Characterization and Scope of S-layer Protein O-Glycosylation in Tannerella forsythia

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Background: Bacterial cell surface glycosylation impacts virulence. Result: Surface layer glycans from T. forsythia are O-linked oligosaccharides that modify also multiple other T. forsythia proteins.

Conclusion: A general protein O-glycosylation system is present in T. forsythia sharing identical sequon requirements as other Bacteroides species.

Significance: Systematic protein O-glycosylation may affect the biology of T. forsythia.

Cell surface glycosylation is an important element in defining the life of pathogenic bacteria. Tannerella forsythia is a Gram-negative, anaerobic periodontal pathogen inhabiting the subgingival plaque biofilms. It is completely covered by a two-dimensional crystalline surface layer (S-layer) composed of two glycoproteins. Although the S-layer has previously been shown to delay the bacterium’s recognition by the innate immune system, we characterize here the S-layer protein O-glycosylation as a potential virulence factor. The T. forsythia S-layer glycan was elucidated by a combination of electrospray ionization tandem mass spectrometry and nuclear magnetic resonance spectroscopy as an oligosaccharide with the structure 4-Me-β-ManpNAcCONH2-(1→3)-[Pse5Am7Gc-(2→4)]-β-ManpN ACA-(1→4)-[4-Me-α-Galp(1→2)]-α-Fucp-(1→4)-[α-Xylp-(1→3)]-β-Glc pA-(1→3)-β-Digp-(1→2)-α-Galp, which is O-glycosidically linked to distinct serine and threonine residues within the three-amino acid motif (D/S/T)(A/L/M/T/V) on either S-layer protein. This S-layer glycan obviously impacts the life style of T. forsythia because increased biofilm formation of an UDP-N-acetylmannosaminuronic acid dehydrogenase mutant can be correlated with the presence of truncated S-layer glycans. We found that several other proteins of T. forsythia are modified with that specific oligosaccharide. Proteomics identified two of them as being among previously classified antigenic outer membrane proteins that are up-regulated under biofilm conditions, in addition to two predicted antigenic lipoproteins. Theoretical analysis of the S-layer O-glycosylation of T. forsythia indicates the involvement of a 6.8-kb gene locus that is conserved among different bacteria from the Bacteroidetes phylum. Together, these findings reveal the presence of a protein O-glycosylation system in T. forsythia that is essential for creating a rich glycoproteome pinpointing a possible relevance for the virulence of this bacterium.

In recent years, bacterial glycosylation systems have come under enhanced scrutiny because of the increasing frequencies with which they are seen in pathogenic and symbiont bacteria as well as their potential for exploitation in recombinant glycosylation engineering (1). Interestingly, in several of the investigated bacteria, a scenario of general glycosylation systems and of overlapping roles for distinct carbohydrate-active enzymes is evolving. For instance, the gastrointestinal pathogen Campylobacter jejuni targets multiple proteins at asparagine residues (2); the intestinal symbiont Bacteroides fragilis (3) and Neisseria species (1) possess general O-glycosylation systems; and in Pseudomonas aeruginosa, distinct stages of LPS, exopolysaccharide, and pilin glycoprotein biosynthesis share common enzymes (4, 5).

It is evident that cell surface-associated glycosylation, representing the contact zone of a bacterium with its immediate environment, is ideally suited to contribute to the bacterial physiology and to the bacterium-host cross-talk. In this context, we are investigating in this study the cell surface layer (S-layer) protein glycosylation of the periodontal pathogen Tannerella forsythia. T. forsythia is a Gram-negative oral anaerobe that, together with the well studied Porphyromonas gingivalis and the spirochaete Treponema denticola, is a member of the “red complex” of microorganisms that inhabits the subgingival plaque biofilm (6) and is considered a major contributor to periodontal disease in humans (7). There are also reports on the

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† The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1 and S2.

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association of periodontitis with systemic diseases, such as cardiovascular diseases and diabetes (8, 9). Thus, there is great medical interest in understanding the bacterium-host cross-talk that forms the basis of health, disease, and healing.

Although a complete genome sequence is available for T. forsythia (available on the Oralgen Web site) relatively little is known about the virulence-associated factors of this bacterium (10). Because adhesion to oral surfaces constitutes the initial step in the bacterial colonization, identification of T. forsythia surface antigens that may play roles in these events is of primary interest. Sharma et al. (11) have identified a leucine-rich repeat BspA surface antigen that binds to fibronectin and other extracellular matrix components, whereas the T. forsythia S-layer was shown to mediate adhesion/invasion to human gingival epithelial cells and to epidermal carcinoma cells of the mouth (12, 13). Data on the virulence potential of the T. forsythia S-layer were supported in our laboratory by investigating the immune responses of human macrophages and gingival fibroblasts upon stimulation with wild-type (WT) T. forsythia and an S-layer-deficient mutant. This mutant induced significantly higher levels of the proinflammatory mediators IL-1β, TNF-α, and IL-8 compared with WT cells, especially at the early phase of response. This suggests that the S-layer attenuates the host immune response to this pathogen by evading its recognition by the innate immune system (14).

S-layers are water-insoluble proteins endowed with an intrinsic capability to self-assemble into a two-dimensional crystalline array that completely covers the bacterial cell surface, thereby obviously providing a selection advantage to the bacterium (15). Comparing T. forsythia with other S-layer-carrying bacteria, its status is unique. It is so far the only known Gram-negative bacterium that is covered with a glycosylated S-layer (16, 17), with, again uniquely, two S-layer glycoproteins being simultaneously present. The S-layer proteins TfsA (TF2661-TF2662; calculated molecular mass, 135 kDa) and TfsB (TF2663; calculated molecular mass, 152 kDa), that are encoded by an operon (18), share 24% amino acid similarity. They do not show overall homology to any other S-layer protein sequence deposited in databases, except for their C-terminal regions, which have profound similarity to putative S-layer glycoproteins of the phylogenetically closely related bacterium Parabacteroides distasonis (19). Although the glycosylation of the TfsA and TfsB proteins was already inferred some years ago against 6M urea in 20 mM Tris/HCl (pH 7.2), the extracellular matrix glycoproteins were concentrated using Amicon Ultra-15 centrifugal filter units on the two S-layer proteins TfsA and TfsB, (iii) analysis of S-layer glycosylation in the T. forsythia wecC mutant with increased biofilm-formation, (iv) determination of the scope of the S-layer protein glycosylation system in T. forsythia, and (v) initial theoretical analysis of the genetic information underlying the glycosylation event.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Medium, and Culture Conditions—** T. forsythia wild-type strain ATCC 43037 (American Type Culture Collection) and defined mutants thereof were grown anaerobically for 4–7 days in enriched tryptic soy broth as described previously (14). The S-layer gene mutants T. forsythia ΔtfsA and ΔtfsB, respectively (13), were kindly provided by Yukitaka Murakami (Aichi-Gakuin University, Nagoya, Japan), and the T. forsythia wecC mutant (6) was obtained from Ashu Sharma (State University of New York at Buffalo). Cells were harvested by centrifugation (5,000 × g, 20 min, 4 °C), washed with 10 mM HEPES (pH 7.2), and stored at −20 °C until further processing.

**SDS-PAGE and Blotting—** SDS-PAGE was carried out on 7.5 or 8% slab gels in a Mini Protein electrophoresis apparatus (Bio-Rad) according to Laemmli (20). Proteins were visualized with colloidal Coomassie Brilliant Blue R-250 staining reagent, carbohydrates were visualized with Pro-Q Emerald 300 fluorescein staining (Invitrogen) (21), and the gels were imaged at 700 nm using the Odyssey imaging system (LI-COR) and at 300 nm using the Infinity-3000 (Vilber-Lourmat) apparatus, respectively. Tank blotting of (glyco)proteins to a polyvinylidene difluoride membrane was performed as described previously (22). Fucosylated structures on the proteins were detected by probing the blot with biotinylated Aleuria aurantia lectin (Szabo Scandic) at a concentration of 2 μg/ml. Detection was performed at 800 nm using streptavidin-conjugated IRDye® (LI-COR).

**Isolation and Purification of the S-layer—** For S-layer extraction, T. forsythia ΔtfsB and ΔwecC cells, respectively, were resuspended in 50 mM HEPES (pH 2.0) for 8 min/25 °C at a ratio of 1:3 (w/v), followed by neutralizing the suspension with 4 M NaOH and centrifugation (25,000 × g, 10 min, 4 °C); the extract was repeatedly at least five times. The pooled supernatants were concentrated using Amicon Ultra-15 centrifugal filter units (50-kDa cut-off; Millipore), and the buffer was exchanged against 6 M urea in 30 mM ethanolamine (pH 10.4) with Pronase E (Sigma) at 37 °C for 24 or 48 h, respectively (23). The degradation products were prepped...
rified using 250 mg of Superclean Envi-Carb cartridges (Sigma) followed by porous graphitized carbon (PGC-HPLC) for isolation and fractionation of glycopeptides, employing a Hypercarb column (100 × 3 mm; Thermo Fisher Scientific) with a gradient of 0–60% of 95% acetonitrile in ammonium formate buffer (0.3% formic acid, pH 3.0; flow rate, 0.6 ml/min).

S-layer O-Glycan Preparation—O-Glycans for MS and NMR analyses were released from purified TsfA S-layer protein by reductive β-elimination with 1 M NaBH₄ in 0.5 M NaOH at 50 °C overnight (24, 25). Excess salt was removed using a 25-mg HyperSep Hypercarb SPE cartridge (Thermo Fisher Scientific) according to published protocols (26–28). For purification of reduced O-glycans, preparative PGC-HPLC was performed as described above.

Furthermore, the TsfA and TsfB S-layer glycoproteins as well as four additional carbohydrate-positive protein bands were excised from SDS-polyacrylamide gels, and the O-glycans were isolated by applying in-gel reductive β-elimination. Briefly, excised gel slices were transferred to a plastic reaction tube, completely covered with 1 M NaBH₄ in 0.5 M NaOH, and incubated at 50 °C overnight, followed by removal of excess salt as described above. Borohydride-reduced glycans were subsequently analyzed by LC-ESI-MS/MS.

Ammonia-based non-reductive β-elimination was done according to a protocol of Huang et al. (29), with slight modifications. Briefly, glycan-positive protein bands were covered with ammonium hydroxide solution (25%), which was saturated with ammonium carbonate. Further, 100 mg of ammonium carbonate were added to the reaction tube, and the mixture was incubated at 60 °C for 24 h. After desalting, the reaction product was analyzed by LC-ESI-MS/MS.

Monosaccharide Analysis—Monosaccharide analysis of the borohydride-reduced S-layer O-glycans was performed on a purified fraction after PGC-HPLC. The glycans were hydrolyzed with 25% TFA for 1 h at 100 °C, and released monosaccharides were analyzed as anthranilic acid derivatives by HPLC (30). Fluorescence detection was performed at 360-nm excitation and 425-nm emission, respectively. To identify the reducing end sugar, labeling was done immediately after non-reductive glycan release (31) and before hydrolysis with TFA.

Labeling with 1,2-diamino-4,5-methylene dioxybenzene dihydrochloride (DMB) and purification of the pseudaminic acid (Pse) derivative for MS and NMR analysis were performed after incubation of a purified O-glycan fraction or, in the case of a large scale preparation, of whole T. forsythia WT cells with 1 M TFA (1 h, 80 °C). Subsequently, cells were centrifuged (15,000 × g, 10 min), and the supernatant was collected and lyophilized. The lyophilisate was subsequently dissolved in a 1:1 (v/v) mixture of solution A (1 mg of DMB in 0.317 ml of MilliQ water) and solution B (1 mg of Na₂S₂O₄, 47 μl of acetic acid, 41 μl of 2-mercaptoethanol in 0.205 ml of MilliQ water) and incubated for 2 h at 56 °C. Insoluble particles were removed by centrifugation (20,000 × g, 5 min), and the supernatant was applied to reverse phase HPLC (Hypersil ODS, 250 × 4 mm). Buffer A consisted of 50 mM ammonium acetate (pH 5.5), and a gradient was performed from 11 to 14% buffer B (95% acetonitrile) within 8 min on a Shimadzu HPLC system consisting of a LC-10AD pump system, a RF-10AXL detector, and a SCL-10Avp controller. Fluorescence detection was done at 373-nm excitation and 448-nm emission (32).

LC-ESI-MS/MS—Borohydride-reduced O-glycans were analyzed by PGC-ESI-MS/MS as described recently (Hypercarb, 0.32 × 150 mm, inner diameter 5 μm) (28, 33). Ammonium formate buffer (0.3% formic acid, pH 3.0) was used as buffer A, and a gradient was performed from 0 to 35% acetonitrile within 35 min using a Dionex Ultimate 3000 (cap flow, 8 μl/min). Detection was done by an ESI-Q-TOF Global Ultima from Micromass (Waters). Data were evaluated using MassLynx 4.0 software. MS/MS experiments were performed at 30% collision energy using CID with argon gas.

High Resolution Mass Spectrometry and Accurate Mass Measurements—High resolution MS and accurate mass measurements were performed on a LTQ Orbitrap-Velos (Thermo Fisher Scientific) at the Thermo Fisher Scientific demonstration center. HPLC-fractionated, borohydride-reduced S-layer O-glycans were analyzed by direct infusion on a nanospray emitter in MS and MS/MS mode.

Proteomics—To identify protein portions of presumptive T. forsythia glycoproteins, an in-gel trypsin digest of the respective bands on SDS-polyacrylamide gels was performed. Extracted proteolytic peptides were further subjected to reverse phase liquid chromatography coupled to ESI-MS/MS (28, 34). The X!tandem algorithm (see the Global Proteome Machine Web site) was used for a protein database search of tandem MS data obtained from tryptic peptides. Results were further evaluated using log(e) values to estimate correctness of peptide assignments.

NMR Spectroscopy—The isolated O-glycans and sugar derivatives were lyophilized and dissolved in D₂O (99.99% D; Sigma-Aldrich) to concentrations of ~500 μg/600 μl. The solutions were transferred into 5-mm NMR sample tubes (Promochem). Spectra were recorded on a Bruker DRX-600 AVANCE spectrometer (Bruker) at 600.13 MHz (1H) using the Bruker Topspin 1.3 software. One-dimensional proton spectra were measured with presaturation during a 1.0-s relaxation delay and acquisition of 32,000 data points. Zero filling to 64,000 data points and Fourier transformation led to spectra with a 7,200-Hz range. Two-dimensional COSY, TOCSY (100 ms mixing time), and ROESY (400-ms mixing time, O-glycans) or NOESY (800-ms mixing time, sugar derivate)s spectra were measured using standard Bruker programs. Therefore, 384 experiments with 2,048 data points and an appropriate number of scans were recorded, each. After linear forward prediction to 512 data points in the f₂ dimension and sinusoidal multiplication in both dimensions, they were Fourier transformed to two-dimensional spectra with a 6,000 Hz range in both dimensions. The diffusion behavior of the monosaccharide was derived from the signal attenuation in a series of stimulated echo spectra with increasing gradient amplitudes by fixed diffusion delay (35, 36). All measurements were performed at 298.1 K. Chemical shifts were referenced to external acetone (δ_H, 2.225 ppm).

Genome Sequence Analysis—A 6.8-kb gene locus comprising ORFs TF2049–TF2055, including the previously described exopolysaccharide operon (TF2053–TF2055) (6), which is predicted to be involved in protein glycosylation, was analyzed and screened for homologous sequences in the phylogenetically
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related organisms *B. fragilis* NCTC 9343, *Bacteroides thetaiotaomicron* VPI 5492, *Bacteroides uniformis* ATCC 8492, *P. gingivalis* ATCC 33277, and *Parabacteroides distasonis* ATCC 8503 using the comparative genomics platform CoGe (available on the World Wide Web). The highest scoring sequences (*E* value <10^-5) were extracted and analyzed for comparable cluster formation within the respective genomes.

### RESULTS

**General Description of the S-layer Proteins TfsA and TfsB**— *T. forsythia* possesses two S-layer glycoproteins that are abundant in a whole cell lysate of the bacterium and exhibit apparent molecular masses of ~230 and 270 kDa, respectively, on an SDS-polyacrylamide gel (Fig. 1A). This is indicative of a modification of the ~135- and ~152-kDa S-layer proteins by glycosylation as confirmed by a strong staining reaction with the Pro-Q Emerald reagent (Fig. 1B). Interestingly, both S-layer proteins are downshifted in *T. forsythia ΔwecC* cells as a result of truncated S-layer glycosylation. B. Pro-Q Emerald carbohydrate-stained gel (8%). C. blot of a *T. forsythia* WT whole cell extract after separation by SDS-PAGE (8% gel) probed with *A. aurantia* lectin. Prominent bands (1–6) giving a positive signal in both Coomassie Brilliant Blue R-250 and carbohydrate-stained gels were excised and subjected to glycoproteomics analysis (see Table 2).

**Glycan Profile of O-Glycans from the S-layer Proteins TfsA, TfsB, and TfsBΔwecC**—In-gel reductive β-elimination was employed to release glycans from the protein backbone. Mass analysis was followed by PGC-ESI-MS/MS. For the TfsA and TfsB proteins, three dominant glycan structures were observed with monoisotopic values of 1,621, 1,751, and 1,897 Da (Fig. 2). CID fragmentation analysis of the 1,621-Da glycan unit revealed it to be a hetero-oligomer consisting of eight different sugar residues (Fig. 3). Mass increments for one pentose, one deoxyhexose, three uronic acids (modified or free), one methylhexose, and one reduced hexose in addition to one or so far unknown glycosylation site were identified. To some extent, the oligosaccharides were substituted by one deoxyhexose and, further, with one more deoxyhexose, leading to the above described mass pattern. Additionally, the ESI-MS spectrum showed a minor peak with a mass difference corresponding to a methylhexose (Fig. 2). Interestingly, glycans analyzed from both TfsA and TfsB S-layer proteins showed identical glycan mass profiles, indicating the presence of one uniform glycosylation pattern. The TfsB protein derived from the *ΔwecC* mutant showed a similar glycan pattern but was devoid of two N-acetylhexosaminuronic acid residues and the yet non-described 361-Da sugar (Fig. 2).

**Monosaccharide Composition Analysis**—To investigate the nature of the monosaccharide constituents of the *T. forsythia* S-layer O-glycans, the isolated and HPLC-purified 1,621-Da O-glycan was hydrolyzed with 4 M TFA to its monosaccharide components and analyzed as anthranilic acid derivatives using reverse phase chromatography. The HPLC profile compared with standard sugars clearly identified the presence of a fucose and a xylose residue. By using non-reductive β-elimination for O-glycan release and anthranilic acid labeling, galactose was identified as reducing sugar, linking the S-layer glycan to the protein backbone (data not shown).

High resolution MS/MS measurements (LTQ Orbitrap-Velos instrument) of the intact and borohydride-reduced O-glycan further allowed the determination of the sum formula for each of the constituting monosaccharides. By this method, the presence of one pentose, one deoxyhexose, one deoxyhexose, one methylated hexose, one hexuronic acid, one N-acetylated hexuronic acid, and one N-acetyl-O-methylhexuronic acid was confirmed (supplemental Table S1).

The structures of all sugar residues were further specified by NMR spectroscopy. Here, we identified the methyl-hexose as methyl-galactose and the two N-acetylhexosaminuronic acid residues as N-acetylmannosaminuronic acid and as O-methyl-N-acetylmannosaminuronic acid, respectively. The deoxyhexose that was already observed in the MS/MS fragmentation profile was identified as a digitoxose.

One final sugar residue remained unclear, even after high resolution MS/MS and NMR analysis of the intact O-glycan. No structure of a known sugar fitted to its exact mass and calculated sum formula (*C_{14}H_{25}O_{9}N_{3}*) as determined by high resolution MS. To unravel this unusual component, a nucleotide sugar screening was performed to identify the pool of activated sugars in the bacterial cytosol (supplemental Fig. S1). A plethora of well known sugar precursors, including GDP-Fuc, UDP-Glc, UDP-Gal, TDP-Rha, UDP-HexNAcs, and UDP-GlcA, were detected (37) in addition to an at first inexplicable peak mass at 683.2 Da (**M** + **H**)^+_. CID fragmentation revealed it to be a CMP-activated sugar with exactly the mass found for the yet unidentified O-glycan constituent. Remarkably, this CMP-activated sugar was present in high concentration exceeding that of house-keeping nucleotide sugars, such as UDP-Glc. Because mainly α-keto sugars are known to be activated by CMP (e.g. CMP-N-acetylneuraminic acid, CMP-KDO, CMP-Pse), this was a first hint of the identity of the unknown compound. Further similarities in the fragmentation pattern to those of sialic acids strongly indicated the presence of an α-keto
Acid. DMB labeling of a hydrolysate of the O-glycan followed by reverse phase HPLC analysis led to a large peak in the elution region of sialic acids. The mass of the DMB-labeled sugar fitted to the unknown sugar residue. Despite its mass differing from that of DMB-labeled N-acetylneuraminic acid, the unknown derivative had a similar retention behavior and also a very similar fragmentation pattern in ESI-MS/MS.

Combined NMR analyses of the purified DMB-labeled sugar residue and of the complete oligosaccharide revealed the presence of a non-2-ulosonic acid carrying two substituents on the amino functions in position C5 and C7. A free methyl group in position 9 was identified by its characteristic 1H NMR shift. Position C5 carries an acetimidol group (Am) showing a weak NOE between the CH3 group and the H in position 5. In position 7, an N-glycolyl group (Gc) is bound, which could also be identified and localized by typical 1H NMR shifts and weak NOEs. This substitution pattern of the DMB-labeled sugar is in agreement with the respective ESI-MS/MS results. Further signals present in the 1H NMR spectra of the DMB-labeled sugar residue were studied by diffusion measurements and were identified to belong to an impurity (35, 36). Comparison of all recorded NMR spectroscopic data of the O-glycan with those of the isolated C14H25O9N3 unit showed good concordance. The configuration of the non-2-ulosonic acid has been proven by the few detectable 3JH-H couplings and NOEs and is in better accordance with those of a pseudaminic acid residue than with those of legosaminic acid. The whole C14H25O9N3 unit can hence be considered as Pse5Am7Gc.

Presence of a Mannosaminuronamide, Which Is Hydrolyzed during β-Elimination to Mannosaminuronic Acid—Upon fragmentation of potential glycopeptides during glycosylation site analysis of the T. forsythia S-layer proteins, a mass difference of 1 Da between released and bound O-glycan was encountered. After comparison of MS/MS data, the mass difference was found to be present at the O-Me-ManNAca residue, which was 1 Da larger after reductive β-elimination. Further experiments showed that the strong base treatment led to this mass change and gave a first indication of its nature. As already shown in a previous study (38), mannosaminuronic acids can be found as amide derivates in bacteria. To verify this, a non-reductive β-elimination was performed using ammonia carbonate (29). Although the previously observed mass shift could not be detected in that case, further exposure to sodium hydroxide for several h at 50 °C led to a deamidation reaction. Further exact mass measurement of ammonia carbonate-released glycans proved our assumption of amide hydrolysis due to strong base exposure (data not shown).

**NMR Spectroscopy of O-Glycans**—Knowing the structure of all nine saccharide units, the interglycosidic connections could be determined. For the determination of such structural details, only 1H NMR (Fig. 4, A and B) and homonuclear two-dimensional NMR spectra were applicable, because the small amounts of purified O-glycans did not allow us to record 13C resonances with adequate signal/noise ratio. Based on mass spectrometric and wet chemical analyses, however, COSY and TOCSY spectra enabled the identification of the monosaccharide units. 1H chemical shifts and coupling constants allowed the analysis of anomeric configurations. ROESY spectra provided further information about spatial closeness of anomeric protons, which enabled the determination of the interglycosidic linkages. The S-layer oligosaccharide isolated from T. forsythia WT cells consists of nine monosaccharide units, which form a branched system (Fig. 4C). This system, however, formed two conformers. They were distinguishable in NMR spectra (and liquid chromatography) and, hence, led to two sets of signals in a ~3:1 ratio, which showed exchange spectroscopy cross-peaks in ROESY spectra. Consequently, not all chemical 1H shifts

![Deconvoluted ESI-TOF-MS spectrum of TfsA, TfsB, and TfsBΔwecC O-glycans. Three main structures were observed after reductive β-elimination, representing variations in Fuc and Dig residues. The terminal O-Me-ManNAca residue was found to be generated from its amide derivative during β-elimination (see “Results”). Minor amounts of each structure missing the O-methylation on this mannosaminuronamide residue were also detected. TfsB O-glycans from the wecC mutant lack the three-sugar branch, consisting of ManNAca, ManNAcCONH₂, and the Pse derivative.](image-url)
could be determined, but all data of the indicative positions were identified.

The carbohydrate unit linked to the protein was identified from a non-reduced sample preparation as an α-Galp (1). This galactose unit carries a terminal β-Digp (2') (39) linked to its position 2 and a 1→3-bound β-GlcA (2). The latter one is part of the oligosaccharide backbone and carries an α-Xylp branch (3') linked to its position 3. The next unit in the backbone has been identified as an α-Fucp (3), which is 1→4-linked to the β-GlcA (2). This fucose carries a 1→2-linked α-Galp (4'), which is methylated on its position 4. A β-ManpNAcA (4, 40), the next unit in the backbone, is 1→4-bound to the fucose residue (3). It carries on its position 3 a β-ManpNAcCONH2-Pse5Am7Gc branch was found. In this case, the α1O glycosidic linkage of Gal was found to serine, which is always preceded by an aspartic acid residue.
The oligosaccharide isolated from the *T. forsythia* wecC mutant consisted only of six glycose units. This smaller oligosaccharide did not show distinguishable signal sets of two conformers and, therefore, has a higher degree of freedom between different conformers. It was identified to be a partial structure of the above described S-layer oligosaccharide. In that structure, the ManpNAcA unit (4) and 4-Me-β-ManpNAcCONH₂ unit (5) as well as the PseAm7Gc (5') were missing. The backbone, hence, consists of the 4-Me-α-Galp-(1→2)-α-Fucp-(1→4)-β-GlcpA-(1→3)-α-Galp-(1→O)-Ser/Thr.

**FIGURE 4.** A, 600-MHz $^1$H NMR spectrum of the S-layer oligosaccharide from *T. forsythia*. B, 600-MHz $^1$H NMR spectrum of a glycopeptide from the ΔwecC strain (see also Fig. 3B). Both spectra were recorded in D₂O at 297 K. Signals of indicative protons are marked with their assignment. In A, overlapping groups of signals from both isomers are marked with one asterisk, whereas in the case of separable singles only the signals of the major isomer are indicated with double asterisks. C, structure of the S-layer oligosaccharide of *T. forsythia* as determined by combined MS and NMR analysis.

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TABLE 1

| Unit         | H-1 | H-2 | H-3 | H-4 | H-5 | H-6 | H-7 | H-8 | H-9 | Ac | Me |
|--------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|----|
| α-Galp (1)   | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND | ND |
| β-GlcPa (2)  | 4.46 | 3.37 | 3.70 | ND  | ND  | ND  | ND  | ND  | ND  | ND | ND |
| β-Digp (2')  | 2.18 | 1.68 | 3.85 | 3.06 | 3.64 | 1.21 | 4.15 | 3.60 | 4.15 | 4.01 | 4.15 |
| α-Fucp (3)   | 3.84 | 3.88 | 4.01 | 4.36 | 1.24 | 4.13 | 1.37 | 1.21 | 3.43 |
| α-XyIp (3')  | 4.08 | ND  | ND  | ND  | 1.99 | ND  | ND  | ND  | ND  | ND | ND |
| 4-Me-α-Galp (4') | 5.06 | 3.71 | 3.44 | ND  | ND  | 3.43 |
| 4-Me-β-ManpNACONH$_2$ (5) | 4.73 | 4.18 | 3.84 | ND  | ND  | 2.06 | 3.46 |
| Glycolyl (of 5')$^b$ | 2.95 | 2.78 | 4.15 | 3.82 | 4.23 | 3.98 | 3.66 | 1.14 | 1.91 | 3.48 |

$^a$ Identified from glycopeptide sample listed in the bottom.
$^b$ Identified from DMP-labeled sample of Pse5Am7Gc.

From two proteins (TF2339 and TF1259) glycopeptides were identified and, upon a Pronase digest (described below), glycosylation sites could be assigned. Due to the low abundance of TF0091 and TF1056, it was impossible to detect glycopeptides by applying that strategy. Notably, TF1150 was co-isolated with TF1056 but without a hint for glycosylation. So far, there are no discrete functions associated with the identified proteins.

Table 2 summarizes all identified proteins (including TfsA and TfsB) together with the detected glycosylation sites. Interestingly, all identified glycosylation sites match the D(S/T)(A/P)L/M/T/V three-amino acid motif that was recently described for the general protein O-glycosylation system in *B. fragilis* (3).

To extend the glycosylation motif analysis to a broader range of proteins, a protein pool out of 1 g of *T. forsythia* cell extract was prepared, and a 24-h proteolytic digest using Pronase was generated to yield incomplete digestion. The pool of prepurified glycopeptides was subsequently analyzed by PGC-ESI-MS/MS. A series of small glycopeptides was identified (supplemental Fig. S2), matching the O-glycosylation motifs found in the work of Fletcher et al. (3).

O-Glycans Are Linked via Galactose to Serine/Threonine Residues—The Pronase digest of the S-layer protein extract from the *T. forsythia wecC* mutant was purified by PGC-HPLC and analyzed by NMR, which showed that galactose is 1O-linked to serine and threonine (data not shown). This is in agreement with the finding of galactose as the reducing sugar (see above).

Theoretical Analysis of Protein O-Glycosylation in *T. forsythia*—The gene locus TF2055-TF2049 contains a predicted WecC (TF2055), a predicted UDP-N-acetylglucosamine 2-epimerase (NeuC, TF2054), three predicted glycosyltransferases (TF2053, TF2050, and TF2049), a predicted acetyltransferase (TF2052), and one ORF with yet unassigned function (TF2051).

As shown in our study, deletion of TF2055 causes truncation of S-layer protein glycans by lacking the 809-Da Pse-containing trisaccharide side branch. This implicates that this genomic region unambiguously carries crucial information for proper surface glycan assembly. We compared the ORFs comprising that 6.8-kb locus with sequences from phylogenetically related...
species found and the said genes present in high homology and clustered within very short distances in the respective genomes (Fig. 6). Interestingly, in B. fragilis, homologues are highly clustered within a locus, which was proven to result in a glycosylation defect of surface proteins upon full knock-out (3). T. forsythia also encodes a flippase upstream of that predicted glycosylation locus (Tf2076), which is highly homologous to the annotated flippases found within the clusters of selected organisms. Although not in immediate proximity to the locus, this enzyme might be crucial for catalyzing the transport of the glycan moiety to the periplasmic space.

**DISCUSSION**

A reduction of oral pathogens and their virulence factors is a prerequisite for the maintenance of both general and oral health. Especially those factors that are associated with the bacterial cell envelope and/or exposed to the environment are prime candidates for mediating virulence through their direct involvement in pathogen-host interactions. In this context, cell surface carbohydrates of various pathogenic species as manifested in the forms of LPS, capsules, or glycoprotein glycans are of primary interest, taking also into account their well estab-

**T. forsythia S-layer O-Glycosylation**

| Band | Protein | Mass (observed/calculated) | Unique peptides | Glycopeptides | Glycosylation site |
|------|---------|----------------------------|----------------|--------------|-------------------|
| 1    | TF1259  | >270/240                   | >5             | NITGDTVNFR   | Thr1529         |
| 2    | TF2339  | >270/200                   | >15            | VPPADIVLGFSDATNTVIK | Thr1529, Thr1528, Thr1523 |
| 3    | TfsB/TF2663 | 270/152               | >25            | IHTDASTSFGK  | Ser1523         |
| 4    | TfsA/TF2661-2 | 230/135             | >25            | YIVDTEFR    | Thr1529, Thr1523 |
| 5    | TF1056  | 110/72                     | >5             | FATDSVFR     | Thr1523, Thr1526 |
| 6    | TF0091  | 80/42                      | >5             | LMVDTLPR     | Thr1523, Thr1526 |

**FIGURE 6.** Predicted O-glycosylation gene locus in T. forsythia (A). Clustering of highly homologous genes was observed in different Bacteroides species, including B. fragilis NCTC 9343 (B), B. thetaiotaomicron VPI 5492 (C), B. uniformis ATCC 8492 (D), P. gingivalis ATCC 33277 (E), and P. distasonis ATCC 8503 (F). Note that the O-glycosylation information of F is not encoded at a single locus. Wzx (gray), flippase (found in all species except for P. gingivalis); X (green), UDP-GlcNac 2-epimerase; Y (pink), acetyltransferase; GTi, GTii, and GTiii (blue), glycosyltransferases. E values of <10^-15 were considered.

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that the terminal Pse5Am7Gc residue participates in the bacterium-host cross-talk, albeit the relevance of the modification of the pseudaminic acid would currently remain unclear. This is supported by the fact that members of this class of sialic acid-like sugars have been found in many Gram-negative bacterial species as constituents of important cell surface glycoconjugates, such as LPS (42), capsules (43), pili (44), and flagella (45, 46), all of which are important to pathogenicity, possibly influencing bacterial adhesion, invasion, and immune evasion (47). It is tempting to speculate that the glycans are recognized by lectin-like receptors that may mediate adhesion to and invasion of specific host cells (13). It is interesting to note that, recently, for T. forsythia a novel sialic acid utilization and uptake system has been described (48), leaving open the principal opportunity of specific host cells (13). It is interesting to note that, recently, for T. forsythia a novel sialic acid utilization and uptake system has been described (48), leaving open the principal opportunity of specific host cells (13).

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In the context of biofilm formation, it is interesting to note that besides the T. forsythia S-layer glycoproteins, the two glycoproteins TF1259 and TF2339 that have been characterized in the course of the present study are up-regulated in biofilm formation (50). This is a further indication that the S-layer protein O-glycosylation system is linked with the biofilm life style of T. forsythia.

The finding that several abundant proteins in T. forsythia are modified with the S-layer glycan is fueled by the recent identification of a rich outer membrane glycoproteome in T. forsythia (34). Our data corroborate and extend a recent study by Veith et al. (34), in which all except the TF0091 protein have been already identified as glycoproteins. Notably, there was no information published about the identity of the glycan structure. All of these glycoproteins are antigenic upon probing with an antisera raised against a T. forsythia outer membrane preparation (34). Interestingly, most of the prevalent T. forsythia glycoproteins identified in this study (the two S-layer glycoproteins TfsA (TF2261-2262) and TfsB (TF2663), TF2339, and its paralog TF1259) are located in the outer membrane of the bacterium, whereas the others (TF1056 and TF0091) are predicted lipoproteins. TF1259 and TF2339 exhibit C-terminal sequence similarity to the CTD family of P. gingivalis (51). TF1056 and TF0091 are predicted non-CTD lipoproteins, showing similarity to TonB-dependent receptor-associated proteins, which are generally known to be important for signal transmission to the cytoplasm and activation of target gene transcription (52).

Overall, the presented data indicate that the periodontal pathogen T. forsythia possesses a general protein O-glycosylation pathway that modifies proteins of yet undefined function at multiple sites with a complex oligosaccharide within the (D)(S/T)(A/I/L/M/T/V) amino acid motif. The underlying glycosylation machinery as well as the glycosylation "sequon" seems to be conserved within Bacteroidetes species. This provokes speculation about the presence of comparable glycosylation patterns, at least in the compared species (compare with Fig. 6). The aspects in which protein O-glycosylation is involved in underpinning the pathogenic strategy of T. forsythia and in its interaction with other bacteria from the oral microflora will be investigated in future studies.

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