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Role of *Treponema denticola* in the pathogenesis and progression of periodontal disease

(Ruolo di *Treponema denticola* nella patogenesi e progressione della malattia paradontale)

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Summary

Periodontal disease refers to the inflammatory processes that occur in the tissues surrounding the teeth in response to bacterial accumulations. Rarely do these bacterial accumulations cause overt infections, but the inflammatory response which they elicit in the gingival tissue is ultimately responsible for a progressive loss of collagen attachment of the tooth to the underlying alveolar bone, which, if unchecked, can cause the tooth to loosen and then to be lost. Various spirochetal morphotypes can be observed in periodontal pockets, but many of these morphotypes are as yet uncultivable. One of the most studied oral spirochetes, *Treponema denticola*, possesses the features needed or adherence, invasion, and damage of the periodontal tissues. The effect of specific bacterial products from oral treponemes on periodontium is poorly investigated. In particular, the Major surface protein (MSP), which is expressed on the envelope of *T.denticola* cell, plays a key role in the interaction between this treponeme and periodontal cells. Oral microorganisms, including spirochetes, and their byproducts are frequently associated with systemic disorders such as cardiovascular disease (CVD). Oral infection models have emerged as useful tools to study the hypothesis that infection is a CVD risk factor. Periodontal infections are a leading culprit, with studies reporting associations between periodontal disease and CVD. Recently our group demonstrated that an oral spirochetes, *T.denticola*, has been detected in atherosclerotic plaques by FISH techniques. This data enlarge the possibility that the oral Spirochetes may disseminate into bloodstream and its relatedness to the CVD.

On the basis of data reported in the literature, the first aim of my research was to investigate the release of proinflammatory mediators in primary human monocytes subseguent stimulation with native MSP. *T.denticola* MSP induced the synthesis of Tumor necrosis-alpha (TNF-α), Interleukin 1-β (IL-1β), Interleukin-6 (IL-6) and Matrix metallo-Proteinase-9 (MMP-9) in a dose- and time-dependent manner. Similar patterns of TNF-α, IL-1β, and IL-6 release were observed when cells were stimulated with 100 and 1000 ng/ mL of MSP . Moreover, the production of MMP-9 was significant only when cells were treated with high concentration of MSP (1000 ng/mL). These results indicate that *T.denticola* MSP , at concentrations ranging from 100 ng/mL to 1.0 µg/mL, activates various intracellular signaling pathways in primary human monocytes, leading to increased production of pro-inflammatory cytokines and chemokines. In addition to other virulence factor, the Major Surface Protein of *T.denticola*, by stimulating the secretion of pro-inflammatory cytokines and chemokynes, can activate the host-mediated destructive process observed during periodontitis.

Considering that endothelial cells are the cellular elements that first come into contact with blood vessel contents, oral treponemes can interact with endothelial cells both in highly vascularized periodontal tissue and outside the periodontal site as consequences of Treponema high motility. In order to determine the direct interactions between treponemes and blood vessels I investigated the ability of the outer membrane of *T.denticola* (OMT) to induce apoptosis and heat shock proteins expression (HO-1 and Hsp70) in porcine aortic endothelial cells (pAECs), compared with results obtained with classical pro-inflammatory lipopolysaccharide (LPS) treatment. Cellular apoptosis was detected when pAECs were treated with either OMT or LPS, suggesting that OMT can damage endothelium integrity by

reducing endothelial cell vitality. Stimulation with OMT, similarly to LPS response, increased HO-1 and Hsp-70 protein expression in a time-dependent manner, correlating with a rise in HO-1 and Hsp-70 mRNA. Collectively, these results support the hypothesis that *T.denticola* alters endothelial cell function. Moreover, these *in vitro* experiments represent a preliminary investigation to further *in vivo* study using a pig model, to elucidate how *T.denticola* leaves the initial endodontic site and participates in the development of several systemic diseases.

In order to further assess the relevance of the spirochetes motility and cell shape during the interaction with phagocytes, I evaluated the capacity of murine macrophage to uptake and kill two *T.denticola* mutant strains: one strain lacked motility due to a knock out of the *flgE* gene thus resulting in a defect of the flagellar system, and a second mutant strain characterized by a filamentation phenotype (extremely long cells) associated with a lack of intermediate-like cytoplasmic filament, due to a knockout of the *cfpA* gene. Macrophages, incubated under aerobic and anaerobic conditions, produced a similar amount of TNF-α when stimulated with *Escherichia coli* LPS. The uptake of FlgE- and CfpA-deficient mutants of *T.denticola* was significantly increased compared to the wild type strain due to cell size or lack of motility. In addition, opsonization with specific antibodies considerably improved the treponemes' uptake. These results suggest that macrophages, in addition to other phagocytes, could play an important role in the control of *T.denticola* infection and that the raising of specific antibodies could improve the efficacy of the immune response toward *T.denticola* either at an oral site or while disseminating.

In the end, I investigated the possible conservation of the sequence encoding the *msp* gene among seventeen clinical samples obtained from patients suffering from acute periodontitis that were identified as positive for *T.denticola* by RT-PCR. Among the different virulence factor, the Major Surface Protein plays a fundamental role in the interaction between *T.denticola* and the host, as previously described. In particular, MSP has cytopathic effects on several cells and is considerate one of the major antigens recognized by the host immune response. Moreover, the MSP region encoded by nucleotides comprised between nucleotide position 567 and 738 has been demonstrated as exposed onto the surface of living treponemes. This portion is considered to be involved in the adhesion to the host cells. So far, the amino acidic sequence of this surface exposed region of the MSP has been determined in a small number of treponemal isolates that have been serially passaged in vitro for several years. The sequence analysis confirmed that each *msp* gene contains two highly conserved 5' and 3' regions (located between nucleotide positions 1 to 600 and 800 to 1632, respectively). The analysis of the central region, comprised between nucleotide positions 600 and 800, showed an degree of variability. A phylogenetic analysis of these central regions of the *msp* gene of these 17samples grouped the specimens in two principal groups; this suggests a low evolutionary rate and an elevated degree of conservation for the *msp* gene also in clinically derived genetic material. In conclusion, the data presented in this thesis, evidence that the surface exposed central region of the MSP molecule could be considered the most prominent target for the host immune system against *Treponema denticola*.

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Chapter I

1. PERIODONTAL DISEASE

1.1. Periodontal Disease

Periodontal diseases are multifactorial infections elicited by a complex of bacterial species that interact with host tissues and cells causing the release of a broad array of inflammatory cytokines, chemokines, and mediators, some of which lead to destruction of the periodontal structures, including the tooth supporting tissues, alveolar bone, and periodontal ligament. The trigger for the initiation of disease is the presence of complex microbial biofilms that colonize the sulcular regions between the tooth surface and the gingival margin through specific adherence interactions and accumulation due to architectural changes in the sulcus (attachment loss and pocket formation). This pocket can extend from 4 to 12 mm and can harbour, depending on its depth and extent, from 10^7 to almost 10^9 bacterial cells (Socransky, 1991).

Most of these microorganisms can produce tissue destruction (Genco, 1992; Bascones, 2003) in two ways: directly, through invasion of the tissues and the production of harmful substances that induce cell death and tissue necrosis; and indirectly, through activation of inflammatory cells that can produce and release mediators that act on effectors, with potent proinflammatory and catabolic activity. This action plays a crucial role in the destruction of

periodontal tissue, while some bacteria also interfere with the normal host defence mechanism by deactivating specific antibodies or inhibiting the action of phagocyte cells (Williams, 1990). The pathogenesis of periodontal destruction involves the sequential activation of different components of the host immune and inflammatory response, aimed in the first place at defending the tissues against bacterial aggression, reflecting the essentially protective role of the response. However, it also acts as a mediator of this destruction (Bascones-Martínez, 2004).

The prevalence of periodontal disease increases with age (Brown, 1996; Grossi, 1995 and 1994; Oliver, 1998) and as more people are living longer and retaining more teeth, the number of people developing periodontal disease will increase in the next decades. About 50% of the adult population has gingivitis (gingival inflammation without any bone loss about teeth and no pockets deeper than 3 mm) around three or four teeth at any given time, and 30% have periodontitis as defined by the presence of three or more teeth with pockets of 4 mm (Albandar, 1999; Oliver, 1998). Between 5 and 15% of those with periodontitis have advanced forms with pockets of 6 mm (Papapanou, 1996). Another 3 to 4% of individuals will develop an aggressive form of periodontal disease, known as early onset periodontitis, between the ages of 14 and 35 years. Any medical condition that affects host antibacterial defense mechanisms, such as human immunodeficiency virus infection HIV (Winkler, 1992), diabetes (Papapanou, 1996, Shlossman, 1990), and neutrophil disorders (Van Dyke, 1985), will predispose the individual to periodontal disease.

1.2. Dental plaque

The dental plaque is unlike any other bacterial ecosystem that survives on the body surfaces, in that it develops on the nonshedding tooth surface and can form complex bacterial communities that may harbour over 400 distinct species and contain over 10^8 bacteria per mg (Moore, 1994). The plaque is divided into two distinct types based on the relationship of the plaque to the gingival margin, supragingival plaque and subgingival plaque. The supragingival plaque is dominated by facultative *Streptococcus* and *Actinomyces* species, whereas the subgingival plaque harbors an anaerobic gram-negative flora dominated by uncultivable spirochetal species (Choi, 1996). It is this gram-negative flora that has been associated with periodontal disease. Since many of its members derive some of their nutrients from the gingival crevicular fluid, a tissue transudate (Cimasoni, 1983) that seeps into the periodontal area, it is possible that their overgrowth is a result of the inflammatory process (Loesche, 1982). Therefore, there is a distinction between the way the host responds to the supragingival plaque and its response to the subgingival plaque. The response to the supragingival plaque has been thoroughly studied in the experimental gingivitis model whereas the response to the subgingival plaque remains under investigation. Host respond to the subgingival plaque as if it were an overgrowth of a bacterial community in which many members produce substances, such as LPS, that are particularly bioactive if they enter the approximating gingival tissue. Moreover the host respond to a plaque in which certain members produce more biologically active molecules, such as butyric acid (Niederman, 1997) or hydrogen sulphide (Ratcliff, 1999), per cell or possess unique proteases, such as are found in *P. gingivalis* and *Treponema denticola*, which can degrade host molecules,

creating a proinflammatory effect (Grenier, 1996; Kuramitsu, 1998; Makinen, 1996; Uitto, 1995). In either case, although bacteria are involved, it is not the scenario of a typical infection, as the offending bacteria generally remain outside the body, attached to the tooth. The most common anaerobic bacteria shown to dominate periodontal plaque associated with disease are *P. gingivalis, B.forsythus*, and *T.denticola*. Anaerobic micro-organisms, such as spirochetes and black-pigmented Bacteroides (classified recently as *Porphyromonas* and *Prevotella*), were identified more than 35 years ago as periodontal pathogens (MacDonald, 1962). As the clinical periodontal parameters worsen, the number and percentage of spirochetes counted by microscopic techniques increase proportionately (Listgarten and Hellden, 1978; Lindlhe, 1980; Loesche and Laughon, 1982; Riviere, 1995). The presence of *T.denticola* and unidentified spirochetes in healthy periodontal sites was also associated with an increased susceptibility to gingival inflammation (Riviere and DeRouen, 1998). Spirochetes were also shown to be significantly elevated (in numbers and pro portions) in dental plaques removed from untreated patients compared with those from patients receiving periodontal treatment. Spirochetes were the overwhelming microbial type in the plaques of adult periodontitis patients, averaging about 45%, of the microscopic count (Loesche ct al., 1985), as well as in microbial samples from patients with acute pericoronitis (Weinberg, 1986) and failing implants (54%) (Listgarten and Lai, 1999). Furthermore, the examination of intra-oral sources of species colonizing dental implants (Lee, 1999) has revealed positive associations between *T.denticola* isolated from bacterial samples taken from implants and teeth at the same visit. Species-specific nested PCR revealed that subjects with periodontitis harbored in their dental plaque strains of *T.denticola, Treponema amylovorum, Treponema maltophilum, Treponema medium, Treponema pectinovorum, Treponema socranskii,* and *Treponema vincentii* (Willis, 1999). Using DNA probes,

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polyclonal antibodies, and culture methods, investigators have also shown that *P. gingivalis*, *B. forsythus, T.denticola*, and other spirochetes were present in 80-100% of plaque samples removed prior to periodontal surgery (Loesche, 1992). In a recent study aimed to determine the association between the levels of granulocyte elastase and prostaglandin E2 in the gingival crevicular fluid and the presence of periodontopathogens in untreated adult periodontitis (Gin, 1999), the predominant combination of species detected was *P. gingivalis, P. intermedia, B. forsythus,* and *T.denticola*. This bacterial combination was significantly higher at periodontitis sites (68%) than at healthy (7%) or gingivitis sites (29%).

1.3. Bacterial dissemination

Oral commensals, particularly those residing in periodontal niches, commonly exist in the form of biofilm communities on either nonshedding surfaces, such as the teeth or prostheses, or shedding surfaces, such as the epithelial linings of gingival crevices or periodontal pockets. A feature that is unique to the oral bacterial biofilm, particularly the subgingival plaque biofilm, is its close proximity to a highly vascularized milieu (Nanci, 2006).
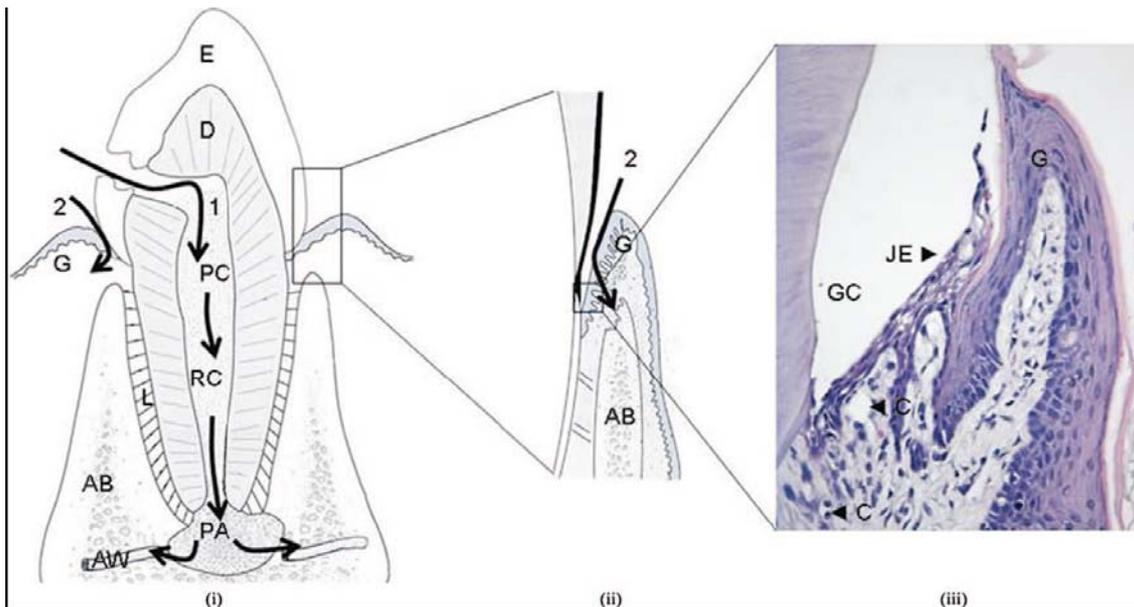
Figure 1. Possible routes of bacterial entry from teeth into the systemic circulation. Pathway 1, entry via the root canal (RC) or from periapical lesions (PA) into the alveolar blood vessels (AW); pathway 2, entry from the periodontium, where bacteria in the gingival crevices (GC) translocate to the capillaries (C) in the gingival connective tissues, possibly through the junctional epithelium (JE). E, enamel; D, dentine; L, periodontal ligament; and AB, alveolar bone. Panel iii Note that the junctional epithelium was detached from the enamel due to processing for microscopy.

This environment is different from other sites where bacteria commonly reside in the human body. For example, the ingress of cutaneous flora into the circulation is prevented by the relatively thick and impermeable keratinized layers of the skin, while the mucosal flora of the gastrointestinal and genitourinary tracts is commandeered by the rich submucosal lymphatics, which keep microorganisms under constant check. Their covering epithelia are continuously shed at a quick rate, denying prolonged colonization by the bacterial flora. Although innate defense by polymorphonuclear neutrophils is highly developed at the

dentogingival junction and backed up by a highly organized lymphatic system, the oral biofilms, if left undisturbed, can establish themselves permanently on nonshedding tooth surfaces subjacent to the dentogingival junction. Under these circumstances, any disruption of the natural integrity between the biofilm and the subgingival epithelium, which is at most about 10 cell layers thick, could lead to a bacteremic state (Samaranayake, 2006).

All too often, in common inflammatory conditions such as gingivitis and chronic periodontitis, which are precipitated by the buildup of plaque biofilms, the periodontal vasculature proliferates and dilates, providing an even greater surface area that facilitates the entry of microorganisms into the bloodstream. Often, these bacteremias are short-lived and transient, with the highest intensity limited to the first 30 min after a trigger episode. On occasions, this may lead to seeding of organisms in different target organs, resulting in subclinical, acute, or chronic infections. Yet there are a number of other organs and body sites that may be affected by focal bacteremic spread from the oral cavity.

4. Relationship between Periodontal disease and Cardiovascular Disease

After two decades of research, it has been firmly established that an association exists between periodontal disease and cardiovascular disease (CVD). The pertinent question, however, is about the nature and relevance of this association.

The fundamental question remain: "*does the infectious and inflammatory periodontal disease process contribute causally to heart attacks and strokes, or are these two conditions coincidentally associated?*"

Cardiovascular diseases such as atherosclerosis and myocardial infarction occur as a result of a complex set of genetic and environmental factors (Herzberg, 1998). The genetic factors include age, lipid metabolism, obesity, hypertension, diabetes, increased fibrinogen levels, and platelet-specific antigen Zwb (P1^{A2}) polymorphism. Environmental risk factors include socioeconomic status, exercise stress, diet, nonsteroidal anti-inflammatory drugs, smoking, and chronic infection. The classical risk factors of cardiovascular disease such as hypertension, hypercholesterolemia, and cigarette smoking can only account for one-half to two-thirds of the variation in the incidence of cardiovascular disease (Scannapieco, 1998). Among other possible risk factors, evidence linking chronic infection and inflammation to cardiovascular disease has been accumulating (Ridker, 1997; Syrjänen, 1990; Valtonen, 1991). It is clear that periodontal disease is capable of predisposing individuals to cardiovascular disease, given the abundance of gram negative species involved, the readily detectable levels of proinflammatory cytokines, the heavy immune and inflammatory infiltrates involved, the association of high peripheral fibrinogen, and the white blood cell (WBC) counts (Kilian, 1982). There are several proposed mechanisms by which periodontal disease may trigger pathways leading to cardiovascular disease through direct and indirect effects of oral bacteria. First, in the case of periodontal disease, the presence of an inflammatory focus in the oral cavity may potentiate the atherosclerotic process by stimulation of humoral and cell-mediated inflammatory pathways. The degree of inflammation in periodontal disease is clearly sufficient to cause a systemic inflammatory response, as evidenced by increases in C-reactive protein. Moderate increases in the level of C-reactive protein (CRP) in serum were predictive of new heart episodes in apparently healthy men (Ridker, 1997). Cross-reactivity of antibodies to periodontal pathogens with antigens present in platelets or endothelial cells might be an additional pro-inflammatory

mechanism. A second hypothesis which explain the association between periodontal disease and may reflect an individual propensity to develop an exuberant inflammatory response to intrinsic (age, sex, genes) or extrinsic stimuli (diet, smoking, etc) that then predisposes to both periodontal disease and atherosclerosis. Another mechanism by which periodontal bacteria could contribute to cardiovascular pathology relates to the antigenic similarity of certain bacterial proteins with host proteins. Recently, a specific heat shock protein, Hsp65, has been reported to link cardiovascular risks and host responses (Xu, 1992 and 1993). Heat shock proteins are important for the maintenance of normal cellular function and may have additional roles as virulence factors for many bacterial species. Many host tissues, including the endothelial lining of blood vessels, produce hsp60 as they respond to certain stressors like high blood pressure and LPS. In animal studies, Xu et al. (Xu, 1993) demonstrated that immunization of rabbits with bacterial Hsp65 induces atherosclerotic lesions. A subsequent large-scale clinical study found a significant association between serum antibody levels to Hsp65 and the presence of cardiovascular disease.
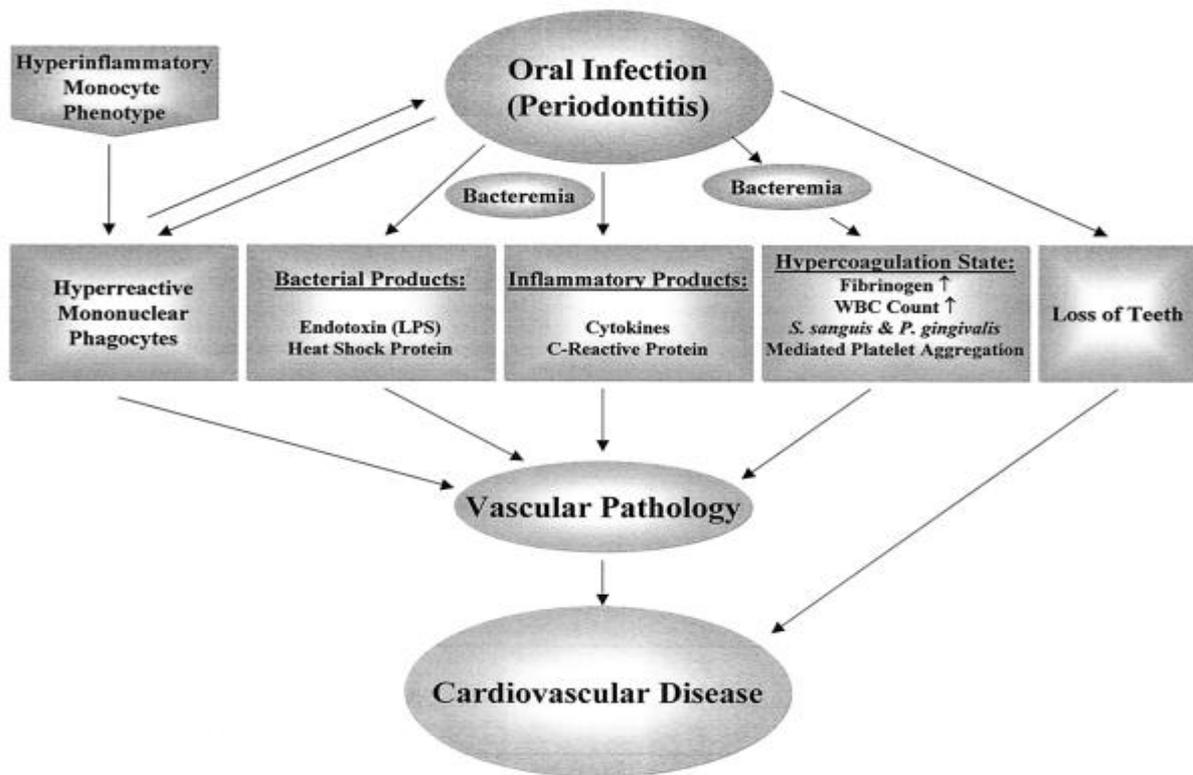
Figure 2. Relationship between Periodontal Disease and Cardiovascular Disease.

Bacterial infection stimulates the host response to Hsp65, which is a major immunodominant antigen of many bacterial species. The interaction between expressed Hsp65 and the immune response induced by bacterial infection is hypothesized to be responsible for the initiation of the early atherosclerotic lesion (Xu, 1993). It has been suggested that chronic oral infection stimulates high levels of Hsp65 in subjects with high cardiovascular risk. Thus, if antibodies directed towards bacterial heat shock proteins cross-react with heat shock proteins expressed in the host tissue, especially if they are found in the lining of blood vessels, then some oral species might well be the link between oral infection and cardiovascular disease (Loesche, 1998).

Moreover, Wick and coworkers have postulated that an autoimmune mechanism in which the host responds to foreign hsp60, such as bacterial hsp, could be important in the development of an atheroma (focal deposit of acellular, mainly lipid-containing material on the endothelial lining of arteries).

In the end, the presence of periodontal infection may lead to brief episodes of bacteremia with inoculation of atherosclerotic plaques by periodontal pathogens such as *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus* and *Treponema denticola*. Subsequent growth of these bacteria would cause inflammation and plaque instability. Indeed, there is evidence using immunostaining and polymerase chain reaction for bacterial rDNA that these pathogens are present in 18% to 30% of carotid atheromas (Haraszthy , 2000; Cavrini, 2005)

As mentioned above, a large number of publications have suggested that oral infection, especially periodontitis, are a potential contributing factor to a variety of clinically important systemic diseases. Endocarditis has been studied most extensively. It appears that dental procedures and oral infection meet currently accepted epidemiological criteria for causation of endocarditis (Hill, 1965). However, there is still not sufficient evidence to claim a causal association between oral infection and other systemic diseases.
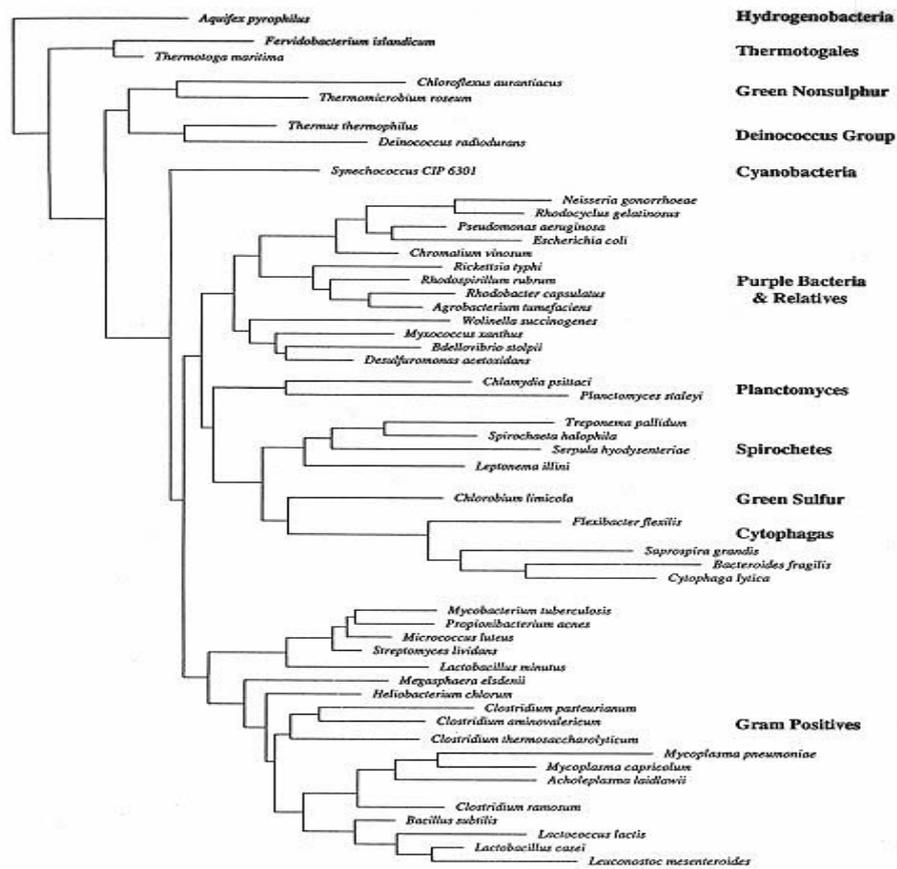
2. SPIROCHETES

2.1. *Spirochaetes*

The genus Treponema belong to a phylum of distinctive Gram-negative bacteria called *Spirochaetales* (Spirochaetes). Spirochaetes derive from a single ancient ancestor and thus represent an example of divergent evolution among bacteria. Characteristic of this phylum is their unique cell architecture. When observed under a dark field microscope Spirochetes present a long and slight cell helically coiled (spiral, helical or serpentine-shaped). These particular morphology causes a twisting motion which allows to penetrate into dense media and some species can penetrate into tissues (Lux, 2001). The capability to invade several tissues is associated with the periplasmic location of the flagellar filaments (Limberger, 2004). In particular, family of Spirochaetes are distinguished from other bacterial phyla by the location of their flagella, *axial filaments*, which run lengthwise between the cell wall (peptydoglican layer) and outer membrane. Flagella filaments wind around the body toward the center of the cell and may or may not overlap with one another in the center. The overall diameter of a filament is usually within the range of 16-25 nm. The typical spirochetes filament consists of multiple FlaB polypeptide species which constitute the core filament, and one or two multiple FlaA polypeptides, which make up the filament sheath. Several species of spirochetes are known to be pathogenic in humans, including *Borrelia burgdorferi*, the etiologic agent of Lyme disease, *Treponema pallidum*, which causes

venereal syphilis, and two other spirochetes related to *T. pallidum* which are the causative

agents of yaws (*T. pertenue*) and pinta (*T. carateum*).

Figure 3. Phylogenetic tree of bacteria. This figure is adapted from

Leptospira, a spirochete found in water and soil, may also be pathogenic to humans and

other mammals (Canale-Parola, 1977; Holt, 1978). Treponemes are also part of the human

normal oral flora. Being anaerobic, they reside mainly in the subgingival area. However,

they may take hold in opportunistic infections such as periodontal diseases, which are

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destructive, inflammatory processes of the tooth attachment tissues, caused by Gram-negative proteolytic anaerobic bacteria. Various spirochetal morphotypes can be observed in periodontal pockets, but many of these morphotypes are as yet uncultivable (Moter, 1998).

Spirochetes are the predominant microorganisms known to proliferate in periodontal disease sites among the bacterial flora. Although the relationship between periodontitis and oral treponemes has been emphasized clinically, cultivation studies of oral treponemes are limited because of the oxygen sensitivity and unique nutritional requirements of these microorganisms and the long cultivation period. The following species of cultivable oral treponemes have been validated: *Treponema amylovorum*, *T.denticola*, *T. lecithinolyticum*, T. maltophilum, *T. medium*, *T. parvum*, *T. pectinovorum*, *T. putidum*, *T. socranskii*, and *T. vincentii* (Umemoto, 1997, 2001; Wyss, 1999, 1996, 1997, 2004; Chan, 1993). These species are classified into two groups according to the fermentation of carbohydrates. The saccharolytic oral treponemes contain six species (*T. amylovorum*, *T. lecithinolyticum*, *T. maltophilum*, *T. parvum, T. pectinovorum*, and *T. socranskii*), and the asaccharolytic oral treponemes contain four species (*T.denticola, T. medium, T. putidum*, and *T. vincentii*). Among the cultivable oral treponemes, T.denticola is frequently isolated from sites of severe infection in patients with periodontitis (Riviere, 1992), and many studies have attempted to elucidate the role of *T.denticola* in periodontitis (Sela, 2001).

3. *TREPONEMA DENTICOLA*

3.1. Introduction

T.denticola is one member of the oral treponemes and play a fundemantal role in the pathogenesis and progression of periodontal disease.
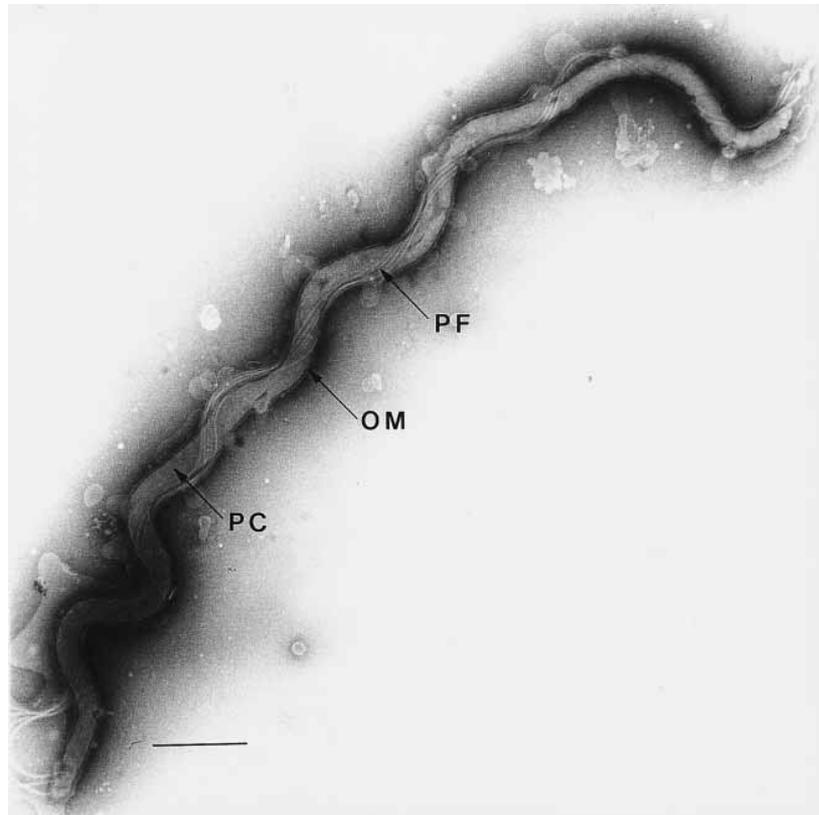

Figure 4. Morphology of an oral spirochete as seen under the transmission electron microscope after negative staining. PF, periplasmic flagella; OM, outer membrane; PC, protoplasmic cylinder. Scale bar 500 nm. This figure is adapted from Chan ()

These obligatory anaerobic bacteria have been recognized since van Leeuwenhoek first observed them almost 350 years ago.

During periods of oral health the number and distribution of these types of bacteria are low or nearly undetectable. However, during gingivitis and the progression to periodontitis there is a large increase in the number, proportion of the total population, and diversity of species. These rapidly motile, obligatory anaerobic gram-negative bacteria have been estimated to account for approximately 50% of the total bacteria present in a periodontal lesion (Moore, 1994). Chemically, the surfaces of gram-positive and gram-negative bacteria are composed of either ipoteichoic acid or lipopolysaccharide, respectively. When examined by electron microscopy, the profile of the outer sheath (outer membrane) is similar, if not identical to that of other gram-negative bacteria. However, Schultz et al. (Schultz, 1998) chemically analyzed the outer sheath lipids of *T.denticola* and found them to lack 3-deoxymanno-octulosonic acid, heptoses, and b-hydroxy fatty acids, typical of the lipopolysaccharides of many of the other gram-negative bacterial species. The proportion of fatty acids in the isolated lipid was also distinctly lower than that found in other lipopolysaccharides. The *T.denticola* outer sheath did contain large amountsh of the iso- and anteiso-fatty acids, supporting a similarity in solute movement across this membrane similar to other membranes. The lipid content of the outer sheath consists mainly of glycerol- based lipids, which are connected to several core sugars. Infrared analysis and Fourier transform studies have indicated that the outer sheath lipid of *T.denticola* is chemically similar to other gram-positive lipoteichoic acids, but similar to lipopolysaccharide in its function.

3.2. Treponemal architecture

A typical treponemal cell ranges between 5 and 20 μm in length and between 0.1 and 0.5 μm in diameter, depending on the species. It posses an outer membrane, a periplasmic space in which flagellar filaments reside, a peptidoglycan layer, and an inner membrane.
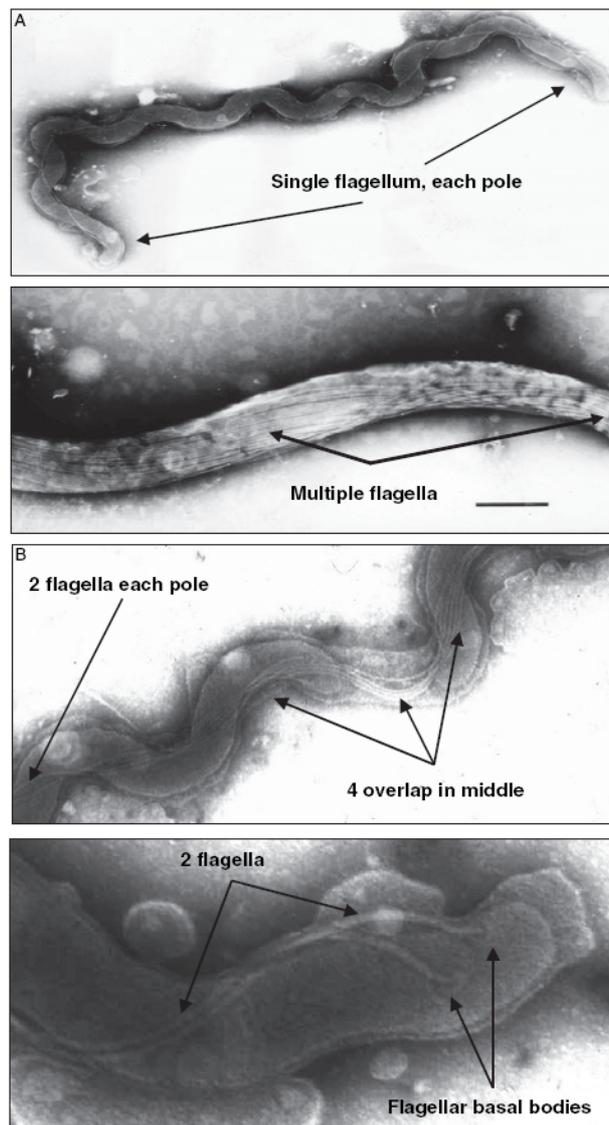

Figure 5. Electron photomicrographs of treponemes. A, Comparison of the size and number of periplasmic flagella in unidentified small (top) and large (bottom). The small spirochete fits into the frame of the figure and has one flagellum originating at each pole, in this case

not overlapping in the middle of the cell. The large spirochete is too long to fit in the frame; it contains multiple flagella originating at each pole. B) Electron photomicrographs of *Treponema denticola*, a small spirochete; two flagella originate at each pole and overlap in the middle of the cell. The cell in the top figure is in the process of dividing; it has already elongated its midsection and separated the cytoplasmic membranes for the daughter cells. They will soon synthesize two flagella at each new pole and the associated proteins that regulate flagellar rotation and cellular motility. This figure is adapted from Ellen and Galimanas (2005).

All natural treponemal cells are flagellated. Flagellar filaments of *Treponema denticola* are attached at one end of the cell, the other being free in the periplasm; the flagellar filaments originating at opposite ends of the same cell overlap at mid-cell.

The formation and rotation of the flagellar filament is associated with its attachment to the flagellar basal body, consisting of the motor, regulator, and export machinery, which is embedded in the cytoplasmic membrane, and protrudes from the cell end (Limberger, 2004). The basal body is composed of a cytoplasmic ring located on the cytoplasmic face of the inner membrane, the rotor-stator element embedded in the inner membrane, and a periplasmic ring located on the periplasmic face of the inner membrane (Limberger, 2004).
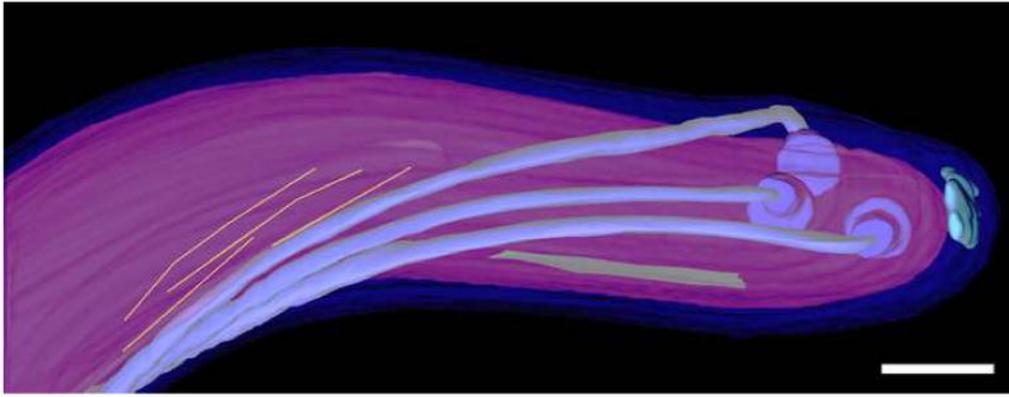
Figure 6. Diagrammatic rapresentation of a *Treponema denticola* cell. The figure show: the flagellar filaments arising from basal bodies (blue), a periplasmic patella-shaped structure (light blue), a plate-like structure (green), and cytoplasmic filaments (yellow). The outer membrane is dark blue, and the cytoplasmic cylinder is purple. This figure is adapted from Izard (2008).

The average diameter, from six periplasmic rings, was 29 nm, while the rotor-stator elements and the cytoplasmic ring were 65 nm and 55 nm in diameter, respectively. These results are in agreement with the described substructures of the flagellar basal bodies of *Treponema primitia* (Murphy, 2006). In contrast with *T. primitia*, *T.denticola* has, on average, two flagella at each cell end. At the cell end, basal bodies were often paired, with a spacing of at least 20 nm and a center-to-center distance of 90–100 nm.

Organized placement of the basal bodies was also observed in *Treponema phagedenis* cells, which have on average five flagella per cell end. This organization within the cell volume may be associated with a structural framework at the ends of the cytoplasmic cylinder.
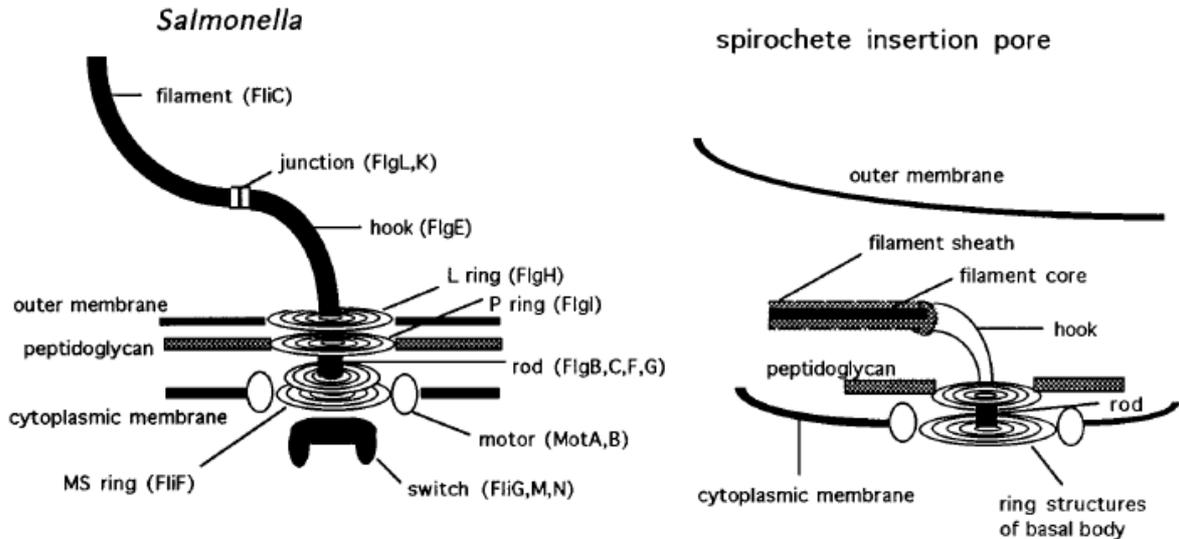
Figure 7. Diagram of the ultrastructural features and component proteins observed in the *S. typhimurium* flagellum and ultrastructural features of the flagellar insertion pore in cultivable treponemes. This figure is adapted from Heinzerling (1997).

The peptidoglycan layer of treponemal cells has not been clearly demonstrated. Treponema posses a cell wall that is at least partly responsibly for maintenance of the cell shape. Analysis of the treponemal cell wall by electron microscopy has revealed an electron-dense layer about 5 nm in thickness. The major constituents are glucosamine, muramic acid, D-glutamic acid, L- and D-alanine, ornithine, and glycine. In addition, whole genome analysis has revealed that most of the genes necessary for the protein and sugar moieties of the peptidoglycan formation has been demonstrated by Shesadri et al (Shesadri, 2004; Fraser, 1998). The outer membrane of the Treponema has been extensively studied using a variety of techniques. The protein components of the membrane are being identified, and their

relation to host immune system are being deciphered (Wang, 2001). Lipoprotein content of treponemal membrane are described in a separate paragraph.

3.3. Genome

A physical map of the *T.denticola* strain ATCC 33520 genome was generated by PFGE and hybridization with probe by MacDougall and Saint Girons (MacDougall and Saint Girons , 1995). The genome is a single circular chromosome of about 3.0 Mb in size and, like those of *T.pallidum* and *T.phagedenis*, contained two rRNA loci.

The genome of ATCC 35405 consisted by a chromosome of 2.8 Mb and differs in several ways from *T.pallidum* subsp. *pallidum* genome, encoding 2786 and 1040 putative ORFs, respectively. Infact, it is approximately 2.5 times larger, and yet exhibits little increase in biosynthetic capabilities. Moreover, the G+C contents is markedly different (37.9% compared to 52.8%), consistent with the genetic divergence between these Treponema species. While approximately 25% of T.denticola ORFs have best matches in *T. pallidum*, over 1000 ORFs did not have homologs in other spirochetes. Half of these are hypothetical proteins, the rest share homology with ORFs from gram-positive organisms such *Streptococcus* (oral and nonoral) and *Clostridium* species, and *Fusobacterium nucleatum*, also a component of dental plaque. As noted by Seshadri et al. (Seshadri, 2004) the larger genomic content of *T.denticola* may have occurred through mechanisms involving gene duplications, as evidenced by tandemly duplicated genes in the chromosome, and horizontal gene transfer exemplified by a 65-kb region that may have originated from phage-mediated transfer.
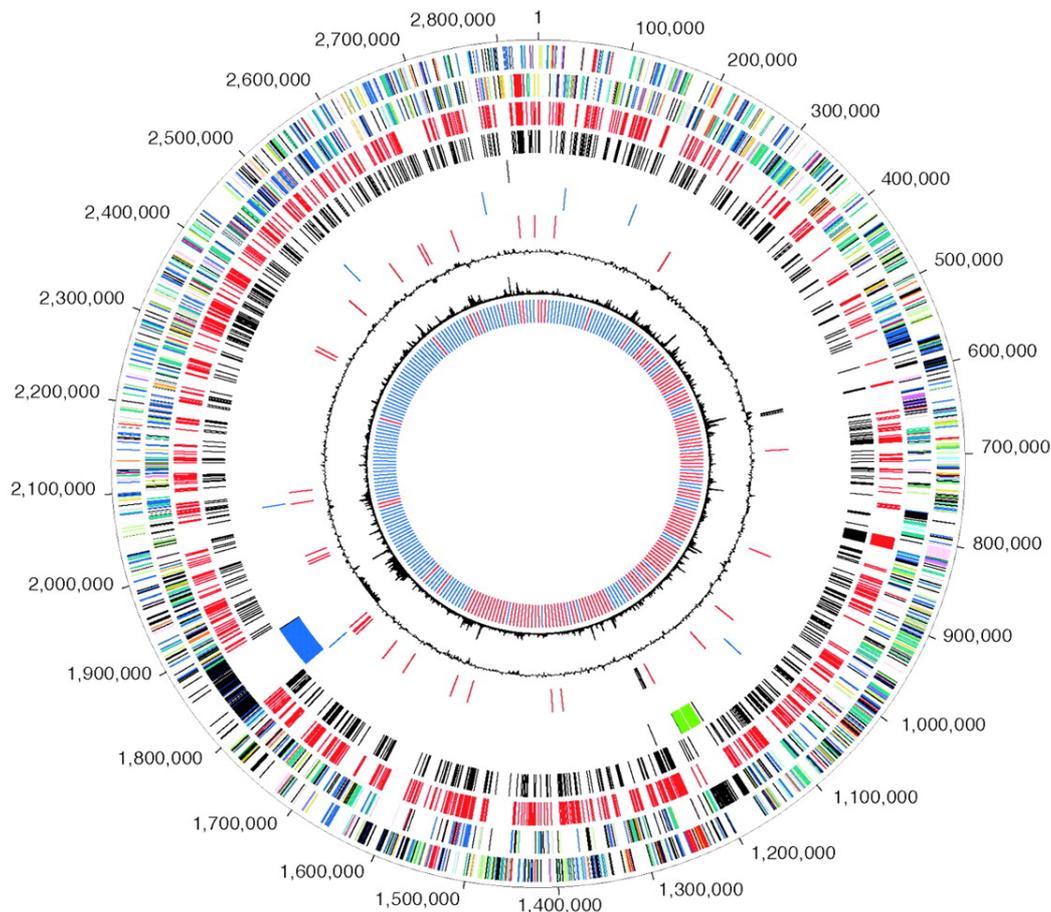
Figure 8. Circular representation of the *T.denticola* (ATCC 35405) overall genome structure. The outer scale designates coordinates in base pairs. The first circle shows predicted coding regions on the plus strand color-coded by role categories: violet, amino acid biosynthesis; light blue, biosynthesis of cofactors, prosthetic groups, and carriers; light green, cell envelope; red, cellular processes; brown, central intermediary metabolism; yellow, DNA metabolism; light gray, energy metabolism; magenta, fatty acid and phospholipid metabolism; pink, protein synthesis and fate; orange, purines, pyrimidines, nucleosides, and nucleotides; olive, regulatory functions and signal transduction; dark green, transcription; teal, transport and binding proteins; gray, unknown function; salmon, other categories; blue, hypothetical proteins. The second circle shows predicted coding regions on the minus strand color-coded by role categories. The third circle shows the core

34

set of CDSs conserved in all other sequenced spirochete genomes. The fourth circle shows CDSs with best matches to predicted CDSs in *T. pallidum*. The fifth circle shows putative phage regions and isolated phage genes. The sixth circle shows IS elements in black. The seventh circle shows rRNA genes in black and tRNA genes in red. The eighth circle shows trinucleotide composition in black. The ninth circle shows percentage G + C in relation to the mean G + C in a 2,000-bp window. The 10th circle shows GC-skew curve in red (positive residues) and blue (negative residues). This figure is adapted from Shesadri et al. (2004)

Of particular note was the finding that T.denticola possesses an unusually large number of genes encoding ABC-type drug efflux functions, 83 proteins representing 47 systems, more than any other sequenced prokaryote. It was proposed that they were involved with secretion of bacteriocin and host-damaging effectors as well as drug efflux systems. The genome sequence also revealed several new surface proteins that could potentially mediate binding to host cells and tissues. Furthermore, it was reported that some of their coding genes contained DNA sequences that may afford the potential for phase variation mechanisms (Seshadri, 2004). However, there have been no reports so far of antigenic heterogeneity of *T.denticola* genes encoding surface proteins, as observed with the *T. pallidum* tprK gene (Centurion-Lara, 2004).

Previously, it was hypothesized that a 65-kb region of the *T.denticola* 35405 genome may have been acquired by lateral gene transfer (Seshadri, 2004), and in a recent analysis the presence of a large integron cassette was discovered within the same region (Coleman, 2004).

Within gramnegative bacteria, integrons are important players in lateral gene transfer because of their ability to capture, rearrange, express, and spread antibiotic resistance genes. The *T.denticola* integron is the first identified outside the Proteobacteria, and is significant because of its large size (58 kb), its similar orientation to the integron integrase gene, and the relatively large size of cassette sequences that are the recombination sites for gene capture. The region possesses all the components for integron functionality, and raises the question of whether it confers on *T.denticola* the capacity to act as a reservoir and disseminator of antibiotic resistance genes (Coleman, 2004; Duncan, 2005).

3.4. Metabolism

T.denticola, such as other Spirochetes, can catabolize glucose via the Embden-Meyerhoff-Parnas pathway. However, *T.denticola*, otherwise from closer related Treponemal species (such as *T.pallidum*), does not utilizes glucose as its primary source but instead catabolises amino acids. In particular *T.denticola* utilizes serine, alanine, valine and cysteine as a energy sources (Canale-Parola, 1977). Canale-Parola (Canale-Parola, 1976) reported extensive studies on the arginine requirements of *T.denticola* cell. Evidence of amino acid fermentation comes from the presence of several selenium-dependent glycine resiductases (TDE0078-9; TDE0239; TDE0745; TDE2119-20). The origin of free amino acids needed for catabolism may be result of several proteolytic enzymes. Uitto et al (Uitto, 1988) reported a chymotrypsin-like protease present in *T.denticola* and suggested that it may play a role in the invasiveness of the organism because of its ability to hydrolyze serum proteins such as transferrin, fibrinogen, serum albumin and specific Immunoglobulin (IgA and IgG). This may partly explain why albumin is required by *T.denticola* for growth (Van Horn,

1983). In addition, Mikx et al (Mikx, 1997) demonstrated that the photeolytic capability of *T.denticola* ATCC 33520 is dependent upon the rate of growth and pH; greater proteolytic activity occurred at higher growth rates and at lower pH. Chu et al (Chu, 1994 and 1995) discovered a novel protein in *T.denticola*, cystalisin (TDE1669), which participated in the destruction of red blood cells and exhibited hemooxidative and haemolytic activities. It was later demonstrated that cystalysin was actually a cysteine desulfhydrase which degraded cysteine to produce H_2S, pyruvate and ammonia. The pyruvate from the breakdown of cysteine could be utilized for ATP production via Glycolisis pathway. Furthermore, cystalisin was shown to be present in several strains of *T.denticola* but not in other oral spirochetes. This enzyme appears to play a critical role both in metabolism and in the pathogenesis of *T.denticola*, see follow. Chu and co-workers (Chu, 2002) began an investigation of the metabolism of the trypeptide glutathione in *T.denticola*. Cystalisin was demonstrated to be incapable of degradating glutathione; cysteine was its sole substrate for catalysis. In 1997, Makinen and Makinen (Makinen, 1997) isolated a α-glutamyltransferase (GGT, TDE0444) supposedly from the outer envelope of *T.denticola*. Chu et al. (Chu, 2002) proposed that this enzyme could serve as the first in the degradation of glutathione to produce glutamate and dipeptide, Cys-Gly. This dipeptide could then be broken down by cysteinyl glycinase to produce glycine and cysteine, the latter being the substrate to cystalysin. In their work, they proposed that metabolism of glutathione could serve multiple biological purposes that are critical for the survival of *T.denticola*. In first, the H_2S produced may be critical for virulence as H_2S plays a major role in hemolysis and hemoxidation. Second, pyruvate, another byproduct of cysteine degradation, has been shown to enhance the growth of *T.denticola*. Lastly, eukaryotic cells usually have high levels of glutathione (up to 4 mM) for macromolecule synthesis, transport and enzyme regulation, so it is an

abundant source for the pathogenic spirochete to use for energy and supply of amino acids. TDE0444 was later cloned and its enzyme activities characterized (Chu, 2003). Of note, PSORT analysis of the amino acid sequence predicts that the enzyme is located in the cytoplasmatic, not the outer, membrane of *T.denticola*.

Genome evidence also support the metabolic data that *T.denticola* preferentially utilizes amino acids as both carbon and energy sources (Seshadri, 2004). 18 genes encoding protease including a serine protease (TDE0672), a glycoprotease ((TDE1468), a prtB protease ((TDE0346), a prtB protease (TDE2140), three genes for CAAX amino terminal proteases (TDE0275, TDE0716, TDE1870), a putative zinc metalloprotease (TDE2341), and five genes encoding ATP-dependent Clp protease (TDE1672, TDE1673, TDE2124, TDE2327, TDE2388). In second, there are at least 49 genes encoding amino acid and peptide permease for the transport of protease-digestion products into *T.denticola* cytoplasm. 33 of these genes code for ABC type permeases for oligo and di-peptide transport and 16 more code amino acid transport. In comparison, the genome of *T.pallidum*, a spirochete whose metabolism prefers to obtain energy and carbon from sugar fermentation, contains only five genes for amino acids transport.

3.5. Oxygen utilization and protective mechanisms

T.denticola is an oral spirochetes normally cultivated in vitro under anaerobic conditions. However anaerobiosis is not an absolute requirements for the cultivation of this spirochetes. *T.denticola* is especially sensitive to oxygen (Loesche, 1969). *T.denticola* cultures grow much better if the air in the head space is replaced with nitrogen or CO_2 and the E_h is below -125 mV. Caldwell and Marquis (Caldwell, 1999) reported that *T.denticola* utilized O_2 at

rate of 0.46 μmoles/hr/mg of cell protein. They found no heme-containing proteins, as cytochromes normally present in other oral spirochetes, but noted that the spirochete has very strong NADH oxidase (TDE1729) and four oxoreductases (TDE0134, TDE0685, TDE0868, TDE2643). Recently Lay (Lay, 2008) reported that demonstrated that *T.denticola* was able to generate microanaerobic environments in growth media for its survival and growth under aerobic conditions, by substitute H_2S with removal of dissolved O_2.

All these findings suggested that, although *T.denticola* lives predominantly in an anaerobic environment and it is capable of utilizing low levels of oxygen and protecting itself from oxygen radicals.

3.6. Motility of the treponema

General characteristics of spirochetes motility have been described in several studies (Canale-Parola, 1978; Charon, 1992; Li, 2000; Limberger, 2004).

Most Spirochetes are elically shaped, but some species have a flat sinuisoidal or meandering waveform. In addition to a typical bacterial plasma membrane surrounded by a cell wall containing peptidoglycan, referred to as the protoplasmatic cell cylinder (PC), they have an outer lipid bilayer membrane, referred to as either an outer membrane sheath, outer membrane, or outer sheath (OS). The space between the protoplasmatic cell cylinder and the outer membrane sheath is referred to as the periplasm or periplasmic space. Treponemal species have flagella that are similar in many respects to the external flagella of rod-shaped bacteria.
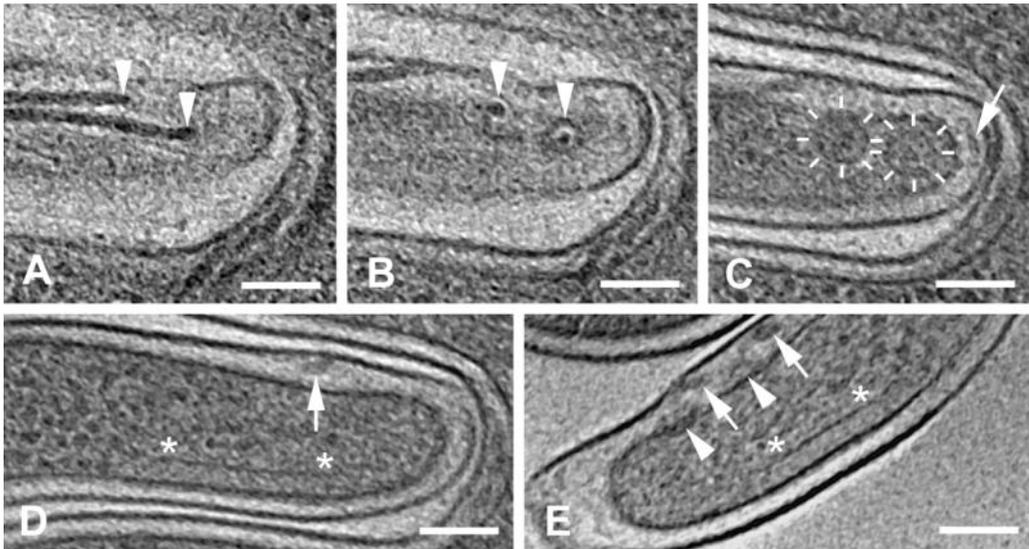
Figure 9. Flagellar filament basal bodies and profiles of a plate-like structure. (A) Slice at the level of the hooks (arrowheads), attaching the flagellar filaments to the basal bodies. (B) Slice 18 nm below A, showing upper rings of the flagellar basal bodies (arrowheads). (C) Slice 32 nm below B showing the larger lower rings of the flagellar basal bodies (indicated by radial line segments). Also seen in C is a cell-end patella-shaped structure. (D) Slice 34 nm below C, showing a flagellar filament (arrow) in a widened periplasmic space, and a profile of a plate-like structure, the two ends of which are marked (*). A–D are from cell WT-10. (E) Side views of basal bodies and hooks from cell WT-8. The periplasmic rings (arrowheads) are below the hooks (barely visible), giving rise to the flagellar filaments (arrows). This cell also has a profile (indicated by * at both ends) of a plate-like structure. Additional membranes from an adjacent cell are seen above and below the cell end of interest in A–D bars = 100 nm. This figure is adapted from Izard (2009).

However, the treponemas are unique in that their flagella located within the periplasm and, hence, are referred to as periplasmic flagella (PF). Each PF is subterminally attached to only

one end of the cell cylinder and extends toward the opposite end. Treponemal species vary with respect to size, number of PFs, and whether the PFs overlap in the centre of the cell. The PFs result to is the organelle necessary to the motility of Treponema. Although it has been directly proven that PFs rotate within the outer membrane sheath, several lines of evidence are strongly suggestive.

T.denticola has two PFs at each end that are long enough to overlap in the center of the cell. The cell bodies as seen in PF-less mutants, are right handed helices (Ruby, 1997). Isolated PFs are left-handed helices, with a helix diameter of about 0.26 µm and a pitch of 0.78 µm. Swimming cells often show a mixture of cell morphologies. Specifically, some cells are right-handed. However, translating cells often have an irregular, twisted morphology that can contain both planar and right-handed helical regions. In certain fraction of swimming cells, the PFs interact with the cell cylinder, and most likely with the PFs at the opposite ends of the cells, to cause An irregular shape. In addition, the outer membrane sheath is hypothesized to be necessary to keep the PFs and cell cylinder in close juxtaposition to one another to bring about this irregularity.

The flagellar structure is complex, with a rotary motor apparatus beginning in the cytoplasm and extending trough the cytoplasmatic membrane, peptidoglycan wall layer, outer membrane, and finally the elical flagellar filaments that contacts the ambient medium.

Several treponema species are known to be highly invasive in host tissue. In addition, quorum sensing, the ability for bacteria to sense density and regulate gene expression, is a key step in the regulation of virulence genes and biofilm formation in other bacterial species (Fuqua, 2001). Treponemes achieve high densities when attached to cells and tissues. In some species, motility plays an important role in biofilm formation. Biofilm formation of a non-motile mutants *T.denticola* is reduced, suggesting that motility may be important for

that species. Clearly, sorting out the relationship between motility, tissue invasion, quorum sensing, and biofilm formation are intriguing areas to understand the process of treponemes infection.

4. VIRULENCE DETERMINANTS OF

TREPONEMA DENTICOLA

4.1. Virulence determinants

Virulence is defined as the capacity of a microorganism to cause damage to its host. Therefore, virulence determinants of a pathogen should include all the properties that foster its colonization, its emergence in the microflora, and its synthesis and delivery of noxious stimuli to a vulnerable host. When considering indigenous species that become opportunistic pathogens under favourable environmental conditions, wheter to include properties that determine colonization and survival *per se* as virulence determinants is debatable, since such microorganisms may develop a longstanding neutral or amphibiotic relationship with the host, without causing damage.

Severity of periodontal tissue inflammation is associated with an increase in the total number and proportional distribution of spirochetes in the adjacent periodontal pocket, whether all or most or some or very few of the species are pathogenic is unknown.

Oral treponemes have evolved to occupy a unique miche. Physically, they concentrate subgingivally, on the biofilms and the epithelium that lines the gingival sulcus. Therefore, they localize in a position where they may bind and parasitize other microorganisms, where they may bind and parasitize host tissues, and where they may forage for nutrients from the gingival crevicular fluid of the periodontal pocket and the extracellular matrix or lysed cell of the periodontium.

Therefore, the capacity to inflict damage on the surrounding tissues is probably a closely linked consequence of their evolution to navigate and survive the conditions of the subgingival environment and to yield sufficient progeny to sustain the population of oral trteponemes. The chronic nature of periodontal disease, which gives rise to deepened, stagnant, anaerobic pockets: prolonged inflammatory degradation of host proteins and proteoglycans; and prolonged flow of a protein-rich serum transudate and blood into contact with subgingival biofilms, ceates ideal conditions for the emergence and survival of diverse treponemes. Therefore, for treponemes indigenous to the oral cavity, the capacity to survive is not only inseparable from their pathogenicity; virulence may be indispensable for survival of treponemal colony.

4.2. Adhesion to extracellular matrix and substrate degradation

Adhesion of bacteria to extracellular matrix (ECM) components is a characteristic shared by many pathogenic bacteria that penetrate peripheral host tissue. In particular, *Treponema denticola* binds several components of the epithelial and connective tissue ECM, including laminin, fibronectin, fibrinogen, and heparin (Dawson and Ellen, 1990; Haapasalo, 1991). Its polar orientation, binding perpendicular to ECM protein-conditioned surfaces, is similar to that of Treponema pallidum, and this characteristic function may provide an advantage that enhances growth (Ellen, 1998). *Treponema denticola* fibronectin-binding adhesins cluster, or cap, toward one pole when the bacterium migrates into contact with a fibronectin-coated surface (Dawson and Ellen, 1994). Therefore, adhesion probably helps *Treponema denticola* and other spirochetes localize close to a source of digestible proteins and peptides.

Prominent among the several *T.denticola* proteins capable to bind matrix-components, result the major surface protein (MSP). MSP have shown to posses the ability to bind a variety of matrix proteins and to bind host cells (Haapasalo, 1992; Mathers, 1996; Fenno 1996 and 1998). Amino acids sequence analysis of the MSP from the type strain ATCC35405 (Chan, 1993) predicts a series of membrane-spanning regions, with a few domains that have characteristics of hydrophilicity and potential immunogenicity and that may be exposed externally. Like porins of some other pathogenic bacteria, MSP has the capacity to insert in and transiently depolarize Hela cell membrane, presumably by establishing a short-lived ion-permeable channel (Mathers, 1996).

There are a number of a alternative ECM adhesins that are expressed by T.denticola. Several unidentified T.denticola polypeptides have been reported to absorb fibronectin from solution (Umemoto, 1993). Fenno and co-workers (Fenno, 2000) identified a 70 KDa T.denticola surface protein Opp A, which binds soluble plasminogen and fibronectin but not bind matrix proteins that are bound to surfaces. There is considerable interest in Opp A both as a potential multifunctional binding protein associated with an ABC transporter for peptides and as a surface protein that may absorb host proteins and thereby decorate the bacterium with proteins that would help it evade immune recognition.

Since enzymes recognize and bind their substrate selectively and stereochemically, it is likely that subtilisin family serine protease of *T.denticola*, dentilisin (PrtP), may serve as an additional adhesion that targets ECM proteins. It is also implicated in the specific binding interaction with the surface fimbriae of Porphyromonas gingivalis (Hashimoto, 2003), one of the other proteolytic species with which *T.denticola* cohabits in biofilms associated with periodontitis. Dentilisin is a lipoprotein that complexes with two accessory proteins (gene products of *prcA*) in the outer sheath to form a chymotrypsin-like protease complex (CTLP,

Uitto, 1988; Rosen, 1995; Lee, 2002). It is the major cell-associated protease of *T.denticola* that is known to degrade native ECM proteins and some other host proteins in the inflammatory cascade. Dentilisin hydrolizes and inactive substance P and angiotensin I (Makinen, 1995). It also has the capacity to active matrix metalloproteinase (MMP) (Sorsa, 1992; Ding, 1996), which in turn may modulate chemokine and cytokine responses as well as degrade ECM proteins like collagen. The signature peptidase activity of dentilisin is to cleave prolyl-phenylalanine bonds, including a crucial peptide bond in the protein encoded by the *prcA* transcript, forming the two necessary proteins, and which is ultimely essential for expression of the active cell-associated dentilisin anzyme (Lee, 2002). During early and exponential growth of *T.denticola*, most of the dentilisin activity is bound to the bacterial surface, and it is released as part of vescicles or shedding outer sheath fragments into the growth medium in older or drying cultures. Its co-localization with MSP may provide the bacterium a readily accessible source of degrade peptides in proximity to a porin that foster their uptake. Therefore, the adhesion of *T.denticola* to ECM proteins by either MSP or dentilisin would serve to promote colonization and subsequent gingival tissue invasion. Dentilisin's efficiency in generating small peptides for transport is probably augmented by *T.denticola*'s elaboration of additional endo-acting peptidases (Mikx, 1992), such as a prolyl oligopeptidase, a FALGPA peptidase (Makinen, 1995), and an arginyl-specific, trypsin like specific peptidase.

4.3. Tissue penetration

Oral spirochetes can be found within the gingival connective tissue in cases of acute necrotizing ulcerative gingivitis (ANUG) and many cases of chronic periodontitis (Riviere,

1991). Protease activity and directed motility would be two properties that are consistent with an invasive phenotype. Oral treponemes also elaborate hyaluronidase and chondroitinase activities that may contribute to degradation of the ECM (Scott, 1996). Dentilisn clearly has the capacity to rpomote the penetration of epithelial tissues by *T.denticola*. When in vitro monolayer of stratified cultures of oral epithelial cells were challenged with *T.denticola* or the native CTLP complex, the enzyme was subsequently found between cells and near the basement membrane. Such cultures became permeable to normally excluded molecules (Uitto, 1995). The CTLP has also been shown to foster the migration of *T.denticola* through an *in vitro* model of the ECM (Grenier, 1990). Inhibition of virulence coincident with the inactivation of a specific gene is considered compelling evidence that encoded bacterial product is a virulence determinant or is at least required for virulence.

Dentilisin (PrtP) is apparently crucial for the virulence of *T.denticola*, at least in terms of abscess formation in a confined wound. Yet, examination of the PrtP mutant K1 yielded a rich variety of pleiotropic effect: 1) slow but dense growth of bacterial cells tightly coiled around each other; 2) reduced surface hydrophobicity; 3) reduced coaggregation with bacteria of the genus *Fusobacterium*; and 4) altered expression of the outer sheath proteins including MSP. Several studies (Isihara, 1998; Fenno, 1998) reported that insertional inactivation of genes encoding either MSP or PrtP causes altered expression of the other surface proteins. Thus, dentilisin may affect virulence directly through its host tissue degradation (enhancing peptide acquisition), or indirectly by post-translational processing of the other virulence factor, or by coupling within outer sheath complexes that have evolved an orientation or conformation of proteins that fosters the optimum expression of virulence.

4.4. Motility

Since all spirochetes are naturally motile, which fosters their colonization of protective enviroments, foraging for nutrients, and avoidance of noxious stimuli, motility *per se* does not discriminate between virulent and avirulent treponemes. Since spirochetal locomotion is influenced by the viscosity of the medium, it is likely that the rather advanced chemotactic and periplasmic flagellar system of treponema motility evolved along with some of the species' invasiveness through the ECM. Experimental evidence indicates that the capacity to invade tissues differs among some groups of oral treponemes (Rivier, 1991). Whether the difference depends of their motility or other factor like adhesion, proteolysis, or differential processing of chemotactic signals remains to be studied. The general capacity of oral spirochetes to penetrate tissues chemotatically through the extracellular matrix probably accounts for the mixed nature of the treponemal morphotypes that have been observed at the forefront of acute periodontal lesions. Among the differ species of treponemes, there are a different innate capacity to penetrate tissues, as observed in the study by Riviere et al (Rivier, 1991).

Chemoattraction is significant in the ability of *T.denticola*, and presumably other oral treponemes, to invade the gingival tissue. The protein that comprise the structure, synthesis, export, orientation, motor and energy systems for flagella, and their function must provide the means for the bacteria to propel themselves there. Once within the tissue, flagellins from disrupted treponems may feasibly interact with cognate receptor, presumably Toll-like, on immunocompetent cells to stimulate innate immunity cascades that modulate inflammatory responses (Hayashi, 2001). How flagellar glycosilation may affect innate and adaptative

immune responses that determine pathogenesis of lesions in which treponemes have invadede remains to be investigated.

4.5. Virulence determinants: Dentilisin and the PrcA-PrtP subtilisin family

Dentilisin is the serine protease of the CTLP complex that clearly has properties of a key virulence determinant of *T.denticola*.

Chymotrypsin-like protease complexes appears to participate as well in the adhesion of T.denticola to host cells. The 72 kDa protein has been shown to be involved in a variety of functions, including the degradation of humoral proteins, basement membrane components (type IV collagen, laminin, and fibronectin, serum proteins such as transferrin, fibrinogen, IgG, IgA, and a1-antitrypsin, as well as bioactive peptides (Bamford, 2007; Makinen, 1995).
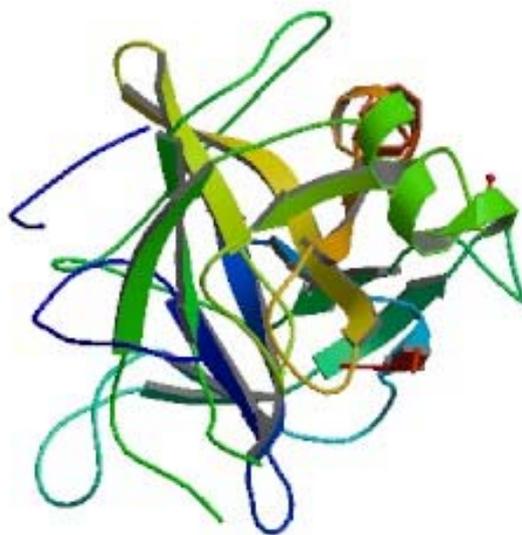
Figure 10. Tridimensional structure of the α Chymotrypsin

The protein has also been shown to be involved in the interaction of T.denticola with epithelial cells (257). Being an enzyme like subtilisin, the dentilisin can function in the destruction (hydrolysis) of substance P and angiotensin 1 (276). It also functions in the activation of selected matrix metalloproteinases, which in the polypeptide state are able to modulate host chemokine and cytokine activity (95, 406).
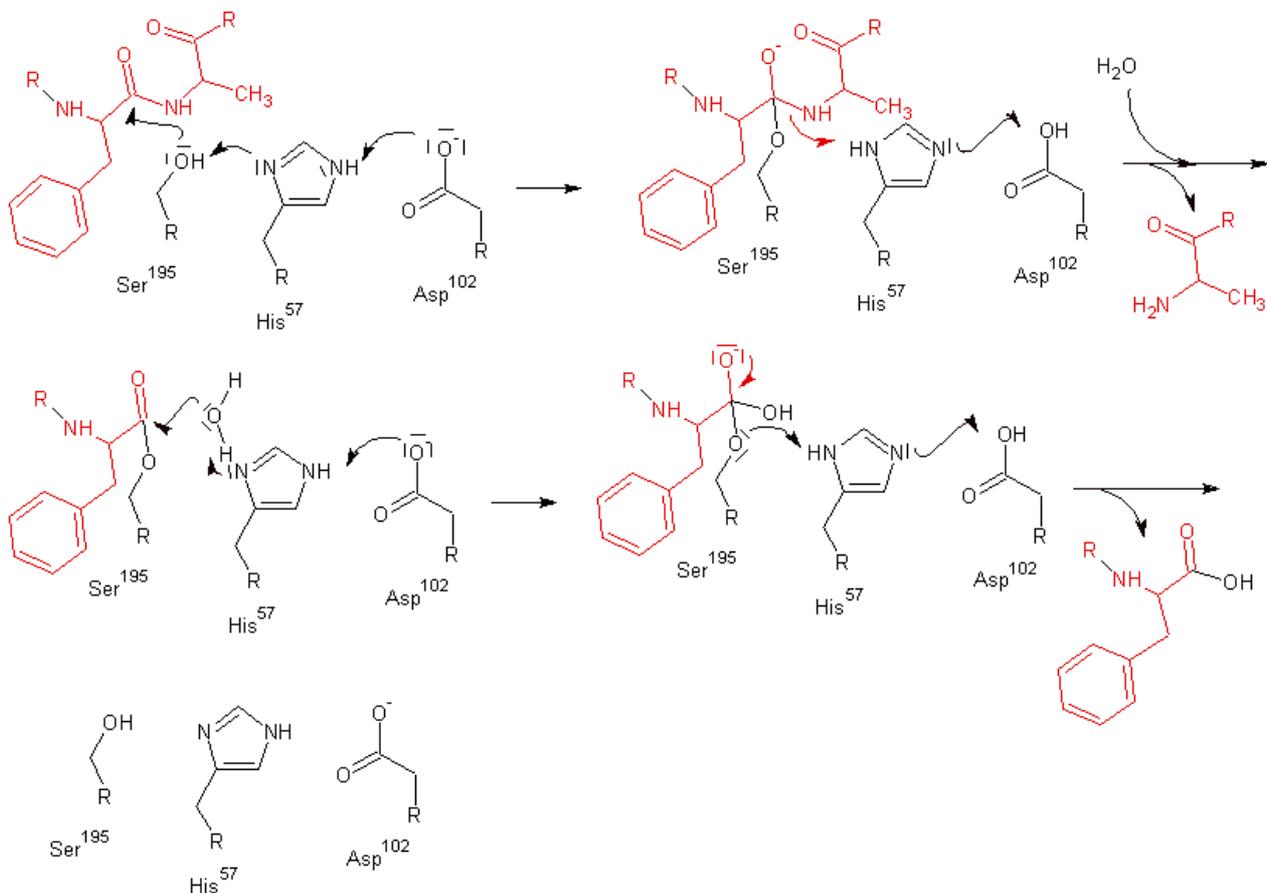
Figure 11. Mechanism of peptide bond cleavage in α-Chymotrypsin

Dentilisin has also been reported to function in the hydrolysis of collagen. While the exact mode of action of this outer sheath-located protein in T.denticola is unclear, it has been found that during exponential phase growth of T.denticola the protein is bound to its outer sheath. As the cells enter early to late stationary growth phase, the enzyme is released into the culture milieu in small, membrane vesicles. These vesicles, which contain numerous other proteins (derivative enzyme such as hyaluronidase, chondroitinase), might be central to the invasion and degradation of host cells and tissues (Fenno, 1998). Exposure of cultured oral epithelial cells to whole cells of T.denticola, or the isolated chymotrypsin-like protease complex, the enzyme appeared to localize between cells, very close to the basement membrane. In addition, these cells became leaky, indicative of an alteration of the membrane transport system. *In vivo*, if dentilisin is functional, it could be a major contributor to the inflammatory and destructive events in the progression of periodontal disease. Grenier and his colleagues (Grenier, 1990) showed that this enzyme (dentilisin) has the ability to move T.denticola across basement membranes, and as a result substantially increases the permeability of these membranes. The presence of intact tight junctions functions to maintain a barrier between the biological compartments on both sides of tissues by interfering with the flow of water, ions, and other small molecules from leaking between the cells.

Damage to the tight junction proteins will result in an alteration in permeability of the epithelial barrier and promote bacterial invasion of these cells, infection and inflammation – all hallmarks of periodontal disease. T.denticola has been shown capable of penetrating both endothelial and epithelial monolayers. Whether dentilisin is the source of this membrane destructive activity has not been clearly determined.

Chi et al. (Chi, 2003) have recently investigated the role of dentilisin in T.denticola penetration of epithelial cell layers. They compared wild-type T.denticola strain 5405 to a prt mutant. The wild-type strain was able to disrupt the epithelial layers by affecting cellular tight junctions and penetrating into the epithelial cells. In contrast, the mutant strain K1 displayed a decreased transepithelial resistance as well as showing poor epithelial cell penetration. Therefore, dentilisin (the chymotrypsin-like protease) more than likely plays a major role in T.denticola tissue penetration. Sorsa et al. (Sorsa, 1992) demonstrated that the dentilisin is capable of acting to direct proteolytic activation of human procollagenases, and therefore playing a potentially important role in host enzyme tissue destruction. Since epithelial cells are the initial barrier that the periodontal microbiota encounters at the gingival margin, the ability of this member of the red complex as well as other bacterial species to disrupt this barrier and penetrate into deeper tissue.

Two accessory proteins, PrcA1 and PrcA2, were first identified biochemically and assigned a variety of masses (Uitto, 1988; Rosen, 1995). Clarity was brought to the relationship between these proteins in terms of biosynthesis and processing, and theoritically in terms of their putative functional relationship, through molecular biology and expression studies: 1) the detailed characterization of the nucleotide and deduced amino acid sequence of the gene encoding dentilisin (*prtP*) and the upstream open reading frame (*prcA*) (Ishiara, 1996); and 2) the likely series of steps for post-translational processing of the *prcA* gene product by dentilisin itself to form the accessory proteins of the CTLP (Lee, 2002).

```
PrtP-I
192YGDKTVVAGIIDTGINMKHKDFK    247IPIGLNWDKGAHGTHCSGTIAAR    436GEDDYETMSGTSMATPFVTGTIA  T. denticola
175YGDKDVVAGIIDTGINMLHEDFK    230VPLNSNWDADGHGTHCSGIMCAL    419DVNSYVSLSGTSMAAPFVTGFVS  T. lecithinolyticum
174YGTNEVVAGIIDTGINMKHEDFK    226VPLDSNWDTDGHGTHCSGIMCAL    419STDSYVSMSGTSMAAPFVTGLIS  T. sp. OMZ 840
171YGDKDVVAGIIDTGINMLHEDFN    224VPLGSNWDTDGHGTHCSGIICAL    412TASSYVSLSGTSMAAPFVTGLIS  T. socranskii ss socranskii
   YG...VVAGIIDTGINM.H.DF.      .P...NWD...HGTHCSG...A.       ....Y...SGTSMA.PFVTG...     Consensus PrtP-I
PrtP-II
210DAKHPVLAAIVDSGVNGLHEDFY    260IPINDNWDDVGHGTHCAGTIAAV    458LRKSYTWMSGTSMATPFVTGTLT  T. lecithinolyticum
199AATNQVLVAIVDTGVNSLHEDLY    250VPINENWDNVGHGTHCSGTIAAV    448VKQSYLWMSGTSMATPFVTGTIA  T. sp. OMZ 839
200DAKYPVLAAIIDTGVNSLHEDFY    251ISLDENWDNHGHGTHCSGTIAAV    449VKSSYRWMSGTSMATPFVTGVVT  T. vincentii
   .A...VL.AI.D.GVN.LHED.Y      .....NWD..GHGTHC.GTIAAV       ...SY.WMSGTSMATPFVTG...     Consensus PrtP-II
Dual consensus
   .....V...I D.G.N..H.D..       .....NWD...HGTHC.G...A.       ....Y...SGTSMA.PFVTG...     Dual consensus
```

Figure 12. Aligned amino acid sequences for the two treponemal subtilisin proteases, PrtPI and PRTPII. Despite the conserved consensus sequence around the putative catalytic residue in both (bold, underlined Aspartic acid, Histidine, Serine), neither protease nor peptidase activity has been identified for PrtPII. This figure is adapted from Correia et al (2003).

```
PrcA-I         ↓                        ↓
        SAAAPL273SNAIFGRVFVIMERVPF290TNNTFS   Treponema denticola
        AAASDL260STCTFDKLSVNTERLPF277SENLFS   Treponema lecithinolyticum gene I
        NSTLDL268SSCSFRNLSAFAERLPY285SEGILS   Treponema sp. OMZ 840
PrcA-II
        PALTDI267SGSSVKDFRIEMHRFPH284SLKLFS   Treponema lecithinolyticum gene II
        PGLKSM268TGARIKKFRVEMHRFPH285SLKLFN   Treponema sp. OMZ 839
        PAIKDM52NGASVKNFRVEMHRFPH69 SLKLFS    Treponema socranskii ss "04"
        PALKTI267EGASIQEFRVEMRRYPH284SLKIFN   "Treponema vincentii"
```

Figure 13. A possible explanation may lie in a nonconservative histidine substitution for phenylalanine of accessory protein PrcAII that is signature target peptide substrate (includin PrcAI) digested by PrtPI subtilisins like dentilisin. This figure is adapted from Correia et al (2003).

It is thought that the co-transcribed PrcA and PrtP have a reciprocal dependence, in that cleavage of the *prcA* gene product by PrtP is necessary to generate the accessory proteins that, in turn, are required for functional "maturation" of the CTLP in the outer sheath (Lee, 2002).
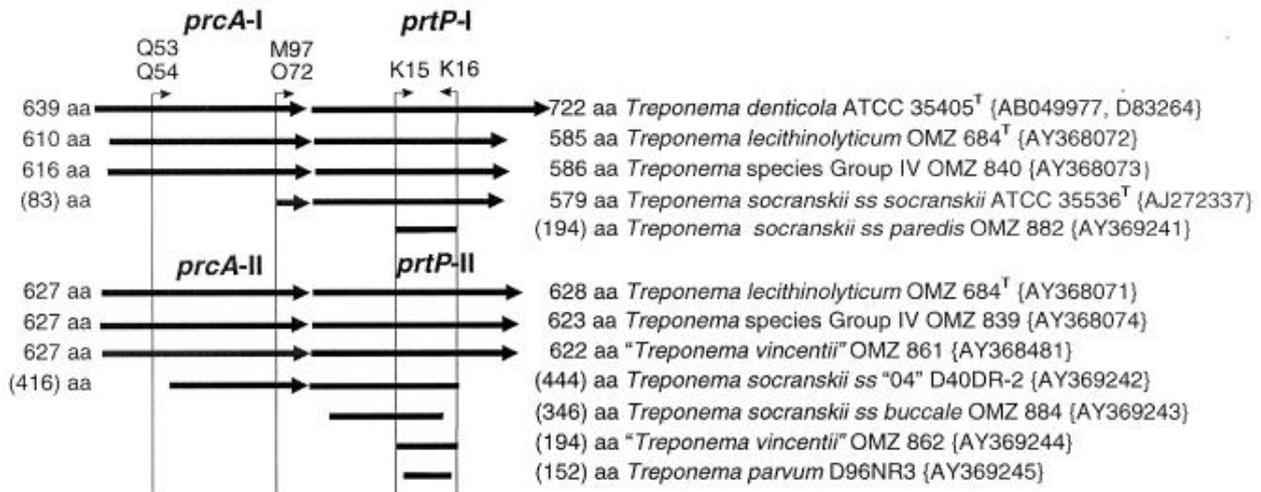
Figure 14. Operon map of sequenced genes *prcA* and *prtB*. Solid lines with arrowheads indicate sequenced portions of each ORF, drawn to scale. The amino acid length of each ORF is indicated, without parentheses for completed sequences and with parentheses for incomplete sequences. The positions of key degenerate primers used to amplify the ORFs are indicated by vertical lines below small, labeled arrowheads. Paralog names and group numbers are indicated above the appropriate ORFs. Genus, species, and strain designations for each ORF are indicated at the right. The prtP gene encodes the active protease dentilisin, and the prcA gene encodes the two accessory proteins of chymotrypsin-like protease complex of treponemal outer sheath. This figure is adapted from Correia et al (2003).

Indeeed, Ishihara and co-workers reported that insertional inactivation of the ORF now called *prcA* yields transcribed but functionally inactive dentilisin. Whether the PrcA proteins serve as chaperones or in some other way sterically integrate dentilisin in a functional orientation in the outer sheath remains to be address specifically.

4.6. Virulence determinants: MSP (Major surface protein)

MSP, the major surface protein of oral spirochetes is the most abundant protein in the outer membrane of *T.denticola*. In its native oligomeric form, MSP is visible as a hexagonal array in the outer membrane of *T.denticola* (Fenno, 1996, 1997, 1998).
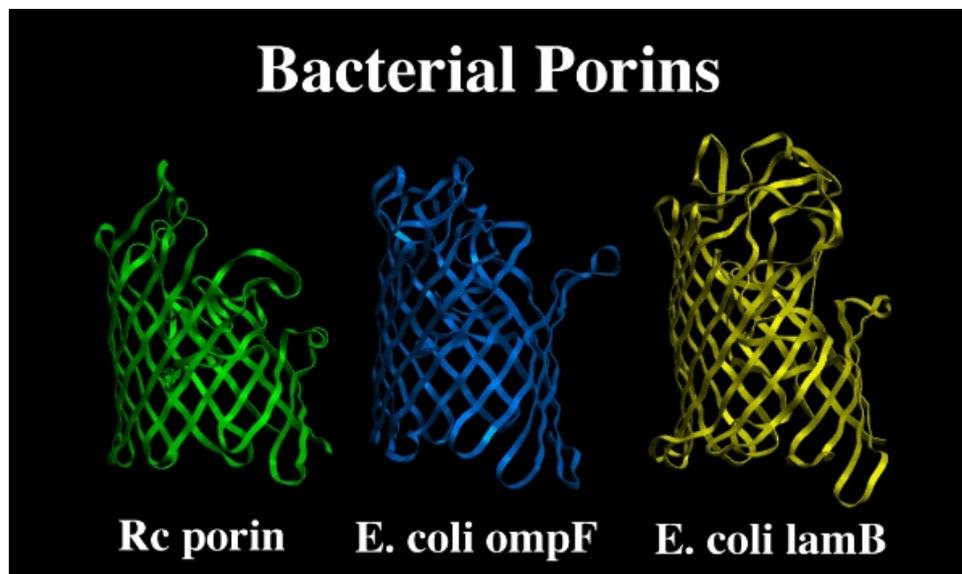
Figure 15. Porins of the outer membrane of Gram-negative bacteria.. The pore structure is formed almost entirely of a beta-barrel; the monomeric protein is matured into a trimeric species which is integrated into the outer membrane.

The apparent molecular weight of monomeric MSP's and MSP -like proteins varies among oral spirochete strains between approximately 42 kDa and 64 kDa. MSP is an adhesin (Hapaasalo, 1992) with pore-forming activity, both in artificial membranes (Egli, 1993) and in epithelial cell membranes (Mathers, 1996).

53 kDa MSP of *T.denticola* ATCC 35405 bound soluble and insoluble forms of fibronectin and laminin (Haapasalo, 1992; Fenno, 1996; Umemoto, 1994). Native and recombinant MSP adhered similarly to extracellular matrix components and to glutaraldehyde fixed periodontal ligament epithelial cells (Fenno, 1996 and 1997). Adherence of *T.denticola*, native MSP or recombinant MSP was inhibited by pretreatment with anti- MSP IgG. MSP bound a 65 kDa HeLa cell surface protein and at least two other apparently cytoplasmic proteins (Mathers, 1996).

Both native and recombinant MSP were highly cytotoxic to epithelial cells and erythrocytes, but fibroblast cultures were relatively resistant to MSP. Cytotoxic effects of MSP were inhibited by preincubation with anti- MSP IgG (Mathers, 1996). Porins of other Gram-negative pathogens have been implicated as mediators of *in vitro* cytopathic effects, including bone resorption, increased cytokine release and decreased leukocyte chemotaxis, and inhibition of phagocytosis by monocytes. A study implicating *T.denticola* outer membrane components in bone resorption activity (Gopalsami, 1993) is significant in this light, and suggests an area of study that requires attention for the characterization of cytopathic effects of MSP. The mechanism of MSP pore formation is not yet known, however the ability of MSP to bind specific epithelial cell receptors suggests that a binding event may mediate the initiation of pore-forming activity of MSP in cell membranes.

```
405  MKKILAILMILVLVGGVAFAQLTPQVTAKASVNWGIDFGAGKNAKAQHGFENLLDAKVVIPLYMGTLNSKTEGDVRMNFDLGVNLAYRFYDQLSNSASPH 100
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
520  MKKILAILMILVLVGGVAFAQLTPQVTAKASVNWGIDFGAGKNAKAQHGFENLLDAKVVIPLYMGTLNSKTEGDVRMNFDLGVNLAYRFYDQLSNSASPH 100

     VAWDSDAEFKLWRKTLSDMSASIHFFGGYMNVYGRPDFSTNYAQIWSPIRDNGVAWGPETSGDITGFGTKLGYASDDLAGTGLKFDAGLKFGSNGSWKAK 200
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     VAWDSDAEFKLWRKTLSDMSASIHFFGGYMNVYGRPDFSTNYAQIWSPIRDNGVAWGPETSGDITGFGTKLGYASDDLAGTGLKFDAGLKFGSNGSWKAK 200

     GTTAQDKFKVVDLKVGDTLIAGATYYKQN....GIDAEGKPI..FSSTAAVFAAPPTVGAGEDGKYLVKTASTATGPAANKYAFGLDLSLGYDKWVTLDF 294
     |||.:.| .:.|.| .|||: |:|.|||:    .:.|:|. : :.:..:. |.:||:|||.|.| ||...|||.|||||||||||||||||||||||||
     GTTSEKK.EIVTLAAGDTVDAAAKYYKFDPSLFTLSADGNLVINLKGKTPFTFAGATVAAGEAGQY.VKVTTTATGSAANKYAFGLDLSLGYDKWVTLDF 298

     GINATFDNVKDFGKAGVHEDVAAGSNPDKPYLGMGLKLGSKPVDGLALTLAMDALMNVGTDSKVAFDLRFDASYKWVALGAYFGNDLSAYAGKDKNNKAI 394
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     GINATFDNVKDFGKAGVHEDVAAGSNPDKPYLGMGLKLGSKPVDGLALTLAMDALMNVGTDSKVAFDLRFDASYKWVALGAYFGNDLSAYAGKDKNNKAI 398

     GDMAAMIAFKSAASGDTNFVEGLAFGVDFRLNHLLSAVPTGDKSTLPMGISAWVNYKYALTDSMWVKPYANFWGETNRKSIDASKTDKFFGVAYKVGATF 494
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     GDMAAMIAFKSAASGDTNFVEGLAFGVDFRLNHLLSAVPTGDKSTLPMGISAWVNYKYALTDSMWVKPYANFWGETNRKSIDASKTDKFFGVAYKVGATF 498

     SPAEKIEIDTYWSQGKLSWNKYEGTEGMISAPAFDAHNGTFVIGVKVIY 543
     |||||||||||||||||||||||||||||||||||||||||||||||||
     SPAEKIEIDTYWSQGKLSWNKYEGTEGMISAPAFDAHNGTFVIGVKVIY 547
```

```
405  MKKILAILMILVLVGGVAFAQLTPQVTAKASVNWGIDFGAG..........KNAKAQHGFENLLDAKVVIPLY....MGTLNSKTEGDVRMNFDLGVNL 85
     |||||.||||||||||||||||:.|:| :.|.:.|||||||          .:| | ||| | : .| :|:: :. ....|:||
OTK  MKKILSILMILVLVGGVAFAQVEPEVRGSATLSWGIDFGAGSETDYTKGGADNHAVATHGFYNTNNFQVRLPFFKRQDFAGGSASKEADV......... 90

     AYRFYDQLSNSASPHVAWDSDAEFKLWRKTLSDMSASIHFFGGYMNVYGRPDFSTNYAQIWSPIRDNGVAWGPE.TSGDITGFGTKLGYASDDLAGTGLK 184
     |.::| .|.|    ..::. . |.:| :.| :||:|:||.|||:||..| | |.||..::. |.:  ..:||:|||:|| ..|| |
     ....YADVSFKATP.....VATGWTESGKAVS.VKAILHFYGAYMTVYGKPDPKANKALGWDPINADDGDQGKLWFNPAFTGWGTKIGYKKADLMG.... 176

     FDAGLKFGSNGSWKAKGTTAQDKFKVVDLKVGDTLIAGATYYKQNGIDAEGKPIFSSTAAVFAAPPTVGAGEDGKYLVKTASTATGPAANKYAFGLDLSL 284
     :|.|||:|||||..|.                                                        :.|.||  ..|||||||::.:
     LDVGLKLGSNGSWHQKA........................................................SDGKDGKRREK........DSKYAFGLDFAM 216

     G.YDKWVTLDFGINAT.........FDNVKDFG.KAGVHEDVAAGSNPDKPYLGMGLKLGSKPVDGLALTLAMDALMNV...GTDSKVAFDLRFDA.... 366
     : :|::.:.|||| ::|. |:| .|| .. :|.:... :.:|| : |.|::::.:.|::|:: .: .::|:. .| :||
     EPVEKYLGVEATINATLGKFFNKTPLENPGDYGYNAGNVPANIGGKEGKSGMVNFGLAFKSEPIEEMTVKLGFDGVTGLTYTDNEGKIQETLGWDAGLSV 316

     SYKWVALGAYFGNDLSAYAG.......KDKNNKAIGDMAAMIAFKS..AASGDTNFVEGLAFGVDFRLNHLL................SAVPTGDK... 437
     :||||. : |:... .|.|    ..| . ::||| ::| | .|. :|.||:|||| |:. .|| :|:.| |
     GYKWVDAALYLCGTGTKYQGYNVAGNDHTKWKNYGANMAAHLGFVSDEEAKKGTGFVDGLAFHVTINAYDLLAKRNSEDIPEATRKANIGALSTYDAALT 416

     ....................................................STLPMGISAWVNYKYALTDSMWVKPYANFWGETN....RKSIDASKTDK.....FFGVAYKV 490
     ..:|:|:. |.|||.|||||:||||:|::|||| : .. |:..|| :|:.| .:
     AYLEANPNKTEADFQADPANAAVVNAFKVAKALFKTKYASIPLGLNLGVSYKYDLTDSMWIKPYAEFYAETNHYKQHADTYAGDPDKMIDKLYLGMAYEL 516

     GATFSPAEKIEIDTYWSQGKLSWNKYE....GTEGMISAP.AFDAHNGTFVIGVKVIY 543
     |..| | |||:::. |.:|||..::||    |.| ||..|  . . |||||:::|:.|
     GVKFQPIEKVQVKASWEHGKLNKDTYEGSFNGGEYMIKTPLNHKMHNGTFVLSLKLTY 574
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Figure 16. Optimal alignments of the deduced MSP amino acid sequences of *T.denticola* ATCC 33520 (520) (A) and OTK (B) with that of ATCC 35405 (405; A and B). Identical residues (P), conservative substitutions (;), and neutral substitutions (I) (7) are indicated between the aligned sequences. This figure is adapted from Fenno (1997).

The MSP proteins of *T.denticola* and *T. vincentii*, while showing considerable interstrain variation in molecular weight and antigenic domains, are encoded by a highly conserved genetic locus. Sequence homology between strains was very high in DNA flanking the *Msp* coding region. Deduced amino acid sequences of three antigenically distinct MSP peptides showed nearly identical signal peptide sequences, while interstrain homology of the mature MSP peptides was as low as 50% (Fenno, 1997). The *Msp* locus was not detected in *T. socranskii* or *T. pectinovorum*, two species of oral spirochetes that have prominent, heat-modifiable MSP-like outer membrane proteins of approximately 42 kDa.

A 42 kDa MSP -like outer membrane protein (designated MompA) of *T. pectinovorum* which is present in the type strain and in a number of clinical isolates had no N-terminal amino acid homology with *T.denticola* MSP (Walker, 1997). These species not been reported to display the hexagonal array outer membrane ultrastructure typical of all strains in which MSP and the gene encoding it were definitively identified.

The MSP peptide is encoded by a single genetic locus in strains of *T.denticola* and *T. vincentii*. MSP peptide was reported to be homologous to predicted products of a number of repetitive sequences present in the *T. pallidum* genome. While there have not yet been reports of expression of these MSP homologues in *T. pallidum*, it is intriguing to speculate on the significance of these homologies and on the possible role that MSP-like proteins could play in chronic infectious diseases other than periodontal disease. There have been no studies describing antigenic variability of outer membrane proteins in *T.denticola* strains, such as is well-known in *Borrelia* species. However, the differences in MSP peptide sequences between some strains of *T.denticola* are confined to the predicted surface-exposed regions (Fenno, 1997), suggesting, at the very least, strong selection pressures for interstrain variation in this prevalent outer membrane protein. Destructive periodontal

disease lesions can contain multiple strains and species of cultivable and uncultivable spirochetes. Even without a genetic mechanism of antigenic variation, the wide variety of MSP proteins present in this heterogeneous population could ensure a continuing source of 'new' (or at least newly predominant) strains following inflammatory responses to successive strains.

All of the MSP peptides characterized thus far have a predicted membrane topology similar to that of bacterial porins and other porin-like molecules, including pore-forming cytotoxins. The N-terminal signal sequence is the only strongly hydrophobic region of any of the MSP peptides. The mature MSP peptide contains a series of regions with predicted amphipathic â-sheet secondary structure that could form a hydrophilic membrane-spanning pore (Fenno, 1996). Sequence variability between MSP peptides is most strongly evident in the region corresponding to MSP residues 200–275 (denominated as V-Region by Ewards) (Ewards, 2005) of ATCC 35405. This region was predicted to form a large, extracellular loop (Fenno, 1997), resulting the major antigenic epitopes of MSP.

Recently Edwards and co-workers reported that the V-region region has three features that could be considered critical to the function of the polypeptide. First this specifically region appears to carry major adhesion-mediating sequences.
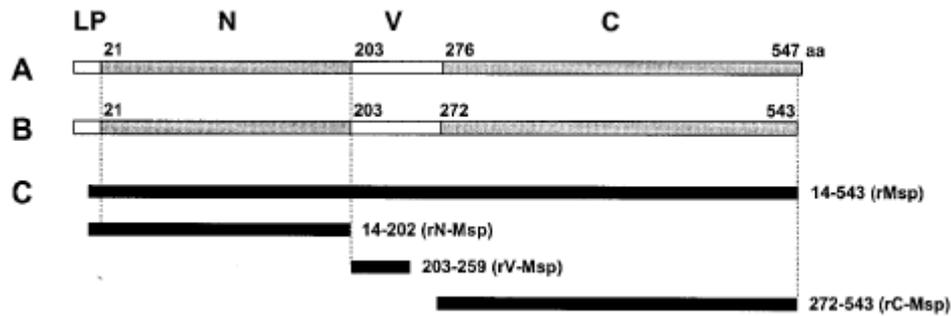
Figure 17. Diagrammatic representation of the *Msp* sequences from *T.denticola* ATCC 35520 (A), strain ATCC 35405 (B), and of recombinant MSP polypeptides r MSP (530 aa residues), rN-MSP (189 aa residues), rV-MSP (57 aa residues), and rN-MSP (272 aa residues), with N-terminal His6 tags derived from the *Msp* sequence of strain ATCC 35405 (C). Designated regions of MSP, based on amino acid sequence conservation between ATCC 35520 (GenBank accession no. U66255) and ATCC 35405 (no. U29399) were as follows: LP, leader peptide (20 aa residues, 100% identical aa sequences); N, amino-terminal region (100% identity); V, variable region (32% identity); and C, carboxy-terminal region (99.6% identity (one aa residue change). This figure is adapted from Ewards (2005).

The second feature demonstrated is antigenic variation. This is a strategy frequently utilized by pathogenic bacteria to evade host immune defenses.

The V region contains the dominant B-cell epitopes for animals immunized with *T.denticola* cells. The V region is thus highly immunogenic and would provide a major target for the host immune system. This provides an explanation for why antibodies raised to closely related MSP proteins, which differ only in their V-region sequences, have been shown to not cross-react. On the other hand, the invariable N and C regions (corresponding to N and C-terminal of the mature MSP protein), which may be important for maintaining structure

and function, are not significantly immunogenic. A third feature is that, on intact cells, there may be a mechanism by which accessibility of MSP is regulated. In vivo this would potentially avoid immune responses that might be harmful to the organism.

The calculated size of the MSP porin channel (3.4 nm) is the largest reported for any bacterial porin, nearly three times that of *E. coli* OmpF (Egli, 1993). Very large diameter porins have been reported in two other spirochetes, the free-living *Spirochaeta aurantia* and the human pathogen *B. burgdorferi*. Similar to *T.denticola* MSP, the oligomeric native form of the 36 kDa porin of *S. aurantia* was by far the most predominant outer membrane protein. A 66 kDa protein (P66) of *B. burgdorferi* had several important characteristics in common with MSP in addition to its similar molecular weight and interstrain heterogeneity. Both P66 and MSP were exposed on the cell surface and localized to the aqueous phase of Triton X-114 surface extracts of the bacteria (Fenno, 1997; Bunikis,1995). While amino acid sequences and predicted secondary structures of both chromosomally encoded proteins were, to varying extents, conserved between strains and species, there appeared to be heterogeneity in surface domains (Fenno, 1997).

Initial characterization of MSP suggested that the extremely large diameter pore might serve in the uptake of large molecules such as oligopeptides (Egli, 1993). The combination of adhesin and cytotoxic activities attributable to MSP greatly expands its role in virulence beyond that of passive nutrient uptake. Conservation of MSP-like genes encoding antigenically distinct MSP molecules is consistent with the evidence that MSP is involved in interactions with host tissue. Important questions remain to be addressed, including the evolution of variability of the MSP, characterization of its functional domains, and further characterization of its interaction with specific host cell molecules.

4.7. Other enzymes and metabolic products

There are a number of other enzymes and metabolic products of oral spirochete metabolism that could have cytotoxic effects in periodontal tissue. These 'normal' products or activities are seldom classified as specific virulence factors, but it is useful to consider them in the discussion of bacterial factors that may contribute to periodontal disease. Several strains of oral spirochetes were shown to secrete a phospholipase C (PLC) activity that might directly or indirectly damage tissue by hydrolysis of membrane phospholipids (Siboo, 1989). The PLC activity was not associated with the bacterial membranes, but was found in culture supernatants. This was confirmed by an agar plate assay in which PLC activity was detected distal to spirochete colonies. A 66 kDa protein with PLC activity was purified by lecithin affinity chromatography from *T.denticola* culture supernatants.

No direct evidence of involvement of the spirochete PLC activity in cytotoxicity has been reported. Oral *Treponema* species produce a variety of short chain fatty acids including acetate, *n*-butyrate, propionate, and succinate (Grenier, 1992). Short chain fatty acids produced by treponemes and other periodontal organisms are present in significant concentrations in subgingival plaque and deep periodontal pockets Butyrate at concentrations typically present in periodontal pockets induced apoptosis in thymocytes and T cells. Similar concentrations of butyrate and propionate inhibited growth of cultured periodontal ligament epithelial cells, fibroblasts and endothelial cells (Pollanen, 1997; Kurita-Ochiai, 1997; Tse, 1992), and inhibited lymphocyte and PMN proliferative and chemotactic responses (Eftimiadi, 1987). Of the total amount of cytopathic short chain fatty acids present in periodontal lesions, the amount due to spirochete metabolism is not

known. In addition to potentially cytopathic fatty acids, oral spirochetes produce several volatile sulfur compounds, including hydrogen sulfide (H2S) and methyl mercaptan (Tanner, 1992). Final H2S concentrations were greater than 0.2 mM in cultures of *T.denticola* grown in human serum for 7 days. Of 75 species of oral bacteria that produced significant amounts of H2S, only *Porphyromonas gingivalis* and two *Prevotella* species produced comparable amounts. H2S has a number of potentially cytotoxic effects that are primarily due to inhibition of cytochrome oxidases, but distinct effects of H2S production by oral spirochetes have not been described. However, the 46 kDa 'hemolysin' of *T.denticola* has been further characterized as having cysteine desulfhydrase activity, producing equimolar amounts of H2S, pyruvate, and ammonia from cysteine (Chu, 1997).

Chapter II

OBJECTIVIES

Oral treponems are indigenous bacteria that become opportunistic pathogens in the mixed microflora that colonizes the periodontal pockets. Among the different bacteria, *Treponema denticola* result to be the most extensively studied oral spirochete. This bacteria is able to bind and to degrade extracellular matrix and theraby probably contributes to the disruption of the gingival epithelial barrier and bacterial penetration of the underlying connective tissue.

On the basis of theese observation, the aim of my research during my PhD work was to understand the effective role of *Treponema denticola* and its major virulent factor in the interaction with several cells tissue and its possible host immune evasion by macrophages uptake.

In particular my study was divided into different study:

1. First study was to assess the role of the *T.denticola* MSP in the stimulation of human peripheral blood monocytes to induce the production of different pro-inflammatory cytokines and MMP-9.

2. The second study of my PhD work was involved in the investigation of HO-1, Hsp70 expression by primary culture of porcine aortic endothelial cells (pAEC), subsequently stimulation with the Outer Membrane of *T.denticola*.

3. The third study principally was involved in the evaluation of the influence of *T.denticola* motility and cell size, and bacteria opsonization on the uptake by mouse peritoneal macrophages, *in vitro*.

4. In the end I evaluate the possible conservation of the *msp* gene and deduced amino acid sequence in a patients affected by periodontal disease at different stages, to determine the possible evolution in the principal T.denticola virulence factor.

Chapter III

MATERIALS AND METHODS

1. MSP induces the production of TNFα, IL-1β, IL-6 and MMP 9 by CD14 positive cells

Bacteria culture and preparation of the MSP. *T.denticola* strain ATCC 35405 was grown in NOS medium (see Supplemental Materials paragraph) at 37°C under anaerobic conditions for four days. The extraction and purification of MSP was performed as described follow in the Supplemental Materials paragraph.

Limulus test. The Limulus ambocyte lisate (LAL test, International PBI, Milan, Italy) assay was performed on MSP preparations, in order to assess the absence of contamination by LPS-like material.

Monocyte isolation. Peripheral blood mononuclear cells obtained from healthy blood donors at the Sant'Orsola-Malpighi central Blood Bank, were separated from buffy coats by Histopaque-1077 (Sigma-Aldrich Chemicals, S.Louis, Mo, USA) density gradient centrifugation according to the manufacturer's instructions. The cells were then selected by using the Miltenyi monocyte isolation kit II human (Miltenyi Biotec, Bergisch Gladbach, Germany) as described follow in Supplemental Materials paragraph, and characterized by

flow cytometry analysis with anti CD14 specific FITC labeled monoclonal antibody and with anti CD163 PE labeled monoclonal antibody (BD Bioscience, Milan, Italy). The resulting enriched CD14 positive cells fraction was re-suspended in Dulbecco modified minimum essential medium (D-MEM, EuroClone – Celbio, Milan, Italy) supplemented with 10% fetal bovine serum (FBS), 1% (vol/vol) l-glutamine (EuroClone-Celbio) and 1% (vol/vol) penicillin plus streptomycin (EuroClone). Cells were plated into individual wells of a 24 well at a concentration of 1.5×10^5 cell/well and incubated for two hours.

Monocyte stimulation. Before performing each stimulus experiment and after 24 hours of incubation, one well per series was stained with Trypan blue to determine the number of surviving cells. CD14 positive cells were stimulated with 1,10, 100 or 1000 ng/ml of the *T.denticola* MSP in D-MEM at 37°C in a 5% CO_2 atmosphere. The culture supernatants were collected after 2, 4, 8 and 24 hours of incubation and stored at -80°C until used for the determination of cytokines and MMP-9 content. Cells incubated in culture medium in the absence of *T.denticola* MSP were used as negative controls.

Cytokine and MMP-9 assay. The amounts of the following pro-inflammatory cytokines, TNF-α, IL-1β, IL-6, and of the matrix metalloproteinase 9 (MMP-9) in the supernatants obtained after each time point of the MSP stimulus, were determined with an enzyme-lynked immunosorbent assay (Istant ELISA Bender MedSystem, Wien , Austria) according to the manufacturer's instructions.

Statistical analysis. Each experiment was performed three times in triplicate. Means and standard deviation (SD) were calculated for group comparisons within and among experiments. The ANOVA test was performed using the GraphPad 4.0 software.

2. *T.denticola* outer membrane induces apoptosis and HO-1, Hsp70 and in PAECs

T.denticola growth and outer membrane preparation. The T.denticola strain ATCC 35405 was grown under anaerobic conditions in NOS medium as described in the Supplement materials. The outer membrane from T.denticola was prepared using the method described in the Supplemental Materials paragraph. The OMT preparation was re-suspended in phosphate-buffered saline and stored frozen until used. OMT was run by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% acrylamide gel. A Comassie Blue stain was performed in order to compare the protein profile in OMT preparation respect to the protein profile of the whole cells. In order to analyze the monomeric form of individual polypeptides, selected outer membrane and whole T.denticola cell preparations were treated at 100°C in boiling water for 10 min.

Cell culture and treatments. pAECs were isolated and maintained as previously described (Bernardini et al. 2005). All experiments were performed with cells from the third to the eighth passage. Apoptosis induction pAECs were grown in 8-well slide chambers (approximately 4×10^4 cells per well; 354631 Becton-Dickinson). Outer membrane from T.denticola or LPS from E. coli 055:B5 (Sigma-Aldrich Co, St. Louis, MO, USA) was added (10 μg/ml) to the pAEC culture medium for 24 h. Treated cells and relative controls were fixed with 1% paraformaldehyde. Endotoxin-induced chromatin fragmentation was evaluated by the ApopTag Fluorescein in situ apoptosis detection kit (Intergen Purchase,

NY, USA) according to the manufacturer's instructions. Briefly, cells were postfixed in pre-cooled ethanol/acetic acid (2:1, v/v) for 5 min and equilibrated with the appropriate buffer; then, cells were incubated with terminal deoxynucleotidyl transferase enzyme for digoxigenin dNTP incorporation (30 min at 37°C) and after several washings with Dulbecco phosphate buffer saline (DPBS), finally incubated with anti-digoxigenin–fluorescein antibody. The reaction was stopped, and fluorescent anti-digoxigenin was added (30 min at room temperature in the dark). After several washings in DPBS, cells were counterstained with propidium iodide and observed under a Nikon epifluorescence microscope. At least a minimum of 200 cells were evaluated for each

treatment.

HO-1 and Hsp70 expression. pAECs were placed in a flat-bottom 24-well plate (approximately 4×104 cells per well; 353813 Falcon, Becton-Dickinson) and grown until confluence. When cells reached the confluence, OMT or LPS (10 μg/ml) was added to the culture medium for 7, 12, 18, and 24 h. At each time point, treated cells and relative control cells were collected and stored at −80°C for analysis of HO-1 and Hsp70 expression.

Real-time PCR. Total RNA was isolated using the RNeasy Mini Kit 50 (Qiagen Sciences Inc, Germatown, MD, USA) and treated with RNase-free DNase set (Qiagen) according to the manufacturer's instruction. RNA concentration was spectrophotometrically quantified (A260 nm), and its quality was determined by gel electrophoresis on 2% agarose gel. One microgram of total RNA was reverse-transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-RAD Laboratories Inc., California, USA) in a final volume of 20 μl. Swine primers [HO-1, Hsp70, hypoxanthine–guanine phosphoribosyltransferase (HPRT)]

were designed using Beacon Designer 2.07 (Premier Biosoft International, Palo Alto, CA, USA). Their sequences, expected polymerase chain reaction (PCR) product length, and accession number in the EMBL database are shown in Table 1A (See Supplement Materials). Real-time quantitative PCR was performed in the iCycler thermal cycler (Bio-RAD) using SYBR green I detection. A master mix of the following reaction components was prepared to the indicated end-concentrations at 1.5 µl forward primer (100 ng/µl), 1.5 µl reverse primer (100 ng/µl), 7 µl water, and 12.5 µl IQ SYBR Green Bio-RAD Supermix (Bio-RAD). cDNA (2.5 µl) was added to 22.5 µl of the master mix. All samples were performed in duplicate. The real-time PCR program was: initial denaturation for 1 min and 30 s at 95°C, 40 cycles of 95°C for 15 s, and 60°C for 30 s, followed by a melting step with ramping from 55°C to 95°C at a rate of 0.5°C/s. The housekeeping HPRT was used to normalize the amount of RNA. The expression of HO-1 and Hsp70 mRNA was calculated as delta CT (HPRT CT–HO-1 CT or Hsp70 CT). Real-time efficiency for each primer set was acquired by amplification of a standardized cDNA dilution series. The specificity of the amplified PCR products was verified by melting curve analyses and an agarose gel electrophoresis.

Western blot Cells were harvested and lysed in SDS solution (Tris-HCl 50 mM pH 6.8; SDS 2%, 5% glycerol). Protein content of cellular lysates was determined using a Protein Assay Kit (TP0300, Sigma-Aldrich Co). Aliquots containing 15 µg of proteins were separated on NuPage 4% to 12% Bis–Tris gels (Gibco-Invitrogen, Paisley, UK) for 50 min at 200 V. Proteins were then electrophotoretically transferred onto a nitrocellulose membrane. Western blot analyses of Hsp70 and HO-1 were performed as previously described (Bernardini, 2005) using a mouse anti-Hsp70 monoclonal antibody (SPA 810,

StressGen, Victoria, BC, Canada) and a rabbit anti-Hsp32 polyclonal antibody (SPA 896, StressGen). In order to normalize Hsp70 and HO-1 protein expression on housekeeping protein, membranes were stripped and re-probed for the antibody against the housekeeping HPRT (1:250 sc-5274 Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). The relative protein content (protein of interest/HPRT) was expressed in arbitrary units.

3. Uptake and Killing of *Treponema denticola* by Macrophages

Bacterial cultures. *T.denticola* strain ATCC 33520 and mutant strains for the *cfpA* gene (Izard, 2001) and for the *flgE* gene (Li, 1996; Limberger, 1999), were grown in NOS medium at 37^0C under anaerobic conditions.

Preparation of macrophages. Peritoneal murine macrophages cells were obtained as described follow, see Supplement materials paragraph. To ensure the viability of macrophages under aerobic and anaerobic (95% CO_2 and 5% N_2) conditions, the cells were incubated in the presence or in the absence of 10 μg/ml of *Escherichia coli* lipopolysaccharides (LPS) (Sigma-Aldrich Chemical Co, USA) in RPMI 1640 medium at 37°C. The supernatant from each well was withdrawn at defined time intervals (1, 2, 4, 8, and 24 hr), and the concentration of released TNF-α was measured by the Mouse TNF-α ELISA kit from Bender Medsystem (Austria), following the manufacturer's protocol. Individual wells containing macrophages were stained with Trypan blue to determine the number of surviving cells.

Preparation of the anti major surface-protein antiserum. The major surface protein (MSP) of *T.denticola* was extracted as described in Supplemental Materials paragraph. Polyclonal rabbit antibodies against MSP were obtained by rabbit immunization as previously reported (Giacani, 2005). The specificity of the anti-MSP rabbit antiserum was tested by immunoblotting using *T.denticola* cell lysate and a purified control protein, as previously described follow in Supplemental Materials paragraph.

Immuno-Fluorescence Assay. The uptake of *T.denticola* by macrophages was assessed by an indirect immunofluorescence assay (IFA) performed with 1:400 diluted anti-MSP rabbit polyclonal antiserum. This method was able to detect both extracelluar bounds and internalized bacteria. Briefly, *T.denticola* cells (2.5×10^7 cells per well) were incubated with adherent macrophages in a final volume of 1 ml (ratio bacteria/macrophage: 100/1), in RPMI 1640 medium, without any antibiotic for 5, 10, 20, 40 and 60 min at 37°C. After each incubation period, supernatants were removed, without previous plate centrifugation, and were serially diluted in NOS medium. The motile treponemal cells were immediately counted under dark-field microscopy. The cells were washed three time with PBS to remove unbound spirochetes and subsequently fixed in cold methanol for 15 min at -20°C. The percentage of anti-MSP positive macrophages was obtained by counting cells in at least 30 different microscopic fields (400x). In order to ensure bacteria viability, three additional wells for each time point were rinsed thoroughly with PBS as described above and the cells were scraped off by shaking with glass beads for 4 minutes. The resulting suspension was cultured in NOS medium at 37°C for up to 14 days, to assess the presence of living spirochetes. In selected experiments performed under anaerobic and aerobic conditions, *T.denticola* strains were opsonized before challenging them with macrophages. This was done by incubating the treponemes in the presence of heat inactivated anti-MSP rabbit polyclonal antiserum (1:100 diluted) for 1 hour, in their cultivation media in anaerobic condition.

Real-time PCR assay. The uptake experiments were performed as above, with the following difference: the ratio between bacteria and macrophage were 10/1 (2.5×10^5 bacterial cells per well), 50/1 and 100/1. DNA of the cellular homogenates of phagocytosis,

was extracted using the NucliSens EasyMag system (bioMérieux, France) following the manufacturer's instructions. The real-time PCR was carried out using a LightCycler system (Roche Diagnostics GmbH, Germany) with SYBR Green I dye.

The specific primers pair targeting *T.denticola* 16S rRNA gene showed in Table 2A (see Supplemental Materials paragraph). DNA standards for *T.denticola* quantification were assessed by a 10-fold scalar dilution of *T.denticola* cell suspension (10^9 treponemes/ml). The sensitivity of real-time PCR was 10^3 treponemes/ml.

Data analysis. Means and standard deviation (SD) were calculated for group comparisons within and among experiments. Analysis of variance, and Student *t* test were performed using the GraphPad 4.0 software.

Imaging movies. Movies of the phagocytosis was obtained by using Nikon Eclipse-Ti microscope and acquired by Nis-Elements 3.1 software.

4. Sequence Heterogeneity in the central region of *msp* gene

To investigate the polymorphism of the *msp* this gene was amplified and sequenced in seventeen clinical samples obtained from periodontal pockets in patients suffering from acute stage periodontal disease. The presence of *T.denticola* in these clinical specimens was determined by PCR-amplification of the 16S rRNA gene before the amplification of the *msp* gene.

Clinical samples. Clinical samples were obtained from diseased sites in subjects affected by periodontitis that were admitted for surgical procedures at the dental clinic of the University of Bologna.

Nucleic acid extraction. Bacterial DNA was purifed from paper conical tip swabs by extraction using the automatic NucliSENS easyMAG (Biomerieux, Marcy l'Etoile, France) extractor according to the manufacturer's instructions.

PCR amplification and sequencing. The amplification was performed using the following set of oligonucleotide primers: KX14 and KX04 primers located in the 5`- and 3`- flanking regions of the *msp* open reading frame (ORF) were used under the conditions previously reported by Fenno and co-workers (Fenno, 1997). Individual nested PCR reactions were used to separately amplify the different regions (5`-, 3`-, and central) by using: primer KX14 in combination with KX09 (Table 3A) for the 5'-end, primer set TD03 and TD06 (Table 3A) for the central fragment, and primers TD05 and TD04 (Table 3A) for the 3' fragment, respectively. For detailed and complete sequence of the primers used in this study

see Table 3A.The amplification mixture (50 µl final volume) contained 2 µM dNTPs, 0,5 µl of each primer (0,2 mM), 3 mM $MgCl_2$, 1 UI of Taq DNA Polymerase (Fermentas, Life Sciences, Burlington, Ont., Canada) and 5 µl of template DNA. Cycling conditions were as following described in the Supplemetal materials paragraph. The amplicons were purified by using the PureLink Quick Gel Extraction Kit (Invitrogen) and sequenced (PRIMM; Milano-Italy).

Sequence and phylogenetic analysis. Sequences were aligned with the CLUSTALW allignement software and a neighbor-joining phylogenetic tree was constructed from the alignment results with the MEGA 4 software (Tamura, 2007). CLC software (Ciblak, 2009) was used to predict the transmembrane, hidrophobicity, and the antigenicity region of the deduced MSP peptide.

Chapter IV

RESULTS

1. MSP induces the production of TNFα, IL-1β, IL-6 and MMP 9 by CD14 positive cells

MSP analysis. The MSP omplex (MSP), obtained by sequential detergent extraction and autoproteolysis of the T.denticola membrane, existed primarily as an oligomeric peptide that had a molecular weight of 53 kDa, as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after heating at 100^0C for 5 min (data not shown). The MSP preparations did not contain any lipopolysaccharide- like material, as measured by Limulus amebocyte lysate assay. After peripheral blood monocyte isolation, more than 87% of the cells were CD14-positive and were 100% viable. After the 24 h incubation, more than 80% of the cells were still viable, even at MSP concentrations as high as 1 ug/mL (data not shown).

CD14 cells stimulation and TNF-α and IL-1β production. The MSP induced the production of proinflammatory cytokines in a dose- and time-dependent manner. After 24 h of incubation in the presence of MSP at 100 ng/mL and 1 ug/ mL, monocytes released TNF-α at a concentration of 240 ± 8 and 260 ± 10 pg/mL, respectively. In contrast, the incubation of monocytes with MSP at concentrations lower than 100 ng/mL did not stimulate TNF-a production (no difference from basal levels) (Fig. 1A, Results paragraph).
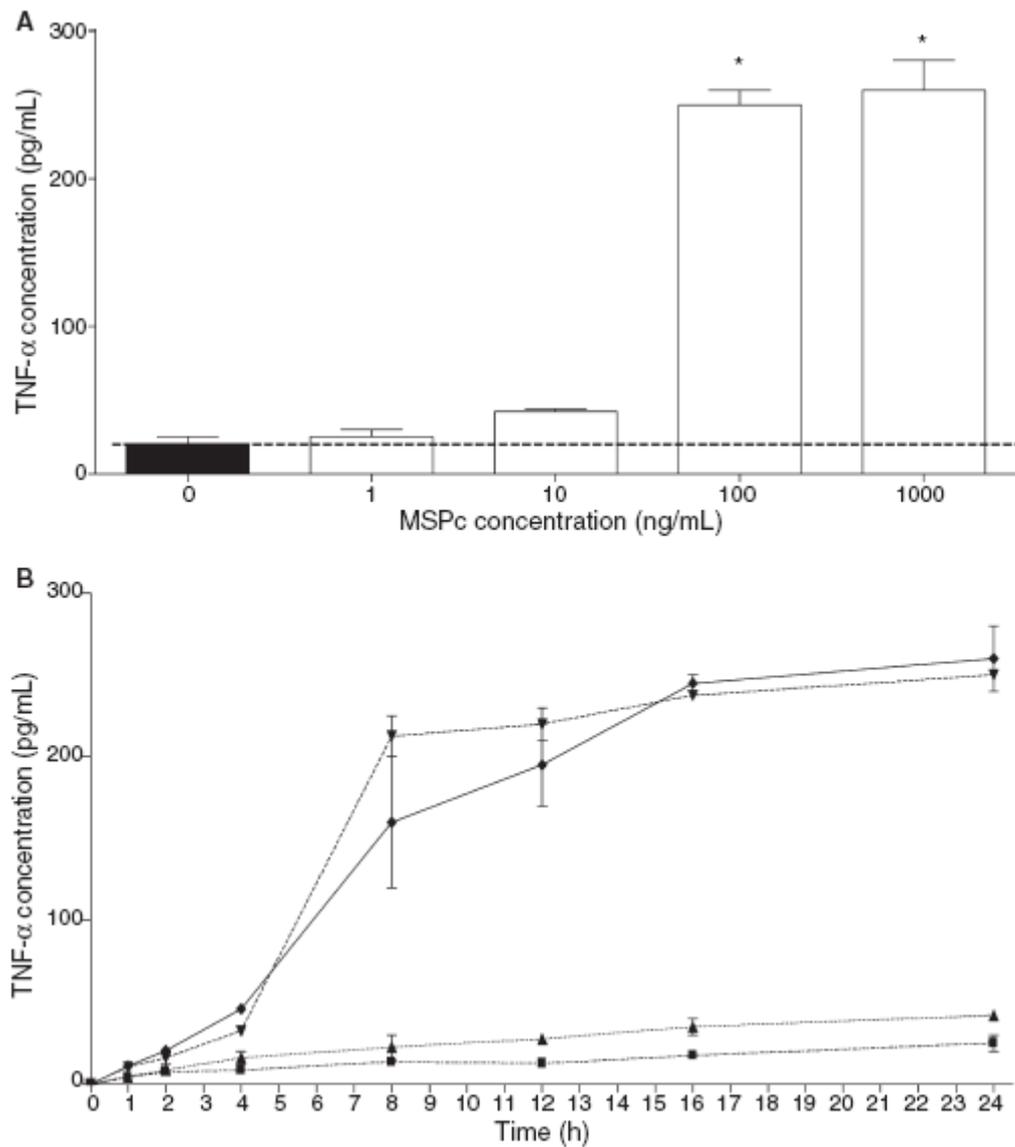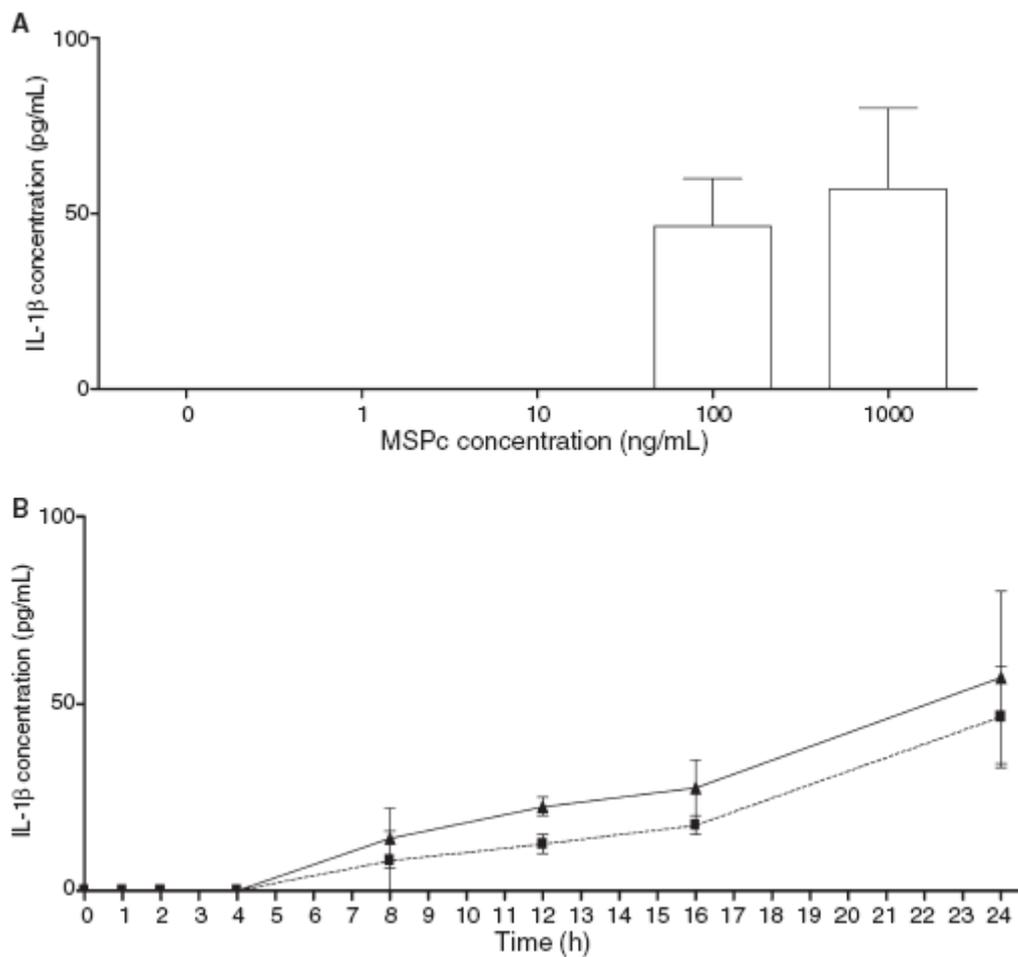
Figure 1. (A) Tumor necrosis factor alpha release by human peripheral blood monocytes stimulated with different concentrations of T.denticola MSP after 24 h. The results are representative of three independent experiments and are expressed as the mean + SD (error bars) of three replicates. The asterisks indicate which MSP concentrations achieved statistically significant differences (p < 0.01) in the cytokine response between the MSP -

These differences were statistically significant ($p < 0.0001$ as demonstrated by the ANOVA test). With regard to the kinetics of cytokine production from cells that were stimulated with MSP at 100 ng/mL and 1.0 ug/mL, TNF-a production increased dramatically up to 8 h and then rose modestly between the remaining time points (Fig. 1B, Results paragraph).

The effect of MSP stimulation on the production of IL-1β by CD14-positive cells was also evaluated. At 100 ng/mL and 1.0 ug/mL, MSP induced a pattern of IL-1b release that was similar to that of TNF-α At low MSP concentrations (1.0 and 10 ng/mL), no IL-1β was released into the supernatant (Fig. 2A, Results paragraph). A significant level of IL-1β was detected in the supernatant only after 8 h of incubation with the MSP (at concentration of 100 ng/mL and 1.0 ug/mL), which increased linearly up to 24 h (Fig. 2B, Results paragraph).

Figure 2. (A) Interleukin-1β release by human peripheral blood monocytes stimulated with different concentrations of T.denticola MSP after 24 h. The results are representative of three independent experiments and are expressed as the mean + SD (error bars) of three replicates. (B) Interleukin-1β levels in the cell supernatant at different time points after stimulation with 100 and 1000 ng/mL of MSP . The continuous line indicates IL-1β

production by monocytes stimulated with 100 ng/mL MSP ; the dashed line indicates IL-1β production by monocytes stimulated with 1000 ng/mL MSP .

IL- 6 production. Interleukin-6 was detectable in the supernatant as early as 4 h after CD14- positive cells were incubated with MSP at concentrations ranging from 100 to 1000 ng/mL.

The release of this cytokine increased rapidly up to 8 h of incubation and slowed between 8 and 24 h with MSP concentrations of 100 and 1000 ng/mL (Fig. 3B, Results paragraph). Concentrations of MSP of 100 ng/mL or greater induced significant IL-6 release, resulting in statistically significant differences ($p < 0.0001$ as demonstrated by the ANOVA test; Fig. 3A, Results paragraph).
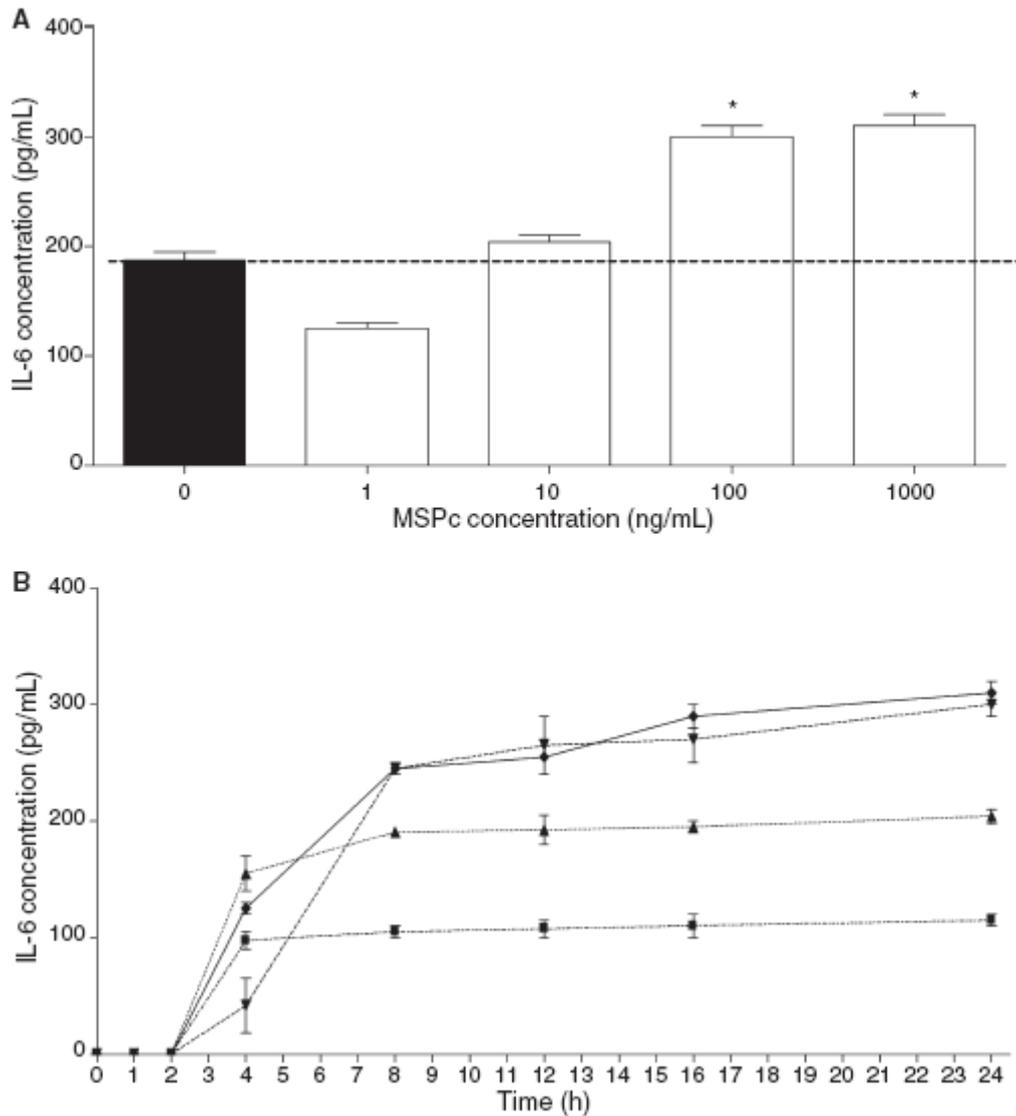
Figure 3. (A) Human peripheral blood monocytes incubated with different concentrations of MSP after 24 h. Supernatants were analyzed for IL-6 release after 24 h. The asterisks indicate which MSP concentrations achieved statistically significant differences ($p < 0.01$) in the cytokine response between the MSP-stimulated monocytes and unstimulated (control) monocytes according to the ANOVA and Bonferroni post hoc test. (B) Interleukin-6 levels in the cell supernatant at different time points after stimulation with different concentrations of MSP. The graph shows interleukin-6 production by monocytes stimulated

with 1 (■), 10 (▲), 100 (▼) and 1000 ng/mL (♦) of MSP . The data presented are from three independent experiments and are expressed as the means ± SD.

MMP9 production. Matrix metalloproteinase 9 was released at a high concentration (240 lg/mL) when the cells were stimulated with MSP at 1000 ng/mL.

This result was statistically different from that obtained with lower concentrations of MSP (p < 0.0023 as demonstrated by the ANOVA test). Lower concentrations of MSP induced MMP-9 release in amounts comparable with basal levels (Fig. 4A, Results paragraph). The MMP-9 release started to increase 2 h after stimulation with 1000 ng/mL. At 100 ng/mL of MSP or lower, the curve showed a marked increase after 4 h of incubation (Fig. 4B, Results paragraph).
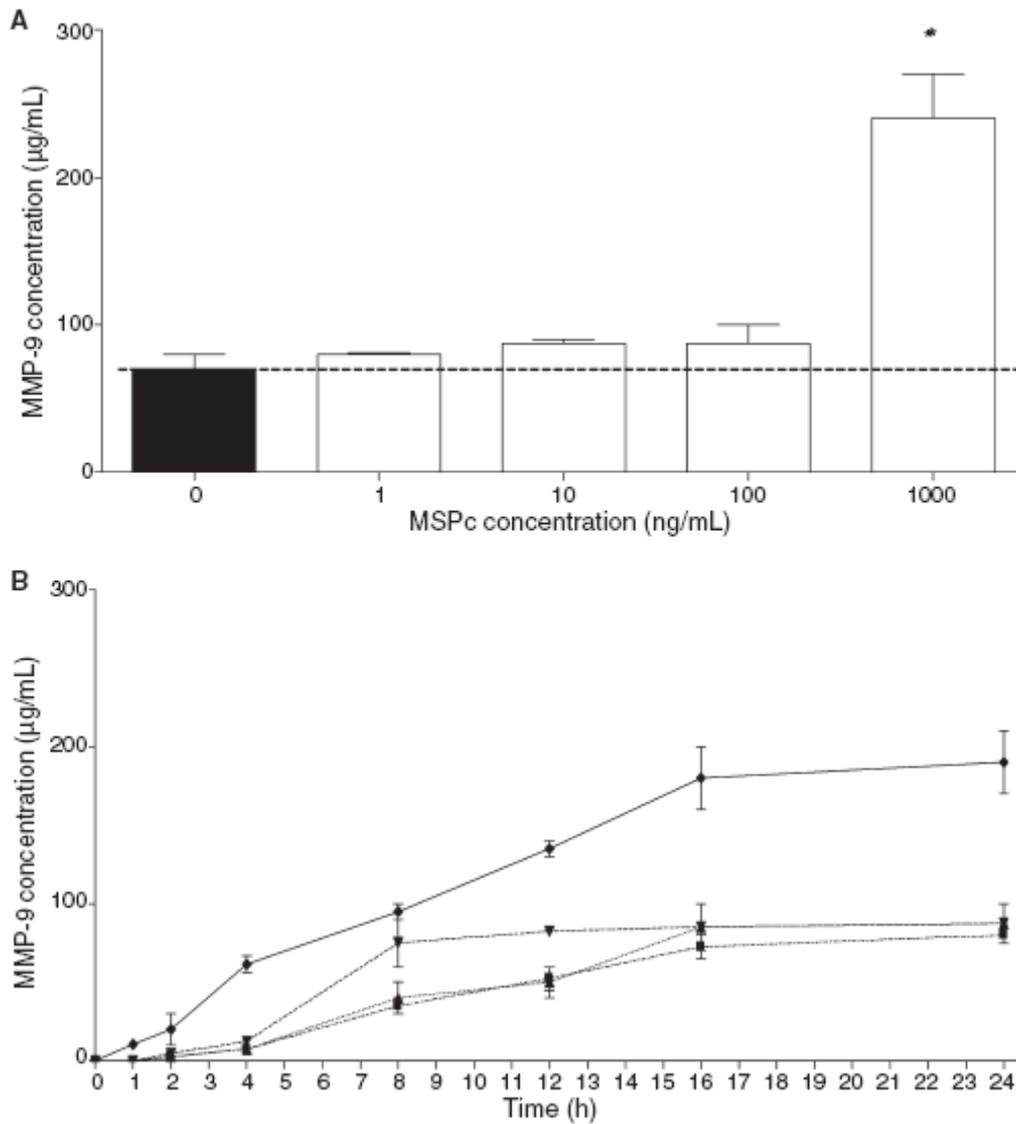
Figure 4. (A) Matrix metalloproteinase 9 release by monocytes stimulated with different concentrations (1, 10, 100 and 1000 ng/mL) of T.denticola MSP . The results are expressed as means + SD of three different cultures. The asterisk indicates which MSP concentration achieved a statistically significant difference ($p < 0.01$) in the cytokine response between the MSP -stimulated monocytes and unstimulated (control) monocytes according to the ANOVA and Bonferroni post hoc test. (B) Matrix metalloproteinase 9 levels in the cell supernatant at different time points after stimulation with different concentrations of MSP .

The graph shows MMP-9 production by monocytes stimulated with 1(■), 10 (▲), 100 ((▼) and 1000 ng/mL (♦) of MSP .

2. *T.denticola* outer membrane induces apoptosis and HO-1 and Hsp70 in   PAECs

SDS-PAGE analysis of the OMT. SDS-PAGE analysis of the OMT extract and whole *T.denticola* cells is shown in Fig. 5, Results paragraph; in OMT preparation after heat treatment (lane 2), the most relevant bands showed a molecular mass of 53 and of 72 kDa corresponding to the monomeric form of MSP and CTLP (Fenno and McBride, 1998).

In unheated samples (lanes 1, 3), a typical double band corresponding to multimeric CTLP (approximately 95 kDa) was detectable (Fenno, 1998) and a higher band, more intense in OMT preparation than whole *T.denticola* cells, ascribable to a CTLP/MSP complex was detected.
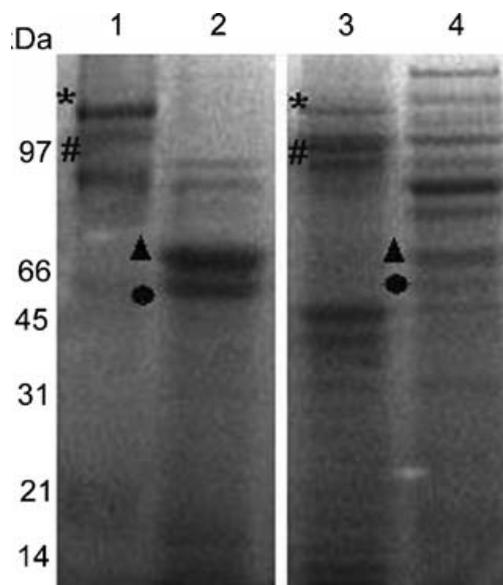
Figure 5. SDS-PAGE analysis of the OMT preparation, before and after treatment at 100°C for 10 min (lanes 1 and 2, respectively), lanes 3, and 4 contain whole *T.denticola* cells, before and after treatment at 100°C for 10 min. Filled circle indicates the position of the monomeric MSP (approximately 53 kDa); filled triangle indicates the position of the monomeric CTLP (approximately 72 kDa); number sign indicates the typical double band of multimeric CTLP (approximately 95 kDa); asterisk indicates the position of the CTLP/MSP complex. The positions of molecular size standards (low-range SDSPAGE standards; Bio-RAD) are shown in kilodalton (kDa) on the left.

Apoptosis. The addition of OMT to pAEC cultures was effective in inducing a significant increase of apoptosis (Fig. 6, Results paragraph) compared with control (14.6±1.7% vs 4.7±0.3%).The rate of apoptosis observed was similar to that induced by LPS treatment (13.8±0.5).
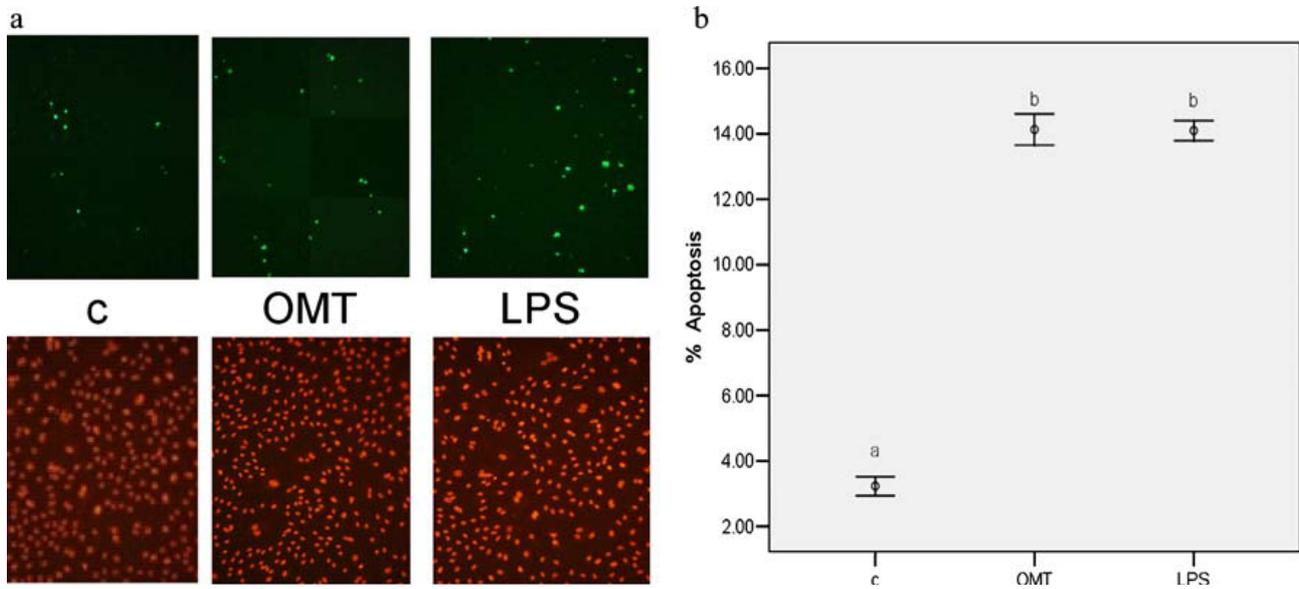
Figure 6. Effect of T.denticola outer membrane in pAECs: apoptosis levels after 24 h of exposure to OMT or LPS. a Apoptosis was assessed by Tunel assay, and cells are counterstained with propidium iodide. Cells with green fluorescence are apoptotic; b percentage of apoptotic cells. A minimum of 200 cells were evaluated for each treatment; data represent the mean±2 SEM of three replicates. Different letters indicate statistically significant differences ($p < 0.05$). C control, OMT T.denticola outer membrane, LPS lipopolysaccharide

HO-1 and Hsp70 expression. The time of culture did not influence HO-1 and Hsp70 expression in control cells (data not shown). Consequently, we used the mean of all the control time points as control (C). OMT was effective in inducing the heat shock response (HSR), and this response was similar to that of LPS. In fact, OMT induced a significant, transient increase in both HO-1 and Hsp70 either at mRNA or at protein levels. HO-1 mRNA increased immediately after stimulation with OMT or LPS (Fig. 7, Results paragraph), peaking at 7 h, then declined slowly but exceeded control levels until 24 h. This increase in HO-1 mRNA was accompanied by a persistent rise in HO-1 protein from 7 to 18 h (Fig. 8, Results paragraph). Hsp70 mRNA increased more slowly and less robustly than HO-1. Hsp70 mRNA reached a maximum at 12 h and declined, returning to control levels at 24 h in OMT and LPS-treated cells (Fig. 9, Results paragraph). Hsp70 protein content reflected a transient increase until 12 h, after which it sharply declined below control levels (Fig. 10, Results paragraph).
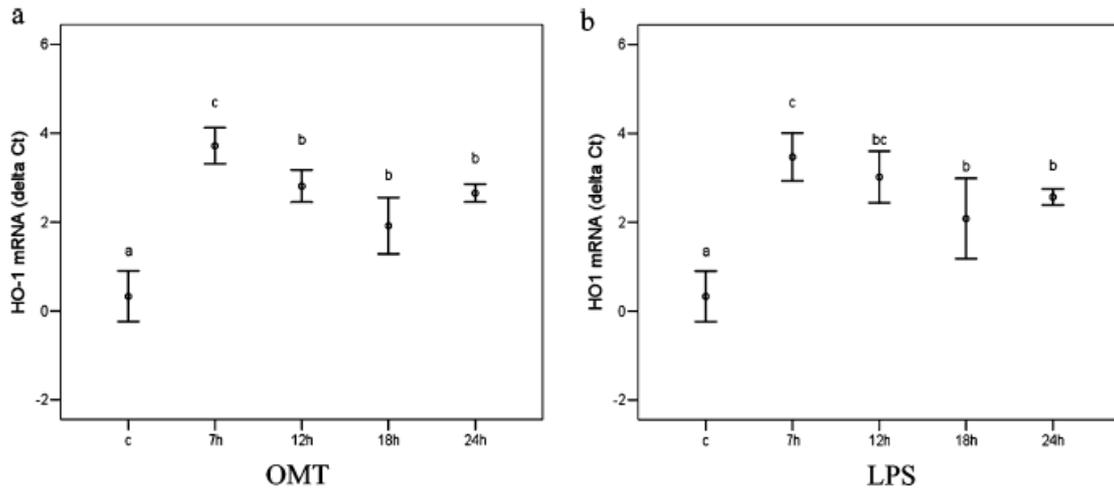
Figure 7. Effect of OMT (a) or LPS (b) HO-1 mRNA levels in pAECs. Data are presented as ΔCt (HPRT Ct–HO-1Ct) ±2 SEM of three replicates. C control, OMT T.denticola outer membrane, LPS lipopolysaccharide
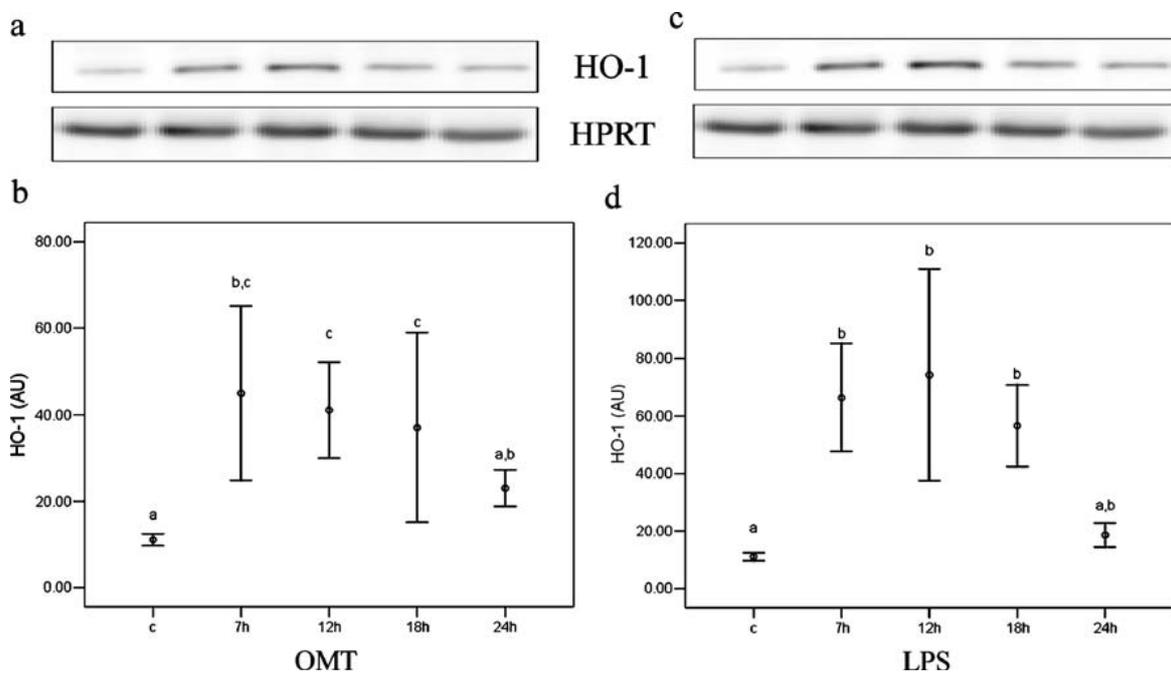
Figure 8. Effect of OMT (a, c) or LPS (b, d) on HO-1 protein levels in pAECs. a, b Representative Western blot of HO-1 and relative housekeeping HPRT are presented. c, d

Data are expressed as arbitrary units (AUs) and represent the mean±2 SEM of three replicates. Different letters indicate statistically significant differences (p<0.05). C control, OMT T.denticola outer membrane, LPS lipopolysaccharide
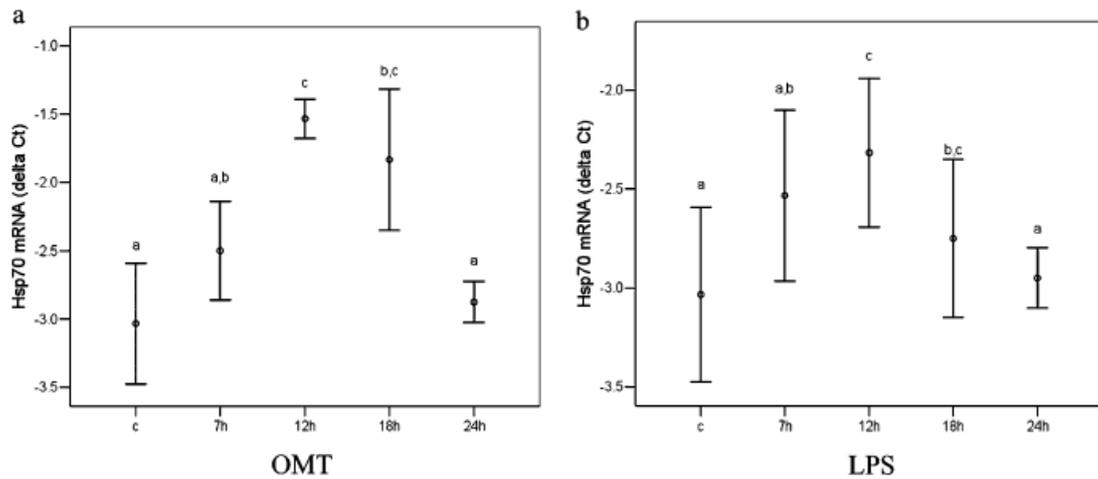
Figure 9. Effect of OMT (a) or LPS (b) Hsp70 mRNA levels in pAECs. Data are presented as ΔCt (HPRT Ct–Hsp70Ct) ±2 SEM of three replicates. C control, OMT T.denticola outer membrane, LPS lipopolysaccharide

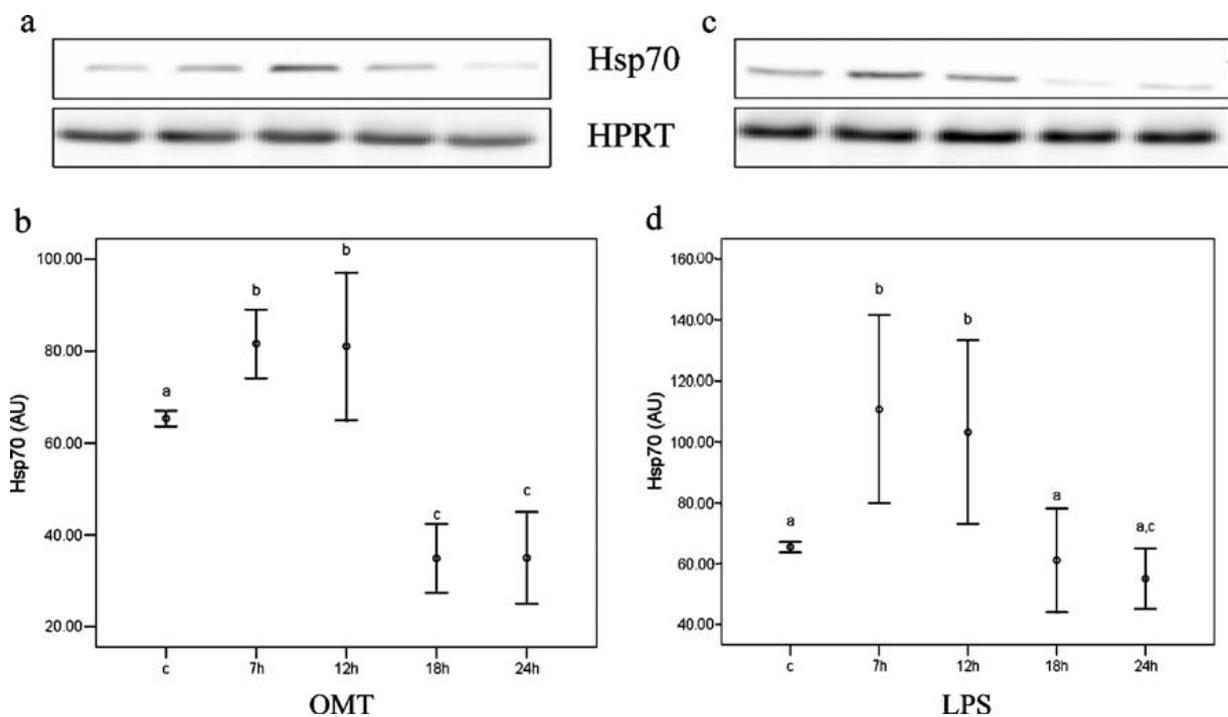**Figure 10.** Effect of OMT (a, c) or LPS (b, d) on Hsp70 protein levels in pAEC. a, b. Representative Western blot of HO-1 and relative housekeeping HPRT are presented. c, d

Data are expressed as arbitrary units (AUs) and represent the mean±2 SEM of three replicates. Different letters indicate statistically significant differences (p<0.05). C control, OMT T.denticola outer membrane, LPS lipopolysaccharide

3. Uptake and Killing of *Treponema denticola* by Macrophages

Uptake and killing of *T.denticola*. To validate our approach, the macrophages were incubated in aerobic atmosphere or under anaerobic conditions. There was no significant difference in the release of TNF-α following the stimulus of macrophages with LPS in either condition (Fig. 11, Results paragraph).

Trypan blue staining of the cells incubated for up to 2 hours under each condition demonstrated that at least 95 % of cells were viable in all the experiments. After one hour of incubation, treponemal motility ratio (motile/total motile + non-motile) was over 97% for experiments performed under anaerobic conditions, whereas the incubation in the presence of normal atmosphere reduced the percentage of motile treponemes to 23%, as observed by dark-field microscopy (Fig. 12, Results paragraph).

The use of macrophages incubated in anaerobic conditions allowed the maintenance of an experimental system with the highest possible viability of the spirochetes during the challenge with phagocytes. The increase of anti-MSP-positive macrophage occurred rapidly in both experimental conditions as shown by IFA (Fig. 15A and B, Results paragraph). A representation of *T.denticola* phagocytosis showed by IFA techniques are visualized in Fig. 13 in Results paragraph.

The difference between the results obtained under aerobic and anaerobic incubation was statistically significant when analyzed by using the Student's t test ($p<0.001$), with the uptake in aerobic condition being more efficient. The association of *T.denticola* cells to the macrophages was a rapid process under both aerobic and anaerobic conditions (Fig. 16C and D, Results paragraph). This process might be saturable for the 50/1 ratio (Fig. 16D. Results paragraph). In anaerobic condition, a decrease in spirochete number was observed

after 40 min by real time PCR (Fig. 16A and C, Results paragraph), but not by IFA (Fig. 15A, Results paragraph).

No living treponemes were detected, after 14 days of broth culture, in tubes inoculated with macrophage homogenates obtained at the end of the challenge with *T.denticola*. Thus suggesting the effective killing of phagocytised treponemes.

In addition, an *in vitro* uptake and subsequent internalization of *T.denticola* cells by macrophages were showed on Movie 1 and 2 (Legend in the Supplemental materials paragraph).
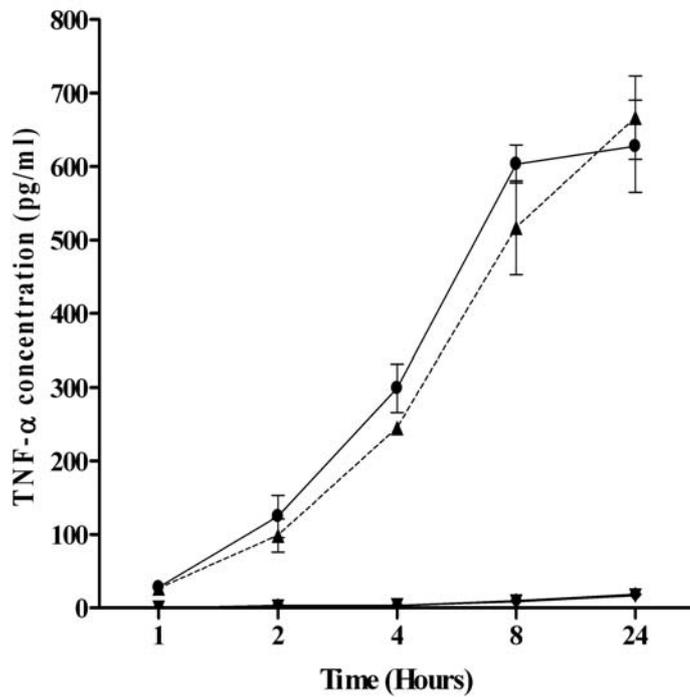
Figure 11. Production of TNF-α by isolated murine peritoneal macrophages stimulated with 10 ug/ml of LPS, for up to 24 hours, under anaerobic (●) and aerobic (▲) conditions of incubation. Production of TNF-α by isolated murine peritoneal macrophages in absence of stimulus, under anaerobic (▼) and aerobic (♦) conditions of incubation. The presented data is from three independent experiments and are expressed as the means ± standard deviations (error bars) of three replicates.
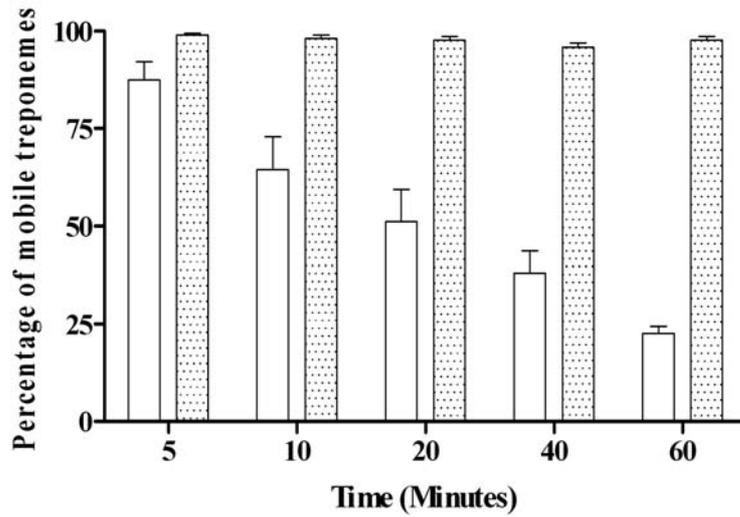
Figure 12. Percentage of motile *T.denticola* cells in the cell culture supernatants after macrophages challenge as observed by dark-field microscopy (400x). Bars indicate the percentage of motile treponemes under anaerobic conditions (dotted bars) and under aerobic conditions (white bars). The presented data is from three independent experiments and are expressed as the means ± standard deviations (error bars) for each condition of incubation.
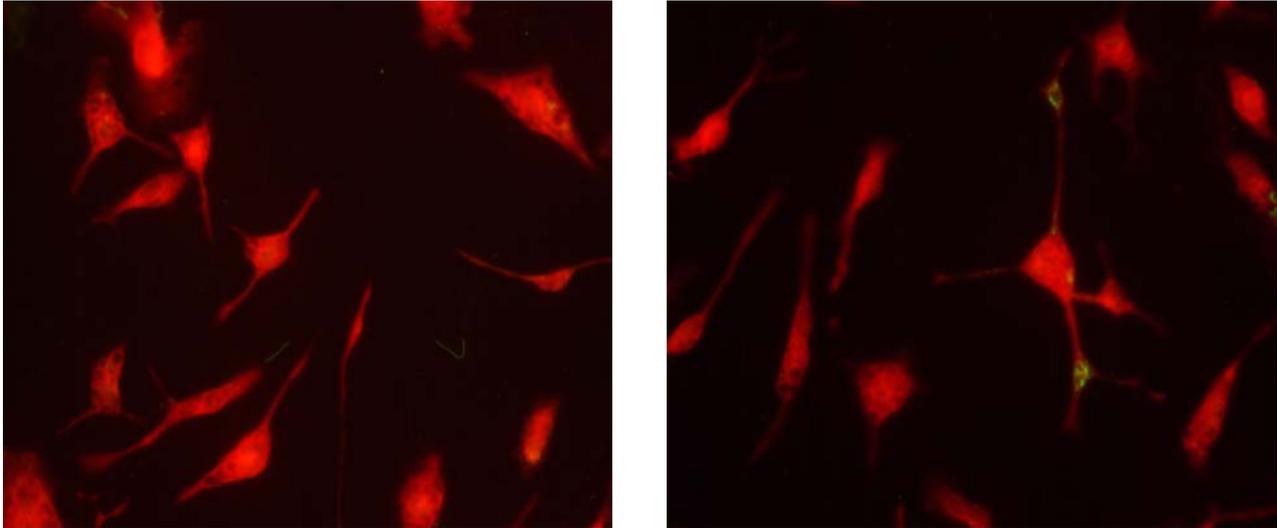
Figure 13. Phagocytosis of *T.denticola* by mice macrophages under anaerobic conditions after 10 minutes of incubation (Right Panel). In the Left Panel are showed the unbound treponemes cells. In green are visible *T.denticola* cells visualized by IFA techniques. In red are observable the mouse peritoneal macrophages.
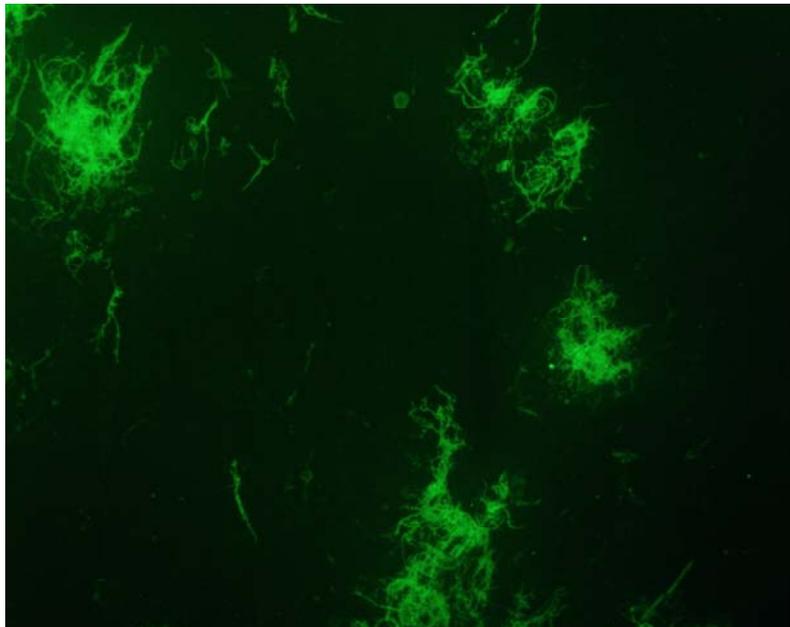
Figure 14. Opsonic *T.denticola* visualized in FITC

Opsonic phagocytosis of *T.denticola.* *T.denticola* treated with anti-MSP antibodies (example of Opsonic Treponemes as show in Fig. 14 in Results paragraph) increased the percentage of anti-MSP-positive macrophage cells significantly when compared to the number of positive macrophages obtained with non-opsonized treponemes for each individual time point ($p < 0.001$). Real-time PCR confirmed (Fig. 16, Results paragraph) the significant increase observed by IFA for both anaerobic (Fig. 15A, Results paragraph) and aerobic (Fig. 15B, Results paragraph) conditions. The Student's t test showed that the difference in the uptake of opsonized treponemes in both atmospheric conditions was statistically significant ($p = 0.05$).
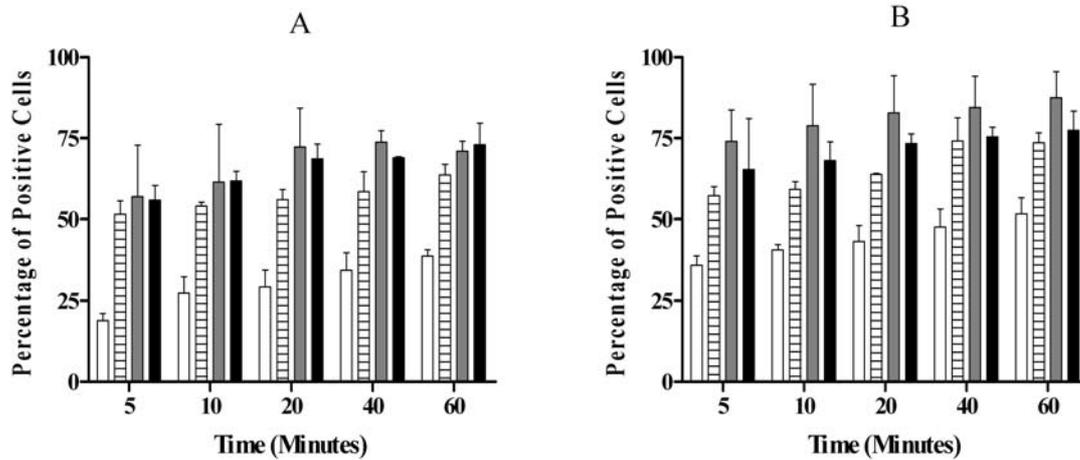
Figure 15. Macrophage uptake of *T.denticola* wild-type and mutant strains analyzed by imunofluorescence in both anaerobic (Part A) and aerobic conditions (Part B). Over-time uptake by isolated peritoneal murine macrophages of the wild-type cells (white bars), of the wild-type cells opsonized with anti major-surface protein (MSP) antibodies (bars with horizontal stripes), of the non-motile mutant cells (grey bars) and of the filamentous mutant cells (black bars). The results are expressed as a percentage of macrophage positive for antibodies against MSP. The difference observed between the number of positive macrophages challenged by the wild type strain and the opsonized wild-type strain (p<0.0001), or the mutant strains (p<0.0001 for either strain) are statistically significant for both anaerobic (Part A) and aerobic conditions (Part B). The results are representative of three independent experiments, each done in triplicate, and are expressed as the means ± standard deviations (error bars) of three replicates.

Phagocytosis of *T.denticola* mutant strains. The observed number of positive macrophages when challenged with either mutant strain was greater than when challenged with the wild-type strain.

The number of positive cells, as observed by IFA, increased rapidly (Fig. 15A and B, Results paragraph) and plateaued after the one-hour time point (data not shown) in both atmospheric conditions. The real-time PCR analysis showed an increase of *T.denticola* cells associated with the macrophage up to the 40 min time point, followed by a decrease, for both atmospheric conditions (Fig. 16A and B, Results paragraph).

The differences among the percentages of *T.denticola* positive phagocytes detected when the macrophages were challenged with either the non-motile mutant strain (*flgE* knockout strain) or the filamenteous mutant strain (*cfpA* knockout strain) compared to the wild type strain were statistically significant in either incubation condition and at each time point (Fig. 15 and 16, Results paragraph).
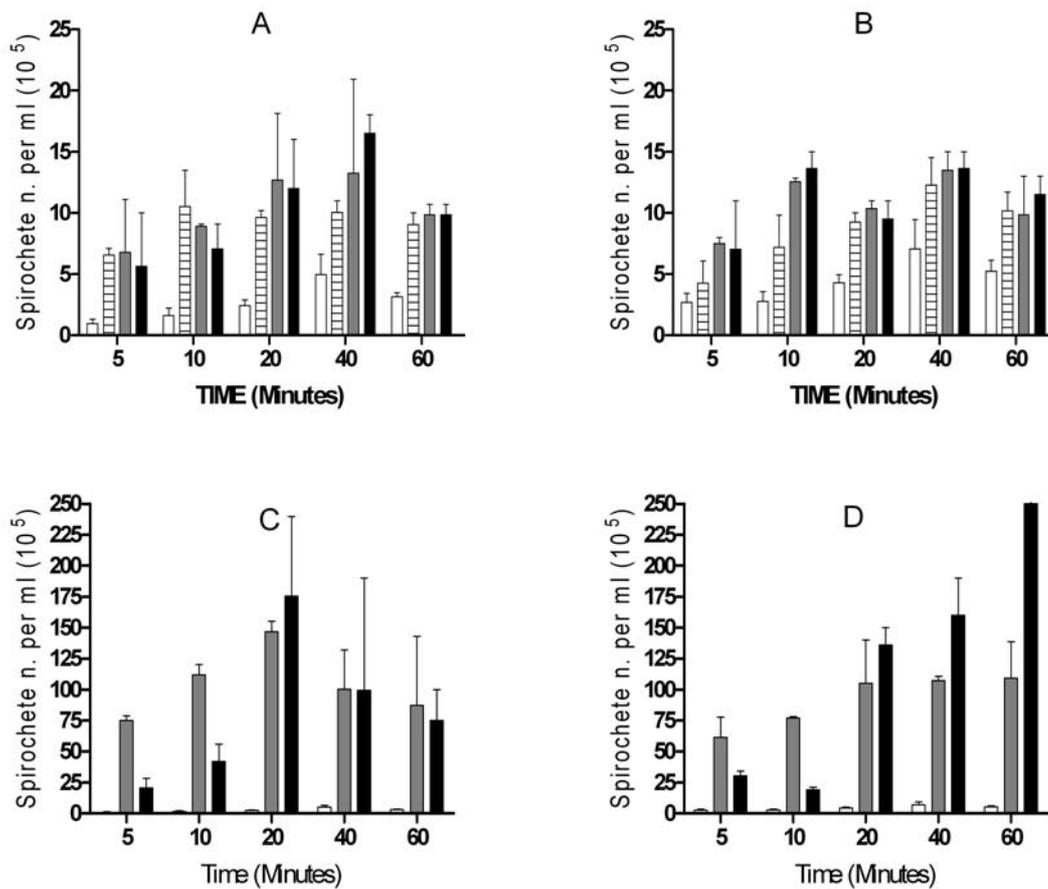
Figure 16. Macrophage uptake of *T.denticola* analyzed by real-time PCR. Panel A: wild-type and mutant strains under anaerobic conditions; Panel B: wild-type and mutant strains under aerobic conditions. The bars indicate the quantitative detection of DNA from the wild-type cells (white bars), the wild-type cells opsonized with anti MSP antibodies (bars with horizontal stripes), the non-motile mutant cells (grey bars) and the filamentous mutant cells (black bars). The difference observed between the number of positive macrophages challenged by the wild type strain and the opsonized wild-type strain (p<0.05), or the mutant strains (p<0.05 for either strain) are statistically significant in both anaerobic (Part A) and aerobic conditions (Part B). Panel C: Effect of different ratios of incubation between macrophages and *T.denticola* wild type strain under anaerobic conditions; Panel D: Effect

of different ratios of incubation between *T.denticola* wild type strain and macrophages under aerobic conditions. White bars indicate 10/1 ratio, grey bars, 50/1, and black bars, 100/1. The results are representative of three independent experiments, each done in triplicate, and are expressed as the means ± standard deviations (error bars) of three replicates.

4. Sequence Heterogeneity in the central region of the *msp* gene

The *msp* sequence of 15 samples showed a very elevated homology (>98%) with that of T.denticola ATCC 35405 (GenBank accession number U29399), while the remaining two matched more closely with the   ATCC 33520 strain (Gen Bank accession number U66255) (Figure 17, Results paragraph). In particular, the whole *msp* gene sequence analysis all the 17 samples evaluated showed that N-Terminal region, comprised between 1 and 600 bp, has an homology of 99% with that of T.denticola ATCC35405, as it was for the the C-terminal region, comprised between the nucleotide positions 800 and 1632. The highest variability was identified in the central region of the *msp* gene, between the nucleotide 600 and 800 (Table 1, Results paragraph).

Sample	*msp* gene	5' end	Central region	3' end	Amino acid
B34	99	99	98	99	99
B46	99	99	98	99	99
B66	99	99	94	99	92
B14	99	100	96	99	99
B16	99	99	97	99	99
B20	99	99	93	99	99
B21	99	99	95	99	98
B37	99	99	100	99	98
B23	98	99	80	99	95
B22	99	99	90	99	99
B6	99	99	97	99	98
B40	98	99	93	99	98
B69	99	99	91	99	97
B74	99	99	90	99	99
B5	99	99	96	99	99
B30*	99	99	99	100	99
B52*	99	98	99	99	98

Table 1. Confront of each isolated samples in confront of cultivable *T.denticola* ATCC 35405 and ATCC 33520 strain([*]). Each sample were confronted with the entire msp gene, the 5' region, the Central Region, and the 3' region. The deduced amino acid sequence were also analyzed in confront of laboratory strains.

The resulting phylogenetic trees (Figure 17, Results paragraph) showed the nucleotide reconstruction of the rooted *msp* sequences. The phylogenetic trees demonstrated a low evolutionary rate among the different samples and the possibility to group the clinical specimens evaluated in to two distinct group: A (including 15 samples) and B (the remaining 2) in which the *msp* nucleotide sequence is closely related to *T.denticola* ATCC35405 and ATCC33520, respectively. The phylogenic analysis of the *msp* nucleotide sequence was also extended to the strain OTK (GenBank accession number U66256), showing that this last isolate has a high divergence from the group A and B samples.
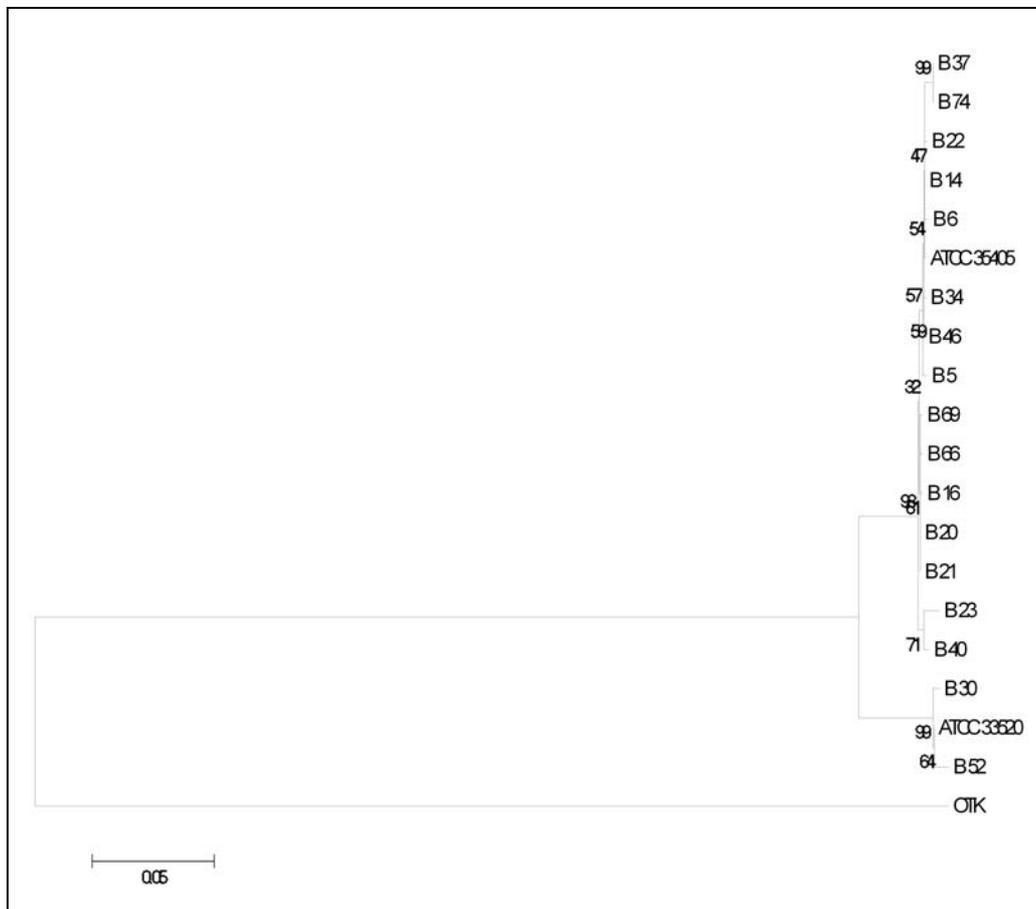
Figure 17. T.denticola *msp* gene evolutionary relationships deduced from each DNA sequence of 17 sample and ATCC 35405, ATCC 33520 and OTK strain. The tree was inferred using the Neighbor-Joining method with a branch length = 0.80466177. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, 2007) and are in the units of the number of base substitutions per site.

To avoid that possibility that silent nucleotide mutations influence evaluation of the sequence diversity, the putative amino acid sequences were derived for each of the *msp* genes obtained from our samples and the resulting sequences were compared with that of MSP from *T.denticola* ATCC35405 and ATCC33520 strains (Table 1, Results paragraph). The level of amino acidic homology ranged between 92% and 99%. In the group A (see figure 17 in the Results paragraph) of the clinical samples closely related with the MSP sequence of *T.denticola* ATCC35405, the majority of mismatches occurred in the amino acid positions between 200 and 270. A amino acid distribution histogram of a representative sample, B14, was shown in figure 18. A predictive hydrophobicity plot of the B14 sample demonstrated that the N-terminal region can be involved in the outer membrane attachment of the MSP, see Figure 20, Results paragraph.

A similar feature in the amino acidic substitution was identified in the group B (B30 and B52) of clinical samples, the one including the specimens with the MSP sequence that were closest to ATCC33520 strain. These results confirmed for the clinical samples the findings reported by Fenno (Fenno, 1997) with the two laboratory adapted strains of *T.denticola* ATCC35405 and ATCC33520. Moreover, the Electrical charge as a function of pH of a representative sample, B14, was deduced by CLC sotware. The deduced PI resulted about 6.5 of pH. In addition the predicted secondary structure of the MSP peptide, see figure 21 in Results paragraph, shown an elevated rate of β-Strand in the N- and C-terminal sequence. A predictive antigenicity plot demonstrated that the highest antigenic secondary structure was in the central region, between 200 to 270 aa, see figure 22 in the Results paragraph.

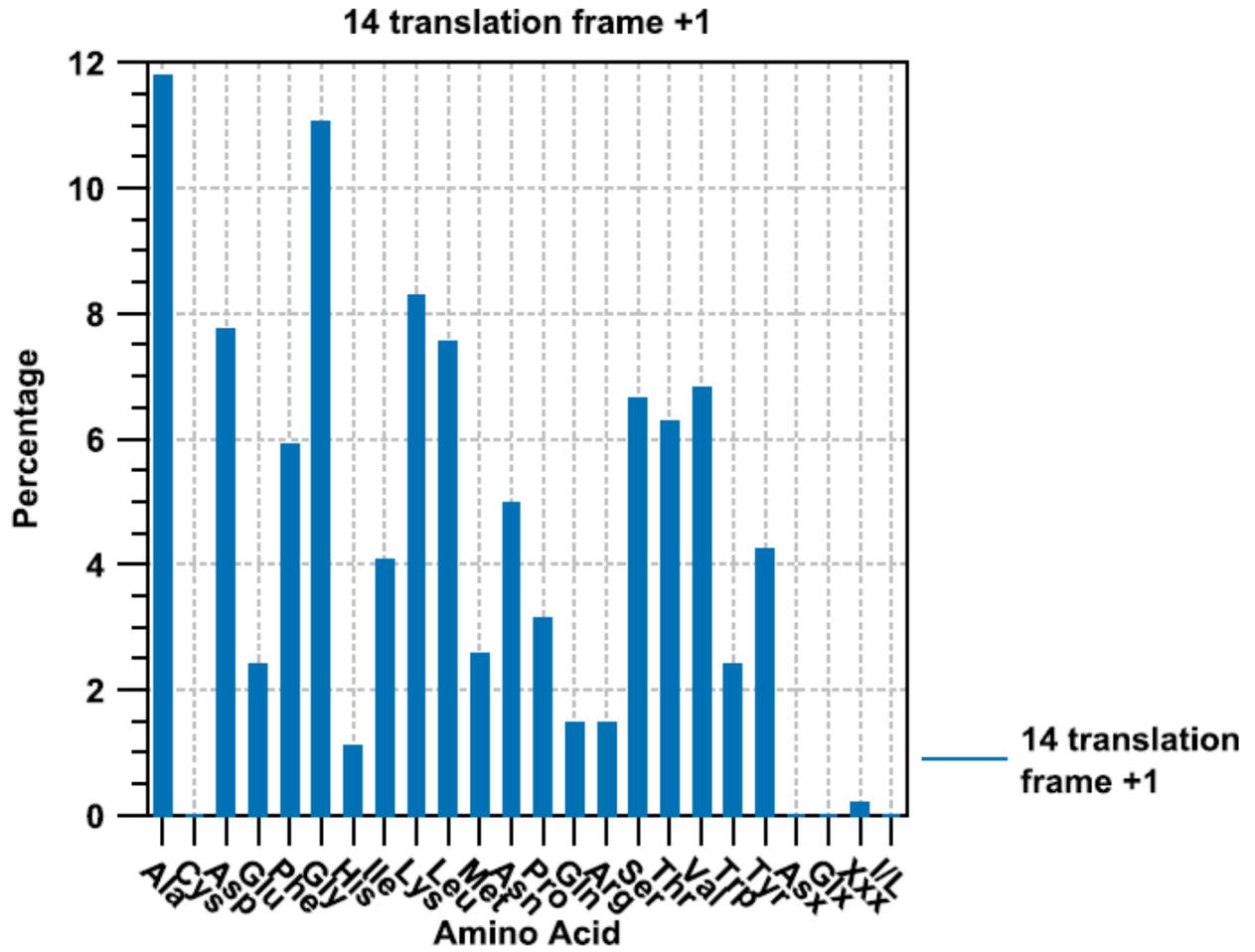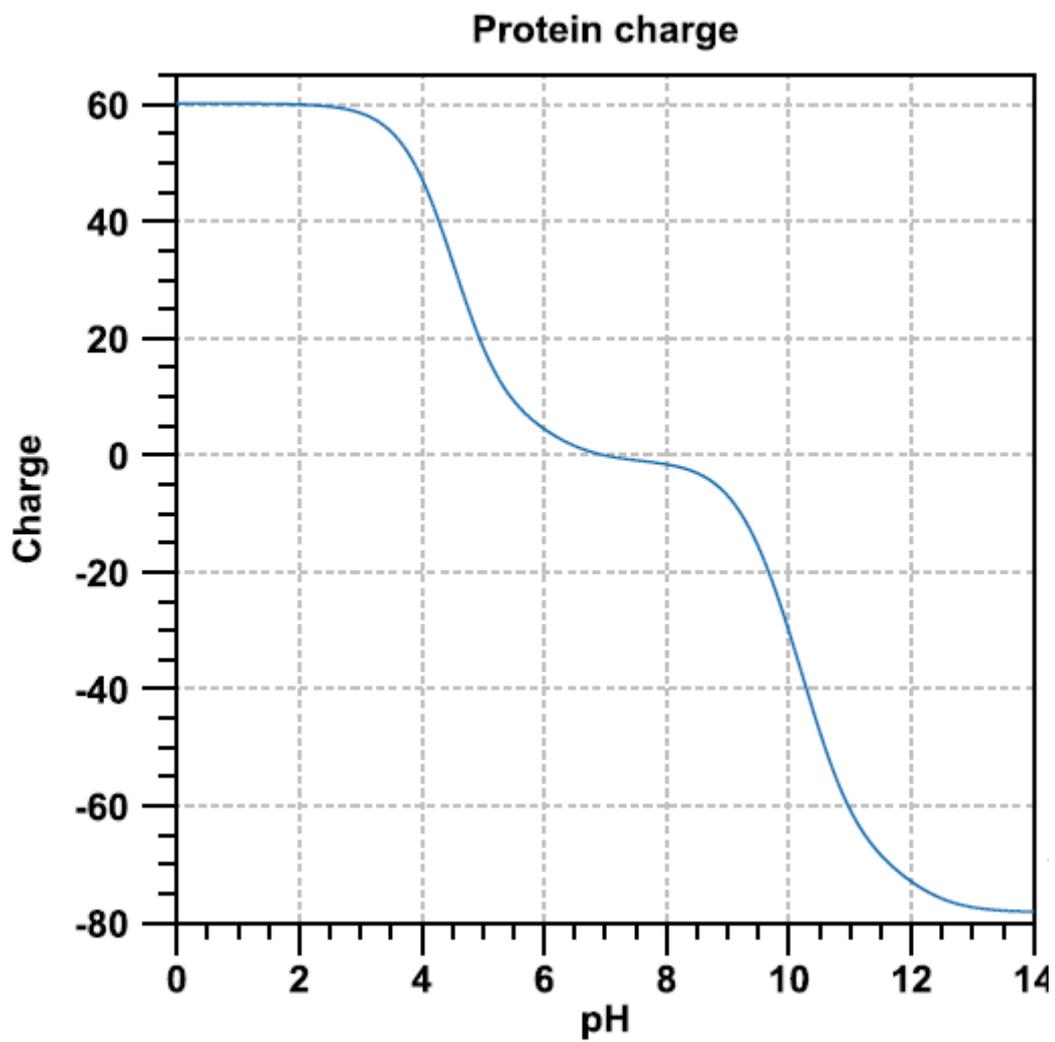Figure 18. Amino acid distribution histogram of the MSP protein obtained from sample B14

Figure 19. Electrical charge as a function of pH of the MSP protein obtained from sample B14

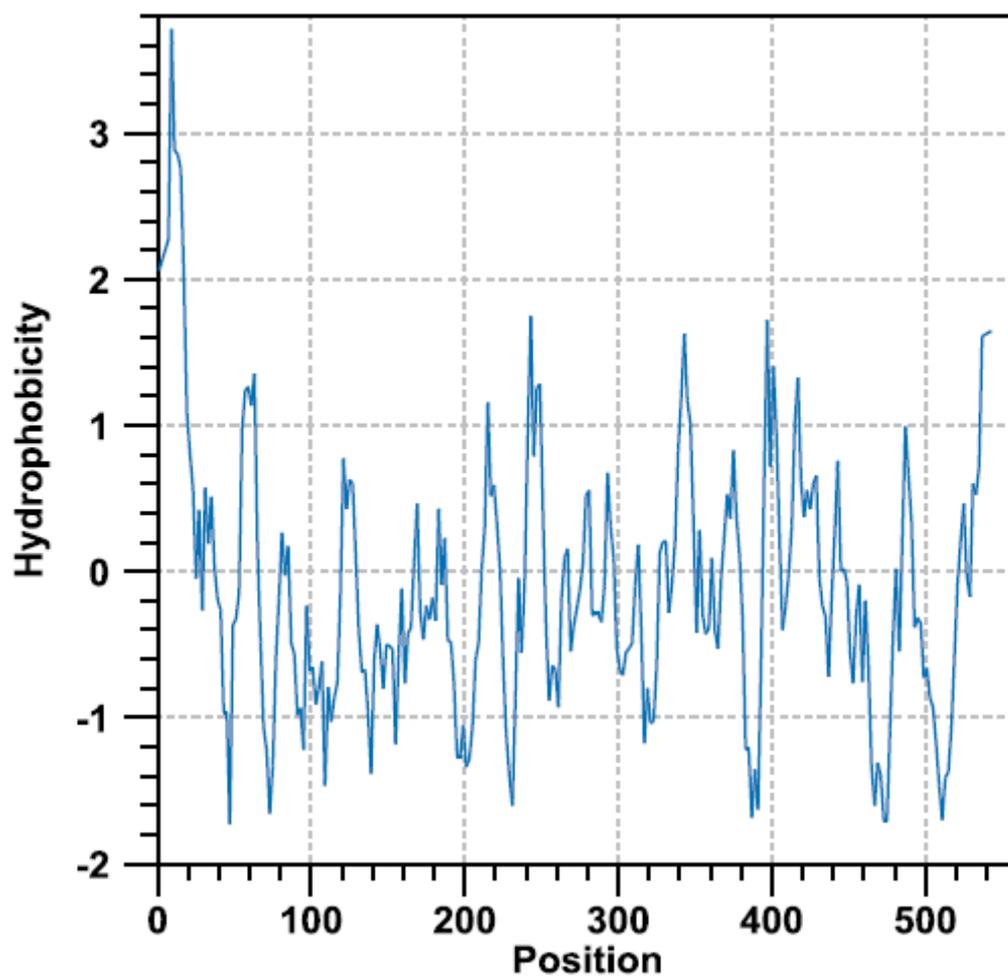Figure 20. Plot of local Hydropathy of the MSP protein obtained from sample B14
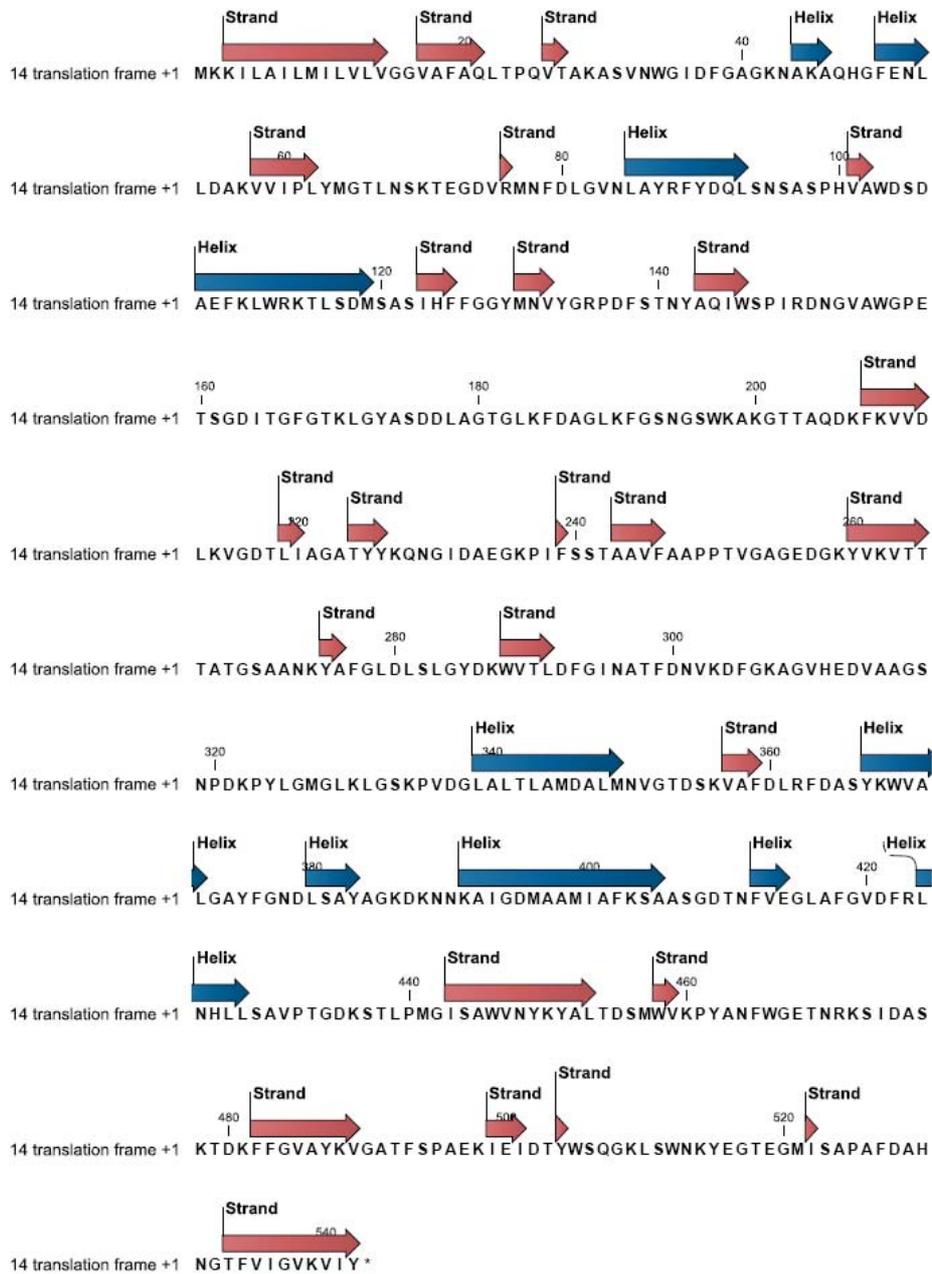
Figure 21. Predicted secondary structure of the MSP peptide of the sample B14.
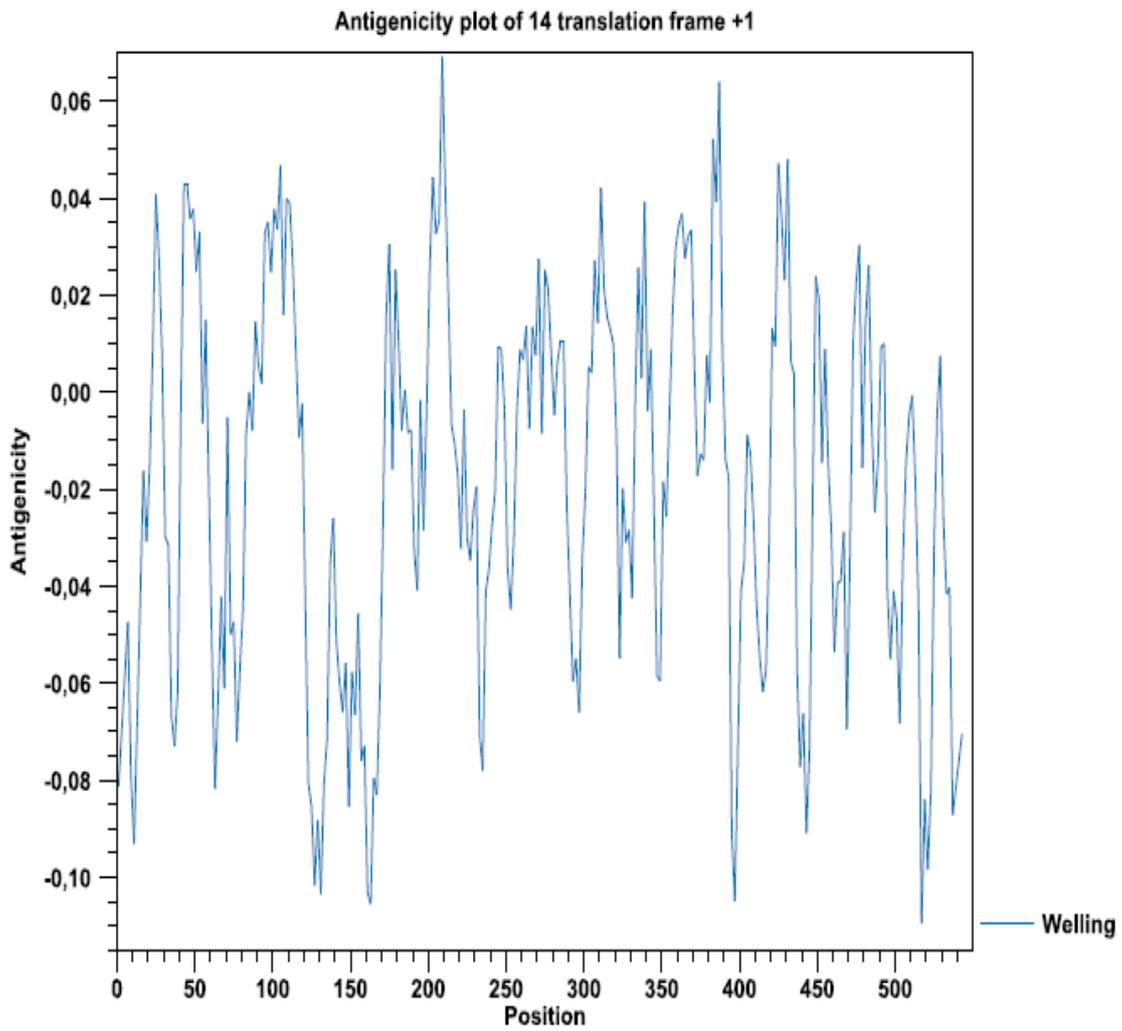
Figure 22. Plot of antigenicity of the deduced MSP protein obtained from the sample B14.

Chapter V

DISCUSSION

Periodontal diseases are widespread pathological conditions that affect millions of individuals worldwide. Among periodontal anaerobic pathogens, the oral spirochetes, and especially *Treponema denticola*, have been associated with periodontal diseases such as early-onset periodontitis, necrotizing ulcerative gingivitis, and acute pericoronitis. Basic research as well as clinical evidence suggest that the prevalence of *T. denticola*, together with other proteolytic Gram-negative bacteria in high numbers in periodontal pockets, may play an important role in the progression of periodontal disease. The accumulation of these bacteria and their products in the pocket may render the surface lining periodontal cells highly susceptible to lysis and damage. The dental biofilm is recognized as one of the major cause of interaction between several different bacteria and the host immune-response. In particular, monocytes are involved in the response against bacteria during infections and are among the cell populations that are detected at sites of active periodontitis (Zappa, 1991). Immune cells synthesize pro-inflammatory mediators that can cause the destruction of tissues (Cochran, 2008; Page, 1991; Silva, 2007). Several bacterial products stimulate host cells to secrete pro-inflammatory mediators; this process is implicated in the development of bone resorption and tissue damage (Rosen, 1999; Page, 1991; Tanabe, 2009). Several studies have shown that the release of cytokines from gingival cells correlates with the progression of periodontitis. Different components of *T.denticola* envelopes, such as CTLP, MSP , lipo-oligosaccharide and peptidoglycan, play specific roles in the progression of

periodontal diseases (Sela, 2001;Fenno, 1998; Mathers, 1996). *In vitro* studies have demonstrated that the MSP –CTLP complex can adhere to and lyse gingival epithelial cells (Fenno, 1998). In addition, isolated MSP is highly cytotoxic to fibroblasts *in vitro* (Wang, 2001). Furthermore, *T.denticola* lipooligosaccharide induces the release of mediators that are involved in the differentiation of osteoclasts (Choi, 2003) from bone marrow cells.

First aim of my study was to demonstrated that the MSP, the major antigen of the outer membrane of *T.denticola*, stimulated the release of pro-inflammatory cytokines from human primary CD14-positive cells. As expected, the production of cytokines was a dose- and time-dependent phenomenon that increased up to 24 h of stimulation. The minimal concentration of MSP that induced significant production of TNF-α, IL-1β and IL-6 by CD14-positive cells was 100 ng/mL. These pro-inflammatory cytokines have been demonstrated to play an important role in the progression of periodontitis (Cochran, 2008; Page, 1991, Silva, 2007), particularly in mechanisms that involve osteoclast differentiation (Boyle, 2003; Kurihara, 1990) and consequent bone resorption (Rodan, 1992). The results presented, evidently confirm the previously reported pro-inflammatory activity of spirochetal outer membrane proteins (Hung, 2006; Sellati, 1998) and clearly suggest that MSP is a strong inducer of the pro-inflammatory response of monocytes. Data collected in this thesis shown that MSP is one of the pathogenic factors that contribute to the pathogenesis of *T.denticola*-related periodontitis. Collagen and numerous extracellular matrix proteins are among the most prominent components of the periodontium (Fenno, 1998). The degradation of nonmineralized portions of periodontal tissues affects the extracellular activity of matrix metalloproteases that are released by intragingival cells (Choi, 2003). This process is the initial step in the progression of periodontal disease. I

demonstrated that MSP is able to release MMP-9, which, indeed, can contribute to the pro-inflammatory response of human blood derived monocytes. Both macrophage-like cells and human primary monocytes released similar amounts of cytokines when they were stimulated with identical concentrations of either MSP or peptidoglycans. In this study I clearly demonstrate that the *T.denticola* bacterial envelope and its virulent factors, such as the Major Surface Protein, plays an important role in the pathogenesis of periodontal disease.

Alsowere, I focused my attention on evaluation on the effects of *T.denticola* outer membrane to alterate vitality and HO-1, Hsp70 expression on a primary porcine aortic endothelial cells (pAEC), in comparison with the classical pro-inflammatory lipopolysaccharide (LPS). Therefore, interesting results were achieved, since data from Western Blot and Real-Time PCR analysis clearly showed a similar apoptotic effect by the outer membrane extract of *T.denticola* and LPS. *T.denticola* outer membrane perturbs the cell cycle of PAEC, leading to the activation of an apoptosis cascade, and this mechanism is a common target utilized by many pathogens (Lee, 2004). Endothelium integrity is fundamental to avoiding the progression of several inflammatory diseases; Seinost and colleagues (Seinost, 2005) hypothesized that the endothelial dysfunction that is associated with severe periodontitis is provoked by direct invasion of the vessel wall by oral pathogens or their bacterial products. The capacity of *T.denticola* outer membrane to perturb the endothelial quiescence was also evidenced by the induction of heat shock proteins. The Heat Shock Response represents a highly conserved mechanism of defense against many physical and chemical stressors, including heat shock, osmotic stress, endotoxin stimulation, shear stress, and heavy metals (Hartl 1996; Morimoto et al. 1994). In the current study, I found

that a preparation of outer membrane of *T.denticola* induces a similar Heat Shock Response in confront with LPS. The different kinetics of HO-1 and Hsp70 induction reflect the disparate roles of these heat shock proteins: Hsp70 prevents protein misfolding and promotes the proteolytic degradation of damaged proteins therefore, a reduction in Hsp70 could be due to functional protein consumption; while the robust and prolonged induction of HO-1 is consistent with a prosurvival significance. Furthermore, the same kinetic trend of Hsp expression by both LPS and OMT could suggest a similar cellular pathway of induction. Although the pathogenicity of T. denticola outer membrane in endothelial cells has not been demonstrated, my results showed the ability of T.denticola to perturb the normal endothelial cells homeostasis.

Moreover, I investigated the interaction of *T.denticola* with phagocytic cells under anaerobic conditions. Anaerobic condition was chosen in order to mimic, as far as possible, the *in vivo* conditions of the periodontal pocket where the level of oxygen is indeed quite low due to modification of gingival microcirculatory function and anatomical position of the oral biofilm. Once uptaken by macrophages *T.denticola* were rapidly and effectively killed, within one hour, independently of the incubation condition. On the other hand, the capability of macrophages to uptake *T.denticola* was influenced by the incubation conditions. When challenge experiments were performed in the presence of atmospheric oxygen concentration, treponemes lossed their motility over time. The number of macrophages showing *T.denticola* intake were greater than those detected in experiments performed under anaerobic conditions. *T.denticola* loss of motility under reduced oxygen condition, was very low and consequently the challenge with macrophages was performed mostly with motile treponemes. These findings suggest that the capability of macrophages

to uptake *T.denticola* in vitro is greatly influenced by the mobility and cellular dimensions of the treponemes. Bacterial motility is likely the major factor influencing cell contact and spatial interaction between macrophages and treponemes in the context of cell sedimentation. In addition, these results suggest that the in vitro interaction between *T.denticola* and macrophages resembles that of other spirochetes, like *Leptospira interrogans* and *Borrelia burgdorferi* that are rapidly and efficiently up taken by macrophages such as by Kupffer cells (Sambri, 1999). The *flgE* non-motile mutant was up taken more efficiently than the parental wild-type strain, independently of the incubation atmosphere. Treponemal motility is a major factor in escaping the uptake by isolated murine macrophages. As expected, another factor that is likely to influence the capability of macrophages to internalize *T.denticola* is the size of the individual bacterial cells. When the surfaces of living cells are coated with specific antibodies, the uptake of *T.denticola* by isolated macrophages in vitro, was highly enhanced. The findings of my study confirmed that the opsonization could indeed increase the ingestion of wild type treponemes by macrophages, as previously suggested for *T. pallidum* (Alder, 1990). The uptake rate increase was independent of the atmospheric condition of the interaction between treponemes and phagocytes. These results suggested that the immune response plays a major role in the control of *T.denticola* spreading from the endodontic site to different anatomical locations. The absence of antibody production against the infection, and the resulting absence of opsonization might have influenced the outcome of the infection and related dissemination. In contrast, opsonization of the two mutant strains did not result in an increased uptake (data not shown), due to an extremely high level of uptake of the two mutant strains in absence of opsonization. Motility and size are capable of influencing the uptake of *T.denticola* by murine macrophage cells, in the in vitro system described in this

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report. Motility greatly facilitates the escape from local immune response, in addition to allowing tissue penetration (Lux *et al.*, 2001) and dissemination to multiple sites (Ehmke, 2004). *T.denticola* could interact with phagocytic cells, in vivo, in to two main locations: within the dental pocket and within the blood stream or in extra-oral tissues, after leaving the oral cavity. Based on theese results, I hypothesize that the presence of a specific antibody response in these sites of infection could greatly contribute to the clearance of the invading treponemes.

In conclusion, in the last part of my thesis I demonstrated that the internal region of the *msp* has the highest degree of variability, when compared with the 5' and 3' portion of the gene, in PCR positive clinical samples from patients suffering from acute periodontal disease. These data confirm that high conservation of the terminal ends of the *msp* gene already reported by Fenno (Fenno, 1997) among *T.denticola* strains cultivated in the laboratory conditions for a long period. The central part of the MSP has been recently demonstrated to be exposed onto the surface of living *T.denticola* cells and to act as a main target for the immune response. In particular, Edwards and co-worker reported that this portion of the protein contain epitopes that bind to an immune serum raised in animal against whole *T.denticola* cells belonging to the same strain ATCC35405 (Edwards, 2005). As previously described, the immune response specific against Msp has a great influence in the efficacy of *T.denticola* phagocytosis by isolated murine macrophages under anaerobic conditions, *in vitro*. The MSP amino acid sequence variability detected among the clinical samples evaluated in this study, could be an important factor influencing the evolution of periodontitis since could hamper the efficiency of the patient immune response during in vivo infections. The interaction between *T.denticola* and the host immune response in vivo

could influence the sequence variation of the MSP central portion. This immune driven sequence variability of one of the most prominent virulence factor of *T.denticola* could underline different pathogenic capabilities during the development of periodontal diseases in vivo. In addition, T.denticola has been demonstrated capable to migrate from the oral cavity to additional anatomical sites. This feature of wild type infecting treponemes could be linked to some variation in the surface exposed portion of the MSP. In addition, the amino acid variation, that usually leads to an antigenic modification, of the surface exposed proteins is a common pathogenic feature of spirochetes. Further studies are necessary to demonstrate that the amino acid difference of *T.denticola* MSP protein could influence the interaction between this treponeme and the immune host response.

Chapter VI

SUPPLEMENTAL MATERIALS

1. Materials and Methods 2

New Oral Spirochete medium (NOS)

Components	g/100 ml medium
BRAIN HEARTINFUSION BROTH	*1.25*
TRYPTICASE (TRYPTONE DIFCO)	*1*
YEAST EXTRACT	*0.25*
ACIDO TIOGLICOLICO	*0.05*
L-CISTEYNE HIDROCHLORIDE	*0.1*
L-ASPARAGYNE	*0.025*
GLUCOSE	*0.2*

Supplements	ml in 100 ml of medium
0.2% (w/v) THIAMINE PYROPHSPHATE (cocacarboxilase)	0.3ml
FAT ACID VOLATLE Per 100 ml di 0.1N di KOH aggiungere: -0.5 ml Isobutyric acid -0.5 ml Isovaleri acid -0.5 ml Valeric acid and D -0.5 ml L-2-Metylbutyrric acid	0.2ml
10% SODIUM BICARBONATE	2ml
NORMAL RABBIT SERUM	2ml

MSP preparation

All procedures were carried out at 48C unless specified. Approximately 8 g (wet weight) of cells per 4-liter culture in late logarithmic growth phase was harvested by centrifugation at 5,000 g for 1 h. The cells were washed twice with phosphate-buffered saline (PBS) (pH 7.3) and once with double-distilled water and then suspended in 25 ml of 20 mM Tris (pH 7.5)–1 mM dithiothreitol–2 mM EDTA–0.1% deoxycholate per liter of culture. The mixture was stirred gently overnight, and undissolved material was collected by centrifugation at 90,000 g for 120 min. The deoxycholate extraction was repeated twice. The pellet was then suspended in the same volume of 10 mM Tris (pH 8.0) containing 1% n-octyl-olyoxyethylene (Octyl-POE; Bachem, King of Prussia, Pa.) and stirred gently overnight.

The Octyl-POE extraction supernatant enriched for MSP was collected after centrifugation at 90,000 g for 45 min at 25^0C. The solution was then incubated for 24 to 48 h at 37^0C. The protein solution was then concentrated approximately 30-fold by Amicon ultrafiltration with an XM50 filter . Detergent was removed from the concentrated protein solution by passing of 10 mM Tris (pH 8.0) through the Amicon unit. The solubilized MSP with the detergent washed out precipitated inside the Amicon unit.

Isolation of CD14 positive cells (Monocytes)

- Isolate PBMC from buffy coat or by using Ficoll-Paque®. To remove clumps, pass cells

- Wash cells, remove supernatant completely and resuspend cell pellet in 30 μl of buffer per 10^7 total cells.

- Add 20 μl of FcR Blocking Reagent per 10^7 total cells and mix well

- Add 20 μl of Biotin-Antibody Cocktail per 10^7 total cells, mix well and incubate at +4 ^{0}C for 10 min.

-Wash cells, remove supernatant completely and resuspend cell pellet in 30 μl of buffer per 10^7 total cells.

- Add 20 μl of Anti-Biotin MicroBeads per 10^7 total cells, mix well and incubate at +4 ^{0}C for 10 min.

-Wash cells, remove supernatant completely and resuspend cell pellet in 30 μl

- Apply cell suspension in a buffer on top of the depletion column (AS: 500 μl)

- Let the negative cells pass through. Rinse with 3−5 column volumes of buffer from top (AS: 3 ml). Collect effluent as CD14 positive cells.

T. denticola **Outer Membrane preparation.**

NOS medium was inoculated with a 3-day culture of *T. denticola* ATCC 35405 at a ratio of 30:1 (fresh medium to inoculum) and incubated at 37°C for 4 days. Bacteria were harvested by centrifugation at 12,000 *g* for 15 min at 4°C and washed twice in PBS. The pellet was weighed, dispersed uniformly, and resuspended at 1.0 g (wet weight) per 10 ml of PBS containing 10 mM MgCl2. Triton X-100 was added to a final concentration of 0.2% (vol/vol). The suspension was incubated with constant mixing at 37°C for 30 min and then repeatedly centrifuged at 12,000 *g* for 6 min until no visible pellet remained. The clear supernatant was dialyzed (molecular mass cutoff, 50 kDa against deionized H2O at 4°C for several days until precipitates formed. The contents of the dialysis tubing were centrifuged at 25,000 *g* for 45 min. The pellet was resuspended in deionized H2O to the predialysis volume and stored at 27°C. Aliquots were also run by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% acrylamide gel and silver stain to compare the migration of OM polypeptides qualitatively with migration patterns of polypeptides from whole cells.

Table 1A

	Gene Sequence (5′–3′)	Length (bp)	Acc. No.
Hsp70 For.:	GTGGCTCTACCCGCATCCC	114 bp	M29506
Rev.:	GCACAGCAGCACCATAGGC		
HO-1 For.:	CGCTCCCGAATGAACAC	112 bp	NM_001004027
Rev.:	GCTCCTGCACCTCCTC		
HPRT For.:	GGACAGGACTGAACGGCTTG	115 b	p AF143818
Rev.:	GTAATCCAGCAGGTCAGCAAAG		

Table 2A

	Gene Sequence (5′–3′)	Length (bp)
Dent01	TAATACCGAATGTGCTCATTTACAT	316 bp
Dent02	TCAAAGAAGCATTCCCTCTTCTTCTTA	

Isolation of mouse peritoneal macrophages.

3 ml quantity of 3% thioglycolate broth (Difco, Detroit, Mich.) was injected intraperitoneally into female mice 7 to 8 weeks of age. Four days after injection, the mice were sacrificed by cervical dislocation, and the peritoneum was washed with PBS. Macrophages were collected by aspiration of the PBS from the peritoneal cavity, washed twice, and counted with a hemocytometer. Cell viability was verified by the trypan blue exclusion technique and was found to be .95% in all experiments. The macrophages were suspended in RPMI 1640 medium containing 100 U of penicillin/ml, 100 mg of streptomycin/ml, 2 mM L-glutamine, and 5% fetal calf serum. The cells were plated in 24-well tissue culture plates (10^6 per well) and incubated for 60 min (37°C; 5% CO2). Non adherent cells were removed by aspiration, and the remaining adherent cells were washed three times with PBS prior to being used in the subsequent experiments.

Preparation of anti-MSP antibodies (Titer Max Adjuvant)

To prepare 1.0 mL of the recommended water-in-oil emulsion, 0.5 mL of aqueous MSP is required. A 50:50 water-in-oil emulsion is usually optimal. Each vial of Adjuvant contains enough product to load a syringe with 500 mL two times.

- After the Adjuvant has been vortexed, load one syringe with 0.5 mL of Adjuvant and load the second syringe with 0.25 mL of MSP in aqueous medium. Set aside the other 0.25 mL of MSP (Adjuvant should be the least 2 small volumes of the aqueous phase)

- Connect the two syringes via the 3-way stopcock. Mix the Adjuvant with the antigen by forcing the materials back and forth through the stopcock for ~2 minutes. Push the antigen into the (Adjuvant syringe first, so that the aqueous phase enters the oil phase rather than vice versa)

- After ~2 minutes, a meringue-like water-in-oil emulsion forms. Push all of the emulsion into one syringe and disconnect the empty syringe.

- Load the empty syringe with the remaining 0.25 mL of aqueous MSP solution. Reconnect the syringes and emulsify for another 60 seconds. Push all of the emulsion into one syringe and disconnect the empty syringe.

- Inject 4 divided doses of 25 mL each into 4 subcutaneous or into intramuscular sites.

- Attempt 40 days collect the blood from the rabbit to determine the titre of anti-MSP specific IgG.

Table 3A

Primer	Nucleotide Sequence (5′–3′)	AmpliconSize	Location	Cycles of Amplification
Dent01 Dent02	5'TAATACCGAATGTGCTCATTTACAT3' 5'TCAAAGAAGCATTCCCTCTTCTTCTTA3'	316 bp	16S rDNA	36 cycles 95^{0}C 30'' 60^{0}C 1' 72^{0}C 1'
Porf2 Porf4	5'AGGCAGCTTGCCATACTGCG3' 5'CTGTTAGCAACTACCGATGT3'	404 bp	16S rDNA	35 cycles 95^{0}C 30'' 58^{0}C 45'' 72^{0}C 30''
Prev2 Prev3	5'CGTGGACCAAAGATTCATCGGT3' 5'CTTTACTCCCCAACAAAGCA3'	256 bp	16S rDNA	35 cycles 95^{0}C 30'' 58^{0}C 45'' 72^{0}C 20''
For1 For2	5'TACAGGGGAATAAAATGAGATACG3' 5'ACGTCATCCCAACCTTCCTC3'	746 bp	16S rDNA	35 cycles 95^{0}C 30'' 61^{0}C 45'' 72^{0}C 40''
E.Fae1 E.Fae2	5'ATCAAGTACAGTTAGTCT3' 5'ACGATTCAAAGCTAACTG3'	941 bp	16S rDNA	35 cycles 94^{0}C 30'' 55^{0}C 45'' 72^{0}C 40''
Kx14 Kx04	5'GCTTGACAAGTGGATTTGGCTGTG3' 5'GAGAATAGCAGCAGAGTCTATTAG3'	1800 bp	(1-24) bp (1777-1754) bp	36 cycles 94^{0}C 1' 45^{0}C 1' 72^{0}C 3'
Kx14 Kx09	5'GCTTGACAAGTGGATTTGGCTGTG3' 5'CGAACGTCACCTTCGGTCTTTGAG3'	294 bp	(1-24) bp (294-271) bp	31 cycles 94^{0}C 1' 55^{0}C 1' 72^{0}C 3'
Td03 Td06	5'CTCAAAGACCGAAGGTGACGTTCG3' 5'GCATATTTGTTTGCTGCG3'	571 bp	(271-294) bp (842-824) bp	31 cycles 94^{0}C 1' 55^{0}C 1' 72^{0}C 3'
Td05 Kx04	5'CCGCAGCAAACAAATATGC3' 5' GAGAATAGCAGCAGAGTCTATTAG3'	875 bp	(824-842) bp (1777-1754) bp	31 cycles 94^{0}C 1' 55^{0}C 1' 72^{0}C 3'

2. Movies

Movie 1 Caption. (Phagocytosis 1). Macrophage uptake of *T. denticola* wild-type cells under aerobic conditions visualized by imaging methdos with a Nikon Microscope at 400x. In particular several Treponemes cells bounds to the membrane of the adhese murine macrophages. Few cells showed an internalizated *T.denticola* cells into macrophages.

Movie 2 Caption. (Phagocytosis 2). Particular of the internalization of *T.denticola* cells by murine peritoneal macrophages, subsequent bound of the treponemes to the membrane of immune cell. In details, is possible to observe the internalization and the morphological change in the macrophages cells with the formation of a vescicle that containing the internalized bacteria.

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