Genetic background of Porphyromonas gingivalis capsule biosynthesis
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Chapter 6

General discussion
Periodontal disease is among the most prevalent infectious diseases world-wide. Chronic periodontitis has been found in a mild to severe form in more than 50% of the adults in the US [1]. Although prevalence may tend to go down the latest decades [1, 2], the disease is thought to play an even more prominent role in the near future as the Western population is ageing rapidly. Chronic periodontitis has been found to be more prevalent in older populations [3].

Periodontal disease can be studied from several different angles, because many factors are involved as described in the introduction of this thesis. Interplay of bacterial species in a complex biofilm is an important factor [4]. In this thesis a small piece of the multifaceted puzzle, namely *Porphyromonas gingivalis* CPS, was chosen as a study object. The anaerobic oral pathogen *P. gingivalis* has been described as one of the causative agents of chronic periodontitis. Many virulence factors of *P. gingivalis* including a part of their mode of action in infection have been described. *P. gingivalis* K-antigen or CPS has also been described as one of the major virulence factors. Encapsulated strains have been reported to be more virulent in infection models than non-encapsulated strains. CPS can consist of polysaccharides of several serotypes of which 6 (K1-K6) have been described [5, 6] and 1 (K7) has been proposed.

Although CPS has been regarded as a virulence factor of *P. gingivalis* since the early 90s, only slow progression has been made in uncovering the role in virulence. The publication of the genome sequence of *P. gingivalis* strain W83 has been an important step in uncovering virulence mechanisms. Further progress in CPS research has been made by determining the CPS biosynthesis locus based on experimental evidence using information from the genome sequence [7]. The first non-encapsulated mutant strains have been constructed proving the involvement of the locus in CPS biosynthesis. After that, mutant studies provided evidence that CPS reduces biofilm formation [8].

*P. gingivalis* CPS has only been characterized to a very limited extent. No structures are available and no repeating unit has been described. Yet, monosaccharide analysis of K1 strain W50 has been performed [9]. No capsular serotype has been linked to increased virulence in the subcutaneous mouse infection model. Using purified CPS from the different serotypes has however revealed that the K1 serotype induces a higher pro-inflammatory immune response in macrophages than all other purified CPSs [10].
The genetic background of the different serotypes has never been studied in a detailed way, whereas in this thesis it is shown that genetic knowledge can be an important step in the development of molecular tools. In chapter 3 we used restriction fragment length polymorphism (RFLP) and sequence analysis to study the CPS locus of a set of K1 strains. The results showed that the CPS locus was conserved among the K1 strains, except for an insertion that was found in 2 strains. Based on those findings and the specific differences between strain W83, HG1703 and FDC381 [7] a K1-specific PCR could be developed. This PCR was found to be highly sensitive and specific. The method could also be used for serotyping *P. gingivalis* from complex patient samples, allowing fast diagnostic screening. Although this method is only detecting a single serotype, instead of the preferable 7, this is a first step towards easier epidemiological studies of *P. gingivalis* capsular polysaccharide types. Developing more serotype-specific primer sets will be a next step.

The specific function of *P. gingivalis* CPS is still unknown. CPS has been shown to induce a pro-inflammatory immune response [10], and to reduce biofilm formation [8]. Its presence has been shown to make a *P. gingivalis* strain likely to be more virulent, as seen by higher invasiveness after subcutaneous inoculation in mice [11]. Furthermore CPS has been described not to be involved in serum killing resistance as was thought earlier [12].

To unravel a part of the mechanism by which *P. gingivalis* CPS acts during infection a mutant was constructed and analyzed using a variety of methods (chapter 2). The constructed epsC mutant was an isogenic mutant of K1 strain W83 only affected in a single gene, as determined by RT-PCR of the neighbouring genes. EpsC is a gene at the 3'-end of the CPS locus of which the annotation, UDP-GlcNAc 2-epimerase, is given by homology. The mutant was conclusively shown to be non-encapsulated by negative staining using India ink on fuchsine-stained *P. gingivalis* cells. Knowing that epsC is essential for CPS biosynthesis and that epsC is present in each so far tested strain, including the ones tested in chapter 5, makes epsC a potential therapeutic candidate. If the gene product could be blocked this would block CPS biosynthesis, thereby potentially reducing virulence. One has to realize however, that CPS is not the only virulence determinant of *P. gingivalis*, which may only result in small changes in virulence. Future research also has to elucidate the actual function of epsC in CPS biosynthesis. The annotated function is not completely in line with the monosaccharide composition described earlier. However, when EpsC would be an
epimerase, but with another than the annotated function, it could potentially provide the cell with GlcNAc which is found in K1 CPS [9].

In chapter 4 the epsC mutant was used in an infection experiment using human gingival fibroblast cells. In these experiments the first evidence was presented that \textit{P. gingivalis} CPS reduces the pro-inflammatory immune response. The experiments were performed using living bacterial cells of wild-type W83, the epsC mutant and the complemented mutant. This experimental design was used to be able to study the role of CPS as a \textit{P. gingivalis} cell component. The finding that \textit{P. gingivalis} CPS reduces the pro-inflammatory immune response does not seem to be in agreement with the earlier findings of K1 CPS being a potent inducer of this response. An explanation could however be that CPS shields/covers even stronger immune inducers, like LPS or fimbriae as is found in other bacterial species [13, 14].

Two ways to get reduction of the host immune response are molecular mimicry of host components by bacteria which hampers recognition by the immune system. A second way is to induce immune suppressive cytokines like IL-10. Both ways have been described to be used by bacterial polysaccharides [15, 16].

The focus in this thesis is CPS that induces an immune response when isolated, but reduces this response when being part of the bacterial cell envelope. CPS reduces the ability to form biofilm but increases the ability to invade tissues. The roles of \textit{P. gingivalis} CPS seem ambiguous. Then, why are encapsulated strains more virulent than non-encapsulated strains? Encapsulated strains have the advantage to induce a low immune response due to which they can survive in the host for a longer period of time. If the bacterium could then down-regulate CPS expression when a suitable niche is found, biofilm formation could be enhanced. Spreading infections could be explained by higher invasion after up-regulating CPS expression again. As proposed previously [8], CPS expression may even be synchronized with expression of other cell surface components thereby being able to switch from a planktonic state to a biofilm state.

CPS export and biosynthesis is a substantial energy investment, which makes capsule expression even more likely to be strictly regulated. CPS biosynthesis regulation upon environmental or genetic influence has been found in a range of bacterial species including \textit{Vibrio vulnificus}, \textit{Neisseria meningitidis}, \textit{Streptococcus pneumoniae} and \textit{Escherichia coli}. [17-21]. In the closely related anaerobic gut pathogen \textit{Bacteroides fragilis} a variety of CPSs can be found on one strain of which the synthesis is controlled by a
reversible ON/OFF system regulated by a DNA invertase [22]. Multiple CPSs are essential for *B. fragilis* to compete with commensal bacteria for colonization [23]. For *P. gingivalis* regulation of CPS expression has been described very recently. The CPS biosynthesis locus contains four transcriptional start sites indicating that complex regulation is possible [8]. Genes up- and downstream of the CPS locus, *PG0104* and *PG0121*, have been implicated in this regulation [24]. Temperature dependent regulation of fimbriae has already been described in *P. gingivalis*. Fimbriae expression is down-regulated during elevated temperatures as found in inflamed tissues [25], making the attached bacterium less vulnerable to the host immune system. It is therefore tempting to speculate about a similar, but reversed regulatory action for CPS expression. Other regulatory stimuli for CPS biosynthesis regulation could be hemin concentration as is the case for a broad spectrum of virulence factors [26].

Neuropathogenic *E. coli* K1 isolates express a polysialic acid capsule which is regulated in an elegant way in a neonatal rat pup K1 infection model. In this model *E. coli* expressing capsule colonizes the gut, after which the bacterium gains access to the blood compartment. Then, *E. coli* crosses the blood-brain barrier where capsule expression is ceased [27]. Prevention of capsule expression by administration of a capsule-selective sialidase has been shown to prevent *E. coli* to cross the blood-brain barrier [28], making it a potential drug to prevent bacterial meningitis.

*Streptococcus pneumonia* serotype 3 capsule is down-regulated when bacteria are in close contact with host cells, thereby uncovering adhesion molecules. This makes bacteria much more adhesive and enhances uptake into the host cell. Bacteria in close proximity, but not in contact with host cells keep expressing capsule. Bacteria recovered from host cells after uptake show a lower capsule expression long after being isolated from the cells [21]. In both cases described above capsule expression is very important in pathogenesis. The exact role of *P. gingivalis* in periodontitis is still not fully understood and the role of its CPS is only slowly being unravelled. *P. gingivalis* CPS has however been shown to reduce adhesion to epithelial cells [29] Oral pathogens including *P. gingivalis* have also been shown to be able to cause recurrent bacteraemia in pigs and periodontitis patients, linking periodontitis with atherosclerosis [30, 31]. CPS may well play a role in *P. gingivalis* bacteraemia by masking strongly antigenic factors as is the case in *E. coli* and pneumococci. *P. gingivalis* could therefore not only play a role in periodontitis but also in the systemic diseases associated with it.
Chapter 5 describes a thorough analysis of the genetic content of a set of representative strains of each capsular serotype (K1-K7) next to a naturally non-encapsulated strain. This comparative genomic hybridization (CGH) study was initiated to study CPS biosynthesis-related genes. The analysis of the hybridization experiments yielded a high quality data set that could be used to study more than just CPS-related genes. Therefore the description of a core *P. gingivalis* gene set was initiated. Microarrays of strain W83 containing all CDSs were hybridized with DNA from eight test strains to look for presence, aberrance or absence of these CDSs in the test strains. The thorough analysis of triplicate hybridizations of arrays designed with quadruplicates for each CDS, gave a high resolution data set. This allowed us to find more differences in the genetic content of the test strains than has been reported before [32]. Making use of the *Arabidopsis thaliana* negative control spots on the array allowed us to define absent genes next to defining aberrant genes.

This thesis initiated the description of the *P. gingivalis* core genome. Core genome research in other bacteria has shown that the more strains are included in the analyses, the smaller the core genome will be. The huge effort in *E. coli* genetic research has resulted in a core genome as small as 48% of an average genome. Other bacterial species have core genomes at the moment covering 52%-85% of an average genome [33-39].

Furthermore, sequencing techniques have rapidly become faster and more cost efficient, allowing complete genome sequencing. Using complete genome data to describe a core genome will potentially include more sequences as besides CDSs also all other sequences can than be included.

The content of the CPS biosynthesis locus in the test strains varied greatly as reported earlier [7, 40, 41]. K3 serotype strain HG1025 was found to almost completely lack the CPS genes as found in strain W83, whereas K7 serotype strain 34-4 possesses 9 of the 14 genes of the W83 CPS locus. Interestingly, the data support the choice for the K1 serotype-specific PCR as described in chapter 3. Genes PG0117 and PG0118 were selected for this PCR on basis of limited sequence data. The data from this CGH study now show that those genes indeed seem K1-specific as they were not detected in any of the test strains.

The non-encapsulated strain FDC381 was found to be most divergent from W83 with almost 14% aberrant CDSs and K4 serotype strain ATCC49417 was found to be least divergent from W83 with 8% aberrant CDSs. These numbers are much higher than previously reported, 3.5% and 0.6% respectively, most likely because our experimental design and analysis
yields higher resolution data. Even small differences can reliably be interpreted as significant.

The next step was to compare the total datasets of all strains in order to describe the core genome of *P. gingivalis*. Our analyses yielded information on the presence, aberrance or absence of each CDS of W83 in the test strains. The core genome that we chose to describe was the conserved core genome. All genes that were called non-aberrantly present were included in this gene set. The conserved core genome as described, using 8 *P. gingivalis* strains, consists of 1476 genes which is around 80% of the W83 genome. 374 genes were aberrant in at least one of the test strains.

The data could then be used in further analyses which showed that most virulence associated genes, e.g. proteases, are part of the conserved core genome. *PG1055*, an annotated thiol protease, is however only non-aberrantly present in strains W83 and ATCC49417. These two strains are members of heteroduplex types which are most strongly associated with disease. This can be seen as an indication that *PG1055* indeed may have a function in virulence, although no functional study was performed.

The description of the *P. gingivalis* core genome as described in this thesis will allow a more focused search for potential important virulence factors. The genetic information is valuable, but experimental data will be needed to support and clarify the findings from a comparative genomics approach. The highly accessed paper will therefore hopefully stimulate *P. gingivalis* this experimental research.

In conclusion the research in this thesis has yielded new tools for further studies of *P. gingivalis* CPS, including a PCR technique, a non-encapsulated mutant and widely applicable integration plasmids. Furthermore, basic knowledge on the CPS biosynthesis locus and the role of CPS in infection was gained. The initiation of the description of a conserved core gene set is likely to be an important step in *P. gingivalis* research. Genetic differences and similarities between strains ensure virulence variation, and this study is therefore a potential starting point of new research.


