., Lantz, M., Marucha, P.T., Kornman, K.S., Trummel, C.L., and Holt, S.C.: Collagenolytic activity associated with Bacteroides species and Actinobacillus actinomycetemcomitans. J. Periodontal. Res. 17:275-283, 1982.

Rudek, W., and Haque, R.V.: Extracellular enzymes of the genus <u>Bacteroides</u>. J. Clin. Microbiol. <u>4</u>:458-460, 1976.

Saglie, R., Newman, M.G., Carranza, F.A., Jr., and Pattison, G.A.: Bacterial invasion of gingiva in advanced periodontitis in humans. J. Periodontol. <u>53</u>:217, 1982.

Slots, J., and Genco, R.J.: Black-pigmented <u>Bacteroides</u> species, <u>Capnocytophaga</u> species and <u>Actinobacillus actinomycetemcomitans</u> in human periodontal disease: Virulence factors in colonization, survival, and tissue destruction. J. Dent. Res. <u>63</u>:412-421, 1984.

Slots, J.: Detection of colonies of <u>B.gingivalis</u> by a rapid fluorescence assay for trypsin-like activity. Oral Microbial. Immunol. <u>2</u>:139-141, 1987.

Slots, J.: Enzymatic characterization of some oral and non-oral gram-negative bacteria with the API ZYM System. J. Clin. Microbiol. <u>14</u>:288-294, 1981.

Slots, J.: in: Host-Parasite Interactions in Periodontal Diseases, 27-45, American Society for Microbiology, Washington, D.C., 1982.

Smith, R.E.: Identification of protease isozymes after analytical isoelectric focusing using fluorogenic substrates impregnated into cellulose membranes. J. Histochem. Cytochem. <u>32</u>:1265-1274, 1984.

Socransky, S.S., and Manganiello, A.D.: The oral microbiota of man from birth to senility. J. Periodontol. <u>42</u>:485, 1971.

Socransky, S.S., Manganiello, A.D., Propas, D., Oram, V., and Van Houte, J.: Bacteriological studies of developing supragingival dental plaque. J. Periodontal. Res. <u>12</u>:90, 1977.

Sorsa, T., Vitto, V-J., Suomalainen, K., Tuto, H., and Lindy, S.: A trypsin-like protease from <u>Bacteroides gingivalis</u>: partial purification and characterization. J. Periodontal. Res. <u>22</u>:375-380, 1987.

Suido, H., Nakamura, M., Mashimo, P.A., et al.: Arylaminopeptidase activities of oral bacteria. J. Dent. Res. 65:1335, 1986.

Sundquist, G., and Johansson, E.: Neutrophil chemotaxis induced by anaerobic bacteria isolated from necrotic dental pulps. Scand. J. Dent. Res. 88:113-121, 1980.

Sundquist, G., Carlsson, J., and Hänström, L.: Collagenolytic activity of black-pigmented Bacteroides species. J. of Periodontal Res. 22:300-306, 1987.

Sundquist, G., Bloom, G.D., Enberg, K., and Johansson, E.: Phagocytosis of Bacteroides melaninogenicus and B. gingivalis in vitro by human neutrophils. J. Periodontal Res. 17: 113-121, 1982.

Theilde, E.: The nonspecific theory in microbial etiology of inflammatory periodontal diseases. J. Clin. Periodontol. 13, 1986.

Tonzetich, J. and McBride, B.C.: Characterization of volatile sulphur production by pathogenic and non-pathogenic strains of oral Bacteroides. Arch. Oral Biol. 26:963-969, 1981.

Van Steenbergen, T.J.M.: Classification and virulence of black pigmented Bacteroides strains. Thesis Vrije Universiteitte, Amsterdam, 1981.

VanDyke, T.E., Bartholemew, E., Genco, R.J., Slots, J., and Levine, M.J.: Inhibition of neutrophil chemotaxis by soluble bacterial products. J. Periodontol. 53:502-508, 1982.

Wikstrom, M.B., Phalen, G., and Linde, A.: Fibrinogenolytic and fibrinolytic activity in oral microorganisms. J. Clin. Microbiol. 17:759-767, 1983.

Wooley, D.E., and Evanson, T.M.: Collagenase in normal and pathological connective tissues. Wiley - Interscience, New York, 1980.

Yanagida, N., Uozumi, T. and Beppu, T.: Specific excretion of Serratia marcesens protease through the outer membrane of Escherichia coli. J. Bacteriol. 166:937-944, 1986.

Yoshimura, F., Nishikata, M., Suzuki, T., Hoover, C.I., and Newbrun, E.: Characterization of a trypsin-like protease from the bacterium Bacteroides gingivalis isolated from human dental plaque. Arch. Oral Biol. 29:559-564, 1984.

Young, R.A., and Davis, R.W. Efficient isolation of genes by using antibody probes. Proc. Natl. Acad. Sci., USA 80:1194-1198, 1983.
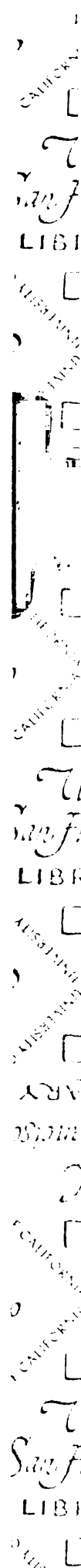
Appendix A: Media, Buffers, and Substrates

Bacterial media:

L Broth

Per liter:
Bacto-tryptone   10g
Bacto-yeast extract 5g
NaCl   5g

Adjust to pH 7.4 with NaOH

plates:
1.2% Bacto agar

soft agar overlays:
0.6% Bacto agar

M-9

Per liter:
Na_2HPO_4 6g
KH_2PO_4 3g
NaCl   0.5g
NH_4Cl 1g

Adjust pH to 7.4, cool, then add:

1 M $MgSO_4$ 2 ml
20% glucose   10 ml
1 M $CaCl_2$ 0.1 ml

M-63

Per liter:
KH_2PO_4 13.6g
(NHy)2SO4   2g
$FeSO_4 \cdot 7H_2O$ 0.5mg

Adjust pH to 7.0 with KOH

Milk Plates

2 x L Broth (1.2% Bacto agar) + 3% dried nonfat milk are added in equal volumes to obtain 1.5% milk plates.

TSB

TSB	30g/liter
Yeast Extract	0.25%
hemin	2.5 µg/ml
menadione	5 µg/ml
DTT	100 µg/ml

Buffers

Ø 80 buffer

5g NaCl
1 ml 1M MgSO4
20 ml 1 M Tris pH 7.4
974 ml H$_2$O
After autoclaving add gelatin to 0.01%

equilibrated phenol

phenol is melted at 68°C, equilibrated several times with 1.0 M Tris (pH 8.0)
followed by 0.1 M Tris (pH 8.0) and 1 mM EDTA until pH = 7.6. Store at 4°C.

10x Eco RI buffer

50 mM NaCl
8.5 mM Tris pH 7.5
5 mM MgCl$_2$

10x ligation buffer

0.66 M Tris pH 7.5
50 mM DTT
10 mM ATP

6x DNA Gel loading buffer

0.25% bromophenol blue
0.25% xylene cyanol
40% (w/v) sucrose in H$_2$O,
Store at 4°C

Agarose gel electrophoresis buffer (1x TBE)

0.089M Tris-borate
0.089M boric acid
0.062M EDTA

agarose type II added to 0.7% and autoclaved to melt. Cool to 50°C then pour gel.

Pentapeptide in auxotrophic selection assay

Z-gly-pro-leu-gly-pro 5 mg/ml in:

sterile H_2O 1 ml
100% Ethanol   1 ml
DMSO   0.2 ml

Appendix B: Bacterial Strains

E. coli:

This laboratory

K10 = E. coli K12 Hfr. Cavalli
(λ^+) tonA$^-$ T$_2$^{R}
pit$^-$ (?) relA$^-$ spoT$^-$ met B$^+$

JF50 = E. coli K12 F$^-$
λ-λ^s mel$^-$ Δlac (mS265)
T$_7$^{s} pro$^+$ nalA^R
SupF = Su III$^+$

SM525 = E. coli K12 F-
λ-λ^s araD139
Δ (ara-leu)$_{7697}$
Δ lac (X74) galE galK
strA relA spoT

RVPI = E. coli RVA
strA^R, Su$^-$, P1^R,
Mu^R and Str^R

C600 = E. coli K12
F$^-$ λ-λ^s
thr$^-$ leu$^-$ lacY$^-$ B1$^-$
tonA$^-$ SuII$^+$

Q359 = E. coli K12
F? λ?
hsd R$_K$$^-$ hsd M$_K$$^+$
$\varnothing$80^R supF (P2)

HB101 = E. coli K12-B hybrid
recA$^-$ r$^-$m$^-$
transformable

AT2457 = E. coli K12
PO1 of Hfr H
pyr^B pil
thi -1, glyA6, relA1, λ^-, spoT$_1$

Mike Hall laboratory, UCSF

MH2 = <u>E.coli</u> K-12
Δ (Lac) x74 hsr⁻
strA pyrF::Tn5
gal E gal K

MH4 = <u>E. coli</u> K-12
Δ (Lac)x74 hsr⁻
strA leuB 600
galE gal K

MC1066 = <u>E. coli</u> K-12
Δ (Lac)x74 hsr⁻
strA galE galK
trpC 9830, leuB600
pyrF::Tn 5

BNN24 = <u>E. coli</u> K-12
hisB⁻

ATCC strain

<u>Bacteroides gingivalis</u>
33277

Figure 4.   BamHI digestion of λL47.1 DNA
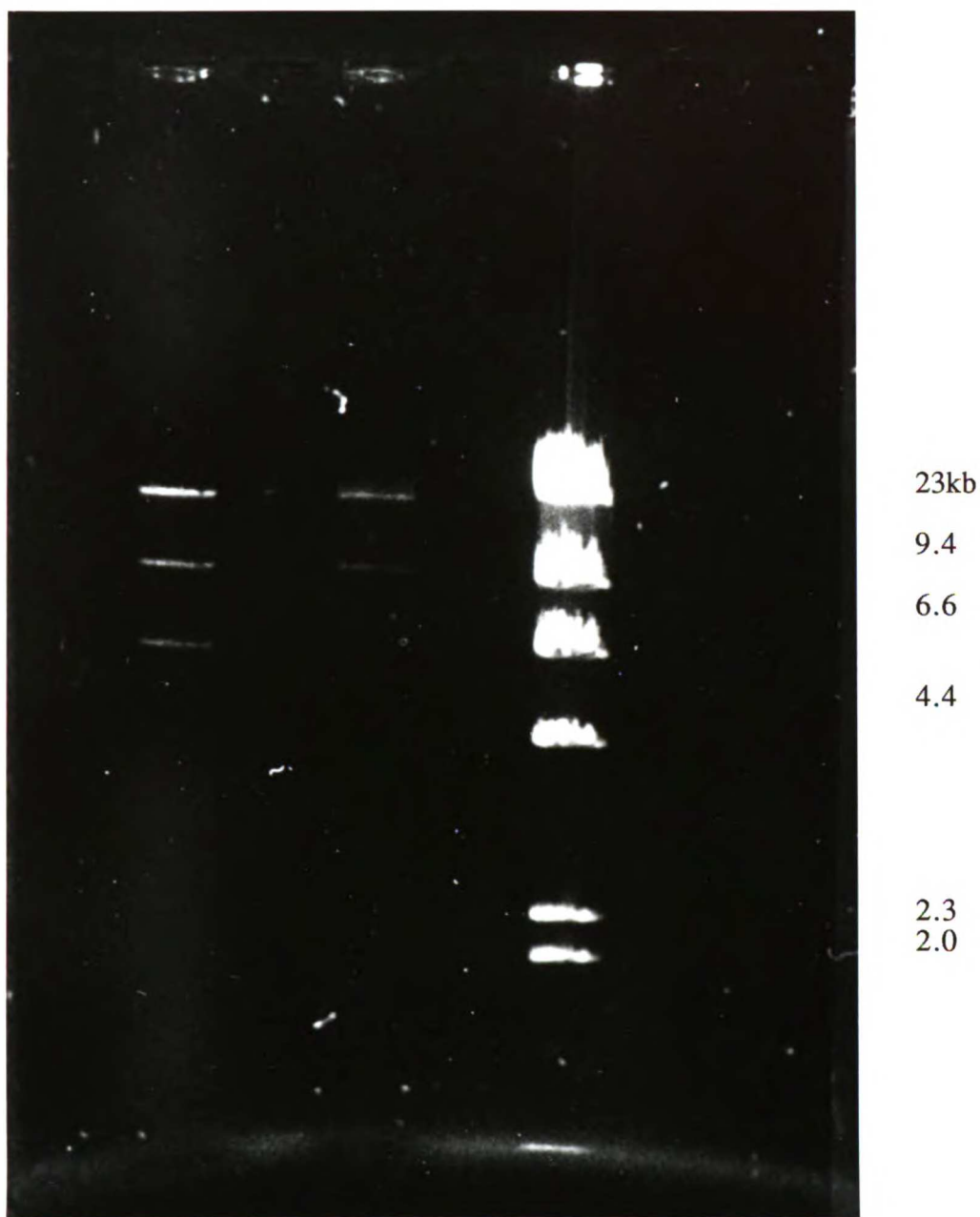
Lane 2 -   3 fragments representing BamHI digest of
         λL47.1 DNA (23kb, 10kb, and 6.5kb
Lane 6 -   λHindIII size standards

Figure 5.   Sau3A digestion of B.gingivalis 33277 DNA

Lane 1,8   λHindIII size standard
Lane 2-6   varying unit of Sau3A (0.015 units to 0.25 units)
Lane 7   uncut B. gingivalis DNA

52

Figure 6.   Sucrose gradient separation of B. gingivalis DNA fragments

Lane   1   2   3   4   5   6   7   8

Size
Standards

23kb
9.4
6.6

4.4

2.3
2.0
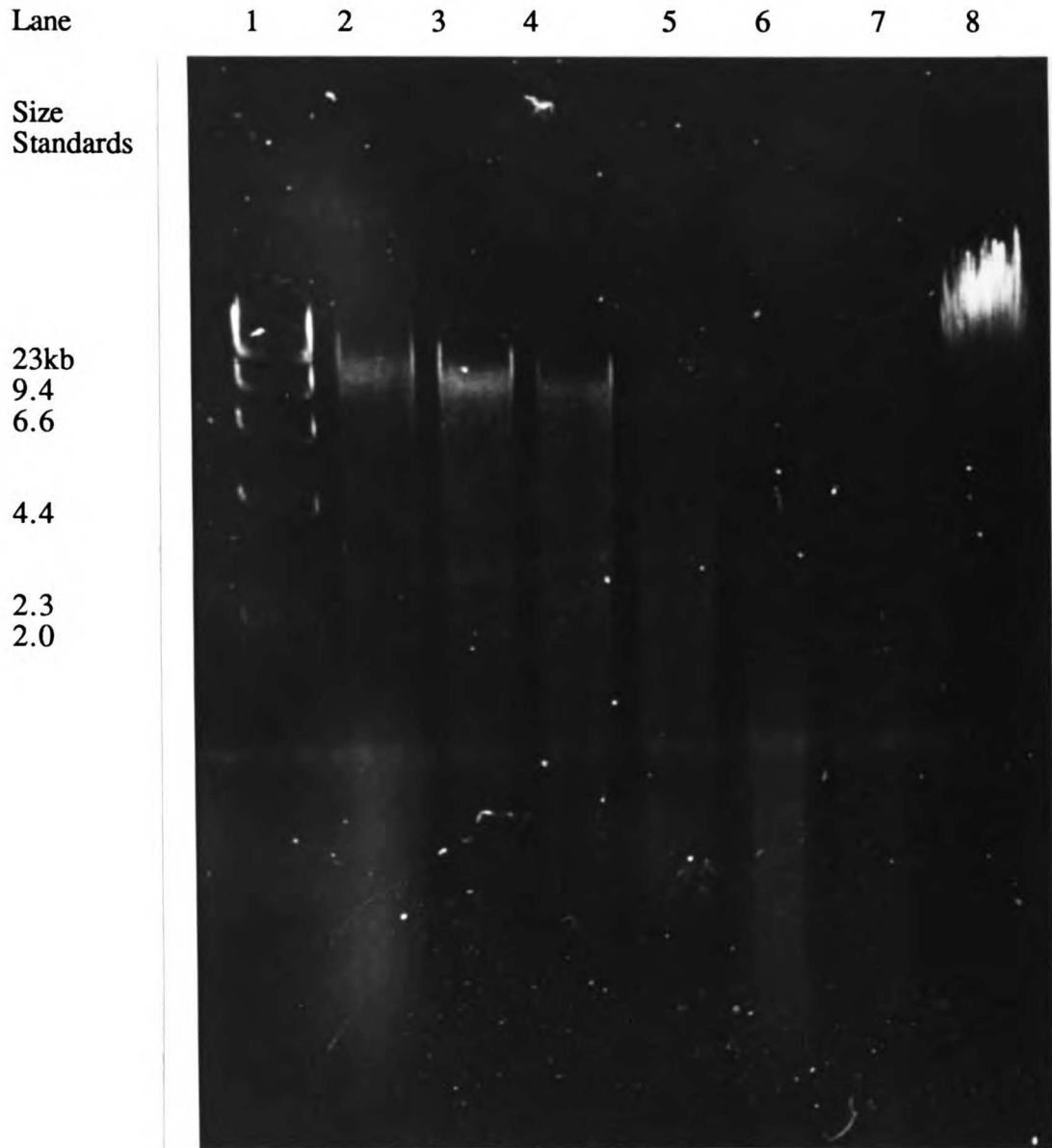
Lane 1   -   λHindIII size standards
Lane 2-7 -   varying sizes of B. gingivalis DNA from
               fractions of sucrose gradient
Lane 8   -   uncut B.gingivalis DNA

53

Figure 7. Confirmation of <u>Sau</u>3A digested <u>B.g.</u> DNA
        4-20 kb in size
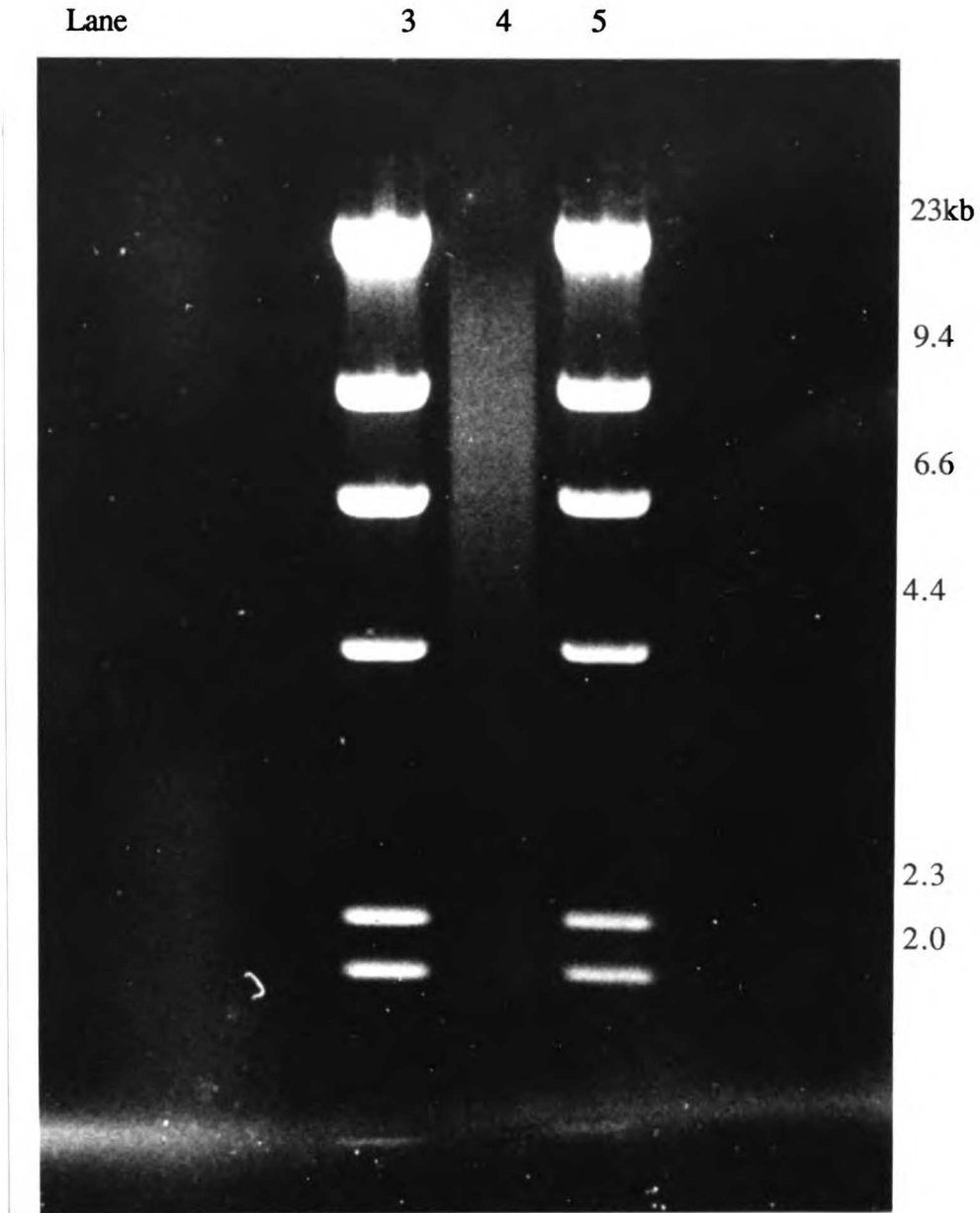
Lane 3,5 - λ<u>Hin</u>dIII size standards
Lane 2   - <u>Sau</u>3A-digested <u>B.g.</u> DNA 4-20 Kb

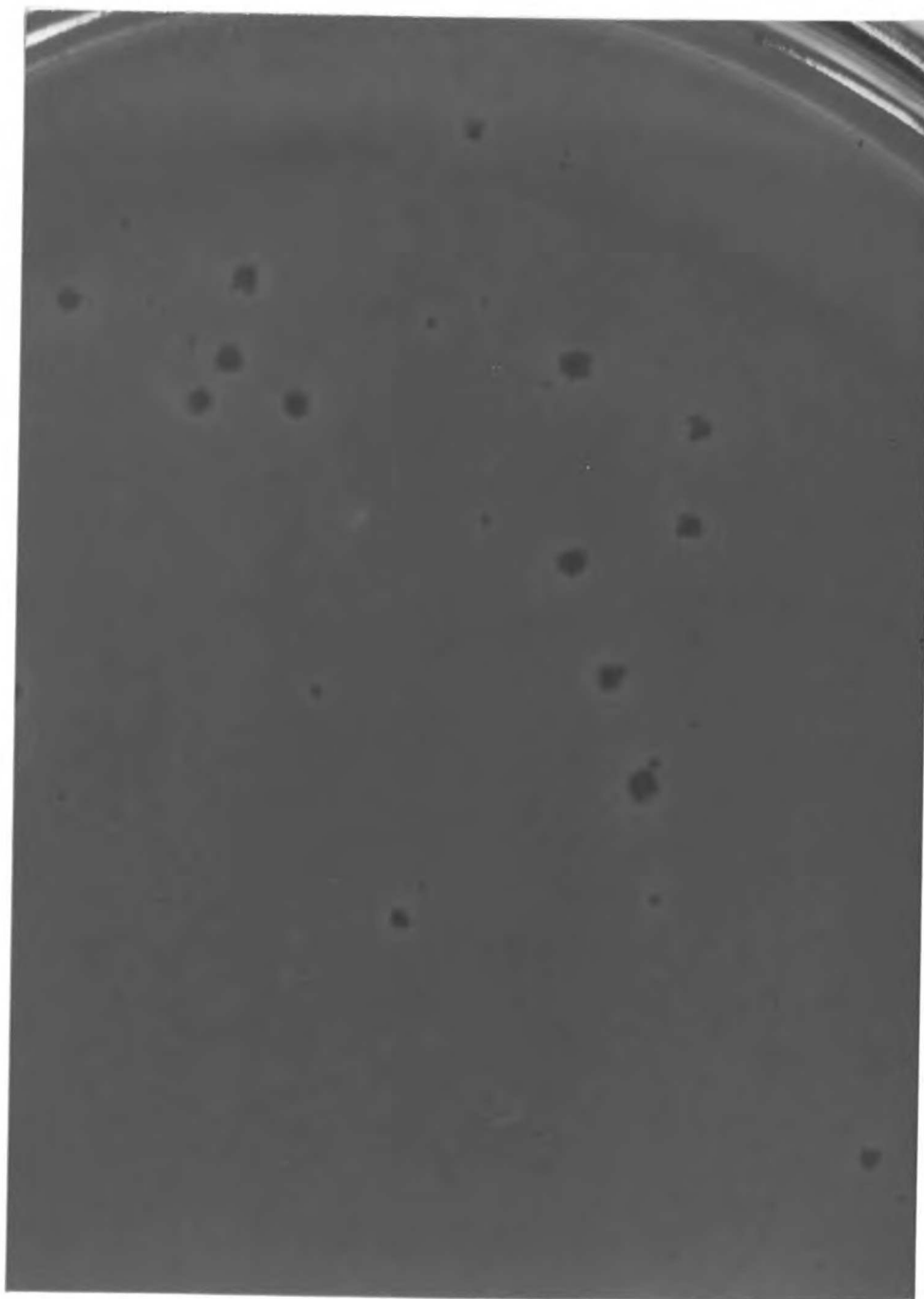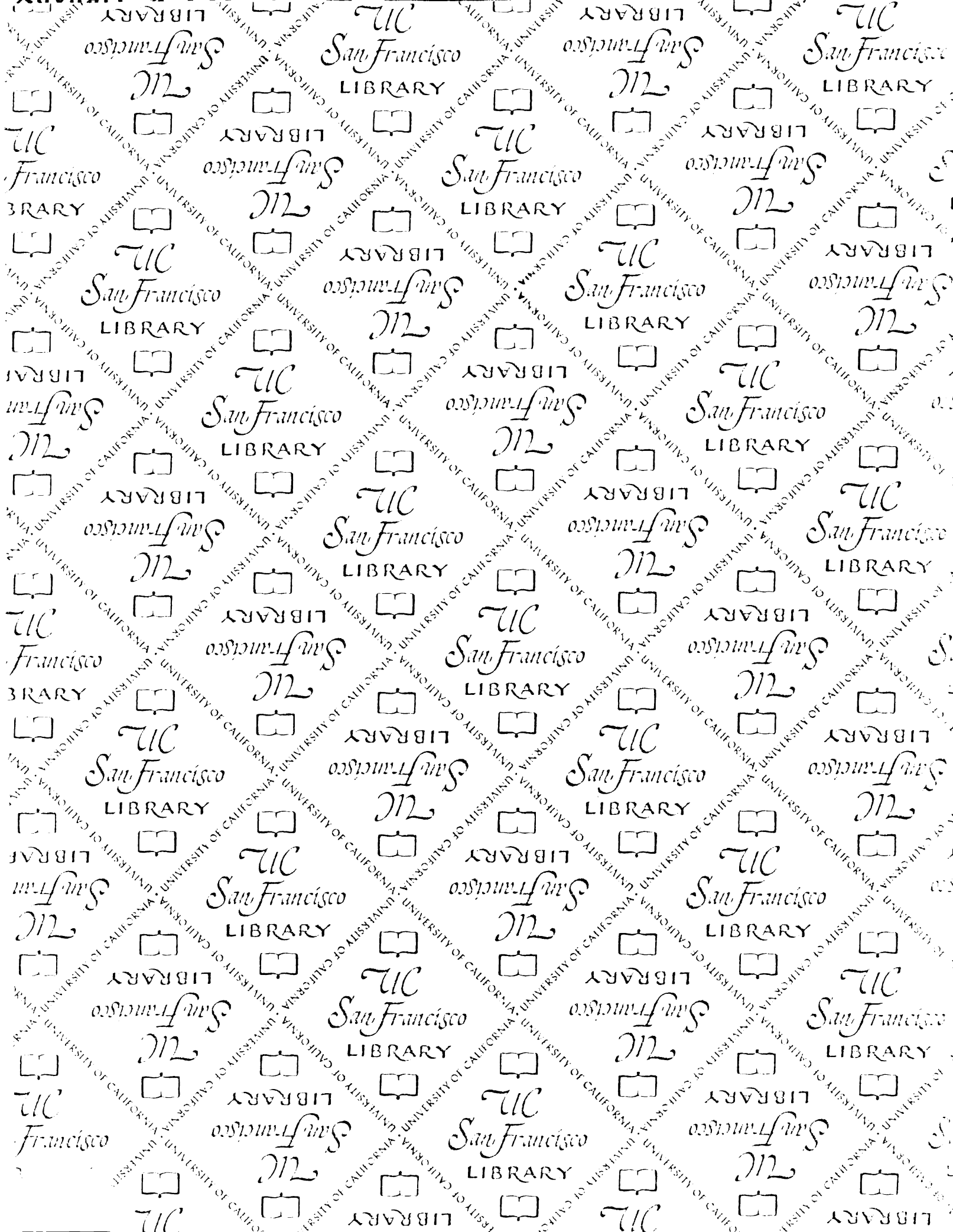Figure 8.   Lawn of <u>E. coli</u> JF50 with recombinant phage plaques and no halo present
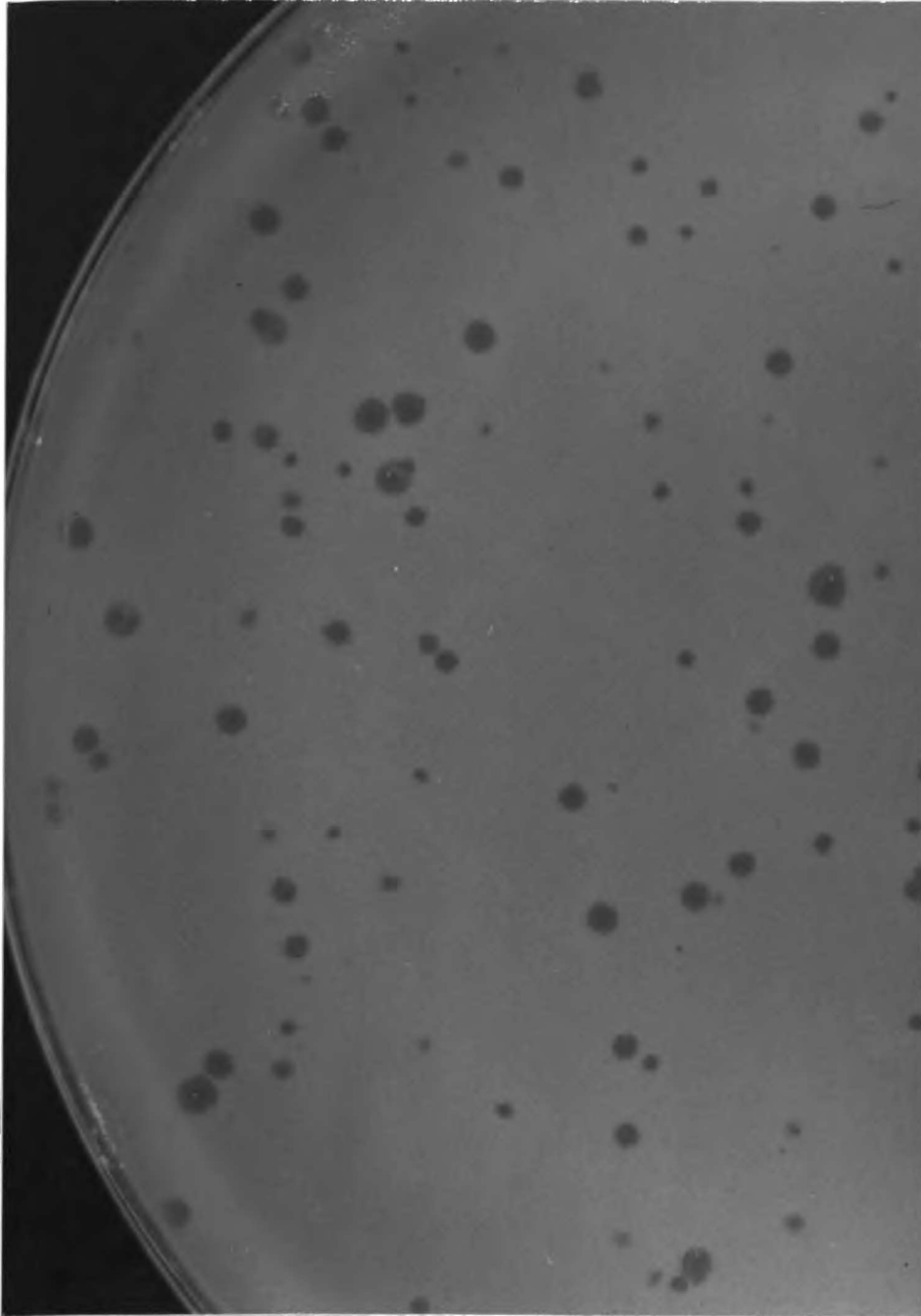
Figure 9.   Lawn of <u>E. coli</u> JF50 with turbid halos around
            recombinant plaques