Chapter 1

General introduction
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Periodontal diseases are among the most prevalent infectious diseases in the world. This thesis concentrates on *Porphyromonas gingivalis*, a causative agent of chronic periodontitis. Chronic periodontitis is defined as an infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment loss, and bone loss [3]. If left untreated, teeth may show exposed root surfaces, in conjunction with red, swollen gums that easily bleed. Dental radiographs reveal periodontal (alveolar) bone loss around the teeth due to the inflammation process; teeth will become mobile and migrate, and can eventually be lost. Patients with periodontitis experience problems with chewing; they have bad breath and suffer from important subjective and objective esthetic problems. Dental practitioners provide labor-intensive diagnostic and treatment sessions to periodontitis patients, including periodontal surgery.

Periodontitis is worldwide highly prevalent. Although, it has been reported that in the US periodontitis might be declining in the last decade [4], it was also concluded recently that the majority (>50%) of the US adult population exhibits, to some extent, mild, chronic periodontitis [4]. Also in Europe a decrease in periodontitis has been reported over the last 30 years, although more high quality studies have to be performed to support these findings [5]. Due to rapid aging and increased prevalence of periodontitis in older populations the problem will, however, be emphasized again. Up to 38% of severe cases have been reported in a male population which was on average 75 years-old [6].

A difficulty in studying the epidemiology of periodontitis is that the definition of the disease is still not well standardized. Therefore, comparing one study with the other is a delicate subject. But knowing that the Western population is ageing and that prevalence of periodontitis is strongly correlated with age, it is a certainty that periodontitis is an important disease now and will be an even more important disease in the decades to come.

Recent data suggest that periodontitis is associated with increased risk for cardiovascular diseases [7], possibly through the elevation of the acute phase reactant C-reactive protein (CRP) or other systemic markers of inflammation [8]. The systemic reactions to periodontitis can also result from dissemination of oral bacteria or its components. There are strong indications that the inflamed and ulcerated periodontal epithelium forms an easy port of entry for oral microorganisms. Short moments of bacteremia occur most likely several times a day. Like any other inflammatory condition,
untreated chronic periodontitis may pose a risk for the overall health of the subject [9].

**Periodontitis etiology**

Although periodontitis has been studied for about 100 years the etiology is still not fully understood. It is considered to be a complex disease. Many risk factors are associated with the disease including microbiological, lifestyle and genetic factors (figure 1).

**Figure 1. Periodontitis as a multifactorial disease** (modified from [10]). Disease development consists of a host response, depending on host genetic factors, to microbiological factors and lifestyle leading to periodontitis.

In the early days (<1930s), oral microbiological research aimed to find pathogens in dental plaque causing periodontitis [24]. Due to the complex nature of the disease and due to limitations of the cultivation methods researchers were not very successful in describing primary pathogens. In the middle of last century plaque was even described as non-specific, which meant that only the amount of plaque and not the composition was seen as a risk factor [28].

The polymicrobial disease periodontitis has now been shown to be triggered by the presence of certain bacteria in subgingival plaque. A shift in microbial composition of the plaque from predominantly Gram positive facultative aerobic bacteria to Gram negative anaerobic bacteria enhances the process. Mostly anaerobic bacteria have been associated with periodontal disease including *Prevotella intermedia* [29, 30], *Fusobacterium*
nucleatum [31], Tannerella forsythia [32, 33, Peptostreptococcus micros [34, Treponema denticola [35] and Porphyromonas gingivalis [33, 36], but also the facultative anaerobic Aggregatibacter actinomycetemcomitans [29].

The most important lifestyle factors in the development of periodontitis are smoking, stress and poor oral hygiene. Smoking has been described to have an association with periodontitis already since the early days of periodontitis research. It increases the risk for the disease 2.5-7 times [11] by weakening the host immune system, systemically as well as locally, but potentially also by affecting bacterial behavior in the subgingival biofilm [12]. Clinical symptoms, i.e. gingival redness and bleeding on probing, are masked by smoking due to dampening of inflammation, and therefore disease severity is regularly underestimated in smoking patients. Furthermore, periodontal therapy is less effective in smokers [13].

Stress can negatively influence general health. Especially associations between infectious disease and stress have been described [14]. Periodontitis researchers also propose a mechanism in which stress is a risk factor [15, 16]. Most research however has great shortcomings due to which evidence is not convincing [16, 17]. For example, the definition of stress is continuously changed, which makes comparison of research impossible. And it is very difficult to have stress as the only variable in an experiment, making data interpretation complicated. Despite that, it would be unlikely that stress is not a risk factor for periodontitis. Oral hygiene is a lifestyle factor directly influencing the plaque formation and plaque composition and thereby the microbiological factors. Poor oral hygiene or poor plaque control has been recognized as one of the risk factors for the development of periodontitis [2]. Poor oral hygiene also decreases success of periodontal treatment [18].

Several systemic diseases have been investigated as risk factors for periodontal disease. Some examples are obesity, osteoporosis, human immunodeficiency (HIV) and diabetes mellitus [19-22]. Although for each of these diseases there have been studies implying them as risk factors, diabetes is the only disease with a solid amount of supporting data. Metabolic control of disease also has been shown to reduce the negative influence of diabetes on periodontal status [23].

The first proposal of a genetic component in the development of periodontal disease was formulated in the thirties of last century [24]. Early recognized signs were potential heritability of susceptibility, but also diversity of clinical outcome when subjects shared all the other risk factors. Only 10-20 years ago however this proposal has got increasing support in
periodontal literature [25, 26]. Putative genetic risk factors have been recognized. Genetic risk factors each playing a small role in periodontitis development have been found to add up in the susceptibility of a subject. Polymorphisms found in genes coding for proteins involved in host immune response have mostly been associated with increased risk to develop periodontitis [27].

**Oral community**

The mouth is a challenging ecosystem to study. Since it is an open system allowing organisms to invade and to be flushed away the community has the potential to change composition rapidly. The huge diversity of species being able to inhabit the oral cavity also makes it a very difficult study object. As many as 700 species have until recently been believed to be present in the oral environment, of which 400 species could be found in the periodontal pocket [37]. New generation sequencing techniques have however changed this idea dramatically. The power to produce and analyze virtually unlimited amounts of sequence data has yielded the idea that the oral cavity harbors actually a much more complex community. It is now suggested that the number of species present is an order of magnitude higher than previously suggested [38].

Many of the species in the oral environment are commensal bacteria, e.g. normal residents. Many of those serve a beneficial purpose for the host and others will not have a negative influence in a stable situation. Altogether these bacteria form a community that is in balance with the host. It is when the balance gets disturbed and pathogens like *P. gingivalis* grow out because of more favorable conditions, that a problem will arise. This shift in biofilm composition could then lead to the triggering of periodontitis. Development of an oral infectious disease is comparable with the development of intestinal diseases where a shift in the extremely complicated microbial community composition can lead to health problems [39].

Despite the fact that the oral microbial community harbors an extensive array of bacterial, fungal, protozoan and archeal species [40-42], the earlier mentioned *P. gingivalis*, *T. forsythia* and *T. denticola* have strongly been associated with periodontitis. Those species have been shown to co-occur in the pockets of periodontitis patients. Together they have been named red complex species as they are most strongly related to periodontitis and its severity, specifically with pocket depth and bleeding on probing [36]. *P. gingivalis* is only found in the presence of both *T. forsythia* and *T. denticola*,


whereas both can be found in the absence of *P. gingivalis* [43]. The red complex species have been thought to be the successors of the orange complex species *F. nucleatum, P. intermedia* and *Prevotella nigrescence* [36, 44] which are also associated with periodontitis, although to a lesser extent.

**Porphyromonas gingivalis**

In this thesis the focus will be on *P. gingivalis* as besides a very strong association with periodontitis it also has a vast arsenal of virulence factors. The anaerobic, non-motile, asaccharolytic, Gram negative rod-shaped bacterium *P. gingivalis* has been implicated as a major causative agent especially in chronic periodontitis. It is present in only 10-25% of healthy subjects and 53-90% in individuals with periodontitis when detection by PCR is used [45–48]. As can be expected the numbers detected by anaerobic culturing are lower [48]. To answer the question how one can be infected with *P. gingivalis* family studies have been performed, showing family members can infect each other. Bacterial load in saliva of the donor as well as genetics of the recipient are determinants in infectivity [49].

*P. gingivalis* is able to interact with its host in several ways. It is able to adhere to salivary components like proline-rich proteins, histatin and fibronectin [50–52]. It can adhere to a variety of cell types, including epithelial cell, erythrocytes and fibroblasts [53, 54]. Interaction with the host is crucial in establishing a chronic infection and molecules involved in interactions with the host are therefore mostly indicated as virulence factors.

Interaction of *P. gingivalis* with the host inevitably induces a host response. Periodontal patients have elevated levels of inflammatory mediators in their crevicular fluid as known from clinical studies. These proteins are produced as a response to the complex infection in periodontitis patients The mediators include receptor activator of NF-κB ligand (RANKL), pro-inflammatory cytokines interleukin-1 (IL-1) and interleukin-6 (IL-6) and tumor necrosis factor (TNF), chemokine IL-8, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1a (MIP-1a), and adhesion molecule intracellular adhesion molecule-1 (ICAM-1) [55]. *P. gingivalis* antigens can stimulate host cells in several different ways. Lipopolysaccharide (LPS), an important surface antigen of Gram negative bacteria, activates a pro-inflammatory response inducing IL-1β, IL-6, IL-8 and TNFα in a wide variety of host cells [56, 57]. Fimbriae have been shown to induce a pro-inflammatory response and a Th1 T-cell phenotype [57, 58]. Capsular polysaccharide also has been shown to be able to induce pro-
inflammatory cytokines and chemokines [59]. Gingipains induce a complex response, including the induction of a Th2 response [60] and inactivation of Th2 cytokines IL-4 and IL-5 [61], but also an induction or attenuation of proinflammatory mediators dependent on RgpA-kgp complex concentration [62]. When using whole viable \textit{P. gingivalis} cells in \textit{in vitro} experiments one can also measure elevated levels of pro-inflammatory mediators [63].

Besides the ability to interact with its host, \textit{P. gingivalis} is also able to interact with a variety of bacteria found in subgingival plaque. Being able to interact with other oral bacteria indicates that bacteria may support each other in establishing a mature biofilm. \textit{P. gingivalis} is described as a late colonizer of the subgingival plaque, which indicates that the environment of the periodontal pocket needs to be “prepared” by other bacteria before being favourable for \textit{P. gingivalis}. The interaction between oral bacteria is extensively reviewed by Kolenbrander \textit{et al.} [64]. They propose some mechanisms used for succession of species. \textit{P. gingivalis} can interact with Gram positive and Gram negative bacteria including streptococci [65], \textit{Peptostreptococcus micros} [66], \textit{Treponema denticola} and \textit{Fusobacterium nucleatum} [67]. Binding between the species is dependent on specific adhesion-receptor interactions.

**Virulence factors**

Virulence is generally defined as the degree of pathogenicity of an organism, which means the relative ability of a pathogen to cause disease. A wide variety of definitions is in use, but all come down to the same idea. For the term virulence factor however the definitions are extremely variable. The two definitions that seem to be most accurate for description of virulence factors are:

1) those components of an organism that determine its capacity to cause disease but are not required for its viability per se [68].

2) molecules expressed and secreted by pathogens (bacteria, viruses, fungi and protozoa) that enable them to achieve the following:

- colonization of a niche in the host (this includes adhesion to cells)
- evasion of the host's immune response.
- entry into and exit out of cells (if the pathogen is an intracellular one).
- obtain nutrition from the host [69].

The first definition is very simple and clear, but it does not describe how a component would act in disease. The second, although much more elaborate, does not exclude vital functions of an organism.
My definition of a virulence factor would be:
A non-vital component of an organism that determines its capacity to cause disease by enabling the pathogen to colonize the host, to evade the immune system, to enter/exit host cells or to obtain nutrition from the host.

But even this definition raises questions when studying polymicrobial infections like periodontitis. Species may not be harmful by nature, but because they are present other species may be able to colonize due to interactions between those species. A polymicrobial consortium acts together as an infectious agent. So, a virulence factor could then also be a component that enables other species to cause disease. Although relevant in the in vivo situation, this is beyond the scope of the studies in which only P. gingivalis strains are studied.

According to the above-mentioned new definition *P. gingivalis* has many virulence factors, ranging from fimbriae involved in colonization and internalization in host cells to proteases involved in nutrient availability and immune system evasion. Below, a set of *P. gingivalis* virulence factors will be described (figure 2).

**Proteases**

*P. gingivalis* has been found to be highly proteolytic, with a wide variety of protease specificities. Some proteases have been studied intensively as they were found to be involved in virulence, but some predicted peptidases from the *P. gingivalis* genome have not been studied yet. Since *P. gingivalis* is asaccharolytic it is dependent on protein degradation for the release of oligopeptides or amino acids that can serve as a nutrition source. A main function of some of the peptidases is therefore food availability. Since *P. gingivalis* is an inhabitant of the subgingival pocket the degradation of host proteins in its surroundings understandably may have an adverse effect on the host. The adverse effects can be degradation of structural components like collagen, but also degradation of immune system components.

The best-studied proteases of *P. gingivalis* are the cysteine proteases called Arg-gingipain (RgpA and RgpB) and Lys-gingipain (Kgp) [71-73] and have been extensively reviewed recently [74].

Their functions have been found to be very broad. The first described function of RgpA was complement activation by hydrolysis of C3 and C5 [75]. The first indicated function of Kgp was hemolysis of erythrocytes [76], enabling heme-acquisition. Later many more functions have been described for each of the proteases including, supplying energy by protein breakdown, involvement in fimbriae expression, hemagglutination, exposing cryptotopes.
for bacterial attachment, involvement in fibroblast apoptosis and complement dysregulation at various stages [77, 78]. In a murine lesion model gingipains were appointed a direct role in virulence as mutant strains lacking the proteases were far less virulent than the wild-type strain [79].

Trp, also a cysteine protease which has been cloned and overexpressed has not been given any function yet. Only some specificities have been described by use of Tpr overexpressed in *Escherichia coli* [80-82]. Another protease PrtT has no appointed role in virulence yet, but it is very similar to the pyrogenic streptococcal exotoxin B. Its proteolytic activity is mostly directed against denatured proteins [83, 84]. A set of peptidases that however do have an appointed function are the di- and tri-peptidyl aminopeptidases, which have been shown to supply the bacteria with nutrients for growth [85].

**Adhesins**

Binding of a bacterium to a host surface, host component or another species is crucial for virulence, as mentioned in the definition of a virulence factor. In *P. gingivalis* several molecules have been indicated to be involved in adhesion. The group of adhesins can be divided into two main groups:
fimbrial and non-fimbrial adhesins [86]. Fimbriae are long protein molecules anchored in the outer membrane (figure 2). They can be visualized by electron microscopy as threat-like structures [54]. Two types of fimbriae have been described for *P. gingivalis*, namely long or major fimbriae and short or minor fimbriae.

The major fimbriae, which can be up to 1 µm long, consist of the 41 kDa fimbrillin FimA. FimA has been shown to be crucial in adherence and invasion of several human cell types including fibroblasts and epithelial cells [86, 88]. Over the years many receptor proteins recognizing *P. gingivalis* FimA have been described. These receptors include salivary proteins, extracellular matrix proteins, hemoglobin, TLR-2 and CD14 [86]. Interaction with the latter two receptors induces cytokine expression, thereby potentially inducing bone-resorption. A rat infection experiment indeed showed that infection with a *fimA* mutant leads to reduced bone loss compared to infection with the wild type [89]. FimA is also involved in binding of other oral bacterial components. Dentilisin from *T. denticola* and GAPDH from *Streptococcus oralis* have both been shown to interact specifically with the major fimbriae of *P. gingivalis* [90, 91].

Minor fimbriae were first discovered when a *fimA* mutant was carefully investigated microscopically [92]. Minor fimbriae consist of the 67 kDa fimbrillin Mfa1 encoded by the *mfaI* gene. Minor fimbriae may also play an important role in periodontal disease as mutants have been shown to induce less alveolar bone resorption in rats [93]. They have only been studied to a limited extent, but roles have been described in immuno stimulation and bacterial interaction [58, 94]. Mfa binds directly to CD14 and TLR-2 thereby inducing a pro-inflammatory response [58].

*P. gingivalis* strain W83 which is used in this thesis has been shown to be deficient in major fimbriae production, although the genetic machinery seems to be present. Recently it has been described however that this strain is affected in both a regulatory FimS protein and in the structural FimA protein [95]. The closely related strain W50 has the same FimA gene, but has been found to poses minor fimbriae [58]. In W83 a disrupted *mfaI* gene has been found.

The second group of adhesins or non-fimbrial adhesins consists of the earlier mentioned gingipains RgpA, RgpB and Kgp by means of their C-terminal adhesion domain, their closely related adhesin hemagglutinin A (HagA) and hemin binding protein 35 (HBP35). Gingipains adhere to laminin, fibronectin, collagen, erythrocytes and hemoglobin by their adhesion domains. After binding the proteinase domain is able to degrade the bound
proteins thereby detaching again [96]. Being able to bind hemoglobin and erythrocytes and subsequently degrade proteins gives them an important role in iron acquisition, although probably not a crucial one [97]. Gingipain complexes have also been shown to be involved in co-aggregation with other bacteria, giving them a role in mixed biofilm formation [98].

**Lipopolysaccharides (LPS)**

LPS has been regarded as a relatively invariant structure of the Gram negative cell envelope (figure 2). It is one of the most intensively-studied bacterial virulence factors. LPS consists of three domains; lipidA, which anchors LPS into the bacterial membrane, a core domain consisting of at least an oligosaccharide connecting lipid A to the third domain, e.g. the O-antigen, which is a repetitive glycan polymer found outside the bacterial membrane. For *P. gingivalis* three O-antigen serotypes have been described O1, O2 and O3 from strains 381, HG1691 and W50 respectively [99, 100]. Patient-derived titers to serotype O3 (W50) have been described as useful in disease classification. Titers to the other serotypes have not been shown to be predictive except when combined with titers to K-antigens (CPS). *P. gingivalis* LPS has however been shown to be different from other Gram negative bacterial LPS structures. The lipid A part can be a mixture of structures that can even be modulated in reaction to exogenous stimuli, thereby changing it’s recognition by and activation of toll-like receptor 4 (TLR4) [101]. Some forms are TLR4 antagonists. *P. gingivalis* LPS is even able to upregulate negative regulators of TLR signalling, thereby suppressing the innate immunity system [102]. TLR2 and TLR4 are both able to recognize *P. gingivalis* LPS, with TLR2 being the dominant receptor. Due to a complex modulation of the immune regulation *P. gingivalis* LPS does not seem to induce a high pro-inflammatory immune response in contrast to the well-studied *E. coli* LPS [56].

A second LPS structure, A-LPS in addition to O-LPS, within a single strain (W50) has been recently described, including genes involved in LPS biosynthesis. PG1051 (*waaL*) an O-antigen ligase and PG1142 an O-antigen polymerase [103, 104] were shown to encode the enzymes linking the repeating units of the O-antigen to the core oligosaccharide and the polymerization of those repeating units respectively. Variation in potency to induce an inflammatory response has been described for the LPSs. A role for this variation in pathogenesis has not been established yet.
Outer membrane vesicles (OMVs)

Electron microscopy studies of *P. gingivalis* revealed the presence of extracellular membrane vesicles, seen as spheres budded off from the bacterial cell. The protein content of these vesicles has been shown to be similar to the outer membrane protein content [105]. When Grenier and Mayrand had shown that the vesicles had a vast protease activity they regarded them as potential virulence factors [106]. Functional characterization of the vesicles then showed that they indeed could be involved in virulence as they protected other species from being killed by the complement system both by protease activity and by the presence of LPS [107]. Next to proteases and LPS also nuclease activity has been found in vesicles [108]. The vesicles have furthermore been found inside host cells in *in vitro* studies thereby interfering with host cell functions [109, 110]. Recently, effort has been put in the direct interaction of membrane vesicles with the immune system. *P. gingivalis* membrane vesicles have now been indicated in modulation of antigen presentation, thereby enabling evasion of the immune system [111]. Another action which has been described for membrane vesicles is the enhancement of attachment of other oral bacteria to host-cells [112]. The many roles that these membrane vesicles can play again show the variety of the *P. gingivalis* virulence factor arsenal.

Capsular polysaccharide (CPS)

Capsules have first been described as external structures surrounding a bacterial cell seen by microscopic examination (figure 3). The bacterial

![Figure 3. Negative staining of *P. gingivalis* capsule using India ink [113]. Bacterial cell bodies are surrounded by a white CPS halo (arrows) unstained by India ink that forms the dark background.](image)
capsule mainly consists of extracellular hydrophilic, negatively charged polysaccharides anchored in the bacterial outer membrane by lipid moieties. It has a role in the rigidity of the cell membrane, but the role for which it has been studied most is its role as a virulence factor in many pathogenic bacteria like *Neisseria meningitides*, *Streptococcus pneumonia* and *E. coli*. Presence of capsular polysaccharide has been associated with resistance to phagocytosis and complement killing thereby giving bacteria a tool to evade the host immune system [113]. *P. gingivalis* can also express a capsule as seen in negative staining using India ink. It can be as thick as 1 µm, which is about the size of an average *P. gingivalis* cell body. The capsule of *P. gingivalis* has also been regarded as an important virulence factor as differences in virulence in a mouse subcutaneous infection model were found between encapsulated and non-encapsulated strains [115]. The strains expressing a capsule mostly caused spreading infections, which often lead to death, whereas non-encapsulated strains caused local abscesses leading to much less dead test animals [113, 115, 116]. Prevalence of encapsulated strains in periodontitis patients has been described to be around 70% as examined by microscopy [117]. So, 30% of these *P. gingivalis* strains are non-encapsulated (K-). One of the most-studied *P. gingivalis* strains, the fully sequenced strain ATCC33277, is a non-encapsulated strain as is its close relative HG91, which is used as a non-encapsulated reference strain in the research described in this thesis.

*P. gingivalis* CPS or K-antigen has antigenic properties that make them typable by serotyping methods. In that way six capsular serotypes have been described and a seventh has been proposed by Schifferle et al. named K1-K7 [114, 116]. The prevalence of typable *P. gingivalis* strains has been shown to be 45% in a Dutch population of periodontitis patients when using K1-K6 antisera. From the encapsulated strains only 58% could be serotyped, suggesting that more CPS serotypes should be present [117]. Serum titers against K-antigens K1 and K6 of periodontitis patients have been shown to be indicative for disease [100].

The structure of *P. gingivalis* CPS until today has not been elucidated. Its monosaccharide composition has been studied, but not in much detail. The most detailed examination of *P. gingivalis* CPS describes the cell-surface polysaccharides of strain ATCC53978 (W50) [118], which supports the earlier findings of *P. gingivalis* polysaccharide [119]. This K1 serotype strain is highly virulent and has a thick capsular layer. The study refers to gel-like properties of the isolated *P. gingivalis* CPS, which make the CPS hardly soluble in heavy water after extraction. The monosaccharide analysis
reveals that mannanuronic acid (ManA), glucuronic acid (GlcA), galacturonic acid (GalA), galactose (Gal) and 2-acetamido-2-deoxy-D-glucose (GlcNac) are the components of *P. gingivalis* K1-type CPS.

Until recently next to protease activity *P. gingivalis* CPS was thought to be one of the main structures in resistance to serum killing. Recently however it has been shown that another anionic polysaccharide (APS) is responsible for this action. A mutant lacking capsule synthesis was still resistant, whereas the APS synthesis mutant was highly sensitive to serum killing [120].

*In vitro* infection studies on macrophages using isolated CPS of each of serotypes K1-K7 showed that K1 CPS triggers the highest pro-inflammatory immune response and most cell migration [59]. However, the differences in response between K1 and the other CPS types have not been explained.

The biosynthesis pathway of *P. gingivalis* CPS has not been described. A start was however made by the publication of the *P. gingivalis* W83 genome sequence including a prediction of potential CPS biosynthesis loci by comparative genomic analysis [121]. W83, a close relative of strain W50, is a highly virulent K1 encapsulated strain [113]. A genomic hybridization study of the non-encapsulated ATCC33277 strain to the W83 genome array revealed a highly divergent region which was already predicted to be a potential CPS synthesis locus [122]. Deletion mutants lacking capsule expression then proved that locus PG0106-PG0120 is the actual CPS biosynthesis locus [124]. Further genetic studies have illustrated the increase in biofilm formation potential of CPS biosynthesis mutants [123]. The role of CPS is not fully understood yet. Strains with CPS seem to have a lower hydrophobicity, which may prevent PMN killing. CPS mutants also show more auto-aggregation and higher hydrophobicity than the parent strains [124].

Studies on *P. gingivalis* have been performed almost exclusively on three strains, namely ATCC33277, W83 and W50. The genomes of the first two have now been sequenced [121, 125]. Because ATCC33277 is non-encapsulated K1 strain W83 was taken as the model strain to study CPS biosynthesis in this thesis.
Outline of this thesis

The focus of this thesis is on *P. gingivalis* CPS and genes involved in CPS biosynthesis. Only little is known about the genetic background of this undoubtedly important virulence indicator of *P. gingivalis* and the role of CPS in the etiology of periodontitis is still unclear.

These studies are performed to better understand how CPS is involved in the etiology of periodontitis and which genes are involved in biosynthesis of the capsular serotypes.

In chapter 2 the construction of molecular tools that can be used in *P. gingivalis* CPS research is described. An isogenic mutant which lacks the epsC gene at the far 3'-end of the CPS locus is presented. EpsC is found in several *P. gingivalis* strains. This epsC mutant is shown to be non-encapsulated by a combination of techniques, showing that epsC is essential for *P. gingivalis* CPS synthesis. This mutant is a good tool to study the role of CPS during an infection as described in chapter 4. Development of some easy-to-use plasmids for targeting of genes to the *P. gingivalis* genome is also described. The plasmids contain two different promoters (CP25 and PG0106) to choose from, two resistance cassettes (tetQ and eryF) which could be introduced at two specific regions of the *P. gingivalis* genome. The plasmids allow for exchange of a gene or a promoter by a single restriction/ligation. Expression cassettes can thus easily be constructed and integrated at the two selected loci in the *P. gingivalis* genome, making the plasmids good tools for complementation and overexpresion experiments.

In chapter 3 the genetic locus of the CPS biosynthesis of a set of K1 serotype strains was analyzed, giving insight in the variation within a group of K1 strains. Using two techniques, restriction fragment length polymorphism (RFLP) analysis and polymerase chain reaction (PCR), only slight differences within the K1 serotype were found. This new knowledge allowed the development of a K-specific PCR based serotyping assay, which was shown to be highly specific and sensitive.

In chapter 4 the epsC mutant constructed in chapter 2 was analyzed for its change in the ability to trigger a pro-inflammatory immune response in human gingival fibroblasts. The study with viable bacteria shows that *P. gingivalis* CPS reduces the immune response of the fibroblasts.

To gain insight on the genetic differences between strains a comparative genomic DNA hybridization (CGH) study is presented using a representative of each capsular serotype. A W83 microarray is used for hybridization. The
description of a \textit{P. gingivalis} core genome was initiated in this study, showing that 80\% of all W83 genes belong to the core genome. Previously described virulence gene sets were used for more in-depth analyses of the data. The presence of almost all virulence related genes in the core genome was seen. As expected, high variation in the CPS locus was seen between he strains.

In chapter 6 this thesis will be overviewed and the findings will be put in a broader perspective. The potential role of \textit{P. gingivalis} CPS will be discussed as well as the potential of regulation of CPS biosynthesis.

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Chapter 1


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