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The S-layer proteins of *Tannerella forsythia* are secreted via a type IX secretion system that is decoupled from protein O-glycosylation

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SUMMARY

Conserved C-terminal domains (CTD) have been shown to act as a signal for the translocation of certain proteins across the outer membrane of *Bacteroidetes* via a type IX secretion system (T9SS). The genome sequence of the periodontal pathogen *Tannerella forsythia* predicts the presence of the components for a T9SS in conjunction with a suite of CTD proteins. *T. forsythia* is covered with a 2-dimensional crystalline surface (S-) layer composed of the glycosylated CTD proteins TfsA and TfsB. To investigate if T9SS is functional in *T. forsythia*, T9SS-deficient mutants were generated by targeting either TF0955 (putative C-terminal signal peptidase) or TF2327 (PorK ortholog), and the mutants were analyzed with respect to secretion, assembly and glycosylation of the S-layer proteins as well as to proteolytic processing of the CTD and biofilm formation. In either mutant, TfsA and TfsB were incapable of translocation, as evidenced by the absence of the S-layer in transmission electron microscopy of ultrathin-sectioned bacterial cells. Despite entrapped within the periplasm, mass spectrometry analysis revealed that the S-layer proteins were modified with the complete, mature glycan found on the secreted proteins, indicating that protein translocation and glycosylation are two independent processes. Further, the T9SS mutants showed a denser biofilm with less voids compared to the wild-type. This study demonstrates the functionality of T9SS and the requirement of CTD for the outer membrane passage of extracellular proteins in *T. forsythia*, exemplified with the two S-layer proteins. In addition, T9SS protein translocation is decoupled from O-glycan attachment in *T. forsythia*.

Keywords

C-terminal domain; protein secretion; C-terminal signal peptidase; T9SS; S-layer glycosylation

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INTRODUCTION

Protein translocation across the cell envelope of Gram-negative bacteria has been intensively studied for decades and up to now, eight types of protein secretion systems (T1SS-T8SS) have been described (Desvaux *et al.*, 2009). In different species of *Proteobacteria*, these systems are employed to secrete proteins into the extracellular environment (T1SS, T2SS, T5SS), inject them into the cytoplasm of eukaryotic cells (T3SS, T4SS) or bacteria (T6SS), and build up cell surface appendages (T7SS, T8SS) (Desvaux *et al.*, 2009). Perplexingly, very little is known of how proteins are secreted by bacteria belonging to other phyla of Gram-negative prokaryotes (Pugsley, 1993, Desvaux *et al.*, 2009, Tseng *et al.*, 2009). Recently, a novel protein secretion system was identified in the crucial periodontopathogen *Porphyromonas gingivalis*, which is referred to as T9SS (Glew *et al.*, 2012).

T9SS is composed of several unique components essential for translocation of proteins with a conserved C-terminal domain (CTD), comprised of 40-70 amino acid residues, across the Gram-negative outer membrane (OM) (Nguyen *et al.*, 2007, Veith *et al.*, 2009, Sato *et al.*, 2013). In total, 31 cargo proteins of T9SS have been identified in *P. gingivalis*, several of which are important virulence factors, including gingipains (RgpA, RgpB and Kgp), peptidylarginine deiminase PPAD, and surface protein hemagglutinin HagA (Veith *et al.*, 2014). All these proteins possess a typical N-terminal signal peptide and traverse the cytoplasmic membrane into the periplasm using the general secretion (Sec) system. Subsequent translocation of these *P. gingivalis* proteins across the OM is enabled by T9SS. T9SS is associated with the cleavage of the CTD prior to carbohydrate modification of the mature protein, probably with anionic lipopolysaccharide (A-LPS) of *P. gingivalis*, which, subsequently, enables anchoring of the protein to the cell surface (Seers *et al.*, 2006, Chen *et al.*, 2011, Shoji *et al.*, 2011, Sato *et al.*, 2013). The modification of *P. gingivalis* CTD proteins with A-LPS was inferred from reactivity with a Man α 1-2Man α 1-phosphate A-LPS antibody, however, the mode of attachment is not yet fully understood (Saiki and Konishi, 2014), as is also the interplay of outer membrane translocation and posttranslational modification of CTD proteins in general. Recent studies revealed that CTD cleavage is catalyzed by a C-terminal signal peptidase PG0022 (PorU), an essential component of the T9SS secretion machinery, the activity of which is required for cell surface display of certain proteins or their release into the extracellular environment. Inactivation of PG0022 as well as any other T9SS component resulted in accumulation of the otherwise secreted proteins in unprocessed form in the periplasm of *P. gingivalis* (Glew *et al.*, 2012). In total, T9SS has been shown to involve 13 components. PorT is a predicted integral OM protein with a β -barrel fold possibly involved in the formation of a channel for protein translocation (Nguyen *et al.*, 2007). To the remaining OM components - PorK, PorL, PorM, PorN, PorP, PorQ, PorV and PorW - no specific functions could be assigned, so far (Sato, 2011). LptO of the *P. gingivalis* T9SS conceivably coordinates the secretion of A-LPS and CTD proteins as well as LPS deacylation (Ishiguro *et al.*, 2009, Chen *et al.*, 2011, Saiki and Konishi, 2014). Sov was found in the OM fraction of *P. gingivalis* and shown to be involved in the secretion of gingipains (Saiki and Konishi, 2014), with PorX and PorY being regulatory proteins additionally involved in secretion (Sato *et al.*, 2010).

Bioinformatics analysis of sequenced bacterial genomes revealed the presence of genes encoding the T9SS machinery as well as CTD-proteins exclusively among members of the *Bacteroidetes* phylum, with a total of 663 such proteins predicted in 21 fully sequenced species (Nguyen *et al.*, 2007, Veith *et al.*, 2013). These also include *Tannerella forsythia* and *Prevotella intermedia*, which together with *P. gingivalis* are recognized as important pathogens implicated in development and progression of periodontal diseases (Socransky and Haffajee, 1992, Socransky *et al.*, 1998, Holt and Ebersole, 2005). Similar to *P. gingivalis*, these periodontopathogens secrete large amounts of CTD proteins, many of which have been identified as virulence factors (Veith *et al.*, 2009, Sharma, 2010, Dashper *et al.*, 2011). In the case of *T. forsythia*, the major proteins carrying CTD are BspA and the S-layer proteins TfsA and TfsB (Lee *et al.*, 2006). BspA belongs to the leucine-rich repeat (LRR) and bacterial immunoglobulin-like protein families. BspA is associated with the cell surface of *T. forsythia* and functions as an important modulator of host innate immune responses through activation of TLR2 in cooperation with TLR1 (Onishi *et al.*, 2008). The S-layer proteins TfsA (TF2661-2) and TfsB (TF2663) are intercalated on the bacterial cell surface forming a so far unique 2-dimensional crystalline monolayer (S-layer) of 22 nm thickness (Sekot *et al.*, 2012). The *T. forsythia* S-layer was shown to mediate adhesion to and invasion of carcinoma cells of the mouth (Sabet *et al.*, 2003) and to delay the bacterium's recognition by the innate immune system of the host, at least at the early stage of infection (Sekot *et al.*, 2011). The S-layer proteins are targeted by the general protein *O*-glycosylation system of *T. forsythia* for display of multiple copies of a complex oligosaccharide (Posch *et al.*, 2011). A trisaccharide branch of that oligosaccharide, composed of two *N*-acetylmannosaminuronic acid residues and one modified nonulosonic acid, was shown to act in modulating dendritic cell effector functions to suppress T-helper 17 responses, thereby ensuring the persistence of the pathogen in the host (Settem *et al.*, 2013).

The identification of proteins which are equipped with typical *Bacteroidetes* CTDs together with the presence of a complete set of genes predicted to encode orthologs of the T9SS apparatus of *P. gingivalis* strongly suggests that *T. forsythia* employs a T9SS. To verify this hypothesis, we have deleted either TF2327 or TF0955 in the *T. forsythia* genome, which are orthologs of PorK and PG0022, respectively (Glew *et al.*, 2012), essential for secretion of proteins via T9SS in *P. gingivalis*. The phenotype of isogenic mutants was analyzed with respect to secretion, glycosylation, proteolytic processing and assembly of the S-layer as well as biofilm formation. Inactivation of TF2327 and TF0955 blocked secretion of the S-layer proteins TfsA and TfsB, cleavage of CTD, and assembly of the composite S-layer on the bacterial cell surface. On the other hand, the retained S-layer proteins were modified with the mature *O*-glycan, indicative of protein glycosylation and protein secretion proceeding independently of each other.

METHODS

Bacterial strains and growth conditions

Tannerella forsythia ATCC 43037 (American Type Culture Collection, USA) and defined T9SS mutants (see below) were grown in 37 g L⁻¹ of Brain-Heart-Infusion (BHI) liquid

media (Oxoid, UK), containing 5 g L⁻¹ yeast extract (Oxoid), 0.5 g L⁻¹ L-cysteine (Sigma, Austria), 2.5 µg mL⁻¹ hemin (Sigma), 2.0 µg mL⁻¹ menadione (Sigma), 10 µg mL⁻¹ N-acetylmuramic acid (Carbosynth, UK) and 5% (v/v) horse serum (Life Technologies, Austria), under anaerobic conditions at 37°C for 4-7 days. For cultivation of *T. forsythia* wild-type and mutants on BHI agar plates (0.8% w/v), the amounts of L-cysteine, hemin, and N-acetylmuramic acid were doubled and plates were incubated under anaerobic conditions in an anaerobic jar (AnaeroJar; Oxoid) at 37°C. Media were supplemented with gentamycin and erythromycin at a concentration of 200 µg mL⁻¹ and 5 µg mL⁻¹, respectively, when appropriate.

Escherichia coli strains were grown under standard conditions in Luria-Bertani (LB) medium supplemented with 100 µg mL⁻¹ ampicillin, when appropriate. *P. gingivalis* W83 is used as a reference strain for comparison with predicted components of the T9SS in *T. forsythia* ATCC 43037.

DNA isolation and PCR amplification

Genomic DNA was isolated from 2 mL of bacterial suspension as published previously (Cheng and Jiang, 2006). Plasmid DNA was purified using the GeneJET Plasmid MiniPrep Kit (Thermo Scientific, Austria). PCR fragments were amplified either by Phusion High-Fidelity DNA Polymerase (Thermo Scientific) or by Herculase II Phusion Polymerase (Agilent Technologies, Germany) according to the protocols provided by manufacturers. PCR fragments were purified with the GeneJET PCR Purification Kit (Thermo Scientific). Oligonucleotide primers (Life Technologies) used in the course of this study are listed in Table 1.

Construction of T9SS deficient mutants (TF 0955, TF 2327)

Gene knock-outs were based on the homologous recombination of a gene knock-out cassette deleting or disrupting the selected gene of the *T. forsythia* T9SS. Positive clones were selected based on transferred erythromycin resistance.

The gene knock-out cassette for constructing the TF 0955 mutant consisted of a 2,188-bp erythromycin resistance gene *ermF-ermAM* (primer pair 415/416) flanked by homologous up- and down-stream regions (Fig. 1A). Genomic DNA from *T. forsythia* *wecC* (obtained from A. Sharma, State University of New York at Buffalo, USA) was used as a template. The 1,035-bp up-stream and 1,027-bp down-stream homology regions were amplified from genomic DNA of *T. forsythia* wild-type cells using the primer pairs 413/414 and 417/418, respectively. The three fragments were joined by overlap extension (OE-) PCR and sub-cloned into the blunt-end cloning vector pJET1.2 (Thermo Scientific) resulting in pJET1.2/TF0955ko. Approximately 5 µg of the circular gene knock-out construct were transferred by electroporation into 100 µL of *T. forsythia* culture grown to the early stationary phase. Cells were regenerated in BHI medium for 24 h before plating on BHI agar plates containing erythromycin as selection marker. Single colonies were picked and used for inoculation of liquid BHI medium. Once bacterial growth was visible, genomic DNA was isolated to confirm the integration of the knock-out cassette via PCR amplification using the primer pair 426/429.

The construction of the TF 2327 mutant was done essentially the same way as described for TF 0955 (Fig. 1B). Genomic integration of the gene knock-out cassette was verified using the primer pair 448/449.

Growth of the T9SS mutants was measured over a period of 5 days and the obtained growth curves were compared to that of *T. forsythia* wild-type.

SDS-PAGE and Western immunoblotting

Crude cell extracts of *T. forsythia* wild-type and mutant cells (TF 0955, TF 2327) were run on 10% SDS-PAGE gels in a Mini Protean electrophoresis apparatus (Bio-Rad, Austria) according to a standard protocol and visualized with colloidal Coomassie Brilliant Blue R-250 (Laemmli, 1970).

For Western immunoblotting, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a Mini Trans-Blot Cell (Bio-Rad). Polyclonal rabbit antiserum raised against the recombinant S-layer proteins TfsA (α -TfsA) and TfsB (α -TfsB) was used in combination with goat anti-rabbit secondary antibody labeled with IRDye 800CW (LI-COR Biosciences, USA). Visualization of the S-layer proteins was done at 800 nm using the Odyssey Infrared Imaging System (LI-COR Biosciences). Staining of carbohydrates was done with the periodic acid-Schiff (PAS) reagent following a standard protocol (Doerner and White, 1990). For semi-quantification of S-layer proteins present in *T. forsythia* wild-type and TF 0955 and TF 2327 mutant cells, equal amounts of total cell protein were loaded on the SDS-PA gels in two different dilutions, each, followed by Western immunoblotting. The integrated intensity of the detected bands was determined using the LICOR Odyssey Application software 3.0.21.

S-layer O-glycan preparation and LC ESI-MS/MS

S-layer O-glycans were isolated from *T. forsythia* mutant cells following a published protocol (Posch *et al.*, 2011). Briefly, S-layer glycoproteins were excised from Coomassie Brilliant Blue R-250-stained SDS-PA gels and O-glycans were released from the protein backbone by in-gel reductive β -elimination followed by removal of excess of salt using a 25-mg HyperSep Hypercarb SPE cartridge (Thermo Scientific). Borohydride-reduced O-glycans were analyzed by PGC-ESI-MS/MS as described recently (Hypercarb, 0.32×150 mm, particle size 5 μ m) (Stadlmann *et al.*, 2008). Detection was done by an ESI-Q-TOF Global Ultima from Micromass (Waters, USA). Data were evaluated using MassLynx 4.0 software. MS/MS experiments were performed at 30% collision energy using CID with argon gas. Slices from the SDS-polyacrylamide gel without sample application were treated in the same way as described for the O-glycan samples and acquired MS spectra were used as a negative control.

Proteomics analysis

S-layer proteins as present in *T. forsythia* wild-type and mutant cells were identified as follows. After in-gel tryptic digest of the respective high molecular-mass bands on SDS-PAGE gels, generated peptides were extracted and subjected to reverse phase ESI-MS/MS peptide mapping using a Bruker IonTrap AmaZon speed ETD and a Bruker Maxis4G.

Protein database search of tandem MS data was done using the ProteinScape 3 software tool (Bruker-Daltonik, Germany) with Mascot 2.3.02 algorithm for peptide identification or the X!tandem algorithm (The Global Proteome Machine Organization).

Scanning electron microscopy

T. forsythia cells with an initial optical density of $OD_{600} = 0.07$ were grown anaerobically on sterile glass slides at 37°C for 2-4 days. After fixation with 2.5% glutaraldehyde in phosphate buffered saline (PBS) at 4°C for 2-4 h, the cells were dehydrated with increasing concentrations of ethanol (20% to 100% ethanol in increments of 10%) for 7 min, each. Subsequently, the cells were treated with 33% and 66% bis(trimethylsilyl)amine (HMDS) in methanol and, eventually, with 100% HMDS for 30 min, each. Glass slides were air-dried and mounted onto 12.5-mm aluminum stubs covered with double-sided carbon tabs (Gröpl, Austria). At one side of the glass slide, one drop of silver solution was applied prior to sputter-coating with gold (EM SDC005, Leica). Biofilms were imaged using an Inspect S50 scanning electron microscope (FEI, The Netherlands).

Ultra-thin sectioning and transmission electron microscopy

Ultrathin-sectioning of *T. forsythia* wild-type and TF 0955 and TF 2327 cells was carried out as described previously (Messner *et al.*, 1984). Briefly, the room-temperature processing included fixation of samples with 2.5% (w/v) paraformaldehyde/ 2.5% (w/v) glutaraldehyde/ 0.5% (w/v) tannin in cacodylate buffer and osmium tetroxide fixation without ruthenium red. Dehydration was performed in an increasing alcohol series before samples were embedded in Epon resin (Gröpl, Austria).

Ultrathin-sectioned samples were investigated in a Tecnai G² 20 Twin transmission electron microscope (TEM; FEI), operating at 80 keV. Pictures were taken with an FEI Eagle 4k CCD camera (4,096 × 4,096 pixels) and images were processed using software developed in house based on Fourier domain techniques (Amos *et al.*, 1982, Crowther *et al.*, 1996).

Confocal laser scanning microscopy

T. forsythia cells were grown under standard conditions as described above until an OD_{600} of 0.5 was reached (~2 days). Subsequently, the bacterial culture was inoculated into fresh growth medium. After 4 days of incubation, 3 ml of that culture were transferred into micro dishes (μ -dishes, 35 mm height; Ibidi, Germany) and biofilm cells were grown anaerobically at 37°C for 3 days. The liquid supernatant containing planktonic cells was removed and the biofilm was stained with Hoechst 33258 (Sigma) at a final concentration of 10 $\mu\text{g mL}^{-1}$ in PBS (stock solution, 10 mg mL^{-1} in distilled water) for 35 min in the dark. The supernatant was discarded and the biofilm was washed with PBS. Images were taken using an inverted TCS-SP5 confocal microscope (Leica Microsystems, Germany) and images were processed with the software ImageJ (Schneider *et al.*, 2012).

RESULTS

Construction of T9SS deficient *T. forsythia*

The Gram-negative periodontopathogen *T. forsythia* encodes a full set of proteins that are orthologs of the *P. gingivalis* components constituting the recently described type IX secretory machinery (T9SS). To investigate, if T9SS is functional in *T. forsythia*, we constructed deletion mutants affecting either TF0955 or TF2327, which are predicted orthologs of the C-terminal signal peptidase (sortase; PG0022) and the PorK component (PG1676), both of which have been shown to be essential for CTD-protein secretion via T9SS in *P. gingivalis* (Chen *et al.*, 2011). The *T. forsythia* T9SS orthologs were identified using the Basic Local Alignment Search Tool BLAST (at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). They are scattered across the bacterial genome and include, besides the genes targeted in the course of this study, TF0188 (PorT), TF0671 (PorY), TF1742 (PorW), TF1930 (PorX), TF1959 (PorQ), TF2326 (PorP), TF2328 (PorL), TF2329 (PorM), TF2330 (PorN), TF2852 (LptO) and TF2901 (Sov) (McBride and Zhu, 2013). In the case of the

TF0955 mutant, no polar effects were expected, since the downstream gene TF0957 is separated by 106 non-coding nucleotides (Fig. 1A). In contrast, the other targeted gene, TF2327, is predicted to constitute an operon (BioCyc) (Caspi *et al.*, 2012) together with the immediate downstream genes TF2328, TF2329 and TF2330, implicating that polar effects might cause the inactivation of the said genes in the mutant (Fig. 1B). However, since these latter genes code for predicted components of the T9SS, even their inactivation would not have adversely affected the usability of the TF2327 mutant for the purpose of this study. For both T9SS mutants, genomic integration of the *ermF-AM* cassette was confirmed by PCR, with the amplicons obtained for TF0955 (primer pair 426/429) and TF2327 (primer pair 448/449) conforming with the calculated size of 7,678 bp (Fig. 1C, lane 3) and 4,277 bp (Fig. 1C, lane 5), respectively. As a control, the same primer pairs were used with wild-type genomic DNA as a template (Fig. 1C, lanes 2 and 4).

The *T. forsythia* T9SS mutants had similar growth characteristics, revealing a slightly decreased growth rate in comparison to the wild-type (data not shown).

S-layer protein expression in T9SS mutants

Among several proteins of *T. forsythia* bearing a conserved CTD and, therefore, predicted to be translocated across the OM via a T9SS comparable to that established for *P. gingivalis* (Glew *et al.*, 2012), are the S-layer proteins TfsA and TfsB. Both are equipped with a typical CTD containing a possible C-terminal signal peptidase cleavage site. The S-layer proteins are modified with *O*-glycans of known structure (Posch *et al.*, 2011) and assemble in equimolar ratio into a 2-dimensional crystalline surface layer completely covering *T. forsythia* cells throughout the whole life-cycle. This translates into a high cellular protein biosynthesis effort being devoted to S-layer protein biosynthesis (Sleytr *et al.*, 1993). Thus, these abundant cell surface proteins are ideal candidates to investigate, if indeed a T9SS is employed in *T. forsythia*. SDS-PAGE analysis of crude cell extracts of *T. forsythia* wild-type shows the two prominent high molecular-mass S-layer protein bands with apparent molecular masses above 250 kDa (Fig. 2A, lane 2). These correspond to TfsA with ~230 kDa and TfsB with ~270 kDa (Posch *et al.*, 2011), the respective protein portions of which

have a calculated molecular mass of 135 kDa and 152 kDa, respectively. The observed apparent molecular masses are in agreement with the migration behavior of the mature and glycosylated forms of TfsA and TfsB on SDS-PAGE gels as reported previously (Posch *et al.*, 2011). Conforming with the glycoprotein nature of TfsA and TfsB, both bands strongly stained for carbohydrates with the PAS reagent (Fig. 2B, lane 2). In both the TF0955 (Fig. 2B, lane 3) and the TF2327 (Fig. 2B, lane 4) mutants, also two prominent protein bands that were carbohydrate-positive, were detected; however, these bands were clearly downshifted compared to the wild-type (Fig. 2AB, lanes 3 and 4). For *T. forsythia* wild-type as well as the T9SS mutants, the corresponding bands were unambiguously identified as S-layer proteins by Western-immunoblotting using polyclonal α -TfsA (Fig. 2C) and α -TfsB antibodies (Fig. 2D), respectively. Note that the α -TfsA antibody recognized a second protein band that co-migrates with the TfsA wild-type protein (Fig. C). In addition, proteomics analysis revealed tryptic peptides originating from either of the S-layer proteins (TfsA and TfsB; see Table 2 below).

Semi-quantification of the S-layer glycoprotein bands as visualized on Western-immunoblots upon separation of equal amounts of cellular protein confirmed that TfsA and TfsB are expressed in equal amounts in the *T. forsythia* wild-type as well as in the TF0955 and the TF2327 mutants. However, the integrated intensity of either S-layer protein expressed in the mutant cells was only about one third compared to that of the wild-type cells, possibly due of feed-back inhibition, since the protein secretion was blocked. Integrated intensity values were 34.5 and 35.5 for TfsB from TF0955 and TF2327, respectively, opposed to 94.7 from wild-type, and 21.3 for TfsA and 22.0 from TF0955 and TF2327, respectively, as opposed to 61.2 from wild-type.

C-terminal domains are not cleaved off S-layer proteins in T9SS deficient strains

The identification of glycosylated forms of the S-layer proteins TfsA and TfsB in the TF0955 and TF2327 mutants - albeit with decreased apparent molecular mass as compared to the *T. forsythia* wild-type glycoproteins (Fig. 2B) - contrasted the situation known from T9SS deficient mutants of *P. gingivalis*, where CTD proteins were shown to be retained in the periplasm in non-glycosylated form (Chen *et al.*, 2011). To investigate this finding in more detail, the S-layer proteins from the wild-type and the T9SS deficient mutants were analyzed for the presence of the CTD. For this purpose, the S-layer glycoprotein bands of *T. forsythia* wild-type and TF0955 and TF2327 mutants as revealed upon SDS-PAGE (marked with an asterisk in Fig. 2A) were digested with trypsin and subjected to MS peptide mapping (Table 2). This clearly confirmed the identity of the investigated bands as TfsA and TfsB S-layer proteins; sequence coverage for TfsA and TfsB, respectively, was 51% and 48% for *T. forsythia* wild-type, 20% and 32% for TF2327, and 16% and 36% for TF0955. For the mutants, several peptides mapping to the CTD were detected both for TfsA and TfsB, whereas no single peptide from this region was derived from the S-layer proteins of *T. forsythia* wild-type (Table 2). This data implicates that no cleavage of the CTD occurs from the periplasmically localized S-layer proteins in the *T. forsythia* secretion mutants, whereas the wild-type proteins are C-terminally fully processed.

T9SS is not coupled with O-glycan assembly and glycan transfer onto proteins

According to our data, in either T9SS deficient *T. forsythia* mutant, essentially the same TfsA and TfsB glycoproteins were produced. These were already N-terminally processed for Sec-mediated translocation across the CM as evident from the MS data obtained in the course of the peptide mapping (data not shown). Inactivation of either the signal peptidase (TF0955) or PorK (TF2327) prevented CTD-cleavage causing entrapment of the S-layer proteins in the periplasm. While glycosylation of TfsA and TfsB from the mutants is in line with the general knowledge of protein glycosylation in Gram-negative bacteria to be elaborated in the periplasm (Wacker *et al.*, 2002), a clear downshift in molecular mass of these glycoproteins in comparison to the wild-type glycoproteins (Fig. 2B) was initially assumed to be due to incomplete protein glycosylation. To shed more light on glycosylation status of the S-layer proteins from the *T. forsythia* secretion mutants, their glycans were analyzed in detail. To this end, in-gel reductive β -elimination was applied to release O-glycans from the protein backbones of TfsA and TfsB from TF2327, TF0955 as well as from the wild-type that served as a reference. Mass analyses presented the wild-type O-glycan with the highest mass peak at 1898, which is in agreement with the known structure of the *T. forsythia* dekasaccharide (Fig. 3) (Posch *et al.*, 2011). Additional glycan fragments at mass to charge ratios of 1752 and 1622, corresponding to the O-glycan devoid of a branching fucose and a digitoxose residue, respectively, were also resolved; this structure are due to previously described O-glycan microheterogeneity (Coyne *et al.*, 2013). Significantly, S-layer glycans from the T9SS deficient *T. forsythia* mutants showed the same pattern and masses on deconvoluted ESI-TOF-MS spectra as the wild-type O-glycan, proving the complete glycan to be attached to the S-layer proteins. In negative controls, performed with SDS-PA gel slices without sample application and Coomassie Brilliant Blue R-250 staining, no glycans were detected. Concluding, full assembly of the *T. forsythia* O-glycan and its transfer onto the proteins occur prior to T9SS-mediated translocation across the OM and, therefore CTD protein glycosylation and CTD protein secretion are independent processes. With respect to the still missing explanation of the downshifted S-layer glycoprotein bands from the TF2327 and TF0955 mutants on SDS-PA gels (Fig. 2), it is tempting to speculate that either less glycosylation sites might be occupied in the mutant S-layer proteins or that the mature, secreted S-layer glycoproteins are complexed with a still to be identified compound, possibly involved in anchoring of the S-layer to the cell surface (Posch *et al.*, 2013).

C-terminal domain proteins of *T. forsythia* are translocated across the T9SS

The intercalating high molecular-mass glycoproteins TfsA and TfsB self-assemble into a 2-dimensional crystalline monolayer on the cell surface of *T. forsythia* (Sekot *et al.*, 2012). The square S-layer lattice has a lattice spacing of approximately 10 nm $\times$ 10 nm and a thickness of about 22 nm (Sekot *et al.*, 2012). To visualize that inactivation of T9SS blocks TfsA and TfsB secretion and, consequently, gives rise to an S-layer deficient cell envelope architecture, ultra-thin cross-sections of whole cell preparations from the TF2327 and TF0955 mutants were investigated in comparison to *T. forsythia* wild-type cells (Fig. 4). Transmission electron micrographs clearly confirmed the absence of the S-layer on either of

the T9SS deficient mutant, while the wild-type cells are fully covered with a serrated S-layer typical of *T. forsythia* (Sekot *et al.*, 2012).

Phenotype of biofilm is largely affected in T9SS deficient *T. forsythia*

Following up the previous finding of the *T. forsythia* S-layer playing a role in biofilm formation (Honma *et al.*, 2007), the capacity of the T9SS deficient mutants to form a monospecies biofilm as well as the architecture of the biofilm was investigated. Scanning electron microscopy of 3 day-old biofilms grown on a glass surface revealed that the *T. forsythia* wild-type strain formed a meshwork of well-connected individual rod-shaped cells of comparable size. The biofilm contained many voids and tunnel-like structures which are known to enable nutrition delivery to the basis of the biofilm (Fig. 5A). The biofilm of the TF0955 and TF2327 mutant, in contrast, revealed an entirely different architecture. The rods were elongated and stuck together, forming fibers and bundles of cells. The matrix was denser compared to the wild-type biofilm with less abundant voids and tunnel-like structures. Individual cells appeared to have a smoother surface indicating the lack of several proteins at the outermost layer of the bacterium (Fig. 5A).

The formation of a much denser biofilm by the mutants compared to the wild-type was confirmed by CLSM analysis (Fig. 5B). Although the overall height of the biofilm was comparable between all analyzed strains, the mutant cells were more densely packed and often stuck together forming lateral string-like structures, most clearly visible in the TF2327 mutant biofilm.

DISCUSSION

Putative CTD proteins have been found in 87 organisms so far, all belonging to the *Bacteroidetes* phylum, and their presence coincides with the occurrence of orthologous genes of essential components encoding the T9SS machinery. Of note, not all species of this phylum have the genetic information for a T9SS and for CTD-bearing proteins, such as, for instance, the human intestinal symbionts *Bacteroides fragilis* and *Bacteroides thetaiotamicron*. In *T. forsythia*, 37 proteins are predicted to possess a CTD, out of which 19 have been verified experimentally (Veith *et al.*, 2013). Here, we have shown for the first time that secretion of these proteins indeed occurs via T9SS.

To this end, we have studied the effect of the inactivation of two *T. forsythia* orthologs of the genes *porK* (TF2327) and *porU* (TF0955) that are essential for T9SS function in *P. gingivalis*, on the secretion of the two CTD-bearing S-layer proteins TfsA and TfsB and their posttranslational modification (glycosylation (Posch *et al.*, 2011)) as well as on the assembly of the 2-dimensional crystalline S-layer on the bacterial cell surface.

In analogy to the T9SS of *P. gingivalis*, TF0955 would be located at the cell surface and is supposed to function as the C-terminal signal peptidase required for cleaving off the CTD from proteins deemed for secretion (Glew *et al.*, 2012). TF2327 is a predicted integral OM protein of unknown function with a β -barrel structure. Despite differences in subcellular location and function of the targeted proteins, both mutants - TF0955 and TF2327 - showed the same phenotype. At the cellular level, the most profound difference in

comparison to the *T. forsythia* wild-type was the loss of the S-layer (Fig. 2). A similar effect was seen for a *P. gingivalis* *lptO* (PG0027) knock-out mutant missing its electron-dense surface layer (EDSL) (Saiki and Konishi, 2014). However, this effect is most probably not attributable to the disruption of *LptO* alone (Glew *et al.*, 2012), but must be associated with inactivation of any other essential component of T9SS. Apart from the lack of the S-layer, T9SS mutants of *T. forsythia* show abnormalities with respect to the shape of individual cells and the architecture of monospecies biofilms as shown by SEM and CLSM analysis (Fig. 5). These abnormalities are most likely a combined effect of the blocked secretion of CTD proteins in the *T. forsythia* TF0955 and TF2327 mutants.

Inactivation of T9SS in *P. gingivalis* resulted in the retention of otherwise secreted proteins in the periplasm in non-glycosylated and unprocessed form still bearing the CTD (Chen *et al.*, 2011). Consequently, also the TfsA and TfsB S-layer proteins from the *T. forsythia* secretion mutants were found with intact CTDs (Table 2). In contrast, the CTDs from the S-layer proteins of the wild-type bacterium are cleaved at the KSPL#ANAE (TfsA) and the EDPV#ANEI (TfsB) motifs (Veith *et al.*, 2013) during or immediately after translocation across the OM. On the other hand, no difference was found in *O*-glycosylation of S-layer proteins derived from the wild-type and T9SS-deficient mutants. Clearly, fully assembled *O*-glycans were attached to both glycoproteins regardless of T9SS functionality, even with glycan microheterogeneity equaling that of the wild-type S-layer glycoproteins (Fig. 3). This finding indicates that glycan assembly and transfer of the glycan to multiple three amino acid motifs D(S/T)(A/I/L/M/T/V) within the CTD S-layer proteins is completed while proteins reside in the periplasm, which is in accordance with the general *O*-glycosylation pathway in *Bacteroidetes* (Coyne *et al.*, 2013).

The same *O*-glycosylation of the S-layer proteins together with the lack of their C-terminal processing indicates that another type of posttranslational modification might be responsible for the accounted molecular mass difference of about 50-kDa between the S-layer glycoproteins derived from the *T. forsythia* wild-type strain and the T9SS-deficient mutants (Fig. 2). In analogy to *P. gingivalis*, we hypothesize that in *T. forsythia*, proteolysis of CTD is coupled with or succeeded by the attachment of a glycan moiety to the protein in a manner different from *O*-glycosylation. Such a glycan would have escaped the MS analysis performed in the course of the present study due to the protocol used for sample preparation and, consequently, could have remained undetected. It is conceivable that, as in *P. gingivalis*, the attached glycan moiety could be a variant of the LPS. This might be involved in anchoring of the TfsA and TfsB S-layer glycoproteins to the OM of *T. forsythia*. This is in line with our assumption of the rough-type LPS of *T. forsythia* being ideally suited for the mediation of protein cell surface attachment because of its defined length and high abundance on the cell envelope (Posch *et al.*, 2013). This hypothesis is currently under investigation in our laboratories.

Yet we have provided the first evidence of the presence of a functional T9SS in *T. forsythia* and confirm the importance of CTDs for successful secretion of two abundant *T. forsythia* proteins. The S-layer glycoproteins TfsA and TfsB were prevented from crossing the OM in T9SS deficient mutants (TF 0955, TF 2327), which, consequently, also lacked the S-layer.

Further, we could show that protein translocation and *O*-glycosylation are decoupled from each other.

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Figure 1.

Strategy for TF0955 gene disruption and TF2327 gene deletion at the *T. forsythia* wild-type locus and confirmation by PCR (not drawn to scale). (A) Genomic organization of the TF0955 gene showing the up- and down-stream homology regions used for homologous recombination and insertion of the selectable *ermF-AM* cassette disrupting TF0955. Primers (compare with Table 1) are indicated with arrows. (B) TF2327 gene deletion and insertion of the selectable *ermF-AM* cassette is shown in the context of its genomic region. The area of homologous recombination is indicated, the position of the primers are marked with arrows. (C) Agarose gel electrophoresis of PCR products from genomic DNA of *T. forsythia* wild-type and mutants with integrated *ermF-AM* cassette. GeneRuler 1 kb Plus DNA Ladder, Thermo Scientific (lane 1), *T. forsythia* wild-type (primer pair 426/ 429) yielding a 5,490-bp PCR-product (lane 2) and with integrated *ermF-AM* cassette, yielding a 7,678-bp PCR-product (lane 3); *T. forsythia* wild-type (primer pair 448/449) yielding a 3,529-bp PCR-product (lane 4) and with integrated *ermF-AM* cassette, yielding a 4,277-bp PCR product (lane 5).

Figure 2.

(A) Coomassie Brilliant Blue R-250 stained SDS gel (10%) of crude cell extracts from *T. forsythia* wild type (lane 2), *TF2327* (lane 3) and *TF0955* (lane 4). The prominent S-layer proteins (identity confirmed by proteomics analyses) are significantly downshifted in both T9SS deficient mutants, which is also evident from the PAS-staining (B) and from Western-immunoblots probing for TfsA (C) and TfsB (D). Note that the α -TfsA antibody recognizes a second protein band that co-migrates with the TfsA wild-type protein. PageRuler Plus PreStained Protein Ladder (lane 1). Asterisks indicate S-layer glycoprotein bands that were subsequently processed for MS analysis (Table 2, Fig. 3).

Figure 3.
(A) Deconvoluted ESI-TOF-MS spectrum of TfsA and TfsB *O*-glycans in *T. forsythia* wild-type and mutant strains. Identical spectra were recorded for the *O*-glycan derived from the *TF0955* and *TF2327* mutants and the wild-type. (B) For clarification, the complete S-layer glycan structure is shown. Gal, galactose; GlcA, glucuronic acid; Fuc, fucose; ManNAcA, N-acetyl-mannosaminuronic acid; Dig, digitoxose; Xyl, xylose; Pse5Am7Gc, 5-acetimidyl-7-glycolyl-pseudaminic acid; Ser, serine; Thr, threonine.

Figure 4.

TEM micrographs showing ultra-thin cross-sectioned cells of *T. forsythia* and T9SS mutants. *T. forsythia* wild-type displays a serrated S-layer, whereas no S-layer is present on *T. forsythia* TF0955 and TF2327 cells. SL, S-layer; OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane; C, cytoplasm.

Figure 5.

(A) SEM micrographs showing altered biofilm and cell morphology in T9SS deficient mutants in comparison to the wild-type. Biofilm was grown anaerobically on glass for two days; (B) CLSM micrographs of *T. forsythia* wild-type and mutant biofilms. Mutant biofilms revealed denser growth and a height comparable to that of the wild-type biofilm. Size bar, 20 μm .

Table 1

Oligonucleotides used in this study

#	Sequence (5' → 3')
413	TCCACGGAGTAATTCCTCAGAGCGATTG
414	GAAGCTATGGGGGTACCTCCCCGGGAGTTGATTTTCCAAGTCCGTAGGCCGGGGTC
415 ^a	CCCGGGGGAGGTACCCCCGATAGCTTC
416 ^a	CCCGGGGGCTAGAGGATCCCCGAAGC
417	GCTTCGGGGATCCTCTAGCCCCGGGCTGCTTTATTGCCGATGATGGAAGCAGCAG
418	AGCCGTTTCGCTTCAAGAGCATTCTGCTCGTC
426	GTGTCGGACGCCTTGGGGCTGCTCTG
429	CAGACATTATCAGGCCTTTC
436	TAGCTTCTGCTTGGGCGCAGGTTGAC
437	GAAGCTATGGGGGTACCTCCCCGGGCTTCCTGATTGTCTATTGTCTGTTCTTATC
438	GCTTCGGGGATCCTCTAGCCCCGGGACTCCAACGACAACAATACATCAG
439	GCAGCAGACGTCCGTAAACCTGATTC
448	CTTTCGAATGACGATCAAACGATTCTG
449	GAGGCTGCTGAGGCGGATACATC

Oligonucleotides used for overlap extension PCR are written in italics

^a(Honma *et al.*, 2007)

Tryptic peptides derived from the C-terminus of TfsA and TfsB as detected in *T. forsythia* wild-type and T9SS mutants. The CTD predicted for TfsA and TfsB according to Veith and coworkers (Veith *et al.*, 2013) is typed in boldface. Peptides derived from the S-layer proteins of *T. forsythia* wild-type are written in red, those derived exclusively from the TfsA and TfsB proteins of the T9SS deficient mutants are written in green.

Table 2

Protein	Unique peptides	log(e) ^a	Score ^b	Sequence ^c
TfsA				
1083-SRGAYTDWIAEAEITWNVKMTKKSPLANAEASVTDVKVYAGAGEVMIRNASGKKVTVSNILGQTIKVVLTSDNQTIAPQGVVVVAIEGENAVKAVVK-1179				
TfsA wild-type	9	-2.8		vist ¹⁰⁸⁴ gaytdwiaetwnvk ¹⁰⁹⁹ mtkk
TfsA 2327	3	-3.1		kmik ¹¹⁰³ ksplanaeasvtdvk ¹¹¹⁷ vvag
	5	-2.4		mtkk ¹¹⁰⁴ splanaeasvtdvk ¹¹¹⁷ vvag
	2	-1.5		asgk ¹¹³⁴ kvtsnilgqtiak ¹¹⁴⁷ vvlt
TfsA 0955	1	-1.7		kmik ¹¹⁰³ ksplanaeasvtdvk ¹¹¹⁷ vvag
TfsB				
1268-RSDVLQAIADAEIFNVEKTKEDPVA NEITPAITEVKVVAENGAVTILNAAKKVVVSNVLGQTLVNTVLTSDRATVAAPQGVVVVVVEGQPAVKAMVK-1364				
TfsB wild-type	39	-3.2		vist ¹²⁶⁸ sdvlqaiadaefnvek ¹²⁸⁴ tked
TfsB 2327	1	-5.1		nvek ¹²⁸⁵ tkedpvaneitpaitevk ¹³⁰² -vvae
	13	-3.2		tevk ¹³⁰³ vvaengavtilnaagk ¹³¹⁸ kvvv
	2		58.2	agkk ¹³¹⁸ vvvsnvlgqtlvntvltedr ¹³³⁹ -atva
TfsB 0955	5		58.4	tsdr ¹³³⁹ atvaapqgvvvvvvvegqpavk ¹³⁶¹ amvk
	3	-3.6		nvek ¹²⁸⁵ tkedpvaneitpaitevk ¹³⁰² -vvae
	1	-3.9		ektk ¹²⁸⁷ edpvaneitpaitevk ¹³⁰² -vvae
	8	-2.6		tevk ¹³⁰³ vvaengavtilnaagk ¹³¹⁸ kvvv
	1	-3.5		tevk ¹³⁰³ vvaengavtilnaagkk ¹³¹⁹ vvvs

^alog(e): the base-10 log of the expectation that any particular peptide assignment was made at random (E-value). If more than one peptide is found average values are shown

^bMascot Score

^cSequence: the sequence of the assigned peptide sequence. The immediate N- and C-terminal sequences of the identified peptides are also shown.