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VIRULENCE FACTORS OF ORAL ANAEROBIC SPIROCHETES

by

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ABSTRACT

The research work presented in this thesis involved the examination of several fundamental questions concerning the role of oral anaerobic spirochetes (OAS) in the etiology of periodontal disease (PD). OAS are unusual, helical, Gram-negative bacteria that are considered putative periodontopathogens due to numerical association with diseased sites and the enzymatic arsenal available to OAS that appears consistent with disease symptoms. *T. denticola* is the most commonly isolated OAS from periodontal pockets and as such is the focus of most investigations into the role of OAS in PD.

As free iron is severely limited in humans the means by which OAS may obtain sufficient iron to prosper in the sub-gingiva was examined. The resultant model suggests *T. denticola* obtains iron through the β-hemolysis of erythrocytes and the binding of liberated hemin by a 47-kDa outer membrane sheath (OMS) protein. The kinetics of the ligand-receptor interaction are presented and the receptor has been purified to apparent homogeneity from *T. denticola*. ^{3}H-labeled hemin is not transported into the cell. Evidence is presented to show that *T. denticola* produces an iron reductase, which may facilitate the transport of ferrous iron across biological membranes. It is also shown that *T. denticola* (Td), *T. vincentii* (Tv) and *T. socranskii* (Ts) do not produce siderophores. In growth assays, under conditions of iron-limitation, *T. denticola* may use inorganic iron, a source unlikely to be available *in vivo*.

Hyaluronidase (Hase) activity is elevated in the gingival crevice during episodes of disease. Hase, when injected into the periodontal cavity under experimental conditions has been shown to result in connective tissue degradation. It is also known that *T. pallidum*, the agent of syphilis, produces a Hase that is critical to pathogenesis. Evidence is presented herein to show that Td, Tv and Ts all produce a hyauronoglucosaminidase (HGase). The identity and specificity of the Td HGase is confirmed through the use of enzyme inhibitors and activators, by electron microscope observations of the enzyme using the Hase inhibitor gold sodium thiomalate and anti-*Apis mellifera* venom antibodies

and examination of the purity of the HA substrate using other polysaccharide degrading enzymes. As the HGase of these OAS would not migrate through a substrate-SDS PAGE system, we have employed hyaluronate (HA)- and chondroitin sulfate (CS)-absorbed nitrocellulose membranes to visualize HGase activity. The 59 kDa HGase of Td has been purified to apparent homogeneity through the conjugation of HA and CS to Affigel 701 beads.

The last subject to be addressed by this thesis pertains to the ultrastructure of oral spirochetes. Using the copper-containing dye, Alcian blue, we have shown that *T. denticola* produces an exopolysaccharide layer, in an electron microscopy investigation. The development of a stain for dark-field microscopy has simplified the observation of this layer. The exopolysaccharide layer may have relevance to the evasion of phagocytosis, to protection against colicins, immunoglobulins and bacteriophages, to adherence and perhaps to the immunogenicity of OAS inhabiting the sub-gingiva.

RÉSUMÉ

Le travail de recherche présenté dans cette thèse implique l'étude de plusieurs questions fondamentales concernant le rôle des spirochètes oraux anaérobies (OAS) dans l'étiologie de la maladie périodontale (PD). Les OAS sont des bactéries Gram-négatives en hélice soupçonnées d'être périodontopathogènes à cause de leurs nombreuses associations avec les sites atteints et l'arsenal enzymatique dont ils bénéficient (qui semble cohérent avec les symptômes de la maladie). *Treponema denticola* est l'OAS le plus souvent extrait des poches périodontales, et de ce fait, est le sujet de la plupart des recherches dans le rôle des OAS dans la PD.

Étant donné que les sources de fer libre sont très limitées, nous avons examiné les moyens par lesquels les OAS peuvent se procurer le fer nécessaire à leur prolifération dans la région sous-gingivale. Le modèle qui en découle suggère que *T. denticola* obtient le fer par son activité β-hémolytique sur des érythrocytes et la liaison de l'hémine libre par une protéine de 47 kDa située sur la gaine de sa membrane externe (OMS).

La cinétique de l'interaction ligand-récepteur est présentée et la protéine de *T. denticola* fut purifiée à homogénéité. L'hémine marquée au ^{3}H n'étant pas transportée à l'intérieur de la cellule, nous présentons ici des évidences de la production d'une réductase du fer par *T.denticola* qui peut- être facilite le transport du fer ferreux à travers les membranes biologiques. *T. denticola* (Td), *T. vincentii* (Tv) et *T. socranskii* (Ts) ne produisent pas de sidérophores. Dans des études de croissance effectuées dans des conditions limitées en fer, *T. denticola* pourrait utiliser le fer inorganique, ce qui semble être une source peu probable *in vivo*.

En période de maladie, l'activité de l'hyaluronidase (Hase) est élevée dans les crevasses gingivales. Lorsqu'injectée expérimentalement dans la cavité périodontale, l'Hase engendre la dégradation du tissu conjonctif. Nous savons également que

T. palladium, l'agent responsable de la syphilis, produit une Hase essentielle à la pathogénèse. Nous avons montré que Td, Tv et Ts produisent tous une hyaluroglucosaminidase(HGase). L'identité et la spécificité de la Td HGase ont été confirmées par l'utilisation des inhibiteurs et des activateurs de l'enzyme, par des observations de la HGase au microscope électronique en utilisantavec l'utilization l'inhibiteur or sodium thiomalate et aussi les anticorps contre le venin d'*Apis mellifera* et l'examen de HA avec d'autres enzymes qui engendrent la dégradation des polysaccharides. Les HGases de ces OAS ne pouvant migrer à travers un système substrat-SDS PAGE, nous avons donc eu recours, pour observer leur activité, à des membranes de nitrocellulose imbibées d'acide hyaluronique (HA) ou de sulfate de chondroitine. L'HGase de 59 kDa de Td fut purifiée à homogénéité en conjugant HA et CS à une matrice de billes Affi-gel 701.

En dernier lieu, nous avons étudié l'ultrastructure des OAS. Nous avons montré, en utilisant le colorant bleu Alcian (contenant du cuivre), que *T. denticola* produit une couche d'exopolysaccharides visible au microscope électronique. La visualisation de cette couche fut facilitée par le dévelopment d'une coloration pour la microscopie à fond noir. La couche d'exopolysaccharide pourrait être importante pour l'évasion à la phagocytose, dans la protection contre les colicines, les immunoglobulines et les bactériophages, dans l'adhérence et probablement dans l'immunogénicité des OAS de la région sous-gingivale.

Acknowledgements

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Many thanks to Antonia Klitorinos, and also to Yu-shan Qiu, Angie DeCiccio and Richard McLaughlin who have shared the 503/508 laboratories with me.

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To my supervisors, Eddie Chan and Russel Siboo, I would like to extend my sincere thanks for their assistance, for their patience and for the opportunity to learn.

I thank my parents and family for their understanding and for ignoring the barrier of the Atlantic Ocean.

For Christopher Gertig, thank-you for making the big world small and the small world big.

Claim of contribution to knowledge

1. *Treponema denticola*, *T. vincentii* and *T. socranskii* were shown to bind hemin and Congo red through a 47-kDa outer membrane sheath (OMS) protein.
2. *T. denticola*, *T. vincentii* and *T. socranskii* were shown to be ß -hemolytic.
3. The kinetics of the 47-kDa hemin-binding protein with both hemin and Congo red interactions were established.
4. *T. denticola* ATCC 35405 can obtain iron from hemin and can utilize ferric ammonium citrate as an inorganic iron source.
5. ^{3}H-Hemin was found not to be transported across the membranes of *T. denticola*.
6. The hemin-binding protein of *T. denticola* was purified to apparent homogeneity by affinity chromatography.
7. The hemin-binding protein of *T. vincentii* was purified to apparent homogeneity by affinity chromatography.
8. *T. denticola* produces an iron reductase.
9. *T. denticola*, *T. vincentii* and *T. socranskii* were shown not to produce siderophores.
10. *T. denticola*, *T. vincentii* and *T. socranskii* were shown to secrete an extracellular hyaluronoglucosaminidase (EC 3. 2. 1. 35).
11. A transblot technique allowing the visualization of hyaluronidase and chondroitinase activities in cell supernatants was established.
12. The hyaluronoglucosaminidase of *T. denticola* was purified to apparent homogeneity by affinity chromatography.

13. The hyaluronoglucosaminidase of *T. denticola* was localized to the proximity of the cell surface by electron microscopy.

14. Electron microscope evidence for the presence of an extracellular polysaccharide layer surrounding *T. denticola* cells was obtained.

15. A stain allowing for the observation of the extrapolysaccharide layer of spirochetes by dark-field microscopy was established.

16. Representative strains of *T. denticola*, *T. vincentii* and *T. socranskii* were distinguished by the use of a lectin- binding ELISA technique.

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CHAPTER 1. LITERATURE REVIEW AND INTRODUCTION

" ... I have had several gentlewomen in my house, who were keen on seeing the little eels in vinegar: but some of them were so disgusted at the spectacle, that they vowed they'd never use vinegar again. But what if one should tell such people that there are more animals living in the scum on the teeth in a man's mouth, than there are men in a whole kingdom?"

Antony van Leeuwenhoek, 1683 (from Stanier, *et al.*, 1983).

The word spirochete, or animated hair from the Greek, was assigned to a spiral bacterium by Ehrenberg in the 1830's (Margulis, *et al.*, 1993). These spiral bacteria were first recognized microscopically by van Leeuwenhoek in the 1600's. Spirochetes are Gram-negative, anaerobic helical bacteria with several complete spiral turns. The oral anaerobic spirochetes (OAS) are classified as members of the genus *Treponema*, of the family *Spirochaetaceae*. Despite the fact that the existence of spirochetes has been established for so long, relatively little is known about this group of bacteria, largely due to difficulties in *in vitro* growth and a lack of a manipulatable genetic exchange mechanism.

Although the spirochetes are a diverse group of bacteria found in widely varied habitats, they have many characteristics in common, including a unique morphology and locomotory apparatus. Free-living spirochetes are thought to inhabit mud (Margulis, *et al.*, 1993), high salt lakes (Greenberg and Canale-Parola, 1975), oceans (Canale-Parola, 1978), and sewage (Johnson, 1977). Many spirochetes form part of the indigenous microflora of eukaryotic hosts, including *Hydra* (Canale-Parola, 1978), the crystalline style of mollusks and the gut of insects (Johnson, 1977) and mammals (Lee and Hampson, 1992, 1994; Johnson, 1977)

There are only a few species of spirochetes known to be pathogenic, causing diarrhea and typhlitis in birds (Swayne, *et al.*, 1995), dysentery in pigs (Glock and Harris, 1972), yaws, pinta and relapsing fever in humans (Schmid, 1989; Steere, *et al.*, 1983). Koch discovered *Treponema pallidum* to be the agent of one of humankind's oldest social diseases, syphilis, in 1905. *Borrelia burgdorferi* causes Lyme

Disease, the prevalence and distribution of which is still being evaluated (Barbour and Fish, 1993).

The oral anaerobic spirochetes (OAS) remain an integral component of theories attempting to explain periodontitis, an inflammatory disease associated with a mixed population of putatively pathogenic bacteria in humans. Opinions differ as to whether OAS are simply part of the natural microflora, opportunistic pathogens or overt mediators of periodontal disease. It is towards the elucidation of the role that these bacteria play in the etiology of human periodontal disease that the work contained in this thesis is addressed.

I. Taxonomy of Spirochetes.

Spirochetes are classified as a single order, *Spirochaetales*, which is divided into two families, *Spirochaetaceae* and *Leptospiraceae*. The *Spirochaetaceae* are comprised of the *Treponema*, *Spirochaeta*, *Borrelia* and *Cristispira* genera. The *Leptospiraceae* are comprised of the genera *Leptospira* and *Leptonema* (Paster, *et al.*, 1991). Recently a new genus has been proposed, *Serpulina (Serpula)* into which *Treponema hyodysenteriae* and related species have been reclassified (Paster, *et al.*, 1991; Stanton, *et al.*, 1991; 1992).

Classical taxonomy, based on morphology, including size, helical wave amplitudes and axial fibril numbers, metabolic profiling and ecology, agrees well with 16s rRNA cataloguing as the defining factor in spirochete phylogeny. This correlation is rare amongst bacteria (Paster, *et al.*, 1991; Woese, 1987). The average difference between rRNA genes of the spirochete genera is 10% (Paster, *et al.*, 1991). An overview of the genera of spirochetes is presented in Table 1.

Spirochetes share unique signature sequences in 16s rRNA genes, which taken together with the cluster patterns derived from rRNA cataloguing, suggest a primordial ancestor that has developed into the present genera and species through divergent evolution (Paster, *et al.*, 1984, 1991). Phototrophic bacterial mats are among the Earth's oldest ecosystems, and mud

Table 1. General characteristics of spirochete genera [a]

	Treponema	*Spirochaeta*	*Borrelia*	*Leptospira / Leptonema*	*Serpulina*	*Cristispira*
Mol % G & C	36 - 54	51 - 66	27 - 32	37 -53	26	?
size ;						
width (μm)	0.1 - 0.7	0.2- 0.75	0.2 - 0.5	0.2	0.3 - 0.4	0.5 - 3
length (μm)	5 - 20	10 - 500	3 - 30	10	7 - 9	30 - 500
number of axial fibrils	2 - 32	2	7 - 30	2	18	>100 - >300
diamino acid of peptidoglycan	ornithine	ornithine	ornithine	diamino-pimelic acid	ornithine	?
optimum growth temp. (^{0}C)	37 - 39	15 - 40	37	15 - 37	36 - 42	?
status relative to oxygen	obligate anaerobes	obligate or facultative anaerobes	micro-aerophilic	aerobic	micro-aerophilic	?
distribution	mouth,genitals, intestines, disseminate in tissues of animals and humans	natural water bodies, mud, sewage	arthropods, wild animals, humans	surface waters, soil, humans and animals	mammalian digestive tract	crystalline style and intestinal tract of fresh and salt water molluscs

[a] The data contained in this table was compiled from Barbour and Fish (1993), Canale-Parola (1978), Chan *et al* (1993), Johnson (1977), Margulis *et al* (1993), Paster *et al* (1991), Paster *et al* (1984) and Schmid (1989).

spirochetes are probably among the original organisms to live in such environments (Margulis, *et al.*, 1993). Margulis and her collaborators, who study the evolution of spirochetes, have reported six other spirochete genera. These are *Clevelandina*, *Diplocalyx*, *Hollandina*, *Mobilifilum*, *Pillotina*, and *Spirosymplokos* (Margulis, *et al.*, 1993).

T. denticola is cultivable *in vitro*, and is the organism chosen for the majority of studies into the role of spirochetes in periodontal disease. It is the most frequently isolated spirochete from diseased sites (Cheng, *et al.*, 1985; Loesche, 1988; Salvador, *et al.*, 1987). The name *"denticola"* was first described by Brumpt in 1925 and literally translates from the Greek to "toothdweller." *T. denticola* is not listed in the Approved Lists of Bacterial names (Chan, *et al.*, 1993; Skerman, *et al.*, 1980) and therefore may be considered an invalid name. However, Bergey's Manual of Systematic Bacteriology does list *T. denticola* (Smibert, 1984). Chan *et al.* (1993) clarified the situation by proposing *Treponema denticola* (ex Brumpt 1925) sp. nov., nom. rev. as a distinct species of *Treponema*. *T. denticola* ATCC 35405 was designated the type strain, with *T. denticola* ATCC 33520 and *T. denticola* ATCC 35404 designated to serve as reference strains (Chan, *et al.*, 1993).

T. denticola is a small to intermediate sized spirochete. An axial filament arrangement of 2:4:2 is common (Chan, *et al.*, 1993) although larger numbers of axial filaments have been reported (Cheng and Chan, 1983). This spirochete does not utilize the glycolytic pathway as a major energy source and is primarily an amino acid fermenter. It can grow at temperatures of 30 to 42 ^{o} C. It has a G and C content of 37-39 mol% (Chan, *et al.*, 1993).

OAS may comprise up to 60% of the microflora at diseased sites in the oral cavity (Reddy, *et al.*, 1986), though many of the oral spirochete species are presently uncultivable (Paster *et al*, 1991). Spirochetes that cross-react with a monoclonal antibody (MAb) specific to a *T. pallidum* antigen (the MAb does not cross-react with any cultivable species of spirochete) are present in the mouth, and are referred to as pathogen-related oral spirochetes (PROS; Riviere, *et al.*, 1991; 1992). Other species that have been

isolated from the periodontium and cultured *in vitro* are *Treponema vincentii, T. socranskii,* and *T. pectinovorum* (Choi, *et al.*, 1994).

II. General Characteristics of Spirochetes.

A spirochete cell is represented diagramatically by Figures 1A, B, and C. Viable cells must be observed by dark-field microscopy with light illumination. A typical dark-field photograph of *Treponema denticola* ATCC 35404 is seen in Figure 2; the characteristic helical shape of spirochetes is apparent.

(i) Mucoid Layer.

Outermost on a spirochete cell is a mucoid layer, which has been little studied. This is a glaring omission in our understanding of pathogenic spirochetes as capsules or slime layers are known to be significant virulence factors in some bacteria. *Haemophilus influenzae* produces six types of polysaccharide capsules, of which type b is thought to be crucial to pathogenesis (Griffiths, *et al.*, 1987; Lee, 1987). *Neisseria meningitidis* strain b polysaccharide is likewise considered important in the etiology of meningitis (Echarti, *et al.*, 1983).

S. hyodysenteriae has been shown to have a mucoid layer (Li *et al.* 1992). As early as 1957 it was noted that mucopolysaccharides accumulate in syphilitic lesions, and the authors proposed that these mucopolysaccharides were the result of capsule formation by *T. pallidum* (Turner and Hollander, 1957). Christiansen (1963) stated that electron microscope (EM) photographs of *T. pallidum* seemed to show an extracellular layer. Other studies in the 1970's also provide evidence for mucopolysaccharide synthesis by *T. pallidum* (Fitzgerald, *et al.*, 1978; Ziegler, *et al.*, 1976).Despite the lack of an *in vitro* growth system, the mucoid layer of *T. pallidum* has received most attention amongst the spirochetes

Until recently, the adherence of a plethora of host proteins to this layer, forming a protective coat against an immune response (Aldrette and Baseman, 1979; Christiansen,

1963) was considered the mechanism by which *T. pallidum* avoided destruction by the immune system. Host proteins reported to be associated with this coat include albumin, immunoglobulins, α-2 macroglobulin, transferrin, and ceruloplasmin. Such a wide array of host proteins suggests non-specific binding (Aldrette and Baseman, 1979). In addition to presenting a barrage of non-foreign proteins to the host, it was also suggested that this mucopolysccharide layer may hide treponemal surface-exposed antigens (Fitzgerald and Johnson, 1979). This model was appealing, as it could explain the remarkable ability of *T. pallidum* to avoid or evade both cellular and humoral immune responses (Bourell, *et al.*, 1994). However, data are now available suggesting a sparcity of outer membrane proteins as the reason for the low antigenicity of *T. pallidum* (Cox, *et al.*, 1992). It is not inconceivable that both a protective coat and limited outer membrane proteins are relevant.

The mucoid layers of the oral spirochetes have not been well studied. Polysaccharides external to the outer membrane have been proposed to aid in the attachment of the putative oral pathogen, *T. denticola*, to epithelial cells (Olsen, 1984).

This gap in our knowledge of the ultrastructure of oral spirochetes is surprising. The mucoid layer will be the first part of the cell to interact with the immune system, with host cells to which spirochetes may adhere, and with other bacteria co-habiting the oral cavity. A study confirming the presence of a mucoid layer in *T. denticola* ATCC 35405 is discussed in Chapter 4.

(ii) Outer Membrane Sheath

An intact outer membrane sheath (OMS) is necessary for spirochete viability. Damage to the OMS, by antibody and complement, in susceptible leptospires, results in the loss of internal components and cell death (Anderson and Johnson, 1968). The use of hypotonic solutions to fully extend the OMS of *Treponema* cells also renders the bacteria non-viable (Johnson, 1977). The OMS has been reported as a three-layered membrane envelope for most spirochetes and as a five-layered envelope for some leptospires (Johnson, 1977). The relationship of the OMS to underlying cellular structures is unclear.

It may be attached to the peptidoglycan layer, as in other Gram-negative bacteria such as *E. coli* (Neidhart, *et al.*, 1990), or it may be a completely independent structure.

The spirochete OMS does not appear to be stabilized by divalent cations (Penn, *et al.*, 1985) and EDTA is not required as an additive to non-ionic detergents, such as Triton-X 100, to remove the OMS from whole cells (Cockayne, *et al.*, 1986; Penn, *et al.*, 1985). This may explain the fragility of the OMS of spirochetes. Much blebbing in the OMS has been noted, especially in old *in vitro* cultures (Johnson, 1977).

The presence of lipopolysaccharide (LPS) in spirochete OMS is probably species variable. *T. pallidum* does not give a positive reaction to the amoebocyte lysate test for LPS (Hardy and Levin, 1983); nor does polymyxin B, a lipid A specific antibiotic, affect the OMS of this bacterium (Penn, *et al.*, 1985). *T. phagedenis* appears to contain smooth type (full length) LPS, whereas *S. hyodysenteriae* appears to possess rough type (truncated O-antigens) LPS (Strugnall, *et al.*, 1990).

It is only recently that the subject of spirochete porins has been addressed. *T. pallidum* contains a protein, TpN 50, that exhibits homology to the OmpA family of outer membrane proteins. This family includes the OmpA protein of *E. coli*, *Pseudomonas* OprF, *Neisseria meningitis* RmpM, and *N. gonorrhoeae* PIII proteins (Hardham and Stamm, 1994). Functions assigned to this protein family are mediation of colicin uptake, maintenance of outer membrane integrity, as bacteriophage receptors, and pore formation (Hardham and Stamm, 1994).

A 53 kDa OMS protein from *T. denticola* has been purified and can bind fibronectin, laminin, and fibrinogen (Egli, *et al.*, 1993; Haapasalo, *et al.*, 1992). The OMS of *T. denticola* is also the location of a 95-kDa chymotrypsin-like enzyme that is able to hydrolyze laminin and fibronectin (Grenier, *et al.*, 1990). The finding that the 53 kDa protein also serves as the only known porin in *T. denticola* is particularly interesting, as it may mediate concentration of a food source near to the channel opening (Egli, *et al.*, 1993). The channel formed by this protein is the largest of all known porins, at 3.4 nm (Egli, *et al.*, 1993). By comparison, the OmpF protein of *E. coli* forms a channel with a diameter of 1.17 nm., and the second largest known channel diameter is formed by the 36.5-kDa protein of *Spirochaeta aurantia*, at 2.3 nm (Egli, *et al.*, 1993; Kropinski, *et al.*,

1987). The findings on the *T. denticola* 53-kDa porin/adhesion are in keeping with the idea that spirochetes are filter feeders whose OMS serves as a crude filtration device as the cell locomotes (Egli, *et al.*, 1993).

The OMS of spirochetes contains less protein than most Gram-negative bacteria; the OMS of *T. pallidum* exhibiting 100-fold less intramembranous protein particles than *E. coli* (Hardham and Stamm, 1994; Radolf, *et al.*, 1989). The intramembranous particles of the *T. pallidum* OMS are not randomly oriented, but seen in arrays spiraling the cells in freeze-fracture and deep-etching electron micrographs, and their mobility within the OMS appears to be limited (Radolf, *et al.*, 1989; Bourell, *et al.*, 1994). Immunoelectron microscopy has provided evidence that the major immunogens of *T. pallidum* are not surface-exposed (Cox, *et al.*, 1992). The low OMS protein content, the non-random orientation which may make antibody cross-linking more difficult, and the apparent lack of surface-expressed major antigens are now considered a better explanation for immune evasion by *T. pallidum* than the previous model of a protective poiysaccharide-host protein coat. However, both models may act in concert.

Such studies of protein arrangements and antigen exposure have not been performed for other spirochete species.

(iii) Axial Fibrils.

Various names have been assigned to the flagella-like structures that originate at the cell tip and wind around the protoplasmic cylinder (the part of the spirochete cell that includes the peptidoglycan layer and below) internal to the OMS, such as axial filaments (AF), periplasmic flagella or fibrils, endoflagella, flagella, and axistyles (Canale-Parola, 1978; Charon, *et al.*, 1992). The notation in use to describe AF arrangements utilizes a three-digit system. A notation of 2:4:2 indicates two AF from each cell end that overlap in the centre.

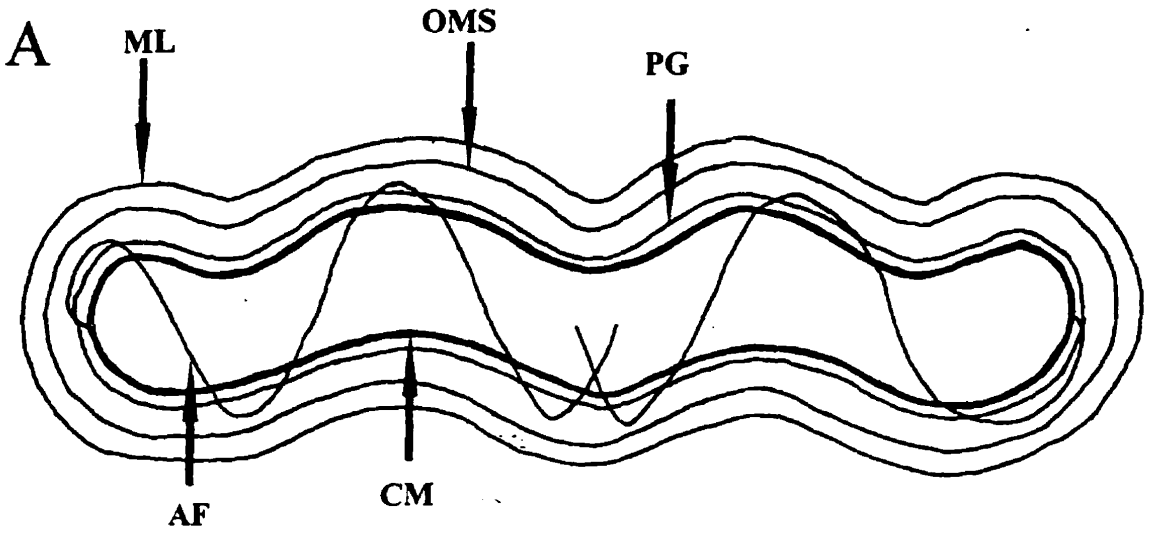
Figure 1. General Characteristics of a spirochete cell.

A Cross-section along the length of a spirochete cell.

B Cross-section through the center of a spirochete cell.
(Adapted from Holt,1978)

C Location of the axial fibrils.
(Adapted from Charon *et al*, 1992)

The locations of the mucoid layer (ML), outer membrane sheath (OMS), axial flagella (AF), basal body (BB), peptidoglycan layer (PG), cytoplasmic membrane (CM) and OMS porin (P) are marked with arrows.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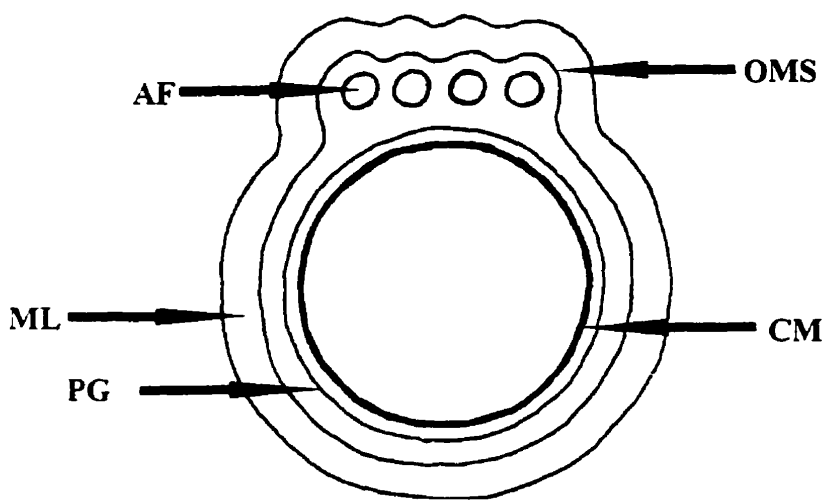
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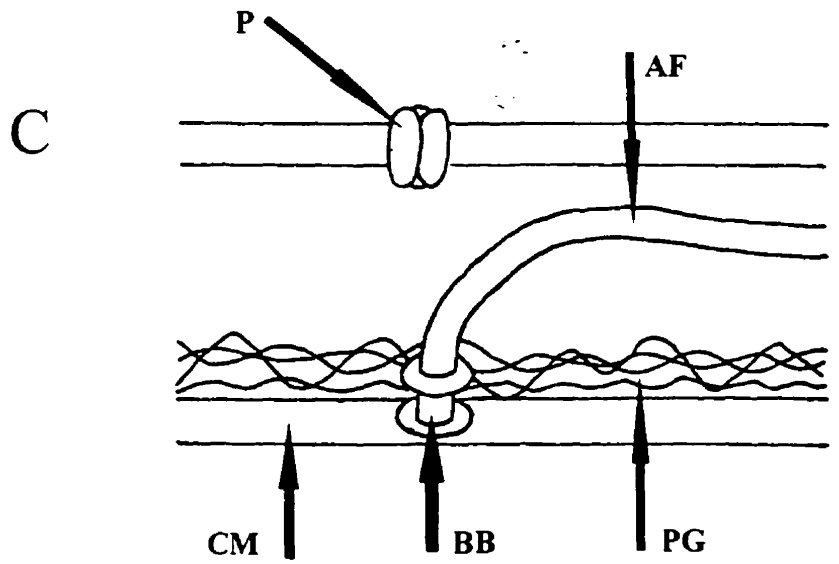
C
P
AF
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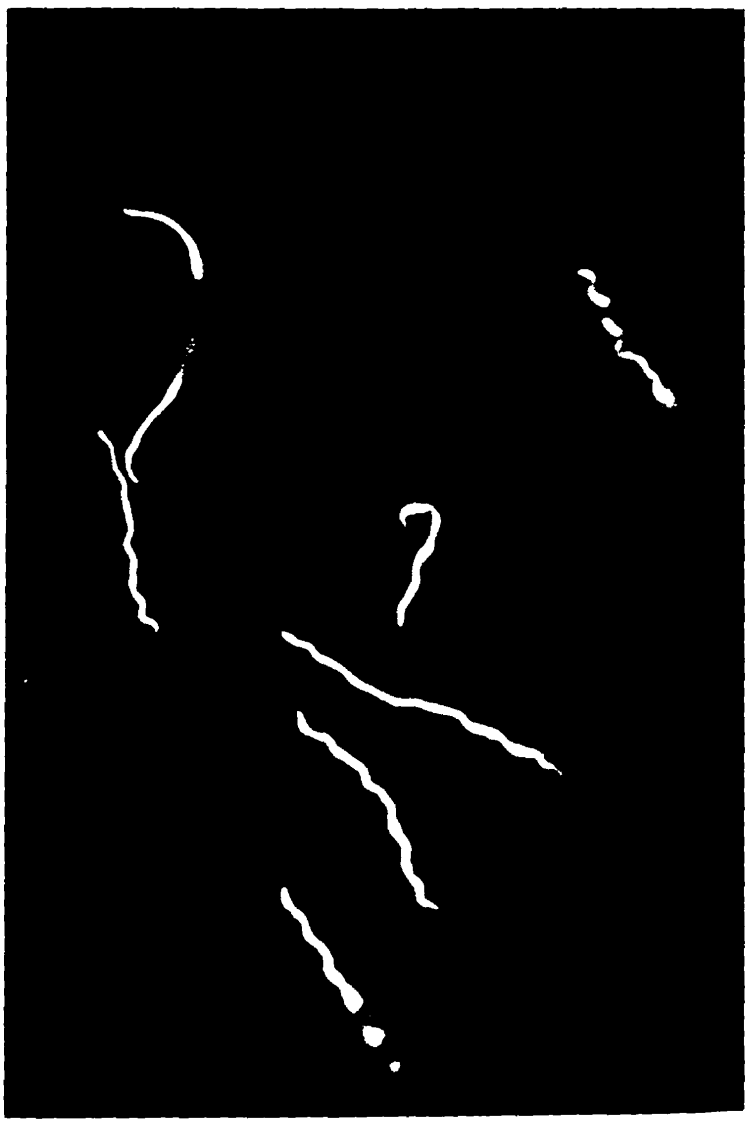
BB
PG

Figure 2. *Treponema denticola* ATCC 35405 seen by dark-field microscopy.



This is the case for some strains of *T. denticola* (Chan, *et al.*, 1993). A notation of 2:0:2 indicates two AF from each cell end that do not overlap, being shorter than half the cell length. Members of the genus *Leptospira* and *T. phagedenis* are the only spirochetes known whose AF do not overlap (Margulis, *et al.*, 1993). The minimal number of flagella reported is two, in *Spirochaeta* spp., and the maximum number is >300, in *Cristispira* (Margulis, *et al.*, 1993). AF appear similar to the flagella of other motile bacteria, but their periplasmic location is a factor that defines spirochetes as a group. Many spirochete species, including most of the *Treponema*, possess cytoplasmic filaments in association with the AF. The cytoplasmic filaments are not well understood (Johnson, 1977; Strugnall, *et al.*, 1990).

Electrophoresis of purified AFs from several diverse spirochete species has revealed that spirochete flagella are chemically more complex than most other bacterial flagella. Exceptions to this rule include *Borrelia burgdorferi* and *Spirochaeta zuelzerae*, whose filaments are composed of a single polypeptide (Charon, *et al.*, 1992). *T. phagedenis* AFs are comprised of a core, containing up to 4 polypeptides, with a surrounding surface protein (Charon, *et al.*, 1992). A similar make-up to the AFs of *T. pallidum*, *S. aurantia*, and *Serpulina hyodysenteriae* is reported (Charon, *et al.*, 1992). The AF's of *Treponema*, *Spirochaeta*, and *Borrelia* have basal bodies resembling the flagella of Gram-positive bacteria; with a single pair of rings (Johnson, 1977). The AF's of *Leptospira* are more analogous to Gram-negative flagella, containing a series of disks (Johnson, 1977).

Video microscopy has shown that the AF rotate, therefore generating a thrust (Charon, *et al.*, 1991; 1992). The role of AFs in locomotion is also implicated by the characterization of *L. illini* motility mutants, whose AFs have lost the coiled configuration of the wild type (Canale-Parola, 1978). The smooth swimming of spirochetes may be considered analogous to the runs of externally flagellated bacteria (Canale-Parola, 1978). Repellents and attractants can be used to induce changes in the rate of flexing (Fosnaugh and Greenberg, 1988; 1989), perhaps analogous to the tumbling of externally flagellated prokaryotes (Charon, *et al.*, 1992).

There is direct evidence for the rotation of *S. aurantia* AF's (Charon, *et al.*, 1991, 1992) and for a proton motive force as the energy source (Fosnaugh and Greenberg, 1988; 1989; Goldbourne and Greenberg, 1980). *S. aurantia* expresses methyl-accepting chemotaxis proteins, suggesting a chemotactic mechanism similar to that described for *E. coli* (Charon, 1992; Nowlin, *et al.*, 1985; Segal, *et al.* 1984). However, ion gradients or potential fluctuations are also involved in signaling to the AF, unlike *E. coli* (Goldbourne and Greenberg, 1981; 1983; Segal, *et al.*, 1984). This is perhaps not surprising as an *S. aurantia* cell is 20-30 μm long with its flagella attached at opposite cell ends

The most interesting aspect of the periplasmic location of the AFs is that it allows the cell to locomote at high viscosities (Klitorinos, *et al.*, 1993; Pietrantonio, *et al.*, 1988). We can therefore hypothesize on several fronts. As flagella are proteinaceous and antigenic, the AF's are hidden from the immune system by the OMS in intact cells. Chemotactic responses in high viscosities could offer advantages to spirochete cells *in vivo*, where the ability to locomote in viscous melieu would allow movement towards food sources or away from repellents. It also helps to explain the presence of spirochetes in highly viscous host tissues, as will be addressed later.

The oral spirochetes *T. denticola*, *T. vincentii*, and *T. socranskii* can locomote through viscosities of up to 700 mPas (Klitorinos, *et al.*, 1993). By comparison, *Bacillus megaterium*, *E. coli*, and *Pseudomonas aeruginosa*, exhibit optimum motility at 2-5 mPa's, and the speed of locomotion of the latter three bacteria decreases with increasing viscosity (Schneider and Doetsch, 1974). Locomotion at high viscosities has been shown for other spirochete species, such as *B. burgdorferi* (Kimsey and Speilman, 1990) and *L. interrogans* (Kaiser and Doetsch, 1975).

(iv) Peptidoglycan Layer.

The peptidoglycan (PG) of spirochetes is located proximal and exterior to the cell membrane (Fig. 1A, 1B, 1C), in common with this general characteristic of Gram-negative bacteria. The PG layer is imperative in maintaining the helical shape of spirochetes. Treatment of spirochete cells with lysozyme or penicillin results in the loss of their spiral shape (Canale-Parola, 1978); conversely, purified PG layers remain helical (Canale-Parola, 1978). The PG is also required for motility (Charon, *et al.*, 1992).

Biochemical analysis of spriochete PG has shown that the PG of *Treponema*, *Leptospira*, and *Spirochaeta* contains muramic acid, glucosamine, alanine and glutamate. Differences in the diamino acid component utilized by spirochetes has been noted. The PG of the genera *Treponema* and *Spirochaeta* contains ornithine, whereas *Leptospira* PG contains diaminopimelic acid (Johnson, 1977). The PG of *T. denticola*, like the PG of other bacteria, may induce complement activation and inflammation, (Grenier and Uitto, 1993), and may therefore play an active role in pathogenesis.

Weigel, *et al.* (1994) showed a *T. pallidum* protein, Tpp47, to be a penicillin-binding protein (PBP), specifically, a zinc-dependent carboxypeptidase. Expression of Tpp47 in *E. coli* proved toxic due to altered PG synthesis. This was perhaps due to limitation of PG cross-linking, by Tpp47 activity removing terminal D-alanine residues (Weigel, *et al.*, 1994).

T. denticola contains approximately 0.1% peptidoglycan in measurements of total dry weight. The PG of *T. denticola* is known to be cytotoxic (Grenier and Uitto, 1993), and shall be considered when addressing the role of the oral spirochetes in periodontal disease.

(v) Cell Membrane.

The spirochete cell membrane (CM) can be visualized as a triple-layered structure beneath the PG layer in electron micrographs (Johnson, 1977). Quantitative studies of *B. burgdorferi* membranes showed that the protein content of the CM is four times that of

the OMS (Bledsoe, *et al.*, 1994). Information on the general features of the CM of spirochetes is lacking. Such functions may include an association with the nucleoid, active transport systems, and cytochrome chains (Neidhardt, *et al.*, 1990).

(vi) Cytoplasm, Nucleoid, and Extrachromosomal Elements.

The cytoplasm of spirochetes appears to be similar to that of most bacteria, with vesicles and inclusion bodies reported (Johnson, 1977). The cultivable spirochetes exhibit a universal resistance to rifampicin (Chan, *et al.*, 1993; Paster, *et al.*, 1984), indicating either an alternative transcription machinery, or a resistance mechanism(s) ubiquitous amongst spirochetes. The G and C content of spirochete DNA ranges from < 30 mol% *(Serpulina;* Paster, *et al.*, 1991) to 66 mol% *(Spirochaeta;* Canale-Parola, 1978), reflecting the wide degree of dissimilarity, in terms of habitat, energy sources and enzymatic activities, despite common locomotory and morphological characteristics.

The spirochete genome size is variable. Taylor *et al.* (1991) estimated the chromosome of *L. interrogans* to be 3,100 kb, and that of *L. biflexa* to be 3,500 kb. These are the largest genome sizes reported for spirochetes. Both *L. interrogans* and *L. biflexa* are less fastidious than the obligate host-associated spirochetes and can survive for prolonged periods in waters (Taylor, *et al.*, 1991). *T. pallidum* has a chromosome of 900 kb (Walker, *et al.*, 1991) and *B. burgdorferi* has a chromosome of 950 kb (Krawiec and Riley, 1990), which are among the smallest known genomes (Krawiec and Riley, 1990; Walker, *et al.*, 1991).

The study of spirochete genetics has produced many puzzling observations. A potential transcription start site was identified for a flagella-associated antigen of *Borrelia burgdorferi*, but no -10 or -35 sequences related to those of *E. coli* could be detected (Arakawa and Kuramitsu, 1994; Wallich, *et al.*, 1993). The antigenic *tdpA* gene of *T. denticola* has been sequenced, but no *E. coli*-like promoter regions could be found upstream (Arakawa and Kuramitsu, 1994; Miyamoto, *et al.*, 1991). Similarly, no *E. coli*-like promoter sequences could be identified on sequencing of the *prtB* gene of

T. denticola, which encodes a chymotrypsin-like protease (Arakawa and Kuramitsu, 1994). A lack of a genetic exchange mechanism hampers such investigations. There is a possibility that the genes described here are transcribed as part of polycistronic mRNA.

Homologues of the heat shock-associated protein, GroEL, are detectable in spirochetes by Western blotting, including *T. pallidum*, *T. denticola*, and *L. interrogans*. The GroEL homologues are not thermoinducible in *T. denticola*, or in *T. pallidum*, which is the only reported microbe that does not exhibit a heat shock response. Yet, its GroEL homologue (Tpn60) is found at higher levels than the basal amount of GroEL described in *E. coli* (Stamm, *et al.*, 1991).

The rRNA genes of spirochetes also present unique characteristics. The 16s rRNA molecules have a 20 to 30 base 5'-extension that, although variable in nucleotide sequence, may form a helix, as suggested by secondary structure analysis (Paster, *et al.*, 1984; 1991). The significance of these putative helices is unknown but may indicate alteration of 16s rRNA transcript processing and maturation (Paster, *et al.*, 1991).

The distribution of genetic information in, and possibly between, *Borrelia burgdorferi* cells is particularly fascinating, suggesting some novel insights into the organization of bacterial DNA. *B. burgdorferi* has a linear maxichromosome (Bergstrom, *et al.*, 1992; Ferdows and Barbour, 1989) with covalently-closed ends (Bergstrom, *et al.*, 1992) and several linear extrachromosomal elements known as minichromosomes (Barbour and Garon, 1987; Bergstrom, *et al.*, 1992), as well as supercoiled circular plasmids (Barbour and Garon, 1987; Bergstrom, *et al.*, *1992*). The termini of one linear minichromosome (LMC) of *B. burgdorferi* shows homology to the iridovirus that causes African swine fever, a member of the pox virus family (Bergstrom, *et al.*, 1992). Indeed, both *B. burgdorferi* and this iridovirus share the same arthropod vector (Bergstrom, *et al.*, 1992). In addition to the linear maxichromosome, LMCs of 50, 40, 20, 20, 15, and 10 kb are known, and circular plasmids of 25 and 5 kb (Bergstrom, *et al.*, 1992). Such DNA organization is similar to that exhibited by certain trypanosomes (Bergstrom, *et al.*, 1992), an ironic finding in view of the fact that historically spirochetes were often confused with trypanosomes (Margulis, *et al.*, 1993).

Nucleic acids are detectable in vesicles secreted by *B. burgdorferi* (Bergstrom, *et al.*, 1992; Garon, *et al.*, 1989, 1991). LMCs and circular plasmids of corresponding size to the extrachromosomal elements of the particular isolate under study may be found in these vesicles (Bergstrom, *et al.*, 1992). It is an interesting possibility that these findings are indicative of a genetic exchange mechanism.

L. interrogans may also contain LMCs. Such linear extrachromosomal elements were not detected in non-pathogenic leptospires, suggesting that the LMCs may encode virulence determinants (Taylor, *et al.*, 1991).

Caudry *et al.* (1995) examined fifteen strains of oral spirochetes belonging to the species *T. denticola, T. vincentii,* and *T. socranskii* and nine newly isolated clinical spirochetes for the distribution of plasmids. A 2.6-kb plasmid was detected in *T. denticola* ATCC 33520 and in *T. denticola* strain e'. The molecular weight, restriction endonuclease digest profiles and southern blotting studies strongly suggest that both strains contain the same, or similar, plasmid. These two *T. denticola* strains are dissimilar in terms of indirect immunofluorescence, serotyping, and DNA-DNA homology studies suggesting a genetic exchange mechanism could occur naturally (Caudry, *et al.*, 1995; Chan, *et al.*, 1993; Cheng, *et al.*, 1985).

Caudry *et al.* (1995) demonstrated that this 2.6-kb plasmid is similar to the 2.6-kb plasmid pTD1, previously reported by Ivic *et al.* (1991), which shows homology to the ssDNA plasmids associated with Gram-positive bacteria. It is 2, 647 bp in total (MacDougall, *et al.*, 1992), with three open reading frames (ORF A, B and C). ORF A showed similarity to sequences encoding the replicative (Rep) proteins of *Bacillus subtilis* plasmid pBAA1, *B. amyloliquefaciens* plasmid pFTB14 and plasmids found in *Lactobacillus hilgardii* and *Streptococcus agalactiae.* Like pBAA1-related plasmids, pTD1 shares a consensus sequence for the origin of replication, also found at the active site of the ϕX174 bacteriophage Rep protein. ORF B has weak homologies to the recombinases of several Gram-positive associated plasmids (MacDougall, *et al.*, 1992). Such ssDNA plasmids replicate through a ssDNA intermediate by rolling circle replication. Regions of pTD1 could putatively serve as the plus origin, minus origin, and Rep proteins essential to this family of plasmids (MacDougall, *et al.*, 1992).

This data holds much promise: it raises the possibility that Gram-positive bacteria may be more appropriate hosts in which to express spirochete proteins, reported to be poor in *E. coli* (MacDougall, *et al.*, 1992), and it could herald the breakthrough that has been long awaited in treponemal genetics. Shuttle vectors between *T. denticola* and Gram-positive bacteria, such as *B. subtilis*, are now a distinct possibility.

III. Spirochete-Associated Diseases.

The majority of spirochete species are free-living or nonpathogenic host-associated bacteria.

In the stomach and intestine of animals spirochetes can be numerous. In ruminants, spirochete counts of 10^{10} ml^{-1} have been reported (Johnson, 1977). Spirochetes are found in the intestines of humans, monkeys, pigs, dog, mice and rats, where they are associated with the epithelium and traditionally considered unimportant pathologically (Glock and Harris, 1972; Johnson, 1977; Neef, *et al.*, 1994).

A long known exception is *Serpulina hyodysenteriae*, which is associated with swine dysentery (Glock and Harris, 1972; Harris, *et al.*, 1972). The disease symptoms are inflammation, over-synthesis of mucus, intestinal necrosis, weight loss, dehydration and hemorrhaging, and can be fatal (Ter Huurne, *et al.*, 1992). Birds are susceptible to infection by *Borellia anserina* (Johnson 1977). This borreliosis is not known to relapse (Johnson, 1977), unlike the borreliosis that affects humans (Greenwood, *et al.*, 1992). In natural populations of rabbits, a syphilis-like venereal disease is caused by *Treponema paraluis-caniculi*. Incidence of this disease has been reported to range from 0.3% to 60% (Johnson, 1977).

Of the many spirochete species only a few are known to be pathogenic to humans. Syphilis, once referred to as the great pox, is an ancient disease that is spread through sexual contact, with initial disease manifestations in the genital area. In the United Kingdom, 3000 new cases were reported between 1981 and 1985 and in the United States, forty thousand cases were reported in 1988 alone (Young, *et al.*, 1989).

Syphilis is caused by *Treponema pallidum* subspecies *pallidum (T. pallidum)*, an organism that, in common with the other pathogenic treponemes, is sensitive to penicillin, with no reports of resistance (Greenwood,*et al.*, 1992; Schmidt, 1989). Syphilis is, therefore, easily resolved if diagnosed early. In approximately one third of untreated individuals, tertiary disease occurs after a period in the region of five years (Schmidt, 1989). Tertiary syphilis can result in arteritis, often leading to strokes, or in treponemal dissemination to the brain, causing dementia. Impotence, bladder problems, destruction of the aortal wall, and sharp pains through the body are also seen in certain cases of tertiary disease (Greenwood, *et al.*, 1992; Schmidt, 1989).

Endemic syphillis, also known as bejel, is transferred by direct contact with an infected person or through food and drinking vessels. It affects mostly children in Africa, Western Asia, and Australia (Greenwood, *et al.*, 1992). The disease, caused by *Treponema pallidum* subspecies *endemicum*, is less severe than venereal syphilis as no effects on the aorta or brain are seen (Greenwood,*et al.*, 1992; Smidt, 1989).

The etiological agent of yaws is *Treponema pallidum* subspecies *pertenue*. This disease occurs in tropical and subtropical regions (Greenwood, *et al.*, 1992). Until recently this was a major health concern with >50 million estimated cases during the 1950's (Guthe and Wilcox, 1954). Since then, the WHO has organised a program of eradication, resulting in a dramatic drop in disease prevalence. Two million cases were estimated during the 1970's. This progress was achieved through the administration of penicillins (Greenwood, *et al.*, 1992; Schmid, 1989). Like endemic syphilis, yaws mainly affects children (Greenwood, *et al.*, 1992; Schmid, 1989).

Pinta was once widespread in the western hemisphere, but is now restricted to remote areas of the central Americas (Schmid, 1989). *Treponema carateum* is responsible for this disease and may be the first pathogenic treponeme to have emerged (Greenwood, *et al.*, 1992; Johnson, 1977; Schmid, 1989). Pinta is limited to the skin and is a disease that can affect all age groups (Schmid, 1989).

Leptospirosis is caused by *Leptospira interrogans* in humans and animals and can be fatal (Greenwood, *et al.*, 1992; Schmid, 1989). *Leptospira interrogans* thrives in the proximal convoluted tubules of the kidney and the liver (Johnson, 1977). Humans become infected through contaminated water or contact with infected animals via openings in the skin (Greenwood, *et al.*, 1992; Johnson, 1977; Lecour, *et al.*, 1989; Schmid, 1989). Leptospires are shed into the urine from their reservoir in the kidney and urinary counts of 10^7 spirochetes ml^{-1} have been found (Johnson, 1977). Excretion of *Leptospira* in urine creates further potential sources of disease: the excreted bacteria can survive for up to 15 days outside a mammalian host (Johnson, 1977). At its extreme, leptospirosis causes jaundice and renal failure, haemoraging of the eyes, skin, and mucous membranes, encephalitis, meningitis and myocarditis (Greenwood, *et al.*, 1992; Schmid, 1989). Icteric disease, caused by serovar *icterohaemorhagiae*, is fatal in 10% of cases (Greenwood, *et al.*, 1992).

Lyme disease, recognized in 1975, is the most recent spirochete-caused disease to be characterized. The name of the disease refers to the town of Lyme, Connecticut, where much attention on the emergence of this disease was centered (Barbour and Fish, 1993; Steere, *et al.*, 1983). Lyme disease is the most common vector-borne infectious disease in temperate climates (Wallich, *et al.*, 1983). Upwards of 6000 new cases are reported annually in North America (France, *et al.*, 1992). Fever, headache, and a characteristic skin rash known as erythema chronicum migrans at the site of the bite of an arthropod vector develop during the month post-infection with *Borrelia burgdorferi* (Schmid, 1989). The rash may grow or several lesions may ensue (Greenwood, *et al.*, 1992; Schmid, 1989).

Secondary disease is characterized by dissemination of spirochetes which may result in swelling, blockage, or failure of the heart, meningitis or encephalitis in a minority of patients (Greenwood, *et al.*, 1992; Schmid, 1989). Approximately 60% of patients develop tertiary symptoms of arthritis, which becomes chronic in 10% of cases (Schmid, 1989). Tertiary disease is therefore disabling, but fatalities are rare (Barbour and Fish, 1993). Spirochetes have been detected in the brain, eyes, joints, nerves and heart of

humans with Lyme disease (Steere *et al.*, 1983). Excretion of *Borrelia burgdorferi* from the kidneys has been reported in humans (Schmid, 1989). *B. burgdorferi* is known to be carried by *Ixodes pacificus* and *I. scapularis*, in North America and by *I. ricinus* and *I. persulcatus* in Europe and Eurasia (Barbour and Fish, 1993).

Louse-borne relapsing fever (LBRF) is caused by *Borrelia recurrentis* and is spread by the human louse, *Pediculus humanus humanus*. Tick-borne relapsing fever (TBRF) is caused by other *Borrelia* species identified in association with their vectors: *B. hermsii*, whose vector is *Orithodoros hermsii*, *B. turicatae (O. turicata)*, and *B. parkeri (O. parkeri;* Johnson, 1977; Schmid, 1989)

Fever, headache and myalgia occur, followed by or concurrent with dissemination of spirochetes to the CNS (Schmid, 1989). In the acute phase of disease, counts of 10^5 to 10^7 spirochetes ml^{-1} can be reached in the blood (Greenwood, 1992; Johnson, 1977). Initial symptoms cease abruptly, with hypotension and shock. Death through heart failure can occur (Greenwood, *et al.*, 1992; Schmid, 1989). Spirochetes can be seen, at autopsy, in the muscle of the heart and inside cardiac vessels (Schmid, 1989). Up to five relapses occur and are thought to be due to the capacity of *Borrelia* to switch serotype (Schmid, 1989). LBRF is thought to have affected 10 million people directly after World War II, of which 5% died from the disease (Johnson, 1977).

The oral spirochetes have been implicated in the etiology of periodontitis. Association studies, potential virulence factors, their effects on the immune system, and interaction with the vast numbers of bacterial species known to inhabit the oral cavity will be discussed in part V of the introduction to this thesis.

IV. The Etiology of Periodontal Disease.

(i) Characterization of periodontal disease.

The term "periodontal disease" (PD) is inclusive of all the bacterial-mediated inflammatory disorders of the periodontium. PD can be distressing as it is painful,

involving bleeding and ulceration, bone destruction and eventually tooth loss. It may be difficult to treat, and may not respond to therapy in some cases.

Historically, there are three hypotheses to describe the relationship of plaque to PD. The specific-plaque hypothesis (Loesche, 1976) states that there is a specific pathogen in plaque that is responsible for the induction of disease. The non-specific plaque hypothesis (Loesche, 1976) works on the proposition that it is the cumulative effects of the many species of bacteria present in plaque and around gingival tissue that leads to clinical manifestations of PD. The third hypothesis is the exogenous hypothesis: here, PD is suggested to be the result of the disruption of the normal plaque microflora by a newly acquired specific pathogen (Genco, 1987).

Gingivitis, a milder disease than periodontitis, is defined by gingival inflammation, and characterized by a red and swollen appearance, with bleeding of the gingiva, either spontaneously or on tooth-brushing or probing (Kleinberg and Wolff, 1986; Loesche, 1993). Localized accumulation of neutrophils (PMNs) and increased vascular permeability are seen (Page and Schroeder, 1981). Whether progression of gingivitis to periodontitis or spontaneous regression to a healthy periodontium occurs varies between individuals (Page and Schroeder, 1981).

Periodontitis, or destructive PD, is characterized by a loss of attachment of epithelial cells, a deepening of the gingival crevice, known as pocket formation, between the tooth and supporting tissues which can reach >10 mm, alveolar bone loss, and increased tooth mobility, eventually leading to tooth loss (Kleinberg and Wolff, 1986; Loesche, 1993). Ulceration of the pocket epithelium and gingival tissues in PD is the result of complex immune and inflammatory reactions to challenge by plaque bacteria. Histological and pathologic alteration to vascular and connective tissues and the epithelium are all apparent during disease progression (Lamster, *et al.*, 1985).

The epithelial barrier is lost, allowing intensification of inflammation (Page and Schroeder, 1981), thereby facilitating bacterial invasion and the movement of cytotoxic and degradative microbial products into the gingival tissues. The clinical subdivisions of PD are largely defined by the age of patients in which disease is manifest, the particular teeth affected and by the speed of disease progression. The early-onset periodontal

diseases (EOP) generally occur after puberty and progress rapidly (Scheinken, 1994). Localized juvenile periodontitis (LJP), which affects the first molars and incisors, generalized juvenile periodontitis (GJP), which can affect large numbers of teeth, and rapidly-progressing periodontitis (RPP) are all forms of EOP. A definitive differentiation has not been established between GJP and RPP (Sheinken, 1994). Pre-pubertal periodontitis (PPP) is another of the EOPs, but, as the name suggests, occurs at an earlier age and usually effects the primary teeth (Scheinken, 1994). The EOPs tend to affect individuals within family groupings (Michalowicz, 1994; Scheinken, 1994).

Acute necrotizing ulcerative gingivitis (ANUG) is a recurrent gingivitis that onsets rapidly and may progress to periodontitis (Riviere, *et al.*, 1991). Adult chronic periodontitis (ACP) is the most common form of PD and primarily affects persons 30 years and older (Michalowicz, 1994; Riviere, *et al.*, 1991). ACP can be a cyclic, episodic condition (Gooman, 1992).

No single bacterial species has been proven to be the direct agent of PD, and many different microbial species inhabit the oral cavity. It is necessary to give a brief overview of the gingival flora and the immune and inflammatory responses to this flora, and the destructive events that occur in the periodontium leading to periods of disease activity, before the significance of the oral anaerobic spirochetes to PD can be properly addressed.

(ii) Treatment of Periodontal Disease.

The ideal periodontal disease therapy would be a treatment that is both preventative and allows regeneration of damaged tissues to a state of full health. Presently, procedures that slow disease progression or help to restore a component of damaged tissues are considered successful (Lynch and Giannobile, 1994). By this yardstick, most cases of periodontal disease can be treated successfully by the conventional therapy of scaling, root planing and by surgery, if required (Oshrain, *et al.*, 1986). Surgical debridement involves elevation of a gingival flap, debridement and scaling of the root surface, removal of granulamatous tissue from the lesion and replacement of the flap, generally leading to clinical improvement (Egelberg, 1987). However, Brown and Garcia (1994) reported that diagnostic and preventative services currently employed in the United States are not predictive of the extent or severity of periodontitis. Periodontal disease remains the leading cause of tooth loss in adults (Lynch and Giannobile, 1994).

As periodontal disease is considered to be mediated by bacteria, the use of antibiotics has been investigated. In reviewing the available data, Gordon and Walker (1993) concluded that tetracyclines are beneficial, in conjunction with debridement, in the treatment of *A. actinomycetemcomitans*-associated disease (LJP and RPP), conventional therapy is sufficient in most adult periodontitis cases, and that the use of metronidazole, amoxicillin/clavulanate potassium, tetracycline or metronidazole/amoxicillin is beneficial in refractory cases. No antibiotic is successful for all cases, therefore culturing and antibiotic sensitivity testing of subgingival flora at diseased sites should be performed. In the United States, tetracyclines (tetracycline hydrochloride, doxycycline and minocycline) are most commonly utilized in the treatment of periodontal disease. These antibiotics are unique in that they reach higher concentrations in the GCF than in serum (Gordon and Walker, 1993).

In a study of 96 patients with adult chronic periodontitis by Al-Joburi *et al.* (1989), all participants received scaling and root planing and tetracycline or spiramycin or a placebo. After 2 weeks the proportions of spirochetes were reduced from 26% to 4%,

28% to 4%, and 30% to 7% for each group, respectively. Short term use of antibiotics in conjunction with conventional therapy was determined not to be of value in controlling oral spirochete growth compared to scaling and root planing alone.

In search of therapies that would permit regeneration of the periodontium to full health, Lehner *et al.* (1994) have reported on the investigation of synthetic peptides with the goal of priming the immune system to putative pathogens by T cell epitope, B cell epitope, and bacterial adhesion molecule epitope mapping. Polypeptide growth factors, such as platelet-derived growth factor, insulin-like growth factor-1, epidermal growth factor, fibroblast growth factor and bone morphogenetic proteins are under investigation for their role in inducing angiogenesis, reconstitution of epithelial cells, ECM and collagen synthesis, osteoclast migration and host cell metabolism with the hope that eventually the application of appropriate growth factors may promote healing (Cho, *et al.*, 1994; Lynch and Giannobile, 1994; Rutherford, *et al.*, 1994). Guided tissue regeneration, in which a barrier is used to separate the soft tissue from the root, with a space between the barrier and the root to allow bone and ligament formation is a clinical reality (Gottlow, 1994). In reviewing therapy for periodontitis, one study by Genco is of note. He found that 43% of people with periodontitis in Buffalo, New York, were untreated (cited in Brown and Garcia, 1994).

(iii) Events in the Periodontium during Periodontitis

The primary barrier between the gingival tissues and plaque, a mixed bacterial population, is the junctional epithelium, extending from the junction formed by the cementum and enamel of the tooth almost to the gingival crest. It shows no pattern of terminal differentiation (Mackenzie, 1987). New cells are formed at the base of the gingival crevice, whilst older cells of the junctional epithelium migrate upwards and are shed (Shultz-Haudt, 1977). A dense plexus of blood vessels, with a concentration of mast cells nearby, is located in the connective tissue just below the junctional epithelium, from which a constant flow of PMNs migrate into the gingival tissue and sulcus. PMNs accumulate between plaque and the gingival tissue (Page and Schroeder, 1981) and are

largely protective against the continuous microbial challenge. The first histopathological symptoms of gingivitis are increased vascular permeability (Page and Schroeder, 1981), reflected in increased flow of gingival crevicular fluid (GCF; Kleinberg and Wolff, 1986), and an increase in accumulating PMNs. Plasma cells eventually become the dominant immune cells at sites of infection (Page and Schroeder, 1981). As the disease progresses to periodontitis, there is a dense population of T cells, B cells and plasma cells, macrophages and PMNs in the infected gingival tissues (Page and Schroeder, 1981).

Collagen Degradation.

Collagen is the major structural component of gingival connective tissues (Lamster, *et al.*, 1985). Type I collagen comprises 85% of connective tissue, lying beneath the epithelium, with fibronectin, acidic glycoproteins, glycosaminoglycans and Type III collagen as the other main components. The connective tissue is synthesized and maintained by a population of fibroblasts resident in the periodontium (Page and Schroeder, 1981). Destruction of collagen fibers is seen at the earliest stages of PD and excessive degradation is a characteristic that helps define periodontitis (Lamster, *et al.*, 1985), particularly lateral to the pocket (Page and Schroeder, 1981). There is an increase in the amount of collagen synthesis as well as breakdown. Poorly cross-linked collagen and a new collagen molecule, a trimer of Type I collagen, is produced (Page and Schroeder, 1981).

The gingival crevicular fluid (GCF) originates in the gingival capillaries of the connective tissue (Lamster, *et al.*, 1985) and migrates upwards, where it is released into the mouth and washed away by saliva. The GCF is composed of serum, connective tissue constituents and factors found in the gingival crevice, including bacteria, bacterial products, and epithelial cells. In healthy periodontia the volume of GCF in a gingival crevice is 0-0.1 ml (Lamster, 1985). This volume increases during episodes of PD (Kleinberg and Wolff, 1986; Lamster, *et al.*, 1985)

Several studies on the specific contents of GCF have been revealing. Bowers *et al.* (1989) measured the amount of osteonectin, a protein with high affinity for

collagen, and N-propeptide α-1-type 1 collagen, an indicator of active collagen synthesis, in the GCF. Both factors increased in concentration in the GCF during periodontal disease, correlated with pocket depth, and are indicative of active alterations to the collagen networks of the connective tissue.

Host collagenases are involved in the deterioration of gingival tissue. Villela *et al.* (1987) tested GCF from diseased sites for collagenases. They found 3/4- 1/4 cleavage patterns of rat tail tendon cartilage, indicative of vertebrate collagenase activity. The use of synthetic collagen peptides also showed active host collagenases in periodontal pockets. Bacterial collagenases preferentially cleave glycine-isoleucine bonds in collagen; whereas vertebrate collagenases cleave glycine-glycine bonds. A synthetic peptide containing the vertebrate collagenase cleavage site was degraded by GCF from diseased sites, and the activity of this collagenase(s) rose by 550% over 4 weeks during observations on the progression of gingivitis in humans (Lamster, *et al.*, 1985). Collagen degradation, as monitored by GCF analysis, precedes obvious clinical signs of disease (Kleinberg and Wollf, 1988). These observations suggest that PD is an interactive disease between the host and challenging bacteria.

Pocket Formation.

Pocket formation is the result of proliferation of the junctional epithelial cells and their apical migration, followed by detachment from the surface of the tooth. The epithelium becomes very thin and disrupted; ulceration occurs (Tokata and Dorath, 1988) and bacteria can penetrate more easily into gingival tissue. Pocket formation is crucial as the Eh of periodontal pockets is estimated to be -100 mV (Loesche and Laughon, 1981) and can therefore support anaerobic growth of bacteria. It is also a protecting site, as bacteria are shielded from masticatory forces, saliva flow, and ingestion.

Alveolar Bone Loss.

Bone formation and absorption is controlled by osteoblasts and osteoclasts.

Osteoclasts are stimulated by IL-1, TNF-α, IL-6 and vitamin D to resorb bone. Differentiation of osteoclasts is inhibited by IFN-γ and TGF-β and IL-1 receptor antagonist. The generation of protons by carbonic anhydrase causes demineralization of the bone, which is subsequently resorbed (Genco, 1992; Stashenko, *et al.*, 1994). Osteoblasts, conversely, are stimulated by TGF-β, osteogenin and osteogenic inducive factor and can synthesize a matrix of collagen, osteocalcin, osteopontin and proteoglycans, which are then mineralized to form new bone (Genco, 1992). This bone forming function of osteoblasts is inhibited by IL-1, TNF-α, and TNF-β (Genco, 1992; Stashenko, *et al.*, 1994). IL-1α, IL-1β, TNF-α and IL-6 are all implicated in the inflammatory response to bacteria (Stashenko, *et al.*, 1994) and are found at significantly elevated levels at diseased sites and in the GCF (Stashenko, *et al.*, 1994). Recombinant human IL-1β stimulates bone resorption in rats *in vivo*, and elevated serum levels of calcium and increases in the number of osteoclasts can be seen (Nguyen, *et al.*, 1991).

(iv) The Role of Bacteria in Periodontal Disease.

Bacterial involvement in PD was suggested in the 1800's (Harlan, 1883; Rawls, 1885). Bacteria are now accepted as the cause of periodontitis and periodontal disease (Frank, 1980; Kleinberg and Wolff, 1986; Listgarten, 1987; Loesche, 1993; Newman, 1990; Page and Schroeder, 1981; Wolff, *et al.*, 1994).

During manifestations of PD it is apparent that a shift has occurred from the normal, predominantly Gram-positive, coccoid microflora to a Gram-negative, anaerobic and largely motile flora in the periodontal pocket (Kleinberg and Wolff, 1986; Listgarten, 1976; Loesche, 1993; Singletary, *et al.*, 1982). Singletary *et al.* (1982) documented 62% coccoid and 5% motile bacteria among the total microflora present at healthy sites, and 18% coccoid and 44% motile cells at diseased sites.

More than 350 species have been found in supra- or subgingival plaque (Wolff, *et al.*, 1994; Listgarten, 1987) and up to 60 species in a single pocket (Wolff, *et al.*, 1994). Of these >350 species, there are perhaps ten bacteria that are considered the most likely periodontopathogens, although additional species that have not yet been cultivated may be

equally important. Those bacteria considered risk indicators for the development of periodontitis are *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Fusobacterium nucleatum, Campylobacter rectus, Eikenella corrodens, Prevotella intermedia,* and the treponemes, including *Treponema denticola, T. socranskii, T. vincentii,* and the uncultivable pathogen-related oral spirochetes (PROs; Newman, 1990; Riviere, *et al.*, 1991, 1992; Wolff, *et al*, 1994).

There is no available method that can accurately quantify and identify all species present in subgingival plaque samples. New oral isolates from the gingival crevice are regularly described in the literature, recently including *Oribaculum catoniae, Catonella morbi, Johnsonella ignava,* and *Hallella seregens* (Moore and Moore, 1994), all Gram-negative bacilli, and nine new oral spirochete isolates (Caudry, *et al.*, 1995).

Frank (1980) showed penetration of spirochetes and other bacteria into the wall of periodontal pockets, although no attempt was made to classify the invading microbes. Research that helps to understand the virulence determinants of individual strains of bacteria, how the immune system responds to them, how the bacteria can affect the immune response and how the bacteria interact with each other is of paramount importance in realizing a more complete picture of periodontitis.

In examining clinical data, it is apparent that gingival inflammation does not necessarily lead to periodontitis, and the putative pathogens associated with periodontitis can be found in both healthy and diseased sites (Barron, *et al.*, 1991; Riviere, *et al.*, 1992; Slots, *et al.*, 1985a\b; Wolff, *et al.*, 1994; Zambon, *et al.*, 1985). The importance of commensal bacteria should not be overlooked. It has long been established that commensal bacteria in general are protective. In the oral cavity, *Streptococcus mitis* inhibits bacilli causing diphtheria (Thomson and Shibuya, 1946), streptococci can inhibit the growth of *Pseudomonas* (Sprunt and Redman, 1968), and *S. sanguis* can inhibit *A. actinomycetemcomitans* (Taubman, *et al.*, 1989).

Certain bacterial factors are found in periodontal pockets that may be involved in pathogenesis. Although specific oral bacteria are known producers, due to the large number of microbial species present in the subgingival plaque, these factors that can be detected in the GCF are of uncertain origin. There is a correlation between endotoxin in

the GCF and gingival inflammation. Endotoxin, as well as lipoteichoic acid and muramyl dipeptide, can stimulate bone resorption *in vitro*. Endotoxin also causes the release of lysosomal enzymes from PMN's which can intensify the inflammatory reactions (Listgarten, 1987). Ammonium, produced through amino acid utilization by plaque bacteria, is found and could be a primary irritant to the gingiva, and can inhibit collagen synthesis (Kleinberg and Wollf, 1986).

The short chain fatty acids (SCFAs) produced by anaerobic bacteria can be detected in periodontal pockets and can diffuse readily and penetrate into gingival tissues (Kleinberg and Wolff, 1986; Tonetti, *et al.*, 1987). The SCFAs found include formate, propionate, isovalerate, succinate and isobuyrate. SCFA levels have been correlated to PD severity (Botta, *et al.*, 1985; Tonetti, *et al.*,1987). SCFAs can inhibit PMN chemotaxis *in vitro*, at concentrations comparable to that found in periodontal pockets (5-30 mM; Botta, *et al.*, 1985), can inhibit lysozyme release by PMNs stimulated with f-met-leu-phe (F-MLP) *in vitro*, and can also partially inhibit superoxide release by PMNs (Tonetti, *et al.*, 1987). Other *in vitro* evidence suggests SCFAs can inhibit human gingival fibroblast proliferation (Singer and Buckner, 1981), affect actin synthesis in fibroblasts (Yamada, *et al.*, 1985), and cause blebbing of PMN cell membranes (Tonetti, *et al.*, 1987).

The metabolism of cysteine and methionine by bacteria can result in the production of hydrogen sulphide (Kleinberg and Wolff, 1986). Methyl mercaptans and H_2S are found in periodontal pockets and the relative amounts of these compounds is reduced on treatment of periodontitis (Horowitz and Folke, 1973; Persson, *et al.*, 1990). H_2S is formed under *in vitro* conditions in a simple experiment where plaque samples are incubated with serum (Persson, *et al.*, 1989). The amount of H_2S formed in periodontal pockets exceeds the amount of free sulfur-containing amino acids available; therefore, proteolysis is probably involved in providing the substrates required (Persson, *et al.*, 1990). Volatile sulfur compounds can cause inactivation of cytochrome oxidase (Nicholls and Kim, 1982), the formation of persulphide groups from disulphide bonds (Valentine, *et al.*, 1987) and inhibit myeloperoxidase (Claesson, *et al.*, 1989) and catalase (Nicholls, 1961).

Hyaluronidases are present in dental plaque and could play a role in tissue

destruction by degrading the hyaluronic acid component of connective tissue ground substance (Tam and Chan, 1985). This could also facilitate bacterial toxin and/or bacterial penetration into gingival tissue.

Phospholipase C (PLC) can participate in the destruction of host cells and the release of arachidonic acid. Arachidonic acid is shunted to the lipoxygenase and cyclooxygenase pathways to produce the inflammatory mediators prostaglandins and leukotrines. Siboo *et al.* (1989) showed that PLC is not present in saliva or in GCF collected from healthy sites, but is detected in sites of active periodontitis. This suggests that PLC-mediated hydrolysis of membrane phospholipids may be an important factor in the etiology of PD.

Adherence, to a host cell or surface, or to other bacteria, is a prerequisite for colonization in the oral cavity. Rambourg (1972) showed that a carbohydrate coat was present at the surface of most cells in plaque adhering to teeth. More recently, adhesion has been shown to be mediated by bacterial adhesins and proline-rich proteins in saliva and mucins (Lie, *et al.*, 1994). *Actinomyces naeslundi, A viscosus, F. nucleatum, P. gingivalis,* and *P. intemedia* can adhere to blood group glycoproteins, which are present on epithelial cells (Lie, *et al.*, 1994).

Prevotella loeschii binds to both bacterial and eukaryotic cells via N-acetyl-D galactosamine residues by means of a fimbrial adhesin, in a similar manner to *A. viscosis* (Gharbia and Shah, 1993). As a specific example of the complex interactions that permit the dynamic population of plaque bacteria to exist in the niche of the subgingiva, *P. loeschii* and *A. viscosis* can aggregate with *Prevotella melaninogenica* and *P. gingivalis. P. melaninogenica* and *P. gingivalis* cannot aggregate with most Gram-positive bacteria. *Streptococcus sanguis* interacts with *P. loeschii* by means of a 77 kDa aspartic acid-glutamic acid rich protein. *F. nucleatum* has a hydrophilic 38.5 kDa outer membrane protein that can interact with LPS and phospholipids of black-pigmented anaerobes, due to its high hydrophilicity (Gharbia and Shah, 1993). *P. gingivalis* can, in turn, interact directly with *T. denticola* and can be seen aggregating with the spirochete *in vitro.* This co-aggregation is bimodal, and can be inhibited by arginine and D-galactosamine (Grenier, 1992a,b). *T. denticola* can attach directly to epithelial cells and

human gingival fibroblasts (Ellen, *et al.*, 1994a; 1994b; Olsen, 1984) in a manner that is fibronectin (Ellen, *et al.*, 1994b) or galactose and mannose (Weinberg and Holt, 1991) mediated. Such interactions are imperative in the establishment of the microflora of periodontal pockets. It is probable that a highly interactive nutritional chain exists and that this mixed population of bacteria exhibit a succession of dominant species as the pocket deepens and periodontal disease progresses.

(v) The Role of the Immune Response in Periodontitis.

The interaction between the host immune response and the challenging bacteria is responsible for PD. It is a combination of immune cell-mediated damage and the direct pathogenesis of bacteria and their products that is destructive. When the immune response does lead to degradation of periodontal tissues, it should be remembered that, even then, it is still playing a protective role against systemic infection. When there is no apparent genetic or pathologic immune deficiency, it is not clear if PD results from a constant challenge, that over a sustained period will result in tissue damage, or if a diseased state results from an immune dysfunction due to specific immunosuppresive agents of pathogenic bacteria, or immune evasion by the pathogens. Overall, however, the immune response is largely protective and primarily beneficial. Several important aspects of the generalized host response to challenge by plaque bacteria will be discussed below.

The immune system and gingival tissue are aided by the shedding of epithelial cells and the upward and outward flow of the GCF, which helps to clear both bacteria and soluble noxious products. In a single pocket there can be 10 to 50 million colony-forming units of bacteria, greater than on any other epithelial surface (Loesche, 1993). The pathogenic microbial components of this population must avoid removal by the GCF, avoid the anti-microbial activities of the PMNs and penetrate the epithelial layer. Once a bacterium has entered the connective tissue it must withstand the higher O_2 tensions of vascularized tissue and a primed immune response.

Sequestration of Iron Sources.

A non-specific aspect of the host defense system is the sequestration of iron, to levels that are incompatible with bacterial life. Free iron in plasma has been estimated at a concentration of $<10^{-18}$ M, while 1 - 4 x 10^{-7} M Fe is generally required by bacteria (Bullen, *et al.*, 1978). In order to circumvent this problem, host-associated bacteria must develop a system(s) that allows their iron requirements to be met. The mechanisms by which the host limits iron availability and how the putative periodontal pathogens are able

to subvert this non-specific defense will be addressed in more detail in chapter 2, which will describe a model to explain how *Treponema denticola* may contribute to the elevated iron concentrations seen in the GCF in adult periodontitis and obtain the iron that is required for growth.

Polymorphonuclear Leukocytes.

In the normal periodontium there is a constant flow of neutrophils from the vascular system to the sulcus. Here, PMNs form a barrier between the bacteria and the tissues underlying the epithelium, forming the first line of defense against plaque (Hart, *et al.*, 1994; Page and Schroeder, 1981).

One percent of the circulating neutrophils leave the blood for the junctional epithelium and gingival sulcus daily (Mayasaki, *et al.*, 1994). The chemotactic influence of bacteria can be observed by application of saline plaque extracts directly to the gingival sulcus, resulting in the accumulation of PMNs (Page and Shroeder, 1981) Host-derived chemotactic agents include kinins, prostaglandins, cytokines, collagen peptides (Page and Schroeder, 1981) and some of the complement proteins, notably C3a, C4a, and C5a (Abbas, 1995). Interaction of neutrophil surface receptors with antibody Fc regions and C3b leads to phagocytosis and ingestion of bacteria (Hart, *et al.*, 1994). Bacteria are engulfed and internalized into the phagosome which fuses with the lysosome to form the phagolysosome (Mayasaki, *et al.*, 1994). Both oxidative and non-oxidative mechanisms are available to the PMNs in order to kill and control bacteria.

Reactive oxygen intermediates are the main means of killing of bacteria by PMNs (Kalmar, 1994). PMNs are generally effective in controlling infection by phagocytosis and release of microbicidal agents by secretion, lysis, and apoptosis, and by the newly recognized bactericidal proteins of the cytosol and nuclear regions (Mayasaki, *et al.*, 1994).

Protein synthesis is limited in mature PMNs. Instead of *de novo* production of antimicrobial molecules, PMNs utilize an arsenal of pre-formed substances stored in primary and secondary granules. The primary, or specific, granules contain lactoferrin, an

iron scavenging protein, cobalophalin, a B-12 binding protein, and lysozyme. The secondary, or azurophilic, granules, contain the defensins (HNP 1 to 4), serpocidins (Elastase, azurocidin, proteinase 3), lysozyme, myeloperoxidases, and Cathespin G (Mayasaki, *et al.*, 1994; Page and Schroeder, 1981). Cathespin G (CG), which is of great interest with respect to treatment of periodontitis, is a neutral serine protease, with a specificity resembling that of chymotrypsin. It is the most potent non-oxidative anti-bacterial agent isolated from neutrophils against periodontal pathogens. Two peptides are currently under investigation as therapeutics and are located in the CG 1-20 and CG 61-80 amino acid regions of the cathespin G molecule. Synthetic peptides representing CG 117-136 and CG 198-223 are also highly bactericidal (Mayasaki, *et al.*, 1994).

The calcium-dependent calprotectin accounts for 45% of the cytosolic protein of periodontal neutrophils. Its antibacterial activity has been estimated to be active for five cell diameters on lysis of PMNs. It is thought to be particularly important in the control of oral health as it is found in neutrophils and gingival keratinocytes and macrophages but not in Langerhans cells, skin, the intestines or other tested cell types (Mayasaki, *et al.*, 1994).

Many studies have highlighted the crucial role of neutrophils in the control of periodontitis. In humans, defects in neutrophil chemotaxis are widely reported in LJP, RPP, and refractory cases of adult periodontitis (Oshrain, *et al.*, 1986; Page and Schroeder, 1981; Sela, *et al.*, 1988; Shenker, 1987) and are evident in peripheral blood neutrophils (Genco, 1992). Double or multiple peak responses of PMN chemotaxis to casein, a chemotactic agent active *in vivo*, indicates a prolonged response that may be due to the inability to target and eliminate the chemoattractant. Such defects could be bacterial-induced or intrinsic. The multiple peak response has been reported *in vivo* in humans with refractory adult periodontitis (Oshrain, *et al.*, 1986) and double peak response has been reported *in vivo* in humans with LJP (Singh, *et al.*, 1984). In normal, healthy periodontia a single peak of accumulating neutrophils to casein is seen (Oshrain, *et al.*, 1986).

PMNs may be defective in adherence, in Leukocyte Adhesion Deficiency (LAD) disease; in locomotion and chemotaxis, in LJP; in phagocytosis, in complement disorders; in degranulation, in Chediak-Higashi syndrome; and in bactericidal activities, in LJP and chronic granulomatous disease. Patients with these disorders are at high risk for periodontitis (Hart, *et al.*, 1994). By comparison, disorders of the primary lymphocytes do not result in greater severity of periodontitis than is normal, and transplant patients immunosupressed by prednisone or cyclosporine also do not have greater risk of periodontitis severity (Genco, 1992; Hart, *et al.*, 1994; Kalmer, 1994; Van Dyke and Hoop, 1991).

In patients with LAD, mutations leading to inherited defects in Mac-1 and p150,95 (the CD11/18 group) lead to dysfunction in neutrophil adherence and has been proposed as the molecular basis to explain generalized PPP and the susceptibility to bacterial infection that results in disease (Hart, *et al.*, 1994; Page, *et al.*, 1984). In LJP there is a reduction in the number of receptors for f-MLP, C5a, IL-8 and Leukotriene B_4 (Hart, *et al.*, 1994; Kalmar, 1994). In LJP patients with defective chemotaxis, there is a 50% drop in the expression of Gp110. Monoclonal antibodies against Gp110 inhibit the chemotaxis of normal neutrophils (Hart, *et al.*, 1994; Kalmar, 1994; Van Dyke, *et al.*, 1987).

PMNs utilize a G-protein signal transduction pathway. On receptor-attractant interaction, the G-proteins signal to activate PLC, which forms diacylglyceride (DAG) and inositol triphosphate (IP_3) from phosphatidylinositol bisphosphate. DAG subsequently activates protein kinase C (PKC); IP_3 activates an intracellular Ca^{++} flux, both leading to a cellular response (Hart, *et al.*, 1994). The Ca^{++} influx is decreased in neutrophils in LJP patients (Daniel, *et al.*, 1993; Hart, *et al.*, 1994). DAG kinase activity falls in neutrophils from patients with LJP, leading to an increased concentration of DAG. This altered signaling could explain the reduced chemotaxis, elevated reactive oxygen intermediates, down regulated f-MLP receptors, and PKC production observed in LJP (Hart, *et al.*, 1994; Tyagi, *et al.*, 1992; Kurihara, *et al.*, 1993). Human PMN DAG kinase cDNA has recently been cloned and sequenced, in order to examine any mutations in

DAG kinase that may be prevalent in diseased individuals, to help explain the molecular basis for certain forms of LJP (Champagne, *et al.*, 1993).

Genetic defects in neutrophil function are instrumental in the etiology of rare forms of PD. Adult chronic periodontitis is the most common form of periodontitis, and observations on genetic defects in the rarer forms of PD have led to a search for bacterial immunosuppressive factors among the putative periodontopathogens.

However, it should be remembered that chronic activation of neutrophils is partly responsible for tissue degradation. The neutrophil-derived factors that can cause injury include the elastins, reactive oxygen intermediates, collagenases, prostaglandins, cationic proteins; and the activation of complement is also important (Listgarten, 1987; Page and Schroeder, 1981).

Macrophages.

Peripheral blood monocytes migrate to periodontal tissue where they differentiate into macrophages. Macrophages, unlike PMNs, can replicate and can synthesize proteins *de novo*. They are long-lived cells with phagocytotic and microbicidal activity, can secrete complement proteins, prostaglandins, cyclic nucleotides, acid hydrolases, PMN chemotactic factors, elastase and activators of plasminogen. They can therefore destroy host tissues as well as bacteria. They can also produce calprotectin, as described in reference to the PMN antibacterial arsenal (Genco, 1992; Mayasaki, *et al.*, 1994; Page and Schroeder, 1981).

The role of macrophages in the control of periodontitis is not as well studied as that of the neutrophil. It is known that the number of macrophages in periodontal tissue is greatly increased during episodes of periodontitis (Page and Schroeder, 1981).

T and B Cells.

In experimental human gingivitis there is a normal $CD4^+$ T cell to $CD8^+$ T cell ratio of 2:1. In adult periodontitis, this ratio is reduced to 1:1 (Seymore, 1987). The autologous mixed lymphocyte reaction (AMLR) is the spontaneous proliferative response seen in peripheral blood leukocytes that have not been exogenously stimulated, when incubated for 7 days. The AMLR does not occur or is depressed in some patients with periodontitis (Genco, 1992; Seymore, 1987; Seymore, *et al.*, 1986), but returns to normal after treatment. Such observations suggest a central role for T cells, and that an imbalance in this control may be associated with disease progression.

T cells are prominent in all periodontal lesions, and $CD8^+$ cells are enriched in severe disease (Taubman, *et al.*, 1994). Isolated T cell clones from the periodontium are major histocompatability complex (MHC) restricted (Ishii, *et al.*, 1992). The $CD4^+$ cells isolated from sites of active adult periodontitis are predominately $CD29^+$, an adhesion molecule, and $CD45RA^+$, suggesting they are mature and activated (Taubman, *et al.*, 1994). Interleukin-2 receptors (IL-2R) and HLA-DR (MHC class II molecules) are elevated in $CD4^+$ T cells present at inflammatory sites in gingival tissue, in comparison to the expression of IL-2R and HLA-DR in peripheral blood lymphocytes. There is further elevation of IL-2R and HLA-DR expression in $CD4^+$ cells isolated from the gingival crevice (Takeuchi, *et al.*, 1991).

Most B cells isolated from gingival tissues are activated, as identified by the maturation and activation antigen FMC7. Indeed, a high level of immunoglobulin (Ig) is produced by B cells isolated from diseased host tissue, without stimulation (Shenker, 1987). Polyclonal B cell activation is noted in the gingiva of 70% of periodontitis patients (Genco, 1992).

Most adults have normal levels of serum Ig (Genco, 1992). It is not known if antibody production is protective, destructive, or irrelevant for PD. Most patients with adult periodontitis have elevated antibody titres to *P. gingivalis* (Genco, 1992), there is variation in reports in antibody responses to *T. denticola* (Jacobs, *et al.*, 1982; Lai, *et al.*,1986; Mangon, *et al.*, 1982; Tew, *et al.*, 1985), and high titres to

A. actinomycetemcomitans are seen in both serum and GCF in patients with LJP (Genco, 1992).

It is difficult to draw conclusions from antibody titres, with respect to the role of antibodies in periodontitis. There is a need to investigate the specific antigens that are recognized by the antibodies, and if the antibodies play a role in neutralizing toxins or in opsonization. Also, titres vary greatly between individuals and cross-reactivity will occur between pathogenic and non-pathogenic strains of related bacteria. However, local Ig production in the gingiva, as measured by Ig to serum albumin ratios, accounts for 75% of the antibody found in the GCF (Lovelace, *et al.*, 1981).

Immunoglobulins can contribute to opsonization, complement fixation, chemotactic factor release and antibody-dependent cellular cytoxicity, and can therefore contribute to periodontal destruction (Fujihashi, *et al.*, 1994). In populations of gingival mononuclear cells (GMCs) isolated from tissues of patients with adult periodontitis, IgG-producing cells are more numerous than IgA-producing cells (Fujihashi, *et al.*, 1994; Ogawa, *et al.*, 1989). IgA is more resistant to proteolytic cleavage than IgG, though some bacteria are capable of hydrolyzing IgA1 (Fujihashi, *et al.*, 1994).

IgG1 is produced by more cells than IgG2, which is produced by more cells than IgG3 and IgG4. IgG3 and IgG4 are produced by similar numbers of cells. The percentage of IgA1 producing cells is greater than IgA2 producing cells, though the numbers of IgA2 producing cells increases with disease severity (Fujihashi, *et al.*, 1994; Ogawa, *et al.*, 1989) This is an unusual pattern of Ig synthesis; and IgG4 and IgA2 have less inflammatory potential as they are not involved in complement activation (Fujihashi, *et al.*, 1994). Chronic antigenic exposure can lead to IgG4 class-switching, as would happen in chronically inflammed tissue (Fujihashi, *et al.*, 1994).

At both the protein and mRNA levels, and confirmed by reverse transcription-PCR, freshly isolated GMC from tissues of adult periodontitis diseased gingiva produce significantly less or no IL-2 and IL-4, in comparison to PBMC or concanavalin A stimulated PBMC and significantly elevated levels of IL-5 and IL-6. High levels of IFN-γ production are also seen (Fujihashi, *et al.*, 1994; Ogawa, *et al.*, 1989).

IL-5 and IL-6 are key cytokines in stimulating B cell growth and differentiation into plasma cells (Abbas, 1994; Fujihashi, *et al.*, 1994). Supernatants of cultured GMCs isolated from surgical specimens removed from patients with adult periodontitis can induce a class switch to IgA and IgG production in PBMCs (Kono, *et al.*, 1991).

This cytokine profile could contribute to the elevated numbers of activated B cells, and local Ig production seen in inflamed gingival tissue (Fujihashi, *et al.*, 1994). A lack of IL-4 and elevated IFN-γ is an unusual profile, and could explain the high percentages of macrophages and monocytes present (Fujihashi, *et al.*, 1994). IFN-γ is a potent macrophage and monocyte activator (Abbas, 1994; Mangan and Wahl, 1991).

It is also known that IL-1 levels are elevated in the GCF during inflammatory episodes. This cytokine can stimulate bone resorption by osteoclasts and prostaglandin E_2 (PGE_2) production by gingival fibroblasts (Genco, 1992). Levels of IL-1β are higher than IL-1α (Masada, *et al.*, 1990; Offenbacher, *et al.*, 1994). PGE_2 concentrations in the GCF of healthy patients have been measured at 20.5 ng/ml, whereas in patients with adult periodontitis it has been measured at 71 ng/ml in pockets of >4 mm. For patients with EOP, PGE_2 levels of 119-157 ng/ml have been found (Offenbacher, *et al.*, 1994). PGE_2 is both inflammatory and a stimulator of bone resorption (Genco, 1992).

V. The Role of Spirochetes in Periodontitis.

(i) Association Studies of Spirochetes and Periodontal Disease.

There are a greater number of studies associating spirochetes with periodontal disease than any other putative oral pathogen (Loesche, 1988). Gornitsky *et al.* (1991) examined the proportions of spirochetes in subgingival plaque. Spirochetes comprised 1-1.5% of the total flora in healthy sites, and 14-16% at sites of periodontitis. Surprisingly, the proportion of *P. gingivalis*, another putative pathogen, dropped from 24% to 12% of the total flora at healthy and diseased sites, respectively.

Other studies have shown even greater proportions of spirochetes to be associated with disease. Lai *et al.* (1986) classified the bacteria in periodontal pockets by

morphotype, and observed a difference in the flora at healthy sites and sites of adult periodontitis. The percentage of coccoid, motile rods, spirochetes and others found were 60, 2, 1, and 37 at healthy sites and 17, 25, 44, and 14 at diseased sites, respectively. In an extensive survey of 423 plaque samples from 112 adult chronic periodontitis and EOP patients by Loesche *et al.* (1985), 70 to 80% of the patients with ACP and EOP had >40% spirochetes, of the total bacteria, in plaque samples. *P. gingivalis* could not be found in the plaque of the majority of ACP patients but constituted approximately 10% of many of the EOP patients. Loesche (1988) averaged the proportions of spirochetes found in many different surveys with the following results. The percentage of spirochetes found at healthy sites was 1.6% (6), at sites with gingivitis, 18% (9), LJP, 12% (3), adult periodontitis, 37% (16), and with EOP, 53% (12). The numbers in brackets refer to the number of studies from which the percentage of spirochetes was averaged.

There are other studies showing a similar trend of increased proportions of spirochetes at diseased sites relative to healthy sites (Armitage, *et al.*, 1982; Evian, *et al.*, 1982; Listgarten and Levin, 1981; Listgarten and Lewis, 1967, and Moore, *et al.*, 1987). These studies heavily implicate spirochetes as etiological agents of periodontitis, especially when it is remembered that >300 bacterial species have been isolated from gingival plaque. The first immunological data implicating a specific species of spirochete as a periodontal pathogen was reported by Simonson *et al.* (1988). Using a monoclonal antibody (IAAII) recognizing an OMS antigen in 8 out of 15 tested strains of *T. denticola* by ELISA it was shown that the *T. denticola*-derived antigen quantities increased from 68-74 $\mu g\ mg^{-1}$ in subgingival plaque from pockets <6 mm to 140 $\mu g\ mg^{-1}$ in subgingival plaque at diseased sites of >6 mm; a ratio of approximately 1:2. This is in keeping with previous studies indicating that the numbers of spirochetes increase with pocket depth (Armitage, *et al.*, 1982; Listgarten and Levin, 1981; Slots, *et al.*, 1985b). The results of Simonson *et al.* (1988) have been confirmed by Loesche (1988).

All cultivable spirochetes isolated from gingival crevices are members of the genus *Treponema*, and *T. denticola* is the most frequently isolated treponeme (Caudry, *et al.*, 1995; Chan, *et al.*, 1993). If *T. denticola*, or another spirochete, is indeed pathogenic, then it would be expected that after treatment the numbers of spirochetes should be

substantially reduced. This is in fact the case and has been documented on many occasions (Henrichs, *et al.*, 1985; Kleinberg and Wolff, 1988; Loesche, 1988; Magnussen, *et al.*, 1984; Mousques, *et al.*, 1980; Wolff, *et al.* 1994). Wolff *et al.* (1994) reported an attachment gain of 1 mm in one month post-root planing and a 70% drop in the proportions of spirochetes, although the spirochete numbers began to rise again by 3 month post-treatment. Singletary *et al.* (1982) showed that a growing percentage of spirochetes are found in subgingival plaque prior to recurrent episodes of disease.

There are limited studies in which an individual spirochete strain has been quantified. Simonson *et al.* (1990) used an IgG2a monoclonal antibody to detect an OMS antigen found only on *T. denticola* serovar c (*T. denticola* ATCC 35404) and *T. denticola* ATCC 33520. The MAb did not cross-react with any of 24 other *Treponema* isolates tested. The ratio of *T. denticola* serovar c at sites of <6 mm to sites of >6 mm was 1:2; however, the absolute amount of *T. denticola* serovar c antigen detected was only 7 μg ml^{-1} plaque in deep pockets, indicating that this serovar may not be a likely candidate for overt pathogenesis. The absolute amount of *T. denticola* antigen recognized my MAb IAAII was 140 μg ml^{-1} plaque at the same pocket depth (Simonson, *et al.*, 1988). Interestingly, chemostat studies in which plaque was inoculated, resulted in a predominance of *T. denticola*, *Bacteroides* spp, *Eubacterium* spp, and *Peptostreptococcus* after one week (Ter Steeg, *et al.*, 1988). Two other studies using plaque inoculated into chemostats resulted in a predominance of spirochetes and *Bacteroides* species (Beighton, *et al.*, 1988; Glenister, *et al.*, 1988)

Two recent publications have been greeted with much interest. Riviere *et al.* (1991, 1992) used a bank of monoclonal antibodies that react with all cultivable treponemes, to specific strains of cultivable treponemes, and to the uncultivable pathogen-related oral spirochetes (PROS), so called as they share several antigenic determinants in common with *T. pallidum*, including a 37 kDa endoflagellar antigen. PROS were detected in plaque samples from patients with ANUG and adult chronic periodontitis. The two publications provided further evidence that spirochetes are more common at diseased sites than at healthy sites. The observation of spirochetes with shared antigenic determinants to *T. pallidum* is very exciting and great efforts should be made to cultivate

PROS. PROS and *T. denticola* accounted for the majority of all spirochetes in subgingival plaque from periodontitis patients (Riviere, *et al.*, 1991; 1992). As PROS are presently uncultivable, *T. denticola* represents the best model in which to study putative virulence mechanisms of spirochetes present in the periodontium.

(ii) Acquisition of Spirochetes.

How, and when, spirochetes become resident in the oral cavity is unclear. They are present in the subgingival plaque of healthy adults and also in children, their proportions increasing dramatically during periodontitis, as described previously. Barron *et al.* (1991) used monoclonal antibodies with specificities for all tested cultivable spirochetes; to *T. denticola* ATCC 33521; to *T. denticola* ATCC 33520 and to *T. denticola* ATCC 35404; and to PROS, in order to investigate the prevalence of oral spirochetes with respect to age. No persons with periodontitis were enrolled in the study. Spirochetes were detected in plaque in 50% of children aged 2-4, 70% of children aged 5-15, and 90% in the 16-25 age group. *T. denticola* ATCC 33521 was detected in 50% and *T. denticola* ATCC 33520 and/or 33504 in 40% of children in the 2-4 age group. The numbers of spirochetes were low, leading the authors to conclude that *T. denticola* may be the first spirochete to colonize the gingiva.

Other studies have shown that spirochetes are present in young children. Loesche (1988) reported spirochetes to be visible in 40% of children age 3-5 and 50% of children aged 6-12, by dark-field microscopy. They comprised >0.5% of the total microflora. Mikx *et al.* (1986) reported spirochetes to be almost ubiquitous in the plaque of 6-12 year old children, both in the Netherlands and in Tanzania. It would seem, therefore, that the acquisition of spirochetes is likely to occur at an early age and that the likelihood of spirochete colonization increases with time. As no oral spirochete strains are known to inhabit any other ecological niche, it is probable that humans acquire oral spirochetes through direct oral contact, or perhaps through shared eating or drinking utensils.

(iii) Adherence and Colonization of Spirochetes.

The first step in colonization of a mammalian host, and probably a prerequisite for invasion, is the interaction of a bacterium with the epithelium or other solid surface. Adhesion to the junctional epithelium, for example, would afford some protection to the bacterium from the outward flow of the GCF and stabilize the cell in the gingival crevice, an environment that supports the proliferation of oral spirochetes.

As the oral spirochetes have no fimbriae, they must utilize alternative mechanisms of adherence to host surfaces. *T. denticola* has the ability to attach to epithelial cells (Olsen, 1984), gingival fibroblasts (Weinberg and Holt, 1990; 1991), gingival keratinocytes (Mikx and Keulers, 1992), laminin (Haapasalo, *et al.*, 1991), fibronectin (Ellen, *et al.*, 1994a; Weinberg and Holt, 1990), gelatin, type I and type II collagen, and fibrinogen (Haapasalo, *et al.*, 1991).

The binding of *T. pallidum* to epithelial cells has been associated with fibronectin, through three fibronectin specific adhesins (32, 37, and 89.5 kDa). The binding of both *T. pallidum* and *S. hyodysenteriae* to epithelial cells may also be due in part to N-acetylneuraminic acid on the epithelial surface (Baseman and Hayes, 1980; Bowden, *et al.*, 1989; Fitzgerald, *et al.*, 1977; Peterson, *et al.*, 1983; Steiner, *et al.*, 1987; Thomas, *et al.*, 1985).

T. denticola appears to have several mechanisms mediating adhesion capabilities. Dawson and Ellen, (1994) reported the tip-oriented adhesion of *T. denticola* ATCC 35405 to fibronectin. This strain can migrate through a methylcellulose matrix and attach to a fibronectin (Fn) coated nitrocellulose membrane. While the bacteria are locomoting through the matrix, Fn-gold conjugates are seen to bind along the entire cell length. On meeting the Fn-coating membrane, the ligand(s) for Fn are seen to cluster at one, adherent cell tip. Polar attachment to host cells has been reported for *T. pallidum* (Thomas, *et al.*, 1985) and leptospiral species (Vinh, *et al.*, 1984). Polar adhesion of *T. denticola* to Fn-coated coverslips has also been reported. Pre-treatment of Fn with anti-Fn antibody resulted in inhibition of adherence, but pre-treatment with soluble Fn enhanced adherence (Ellen, *et al.*, 1994a). *T. denticola* ATCC 35405 can attach to the surface of human gingival fibroblasts and cause their detachment from plastic surfaces *in vitro* (Ellen, *et al.*, 1994b). Phenylmethylsulfonyl fluoride (PMSF), an inhibitor of chymotrypsin-like

enzymes, inhibited detachment of HGFs but not attachment of *T. denticola* to the cells (Ellen, *et al.*, 1994b). *T. denticola* ATCC 35405 is known to produce a 95 kDa chymotrypsin-like enzyme that hydrolyzes many host-derived proteins, including transferrin, fibrinogen, and gelatin (Uitto, *et al.*, 1988).

Several Fn-binding proteins are reported to be synthesized by *T. denticola*. Haapasalo *et al.* (1992) showed that the 53 kDa and 72 kDa major OMS proteins of *T. denticola* ATCC 35405 can bind laminin, Fn, and fibrinogen (Fb). Haapasalo *et al.* (1992) also reported four other laminin, Fn, and Fb binding proteins, ranging from 75 to 95 kDa that did not react with antibodies prepared against the 53 kDa protein or anti-whole cell antibodies, indicating these proteins are not surface exposed and probably not involved in binding to host cells. The 53 kDa protein was seen to readily form aggregates that were highly resistant to proteolytic degradation.

Haapasalo *et al.* (1991) had previously shown *T. denticola* ATCC 35405 to bind to laminin, Fn, Fb, gelatin, type I and type IV collagen and the RGD peptide (Gly-Arg-Gly-Asp-Ser). Ellen *et al.* (1994a) reported that *T. denticola* could bind to RGD-containing hexa- and heptapeptides, but not to control peptides, in a polar orientation. The RGD peptide is the binding site for VLA α_5/β_1 integrin and the α_{IIb}/β_3 cytadhesin (Albelda,1991). Haapasalo *et al* (1991) used the sulphydryl reagents p-chloromercuribenzoate (PCMB), which replaces the H atom in -SH groups, and oxidized glutathione, which induces sulphide bond formation between -SH groups, to reduce binding of *T. denticola* to each of the substrates by 70 - 99%, depending on the substrate. Heating of the spirochetes reduced binding by 70%, indicating cell viability is required. Glycosidase treatment also reduced binding by up to 80%, suggesting sugar residues to be important. Cultivated porcine gingival epithelial cells (GEC) produce Fn, type IV collagen and laminin. *T. denticola* ATCC 35405 bound to GECs initially in high numbers. After 8 hours, however, the spirochetes seemed to be attached to cell products and not to the cells themselves (Haapasalo, *et al.*, 1991) with migration of the GECs away from the spirochetes noted. It is interesting to note that *T. denticola* ATCC 35405 should attach to such differing proteins as reported by Haapasalo *et al.* (1991). These proteins are highly variable in terms of molecular weight, primary structure, charge, carbohydrate

content and free thiol groups. This indicates that several mechanisms of attachment may be available to the spirochete.

Weinberg and Holt (1990) observed the adherence of three clinical isolates of *T. denticola* (GM-1, TD-4, and MS25) to human gingival fibroblasts (HGFs) under both aerobic and anaerobic conditions. Trypsin had no effect, but proteinase K inhibited binding by 80% in all three strains. Sugars and lectins confirmed that carbohydrate residues are important in spirochete adherence. D-galactose, D-mannose, and N-acetyl-D-galactosamine inhibited the binding of GM-1 to HGFs in a dose-dependent manner. In addition, lectins with specificities for D-mannose and α-D-glucose and for β-D-galactose could inhibit the attachment of all three strains to HGFs. Periodate oxidation of HGF carbohydrate residues caused a 50% inhibition of GM-1 and MS25, but not TD-4, binding to HGFs. Anti-Fn antibodies inhibited the binding of TD-4, but not GM-1 or MS25.

In another study on spirochete adherence a 64 kDa OMS protein was isolated from *T. denticola* GM-1, MS25, and SR-5. Cyanogen bromide treatment of the 64 kDa protein resulted in the formation of a 42 kDa fragment. Sequencing of a 16 amino acid fragment revealed a stretch of amino acids with homology to a region of the human leukocyte adhesion molecule, p150.95 (TLDLALDLGEAXINAT and TLDLALDPGRLSPRAT, respectively) Antibodies generated against the 64 kDa protein caused inhibition of *T. denticola* adherence to HGF. This 64 kDa protein did not cluster to the tips (Weinberg and Holt, 1991).

It appears that *T. denticola* binds to host cells by mechanisms that vary between strains. Mannosyl and galactosyl moieties on host cell surfaces are involved, there are several proteins produced by *T. denticola* with affinity for host cell surface and connective tissue proteins and the data strongly suggests that a spirochete adhesin(s) with specificity to fibronectin is involved in forming a bridge between *T. denticola* and host cell surfaces. The adherence of *T. denticola* to host cells is not without cytopathological consequences, at least *in vitro*. *T. denticola* is cytotoxic to epithelial cells (Baehni, *et al.*, 1992) and can induce microulceration of sulcular epithelial cells (Makinen, *et al.*, 1994), inhibit fibroblast proliferation (Boehringer, *et al.*, 1984) and endothelial cell proliferation (Taichman, *et al.*, 1984).

Baehni *et al.* (1992) showed that *T. denticola* ATCC 35405, and two other strains, e and e', preferentially bind to the dorsal surface of HGFs rather than the ventral surface. *T. denticola*-exposed HGFs exhibited morphological changes from a flattened, stellate appearance to a fusiform shape within one hour. The HGFs rounded up and detached, membrane blebbing was apparent and dense ruffling of the HGF surface was seen. The spirochetes were often seen associated with HGF membrane invaginations. No detachment of HGFs was noted using cell-free culture supernatants, or when the spirochetes were heat killed or the HGFs rendered non-viable, indicating the pathological alterations induced on HGFs is a dynamic process in both the spirochetes and the HGFs. *T. denticola* induced rapid actin filament rearrangement within minutes. Control HGFs had normal actin filament arrays. Detachment was dependent on the time of incubation and concentration of spirochetes used. PMSF, the chymotrypsin-like enzyme inhibitor, reduced detachment, whereas N-α-p-tosyl-L-lysine chloromethylketone (TLCK), a trypsin-like enzyme inhibitor, did not. The chymotrypsin-like enzyme of *T. denticola* is localized to the bacterial surface and can degrade fibrinogen (Uitto, *et al.*, 1988; Grenier, *et al.*, 1990). Actin filaments are required for contact with the extracellular matrix (Baehni, *et al.*, 1992), therefore, if the pathological alterations induced in HGFs by *T. denticola* are active *in vivo*, repair mechanisms of the HGFs could be effected. Fibronectin is present on the surface of HGFs and interacts with integrins and integral membrane proteins and so may be involved in cell signaling (Ellen, *et al.*, 1994b). The ability of *T. denticola* to bind to Fn and HGFs and induction of actin filament rearrangement could therefore have serious pathological consequences.

Peptidoglycan from *T. denticola* was shown to be a potent toxin active against porcine periodontal epithelial cells *in vitro*. This cytotoxicity was enhanced by serum. Epithelial cells exposed to *T. denticola* were rendered nonviable and did not recover (Grenier and Uitto, 1993).

A recent report has shown 6 different fibrinolytic proteins to be produced by *T. denticola*. These proteases have apparent molecular weights of 91, 123, 138, 151, 173 and 228 kDa and have been referred to as T1-T6 (Rosen, *et al.*, 1994). T1, T5 and T6 are secreted into the supernatants of *T. denticola* ATCC 35404, and T5 and T6 into the

supernatants of ATCC 35404, 33520, and GM-1. T1 and T5 can also degrade albumin. T1, T5 and T6 can degrade human umbilical cord collagen type IV. The activity of T1-T6 against fibronectin was not tested. These fibrinolytic proteases were also sulphydryl dependent, as they were inhibited by PCMB and $HgCl_2$. Rosen *et al.* considered the T1 protease to be the same protein as the chymotrypsin-like enzyme described by Uitto *et al.* (1988).

T. denticola produces 6 fibrinogen degrading enzymes, T1-T6 (Rosen, *et al.*, 1994), can bind to fibrinogen to a similar degree as to fibronectin (Haapasalo, *et al.*, 1991), inhibition of chymotrypsin-like enzymes with PMSF reduces binding of *T. denticola* to host cells and ground substance proteins (Baehni, *et al.*, 1992; Ellen, *et al.*, 1994b), sulphydryl reagents inhibit substrate binding and fibrinolytic activity in independent studies (Haapasalo, *et al.*, 1991; Rosen, *et al.*, 1994), several fibrinogen binding proteins of *T. denticola* have been reported (Egli, *et al.*, 1993; Haapasalo, *et al.*, 1992), and the activity of the chymotrypsin-like enzymes against fibrinogen is unknown; therefore the role of fibrinogen and fibrinolytic enzymes of *T. denticola* in adherence and toxicity is deserving of investigation.

(iv) Invasion of Periodontal Tissue by Oral Spirochetes.

In considering the vast numbers of bacteria that are found in periodontal pockets, the amount of cells invading the underlying tissues is surprisingly low. For this reason it has been proposed that the infiltration of bacterial metabolites into gingival tissues may be more significant than frank invasion (Newman, 1990). However, several types of bacteria do penetrate the tissues and would therefore permit easier access of microbial metabolites or other plaque bacteria across a damaged junctional epithelium.

Listgarten's (1976) microscopic studies of the microbial flora associated with periodontal tissues in varying states of health revealed a generally intact junctional epithelium in healthy sites, with a proximal layer of bacteria reaching 60 μm thick. In

periodontitis patients, spirochetes, as well as concave flagellated Gram-negative bacteria, were concentrated next to the gingival tissue and below the main plaque mass.

Tissue biopsies from patients with adult periodontitis exhibiting severe bone loss and pocket depths of > 8 mm were examined for bacterial invasion by Frank (1980). Bacterial invasion was noted only in the apical wall of pockets, at the lower base of subgingival plaque. Spirochetes were observed in enlarged junctional epithelial spaces. Previously, Listgarten (1965) had shown spirochetes to invade the underlying connective tissue in ANUG. Listgarten had observed spirochetes be in direct contact with leukocytes during periodontal disease (Listgarten, 1976). Saglie *et al.* (1982) showed spirochetes to be in direct contact with alveolar bone in electron microscopic examination of periodontally diseased tissue. The frequent presence of spirochetes in gingival tissue was confirmed by Listgarten (1988). *Treponema denticola* has been shown to migrate through reconstituted basement membranes *in vitro.* Degradation of collagen occurred and the chymotrypsin-like enzyme was considered an important factor in migration (Grenier, *et al.*, 1990; Uitto *et al.*, 1988). The only reported intracellular invasion of oral spirochetes was that of *T. denticola* into human gingvial fibroblasts (HGFs) *in vitro. T. denticola* cells wound around actin filaments in proximity to the HGF nucleus (Ellen, *et al.*, 1994a). This observation is particularly noteworthy in view of the pathological effects of *T. denticola* on HGFs noted by Baehni *et al.* (1992) and Ellen *et al.* (1994a, 1994b).

(v) Virulence Factors and the Effects of Oral Spirochetes on the Immune System :

There are conflicting reports on the production of antibodies to oral spirochetes. Jacob *et al.* (1982) reported elevated serum IgG titres to *T. denticola* and *T. socranskii* in individuals with advanced periodontitis. Lai *et al.* (1986) demonstrated higher IgA and IgG antibodies to specific spirochete strains *(T. denticola* CD-1 and *T. vincentii* Ritz-A and LA-1) in patients with adult periodontitis compared to individuals with juvenile periodontitis or healthy periodontia. Significantly elevated proportions of spirochetes in subgingival plaque, compared to healthy controls, was also noted in the adult periodontitis samples (44% versus 1%). In another study, no significant elevation of IgM

or IgG titres to *T. denticola* in patients with moderate to advanced periodontitis was reported, by contrast with Jacob *et al.* (1982) and Lai *et al.* (1986), although elevated levels of IgA compared to healthy individuals were observed (Mangon, *et al.*, 1982).

Tew *et al.* (1985) showed that patients with juvenile periodontitis were more frequently seropositive to *T. socranskii* ss. *buccale, T. denticola* D3A9 and *T. socranskii* ss. *paredis* compared to healthy controls, but low levels and even lack of antibody in patients with severe periodontitis was noted. Steinberg and Gershoff (1968) first reported the phenomenon of a lack of antibody response to spirochetes in patients with severe periodontitis. The low antibody titres to oral spirochetes in severe disease may be explained by antigen masking by bound fibronectin or fibrinogen or other host factors, in a manner similar to that proposed for *T. pallidum* (Aldrette and Baseman, 1979; Bourell, *et al.*, 1994; Strugnall, *et al.*, 1990). It also raises the possibility of immune suppression allowing *T. denticola,* or other oral spirochete, to escape immune surveillance. It has not been established, however, that elevated antibody titres to any bacterial species correlates with the status of a primary pathogen or, indeed, exonerates any bacterial species from a role in periodontal disease.

Adsorption of complement proteins by *T. denticola* in serum and Mg-EDTA-chelated serum indicates that this oral spirochete can activate both the alternative and the classical pathways (Shenkein and Berry, 1991). Cleavage of C3 to C3b rendered the molecule susceptible to proteolytic degradation by *T. denticola.* The C3a, C4a and C5a proteins generated in complement activation can induce smooth muscle contraction, increase vascular permeability, are chemotactic for PMNs and cause histamine release from mast cells and basophils (Genco, 1992) and are therefore inflammatory mediators.

However, as the numbers of spirochetes are highly elevated at periodontally diseased sites it would seem that the antibody/complement-mediated opsonization process and lysis is not effective against the oral spirochetes. *T. pallidum* and *T. vincentii* also activate both pathways (Fitzgerald, 1987). *T. pallidum* is known to be resistant to antibody and complement-mediated immobilization and agglutination (Cox, *et al.*, 1992). Thus, activation of complement by *T. denticola* may lead to tissue destruction through chronic activation of the complement system's inflammatory mediators.

The potential for immune suppression by *T. denticola* was highlighted by the observation of the inhibition of the proliferative response of human peripheral blood mononuclear cells (HPBMs) in response to mitogen and antigen *in vitro*. Sonicated extracts of *T. denticola* strains CD-1, LL2513, and LL2516 caused a dose-dependent inhibition of the proliferation of HPBMs isolated from healthy individuals, as measured by ^{3}H-thymidine incorporation in response to conconavalin A. The extract from strain LL2513 also inhibited HPBM proliferation in response to poke weed mitogen. No effect on HPBM viability was noted. Inhibition was monocyte-dependent, and the suppressive effects were blocked by catalase and indomethacin, indicating the mechanism of suppression may involve prostaglandins and H_2O_2 (Shenker, *et al.*, 1984). Fractionation of sonic extracts by molecular weight, revealed that the suppressive factor was contained in the 100 kDa fraction (Shenker, *et al.*, 1984). These observations of Shenker *et al.* shed some light on the lack of antibody response to oral spirochetes in severe disease where the proportion of spirochetes in subgingival plaque is highest, although the specific effects of this dose-dependent 100 kDa suppressive factor on B cells has not been tested.

As PMNs form the primary line of defense against plaque bacteria in gingival pockets, any suppressive factor(s) produced by oral spirochetes blocking the production of reactive oxygen intermediates would offer the spirochetes protection against these bactericidal molecules and a selective advantage over bacteria with no such defense mechanism. The *in vitro* production of superoxide (O_2^-) by freshly isolated human peripheral blood PMNs was inhibited by a low passage clinical oral spirochete isolate, and by *T. denticola* ATCC 35405 (Sela, *et al.*, 1988). Superoxide production, measured by superoxide dismutase-inhibitable cytochrome c reduction by O_2^-, was inhibited by sonic extracts of both strains and more so by cell-free culture supernatants and phenol extracts. The production of H_2O_2 by PMNs was similarly inhibited, growth phase related and stable well beyond the onset of the decline phase of growth. The inhibitory factor is proteinaceous in nature as inhibition of O_2^- was suppressed by trypsin treatment. It is possible that it is an LPS-like molecule-associated protein which would be liberated on lysis of the spirochetes, therefore explaining the suppressive effect of culture supernatants

after 5 weeks, when most cells would be non-viable (Sela, *et al.*, 1988). No further characterization of the PMN suppressive factor has yet been reported.

T. denticola can by phagocytosed by neutrophils *in vitro*, but is not killed and the lysosomal granules do not granulate, thus *T. denticola* may block the fusion of lysosomes to phagosomes (Boehringer, *et al.*, 1986; Loesche, 1993).

Persson *et al.* (1990) tested 163 bacterial strains for the production of hydrogen sulphide in serum. *T. denticola*, *P. gingivalis*, and other strains of black-pigmented *Bacteroides* produced >200 mg H_2S dm^{-3} in serum and were the most potent producers observed. Volatile sulfur compounds produced *in vivo* could have significant cytotoxic effects and can inhibit myeloperoxidase, an important oxidative weapon of PMNs (Claesson, *et al.*, 1989; Persson, *et al.*, 1990).

Oral spirochetes can also inhibit the proliferation of fibroblasts *in vitro* and could therefore impede the repair of degraded collagen. Sonic extracts of six tested *T. denticola* strains caused a dose-dependent inhibition of murine and human skin fibroblasts, monitored by ^{3}H-thymidine, ^{3}H-uracil and ^{3}H-leucine incorporation. Despite the effect on DNA, RNA and protein synthesis, the fibroblasts remained viable (Boehringer, *et al.* 1984). Minimal inhibition by a *T. vincentii* strain extract was also observed. The inhibiting factor produced by *T. denticola* was found in a single peak of approximately 50-kDa on separation of sonic extract components by molecular weight. Fibroblast inhibitory factors have been reported to be produced by several other oral bacteria including *A. actinomycetemcomitans* (Shenker, *et al.*, 1982), *A. viscosis*, *Streptococcus mutans* and *S. intermedius* (Higerd, *et al.*, 1978).

T. denticola can therefore activate complement, suppress the proliferation of fibroblasts and leukocytes, avoid destruction even when opsonized by PMNs, inhibit superoxide and H_2O_2 production by PMNs, and produce inflammatory hydrogen sulphide in large quantities *in vitro*. These effects, if active *in vivo*, could allow spirochetes to avoid some of the immune systems killing mechanisms, to participate in tissue destruction, and to block the repair of damaged collagen.

A number of host proteins are degraded by the 95 kDa chymotrypsin-like protease of *T. denticola* ATCC 35404, isolated by Uitto *et al.* (1988). This enzyme is not produced

by *T. vincentii* and was able to completely degrade fibronectin, fibrinogen, and gelatin and partially degrade IgA, IgG, and α-1-antitrypsin. The chymotrypsin-like enzyme was sulphydryl-dependent as activity was increased in the presence of cysteine and inhibited by $HgCl_2$, PCMB and oxidized glutathione, and defined as a chymotrypsin-like protease as it was inhibited by PMSF and not TLCK. The ability to degrade IgA and IgG would allow the cells to avoid opsonization and the effects of the classical complement pathway, and can therefore be considered a virulence factor. Degradation of α-1-antitrypsin may be important *in vivo* as trypsin has been reported to activate host gingival tissue collagenase by destruction of serum collagenase inhibitors, to activate the alternative pathway of complement fixation and to disrupt cell-cell and cell-ECM adhesions (Loesche and Laughon, 1981).

In a later study (Grenier, *et al.*, 1990), binding of antibodies generated against the purified protease by *T. denticola* ATCC 35405 cells indicated the chymotrypsin-like protease is present at the cell surface. The chymotrypsin-like enzyme was considered to be important in migration of the spirochete through a reconstituted gel-like basement matrix. Migration was enhanced by dithiothreitol, which also increased chymotrypsin-like activity, whereas PCMB suppressed migration. Degradation of fibronectin, laminin, and type IV collagen, important components of the basement membrane, was apparent; therefore, active chymotrypsin-like protease *in vivo* could reduce the ability of the basement membrane to support epithelial cell function and facilitate cell and particulate matter access (Grenier, *et al.*, 1990).

There is evidence to suggest that *T. denticola* ATCC 35405 produces more than one chymotrypsin-like enzyme. Que and Kuramitsu (1990) purified a 67 kDa protease capable of degrading the synthetic chymotrypsin substrate succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalinine-p-nitroanilide. This 67 kDa enzyme was able to degrade type IV collagen, laminin, and fibronectin, but could not degrade azocoll or azocasein, as reported for the 95 kDa enzyme (Grenier, *et al.*, 1990; Que and Kuramitsu, 1990). However, antibodies against the 95 kDa protein reacted strongly with the cloned 67 kDa enzyme.

Arakawa and Kuramitsu (1994) identified a clone expressing a chymotrypsin-like protease from *T. denticola* ATCC 35405. Purified protein of this *prt* b gene product degraded BSA, horse serum albumin, and casein but not type IV collagen, fibronectin, gelatin, IgG or transferrin. TPCK, but not TLCK, a trypsin inhibitor, inhibited the *prt* b gene product activity. The enzyme specificities suggest a different chymotrypsin-like protease to those described by Uitto *et al.* (1990) and Que and Kuramitsu (1990). It is difficult to speculate on an *in vivo* role for the *prt* b product as the location of the enzyme in *T. denticola* is unknown and the proteins known to be degraded by the protease are not found in the periodontal pocket.

Makinen *et al* (1990) reported a 100 kDa protease from a *T. denticola* clinical isolate that could hydrolyze phenyl-azobenzyl-oxycarbonyl-L-pro-L-leu-gly-L-pro-D-arg, collagen and collagen-derived substrates.

T. denticola also produces a trypsin-like enzyme. The molecular weight of purified trypsin-like enzyme was estimated as 69 kDa by SDS-PAGE and 50 kDa by sephadex-G100 fractionation. The enzyme hydrolyzed N-benzoyl-DL-arginine-2-naphthylamide (BANA) and other synthetic trypsin substrates, is inhibited by TLCK but not TPCK, enhanced by Ca^{++}, Mg^{++}, Ba^{++} and Cu^{++} but inhibited by Zn^{++}, Hg^{++} and Co^{++}, and is unaffected by sulphydryl reagents. The trypsin-like enzyme preferentially hydrolyzed ester, amide, and peptide bounds involving arginine and lysine, as seen by the profile of activity against synthetic substrates (Ohta, *et al.*, 1986).

The trypsin-like enzyme of *T. denticola*, or other oral spirochetes, appears to be produced in large amounts *in vivo*. Although other oral bacteria species also produce a trypsin-like enzyme (Loesche, 1988; 1993), a striking correlation between the percentage of BANA hydrolyzing plaques and the percentage of spirochetes present in plaque samples is seen. BANA hydrolysis has thus been considered a primarily treponemal reaction (Loesche, 1987). Although the significance of the *T. denticola* trypsin-like enzyme to the etiology of periodontal disease is unknown, the measurement of BANA hydrolysis by subgingival plaque is an accurate indicator of clinical disease status and the numbers of spirochetes at individual gingival plaques (Schmidt, *et al.*, 1988).

Loesche *et al.* (1990) examined 702 plaque samples from 117 untreated periodontal patients. They found that both examination for BANA-hydrolyzing plaque and ELISA detection of *P. gingivalis* and *T. denticola* could distinguish between clinically diseased and healthy sites. It was also found that BANA-hydrolyzing plaque correlated to detection of *T. denticola* and *P. gingivalis* by ELISA at clinically healthy sites and suggested treatment should be considered to prevent the onset of clinical symptoms in such cases. Loesche *et al.* (1992) reported that if *P. gingivalis, T. denticola,* and *B. forsythus,* all capable of BANA hydrolysis, are appropriate periodontopathic markers, then BANA hydrolysis was equally accurate in diagnosing infection as DNA probing and immunological methods using species specific antibodies.

A cell-associated 75-77 kDa endo-acting proline-specific oligopeptidase (POPase) is produced by *T. denticola* ATCC 35405. The POPase hydrolyzes carbobenzoxy-gly-pro-p-nitroanilide and cleaves at proline residues of bradykinin, substance P, neurotensin, angiotensin, oxytocin, vasopressin, and human endothelin fragment 22-38, all human bioactive peptides of 4-17 amino acids (Makinen, *et al.*, 1994). Information is lacking on the role of bacterial POPases in inflammatory diseases, but the high specificity for and high affinity to proline residues of the human bioactive peptides would suggest that the POPase of *T. denticola* is beneficial to the spirochete and could supply peptides for growth substrates and/or effect the control of host bioactive peptides by human POPases. Some amino acid homology of the *T. denticola* POPase to the pig brain POPase is seen (50% between amino acids 2-45 of the spirochete POPase and amino acids 523-566 of the porcine POPase; Makinen, *et al.* 1994).

A second endopeptidase that cleaves bradykinin is produced by *T. denticola* ATCC 35405. This 62 kDa metallopeptidase cleaves Leu-gly bonds in the synthetic collagen peptide furylacryloyl-leu-gly-pro-ala and phe5-ser6 bonds in bradykinin, bradykinin-related peptides and a bradykinin antagonist. All substrates required a proline residue at position P'_2 (the amino acid sequence of bradykinin is arg-pro-pro-gly-phe-ser-pro-phe-arg) to be suitable for hydrolysis by the 62-kDa peptidase (Makinen, *et al.* 1992). Bradykinin is a plasma peptide that can increase vascular permeability.As the bradykinin antagonist was also cleaved, the *in vivo*

consequences of the 62-kDa peptidase is unclear. The natural *in vivo* substrate for this *T. denticola* enzyme may be small collagen-derived peptides due to its high activity against furylacryloyl-leu-gly-pro-ala. This would complement the activity of the 95-kDa enzyme described by Grenier *et al.* (1990) and the 67-kDa enzyme described by Que and Kuramitsu (1990), which were capable of hydrolyzing native collagen.

Using FITC-labelled keratin, oral bacterial species were tested for keratinolytic activity by Mikx and deJong (1987). *S. epidermidis, S. haemolyticus,* and *P. gingivalis,* in addition to a *T. denticola* clinical isolate, were able to degrade native human keratin. The enzyme responsible for keratin degradation has not been characterized. However, keratinolytic activity by *T. denticola in vivo* would be a further weapon in the attack of the epithelial barrier and tissues, and presumably in obtaining amino acids or peptides for growth.

A 53-kDa protein is encoded by the *tdp* A gene, with an open reading frame of 1419 base pairs, and has a conserved signal peptidase II signal sequence (leu-ser-ser-cys). The gene was isolated from *T. denticola* Johnson and a *tdp* A specific DNA probe hybridized intensively with *T. denticola* ATCC 35405, ATCC 35404, ATCC 33520 and other tested strains, but not to *T. vincentii* or *T. pallidum* chromosomal DNA, indicating the gene is conserved amongst *T. denticola* strains (Miyamoto, *et al.* 1991). The cellular location of this protein in *T. denticola* has not been established and no function assigned.

T. denticola displays wide ranging peptidase activities, presumably allowing degradation of smaller peptides derived from native host proteins. Mikx (1991) examined 20 oral and non-oral treponemes and observed the highest endo- and exopeptidase activities in *T. denticola.* The four strains of *T. denticola* tested exhibited aminoarylamidase (hydrolysis of amino acid-2-napthylamide to 2-napthylamine and the amino acid) activity against 18/19 substrates and 21/23 dipeptidylarylamidase substrates between them. Oligopeptidyl arylamidase activity against 2-5 amino acid derivatives again was highest for *T. denticola* strains, which hydrolyzed all 9 substrates tested. The most frequently noted activities of the 20 treponemes was prolyl- and arginyl-amidase. Arginine is an important energy source for *T. denticola* (Blakemore and Canale-Parola, 1976; Loesche and Laughan, 1981).

The ability of *T. denticola* to bind to epithelial cells (Olsen, 1984) and gingival fibroblasts (Ellen, *et al.*, 1994a; 1994b; Weinberg and Holt, 1990; 1991) and to host proteins (Dawson and Ellen, 1994; Ellen, *et al.*, 1994a; Haapsalo, *et al.*, 1991), the proteases produced that can degrade native proteins such as fibrinogen, laminin, elastin, immunoglobulins, transferrin, fibronectin, α-1-antitrypsin, type IV collagen and gelatin (Ellen, *et al.*, 1994b; Grenier, *et al.*, 1990; Makinen, *et al.*, 1990; Que and Kuramitsu, 1990; Uitto, *et al.*, 1988); the wide ranging endo- and exopeptidase activities against human bioactive peptides (Makinen, *et al.*, 1992; 1994) and 48 different amino-, diamino-, and oligoaminoarylamidase specificities (Mikx, 1991) are a strong indication that *T. denticola* is actively involved in periodontal destruction through proteolytic degradation of host tissues.

Other metabolites of the oral spirochetes are relevent to periodontal tissue destruction. *T. denticola* ATCC 35405 and *T. vincentii* ATCC 35580 may contribute to the increased phospholipase C (PLC; phosphatidylcholine cholinesphosphohydrolase [E.C. 3 1.4.3]) levels found in the GCF of patients with periodontitis (Siboo, *et al.*, 1989). The PLC produced by *T. denticola* ATCC 35405 is secreted across the bacterial membranes, thus has the potential to hydrolyze membrane phospholipids and destroy epithelial cells and potentiate inflammation through the production of prostaglandins and leukotrienes from released arachadonic acid. The PLC of *T. denticola* ATCC 35405 has been purified by affinity chromatography using L-α-phosphatidylcholine (lecithin) - conjugated actyl-sepharose and has an apparent molecular mass of 66 kDa (Chan, *et al.*, 1991).

Esterase activity against C_4 to C_{18} fatty acids and strongest against C_6 to C_{10} fatty acids, has been reported for *T. denticola*. *T. vincentii* is also capable of fatty acid degradation, (Mikx, 1991). Esterases could act in conjunction with PLC in causing tissue destruction.

T. denticola strains also exhibit glycosidase activity against α-D-galactose, β-D-galactose, α-D-glucose, β-D-glucose, α-L-fucose, β-D-fucose and N-acetyl-β-D-glucosamine p-nitrophenol derivatives. Such activity is rare in other *Treponema* species (Mikx, 1991). Homer *et al.* (1992) reported neuraminidase activity, which could

conceivably, along with the other reported glycosidase activities (Mikx, 1991) render glycoproteins more susceptible to proteolytic cleavage following the removal of oligosaccharide moieties.

Fiehn (1986, 1987) observed both hyaluronidase and chondroitinase activity by 9 unidentified spirochetes with a 1:2:1 endoflagellar arrangement and 8 with a 2:4:2 endoflagellar arrangement. Such enzyme(s) could also be important virulence factor(s), facilitating penetration of the spirochetes through the extracellular matrix and disrupting the nutrition and locomotion of host cells.

Oral spirochetes produce a neutral (Isahova and Kuramitsu, 1995), acidic (Fiehn, 1986, 1987) and alkaline (Fiehn, 1986, 1987) phosphatase which could be involved in mineralization and bone resorption (Isahova and Kuramitsu, 1995).

T. denticola has the potential to produce inflammatory mediators such as H_2S (Perrson, *et al.*, 1990; Kleinberg and Wolf, 1986), ammonium (Loesche and Laughon, 1981) and volatile fatty acids (Tonetti, *et al.*, 1987), to activate complement (Schenkein and Berry, 1991), degrade host proteins and peptides through a rich proteo- and peptidolytic arsenal, including collagen, ground substance and basement membrane components (Arakawa and Kuramitsu, 1994; Ellen, *et al.*, 1994a; 1994b; Grenier, *et al.*, 1990; Makinen, *et al.*, 1992; Mikx and deJong, 1987; Que and Kuramitsu, 1990; Rosen, *et al.*, 1994; Uitto, *et al.*, 1988); and is cytotoxic to epithelial and fibroblast cells (Baehni, 1992; Ellen, *et al.*, 1994a; 1994b; Weinberg and Holt, 1990).

T. denticola can attach to host cells and host proteins (Egli, *et al.*, 1993; Ellen, *et al.*, 1994a; 1994b; Haapasalo, *et al.*, 1991; 1992; Weinberg and Holt, 1990; 1991), can locomote at high viscosities (Klitorinos, *et al.*, 1993; Pietrantonio, *et al.*, 1989) and can penetrate into the underlying connective tissue (Frank, 1980; Listgarten, 1988; Saglie, *et al.*, 1982). *T. denticola* is able to degrade IgA and IgG (Uitto, *et al.*, 1988), avoid killing by PMNs (Boehringer, *et al.*, 1986) and exert suppressive effects on host cells (Boehringer, *et al.*, 1984; Persson, *et al.*, 1990; Sela, *et al.*, 1988; Shenker, *et al.*, 1984), and may suppress antibody production, or avoid detection, in severe disease (Tew, *et al.*, 1985; Steinberg and Gershoff, 1968). Elevated proportions of spirochetes in subgingival plaque are found at diseased sites (Armitage, *et al.*, 1982; Gornitsky, *et al.*, 1991; Lai,

et al., 1986; Loesche, 1988) and the numbers of spirochetes are reduced on treatment (Loesche, 1988; Wolff, *et al.*, 1994). All of these observations would indicate that *T. denticola*, or perhaps other oral spirochetes that are not as well studied or are uncultivable, play a significant role in the deterioration of gingival health in periodontitis and are not simply taking advantage of a niche that supports spirochete proliferation.

In 1992, at the onset of the research that forms this thesis, certain basic questions remained unanswered. A glaring omission in our knowledge of the oral spirochetes was the mechanisms by which they could obtain sufficient iron for growth and the potential for haemolytic activity. In humans, extracellular iron is limited by the high-affinity iron-binding proteins transferrin, lactoferrin, haemoglobin and myoglobin, the hemin-binding proteins hemopexin and albumin (Grenier, 1991); and low solubility of iron under physiological conditions (Tai, *et al.*, 1993). Pathogenic bacteria can circumvent this non-specific defense mechanism by the production of siderophores, low molecular weight iron-chelating molecules, such as aerobactin and enterobactin by *E. coli* (Griffiths, 1991) and malleobactin by *Pseudomonas pseudomallei* (Yang, *et al.*, 1991). Transferrin and lactoferrin can support the iron requirements of *Bordetella pertussis* (Menozzi, *et al.*, 1991) and *Neisseria meningitidis* (Griffiths, 1991). Heme molecules, which contain a central iron moiety, can be used as an iron source by *Pleisiomonas shigelloides* (Daskaleros, *et al.*, 1991) and *Haemophilus influenzae* (Hanson and Hansen, 1991). Heme may be liberated from erythrocytes by the production of a hemolysin. Hemolysins have been reported to be produced by *Fusobacterium nucleatum* (Falker, *et al.*, 1983), *P. gingivalis* (Kay, *et al.*, 1990), and *Streptococcus pneumoniae* (Tai, *et al.*, 1993). Iron reductases have been shown to be important in the acquisition of iron by *Legionella pneumophila* (Johnson, *et al.*, 1991), *Pseudomonas aeruginosa* (Cox, 1980), and *Listeria monocytogenes* (Cowart and Foster, 1985). Known methods employed by bacteria to sequester iron were examined and those mechanisms available to *T. denticola* are incorporated into a model presented in Chapter 2.

There were several reports on the production of a hyaluronidase (Hase) by *T. pallidum* and the importance of this enzyme in the progression of syphilitic infections

(Fitzgerald, *et al.*, 1979; Fitzgerald and Gannon, 1983; Fitzgerald and Johnson, 1979; Fitzgerald and Repesh, 1987). Animals treated with Hase exhibit degradation of the extracellular matrix, intravascular thrombosis and changes in metachromatic staining (Fitzgerald and Gannon, 1983; Szabo and Magyur, 1958; Williams, 1955), observations also reported in syphilitic lesions (De Lameter, *et al.*, 1952; Fitzgerald and Gannon, 1983; Turner and Hollender, 1954; 1957). The Hase may be involved in dissemination, which can be rapid. In rabbits, *T. pallidum* can be found in the bloodstream only 5 minutes post-inoculation of the testicles (Fitzgerald and Repesh, 1987). Anti-Hase antibodies retarded invasion of *T. pallidum* across rabbit amnions in an *in vitro* penetration model (Fitzgerald and Repesh, 1987). Anti-Hase antibodies also reduced the capacity of *T. pallidum* to disseminate *in vivo*. The movement of treponemes inoculated into one rabbit testicle to the second testicle was significantly diminished with the co-administration of anti-Hase antibodies, in comparison to untreated controls (Fitzgerald and Repesh, 1987). Thus, Hase is probably a major virulence determinant of *T. pallidum*.

With the exception of Fiehn (1986, 1987), who showed chrondroitinase and hyaluronidase activity in uncharacterized oral spirochetes, no reports on the production of these enzymes by the oral spirochetes were available. Fiehn observed hyaluronidase and chondroitinase production by growing spirochetes in a substrate-containing medium but did not characterize the enzyme(s) at all. The production of a hyaluronic acid and chondroitin sulfate - degrading enzyme by *T. denticola*, *T. vincentii*, and *T. socranskii* and the potential *in vivo* role for this enzyme is addressed in Chapter 3.

The polysaccharide layer external to the outer membrane of oral spirochetes had not been studied. Chapter 4 contains a dark-field and electron microscope study of this layer in *T. denticola*. Considering the role that capsules and slime layers are known to play in preventing desiccation, avoidance of opsonization, adherence, and the binding of host proteins that has been proposed as part of the reason by which *T. pallidum* can avoid immunological detection and survive in human hosts, it was surprising that this issue had been largely ignored previously.

Guidelines Regarding Doctoral Theses Containing Quotations From Published or Submitted Manuscripts.

Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidates interest to make perfectly clear the responsibilities of all authors of the co-authored papers. **Under no circumstances can a co-author of any components of such a thesis serve as an examiner for that thesis.**

According to the University regulations, the above text has been quoted in full from Guidelines for Thesis Preparation. Four manuscripts have been included as the main body of this thesis, and appear as Chapters 2, 3, and 4. A common abstract, introduction, literature review, and discussion are included as required in the above guidelines. Each chapter represents an individual study on unrelated aspects of the biology of oral

anaerobic spirochetes, therefore bridging text between each chapter is included to allow the thesis to flow. Following as per the guidelines, the specific contributions of other authors are presented below.

Chapter 2 of this thesis includes the text of a manuscript published in Oral Microbiology and Immunology **8**: 245-259, 1993, entitled "Binding of hemin and Congo red by oral hemolytic spirochetes", by D. Scott, I.R. Siboo, E.C.S. Chan, A. Klitorinos, and R. Siboo. I.R. Siboo and A. Klitorinos were responsible for preliminary experiments establishing the binding of hemin and congo red to intact cells shortly before my arrival to the department. A. Klitorinos contributed the SDS-PAGE analysis of the spirochetal outer membrane extracts (Fig. 2). All other experiments were designed in consultation with Dr. Chan and Dr. Siboo and carried out by myself. The manuscript was written by myself and Dr. Siboo and edited by Dr. Siboo and Dr. Chan. The remainder of Chapter 2 is a manuscript accepted for publication in the Canadian Journal of Microbiology, entitled "Iron acquisition by oral hemoytic spirochetes : isolation of a hemin-binding protein and identification of iron reductase activity.", by D. Scott, E.C.S. Chan, and R. Siboo. The data on the growth of *T. denticola* in hemin-depleted New Oral Spirochete medium was supplied by A. Klitorinos. All other experiments were designed in consultation with Dr. Chan and Dr. Siboo and performed by myself. The manuscript was written by myself and edited by Dr. Siboo and Dr. Chan.

Chapter 3 of this thesis is the text of a manuscript entitled "An exoenzyme of some oral spirochetes with hyaluronidase and chondroitinase activities" which has been accepted for publication in Microbiology. The authors are D. Scott, I.R. Siboo, E.C.S. Chan, and R. Siboo. I.R. Siboo aided in the electron microscopy, specifically the experiment involving the binding of gold-labelled rabbit IgG to intact cells. All other experiments were performed by myself and designed in consultation with Dr. Chan and Dr. Siboo. The manuscript was written by myself and Dr. Siboo, and edited by Dr. Siboo and Dr. Chan.

Chapter 4 of this thesis is comprised of a manuscript entitled "Visualization of an extracellular mucoid layer of *Treponema denticola* ATCC 35405 and surface sugar lectin-analysis of some oral spirochete species" which has been accepted for publication in Oral Microbiology and Immunology. The authors are D. Scott, A. Klitorinos, E.C.S. Chan and R. Siboo. A. Klitorinos was of great assistance in the lectin-agglutination experiments (Table 1). All other experiments were performed by myself and designed in consultation with Dr. Chan and Dr. Siboo. The manuscript was written by myself and edited by Dr. Siboo and Dr. Chan.

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Binding of hemin and Congo red by oral hemolytic spirochetes

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Colony-forming units or cells in suspension of oral anaerobic spirochetes (*Treponema denticola*, *Treponema vincentii* and *Treponema socranskii*) bind hemin and Congo red. Hemin or Congo red binds to a hydrophobic polypeptide receptor that is located in the outer membrane of the bacterial cells and it has a relative molecular mass of 47 kDa. These oral spirochetes also lyse sheep erythrocytes to produce beta-hemolytic zones around colony-forming units. The oral spirochetes may acquire iron for growth when they lyse erythrocytes and bind heme from which they may sequester and transport iron into the cells.

Key words: oral spirochete, hemin and Congo red binding, hemolysis

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There is substantial evidence that oral spirochetes multiply successfully in periodontal pockets to establish extracellular infections (1, 16, 17, 27, 28). A critical step in colonization of periodontal pockets relies on the ability of oral spirochetes to acquire iron for growth. Several bacterial iron-uptake systems have been reported (13) and heme, either free or complexed to carrier proteins (30, 32), can serve as an iron source. The term heme (hemin) is used to describe all iron-protoporphyrin complexes. The ability of certain pathogenic bacterial species to bind Congo red correlates strongly with their ability to bind heme (8, 15, 31, 34). We speculated that oral spirochetes may use heme from hemoglobin or other hemoproteins as an iron source since these bacteria produce phosphotidylcholine-hydrolyzing phospholipase C (26), which could hemolyze erythocytes (2). Accordingly, we undertook to determine whether oral spirochetes could bind hemin and Congo red.

Material and methods

Growth of oral spirochetes

Treponema denticola serovar a and c were isolated, characterized by us and maintained in this laboratory and carry the accession numbers ATCC 35405 and ATCC 35404 (7). *Treponema vincentii* ATCC 35580, *T. denticola* ATCC 33520 and *Treponema socranskii* ATCC 35536 were purchased from the American Type Culture Collection (Rockville, MD). The spirochetes were grown as suspension cultures in new oral spirochete medium (6) or as colony-forming units (CFU) in new oral spirochete 0.7% sea plaque agarose (FMC BioProducts, Rockland, ME) medium as described recently (5).

Congo red and hemin binding to CFU

Tubes containing sterile new oral spirochete medium and agarose (20 ml/tube) were held at 37°C in a water bath. *T. denticola* ATCC 35405, *T. vincentii* and *T. socranskii* grown in new oral spirochete medium broth were diluted serially, inoculated into the tubes and poured into petri dishes (5). The agar was allowed to gel for 30 min at 4°C and the dishes were incubated in an anaerobic chamber (Coy Laboratories Products, Ann Arbor, MI) until the CFU were visible. Congo red (85% dye content, ICN Biochemicals, Cleveland, OH) was dissolved in 20 mM Tris-HCl buffer, pH 7.5 to a final concentration of 20 $\mu g \cdot ml^{-1}$ and poured onto the surface of the dishes. The dishes were incubated for 1–2 h at room temperature and Congo red binding to the CFU observed. The procedure was repeated with hemin (Sigma Chemical Co., St. Louis, MO). The hemin was solubilized in 1 M NaOH (2 $mg \cdot ml^{-1}$) and diluted to a concentration of 20 $\mu g \cdot ml^{-1}$ Tris-HCl buffer. *Escherichia coli* NCTC 9001, which did not bind Congo red, was used as a negative control.

Hemolytic activity

Polystyrene tissue culture flasks (ICN Biochemicals, Mississauga, Ontario, Canada) with new oral spirochete agarose medium were held at 37°C in a water bath (5). Sterile sheep blood was added to a final concentration of 1.0%, and an inoculum of *T. denticola* ATCC 35405 (approximately 30–50 CFU) was added. The contents of the flasks were mixed and the agar allowed to gel for 30 min at 4°C, the flasks were incubated at 35°C in an anaerobic chamber and observed for zones of hemolysis around CFU. The procedure was repeated with *T. vincentii*, *T. socranskii*, *T. denticol*

ATCC 35404 and *T. denticola* ATCC 33520.

Binding of Congo red and hemin to bacterial cells

New oral spirochete broth suspension cultures of oral spirochetes were grown to a density of 1×10^9 cells $\cdot$ ml^{-1}. The cells were sedimented by centrifugation at $4000 \times g$ at 4°C for 15 min; the pelleted cells were washed once with Tris-HCl buffer, once with Tris-HCl buffer containing 0.01% Tween 20, twice with Tris-HCl buffer by centrifugation (same as above) and resuspended in Tris-HCl buffer to a density of 1×10^8 cell $\cdot$ ml^{-1}. The suspension of each bacterial species was dispensed separately in microcentrifuge tubes (2 ml of cells per tube); the cells were pelleted by centrifugation at $14.000 \times g$ for 5 min and the supernatants were discarded. Hemin (2 ml) of varying concentrations (0.1 to 20 μg $\cdot$ ml^{-1}) was added to the pellets and the cells were resuspended and incubated, with shaking, for 1 h at 37°C. The incubation time was selected to ensure maximal binding at, or near, equilibrium. Preliminary experiments had shown that, after 10 min of incubation, no further binding of hemin could be detected by the assay used (3, 8). The cells were sedimented by centrifugation ($14,000 \times g$, 5 min), the absorbance of the unbound hemin in the supernatants was determined at 400 nm and the bound hemin was determined by subtraction. The binding of Congo red to spirochetes was carried out identically, except that Congo red was solubilized in Tris-HCl buffer and the absorbance of the unbound Congo red was determined at 480 nm. Triplicate assays were carried out with each concentration of Congo red or hemin. The concentrations of unbound Congo red or hemin were determined from calibration curves of concentration versus absorbance. The lower limit of sensitivity for the detection of hemin or Congo red is 0.2 μg $\cdot$ ml^{-1}. In competition experiments, Congo red binding (0.1 to 20 μg) was carried out as described. The bacterial cells were then sedimented by centrifugation, washed with Tris-HCl buffer, pelleted by centrifugation and the supernatants discarded. Hemin (10 μg $\cdot$ ml^{-1}) was added to each of the cell pellets, incubated and the unbound hemin in the supernatants was determined as described above.

Binding of Congo red or hemin to treated cells

Two ml of *T. denticola* ATCC 35405 (1×10^9 cells $\cdot$ ml^{-1}) was pipetted into each of a series of microcentrifuge tubes. The cells were sedimented by centrifugation and the supernatants discarded. Papain was solubulized in Tris-HCl buffer, pH 6.5 containing 1.3 mM cysteine-HCl (500 μg $\cdot$ ml^{-1}), pepsin was dissolved in 10 mM HCl (500 μg $\cdot$ ml^{-1}), trypsin was solubilized in Tris-HCl buffer, pH 8.5 (100 μg $\cdot$ ml^{-1}), proteinase K was dissolved in Tris-HCl buffer containing 0.1% sodium dodecyl sulfate (100 μg $\cdot$ ml^{-1}), polymyxin B was dissolved in Tris-HCl buffer containing 0.15 M NaCl (300 μg $\cdot$ ml^{-1}), 8-anilino-1-naphthalenesulfonic acid was dissolved in 0.15 M NaCl containing 0.1% sodium dodecyl sulfate (500 μg $\cdot$ ml^{-1}) and Triton X-100 was prepared in Tris-HCl buffer (1.0% v/v). Two ml of each of these solutions was added separately to the sedimented bacteria cells that were resuspended and incubated, with shaking, for 30 min at 37°C. The cells were treated with 0.2 M meta-periodate in 0.1 M acetate buffer, pH 4.5 for 24 h at 4°C. The treated cells were sedimented by centrifugation and the supernatants discarded. Then, the cells were washed with Tris-HCl buffer by centrifugation and resuspended in Tris-HCl buffer. The binding-assay for hemin or Congo red was carried out as described above. Assays that served as 100% binding controls were carried out with each of the solvents less the test substance. All the assays were carried out in triplicate. The binding values of the treated cells (i.e., with test substances) were calculated as a percentage of the 100% binding controls. *T. vincentii* and *T. socranskii* were treated as described above and assayed for hemin but not Congo red binding. All reagents were purchased from Sigma Chemical Co.

Electrophoresis

T. denticola ATCC 35405 (1×10^9 packed cells) was mixed with 1 ml of hemin (20 μg) or 1 ml of Congo red (20 μg) and incubated, with shaking, for 1 h at 37°C. Unbound Congo red or hemin was removed by centrifugation ($14,000 \times g$, 5 min) and the cells washed twice with Tris-HCl buffer. The outer membrane proteins were extracted with Triton X-100 (21) and the hemin-bound or Congo red-bound protein(s) was detected under reducing conditions by the discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique as described previously (4) and the separated proteins electroblotted onto nitrocellulose membranes, with the Mini-Protein II system (Bio-Rad Laboratories, Mississauga, Ontario, Canada). The hemin-bound or Congo red-bound protein(s) of *T. denticola* ATCC 35404 and ATCC 33520 were also extracted from the outer membranes, separated by SDS-PAGE and electroblotted.

Assay for siderophores

T. denticola, ATCC 35405, *T. vincentii* and *T. socranskii* were grown in serum-free new oral spirochete broth medium with ethylenediamine-di(o-hydroxyphenylacetic acid) at a concentration of 100 μg $\cdot$ ml^{-1}. The spent media after cell harvest by centrifugation ($5000 \times g$, 30 min, at 4°C) were assayed for siderophores by the shuttle chrome azurol S method (24). New oral spirochete broth medium without serum and deferroxamine (25 μM) were used respectively as negative and positive controls.

Results

Binding of hemin and Congo red to CFU

CFU were visible on day 6 (*T. denticola* ATCC 35405) and day 9 (*T. vincentii* and *T. socranskii*) of incubation. Colonies of *T. denticola*, *T. vincentii* and *T. socranskii* became visibly red or visibly brown-black when they bound respectively Congo red or hemin from solution. None of the 3 spirochete species produced variant CFU that did not bind Congo red or hemin.

Hemolysis of erythrocytes

Beta-hemolytic zones were visible around CFU of *T. denticola* species at day 9 (Fig. 1) and *T. vincentii* (day 12) and *T. socranskii* (day 16) of growth. All species tested secrete a hemolysin(s) that lyses erythrocytes in about 3–7 days after the appearance of the CFU. This may be either because of the secretion of the hemolysin later in the growth phase of the organisms or because low levels of the hemolysin are secreted and must reach a critical concentration.

Siderophore production

Siderophores were not detected in the spent medium after harvest of the oral

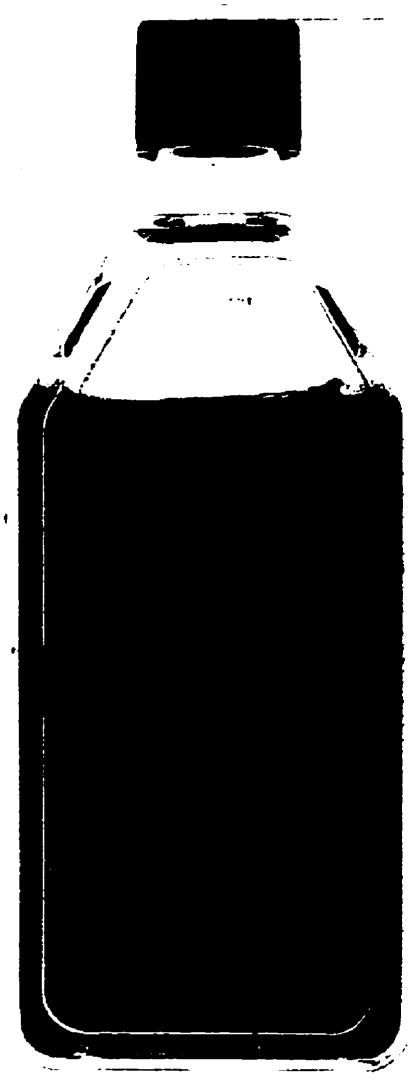

Fig. 1. Beta hemolytic zones (arrow) around colony-forming units of *T. denticola* ATCC 35405

spirochetes (*T. denticola*, *T. vincentii* and *T. socranskii*). Even chelation of the iron in the growth medium did not induce siderophore production.

Binding of Congo red and hemin to cells

The interaction between the chromogens (Congo red and hemin) and bacterial cells (1×10^8 ml^{-1}) was assessed with varying concentrations of the chromogens (0.1 to 20 μg · ml^{-1}). Higher concentrations of the chromogens could not be used, as they precipitated out of solution. Precipitation of these chromogens at concentrations above 25 μg · ml^{-1} has been reported by others (15). The density of the bacteria was selected (based on preliminary experiments) to bind near saturation levels of either chromogen. Specific binding increased as a function of the chromogen concentration, attesting to the saturability of the binding under the selected conditions of the assay. The binding of 2.4 μM of Congo red and 2.9 μM of hemin (Fig. 2) by *T. denticola* appears to be close to saturation levels. The data was further analyzed by an Eadie-Hofstee plot (35). The intersection of the regression line with the y-axis is equivalent to the maximal binding concentration (B_{max}) of chromogen bound to the cells and the slope of the line gives the K_d (dissociation constant). The regression lines of the Eadie-Hofstee plots show that *T. denticola* cells bind 2.1 μM of Congo red and 2.9 μM of hemin that has a K_d of 0.16 μM. The B_{max} of Congo red and hemin bound by *T. denticola* are similar when determined from the regression line (Fig. 3) and the curves (Fig. 2).

To simplify the presentation of the binding data, only the B_{max} and the K_d of the physiological ligand, i.e., hemin are reported. The B_{max} and K_d of *T. vincentii* are 2.8 μM and 0.8 μM respectively. The B_{max} and K_d of *T. socranskii* are 0.8 μM and 0.03 μM respectively

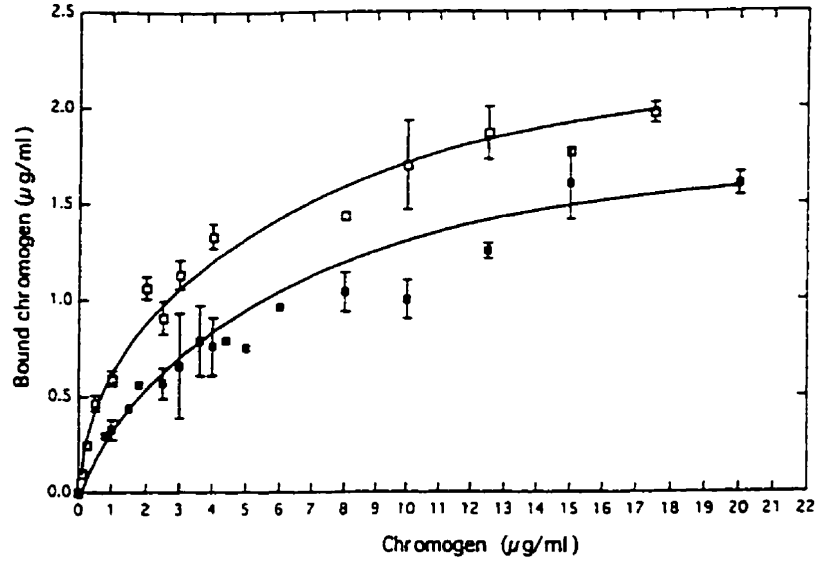

Fig. 2. Concentration-dependent binding of the chromogens Congo-red (■) and hemin (□) to *T. denticola* ATCC 35405. The bars indicate the standard deviation. The method is described in the text.

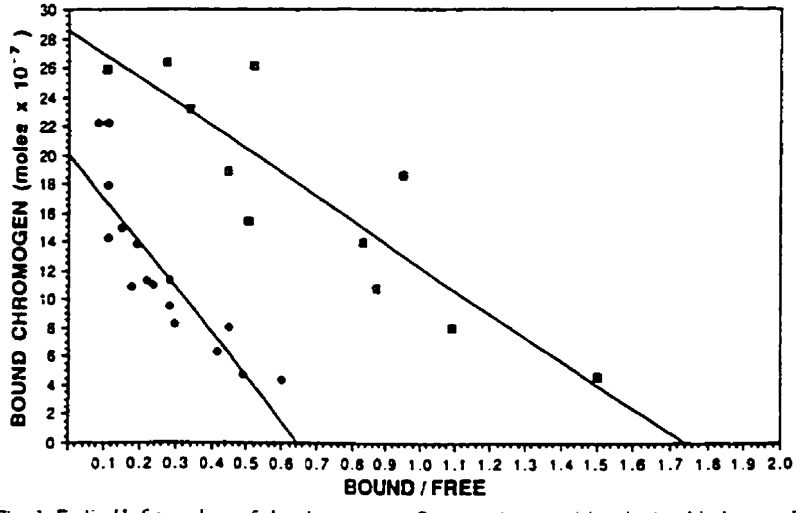

Fig. 3. Eadie-Hofstee plots of the chromogens, Congo-red (■) and hemin (□) binding to *T. denticola* ATCC 35405 [$r = 0.87$ (■), $r = 0.88$ (□)]. The method is described in the text.

(the regression curves for these two species are not shown). *T. socranskii* does not bind as much hemin as the other species, but binds hemin more avidly.

The data from the inhibition studies show that increasing concentrations of Congo red effectively block the binding of hemin to the bacterial cells (Fig. 4). Approximately 7.5 μg of Congo red block 96% (9.6 μg) of hemin from binding to 2×10^8 bacterial cells.

Congo red and hemin binding to treated cells

To characterize the biochemical nature of the binding site, the treated cells were analyzed for their ability to bind the chromogens. Treatment of the cells with papain, trypsin, periodic acid and polymyxin B did not reduce binding of the chromogens. However, treatment of the cells with pepsin, proteinase K, Triton X-100 and 8-anilino-1-naphthalenesulfonic acid reduced the binding of the chromogens (Table 1). 8-anilino-1-naphthalenesulfonic acid binds to hydrophobic amino acids. Pepsin preferentially hydrolyzes peptide linkages that involve the amino groups contributed by the hydrophobic amino acids (leucine, phenylalanine and tryptophan) in addition to tyrosine and pairs of nonpolar amino acids. Proteinase K predominantly cleaves the peptide bond adjacent to the carboxylic group of the hydrophobic amino acid alanine in addition to aliphatic and aromatic amino acids (9). Polymyxin B binds to lipid A and periodic acid oxidizes sugars.

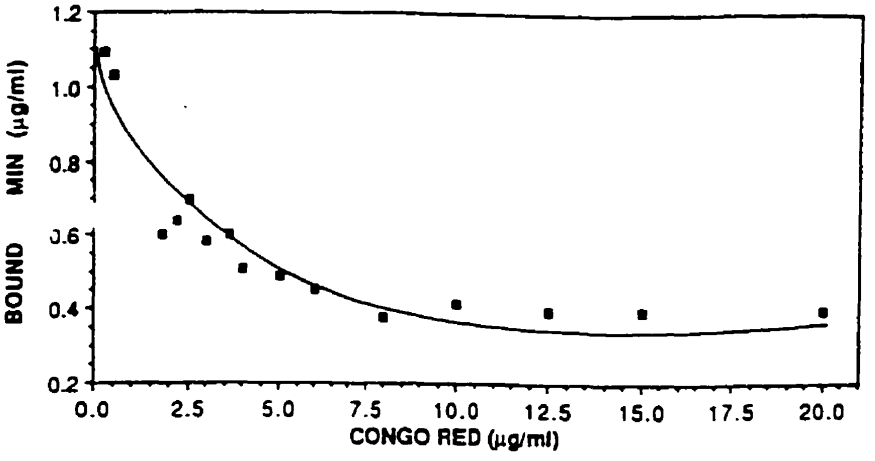

Fig. 4. Congo-red inhibition of hemin binding to *T. denticola* ATCC 35405. The method is described in the text.

Table 1. Effect of pre-treatment of *T. denticola* ATCC 35405 on hemin and Congo red binding

Treatment	Binding relative to control (%)[a]	
	Hemin[b]	Congo red[b]
ANSA[c]	59	48
Proteinase K	47	45
Papain	100	100
Pepsin	75	54
Trypsin	100	100
Sodium periodate	100	100
Triton X-100	30	39
Polymyxin B	> 100	> 100

[a] Assays carried out with the buffers only served as 100% controls. The concentrations of the test substances are given in the text. [b] The results are the mean of triplicate assays. [c] 8-anilino-1-naphthalenesulfonic acid.

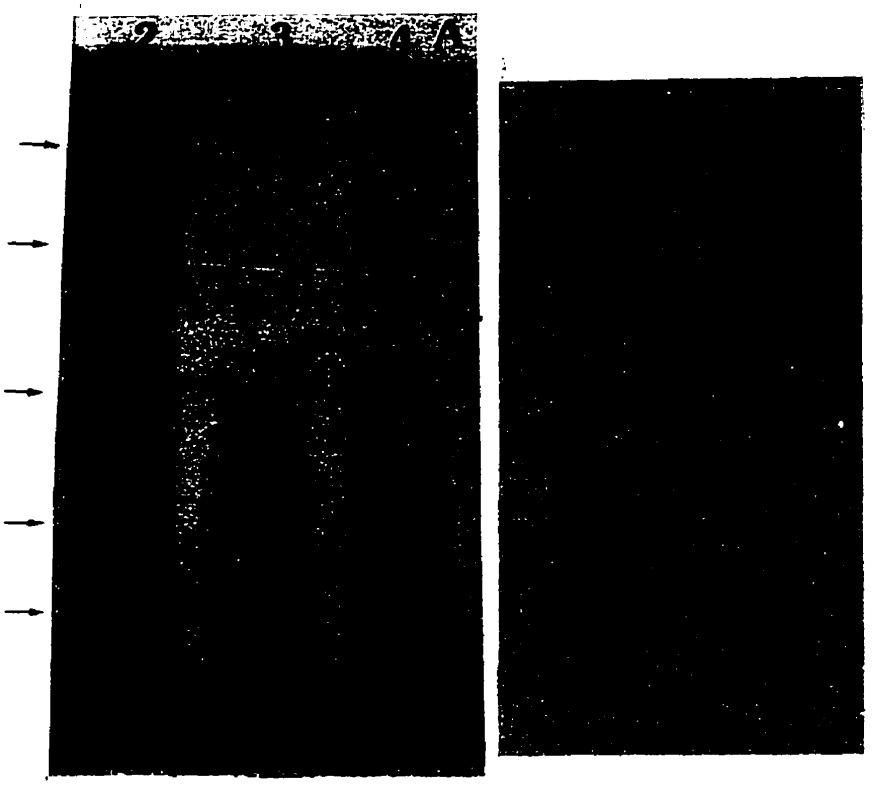

Fig. 5. SDS-PAGE and Western blot analysis of the outer membrane extracts of oral spirochetes. A. Silver-stained gel. B. Western blot of the same gel. The extracts from *T. denticola* ATCC 35405, ATCC 35404 and ATCC 33520 are in lanes 2, 3 and 4 respectively. The position of the molecular weight markers (106, 80, 49.5, 32.5, 27.5 and 18.5 kDa from top to bottom) are indicated by arrows in (A) and lane 1 in (B).

Electrophoresis

The protein binding Congo red or hemin migrated with an average molecular weight of 47 kDa. The Congo red-bound protein is visible in the polyacrylamide gel but the signal is enhanced, for photography, when the Congo red-protein complex is electroblotted onto nitrocellulose paper (Fig. 5B). The enhanced signal is most likely due to the thinness of the nitrocellulose membrane as compared with the thickness of the polyacrylamide gel. The electroblot of the hemin-bound protein is not shown, as the signal is weak and not readily apparent in a photographic reproduction.

Discussion

The Congo red binding site of oral spirochetes also binds hemin. These chromogens apparently bind to a receptor(s) that is very similar structurally, since Congo red inhibits the binding of hemin to the bacterial cells. The receptor is located in the outer membrane of the cells, as Triton X-100 extraction drastically reduces binding of the chromogens; it has a molecular weight of 47 kDa and the binding site of the receptor is a peptide rather than an oligosaccharide or lipid A. This is based on the fact that 8-anilino-1-naphthalenesulfonic acid and the proteases, pepsin and proteinase K, but not polymyxin B nor periodation of the cells, significantly reduced the binding of the chromogens. It is feasible, but speculative, that the binding site is represented by a stretch of hydrophobic aromatic amino acids. This argument is based on the ability of 8-anilino-1-naphthalenesulfonic acid to bind to hydrophobic amino acids, and hydrolysis of the peptide bonds by pepsin and proteinase K but not by trypsin and papain and the known hydrophobic interaction of heme to hemoglobin. Since oral spirochetes do not produce siderophores, the heme receptor could have evolved through physiological adaptation of the oral spirochetes to bind the ligand hemin, from which it acquires iron. The mechanisms of iron uptake from hemin by the organisms is not yet known. Hemin is not, however, taken into the bacteria since >90% of prebound hemin was obtained in the Triton X-100 extract (data not shown) and the Western blot (Fig. 5B) shows that the chromogens remain bound to the receptor of the outer membrane (Triton X-100 extract). Congo red can be considered an "opportunistic ligand" because of its structural similarity to protoporphyrin (15). Nevertheless, Congo red binding can serve as a useful marker to identify bacterial species or strains that bind heme.

The growth of oral spirochetes in subgingival plaque may be determined, in part, by their ability to bind the heme prosthetic group of hemoproteins in crevicular fluid. Since hemin remains bound to the 47 kDa in the outer membrane of spirochetes, these bacteria may have a mechanism for sequestering iron from the bound hemin and transporting the iron into the bacteria. These hemoproteins can accumulate in crevicular fluid as a result of inflammation and degradation of gingival tissue by bacteria of the subgingival plaque (such as oral spirochetes, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*). Cytotoxic substances and proteases are produced by oral spirochetes (18, 22, 23, 29, 33), *P. gingivalis* (19) and *F. nucleatum* (29). As a result of inflammation, erythrocytes may extravasate into the crevicular fluid and can be lysed by phospholipase C (26) that is secreted by oral spirochetes (Fig. 1) or another hemolysin (12) as well as hemolysins produced by *P. gingivalis* (14, 25) and *F. nucleatum* (10). The hemoglobin and other hemoproteins of degrated epithelial cells and fibroblasts can account for the increased iron concentration in crevicular fluid of patients with adult periodontitis (20). *P. gingivalis* has also been reported to bind hemin by an outer membrane protein (3) and the lipid A component of lipopolysaccharide (11). The hemin receptor of oral spirochetes is not, however, lipid A, as polymyxin B enhances binding of the chromogens.

The ability of oral spirochetes and *P. gingivalis* to lyse erythrocytes and bind hemin may account for the interesting bacterial synergy between *T. denticola* and *P. gingivalis* (28). These organisms are always found in association in the periodontal pockets of patients with adult periodontitis. They may, by their combined action, provide each other with a ready source of free heme before the free heme is complexed by hemopexin. It is not known at the present time whether oral spirochetes and *P. gingivalis* use the heme-hemopexin complex as a source of iron (34), as do other bacterial species.

Acknowledgement

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CHAPTER 2

PART II

Iron acquisition by oral hemolytic spirochetes : isolation of a hemin binding protein and identification of iron reductase activity.

Further studies on the mechanism of iron acquisition by OAS are now presented in manuscript form, as accepted for publication in the Canadian Journal of Microbiology. These data and conclusions are a progression of the first section of Chapter 2. In Part II, the outer membrane sheath hemin-binding protein, the fate of the bound hemin molecules at the outer membrane sheath, the iron sources that can support the growth of *Treponema denticola* and the production of a ferric iron reductase are investigated.

CHAPTER 2

PART II

Iron acquisition by oral hemolytic spirochetes : isolation of a hemin-binding protein and identification of iron reductase activity.

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ABSTRACT.

Oral anaerobic spirochetes (OAS) have been implicated in the etiology of periodontal disease. In order to adapt to the environment of the subgingiva, OAS must be able to acquire iron from limited sources. OAS have previously been shown not to produce siderophores but are β-hemolytic and can bind hemin via a proteinaceous 47-kDa outer membrane sheath receptor. Present studies show that ^{3}H-hemin is not transported into the cytoplasm, that hemin, and ferric ammonium citrate, can support the growth of OAS as the sole iron sources, yet protoporphyrin IX and Congo red are inhibitory, thereby implying an important *in vivo* role for hemin as an iron source. *Treponema denticola* ATCC 35405 produces an iron reductase. The iron reductase can reduce the central ferric iron moiety of hemin. The 47-kDa outer membrane sheath hemin-binding protein has been purified to apparent homogeneity by methanol/chloroform extraction of cellular lipoproteins and the use of a hemin-agarose bead affinity column. A model of iron acquisition by OAS is presented.

INTRODUCTION

Oral anaerobic spirochetes (OAS) are considered periodontal pathogens due to the production of a range of peptidolytic and proteolytic enzymes (Makinen *et al*, 1994, Ohta *et al*, 1986; Uitto *et al*,1989); phospholipase C (Siboo *et al*, 1989); hyaluronidase and chondroitinase (Fiehn, 1986) and immunosuppressive factors (Sela *et al*,1988; Shenker *et al*,1984). OAS have been numerically associated with diseased sites (Loesche,1988; Simonson *et al*, 1988), are reduced in numbers following therapy (Loesche,1988; Wolff *et al*,1994), increase in percentage prior to recurrent episodes of disease (Singletary *et al*,1982) and have been reported to penetrate gingival tissues (Frank,1980).

In humans, extracellular iron is limited by high-affinity iron- and hemin-binding proteins and the low solubility of ferric iron under physiological conditions reduces iron concentrations to levels below that required for bacterial viability (Bullen,1978; Grenier,1991a). Several mechanisms are known by which host-associated bacteria can scavenge their iron requirements. Bacteria may produce outer membrane transferrin- or lactoferrin-receptors (McKenna *et al*,1988). Other species produce iron-chelating siderophores (Yang et al,1991). Hemagglutinins (Kay *et al*,1990) and hemolysins (Grenier 1991a) can liberate hemoglobin from erythrocytes and several bacterial species can utilize hemin as an iron source (Coulton and Pang,1983). The production of iron reductases, allowing iron in the form of Fe^{++} to cross biological membranes, has also been reported (Deneer *et al*,1995). Bacteria may utilize one or several of the above mechanisms. The methods of iron acquisition available to OAS have only recently been addressed. *Treponema denticola* has been shown to possess hemagglutinin

(Grenier,1991b), hemoxidative (Chu *et al.*,1994b) and hemolysin (Chu *et al.*,1994b; Chu and Holt,1994; Scott *et al.*,1993; Grenier,1991b) activities. *T. denticola* constitutively produces a 47-kDa outer membrane sheath (OMS) hemin-binding protein (Scott *et al.*,1993) and two hemin-binding proteins under conditions of iron-limitation (Chu *et al.*,1994a). *T. denticola* has also been reported to utilize lactoferrin, but not transferrin (Russell *et al.*, 1992).

We herein report on the purification of the 47-kDa hemin-binding OMS protein by hemin-agarose affinity chromatography, the observation that ^{3}H-labeled hemin is not transported into the cytoplasm, growth in iron-limited medium and on the production of an iron reductase by *T. denticola* ATCC 35405.

MATERIALS AND METHODS

Growth of Oral Anaerobic Spirochetes

Treponema denticola ATCC 35405 was originally isolated and maintained by our laboratory (Cheng and Chan,1983). *T. vincentii* ATCC 33520 was purchased from the American Type Culture Collection, Rockville, MD. The OAS were grown in liquid New Oral Spirochete (NOS) medium, as described previously (Cheng and Chan,1983), in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI).

Lipoprotein extraction

Fifty ml, 6-day NOS cultures of *T. denticola* and *T. vincentii* were centrifuged (4 000 x g, 15 min, 4°C) and the harvested cells washed twice with 10mM phosphate-buffered saline, pH 7.2 (PBS). Cellular lipoproteins were extracted from cell pastes by two 12 h, 37°C, incubations with 4 ml methanol / chloroform (2:1 v/v) followed by a 6h, 37°C, methanol / chloroform / H_2O (2:1:0.8 v/v) extraction. The combined supernatants were dialyzed in 3500 MW cut-off tubing against 0.2 % saline. The lipoproteins were precipitated by the addition of 10% dextran sulfate (2% v/v) and 1M $CaCl_2$ (1% v/v)), and 5 min mixing, then centrifuged at 5 000 x g, 4°C, 15 min. The precipitate was redissolved in 10 ml 5% NaCl then reprecipitated with 90ml H_2O and 10 ml 1M $CaCl_2$. This precipitation process was repeated twice and the final precipitate redissolved in 5 ml 1M sodium oxalate. Two g NaCl and 2.5 ml 2% protamine sulfate

were added to remove dextran residues. The extracted lipoproteins, contained in the aqueous phase, were then dialyzed against 0.15% saline at 4°C and concentrated with polyethylene glycol.

Purification of the 47kDa hemin-binding protein

The extracted cellullar lipoproteins were passaged through a column packed with hemin conjugated to 4% agarose beads (Sigma Chemical Co., St. Louis, Mo) using a PBS eluant. Two-ml fractions were collected with a Bio-Rad 2110 fraction collector and the absorbance of each fraction at 280 nm monitored for protein content. Hemin-binding proteins were then eluted with 0.5M glycine-NaCl-HCl buffer, pH 2.7. Protein concentrations were determined using the Bio-Rad protein assay kit. Total cellular lipoproteins and column-purified hemin-binding lipoproteins were then subjected to SDS-PAGE. The proteins were visualized by silver-staining.

Preparation of ^{3}H-labeled hemin

The carboxyl groups of hemin (Sigma) were labeled with tritium by a 48-h, 25°C incubation of 10ml 40 μg ml^{-1} hemin (dissolved in 1 ml 0.1 M NaOH with 9 ml H_2O added subsequently) and 0.3 ml $NaB_3{}^3H_4$ (Amersham International, Oakville, Ont.) with gentle shaking. Unincorporated label was removed by passage through a Sephadex G10 column using 0.01 M NaOH as the eluant. Hemin levels collected in 1 ml fractions were

measured photometrically at $O.D._{400\ nm}$. Twenty µl aliquots of each fraction were spotted on Whatman glass fibre filters and counted for tritium levels on a Wallac RackBeta scintillation counter. Ten ml of unlabeled hemin (40 µg ml^{-1} in 0.1 M NaOH) was also passaged through the Sephadex G10 column and the elution profiles compared to ensure no degradation of the labeled hemin had occurred.

Binding assays for ^{3}H-hemin by *T. denticola* ATCC 35405.

Four-day (exponential growth phase) and 10-day (stationary phase) NOS cultures of *T. denticola* were washed in PBS and adjusted to 1 x 10^{8} cells ml^{-1}. One ml cells were incubated with 1 ml 20 µg ml^{-1} ^{3}H-hemin (specific activity = 5.704 x 10^{6} cpm ml^{-1}) for 30, 60 or 180 min, at 37°C. Each sample was then centrifuged, 10 min, 4°C, 5 000 x g. The amount of hemin bound to the cells was measured by subtractive photometery at 400nm, as described previously (Scott *et al*, 1993). A 10 µl aliquot of each supernatant was spotted on a glass fiber filter. Ten µl of the OMS proteins, prepared by incubating cell pellets with 1 ml 2% Triton-X 100 following two washes in PBS (Scott *et al*, 1993), from each sample were then similarly spotted. The resultant protoplasmic cylinders (PC) were washed twice in PBS, resuspended in 0.5 ml PBS and cracked open using a bead beater (Xymotech Biosystems, Mount Royal, Que.). The PC constituents were collected from the bead beater and any residual cytoplasmic material was washed off the beads with 0.5 ml PBS. Twenty µl of the cracked PC fractions were spotted on glass fiber filters. Filters were washed and placed in scintillation vials. Tritium levels on all filters were counted

and the percentage, and absolute levels, of ^{3}H-hemin found at the OMS, and in the PC calculated over a time period of 180 min. All assays were performed in triplicate.

Growth of *T. denticola* ATCC 35405 under iron limitation and supplemented with hemin, protoporphyrin IX, Congo red, ferric chloride and ferric ammonium citrate as iron sources.

T. denticola (1 x 10^5 cells) was inoculated into 10 ml of NOS and into NOS containing 5, 10 or 100 µl of a stock solution of 10 mg ml^{-1} of the iron chelator deferoxamine (desferrioxamine mesylate, Sigma) and incubated at 37°C. The growth of the spirochetes was monitored photometrically at O.D. $_{620nm}$. NOS containing 10 µl of the deferoxamine stock (NOS-D) permitted only minimal growth, therefore 1 x 10^5 cells of *T. denticola* was inoculated into 10 ml NOS-D and into NOS-D supplemented with either 10 µg ml^{-1} ferric ammonium citrate, 10 µg ml^{-1} ferric chloride, 20 µg ml^{-1} protoporphyrin IX or 12 µg ml^{-1} hemin as iron sources. *T. denticola* was also inoculated into NOS and NOS containing 20 µg ml^{-1} protoporphyrin IX or 20 µg ml^{-1} Congo red. The growth of the spirochetes was measured periodically by photometry at O.D. $_{620\ nm}$.

Replacement of serum with hemin in NOS medium.

T. denticola (1 x 10^5 cells), was inoculated into NOS medium in which rabbit serum was omitted and replaced with 12 µg ml^{-1} hemin. Growth was monitored at 620 nm. An inoculum of 1 x 10^5 cells from this culture was subsequently passaged into

serum-depleted, hemin-supplemented NOS. Growth of *T. denticola* was monitored over a period of 10 passages under identical conditions.

Photometric and solid-phase assays for iron reductase activity.

Fe^{+++} to Fe^{++} iron reductase activity was monitored photometrically essentially according to the method of Dailey and Lascelles (1977). A 10 ml, 5-day NOS culture of *T. denticola* ATCC 35405 and a 10 ml, 24-h nutrient broth culture of *Escherichia coli* (McGill University, Department of Microbiology and Immunology, Teaching Collection) were harvested (4 000 x g, 15 min, 4°C) and washed twice with 10 mM Tris-HCl, pH 7.6. Ferrozine [3-(2-pyridyl-5,6-bis-(4-phenylsulfonate)-1,2,4-triazine)], which is water-soluble and remains colorless in the presence of ferric iron, forms a purple chelate in the presence of ferrous iron. Ferrozine does not react with any other divalent ion, with the exception of copper, and does not interfere with the activity of nicotinamide adenine dinucleotide (NADH; Dailey and Lascelles,1977; Deneer and Boychuk, 1993). A 2-ml suspension of 1 x 10^9 cells ml^{-1} in 10 mM Tris-HCl, pH 7.6, was placed in the bottom of a Thunberg cuvet. One ml of 5 μM NADH, 3 μM flavin mononucleotide (FMN), 2 μM ferrozine, 10 mM $MgCl_2$ and 0.4 μM ferric ammonium citrate or 0.6 μM ferric chloride (final concentrations in 10 mM Tris-HCl, pH 7.6) was placed in the neck of the cuvet. The cuvets were vacuumed and re-filled with 85% nitrogen, 10% hydrogen and 5% CO_2. The reaction was initiated by inverting the Thunberg cuvets and iron reductase activity monitored by the increase in absorbance at 562nm due to the formation of a ferrous iron - ferrozine complex. Cuvets containing all reagents, but no cells, served as controls.

A second assay for the production of iron reductase by *T. denticola* was devised, taking advantage of the colorimetric reaction of ferrozine in the presence of Fe^{++} and a recently published technique that permits consistent and reproducible enumeration of viable, cultivable spirochetes using NOS-0.7% Sea-plaque agarose in tissue culture flasks (Qiu *et al.*, 1994). Ten ml of 0.7% agarose containing 5 μM NADH, 3 μM FMN, 2 μM ferrozine and 10 mM $MgCl_2$ were placed at the bottom of a 45-ml tissue culture flask and allowed to gel. An overlay of 15 ml of molten (40°C) NOS-0.7% Sea plaque agarose or NOS-0.7% Sea-plaque agarose containing 15 μl of 10 mg ml^{-1} deferoxamine (NOS-SD) or NOS-SD with 12 μg ml^{-1} hemin or NOS-SD with 20 μg ml^{-1} ferric ammonium citrate was added and inoculated with 1 x 10^5 cells *T. denticola* prior to gelling. A third layer, identical to the first layer, was then overlaid. The tissue culture flasks were sealed with 0.5% Noble agar and incubated anaerobically at 37°C and examined for a colorimetric change in the ferrozine indicator. Ferrozine, FMN and NADH were supplied by Sigma.

RESULTS

Purification of the 47 kDa hemin-binding protein

Glycine-eluted fractions 18 - 21 of the methanol / chloroform-extracted lipoproteins of *T. denticola* passed through a hemin-agarose column contained functional hemin-binding protein (Figure 6). The hemin-binding protein was purified to apparent homogeneity, seen as a single band of 47-kDa on SDS-PAGE analysis (Figure 7). Protein estimation using the Bio-Rad kit determined the yield of hemin-binding protein to be 9% of the total cellular lipoproteins. Similar results were obtained using *T. vincentii*. The hemin-binding protein of *T. vincentii* was eluted in fractions 25 - 27 (Figure 6), and was visualized as a single protein species, also of 47-kDa, on electrophoresis (Figure 7). The hemin-binding protein of *T. vincentii* constituted 6% of the total extracted cellular lipoproteins.

Binding and cellular distribution of ^{3}H-labeled hemin

The elution profiles of labeled and unlabeled hemin through a Sephadex G-10 column were identical (data not shown) indicating that no degradation of the hemin molecules had occurred during the labeling procedure.

The cellular location of the ^{3}H-hemin bound by 4-day and 10-day cultures of *T. denticola* is shown in Table 2. The amount of hemin bound by whole cells and the

Figure 6. The elution profiles of the cellular lipoproteins of *T. denticola* and *T. vincentii* from a hemin-agarose bead affinity column. The elution profiles, in 2 ml fractions as monitored for protein content at $O.D._{280nm}$, of the methanol/chloroform extracted lipoproteins of *T. denticola* (■) and *T. vincentii* (□). The arrows represents the switch of eluant from PBS, pH 7.2 to glycine-NaCl-HCl buffer, pH 2.7. The full protocol is described in the text.

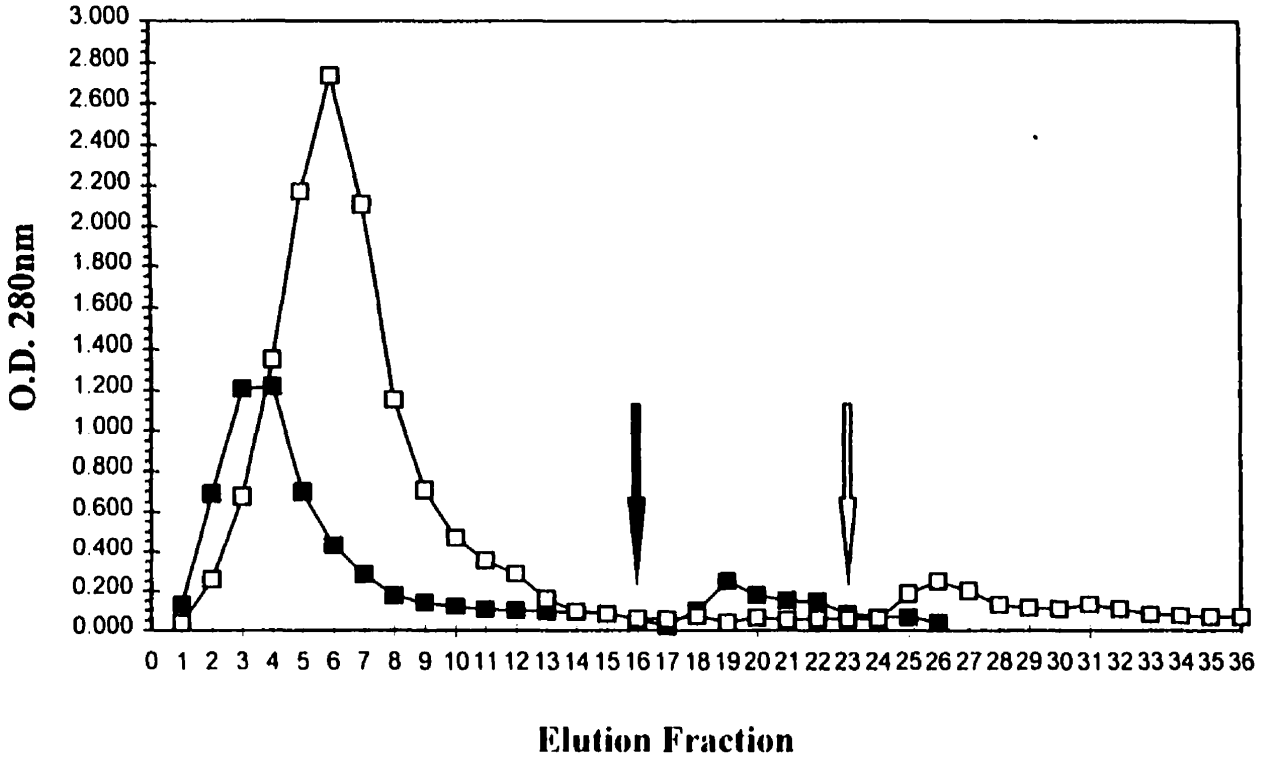

O.D. 280nm
3.000
2.800
2.600
2.400
2.200
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
Elution Fraction

Figure 7. SDS-PAGE analysis of the purified 47-kDa hemin-binding proteins of *T. denticola* and *T. vincentii*. The affinity purified hemin-binding proteins of *T. denticola* and *T. vincentii* were electrophoresed through a 10% SDS-PAGE system and silver stained. The total extracted proteins of *T. denticola* and *T. vincentii* are in Lane 1 and Lane 2, respectively. The affinity purified hemin-binding proteins of *T. denticola* and *T. vincentii* are in Lane 3 and Lane 4, respectively. The molecular weight markers (MW) are 84, 53.2,34.9, 28.7 and 20.5 kDa, respectively, from top to bottom.

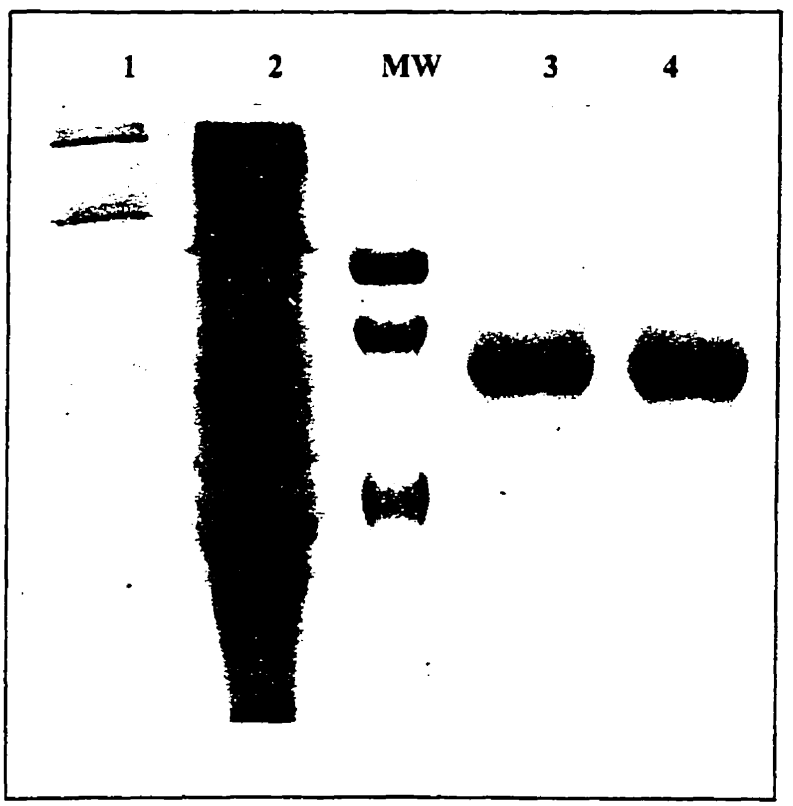

1
2
MW
3
4

Table 2. The binding and distribution of ^{3}H-hemin in whole cells of *T. denticola* ATCC 35405.

Age of culture	Time of assay (min)	%label at OMS(¶)	%label in PC (‡)	total hemin bound (†) (μg)	absolute ^{3}H count in PC (cpm /10 μl)
4 day	30	92.3	7.7	2.0	20.3
	60	91.7	8.3	2.0	21.0
	180	94.5	5.5	2.0	22.2
10 day	30	92.7	7.3	2.0	19.7
	60	88.8	11.2	2.0	29.5
	180	94.8	5.2	1.8	22.7

(¶) The percentage of ^{3}H-hemin found in the outer membrane sheath (OMS) extracted from whole cells treated with Triton-X 100. Results are the average of triplicate assays. The incubation mixture contained 20 μg ml^{-1} ^{3}H-labeled hemin with a specific activity of 5.704 x 10^{6} cpm ml^{-1}.

(‡) The percentage of ^{3}H-hemin found in the protoplasmic cylinder (PC) following removal of the OMS, washing with PBS, pH 7.2 and cracking of the PC by use of a bead breaker.

(†) Absolute amount of ^{3}H-hemin bound by 1 x 10^{8} whole cells of *T. denticola* per ml calculated by subtractive photometric measurements.

All the results are the average of triplicate assays.

Full details of the assay are given in the text.

percentage and absolute amounts of tritium detected on the outer membrane sheath (OMS) and in the protoplasmic cylinder (PC) was constant over the 3 hours of the assay. This would suggest that hemin is not transported into the PC of the spirochete cells. No significant difference was observed in the localization of the ^{3}H-label in the 10 day cultures, compared to the 4 day cultures, when competition for iron would be expected to be more intense.

Growth of *T. denticola* under iron limitation and supplemented with hemin, protoporphyrin IX, Congo red, ferric chloride and ferric ammonium citrate as iron sources.

The growth of *T. denticola* was monitored by spectrometry under varying conditions of iron availability. As can be seen in Figure 8, iron depletion due to chelation by deferoxamine resulted in the severe limitation of growth of *T. denticola*. However, the spirochetes were able to utilize inorganic iron, in the form of ferric ammonium citrate, to meet their iron requirements. Hemin was also able to serve as the sole iron source (Figure 8) although the amount of growth was reduced in comparison to complete NOS medium. Both Congo red, a tetrapyrolle analogue, and protoporphyrin IX, which has the same structure as hemin but lacks a central iron moiety, were inhibitory to the growth of *T. denticola* cells when added to complete NOS. Ferric chloride proved incompatible with NOS medium as it precipitated rapidly.

Figure 8. The growth of *T. denticola* with various iron sources. The growth curves of *T. denticola* grown in 10 ml New Oral Spirochete medium (●), NOS containing 10 µl of 10 mg ml^{-1} deferoxamine[NOS-D] (■), NOS-D with 12 µg ml^{-1} hemin(□), NOS-D with 10 µg ml^{-1} ferric ammonium citrate (◇),and in NOS-D with 20 µg ml^{-1} protoporphyrin IX (▲). Growth curves in NOS with 20 µg ml^{-1} protoporphyrin IX (◆) and in NOS with 20 µg ml^{-1} Congo red (✖)are presented. The full protocol is given in the text.

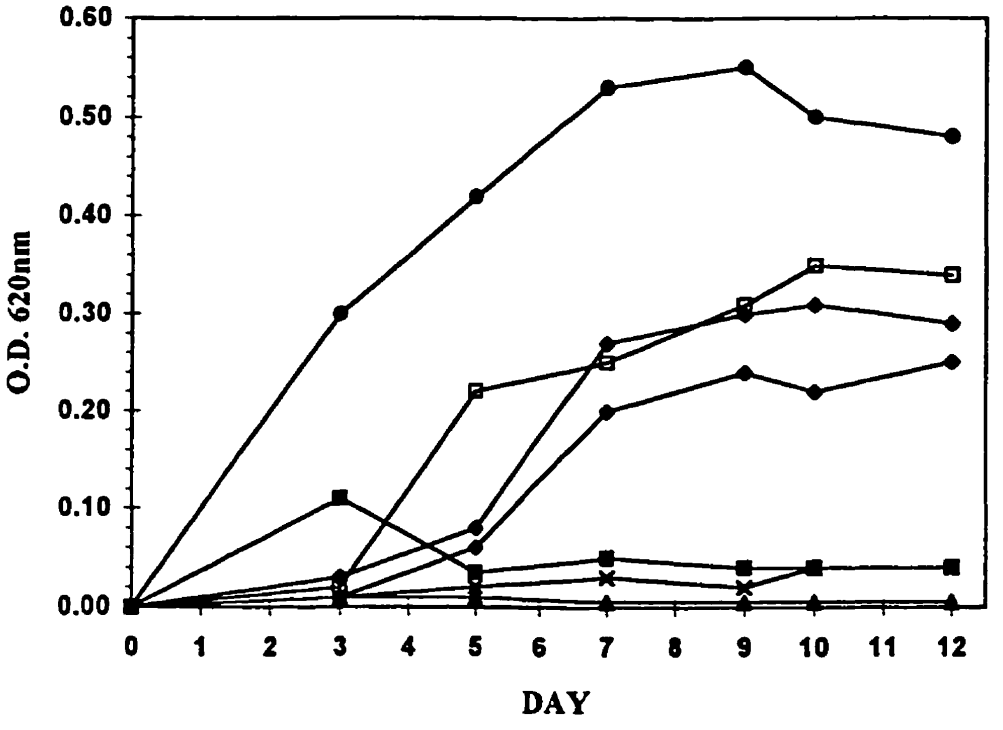
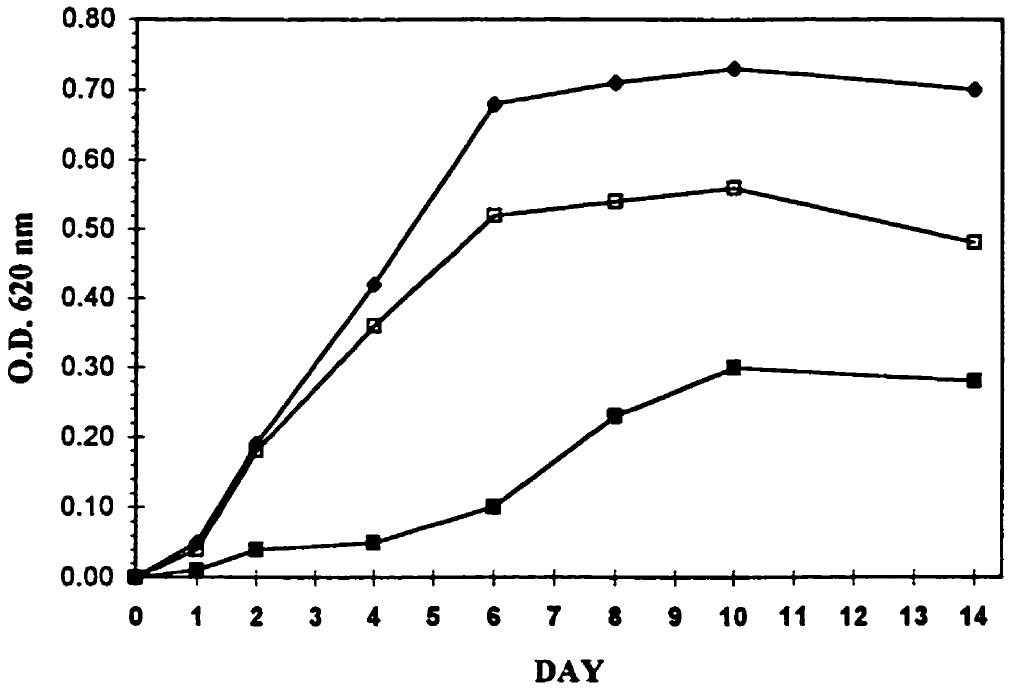

0.60
0.50
0.40
0.30
0.20
0.10
0.00
O.D. 620nm
0
1
2
3
4
5
6
7
8
9
10
11
12
DAY

Figure 9. The growth of *T. denticola* ATCC 35405 in New Oral Spirochete medium with hemin but no serum. The growth curves of *T. denticola* grown in 10 ml New Oral Spirochete medium with rabbit serum omitted, and 12 μg ml^{-1} hemin added, for passage 2(□). for passage 10 (■) and in complete NOS (◇) are presented. The full protocol is given in the text.


0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
O.D. 620 nm
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
DAY

Replacement of serum with hemin in NOS growth medium

Hemin, in place of rabbit serum in NOS medium, was initially able to support 75% of the growth of *T. denticola* ATCC 35405 in comparison to complete NOS medium (Passage 2). However, as can be seen in Figure 9, the growth of *T. denticola* was reduced to only 31% of that in complete NOS after 10 passages in the serum-depleted medium.

Iron reductase activity

Iron reductase (Fe^{+++} to Fe^{++}) was observed in the photometric assay using ferric chloride or ferric ammonium sulfate as the source of ferric iron (Figure 10). The formation of ferrous iron in the Thunberg cuvets, from the ferric salts, occurred immediately on the initiation of the assay. This rapid colorimetric shift was not observed without the presence of *T. denticola* cells or with *E. coli* cells. The shift in $O.D._{562\ nm}$ readings, although in keeping with results reported for the iron reductase activity of *Spirillum itersonii*, *Rhodobacter capsulata* and *Staphylococcus aureus* (Dailey and Lascelles, 1977), was rather small (from 0.020 to 0.074 over 10 min). Therefore the presence of the iron reductase was confirmed by a second method.

A Petri plate assay has been employed to observe the iron reductase activity of *Listeria* species (Deneer and Boychuk, 1993) but it cannot be applied directly to OAS as they do not grow well in Petri plates. In the triple-layered tissue culture flask adaptation of the assay, with ferrozine-containing agarose sandwiching a NOS-Sea plaque agarose

Figure 10. Iron reductase activity in whole cells of *T. denticola*. Whole cells of *T. denticola* were assayed in Thunberg cuvets with ferrozine, for iron reductase activity, as detailed in the text. The activities of *T. denticola* cells (□), *E. coli* cells (■), buffers and reagents only (◇), with ferric chloride as the iron source are presented. The activity of *T. denticola* cells with ferric ammonium citrate as the iron source(◆) is also presented. The bars represent the mean and standard deviation of the data.

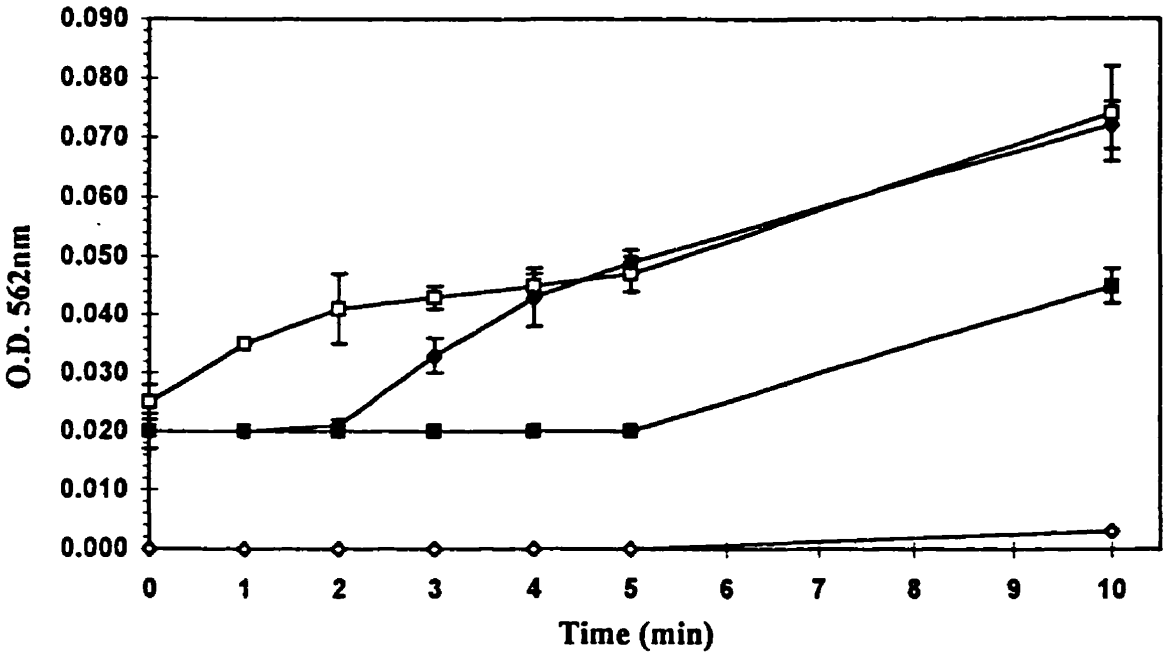
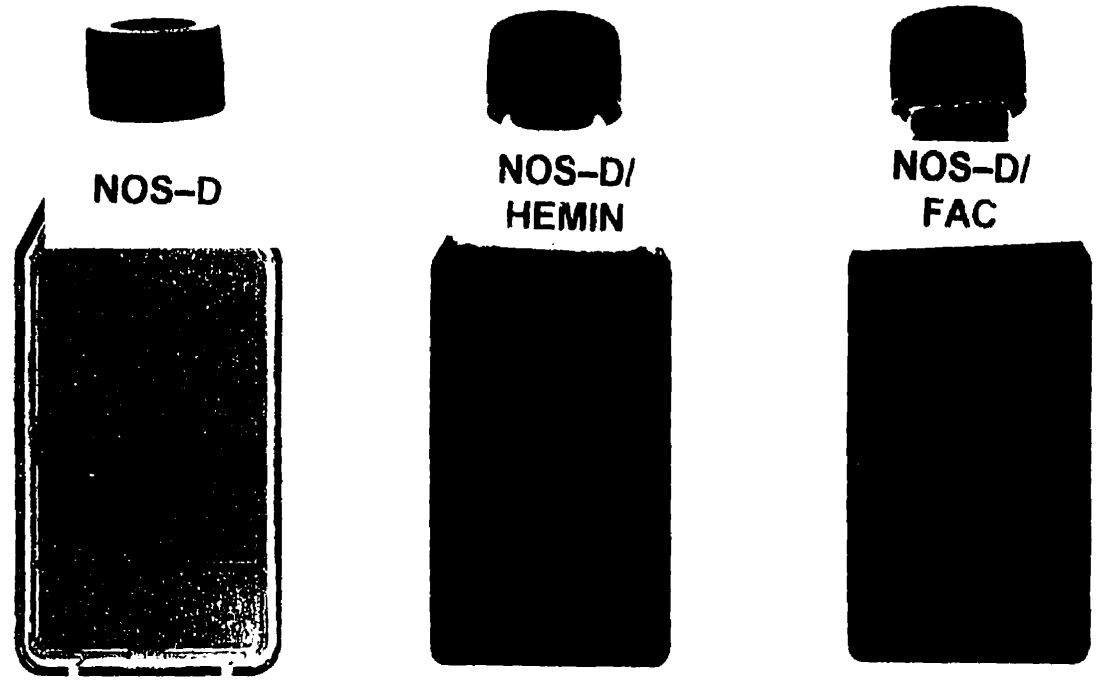

0.090
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
O.D. 562nm
0
1
2
3
4
5
6
7
8
9
10
Time (min)

Figure 11. The iron reductase activity of *T. denticola* grown in tissue-culture flasks. Colony-forming units of *T. denticola* were assayed for iron reductase activity as detailed in the text. *T. denticola* in New Oral Spirochete - 0.7 % agarose and deferoxamine is presented on the left. *T. denticola* in New Oral Spirochete - 0.7 % agarose and deferoxamine with 12 μg ml^{-1} hemin is presented in the center. *T. denticola* in New Oral Spirochete - 0.7 % agarose and deferoxamine with 20 μg ml^{-1} ferric ammonium citrate presented on the right.


NOS–D
NOS–D/
HEMIN
NOS–D/
FAC

layer, which allowed growth in a solid medium, we were able to observe iron reductase activity. As can be seen in Figure 11, *T. denticola* using hemin or ferric ammonium citrate as the sole iron sources caused a deep purple colorimetric reaction between ferrozine and ferrous iron, due to the reduction of Fe^{+++} from hemin or from ferric ammonium citrate. In this solid phase assay, the development of a purple color, indicative of iron reductase activity, was not due to any component of the NOS medium as ferrozine did not react with the iron-chelated medium nor does it react with ferric iron (Dailey and Lascelles, 1977; Deneer and Boychuk, 1993). In addition, should ferrous iron have been present in the medium, an immediate colorimetric reaction with ferrozine would have been observed.

DISCUSSION

The purificaton of a hemin-binding protein confirms our earlier report of a 47-kDa hemin-binding protein in *T. denticola* ATCC 35405, *T. vincentii and T. socranskii* (Scott *et al*, 1993). The hemin-binding protein constituted 9% and 6% of the total chloroform/methanol extracted cellular lipoproteins of *T. denticola* and *T. vincentii*, respectively. Two hemin-binding proteins have been isolated from *T. denticola* ATCC 33520 and ATCC 33404 of 44-kDa and 43-kDa (Chu *et al*, 1994a). We did not detect the 44-kDa or 43-kDa hemin-binding proteins in *T. denticola* ATCC 35405, in *T. vincentii* or in *T. socranskii* by SDS-PAGE, in an earlier investigation (Scott *et al*, 1993). Only one hemin-binding protein was isolated on passage of cellular lipoproteins through a hemin-agarose column (Figure 7). It appears, therefore, that there may be variation in the molecular weight of hemin-binding proteins produced by different strains of *T. denticola*. Alternatively, the 47-kDa hemin-binding protein is constitutively expressed, as we did not deplete the iron content of the growth medium, whereas the 44-kDa and 43-kDa proteins were observed under conditions of iron-limitation (Chu *et al* 1994a).

We had previously established that the maximal binding of hemin, and of Congo red, by OAS occurred within 30 minutes (Scott *et al*, 1993). For this reason, if hemin was to be transported into the cytoplasm of *T. denticola* we would have expected to observe an increase in ^{3}H counts in the PC of the cells over the assay period of 3 h . Coulton and Pang (1987) have shown that the intracellular transport of ^{59}Fe- and ^{14}C-labeled hemin by *Haemophilus influenzae* type b was linear with time. Our results confirm that maximal binding of hemin by *T. denticola* cells occurred by 30 min (Table 2). As >90% of the ^{3}H-label was found in the OMS after 30, 60 and 180 min after exposure to 20 $\mu g\ ml^{-1}$

^{3}H-hemin these data strongly suggest that hemin was not transported into the cytoplasm. Only a small percentage of the total label bound by the cells could be detected in the PC fractions, even after 180 min of hemin-binding at the maximal capacity of the cells (approximately 6%), and this might be due to dissociation of hemin from the OMS during Triton-X 100 extraction or to small amounts of contaminating OMS in the PC fractions due to limitations in the fractionation procedure.

In the presence of the iron chelator, deferoxamine, growth of *T. denticola* was limited. Hemin was able to support growth as the sole iron source (Figure 8). As Protoporphyrin IX and especially Congo red were inhibitory to the growth of *T. denticola* (Figure 8), this infers an important *in vivo* role to hemin as an iron source of OAS. Congo red has been previously shown to act as a competitive inhibitor of hemin-binding by intact cells of *T. denticola* (Scott, *et al*, 1993).

We found that ferric ammonium citrate could also support the growth of *T. denticola* (Figure 8). These data confirm the findings of Wyss (1992) who grew *T. denticola* in a chemically defined medium using only inorganic iron ($FeSO_4$) and hemin as iron sources. Inorganic iron sources, however, are unlikely to be available to bacteria in the periodontium.

Transport of iron sequestered from hemin may be facilitated by the iron reductase. We have observed the reduction of ferric ammonium citrate, ferric chloride and the iron moiety of hemin by *T. denticola* in the ferrozine assays for detection of iron reductases. The reduction of Fe^{+++} in the liquid assay, using whole cells of *T. denticola* was detected exceedingly rapidly. The observation of the rapid reduction of Fe^{+++} has also been

reported for the iron reductase produced by *L. monocytogenes* in an almost identical assay (Deneer *et al*, 1995).

Iron reductases have been shown to allow bacteria to utilize the iron-binding capacity of siderophores (Johnson *et al*, 1991), raising the possibility that *T. denticola* may have the ability to scavenge iron from siderophores produced by other members of the oral flora. This hypothesis remains to be tested.

It is known that *T. denticola* strains can hemagglutinate human, horse, bovine, rabbit and sheep erythrocytes (Chu and Holt, 1994; Grenier, 1991b; Mikx and Keuhlers, 1992). Several strains of *T. denticola* have been shown to elicit hemoxidative and hemolytic potential, both cell-associated and secreted (Chu *et al*,1994b; Grenier 1991b). The hemoxidative activity reported for *T. denticola* ATCC 35404, ATCC 33520, GM-1 and MS25 (Chu *et al*,1994b) at first appears contradictory to the iron reductase reported in this communication. However Chu *et al* (1994b) point out that hemoxidation can result in oxidative damage and we have shown that substantial amounts of hemin can be bound by OAS (Scott *et al*, 1993). The iron reductase may, therefore, function both in the acquisition of iron and in tandem with the hemoxidative activity to protect the spirochete cell.

From the available data we can formulate a working model to explain the ability of *T. denticola* to scavenge and compete for the iron required to survive in the subgingiva. *T. denticola* does not produce siderophores (Chu *et al* ,1994a; Scott, *et al*,1993). and cannot employ transferrin as an iron source (Russell *et al* 1992). *T. denticola* produces phospholipase C and several cell-associated and extracellular factors capable of the hemagglutination, hemoxidation and hemolysis of erythrocytes.

Through the action of these factors hemin is liberated and therefore available to *T. denticola*, or other periodontal bacteria. *T. denticola* ATCC 35405 can then bind hemin through a 47-kDa outer membrane sheath receptor that is constitutively expressed. The expression of proteins of 16, 31, 43, 44, 54 and 73-kDa are altered in response to iron limitation, with the 73-kDa cytoplasmic membrane protein being down-regulated. The 43-kDa and 44-kDa hemin-binding proteins are up-regulated (Chu *et al* ,1994a). In addition to hemoxidative activity, *T. denticola* can reduce iron from hemin and inorganic sources. Inorganic iron is likely not available *in vivo*. The hematoporphyrin backbone is not transported into the cells but, instead, iron is stripped from the center of the hemin molecule which may be facilitated through the action of its ferric reductase. As an alternative source of iron, *T. denticola* can utilize lactoferrin, an iron-binding protein found in saliva, through the expression of 17-kDa and 43-kDa OMS receptors (Russell *et al* 1992).

The study of the iron acquisition mechanisms of OAS is important, not only in understanding how the spirochetes are able to grow in the environment of the oral cavity, but also has relevance to the progression of peridontitis, where substantial bleeding is a main characteristic of active disease.

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CHAPTER 3

An extracellular enzyme of some oral anaerobic spirochaetes with hyaluronidase and chondroitinase activities

Chapter three represents observations on an entirely different aspect of the physiology of *Treponema denticola*, *T. vincentii* and *T. socranskii* than iron acquisition. This chapter contains a report on the production of an enzyme capable of degrading both hyaluronic acid and chondroitin sulfate, glycosaminoglycans that are present in the periodontal tissues. The report includes an analysis of the properties of the hyaluronoglucosaminidase produced by *Treponema denticola* ATCC 35405, enzyme purification and localization of the enzyme by electron microscopy.

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ABSTRACT

Treponema denticola, *Treponema vincentii* and *Treponema socranskii* produce an enzyme that hydrolyses hyaluronic acid (HA) and chondroitin sulphate (CS). The secreted enzyme is inhibited specifically by gold sodium thiomalate and anti-bee venom antibodies. The use of saturated substrate (HA or CS) transblots allowed for the visualisation of active enzyme directly from culture supernatants and is a useful tool in clarification of complex polysaccharide-degrading enzyme specificities. The affinity-purified extracellular enzyme of *T. denticola* contains a single molecular species with an M_r of 59 kDa. Since it hydrolyses both HA and CS, it can more appropriately be termed a hyaluronoglucosaminidase (HGase). The HGase has been localised at the cell surface by electron microscopy and may play an active role in the degradation of connective tissue ground substance in the initiation and progression of periodontal disease.

Abbreviations : HA (hyaluronic acid), CS (chondroitin sulphate), Hase (hyaluronidase), HGase (hyaluronoglucosaminidase), Case (chondroitinase) GAG (glycosaminoglycan), anti-venom (anti-*Apis mellifera* venom antibody), Td, Tv, Ts (*Treponema denticola*, *T. vincentii*, *T. socranskii*, respectively), HA-gel (hyaluronate-Affi-gel 701 conjugate), C6S-gel (chondroitin-6-sulphate-Affi-gel 701 conjugate).

INTRODUCTION

Extracellular products of oral bacteria have been implicated in the initiation and progression of periodontal disease (Loesche, 1993 ; Stevens and Hammond, 1988). Bacteria isolated from saliva (Mahler and Lasanti,1952), gingival sulci (Hershan, 1974; Rosan and Williams, 1964; Schultz-Haudt and Scherp, 1955) and periodontal pockets (Tam *et al*, 1988) produce hyaluronidase (Hase). The severity of gingivitis in young male adults correlated with increased Hase activity in their saliva (Rovelstad *et al*, 1958). Injection of Hase into interdental papillae of monkeys produced gingivitis (Aisenberg *et al*, 1951) and administration of Hase into gingival sulci of healthy human adults resulted in a loss of cementing ground substance, tract formation, and invasion of the corium by bacteria (Schultz-Haudt *et al*, 1953). It was consequently postulated that disruption of the crevicular epithelium might be the first step in the pathogenesis of periodontal disease (Schultz-Haudt and Scherp, 1955). Most putative periodontopathogens do not produce Hase (Grenier and Michaud, 1993) but the production of Hase and the role that this enzyme may play in periodontal disease has been largely ignored in recent years.

Glycosaminoglycans (GAGs), polymers of a hexosamine and a uronic acid disaccharide (Iozzo, 1985), are constituents of the extracellular matrix of connective tissues (Last *et al*, 1988). HA (D-glucuronic acid and N-acetylglucosamine repeating units) is commonly detected in the gingival crevicular fluid (GCF) and is the only major GAG found in GCF collected from patients with chronic gingivitis (Last *et al*, 1985). Chondroitin-4-sulphate (C4S) is found in the GCF during advanced periodontal disease (Last *et al*, 1985;1988; Smith *et al*, 1995). Variation in the GAG content of the GCF has, therefore,

been proposed as a marker of degradative changes in the periodontium due to disease or to orthodontic treatment (Last *et al*, 1985;1988; Smith *et al*,1995; Waddington and Embery,1991). In periodontal tissues the major GAGs are HA, CS and dermatan sulphate, with C4S present in significantly higher amounts than chondroitin-6-sulphate (C6S) in alveolar bone, cementum, periodontal ligaments and gingiva (Last *et al*, 1985;1988;Okazaki *et al*, 1993; Smith *et al*,1995; Waddington and Embery,1991). HA, the non-sulphated GAG (Iozzo, 1985), is found in high amounts in the gingiva (Last *et al*, 1988). CS is a polymer of D-glucuronic acid and N-acetylgalactosamine repeating units. GAGs are thought to be involved in maintaining the structural integrity of connective tissue, in water retention, and in cell migration (Iozzo, 1985). Sulphated GAGs are protein-linked in connective tissues, forming proteoglycans (Iozzo,1985; Issacs, 1994) which tend to be aspartate, glycine and glutamate rich (Waddington and Embery, 1991). Proteoglycan can be associated with HA via link-proteins (Iozzo, 1985). Streptococcal Hase, bovine Hase and flavobacter Case (Dell'Orbo *et al*,1995; Quacci *et al*,1992) have been shown to cause structural alterations to collagen fibrils. Hase, Case and collagenase producing bacteria have also been associated with infections of root canals (Hashioka *et al*, 1994).

The nomenclature in use to describe GAG-degrading enzymes is in need of clarification. There are many names in press used to describe GAG-degrading enzymes, such as hyaluronate lyase, hyaluronidase and chondroitin sulfatase. Care should be taken before assigning a descriptive name to the enzyme under investigation. Chondroitin sulfatase should only be used where sulphate moieties are, in fact, removed from the core GAG. Hyaluronidase is a general term which should be refined to better describe enzymatic

activity, for example, a Hase whose specificity is restricted to the cleavage of β-glucuronate-[1→3]-N-acetylglucosamine glycosidic bonds in HA should be referred to as a hyaluronoglucuronidase, whereas an enzyme that is able to cleave the β-N-acetyl-hexosamine-[1→4] glycosidic bonds in both HA and in chondroitin sulphates is a hyaluronoglucosaminidase. A review of the nomenclature of GAGs and their relationship to GAG-degrading enzymes has recently been published (Scott, 1993).

There has also been some confusion as to the specificity of complex polysaccharide-degrading enzymes in the literature. This has been partly due to the impurity of bovine submaxillary mucin, the commonly used substrate to monitor neuraminidase activity (Pritchard and Lin, 1993). Characterisation of the degradation products of a group B streptococcal enzyme thought to be a neuraminidase due to its ability to hydrolyse this substrate (Hayano and Tanako,1969 ; Milligan *et al* ,1980; Brown and Straus, 1987) revealed that a hyalubiuronic acid derivative was liberated from HA contaminating bovine submaxillary mucin preparations and that the enzyme is, in fact, a hyaluronidase (Pritchard and Lin, 1993). Affinity columns containing N-(p-aminophenyl)oxamic acid-agarose have been employed to purify bacterial neuraminidase but are now known also to bind Hase (Pritchard and Lin, 1993). Interference by proteases in the bovine serum albumin-precipitation method for the assay of Hase may give rise to false positive results (Grenier and Michaud, 1993). In addition, Hase may not migrate through HA-containing gels (Hotez *et al*, 1993) due to binding of the enzyme to its substrate. This may result in a significant overestimation of the molecular weight.

We conjectured that oral anaerobic spirochaetes may produce Hase as they are found

in high numbers in the most apical aspect of periodontal pockets (Omar *et al*, 1990) and are seen intercellularly in gingival tissue sections of patients with periodontitis (Loesche, 1988).

We have identified the secreted HA- and CS-degrading enzyme of *T. denticola* ATCC 35405 as a hyaluronoglucosaminidase (EC 3.2.1.35). This enzyme may function as a virulence factor by breaking down the extracellular matrix of the gingiva, thus contributing to the progression of periodontal disease.

(Part of the data were presented at the 94th General Meeting of the American Society for Microbiology, Las Vegas, Nev. ,23 to 27 May, 1994).

MATERIALS AND METHODS

Reagents

Purified bovine hyaluronidase, hyaluronic acid (HA), chondroitin-4-sulphate (C4S), chondroitin-6-sulphate (C6S), Stains-all, Alcian blue, rabbit anti-*Apis mellifera* venom antibodies, gold-labelled protein G, *Clostridium perfringens* neuraminidase, *Penicillium* dextranase and routine chemicals were purchased from Sigma Chemical Company. Gold sodium thiomalate was obtained from Aldrich Chemical Company. Nitrocellulose membranes, molecular weight markers, protein assay-kit, Affi-Gel 701 and gold-labelled rabbit anti-human IgG were supplied by Bio-Rad Laboratories. It should be noted that the commercial substrates, HA, CS-A and CS-C are impure. Hyaluronic acid contains 2-3% chondroitin sulphate, and traces of heparin (Sigma), and as much as 20% CS (United States Biochemical). In addition, C4S contains C6S and C6S contains C4S.

Bacteria and culture conditions

Oral spirochaetes *Treponema denticola* ATCC 35405 (Td), *Treponema vincentii* ATCC 35580 (Tv) and *Treponema socranskii* ATCC 35536 (Ts) were grown anaerobically in new oral spirochaete (NOS) broth as described previously (Cheng and Chan, 1983). *Streptococcus agalactiae* was obtained from McGill University, Department of Microbiology and Immunology Culture Collection for Teaching and was grown in Robertson's Cooked Meat medium, at 37°C, under aerobic conditions. The spirochaetes were harvested at their late log growth phases (Td, 7 days; Tv and Ts, 12 days) and *S. agalactiae* after 24 h, by centrifugation (4000 x g, 15 min, 4° C). Preliminary experiments

had established that maximal Hase activity was present in the growth medium at these times. The cell-free culture supernatants were collected and their protein content estimated with the Bio-Rad protein assay kit. Samples of the supernatants were used immediately or frozen and used within 48 h to assay for Hase and chondroitinase (Case) activity.

Assays for Hase and Case Activities

The enzymatic activity of each culture supernatant was determined with the substrates HA, C4S and C6S essentially by the method of Hotez *et al* (1993). Triplicate assays were performed in 1.5 ml disposable plastic cuvettes on each batch of supernatants. Each cuvette received 100 µl of HA in 0.1M sodium acetate buffer, pH 6.0, to give a final concentration of 40 µg ml^{-1}, and 10 µl of the Td supernatant. The cuvettes were sealed, shaken and incubated for 3 h at 37° C. Thereafter 890 µl of a stock solution of Stains-all (50 µg ml^{-1} in 50% formamide-0.06% acetic acid solution) were added, the contents of the cuvettes mixed and the absorbance measured immediately at 640 nm. The assay is based on the shift to a longer wavelength when Stains-all binds to hyaluronic acid or chondroitin sulphate. The Tv and Ts culture supernatants were assayed for Hase activity in an identical manner as described. The Case activity of the Td supernatant was determined as described above with C4S and C6S as substrates and the Case activity of Ts, and Tv supernatants was assayed with C6S. The amount of substrate cleaved was determined by subtraction (i.e., absorbance of the substrate at $t_{0\ min}$ – absorbance of the substrate at $t_{180\ min}$ = cleaved substrate). The concentration of the substrates was determined from standard curves prepared with HA, C4S or C6S in acetate buffer, and Stains-all. The enzyme activity is expressed as µg of

hyaluronic acid or chondroitin sulphate cleaved per hour per ml of the supernatant. Control assays were carried out with bovine Hase, uninoculated NOS supernatant and acetate buffer with each of the substrates. Preliminary assays were carried out at a pH range of 4.0 to 9.0 and the enzyme was found to hydrolyse HA, C6S and C4S optimally at pH 6.0. The experiment was repeated with Td and *S. agalactiae* culture supernatants (0 to 40 µl), with a 3-h digestion period. Enzyme kinetics were not investigated due to the impurity of the commercial substrates HA, CS-A and CS-C.

Inhibition assays

The specificity of the Hase and Case activities was determined initially with gold sodium thiomalate, a known inhibitor of hyaluronidases (Hotez *et al.* 1993). Forty µl of Td, Tv and Ts supernatants were each pipetted into separate microcentrifuge tubes. 40 µl of gold sodium thiomalate (1 mg ml^{-1} in acetate buffer) were added to each tube and they were shaken continuously for 20 min at 37°C. Each of the mixtures was then assayed for Hase and Case activities with HA, C4S and C6S by the Stains-all method. Td, Tv and Ts supernatants incubated separately with acetate buffer only served as controls for enzyme degradation over the incubation period. The Td, Tv and Ts supernatants, with and without gold sodium thiomalate, were assayed at the same time.

The effect of several ions (Cd^{--}, Hg^{--}, Cu^{--}, and Mn^{--}) and of EDTA, SDS, p-chloromercuribenzoate (pCMB), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), soybean trypsin inhibitor, phenylmethylsulfonyl flouride, dithiothreitol, glutathione, and L-cysteine hydrochloride on the Hase activity of Td supernatant was determined using the

Stains-all assay, with appropriate controls, over a 3 h period as described above, and 20 µl Td supernatant was used. The concentration of each inhibitor / activator is given in Table 2. The Hase activity of Td culture supernatant alone served as the 100% control.

As a further means of examining the specificity of the Hase activity, 0 to 40 µl of Td culture supernatant was used and the degradation of HA was, again, measured by the Stains-all assay after a 3 h incubation. Under identical conditions, the effect of 20 µl gold sodium thiomalate (1 mg ml^{-1} in acetate buffer) and 20 µl of a 1/100 and a 1/500 of commercial rabbit anti-*Apis mellifera* (honey bee) venom antibodies in acetate buffer at each concentration of Td supernatant was monitored. The effect of gold sodium thiomalate and of rabbit anti-*Apis mellifera* venom antibody on increasing concentrations of *S. agalactiae* supernatant (0 to 20 µg ml^{-1}) was also examined, under identical conditions. The effect of dextranase (3 Units) and neuraminadase (1 Unit) on the HA substrate was monitored by the Stains-all assay after a 3h incubation at 37°C.

Conjugation of HA and C6S to Affi Gel 701

Five ml of Affi Gel 701 were sedimented by centrifugation (4000 x g, 10 min). The resin was washed twice with HCl-acidified water, pH 4.7, by centrifugation, resuspended in 2.0 ml of acidified water and 2.0 ml of HA (1 mg ml^{-1} acidified water) were added to the resin. A total of 4 mg of carbodiimide were added, in small aliquots, to the mixture and the tube vortexed after the addition of each aliquot. The mixture was shaken continuously for 1 h at 37°C. An additional 4 mg of carbodiimide were added to the tube and the pH of the reaction mixture maintained at 4.7 for 4 h. Conjugation was allowed to proceed with

continuous shaking of the tube for 18 h at 4°C. The HA-conjugated Affi-Gel 701 (HA-Gel) was packed by centrifugation, the supernatant collected and assayed for unbound HA. The HA bound to the Affi-Gel 701 was determined by subtraction. The packed HA-Gel was washed twice with acetate buffer (5 min per wash) by inversion of the tube and centrifugation, and resuspended to a total volume of 5.0 ml in acetate buffer. C6S was conjugated to Affi-Gel 701 (C6S-Gel) and assayed for unbound C6S as described for HA. Td supernatant (500 µl) was pipetted into each of two microcentrifuge tubes. HA-Gel (500 µl) was added to one of the two tubes, C6S-Gel (500 µl) was added to the other tube and they were shaken continuously for 2 h at 4°C. The tubes were centrifuged and the supernatants (unbound proteins) collected. The gels were washed with acetate buffer to remove proteins trapped in the gels. Each of the two washed gels were resuspended in 1.0 ml of a 0.5 M NaCl-0.25M glycine, pH 3.2 buffer, mixed continuously for 15 min by inversion and centrifuged (4000 x g, 10 min). The eluted protein(s) was collected and dialysed against acetate buffer. The unbound and the eluted protein(s) were assayed for both Hase and Case activities by the Stains-all method (described above). Td supernatant was incubated with acetate buffer, instead of HA-or C6S-Gels, and served as a control for the dilution effect and stability of the Hase and Case in the Td supernatant during the adsorption-incubation period. Bovine Hase served as a positive control to ascertain the efficacy of the HA-and C6S-Gels in the adsorption of Hase and Case activities.

Electrophoresis and electroblotting

Td, Tv and Ts culture supernatants, supernatants treated with gold sodium thiomalate (as

described above) as well as the unbound and eluted protein(s) after adsorption of the supernatants with HA- or C6S-Gels were subjected to SDS-PAGE under nonreducing conditions and transblotted onto nitrocellulose membranes as described previously (Chan *et al.* 1991). The membranes were washed twice (5 min per wash) in acetate buffer and then immersed in a solution of either HA or C6S (20 μg ml^{-1} in acetate buffer) for 15 min at room temperature. Unbound HA and C6S were drained from the membranes which were dried for 30 min at 37° C. Thereafter, the membranes were soaked in acetate buffer for 2 to 3 min and drained of excess buffer. The saturated membranes were incubated for 24 h at 37°C in a closed container and then immersed in a solution of Alcian blue (20 μg ml^{-1} in acetate buffer, pH 4.7) for 1 h at room temperature. Thereafter the membranes were washed extensively with acetate buffer, pH 4.7 and examined for unstained degradative-bands of HA and C6S. Alcian blue binds to the membrane-bound HA and C6S but it does not bind to the degradative products of HA and C6S. The molecular weight of the enzyme(s) responsible for HA- and CS-degradation were thus established.

Preliminary experiments, with the Stains-all assay, showed that neither Hase nor Case were inactivated by the concentrations of SDS and methanol in the buffers used in the SDS-PAGE and transblot techniques. Miura, *et al* (1995) have previously reported that SDS did not inhibit the activity of several GAG-degrading enzymes. Td culture supernatant, and the HA-gel and C6S-gel purified Td Hase, were subjected to electrophoresis through a 10% SDS-Page system at 200V, as described previously (Chan et al. 1991) and double-stained with Coomassie blue and silver to assess purity.

To determine if the spirochaetes Hase could be recognised by anti-bee venom

antibodies, which could then be used in electron microscope studies, a western immunoblot was also performed after electrophoresis of purified Td Hase, Bovine Hase and *Apis mellifera* venom through a 10% SDS-PAGE system. The immunoblot was developed using rabbit anti-*Apis mellifera* venom antibodies, alkaline phosphatase-conjugated goat anti-rabbit IgG, *p*-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Electron microscopy

T. denticola was sedimented from NOS culture medium by centrifugation (4000 x g, 10 min), the cells resuspended in acetate buffer, washed by inversion of the tube and sedimented by centrifugation. The cells were resuspended in acetate buffer and adjusted to 10^7 cells ml^{-1}. Two aliquots of the cells (40 µl per aliquot) were placed onto two separate areas of a plastic Petri dish. To one aliquot was added 40 µl of HA (1 mg ml^{-1} in acetate buffer) and to the second aliquot was added 40 µl of acetate buffer. Each aliquot was stirred and incubated for 15 min at 37°C. Then, 40 µl of gold sodium thiomalate (1 mg ml^{-1} in acetate buffer) were added to each of the aliquots which were stirred and incubated for 30 min at 37°C. Nickel formvar grids (mesh size 300) were submerged separately into each sample preparation for 1 min, the grids were washed twice with acetate buffer, blotted dry on bibulous paper, and the cells fixed with 2% glutaraldehyde. Gold-labelled rabbit anti-human IgG was incubated with Td, as described above, and served as a control.

A suspension of 10^7 cells ml^{-1} of *T. denticola* was also prepared as described above. An equal volume of a 1/100 dilution of anti-*Apis mellifera* venom antibody in acetate buffer

was added and the cells incubated for 1 h, at 37°C. The cells were then washed twice with acetate buffer, then incubated with a 1/10 dilution in acetate buffer of gold-labelled protein G for 1 h. at 37°C. The gold-labelled protein G was first centrifuged at 14,000 x g, 5 min, to remove any gold aggregates. The protein G preparation contained 2.7×10^{13} gold particles ml^{-1}, as purchased, with a mean diameter of 9.6 nm. The cells were washed twice with acetate buffer. Nickel formvar grids were submerged for 1 min., the grids rinsed with acetate buffer, submerged into 0.2% phosphotungstate for 1 min, re-rinsed and blotted, then fixed with 2% glutaraldehyde. Cells were also prepared for examination as above after first being incubated with 1 mg ml^{-1} HA in acetate buffer and as above using gold-labelled protein G without the prior incubation with anti-venom antibodies, as negative controls. The prepared grids were examined with a Philips 410 electron microscope.

RESULTS

Hase and Case activities in culture supernatants

The enzyme(s) secreted by Td, Tv and Ts, and active in the culture supernatants, each hydrolysed HA and C6S (Table 1). Td produces about three times and five times more of the HA-hydrolysing enzyme than Ts and Tv, respectively. The Td enzyme, however, hydrolysed C6S and C4S less efficiently than HA. The enzyme(s) of Tv and Ts hydrolysed HA and C6S at a much lower rate than the enzyme of Td. The hydrolysis of C4S by the enzyme of Tv and Ts was not tested. Freezing of the supernatants did not appear to affect enzyme activity. Gold sodium thiomalate, an inhibitor of Hase (Hotez, *et al*, 1993), inhibited completely the hydrolysis of HA and C6S by the enzyme produced by Td, Tv and Ts (Table 1). The apparent higher rate of hydrolysis of HA and C6S by the culture supernatants of Td, in comparison with the supernatants of Ts and Tv, may be due to the amount of enzyme produced and secreted by each species. Although the supernatants were harvested at the time of maximal enzyme production, it is recognised that the apparent higher rate of hydrolysis may also reflect differences in growth yields of the three oral spirochaete species.

Affinity chromatography

The Td-eluate from the HA-Gel (affinity purified) hydrolysed 650 μg HA/h/ml and 208.3 μg C6S/h/ml. Likewise, the Td-eluate from the C6S-Gel also hydrolysed 633.3 μg HA/h/ml and 233.3 μg C6S/h/ml.

Table 1. The hydrolysis of hyaluronic acid (HA), chondroitin-4-sulphate (C4S) and chondroitin-6-sulphate (C6S) by the culture supernatants of *Treponema denticola*, *T. socranskii* and *T. vincentii*, and inhibition of hyaluronidase and chondroitinase activities with gold sodium thiomalate (GST).

culture supernatant of	μg of substrate hydrolysed / hr / ml of supernatant		
	HA	C6S	C4S
Td	312.0 ± 2.5 (5)	108.9 ± 8.3 (5)	53.5 ± 3.5 (3)
Td + GST	0	0	0
Ts	111.0 ± 1.8 (2)	44.9 ± 6.9 (3)	----
Ts + GST	0	0	----
Tv	59.5 ± 4.0 (2)	27.0 ± 4.9 (2)	----
Tv + GST	0	0	----

( ), number of culture supernatants tested and standard deviation (±); 0, complete inhibition of Hase and Case activities; ----, not done. Details of the assay are given in the text.

Table 2. The effect of various ions and inhibitor molecules on the hydrolysis of hyaluronic acid (HA) by the culture supernatant of *T. denticola* and of neuraminidase and dextranase treatment of the HA substrate.

culture supernatant with [†]	% hydrolysis of HA [‡], [§]
no additive	100
Hg^{--} (2 mM HgCl)	93
Cd^{--} (2 mM $CdSO_4$)	95
Cu^{--} (2 mM $CuSO_4$)	82
Mn^{--} (2mM $MnSO_4$)	98
SDS (0.1%)	93
p-chloromercuribenzoate (2 mM)	96
EDTA (1 mM)	103
TPCK (2 mM)	99
Soybean Trypsin Inhibitor (10μg ml^{-1})	101
Dithreitol (5 mM)	98
L-cysteine hydrochloride (5mM)	99
glutathione (5mM)	97
PMSF (0.1mM)	96
pH 4.0	59
pH 8.5	12
Neuraminidase [¶]	3
Dextranase [*f*]	0

[†] Three-hour digestions of HA by 20 μl Td supernatant with sodium acetate buffer, pH 6.0, as described in the text.

[‡] The amount of HA hydrolysed in 3 h by 20 μl Td supernatant only serves as the 100% control.

[¶] Three-hour digestion of HA by 1 Unit of *C. perfringens* neuraminidase in acetate buffer, pH 6.0 as described in the text.

[*f*] Three-hour digestion of HA by 3 Units *Penicillium* dextranase in acetate buffer, pH 6.0 as described in the text.

[§] All results are the average of triplicate assays.

Effect of inhibitors

The effects of the polysaccharide-degrading enzyme inhibitors, ions, sulphydryl-reagents and protease inhibitors on the Hase activity of Td supernatant is given in Table 2. There was no effect on Td supernatant Hase activity observed by sulphydryl-reagents, protease inhibitors, SDS, pCMB, EDTA or by Mn^{++}, Cd^{++} or Hg^{++}. The protease inhibitors were used to confirm that HA hydrolysis was not due to proteolytic degradation of proteoglycans in commercial preparations of hyaluronate. The Hase activity was inhibited by approximately 20% by 2 mM Cu^{++}. Neuraminidase and dextranase exhibited negligible ability to degrade HA, as determined by the Stains-all assay. In preliminary studies, the optimum pH of the Td Hase was determined to be 6.0, unlike the bovine Hase which hydrolyses HA over a broad range (pH 4.5 to 6.0).

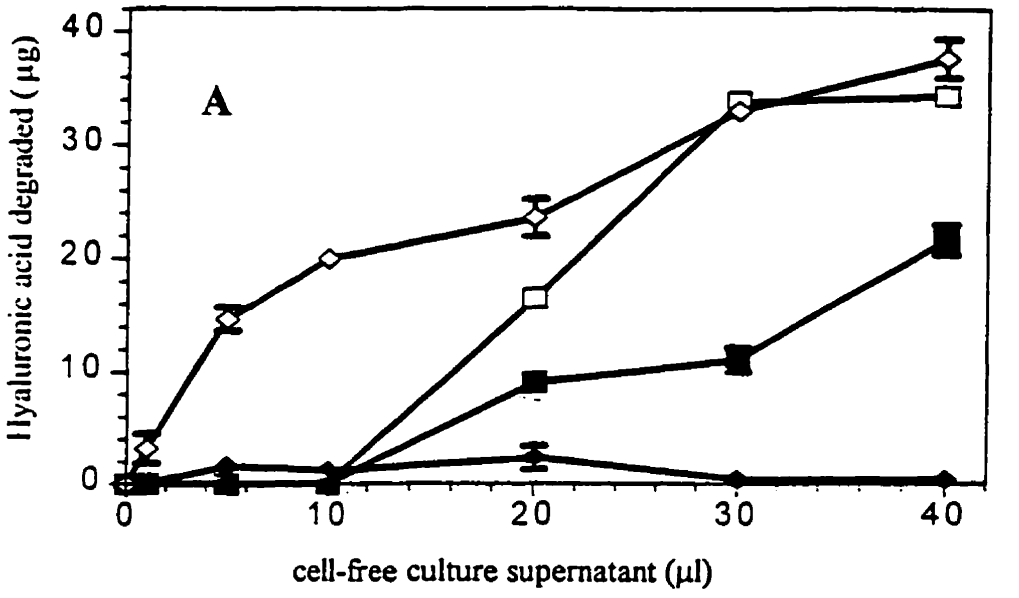
The inhibiting effect of gold sodium thiomalate and of rabbit anti-*Apis mellifera* venom antibodies on the ability of Td and *S. agalactiae* culture supernatants to degrade HA over a 3 h period is presented in Figure 1.

SDS-PAGE, transblots, and hydrolysis of HA and C6S

The secreted enzyme(s) of Td, Tv and Ts separated by SDS-PAGE and transblotted onto membranes hydrolysed HA or C6S that were adsorbed subsequently onto the membranes. The hydrolysis of HA and C6S results in degradative products that are not stained by Alcian blue. Each of the enzyme(s) secreted by Td, Tv and Ts hydrolysed both HA and C6S (Figure 2A and C) and produced discrete single unstained (clear) bands against a blue background. From the location of the bands, the molecular weight of the enzyme was

Figure 1. Inhibition of the hyaluronidase activity of the culture supernatants of *T. denticola* and *S. agalactiae*.

The effect of gold sodium thiomalate and anti-*Apis mellifera* venom antibodies on the amount of hyaluronic acid degraded by the culture supernatants of *T. denticola* (A) and *S. agalactiae* (B). Panel (A); culture supernatant alone(◊); supernatant incubated with a 1/100 dilution of anti-venom antibody(♦), supernatant with a 1/500 dilution of anti-venom antibody (□); supernatant incubated with 1 mg ml^{-1} gold sodium thiomalate (■). Panel (B) culture supernatant alone(◊); supernatant incubated with a 1/100 dilution of anti-venom antibody (♦). The error bars represent the standard error of the mean. All results are the average of triplicate assays.


A
Hyaluronic acid degraded (μg)
0
10
20
30
40
cell-free culture supernatant (μl)

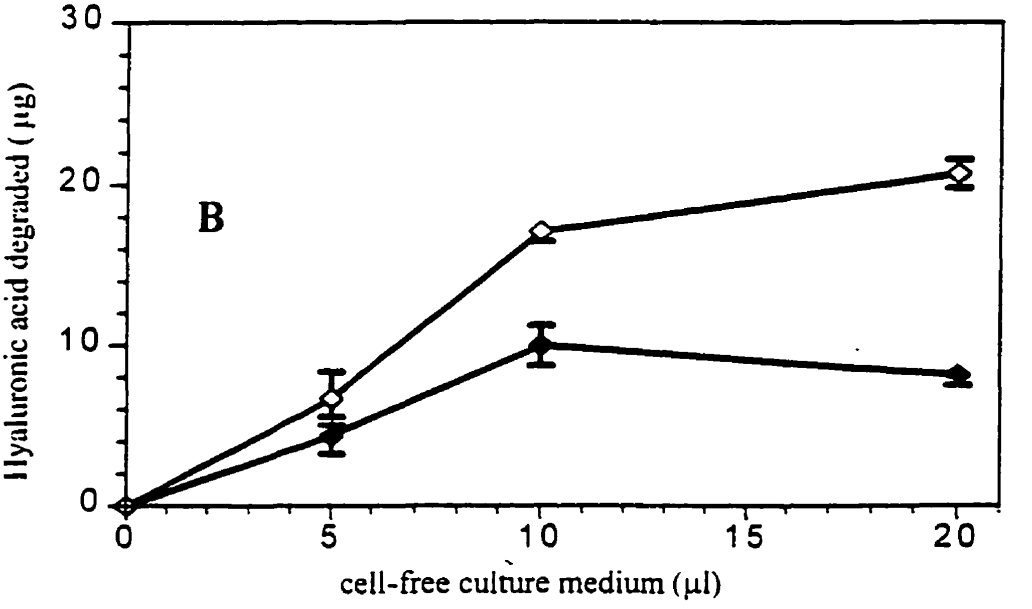
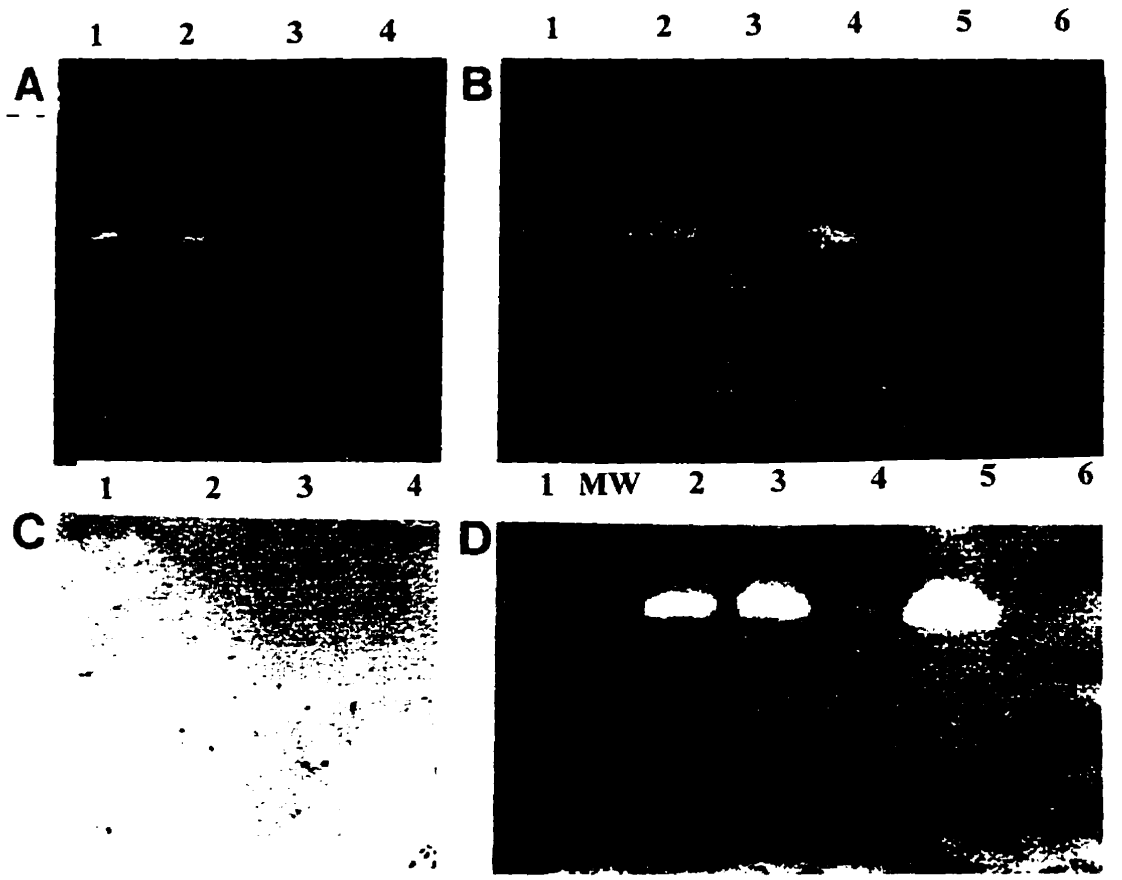

B
Hyaluronic acid degraded (μg)
0
10
20
30
5
15
cell-free culture medium (μl)

Figure 2. Analysis of the enzymatic activity of the Td, Ts, and Tv culture supernatants by SDS-PAGE, transblot and degradation of HA or C6S absorbed onto the transblot membranes. A. Culture supernatant of Td (lane 1), Ts (lane 2), Tv (lane 3); bovine Hase (lane 4); HA is absorbed onto the membrane. C. The supernatants in lanes 1,2,3 and 4 same as in A but C6S is absorbed onto the membrane. B. Culture supernatant of Td (lane 1), after absorption with HA-Gel (lane 3) and C6S-Gel (lane 5), after incubation with gold sodium thiomalate (lane 6), and incubation with buffer without HA-Gel (lane 2) and without C6S-Gel (lane 4); HA is absorbed onto the membrane. D. Culture supernatant of Td (lane 2), after absorption with HA-Gel (lane 4) and C6S-Gel (lane 6), after incubation with gold sodium thiomalate (lane 1); incubation with buffer without HA-Gel (lane 3) and without C6S-Gel (lane 5); lane MW, molecular weight markers (110, 84, 47, 33, 24 and 16 kDa from top to bottom); C6S is adsorbed onto the membrane. See material and methods for details.

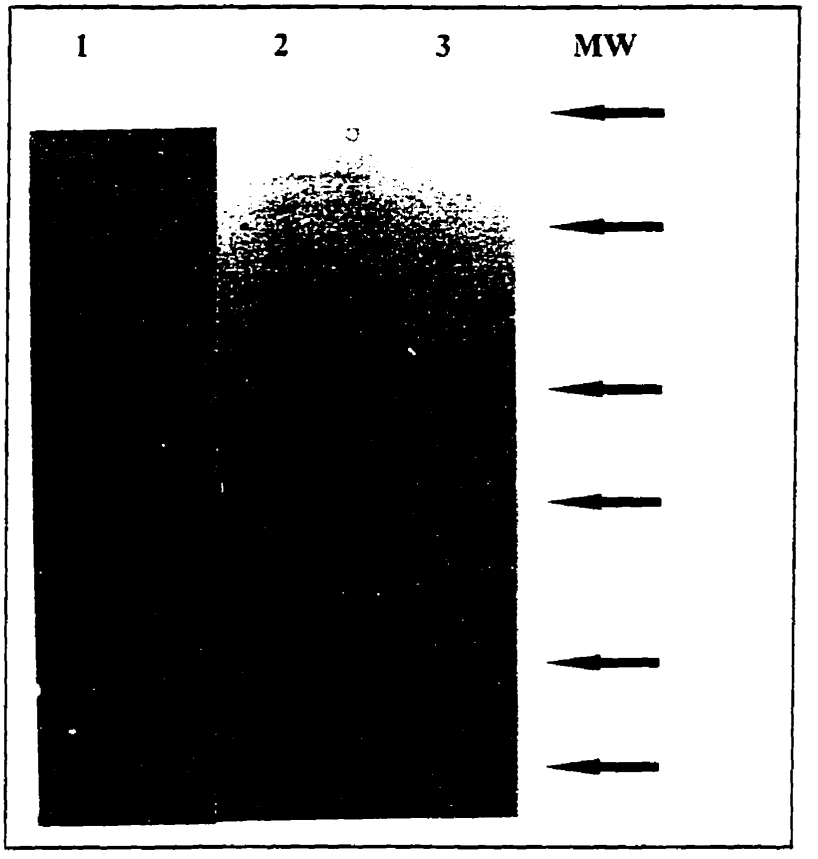

1 2 3 4
A
1 2 3 4 5 6
B
1 2 3 4
C
1 MW 2 3 4 5 6
D

estimated as 59 kDa. The Td culture supernatant treated with gold sodium thiomalate did not hydrolyse either HA or C6S, i.e. unstained bands were not evident (Figure 2B and D). The inhibition of the Td enzyme indicates that gold sodium thiomalate binds firmly to the extracellular enzyme as neither SDS-PAGE nor transblotting reversed the effect of gold sodium thiomalate. The unbound protein which passed through the HA-Gel or the C6S-Gel affinity columns did not hydrolyse either HA or C6S (Figure 2B and D).

The eluates from the HA-Gel and the C6S-Gel each hydrolysed both HA and C6S. Each biologically active eluate contained a single molecular species with an M_r of 59 kDa as detected in doubly stained SDS-PAGE gels with Coomassie blue and silver (Figure 3). The affinity purified enzyme and the enzyme in the supernatants have comparable molecular weights (compare Figures 2 and 3). Taken together these data suggest that Hase and Case activities may be attributed to a single protein in the Td culture supernatant with dual activity towards both GAGs. HA and CS share a common hexuronyl-hexosaminyl glycosidic linkage (Iozza, 1985), and this linkage would appear the best candidate target for the enzymatic action. Further studies involving infra red spectroscopy and high performance liquid chromatography are required, however, in order to accurately determine the mechanism of degradation. Affinity purification of Ts, and Tv supernatants was not carried out.

On western blotting, the affinity purified enzyme from the HA- conjugated Affi-gel 701 beads produced a single line of 59 kDa, seen in Lane 1 of Figure 6. The honeybee venom , in lane 3, gave two major bands, of 48 kDa and 11 kDa.

Figure 3. SDS-PAGE of Td culture supernatant and the protein(s) of Td supernatant bound to HA-Gel or C6S-Gel and eluted. The Td culture supernatant (lane 2), the eluate of the protein from the C6S-Gel (lane 1), and the HA-Gel (lane 3); MW, molecular weight markers (110, 84, 47, 33, 24 and 16 kDa from top to bottom).


1
2
3
MW

Electron microscopy

Anti-*Apis mellifera* antibodies bound on, or near to, the surface of Td, as seen in Figure 4. The 10 nm particle-sized gold-labelled protein G did not bind to the Td cells without the prior addition of the anti-venom antibodies. Prior incubation of the Td cells with 1 mg ml^{-1} of HA completely blocked the binding anti-*Apis mellifera* venom antibodies, as determined by electron microscopy. Gold sodium thiomalate binds to the extracellularly located enzyme of Td, Tv and Ts and HA also blocks the binding of gold sodium thiomalate. Gold-labelled rabbit anti-human IgG does not bind to the cells. The cluster-density of the gold sodium thiomalate molecules is more abundant nearer to than farther away from the outer membrane of the spirochaetes (Figure 5). The enzyme appears to the trapped around the periphery of cell in the exopolysaccharide layer or slime layer (Johnson,1977), as well as clumps of polysaccharide that are shed from the surface of the cells, for some time before ending up in the supernatant.

Figure 4. Electron micrograph of anti-*Apis mellifera* venom antibody and gold-labelled protein G binding along the periphery of Td. A. Td incubated with anti-venom antibody and gold-labelled protein G. B. Td pre-incubated with hyaluronic acid prior to the addition of anti-venom antibody and gold-labelled protein G.

Gold-labelled protein G alone did not bind to Td (data not shown). Bar = 0.1 μm.

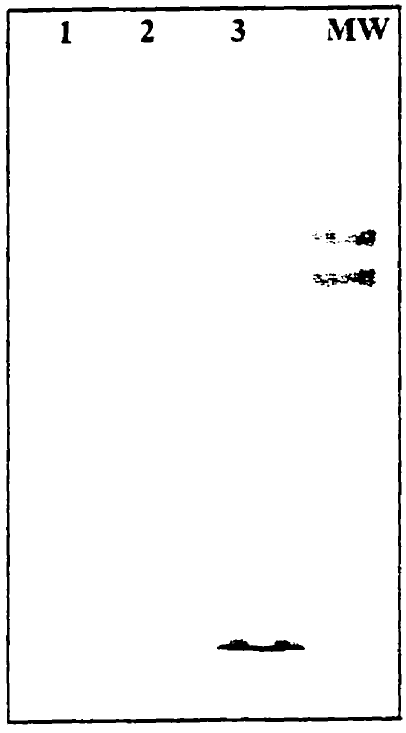

A


B

Figure 5. Electron micrographs of gold sodium thiomalate binding along the periphery of Td. A. Td incubated with gold sodium thiomalate (x32,000). B. Td incubated with a mixture of HA and gold sodium thiomalate (x20,000). Arrow points to one of many sites to which gold sodium thiomalate binds. Bar = 0.1 μm.


A
B

Figure 6. Western immunoblot detection of antigens cross-reacting with anti-*Apis mellifera* venom antibodies. Lane 1 shows the HA-column affinity purified Hase from Td ; Lane 2 shows Bovine hyaluronidase; *Apis mellifera* venom is in Lane 3. The molecular weight markers, MW, are 112 ,84, 53.5,27.9 and 21 kDa, from top to bottom.


1
2
3
MW

DISCUSSION

Low levels of Hase and Case activities have been reported previously to be produced by nine small-sized unclassified oral spirochaetes (Fiehn, 1987). The insensitivity of the turbidimetric plate assay (Fiehn, 1987) could account for the reported low Hase and Case activities of these spirochaetes or this may be due to a false positive reaction that results from the degradation of BSA by protease secreted by the spirochaetes (Grenier and Michaud, 1993). Others have reported that *T. vincentii* and *T. socranskii* did not produce Hase (Fitzgerald and Gannon, 1983). These investigators incubated the viable spirochaetes cells in 500 μg ml^{-1} of HA for a maximum of 40 h and measured the relative viscosity of the mixture for Hase activity (Fitzgerald and Gannon, 1983). With such a short incubation period, these two oral spirochaetes might not have produced sufficient extracellular Hase to effect changes in the viscometric flow of 500 μg of HA. For example, Hase and Case activities were detected after two weeks of incubation by the turbidimetric plate assay (Fiehn, 1987) and in our investigation Hase activity was detected in the supernatants after 7 days (Td) and 12 days (Ts and Tv) of growth. Moreover, both the plate and viscometric assays are not as sensitive as the Stains-all spectrophotometric assay which has been reported to detect Hase activity in 60 ng of protein from *Ancylostoma braziliense* (Hotez *et al*, 1993). In our hands, the lower limit of Hase activity was detected in 150 ng of protein of the culture supernatant. The extracellular enzyme produced by Td, Tv and Ts readily hydrolysed HA and C6S. The affinity purified 59 kDa enzyme of Td hydrolysed both HA and CS and a specific inhibitor of hyaluronidase, gold sodium thiomalate (Hotez *et al*, 1993), inhibited the extracellular enzyme hydrolysis of both HA and CS. There are only two

major proteins present in honeybee venom, a hyaluronidase and a phospholipase A (Gmachl and Kreil, 1995; King *et al*, 1993) and anti-*Apis mellifera* venom antibodies also inhibited the Hase activity of Td. A single band of 59 kDa was observed on electrophoresis and transblotting of the affinity purified Td Hase. That component(s) of the anti-venom antibodies exhibit specificity to the Hase of Td is also suggested by the ability of HA to block the binding of the antibodies to cells of Td, as observed by electron microscopy and the inhibition of Hase activity in the Stains-all assay.

The inhibition assays confirm that enzymatic activity is due to hyaluronidase. The Td Hase is not activated by reducing agents, or ions, since dithiothreitol, glutathione and EDTA did not inhibit the activity. The lack of a requirement for activating factors has also been reported for the hyaluronidase of *Clostridium perfringens*. Information on inhibitors and activators of polysaccharide-degrading enzymes was obtained from commercial suppliers and from Colowick and Kaplan (1972).

We selected a wide range of potential inhibitors and activators as there has been some confusion in the literature over the identity and specificities of polysaccharide-degrading enzymes. It has recently been reported that *Porphyromonas gingivalis* does not produce a hyaluronidase (Grenier and Michaud, 1993), contrary to previous reports (Seddon and Shah, 1989 ; Steffen and Hentges, 1981). The degradation of bovine serum albumin, used to form a precipitate under acidic conditions with HA, by bacterial proteases might have led to false positive results (Grenier and Michaud, 1993). It has also been shown that group B streptococci produce a hyaluronidase (Pritchard and Lin, 1993), an enzyme previously considered to be a neuraminidase (Hayano and Tanaka, 1969; Milligan *et al*, 1980; Brown

and Straus, 1987). The confusion surrounding the specificity of this enzyme associated with neonatal meningitis and sepsis caused by group B streptococci (Milligan *et al*, 1978) was probably due to the impurity of the substrate employed, that is, bovine submaxillary mucin which contains HA (Pritchard and Lin, 1993). *S. agalactiae*, one of the group B streptococci, is a known hyaluronidase producer (Ozegowski *et al*, 1994) and, therefore, selected as a positive control of bacterial origin, to complement the use of purified bovine hyaluronidase in our Stains-all assays.

The hyaluronidase of *Ancylostoma braziliense* larvae can migrate through substrate-containing polyacrylamide gels on electrophoresis (Hotez *et al*, 1993). In initial experiments we found that the Hase of the oral anaerobic spirochaetes under study would not migrate through HA-containing polyacrylamide gels by SDS-PAGE at 200V. All lytic activity was observed only at the top of the gel. This can be explained by retardation of the enzyme mobility due to interaction of the Hase with the substrate incorporated into the gel. For this reason, the technique was adapted to adsorb HA or CS onto the nitrocellulose membrane after the separation of the proteins of the supernatants by SDS-PAGE and transblotting. The migration of the enzyme was then observed by the localised degradation of HA and CS. This gives a more accurate estimation of the Mr of the enzyme. The use of this technique by other investigators should permit the visualisation of Hase whilst giving rise to more accurate estimations of molecular weight.

The term hyaluronidase has been used generically for hyaluronate lysate (EC 4·2·2·1), hyaluronoglucuronidase (EC 3·2·1·36) and hyaluronoglucosaminidase (EC 3·2·1·35). The latter hydrolyses both HA and CS while the two other enzymes

specifically hydrolyse HA. Therefore, Td, Tv and Ts produce a hyaluronidase that can more appropriately be termed a hyaluronoglucosaminidase (HGase).

The HGase produced by Td, Tv and Ts might function as a virulence factor. Spirochaetes have been isolated from the gingival sulci (Gornitsky *et al*, 1991) and periodontal pockets (Gornitsky *et al*, 1991; Omar *et al*, 1990). They locomote in highly viscous environments (Klitorinos *et al*, 1993) and are located histologically in the intercellular matrix of diseased gingival tissue (Loesche, 1988). The HGase could disrupt the integrity of the gingival epithelium and could continue to degrade the extracellular matrix as the spirochaetes locomote through the gingival matrix. The oral spirochaetes may create avenues through which other bacteria and their products penetrate into the deeper tissue.

Acknowledgements

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CHAPTER 4.

Visualization of an extracellular mucoid layer of *Treponema denticola* ATCC 35405 and surface sugar lectin-analysis of some oral spirochete species.

This chapter, which represents a manuscript that has been accepted for publication in Oral Microbiology and Immunology, reports that oral anaerobic spirochete cells are surrounded by an extracellular mucoid layer which may play a significant role *in vivo*. Two morphotypes of *T. denticola* ATCC 35405 are referred to, which are now described briefly.

Through the development of a technique that allows the reproducible and consistent enumeration of colony-forming units of spirochetes (Chan *et al*, 1995a ; Qiu *et al*, 1994), two colony morphologies of the type strain were observed (Scott, *et al*, 1995). Morphotype 1 (M1) and Morphotype 2 (M2) exhibited different enzyme profiles in the API 20A system, notably the production of catalase, urease and gelatinase by M2, but not M1, and the production of indole from tryptophan by M1, but not M2 (Scott *et al*, 1995). Antibodies developed against the parental strain (P), *T. denticola* ATCC 35405, were used to probe the outer membrane sheath (OMS) antigens of M1 and M2, in order to compare those antigenic determinants recognized. Again M1 and M2 could be differentiated by OMS antigens of 31, 24 and 15-kDa found in the OMS extracts of M1 but not M2 and antigens of 56 and 34-kDa, absent from M1 but present in the M2 OMS extracts (Scott *et al*, 1995). The measurements of the cell length, width and wavelength of the helical wave, taken from electron micrographs (Scott *et al*, 1995), also varied between M1 and M2 (7.68 ± 1.8 μm, 0.18 ± 0.04 μm and 1.03 μm for M1 and 10.12 ± 3.15 μm, 0.21 ± 0.05 μm and 0.90 μm for M2, respectively). However, as there is little available data on the variation of the size of spirochetes within the same strain, it is not clear how appropriate it is to differentiate the two morphotypes by this method.

In future studies genomic fingerprinting, as established by the restriction fragment end-labeling method developed by Van Steenbergen *et al* (1995), will be employed to compare the chromosomes of M1 and M2, in order to monitor potential genomic variation that may offer an explaination to the phenotypic variation so far observed. It should be noted that *T. denticola* ATCC 35405 is plasmid free (Caudry *et al*, 1995).

Both morphotypes are members of the same species, *T. denticola*, due to a homology of 87.5% determined by DNA-DNA reassociation studies (Scott, *et al*, 1995). The growth of newly isolated spirochetes in NOS-0.7% agarose in tissue culture flasks allows both consistent and reproducible enumeration of cultivable, viable cells and the visualiziation of different colony morphologies (Chan *et al*, 1995a ; Qiu *et al*, 1994), thereby facilitating the observation of mixed strains that may not otherwise be detectable, especially in liquid media.

The colony-forming units of M1, grown in NOS-0.7% agarose are large and diffuse, whereas M2 forms small, compact colonies. With this in mind, both morphotypes were examined for the presence of an extracellular polysaccharide layer. No differences were observed between M1 and M2 in any aspect relating to Chapters2 and 3 of this thesis (see Appendix 2).

Visualization of an extracellular mucoid layer of *Treponèma denticola* ATCC 35405 and surface sugar lectin - analysis of some *Treponema* species.

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ABSTRACT

Slime layers and capsules are common amongst medically relevant bacteria. We herein report that *Treponema denticola*, which has been associated with periodontitis, synthesizes or aquires an extracellular polysaccharide layer that we have observed through electron microscopy using the polysaccharide-specific dye, Alcian blue, and phosphotungstate. We have also visualized this extracellular layer by dark-field microscopy of Alcian blue-stained spirochete cells. A representative strain of each of the oral spirochete species *T. denticola*, *Treponema vincentii* and *Treponema socranskii* were differentiated by concanavalin A, phaseolus, lotus A and arachis lectins in a microtitre plate immunoassay for the detection of surface sugars.

INTRODUCTION

Polysaccharides external to the outer membrane of bacteria can be arranged to form a capsule or as a slime layer, a term indicating a looser, less compact organization than a capsule (Deighton and Borland, 1993; Schifferle, 1994). The exopolysaccharides of capsulated bacteria are thought to be anchored in the outer membrane covalently (Schifferle, 1994) and by hydrophobic interactions (Bayer and Bayer, 1994; Kuo, *et al*, 1985).

Fitzgerald and Johnson (Fitzgerald and Johnson, 1979) suggested the extracellular layer of *Treponema pallidum*, first visualized by Zeigler, *et al.* (1976), to be constituted of glycosaminoglycans. However, radiolabelled sulfated, and non-sulfated, glycosaminoglycan precursors are not incorporated into any degradable material of *T. pallidum* (Strugnall, *et al*, 1990). It was proposed that *T. pallidum*, which is found in tissues containing high levels of mucopolysaccaride, may obtain the components required for capsule formation from the host through the action of a *T. pallidum* glycosaminidase (Fitzgerald and Johnson, 1979). *T. denticola* possesses neuraminidase activity (Mikx, 1991), hyaluronidase and chondroitinase activity (Fiehn, 1986;1987) and several glycosidases including those with specificities for α-D-galactose, β-D-galactose, α-D-glucose, β-D-glucose, α-L-fucose, and β-D-fucose (Mikx, 1991).

Slime layer production, or acquisition, by *T. denticola* may have relevance to adherence, to immune evasion, and to virulence. Inhibition of slime layer synthesis may serve to inhibit colonization of periodontal pockets by *T. denticola*. In order to investigate

whether or not an extracellular mucoid layer is present at the cell surface of *T. denticola*, the most frequently isolated species of oral spirochete (Caudry, *et al*, 1995; Chan, *et al*, 1993), we employed electron and dark-field microscopy using Alcian blue, a polysaccharide specific, electron dense dye.

T. denticola ATCC 35405 has been shown to contain two locomotory phenotypes (Chan, *et al*, 1995b) and two distinct colony morphologies when grown in New Oral Spirochete (NOS)-agarose medium that can be differentiated by growth parameters in NOS medium, enzyme profiling in the API20A system and outer membrane protein antigens that are recognized by anti-*T. denticola* antibodies generated in rabbits (Scott, *et al*, 1995). *T. denticola* ATCC 35405 and its two colony types with distinctive reproducible morphologies, named Morphotype 1 (M1) and Morphotype 2 (M2), were originally isolated and maintained by our laboratory (Cheng and Chan, 1983; Scott, *et al*, 1995).

Electron microscopy and dark-field microscopy

In order to prepare the spirochete cells for electron microscopy, the supernatants from 10 ml, 10-day NOS cultures (Cheng and Chan, 1983) of *T. denticola* ATCC 35405, parental strain (P), Morphotype 1 and Morphotype 2, were decanted and the pelleted cells re-suspended in 2ml 0.1 M sodium acetate buffer, pH 4.7 (SAB). After enumeration of spirochete cells by dark-field microscopy using a counting chamber, the cells were adjusted to 1×10^7 cells ml^{-1} in SAB and one drop of each cell suspension placed onto the

surface of separate petri plates. To each drop was added an equal aliquot of Alcian blue (30 $\mu g ml^{-1}$ in SAB). After 4 min, a 400-mesh formvar-coated nickel grid was immersed for 1 min and blotted dry with bibulous paper. A second set of grids were prepared, as above, with an additional step of a 1 min immersion in 2% phosphotungstic acid following Alcian blue staining. The grids were examined with a Philips 410 electron microscope.

With the aim of observing the mucoid layer by dark-field microscopy, 10 ml, 10-day NOS cultures of *T. denticola* ATCC 35405 were prepared and adjusted to 1 x 10^7 cells ml^{-1}, as described above. The cell suspensions were then spotted onto glass microscope slides and fixed with 95% ethanol. The ethanol was allowed to evaporate completely. The cells were then stained with 30 $\mu g\ ml^{-1}$ Alcian Blue in SAB, for 4 min, and rinsed with ddH_20. A drop of glycerol was added and a cover slip was placed on top. The slides were then examined by dark-field microscopy.

The extracellular mucoid layer of Morphotypes 1 and 2 of *T. denticola* ATCC 35405, visualized by electron microscopy, is presented in Figures 1, 2, 3 and 4. In Figure 1, which is not stained with phosphotungstate, the copper moieties of Alcian blue are seen as an amorphous, electron dense pattern of dots surrounding the M1 spirochete cell.

The mucoid layer is seen as a dense amorphous layer surrounding the entire cell when stained with Alcian blue and phosphotungstate (Figure 2). Figures 3 and 4 are typical electron micrographs of M1 and M2, respectively. The staining technique is

Figure 1. The exopolysaccharide layer of Morphotype 1 of *T. denticola* ATCC 35405 visualized by Alcian blue staining. Bar = 0.5 μm.

Figure 2. The exopolysaccharide layer of *T. denticola* ATCC 35405 visualized by Alcian blue staining and phosphotungstic acid. Bar = 0.5 μm.

Figure 3. The exopolysaccharide layer of Morphotype 1 of *T.denticola* ATCC 35405 visualized by Alcian blue staining and phosphotungstic acid. Bar = 0.2 μm.

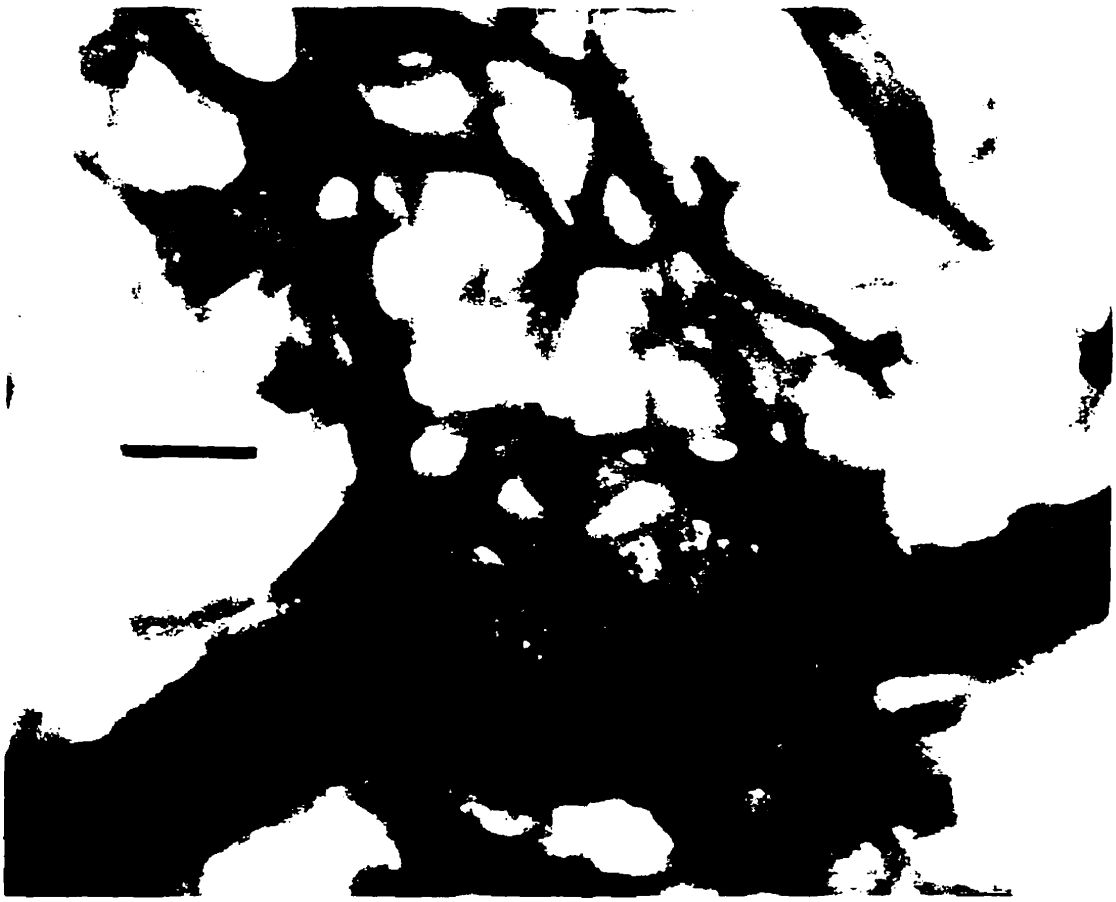
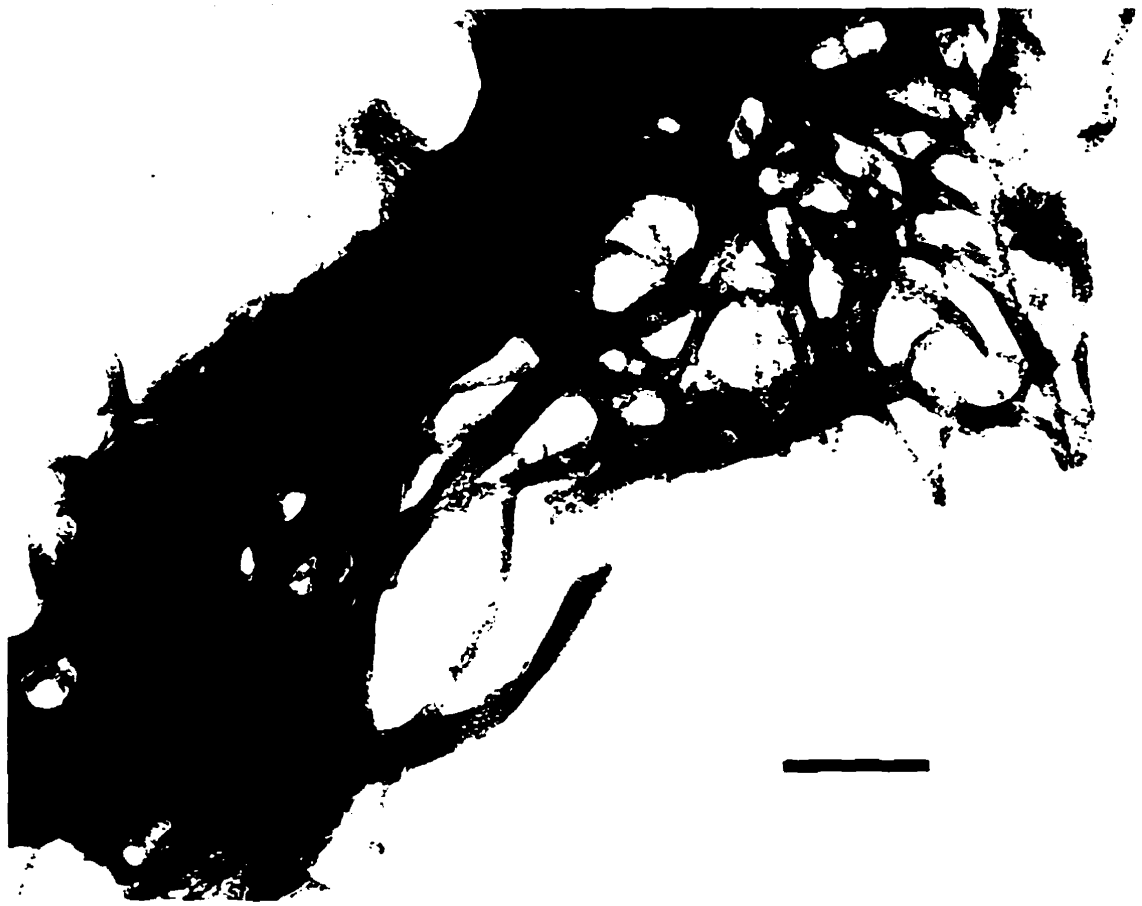


Figure 4. The exopolysaccharide layer of Morphotype 2 of *T. denticola* ATCC 35405 visualized by Alcian blue staining and phosphotungstic acid. Bar = 0.2 μm.

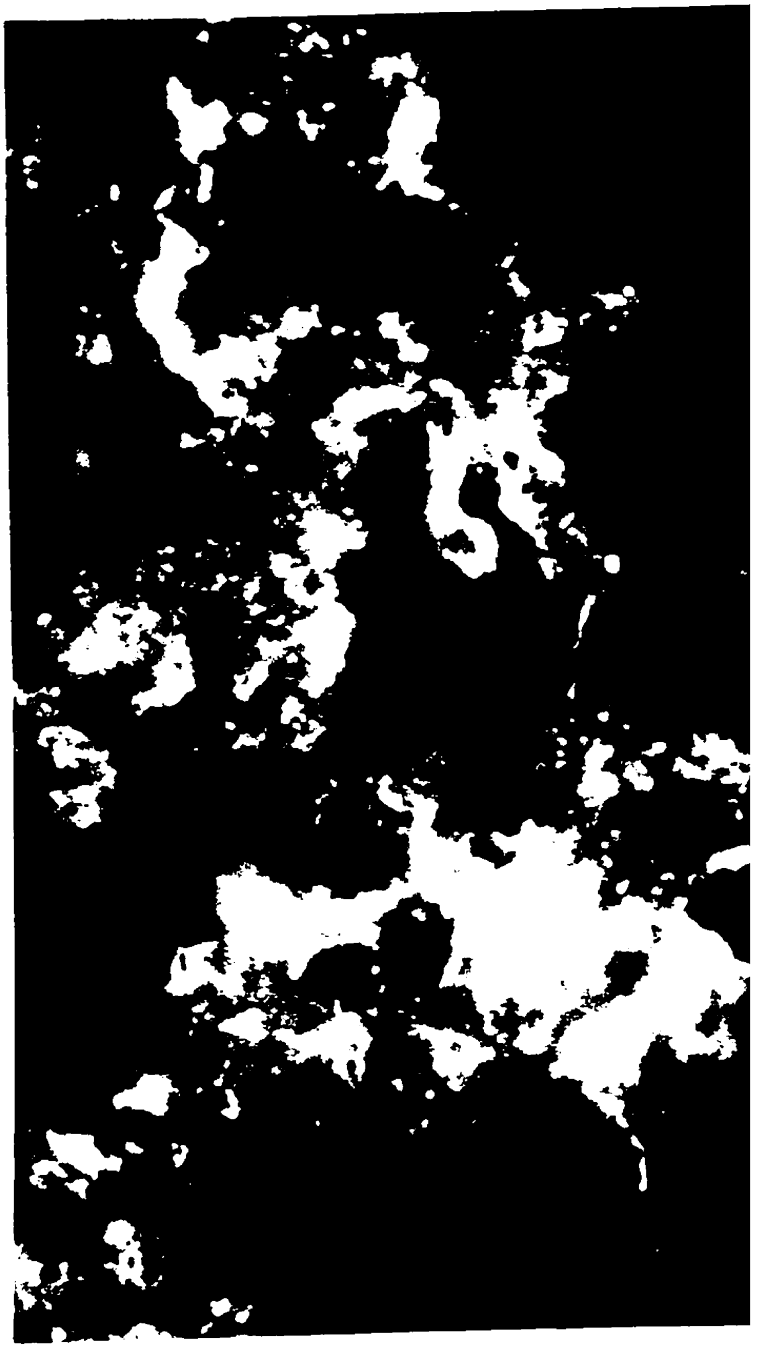


identical to Figure 2. The difference is in the magnification to obtain greater detail of the mucoid layer. The extracellular mucoid layer of both morphotypes can be seen as a series of interconnected strands rising outward from the outermost membrane of the spirochetes. This is consistent with the description of bacterial extracellular polysaccharide capsule organization as being composed of "numerous fibers emerging from the plane of the outer membrane more or less vertically" reported by Bayer and Bayer (1994).

Alcohol-treatment and Alcian blue-staining of *T. denticola* ATCC 35405 allowed the extracellular carbohydrate layer of the bacteria to be observed by dark-field microscopy. The parental strain, and both morphotypes were all stained by Alcian blue. As is shown in Figure 5, the mucoid layers of spirochete cells of the parental strain appear as an amorphous red zone, when stained with Alcian blue, that masks the characteristic helical shape that is normally observed by dark field microscopy of untreated oral anaerobic spirochetes (OAS). The mucoid layer visualized by this method was present on the surface of most, but not all, spirochete cells. This method is not useful for the identification and enumeration of oral spirochetes in smears of plaque.

Alcian blue staining of the carbohydrate layers of oral spirochetes was performed when the cells had reached late stationary phase. At this stage of growth, it is likely that the bacteria produce greater amounts of slime layer polysaccharides (Deighton and Borland, 1993; Neidhardt, *et al*, 1990).

Figure 5. The exopolysaccharide layer of *T. denticola* ATCC 35405 visualized by Alcian blue staining and dark-field microscopy.



Surface sugar lectin-analysis of *Treponema denticola*, *T. vincentii* and *T. socranskii*

As a preliminary study of possible variations in the sugar moieties comprising the extracellular mucoid layer of OAS, an examination of the cell surfaces of *T. vincentii* ATCC 35580, *T. socranskii* ATCC 35536, and *T. denticola* ATCC 35405 was performed by lectin-analysis. Recognition of the sugar moieties may be useful for determining the composition of the slime layer and differentiation between strains and species (Mansheim and Kasper, 1977; Okuda, *et al*, 1987; Schifferle, *et al*, 1988; Schifferle, 1994) of OAS.

Ten-day NOS cultures of *T. denticola*, parent strain, Morphotypes 1 and 2, *T. vincentii*, and *T. socranskii*, were sedimented at 1,000 x g, 10 min, and washed twice, with 20mM Tris-HCl buffer, pH 7.5 (TB). *T. vincentii* ATCC 35580 and *T. socranskii* ATCC 35536 were purchased from the American Type Culture Collection (Rockville, MD). The prescence of an extracellular mucoid layer was confirmed by Alcian blue staining and dark-field microscopy. The cells were adjusted to 1 x 10^8 cells ml^{-1} in TB and 50 µl aliquots of each cell suspension added to the wells of a microtitre plate. The cells were air dried for 1 h, placed in a 37 °C incubator for 2 hours, then fixed with 1% formaldehyde in 0.01 M phosphate-buffered saline, pH 7.5, 150 µl per well. The microtitre plates were then incubated at 4 °C for 12 h. The plates were placed at 37 °C for 1h and washed twice with 0.1% bovine serum albumen (BSA), 0.1% Tween 20 in TB (BT). Two hundred µl of 1% BSA in TB were added to each well and the plates incubated at 4 °C for 12 h. Following the incubation, 50 µl BT was added to each well.

Fifty µl of 2 mg ml^{-1} concanavalin A were added and serially diluted to a final dilution of 1 in 1024. The plates were incubated for 1 h at 37 °C and washed twice in BT. A 1 in 50 dilution of rabbit anti-concanavalin A in BT was added to each well in 50 µl aliquots, and the plates incubated for 1 h at 37 °C, then washed three times with 100 µl BT. Alkaline phosphatase-conjugated goat anti-rabbit IgG was added at a 1:800 dilution in BT, the plates incubated for 1 h, 37°C, then washed three times with 100 µl phosphate-buffered saline, pH 7.5 (PBS) and once with 100 µl 5 mM $MgCl_2$ in PBS, 5 min per wash, prior to the addition of 50 µl of 1 mg ml^{-1} Sigma 104 alkaline phosphatase substrate. The plates were incubated in the dark for 30 minutes, the reaction stopped with 100 µl 3 M NaOH and the OD_{405nm} read immediately on a Bio-Rad model 2550 EIA microtitration plate reader (Bio-Rad Lab. Ltd., Hercules, CA). The assay was repeated using phaseolus, arachis and lotus A lectins with their homologous antibodies. All reagents were purchased from Sigma Chemical Co., St. Louis, MO. Each assay was performed in triplicate.

The peak titres, or the dilution of lectin giving the highest absorbance on development of the microtitre plate wells with alkaline phosphatase substrate, represent the comparative quantities of each lectin-specific sugar found on the cell surfaces of *T. denticola* ATCC 35405 (M1, M2, and parental strain), *T. socranskii* ATCC 35536, and *T. vincentii* ATCC 35580 that are available for binding by each individual lectin (see Table 1). *T. vincentii* ATCC 35580 may be differentiated from *T. denticola* P, M1, and M2, and from *T. socranskii* ATCC 35536, by peak titres to concanavalin A and phaseolus, lectins with

Table 1. Lectin analysis of surface sugars of some oral spirochetes [(a)]

Lectin	Carbohydrate specificity	*T. denticola* ATCC 35405 I	Morphotype	Morphotype 2	*T. vincentii*	*T. socranskii*
ConcanavalinA	α-mannose / α-glucose	32	32	64	16	NB
Arachis	β-galactose	32	32	32	64	128
Phaseolus	β-galactose/ β-galactosamine	128	128	128	2	1
Lotus A	α-fucose	8	8	8	16	32

(a) The results are the average of triplicate assays of the dilution of lectin, from an initial concentration of 1 mg ml^{-1}, giving the peak optical density at 405nm on binding of the lectin to whole cells, following the addition of homologous rabbit anti-lectin antibodies, alkaline phosphatase-conjugated anti-rabbit IgG and alkaline phosphatase substrate in microtitre plates, as described in the text.

(NB) represents no binding.

specificities for α-D-mannose and α-D-glucose and for β-galactose and β-galactosamine, respectively.

T. socranskii ATCC 35536 can be differentiated from *T. vincentii* ATCC 35580, P. M1, and M2 as it does not bind concanavalin A. It was not possible to differentiate the parental strain and Morphotypes 1 and 2 of *T. denticola* ATCC 35405 by comparative analysis of surface sugars with the lectins employed.

It should be noted that definitive speciation of OAS by lectin-analysis will require large numbers of clinical isolates that should be compared to reference strains. In addition, several lectins with identical specificity should be utilized as the molecular weight of the lectin employed and the exact location of the target carbohydrates are important factors, therefore a single lectin may give rise to a false negative reaction (Grenier, *et al*, 1993). Positive reactions should be confirmed using inhibiting sugars. However, these initial results using a representative strain of three OAS species suggest that such an undertaking may prove worthwhile.

In conclusion, the presence of an extracellular mucoid layer on oral spirochetes should be taken into account when planning and executing experimental protocols involving investigations into aspects of the physiology of the spirochetal outer membrane sheath. The production of an extracellular polysaccharide layer by OAS may protect against phagocytosis, bacteriocins, and immunoglobulins and act as an additional mechanism of adherence. Further investigation into the composition and role of the mucoid layer of oral *Treponema* species is warranted and may represent a hitherto

unappreciated virulence factor.

Acknowledgements

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Chapter 5. SUMMARY AND CONCLUSIONS

The oral anaerobic spirochetes form part of any hypothesis attempting to explain the etiology of periodontal disease (PD). As *Treponema denticola* is the most frequently isolated spirochete from sites of active disease (Chan *et al.* 1993 ; Caudry *et al.* 1995), it is the most frequently employed species in research into the role played by oral anaerobic spirochetes (OAS) in the progression of disease, and *Treponema denticola* ATCC 35405 is the designated type strain (Chan et *al.* 1993).

At the time that the work contained in this thesis was initiated the potential iron acquisition mechanisms of the OAS had not been addressed. It was known that bacteria can obtain their necessary iron by a variety of means. Siderophores are extensively employed by human pathogens and commensal bacteria (Agiato and Dyer, 1992; Cox, 1980; Griffiths, 1991; Yang *et al.* 1991). Using the chrome azurol S shuttle (CAS) photometric assay we established that three OAS species, *T. denticola* (Td), *T. vincentii* (Tv), and *T. socranskii* (Ts), do not produce siderophores, even under conditions of iron limitation. The CAS assay allows for the observation of siderophore activity of both the hydroxymate and phenolate types (Schwyn and Nielands, 1987).

Some bacteria possess the machinery to obtain iron from hemin (Tai *et al.* 1993; Stojiljkovic, *et al.* 1992). Hemin is liberated from erythrocytes when lysed. The lysis of erythrocytes may be due to the action of hemolysins and phospholipase C (PLC). Our laboratory had previously demonstrated the presence of PLC in gingival crevicular fluid

at sites of active PD, the production of PLC by OAS and had purified the PLC of *T. denticola* by lecithin-PLC affinity chromatography (Chan *et al*, 1991; Siboo *et al*, 1989). We have shown, in Chapter 2, that Td, Tv and Ts are capable of the β-hemolysis of erythrocytes (Figure 1). The combined lytic activity against erythrocytes exerted by other bacteria co-inhabiting the subgingiva, such as *Porphyromonas gingivalis* (Shah and Gharbia, 1989; Grenier, 1991), and *Fusobacterium nucleatum* (Falker *et al*, 1983), likely contributes to the liberation of hemin.

Hemin and Congo red, a tetrapyrole analogue, bind to colony-forming units of Td, Tv, and Ts . Hemin (H) and Congo red (CR) bind to a 47-kDa outer membrane sheath receptor of these three OAS species and remains bound to a single protein on electrophoresis through a SDS-PAGE system and transblotting onto nitrocellulose membranes (Chapter 2, Figure 5). Only a single protein was found to bind hemin and CR by this method.

Pretreatment of whole cells of *T. denticola* with 8-anilino-naphthalene-1-sulfonic acid (ANSA), proteinase K, papain, pepsin, trypsin, sodium periodate, Triton X 100 and polymyxin B and our observations of the effect of these pretreatments on the ability of whole cells to bind H and CR led us to conclude that the 47-kDa H-receptor is truly an OMS protein, as Triton X 100 removes the OMS and inhibits H and CR binding (Chapter 2, Table 1). Periodation and polymyxin B had no effect on H and CR binding but inhibition was noted using ANSA, pepsin and proteinase K, leading us to believe that the binding site is a peptide and not lipid A or an oligosaccharide. As ANSA binds to hydrophobic amino acids, proteinase K cleaves

peptide bonds that are adjacent to carboxylic group of alanine, a hydrophobic amino acid, as well as aliphatic and aromatic amino acids, and because pepsin targets peptide bonds involving the amino groups of leucine, phenylalanine and tryptophan, which are hydrophobic amino acids, we speculate that the binding site may be comprised of a hydrophobic, aromatic-rich stretch of amino acids.

Through the use of subtractive photometric measurements it was possible to monitor the amount of H or CR binding to whole cells of Td, Tv and Ts. Eadie-Hofstee plots of the ratio of bound to free chromogen and the molar quantity of ligand bound allowed us to calculate the kinetics of the ligand-receptor interactions in intact cells (Chapter 2, Figures 2 and 3). The maximal binding capability (Bmax) for hemin was calculated to be 2.9 μM, 2.8 μM, and 0.8 μM for Td, Tv and Ts, respectively. The dissociation constants (Kd) for the interaction of hemin and the hemin-receptor were found to be 0.16 mM, 0.8 mM and 0.03 mM for Td, Tv, and Ts respectively. From this information it can be seen that although Ts binds less hemin than Td or Tv, the avidity of hemin-receptor interaction with its ligand, hemin, is stronger.

Using a methanol / chloroform lipoprotein extraction procedure and a hemin-agarose bead affinity column we were able to purify the 47-kDa hemin receptor of Td and Tv to apparent homogeneity (Chapter 2,Figure 7). *T. denticola* ATCC 35404 produces two hemin-binding proteins of 43-kDa and 44-kDa under conditions of iron-limitation (Chu *et al*, 1994b). N'-terminal sequence analysis of the first 21 amino acids of the 44-kDa protein suggests that this inducible protein represents a novel class of

hemin-binding receptor (Chu *et al*, 1994a). It will be of great interest to compare the 44-kDa protein with the N'-termini of the Td and Tv 47-kDa hemin-binding protein(s).

In initial photometric experiments it was found that >90% of the hemin bound by *T. denticola* cells remained bound to Triton-X 100 OMS extracts, suggesting hemin may not be transported into the cell. To confirm this hypothesis we labeled hemin with tritium and followed the fate of the label in whole cells, as to its distribution amongst OMS and cytoplasmic cellular fractions. The levels of ^{3}H-labeled hemin found in the cytoplasm were unchanged after 3 hours incubation with the ^{3}H-hemin in 4-day and 10-day cultures (Chapter 2, Table 2). The hemin-binding capacity of the *T. denticola* cells is saturated by 30 min. As hemin is thought to be transported across bacterial membranes linearly with time in bacteria that localize extracellular hemin to the cytoplasm (Coulton and Pang, 1983), we have concluded that hemin is not transported into the cytoplasm of Td and the small percentage of label detected in the cytoplasmic fractions is likely the result of the limitations in cell fractionation.

NOS medium treated with deferoxamine to reduce iron levels to support only minimal growth of *T. denticola* allowed us to observe various iron sources that could permit the growth of Td to be rescued to approach the parameters of Td growth in complete NOS medium (Chapter 2, Figure 8). It was found that hemin, but not protoporphyrin IX, and ferric ammonium citrate (FAC) could supply the iron required to increase the growth yields of *T. denticola* in 10 ml iron-limited NOS cultures. Protoporphyrin IX represents the hemin molecule that is lacking a central iron moiety. It

is interesting that the competitive inhibition of hemin binding by Congo red inhibited the growth of Td, Tv and Ts, suggesting that hemin may be an important iron source *in vivo*.

Hemin and FAC were used as the iron source for the growth of Td in a three layered tissue-culture flask assay for iron reductases making use of the colorometric reaction that occurs between Fe^{++} and ferrozine, which does not occur between Fe^{+++} and ferrozine (Chapter 2, Figure 11). NOS-deferoxamine medium supported minimal growth of *T. denticola* and no colorimetric changes were observed after 14 days incubation. However, with hemin or FAC as iron sources, a deep purple color developed in the flasks indicating the reduction of the ferric form of iron to the ferrous state. This could allow the transfer of iron across the bacterial membrane (Cowart and Foster, 1985; Deneer *et al*, 1995). Iron in the ferric form is insoluble under physiological conditions (Griffiths, 1991).

One of the clinical manifestations of PD is the break-down of connective-tissues, which include hyaluronates (HA) and chondroitin sulfates (CS) as constituents. As most periodontal pathogens do not produce hyaluronidase (Hase : Grenier and Michaud, 1993), because *in vivo* administration of Hase to human volunteers results in degeneration of connective tissue (Schultz-Haudt *et al*,1953)) and because *T. pallidum* is a known producer of Hase (Fitzgerald *et al*,1979; Fitzgerald and Gannon,1983; Fitzgerald and Johnson, 1979; Fitzgerald and Repesh,1987), it was decided to examine OAS for the production of a Hase.

Hyaluronidase activity was initially observed in preliminary experiments using HA-containing gels through which cell free culture supernatants and homogenized cell extracts were electrophoresed. Lytic activity was seen only at the top of the gels in proximity to the loading lanes. We, therefore, developed a method of observing Hase activity by using HA- or C6S-absorbed membranes following transblotting. The polysaccharide staining dye, Alcian blue, bound to the entire membranes, except at points of HA or of C6S degradation. The cell-free culture supernatants of Td, Tv and Ts were seen to contain a single band with lytic activity at 59 kDa (Chapter 3, Figure 3).

Photometric assays of the hydrolysis of HA, C6S and C4S allowed for quantification of degradative activity contained in the CFC-supernatant (Chapter 3, Table 1). The CFC-supernatant of Td could degrade 312 ± 2.5 μg hyaluronate per hour per ml, and 108.9 ± 8.5 and 53.5 ± 3.5 μg per hour per ml of C6S and C4S, respectively. Tv and Ts produced significantly lower amounts of the secreted enzyme (111.1 ± 1.8 and 59.5 ± 4.0 μg HA degraded per hour per ml, respectively)

The enzyme responsible for HA and CS degradation was a hyaluronoglucosaminidase (E.C. 3.2.1.35) as it degraded both substrates. Hyaluronate lyases (E.C. 4.2.2.1) and hyaloglucoronidases (E.C. 3.2.1.36) specifically degrade HA only. The enzymatic activity was not due to proteolytic degradation of proteoglycans that can contaminate commercially available HA substrates as protease inhibitors did not affect activity. Dextranase and neuraminidase were not able to degrade any component of the HA-substrate detectable by the Stains-all photometric assay. Moreover, the enzyme is

likely not a lysozyme as it is not inhibited by SDS. The Hase activity of cell-free culture supernatant was unaffected by Cd^{++}, Mn^{++}, Hg^{++}, or p-chloromercuribenzoate (pCMB) and had an optimal pH of 6.0. The optimal pH of *Clostridum perfringens* neuraminidase is 5.0-5.1, a pH optimum of 7.5 has been reported for heparinase, and of 9.2 for lyzosyme. Heparinase is inhibited by Hg^{++} and neuraminidase is inhibited by pCMB. It was necessary to ensure the identity of the hyaluronoglucosaminidase of Td by these methods as *P. gingivalis* has been shown not to produce a Hase (Grenier and Michaud, 1993), as had been previously reported (Seddon and Shah, 1989; Steffen and Hentges, 1981). The reports of the neuraminidase activity of group B streptococci (Hayano and Tanaka,1969; Milligan *et al.*1980; Brown and Straus, 1987) have also been proven to be incorrect, as the enzyme is, in fact, a Hase (Pritchard and Lin, 1993). These conflicting reports are likely due in part to proteolytic degradation of bovine serum albumin used in a plate assay for Hase activity, as it precipitates with HA under acidic conditions (Grenier and Michaud, 1993) and to the impurity of bovine submaxillary mucin used as a substrate for neuraminidase activity but also containing HA (Pritchard and Lin, 1993). It was necessary to rule out heparinase activity due to structural similarities of HA and heparin.

The hyaluronidase of group B streptococci has been fortuitously purified, essentially by the use of a single affinity column containing N -(p-aminophenyl) oxamic acid-agarose beads (Pritchard and Lin, 1993). It was the author's intention to purify what had been considered a neuraminidase. We were able to purify the Td Hase through the use of HA- or CSC-conjugated to Affi-gel 701 beads (Chapter 3, Figure. 4). The affinity purified Hase of Td ran as on single species of 59 kDa

in a 10% SDS-PAGE gel. The affinity purified Hase of Td cross-reacted with commercial antibodies generated against *Apis mellifera* venom at a single point on a nitrocellulose membrane. The single band recognized by the anti-honeybee venom antibodies was calculated to have an apparent MW of 59 kDa (Chapter 3, Figure 6). *Apis mellifera* venom contains only two major proteins, a Hase and a phospholipase A (Gmachl and Kreil,1995; King *et al*,1993).

Gold sodium thiomalate (GST), an electron dense inhibitor of Hase, and gold-labeled protein G, which binds to the anti-*Apis mellifera* antibodies, allowed the visualization of the Hase on, or near to, the cell surface on examination by electron microscopy. As was demonstrated in Chapter 4 (Figs. 1, 2, 3 4 and 5), Td produces an extracellular mucoid layer. It appeared from the electron micrographs of GST- and anti-*Apis mellifera* venom / gold labeled protein G-tagged cells of Td that the hyaluronidase is localized to the cell surface and may be trapped in the exopolysaccharide layer and in clumps of polysaccharide that are shed from the cell surface (Chapter 3, Figs. 5 and 6). Prior incubation of Td cells with HA blocked binding of GST and anti-bee venom antibodies, indicating the specificity of GST and the anti-bee venom to Hase.

The hyaluronoglucosaminidase produced by Td, Tv, and Ts may represent a virulence factor, and compliment the other reported enzymes with a potential for host tissue degradation, such as collagenase (Grenier *et al*,1990; Que and Kuramitsu,1990; Uitto *et al*,1988), keratinase (Mikx and DeJong,1987), neuraminidase (Homer *et al*, 1992), PLC (Chan *et al*, 1991; Siboo *et al* 1989) and hemolysins (Chu and

Holt,1994; Scott *et al*,1993) that are produced by oral anaerobic spirochetes. Spirochetes have been found in the intercellular matrix at diseased sites (Frank,1980; Loesche, 1988). The degradation of HA and CS by the hyaluronoglucosaminidase of OAS may be directly involved in the disruption of gingival epithelium and the creation of avenues of access into deeper tissue for both OAS and other periodontal bacteria.

Finally, in Chapter 4, evidence is presented confirming the presence of a polysaccharide layer external to the outer membrane sheath of *Treponema denticola*.

We were able to observe this layer on two morphotypes of *T. denticola* ATCC 35405 by electron microscopy, utilizing Alcian blue, an electron dense dye that binds to polysaccharides, and phosphotungstate (Chapter 4, Figs. 1, 2, 3 and 4). We were also able to visualize this layer by dark-field microscopy by the development of a stain which involves polysaccharide fixation with ethanol followed by Alcian blue staining.

Although the results presented in Chapter 4 allow us only to speculate on the role of the exopolysaccharide layer *in vivo*, they are important in consideration of mechanisms that may permit the evasion of the immune system *in situ* by OAS (Boehringer *et al*,1986; Loesche,1993, Tew *et al*,1985). The exopolysaccharide layer may protect against immunoglobulins, against phagocytosis and against bacteriocins, and as such act as a virulence factor. It is also possible that this layer may have antigenic potential. It is known that capsule or slime layer production is a significant factor in the pathogenesis of

many other bacteria, e.g. *Escherichia coli* (Schifferle,1994), and *Staphylococcus epidermidis* (Deighton and Borland, 1993).

The visualization of the exopolysaccharide layer opens up many possible avenues for further research into the role of OAS in PD, as it represents confirmation of a component of the ultrastructure of the oral spirochetes that has been hitherto unappreciated. In addition, as lectin analysis of the surface sugars of Td, Tv, and Ts allowed us to distinguish these 3 species of OAS, it may be possible to differentiate strains of OAS on a more extensive application of this technique.

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Appendix 1 ; Further observations of the two morphotypes of *Treponema denticola* ATCC 35405.

With respect to the results and conclusions presented in Chapter 2 concerning the iron aquisition mechanisms of *T. denticola* ATCC 35405 it should be noted that both Morphotype 1 (M1) and Morphotype 2 (M2) were examined for differences in β-hemolysis, binding of Congo red and hemin to whole cells and the kinetics of the receptor-ligand interactions. Whole cells of M1 and of M2 were able to bind Congo red and hemin via a 47 kDa OMS receptor, as determined by transblotting of Triton-X 100 extracted OMS proteins. The ligands remained bound to the receptor throughout the SDS-PAGE and transblotting process. Only a small difference in the Bmax and Kd for hemin binding by intact cells was observed. The Bmax for hemin binding by M1 and M2 were calculated from Eadie-Hofstee plots to be 3.1 μM and 2.7 μM, respectively. The Kd values for hemin binding by M1 and M2 were calculated as 0.16 μM and 0.15 μM, respectively.

Both M1 and M2 were β-hemolytic.

M1 and M2 could both reduce iron from ferric ammonium citrate and from hemin in the solid-phase assay for iron reductase activity. In the Thunberg cuvet assay the O.D. 562nm was raised from 0.018 to 0.078 and from 0.022 to 0.056 over 10 minutes by whole cells of M1 and M2, respectively. There was no increase in the percentage, or absolute amounts, of ^{3}H-hemin detected in the protoplasmic cylinders of M1 or M2, indicating that hemin was not transported across the cellular membranes of either morphotype.

In addition ferric ammonium citrate and hemin could support the iron requirements of both morphotypes. These results suggest that there are no major differences in the mechanisms of iron aquisition available to the parental strain, ATCC 35405, and the two isolated morphotypes.

With respect to Chapter 3, the activities of M1 and M2 against hyaluronate and chondroitin sulfate were determined by the Stains-all assay in an identical manner to the results presented for the parent strain in Table 1 of Chapter 3. The activity of the cell-free culture (CFC) supernatants of M1 and M2 were found to be 322.5 μg and 280.0 μg substrate degraded per hour per ml CFC supernatant against hyaluronic acid and 124.0 μg and 97.6 μg substrate degraded per hour per ml CFC supernatant against chondroitin sulfate-type C, respectively. M1 and M2 also exhibited only a single lytic band of activity on hyaluronate absorbed nitrocellulose membranes, as presented for the parent strain in Figure 3 of Chapter 3. Both M1 and M2, therefore, may contribute to the breakdown of the extracellular matrix in the gingiva and play a role in the pathologic degradation of peridontal tissue through the action of their extracellular hyaluronoglucosaminidases.

Appendix 2. Abbreviations used in thesis.

ACP ; adult chronic periodontitis

AF ; axial fibril

ANSA ; 8-anilino-naphthalene-1-sulfonic acid

anti-venom ; anti-*Apis mellifera* venom antibodies

ANUG ; acute necrotizing ulcerative gingivitis

BANA ; N-benzoyl-DL-arginine-naphthylamide

BB ; basal body

Bmax ; maximum binding

BSA ; bovine serum albumin

BT ; 0.1% BSA / 0.1% Tween 20 in TB

CFC ; cell-free culture (supernatant)

CFU ; colony-forming unit

CG ; cathespin G

CM ; cytoplasmic membrane

CNS ; central nervous system

CR ; Congo red

CS ; chondroitin sulfate

Case ; chondroitinase

C6S-gel ; chondroitin sulfate-Affi-gel-conjugate

CTL ; chymotrypsinlike enzyme

DAG ; diacylglyceride

ECM ; extracellular matrix

EDTA ; ethylene diamine tetraacetic acid

EM ; electron microscope

EOP ; early-onset periodontitis

FMN ; flavin mononucleotide

GAG ; glycosaminoglycan

GCF ; gingival crevicular fluid

GEC ; gingival epithelial cell

GJP ; generalized juvenile periodontitis

GST ; gold sodium thiomalate

H ; hemin

HA ; hyaluronic acid

Hase : hyaluronidase

HA-gel ; hyaluronic acid-Affi-gel-conjugate

HGase ; hyaluronoglucosaminidase

HGF ; human gingival fibroblast

HPBM ; human peripheral blood mononuclear cell

Kd ; dissociation constant

LAD ; leukocyte adhesion deficiency

LBRF ; louse-borne relapsing fever

LJP ; localized juvenile periodontitis

LMC ; linear minichronosome

LPS ; lipopolysaccharide

M1 ; Morphotype 1 of *T. denticola* ATCC 35405

M2 ; Morphotype 2 of *T. denticola* ATCC 35405

MAb ; monoclonal antibody

NADH ; nicotinamide adenine dinucleotide

NOS ; New Oral Spirochete medium

NOS-SD ; New Oral Spirochete medium-Sea-plaque agarose-deferoxamine

OAS ; oral anaerobic spirochete

OMS ; outer membrane sheath

ORF ; open reading frame

P ; parent culture (*T. denticola* ATCC 35405)

PBP ; penicillin-binding protein

PBS ; phosphate-buffered saline

PC ; protoplasmic cylinder

PCMB ; p-chloromercuribenzoate

PD ; periodontal disease

PG ; peptidoglycan

PGE_2 ; Prostaglandin E_2

PIP_2 ; phosphatidylinositol bisphosphate

PLC ; phospholipase C

PKC ; protein kinase C

PMN ; polymorphonuclear cell / neutrophil

PMSF ; phenylmethylsulfonic acid

POPase ; endo-acting proline-specific oligopeptidase

PROS ; pathogen-related oral spirochete

PWM ; pokeweed mitogen

Rep protein ; replicative protein

RPP ; rapidly-progressing periodontitis

Sa; *Streptococcus agalactiae*

SAB ; sodium acetate buffer

SCFA ; short chain fatty acids

TB ; Tris-HCl buffer

TBRF ; tick-borne relapsing fever

Td ; *Treponema denticola*

TLCK ; N-α-p-tosyl-L-lysine chloromethylketone

Ts ; *Treponema socranskii*

Tv ; *Treponema vincentii*

VLA ; very late antigen

WHO ; World Health Organization

IMAGE EVALUATION
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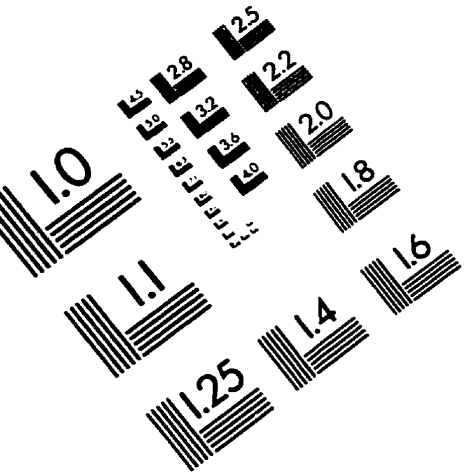

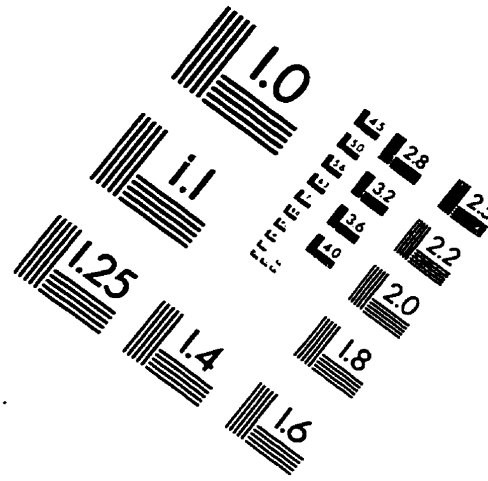

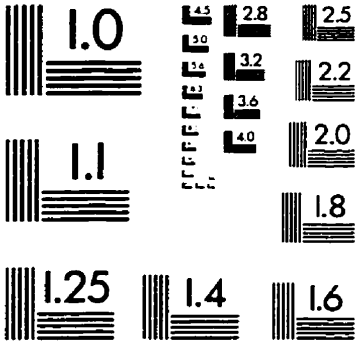

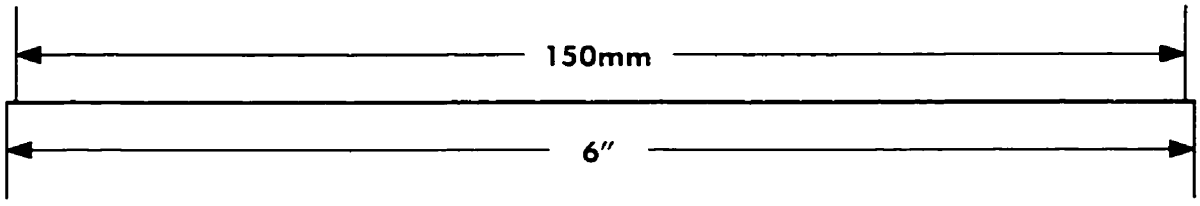

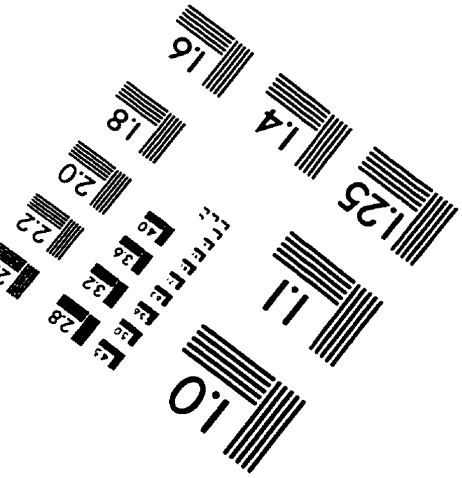

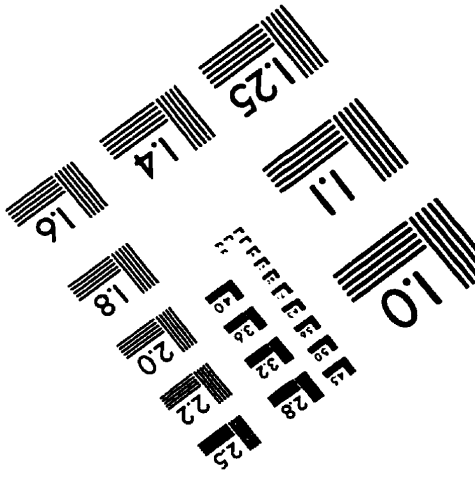

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