Genetic background of Porphyromonas gingivalis capsule biosynthesis
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Chapter 2

Molecular tools for use in Porphyromonas gingivalis capsular polysaccharide research

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Abstract

Molecular tools can be used to functionally study genes, gene products, regulation and bacterial cell components. In this chapter some new molecular tools for *Porphyromonas gingivalis* research will be presented. The construction of an isogenic epsC mutant is described. This mutant is shown to lack CPS by a range of methods. The other tool that is described is a set of integration plasmids for *P. gingivalis*. These plasmids allow integration of expression cassettes into specific intergenic regions in a wide range of strains. The expression cassettes can easily be modified. Potential applications of these plasmids are foreign protein expression, promoter studies but also complementation without the need for antibiotics pressure.

Introduction

Since *P. gingivalis* has been strongly associated with periodontitis more and more research has been focusing on this Gram negative pigmented oral anaerobic rod in the latest years. This increased interest is supported by the fact that in November 2005 2838 articles could be found in the PubMed database when using the query *Porphyromonas gingivalis*, whereas just over five years later (April 2011) this number was increased with 58% to 4479 articles. In the meantime also an extra genome sequence besides the genome of the well-known W83 strain of *P. gingivalis* strain has become available [1]. The availability of those genome sequences is a very important tool for advanced research to unravel the molecular mechanisms behind *P. gingivalis* virulence.

The first genome publication already gave rise to speculation which of the genetic loci was the capsular polysaccharide (CPS) biosynthesis locus in *P. gingivalis*. Further studies using genomic hybridization yielded more indications towards defining the CPS biosynthesis locus [2]. But final evidence was presented in a molecular study in which a part of a putative CPS biosynthesis locus was deleted yielding a non-encapsulated mutant [3].

This example shows the power of molecular genetics in microbiology. In this case the method used for introduction of the antibiotic resistance marker into the CPS locus was electroporation of linearized plasmid DNA. Electroporation has been shown to be very successful when used as a tool for introduction of DNA into the genome of *P. gingivalis* also in several other studies [4], usually to make knockouts of genes of interest. Gene function
can then be examined by studying the change in phenotypic appearance of the bacteria lacking a functional gene copy.

Problems however arise when intact plasmid DNA is electroporated into *P. gingivalis*. From early studies it became apparent that *P. gingivalis* has an extensive restriction-modification system to get rid of introduced DNA [5]. It was shown that plasmid DNA isolated from *Escherichia coli* could not be maintained by *P. gingivalis* when introduced by electroporation, while the same plasmid DNA when isolated from *P. gingivalis* could be maintained. To overcome the problem of restriction-modification a plasmid vector (pYH400) had been constructed which could be introduced by electroporation into a restriction-modification transposon mutant *P. gingivalis* strain YH522 [6]. Strain YH522 could therefore be used as an easy-to-use host system.

For general use a more diverse host system would be more attractive, however. Therefore a search for other plasmids that could be maintained in *P. gingivalis* yielded a plasmid named pT-COW [7]. This plasmid, designed

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**Figure 1.** Schematic representation of the knockout strategy to construct the epsC insertional mutation in W83. **A.** The genetic arrangement of the 3'-end of the CPS locus in the W83 wild type strain with the grey rectangles representing the genes present. **B.** Construct pΔepsC for insertional inactivation of epsC. The 1.2 Kb epsC was inserted into *BamHl*-EcoRI digested pGEX-6-p3 (oval) and interrupted by insertion of a 1.2Kb EryF (shaded rectangle) in the single *ClaI* restriction site present. The dashed lines between A and B show the homologous crossover regions between the plasmid and W83 CPS locus. **C.** The final arrangement of the 3'-end of the *P. gingivalis* CPS locus after double crossover showing the insertional inactivation of epsC. Arrows represent the primers used to confirm the integrity of the epsC mutant.
for use in *Prevotella ruminicola*, was not restricted to a certain host strain as long as it was introduced into *P. gingivalis* by conjugation in stead of electroporation. It has been used in several complementation experiments [4, 8], but also in regulatory studies using promoter-*lacZ* fusions inserted into the pT-COW vector [9].

The vector contains three antibiotic resistance markers, of which two (*tetQ* and *eryF*) are for tetracycline and erythromycin/clindamycin selection in *P. gingivalis* and the other one (*bla*) is for ampicillin selection in *E. coli* making it a very suitable shuttle plasmid. A drawback of pT-COW is however that there are only very few restriction sites usable for insertion of your DNA fragment of interest.

The aim of this study was to design molecular tools for use in *P. gingivalis* CPS research. The first goal was to create an isogenic CPS mutant which is only affected in a single gene. The next step would then be to complement that mutant with an intact copy of the knocked-out gene. These tools would allow functional studies of CPS in *P. gingivalis*.

Besides that, the research has been focusing on the design of vectors which could be used for expression of a variety of proteins in *P. gingivalis* under control of exchangeable promoters. For application of reporter proteins for the detection of *P. gingivalis* in for example a multi-species biofilm it is necessary to integrate the expression cassette into the genome of *P. gingivalis* as only then the bacteria can be maintained without losing its construct of interest in the absence of antibiotics for selection. Therefore specific integration plasmids were constructed. These vectors carrying a suitable reporter protein in combination with a CPS mutant would for example allow investigation of the role of CPS in biofilm formation.

**CPS mutant and complementation**

Here we describe the construction of an insertional isogenic *P. gingivalis* knockout in the epimerase-coding gene *epsC* that is located at the end of the CPS biosynthesis locus (figure 1). This mutant was subsequently shown to be non-encapsulated. K1 capsule biosynthesis could be restored by *in trans* constitutive expression of an intact *epsC* gene on an derivative of plasmid pT-COW.
Construction rationale of *P. gingivalis* integration vectors (pINT1-8)

Integration plasmids for *P. gingivalis* were designed in a way that when linearized before electro-transformation they could integrate into two distinct regions in the *P. gingivalis* genome. These intergenic regions IGR1 between genes *galK* and *PG1634* and IGR2 between *pepC* and *kch* were chosen for their potential lack of interference with surrounding gene functions, as the 3’-end of both neighboring genes are pointed towards the IGRs (figure 2). Another advantage is that the regions are present in both sequenced *P. gingivalis* strains [2, 10], which makes the plasmids potentially widely applicable in *P. gingivalis* strains as strains W83 and ATCC33277 have been shown to be genetically highly divergent [11].

Two antibiotic resistance cassettes *tetQ* and *eryF* known to be functional in *P. gingivalis* were used for integration selection of the constructs. Two promoters were used to drive transcription. Promoter pCP25 is a 120 bp artificial strong constitutive promoter used in the Gram positive bacteria *Lactococcus lactis* and *Streptococcus mutans*, but also in the Gram negative bacterium *E. coli* [12, 13]. The CP25 promoter was used successfully for the first time in a complementation experiment in *P. gingivalis* in a recent study.
in our group [14]. Promoter pPG0106 is the 430 bp region upstream of gene PG0106 which potentially is the CPS synthesis promoter of *P. gingivalis*. The promoters were driving the transcription of a gene encoding a fluorescent protein (BsFbFP) which does not need any oxygen for its fluorescence [15]. The potential of this protein as a fluorescent marker in *P. gingivalis* will be investigated.

Here, the construction of the integration plasmids (pINT1 to pINT8) from which promoter and gene can easily be replaced for any other fragment using a single restriction/ligation step will be described.

**Material and methods**

**Bacterial strains and maintenance**

The *E. coli* strain DH5α was used for maintenance of plasmids. *E. coli* strains carrying plasmids with the *bla* gene encoding β-lactamase were cultured in liquid or solid Luria Bertana (LB) medium supplemented with 100 µg/ml ampicillin. *E. coli* S17-1, used for conjugation of plasmids to *P. gingivalis*, was grown on LB additionally supplemented with 5 µg/ml tetracycline.

*P. gingivalis* strains were grown either on 5% horse blood agar plates (Oxoid no. 2, Basingstoke, UK) supplemented with hemin (5 µg/ml) and menadione (1 µg/ml) (BA+H/M plates) or BHI+H/M, both, at 37°C in an anaerobic atmosphere of 80% N₂, 10% H₂, and 10% CO₂.

Strain W83 was used for transformation with integration plasmids. Selection for transformants carrying the integration fragments was performed using BA+H/M plates and 1 µg/ml tetracycline or 5 µg/ml erythromycin depending on the inserted resistance cassette. Purity of *P. gingivalis* liquid and plate-grown cultures was routinely checked by Gram staining and microscopic examination.

**P. gingivalis** transformation

After purification of the linearized plasmid using a GE Healthcare PCR purification kit, between 200 ng and 400 ng DNA was added to 200 µl electrocompetent *P. gingivalis* W83 cells (cells were grown to mid-log phase, harvested by centrifugation (5000 rpm for 15 minutes), 3x washed in electroporation buffer (EPB; 10% glycerol, 1 mM MgCl₂) and concentrated 80-100 times compared to the starting volume when resuspended in electroporation buffer (EPB; 10% glycerol, 1 mM MgCl₂) and concentrated
80-100 times compared to the starting volume when resuspended in EPB). The mixture was transferred to 0.2 cm electroporation cuvettes (Bio-Rad) and electroporated using a Gene PulserXcell (Bio-Rad). Settings: 2.5kV potential difference, 200 Ω resistance, and 25 μF capacitance. O/N anaerobic recovery was performed at 37°C after addition of 1 ml BH + H/M. Cells were then plated on BA + H/M plates containing the proper antibiotics.
for selection of the transformants. After 4-6 days transformants could be checked for proper integration of the inserted DNA.

Construction of knockout plasmid pΔEpsC

To make an insertional knockout of epsC in the W83 wild type strain we constructed plasmid pΔEpsC. Primers epsC BamHI-F and epsC EcoRI-R (see table 1 for details) were used to amplify the 1.2 Kb epsC gene from P. gingivalis W83 genomic DNA in a PCR reaction. Pfu polymerase (Fermentas, GmbH, St. Leon-Rot, Germany) was used according to the manufacturer's protocol with 100 ng of genomic DNA. The PCR program started with 95°C for 5 min and then 25 cycles of 95°C, 55°C and 72°C for 30 s, 30 s and 2.5 min respectively and was ended by one step of 72°C for 5 min. The amplified fragment was cleaned using the Qiagen PCR purification kit (Qiagen Benelux B.V.) and restricted with BamHI and EcoRI. This restricted epsC gene fragment was ligated into BamHI-EcoRI restricted pGEX-6p-3 plasmid to yield pGEX-PG0120. The 1.2 Kb EryF erythromycin resistance cassettes for use in P. gingivalis was amplified from plasmid pEP4351 using primers EryF ClaI F and EryF ClaI R. and after restriction with ClaI this fragment was ligated into the ClaI-restricted pGEX-PG0120 plasmid yielding pΔEpsC. Scal-linearized pΔEpsC plasmid was used for insertional inactivation of epsC in P. gingivalis strain W83.

Construction of complementation plasmid pT-PG0120

The 120 bp artificial constitutive CP25 promoter [13] was amplified from plasmid pDM15 [12] using primers CP25 ClaI F and CP25 Ascl R (table 2). The intact epsC 1.2 Kb gene was amplified from genomic DNA of P. gingivalis strain W83 using primers epsC Ascl F and epsC SpeI R. After ligation of these fragments into cloning vector pJET1.2 (Fermentas, GmbH, St. Leon-Rot, Germany) the constructed expression cassette was cut out with XhoI and HindIII and ligated into the SalI and HindIII digested pT-COW shuttle plasmid [7] to yield the complementation construct pT-PG0120.

Conjugation of P. gingivalis

To complement the epsC mutant, plasmid pT-PG0120 was transferred into the mutant by conjugation following a protocol described earlier [16], with slight modifications. For selection of P. gingivalis after the over-night conjugation we used 50 µg/ml of gentamycin in our blood agar plates instead of 150 µg/ml.
**P. gingivalis** genomic DNA isolation

To isolate genomic DNA five-day-old *P. gingivalis* cells were scraped from BA + H/M plates and re-suspended in 500µl PBS buffer. *P. gingivalis* genomic DNA was extracted according to the protocol for Gram negative bacteria using the DNeasy Blood and Tissue Kit (Qiagen).

**Real-time PCR of neighbouring genes**

Furthermore, using Real-Time PCR, the expression of the downstream gene *hup-1* in both W83 and the *epsC* mutant was monitored using primers *hup-1* F and *hup-1* R (table 2) to exclude polar effects. W83 and the *epsC* mutant were grown till early exponential phase. The cell pellets were collected by centrifugation and resuspended in RLT buffer (Qiagen, Benelux B. V.). The cells were disrupted using a Fast Prep Cell Disrupter (Bio 101, Thermo electron corporation, Milford, USA) and centrifuged, the total RNA was extracted from the supernatant according to the manufacturer's protocol of Qiagen RNeasy® mini kit (Qiagen Benelux B.V.). The residual contaminating genomic DNA was removed by Turbo DNA-free™ kit.

**Table 2. Primers used in the present study.**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5'-3' a</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG0120 BamHI F</td>
<td>ATATAGGATCCATGAAGAATATGGATTTGTC</td>
</tr>
<tr>
<td>PG0120 EcoRI R</td>
<td>CTAAGAATTCATCTTGCCGATAATGCATCG</td>
</tr>
<tr>
<td>EryF F Clal</td>
<td>CCACCATCGATCGATCCTCCGTCATATTCGC</td>
</tr>
<tr>
<td>EryF R Clal</td>
<td>CCACCATCGATGTTCGCTCAGCTCCATCAAGGACA</td>
</tr>
<tr>
<td>pepC F</td>
<td>GAAGCAAGGCTTTATATCGGCAACAGCAATAGAAG</td>
</tr>
<tr>
<td>pepC R NotI</td>
<td>CTTATTGCTGGTCCTGGCAGGCCGCCCCGATTAATACATTTG</td>
</tr>
<tr>
<td>kch F NotI</td>
<td>GCCAATGTATTTATACTCGGGGCAGCCAACAGCAGAATAGAAG</td>
</tr>
<tr>
<td>kch R</td>
<td>GAAGTAAAAGCTTTGTTGGGTGATGATTG</td>
</tr>
<tr>
<td>galK F</td>
<td>TAAACGACGTAGTCTGCGAAATGTGGTC</td>
</tr>
<tr>
<td>galK R NotI</td>
<td>CCACTATCTCTTTGCGCTGGCGGCCGCGCATATCCTCT</td>
</tr>
<tr>
<td>PG1634 F NotI</td>
<td>AGAGGATATGCGCCGACTCGGGGCCGCCGAAACGAGAGAGATAGG</td>
</tr>
<tr>
<td>PG1634 R</td>
<td>GATCAAGGCTTGTGATGCTGACCTCTT</td>
</tr>
<tr>
<td>pPG0106 F Clal</td>
<td>CCAATCGTAGAAACTCGCTGAGT</td>
</tr>
<tr>
<td>pPG0106 R Ascl</td>
<td>TAAACGACGTAGTCTGCGAAATGTGGTC</td>
</tr>
<tr>
<td>EryF-AM F Spel</td>
<td>GGGGACTAGTTCCGATAGCTTCCGCTATGG</td>
</tr>
<tr>
<td>EryF-AM R Xbal</td>
<td>GTCGACTCTAAGAGATCTCCCGAGATGAAATAGTACCT</td>
</tr>
<tr>
<td>CP25 F Clal</td>
<td>GCCATATGATGCATCGCAGATCCATTAGT</td>
</tr>
<tr>
<td>CP25 R Ascl</td>
<td>CTTTAAGCGCCGCCTTAATTTTCTC</td>
</tr>
<tr>
<td>BsFbfP F Ascl</td>
<td>GAAAGAAGCCGCGCAATGGGAGAGAGACCCATCG</td>
</tr>
<tr>
<td>BsFbfP R Spel</td>
<td>GAATTCCGAGTTTACATATCGGAAGCACCTT</td>
</tr>
<tr>
<td>TetQ F Spel</td>
<td>GGGGACTAGTTAAATTTAAATAAACAC</td>
</tr>
<tr>
<td>TetQ R Xbal</td>
<td>CTTCACTAGTATTTTATTTTATTGCAAAG</td>
</tr>
<tr>
<td>PG0120 Ascl F</td>
<td>GAATATGGCCGCATGAAAAAGTAGTATGTTG</td>
</tr>
<tr>
<td>PG0120 Spel R</td>
<td>CTAAGTACTATTTTCGCTAAATGCATCG</td>
</tr>
<tr>
<td>hup1 F</td>
<td>GAAAGCGCAACCTCACAAG</td>
</tr>
<tr>
<td>hup1 R</td>
<td>TCCGATGAGCGATTTTCT</td>
</tr>
<tr>
<td>glk F</td>
<td>ATGAATCCGCCATCGCACCAC</td>
</tr>
<tr>
<td>glk R</td>
<td>GCCCTCGATCCGAAAAGCCT</td>
</tr>
</tbody>
</table>

a Restriction sites are indicated in bold.
mRNA was then reverse transcribed using the Fermentas first-strand cDNA synthesis kit (Fermentas GmbH, St. Leon-Rot, Germany) according to the manufacturer's protocol. The synthesized cDNA was further analyzed using Real-Time PCR with gene-specific primers on an ABI Prism 7000 Sequence Detecting System (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Gene expression was normalized to the expression of glucokinase (glk), amplified with primers glk F and glk R (table 2) (40). The relative hup-1 expression levels of W83 from three independent experiments were compared in duplicate to those of the epsC mutant.

**P. gingivalis** serotyping

Serotyping of *P. gingivalis* was based on the detection of the six described K-antigens (8,9). In short, serotype-specific, polyclonal antisera were obtained after immunization of rabbits with whole bacterial cells of the six *P. gingivalis* type strains (42). Bacterial antigens for double immunodiffusion tests were prepared as described previously (8). Immunodiffusion was carried out in 1% agarose (Sigma Chemical Co., St. Louis, MO, type 1, low EEO) in 50 mM Tris-HCl buffer (pH 8.6). 10 µl antiserum and 10 µl of antigen were loaded and allowed to diffuse and precipitate for 48 hours at room temperature.

**India ink negative staining**

*P. gingivalis* cells were taken from 4 day-old plates and resuspended in 1 ml of PBS. On a glass slide 10 µl of this suspension was mixed with 10 µl of India ink (Talens, Apeldoorn, the Netherlands) and using another glass slide a thin film was made. The film was air-dried. A drop of 0.2% fuchsine was carefully added onto the film and removed after 2 minutes by decanting. Then the film was air-dried. Pictures were taken with a Leica DC500 camera on a Zeiss Axioskop using phase-contrast.

**Percoll density gradient centrifugation**

Percoll density gradients were in principle prepared as described by Patrick and Reid (24). In short, a 9:1 stock solution of Percoll (Pharmacia, Biotech AB, Uppsala, Sweden) was prepared with 1.5 M NaCl. Solutions containing 80, 70, 60, 50, 40, 30, 20 and 10% Percoll in 0.15 NaCl were prepared from the stock. In an open top 14 ml polycarbonate tube (Kontron instruments, Milan, Italy) 1.5 ml of each of the solutions was carefully layered on top of the previous starting with 80%. 1 ml of an anaerobically
grown over night culture of wild type and the epsC mutant concentrated to an OD690 of 4 in PBS was added to the top of the 10% layer and centrifuged for one hour at 8000 × g at 20°C in a Centrikon TST 41.14 rotor (Kontron instruments, Milan, Italy) using a Centrikon T-1170 (Kontron instruments, Milan, Italy) centrifuge.

**Hemagglutination of P. gingivalis**

W83 and the EpsC mutant were cultured anaerobically for 1, 2 or 3 days at 37°C. The cells were washed three times in PBS and the OD₆₀₀ was set to 1 using PBS. A serial dilution of this suspension was made in PBS. 50 µl of the 1:8 – 1:16384 dilutions was used to add to 50 µl of 0.8% PBS-washed sheep erythrocytes in a 96-well-U-bottom plate (Costar, Cambridge, cat. no. 3799). The plate was incubated at 37°C for 1 hour and then at 4°C over night.

**Hydrophobicity of P. gingivalis**

W83, the epsC mutant and the complemented mutant were grown 18 hours in BHI+H/M. The bacteria were washed twice in PBS after which the
OD600 was set to 0.5. After addition of 150 µl n-hexadecane to 3 ml of this suspension the mix was vortexed 30 seconds, rested for 5 seconds and vortexed for 25 seconds. After exactly 10 minutes incubation at room temperature a sample was taken to measure the OD<sub>600</sub> of the aqueous phase. The percentage of bacteria adhered to hexadecane was calculated by the formula: (OD<sub>600</sub> before-OD<sub>600</sub> after)/ OD<sub>600</sub> before × 100%. Data were collected from two experiments using triplicate measurements.

Construction of pINT-plasmids

The building blocks to construct the integration plasmids pINT1-8 (figure 3) include two promoters pCP25 and pPG0106, an anaerobic fluorescent protein gene, two antibiotic resistance cassettes tetQL and eryF and two 5’ and 3’ homologous recombination fragments flanking IGR1 and IGR2 for insertion into the <i>P. gingivalis</i> genome (figure 2). The cloning strategy which is followed is described below and schematically presented in figure 4.

PCR amplifications

pCP25 was amplified by PCR from plasmid pDM35r (CP25 F ClaI x CP 25 R Ascl). pPG0106 (pPG0106 F ClaI x pPG0106 R Ascl) and all four homologous recombination fragments (pepC F x pepC R NotI, kch F NotI x kch R, galK F x galK R NotI and PG1634 F NotI x PG1634 R) were amplified by PCR from <i>P. gingivalis</i> W83 genomic DNA. EryF was amplified from plasmid pEP4351 (EryF-AM F SpeI x EryF-AM R XbaI) [17]. TetQ was amplified from pT-COW (TetQ F SpeI x TetQ R XbaI) [17]. For amplification
of fragments by PCR the proofreading *Pfu* polymerase (Fermentas, GmbH, St. Leon-Rot, Germany) was used except when cloning to pGEM-T easy is described. Then Taq polymerase (Fermentas, GmbH, St. Leon-Rot, Germany) was used to make use of the additional A-overhang for easy cloning. In all cases the reactions was performed according to the manufacturer’s protocol. Primer names and sequences used to amplify each target fragment are listed in table 1.

**pGP and pPK construction**

The IGR-flanking 5’ and 3’ regions were amplified using primers allowing sewing PCR by an extension of the primer with the reverse and complement sequence of the accessory fragment. The primers also contained an integrated *NcoI* restriction site for insertion of the expression cassettes later in the cloning procedure.

After amplification of the IGR-flanking 5’ and 3’ regions as mentioned in figure 3b, these fragments were PCR-cleaned (Qiagen Benelux BV) and 1 µl of each was used in a sewing PCR using the forward primer of the 5’ fragment with the reverse primer of the 3’ fragment. The products with the expected sizes of about 1 kb were isolated from gel (Qiagen Benelux BV) and ligated into vector pJET1.2/blunt (Fermentas, GmbH, St. Leon-Rot, Germany). After *HindIII* digestion the 1 kb fragment was ligated into the dephosphorylated *HindIII*-digested pUC19, yielding pGP and pPK.

**pCA-Tet, pCA-Ery, pPA-Tet and pPA-Ery construction**

The pCP25 and pPG0106 fragments were separately cloned into pGEM-T easy yielding plasmids pGEM-pCP25 and pGEM-pPG0106 respectively, which were then *AseI*-SpeI digested. The *AseI*-SpeI digested AnFP fragment was ligated into the plasmids to yield pCA and pPA respectively. After *SpeI* digestion

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Fragments and sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>pINT-1</td>
<td>5 fragments: 3191bp, 2053bp, 1029bp, 572bp and 486bp</td>
</tr>
<tr>
<td>pINT-2</td>
<td>4 fragments: 3191bp, 2591bp, 572bp, 486bp</td>
</tr>
<tr>
<td>pINT-3</td>
<td>5 fragments: 3191bp, 2053bp, 1029bp, 869bp, and 486bp</td>
</tr>
<tr>
<td>pINT-5</td>
<td>5 fragments: 3203bp, 2053bp, 1029bp, 572bp and 548bp</td>
</tr>
<tr>
<td>pINT-6</td>
<td>4 fragments: 3203bp, 2591bp, 572bp and 548bp</td>
</tr>
<tr>
<td>pINT-7</td>
<td>5 fragments: 3203bp, 2053bp, 1029bp, 869bp, and 548bp</td>
</tr>
</tbody>
</table>
digestion of the plasmids the SpeI-XbaI digested eryF and tetQ antibiotic resistance cassettes were ligated yielding the four plasmids pPA-Tet, pCA-Tet, pPA-Ery and pCA-Ery.

**Construction of integration plasmids pINT1-pINT8**

The four expression cassettes from the above mentioned plasmids were cut out by a NotI digestion to be ligated into the NotI linearized plasmids pGP and pPK. The ligation then yields eight integration plasmids carrying expression cassettes with AnFP under control of two promoters which can be selected for with two different antibiotics after integration in two distinct IGRs in the *P. gingivalis* genome. The plasmids could then be checked by a digestion using EcoRI yielding fragment sizes as mentioned in table 3.

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**Figure 5. Hydrophobicity of *P. gingivalis* strains.** Percentage of bacterial cells adhered to hexadecane after extensive vortexing and 10 minutes incubation. 3.4%, 61% and 19% of the cells was adhered to hexadecane for W83, the epsC mutant and the complemented mutant respectively, indicating increased hydrophobicity for the epsC mutant. The data are the averages of two experiments comprised of triplicate measurements. The bars show the standard deviations.
After transformation of the linearized plasmid pΔEpsC to *P. gingivalis* W83 the epsC insertional mutation was confirmed by specific PCR amplifications and agarose gel electrophoresis of the products (data not shown). Primer combinations PG0119 F × epsC BamHI R and EryF F × epsC EcoRI R (table 1) ensured that a 1.2 Kb fragment of pΔEpsC had been integrated by double crossover at PG0120 (epsC) as expected, replacing the intact copy with the insertional inactivated copy (figure 1).

To examine if the mutation had an influence on the growth characteristics of the epsC mutant both W83 and the epsC mutant were grown in brain heart infusion broth supplemented with hemin (5 µg/ml) and menadione (1 µg/ml) (BHI+H/M). Phase-contrast microscopy revealed that the mutant grows in aggregates, but no difference in growth rate was observed.

**EpsC mutant characterization**

The potential polar effect of the insertional inactivation on the down
Molecular tools for *P. gingivalis* research

stream gene of *epsC* named *hup-1* was examined. Total RNA was extracted from W83 and the *epsC* mutant in the early exponential phase and the *hup-1* expression levels were evaluated by Real-Time PCR. No significant difference in expression of *hup-1* was found between W83 and the *epsC* mutant (data not shown). To show the effect of capsule-loss on the surface structure of *P. gingivalis* the hydrophobicity of the *epsC* mutant was tested by the capacity to adhere to hexadecane. While 3% of W83 cells was shown to adhere to hexadecane more than 60% of the *epsC* mutant cells was adhered to hexadecane. Cells from the complemented mutant again showed a much lower adherence to hexadecane of 19% (figure 5).

Reactivity with the CPS-specific polyclonal rabbit antisera against *P. gingivalis* serotypes K1-K6 (8,9) was examined for W83, the *epsC* mutant and the complemented mutant. The *epsC* mutant was not recognized by any of the antisera including the K1 antiserum, whereas the wild type strain was only recognized by the K1 antiserum as expected (figure 6). Introduction of an intact *epsC* gene copy into the *epsC* mutant restored the recognition by the K1 antiserum. In hemagglutination tests no differences could be seen between W83 and the *epsC* mutant (figure 7).

Differences in CPS characteristics were also studied by Percoll density gradient centrifugation, which can reveal density differences between encapsulated and non-encapsulted *bacteroides* strains (24). Percoll density gradient centrifugation analyses of W83 and the *epsC* mutant showed that the density of the mutant had been changed (figure 8). Where W83 mostly settled at the 20-30% interface, the *epsC* mutant settled at the 50-60%
interface. Note that the appearance of W83 is diffuse and not restricted to the 20-30% interface. The mutant settles as a compact and granulous layer.

To conclusively examine the absence of CPS in the epsC mutant, light microscopy was performed using India ink in combination with fuchsin staining (figure 9). The negative India ink staining allows direct visualization of the capsule, appearing as a light halo surrounding the *P. gingivalis* cell. Fuchsin is used to stain the cell body. The halos around the W83 wild type strain are clearly visible in the phase contrast microscopic picture, whereas halos are absent around the epsC mutant. The intact epsC gene in *trans* under control of the CP25 promoter rescues the wild-type phenotype enabling the complemented mutant to produce capsule again.

**Integration plasmids**

The construction of the pINT constructs was performed as described above and restriction checks were performed to ensure their integrity. From the 8 possible plasmids 6 were successfully constructed in the course of this
study, e.g. pINT1, pINT2, pINT3, pINT5, pINT6 and pINT7. The digested plasmids showed the expected restriction patterns after EcoRI digestion as mentioned in table 1. Next to that a PCR screening with a promoter-specific forward primer and an IGR1- or IGR2-specific primer was performed to check for the orientation of the expression cassette as inserted between IGR1 and IGR2, the homologous regions for recombination into the P. gingivalis genome.

Figure 9. Negative capsule staining of fuchsine-stained P. gingivalis cells with India ink. Phase contrast microscopic picture at a 1000x magnification of (A) W83 wild type strain, (B) epsC mutant and (C) the complemented epsC mutant in an India ink preparation which reveals the capsule as a white halo (arrow). The inset shows an extra six times magnification.
When the plasmids were transformed to W83 electrocompe tent cells, transformation efficiencies were found to be low. 7 to 14 transformants were recovered after transformation of 300 ng of linearized plasmid DNA, whereas the survival of the competent W83 cells could not be calculated as non-selective plates were full with colonies.

After transformants were grown for at least 1 week, colonies were picked and streaked onto a new selective plate containing either 5 µg/ml erythromycin or 1 µg/ml tetracycline. First a fluorescence microscopic examination was performed, as the newly introduced plasmid DNA contains an expression cassette carrying a fluorescent protein encoding gene. No fluorescence was detected.

To check if the transformants contained the expected linearized plasmid DNA PCR’s on genomic DNA were performed in which a distinction between wild type and transformed situation could be made by a clear size difference of the PCR product. IGR1 and IGR2 or the insertional replacements were amplified by using primers galK F x PG1634 R and pepC F x kch R respectively. The wild type situation yielded products of about 1 Kb and the

Figure 10. Agarose gel analysis to check for integration of a subset of pINT plasmids. Wild type IGR1 and IGR2 PCR fragments are roughly 1 Kb. After integration of the pINT constructs the same primer combinations yield a product of roughly 4.2 Kb for pINT2 and pINT6, 4.5 Kb for pINT5 and 4.8 Kb for pINT3. M: GeneRuler 1 kb DNA Ladder
transformed situation yielded fragments of 4.2 Kb to 4.8 Kb depending on the construct (figure 10). Integration of pINT1 and pINT7 also yielded the expected 4.5 Kb and 4.8 Kb fragments respectively (data not shown).

Discussion

This study was initiated to develop tools for use in *P. gingivalis* capsule studies. The first goal was to make a non-encapsulated isogenic mutant from an encapsulated strain which is only affected in a single gene. We chose *epsC*, which is the last gene at the 3'-end of the CPS biosynthesis locus. *EpsC* is present in *P. gingivalis* strains of different serotypes. The *Listeria monocytogenes* homologue *lmo2537* has been shown to be essential for survival and has been suggested to be involved in the maintenance of cell shape by providing a precursor of the teichoic acid linkage unit that serves as an acceptor for the main teichoic acid chain assembly [18]. Here we show that by insertion of an *eryF* resistance cassette we were able to inactivate *epsC*, showing the gene not to be essential for survival in *P. gingivalis*. The insertional inactivation did not have a polar effect on *hup-1*, the gene downstream of *epsC*, as was shown by gene transcription analysis using real-time PCR.

The *epsC* mutant was shown to be nonencapsulated by double immunodiffusion, density gradient centrifugation and India ink staining. Hemagglutination was shown not to be influenced by capsule-loss, whereas hydrophobicity was strongly influenced. Complementation resulted in rescue of wild-type K1 capsule biosynthesis. Although the exact role of EpsC remains to be elucidated, this finding provides evidence that EpsC is essential in *P. gingivalis* CPS biosynthesis.

We can conclude that this *epsC* mutant is an excellent tool to study the involvement of CPS in virulence. Infection experiments comparing encapsulated wild type and non-encapsulated mutant are now feasible as will be described in chapter 4.

The construction of integration plasmids pINT1-8 resulted in six broadly applicable plasmids carrying AnFP expression cassettes. Although no expression of the anaerobic fluorescent protein could be detected by fluorescence microscopy, the integration of the expression cassettes into the genome of *P. gingivalis* was successful as found by PCR analysis. The plasmids in which the constitutive promoter pCP25 or the potential CPS promoter pPG0106 and AnFP can easily be exchanged for any other promoter or gene are a useful tool in *P. gingivalis* research. Examples of
potential applications are foreign protein expression, promoter studies but also complementation without the need for antibiotics pressure.

Real-time PCR studies on the expression levels of the neighbouring genes will have to be performed to make sure that the insertions into the IGRs do not influence their transcription.

Finding a fluorescent marker that can be expressed in *P. gingivalis* would broaden the potential application of these plasmids with biofilm and mixed culture studies. Very recently SNAP-tag-mediated live cell labelling was reported for *P. gingivalis* [19]. This labelling is based on SNAP26b a protein which covalently binds a fluorescent dye added to the medium. It was shown to be suitable for studying a dual species biofilm. In combination with the constitutive *trxB* promoter this would make a great candidate for use in the pINT plasmids described here. The ideal fluorescent marker would however not need a substrate for fluorescence.

**References**


