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Isolation and characterization of Trypsin-like protease-deficient mutants of Porphyromonas Gingivalis

Author
Hoover, Charles Irvin

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ISOLATION AND CHARACTERIZATION OF TRYPSIN-LIKE PROTEASE-DEFICIENT MUTANTS OF PORPHYROMONAS GINGIVALIS

by

CHARLES I. HOOVER

DISSERTATION

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PREFACE

The accumulation of knowledge and the establishment of scientific paradigms occurs through the efforts of many individuals. The studies described in this dissertation were begun in 1988. Since that time other researchers have made contributions relevant to the investigations described herein. Those literature reports, which have appeared since 1988, that have direct relevance to the experimental results presented in this dissertation are discussed in the pertinent chapters rather than in the Introduction. Otherwise the Introduction presents an up-to-date review of the biology of Porphyromonas gingivalis and provides a comprehensive background for the specific aims stated in the Research Goals.

The findings of this investigation are reported in three chapters: I. Isolation and Characterization of Trypsin-Like Protease-Deficient Mutants; II. Strategies to Clone the Trypsin-Like Protease Gene; and III. Development of Molecular Genetic Techniques. Specific discussions of the results are presented in each chapter. The Summary reviews the significant findings of this investigation and outlines areas for future inquiry.

Portions of this dissertation have been communicated in the following literature reports:

> Li, J., Ellen, R.P, Hoover, C.I., and Felton, J.R., 1991. Association of proteases of Porphyromonas (Bacteroides) gingivalis with its adhesion to Actinomyces viscosus. J. Dent. Res. 70:82-86.
>
> Hoover, C.I., Ng, C.Y., and Felton, J.R., 1992. Correlation of haemagglutination activity with trypsin-like protease activity of Porphyromonas gingivalis. Arch. Oral Biol. 37:515-520.

Hoover, C.I., Abarbarchuk, E., Ng, C.Y., and Felton, J.R., 1992. Transposition of Tn4351 in Porphyromonas gingivalis. Plasmid 27:246-250.

Hoover, C.I., Kapila, Y.L., Ng, C.Y., and Felton, J.R., 1992. Restriction mapping and subcloning of a plasmid from P. endodontalis. J. Dent. Res. 71(Abstracts of Papers):293, Abst. #1498.

ABSTRACT

ISOLATION AND CHARACTERIZATION OF TRYPSIN-LIKE PROTEASE-DEFICIENT MUTANTS OF PORPHYROMONAS GINGIVALIS

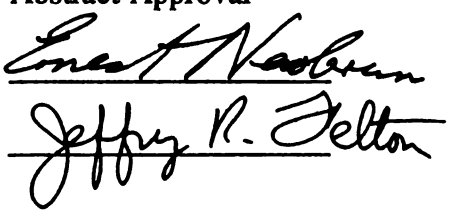
CHARLES I. HOOVER

Numerous in vitro studies suggest that the trypsin-like protease of Porphyromonas gingivalis contributes to its pathogenicity. Techniques were developed to isolate trypsin-like protease-deficient mutants. Twenty-one trypsin-like protease-deficient mutants isolated following mutagenesis with nitrosoguanidine were found to also exhibit reduced black pigmentation (heme accumulation). Four of these mutants were chosen for further study. A quantitative trypsin-like protease activity assay indicated that they possessed less than 2% of the trypsin-like protease activity of their wild-type parent strain. SDS-PAGE zymograms with the parent strain and the mutants suggested that most of the activity observed with the parent strain was due to a single trypsin-like protease. Subsequent studies found that the trypsin-like protease-deficient mutants also exhibited significantly reduced hemagglutination activity and significantly reduced adherence to Actinomyces viscosus. In addition, inhibitors and enhancers of trypsin-like protease activity significantly reduced and increased, respectively, both the hemagglutination activity of wild-type P. gingivalis strains and the adherence of wild-type P. gingivalis strains to A. viscosus. R751::*Ω4, an Escherichia coli suicide vector that contains transposon Tn4351, was transferred to P. gingivalis by conjugation, and transposition of Tn4351 to different

chromosomal locations in independent transconjugants was demonstrated by Southern blot hybridization analysis. Six transconjugants with reduced black pigmentation were observed among approximately 20,000 erythromycin-resistant transconjugants screened. These six transconjugants were found to exhibit the same pleiotropic phenotype as the nitrosoguanidine-induced trypsin-like protease-deficient mutants. These results indicate that three important virulence characteristics of P. gingivalis – trypsin-like protease activity, adherence (hemagglutination), and heme accumulation (black pigmentation) – are physiologically linked, genetically co-regulated, or the result of a single multifunctional gene product.

Efforts were also made to develop gene replacement techniques for use in P. gingivalis. In this regard, attempts to clone the trypsin-like protease gene, to amplify putative protease gene fragment homologs using the polymerase chain reaction technique, and to transform P. gingivalis by electroporation with colonic Bacteroides-based plasmids were unsuccessful. However, the isolation, restriction mapping, and partial cloning of a unique naturally occurring plasmid from P. endodontalis 27067 may allow the development of a Porphyromonas cloning vector and an E. coli-Porphyromonas shuttle vector in the future.

Abstract Approval



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INTRODUCTION

Microbial Etiology of Periodontal Disease

Periodontal disease is an inflammatory disease of the periodontium, characterized by apical migration of the junctional epithelium (pocket formation), marked degeneration of connective tissue (including the periodontal ligament), resorption of alveolar bone, and tooth loss. Various forms of periodontal disease have been defined by clinical criteria, however no classification scheme has been universally accepted. Recently, an attempt to institute standard clinical criteria for the classification of periodontal disease(s) was formulated at the 1989 World Workshop in Clinical Periodontics (128). Five general disease classifications were proposed; adult periodontitis, early-onset periodontitis, refractory periodontitis, necrotizing ulcerative periodontitis, and periodontitis associated with systemic disease. Periodontal diseases are among the most common bacterial infections of humans. A recent national epidemiologic survey (115) found that 7.6% of individuals under 65 and 34% of individuals over 65 had at least one site with attachment loss greater than 6 mm. Considering the limited number of sites examined by probing in each individual this is probably an underestimation of the prevalence of significant attachment loss.

The observations that germ-free animals do not develop typical periodontal disease and that antibiotics modulate periodontal disease progression provide strong evidence that bacteria play a crucial role in inducing and maintaining the host inflammatory processes that are thought to mediate destruction of periodontal tissues. Historically, two prevailing

theories, the nonspecific plaque hypothesis and the specific plaque hypothesis, have been advanced to explain the bacterial etiology of periodontal disease. The nonspecific plaque hypothesis (204, 205) proposes that the bacterial mass of plaque is the most important contributory factor to the development of periodontal disease and that all bacterial species contribute equally to periodontal pathogenesis. In this theory, when bacterial mass is below a certain threshold the host defense mechanisms are able to neutralize bacterial irritants and suppress periodontal tissue destruction, but when bacterial mass exceeds the threshold the host defense mechanisms are overwhelmed or subverted and periodontal tissue destruction proceeds. The specific plaque hypothesis (102, 201) proposes that the presence of specific bacterial species (pathogens) is more important than the bacterial mass of plaque. These periodontopathic bacterial species possess specific attributes (virulence factors) which initiate and maintain the progression of periodontal disease. Several clinical observations suggest that the presence of specific bacterial species is more important than plaque mass in some forms of periodontal disease, and thus support the specific plaque hypothesis. For example, individuals with localized juvenile periodontitis (a form of early-onset periodontitis) exhibit little plaque mass but have rapidly progressive disease. In addition, individuals with adult periodontitis, the most common form of periodontal disease, usually exhibit greater plaque mass but have slowly progressive disease.

A myriad of studies have sought to identify periodontopathic bacterial species by comparing the bacterial species present in "diseased" periodontal sites with those present in "healthy" periodontal sites (see 117 for review). These association studies lack

standardization and have been plagued by numerous inherent methodological problems. The foremost of which is the inability of investigators to identify "diseased" subgingival sites exhibiting "active" progression at the time of plaque sampling. Nevertheless, a relatively small subset (Table I) of the more than 300 bacterial taxa isolated from periodontal sites have been implicated as putative periodontal pathogens (118). For a review of the microbiology of periodontal disease see Slots and Rams (182).

Numerous association studies have implicated Porphyromonas gingivalis, a nonmotile Gram-negative anaerobic coccobacillus, as a major periodontal pathogen. It has been isolated more frequently and in greater proportion from "diseased" periodontal sites than from "healthy" sites (34, 35, 118, 177, 178, 202) and many individuals with periodontitis exhibit elevated serum (37, 120, 123) and local (134, 221) antibody levels to P. gingivalis. Although the cellular infiltrate of periodontal lesions is dominated by polymorphonuclear leukocytes (PMNs), lymphocytes, and plasma cells (141) and most patients with periodontal disease mount a humoral response to P. gingivalis, the antibody produced is apparently ineffective at limiting continued disease progression (2). Due to the complexity of the periodontal microbiota, the inability to recognize actively progressive periodontal sites at the time of microbial sampling, and the infrequent and apparent sporadic progression of periodontal tissue destruction, human clinical studies are at present unable to demonstrate a causative role for any specific microbe (including P. gingivalis) in periodontal disease.

Traditionally animal pathogenicity testing has played a major role in implicating specific microorganisms as the causative agents of many human diseases and in defining

Table I. Consensus List of Oral Bacterial Taxa Implicated as Periodontal Pathogens.

Porphyromonas gingivalis
Actinobacillus actinomycetemcomitans
Prevotella intermedia
Bacteroides forsythus
Campylobacter rectus
Spirochetes (e.g. Treponema denticola)
Eikenella corrodens
Fusobacterium species
Peptostreptococcus species
Eubacterium species

Compiled from Dzink et al. (34, 35), Moore (117), and Slots and Rams (182) using updated taxonomy.

microbial virulence factors. A variety of animal models have been employed to study the microbial etiology of periodontal disease. Initial studies with conventional animals (hamsters and rice rats) found that certain Gram-positive bacteria, particularly Actinomyces viscosus, were capable of transmitting periodontal disease from animal to animal (30, 75). Germ-free animals slowly lose alveolar bone and exhibit slow apical migration of the junctional epithelium (5, 6), however it is generally thought that this is due to mechanical irritation caused by hair and food impaction (151). Early studies with gnotobiotic rats maintained on high sucrose diets found that infections with a variety of both Gram-positive bacteria (Streptococcus mutans, Streptococcus salivarius, Actinomyces naeslundii, and A. viscosus) and Gram-negative bacteria (oral Bacteroides species, Capnocytophaga species, Eikenella corrodens, and Fusobacterium nucleatum) led to accelerated loss of alveolar bone (24). While this animal model indicates the potential of many specific bacteria to induce destruction of periodontal tissues it may lack sufficient discrimination to recognize those species important in human periodontal disease. For example, the experimental conditions are artificial in that the animal is exposed to a pure culture of the infecting organism without the possible modulating effects of other plaque bacteria. In addition, clinical and histopathological examination has revealed significant differences between these infections in gnotobiotic rats and human periodontal disease (100). The lesions in gnotobiotic rats exhibit minimal inflammation and few lymphocytes and plasma cells are present. In contrast, periodontal lesions in humans usually exhibit marked inflammation and contain numerous lymphocytes and plasma cells (141). Studies employing conventional animals and human

isolates also present problems in that it has been difficult to establish human isolates in conventional animals (186). Human periodontal pathogens may lack specific factors needed to colonize other animals and/or may not be able to effectively colonize in the presence of an established "normal" oral flora. However, the development of ligature-induced periodontitis models to study longitudinal ecological shifts of indigenous microbes which precede periodontal destruction has provided some evidence implicating specific bacterial species as periodontal pathogens. Ligature-induced periodontitis models have been developed in dogs (160) and in nonhuman primates (63, 84). Perhaps the strongest evidence indicating that P. gingivalis is a causative agent of periodontal disease comes from studies using the ligature-induced periodontitis model in cynomolgus monkeys (69, 84). In this regard, Holt et al. (69) have reported that exogenous infection with P. gingivalis induces rapid loss of attachment and bone resorption, as well as an increase in specific antibody against P. gingivalis. The disease produced by exogenous infection with P. gingivalis is identical to "spontaneous" periodontal disease in ligated monkeys. In addition to indicating that P. gingivalis "is capable of functioning as a primary pathogen in periodontal disease", these findings establish a basis for the future use of the ligature-induced periodontitis model to identify bacterial virulence factors that promote colonization and pathogenicity of P. gingivalis.

In addition to the animal periodontitis models discussed above, general animal pathogenicity models have been used to investigate the virulence characteristics of P. gingivalis. These rodent-based animal pathogenicity models are discussed in a latter section (Cellular Components and Pathogenicity).

BIOLOGY OF PORPHYROMONAS GINGIVALIS

Taxonomy and Phylogeny

Oliver and Wherry (138), in 1921, were the first to isolate and describe pleomorphic Gram-negative anaerobic rods which produce black-pigmented colonies on blood-containing media. They obtained isolates from a variety of anatomical sites, including the oral cavity. They considered the black pigment to be melanin and designated their isolates Bacterium melaninogenicum. Subsequent early studies, reflecting then current taxonomic criteria, suggested a variety of other names (Table II). All of these names were based on production of black pigment and until 1977 the production of black pigment by a Gram-negative anaerobic rod was sufficient for its identification as Bacteroides melaninogenicus. However, several studies indicated heterogeneity among strains of B. melaninogenicus. Sawyer et al. (157) reported biochemical heterogeneity among 31 strains of B. melaninogenicus. They found that the final pH in glucose-containing broth and chromatographic analysis of volatile fatty acid endproducts could be used to divide the 31 strains into strongly fermentative, weakly fermentative, and nonfermentative groups. They also noted that isolates of intestinal origin were uniformly nonfermentative, whereas oral isolates represented all three groups. In the 1970s a variety of biochemical studies (59, 66, 170, 228) recognized three subspecies of B. melaninogenicus. Fermentative strains were designated B. melaninogenicus subspecies melaninogenicus and B. melaninogenicus subspecies intermedius. Nonfermentative strains were designated B. melaninogenicus subspecies asaccharolyticus. In brief, strains that produced acid from glucose but did not produce indole were placed in subspecies melaninogenicus, those that produced acid from glucose and also produced indole were

Table II. Nomenclature of Pophyromonas gingivalis.

Bacterium melaninogenicum	Oliver & Wherry, 1921 (138)
Hemophilus melaninogenicus	Bergey et al., 1930 (10)
Ristella melaninogenica	Prevot, 1938 (144)
Bacteroides melaninogenicus	Roy & Kelley, 1939 (152)
Fusoformis nigrescens	Schwabacher et al., 1947 (162)
Bacteroides melaninogenicus	Kelley, 1957 (78)
Bacteroides melaninogenicus subsp. asaccharolyticus	Holdeman & Moore, 1970 (67)
Bacteroides asaccharolyticus	Finegold & Barnes, 1977 (41)
Bacteroides gingivalis	Coykendall et al., 1980 (23)
Porphyromonas gingivalis	Shah & Collins, 1988 (167)

placed in subspecies intermedius, and those that did not produce acid from glucose were placed in subspecies asaccharolyticus. However, several investigators suggested that the asaccharolytic strains should be assigned to a separate species. Werner et al. (224) argued that "including fermentative and non-fermentative organisms within the same species is irreconcilable with any established rule of bacterial taxonomy".

In 1977, Finegold and Barnes (41) officially proposed that the subspecies asaccharolyticus be elevated to the species level as B. asaccharolyticus. They concluded that the inclusion of strains with different energy-yielding metabolisms and different DNA base ratios in one species, primarily defined by the production of black pigment, was inappropriate. Asaccharolytic strains were differentiated from saccharolytic strains by their failure to produce a lower pH in glucose-containing media than in non-glucose containing media, by their volatile fatty acid endproducts, and by their guanine plus cytosine (G+C) content. Asaccharolytic strains produced propionic, n-butyric, and isobutyric acid, whereas saccharolytic strains produced primarily isobutyric and succinic acid. Asaccharolytic strains had a G+C content of 50 to 54% and saccharolytic strains had a G+C content of 40 to 45%. However, it soon became apparent that two distinct groups existed among B. asaccharolyticus strains. Shah et al. (170) examined thirteen asaccharolytic strains and found that they formed two distinct groups based on their G+C content and electrophoretic mobility of their malate dehydrogenase. van Steenbergen et al. (217) also recognized two distinct groups of asaccharolytic strains based on their G+C content. One group had a G+C content of about 48% and the other group had a G+C content of about 53-54%. van Steenbergen et al. argued that "since there is a difference

of about 5-6% between the two groups, they should not be classified together in one species". Other investigators also observed phenotypic differences among B. asaccharolyticus strains. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of total cellular proteins suggested the presence of two distinct groups of asaccharolytic strains (196) and asaccharolytic strains isolated from the oral cavity hemagglutinated sheep red blood cells, whereas asaccharolytic strains isolated from other anatomical sites did not (179).

In 1980, Coykendall et al. (23) officially proposed that the differences between oral and non-oral strains of B. asaccharolyticus were large enough to justify placement of the oral asaccharolytic strains in a new species. B. gingivalis (oral) strains were differentiated from B. asaccharolyticus strains by their ability to hemagglutinate sheep red blood cells, their production of phenylacetic acid (110), and their approximately 5% lower G+C content (48%). A third species of asaccharolytic black-pigmented Bacteroides was described in 1984 by van Steenbergen et al. (220). They named the new species B. endodontalis, since most of their original strains were isolated from infected dental root canals. B. endodontalis was differentiated from B. gingivalis by its inability to hemagglutinate sheep red blood cells, by its lack of trypsin-like protease activity, and by the absence of N-acetyl-β-glucosaminidase activity. B. endodontalis was differentiated from B. asaccharolyticus by its lack of α-fucosidase activity, its requirement for menadione for growth, and its inability to grow in an atmosphere of 95% N_2-5% CO_2.

In 1988, Shah and Collins (167) proposed that the three species of asaccharolytic black-pigmented Bacteroides be reclassified in a new genus, Porphyromonas. They

argued that B. asaccharolyticus, B. gingivalis, and B. endodontalis are well-defined species and form a relatively homogenous group that is quite different from the type species of the genus Bacteroides, Bacteroides fragilis. Production of black pigment, the presence of a greater amount of 13-methyl-tetradecanoic acid rather than 12-methyl-tetradecanoic acid, and the lack of diaminopimelic acid were the major differences reported between Porphyromonas and Bacteroides.

This review of taxonomic history has concentrated only on black-pigmented asaccharolytic species. Similar taxonomic upheavals have occurred among the black-pigmented saccharolytic strains as well. They have also been separated from the genus Bacteroides and placed in a new genus, Prevotella (168). Isolates identified as B. melaninogenicus in 1977 would, at present, be classified in two genera and at least nine species.

Phylogenetic studies, in general, support the conclusions of phenotypic classification schemes. Both 16S and 5S rRNA sequence analysis indicate that Porphyromonas are closely related to the colonic Bacteroides. Woese (232), based on "sequence distance and parsimony analysis" of 16S rRNA sequences grouped the Bacteroides (including oral species), the Cytophaga, and the Flavobacterium in a single phylum. A relatively "distinct signature" defined this grouping. For example, all members of the group contain a U residue at position 570 that is not present in any other eubacteria (except the plactomyces). In addition, the A residue at position 995 is unique (except for the green sulfur bacteria) to the Bacteroides-Cytophaga-Flavobacterium phylum and the A residue at position 1532 sets the group apart from all other eubacteria,

as well as the archaebacteria and eukaryotes. 5S rRNA sequence analysis also groups Porphyromonas species with the Bacteroides-Cytophaga-Flavobacterium phylum (212). Of the species studied, P. gingivalis was most closely related to B. fragilis and B. thetaiotaomicron. The dissimilarity index between P. gingivalis and these colonic-Bacteroides was approximately 0.15.

To date, the most definitive phylogenetic study of Porphyromonas is that of Paster et al. (143). They compared 16S rRNA sequences of Bacteroides fragilis, Bacteroides thetaiotamicron, Bacteroides forsythus, Bacteroides vulgatus, Prevotella disiens, Prevotella corporis, Prevotella melaninogenica, Prevotella intermedia serotype II, Porphyromonas asaccharolyticus, Porphyromonas endodontalis, and Porphyromonas gingivalis. Sequence analysis of 16S rRNA indicated that these species formed a coherent cluster divided into three major groups. The first group consisted of B. fragilis, B. thetaiotamicron, and B. vulgatus with an average similarity of 92%. The second group consisted of P. corporis, P. disiens, P. melaninogenica, and P. intermedia with an average similarity of 91%. P. asaccharolyticus, P. endodontalis, and P. gingivalis formed the third major group with an average similarity of 85%.

It is noteworthy that these results conform to the present taxonomy which separates all these species, which were formerly members of the genus Bacteroides, into three genera: Bacteroides, Prevotella, and Porphyromonas. It is also of interest that the purple bacteria, to which E. coli belongs, are more closely related to the Gram-positive bacteria than to the Bacteroides-Cytophaga-Flavobacterium phylum.

Biochemical Characterization of Proteases

Trypsin-like protease(s).

A relatively unique aspect of P. gingivalis, in comparison to most other oral bacteria, is the production of an array of proteolytic enzymes. In fact, production of trypsin-like protease activity is a key phenotypic determinant for differentiation of P. gingivalis from other Porphyromonas and Prevotella species. Several studies have employed synthetic chromogenic and fluorogenic protease substrates to characterize P. gingivalis proteases. Laughon et al. (90) were the first to report that P. gingivalis hydrolyzed benzoyl-L-arginine-ß-naphthylamide (BANA). They described this activity as "trypsin-like" because like mammalian trypsin, which is a paradigm for serine proteases (EC 3.4.21), it exhibited substrate specificity for arginine. According to the terminology of Berger and Schechter (9) for describing the active sites of endopeptidases and the complementary features of their substrates, the trypsin-like protease of P. gingivalis exhibits activity with synthetic substrates that contain arginine at the P_1 site. The P_1 site is the substrate residue that is subject to nucleophilic attack of its carbonyl carbon. Several subsequent investigations have "partially purified" trypsin-like protease(s) from P. gingivalis (42, 139, 183, 188, 208, 235). Direct comparison of the results of these studies is problematic. The use of different purification procedures, different inhibitor concentrations, different enzyme activity assay conditions, and different analytical methods for estimation of molecular weights has produced conflicting results. Estimated molecular weights reported for these partially purified trypsin-like protease(s) range from

35,000 (188) to 300,000 (42).

Substrate specificity is not generally considered an acceptable basis for naming and classifying proteases. Usually proteases are classified by their catalytic mechanism (see PCR Amplification of Putative Protease Gene Homologs). Specific protease inhibitors and enhancers are commonly used to characterize the catalytic mechanisms of proteases. Based on enhancement of activity by reducing agents (dithiothreitol {DTT} and cysteine), inhibition by sulfhydryl-blocking agents (N-ethylmaleimide {NEM}) and by ethylenediaminetetraacetic acid (EDTA) most studies have categorized the trypsin-like protease as a cysteine (thiol) protease that requires divalent cations (42, 139, 235). However, two studies based on inhibition by diisopropylfluorophosphate (208) and phenylmethylsulfonyl fluoride (188) suggest that the trypsin-like protease may be a serine protease. See Table III for a summary of studies examining the effect of inhibitors and enhancers on trypsin-like protease activity of P. gingivalis. In conclusion, it appears that the trypsin-like protease is "trypsin-like" in its specificity for arginine at the P_1 site in the substrate, but is not "trypsin-like" in regard to the amino acid composition of its catalytic domain (catalytic mechanism). At present it is unclear whether P. gingivalis produces one or more than one protease with trypsin-like substrate specificity. The cloning and analysis of protease genes should help resolve these ambiguities.

Collagenase(s).

Numerous studies report that P. gingivalis has collagenolytic activity (12, 91, 111, 150, 188, 192, 207). Sorsa et al. (188) isolated a trypsin-like protease and a collagenase

Table III. Effect of various protease inhibitors[a] and enhancers[b] on trypsin-like protease activity of P. gingivalis.

Inhibitor/ Enhancer	Suido et al. (191)	Fujimura and Nakamura (42)	Sorsa et al. (188)	Ono et al. (139)	Tsutsui et al. (208)
NEM		⇓[c]	⇓	⇓	
BZMD			⇓		
TLCK	⇓	⇓		⇓	⇓
TPCK	⇓			⇓	—[d]
PMSF	—		⇓		
EDTA	⇑[e]	⇓	⇓	⇑	⇓
Hg^{2+}	⇓	⇓			
Zn^{2+}	⇓				⇓
β-mercaptoethanol			⇑	⇑	
Cysteine				⇑	
Dithiothreitol			⇑	⇑	

[a] Protease inhibitors: BZMD, benzamidine; NEM, N-ethylmaleimide; TLCK, tosyl-L-lysine chloromethyl ketone; TPCK, tosyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; & EDTA, ethylenediaminetetraacetic acid.

[b] Protease enhancers: β-mercaptoethanol, cysteine, & dithiothreitol.

[c] Decrease of > 30% compared to control.

[d] Change of < 30% compared to control.

[e] Increase of > 30% compared to control.

from P. gingivalis 33277. The trypsin-like protease degraded type IV collagen and denatured type I collagen but not native type I collagen, whereas the collagenase degraded native type I collagen. Degradation of type IV collagen is not sufficient to classify an enzyme as a "genuine" collagenase, since type IV collagen has several non-helical segments interrupting the triple helix of the molecule (85, 161) and these segments are susceptible to the action of non-specific proteolytic enzymes such as trypsin, elastase(s), and gelatinase(s) (98, 107, 210). Birkedal-Hansen et al. (12) have stated that to characterize a protease as a collagenase it is "necessary to establish that it (i) dissolves collagen fibrils completely, rather than partially, and (ii) attacks the helical domain of intact native, triple helical molecules". They reported the purification of a genuine collagenase from P. gingivalis that degrades reconstituted type I, type II, and type III collagen at $\leq$ 20°C. Lawson and Meyer (91) have also recently reported the purification of a "collagenase" from P. gingivalis; however collagenolytic activity was assayed only with type IV collagen and a synthetic substrate for eukaryotic collagenases.

Several studies suggest that the trypsin-like protease from P. gingivalis possesses collagenolytic activity and may be a genuine collagenase. Sundqvist et al. (192) have reported that both trypsin-like protease activity and activity against reconstituted rat skin collagen responded similarly to specific protease inhibitors. Both trypsin-like protease activity and collagenolytic activity were found to be inhibited by thiol-blocking agents and chelating agents. Smalley et al. (183) partially purified a "soluble trypsin-like enzyme" from culture supernatants of P. gingivalis W50. The purified enzyme was capable of degrading type I collagen at both 37°C and 30°C whereas mammalian trypsin

only degraded type I collagen at 37°C.

Kato et al. (77) have suggested that P. gingivalis may produce two distinct collagenases. Two fractions exhibiting activity against reconstituted type I collagen were detected when crude extracts of P. gingivalis were subjected to Mono Q anion-exchange chromatography. An active fraction not retained by Mono Q columns contained a 35 kD protein which appeared to correspond to the product of the prtC gene (see section on E. coli and Colonic Bacteroides Plasmid Vector Cloning Systems). The second active fraction was retained by Mono Q columns and was eluted with a salt gradient. Kato et al. (77) suggest that the retained enzyme may be a trypsin-like protease; however the retained enzyme was not extensively characterized, genetically or enzymatically. At present it is unclear how many enzymes with collagenolytic activity are produced by P. gingivalis and how many of them are genuine collagenases.

Dipeptidylaminopeptidases.

P. gingivalis also hydrolyzes several synthetic arylaminopeptide substrates; gly-pro-NA, arg-arg-NA, gly-arg-NA, gly-phe-NA, leu-gly-NA, lys-ala-NA, and lys-pro-NA (1, 190). It is unknown how many different proteases are responsible for hydrolysis of these arylaminopeptide substrates. To date, only the gly-pro dipeptidylaminopeptidase activity has been investigated. Several studies (1, 8, 54, 191) have partially purified gly-pro dipeptidylaminopeptidase(s) from P. gingivalis. These studies suffer from the same difficulties as those of the "trypsin-like" protease. Reported molecular weights vary from 29,000 (54) to 160,000 (1) and results of inhibitor studies, even from the same research

group (51, 54), have been inconsistent. Based on inhibition by diisopropylfluorophosphate, N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), and phenylmethylsulfonyl fluoride (PMSF) the gly-pro dipetidylaminopeptidase has been categorized as a serine protease.

Cellular Components and Pathogenicity

A variety of potential virulence factors have been attributed to P. gingivalis (for review see 68, 112, 180). Bacterial pathogens must meet four basic criteria: they must adhere to host tissue(s), avoid host defense systems, multiply in the host environment, and cause tissue damage. In the broadest sense any bacterial component that is essential for any of these processes can be considered to be a virulence factor. However, all commensal bacteria, which normally colonize the oral cavity, must accomplish the first three criteria of adherence, avoidance of host defense systems, and multiplication. Damage to the host is what truly differentiates pathogenic bacteria from commensal bacteria. Pathogenicity may be considered to be a subset of colonization in which damage to the host occurs. Therefore, in a sense, colonization is the most important biological process. There is a much greater difference between bacteria that colonize humans and those that do not, than there is between pathogenic and commensal bacteria.

Fimbriae

Within the oral cavity the ability to attach to mucosal surfaces, bacterial plaque constituents, and/or adsorbed salivary components is considered a prerequisite for

successful colonization and subsequent production of disease (198). In this regard, P. gingivalis has been shown to possess fimbriae (58, 136) and hemagglutinin(s) (13, 135, 136) as well as other potential adhesins. P. gingivalis has been reported to adhere to buccal and crevicular epithelial cells, erythrocytes, and Gram-positive bacteria (135, 181). Early studies inferred that fimbriae were responsible for the hemagglutination activity of P. gingivalis. However, preparations of purified fimbriae from P. gingivalis neither possess hemagglutination activity nor inhibit hemagglutination by whole bacterial cells (236).

Some recent studies suggest that fimbriae of P. gingivalis may mediate its adhesion to oral mucosal cells and to salivary constituents. Isogai et al. (73) have reported that Fab fragments of monoclonal antibodies against fimbriae inhibit binding of P. gingivalis to buccal epithelial cells. Lee et al. (92) have investigated the importance of fimbriae in adherence of P. gingivalis to saliva-coated hydroxyapatite (sHAP). Binding to sHAP of a fully fimbriated strain (2561/33277) and a sparsely fimbriated strain (W50) was compared. Although there was only a one log reduction in binding to sHAP by the sparsely fimbriated strain, compared to the fully fimbriated strain, Lee et al. (92) concluded that the sparsely fimbriated strain bound poorly to sHAP. In addition, purified fimbriae and synthetic peptides corresponding to fimbrilin sequences inhibited binding of P. gingivalis to sHAP beads, whereas a purified 75kD outer membrane protein of P. gingivalis did not. While these inhibition studies provide strong evidence for the participation of fimbriae in binding to sHAP, any conclusions based on comparison between binding by the fully fimbriated and sparsely fimbriated strains are suspect since

the presence of unknown confounding differences between these two strains cannot be evaluated.

Capsular polysaccharide and phagocytosis

The ability to avoid or neutralize phagocytic cells is a consistent feature of successful pathogens. Therefore an intriguing paradox of bacterial pathogenicity in the oral cavity is that while adherence to oral epithelium and/or plaque bacteria is required for colonization, resistance to binding by phagocytic cells is also usually essential. The cellular infiltrate of periodontal lesions is dominated by PMNs, lymphocytes, and plasma cells (104, 141) depending on the stage of the lesion. Normal PMN function appears to be critical in host defense against periodontal disease since the presence of defects in PMN function correlates strongly with the occurrence of rapidly progressive forms of periodontal disease (64, 215). In many bacterial species the production of capsular polysaccharide is essential for resistance to phagocytosis and for pathogenesis. In this regard, electron-dense capsular material has been observed to coat the outer membranes of many strains of P. gingivalis (58, 101, 233).

Several in vitro studies have investigated the interaction between P. gingivalis and PMNs. van Steenbergen et al. (219) used luminol-dependent chemiluminescence (CL) of human leukocytes as a measure of phagocytosis of P. gingivalis in the presence of pooled "normal" human sera. P. gingivalis strains W83(HG66) and W50(HG76) produced the lowest CL response of the seven strains studied. Electron microscopic studies were reported to show a more pronounced capsule surrounding strains W83(HG66) and

W50(HG76) than the other five P. gingivalis strains tested. These two strains were also found to exhibit the lowest hydrophobicity as measured by the partition of bacterial cells in a two-phase system (xylene/water). Since capsular polysaccharide is known to be hydrophilic, van Steenbergen et al. (219) suggested that the low hydrophobicity of strains W83(HG66) and W50(HG76) and the low CL response to them may be due to capsular polysaccharide. It is of interest that these two strains had been previously found to be the most virulent in the mouse subcutaneous abscess model (218). General animal pathogenicity models are discussed at the end of this section.

Effective phagocytosis of bacteria by PMNs is often dependent upon opsonization by complement and/or antibody. Recently, Cutler et al. (26, 27) investigated the phagocytosis of four strains of P. gingivalis by human PMNs in the presence of normal rabbit sera, hyperimmune rabbit antisera against P. gingivalis, or sera from human adult periodontitis patients. Three strains of P. gingivalis (A7436, W83, & HG405), which have been classified as virulent, were not opsonized by normal sera but required strain-specific hyperimmune antisera for effective opsonophagocytosis. Although specific antibody was required, complement activity was also essential for optimal opsonophagocytosis. It appeared that specific antibody stimulated both the classical and alternate complement pathways (26, 27). The strain-specific antibody required for opsonophagocytosis of these strains may be against capsular polysaccharide. Schifferle et al. (159) have demonstrated that purified capsular polysaccharide from strain A7A1-28 reacted only with homologous antisera, whereas antigens present in phenol-water extracts reacted with antisera against three heterologous strains.

Cutler et al. (27) have reported, that in contrast to the three virulent strains studied, P. gingivalis 33277, which has been classified as avirulent in mouse models, was opsonized by normal sera to a significantly greater extent. Since the opsonophagocytosis of P. gingivalis 33277 was heat-sensitive and occurred in the absence of specific antibody, Cutler et al. (27) concluded that it was complement-dependent and involved primarily the alternate complement pathway. The opsonophagocytosis of strain 33277 may result from accumulation of complement component C3 on its surface. In this regard, Schenkein (158) has reported that P. gingivalis 33277 accumulates C3 on its surface whereas strain W83 does not. While C3 accumulation is known to enhance phagocytosis, it may not be required for phagocytosis of strain 33277. Armitage et al. (3) have observed marked phagocytosis of P. gingivalis 33277, in the absence of serum, by retinoic acid-induced and DMSO-induced HL-60 cells.

Various investigators have suggested that protease activity, as well as capsular polysaccharide, may contribute to resistance of P. gingivalis to opsonophagocytosis by degrading oposonins. Addition of the protease inhibitor TLCK increases binding of C3 to P. gingivalis W83 (158), and variants of P. gingivalis W50 that exhibit 3-fold less trypsin-like protease activity and 3- to 4-fold less capsular polysaccharide are avirulent in the mouse subcutaneous abscess model (109). However, the importance of protease activity in virulence and resistance to phagocytosis remains unclear since P. gingivalis 33277, although it accumulates C3 on its surface, possesses protease activity comparable to "virulent" strains.

Unquestionably virulence of P. gingivalis is multifactorial. Considerable caution

must be exercised in forming analogies from results with mouse virulence models and in vitro assays, such as phagocytosis, which purport to be in vitro correlates of pathogenicity. For example, although P. gingivalis HG405 is only moderately virulent in the mouse abscess model (216) and avirulent in the mouse chamber model (45) it was the most resistant to in vitro phagocytosis of the four strains studied by Cutler et al. (27).

P. gingivalis may also resist phagocytosis by affecting various cellular functions of PMNs. Van Dyke et al. (214) have reported that culture supernatants and sonic extracts of P. gingivalis 10-2-1 inhibited PMN chemotaxis and inhibited binding of the chemotactic peptide, N-formylmethionylleucylphenylalanine (FMLP). Culture supernatants and whole cells of P. gingivalis W83 also fail to induce chemotaxis (124). The lack of chemotactic induction of PMNs by P. gingivalis may be due to proteolytic degradation of PMN chemotaxis receptors. In this regard, Maeda et al. (106) have observed that incubation of culture supernatants of P. gingivalis 381 with PMNs resulted in the reduction of FMLP receptors. Other effects on PMN membranes by P. gingivalis which could interfere with PMN motility and phagocytosis have also been reported. P. gingivalis products have been found to inhibit receptor capping by FMLP-receptors, ConA-receptors, and C3b-receptors of PMNs (195, 229). In addition, Scragg et al. (165, 166) have observed that culture supernatants of P. gingivalis cause depolarization of PMN membranes. This could interfere with PMN migration since polarization of PMNs is an initial event in PMN activation. Scragg et al. (166) suggested that the trypsin-like protease of P. gingivalis may be responsible for PMN depolarization since heat treatment of bacterial supernatants abolished the effect and incubation of PMNs with mammalian

trypsin resulted in depolarization.

Lipopolysaccharide

Like all other Gram-negative bacteria, P. gingivalis contains lipopolysaccharide (LPS, endotoxin) as a component of its outer membrane. Studies with LPS from enteric bacteria indicate that LPS can mediate over 30 host-related biological responses, mainly associated with host defense systems (103, 225). Numerous studies have examined the role of bacterial LPS in periodontal disease (see 28 for review). Lipopolysaccharide (LPS) of P. gingivalis may contribute to avoidance of host defense mechanisms, as well as to destruction of periodontal tissues. SDS-PAGE analysis of LPS from several P. gingivalis strains indicates the presence of high molecular weight "smooth" LPS (16, 29). In enteric bacteria the presence of high molecular weight LPS, in contrast to low molecular weight "rough" LPS, has been associated with virulence and resistance to the bactericidal action of serum (93, 203). Therefore, it is tempting to speculate that the resistance of P. gingivalis to the bactericidal action of serum (194) is also mediated by high molecular weight LPS. Evidence supporting a role for LPS in resistance to the bactericidal action of antibody and complement will be discussed later in relation to outer membrane vesicles or "blebs".

P. gingivalis LPS is likely to interact with the host immune system in a number of other ways. Several investigators have found that P. gingivalis LPS possesses mitogenic activity and induces the production of interleukin-1 (IL-1), as well as other lymphokines (16, 43, 57, 83). IL-1 is known to enhance the immune response, stimulate

thymocyte proliferation, activate B cells, stimulate prostaglandin production, and enhance bone resorption. It has also been reported that LPS of P. gingivalis enhances natural killer (NK) cell activity (96, 97). However, it is unclear whether this enhancement occurs through direct action of LPS on NK cells or indirectly through the action of LPS-stimulated cytokine production by monocytes and macrophages. Besides its effects on leukocytes, P. gingivalis LPS has effects on other host cell types. In this regard, P. gingivalis LPS has been reported to inhibit fibroblast proliferation (89).

In general the LPS molecule can be divided into two main components; a lipid component known as Lipid A and a heteropolysaccharide component, composed of a short core polysaccharide (R core) and a repeating oligosaccharide unit (O-antigen). Chemical analysis of LPS from P. gingivalis (16, 108) indicates that the Lipid A contains a higher proportion of fatty acid (50-60% by weight) than Lipid A from enteric bacteria (<30%). The predominant fatty acids found in Lipid A from P. gingivalis are 13-methyl-tetradecanoate, 3-OH-heptadecanoate, hexadecanoate, and 12-methyl-tetradecanoate. The core heteropolysaccharide of P. gingivalis LPS also appears to be structurally different than that of LPS from enteric bacteria. Although several studies have reported that P. gingivalis LPS contains little, if any, heptose and 3-keto-2-deoxyoctonoate (KDO; 16, 65, 74, 83, 108), they may have underestimated the amount of KDO present since KDO occurs as a phosphorylated molecule in P. gingivalis LPS and requires strong acid hydrolysis for detection (16, 43, 86).

P. gingivalis LPS has been generally found to have low potency, compared to LPS from enteric bacteria, in the dermal Schwartzman reaction (43, 108), chick embryo

lethality test (108), and rabbit pyrogenicity test (121). In addition, some studies have also reported that P. gingivalis LPS is less potent than LPS from enteric bacteria in the Limulus lysate test (43, 108); however others have found comparable activity (43, 83).

Although P. gingivalis LPS has low potency in many of these biological assays, it has been found to exhibit strong activity in bone resorption assays (70, 121) and to inhibit bone collagen-formation (114). This is of particular interest since loss of alveolar bone is a significant clinical feature of periodontal disease. It has been hypothesized that P. gingivalis LPS may stimulate bone resorption through direct effects on osteoclastic cells, as well as through secondary pathways. P. gingivalis LPS has been reported to stimulate B-lymphocytes to produce osteoclast activating factor (11), to stimulate monocytes and macrophages to produce prostaglandins (44, 226), and to potentiate bone resorption in the presence of prostaglandins (148). Prostaglandins are known to stimulate bone resorption. However, Iino and Hopps (70) determined that prostaglandins did not contribute significantly to P. gingivalis LPS-mediated bone resorption in their in vitro assay, since inclusion of indomethacin (a prostaglandin synthetase-inhibitor) did not appreciably lower bone resorption.

Outer membrane vesicles

Electron-microscopic studies have shown that many, if not all, Gram-negative bacteria, release extracellular vesicles ("blebs") from their outer membranes. Several studies have reported the production of extracellular vesicles by P. gingivalis (52, 101, 227). These studies have indicated that the vesicles consist of primarily outer membrane

and possess outer membrane-associated biological activities. In general, SDS-PAGE profiles of isolated vesicles and of purified outer membranes suggest that they contain similar, but not identical, protein constituents (29, 52, 227). Grenier and Mayrand (52) observed primarily quantitative differences in SDS-PAGE protein profiles of vesicles and outer membranes, however the outer membranes appeared to contain fewer proteins. They hypothesized that this could be due to concentration of specific proteins during extracellular vesicle formation or by the action of protease(s) on vesicle-associated proteins. It is possible that limited proteolytic degradation of both outer membrane proteins and vesicle-associated proteins occurred in the study of Grenier and Mayrand (52) since Deslauriers et al. (29) observed many more protein bands in SDS-PAGE profiles of vesicles and Sarkosyl-insoluble membranes. In this regard, the SDS-PAGE profiles of outer membrane proteins reported by Deslauriers et al. (29) are more consistent with those reported by other investigators (16, 79).

Numerous investigators have suggested that vesicle formation could be an important virulence factor in periodontal pathogenesis. It has been postulated that their small size (approx. 50 nm) could enable them to cross anatomical barriers, such as the sulcular epithelium, that are usually impermeable to whole bacterial cells. Therefore vesicles could serve as a vehicle for delivery of proteases and toxins, such as LPS, into connective tissues. Once in connective tissue, vesicles could interact with immune cells to heighten the inflammatory response and exacerbate destruction of periodontal tissues.

Extracellular vesicles of P. gingivalis have been found to contain LPS (16, 29) and trypsin-like protease activity (52, 183). Vesicles of P. gingivalis may interfere with

humoral immune defenses. Grenier and Belanger (50) investigated the ability of vesicles of P. gingivalis to inhibit the bactericidal action of serum. They observed that both heat-labile and heat-stable components of P. gingivalis vesicles were capable of inhibiting the bactericidal action of serum on Capnocytophaga ochracea. The heat-labile component (assumed to be protease) produced complete inhibition at a vesicle concentration of 0.3 mg/ml, was inhibited by a thiol-blocking reagent, and enhanced by a reducing agent. The heat-stable component produced partial inhibition at 1.5 mg/ml and was concluded to be LPS, since purified P. gingivalis LPS had a similar effect on the bactericidal action of serum. These results suggest that vesicle production could contribute to compromising host immune defenses by sequestering and/or degrading complement and specific antibody directed at intact bacterial cells. This activity could protect other bacteria, as well as P. gingivalis, against host defense mechanisms, thereby contributing to periodontal pathogenesis.

Extracellular vesicles of P. gingivalis could also contribute directly to the accumulation of plaque. Vesicles of P. gingivalis have been reported to enhance attachment of P. gingivalis to saliva-coated hydroxyapatite (176), to mediate coaggregation between P. gingivalis and Actinomyces species (38), and to induce coaggregation between bacterial cells of normally non-coaggregating species, Eubacterium sabureum and Capnocytophaga ochracea (52).

Although many in vitro assays suggest possible functions for vesicles in pathogenesis, the results of studies in the mouse subcutaneous abscess model appear contradictory. McKee et al. (113) have reported that P. gingivalis cells grown in hemin-

limited conditions produced large amounts of vesicles but were avirulent, whereas cells grown in hemin-excess produced few vesicles and caused spreading infections. However, the meaning of these results is difficult to interpret, since cells grown in hemin-excess accumulate hemin. Hemin stored by cells grown in hemin-excess could be utilized for growth and to prolong the survival of cells in the hemin-limited environment encountered in the mouse virulence model. Therefore the differences in virulence reported by McKee et al. (113) may be due to differences in hemin storage rather than differences in the quantity of extracellular vesicles produced.

Protease activity

It has been hypothesized that proteases of P. gingivalis contribute to avoidance of host defense mechanisms. P. gingivalis proteases are capable of degrading most serum proteins involved in host defense. In this regard, they have been reported, in vitro, to degrade immunoglobulins, including secretory IgA, and complement components C3 and C5 (80, 129, 156, 193). In vivo, proteolytic destruction of immunoglobulins and complement components could serve to increase bacterial adherence, decrease bacterial lysis due to complement, decrease phagocytosis, and decrease antibody-mediated neutralization of bacterial toxins and enzymes.

P. gingivalis proteases may also contribute to invasiveness and tissue destruction. In this regard, P. gingivalis has been demonstrated in vitro to adhere to and degrade an intact basement membrane-like matrix (230, 231). In vitro, P. gingivalis has been found to degrade fibrinogen (88, 132), keratin (116), and several other high molecular weight

cell surface glycoproteins (209). In addition, P. gingivalis proteases may contribute indirectly to tissue destruction. P. gingivalis proteases have been reported, in vitro, to degrade human plasma protease inhibitors (17). In vivo, these protease inhibitors may contribute to host defense by neutralization of bacterial proteases, but their major function is to modulate the activity of mammalian proteases secreted by fibroblasts and PMNs. In this regard, degradation of host plasma protease inhibitors could lead to increased degradation of host tissue by "deregulated" host proteases. Fibroblasts cultured in the presence of partially purified trypsin-like protease from P. gingivalis have also been observed to secrete increased amounts of collagenase and plasminogen activator (209).

In addition, P. gingivalis has been reported to degrade serum iron transport proteins such as albumin, haptoglobin, hemopexin, and transferrin (18). Inoshita et al. (72) have reported that P. gingivalis can utilize transferrin as an iron source in "iron-free" media. Bramanti and Holt (15) also investigated the ability of P. gingivalis to utilize host iron-sequestering proteins as iron sources. They reported that hemin-saturated serum albumin, lactoperoxidase, haptoglobin-hemoglobin, hemopexin-hemin, transferrin, and lactoferrin can all serve as sources of iron for P. gingivalis. Sequestration of iron, in order to make it unavailable to bacteria, is considered to be an important aspect of the host's innate defense against bacterial infection. Therefore, the ability of bacteria to acquire iron from the host is essential for pathogenicity. In this regard, growth of E. coli and Vibrio cholerae in chambers implanted in animals is limited by the availability of iron and results in the induction of specific iron-regulated outer membrane proteins (55, 164). P. gingivalis has also been reported to modulate its outer membrane proteins when grown

in vitro under iron-restricted conditions (14). As many as 10 surface proteins were found to be induced under iron-restricted growth conditions. One of these proteins, the 26 kD protein, appears to be involved in hemin uptake by P. gingivalis (T.E. Bramanti, personal communication).

General animal pathogenicity models

A number of studies have used general animal pathogenicity models to investigate the virulence characteristics of P. gingivalis. Although these models are not periodontitis models, and therefore can not directly assess the ability of P. gingivalis strains to cause periodontal disease, they do test the ability of P. gingivalis to colonize the host, avoid immune defenses, and produce tissue damage. In 1965, Socransky and Gibbons (187) subcutaneously injected mixtures of anaerobic bacteria into guinea pigs and demonstrated that B. melaninogenicus was required for production of infection. Subsequently, subcutaneous injection of certain B. melaninogenicus strains, alone, were found to be able to induce experimental infections in rabbits (199), mice (76), and guinea pigs (76, 199). Van Steenbergen et al. (218) injected bacterial suspensions subcutaneously into the backs of mice. P. gingivalis strains produced a "spreading type of inflammation, mostly resulting in a gravity abscess or phlegmonous abscess", whereas Prevotella intermedia strains only induced a local abscess at the site of injection. In addition, differences in the degree of virulence were observed among the P. gingivalis strains studied. P. gingivalis W83 and W50 were the most virulent of the eight P. gingivalis strains tested. This model has become known as the subcutaneous abscess model and has been used in several

subsequent investigations concerning putative virulence factors of P. gingivalis (see Outer membrane vesicles and Capsular polysaccharide and phagocytosis).

Recently, Genco et al. (45, 46) have developed and employed a mouse subcutaneous chamber model to study pathogenicity of P. gingivalis. This model requires smaller inocula than the subcutaneous abscess model and supposedly allows better assessment of the local interactions between the host and bacteria during the course of infection. Using this model, Genco et al. (46) have shown that immunization with either invasive strains (A7436 and W83) or non-invasive strains (33277, HG405, and 381) resulted in the appearance of specific antibody in the subcutaneous chambers 21 days post-immunization and protected the mice against secondary abscess formation when subsequently challenged with strain A7436. However, the presence of specific antibody did not prevent colonization of the chambers by P. gingivalis.

In many cases, general animal pathogenicity models have been used to compare the virulence of different strains of P. gingivalis. These studies have attempted to correlate the variability of the level of virulence observed among various strains of P. gingivalis with specific phenotypic differences, such as protease production (53, 125). A major difficulty with these investigations is that the strains studied are likely to differ in several phenotypic characteristics. This confounds the interpretation of results and prevents absolute correlation between any specific phenotypic characteristic and virulence in the model. The use of molecular genetic techniques to isolate strains of P. gingivalis which are isogeneic, except for the engineered deficiency in a specific phenotypic characteristic, will allow more rigorous assessment of the contribution of specific factors to pathogenesis.

RESEARCH GOALS

Numerous in vitro studies suggest that proteases of P. gingivalis contribute to its pathogenicity. These studies suggest that proteases, particularly the trypsin-like protease, of P. gingivalis are involved in avoidance of host defense systems, acquisition of heme, and destruction of periodontal tissues. Strong circumstantial evidence also suggests that trypsin-like proteases are involved in periodontal pathogenesis. Of the more than 300 bacterial taxa present in the oral cavity only three (< 1%) possess strong trypsin-like protease activity. Remarkably, these three species (P. gingivalis, B. forsythus, and T. denticola) are among the limited number (10 to 12) of bacterial taxa implicated as periodontal pathogens (Table I). Thus bacterial species with trypsin-like protease activity represent approximately 30% of the bacterial taxa implicated as periodontal pathogens.

The overall aim of this investigation was to develop genetic techniques to study the role of the trypsin-like protease in the physiology and virulence of P. gingivalis. The primary specific goal was to isolate and phenotypically characterize trypsin-like protease-deficient mutants of P. gingivalis. Mutants can be generated by a number of techniques, including classical chemical mutagenesis, transposon-induced mutagenesis, and replacement of wild-type alleles by cloned genes that have been inactivated in vitro.

Initial experiments employed chemical mutagenesis to generate trypsin-like protease-deficient mutants, since transposon mutagenesis techniques and genetic transfer systems which function in P. gingivalis had not been previously reported. Although there are certain drawbacks to the use of chemical mutagenesis, the primary one being the introduction of random secondary mutations which may confound interpretation of any

phenotypic differences observed, it is a widely applicable technique and the isolation of trypsin-like protease-deficient mutants is practically assured.

In order to generate isogeneic trypsin-like protease deficient mutants, subsequent experiments were directed toward developing genetic transfer systems and transposon mutagenesis techniques for use in P. gingivalis, as well as toward cloning the trypsin-like protease gene. Attempts to develop genetic transfer systems and transposon mutagenesis techniques concentrated on the use of systems developed for colonic Bacteroides species. Colonic Bacteroides systems were chosen because P. gingivalis is phylogenetically more closely related to the colonic Bacteroides than to E. coli and no plasmid replication origins or antibiotic resistance genes which function in E. coli are known to function in colonic Bacteroides, thus they are also unlikely to function in P. gingivalis. A variety of strategies were employed to clone the trypsin-like protease gene. These included the use of E. coli and colonic Bacteroides plasmid cloning vectors, as well as PCR techniques to amplify protease gene fragment homologs.

The generation, by transposon mutagenesis or gene replacement, of isogeneic mutant strains of P. gingivalis that differ from their wild-type parent by deficiency in trypsin-like protease activity will allow rigorous assessment of the contribution of the trypsin-like protease to pathogenicity in in vivo periodontitis models in the future. The development of genetic transfer systems and transposon mutagenesis techniques for use in P. gingivalis will have wide applicability beyond the interests of this investigation.

I. Isolation and Characterization of Trypsin-Like Protease-Deficient Mutants

The following chapter describes the isolation and characterization of nitrosoguanidine-induced trypsin-like protease-deficient mutants of P. gingivalis.

Nitrosoguanidine Mutagenesis, Isolation of Mutants, and Protease Activity Assays

At the onset of this investigation no transposable elements or plasmids were known to function in P. gingivalis and no methods for introducing DNA into P. gingivalis had been reported. Therefore in our initial studies a classic chemical mutagen, N-methyl-N'-nitro-N-nitrosoguanidine, was used to generate trypsin-like protease-deficient mutants.

Methods

Nitrosoguanidine mutagenesis. P. gingivalis 3079.03 was grown anaerobically for 18 to 36 h (A_{660} = 0.8 to 1.2) in 50 ml of sTSB (Trypticase soy broth supplemented with 0.25% yeast extract, 2.5 μg/ml hemin, 5.0 μg/ml menadione, and 0.01% DTT). Portions (0.1 ml) of the undiluted bacterial broth culture and of 10-fold serial dilutions in sTSB were plated on LRBB agar (Brucella agar supplemented with 5% laked rabbit blood, 2.5 μg/ml hemin, 5.0 μg/ml menadione, and 0.01% DTT) to obtain an initial viable count and on LRBB agar containing 0.15 μg/ml rifampicin to determine the initial frequency of rifampicin resistance (an easily scored selectable phenotypic marker). The 50 ml broth

was then divided into two 25-ml portions; one portion served as a viability control, and N-methyl-N'-nitro-N-nitrosoguanidine (40 to 80 μg/ml final concentration) was added to the other portion. Both portions were incubated anaerobically at 37°C for 2 to 3 h. Another viable cell count was then obtained from each portion to determine the extent of killing due to mutagenesis, and undiluted aliquots (0.1 ml) of the mutagenized portion were plated and "grown out" on LRBB agar for 3 to 5 days to allow segregation and expression of the induced mutations. The resulting confluent bacterial growth from these plates was harvested with a cotton swab and resuspended in 1 ml sTSB, serially diluted, and plated on LRBB agar to obtain viable counts and to isolate colonies for subsequent phenotypic testing. Aliquots were also cultured on LRBB agar containing 0.15 μg/ml rifampicin to determine the nitrosoguanidine-induced increase in the frequency of mutation to rifampicin resistance. The increase in the frequency of mutation to rifampicin resistance induced by nitrosoguanidine was determined by dividing the frequency of rifampicin resistance after exposure to nitrosoguanidine by the frequency of rifampicin resistance of the unmutagenized portion of the culture.

To screen for trypsin-like protease-deficient mutants, randomly chosen individual colonies (50 to 150 colonies/mutagenesis) obtained from plating dilutions of the "grown out" mutagenized culture were patched onto sBRU agar (LRBB agar without laked rabbit blood). Following anaerobic incubation at 37°C for 5-7 days, the patched sBRU plates were overlaid with cellulose acetate sheets impregnated with the synthetic fluorogenic protease substrate N-α-carbobenzoxy-arginine-7-amino-4-trifluoromethylcoumarin (CBz-arg-AFC; Enzyme Systems Products, Dublin, CA) to detect differences in trypsin-like

protease activity. Patches of the wild-type parent strain (3079.03) of the mutants and of the type strain of P. gingivalis (ATCC 33277) were used as positive controls. Patches of the type strain of Prevotella intermedia (ATCC 25611) were used as a negative control. The overlaid patch plates were incubated at 37°C and examined for evidence of trypsin-like protease activity under long wavelength UV light at 15-min intervals for up to 4 h. Under long wavelength UV light, uncleaved substrate weakly fluoresces blue-violet and cleaved substrate fluoresces bright blue-green. Putative protease-deficient mutants were restreaked for single-colony isolation on LRBB agar and then retested for protease activity as above.

Preparation of bacterial cells for protease activity assays. All bacterial strains were grown anaerobically at 37°C in sTSB for 18 to 36 h (A_{660} = 0.8 to 1.2). Bacterial cells were collected by centrifugation (16,000 x g, 10 min, 4°C) and washed 3 times with activity buffer (5 mM $CaCl_2$, 5 mM NaCl, 5 mM $MgCl_2$, 0.01% DTT, 50 mM Tris-HCl, pH 7.5). The cells were then resuspended (100 mg wet weight/ml) in activity buffer and 1 ml aliquots were stored at -20°C until needed.

Quantitative trypsin-like protease activity assay. Trypsin-like protease activity was determined by hydrolysis of benzoyl-DL-arginine-p-nitroanilide (BAPNA). Bacterial cells (5.6 mg of the wild-type strains or 70 mg of the trypsin-like protease-deficient mutant strains) were added to 20 ml of prewarmed (10-15 min at 37°C) BAPNA assay buffer (350 μg/ml BAPNA, 1.5 mM $CaCl_2$, 100 mM Tris-HCl, pH 8.0) containing 50 mM cysteine. After 0, 2, 4, 6, 8, and 10 min of incubation a 3-ml portion was taken from the assay mixture and added to 2 ml of 20% acetic acid to stop the reaction. The A_{405} for

each sample was then determined and the 0 time value was used as a blank. One unit of trypsin-like protease activity was defined as the amount of enzyme required to release 1 μmol of p-nitroaniline per minute under the conditions described above. Specific activity was defined as units of trypsin-like protease activity per milligram wet weight of bacterial cells.

Assay of protease activities via zymograms. The proteolytic activities of the wild-type parent strain (3079.03) and of the trypsin-like protease-deficient mutants were also compared by two SDS-PAGE zymogram techniques: (i) incorporation of gelatin (biosubstrate) into polyacrylamide gels and (ii) overlaying of polyacrylamide gels with cellulose acetate membranes containing a variety of synthetic fluorogenic substrates with arginine at the P_1 site (CBz-arg-AFC, CBz-arg-arg-AFC, CBz-gly-gly-arg-AFC, and CBz-val-lys-lys-arg-AFC).

SDS-PAGE was performed using the discontinuous buffer system of Laemmli (87). For biosubstrate polyacrylamide gel electrophoresis, gelatin (1 mg/ml) was incorporated into the separating gel (7.5% polyacrylamide) during polymerization. The stacking gel contained 6% polyacrylamide without added gelatin. Washed bacterial cells (12.5 mg wet weight) were extracted at room temperature for 30 min in 1 ml SDS-PAGE sample buffer (2% SDS, 20% glycerol, 0.1% bromophenol blue, 0.0625 M Tris-HCl, pH 6.8). Aliquots of the samples were loaded into the gel wells and constant current (30 mA) was applied until the bromophenol blue dye front reached the bottom of the separating gel (4 to 6 h). After electrophoresis, SDS was removed from the gels by sequential washing (three washes, 150 ml, 30 min each) in renaturation buffer (2.5%

Triton X-100, 0.01% DTT, 50 mM Tris-HCl, pH 7.5). After the third wash, the gels were placed in fresh activity buffer and incubated overnight at 37° C in gassed (85% N_2, 10% H_2, 5% CO_2) and sealed plastic bags. The gels were then stained for 36-48 h with Coomassie blue. After destaining, proteolytic activity against gelatin was apparent as clear bands against a uniformly stained blue background. A similar method was used for SDS-PAGE zymograms with synthetic fluorogenic protease substrates, except that no substrate (gelatin) was incorporated into the gels. After the third wash in renaturation buffer these gels were washed two times in activity buffer (150 ml, 15 min, room temperature), placed on glass plates, and overlaid with cellulose acetate membranes containing the specific synthetic fluorogenic protease substrates. The overlaid gels were then covered with plastic wrap, incubated at 37°C, and examined periodically (for up to 4 h) with long wavelength UV light for the appearance of bright blue-green fluorescent bands (protease activity).

Results

Characterization of nitrosoguanidine mutagenesis procedure and phenotypic characterization of trypsin-like protease-deficient mutants. The nitrosoguanidine-mutagenesis procedure reduced the viable count by approximately 92% (from $1.9 \times 10^9 \pm 8.3 \times 10^8$ to $1.6 \times 10^8 \pm 1.4 \times 10^8$) and increased the frequency of rifampicin resistance (an easily scored mutation) by 1,300-fold ($2.4 \times 10^{-8} \pm 2.2 \times 10^{-8}$ to $3.2 \times 10^{-5} \pm 2.7 \times 10^{-5}$). These results, from ten independent experiments, which estimate the extent of mutagenesis are comparable to those obtained in numerous other reports which have used

nitrosoguanidine mutagenesis with other bacterial species (for review see 48). The frequency of trypsin-like protease-deficient mutants was 1.5% (21/1,423). All twenty-one trypsin-like protease-deficient mutants exhibited visibly reduced black pigmentation. Four independent nitrosoguanidine-induced trypsin-like protease-deficient mutants were randomly chosen for further phenotypic testing. These four trypsin-like protease-deficient mutants produced positive results, identical to their wild-type parent (3079.03), for 11 of 14 phenotypic tests (Table IV). In addition to deficiency in trypsin-like protease activity and reduced black colonial pigmentation, the four nitrosoguanidine-induced mutants also exhibited reduced hemagglutination activity. The association between trypsin-like protease activity and hemagglutination activity is investigated in a later section of this chapter. Based on the observation that all twenty-one nitrosoguanidine-induced trypsin-like protease-deficient mutants exhibited reduced black colonial pigmentation, a spontaneous mutant (SW3A) with reduced black colonial pigmentation was isolated, by inspection, from an unmutagenized culture of strain 3079.03. This spontaneous mutant also exhibited reduced trypsin-like protease activity and reduced hemagglutination activity. Subsequent experiments indicated that similar spontaneous reduced black colony pigment mutants occurred with strain 3079.03 at a frequency of 1.3×10^{-3} (53/39,471). Attempts to obtain revertants from strain SW3A have been unsuccessful (0/184,000; reversion frequency $<5.4 \times 10^{-6}$).

Quantitative assay of trypsin-like protease activity. The trypsin-like protease activity of the type strain of P. gingivalis (33277), the parent strain of the mutants (3079.03), and four nitrosoguanidine-induced trypsin-like protease-deficient mutants are

Table IV. Phenotypic characterization of P. gingivalis 3079.03 and nitrosoguanidine-induced trypsin-like protease-deficient mutants.

	3079.03	NG4B19	NG5B2	NG19A18	NG22B8
Trypsin-like protease[d]	+[a]	w[b]	w	w	w
Black pigmentation	+	-[c]	-	w	w
Hemagglutination	+	-	-	w	w
Gelatinase[e]	+	+	w	+	+
Indole production[e,f]	+	+	+	+	+
Leucylglycylaminopeptidase[f]	+	+	+	+	+
TTZ[g] reduction[f]	+	+	+	+	+
Pyrrolidonylaminidase[f]	+	+	+	+	+
Alkaline phosphatase[d]	+	+	+	+	+
Acid phosphatase[d]	+	+	+	+	+
Phosphohydrolase[d]	+	+	+	+	+
Esterase[d]	+	+	+	+	+
Esterase lipase[d]	w	w	w	w	w
N-acetyl-β-glucosaminidase[d,f]	+	+	+	+	+

[a] Moderate to strong positive reaction
[b] Weak or delayed reaction
[c] Negative reaction
[d] Determined by API ZYM kit (Analytab Products, Plainview, NY)
[e] Determined by API 20A kit (Analytab Products, Plainview, NY)
[f] Determined by RapID ANA kit (Innovative Diagnostic Systems, Atlanta, GA)
[g] Triphenyltetrazolium

compared in Table V. The type strain of P. gingivalis (33277) and the parent strain (3079.03) of the trypsin-like protease-deficient mutants exhibited similar amounts of trypsin-like protease activity (specific activities of 24.9 $\pm$ 5.2 and 28.0 $\pm$ 4.5 units/mg cells, respectively). All four nitrosoguanidine-induced trypsin-like protease-deficient mutant strains exhibited less than 2% of the trypsin-like protease activity (< 0.52 $\pm$ 0.07 specific activity) of the parent strain.

Comparison of proteolytic activities via zymograms. In SDS-polyacrylamide gels containing 1 mg/ml gelatin and stained with Coomassie blue, numerous (10 to 12) discrete clear (negatively stained) bands, indicative of protease activity, were apparent with the parent strain (Fig. 1a). The position of proteolytic bands in the SDS-PAGE gels ranged from apparent molecular weights of 30,000 kD to greater than 150,000 kD when compared with the relative mobility of protein molecular weight standards. The majority (8 to 10) of the proteolytic bands were closely and evenly spaced in the 50,000 kD to 75,000 kD region of the gel. The four independent nitrosoguanidine-induced trypsin-like protease-deficient mutant strains exhibited greatly reduced proteolytic activity against gelatin compared to the parent strain (Fig. 1a). The proteolytic bands between 50,000 kD and 75,000 kD were barely visible and only one or two of the proteolytic bands in the low molecular weight (< 45,000 kD) region of the gels were observed with the mutant strains.

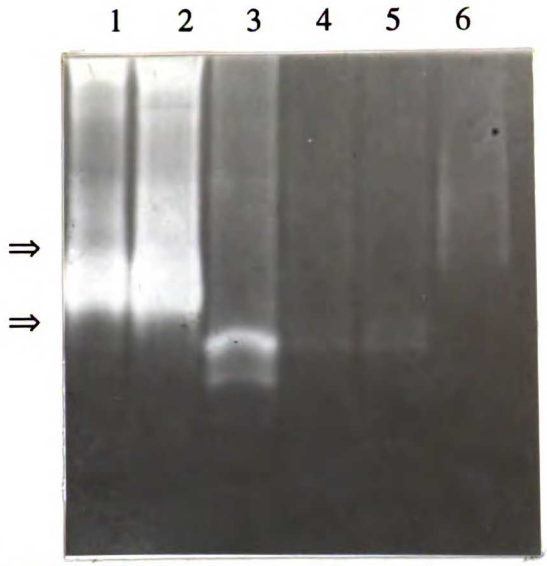
To determine whether all of the proteolytic bands in the gelatin-containing gels represented trypsin-like protease activity, gels without gelatin were overlaid with cellulose acetate membranes containing a variety of synthetic fluorogenic protease substrates with

Table V. Trypsin-like protease activity of wild-type and nitrosoguanidine-induced trypsin-like protease-deficient mutants of P. gingivalis.

Strain	Trypsin-like protease activity[a]	% activity of strain 3079.03
33277	24.9 ± 5.2	
3079.03	28.0 ± 4.5	100
NG4B19[b]	0.20 ± 0.05	0.7
NG5B2[b]	0.11 ± 0.02	0.4
NG19A18[b]	0.52 ± 0.07	1.9
NG22B8[b]	0.16 ± 0.01	0.6

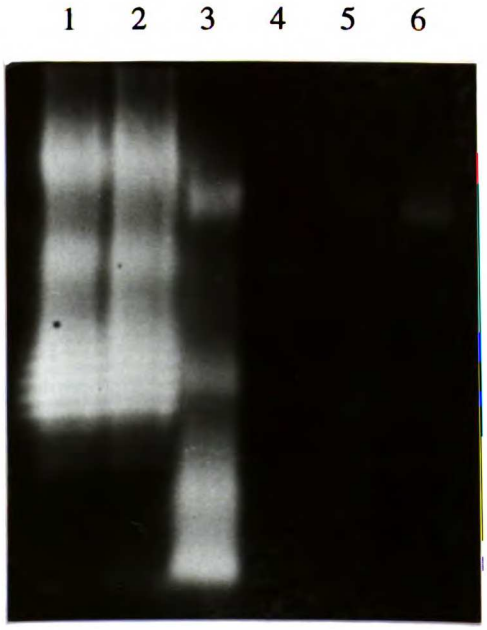
[a] Specific activity was defined as units of trypsin-like protease activity per milligram wet weight of bacterial cells. One unit of trypsin-like protease activity was defined as the amount of enzyme required to release 1 μmol of p-nitroaniline per minute as described in Methods. Mean ± standard deviation of at least three independent experiments ($n \geq 3$).

[b] Trypsin-like protease-deficient mutant isolated from strain 3079.03 following nitrosoguanidine mutagenesis.



Lane 1 - 300 µg of 3079.03
Lane 2 - 625 µg of 3079.03
Lane 3 - 300 µg of NG4B19
Lane 4 - 300 µg of NG5B2
Lane 5 - 300 µg of NG19A18
Lane 6 - 300 µg of NG22B8

(A) SDS-PAGE zymogram with gelatin.



Lane 1 - 625 µg of 3079.03
Lane 2 - 1 mg of 3079.03
Lane 3 - 1 mg of NG4B19
Lane 4 - 1 mg of NG5B2
Lane 5 - 1 mg of NG19A18
Lane 6 - 1 mg of NG22B8

(B) SDS-PAGE zymogram with CBz-gly-gly-arg-AFC.

Figure 1. Zymograms of wild-type and nitrosoguanidine-induced trypsin-like protease-deficient mutants of P. gingivalis[a].

[a] Arrows indicate the region of the gel that corresponds to protein molecular weights of 50,000 to 75,000.

arginine at the P_1 site (CBz-arg-AFC, CBz-arg-arg-AFC, CBz-gly-gly-arg-AFC, and CBz-val-lys-lys-arg-AFC). The parent strain and the mutant strains produced banding patterns with all of these synthetic fluorogenic substrates that were nearly identical to those observed with gelatin, except that the absence of gelatin resulted in increased mobility and greater separation of the proteolytic bands (Fig. 1b; data shown for CBz-gly-gly-arg-AFC only). The appearance of additional proteolytic bands in the low and high molecular weight regions of the overlaid gels may be due to increased sample loading and greater sensitivity of the fluorogenic substrates. The CBz-gly-gly-arg-AFC and CBz- val-lys-lys-arg-AFC membranes appeared to be the most sensitive indicator(s) of trypsin-like protease activity in that they exhibited bright blue-green fluorescence more quickly than membranes containing the other substrates.

Discussion

As an initial approach to investigate the function of the trypsin-like protease in the physiology and virulence of P. gingivalis, a nitrosoguanidine mutagenesis procedure and a trypsin-like protease activity screening technique were developed to isolate trypsin-like protease-deficient mutants. Quantitative trypsin-like protease activity assays with BAPNA indicated that the mutants possessed less than 2% of the trypsin-like protease activity of the parent strain. Zymogram techniques (SDS-PAGE gels containing gelatin and SDS-PAGE gels overlaid with specific synthetic fluorogenic protease-substrates) were also used to compare the proteolytic activity of the mutants with that of the parent strain. The parent strain produced a complex pattern consisting of multiple (> 10) proteolytic bands

with both gelatin-containing SDS-PAGE gels and with fluorogenic protease-substrate overlays with arginine in the P_1 site. Since the samples for zymograms cannot be boiled in SDS-PAGE loading buffer and since the complex protease banding pattern corresponds to the ladder-like banding pattern of lipopolysaccharide (14, 16), it is hypothesized that the complex protease banding pattern represents non-covalent association of the trypsin-like protease with lipopolysaccharide and/or other outer membrane components.

SDS-PAGE zymograms with the nitrosoguanidine-induced trypsin-like protease-deficient mutants exhibited considerably less total proteolytic activity than with the parent strain. In all four mutants negligible activity remained in the ladder-like region (50,000 kD to 75,000 kD) of the gel. The mutant strains exhibited two proteolytic bands, in the low molecular weight region (< 45,000) of the gel, that were more pronounced than those observed in this same region with the parent strain (Fig. 1a & 1b). These low molecular weight bands in the mutant strains could reflect reduced association between the trypsin-like protease and lipopolysaccharide, degradation products of the protease which retain activity, or overproduction of other proteases to compensate for the deficiency in the trypsin-like protease.

Other investigators have also used zymogram techniques to study proteolytic activity of P. gingivalis. Grenier et al. (51) reported the presence of eight proteolytic bands with SDS-PAGE gels containing covalently-linked bovine serum albumin. Similar results were obtained with IgA, IgG, α_1-antitrypsin, transferrin, and fibrinogen as substrates. Based on differential responses of the proteolytic activities in different bands to reducing agents and protease inhibitors, Grenier et al. (51) suggest that the eight

proteolytic bands are the result of at least three different proteases. However, they also state that "the possibility that the different zones of hydrolysis are the result of autodigestion products ... can not be ruled out by the experiments described".

Several observations in the present investigation suggest that a single protease may be responsible for most, if not all, the proteolytic activity observed with our zymograms. Practically all of the proteolytic bands (noted in zymograms with the parent strain) were completely absent or exhibited considerably less activity in zymograms with the trypsin-like protease-deficient mutant strains. All of the proteolytic bands, observed with gelatin, with the parent strain appear to exhibit activity with synthetic fluorogenic protease substrates with arginine in the P_1 position, indicating that they share the same substrate specificity and therefore could result from the action of a single protease. In addition, synthetic fluorogenic substrates for collagenase-like protease(s) (CBz-gly-pro-leu-gly-pro-AFC) and glycylprolyl-dipeptidylaminopeptidase(s) (gly-pro-AFC) failed to demonstrate these activities in the zymograms, although these substrates did produce positive reactions with overlays of bacterial colonies of the parent and mutant strains. Although at present it is unclear how many different proteases produced by P. gingivalis hydrolyze substrates with arginine at the P_1 active site, the results of the present study suggest that the majority of protease activity detected with BAPNA and most of the proteolytic activity detected with the zymogram techniques results from the action of a single trypsin-like protease.

Since nitrosoguanidine, like other chemical mutagens, produces fairly random mutations, it is likely that the nitrosoguanidine-induced trypsin-like protease-deficient mutants contain unknown secondary mutations. This is of particular concern since these

trypsin-like protease-deficient mutants exhibit a pleiotropic phenotype. The pleiotropic phenotype of the nitrosoguanidine-induced trypsin-like protease-deficient mutants and other concerns regarding nitrosoguanidine mutagenesis are addressed more completely in the following section.

Trypsin-like Protease Activity and Hemagglutination

Adherence is a prerequisite for successful colonization and initiation of disease by bacteria in the oral cavitity. The bacterial adhesin(s) responsible for adherence of P. gingivalis to oral mucosal cells, erythrocytes, and Gram-positive bacteria have not been definitively identified. Unlike bacterial lectins, the hemagglutinin(s) of P. gingivalis are not inhibited by sugars but are inhibited by L-arginine and L-arginine-containing peptides (71, 137). Numerous potential virulence factors have been attributed to P. gingivalis, including the production of a variety of proteases. Recently, L-arginine, some L-arginine-related compounds, and a few irreversible protease inhibitors have been reported to qualitatively reduce both trypsin-like protease activity and hemagglutination activity of total membrane preparations from P. gingivalis (131). These observations suggest that the trypsin-like protease of P. gingivalis may directly mediate hemagglutination. To investigate this hypothesis we employed both a genetic and a biochemical approach. Quantitative assays were used to compare the trypsin-like protease activity and hemagglutination activity of a battery of trypsin-like protease-deficient mutants of P. gingivalis and to compare the effect of several protease enhancers and inhibitors on trypsin-like protease activity and hemagglutination activity of wild type P. gingivalis cells.

Methods

The bacterial strains, growth conditions, preparation for storage, and the BAPNA assay for trypsin-like protease activity have been previously described. Hemagglutination activity was assayed in conical-bottom microtiter plates (Flow Laboratories, Hamden, CT). Sheep red blood cells (SRBC) were washed 3 times by centrifugation (7,500 x g, 15 min, 4°C) with phosphate-buffered saline (PBS: 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3) and resuspended to a final concentration of 0.2%. Washed suspensions of P. gingivalis cells were adjusted to an A_{660} of 1.0 in PBS. Homogenous suspensions of intact bacterial cells were obtained with each strain and no autoagglutination of bacterial strains was observed. Portions of the adjusted cell suspensions (50 µl) were then serially diluted (1/2 to 1/2048) with PBS in microtiter plates. Where appropriate, enhancers and inhibitors of trypsin-like protease activity were added (at the concentrations indicated in Table VII and Fig. 3) to the PBS used to prepare the initial bacterial cell suspensions and to the PBS used to prepare the twofold serial dilutions. After serial dilution of the bacterial cell suspensions, 50 µl of the 0.2% SRBC suspension was added to each microtiter well. The hemagglutination assay plates were then incubated at room temperature for 16 to 24 h. The hemagglutination titer was defined as the reciprocal of the highest dilution exhibiting positive hemagglutination.

Results

Quantitative assay of trypsin-like protease activity and hemagglutination activity.

The trypsin-like protease activity and hemagglutination activity of two wild-type strains, four nitrosoguanidine-induced trypsin-like protease-deficient mutants, and a spontaneous trypsin-like protease-deficient mutant of P. gingivalis are compared in Table VI. The mutants possess less than 2% of the trypsin-like protease activity of wild-type strains and exhibit a reduction in mean $\log_2$ reciprocal hemagglutination titer of greater than eight logs (> 256-fold).

Effect of protease enhancers and inhibitors on trypsin-like protease activity and hemagglutination activity. The effects of the protease enhancers and inhibitors on trypsin-like protease activity and hemagglutination activity of wild-type strains of P. gingivalis are summarized in Table VII. Enhancers of trypsin-like protease activity, DTT and cysteine, significantly increased both trypsin-like protease activity and hemagglutination activity of wild-type P. gingivalis strains ($P < 0.05$, paired t-test), whereas protease inhibitors (NEM, TLCK, and PMSF) significantly reduced both trypsin-like protease activity and hemagglutination activity ($P < 0.05$, paired t-test). The protease enhancers and inhibitors, however, did not significantly change the hemagglutination activity of a control, F. nucleatum strain FN2 (Table VII). Statistical analysis of the results (data from Tables VI and VII) indicates a strong correlation ($r = 0.85$, $P < 0.001$, Spearman rank correlation) between hemagglutination activity and trypsin-like protease activity of P. gingivalis (Fig. 2). The effects of varying the concentration of one of the protease enhancers (cysteine) and one of the protease inhibitors (NEM) on trypsin-like protease activity and hemagglutination activity were also determined (Fig. 3). Varying the concentration of cysteine from 0 to 50 mM produced a concordant increase in both

TABLE VI. Trypsin-like protease activity and hemagglutination activity (HA) of wild-type and trypsin-like protease-deficient mutants of P. gingivalis.

Strain	Trypsin-like protease activity[a]	Mean $\log_2$ reciprocal HA titer
33277	24.9 ± 5.2	9.0
3079.03	28.0 ± 4.5	9.5
SW3A[b]	0.03 ± 0.01	0
NG4B19[c]	0.20 ± 0.05	0
NG5B2[c]	0.11 ± 0.02	0
NG19A18[c]	0.52 ± 0.07	1.5
NG22B8[c]	0.16 ± 0.01	0.7

[a] Specific activity was defined as units of trypsin-like protease activity per milligram wet weight of bacterial cells. One unit of trypsin-like protease activity was defined as the amount of enzyme required to release 1 μmol of p-nitroaniline per minute as described in Materials and Methods. Mean ± standard deviation of at least three independent experiments ($n \geq 3$).

[b] Spontaneous trypsin-like protease-deficient mutant isolated from strain 3079.03.

[c] Trypsin-like protease-deficient mutant isolated from strain 3079.03 following nitrosoguanidine mutagenesis.

TABLE VII. Effect of enhancers and inhibitors on trypsin-like protease activity and hemagglutination activity (HA) of wild-type strains.

Strain & assay condition	Trypsin-like protease activity	Mean $\log_2$ reciprocal HA titer
P. gingivalis 33277		
Buffer alone	8.2 ± 3.8	7.0 ± 0.8
0.5% dithiothreitol	15.4 ± 2.2[a]	8.2 ± 0.5[b]
50 mM cysteine	24.9 ± 5.2[b]	9.0 ± 0[b]
10 mM NEM	0.1 ± 0.05[b]	3.0 ± 0[b]
1 mM TLCK	0.1 ± 0.1[b]	3.2 ± 1.5[a]
2 mM PMSF	3.6 ± 1.6[b]	5.8 ± 1.3[b]
P. gingivalis 3079.03		
Buffer alone	7.9 ± 3.4	7.0 ± 0.7
0.5% dithiothreitol	13.2 ± 1.7[a]	8.2 ± 0.8[b]
50 mM cysteine	28.0 ± 4.5[a]	9.5 ± 0.6[a]
10 mM NEM	0.9 ± 0.5[b]	3.0 ± 1.2[a]
1 mM TLCK	0.2 ± 0.1[a]	3.0 ± 1.4[a]
2 mM PMSF	3.7 ± 1.9[a]	5.6 ± 1.4[b]
F. nucleatum FN2		
Buffer alone	not done	7.0 ± 0
0.5% dithiothreitol	not done	7.0 ± 0
50 mM cysteine	not done	6.7 ± 0.6
10 mM NEM	not done	7.0 ± 0
1 mM TLCK	not done	7.0 ± 1
2 mM PMSF	not done	6.3 ± 0.6

[a] $P < 0.01$ versus buffer alone (paired t-test).

[b] $P < 0.05$ versus buffer alone (paired t-test).

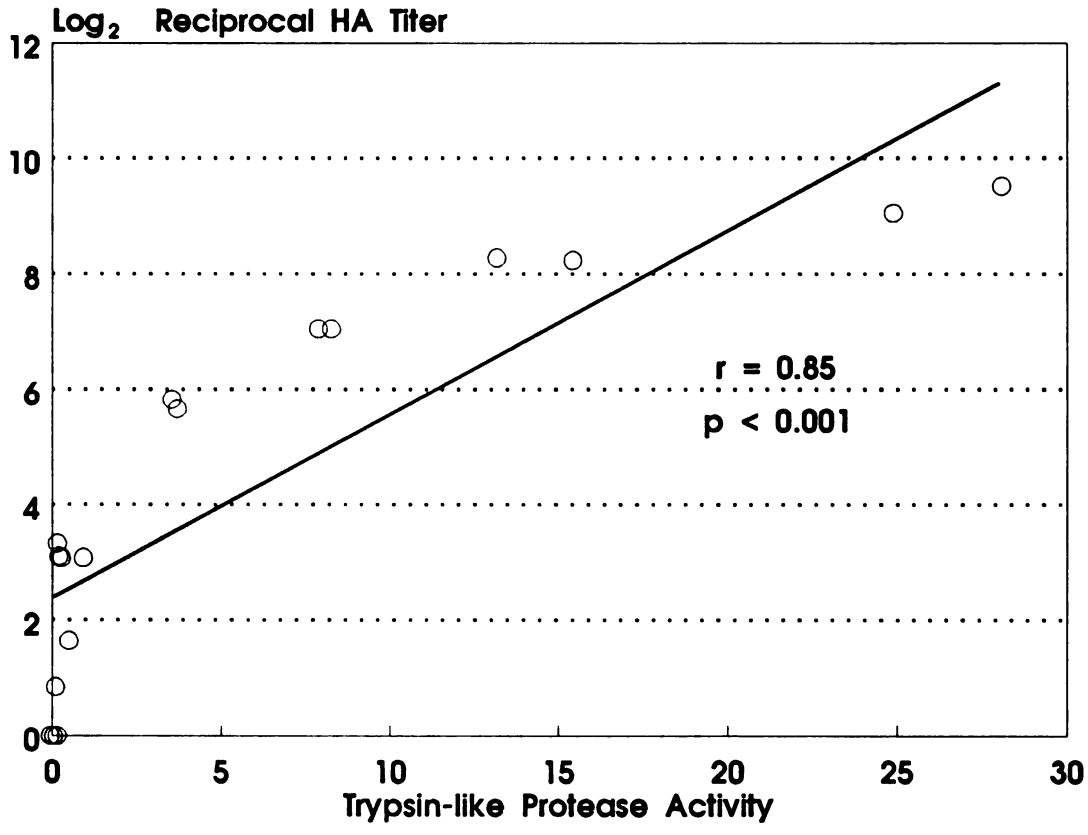


Figure 2. Relationship between trypsin-like protease activity and hemagglutination activity[a].

[a] Data from Tables VI and VII evaluated by Spearman rank correlation and regression line plotted (solid line).

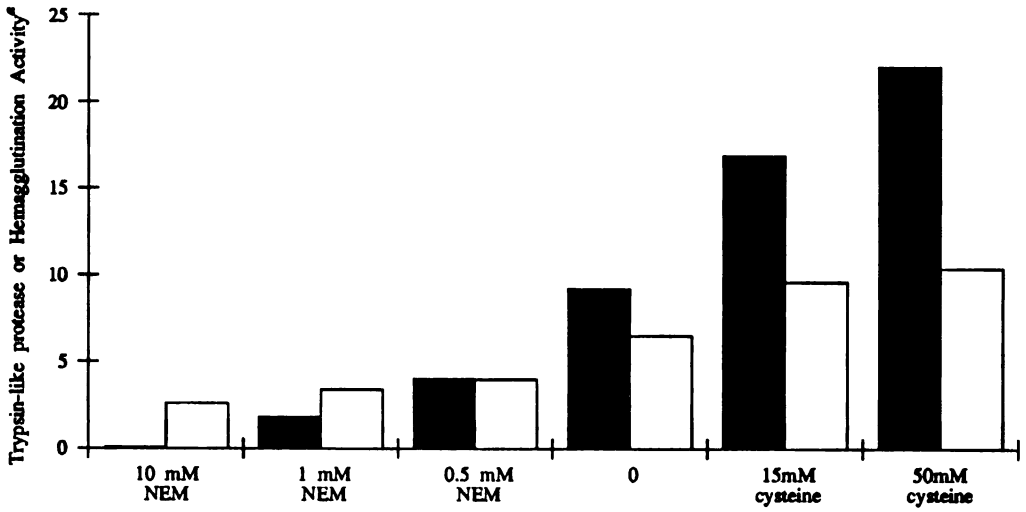


Figure 3. Effect of varying the concentration of NEM and cysteine on trypsin-like protease activity and hemagglutination activity of wild-type P. gingivalis cells.

[a] Trypsin-like protease activity ■; hemagglutination activity □

trypsin-like protease activity and in hemagglutination activity; whereas, varying the concentration of NEM from 0 to 10 mM produced a concordant decrease in both trypsin-like protease activity and hemagglutination activity.

Discussion

Okuda and Takazoe (136) were the first to report that some strains of black-pigmented Bacteroides isolated from the oral cavity possessed hemagglutinating activity. They observed the presence of fimbria-like structures (pili) on hemagglutinating strains and suggested that fimbriae mediated hemagglutination. Other early reports (135, 181) also suggested that fimbriae may be responsible for hemagglutination since partially purified preparations of fimbriae possessed strong hemagglutinating activity. More recent reports have presented conflicting evidence. Highly purified preparations of fimbriae do not exhibit hemagglutinating activity (119, 236) or competitively inhibit binding of specific polyclonal anti-hemagglutinin antibodies to cell-surface antigens of P. gingivalis (119).

Presently it is not known whether P. gingivalis possesses one or more than one hemagglutinin. Progulske-Fox et al. (147) have cloned two different DNA sequences from P. gingivalis that express hemagglutination activity in E coli. In addition, Inoshita et al. (71) have reported the presence of three major proteins in affinity-purified hemagglutinin preparations, and Mouton et al. (119) have detected two major protein antigens in immunoblots of cell-surface extracts of P. gingivalis with monospecific polyclonal and monoclonal anti-hemagglutinin antisera.

In this study five independent trypsin-like protease-deficient mutants were shown to exhibit concomitant reduction in hemagglutination activity. In addition, enhancers and inhibitors of trypsin-like protease activity were shown, respectively, to significantly enhance and inhibit hemagglutination activity of wild-type P. gingivalis cells. Statistical analysis indicated a strong correlation between levels of hemagglutination activity and trypsin-like protease activity in all the situations examined. These results suggest that the trypsin-like protease may be the major hemagglutinin of P. gingivalis and that the proteolytic active site of the trypsin-like protease enzyme is directly involved in hemagglutination. However, it is possible that the trypsin-like protease could indirectly participate in hemagglutination by modifying erythrocyte (and/or P. gingivalis) surface proteins to expose cryptic adherence epitopes. In this regard, it has been reported that mild trypsin treatment of buccal epithelial cells enhances binding of P. gingivalis (19, 20).

Both a genetic and a biochemical approach were used to establish a strong correlation between the levels of trypsin-like protease activity and hemagglutination activity of P. gingivalis. The combination of these two approaches provided complementary results. Four of the five trypsin-like protease-deficient mutants used in this study were induced by nitrosoguanidine mutagenesis. Since nitrosoguanidine, like other chemical mutagens, produces random mutations, it is likely that the trypsin-like protease-deficient mutants contain unknown secondary mutations. The isolation and testing of several independent trypsin-like protease-deficient mutants greatly decreases the likelihood that any consistently observed phenotypic changes (e.g. reduced hemagglutination activity) are due to unknown secondary mutations induced by

nitrosoguanidine mutagenesis. In addition, biochemical testing of these mutants established that secondary mutations did not occur in 11 other phenotypic characteristics. Furthermore, spontaneous mutants of strain 3079.03 deficient in both trypsin-like protease activity and hemagglutination activity occur at a high frequency (1.3×10^{-3}). This suggests that both of these phenotypes are due to a single mutation rather than multiple mutations. Shah et al. (169) have also isolated similar spontaneous pleiotropic mutants from prolonged chemostat cultures of P. gingivalis W50, although they did not propose an association between trypsin-like protease activity and hemagglutination activity.

Another inherent difficulty with the genetic approach is that our mutants could occur by mutation in a gene that co-regulates expression of genes for trypsin-like protease activity and hemagglutination activity rather than by mutation in a single structural gene that encodes both activities. However, the observation that protease enhancers and inhibitors, respectively, enhance and inhibit hemagglutination activity strongly supports the concept of a bifunctional protein rather than a global regulatory gene hypothesis. In addition, Nishikata and Yoshimura (130) have recently purified, from membrane fractions of P. gingivalis, a hemagglutinin that possesses protease activity.

The presence of a bifunctional protein with protease activity and hemagglutinating activity would not be unique to P. gingivalis. Two other mucosal pathogens, Vibrio cholerae and Pseudomonas aeruginosa, have been reported to possess metalloproteases with hemagglutinating activity (61).

Trypsin-like Protease Activity and Adherence to Actinomyces viscosus

In addition to erythrocytes, P. gingivalis adheres to a variety of Gram-positive bacteria (181). Gram-positive bacteria, like A. viscosus, that rapidly adhere to cleaned tooth surfaces promote accumulation of P. gingivalis on tooth surfaces in vivo and on saliva-coated hydroxyapatite in vitro. In a collaborative study (95), with colleagues at the University of Toronto, we investigated the association between trypsin-like protease activity and adherence of P. gingivalis to A. viscosus. The relative adherence of wild-type and nitrosoguanidine-induced trypsin-like protease-deficient mutants of P. gingivalis to A. viscosus was compared. In addition, we determined the effect of inhibitors and enhancers of trypsin-like protease activity on the relative adherence of wild-type P. gingivalis to A. viscosus.

Methods

Bacterial strains and culture conditions. The P. gingivalis strains used and conditions for their growth have been described in previous sections. A. viscosus WVU627 (originally obtained from Dr. M.A. Gerencser) was maintained on brain heart infusion agar slants (Difco Laboratories, Detroit, MI) stored at 4°C. A. viscosus was cultured for 48 h in a chemically defined medium as described by Schwarz et al. (163) for use in binding studies.

Porphyromonas-Actinomyces binding assay. A binding assay similar to that of Li and Ellen (94) was used to compare the binding of wild-type and trypsin-like protease-deficient mutants of P. gingivalis to A. viscosus. In brief, A. viscosus cells were

adsorbed to hydroxyapatite beads that had been coated with clarified whole human saliva. P. gingivalis cells of various strains were radiolabeled, in growth medium, with 10 μCi/ml (methyl-^{3}H)-thymidine. Radiolabeled P. gingivalis cells were harvested and washed by centrifugation. Washed cell suspensions were spectrophotometrically adjusted to contain 2.0 x 10^7 cells/ml. These adjusted cell suspensions of P. gingivalis were incubated with the A. viscosus-saliva-coated-hydroxyapatite beads ("actinobeads") for 60 min. The actinobeads were then washed with buffer to remove unbound and loosely-attached P. gingivalis cells. The percent of P. gingivalis cells bound was determined by liquid scintillation counting. As an internal control to account for day-to-day experimental variation, P. gingivalis 33277 was included in every experiment. The percent adherence of specific strains was then determined relative to that of P. gingivalis 33277.

Effect of temperature on adherence of P. gingivalis to actinobeads. Washed cell suspensions of radiolabeled P. gingivalis 33277 cells were preincubated for 60 min at 4°C, 22°C, or 37°C. The cell suspensions were then incubated for 60 min with actinobeads, at the corresponding temperature. In addition, washed cell suspensions of radiolabeled P. gingivalis cells were preincubated at 70°C for 45 min, and then incubated with actinobeads at 22°C for 60 min.

Effect of inhibitors and enhancers of trypsin-like protease activity on adherence to actinobeads. The effect of protease inhibitors (PMSF, TLCK, p-chloromercuriphenyl sulfonate {CMPS}, TPCK, NEM, and BZMD) and protease enhancers (β-mercaptoethanol, cysteine, and DTT) on adherence of P. gingivalis to actinobeads was determined relative to phosphate buffered saline (PBS). Washed radiolabeled P.

gingivalis 33277 cells were preincubated at 22°C for 60 min with each test compound at a concentration of 10mM. The P. gingivalis cells were then incubated with actinobeads at 22°C for 60 min.

Results

Adherence of trypsin-like protease-deficient mutants to actinobeads. The relative adherences of two independent nitrosoguanidine-induced trypsin-like protease-deficient mutants of P. gingivalis are compared with the relative adherence of the wild-type parent strain (3079.03) in Table VIII. The two trypsin-like protease-deficient mutants studied exhibited only 15% (NG4B19) and 4% (NG5B2) of the adherence to actinobeads found with their parent strain (3079.03).

Effect of temperature on adherence of P. gingivalis to actinobeads. The adherence of wild-type P. gingivalis 33277 to actinobeads was significantly influenced by temperature (Table IX). Relative to room temperature (22°C), the adherence of P. gingivalis increased by more than 75% at 37°C, but decreased by approximately 60% at 4°C. Pretreatment of P. gingivalis at 70°C for 45 minutes reduced the relative adherence to actinobeads by more than 80%.

Effect of inhibitors and enhancers of trypsin-like protease activity on adherence to actinobeads. As summarized in Table X, several protease inhibitors significantly reduced adherence of P. gingivalis 33277 to actinobeads. PMSF, TLCK, CMPS, and TPCK all reduced relative adherence by more than 30%, whereas NEM and benzamidine did not significantly reduce relative adherence of P. gingivalis to A. viscosus. Enhancers

Table VIII. Relative adherence of wild-type and trypsin-like protease-deficient mutants of P. gingivalis[a] to actinobeads.

Strain	% adherence relative to strain 33277	% adherence relative to strain 3079.03
33277[b]	100	
3079.03[c]	75.6 ± 7.2	100
NG4B19[d]	11.2 ± 0.4	14.8
NG5B2[d]	3.2 ± 0.1	4.2

[a] Adherence was expressed as percent of cells bound to 10 mg actinobeads at an input concentration of 2.0 x 10^7 cells/ml. Data expressed as the mean relative adherence ± standard error.

[b] Type strain of P. gingivalis.

[c] Parent strain of trypsin-like protease-deficient mutants.

[d] Nitrosoguanidine-induced trypsin-like protease-deficient mutants.

Table IX. Effect of temperature on adherence of P. gingivalis 33277 to actinobeads.

Temperature	% Relative adherence[a]
22°C	100
37°C	176 ± 21[b]
4°C	42 ± 6[b]
70°C	19 ± 4[b]

[a] Data are expressed as mean percent relative adherence ± standard error.

[b] Groups statistically different ($p < 0.05$) from control (22°C). Duncan's multiple range test was used to test differences between specific groups.

Table X. Effect of protease inhibitors[a] on adherence of P. gingivalis 33277 to actinobeads.

Test compound	% Relative adherence[b]
Phosphate buffered saline (PBS)	100
Phenylmethylsulfonyl fluoride (PMSF)	69 ± 8[c]
Tosyl-L-lysine chloromethyl ketone (TLCK)	66 ± 5[c]
p-chloromercuriphenyl sulfonate (CMPS)	56 ± 13[c]
Tosyl-L-phenylalanine chloromethyl ketone (TPCK)	49 ± 15[c]
N-ethylmaleimide (NEM)	98 ± 9
Benzamidine (BZMD)	89 ± 1.5

[a] All inhibitors used at a concentration of 10 mM.

[b] Data are expressed as mean percent relative adherence ± standard error.

[c] Groups statistically different ($p < 0.05$) from control (22°C). Duncan's multiple range test was used to test differences between specific groups.

of trypsin-like protease activity (reducing agents) significantly increased the relative adherence of P. gingivalis 33277 to actinobeads (Table XI). β-mercaptoethanol, cysteine, and DTT all increased the relative adherence to actinobeads by more than 50%.

Discussion

All the experimental results obtained in this study suggest that the trypsin-like protease is an important factor in adherence of P. gingivalis to A. viscosus. With the actinobead binding assay, two trypsin-like protease-deficient mutants of P. gingivalis exhibited relative adherence that would place them among the least adherent Porphyromonas and Bacteroides strains of any species previously tested (94). In contrast, the relative adherence of the parent strain of the mutants (3079.03) was comparable to that of other P. gingivalis strains, including the type strain of P. gingivalis (33277). The sensitivity to temperature also suggests that adherence of P. gingivalis to A. viscosus may be dependent on enzymatic action of the trypsin-like protease. In addition, inhibitors and enhancers of trypsin-like protease activity, respectively, reduce and increase adherence of P. gingivalis to A. viscosus.

These findings, obtained with three different experimental approaches, are consistent with the hypothesis that the trypsin-like protease of P. gingivalis participates in adherence of P. gingivalis to A. viscosus. However, further investigation is needed to determine the exact mechanism by which the trypsin-like protease facilitates adherence to A. viscosus. The proteolytic active site of the trypsin-like protease may modify or unmask hidden ligands on adhesin or receptor molecules and/or function in a specific

Table XI. Effect of protease enhancers[a] on adherence of P. gingivalis 33277 to actinobeads.

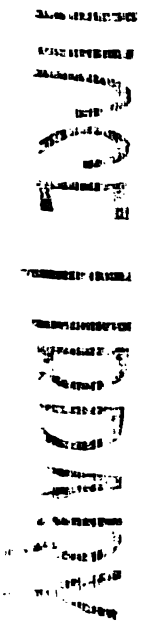
Test compound	% Relative adherence[b]
Phosphate buffered saline	100
β-mercaptoethanol	164 ± 22[c]
Cysteine	152 ± 14c
Dithiothreitol	152 ± 13[c]

[a] All enhancers were used at a concentration of 10 mM.

[b] Data are expressed as mean percent relative adherence ± standard error.

[c] Groups statistically different ($p < 0.05$) from control (22°C). Duncan's multiple range test was used to test differences between specific groups.

enzyme-like stereochemical binding interaction with complementary receptors (arginine-containing proteins) on the surface of A. viscosus cells. In this regard, Gibbons (47) has proposed that "cryptitopes" (hidden or conformationally incorrect cell surface components) could serve as receptors for bacterial attachment once they have been conformationally altered. In support of this hypothesis, Childs and Gibbons (19, 20) have reported that mild treatment of oral epithelial cells with trypsin, papain, or neuraminidase enhances binding of P. gingivalis. In addition, Naito and Gibbons (122) have reported that gentle treatment of fibronectin-collagen complexes with trypsin enhanced binding of P. gingivalis, whereas treatment with cysteine protease inhibitors or serum-protease inhibitors reduced binding of P. gingivalis.



II. Strategies to Clone the Trypsin-Like Protease Gene

A variety of strategies were employed in efforts to clone the trypsin-like protease gene. These included the use of two E. coli plasmid cloning vector systems, a colonic Bacteroides plasmid cloning vector system, and polymerase chain reaction techniques to amplify protease gene fragment homologs. Current efforts to clone the trypsin-like protease gene are directed toward the use of recently isolated putative transposon-induced trypsin-like protease-deficient mutants (see Transposition of Tn4351 in P. gingivalis).

E. coli and Colonic Bacteroides Plasmid Cloning Vector Systems.

Methods

Bacterial strains and plasmids. E. coli XL1-Blue and pBluescript were obtained from Stratagene (La Jolla, CA). E. coli X-90 was obtained from Dr. L.B. Evnin (University of California, San Francisco; 40). Both E. coli strains were maintained on Luria-Bertani (LB) agar. B. fragilis 638 and pBI191 were obtained from Dr. C.J. Smith (East Carolina University; 184). pBI191 is selectable in Bacteroides species by resistance to erythromycin. B. uniformis 1001 was obtained from Dr. N.B. Shoemaker (University of Illinois, Urbana; 173). Both Bacteroides strains were maintained on LRBB agar. The construction of pBlueI192 is described in a later section (Strategies for Transformation by Electroporation). pBlueI192 is selectable in E. coli by resistance to ampicillin and in Bacteroides by resistance to erythromycin. The strains of P. gingivalis used and the methods of propagation have been described in a previous section.

Isolation of P. gingivalis genomic DNA, partial digestion with Sau3A, and size

selection using sucrose density gradient centrifugation. High molecular weight genomic DNA was isolated from either P. gingivalis 33277 or 3079.03 by the guanidine-isothiocyanate method of Lippke et al. (99). Preliminary restriction digests of the isolated genomic DNA with Sau3A were conducted and analyzed by agarose gel electrophoresis to determine optimal conditions to maximize the amount of 2 to 6 kb Sau3A fragments (data not shown). For genomic library construction, Sau3A fragments (2 to 6 kb) were then isolated from restricted P. gingivalis genomic DNA by sucrose density gradient centrifugation (See Appendix {I}).

Ligation of size-selected genomic DNA into plasmids. Plasmid DNA was isolated using the method of Godson and Vapnek (49) and purified by CsCl-ethidium bromide equilibrium density gradient centrifugation (Appendix {II}). Purified plasmid DNA was then digested with BamHI, precipitated with ammonium acetate-isopropanol, and dissolved in a minimal volume of 1X ligation buffer. In most instances the restricted plasmid DNA was treated with calf intestinal alkaline phosphatase (Appendix {III}) prior to use in ligation reactions. Plasmid DNA and 2 to 6 kb Sau3A-fragments of P. gingivalis genomic DNA, selected by sucrose density gradient centrifugation, were then ligated with T4 ligase overnight at 12°C, precipitated with ammonium acetate-isopropanol, washed 2X with ice-cold 70% ethanol, and dissolved in 20 µl of sterile water (Appendix {IV}).

Transformation by electroporation. DNA from the ligation reactions was introduced into the various host strains by electroporation (Appendix {V}). In brief, 40 µl of washed bacterial cells (10^9 to 10^{10}/ml) and 10 µl of each ligation reaction (containing

≥ 1 μg DNA) were added to ice-chilled electroporation cuvettes (0.2 cm gap, Bio-Rad Laboratories, Richmond, CA). Electroporation was accomplished using a Gene Pulser System (Bio-Rad Laboratories, Richmond, CA) equipped with a pulse controller. All electroporations were conducted at a field strength of 12.5 kV/cm. Resistance was varied from 100 ohms to 200 ohms and capacitance was held constant at 25 microfarads (μF) in individual experiments, therefore RC time constants varied, in general, between 2.5 msec and 5 msec (for explanation of RC time constant see Appendix {V}). Following electroporation, the contents of the electroporation cuvette were immediately diluted in 1 ml fresh growth medium. E. coli strains were diluted in SOC broth (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose, pH 7.0) and incubated at 37°C for 1 h. Colonic Bacteroides strains were diluted in sTSB and incubated under anaerobic conditions at 37°C for 3 h. After incubation, 10-fold serial dilutions were performed and aliquots were plated on various media to determine viability, to determine the number of transformants, and to screen or select for protease-producing clones.

E. coli XL1-Blue and pBluescript. Viability of E. coli XL1-Blue, following electroporation, was determined on LB agar. The number of transformants and the percentage with inserts (non-pigmented colonies) was determined by plating on LB-TAXI agar (LB agar containing 12.5 μg/ml tetracycline, 100 μg/ml ampicillin, 40 μg/ml X-Gal, and 5 mM IPTG). Transformants of E. coli XL1-Blue were screened for protease activity using two different techniques. The genomic libraries were primarily screened by direct plating on sBRU agar (described previously) containing 1% skim milk and 100 μg/ml

ampicillin (SK-AMP agar). All plates were incubated anaerobically at 37°C, SK-AMP cultures were observed for 7 days for evidence of proteolytic activity. Proteolytic activity should be detectable as clearing (hydrolysis of milk proteins) surrounding isolated colonies. Transformants were also screened for trypsin-like protease activity by overlaying transformants on LB-AMP agar (LB agar containing 100 μg/ml ampicillin) with cellulose acetate membranes impregnated with Z-arg-AFC.

E. coli X-90 and pBluescript. Viability of E. coli X-90, following electroporation, was determined on LB agar. Transformants of E. coli X-90 were plated on LB-AMP agar to determine the number of transformants and on MOPS minimal media (126) containing 0.5 mM arginine-β-naphthylamide, 5 mM IPTG, 1 μg/ml thiamine, and 100 μg/ml ampicillin (MOPS-ARG-AMP) to select for trypsin-like protease producing clones.

Colonic Bacteroides and pBI191-based plasmids. Viability of colonic Bacteroides strains, following electroporation, was determined by plating on LRBB agar and the number of transformants was estimated by plating on LRBB agar containing 20 μg/ml erythromycin. Libraries constructed in colonic Bacteroides were screened for proteolytic activity using methods similar to those described above, with the exception that direct plating was performed on sBRU agar containing 1% skim milk and 20 μg/ml erythromycin. Cultures were incubated anaerobically and examined for proteolytic activity for 14 days. In some instances, screening for proteolytic activity was also performed with Z-arg-AFC overlay membranes on sBRU agar containing 20 μg/ml erythromycin.

Results

E. coli XL1-Blue and pBluescript. Six libraries of P. gingivalis genomic DNA were constructed in pBluescript and introduced into E. coli XL1-Blue by electroporation (Table XII). The number of transformants in each library ranged from 2.1 x 10^3 to 5.1 x 10^4. Non-pigmented transformant colonies (indicative of plasmids containing insert DNA) accounted for 32.5 to 65.2% of the transformants. Blue-pigmented colonies (containing intact pBluescript) most likely occurred due to incomplete restriction and/or incomplete phosphatase treatment of pBluescript prior to ligation. In most instances, each genomic library was screened without amplification of clones. A total of approximately 7.5 x 10^4 non-pigmented transformant colonies were screened for proteolytic activity. Only one putative protease-producing transformant which exhibited a zone of clearing on SK-AMP agar was observed. The transformant hydrolyzed Z-gly-gly-arg-AFC when induced with isopropyl-β-D-thiogalactopyranoside (IPTG) but had minimal activity with Z-gly-gly-arg-AFC in the absence of IPTG. Unfortunately the transformant proved to be genetically unstable and subsequent experiments were unable to demonstrate protease activity or the presence of inserted DNA.

E. coli X-90 and pBluescript. Two libraries of P. gingivalis genomic DNA were constructed in pBluescript and introduced into E. coli X-90 by electroporation (Table XII). Plating on LB-AMP indicated the presence of 8.8 x 10^3 and 9.0 x 10^2 transformants in each genomic library (total of 9.7 x 10^3 transformants). Aliquots of the genomic libraries were plated on MOPS-ARG-AMP agar to select for transformants with trypsin-like protease activity. Numerous ($\geq$ 400) large colonies (surrounded by small satellite

Table XII. Summary of P. gingivalis genomic libraries constructed in E. coli.

Plasmid	Insert DNA[a]	Recipient strain	RC time constant[b]	Transformants/ml
pBluescript[c]	P. gingivalis 3079.03	E. coli XL1-Blue	4.2 msec.	4.0×10^3 (32.5%)[d]
pBluescript[c]	P. gingivalis 3079.03	E. coli XL1-Blue	4.2 msec.	2.1×10^3 (47.6%)[d]
pBluescript[c]	P. gingivalis 3079.03	E. coli XL1-Blue	2.3 msec.	4.4×10^4 (59.1%)[d]
pBluescript[c]	P. gingivalis 3079.03	E. coli XL1-Blue	4.1 msec.	3.8×10^3 (65.8%)[d]
pBluescript[c]	P. gingivalis 3079.03	E. coli XL1-Blue	0.9 msec.	4.2×10^3 (47.6%)[d]
pBluescript[c]	P. gingivalis 33277	E. coli XL1-Blue	2.3 msec	5.1×10^4 (47.1%)[d]
pBluescript[c]	P. gingivalis 33277	E. coli X-90	1.7 msec.	8.8×10^3
pBluescript[c]	P. gingivalis 33277	E. coli X-90	2.3 msec	9.0×10^2

[a] 2 to 6 kb sucrose-sized Sau3A partial digest
[b] Electroporation RC time constant (see Appendix)
[c] Plasmid treated with calf intestinal alkaline phosphatase (see Appendix)
[d] % transformants with inserts (white-pigmented)

colonies) were observed on MOPS-ARG-AMP agar plates after 24 to 48 h anaerobic incubation. All of these colonies were patched to SK-AMP plates to test for protease activity. No protease activity was detected during 7 days of anaerobic incubation. Subsequent experiments (plating on minimal media without a source of arginine) indicated that these transformants had lost their auxotrophic requirement for arginine and that reversion to arginine prototrophy occurred frequently with E. coli X-90.

Colonic Bacteroides and pBI191-based plasmids. Three genomic libraries of P. gingivalis genomic DNA were constructed in pBI191-based plasmids and introduced into colonic Bacteroides strains (B. fragilis 638 and B. uniformis 1001) by electroporation (Table XIII). The number of transformants obtained ranged from 1.3×10^2 to 2.7×10^4. All transformants (total 3.0×10^4) were screened for protease activity as outlined above. No putative protease-producing transformants were detected.

Discussion

Initial attempts to clone the trypsin-like protease gene were conducted with pBluescript and E. coli XL1-Blue. This combination allows blue-white colony screening for transformants containing plasmids with genomic DNA inserts. In addition to constructing libraries to screen for protease producing transformants this system also permits evaluation of ligation and transformation efficiencies. This information could be useful in evaluating construction of P. gingivalis genomic libraries in other systems that do not offer this advantage (E. coli X-90 and colonic Bacteroides). Since the multiple

Table XIII. Summary of P. gingivalis genomic libraries constructed in colonic Bacteroides.

Plasmid	Insert DNA[a]	Recipient strain	RC time constant[b]	Transformants/ml
pBI191[c]	P. gingivalis 33277	B. fragilis 638	2.4 msec.	1.3×10^2
pBlueI192	P. gingivalis 3079.03[c]	B. uniformis 1001	2.3 msec.	2.4×10^3
PBlueI192[c]	P. gingivalis 3079.03	B. uniformis 1001	4.6 msec.	2.7×10^4

[a] 2 to 6 kb sucrose-sized Sau3A partial digest

[b] Electroporation RC time constant (see Appendix)

[c] Treated with calf intestinal alkaline phosphatase (see Appendix)

cloning site of pBluescript is preceded by the lac promoter, pBluescript may serve as an expression vector following induction with IPTG. Assuming that the genome size of P. gingivalis is similar to that of E. coli and that the average insert size is 4 kb, a representative library (99% confidence interval) should contain approximately 4.6 x 10^3 recombinants. If expression of a fusion protein from a vector-driven promoter is required, the theoretical number of recombinants is increased by at least 6-fold (2.6 x 10^4) to allow for out-of-frame recombinants and/or misorientation of the insert. One protease-producing transformant was detected among approximately 7.5 x 10^4 transformants screened for degradation of skim milk proteins. Unfortunately the transformant was genetically unstable and lost its proteolytic phenotype before extensive characterization could be performed. Preliminary experiments suggested that expression of proteolytic activity was dependent upon and regulated by the upstream lac promoter present in pBluescript.

In subsequent experiments, an E. coli-based auxotrophic selection technique was used to attempt to clone the trypsin-like protease gene. Libraries of P. gingivalis DNA were constructed using pBluescript and E. coli X-90. E. coli X-90 is auxotrophic due to an amber mutation in an arginine biosynthesis gene and, like all E. coli K-12 strains, is unable to hydrolyze arginine-β-naphthylamide. The trypsin-like protease of P. gingivalis readily cleaves arginine-β-naphthylamide; therefore the selection relied on the ability of transformants expressing trypsin-like protease activity to release arginine from arginine-β-naphthylamide and thus complement the arginine auxotrophy of E. coli X-90. It was hoped that the application of selective pressure would help to stabilize any protease-producing transformants obtained. However, the high reversion frequency to arginine

prototrophy by E. coli X-90, observed in this study, made the identification of any transformants with trypsin-like protease activity unlikely. Perhaps the use of an E. coli strain with a "tighter" mutation in an arginine biosynthesis gene would yield better results.

Numerous observations suggest that E. coli is not the optimal host for cloning and expression of Porphyromonas genes. Some reports have noted that Porphyromonas and Bacteroides contain DNA sequences that are toxic for E. coli and that DNA from Porphyromonas and Bacteroides is unstable in E. coli (31, 172). In addition, as reported below for several recently cloned P. gingivalis genes, it appears that P. gingivalis promoters are usually not recognized, or are poorly recognized, by the E. coli transcription system. Therefore, since Porphyromonas species are phylogenetically more closely related to the colonic Bacteroides than to E. coli, an attempt was made to clone P. gingivalis protease genes in colonic Bacteroides strains. It was hypothesized that P. gingivalis DNA may be more stable in colonic Bacteroides and that P. gingivalis promoters may function better in colonic Bacteroides. This would increase the chance of detecting trypsin-like protease gene expression; however no protease-producing clones were identified. A serious drawback to this approach is that restriction-negative strains of colonic Bacteroides are not presently available; therefore restriction of insert DNA is likely. In this regard, Smith (185) has reported that the transformation frequency of colonic Bacteroides strains with E. coli-Bacteroides shuttle vectors is 1,000-fold lower when the vectors are isolated from E. coli than when they are isolated from the homologous colonic Bacteroides strain. Similar results were obtained in this investigation (see Strategies for Transformation of P. gingivalis by Electroporation).

Although the efforts described in this investigation failed to result in the cloning of any protease genes from P. gingivalis, recent publications have reported the cloning of a few P. gingivalis genes, including protease genes. Dickinson et al. (31) were the first to clone a gene from P. gingivalis. They used the N-terminal amino acid sequence of purified fimbrilin to construct 17- to 20-residue mixed-base oligonucleotide probes. The synthesized probes were tested by Southern blot hybridization analysis of P. gingivalis genomic DNA. A 20-mer probe (BGFIM2) was found that hybridized at high stringency with P. gingivalis DNA but not with E. coli DNA. Initially, a library of P. gingivalis DNA was constructed in a cosmid vector (pJB8). No clones which hybridized with BGFIM2 were detected among more than 2 x 10^3 recombinants. Dickinson et al. (31) theorized, since the cosmid library contained large P. gingivalis DNA inserts, the presence of P. gingivalis sequences that were toxic to E. coli might be one reason for their failure to detect fimbrilin gene sequences in the cosmid library. Therefore, they examined the ability of BGFIM2 to hybridize with P. gingivalis DNA that had been digested with several different restriction enzymes, individually. Digestion with SacI was found to produce a 2.5 kb fragment that hybridized with BGFIM2. A second genomic library was then constructed in pUC13 with 2 to 3 kb SacI fragments of P. gingivalis DNA, isolated from agarose gels. One out of the first twenty-two transformants tested from this library was found to hybridize with BGFIM2. Subsequent analysis and DNA sequencing indicated that the cloned fragment contained an open reading frame that extended 337 codons downstream from the N-terminal alanine of purified fimbrilin. The first 24 codons were found to correspond exactly with the N-terminal sequence determined from purified

fimbrilin. In support of the hypothesis that P. gingivalis contains DNA sequences that are toxic for E. coli, DNA sequences between the 3' end of the cloned fimbrilin gene and the downstream SacI site were found to be deleterious for E. coli when inserted in the opposite orientation from that of the parent clone.

Progulske-Fox et al. (147) were the first to report expression of a cloned P. gingivalis gene in E. coli. Polyclonal antisera against P. gingivalis 381 detected two strongly reactive transformants, out of 1,500 screened. One transformant (ST2), chosen for further study, was found to contain a 3.2 kb insert. This transformant hemagglutinated sheep erythrocytes and antisera raised against ST2 inhibited hemagglutination by P. gingivalis. The authors suggest that these results indicate that some P. gingivalis surface antigens are not only expressed in E. coli, but can be processed and translocated to the cell surface as well. The observation that expression was enhanced by IPTG was interpreted as evidence that the cloned gene was oriented in the direction of transcription from the lac promoter of the vector; however the effect of inverting the gene was not investigated.

Watanabe et al. (222) have also cloned and expressed a P. gingivalis outer membrane protein in E. coli. They cloned a major immunodominant surface protein (the 75 kD protein) of P. gingivalis. Two 20-mer mixed base oligonucleotide probes were synthesized to correspond to the N-terminal amino acid sequence of the purified 75 kD protein. One transformant, out of 5,000 examined, was detected that hybridized strongly with the 75 kD protein mixed base oligonucleotide probes. Subsequent experiments, after subcloning downstream from the T7 promoter, indicated expression of the cloned 75 kD

protein in E. coli was orientation-dependent. This suggests that expression of the cloned 75 kD protein was mediated by the T7 promoter in the cloning vector.

Madden and Clark (105) were the first to report the cloning of a protease gene from P. gingivalis. A genomic library of P. gingivalis 33277 was constructed in a temperature-regulated expression vector (pCQV2) and E. coli DH5-α-mcr. One proteolytic transformant colony was detected among 30,000 transformants by plating on LB-AMP agar containing 2% sodium caseinate. This transformant was reported to hydrolyze type I collagen, azocoll, and azocasein. Unfortunately the proteolytic transformant proved to be very unstable, even at low temperature (32°C), and was lost prior to characterization of the inserted P. gingivalis sequences (T.E. Madden, personal communication).

Arnot et al. (4) have also reported cloning a P. gingivalis protease gene. A library of genomic DNA from P. gingivalis W83 was constructed in phage λ (EMBL4) and E. coli LE392. Three recombinant proteolytic clones, out of 3 x 10^4 recombinants, were detected by overlaying plaques on LB-AMP plates with 1% casein containing β-mercaptoethanol. No proteolytic recombinants were detected with casein overlays without β-mercaptoethanol. Restriction analysis of the three proteolytic recombinants revealed that they contained a nested set of overlapping cloned DNA fragments in excess of 15 kb from a single region of the P. gingivalis chromosome. The location of the protease-producing gene within this 15 kb region was not reported, nor was a restriction map of the 15 kb region presented. Activity against benzoyl-arginine-7-amino-4-methyl-coumarin was minimal after 60 min incubation (control, A_{412} = 0.01; recombinant A_{412} = 0.12);

however Arnot et al. (4) concluded that they had cloned a thiol-dependent protease with trypsin-like protease activity. Subcloning into pACYC184 resulted in even lower protease activity; therefore it appears that expression of the protease was dependent on read-through transcription from the phage lambda promoter.

Park and McBride (142) have also reported the cloning of a thiol-dependent protease from P. gingivalis. Sau3A-digested DNA fragments (2 to 10 kb) from P. gingivalis W83 were ligated into the BamHI site of pT218R and transferred into E. coli JM83. LB agar containing 1% skim milk was used to screen transformants for proteolytic activity. Initially, cultures were incubated aerobically overnight, exposed to chloroform vapors to lyse bacterial cells, and then reincubated to detect proteolytic activity. One transformant with proteolytic activity was detected among 2,000 transformants tested after 2 weeks incubation. Subsequent experiments, which reincubated cultures anaerobically after chloroform lysis, resulted in detection of proteolytic activity after only 3 days incubation. The authors stated that these results "suggest that the cloned protease is only active under reducing conditions". However, in subsequent SDS-PAGE zymography experiments with whole cell extracts, proteolytic activity was detected only in the absence of β-mercaptoethanol. Park and McBride (142) did not address these seemingly contradictory results. The recombinant plasmid (pYS307) was found to contain a 5.2 kb insert and the cloned gene was localized in a 2.4 kb BamHI-HindIII fragment. In contrast to all other P. gingivalis genes that have been cloned to date, the orientation of the BamHI-HindIII fragment was reported not to influence expression of protease activity. This suggests that the P. gingivalis promoter of this gene is recognized by E. coli

transcriptional systems or that an upstream sequence can function as a fortuitous promoter in E. coli.

By far, the most convincing reports regarding cloning protease genes from P. gingivalis are from Kuramitsu and colleagues (77, 140, 197). They constructed a genomic library of P. gingivalis 53977 (A7A1-28) DNA in pP_L-lambda using E. coli HB101 as host. Transformants were selected and screened for protease activity on LB agar plates containing 1% skim milk and 50 μg/ml ampicillin. Two transformants, out of approximately 1,500 screened, were identified based on the presence of clear zones surrounding the colonies. Restriction endonuclease analysis of these recombinant plasmids indicated that they both contained identical 5.9 kb inserts. Protease activity was detected with PZ-PLGPA (synthetic collagenase substrate), type 1 collagen, azocoll, gelatin, and BAPNA (synthetic trypsin-like protease substrate). Subcloning and deletion analysis mapped the prtC (collagenase gene) at one end of the 5.9 kb fragment. Protease activity of the prtC gene was reported to be orientation-dependent, indicating that transcription initiation was most likely due to the tetracycline promoter present upstream of the cloning site. Surprisingly, subclones of the prtC gene expressed proteolytic activity against type I collagen and azocoll but no longer hydrolyzed PZ-PLGPA, gelatin, or BAPNA. In this regard, the 5.9 kb fragment has been reported to contain three additional open reading frames; these have been identified as a superoxide dismutase gene (21), a trypsin-like protease gene (140), and a hemagglutinin gene (140). Preliminary characterization of the prtT gene (trypsin-like protease gene) indicates that its gene product hydrolyzes gelatin and BAPNA. Like the prtC gene, transcription of the prtT

gene was orientation-dependent in E. coli vectors.

Several observations in the reports described above have implications for future genetic studies of P. gingivalis. The apparent instability of P. gingivalis DNA in E. coli and the inability of E. coli transcription systems to recognize P. gingivalis promoters suggest that the use of colonic Bacteroides host-vector systems and/or the development of DNA transfer systems that function in P. gingivalis may be necessary to accurately identify and study P. gingivalis promoters. In addition, the development of suitable vectors and DNA transfer systems which function in P. gingivalis, coupled with specific mutagenic techniques, will permit cloning of P. gingivalis genes by complementation.

PCR Amplification of Putative Protease Gene Homologs

Hartley (60) in 1960 observed that proteases appeared to act through four distinct catalytic mechanisms and could be classified as "serine", "thiol", "acid", or "metal" proteases. Although much more is presently known about the catalytic sites of proteases, Hartley's system is still valid and the four groups of proteases are known as "serine", "cysteine", "aspartic", and "metallo" proteases.

As discussed in a previous section various research groups, using protease inhibitors, have classified the trypsin-like protease of P. gingivalis as either a serine or a cysteine protease. Proteases can been assigned to various superfamilies based on structural similarity of their catalytic domain. Serine proteases appear to be the most common and widespread in nature. Most serine proteases studied to date belong to the chymotrypsin superfamily or the subtilisin superfamily. Chymotrypsin-related proteases

have been found in both prokaryotic and eukaryotic microorganisms, as well as in plants and animals (7). Subtilisin-related proteases were initially observed in prokaryotes, but recent studies have demonstrated their presence in fungi (189), insects (62), and mammals (25). Cysteine proteases also occur in both prokaryotic and eukaryotic microorganisms, as well as plants and animals. At least four superfamilies of cysteine proteases have been described, however only the papain superfamily has been studied extensively. Accordingly, the papain superfamily contains most of the known cysteine proteases, including many of the cathepsins from mammalian lysosomes.

Comparative studies have indicated strong conservation of amino acid sequence in the catalytic domains within the superfamilies of both serine and cysteine proteases. Sakanari et al. (153) used this conservation of amino acid residues at the catalytic site to construct synthetic degenerate oligonucleotide primers corresponding to consensus regions surrounding the catalytic serine and histidine residues of the chymotrypsin-like superfamily of serine proteases. These degenerate primers were used in the polymerase chain reaction (PCR) with genomic DNA from nematode and protozoan parasites to amplify and isolate previously unidentified serine protease gene fragments. Eakin et al. (36) used a similar approach, with degenerate primers based on the consensus regions surrounding the catalytic cysteine and asparagine residues of the papain superfamily of cysteine proteases, to amplify and isolate cysteine protease gene fragments from Trypanosoma species and Entamoeba histolytica.

The following section describes attempts to employ PCR and degenerate oligonucleotide primers to amplify and isolate protease gene fragment homologs from P.

gingivalis. Primer design was based on the consensus sequences surrounding the catalytic residues of chymotrypsin-like, subtilisin-like, and papain-like proteases.

Methods

Isolation of genomic DNA, design of oligonucleotide primers, and PCR conditions. High molecular weight genomic DNA was isolated from four strains of P. gingivalis (3079.03, 5083.03, 33277, & 381) by the guanidine-isothiocyanate method (99). Synthetic degenerate oligonucleotide primers based on catalytic-site consensus regions of chymotrypsin-like and subtilisin-like serine proteases were obtained from Dr. C.S. Craik (University of California, San Francisco; 153). The sequences of the chymotrypsin-like serine protease PCR primers are shown in Figure 4a. Degenerate oligonucleotide primers, similar to those used by Eakin et al. (36) were designed based on the catalytic-site consensus regions of papain-like cysteine proteases. These primers were synthesized commercially (Operon, Inc., Alameda, CA) and their sequences are shown in Figure 4b. PCR was performed essentially as described by Sakanari et al. (153). In brief, 100 µl reaction volumes contained 1 to 2 µg of genomic DNA, dNTP's (400 µM each), 750 to 1000 ng of each primer, and 5 units of Taq DNA polymerase. DNA was amplified for 60 cycles of PCR with denaturation at 93°C for 1 min, annealing at 25°C for 2 min, and primer extension at 72°C for 1.5 min. pTN, a plasmid that contains a cDNA insert of rat anionic trypsin II (39), was used as a positive control for chymotrypsin-like serine protease gene fragments, and p527, a plasmid that contains a 527 bp insert of a cysteine protease gene homolog isolated from Trypanosoma species, was used as a positive control

(a)

His[57] sense strand primer - 5′-ACA GAA TTC TGG GTI GTI ACI GCI GCI CAY TG-3′
EcoRI trp val val thr ala ala his cys

Ser[195] antisense strand primer - 3′-CC ICT RWS ICC ICC IGG IRA TTC GAA ACA-5′
gly asp ser gly gly pro leu HindIII

(b)

Cys[25] sense strand primer - 5′-ACA GAA TTC CAR GGI WSI TGY GGI WSI TGY TGG-3′
EcoRI glu gly ser cys gly ser cys trp

Asn[175] antisense strand primer - 3′- ATR ACC KAI YAI TTY TTR WSI ACC TTC GAA ATT-5′
tyr trp leu val lys asn ser trp HindIII

Fig. 4. Degenerate primers for PCR amplification of chymotrypsin-like (a) and papain-like (b) protease gene fragments[a].

[a] I = inosine; K = G or T; R = A or G; S = G or C; W = A or T; and Y = T or C

for papain-like cysteine protease gene fragments. pTN and p527 were obtained from Dr. C.S. Craik (University of California, San Francisco; 36, 39). PCR-amplified DNA fragments were visualized in 4% agarose gels (3% Nusieve GTG/1% SeaKem GTG) by staining with ethidium bromide.

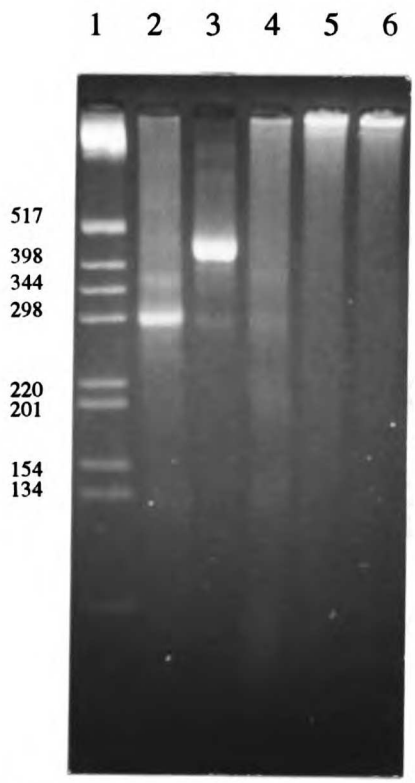
Subcloning and DNA sequence analysis. To aid in subcloning PCR products, the degenerate oligonucleotide primers used were specifically designed to contain an EcoRI site in the "sense strand primer" and a HindIII site in the "antisense strand primer". For subcloning, the PCR products were digested with EcoRI and HindIII and electrophoretically separated in 4% agarose gels. Appropriately sized (>300 bp but <600 bp) DNA fragments were isolated from excised agarose gel slices using GeneClean (BioLabs 101, La Jolla, CA), cloned into EcoRI-HindIII digested pBluescript, and their DNA sequence was determined by the dideoxy termination method (see Appendix {VI}). Since the amplified 500 bp DNA fragment (see Results) obtained with primers based on papain-like cysteine proteases was found to contain a "naturally occurring" EcoRI site it was cloned into pCR1000 (Invitrogen Inc., San Diego, CA), a specifically designed plasmid for subcloning PCR products. Plasmid pCR1000 takes advantage of the fact that Taq polymerase adds an overhanging adenosine to the 3' ends of duplex DNA. Therefore, PCR products can be subcloned directly, without restriction, into a site in pCR1000 that contains 3' thymidine overhangs. The DNA sequence of the amplified 500 base pair fragment was determined by the Biochemistry Research Center (UCSF) using dye termination chemistry and an automated sequencing system (Applied Biosystems, Foster City, CA).

Results & Discussion

Agarose gel analysis of the PCR products obtained with DNA from P. gingivalis 3079.03 and degenerate primers for the consensus regions of chymotrypsin-like and subtilisin-like proteases are shown in Fig. 5. pTN was used as a positive control since it contains a cDNA insert encoding rat trypsin. A single strongly staining DNA fragment, of the predicted size (434 bp) was obtained with pTN DNA and primers for chymotrypsin-like proteases. A single strongly staining 300 bp DNA fragment was obtained with P. gingivalis 3079.03 DNA and the primers for chymotrypsin-like serine proteases. Subsequent PCR experiments with DNA from two other P. gingivalis strains (33277 and 5083.03) failed to amplify a comparable 300 bp fragment (data not shown). Following cloning of the PCR-amplified 300 bp fragment from P. gingivalis 3079.03 DNA sequence analysis of the 5' end of the putative sense strand indicated that the fragment contained codons for all of the eight amino acid residues encoded by the His^{57} sense strand primer; however two stop codons appeared within the next 15 codons (Fig. 6).

No PCR products were obtained from P. gingivalis 3079.03 DNA with primers designed from the consensus regions of subtilisin-like serine proteases (Fig. 5).

Agarose gel analysis of the PCR products obtained with DNA from four P. gingivalis strains (5083.03, 3079.03, 33277, & 381) and degenerate primers for the consensus regions of papain-like cysteine proteases are shown in Fig. 7. The electrophoretic banding pattern of the PCR products differed somewhat among the strains. The two small (< 250 bp) DNA fragments obtained with all four stains were not cloned since they did not correspond to the expected size (approx. 450 bp) of papain-like



Lane 1 1 kb ladder DNA standard
Lane 2 P. gingivalis 3079.03 DNA and chymotrypsin-like protease primers
Lane 3 pTN DNA and chymotrypsin-like protease primers
Lane 4 Negative control (PCR mixture with chymotrypsin-like protease primers)
Lane 5 P. gingivalis 3079.03 DNA with subtilisin-like protease primers
Lane 6 Negative control (PCR mixture with subtilisin-like protease primers)

Fig. 5. PCR products obtained with degenerate primers for chymotrypsin-like and subtilisin-like protease gene fragments.

His^{57} sense strand primer

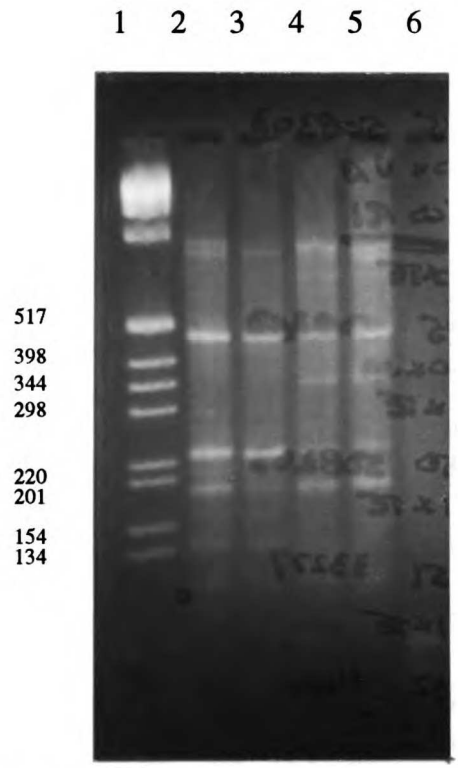
```
5'-ACA GAA TTC TGG GTI GTI ACI GCI GCI CAY TG-3'
       EcoRI  trp val val thr ala ala his cys
```

PCR-amplified 300 bp fragment from P. gingivalis 3079.03

```
5'-CAG GAA TTC TGG GTG GTG ACG GCG GCG CAT TGC AGA ATG
       EcoRI  trp val val thr ala ala his cys arg met

GGA CAA GAC ATT CCC CAA AAG CAG TAA  GGT GGA ACA TAA ...-3'
gly gln asp ile pro gln lys gln stop            stop
```

Fig. 6. Comparison of His^{57} sense strand primer for chymotrypsin-like protease gene fragments and the DNA sequence of the PCR-amplified 300 bp fragment from P. gingivalis 3079.03.



Lane 1 1 kb DNA ladder standard
Lane 2 P. gingivalis 5083.03 DNA and papain-like protease primers
Lane 3 P. gingivalis 3079.03 DNA and papain-like protease primers
Lane 4 P. gingivalis 33277 DNA and papain-like protease primers
Lane 5 P. gingivalis 381 DNA and papain-like protease primers
Lane 6 Negative control (PCR mixture with papain-like protease primers)

Fig. 7. PCR products obtained with degenerate primers for papain-like protease gene fragments.

cysteine protease gene homologs. A strongly staining 500 bp DNA band was observed with all four strains, however only the PCR products with strains 33277 and 381 (human strains) contained a 350 bp DNA fragment. The 500 bp and 350 bp fragments obtained with strain 33277 were cloned for DNA sequence analysis. The 350 bp fragment was cloned into pBluescript (as described above) and the sequence of the 5' end of the putative sense strand was determined. Although the expected EcoRI site was present, none of the subsequent codons matched those present in the Cys^{25} sense strand primer and a stop codon appeared nine codons after the EcoRI site (Fig. 8). Since the amplified 500 bp fragment contained an internal EcoRI site it was cloned into pCR1000 rather than pBluescript for DNA sequencing. Analysis of the 5' end of the putative sense strand indicated the presence of the EcoRI site and codons for five out of the eight amino acids encoded by the Cys^{25} sense strand primer; however two stop codons appeared within the next thirty codons (Fig. 8). The 500 bp amplified fragment most likely encodes a portion of a gene (open reading frame), since in one reading frame the strand amplified by the Asn^{175} antisense strand primer was found to encode an uninterrupted run of 155 amino acids (Fig. 9).

Cys[25] sense strand primer

```
5'-ACA GAA TTC CAR GGI WSI TGY GGI WSI TGY TGG-3'
       EcoRI  glu gly ser cys gly ser cys trp
```

PCR-amplified 350 bp fragment from P. gingivalis 33277

```
5'-CAG GAA TTC CAC TTG CCT TTA ACA ACT GTA TTT CAG
       EcoRI  his leu pro leu thr thr val phe gln

   ACG TTA TTT TAG ... -3'
   thr leu phe stop
```

PCR-amplified 500 bp fragment from P. gingivalis 33277

```
5'-TCA GAA TTC CAA GGG TGG TGC GGG ACG TGT TGG AGA
       EcoRI  gln gly trp cys gly thr cys trp arg

   CTG TTG CGC CCA GTA TAT TAC CAC CCA TTT GGG GTA
   leu leu arg pro val tyr tyr his pro phe gly val

   GAT ATT CCT GAC TGA  TAC GTT CGT TGG AAT GTC TTG
   asp ile pro asp stop tyr val arg trp asn val leu

   CCA CAG CAT GGC TGA ... -3'
   pro gln his gly stop
```

Fig. 8. Comparison of Cys[25] sense strand primer for papain-like protease gene fragments and DNA sequences of PCR-amplified 350 bp and 500 bp fragments from P. gingivalis 33277.

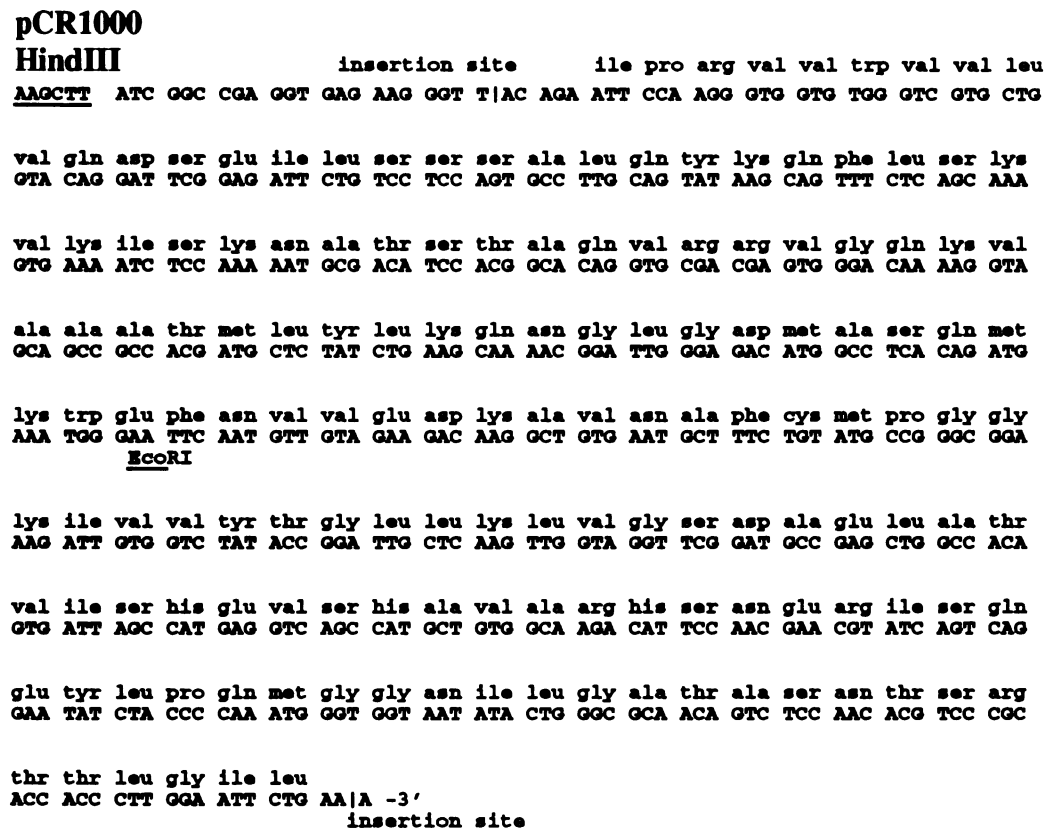


Fig. 9. Open reading frame encoded by PCR-amplified 500 bp fragment from P. gingivalis 33277.

III. Development of Molecular Genetic Techniques

The following chapter describes efforts to develop genetic transfer systems for use in P. gingivalis. The colonic Bacteroides are phylogenetically the closest group to P. gingivalis in which genetic transfer systems have been developed. Therefore a variety of conjugation and electroporation techniques, using colonic Bacteroides systems, were explored for use in P. gingivalis. A simple mating procedure was used to demonstrate that R751, an IncP E. coli plasmid, can be conjugated from E. coli to P. gingivalis and that Tn4351, a transposon originally found on B. fragilis plasmid pBF4, can transpose from R751 to the P. gingivalis chromosome. This represents the first documented observation of transposition in P. gingivalis. In addition, a unique naturally occurring plasmid from P. endodontalis 27067 was isolated, restriction mapped, and partially cloned.

Transposition of Tn4351 in P. gingivalis

A powerful approach to assess the role of P. gingivalis virulence factors in vivo would be to isolate mutants deficient in specific phenotypic characteristics and compare their pathogenicity with that of the wild-type parent strain in the ligature-induced cynomolgus monkey model of periodontitis (69).

To construct well-defined strains of P. gingivalis, having only a single known mutation in a chosen gene, genetic tools such as transposons and shuttle vectors which function in P. gingivalis are needed. Unfortunately, no naturally occurring bacteriophages, transposable elements, or plasmids have been described to date in P.

gingivalis (32, 154, 213). In order to develop genetic tools for use in P. gingivalis a simple mating procedure was used to investigate the ability of several different conjugal and transpositional systems found in colonic Bacteroides species to function in P. gingivalis. Colonic Bacteroides based systems were chosen for the following reasons: (a) Porphyromonas is phylogenetically more closely related to Bacteroides than to Escherichia coli (143, 232), (b) RNA polymerase(s) from P. gingivalis and E. coli are reported to have different subunit structure and to exhibit different DNA template specificities (81), and (c) no antibiotic resistance genes known to function in E. coli also function in Bacteroides (133, 155); therefore they are unlikely to function in P. gingivalis.

Methods

Bacterial strains, plasmids, and culture conditions. E. coli HB101 (R751::*Ω4) was kindly provided by Dr. N.B. Shoemaker (Univ. of Illinois, Urbana; 173). E. coli HB101 (R751::*Ω4) was maintained on Luria-Bertani (LB) agar containing 10 μg/ml tetracycline. For conjugation experiments, one or two colonies were transferred to 5 ml LB broth containing 10 μg/ml tetracycline and incubated aerobically at 37°C for 16-18 h. P. gingivalis 33277 was maintained on LRBB agar as previously described. For conjugation experiments, P. gingivalis was sequentially cultured two times in sTSB. In brief, several bacterial colonies from an LRBB plate (incubated anaerobically at 37°C for 3-5 days) were collected on a cotton swab, resuspended in 5 ml sTSB, and incubated anaerobically overnight at 37°C. Two ml of the overnight broth culture was then inoculated into 50 ml of sTSB and incubated anaerobically at 37°C for 18-36 h (A_{660} =

0.8 to 1.2). Ten-fold serial dilutions were prepared from both broth cultures (E. coli and P. gingivalis) and plated on LRBB agar to determine the initial viable counts. For conjugation experiments, bacterial cells were harvested as described below.

Mating conditions. Two ml of donor cell culture (E. coli HB101 R751::*Ω4) and 2 ml of recipient cell culture (P. gingivalis 33277) were harvested by centrifugation for 5 min in a single microcentrifuge tube (conjugation mixture). Two ml of recipient cell culture alone was harvested in a separate microcentrifuge tube (recipient control). The bacterial cell pellet in each tube was then resuspended in 200 µl of sTSB, and 100 µl of each cell suspension was spotted on an LRBB plate to allow mating to occur. The LRBB plates spotted with the bacterial cell suspensions were incubated aerobically at 37°C for 2 to 4 h. Following this brief aerobic incubation the spotted LRBB plates were incubated anaerobically at 37°C for 36-48 h. The cultured bacterial spots were then harvested with a cotton swab and resuspended in 1 ml of sTSB. Portions (100 µl) of each suspension and of 10-fold serial dilutions of each suspension were plated on LRBB agar containing 20 µg/ml erythromycin and 100 µg/ml gentamicin to select for transconjugants. Portions (100 µl) were also plated on LRBB containing 100 µg/ml gentamicin alone to enumerate recipient cells. After anaerobic incubation at 37°C for 7 days the number of transconjugants and recipients was determined.

Isolation of bacterial DNA. DNA was extracted from P. gingivalis 33277 and from erythromycin-resistant (Em^r) transconjugants of P. gingivalis 33277 by the guanidine-isothiocyanate technique as described by Lippke et al. (99). Plasmid DNA was isolated from E. coli HB101 (R751::*Ω4) by the NaCl-SDS procedure of Godson and

Vapnek (49) and further purified by CsCl-ethidium bromide equilibrium density gradient centrifugation.

Southern blot hybridization analysis and biotinylation of R751::*Ω4 DNA. Two micrograms of high molecular weight DNA from each strain was digested with 10 units of AvaI for 1 h in buffer supplied by the manufacturer (Stratagene, La Jolla, CA). Restriction fragments were electrophoretically separated in 1% agarose gels submerged in TBE buffer (0.089 M Tris, 0.89 M boric acid, 0.002 M EDTA, pH 8.0) and blotted onto nylon membranes. Purified R751::*Ω4 DNA was biotinylated using the BioNick™ Labelling System (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions and used to probe the Southern blots. Hybridized biotinylated R751::*Ω4 DNA was detected with the PhotoGene™ Nucleic Acid Detection System (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions, except that the hybridization stringency was increased by raising the temperature of the 0.1X SSC, 1% SDS wash from 50°C to 65°C.

Results

Frequency of Em^r transconjugants. Black-pigmented colonies which grew on LRBB plates containing erythromycin and gentamicin were considered to be transconjugants. Gentamicin inhibits growth of donor (E. coli) cells and erythromycin selects for recipients (P. gingivalis) that have acquired Tn4351. P. gingivalis is naturally resistant to gentamicin. No spontaneous Em^r colonies were detected with the recipient control spots. The frequency of transconjugants varied from 8.2×10^{-7} to 9.8×10^{-9}

(mean 1.6 x 10^{-7} for 10 independent matings). The frequency of P. gingivalis transconjugants obtained was approximately 10-fold lower than that previously reported for colonic Bacteroides species (173). Most matings were done with a donor-to-recipient ratio ranging from 0.25 to 0.5. Spots of the cell suspensions initially contained 10^8 to 10^9 recipient cells. Increasing the donor-to-recipient ratio to 1.0 adversely affected the viability of recipient cells and therefore reduced the number of transconjugants; whereas increasing the length of the aerobic incubation from 4 h to 24 h appeared to slightly increase the number of transconjugants (data not shown).

Southern blot hybridization analysis of Em^r transconjugants. To confirm that Tn4351 was inserted into the P. gingivalis chromosome, DNA was isolated from 10 Em^r transconjugants, digested with AvaI, electrophoretically separated in agarose gels, transferred by blotting to nylon membranes, and probed with biotinylated R751::*Ω4 DNA. Since AvaI cuts twice within Tn4351, simple insertions of Tn4351 should yield three hybridizing AvaI DNA restriction fragments; one 3.8-kb fragment corresponding to the 3.8-kb internal fragment of Tn4351, and two AvaI fragments of varying size corresponding to junction fragments between Tn4351 and the P. gingivalis chromosome (Fig. 10). Digestion of chromosomal DNA with AvaI allowed easier visualization of both of the junction fragments from transconjugants containing simple insertions of Tn4351 than digestion with EcoRI, which was used in previous studies with colonic Bacteroides (173). AvaI digestion leaves 0.5 kb of IS4351$_L$ and 0.6 kb of IS4351$_R$ attached to the two chromosomal junction fragments; whereas EcoRI digestion leaves less than 10 base pairs of IS4351$_R$ attached to one of the chromosomal junction fragments (Fig. 10).

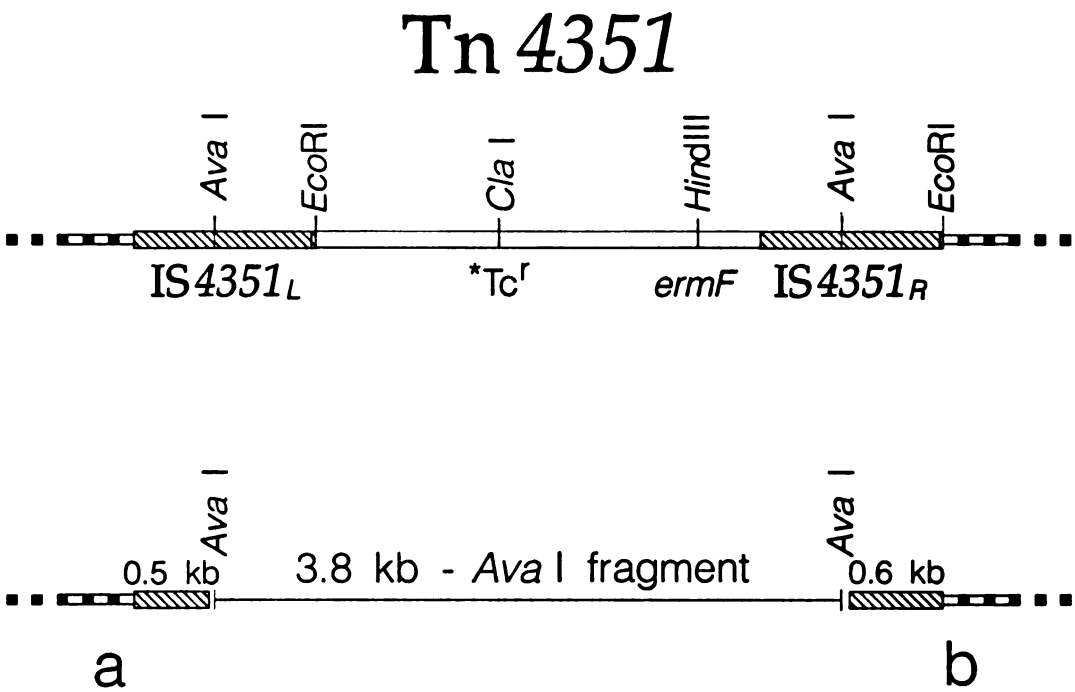


Fig. 10. Restriction endonuclease map of integrated Tn4351[a].

[a] In Tn4351, insertion elements (IS4351$_L$ and IS4351$_R$) are indicated by the hatched areas and the approximate location of the erythromycin (ermF) and aerobic tetracycline resistance (*Tc^r) genes are shown (149). AvaI digestion of integrated Tn4351 results in a 3.8-kb fragment and two junction fragments (a and b). AvaI digestion leaves 0.5 kb of IS4351$_L$ attached to junction fragment a and 0.6 kb of IS4351$_R$ attached to junction fragment b.

Therefore usually only one junction fragment is visible in Southern blots with EcoR1 digested DNA.

Ten Em^r transconjugants were analyzed by Southern blot hybridization for evidence of transposition of Tn4351, results for five representative transconjugants are shown in Fig. 11. Three of the Em^r transconjugants (Fig. 11; lanes 3, 6, and 7) exhibit hybridization patterns consistent with simple insertion of Tn4351. The fact that the junction fragments of these three transconjugants are different sizes reflects insertion of Tn4351 at different sites within the P. gingivalis chromosome. Two of the Em^r transconjugants shown in Fig. 11 (lanes 4 and 5) contain R751-derived DNA sequences, in addition to Tn4351 DNA sequences. Analysis of DNA extracts from these transconjugants by agarose gel electrophoresis and CsCl-ethidium bromide equilibrium density gradient centrifugation failed to demonstrate the presence of autonomous plasmid (data not shown). This is not unexpected, since R751 is known to be unable to replicate in colonic Bacteroides (173). Therefore, the Southern blot hybridization patterns of these two transconjugants most likely reflect Tn4351-mediated cointegration of R751 into the P. gingivalis chromosome. In addition, one putative junction fragment was visible within the complex banding pattern of each of these transconjugants (Fig. 11; lanes 4 and 5). No hybridization occurred between the R751::*Ω4 probe and DNA from the parental recipient (P. gingivalis) strain alone (Fig. 11, lane 8).

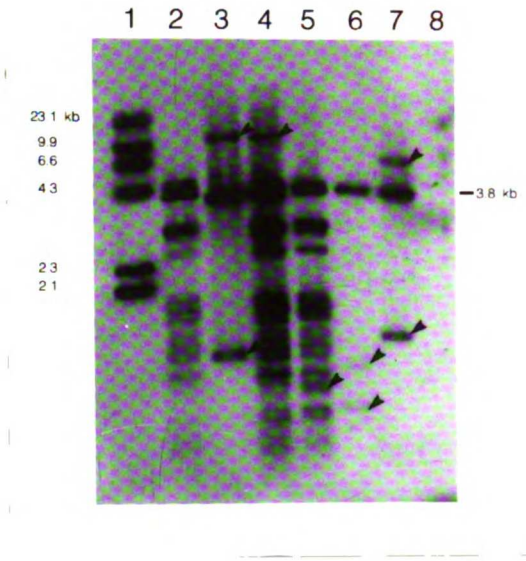


Fig. 11. Southern blot hybridization analysis of AvaI-digested chromosomal DNA from five independent Em^r transconjugants of P. gingivalis 33277[a].

[a] HindIII-digested biotinylated λ DNA (lane 1) was used for molecular sizing (numbers at left). The blot was probed with biotinylated R751::*Ω4 DNA. Lane 2 contains AvaI-digested R751::*Ω4 DNA and lane 8 contains AvaI-digested DNA from P. gingivalis 33277 (recipient control, no hybridization with R751::*Ω4). Lanes 3 through 7 contain AvaI-digested chromosomal DNA from five independent Em^r transconjugants of P. gingivalis. The 3.8 kb at the right indicates the position of the internal 3.8 kb AvaI fragment of Tn4351. Junction fragments between Tn4351 and the P. gingivalis chromosome are indicated by arrows (only one putative junction fragment is distinguishable in transconjugants with co-integrated R751 sequences; junction fragments in lane 6 are more apparent with prolonged exposure).

Discussion

A simple mating procedure was used to investigate the ability of conjugal and transpositional systems which function in colonic Bacteroides to function in P. gingivalis. Shoemaker et al. (173) have previously demonstrated that Tn4351 transposes in colonic Bacteroides. Plasmid R751, containing Tn4351, was transferred by conjugation from E. coli to colonic Bacteroides species. R751 is a conjugal broad-host-range IncP plasmid that can transfer itself from E. coli to Bacteroides but does not replicate in Bacteroides (173). Tn4351 is a composite transposon that contains two antibiotic-resistance genes flanked on either side by two directly repeated insertion sequences (Fig. 10); one gene codes for erythromycin resistance (Em^r) which is expressed in Bacteroides but not in E. coli and the other gene codes for tetracycline resistance which is expressed in aerobically grown E. coli but not in anaerobically grown E. coli or Bacteroides (175). Since R751 does not replicate in Bacteroides, Em^r transconjugants obtained from transferring R751 containing Tn4351 into Bacteroides from E. coli result from transposition of Tn4351 into the Bacteroides chromosome or from Tn4351-mediated cointegration of the plasmid. Southern blot hybridization analysis of Em^r transconjugants indicated that Tn4351 inserted into the Bacteroides chromosome at several different locations.

In this study R751::*Ω4 was used to maximize the chance of detecting transposition in P. gingivalis because it contains a partial duplication of Tn4351 and was found to transpose in colonic Bacteroides two- to four-fold more frequently than R751 containing single insertions of Tn4351 (173). The results obtained with R751::*Ω4 and P. gingivalis are very similar to those previously reported with colonic Bacteroides (173).

Both simple transpositions of Tn4351 and Tn4351-mediated cointegration of R751 into the P. gingivalis chromosome were observed. As reported with colonic Bacteroides, approximately half of the Em^r transconjugants of P. gingivalis (5 out of 10 analyzed) contained simple insertions of Tn4351 and half contained both Tn4351 and R751-derived sequences.

The varying sizes of the junction fragments observed in transconjugants with simple insertions of Tn4351 indicate that insertion of Tn4351 has occurred at several different locations in the P. gingivalis chromosome. Tn4351 may therefore be useful as a mutagen to generate well-defined mutants of P. gingivalis deficient in specific gene functions. Thus far six independent black-pigment deficient mutants of P. gingivalis 33277 have been isolated by screening approximately 20,000 Em^r transconjugants produced from matings with E. coli HB101 containing R751::*Ω4. Preliminary phenotypic characterization of four of these mutants indicate that they are also deficient in trypsin-like protease activity and hemagglutination activity (Table XIV). Therefore, they exhibit the same pleiotropic phenotype observed with the nitrosoguanidine-induced and spontaneous black-pigment deficient mutants of P. gingivalis 3079.03 previously described. It remains to be rigorously confirmed that the trypsin-like protease-deficient phenotype of these mutants is a consequence of the transposon insertions they have sustained. Transposon-induced mutants should not only be useful for studying the effect of specific gene products on the physiology and virulence of P. gingivalis but could also aid in the cloning of specific genes, since transposon insertion physically links the mutated gene to a DNA sequence which can be detected by DNA hybridization or

TABLE XIV. Trypsin-like protease activity and hemagglutination activity (HA) of black pigment-deficient mutants from matings between P. gingivalis 33277 and E. coli containing R751::*Ω4.

Strain	Trypsin-like protease activity[a]	Mean $\log_2$ reciprocal HA titer
33277	24.9 ± 5.2	9.0
TN6-5	8.43 ± 0.38	2.4
TN7-1	0.46 ± 0.05	0
TN7-3	0.08 ± 0.02	0
TN10-4	0.22 ± 0.04	0

[a] Specific activity was defined as units of trypsin-like protease activity per milligram wet weight of bacterial cells. One unit of trypsin-like protease activity was defined as the amount of enzyme required to release 1 μmol of p-nitroaniline per minute as described in Materials and Methods. Mean ± standard deviation of at least three independent experiments ($n \geq 3$).

selected in vivo by antibiotic resistance. Future studies will be directed towards characterizing the location of Tn4351 insertions in a collection of (> 20) trypsin-like protease-deficient mutants. In addition to assisting in cloning the trypsin-like protease structural gene, these mutants may also aid in the identification of associated regulatory and accessory genes.

Some strains of colonic Bacteroides also contain conjugal transposable elements which are thought to be chromosomal. These elements transfer either Tc^r and Em^r or Tc^r alone (133, 155) and can mobilize the transfer of co-resident plasmids (174, 211). One of these is Tc^r-ERL, a conjugal chromosomal transposable element originally observed in B. fragilis. Based on the results obtained with R751::*Ω4 and Tn4351, the ability of Tc^r-ERL to transfer itself and/or co-resident plasmids containing Em^r genes to P. gingivalis recipients was tested. No Tc^r or Em^r transconjugants were detected from matings between B. uniformis 1004 (Tc^r-ERL) and P. gingivalis or between B. fragilis TM4003 (Tc^r-ERL, pBFTM10) and P. gingivalis (data not shown). These results suggest that, with the current mating procedure, Tc^r-ERL is unable to efficiently transfer itself and/or mobilize co-resident plasmids from Bacteroides to P. gingivalis. pBFTM10 contains Tn4400, a composite transposon that is very similar to Tn4351 (133, 155). Based on our results with Tn4351, if pBFTM10 was efficiently transferred from Bacteroides to P. gingivalis one would expect to obtain Em^r transconjugants even in the absence of pBFTM10 replication, due to transposition of Tn4400 to the P. gingivalis chromosome.

Only one previous study has reported the conjugal transfer of DNA into P.

gingivalis. Progulske-Fox et al. (146) found that R751 could mobilize the transfer of plasmid pE5-2 from E. coli to P. gingivalis. pE5-2 (175) is a chimeric E. coli-Bacteroides shuttle vector constructed from pB8-51 (a cryptic colonic Bacteroides plasmid), the 3.8-kb EcoR1-D fragment of pBF4 (contains most of Tn4351), and RSF1010 (an IncQ E. coli plasmid). Em^r transconjugants occurred at a frequency of 2 x 10^{-7}. Dot blot hybridizations of DNA from 19 Em^r transconjugants with probes specific for Tn4351 and RSF1010 demonstrated that most of the transconjugants (14/19) contained both Tn4351 and RSF1010 sequences. Since pE5-2 contains an origin of replication (from pB8-51) that functions in Bacteroides and since the authors claimed that agarose gels of DNA extracts from transconjugants containing both Tn4351 and RSF1010 sequences indicated the presence of plasmid DNA they concluded that the majority of their Em^r transconjugants resulted from autonomous replication of pE5-2 in P. gingivalis. Some Em^r transconjugants (2/19) were observed that contained Tn4351 sequences but no RSF1010 sequences. Although these results are consistent with transposition of Tn4351, Progulske-Fox et al. (146) suggested that these transconjugants may have occurred by homologous recombination. However, they did not suggest which sequences on pE5-2 might have provided the homology to the P. gingivalis chromosome. Presumably transposition was not considered possible, since the 3.8-kb EcoR1-D fragment of pBF4, used in constructing pE5-2, is missing all but 9 base pairs of one insertion sequence ($IS4351_L$) as well as the distal inverted repeat of the remaining insertion sequence ($IS4351_R$) of Tn4351. We have also obtained Em^r transconjugants from matings with pE5-2 and P. gingivalis (data not shown); however we have been unable to demonstrate

the presence of autonomous plasmid. It would be interesting to determine if the 3.8 kb EcoRI-D fragment of pBF4, used in constructing pE5-2, is capable of promoting transposition and/or cointegration.

Strategies for Transformation of P. gingivalis by Electroporation

The use of an electric field to transiently render cells permeable to exogenous macromolecules (e.g. DNA) has been termed "electroporation" (127). Exposure of cells to an electric field causes cellular membrane components to become polarized and results in the development of a voltage potential across the cell membrane. When the potential difference exceeds a threshold level, localized areas of the cell membrane temporarily break down and the cell becomes transiently permeable to exogenous molecules (82). Electroporation has become a commonly used technique for the introduction of DNA into eukaryotic and prokaryotic cells. Electroporation has been demonstrated, in many instances, to be a more efficient means of transformation than traditional chemical methods (22, 33, 200). In addition, electroporation has been used to successfully transform a variety of Gram-positive and Gram-negative bacterial species that have not been transformed by chemical methods (171).

Molecular genetic studies of P. gingivalis have been hindered both by the lack of any naturally-occurring plasmids and/or bacteriophages and by the lack of any DNA transfer techniques. Phylogenetically, the colonic Bacteroides are the closest group to P. gingivalis in which DNA transfer techniques have been developed. Conjugation procedures (56, 145, 175, 223) and a chemical transformation procedure (184) have been

reported. At the onset of this investigation, electroporation techniques to transform colonic Bacteroides had not been described. Therefore, initial experiments were performed to assess the utility and efficiency of transformation of colonic Bacteroides by electroporation. Subsequently, similar electroporation conditions were used to attempt transformation of P. gingivalis with plasmids from colonic Bacteroides. In addition, single-stranded DNA was used in some electroporation experiments in an attempt to avoid restriction of heterologous DNA.

Methods

Bacterial strains, culture conditions, and preparation of bacterial cells for electroporation. The bacterial strains used are listed in Tables XV, XVI, XVII, and XVIII. P. gingivalis strains GA64 and EN7 are clinical isolates from patients at the UCSF Periodontology Clinic. The sources of the other bacterial strains and culture conditions for their propagation have been presented in previous sections. Preparation of bacterial cells for electroporation is described in Appendix {V}.

Plasmids. pBI191 (184) is a colonic Bacteroides cloning vector which contains a replication origin from pB143 (a cryptic 2.7 kb Bacteroides plasmid), an erythromycin resistance gene from pBF4 (a B. fragilis plasmid), and a multiple cloning site from pUC19. We constructed pBlueI191 (an E. coli-Bacteroides shuttle vector) from pBI191 and pBluescript (Stratagene, La Jolla, CA). In brief, pBI191 and pBluescript were digested with BamHI, ligated overnight, and electroporated into E. coli XL1-Blue. Ampicillin-resistant transformants were selected on L-TAXI agar (described previously).

Plasmids isolated from individual non-pigmented transformant colonies were digested with BamHI and analyzed by agarose gel electrophoresis for the presence of a 5.3 kb band (pBI191) and a 2.9 kb band (pBluescript). A restriction fragment map of pBlueI191 is shown in Fig. 12. pE5-2 (175) is an E. coli-Bacteroides shuttle vector which contains replication origins from p8-51 (a widely distributed cryptic 4.2 kb plasmid from colonic Bacteroides) and RSF1010 (an IncQ E. coli plasmid). pE5-2 also contains an erythromycin resistance gene from pBF4.

Preparation of single-stranded pBlueI191 and hybridization with M13-40 primer. pBluescript, and thus pBlueI191, contain an f1 filamentous phage origin of replication that allows recovery of single-stranded plasmid DNA when transformed E. coli XL1-Blue cells are co-infected with a helper phage (VCS-M13; Stratagene, La Jolla, CA). The procedure used for production and isolation of single-stranded DNA was essentially that suggested by the supplier. In brief, an early exponential phase culture of E. coli XL1-Blue (A_{660} = 0.3) containing pBlueI191 was infected with phage VCS-M13 at an multiplicity of infection of 20:1 (phage:bacteria) and incubated at 37°C for 8 h. The culture was then heated at 65°C for 15 min and centrifuged (10,000 x g, 4°C, 15 min) to remove bacterial cells and debris. Phage were precipitated in the presence of ammonium acetate and polyethylene glycol and harvested by centrifugation. The resulting phage pellet was resuspended in TE buffer and extracted with phenol-chloroform-isoamyl alcohol. Single-stranded DNA was then precipitated with ammonium acetate-isopropanol, washed 2X with cold 70% ethanol, air-dried, and redissolved in a minimal volume of 1X TE. The M13-40 primer was hybridized to single-stranded pBlueI191 (ss-pBlueI191) by heating a mixture

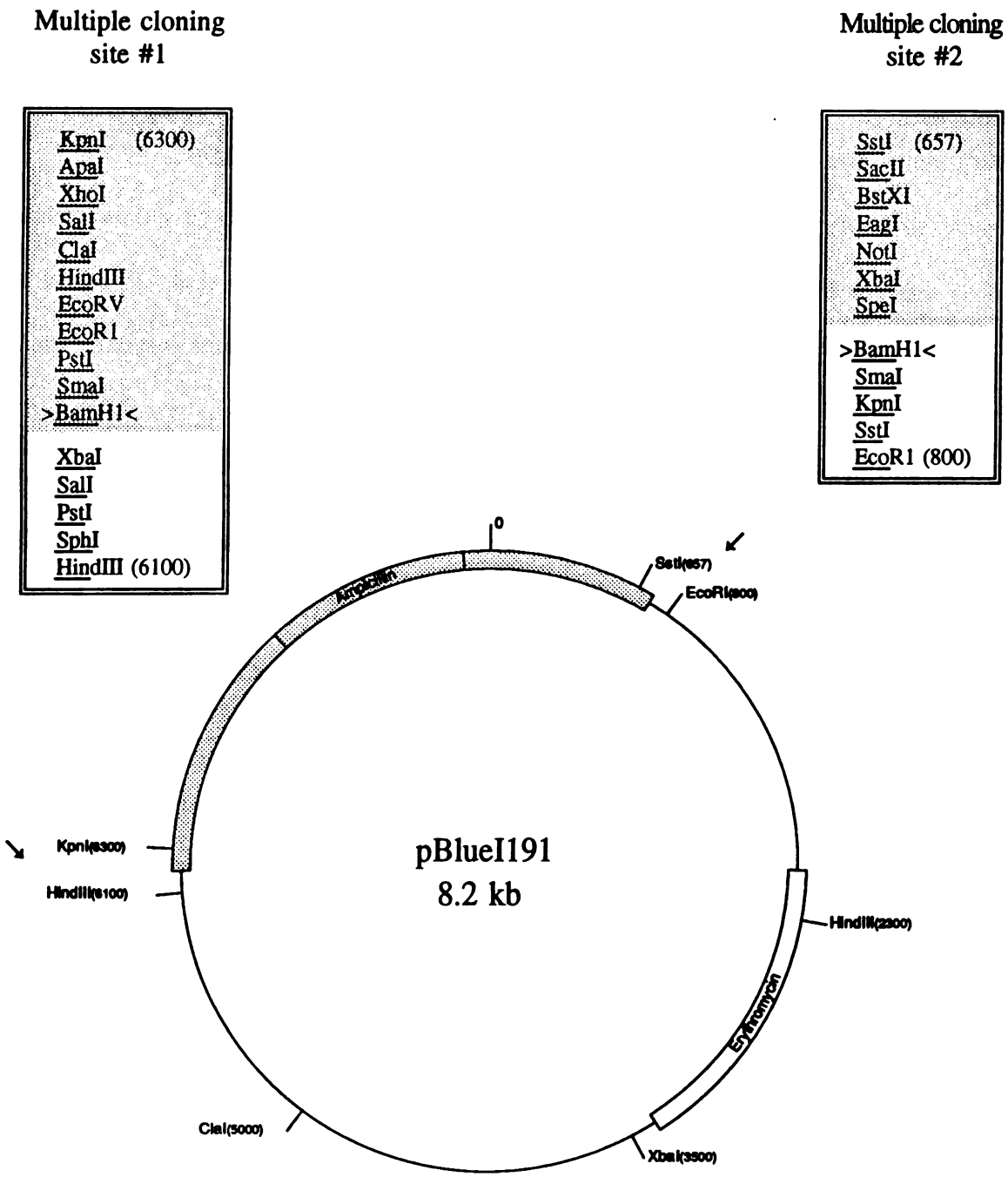


Fig. 12. Restriction map of pBlueI191[a].

[a] E. coli-Bacteroides shuttle vector constructed from pBluescript (shaded) and pBI191 by digestion with BamH1 followed by ligation. pBlueI192 was constructed from pBlueI191 by digestion with SstI, to remove multiple cloning site #2, followed by ligation (See E. coli and Colonic Bacteroides Plasmid Vector Cloning Systems).

of M13-40 primer (0.5 pmol) and ss-pBlueI191 (1 μg) at 65°C for 15 min and then cooling slowly to room temperature.

Electroporation procedures. Previous studies with E. coli (33) have found that transformability by electroporation is sensitive to both initial field strength of the pulse and to the RC time constant. Dower et al. (33) found that with a field strength of 7 kv/cm maximum transformability occurred at an RC time constant of 20 msec and that with a field strength of 11 kv/cm maximum transformability occurred at an RC time constant of 5 msec. However, the same maximum level of transformants was obtained with either set (field strength and RC time constant) of electroporation conditions. In the present study field strength (12.5 kv/cm) and capacitance (25 μF) were held constant. To determine efficient electroporation conditions for transformation of B. fragilis, resistance was varied (100 to 400 Ω)to obtain RC time constants between 2.5 to 12.5 msec. The general electroporation procedure has been described in a previous section (E. coli and Colonic Bacteroides Plasmid Vector Cloning Systems).

Results

Electroporations with pBI191. Table XV summarizes the results of electroporations with pBI191. In initial experiments pBI191, isolated from B. fragilis 638, was used to determine suitable electroporation conditions for transformation of B. fragilis 638. At an RC time constant of 2.4 msec the frequency of transformants was 2.3 x 10^{-5}. Increasing the RC time constant to 4.6 and 8.4 msec increased the frequency of transformants by approximately 5-fold and 10-fold respectively, compared to an RC time

Table XV. Summary of electroporations with pBI191.

Plasmid[a]	Recipient	RC Time Constant	Transformants/ug DNA	Frequency[b]
pBI191	B. fragilis 638	2.4	3.2×10^{4}	2.3×10^{-5}
pBI191	B. fragilis 638	4.6	1.9×10^{5}	1.1×10^{-4}
pBI191	B. fragilis 638	8.4	1.8×10^{4}	1.9×10^{-4}
pBI191	B. fragilis 638	12.3	1.2×10^{3}	4.6×10^{-5}
pBI191	P. gingivalis 33277	2.4	0	0
pBI191	P. gingivalis 33277	2.4	0	0
pBI191	P. gingivalis 32277	2.4	0	0
pBI191	P. gingivalis 33277	4.4	0	0
pBI191	P. gingivalis 33277	4.7	0	0

[a] Isolated from B. fragilis 638.

[b] Frequency of transformants per surviving recipient cell.

constant of 2.4 msec. Increasing the RC time constant to 12.3 msec resulted in a decrease in the frequency of transformants. No transformants were obtained from six electroporations of P. gingivalis 33277 with pBI191 (RC time constants ranging from 2.4 to 4.7 msec).

Electroporations with pBlueI191. Table XVI summarizes the results of electroporations with pBlueI191 isolated from E. coli XL1-Blue. Electroporation of E. coli XL1-Blue with pBlueI191, at an RC time constant of 1.7, produced a high frequency of transformants (5.3×10^{-3}). Electroporation of B. uniformis 1001 and B. fragilis 638 at RC time constants of 2.4 and 4.5, respectively, resulted in more than a 1,000-fold decrease in the frequency of transformants (B. uniformis 1001, 4.4×10^{-6}; B. fragilis 638, 8.3×10^{-8}) compared to E. coli XL1-Blue. No transformants were obtained from three electroporations of P. gingivalis 33277 with pBlueI191 (RC time constants ranging from 2.4 to 7.7 msec).

Electroporations with ss-pBlueI191. Table XVII summarizes the results of electroporations with ss-pBlueI191. The frequency of transformants obtained by electroporation of E. coli XL1-Blue with ss-pBlueI191 was 6.9×10^{-6} (RC time constant of 2.4 msec). Hybridization of ss-pBlueI191 with M13-40 primer, prior to electroporation, increased the frequency of transformants to 1.4×10^{-4}, presumably by enhancing the efficiency of conversion of single-stranded to double-stranded plasmid DNA inside transformed cells. Electroporation of colonic Bacteroides with ss-pBlueI191 and with ss-pBlueI191 hybridized with M13-40 primer produced transformants at relatively low frequencies (4.2×10^{-8} to 5.4×10^{-9}). No transformants were obtained from

Table XVI. Summary of electroporations with pBlueI191.

Plasmid[a]	Recipient	RC Time Constant	Transformants/ug DNA[b]	Frequency[c]
pBlueI191	E. coli XL1-Blue	1.7	3.1×10^{6}	5.3×10^{-3}
pBlueI191	B. uniformis 1001	2.4	2.1×10^{4}	4.4×10^{-6}
pBlueI191	B. fragilis 638	4.5	1.0×10^{2}	8.3×10^{-8}
pBlueI191	P. gingivalis 33277	2.4	0	0
pBlueI191	P. gingivalis 33277	4.3	0	0
pBlueI191	P. gingivalis 33277	7.7	0	0

[a] Isolated from E. coli XL1-Blue.
[b] Transformants selected by ampicillin resistance in E. coli and by erythromycin resistance in Bacteroides and Porphyromonas.
[c] Frequency of transformants per surviving recipient cell.

Table XVII. Summary of electroporation with single-stranded pBlueI191.

Plasmid[a]	Recipient	RC Time Constant	Transformants/ug DNA[b]	Frequency[c]
ss-pBlueI191	E. coli XL1-Blue	2.4	5.6×10^{3}	6.9×10^{-6}
ss-pBlueI191	B. uniformis 1001	2.0	5.0×10^{1}	4.2×10^{-8}
ss-pBlueI191	P. gingivalis 33277	2.4	0	0
ss-pBlueI191[d]	E. coli XL1-Blue	2.4	7.9×10^{4}	1.4×10^{-4}
ss-pBlueI191[d]	B. fragilis 638	4.6	2.0×10^{1}	5.4×10^{-9}
ss-pBlueI191[d]	P. gingivalis 33277	2.4	0	0

[a] Single-stranded pBlueI191 isolated from E. coli XL1-Blue.
[b] Transformants selected by ampicillin resistance in E. coli and by erythromycin resistance in Bacteroides and Porphyromonas.
[c] Frequency of transformants per recipient cell.
[d] Hybridized with M13-40 primer prior to electroporation.

electroporations of P. gingivalis 33277 with ss-pBlueI191 or with ss-pBlueI191 hybridized with M13-40 primer.

Electroporations with a mixture of pE5-2 and R751. Table XVIII summarizes the results of electroporations with a mixture of pE5-2 and R751. Electroporation of E. coli XL1-Blue with a mixture of pE5-2 and R751 resulted in a moderate frequency of R751 transformants (2.5×10^{-5}). Electroporation of colonic Bacteroides with a mixture of pE5-2 and R751 resulted in low frequencies of pE5-2 transformants (4.5×10^{-8} to 7.9×10^{-8}). No transformants were obtained from electroporations of four different strains of P. gingivalis with a mixture of pE5-2 and R751.

Discussion

Since at the onset of this study transformation of colonic Bacteroides by electroporation had not been reported, initial experiments were directed at determining suitable electroporation conditions for transformation of colonic Bacteroides before attempting to transform P. gingivalis. With pBI191, isolated from B. fragilis 638, a moderately high number of transformants (1.2×10^{3} to 1.9×10^{5}) was obtained with B. fragilis 638 over a range of RC time constants (2.4 to 12.3 msec). The frequency of transconjugants increased as the RC time constant increased from 2.4 to 8.4 msec. However, at an RC time constant of 12.3 msec both the number of transformants and the frequency of transformants decreased. The use of high RC time constants ($\geq$ 10 msec) occasionally resulted in arcing between the electrodes in the electroporation cuvettes. Therefore most electroporations were conducted at RC time constants < 10 msec.

Table XVIII. Summary of electroporations with a mixture of pE5-2 and R751[a].

Plasmid	Recipient	RC Time Constant	Transformants/ug DNA[b]	Frequency[c]
pE5-2 & R751	E. coli XL1-Blue	0.5	4.7×10^{4}	2.5×10^{-5}
pE5-2 & R751	B. fragilis 638	2.4	1.0×10^{2}	4.5×10^{-8}
pE5-2 & R751	B. uniformis 1001	2.4	3.0×10^{2}	7.9×10^{-8}
pE5-2 & R751	P. gingivalis 33277	2.4	0	0
pE5-2 & R751	P. gingivalis 3079.03	2.4	0	0
pE5-2 & R751	P. gingivalis GA64	2.0	0	0
pE5-2 & R751	P. gingivalis EN7	2.4	0	0

[a] Isolated from a derivative of E. coli HB101 that contains both pE5-2 and R751.

[b] Transformants selected by the trimethoprim resistance of R751 in E. coli and by the erythromycin resistance of pE5-2 in Bacteroides and Porphyromonas.

[c] Frequency of transformants per surviving recipient cell.

Isolation of sufficient amounts of pBI191 from B. fragilis 638 was tedious. This may have been due to low copy number of pBI191 and/or inefficient plasmid isolation from B. fragilis. To overcome this difficulty, a chimeric shuttle vector (pBlueI191) was constructed from pBI191 and pBluescript. This permitted the rapid isolation of large amounts of plasmid from E. coli since replication of pBlueI191 in E. coli was dependent on the pBluescript replication origin. Electroporation of E. coli XL1-Blue with pBlueI191 propagated in E. coli resulted in a high frequency of transformants (5.3×10^{-3}), however the frequency of transformants with colonic Bacteroides recipients was about 1,000-fold less. These results are similar to those described in two recent reports concerning transformation of colonic Bacteroides by electroporation. Thomson and Flint (206) used electroporation, under anaerobic conditions, to transform B. uniformis with pDP1 (a 19 kb E. coli-Bacteroides shuttle vector). When pDP1 was isolated from B. uniformis, they obtained up to 10^6 transformants per μg of DNA. Smith et al. (185) used a variety of plasmids isolated from colonic Bacteroides to transform several different strains of colonic Bacteroides. At an RC time constant of 5 msec up to 1.8×10^6 transformants per μg DNA were obtained with B. fragilis 638 and pBI191. Increasing the RC time constant up 10 msec increased the number of transconjugants by about 4-fold. However, as observed in the present investigation, occasional arcing between electrodes was reported with RC time constants $\geq$ 10 msec. Also as observed in the present study, both Smith et al. (185) and Thomson and Flint (206) reported approximately a 1,000-fold reduction in the number of transformants obtained with colonic Bacteroides when plasmids were isolated from E. coli rather than from homologous colonic Bacteroides strains. It is likely

that differences in host-controlled restriction/modification systems between E. coli and colonic Bacteroides strains account for the observed decrease in efficiency of transformation.

The presence of an even greater restriction barrier in P. gingivalis is one factor that could contribute to the inability to transform P. gingivalis with pBI191, pBlueI191, and pE5-2. To circumvent and/or limit the effect of restriction, ss-pBlueI191 was used to attempt transformation of P. gingivalis. It was reasoned that since single-stranded DNA is transferred during conjugation and since conjugation is usually not affected by restriction, then electroporation with single-stranded DNA might result in successful transformation of P. gingivalis. Although transformants of both E. coli and colonic Bacteroides were obtained with ss-pBlueI191 no transformants of P. gingivalis were detected. Transformation frequency of E. coli with ss-pBlueI191 was approximately 1,000-fold lower than with double-stranded pBlueI191. One possible explanation for this large decrease in transformation efficiency could be that ss-pBlueI191 DNA was not being efficiently converted to double-stranded DNA after electroporation. In subsequent experiments, the M13-40 primer was hybridized to ss-pBlueI191 to provide a primer for conversion, after electroporation, of ss-pBlueI191 into double-stranded pBlueI191. This increased the frequency of transformants obtained with E. coli XL1-Blue by a factor of 20. Electroporation with primer-hybridized ss-pBlueI191 DNA also yielded transformants of colonic Bacteroides, but none were obtained with P. gingivalis.

Another reason for the inability to obtain transformants of P. gingivalis with pBI191 and pBlueI191 could be that the replication origin of pBI191 does not function

in P. gingivalis. However, this would not explain the inability to transform P. gingivalis with pE5-2 by electroporation, since the replication origin of pE5-2 (from the cryptic Bacteroides plasmid p8-51) has been reported to function in P. gingivalis (146). Other factors, besides restriction and the presence of a functional replication origin, may have contributed to the inability to transform P. gingivalis with pE5-2 by electroporation. Smith et al. (185) have reported that plasmid size influences the efficiency of transformation by electroporation. Electroporation of Bacteroides with pBFTM10 (a 14 kb plasmid) resulted in 95% fewer transformants than electroporation with pBI191 (a 5.4 kb plasmid). In the present study, the results obtained with R751 (a 51.4 kb plasmid) and pBlueI191 (an 8.3 kb plasmid) also suggest that plasmid size influences the efficiency of transformation by electroporation. Electroporation of E. coli with R751 resulted in approximately a 200-fold decrease in the frequency of transformants compared to electroporation with pBlueI191. However, the fact that transformants were obtained with pE5-2 and colonic Bacteroides suggests that plasmid size is probably not the primary reason for the inability to transform P. gingivalis with pE5-2.

The electroporation conditions used with P. gingivalis were based on results obtained with B. fragilis. It is possible, but not likely, that these conditions may not be suitable for transiently rendering the membranes of P. gingivalis permeable to DNA.

Future studies will be directed towards the construction and use of pPE1-based vectors to attempt transformation of P. gingivalis by both conjugation and electroporation (see Genetic Studies of a Naturally Occurring Plasmid from P. endodontalis).

Genetic Studies of a Naturally Occurring Plasmid from P. endodontalis.

The development of genetic tools, such as shuttle vectors, is vital for examining the role of specific gene products in the physiology and virulence of Porphyromonas species. Shuttle vectors are chimeric plasmids that can replicate in two different organisms because they contain two DNA replication regions, one from each organism. The construction of an E. coli-Porphyromonas shuttle vector has been hindered by an apparent lack of naturally occurring plasmids in Porphyromonas species (32, 154, 213).

As a preliminary step in the construction of an E. coli-Porphyromonas shuttle vector a unique 4.9 kb plasmid (pPE1) from P. endodontalis ATCC 27067 was restriction mapped and subcloned. Yoshimoto and Umemoto (234) were the first to report the presence of a plasmid, which they named pYHBA1, in strain ATCC 27067. No further studies of this plasmid have appeared to date. Strain ATCC 27067, formerly Bacteroides asaccharolyticus, has recently been reclassified as P. endodontalis (based on unpublished data submitted to the ATCC by Dr. W.E.C. Moore). To conform with the new taxonomy and to indicate the original source of this plasmid we refer to it as pPE1.

Methods

Bacterial strains & culture conditions. P. endodontalis ATCC 27067 was obtained from the American Type Culture Collection (Rockville, MD) and was maintained on LRBB agar. For large-scale plasmid isolation, P. endodontalis ATCC 27067 was sequentially cultured three times in HS-sTSB broth (Trypticase soy broth supplemented with 5% horse serum, 0.25% yeast extract, 2.5 μg/ml hemin, 5 μg/ml menadione, and 0.01% DTT). In brief, several bacterial colonies from an LRBB plate (incubated

anaerobically at 37°C for 3-5 days) were collected on a cotton swab, resuspended in 5 ml HS-sTSB, and incubated anaerobically overnight at 37°C. Two ml of the overnight broth culture was inoculated into 50 ml of HS-sTSB and incubated anaerobically at 37°C for 18-36 h. Twenty ml of the 50 ml broth was then inoculated into 500 ml of HS-sTSB and incubated anaerobically at 37°C for 18-36 h (A_{660} = 0.8 to 1.2). E. coli XL1-Blue (Stratagene, La Jolla, CA) was maintained on Luria-Bertani (LB) agar containing 10 µg/ml tetracycline. pBluescript (Stratagene, La Jolla, CA) was used to clone pPE1 sequences in E. coli XL1-Blue.

Plasmid isolation. Plasmid was extracted from P. endodontalis ATCC 27067 by the NaCl-SDS procedure of Godson and Vapnek (49) and purified by CsCl-ethidium bromide equilibrium density gradient centrifugation.

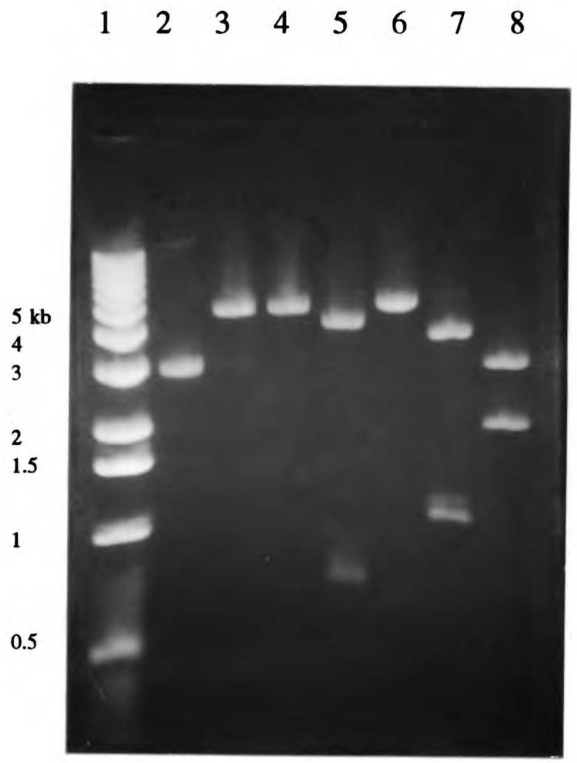
Restriction endonuclease digestion & agarose gel electrophoresis. In general, 0.25 to 0.5 µg of purified plasmid DNA was digested with 10 units of each restriction endonuclease for 1 h at 37°C in 20 µl of 1X buffer supplied by the manufacturer. The size of the resulting DNA restriction fragments was estimated by agarose gel electrophoresis. DNA fragments larger than 500 base pairs were separated by electrophoresis in 1% agarose gels submerged in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). DNA fragments smaller than 500 base pairs were separated by electrophoresis in 4% agarose gels submerged in TAE buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 8.0). A 1 kb DNA ladder (Gibco BRL, Gaithersburg, MD) was used as a standard for estimating the size of the DNA restriction fragments.

Cloning of pPE1 in pBluescript. Purified pPE1 and pBluescript were digested

with the restriction endonucleases listed in Table XIX, precipitated with isopropanol, washed twice with cold 70% ethanol, air dried, and redissolved in 20 µl of 1X ligase buffer. Following overnight ligation at 12°C, the ligation mixture was precipitated and washed as above, except that the DNA was redissolved in 20 µl of water. Ten µl was then used to transform E. coli XL1-Blue by electroporation. Transformants were plated on L-TAXI agar (described previously) to allow blue-white screening of transformants. Several non-pigmented (white) transformants from each ligation were subcultured and evaluated by restriction endonuclease digestion and agarose gel electrophoresis to detect the presence of pBluescript containing inserts of pPE1 DNA segments.

Results

Isolation of pPE1 and restriction mapping. pPE1 appears to be a relatively high-copy number plasmid in that good yields were routinely obtained from P. endodontalis both with miniprep (5 ml) and maxiprep (500 ml) plasmid isolation procedures. Fig. 13 depicts a 1% agarose gel of pPE1 in its intact conformation and also digested with three different restriction endonucleases, individually and in pairs. Lane 2 shows one DNA band migrating at an apparent molecular size of 3.0 kb representative of uncut supercoiled pPE1. Plasmid pPE1 contains one restriction site for ClaI (Lane 3), BamHI (Lane 4), and SacI (Lane 6). A few examples of double digests of pPE1 are also shown in Fig. 13. Lane 5 shows two bands (0.8 and 4.1 kb) obtained with digestion by ClaI and BamHI, Lane 7 shows two bands (1.1 and 3.8 kb) obtained with ClaI and SacI, and Lane 8 shows two bands (1.9 and 3.0 kb) obtained with BamHI and SacI. These results indicate that

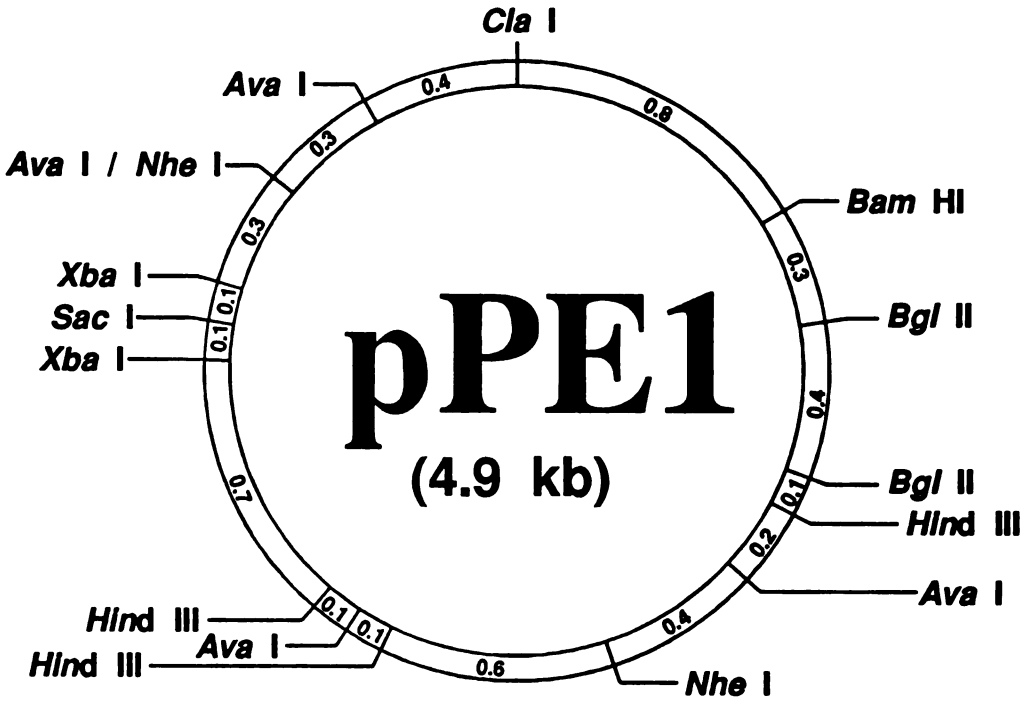


Lane 1	1 kb ladder	
Lane 2	pPE1 (unrestricted)	3.0 kb
Lane 3	pPE1 (ClaI)	4.9 kb
Lane 4	pPE1 (BamHI)	4.9 kb
Lane 5	pPE1 (ClaI & BamH1)	0.8 & 4.1 kb
Lane 6	pPE1 (SacI)	4.9 kb
Lane 7	pPE1 (ClaI & SacI)	1.1 & 3.8 kb
Lane 8	pPE1 (BamH1 & SacI)	1.9 & 3.0 kb

Fig. 13. Agarose gel electrophoresis of purified pPE1, before and after restriction endonuclease digestion.

the size of pPE1 is 4.9 kb. To construct a more detailed restriction endonuclease map, pPE1 was digested with a battery of eight restriction endonucleases, used individually, and in combinations of two. The size of the resulting fragments was determined by agarose gel electrophoresis, and an internally consistent restriction map of pPE1 was deduced (Fig. 14). Sixteen restriction sites were mapped: pPE1 contains one restriction site for ClaI, BamHI, and SacI; two restriction sites for BglII, NheI, and XbaI; three restriction sites for HindIII; and four restriction sites for AvaI.

Cloning of pPE1 in pBluescript. Attempts were made to clone all or part of pPE1 into pBluescript. Plasmid pPE1 was digested with BamHI, ClaI, BamHI plus ClaI, or ClaI plus SacI and ligated into pBluescript digested with the corresponding restriction endonucleases. Plasmid preparations from $\geq$10 non-pigmented transformant colonies from each ligation were digested with the appropriate restriction endonucleases and the resulting DNA fragments were analyzed by agarose gel electrophoresis. As shown in Table XIX, initial attempts to obtain a chimeric plasmid containing the entire 4.9 kb of pPE1 were unsuccessful. From experiments using double-digested (ClaI & BamHI) pPE1 and pBluescript, only the 0.8 kb ClaI-BamHI fragment of pPE1 was obtained (8 out of 20 transformants analyzed). In subsequent experiments with ClaI-SacI, only the 3.8 kb ClaI-SacI fragment of pPE1 was obtained (9 out of 20 transformants analyzed). One transformant from ligation 10/31-B (Table XIX), containing the 3.8 kb ClaI-SacI fragment of pPE1 inserted into the ClaI-SacI site of pBluescript, was designated pBlueEndo1 and propagated for further study. The restriction endonuclease fragment map of pBlueEndo1 (6.8 kb) is shown in Fig. 15.



Restriction endonuclease	Number of sites	Size of fragments
ClaI	1	4.9
BamHI	1	4.9
Sac I	1	4.9
BglII	2	0.4, 4.5
NheI	2	2.0, 2.9
XbaI	2	0.2, 4.7
HindIII	3	0.2, 1.2, 3.5
AvaI	4	0.3, 1.1, 1.3, 2.2

Fig. 14. Restriction map of pPE1.

Table XIX. Summary data for cloning pPE1 sequences in pBluescript.

Ligation	Restriction sites used	Total transformants obtained	fraction of analyzed transformants which contain pPE1 DNA
9/16	BamHI	4.2×10^4 (10)[a]	0/10
11/1	ClaI	9.0×10^4 (1.2×10^4)[a]	0/15
10/31-A	ClaI & BamHI	3.6×10^4 (3.6×10^4)[a]	8/20[b]
10/31-B	ClaI & SacI	4.0×10^4 (2.0×10^4)[a]	9/20[c]

[a] Number of non-pigmented transformants.
[b] All eight of these transformants contained the 0.8 kb ClaI-BamHI fragment of pPE1.
[c] All nine of these transformants contained the 3.8 kb ClaI-SacI fragment of pPE1.

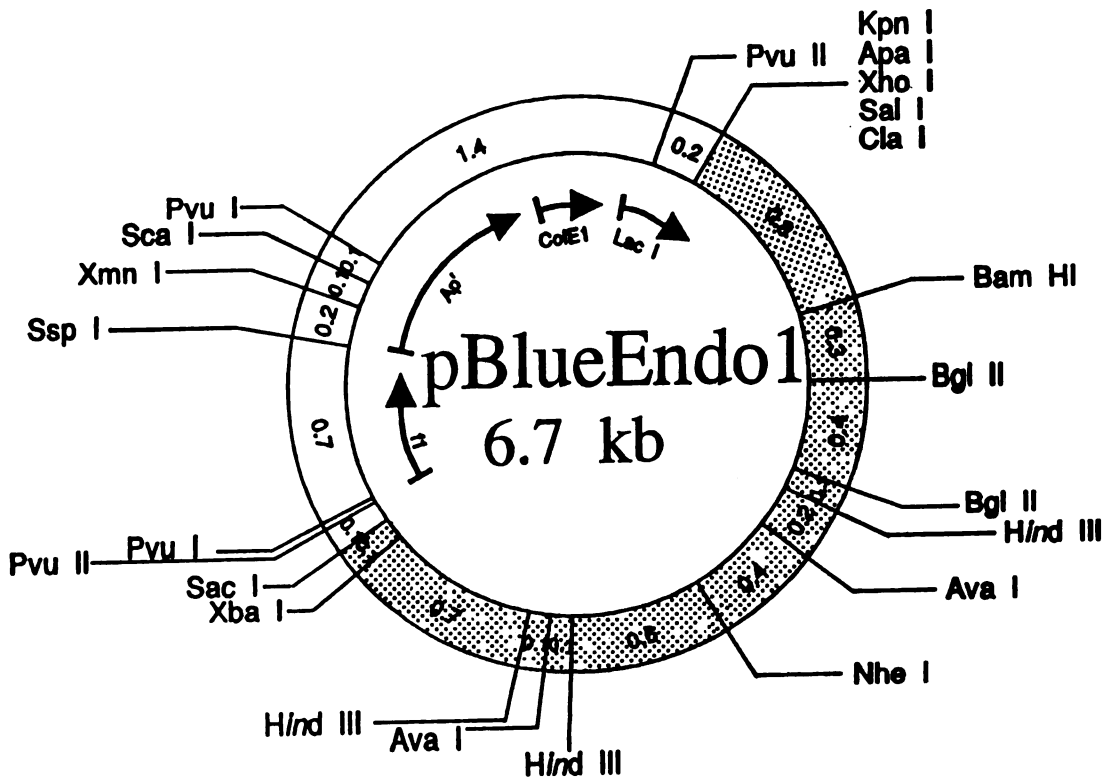


Fig. 15. Restriction map of pBlueEndo1[a].

[a] Shaded area is 3.8 kb ClaI-SacI fragment of pPE1. Open area is 2.9 kb ClaI-SacI fragment of pBluescript.

Discussion

To date, pPE1 (formerly pYHBA1) from P. endodontalis ATCC 27067 is the only known naturally occurring plasmid that has been found in the genus Porphyromonas. Because of its relatively small size (4.9 kb) and its apparent high copy number, pPE1 may serve as a replicon for the construction of a Porphyromonas cloning vector and an E. coli-Porphyromonas shuttle vector. In order to clone full-lenght pPE1 and/or pPE1 fragments, a restriction map of pPE1, with sixteen restriction sites for eight restriction endonucleases was determined. Initial attempts to obtain chimeric plasmid(s) containing the entire 4.9 kb sequence of pPE1 were unsuccessful; therefore subsequent experiments utilized double-digested pPE1 and pBluescript. The results of these experiments, summarized in Table XIX, suggest that pPE1 contains DNA sequences that are toxic for E. coli. These toxic sequences appear to be located somewhere within the 1.1 kb ClaI-SacI fragment of pPE1, since no transformants containing this region of pPE1 were obtained (0 out 65 analyzed). A chimeric plasmid containing the 3.8 kb ClaI-SacI fragment of pPE1 was designated pBlueEndo1. Present efforts are directed toward inserting the erythromycin resistance gene from Tn4351 into pBlueEndo1. This would provide a selectable marker necessary to attempt transformation of Porphyromonas species by electroporation. This approach may prove unsuccessful since the position of the origin of DNA replication of pPE1 is unknown it may not be present in pBlueEndo1. In addition, the 1.1 kb ClaI-SacI fragment of pPE1 may contain part or all of a gene which is essential for the maintenance of pPE1 in Porphyromonas species. DNA sequence analysis of pPE1 is in progress to determine the location of open reading frames (genes) and of putative nonessential regions

that may serve as potential cloning sites. These studies may help locate the DNA replication origin of pPE1, as well as provide valuable information regarding gene organization and promoter structure in Porphyromonas.

SUMMARY

The accumulation of scientific knowledge is an ongoing process, thus the results of any investigation pose areas for future inquiry. The results of the investigations described in this dissertation and afford insights into the function of the trypsin-like protease in the physiology and virulence of P. gingivalis and provide a foundation for in vivo assessment of the importance of the trypsin-like protease in periodontal pathogenesis.

Initially a nitrosoguanidine mutagenesis procedure and a trypsin-like protease activity screening technique were developed to isolate trypsin-like protease-deficient mutants of P. gingivalis. Quantitative assays of trypsin-like protease activity demonstrated that these mutants contained less than 2% of the trypsin-like protease activity of their parent strain, and SDS-PAGE zymogram techniques indicted that the major fraction of the proteolytic activity observed in zymograms was due to a single trypsin-like protease. Phenotypic characterization of these mutants indicated that, in addition to being deficient in trypsin-like protease activity, they exhibited significantly lower hemagglutination activity, diminished adherence to Actinomyces viscosus, and dramatically reduced black pigmentation. Subsequent studies with inhibitors and enhancers of trypsin-like protease activity using wild-type P. gingivalis also indicated a correlation between trypsin-like protease activity and hemagglutination, as well as between trypsin-like protease activity and adherence to A. viscosus. Spontaneously occurring black pigment-deficient mutants of P. gingivalis and black pigment-deficient mutants containing chromosomal insertions of transposon Tn4351 were also found to be

deficient in trypsin-like protease activity and in hemagglutination activity. Therefore, it appears that three important virulence characteristics of P. gingivalis – trypsin-like protease activity, hemagglutination activity (adherence), and accumulation of heme (black pigment) – are physiologically linked, genetically co-regulated, or the result of a single multifunctional gene product.

It is hypothesized that the trypsin-like protease of P. gingivalis may function as a "dynamic adhesin". Protease-substrate interactions can be simplistically divided into three steps; substrate binding, substrate hydrolysis, and product release. Each step is associated with its own reaction rate constant. If the rate constant for substrate-binding is comparable to the rate constants for substrate hydrolysis and product release, each protease molecule will spend a significant fraction of its time bound to substrate. Each P. gingivalis cell is likely to contain hundreds or thousands of surface associated trypsin-like protease molecules, many of which are likely to be in the bound state at any given moment. These could mediate attachment of P. gingivalis to receptor molecules. The proposed dynamic adherence mechanism could be of ecological benefit to P. gingivalis, since this bacterium requires amino acids and/or peptides for growth. As long as protein is available, the trypsin-like protease would maintain contact between the bacterium and substrate, as well as release peptides necessary for growth. When the protein substrate has been completely consumed the bacterium would be free to locate a new protein source.

The results obtained with R751::*Ω4, an E. coli-Bacteroides suicide vector that contains Tn4351, represent the first documentation of transposition in P. gingivalis. Since

Southern blot hybridization analysis indicated insertion of Tn4351 at different chromosomal locations in independent transconjugants, it is likely that Tn4351 can be used as a mutagen to inactivate specific genes by insertion and to isolate well-defined mutants of P. gingivalis. In addition to studying the effect of specific mutations on the physiology and virulence of P. gingivalis, transposon-induced mutants will aid in the cloning specific genes. Future efforts will be directed toward analyzing the sites of Tn4351 insertions in a collection of trypsin-like protease-deficient erythromycin resistant mutants isolated from matings between E. coli containing R751::*Ω4 and P. gingivalis. In addition to assisting in cloning the trypsin-like protease gene, analysis of these mutants may identify accessory genes involved in the regulation and expression of trypsin-like protease activity.

To date pPE1, isolated from P. endodontalis 27067, is the only known naturally occurring plasmid from Porphyromonas species. Future efforts will be directed towards the use of pPE1 to construct a Porphyromonas cloning vector and an E. coli-Porphyromonas shuttle vector. The development of these vectors should allow the cloning of Porphyromonas genes by complementation of appropriate mutants, as well as aid in studying the effect of specific mutations in cloned P. gingivalis genes.

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APPENDIX

{I}. SIZE SELECTION OF Sau3A-DIGESTED DNA BY SUCROSE DENSITY GRADIENT CENTRIFUGATION.

1) Layer 6.0 ml of 40% sucrose/TE-NaCl solution into bottom of Beckman polyallomer ultracentrifuge tube (#331372).

2) Carefully overlay with 6.0 ml of 10% sucrose/TE-NaCl solution.

3) Seal top of tube with Parafilm. Gently lay tube in secure horizontal position. Leave at room temperature for 2 h to form gradient.

4) Gently return tube in vertical position.

5) Carefully layer 0.2 ml of partially digested DNA sample (approx. 200 µg) onto top of gradient.

6) Centrifuge in SW41Ti rotor at 28,000 rpm (100,000 g) for 18 h @ 20° C.

7) Fractionate contents of tube into 500 µl aliquots.

8) Determine DNA fragment size(s) in each fraction by agarose gel electrophoresis (3-5 kb DNA usually in fractions 10 to 20).

9) Pool fractions containing appropriate sizes, precipitate with isopropanol, and resuspend in 100 µl of 1X TE, pH 8.0.

10% sucrose/TE-NaCL solution
0.24 g Tris (20 mM final conc.)
0.19 g disodium EDTA (5 mM final conc.)
5.8 g NaCl (1 M final conc.)
10 g sucrose
Dissolve in 60 ml distilled water
Adjust pH to 8.0 with HCl
Bring up to 100 ml and store @ 4°C

40% sucrose/TE-NaCL solution
Same as above except with 40 g of sucrose.

{II}. PURIFICATION OF PLASMID DNA BY CsCl-ETHIDIUM BROMIDE EQUILIBRIUM DENSITY GRADIENT CENTRIFUGATION

1) Dissolve 4.2 g of CsCl in 4.2 ml of DNA solution in TE buffer (10/1, pH 8.0).

2) Add 0.2 ml of 10 mg/ml ethidium bromide. Mix well. Density should be approximately 1.55 g/ml.

3) Pipet DNA/CsCl/EtBr solution into Beckman quick-seal ultracentrifuge tubes (#342412-polyallomer). Full volume for tubes is 5.3 ml.

4) Seal tubes with Beckman quick-sealer and recheck density.

5) Place tubes in VTi65.2 Beckman rotor, insert spacers, and torque plugs to 120 in.-lbs.

6) Centrifuge at 60,000 rpm (325,000 g) for 5 h at 20° C. Use slow deceleration (brake to 800 rpm).

7) After ultracentrifugation support tube with clamp, insert needle into top of tube to vent, and illuminate with UV light to visualize DNA bands.

8) Puncture tube with 1 ml syringe just below lower DNA (plasmid) band and withdraw plasmid band (approximately 0.5 to 1 ml).

9) Remove ethidium bromide from plasmid band by sequential extraction (3X) with n-butanol (saturated with water).

10) Dialyze plasmid DNA sample overnight vs. 2 liters of TE (10/1, pH 8.0).

11) Run agarose gel of portion of plasmid DNA sample to determine yield and purity.

{III}. TREATMENT OF RESTRICTED DNA WITH CALF INTESTINAL ALKALINE PHOSPHATASE

1) Dissolve ethanol-washed DNA (5 to 20 µg) in 50 µl 1X CIAP buffer.
2) Add 1 unit of calf intestinal alkaline phosphatase per 100 pmol ends of DNA. (1 µg of restricted pBluescript {3 kb} = 1 pmol of 5' termini)
3) Incubate at 37°C for 30 min.
4) Add EDTA to final concentration of 5 mM and incubate at 70°C for 30 minutes.
5) Cool to room temperature, phenol-chloroform extract (1X), precipitate with ammonium acetate/isopropanol, wash 2X with cold 70% ethanol, and dissolve in 50µl distilled H_2O.

10X CIAP Buffer

500 mM TRIS, pH 8.0
10 mM $MgCl_2$
10 mM $ZnCl_2$
Sterilize by autoclaving

{IV}. LIGATION WITH T4 DNA LIGASE

For 50 µl of 1X ligation buffer containing 1 to 10 µg DNA
Add 2 µl of 25X ATP
Add 2 µl of 25X DTT
Add 1 U T4 DNA ligase per µg of DNA
Incubate @ 12° C for 16 h
Stop reaction by heating @ 70° C for 15 min
Precipitate DNA with ammonium acetate-isopropanol, wash 2X with 70% ethanol, air dry, and dissolve in minimal volume distilled H_2O.

10X T4 DNA Ligase Buffer
500 mM Tris, pH 7.5
100 mM $MgCl_2$
500 µg/ml BSA
Filter sterilized & stored at 4°C

25X DTT / 10% DTT
0.5 g DTT in 5 ml distilled water
Filter sterilized & stored at 4°C

25X ATP / 25 mM ATP
0.14 g ATP in 10 ml distilled water
Filter sterilized & stored at 4°C

{V}. PROCEDURES FOR TRANSFORMATION OF BACTERIA BY ELECTROPORATION.

All electroporations were conducted with a Gene Pulser System (Bio-Rad Laboratories, Richmond, CA) equipped with a pulse controller. An electric field is generated that then undergoes an exponential decay. The RC time constant is an expression of the time required for the voltage to decline to approximately 37% of its peak value. The RC time constant = R x C = (resistance in ohms) x (capacitance in microfarads). Previous studies (33, 171) with E. coli have demonstrated that transformation efficiency is dependent on two characteristics of the electrical pulse: the electric field strength and pulse length (RC time constant). Maximum transformation efficiency was obtained with either low field strengths (7 kV/cm) and long RC time constants (20 msec) or high field strengths (10 kV/cm) and short RC time constants (5 msec).

In the present study field strength (12.5 kV/cm) and capacitance (25 μF) were held constant while resistance was varied from 100 to 400 ohms to produce RC time constants ranging, in general, from 2.5 to 10 msec. Electroporations were conducted in 0.2 cm gap electroporation cuvettes (Bio-Rad Laboratories, Richmond, CA) containing 40 μl of washed bacterial cells and 10 μl of DNA solution (containing $\geq$ 1 μg DNA).

Preparation of bacterial cells for electroporation[a].

1) Harvest 500 ml of bacterial cells (A_{660} = 0.8 to 1.2) by centrifugation (7,000 x g, 15 min, 4°C).
2) Wash harvested bacterial cells with 500 ml of ice-cold 1 mM Hepes (pH 7.0) and pellet by centrifugation as above.
3) Pool bacterial pellets and wash 2X with 150 ml of ice-cold 1 mM Hepes (pH 7.0).
4) Resuspend bacterial pellet in 10 ml ice-cold 1 mM Hepes-20% glycerol (pH 7.0) and harvest by centrifugation as above.
5) Resuspend bacterial pellet in 2 ml ice-cold 1 mM Hepes-20% glycerol (pH 7.0), aliquot into 100 μl portions, and store at -80°C until use.

[a] When preparing colonic Bacteroides or Porphyromonas cells DTT (0.01% final concentration) was included in the 1 mM Hepes and 1 mM Hepes-20% glycerol solutions.

{VI}. DNA SEQUENCING BY THE DIDEOXY TERMINATION METHOD.

DNA sequencing was performed with the Sequenase Version 2.0 kit according to instructions supplied by the manufacturer (United States Biochemical, Cleveland, OH).

A) Alkaline denaturation of double-stranded plasmid DNA (purified by CsCl-ethidium bromide equilibrium density gradient centrifugation).

1) To 36 µl of distilled water containing 5 to 10 µg of DNA add 4 µl of 2M NaOH containing 2mM EDTA. Incubate at 37°C for 15 min.
2) Precipitate DNA by addition of 10 µl of 10M ammonium acetate and 75 µl of isopropanol. Incubate in dry ice-ethanol bath for 30 min.
3) Harvest DNA by centrifugation in a microfuge (15 min, 4°C) and wash 2 times with 100 µl ice-cold 70% ethanol.
4) Dissolve DNA in 7 µl of distilled water. Then add 1 µl (0.5 pmol) of the appropriate sequencing primer and 2 µl of 5X Sequenase® buffer.
5) Mix well, incubate at 65°C for 15 min, and cool slowly to room temperature. Store on ice until use.

B) Labelling reaction

1) Dilute 1 µl of Sequenase® labelling mix with 9 µl distilled water. Store on ice until use.
2) Dilute 1 µl Sequenase® Version 2.0 DNA polymerase with 7 µl of ice-cold Sequenase enzyme dilution buffer. Store on ice until use.
3) Set up labelling reaction mixture on ice:

Template-primer mix	10 µl
0.1M DTT	1 µl
Diluted labelling mix	2 µl
^{35}S-dATP (5 µCi)	4 µl
Diluted Sequenase® Version 2.0 DNA polymerase	2 µl

Mix well and incubate at room temperature for 5 min.

C) Termination reaction

1) Label 1 microfuge tube each G, A, T, and C. Place 2.5 µl of the appropriate ddNTP solution in each tube. Warm to 37°C for 1-2 min.
2) Add 3.5 ul of the labelling reaction to each tube and incubate at 37°C for 5 min.

3) Stop termination reaction by addition of 6 ul of Sequenase® stop solution to each tube. Store on ice until ready to load sequencing gel or at -20°C for up to 1 week).

D) Preparation of DNA sequencing gel

1) Acrylamide	5.7 g
Bis-acrylamide	0.3 g
Urea	42 g
10X long running buffer	10 ml

Bring up to 100 ml with distilled water.

2) Degas gel mixture on ice under vacuum for 10 min. Then add 800 µl of fresh 10% ammonium persulfate and 100 µl of TEMED.
3) Introduce mixture between glass support plates and allow to polymerize for $\geq$ 45 min.

10X Long Running Buffer
0.45 M boric acid
1.35 M Tris
25 mM EDTA
pH 8.9

E) Electrophoresis

1) Pre-run gel for 1 h at 110 watts.
2) Heat termination reactions at 75°C for 2 min.
3) Insert sharks-tooth comb above sequencing gel and load 2.5 ul of each termination reaction specimens.
4) Run samples into gel at 10 watts for 5 min, then increase to 100 watts.
5) After completion of electrophoresis, remove one glass support plate and wash gel with 1 L of fixer (10% acetic acid, 12% methanol, 1.5 % glycerol).
6) Transfer fixed gel to Whatman filter paper (3 MM), dry with gel-drying apparatus, develop autoradiogram, and read DNA sequence.