Identification of the Linkage between A-Polysaccharide and the Core in the A-Lipopolysaccharide of *Porphyromonas gingivalis* W50

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ABSTRACT

Porphyromonas gingivalis synthesizes two lipopolysaccharides (LPSs), O-LPS and A-LPS. The structure of the core oligosaccharide (OS) of O-LPS and the attachment site of the O-polysaccharide (O-PS) repeating unit [$\rightarrow 3$]- α -D-Galp-(1 $\rightarrow$ 6)- α -D-Glcp-(1 $\rightarrow$ 4)- α -L-Rhap-(1 $\rightarrow$ 3)- β -D-GalNAcp-(1 $\rightarrow$) to the core have been elucidated using the Δ PG1051 (WaaL, O-antigen ligase) and Δ PG1142 (Wzy, O-antigen polymerase) mutant strains, respectively. The core OS occurs as an “uncapped” glycoform devoid of O-PS and a “capped” glycoform that contains the attachment site of O-PS via β -D-GalNAc at position O-3 of the terminal α -(1 $\rightarrow$ 3)-linked mannose (Man) residue. In this study, the attachment site of A-PS to the core OS was determined based on structural analysis of SR-type LPS (O-LPS and A-LPS) isolated from a *P. gingivalis* Δ PG1142 mutant strain by extraction with aqueous hot phenol to minimize the destruction of A-LPS. Application of one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy in combination with methylation analysis showed that the A-PS repeating unit is linked to a nonterminal α -(1 $\rightarrow$ 3)-linked Man of the “capped core” glycoform of outer core OS at position O-4 via a $\rightarrow 6$)-[α -D-Man- α -(1 $\rightarrow$ 2)- α -D-Man-1-phosphate $\rightarrow$ 2]- α -D-Man-(1 $\rightarrow$) motif. In order to verify that O-PS and A-PS are attached to almost identical core glycoforms, we identified a putative α -mannosyltransferase (PG0129) in *P. gingivalis* W50 that may be involved in the formation of core OS. Inactivation of PG0129 led to the synthesis of deep-R-type LPS with a truncated core that lacks α -(1 $\rightarrow$ 3)-linked mannoses and is devoid of either O-PS or A-PS. This indicated that PG0129 is an α -1,3-mannosyltransferase required for synthesis of the outer core regions of both O-LPS and A-LPS in *P. gingivalis*.

IMPORTANCE

Porphyromonas gingivalis, a Gram-negative anaerobe, is considered to be an important etiologic agent in periodontal disease, and among the virulence factors produced by the organism are two lipopolysaccharides (LPSs), O-LPS and A-LPS. The structures of the O-PS and A-PS repeating units, the core oligosaccharide (OS), and the linkage of the O-PS repeating unit to the core OS in O-LPS have been elucidated by our group. It is important to establish whether the attachment site of the A-PS repeating unit to the core OS in A-LPS is similar to or differs from that of the O-PS repeating unit in O-LPS. As part of understanding the biosynthetic pathway of the two LPSs in *P. gingivalis*, PG0129 was identified as an α -mannosyltransferase that is involved in the synthesis of the outer core regions of both O-LPS and A-LPS.

Porphyromonas gingivalis, a Gram-negative anaerobe, is frequently isolated from the subgingival plaque of periodontitis patients and is considered to be an important etiologic agent in periodontal disease. Among the major virulence factors of the organism are the cysteine proteases Arg gingipains (Rgps) and Lys gingipain (Kgp), specific for Arg-X and Lys-X peptide bonds, respectively, which are capable of degrading several host proteins (1), and lipopolysaccharide (LPS), which has the potential to alter the responses of the host innate defense system specifically through its lipid A component (2, 3).

P. gingivalis W50 synthesizes two LPSs, namely O-LPS and A-LPS (4–6). Monoclonal antibody (MAb) 1B5, raised against one (RgpA_{cat}) of the isoforms of Arg gingipain, which are differentially glycosylated proteins, cross-reacts with A-LPS, suggesting that they share an epitope (7). Our group has identified the Man α 1-2Man α 1-phosphate fragment present in A-polysaccharide (A-PS) as part of the epitope recognized by MAb 1B5 (5). Thus, there appear to be common steps in the biosynthesis of A-LPS and the glycosylation of Arg gingipains in *P. gingivalis*.

Analysis of an R-type LPS isolated from the *P. gingivalis* Δ PG1051 mutant strain (*waaL*, encoding O-antigen ligase) enabled us to solve the structure of the core region of LPS (Fig. 1) (8).

The inner core region lacks L-(D)-glycero-D(L)-manno-heptosyl residues and is linked to the outer core via 3-deoxy-D-manno-octulosonic acid (Kdo), which is attached to a glycerol (Gro) residue in the outer core via a monophosphodiester bridge (8). The outer region of the “uncapped core” is composed of a linear α -(1 $\rightarrow$ 3)-linked D-Man OS with four or five members, one half of which are modified by phosphoethanolamine (PEA) at position O-6, which is attached to the glycerol at position O-2. In addition,

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FIG 1 Structure of the outer core OS of O-LPS of *P. gingivalis*. Uncapped core OS is devoid of O-antigen and additional α -(1 $\rightarrow$ 3)-linked D-Man residues. Capped core OS provides the site for O-antigen attachment through a terminal α -D-Man residue.

an amino sugar, α -D-allosamine, is attached to the glycerol at position O-3 (Fig. 1). Studies on the SR-type LPS (core plus 1 repeating unit) of O-LPS, which was isolated from the *P. gingivalis* Δ PG1142 mutant strain (*wzy*, encoding O-antigen polymerase) using the procedure of Darveau and Hancock (9), which yields only O-LPS (8), enabled us to solve the structure of the “capped” core glycoform. In the capped core, there is a 3- to 5-residue extension of α -(1 $\rightarrow$ 3)-linked Man residues glycosylating the outer core region of the uncapped core at the nonreducing terminal Man residue (Fig. 1).

A-LPS is probably destroyed under the conditions employed during the isolation of LPS (5, 6). Analysis of SR-type O-LPS enabled us to determine that O-PS is attached to the outer core region at position O-3 of the nonreducing terminal Man via β -D-GalNAc (8). Hence, in this study, we describe the structural investigation of SR-type A-LPS, which was prepared from the *P. gingivalis* Δ PG1142 mutant strain using aqueous-hot-phenol extraction (10), which allowed purification of both O-LPS and A-LPS without significant loss of the latter. Application of one-dimensional (1D) and 2D nuclear magnetic resonance (NMR) spectroscopy, including the NMR simulation database program glycosciences.de (<http://www.glycosciences.de/>), and methylation analysis using gas chromatography-mass spectrometry (GC-MS) to purified core-containing OS enabled us to localize the attachment site of A-PS to the core region in A-LPS of *P. gingivalis*.

The role of *P. gingivalis* mannosyltransferase (PG0129) in the biosynthesis of the outer core region of LPS was also investigated. Interrogation of the fully annotated genomes of *P. gingivalis* W83 and ATCC 33277 (11, 12) using the Carbohydrate-Active En-

zymes (CAZy) database (<http://www.cazy.org/>) has recognized a number of potential glycosyltransferases that are classified into 28 and 29 groups for *P. gingivalis* strains W83 and ATCC 33277, respectively. In addition, the structures and organizations of open reading frames of the PG0129 genomic loci in all sequenced strains, in particular *P. gingivalis* W83 (11), ATCC 33277 (12), TDC60 (13), JCVI SC001 (14), HG66 (15), and W50, are identical, i.e., the sequences are homologous and synteny is preserved. Although the complete genome sequence of *P. gingivalis* W50 is not yet published, the strain appears indistinguishable from *P. gingivalis* W83 with respect to a range of molecular and biochemical characteristics (16). PG0129 in *P. gingivalis* W83 and PGN_0242 in *P. gingivalis* ATCC 33277 belong to the glycosyltransferase 4 family (CAZy), of which there are 8 members in W83 and 7 members in ATCC 33277. The family also includes PG0110 (capsule locus) and PG1141 (*porR* locus) (11, 16, 17). Basic Local Alignment Search Tool (BLAST) analysis of the PG0129 protein indicated the presence of a conserved domain classed under glycosyltransferase B (GT-B fold) and a glycosyltransferase 1 motif (see the Pfam database [<http://pfam.xfam.org/>]) at the C terminus. Structural examination of PG0129 using PHYRE 2 (Protein Homology/analogy Recognition Engine) (18) further classified the protein as a sugar transferase, with the best hit with MshA, which is in the same fold group (GT-B) as PG0129 and catalyzes the transfer of *N*-acetylglucosamine from UDP-GlcNAc to myo-inositol-1-phosphate in *Corynebacterium glutamicum* (19).

The PG0129 locus in *P. gingivalis* W50 (Fig. 2) encodes a potential Kdo kinase (PG0128) and a putative ferrochelatase (HemH; PG0127) (11). The presence of genes likely to be involved

FIG 2 Genomic organization of the PG0129 locus in *P. gingivalis* W83 (PG_0242 in ATCC 33277). *, Orf56 is not annotated in the J. Craig Venter Institute sequence; **, gene marked as hypothetical.

in glycerol metabolism, a phosphoglycerol mutase (PG0130) and a CDP-glycerol-glycerophosphotransferase (Orf56), also indicated that the locus may be involved in the synthesis of both the inner and outer core regions of LPS (8).

We describe the effects of gene inactivation of PG0129, which produced deep-R-type LPS (see below). Analysis of the LPS isolated from the *P. gingivalis* ΔPG0129 mutant strain allowed us to propose a structure for the truncated core OS, which confirms that PG0129 is an α-mannosyltransferase that is responsible for the biosynthesis of the outer core of *P. gingivalis* LPS.

MATERIALS AND METHODS

Materials. A solution containing 30% acrylamide-bisacrylamide (37.5:1) was obtained from Bio-Rad Laboratories (Hercules, CA). Horseradish peroxidase-labeled mouse immunoglobulin was purchased from Dako A/S, High Wycombe, Buckinghamshire, United Kingdom. All other chemicals were from VWR, Lutterworth, Leicestershire, United Kingdom, or from Sigma-Aldrich Co. Ltd., Poole, Dorset, United Kingdom, and were the purest grades available unless otherwise stated. Restriction and modification enzymes were purchased from New England Biolabs, and DNA purification reagents were obtained from Qiagen.

Bacterial growth conditions. *P. gingivalis* W50 and the ΔPG0129, ΔPG1051, and ΔPG1142 mutant strains were grown either on blood agar plates containing 5% defibrinated horse blood or in brain heart infusion (BHI) broth supplemented with hemin (5 μg/ml) in an anaerobic atmosphere of 80% N₂, 10% H₂, and 10% CO₂ (Don Whitley Scientific). The cultures were harvested, and the cell pellets were washed with phosphate-buffered saline (PBS) and freeze-dried for use in the preparation of LPS.

The *P. gingivalis* ΔPG1051 (*waal*) and ΔPG1142 (*wzy*, encoding a putative O-antigen polymerase) mutant strains have been described previously (6, 8). *Escherichia coli* XL-1 Blue or XL-10 Gold (Stratagene) was used for the maintenance of plasmids and cloning purposes. Antibiotics were added for cell (tetracycline HCl, 20 μg/ml) and plasmid (ampicillin [Na⁺ salt; 100 μg/ml] or erythromycin [300 μg/ml]) selection.

Generation of the *P. gingivalis* ΔPG0129 mutant strain. For allelic-exchange mutagenesis of PG0129, a procedure similar to that used for the generation and characterization of *P. gingivalis* ΔPG1051 was followed (8). The 3' and 5' amplicons of PG0129 were tagged at the ends of an *erm* cassette via PCR and DNA manipulations using primer pairs (see Table S1 in the supplemental material), which resulted in *in vivo* replacement of an internal 227 bp of the gene with the *erm* cassette via homologous recombination. A schematic depiction of the organization of the locus for *P. gingivalis* W83 is shown in Fig. 2.

Complementation of the *P. gingivalis* ΔPG0129 mutant strain. Complementation of the ΔPG0129 mutant strain was performed by initial cloning of PG0129 possessing an additional 550 bp of DNA at the 5' end into the NotI-BglII sites of pUCET1 (20) via high-fidelity DNA amplification, manipulation, and cloning. Integration of the appropriate part of the resulting plasmid (pExp129) into the genome of PG0129 was confirmed by PCR using a combination of primer sets (see Table S1 in the supplemental material) (6). Restoration of wild-type functions in the complemented strain was confirmed by the phenotype and immunoreactivity with MAb 1B5.

Gel electrophoresis. SDS-PAGE was carried out in 12.5% slab gels at 8°C using the Hoefer SE 600 series gel system. LPSs from the *P. gingivalis* W50, ΔPG1051, ΔPG1142, ΔPG0129, and ΔPG0129 strains were subjected to SDS-urea-PAGE (21) using the Hoefer SE 600 series gel system as described previously (6).

Silver staining of gels was performed using a silver-staining kit (Sigma Chemical Co.) according to the manufacturer's instructions. For Western blotting, nitrocellulose membranes were used and probed with MAb 1B5 as described previously (6).

Isolation of LPS. LPSs from *P. gingivalis* W50 and the complemented strain ΔPG0129 for use in SDS-urea-PAGE were prepared using an LPS extraction kit (Intron Biotechnology, South Korea) as previously de-

scribed (6). LPS required for SDS-(urea)-PAGE/Western blotting experiments does not need to be at a high level of purity, and hence, the commercial LPS extraction kit was used for these preparations. However, for structural analysis of LPSs from the ΔPG1142 and ΔPG0129 mutant strains, the hot-phenol-water method of Westphal and Jann (10) was used to yield LPS of high purity.

We have previously described the isolation of LPS from the *P. gingivalis* mutant strain ΔPG1142 (8) using the procedure described by Darveau and Hancock (9), which yields only O-LPS and no A-LPS, probably due to the conditions employed during LPS isolation, which involves exposure of cell lysates to pH ~9.5 for prolonged periods for the removal of peptidoglycans and heating at 85°C to denature SDS-resistant proteins, followed by digestion with pronase. A-LPS is sensitive to both high pH (unpublished observations) and low pH (5, 6). Hence, in this study, the hot-phenol procedure described by Westphal and Jann (10) was used to purify LPS from the *P. gingivalis* ΔPG1142 mutant strain to determine whether A-LPS was being synthesized.

LPSs from the *P. gingivalis* ΔPG1142 and ΔPG0129 mutant strains were isolated from 2.5 g and 2 g (dry weight) of cells, respectively, using the aqueous-hot-phenol extraction procedure of Westphal and Jann (10) with some modifications, as previously described for the purification of LPS from the *P. gingivalis* mutant strain ΔPG1051 (8). Yields of 95 mg and 120 mg of LPS were obtained from the *P. gingivalis* ΔPG1142 and ΔPG0129 mutant strains, respectively.

Isolation of the core oligosaccharide (OS-1142). A suspension of LPS (80 mg) from the ΔPG1142 mutant strain in 2.5 ml of 2% aqueous acetic acid was heated at 100°C for 2 h, and undigested LPS and insoluble lipid A were removed by ultracentrifugation (105,000 × g) at 14°C for 3 h. The supernatant containing the core OS was freeze-dried, and the residue was dissolved in 2 ml of 0.05% (vol/vol) acetic acid and subjected to gel permeation chromatography on a Fractogel TSK HW-40 (S) column (2-cm inside diameter [i.d.] by 80 cm) equilibrated in the same solvent. The column effluent was monitored for changes in the refractive index (RI) with a Knauer Wellchrom K-2400 RI detector (Wissenschaftliche Gerätebau, Dr. Ing. Herbert Knauer GmbH, Berlin, Germany). Material eluting in the void volume was discarded, and the low-molecular-weight fractions were pooled and lyophilized to give 14.4 mg of the core OS, referred to as OS-1142, and used for structural analysis.

Monosaccharide composition. The monosaccharide composition of LPS from the *P. gingivalis* ΔPG0129 mutant strain was determined by methanolysis, followed by analysis of the methyl glycosides as *O*-trimethylsilyl ethers by GC-MS (22, 23).

Methylation analysis. OS-1142 and LPS from *P. gingivalis* ΔPG0129 were methylated using the procedure of Kvernheim (24), followed by hydrolysis with 0.5 M trifluoroacetic acid for 1.5 h at 120°C and reduction with NaBD₄ (22°C; 4 h), and acetylated with pyridine-acetic anhydride (1:1 [vol/vol]) at 60°C for 1 h. Partially methylated alditol acetates were analyzed by GC-MS.

NMR spectroscopy. The oligosaccharide sample OS-1142 was deuterium exchanged by lyophilizing twice from 99.6% D₂O and finally dissolved in 0.52 ml of 99.9% D₂O containing a trace of acetone as an internal standard (δ_H 2.225; δ_C 31.45). 2D ¹H-¹H- and ¹H-¹³C-decoupled NMR spectra were recorded at 25°C on a Bruker AV600 spectrometer, and spectral data were acquired and processed using the Bruker software TOPSPIN (v.2.0). The 2D NMR pulse programs were as follows: ROESY (rotating-frame nuclear Overhauser effect spectroscopy) (25) and ¹H-¹³C HMQC (heteronuclear multiple-quantum correlation)-TOCSY (total correlation spectroscopy) (26).

The assignment of the proton and carbon chemical shifts of the model core OS-containing fragment was performed using the simulation program glycosciences.de (<http://www.glycosciences.de/>).

RESULTS

SDS-urea-PAGE analysis of LPS. In earlier studies by our group (8), LPS from *P. gingivalis* ΔPG1142 was prepared by the Darveau and Hancock procedure (9), which yielded mainly O-LPS due to

FIG 3 SDS-urea-PAGE, followed by silver staining (A) and Western blotting versus MAb 1B5 (B), of LPSs from *P. gingivalis* W50, ΔPG1051, and ΔPG1142 strains. SDS-urea-PAGE was performed as described in Materials and Methods. (A) Lanes: 1, molecular mass markers; 2, W50 LPS; 3, 4, and 5, ΔPG1051 LPS (0.5, 1, and 2 μg); 6, W50 LPS; 7 and 8, ΔPG1142 LPS (0.5 and 1 μg). (B) ΔPG1142 LPS. *, capped core plus one repeating unit; **, uncapped core.

loss of the core plus one repeating unit of A-PS because of the harsh conditions involved in the purification protocol (see Materials and Methods). Hence, in this study, we employed the procedure of Westphal and Jann (10), which appears to have caused less destruction of the SR-type A-LPS (core plus one repeating unit of A-PS) purified from the *P. gingivalis* ΔPG1142 mutant strain.

SDS-urea-PAGE analysis and silver staining of LPS from the *P. gingivalis* ΔPG1051 and ΔPG1142 mutant strains showed the presence of banding patterns typical of R-type LPS (core) and SR-type LPS (core plus one repeating unit), respectively (Fig. 3), as was shown previously (8).

Western blotting of LPS from *P. gingivalis* ΔPG1142 and probing with MAb 1B5 showed the presence of an immunoreactive component, which migrated as a low-molecular-mass band (<12 kDa), suggesting that the ΔPG1142 LPS preparation contains core plus one A-PS repeating unit. These data indicate that the *P. gingivalis* ΔPG1142 mutant strain (wzy, encoding O-antigen polymerase) synthesizes SR types of both O-LPS and A-LPS, which suggests that Wzy apparently possesses dual specificity for the polymerization of both O-PS and A-PS. Thus, the presence of SR-type O-LPS or both SR forms of O-LPS and A-LPS in the LPS preparations from the ΔPG1142 mutant strain depends on the method of their isolation (8, 9).

Methylation analysis of OS-1142. The glycosyl linkage analysis of the capped core plus one repeating unit, OS-1142, revealed 4-substituted rhamnose, terminal mannose (term-Man), terminal galactose, 6-substituted glucose, 2-substituted mannose, 3-substituted mannose, 2,6-disubstituted mannose, 3,4-disubstituted mannose, and 3-substituted *N*-acetylgalactosamine, along with traces of 6-substituted mannose (Table 1). Thus, OS-1142 contained components of both O- and A-PS repeating units and of the outer core.

NMR analysis of OS-1142. Analysis of the ¹H NMR spectrum of OS-1142 revealed, *inter alia*, the presence of signals of high

intensity in the anomeric region at δ_H 5.14, 5.06, 4.99, and 4.88 and of relatively low intensity at δ_H 5.24, 5.16, 5.15, 5.11, 5.09, 5.04, 5.03, and 4.89 (Table 2 and Fig. 4). The former set of signals for anomeric protons was assigned to the spin systems for 3 or 4 residues of →3)-α-D-Manp, which “caps” the outer core OS, and

TABLE 1 Methylation analysis data for OS-1142

OS-1142 methylation analysis ^a	Peak area ^b
1,4,5-Tri- <i>O</i> -acetyl-6-deoxy-1-deuterio-2,3-di- <i>O</i> -methylrhamnitol [→4)-Rha] ^c	5.9
1,5-Di- <i>O</i> -acetyl-1-deuterio-2,3,4,6-tetra- <i>O</i> -methylmannitol (term-Man) ^{d,e}	1.3
1,5-Di- <i>O</i> -acetyl-1-deuterio-2,3,4,6-tetra- <i>O</i> -methylgalactitol (term-Gal) ^c	13.2
1,2,5-Tri- <i>O</i> -acetyl-1-deuterio-3,4,6-tri- <i>O</i> -methylmannitol [→2)-Man] ^d	0.2
1,3,5-Tri- <i>O</i> -acetyl-1-deuterio-2,4,6-tri- <i>O</i> -methylmannitol [→3)-Man] ^e	10.0
1,5,6-Tri- <i>O</i> -acetyl-1-deuterio-2,3,4-tri- <i>O</i> -methylmannitol [→6)-Man] ^d	0.3
1,5,6-Tri- <i>O</i> -acetyl-1-deuterio-2,3,4-tri- <i>O</i> -methylglucitol [→6)-Glc] ^c	15.8
1,2,5,6-Tetra- <i>O</i> -acetyl-1-deuterio-3,4-di- <i>O</i> -methylmannitol [→2,6)-Man] ^d	1.0
1,3,4,5-Tetra- <i>O</i> -acetyl-1-deuterio-2,6-di- <i>O</i> -methylmannitol [→3,4)-Man] ^e	0.6
1,3,5-Di- <i>O</i> -acetyl-2-(acetylmethylamino)-2-deoxy-1-deuterio-4,6-di- <i>O</i> -methylgalactitol [→3)-GalNAc] ^c	1.2

^a The reduced amounts of partially methylated derivatives of *N*-acetyl-hexosaminitol are a result of the resistance of the residue to acid hydrolysis.

^b Peak area refers to the relative detector response; the values are not corrected for differences in detector response factors.

^c Derived from the O-PS repeating unit.

^d Derived from the A-PS repeating unit.

^e Derived from core OS.

TABLE 2 ^1H NMR data for the anomeric region of the A-PS repeating unit in OS-1142

Proton	Chemical shift (δ_{H} [ppm])	Mannose residue ^a
H-1	5.24	$\rightarrow 2$)- α -Man
H-1	5.16	term- α -Man
H-1	5.15	term- α -Man
H-1	5.04	$\rightarrow 2,6$)- α -Man
H-1	5.03	$\rightarrow 2,6$)- α -Man
H-1	4.89 ^b	$\rightarrow 6$)- α -Man

^a Data from reference 5.^b The residue at 4.89 ppm results from the removal of the Man- α -(1 $\rightarrow$ 2)-Man- α -1-phosphoryl group from position C-2 of the backbone mannopyranosyl residue in the native A-PS.

the components of the O-PS repeating unit: $\rightarrow 6$)- α -D-Glcp plus $\rightarrow 4$)- α -L-Rhap(2 $\leftarrow$ PEA), term- α -D-Galp, and $\rightarrow 4$)- α -L-Rhap (cf. the ^1H NMR spectral data for the core plus one O-PS repeat [8]).

Comparison of the ^1H NMR spectral data for the set of signals of low intensity in the anomeric region and those for both A-PS (5) and exopolysaccharide from *Pseudomonas syringae* pv. *ciccaronei* (27) allowed us to suggest that OS-1142 also included components of the A-PS repeating unit, namely, $\rightarrow 2$)- α -mannose, 2 residues of term- α -mannose, and 2 residues of $\rightarrow 2,6$)- α -mannose (Table 2). The partly overlapped signal at δ_{H} 4.89 in the proton spectrum was assigned to $\rightarrow 6$)- α -mannose, while the signal at δ_{H} 5.09 was attributed to $\rightarrow 3$)- α -mannose of the uncapped core glycoform (cf. the ^1H NMR spectral data for OS-1142 [8]). This was in agreement with glycosidic-linkage analysis of OS-1142, which revealed components of both O-PS and A-PS repeating units, as well as the monosaccharides present in the core region of the LPS (Table 1). Taking into account the data described above and those for the analysis of the core region of *P. gingivalis* (8), we suggest that 6-linked mannose represents a backbone residue of the A-PS repeating unit that is formed following the loss of the Man- α -(1 $\rightarrow$ 2)-Man- α -1-phosphate group as a result of the high acid lability of the glycosidic linkage of Man- α -1-phosphate (28). The 6-linked mannose, therefore, provides the link residue between A-PS and the core region in OS-1142. The presence of 3,4-disubstituted Man detected by methylation analysis (Table 1), which was not observed during the analysis of OS-1142 (8), could be accounted for by a linkage between A-PS and the core region. There is no structural evidence for the presence of 3,4-disubstituted α -D-mannose as a branching point in mannose-containing polymers. Therefore, we applied the database search program glycosciences.de (<http://www.glycosciences.de/>) to the model branched oligosaccharide $\rightarrow 3$)- α -D-Man [$\rightarrow 6$)- α -D-Man(1 $\rightarrow$ 4)](1 $\rightarrow$ 3)- α -D-Man(1 $\rightarrow$ 3)- α -D-Man in order to compare the experimental proton and carbon chemical shifts obtained for the glycosidic fragment containing the 3,4-disubstituted mannosyl motif, which represents the putative attachment site within the core region. The residues of the putative manno-oligosaccharide fragment [$\rightarrow 6$)- α -D-Man(1 $\rightarrow$ 4)] $\rightarrow 3$)- α -D-Man(1 $\rightarrow$ 3)- α -D-Man-, which is present in OS-1142, were designated A, B, and C, respectively.

The 2D ^1H - ^1H ROESY spectrum of OS-1142 (Fig. 5) contained interresidue nuclear Overhauser effect (NOE) cross-peaks, the positions of which were in accordance with those described during the determination of the attachment site of the O-PS repeating unit (8).

Analysis of the ^1H - ^1H ROESY data upon preirradiation of H-1 of residue A at δ_{H} 4.89 revealed a cross-peak at δ_{H} 3.77, which was assigned to H-4B despite being partly overlapped by those arising from preirradiation of H-1 of the α -L-rhamnose residue (cf. ROESY data for OS-1142 [8]).

The interresidue NOEs observed at δ_{H} 3.84 and 3.90 on the track of H-1 of the 2,6-disubstituted α -D-mannopyranosyl residue at δ_{H} 5.04 were found to correspond to the resonances for H-6'A and H-6A, respectively. These observed H-1/H-6'A and H-1/H-6A NOEs resulted from the glycosylation of residue A at position O-6 by the $\rightarrow 2,6$)- α -D-mannose residue, which originated from the backbone of the A-PS repeating unit after loss of the Man- α -(1 $\rightarrow$ 2)-Man- α -(1 $\rightarrow$ 2)-Man-1-phosphate motif.

The assignment of the ^{13}C NMR signals for both $\rightarrow 3,4$)- α -D-mannose (B) and $\rightarrow 6$)- α -D-mannose (A) residues resulted from analysis of the ^1H - ^{13}C correlations in the HMQC-TOCSY NMR spectrum of OS-1142 (Table 3 and Fig. 6). The positions of H-1A/C1A-H-6/C6A and H-1B/C1B-H-4/C4B correlations were found to be in accordance with data obtained using the database program glycosciences.de.

Analysis of the α and β effects (29) caused by glycosylation of the $\rightarrow 3,4$)- α -D-mannose residue (B) at positions O-3 and O-4 and the $\rightarrow 6$)- α -D-mannose residue (A) at position O-6 in OS-1142 was performed by comparison of changes in the ^{13}C NMR chemical shifts of the linkage carbons and the carbons adjacent to the linkage carbons, respectively, with those in free sugar residues (Table 3) (30). Glycosylation of residue B by residue A at position O-4 resulted in relatively large positive α -glycosylation effects of 7.5 ppm and 7.3 ppm on C-3 and C-4 of residue B, respectively. These data indicate that both residues A and B have the same absolute configuration, i.e., D-D or L-L (29). The α and β effects of glycosylation on C-2, C-3, and C-4 of residue B would have values of -3.3 ppm, $+4.2$ ppm, and $+9.0$ ppm, respectively, if residues A and B had the opposite absolute configurations—D-L or L-D. In addition, for glycosylating residue A, the δ_{C} value for C-1 of 101.8 was in accordance with the glycosylation effect of $+6.9$ ppm, which confirmed that residues A and B have the same absolute configuration. The relatively small α effect of glycosylation of

FIG 4 Part of the ^1H NMR spectrum of OS-1142. The signals for anomeric protons for residues A and B are annotated in boldface. The signals for the anomeric protons of the A-PS repeating unit are indicated by asterisks.

FIG 5 2D ^1H - ^1H ROESY NMR spectral plot for core plus one repeating unit OS-1142. Part of the ^1H - ^1H ROESY NMR spectrum for core plus one repeating unit OS-1142 is shown. Residue A is $\rightarrow 6$)- α -D-Manp (1-, and residue B is $\rightarrow 3,4$)-linked α -D-Manp. X, the inter- and intrareidue NOEs observed under preirradiation of H-1 of residue A and H-1 of $\rightarrow 2,6$)- α -D-Manp-(1-. The structural diagram shows the α -(1-4) linkage between the 6-linked Man residue (A) of the A-PS repeating unit and the 3-linked Man residue (B) of the capped core OS.

+3.6 ppm on C-6 at δ_{C} 65.5, caused by glycosylation of residue A at position O-6 by the α -(1 $\rightarrow$ 6)-linked mannopyranosyl residue of the backbone of the A-PS repeating unit, indicates that these α -mannopyranosyl residues have the same absolute configuration (29).

Thus, we conclude that the α -(1 $\rightarrow$ 3)-linked Man residues of the outer core region and those of the A-PS repeating unit have the same, i.e., D, absolute configuration (cf. data from references 5 and 8).

We have established that the A-PS repeating unit linked to the core OS in OS-1142 contains a $\rightarrow 6$)- α -D-mannose backbone residue that was not detected during the analysis of the native A-LPS using both ^1H NMR and compositional data. However, $\rightarrow 6$)- α -D-mannose was present in dephosphorylated A-LPS (5). In addition, the absence of both proton and carbon signals for the α -D-Man-(1 $\rightarrow$ 2)- α -D-Man-1-phosphate moiety in the NMR spectra of OS-1142 indicates that it was probably destroyed/lost during isolation of OS-1142 (31). Taking into account the above data, we propose that in the SR-type LPS of the *P. gingivalis* Δ PG1142 mutant strain, the A-PS repeating unit is attached to the nonterminal residue of the α -(1 $\rightarrow$ 3)-mannose-containing extension of the capped core glycoform at position O-4 via the $\rightarrow 6$ - α -D-mannose

residue, which carries the α -(1 $\rightarrow$ 2)-D-Man- α -(1 $\rightarrow$ 2)-Man-1-phosphate motif (Fig. 7).

We suggest that O-PS and A-PS repeating units are attached to structurally distinct capped core glycoforms (Fig. 1 and 7) that differ by the lack of one $\rightarrow 3$)- α -D-mannose residue, which is linked to a monophosphorylated glycerol residue at position O-2, the presence of which was detected by NMR spectral analysis of the core OS from the *P. gingivalis* Δ PG1051 mutant strain (8). This subtle structural difference within the outer core region may give rise to the formation of O-LPS and A-LPS produced by *P. gingivalis* W50 (6, 32).

Properties of the *P. gingivalis* Δ PG0129 mutant strain. Based on these data, we hypothesized that deletion of α -1,3-mannosyltransferase activity responsible for the formation of the capped core OS would cause the loss of the attachment site for both O-LPS and A-LPS. We identified a putative α -mannosyltransferase (PG0129) in *P. gingivalis* W50 that could be involved in the biosynthesis of the core OS.

Gel electrophoresis of LPS from the *P. gingivalis* Δ PG0129 mutant strain. LPSs isolated from the *P. gingivalis* W50, Δ PG1051, and Δ PG0129 strains were subjected to SDS-urea-PAGE, followed by silver staining (Fig. 8A). LPS from the *P. gingivalis* Δ PG0129

mutant strain does not show the characteristic laddering pattern of S-type LPS and appears to migrate faster than the R-type LPS synthesized by the *P. gingivalis* ΔPG1051 mutant strain, which is shown for comparison (Fig. 8A). Thus, LPS from the *P. gingivalis* ΔPG0129 mutant strain is smaller, which we refer to as deep-R-type LPS (containing truncated core OS), and does not show immunoreactivity with MAb 1B5 (Fig. 8C).

However, LPS isolated from the complemented CΔPG0129 strain shows the characteristic laddering pattern on SDS-urea-PAGE and immunoreactivity with MAb 1B5 on Western blotting (Fig. 8B and C), indicating that the synthesis of S-type LPS was restored on complementation.

Monosaccharide and methylation analyses of LPS from the *P. gingivalis* ΔPG0129 mutant strain. Monosaccharide analysis of LPS from the *P. gingivalis* ΔPG0129 mutant strain showed the presence of mannose and Kdo in the molar ratio of 3:2 and minor amounts of allosamine (0.3 molar ratio relative to Kdo). Monosaccharide analysis of OS-1051 (the uncapped core OS from the *P. gingivalis* ΔPG1051 mutant strain [8]) showed the presence of mannose-Kdo-hexosamine in the molar ratio 67.3:1:4.0. The levels of Kdo present in OS-1051 appeared to be very low, probably due to losses during the preparation of the core OS from ΔPG1051 LPS (33). Nevertheless, compositional analysis of LPS from ΔPG0129 showed drastically reduced amounts of mannose compared to OS-1051. The methylation analysis of LPS from the *P. gingivalis* ΔPG0129 mutant strain (Table 4) indicates the presence of terminal mannose and terminal allosamine, and no 3-linked mannose was detected.

However, the methylation analysis of OS-1051 (Table 4) (8) clearly shows the presence of 3-linked mannose, and this is the major difference between the LPSs from the *P. gingivalis* ΔPG0129 and ΔPG1051 mutant strains. Based on the results obtained, the *P. gingivalis* ΔPG0129 mutant strain synthesizes a deep-R-type LPS with a truncated core region lacking 3-linked mannose residues, which constitutes the complete outer core region of the uncapped core and capped core glycoforms (Fig. 7). Thus, PG0129 in *P. gingivalis* is an α-1,3-mannosyltransferase, and inactivation leads to the formation of modified LPS and loss of the attachment sites within the outer core region, namely, the nonreducing terminal mannose and the nonterminal mannose residues to which O-PS repeating units and A-PS repeating units, respectively, are normally attached (Fig. 7) (8).

DISCUSSION

P. gingivalis W50 synthesizes two structurally distinct LPSs, namely, O-LPS and A-LPS (4–6). Previous studies had failed to elucidate whether the same core OS glycoform is present in these two macromolecules. There are common steps in the biosynthesis of A-PS and multiple glycosylated proteases, namely, Arg gingipains, in *P. gingivalis* (1, 8, 34). Thus, elucidation of the full structures of the two LPSs may also shed light on the glycosylation process in the organism.

Earlier studies by our group revealed that the core OS exists in two glycoforms, namely, an uncapped core OS that was devoid of the attachment site for O-PS and a capped core OS that contains the attachment site of the O-PS repeating unit. The outer core region of the uncapped core was composed of an α-(1→3)-Man-containing oligomer in which alternate residues of Man are substituted by PEA at position O-6 (Fig. 1), (8). The capped core is formed by glycosylation of the terminal nonreducing residue of

TABLE 3 ^1H and ^{13}C NMR spectral data for the $[-\rightarrow 6](\alpha\text{-D-Man}(1\rightarrow 4))[(1\rightarrow 3)(\alpha\text{-D-Man}(1\rightarrow 3))\text{glycosidic fragment present in OS-1142}]^a$

Residue	^1H - ^{13}C correlation ^b	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6	H-6'/C-6
A $[-\rightarrow 6](\alpha\text{-D-Man}(1\rightarrow 4))$		4.89 (4.99)/ 101.8 (101.0) [+6.9]	3.98 (4.03)/ 72.0 (70.7)	3.86 (3.87)/ 72.1 (71.4)	3.77 (3.74)/ 69.5 (70.9)	3.84 (3.83)/ 68.5 (67.2)	3.90 (3.96)/ 65.5 [+3.6]	3.84 (3.85)/ (66.6)
B $[-\rightarrow 3,4](\alpha\text{-D-Man}(1\rightarrow 3))$		5.11 (5.12)/ 102.40 (102.2)	4.08 (4.07)/ 70.9 (70.3) [+0.2]	3.94 (3.93)/ 78.9 (78.8) [+7.5]	3.77 (3.76)/ 74.3 (74.5) [+7.3]	3.68 (3.64)/ 70.8 (71.4) NA	3.92 (3.93)/61.9	3.85 (3.85)/(61.3)

^a The oligosaccharide $[-\rightarrow 3](\alpha\text{-D-Man}(1\rightarrow 4))[\alpha\text{-D-Man}(1\rightarrow 3)]\alpha\text{-D-Man}$ (residues A, B, and C, respectively) represents the attachment of the 6-linked backbone residue of the A-PS repeating unit to the 3-linked mannopyranose at position O-4 of the capped outer core region of the LPS of *P. gingivalis* W50.

^b The chemical shifts of glycosylated carbon atoms are indicated in boldface; the α and β effects of glycosylation for carbon atoms are shown in brackets; the estimated ^1H and ^{13}C shifts for the corresponding model branched oligosaccharide (obtained using the database at <http://www.glycosciences.de/>) are shown in parentheses. NA, not applicable.

FIG 6 2D ^1H - ^{13}C HMQC-TOCSY NMR spectral plot for OS-1142. Part of the 2D ^1H - ^{13}C HMQC-TOCSY NMR spectral plot for OS-1142 is shown. The traces between intraresidue cross-peaks for $\rightarrow 6$ - α -D-Man(1 $\rightarrow$ (residue A) and $\rightarrow 3,4$ - α -D-Man(1 $\rightarrow$ (residue B) are shown by solid lines.

the uncapped core at position O-3 with a linear three- to five-member α -(1 $\rightarrow$ 3)-mannopyranosyl OS. Structural analysis of the uncapped core OS derived from LPS of the *P. gingivalis* Δ PG1051 mutant strain revealed the presence of two core glycoforms that differ by the lack of one of the α -(1 $\rightarrow$ 3)-D-mannopyranosyl residues in the linear α -1,3-linked disaccharide fragment, which is linked to the glycerol residue at position O-2 (Fig. 1) (8).

This is analogous to *Pseudomonas aeruginosa*, which synthesizes two glycoforms of the core OS that differ by the linkage of an L-Rha to different D-Glc residues in the outer core region. The capped core glycoform consists of L-Rha attached via (1 $\rightarrow$ 3) linkage to β -D-Glc of the core side chain and acts as the acceptor molecule for the covalent attachment of either A- or B-band O-antigen (35). The other core glycoform, called the uncapped core, contains L-Rha linked to the backbone by a D-Glc residue at position 6 and is not substituted by O-antigen but by β -D-Glc (36, 37). Poon et al. (35) showed that MigA, an α -1,6-rhamnosyltransferase, and WapR, an α -1,3-rhamnosyltransferase, are the key enzymes responsible for attaching L-Rha residues to the different D-Glc residues in the outer core OS in *P. aeruginosa* and could play an important role in regulating the relative amounts of uncapped core and capped core glycoforms, respectively, on the cell surface.

In this study, LPS was isolated from *P. gingivalis* Δ PG1142 (*wzy*, encoding O-antigen polymerase) using extraction with hot

phenol (10), which yielded an SR-type LPS containing both SR-type O-LPS and SR-type A-LPS and showed immunoreactivity to MAb 1B5 on Western blotting. This also confirmed that PG1142 (*Wzy*; O-antigen polymerase) possesses dual specificity and causes polymerization of both O-PS and A-PS chains.

Methylation analysis of OS-1142 showed not only the presence of O-PS and A-PS components but also 3-substituted and 3,4-disubstituted mannoses, which were thought to be derived from the core region of LPS of *P. gingivalis*. Since 3,4-linked Man was not detected during the structural determination of the attachment site of O-PS to core lipid A (cf. NMR and MS data for OS-1142 in reference 8), the presence of this carbohydrate component in OS-1142 could be due to a change in the glycosylation pattern of the core caused by attachment of the A-PS repeating unit. The ^1H NMR spectrum of OS-1142 contained anomeric signals for components of both the O-PS and A-PS repeating units, as well as those for the protons that were specific for the α -1,3-linked Man residue of the outer core region (8). There was no signal in the region δ_{H} 5.4 to 5.5 observed in the ^1H NMR spectrum of OS-1142 that had the splitting pattern that would be caused by vicinal coupling of the anomeric proton by a phosphorus nucleus. This indicated the absence of an α -D-Man-(1 $\rightarrow$ 2)- α -D-Man-1-phosphate moiety attached to the α -(1 $\rightarrow$ 6)-D-mannose-containing backbone of the repeating unit of A-PS of *P. gingivalis* W50 (5). As

FIG 7 Structure of the core region of LPS from *P. gingivalis* W50 showing the proposed attachment site of the A-PS repeating unit to the core oligosaccharide in the A-LPS of *P. gingivalis* and structure of the truncated core OS in the *P. gingivalis* ΔPG0129 mutant strain. The linkage between the A-antigen and one of the residues of the $[\alpha\text{-D-Manp}]_{1-2}$ extension of the capped core is indicated by a dashed arrow. The A-PS repeating unit is shown in the shaded box. The thick vertical arrow indicates the probable site of action of PG0129.

judged from the comparative structural analysis of the core plus one O-PS glycoform obtained from LPS of *P. gingivalis* ΔPG1142 described previously (8) and that of OS-1142 in this study, the latter showed the presence of $\alpha\text{-D-Manp}$, which was

derived from the distinct linkage of the A-PS repeating unit to the outer core.

The major components of yeast cell walls are mannans and phosphomannans, which are branched polymers of D-mannose com-

FIG 8 SDS-urea-PAGE followed by silver staining and Western blotting versus MAb 1B5 of LPS from *P. gingivalis* strains. (A) Silver staining of LPSs from *P. gingivalis* W50, ΔPG1051, and ΔPG0129. Lanes: 1, *P. gingivalis* W50 LPS; 2, 3, and 4, ΔPG1051 LPS (1, 2, and 4 μg); 5, 6, and 7, ΔPG0129 LPS (1, 2, and 4 μg). (B) Silver staining of LPSs from *P. gingivalis* W50, ΔPG0129, and CΔPG0129. Lanes: 1 and 2, W50 LPS; 3, ΔPG0129 LPS; 4, CΔPG0129 LPS. (C) Western blotting versus MAb 1B5 of LPSs from *P. gingivalis* W50, ΔPG0129, and CΔPG0129. Lanes: 1, molecular mass markers; 2, W50 LPS; 3, ΔPG0129 LPS; 4, CΔPG0129 LPS.

TABLE 4 Methylation analysis data for LPS from *P. gingivalis* ΔPG0129 and OS-1051 from LPS of *P. gingivalis* ΔPG1051 (*waaL*)

Component	Peak area ^a
LPS from ΔPG0129	
1,5-Di- <i>O</i> -acetyl-1-deuterio-2,3,4,6-tetra- <i>O</i> -methylmannitol	1.5
1,5-Di- <i>O</i> -acetyl-2-(acetylmethylamino)-2-deoxy-1-deuterio-3,4,6-tri- <i>O</i> -methylallositol	0.6 ^b
OS-1051 (core OS from ΔPG1051) ^c	
1,5-Di- <i>O</i> -acetyl-1-deuterio-2,3,4,6-tetra- <i>O</i> -methylmannitol	1.7
1,3,5-Tri- <i>O</i> -acetyl-1-deuterio-2,4,6-tri- <i>O</i> -methylmannitol	3.2
1,5-Di- <i>O</i> -acetyl-2-(acetylmethylamino)-2-deoxy-1-deuterio-3,4,6-tri- <i>O</i> -methylallositol	0.4 ^b

^a The reduced amount of partially methylated derivatives of 2-acetamido-2-deoxyhexosols is due to resistance of their glycosidic bonds to acid hydrolysis.

^b Peak area refers to the relative detector response; the values are not corrected for differences in detector response factors.

^c Data from reference 8.

prised of α -(1 $\rightarrow$ 6)-linked backbones and side chains having α -(1 $\rightarrow$ 2), α -(1 $\rightarrow$ 3)-glycosidic, and, rarely, β -(1 $\rightarrow$ 2)-glycosidic linkages (38).

The extracellular polysaccharide that is produced by *Pichia* (*Hansenula*) *holstii* NRRL Y-2448 was found to have the structural pattern α -D-Man α -(1-phosphate-6)- α -D-Man, specific for a wide range of phosphomannans (39–41). There is no current structural evidence that mannose-containing polymers contain 3,4-disubstituted α -D-mannose as a branch point residue. To resolve the uncertainty in the structural analysis of OS-1142, the NMR database glycosciences.de was employed to compare the experimental proton and carbon NMR spectral data obtained for OS-1142 to those for the tetrasaccharide $\rightarrow$ 3) α -D-Man [$\rightarrow$ 6) α -D-Man(1 $\rightarrow$ 4)](1 $\rightarrow$ 3) α -D-Man(1 $\rightarrow$ 3) α -D-Man-, which was used as a model oligomer. The latter mimics part of the capped outer core that carries an A-PS repeating unit (lacking the epitope immunoreactive with MAb 1B5) attached via a $\rightarrow$ 6) α -D-Man residue at position O-4.

The combined analyses of ¹H and ¹³C NMR spectral data for OS-1142 have shown that both experimental proton and carbon chemical shifts for residue A, ($\rightarrow$ 6)- α -D-Man-(1 $\rightarrow$ 4), and residue B, ($\rightarrow$ 3,4)- α -D-Man-(1 $\rightarrow$ 3), were in accordance with those determined for the model oligosaccharide using the NMR database (Table 3). Moreover, the analysis of α - and β -glycosylation effects for residue B at C-4, C-3, and C-2, which were caused by glycosylation with residue A at O-4 and with the nonreduced terminal α -D-Man at O-3, confirmed that both residues A and B have the same absolute configuration, D, as previously described (5, 8). Thus, on the basis of the data obtained, A-PS in *P. gingivalis* is linked to the nonterminal α -(1 $\rightarrow$ 3)-D-Man residue of the three- to five-member α -1,3-mannopyranosyl capped core extension at position O-4 via the backbone α -(1 $\rightarrow$ 6)-D-Man, which carries the Man- α -(1 $\rightarrow$ 2)-Man- α -1-phosphate group as a side chain.

To confirm that the α -(1 $\rightarrow$ 3)-linked Man, as a major component of the outer core, provides the attachment sites for both O-PS and A-PS, we aimed to determine the gene(s) responsible for the biosynthesis of the outer core regions of both O-LPS and A-LPS in *P. gingivalis*.

Genes encoding a putative α -mannosyltransferase (PG0129) and an α -1,3-mannosidase (PG1711) are both present in the genome of *P. gingivalis* W83 (11). Inactivation of PG1711 in *P. gingivalis* has no effect on the synthesis of O-LPS, A-LPS, and the Arg gingipains (20). In this study, therefore, we focused upon the influence of PG0129 on core OS biosynthesis and O- and A-LPS formation.

BLAST analysis of PG0129 indicated the presence of a conserved domain classed under glycosyltransferase B (GT-B fold) and a glycosyltransferase 1 motif (see the Pfam database [<http://pfam.xfam.org/>]) at the C terminus. In *E. coli* O-antigen serotype O9a, the enzyme (WbdB) is an α -1,3 mannosyltransferase required for the attachment of 2 Man residues onto the disaccharide α -Manp-(1 $\rightarrow$ 3)- β -Glc pNAc-PP-undecaprenyl intermediate (42), giving rise to α -Manp-(1 $\rightarrow$ 3)- α -Manp-(1 $\rightarrow$ 3)- α -Man-(1 $\rightarrow$ 3)- β -Glc pNAc-PP-undecaprenyl, and also possesses a similar fold. Thus, WbdB can transfer Man residues to Man linked either to position 3 of β -Glc pNAc or to position 3 of Man, highlighted above.

The LPS isolated from the *P. gingivalis* ΔPG0129 mutant strain was a deep-R-type LPS with a truncated core lacking 3-linked mannose, which constitutes the complete outer core region of the uncapped core and capped core glycoforms of O-LPS (8) (Fig. 7). This suggests that PG0129 of *P. gingivalis* W50 is an α -1,3-mannosyltransferase that catalyzes the transfer of an α -mannopyranosyl residue(s) to the α -mannose linked to monophosphorylated glycerol at position O-2, forming an α -(1 $\rightarrow$ 3)-linkage(s), and thus extends the outer core region of LPS (Fig. 7). Thus, there are at least two α -mannosyltransferases in *P. gingivalis* that are involved in the biosynthesis of the outer core region: (i) one that can transfer a mannose residue to position 2 of glycerol-1-phosphate and (ii) PG0129, described in this study, which catalyzes the attachment of a mannose residue at position O-3 of the mannose linked to position 2 of glycerol and probably the formation of a linear α -(1 $\rightarrow$ 3)-linked Man extension of both uncapped and capped core glycoforms of LPS.

Since previous studies have demonstrated that it is possible to independently purify and characterize O-LPS (4) and A-LPS (5, 8) from *P. gingivalis* W50, the two LPSs are therefore two different macromolecules. Taking into account these findings and studies on the formation of enterobacterial common antigen and B-band LPS of *P. aeruginosa* (35, 37), we suggest that in *P. gingivalis*, the biosynthesis of both O-LPS and A-LPS includes the ligation of O-PS and A-PS repeating units to two capped core OS glycoforms, which are structurally very similar.

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REFERENCES

- Curtis MA, Aduse-Opoku J, Rangarajan M. 2001. Cysteine proteases of *Porphyromonas gingivalis*. Crit Rev Oral Biol Med 12:192–216. <http://dx.doi.org/10.1177/10454411010120030101>.
- Coats SR, Jones JW, Do CT, Braham PH, Bainbridge BW, To TT, Goodlett DR, Ernst RK, Darveau RP. 2009. Human Toll-like receptor 4 responses to *P. gingivalis* are regulated by lipid A 1- and 4'-phosphatase activities. Cell Microbiol 11:1587–1599. <http://dx.doi.org/10.1111/j.1462-5822.2009.01349.x>.
- Trent MS, Stead CM, Tran AX, Hankins JV. 2006. Diversity of endotoxin and its impact on pathogenesis. J Endotoxin Res 12:205–223. <http://dx.doi.org/10.1179/096805106X118825>.
- Paramonov N, Bailey D, Rangarajan M, Hashim A, Kelly G, Curtis MA, Hounsell EF. 2001. Structural analysis of the polysaccharide from the lipopolysaccharide of *Porphyromonas gingivalis* strain W50. Eur J Biochem 268:4698–4707. <http://dx.doi.org/10.1046/j.1432-1327.2001.02397.x>.
- Paramonov N, Rangarajan M, Hashim A, Gallagher A, Aduse-Opoku J, Slaney JM, Hounsell E, Curtis MA. 2005. Structural analysis of a novel anionic polysaccharide from *Porphyromonas gingivalis* strain W50 related to Arg-gingipain glycans. Mol Microbiol 58:847–863. <http://dx.doi.org/10.1111/j.1365-2958.2005.04871.x>.
- Rangarajan M, Aduse-Opoku J, Paramonov N, Hashim A, Bostanci N, Fraser OP, Tarelli E, Curtis MA. 2008. Identification of a second lipopolysaccharide in *Porphyromonas gingivalis* W50. J Bacteriol 190:2920–2932. <http://dx.doi.org/10.1128/JB.01868-07>.
- Curtis MA, Thickett A, Slaney JM, Rangarajan M, Aduse-Opoku J, Shepherd P, Paramonov N, Hounsell EF. 1999. Variable carbohydrate modifications to the catalytic chains of the RgpA and RgpB proteases of *Porphyromonas gingivalis* W50. Infect Immun 67:3816–3823.
- Paramonov NA, Aduse-Opoku J, Hashim A, Rangarajan M, Curtis MA. 2009. Structural analysis of the core region of O-lipopolysaccharide of *Porphyromonas gingivalis* from mutants defective in O-antigen ligase and O-antigen polymerase. J Bacteriol 191:5272–5282. <http://dx.doi.org/10.1128/JB.00019-09>.
- Darveau RP, Hancock RE. 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J Bacteriol 155:831–838.
- Westphal O, Jann K. 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure, p 83–91. In Whistler RL (ed), Methods in carbohydrate chemistry. Academic Press, New York, NY.
- Nelson KE, Fleischmann RD, DeBoy RT, Paulsen IT, Fouts DE, Eisen JA, Daugherty SC, Dodson RJ, Durkin AS, Gwinn M, Haft DH, Kolonay JF, Nelson WC, Mason T, Tallon L, Gray J, Granger D, Tettelin H, Dong H, Galvin JL, Duncan MJ, Dewhirst FE, Fraser CM. 2003. Complete genome sequence of the oral pathogenic bacterium *Porphyromonas gingivalis* strain W83. J Bacteriol 185:5591–5601. <http://dx.doi.org/10.1128/JB.185.18.5591-5601.2003>.
- Naito M, Hirakawa H, Yamashita A, Ohara N, Shoji M, Yukitake H, Nakayama K, Toh H, Yoshimura F, Kuhara S, Hattori M, Hayashi T. 2008. Determination of the genome sequence of *Porphyromonas gingivalis* strain ATCC 33277 and genomic comparison with strain W83 revealed extensive genome rearrangements in *P. gingivalis*. DNA Res 15:215–225. <http://dx.doi.org/10.1093/dnares/dsn013>.
- Watanabe T, Maruyama F, Nozawa T, Aoki A, Okano S, Shibata Y, Oshima K, Kurokawa K, Hattori M, Nakagawa I, Abiko Y. 2011. Complete genome sequence of the bacterium *Porphyromonas gingivalis* TDC60, which causes periodontal disease. J Bacteriol 193:4259–4260. <http://dx.doi.org/10.1128/JB.05269-11>.
- McLean JS, Lombardo MJ, Ziegler MG, Novotny M, Yee-Greenbaum J, Badger JH, Tesler G, Nurk S, Lesin V, Bami D, Hall AP, Edlund A, Allen LZ, Durkin S, Reed S, Torriani F, Nealon KH, Pevzner PA, Friedman R, Venter JC, Lasken RS. 2013. Genome of the pathogen *Porphyromonas gingivalis* recovered from a biofilm in a hospital sink using a high-throughput single-cell genomics platform. Genome Res 23:867–877. <http://dx.doi.org/10.1101/gr.150433.112>.
- Siddiqui H, Yoder-Himes DR, Mizgalska D, Nguyen K-A, Potempa J, Olsen I. 2014. Genome sequence of *Porphyromonas gingivalis* strain HG66 (DSM 28984). Genome Announc 2:e00947-14. <http://dx.doi.org/10.1128/genomeA.00947-14>.
- Aduse-Opoku J, Slaney JM, Hashim A, Gallagher A, Gallagher RP, Rangarajan M, Boutaga K, Laine ML, Van Winkelhoff AJ, Curtis MA. 2006. Identification and characterization of the capsular polysaccharide (K-antigen) locus of *Porphyromonas gingivalis*. Infect Immun 74:449–460. <http://dx.doi.org/10.1128/IAI.74.1.449-460.2006>.
- Davey ME, Duncan MJ. 2006. Enhanced biofilm formation and loss of capsule synthesis: deletion of a putative glycosyltransferase in *Porphyromonas gingivalis*. J Bacteriol 188:5510–5523. <http://dx.doi.org/10.1128/JB.01685-05>.
- Kelley LA, Sternberg MJ. 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc 4:363–371. <http://dx.doi.org/10.1038/nprot.2009.2>.
- Vetting MW, Frantom PA, Blanchard JS. 2008. Structural and enzymatic analysis of MshA from *Corynebacterium glutamicum*: substrate-assisted catalysis. J Biol Chem 283:15834–15844. <http://dx.doi.org/10.1074/jbc.M801017200>.
- Rangarajan M, Aduse-Opoku J, Hashim A, Paramonov N, Curtis MA. 2013. Characterization of the α - and β -mannosidases of *Porphyromonas gingivalis*. J Bacteriol 195:5297–5307. <http://dx.doi.org/10.1128/JB.00898-13>.
- Inzana T, Apicella M. 1999. Use of a bilayer stacking gel to improve resolution of lipopolysaccharides and lipooligosaccharides in polyacrylamide gels. Electrophoresis 20:462–465. [http://dx.doi.org/10.1002/\(SICI\)1522-2683\(19990301\)20:3<462::AID-ELPS462>3.0.CO;2-N](http://dx.doi.org/10.1002/(SICI)1522-2683(19990301)20:3<462::AID-ELPS462>3.0.CO;2-N).
- Altman E, Perry MB, Brisson JR. 1989. Structure of the lipopolysaccharide antigenic O-chain produced by *Actinobacillus pleuropneumoniae* serotype 4 (ATCC 33378). Carbohydr Res 191:295–303. [http://dx.doi.org/10.1016/0008-6215\(89\)85072-4](http://dx.doi.org/10.1016/0008-6215(89)85072-4).
- Kakehi K, Honda S. 1989. Analysis of carbohydrates by GLC and MS. CRC Press, Boca Raton, FL.
- Kvernheim A. 1987. Methylation analysis of polysaccharides with butyllithium in dimethylsulphoxide. Acta Chem Scand B Org Chem Biochem 41:150–152.
- Bax A, Davis DG. 1985. Practical aspects of two-dimensional transverse NOE spectroscopy. J Magn Reson 63:207–213.
- Kövér KE, Hruby VJ, Uhrin D. 1997. Sensitivity- and gradient-enhanced heteronuclear coupled/decoupled HSQC-TOCSY experiments for measuring long-range heteronuclear coupling constants. J Magn Reson 129:125–129. <http://dx.doi.org/10.1006/jmre.1997.1265>.
- Corsaro MM, Evidente A, Lanzetta R, Lavermicocca P, Molinaro A. 2001. Structural determination of the phytotoxic mannan exopolysaccharide from *Pseudomonas syringae* pv. ciccaronei Carbohydr Res 330:271–277. [http://dx.doi.org/10.1016/S0008-6215\(00\)00279-2](http://dx.doi.org/10.1016/S0008-6215(00)00279-2).
- Hernandez LM, Ballou L, Alvarado E, Tsai PK, Ballou CE. 1989. Structure of the phosphorylated N-linked oligosaccharides from the mnn9 and mnn10 mutants of *Saccharomyces cerevisiae*. J Biol Chem 264:13648–13659.
- Lipkind GM, Shashkov AS, Knirel YA, Vinogradov EV, Kochetkov NK. 1988. A computer-assisted structural analysis of regular polysaccharides on the basis of ^{13}C -N.M.R. data. Carbohydr Res 175:59–75. [http://dx.doi.org/10.1016/0008-6215\(88\)80156-3](http://dx.doi.org/10.1016/0008-6215(88)80156-3).
- Jansson PE, Kenne L, Widmalm G. 1989. Computer-assisted structural analysis of polysaccharides with an extended version of CASPER using ^1H - and ^{13}C -N.M.R. data. Carbohydr Res 188:169–191. [http://dx.doi.org/10.1016/0008-6215\(89\)84069-8](http://dx.doi.org/10.1016/0008-6215(89)84069-8).
- Caroff M, Tacken A, Szabó L. 1988. Detergent-accelerated hydrolysis of bacterial endotoxins and determination of the anomeric configuration of the glycosyl phosphate present in the “isolated lipid A” fragment of the *Bordetella pertussis* endotoxin. Carbohydr Res 175:273–282. [http://dx.doi.org/10.1016/0008-6215\(88\)84149-1](http://dx.doi.org/10.1016/0008-6215(88)84149-1).
- Raetz CR, Whitfield C. 2002. Lipopolysaccharide endotoxins. Annu Rev Biochem 71:635–700. <http://dx.doi.org/10.1146/annurev.biochem.71.110601.135414>.
- Holst O. 2011. Structure of the lipopolysaccharide core region, p 21–39. In Knirel YA, Valvano MA (ed), Bacterial lipopolysaccharides: structure, chemical synthesis, biogenesis and interaction with host cells. Springer, New York, NY.
- Gallagher A, Aduse-Opoku J, Rangarajan M, Slaney JM, Curtis MA. 2003. Glycosylation of the Arg-gingipains of *Porphyromonas gingivalis* and comparison with glycoconjugate structure and synthesis in other bacteria. Curr Protein Pept Sci 4:427–441. <http://dx.doi.org/10.2174/1389203033486974>.
- Poon KK, Westman EL, Vinogradov E, Jin S, Lam JS. 2008. Functional characterization of MigA and WapR: putative rhamnosyltransferases in-

- volved in outer core oligosaccharide biosynthesis of *Pseudomonas aeruginosa*. J Bacteriol 190:1857–1865. <http://dx.doi.org/10.1128/JB.01546-07>.
36. Sadovskaya I, Brisson JR, Lam JS, Richards JC, Altman E. 1998. Structural elucidation of the lipopolysaccharide core regions of the wild-type strain PAO1 and O-chain-deficient mutant strains AK1401 and AK1012 from *Pseudomonas aeruginosa* serotype O5. Eur J Biochem 255:673–684. <http://dx.doi.org/10.1046/j.1432-1327.1998.2550673.x>.
 37. Sadovskaya I, Brisson JR, Thibault P, Richards JC, Lam JS, Altman E. 2000. Structural characterization of the outer core and the O-chain linkage region of lipopolysaccharide from *Pseudomonas aeruginosa* serotype O5. Eur J Biochem 267:1640–1650. <http://dx.doi.org/10.1046/j.1432-1327.2000.01156.x>.
 38. Nikolaev AV, Botvinko IV, Ross AJ. 2007. Natural phosphoglycans containing glycosyl phosphate units: structural diversity and chemical synthesis. Carbohydr Res 342:297–344. <http://dx.doi.org/10.1016/j.carres.2006.10.006>.
 39. Bretthauer RK, Kaczorowski GJ, Weise MJ. 1973. Characterization of a phosphorylated pentasaccharide isolated from *Hansenula holstii* NRRL Y-2448 phosphomannan. Biochemistry 12:1251–1256. <http://dx.doi.org/10.1021/bi00731a002>.
 40. Parolis LA, Duus JO, Parolis H, Meldal M, Bock K. 1996. The extracellular polysaccharide of *Pichia (Hansenula) holstii* NRRL Y-2448: the structure of the phosphomannan backbone. Carbohydr Res 293:101–117. [http://dx.doi.org/10.1016/0008-6215\(96\)00190-5](http://dx.doi.org/10.1016/0008-6215(96)00190-5).
 41. Parolis LA, Parolis H, Kenne L, Meldal M, Bock K. 1998. The extracellular polysaccharide of *Pichia (Hansenula) holstii* NRRL Y-2448: the phosphorylated side chains. Carbohydr Res 309:77–87. [http://dx.doi.org/10.1016/S0008-6215\(98\)00101-3](http://dx.doi.org/10.1016/S0008-6215(98)00101-3).
 42. Greenfield LK, Richards MR, Li J, Wakarchuk WW, Lowary TL, Whitfield C. 2012. Biosynthesis of the polymannose lipopolysaccharide O-antigens from *Escherichia coli* serotypes O8 and O9a requires a unique combination of single- and multiple-active site mannosyltransferases. J Biol Chem 287:35078–35091. <http://dx.doi.org/10.1074/jbc.M112.401000>.