Glycoprotein biosynthesis and cell surface translocation in the oral pathogen *Tannerella forsythia*

Dissertation

in fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

at the University of Natural Resources and Life Sciences, Vienna, Austria

submitted by

Dipl.-Ing. Bakk. techn. Markus Bernhard TOMEK

Supervisor: Ao. Univ.-Prof. Dipl.-Ing. Dr. Christina SCHÄFFER

performed at the

Department for NanoBiotechnology, *NanoGlycobiology unit*

Head of Department: Univ.-Prof. Dr. José Luis Toca-Herrera

University of Natural Resources and Life Sciences, Vienna, Austria

Vienna, February 2017

Η φύση δεν κάνει τίποτα άχρηστο.

Nature does nothing uselessly.

Aristotle

Acknowledgements

First, I would like to express my gratitude to my supervisor Christina Schäffer for giving me the opportunity to perform my PhD thesis in the *NanoGlycobiology* research group and for her supervision thereof. I would like to thank her for providing an environment where I was able to learn and develop my laboratory skills as well as the planning and processing of experimental based studies. This working atmosphere encouraged me to create new research ideas and realize them.

All members of my thesis committee, Lukas Mach and Paul Messner from BOKU University, Oleh Andrukhov and Peter Steinberger from the Medical University in Vienna as well as Jan Potempa from the University of Louisville and the Jagiellonian University in Kraków are acknowledged for critical evaluation of my thesis progress and fruitful discussions.

I am glad to have participated in the international PhD programme BioToP (Biomolecular Technology of Proteins) and I want to thank Christian Obinger for initiating this great programme and Christa Jakopitsch for excellent administration. Thanks to BioToP, I was able to get to know many great PhD colleagues from different scientific areas and I was able to spend several months abroad in the research group of Jan Potempa. Many thanks to Jan and his wife Barbara for hosting me at the University of Louisville and the Jagiellonian University in Kraków; I really enjoyed my time there and I am grateful for inspiration and scientific discussions we had (sometimes over a glass of bourbon). Danuta Mizgalska was a great help and inspiration in the Kraków laboratory and is a great person, too. Thanks a lot for discussing several aspects concerning our diva, *Tannerella forsythia.*

I want to thank our cooperation partners from the Department of Chemistry at BOKU University for mass spectrometry analyses. I am much obliged to Daniel Maresch, Markus Windwarder, Laura Neumann and Friedrich Altmann for taking their time to analyse my samples and interpret MS results with me.

Although data from an ongoing project with the St. Anna Children´s Cancer Research Institute in Vienna are not included in this thesis, I already want to cordially thank Klara Soukup for the immunological analyses of the *T. forsythia* mutants, interpretation of data and the shared supervision of the Master thesis of Kristina Fuchs.

In the course of my thesis I supervised several Bachelor and Master students. I am glad for this experience, because I think that knowledge transfer is essential at university. I enjoyed working with my Master students Irene Nimeth and Kristina Fuchs as well as with my Bachelor students Vanessa Traxler, Johanna Bacher, Carmen Negrau, Lena Flörl and Anna-Sophia Kampl. I hope I could promote their interest for (natural) sciences and I am thankful for their assistance in my research.

For the great working environment in the laboratory and in our office I would like to thank all my present and former colleagues/friends from the *NanoGlycobiology* group. In particular I want to thank my fellow workers from the *Tannerella* field; Gerald Posch (he taught me everything about the organism at the beginning and is still my *Tannerella* hero), Valentin Friedrich (for scientific accomplishments and non/scientific fun we had over the last years), Andrea Koerdt, Matthias Braun, Susanne Bloch and Zoë Megson.

Special thanks go to Bettina Janesch and Cornelia Rath for critical evaluation of results, for listening and for motivation as well as for fun times we had in the lab, in the office and outside of work.

Further colleagues I want to acknowledge are Julia Anzengruber, Arturo Lopez-Guzman, Eva Bönisch, Andrea Lassenberger, Fiona Hager, Philipp Andesner and Heinrich Schuster. I thank Erik Reimhult for being an excellent former Department head and for late night discussions, Dieter Jäger is acknowledged for being an excellent Department secretary and for motivation throughout the years. Sure enough, many more people have accompanied and influenced me in various ways during the last years. Please feel addressed even if your name is not mentioned here.

Finally, I thank my family and friends for encouragement and support over the last years, especially Petra.

Summary

Tannerella forsythia is a keystone pathogen critically implicated in the onset and progression of periodontal diseases in humans. Thus, its occurrence, virulence mechanisms and the role(s) of its recently demonstrated protein *O*-glycosylation potential are of particular interest. This thesis is divided into two main projects, both dealing with novel aspects of the general protein *O*-glycosylation system of this human oral pathogen, exemplified with two distinct clinical isolates *Tannerella forsythia* ATCC 43037 and *Tannerella forsythia* UB4.

- In the first project, the basic principle governing glycoprotein biosynthesis was investigated focusing on the strain-specific transfer of a nonulosonic acid derivative as a terminal residue onto the preformed *T. forsythia* oligosaccharide.
- In the second project, the translocation of (glyco)proteins across the bacterium´s outer membrane via a novel secretion system (termed type IX secretion system) and the interplay of this secretion system with protein *O*-glycosylation were studied.

Project 1

The occurrence of nonulosonic acids in bacteria is wide-spread and increasingly linked to pathogenicity. In the periodontopathogen *T. forsythia*, several proposed virulence factors carry strain-specifically a derivative of either a pseudaminic acid (exemplified with strain ATCC 43037) or of a legionaminic acid (exemplified with strain UB4) as terminal residue on a protein-bound oligosaccharide that is synthesized by the bacterium´s general protein *O*-glycosylation system. Comprehensive molecular, biochemical and bioinformatic analyses performed in the course of this PhD thesis added genes encoding further glycosyltransferases, methyltransferases and carbohydrate modifying enzymes to the already described general protein *O*-glycosylation gene cluster of the bacterium.

A specific aim of the work was to shed light on the transfer of either nonulosonic acid derivative on a proximal *N*-acetyl mannosaminuronic acid residue within the *O*-glycan structure, exemplified with the bacterium's abundant S-layer glycoproteins. Bioinformatic analyses provided the candidate genes *Tanf_01245* (strain ATCC 43037) and *TFUB4_00887* (strain UB4), encoding a putative pseudaminic and a legionaminic acid derivative transferase, respectively. These transferases are identical in their C-termini and contain motifs typical of glycosyltransferases (DXD) and bacterial sialyltransferases (D/E-D/E-G and HP motif). They share homology with type B glycosyltransferases and TagB, an enzyme catalysing glycerol transfer to an *N*-acetylmannosamine residue in teichoic acid biosynthesis. Analysis of a cellular pool of nucleotide-activated sugars

confirmed the presence of the CMP-activated nonulosonic acid derivatives, which are most likely serving as substrates for the corresponding transferase. Single gene knock-out mutants targeted at either transferase were analysed for S-layer *O*-glycan composition by ESI-MS, confirming the loss of the nonulosonic acid derivative. Cross-complementation of the mutants with the non-native nonulosonic acid transferase was not successful, indicating high stringency of the enzymes.

Results from this study identified plausible candidates for so far unknown pseudaminic and legionaminic acid transferases.

Project 2

Protein translocation across the cell envelope of Gram-negative bacteria is a central event in the overall lifestyle of an organism and has been intensively studied for decades. Recently, a novel protein secretion system (type IX secretion system, T9SS) has been identified in the crucial periodontopathogen *Porphyromonas gingivalis*. The second part of this thesis was to investigate the presence and functionality of the T9SS in the phylogenetically related bacterium *T. forsythia*. Conserved C-terminal domains (CTD) have been shown to act as a signal for the translocation of proteins across the outer membrane of *Bacteroidetes* via the multi-component T9SS.

The genome sequence of *T. forsythia* predicts the presence of the components for a T9SS in conjunction with a suite of CTD proteins. *T. forsythia* cells are characteristically covered with a 2D cell surface (S-) layer composed of the two glycosylated proteins TfsA and TfsB. The S-layer is a proven virulence factor of the bacterium and both glycoproteins are among the group of CTD proteins. To investigate, if a T9SS is functional in *T. forsythia*, T9SS-deficient mutants were generated by targeting either Tanf_02580 (putative C-terminal signal peptidase) or Tanf_02360 (PorK ortholog). Subsequently, the mutants were analysed with respect to secretion, assembly and glycosylation of the S-layer proteins as well as proteolytic processing of the CTD and biofilm formation. In either mutant, TfsA and TfsB were incapable of cell surface translocation, as evidenced by the absence of the S-layer in transmission electron microscopy of ultrathin-sectioned bacterial cells. Despite being entrapped within the periplasm, mass spectrometry analysis revealed that the S-layer proteins were modified with the 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.

As a result, the presence and the functionality of the T9SS as well as the requirement of a CTD for the outer membrane passage of extracellular proteins in *T. forsythia*, exemplified with the two S-layer proteins, were demonstrated.

Novel data presented within the frame of this PhD thesis may serve as a basis for the design of tailored glycoconjugates containing nonulosonic acids and suggest the T9SS as novel target for treatment strategies against periodontal diseases.

Zusammenfassung

Tannerella forsythia ist ein oralpathogenes Bakterium und ein entscheidender Erreger von Parodontitis. Daher ist es wichtig die Verbreitung, das Virulenzpotenzial sowie die Rolle der kürzlich beschriebenen Proteinglykosylierung zu untersuchen. Die vorliegende Dissertation gliedert sich in zwei Hauptprojekte, die sich mit neuartigen Aspekten der allgemeinen Proteinglykosylierung dieses oralen humanen Pathogens beschäftigen. Anhand von zwei verschiedenen klinischen Isolaten, *Tannerella forsythia* ATCC 43037 und *Tannerella forsythia* UB4, werden diese Aspekte veranschaulicht.

- Das erste Projekt befasste sich mit dem Mechanismus der Glykoproteinbiosynthese in *T. forsythia*, mit Fokus auf dem stammspezifischen Transfer von Nonulosonsäurederivaten als terminale Zuckereinheit auf ein *T. forsythia* Oligosaccharid.
- Im zweiten Projekt wurde die Translokation von (Glyko)proteinen durch die äußere Zellmembran des Bakteriums anhand eines neuartigen Sekretionssystems (Typ-IX-Sekretionssystem, T9SS) und das Zusammenspiel dieses Sekretionssystems mit der Proteinglykosylierung untersucht.

Projekt 1

Das Auftreten von Nonulosonsäuren ist weit verbreitet und steht in direktem Zusammenhang mit bakterieller Pathogenität. Virulenzfaktoren des mit Parodontitiserkrankungen assoziierten Pathogens *T. forsythia* tragen stammspezifisch entweder ein Derivat einer Pseudaminsäure (veranschaulicht mit dem Stamm ATCC 43037) oder einer Legionaminsäure (veranschaulicht mit dem Stamm UB4) am nicht-reduzierenden Ende eines Protein-gebundenen Glykans. Umfassende molekulare, biochemische und bioinformatische Analysen, die im Zuge dieser Dissertation durchgeführt wurden, identifizierten zu dem bereits beschriebenen allgemeinen Proteinglykosylierungscluster des Bakteriums weitere Gene für Glykosyltransferasen, Methyltransferasen und kohlenhydratmodifizierende Enzyme.

Ziel dieser Arbeit war es, den Transfer der Nonulosonsäurederivate auf die proximale *N*-Acetylmannosaminsäure in der Glykanstruktur anhand der S-Schicht Glykoproteine zu untersuchen und erstmals zu beschreiben. Bioinformatische Analysen zeigten Gene auf, die für den Transfer einer Pseudaminsäure oder Legionaminsäure verantwortlich sein könnten, nämlich *Tanf_01245* (Stamm ATCC 43037) beziehungsweise *TFUB4_00887* (Stamm UB4). Die C-Termini beider Transferasen sind ident und beinhalten ein typisches Glykosyltransferasemotiv (DXD) sowie Motive, die Signaturen bakterieller Sialinsäuretransferasen darstellen (D/E-D/E-G und HP). Beide

Transferasen haben Aminosäuresequenzhomologien zu Glykosyltransferasen vom Typ B sowie zu TagB, einem Enzym das Glycerol an *N*-Acetylmannosamin im Zuge der Teichonsäurebiosynthese transferiert. Weiters wurden nukleotidaktivierte Zucker untersucht, wobei das Vorhandensein von CMP-aktivierten Nonulosonsäurederivaten, welche die natürlichen Substrate der untersuchten Transferasen darstellen, bestätigt werden konnte. Knock-out Mutanten beider Transferasegene zeigten den Verlust der terminalen Nonulosonsäurederivate am Beispiel des S-Schicht *O*-Glykans was mit Hilfe von ESI-MS experimentell bewiesen werden konnte. Kreuzkomplementierungen der Transferasemutanten mit der jeweiligen nicht-nativen Transferase waren nicht erfolgreich, was auf eine hohe Spezifität der Enzyme schließen läßt.

Im Rahmen dieser Arbeit konnten zum ersten Mal Transferasen für Pseudaminsäure- und Legionaminsäurederivate erfolgreich identifiziert und untersucht werden.

Projekt 2

Die Translokation von Proteinen durch die Zellwand von Gram-negativen Bakterien ist essenziell für die Lebensweise der Bakterien und wurde in den letzten Jahrzehnten intensiv beforscht. Ein bis vor kurzem unbekanntes Proteinsekretionssystem (Typ-IX-Sekretionssystem, T9SS) wurde nun im Parodontitis-assoziierten Stamm *Porphyromonas gingivalis* identifiziert. Ziel der vorliegenden Arbeit war es zu untersuchen, ob dieses Sekretionssystem auch in dem phylogenetisch verwandten Bakterium *T. forsythia* vorhanden und funktionell ist. Eine Voraussetzung die das Sekretionssystem an Proteine stellt, ist das Vorhandensein einer konservierten C-terminalen Domäne (CTD), die als Signalsequenz für die Translokation durch die äußere Zellmembran dient.

Erste Untersuchungen des Genoms von *T. forsythia* zeigten sowohl das Vorhandensein von CTD Proteinen, als auch von Komponenten für den Aufbau des Sekretionssystems selbst. Die Zelloberfläche von *T. forsythia* besteht aus einer zweidimensional kristallinen Schicht, welche aus den beiden glykosylierten CTD Proteinen TfsA und TfsB aufgebaut ist. Um die Funktionalität des T9SS zu untersuchen, wurden T9SS defiziente Mutanten hergestellt, wobei entweder Tanf_02580 (mutmaßliche C-terminale Signalpeptidase) oder Tanf_02360 (PorK Ortholog) ausgeknockt wurde. Darauf folgte eine Untersuchung beider Mutanten hinsichtlich der Sekretion, des Zusammenbaus der S-Schicht und der Glykosylierung der S-Schicht Proteine sowie einer zu erwartenden proteolytischen Abspaltung der CTD sowie der Biofilmbildung. In beiden Mutanten konnten weder TfsA noch TfsB erfolgreich über die äußere Zellmembran transportiert werden und bildeten als Konsequenz keine S-Schicht aus. Dies wurde bei einer Transmissionselektronenmikroskopie von Ultradünnschnitten bakterieller Zellen klar sichtbar. Obwohl beide S-Schicht Proteine im

periplasmatischen Raum zurückgehalten wurden, konnte mittels Massenspektrometrie gezeigt werden, dass die Proteine entsprechend der nativen Situation vollständig glykosyliert waren. Demnach sind die Proteintranslokation und die Proteinglykosylierung voneinander entkoppelte Prozesse. Außerdem konnte ein dichterer Biofilm mit vergleichsweise wenigen Hohlräumen bei beiden Sekretionssystem-defizienten Mutanten festgestellt werden.

Zusammenfassend konnte mit dieser Arbeit bewiesen werden, dass das T9SS in *T. forsythia* vorhanden und funktionell ist sowie die CTD einen essenziellen Bestandteil für die erfolgreiche (Glyko)proteintranslokation durch die äußere Zellmembran darstellt.

Neue Erkenntnisse, die im Rahmen dieser Doktoratsarbeit gewonnen wurden, sind für das Design von neuen Glykokonjugaten, die Nonulosonsäuren beinhalten, wertvoll und präsentieren das T9SS als mögliches neuartiges Target für Behandlungsstrategien gegen Parodontitis-Erkrankungen.

Abbreviations

Ac	*N*-acetyl or acetamido
Am	*N*-acetimidoyl or acetamidino
BspA	*Bacteroides* surface protein A
CMP	cytidine-5'-monophosphate
CTD	C-terminal domain
Dig	digitoxose
ESI-IT MS	electrospray ionization ion-trap mass spectrometry
ESI-TOF MS	electrospray ionization time-of-flight mass spectrometry
Fuc	fucose
Gc	*N*-glycolyl
GDP	guanosine-5'-diphosphate
Gra	*N*-glyceroyl or *N*-2,3-dihydroxypropionyl or glycerate group
GT	glycosyltransferase
IL	interleukin
LC	liquid chromatography
Leg	legionaminic acid, (Leg5,7Ac_2), 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-nonulosonic acid
ManNAcA	*N*-acetyl mannosaminuronic acid
ManNAcCONH_2	*N*-acetyl mannosaminuronamide
MT	methyltransferase
NeuAc	*N*-acetylneuraminic acid
OTase	oligosaccharyltransferase
Pse	pseudaminic acid, (Pse5,7Ac_2), 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-nonulosonic acid
Sia	sialic acid, (Neu5Ac), 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid
S-layer	surface layer
T9SS	type ix secretion system
TfsA	*Tannerella forsythia* surface layer protein A
TfsB	*Tannerella forsythia* surface layer protein B
Th17	T-helper 17 cells
UDP	uridine 5’-diphosphate
Xyl	xylose

Table of contents

Acknowledgements v

Summary vii

Zusammenfassung xi

Abbreviations xv

1 Aims of the thesis 1

2 *Tannerella forsythia* - a keystone periodontal pathogen 7

2.1 Occurrence of *Tannerella forsythia* 9

2.2 Cellular integrity of *Tannerella forsythia* 9

2.3 Virulence potential of *Tannerella forsythia* 11

2.4 Glycobiology aspects of *Tannerella forsythia* 12

2.5 *Tannerella forsythia* is a periodontal pathogen 14

3 Glycoprotein biosynthesis in *Tannerella forsythia* 19

3.1 Bacterial protein glycosylation in general 22

3.2 Biological roles of bacterial glycoproteins 23

3.3 Bacterial glycoprotein biosynthesis 24

3.4 Bacterial glycosyltransferases 25

3.5 General protein *O*-glycosylation systems 26

3.6 The protein *O*-glycosylation gene cluster in *Tannerella forsythia* 26

3.7 Publication 1: A pseudaminic acid or a legionaminic acid derivative transferase is strain-specifically implicated in the general protein *O*-glycosylation system of the periodontal pathogen *Tannerella forsythia* 33

4 Cell surface glycoprotein translocation in *Tannerella forsythia* 91

4.1 Protein secretion systems in Gram-negative bacteria 94

4.2 The type IX secretion system in *Bacteroidetes* 97

4.3 Publication 2: S-layer glycoproteins of *Tannerella forsythia* are secreted via a type IX secretion system that is decoupled from protein *O*-glycosylation. 103

5 Conclusions of the thesis 121

6 Conference contributions 127

7 References 145

8 *Curriculum vitae* 159

1

Aims of the thesis

Glycobiology is the study of the structure, biosynthesis, biology and evolution of saccharides that are widely distributed in nature, and the proteins that recognize them (Varki *et al.* 2009). As this definition implicates, carbohydrates are an inherent part of living cells from all domains of life, the *Archaea*, *Bacteria* and *Eukarya*. Focusing on archaeal glycosylation, first discoveries were presented in the 1970s when Mescher and Strominger purified and characterized a glycoprotein from the cell envelope of *Halobacterium salinarum* (Mescher and Strominger 1976). An early example of a bacterial glycoprotein was reported by Sleytr and Thorne in describing glycosylated surface (S-) layers in the bacteria *Clostridium thermosaccharolyticum* and *Clostridium thermohydrosulfuricum* (Sleytr and Thorne 1976). Since then, many more cases showed that bacterial glycosylation is not a *lusus naturae*, but the most prevalent post-translational modification of proteins across the whole domains of *Archaea* and *Bacteria* (Messner 1997; Nothaft and Szymanski 2010; Messner *et al.* 2013; Tytgat and Lebeer 2014).

In *Bacteria*, protein glycosylation is a common modification of secreted and cell-surface associated proteins, such as flagella and pili as well as S-layers (Moens and Vanderleyden 1997; Schäffer and Messner 2001; Upreti *et al.* 2003; Logan 2006; Messner *et al.* 2008; Schäffer and Messner 2017). Besides glycosylated proteins, additional glycoconjugates such as lipopolysaccharides (LPS), lipooligosaccharides (LOS), peptidoglycan (PG), capsular polysaccharides and secondary cell-wall polymers (SCWP) can be found in bacteria, especially in bacterial cell envelopes (Messner *et al.* 2013). The finding of glycosylated proteins on the cell surface of pathogenic bacteria, such as *Campylobacter* spp., *Pseudomonas aeruginosa*, *Helicobacter pylori* and *Streptococcus* spp. (Schmidt *et al.* 2003; Valguarnera *et al.* 2016), strengthened previous research results. In particular, to one class of sugars, nonulosonic acids, high relevance for bacterial pathogenicity and human physiology is attributed (Varki 2008).

Potential biological roles of glycosylated proteins and glycoconjugates can be very diverse and cannot be predicted *a priori*. Mediation of specific recognition events or modulations of biological processes seem to be common features of oligosaccharides, affecting the development and differentiation of complex organisms as well as inter- and intraspecific interactions (Varki 1993, 2016; Tytgat and de Vos in press). Likewise, adhesion processes can be regulated by carbohydrate-carbohydrate interactions, which is an essential step in the colonization of a specific target host region by (pathogenic) bacteria. The employment of molecular mimics of host glycans is an evolutionary successful strategy of pathogenic bacteria to evade host immune responses, again demonstrating the vast influence of carbohydrates on the bacterial lifestyle and disease progression (Comstock and Kasper 2006; Varki 2008; Woolard and Frelinger 2008; Varki *et al.*

2009). Understanding these bacterial mechanisms is crucial, both for understanding the glycobiology of pathogenic bacteria and for applying this knowledge to the development of new drugs and treatment strategies.

Work presented in this PhD thesis intends to unravel essential steps in the lifestyle of the oral pathogen *Tannerella forsythia*. Investigations into the glycoprotein biosynthesis and the translocation of glycoproteins across the cell wall are the focuses of this thesis. The obtained insights may lead to the identification of novel drug targets for interfering with the lifestyle and disease progression of this worldwide appearing human pathogen. This thesis is divided into three main chapters and is finalized by general conclusions. The chapter **"*Tannerella forsythia* – a keystone periodontal pathogen"** introduces the organism and focuses on its prevalence, virulence potential and the potential roles of its protein *O*-glycosylation.

The chapter **"Glycoprotein biosynthesis in *Tannerella forsythia*"** provides an overview of proposed mechanisms of protein *O*-glycosylation in bacteria, highlighting the roles of glycosyltransferases. One particular glycosyltransferase present within the general *O*-glycosylation gene cluster of *T. forsythia*, a nonulosonic acid transferase, was investigated in detail and is subject of the first publication.

> **Tomek, M.B.**, Janesch, B., Maresch, D., Windwarder, M., Altmann, F., Messner, P. and Schäffer, C. A pseudaminic acid or a legionaminic acid derivative transferase is strain-specifically implicated in the general protein *O*-glycosylation system of the periodontal pathogen *Tannerella forsythia*. *Glycobiology,* in press. doi: 10.1093/glycob/cwx019

The chapter **"Cell surface glycoprotein translocation in *Tannerella forsythia*"** is introduced by describing known bacterial protein translocation systems and giving examples thereof. Special emphasis is on the only recently described type IX secretion system (T9SS) and its relation to proteins with conserved C-terminal domains (CTD). In the second publication, the presence and functionality of the T9SS in *T. forsythia* and the involvement of conserved C-terminal domains in this context was investigated. Interplay of protein *O*-glycosylation and protein translocation was an additional question addressed.

> **Tomek, M.B.**, Neumann, L., Nimeth, I., Koerdt, A., Andesner, P., Mach, L., Messner, P., Potempa, J.S., Schäffer, C. (2014) S-layer glycoproteins of *Tannerella forsythia* are secreted via a type IX secretion system that is decoupled from protein *O*-glycosylation. *Mol Oral Microbiol*, 29:307-320. doi: 10.1111/omi.12062

2

Tannerella forsythia - a keystone periodontal pathogen

2.1 Occurrence of *Tannerella forsythia*

2.2 Cellular integrity of *Tannerella forsythia*

2.3 Virulence potential of *Tannerella forsythia*

2.4 Glycobiology aspects of *Tannerella forsythia*

2.5 *Tannerella forsythia* is a periodontal pathogen

2.1 Occurrence of *Tannerella forsythia*

Tannerella forsythia was Isolated from patients with advanced periodontitis in the mid-1970s and initially described in 1986 by Anne Tanner as *Bacteroides forsythus* (Tanner *et al.* 1979; 1986b). A 16S rRNA phylogenetic analysis groups this Gram-negative bacterium within the phylum *Bacteroidetes*, which is a member of the *Cytophaga-Flavobacterium-Bacteroides* family of bacteria. Phylogenetically closely related to *T. forsythia* are *Porphyromonas*, *Prevotella* and *Bacteroides* species (Tanner and Izard 2006). In addition to isolates from periodontitis patients, of which only few have been sequenced to date (Tanner and Izard 2006; Friedrich *et al.* 2015c; Stafford *et al.* 2016), *T. forsythia* strains were also isolated from the subgingival plaque of monkeys and from cat and dog bite wounds of humans (Tanner and Izard 2006).

Interestingly, despite the general association of *T. forsythia* with periodontal diseases, another human *Tannerella* sp. phylotype, named BU063, was isolated from periodontally healthy sites of the human oral cavity. This isolate is particularly interesting for comparative genomics and transcriptomics analyses in relation to the periodontitis-associated isolates (Fodor *et al.* 2012; Beall *et al.* 2014). However, cultivation of *Tannerella* BU063 is laborious and was only recently successful on culture plates when cross-streaked with helper strains (*Propionibacterium acnes* and *Prevotella intermedia*) (Vartoukian *et al.* 2016).

2.2 Cellular integrity of *Tannerella forsythia*

Tannerella forsythia is a non-motile anaerobic bacterium with a typical Gram-negative cell envelope profile, including an inner and an outer membrane. In addition, the cell is covered by a characteristic 2D crystalline cell surface layer (S-layer) (Kerosuo 1988; Sekot *et al.* 2012) that is formed by an entropy-driven self-assembly event of constituting subunits (Sleytr and Messner 1983; Messner *et al.* 2010). To bacterial S-layers different biological functions have been attributed, including acting as a permeability barrier or as a protective coat, as adhesion and surface receptor recognition mechanism as well as involvement in mediation of virulence (Beveridge *et al.* 1997; Fagan and Fairweather 2014). The S-layer of *T. forsythia* is so far unique, since it is composed of two intercalating high molecular-weight glycoproteins, TfsA (Tanf_03370; TF2661-2662[1]) and TfsB (Tanf_03375; TF2663). This circumstance makes *T. forsythia* an exciting model organism, being the first Gram-negative prokaryote known so far, to possess a glycosylated S-layer which is in addition structurally unique due to the simultaneous

[1] The draft genome sequence of the *T. forsythia* strain ATCC 43037 includes a "*Tanf*" locus tag. The previously available genome of this designation (NCBI reference sequence NC_016610.1) was discovered to be derived from a different strain, FDC 92A2 (=ATCC BAA-2717) including a "*TF*" locus tag (Friedrich *et al.* 2015c). For traceability, within publications before 2015, both annotations are given, if applicable.

presence of two S-layer proteins (Lee *et al.* 2006; Sekot *et al.* 2012). The lattice constant of the square S-layer lattice was determined to be 10.1 ± 0.7 nm, the thickness of the layer was measured to be approximately 22 nm (Sekot *et al.* 2012). Both S-layer proteins possess a so-called C-terminal domain (CTD) sequence putatively involved in a CTD-dependent novel secretion system in addition to known S-layer domains such as the self-assembly (SA) and the cell-wall anchoring domain (Nguyen *et al.* 2007; Veith *et al.* 2009).

In its native environment, *T. forsythia* is present in a complex and dynamic multispecies biofilm of the oral cavity. In general, oral biofilm formation is a complex process involving multiple adhesion and dispersion events which takes place from initial surface contact to tridimensional maturation (Kolenbrander *et al.* 2010). It is important to note that the biofilm lifestyle of the organism is crucial for conferring its virulence potential (Socransky *et al.* 1998; Hajishengallis and Lamont 2012). A monospecies biofilm of *T. forsythia* ATCC 43037 is shown in Figure 1.

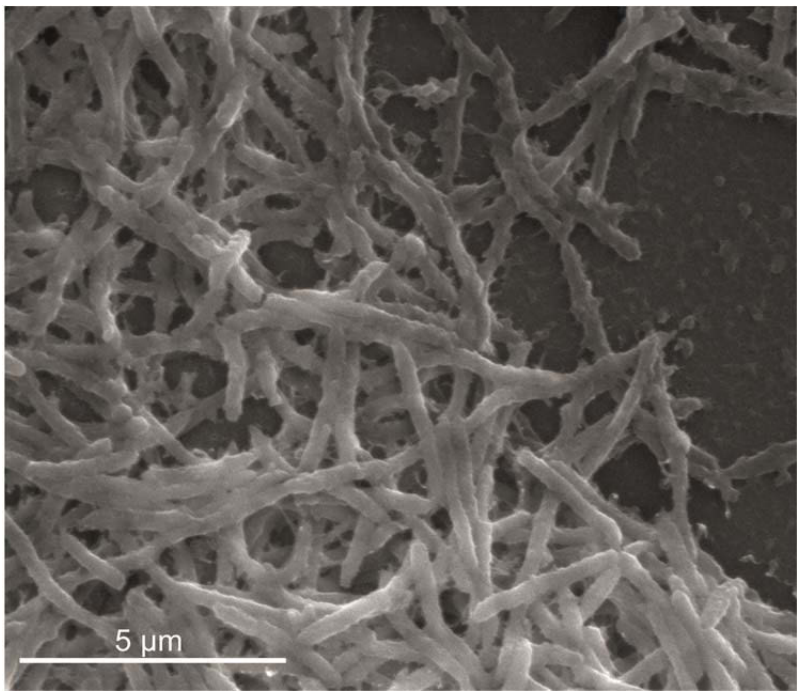


Figure 1 Scanning electron micrograph of a monospecies *T. forsythia* biofilm showing the typical rod shaped appearance of non-motile cells forming a biofilm with several voids. The biofilm was grown anaerobically on a glass slide for two days before analysis. [Taken from and modified after (**Tomek *et al.* 2014**)].

2.3 Virulence potential of *Tannerella forsythia*

Virulence defines the ability of an organism to cause disease or to interfere with its host metabolic or physiologic functions. Virulence factors contribute to the pathogenicity of the organism including the ability to induce microbe-host interactions, to invade and to grow in a niche of the host as well as to evade or interfere with host defence mechanisms (Holt and Bramanti 1991; Holt and Ebersole 2005).

Due to fastidious growth requirements and the difficulty to genetically modify the organism, only a few virulence factors have been identified in *T. forsythia*, so far. Several studies deal with the virulence of both S-layer glycoproteins TfsA and TfsB. They are strongly antigenic and mediate hemagglutination (Sabet *et al.* 2003; Lee *et al.* 2006). Adherence to and invasion of oral epithelial (KB) and human gingival epithelial cells was demonstrated in different studies (Sabet *et al.* 2003; Lee *et al.* 2006; Sakakibara *et al.* 2007). Immune responses of human macrophages and gingival fibroblasts upon stimulation with *T. forsythia* cells suggest that the S-layer attenuates the host immune response by evading its recognition by the innate immune system of the host, at least in the early phase of infection (Sekot *et al.* 2011). Furthermore, there are indications that the surface glycosylation is crucial for the modulation of host immunity. In contrast to the wild-type strain, infection with a mutant strain with a truncated S-layer glycan, induced robust T-helper (Th)17-linked mobilization of neutrophils and reduced periodontal bone loss in mice (Settem *et al.* 2014).

Other virulent surface components of *T. forsythia* include the *Bacteroides* surface protein A (BspA) and surface lipoproteins. BspA is glycosylated and belongs to the leucine-rich repeat family often associated with protein-protein interactions (Sharma *et al.* 1998; Kobe and Kajava 2001). A BspA deficient mutant was significantly less potent in inducing alveolar bone loss in mice when compared to the wild-type strain (Sharma *et al.* 2005). *Tannerella forsythia* surface lipoproteins are able to release proinflammatory cytokines (interleukin-6) and tumor necrosis factor alpha (TNFα) upon stimulation of human gingival fibroblasts and monocytic cells (Hasebe *et al.* 2004). Thus, lipoproteins have virulent potential by induction of host cell activity and apoptosis.

Proteases contribute to bacterial virulence in various ways, including the degradation of host periodontal tissues and the activation of host degradative enzymes, the modification of host cell proteins and the cleavage of components involved in innate and adaptive immunity (Holt and Bramanti 1991; Potempa and Pike 2009). *Tannerella forsythia* employs multiple proteases which may contribute to its virulence. A group of proteases, termed KLIKK proteases, appears to be a potent virulence factor (Ksiazek *et al.* 2015b). Karilysin, mirolysin and mirolase are most comprehensively studied proteases

from this group which belong to CTD-containing proteins. Karilysin, related to human matrix metalloproteases, can inhibit all pathways of the complement system and sheds fully active TNFα from macrophage surfaces (Jusko *et al.* 2012; Bryzek *et al.* 2014). Mirolysin, a metalloprotease, can degrade an array of host proteins, including human fibronectin, fibrinogen, the complement proteins C3, C4 and C5 and the antimicrobial peptide LL-37 (Koneru *et al.* in press). Mirolase is a calcium-dependent serine protease involved in the hydrolysis of human fibrinogen, haemoglobin and LL-37 (Ksiazek *et al.* 2015a). Other KLIKK-proteases, forsilysin, miropsin-1 and miropsin-2, were able to degrade protein substrates, but need further research to identify their exact biological functions (Ksiazek *et al.* 2015b). Levels of the cysteine protease PrtH correlate with the loss of periodontal attachment and PrtH is able to detach adherent cells from the substratum including the stimulation of interleukin-8 (IL-8) expression (Nakajima *et al.* 2006; Hamlet *et al.* 2007).

Further putative virulence factors identified in *T. forsythia* include outer membrane vesicles (OMV) (Friedrich *et al.* 2015a; Veith *et al.* 2015), the sialidases NanH and SiaH (Honma *et al.* 2011; Roy *et al.* 2011), an α-L-fucosidase (Megson *et al.* 2015), the pathogenic mediator methylglyoxal (Maiden *et al.* 2004) and the molecular chaperon GroEL which is homologous to the human heat shock protein 60 (Jung *et al.* in press).

2.4 Glycobiology aspects of *Tannerella forsythia*

Tannerella forsythia employs a glycosylation system to modify several proteins with an *O*-linked glycan. The first and best studied glycoproteins are the S-layer proteins TfsA and TfsB. Further experimentally identified glycoproteins include BspA and the predicted outer membrane proteins Tanf_02425 (TF2339) and its paralog Tanf_08965 (TF1259) as well as the predicted lipoproteins Tanf_08330 (TF1056) and Tanf_13485 (TF0091) (Posch *et al.* 2011). The 1179-amino acid S-layer protein TfsA carries four glycosylation sites (Ser^{197}, Thr^{819}, Ser^{943}, Thr^{979}) and five sites of the 1364-amino acid S-layer protein TfsB are glycosylated (Ser^{473}, Thr^{493}, Thr^{752}, Thr^{786}, Thr^{1069}). The glycan is *O*-glycosidically linked to distinct threonine or serine residues within a three-amino acid motif Asp-(Ser/Thr)-(Ala/Ile/Leu/Met/Thr/Val) (Posch *et al.* 2011). This unique glycosylation sequon is predicted to be valid phylum-wide and has been experimentally proven to be targeted by the protein *O*-glycosylation systems of *T. forsythia* and *Bacteroides fragilis*, where a protein non-native to the other organism could be successfully cross-glycosylated (Posch *et al.* 2013b). It seems that not only the glycosylation sequon but also the principal operation mode of the protein glycosylation machinery is conserved within *Bacteroidetes* species, since predicted protein *O*-glycosylation gene loci were found in the above mentioned *B. fragilis* NCTC 9343 as well as in *Bacteroides thetaiotaomicron* VPI 5492, *Bacteroides*

uniformis ATCC 8492, *Porphyromonas gingivalis* ATCC 33277 and *Parabacteroides distasonis* ATCC 8503 (Posch *et al.* 2011). The proposed phylum-wide general protein *O*-glycosylation system comprises the formation of a conserved *Bacteroidetes* core glycan and a species-specific outer glycan, which might be exported separately prior to final assembly of the mature glycoprotein (Fletcher *et al.* 2009; Coyne *et al.* 2013). An elaborate description of the general protein *O*-glycosylation gene cluster present in *T. forsythia* is given in chapter 3.6, where genes and their predicted biological functions are discussed.

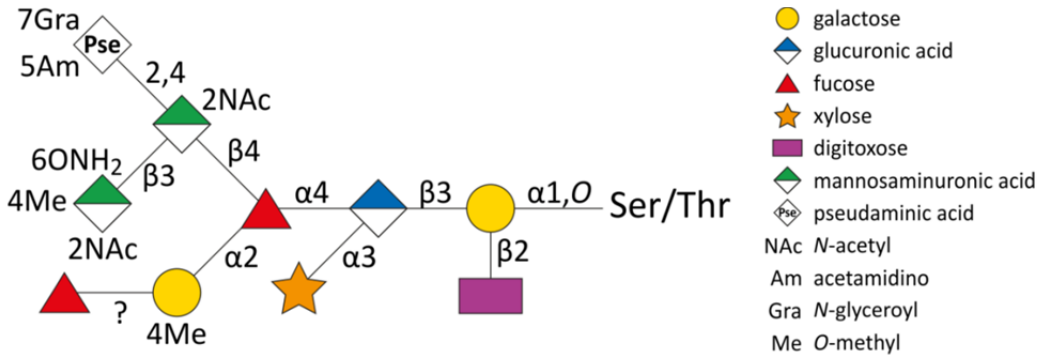


Figure 2 Schematic drawing of the abundant protein *O*-glycan in *T. forsythia* ATCC 43037. [Modified after (Posch *et al.* 2011)].

The composition and structure of the *T. forsythia O*-glycan was elucidated by a combination of mass spectrometric and nuclear magnetic resonance (NMR) spectroscopic analyses to be an oligosaccharide with the structure 4-Me-β-Man*p*NAcCONH$_2$-(1→3)-[Pse5Am7Gra-(2→4)-]-β-Man*p*NAcA-(1→4)-[4-Me-α-Gal*p*-(1→2)-]-α-Fuc*p*-(1→4)-[-α-Xyl*p*-(1→3)-]-β-Glc*p*A-(1→3)-[-β-Dig*p*-(1→2)-]-α-Gal*p* (Posch *et al.* 2011) (Figure 2). Until that finding, bacterial S-layer glycans had been described as long-chain heteropolysaccharides composed of individual repeating units being reminiscent to archaeal S-layer glycans (Ristl *et al.* 2011; Messner *et al.* 2013). With the *T. forsythia* S-layer *O*-glycan, the uncommon sugar residues digitoxose (Dig), xylose (Xyl), *N*-acetyl mannosaminuronic acid (ManNAcA), *N*-acetyl mannosaminuronamide (ManNAcCONH$_2$) and a modified pseudaminic acid (Pse5Am7Gra)[2], have been reported for the first time in a bacterial S-layer glycan.

The defined biological functions of the individual carbohydrate residues and their modifications remain elusive. However it is tempting to assume that the modified pseudaminic acid residue participates in the bacterium-host cross-talk. Pseudaminic acids belong to the chemical class of nonulosonic acids (sialic acids), with the *N*-acetylated form being the most common member of this group (*N*-acetylneuraminic acid, Neu5Ac) (Varki 1992; Angata and Varki 2002; Knirel *et al.* 2003). Many cases

[2] The modification at carbon position 7 was initially described as a glycolyl (Gc) group. However, it turned out to be a glyceroyl (Gra) group instead (Friedrich *et al.* 2017).

demonstrate the importance of sialic acids to bacterial pathogenicity in mediating adhesion to and invasion of specific host cells as well as evading the host immune system (Varki 2008; Varki and Gagneux 2012; Zunk and Kiefel 2014). Few experiments were performed in order to understand the biological roles of the *T. forsythia* protein *O*-glycan. A *T. forsythia* mutant possessing an insertional inactivation in the WecC (UDP-*N*-acetylmannosaminuronic acid dehydrogenase; Tanf_01280, TF2055) gene (Δ*wecC*), resulting in the production of a truncated protein *O*-glycan, has increased biofilm formation capacity (Honma *et al.* 2007). In addition, the gene was found to be located within a putative exopolysaccharide operon. Further investigations detect that the three sugar branch, including the terminal pseudaminic acid and both mannosaminuronic acids, was missing on the glycan of this mutant (Posch *et al.* 2011). Besides of increased biofilm formation, the absence of the three sugar branch resulted in a robust Th17 induction and lead to a reduced periodontal bone loss in mice, in contrast to the wild-type strain (Settem *et al.* 2013a). This finding demonstrates that the terminal motif of the glycan acts in modulating dendritic cell effector functions in suppressing Th17 responses (Honma *et al.* 2007; Settem *et al.* 2013a; 2014).

2.5 *Tannerella forsythia* is a periodontal pathogen

Tannerella forsythia meets all criteria to be classified as an oral pathogen (Socransky 1979), because (i) the bacterium is enriched at the site of pathology (Socransky *et al.* 1998; Colombo *et al.* 2009); (ii) cellular and humoral immune responses to its antigens are documented (Gosling *et al.* 2005; Darveau 2010; Settem *et al.* 2013b); (iii) periodontal disease can be initiated in experimental animals (Sharma *et al.* 2005; Settem *et al.* 2013a; 2014; Jung *et al.* in press); and (iv) virulence factors for the progression of destructive periodontal disease are expressed (see 2.3 Virulence potential of *T. forsythia*).

Periodontitis is a chronic inflammatory disease leading to the destruction of the periodontium. That is the tooth-supporting structure comprised of the gingiva, the periodontal ligament and the underlining alveolar bone. The tooth-associated bacterial biofilm (dental plaque) is required but not sufficient to induce the chronic disease, because it is the host inflammatory response to this microbial challenge that ultimately can cause destruction of the periodontium (Hajishengallis 2015) (Figure 3). Few pathogens, grouped into five microbial complexes, have traditionally been considered as causative agents of periodontitis. These microbial communities were characterized based on a color-coded system reflecting their time of appearance, association between complexes and relation to disease severity. In particular the group of “red complex” bacteria (*T. forsythia*, *P. gingivalis* and *Treponema denticola*) are strongly associated with each other and with disease progression (Socransky *et al.* 1998; Holt and Ebersole 2005). Recent advances in understanding the pathogenesis of periodontitis suggest

that the disease is not caused by distinct pathogens (periopathogens). The transition from a healthy to a diseased state is associated with a shift from a symbiotic to a dysbiotic microbial community (Szafranski *et al.* 2015), termed the polymicrobial synergy and dysbiosis (PSD) model (Hajishengallis and Lamont 2012; Lamont and Hajishengallis 2015) (Figure 4). Low abundance keystone pathogens are crucial as they initially subvert host immune responses, further leading to homeostasis breakdown and destructive inflammation (Hajishengallis *et al.* 2012; 2014). Inflammation and dysbiosis positively reinforce each other resulting in a continuing periodontal tissue destruction including resorption of the supporting alveolar bone.

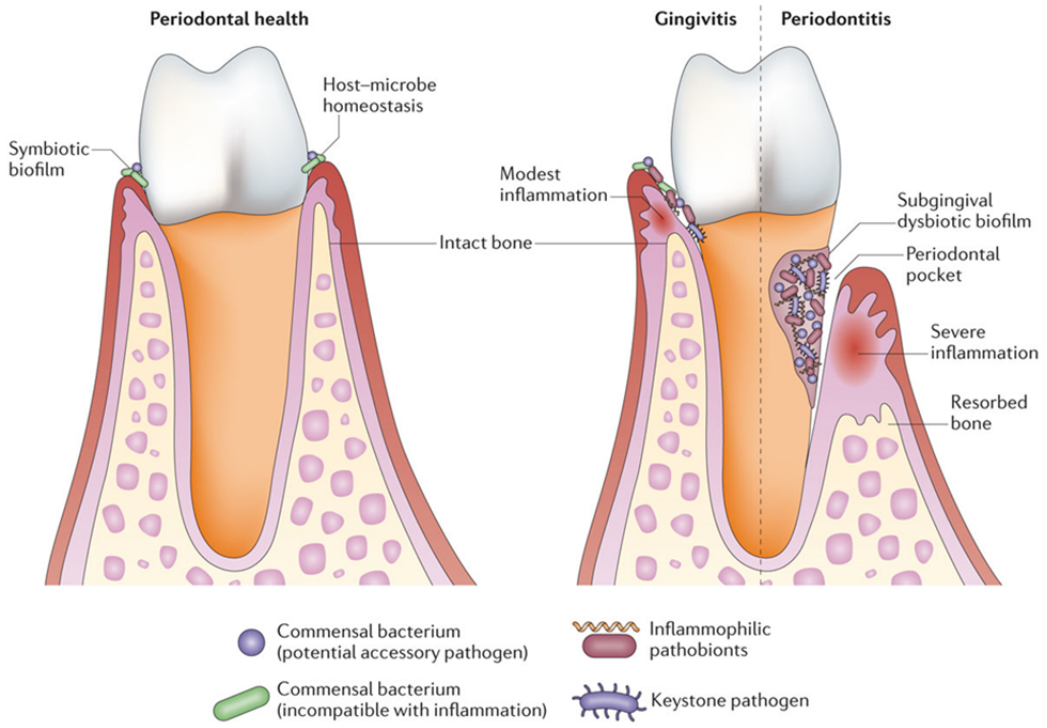


Figure 3 The progression from a periodontal healthy site to gingivitis and periodontitis is depicted. Keystone pathogens initially subvert the host response leading to a dysbiotic microbiota which, together with pathobionts, over-activates the inflammatory response and causes periodontal tissue destruction. [Taken from and modified after (Hajishengallis 2015)].

Periodontitis patients have a higher risk for atherosclerosis, adverse pregnancy outcomes, rheumatoid arthritis, aspiration pneumonia and cancer (Teng *et al.* 2002; Otomo-Corgel *et al.* 2012). The disease is highly prevalent among adult populations in all regions of the world and 10-15% are affected by an advanced state of the disease (severe periodontitis) (Petersen and Ogawa 2005; Dye 2012; Kassebaum *et al.* 2014). Periodontal diseases are classified into eight conditions with gingivitis (periodontal inflammation without bone loss; pocket depth ≤3mm) and periodontitis (formation of periodontal pockets ≥4mm and inflammatory bone loss) being the most prevalent ones (Highfield 2009).

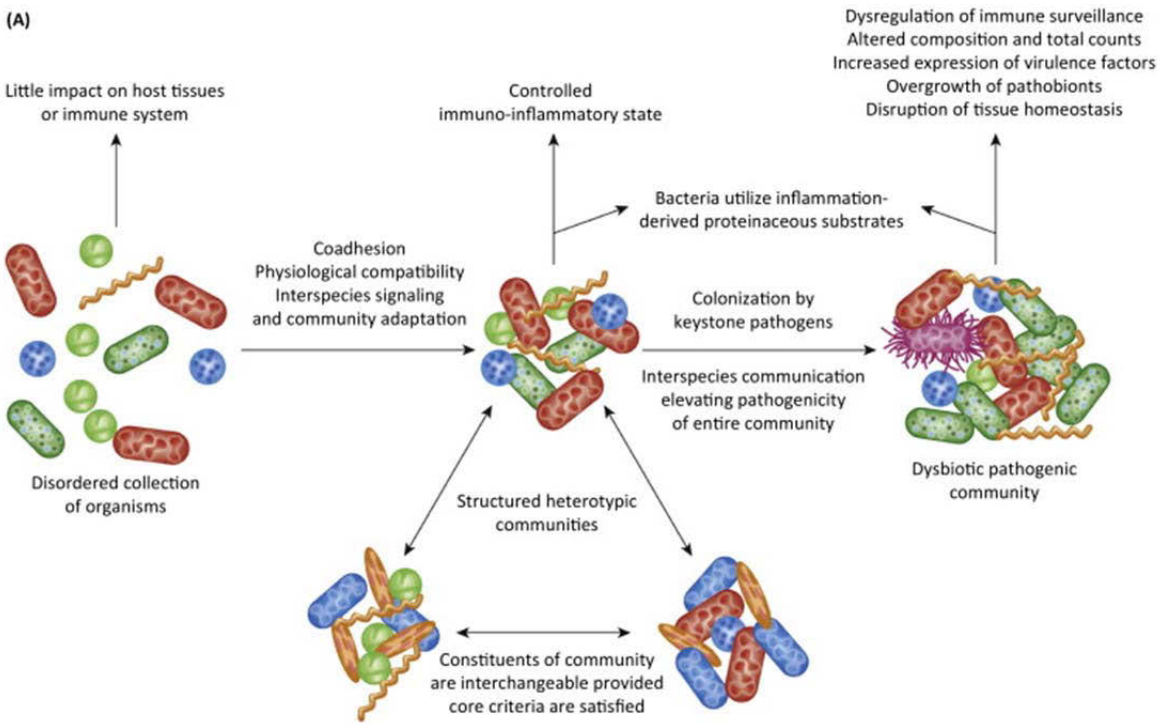


Figure 4 The polymicrobial synergy and dysbiosis model of periodontal disease aetiology. The microbial constituents of the communities can vary over time and colonization with keystone pathogens elevates the virulence of the whole community. Host homeostasis is disrupted and the dysbiotic community increases in number causing destruction of periodontal tissue while progression of the disease. [Taken from (Lamont and Hajishengallis 2015)].

3

Glycoprotein biosynthesis in *Tannerella forsythia*

3.1 Bacterial protein glycosylation in general

3.2 Biological roles of bacterial glycoproteins

3.3 Bacterial glycoprotein biosynthesis

3.4 Bacterial glycosyltransferases

3.5 General protein *O*-glycosylation systems

3.6 The protein *O*-glycosylation gene cluster in *Tannerella forsythia*

Since the end of the almost dogmatic belief, stating that only eukaryotes are able to glycosylate their proteins, many studies have demonstrated, that several Gram-negative and Gram-positive bacteria as well as archaea employ protein glycosylation systems. The combination of advances in analytical techniques and the availability of bioinformatic tools has led to the identification of key enzymes of protein glycosylation (such as glycosyltransferases and oligosaccharyltransferases) and whole protein glycosylation pathways. Studying glycosylation pathways in pathogenic bacteria is essential for understanding disease progressions and has been partially or completely elucidated for *e.g.*, *Campylobacter jejuni*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Neisseria meningitidis* and *Neisseria gonorrhoeae* (Benz and Schmidt 2002; Faridmoayer and Feldman 2010; Nothaft and Szymanski 2010).

Although structure-function relationships of glycoproteins have been established only for a few bacterial glycoproteins, the diversity of observed structures indicates great potential effects of glycoproteins during pathogenesis (Schäffer *et al.* 2001; Schmidt *et al.* 2003; Comstock and Kasper 2006; Tytgat and Lebeer 2014; Schäffer and Messner 2017). Subsequently, the development and production of therapeutic proteins including tailor-made glycans is a promising approach in targeting or interfering with pathogenic bacteria. In understanding and manipulating protein glycosylation processes, the engineering of customized glycoproteins is technically feasible within bacterial expression systems (Baker *et al.* 2013; Merritt *et al.* 2013; Anyaogu and Mortensen 2015; Price *et al.* 2016; Valguarnera *et al.* 2016).

3.1 Bacterial protein glycosylation in general

A categorization of protein glycosylation is based on glycosidic linkages between amino acids and carbohydrate residues (Spiro 2002). Glycans can be covalently attached to the amide nitrogen of an asparagine residue (*N*-glycosylation), to the hydroxyl group of a serine, threonine, hydroxyproline or tyrosine residue (*O*-glycosylation), and in rare cases, to the thiol sulphur of cysteine residues (*S*-glycosylation) (Upreti *et al.* 2003; Hitchen and Dell 2006; Nothaft and Szymanski 2010; Messner *et al.* 2013).

The prokaryotic *N*-linked glycosylation system is best studied in the food-borne pathogen *C. jejuni* (Wacker *et al.* 2002; Szymanski *et al.* 2003). Here, a heptasaccharide is built onto a lipid-linked precursor, undecaprenyl phosphate (und-P), on the inner membrane facing the cytosol. The lipid-linked oligosaccharide is then flipped across the inner membrane into the periplasm where an oligosaccharyltransferase links the carbohydrate onto the asparagine residue of the target protein via the *en bloc* mechanism (Kelly *et al.* 2006). In comparison to eukaryotic *N*-glycosylation, prokaryotes have an extended consensus sequence motif, Asp/Glu-X_1-Asn-X_2-Ser/Thr (X can be any amino acid except proline). More than 65 *C. jejuni* proteins of various functions have been shown to be *N*-glycosylated using this general glycosylation mechanism in *C. jejuni* (Nothaft and Szymanski 2013). Similar *N*-linked protein glycosylation systems and corresponding orthologous gene clusters have been found in other *Campylobacter* species as well as in *Desulfovibrio* and *Wolinella* species (Nothaft and Szymanski 2010).

O-linked glycosylation is a post-translational protein modification prevalent to bacteria. Sugar donors are usually available as nucleotide-activated monosaccharides linked either to UDP (uridine diphosphate), GDP (guanine diphosphate) or CMP (cytosine monophosphate) (Varki *et al.* 2009). So far, no consensus sequences for protein *O*-glycosylation could be defined. The few investigated *O*-glycosylation systems secrete mostly virulence factors or translocate glycoproteins to the cell surface. General protein *O*-glycosylation systems have been described in *Campylobacter* spp. (Szymanski *et al.* 1999; Szymanski *et al.* 2003), *Neisseria* spp. (Ku *et al.* 2009; Vik *et al.* 2009), *Bacteroides* spp. (Fletcher *et al.* 2009), *Acinetobacter* spp. (Iwashkiw *et al.* 2012; Harding *et al.* 2015), *Francisella tularensis* (Egge-Jacobsen *et al.* 2011) and in *Actinomycetes* (Espitia *et al.* 2010).

3.2 Biological roles of bacterial glycoproteins

The majority of known bacterial glycoproteins is either secreted or located at the outer membrane or cell surface. Thus, the involvement of carbohydrates in interaction processes is a clearly suggested role (Benz and Schmidt 2002; Comstock and Kasper 2006). In analogy to eukaryotic glycoproteins, speculative but crucial roles of prokaryotic glycoproteins are for example, conferring protein structure and stability, protection against proteolytic digestion (Herrmann *et al.* 1996), surface recognition and involvement in attachment processes (Kuo *et al.* 1996; Lindenthal and Elsinghorst 2001) or mediating immune responses (van Sorge *et al.* 2009; Varki and Gagneux 2012; Varki 2016).

Depending on their function, glycoproteins can be divided into two groups. Carbohydrate modifications of glycoproteins belonging to the first group of glycoproteins directly affect protein functions, such as subunit interactions or assembly of flagellins and the adherence to host cells. In most of the studied cases, flagellar glycosylation is also linked to bacterial motility and biofilm formation (Szymanski and Wren 2005). The modification of flagellin subunits with Pse5Ac7Ac is absolutely necessary for the assembly of a functional flagellum and consequently bacterial motility as discovered in two *C. jejuni* strains (Goon *et al.* 2003). The necessity for carbohydrate modification of the major outer membrane protein (MOMP) from *Chlamydia trachomatis* for attachment of the infectious elementary body (EB) to HeLa cells was demonstrated (Kuo *et al.* 1996). A further example for interaction processes between glycoproteins and a host cell is given by *Neisseria* spp. Here, the carbohydrate modification of the type IV pili has been identified as an important feature for cell adhesion to eukaryotic cells (Banerjee and Ghosh 2003). Glycoproteins from the second group interact with the immune system of the host. For instance, the degree of glycosylation of the 45/47-kDa Apa (alanine- and proline-rich antigenic) glycoprotein of *Mycobacterium tuberculosis* influences the induction of delayed type hypersensitivity (DTH) responses (Horn *et al.* 1999).

Some pathogenic bacteria employ a different strategy for evading the immune system, termed molecular mimicry. *Neisseria meningitidis* capsules are decorated with sialic acids, which inhibits activation of the complement system and, thus, allows these bacteria to survive in the bloodstream (Kugelberg *et al.* 2008).

3.3 Bacterial glycoprotein biosynthesis

The genetic information for a bacterial protein glycosylation system is generally organized within a protein glycosylation (pgl) gene cluster (Nothaft and Szymanski 2010). Such a gene cluster includes all necessary information for glycoprotein biosynthesis, including nucleotide sugar biosynthesis pathways, glycosyltransferase genes, glycan processing genes and transporter genes. A classification of biosynthetic pathways depending on the presence or absence of a bacterial oligosaccharyltransferase (OTase) is made for *N*- and *O*-glycosylated proteins (Nothaft and Szymanski 2010).

3.3.1 Sequential glycosylation

Simple glycan structures, consisting of a single or only a few different sugar building blocks are assembled by sequential glycosylation. Glycosyltransferases transfer sugars from nucleotide-activated sugar donors in a step-wise manner onto the acceptor protein in the cytoplasm. Once synthesized, the glycoconjugate is transported across the inner membrane into the periplasm. OTase-independent *O*-glycosylation is primarily utilized to decorate flagella and adhesin structures and is best studied in *Campylobacter* spp. and *Pseudomonas* spp. (Logan 2006; Iwashkiw *et al.* 2013).

3.3.2 *En bloc* transfer

The biosynthesis of more complex glycoconjugates is characterized by the *en bloc* transfer and the involvement of an oligosaccharyltransferase. OTases are enzymes transferring a preassembled glycan *en bloc* onto an acceptor protein. An initiating glycosyltransferase attaches a first nucleotide-activated monosaccharide onto the lipid carrier, und-P, residing on the inner side of the cytoplasmic membrane. Subsequently, monosaccharides are added by glycosyltransferases from the corresponding nucleotide-activated monosaccharides onto the growing glycan chain until the complete structure is built up. Upon completion of the fully synthesized glycan, the lipid-linked oligosaccharide is flipped across the cytoplasmic membrane by either a Wzx-like flippase or an adenosine triphosphate (ATP)-binding cassette transporter (ABC transporter) (Greenfield and Whitfield 2012) before the OTase transfers and ligates the glycan to selected serine or threonine residues on the acceptor protein (Iwashkiw *et al.* 2013; Tytgat and Lebeer 2014). The presence of an OTase-dependent mechanism has so far only been described for *N*- and *O*-glycosylation in bacteria, with protein *N*-glycosylation in *C. jejuni* serving as model organism (Szymanski *et al.* 2003; Nothaft and Szymanski 2010).

3.4 Bacterial glycosyltransferases

Glycosyltransferases (GTs) catalyse the formation of glycosidic bonds between an activated donor sugar and a saccharide or non-saccharide acceptor. Consequently, GTs are key enzymes in the biosynthesis of glycoconjugates. Based on sequence homology, 101 families have been classified in the Carbohydrate Active Enzyme database (CAZy; http://www.cazy.org/) to date (Coutinho *et al.* 2003; Lombard *et al.* 2014). However, predicting the sugar donor and the acceptor for a GT of unknown function is challenging and, thus, requires experimental verification.

Two major structural folds have been revealed for glycosyltransferases by protein X-ray crystallography, GT-A and GT-B (Lairson et al. 2008; Gloster 2014). GT-A enzymes are generally metal-dependent with a characteristic DXD motif and consist of two closely linked α/β/α nucleotide binding domains (Rossmann fold). GT-B enzymes are metal-independent and consist of two α/β/α domains separated by a flexible linker region and an interdomain cleft. The DXD motif interacts primarily with the phosphate groups of the nucleotide donor through the coordination of a divalent cation (Breton 2005). Additionally, novel folds are being uncovered, as exemplified, for instance, by the bacteriophage-lysozyme-fold reported for the peptidoglycan synthase PBP2 (penicillin-binding protein 2) (Lovering *et al.* 2007), the fold for the oligosaccharyltransferase STT3 found in *Pyrococcus furiosus* (Igura *et al.* 2008) or a sialyltransferase CstII from *C. jejuni* (Chiu *et al.* 2004).

Based on the stereochemical outcome of the formation of a new glycosidic bond, catalysis by glycosyltransferases can be categorized into two mechanisms. First, inverting glycosyltransferases utilize a S_N2-like mechanism where one bond is broken and one bond is formed simultaneously, involving an enzymatic base catalyst and Lewis acid activation of the substituted phosphate leaving group. As a result, the anomeric configuration of the reaction center from the donor substrate is inverted (*e.g.*, UDP-glucose → β-glucoside) (Lairson *et al.* 2008; Breton *et al.* 2012). Retaining glycosyltransferases retain the stereochemistry of the donor glycosidic linkage after transfer to an acceptor molecule (*e.g.,* UDP-glucose → α-glucoside). The mechanism of retaining glycosyltransferases is less clear. They employ a proposed double-displacement mechanism involving a short-lived covalently bound glycosyl-enzyme intermediate (Lairson *et al.* 2008; Breton *et al.* 2012). A report based on re-evaluation of structural and kinetic data, however, suggests a single step orthogonal (front-side) displacement reaction (Schuman *et al.* 2013). Interestingly, there is no correlation of the overall fold with catalytic mechanism. Inverting and retaining enzymes are known with both GT-A and GT-B topologies.

3.5 General protein *O*-glycosylation systems

General *O*-linked protein glycosylation systems are encoded in protein glycosylation gene clusters and target structurally and functionally diverse groups of proteins for glycosylation (Nothaft and Szymanski 2010). In *Neisseria gonorrhoeae*, 11 glycoproteins have been identified to carry a diNAcBac-$(Gal)_2$ trisaccharide (Vik *et al.* 2009; Hartley *et al.* 2011). Most of these glycoproteins have a predicted localization within the cell and are implicated in activities such as protein folding, disulphide bond formation as well as aerobic and anaerobic respiration processes (Vik *et al.* 2009). Key enzymes of the glycosylation system are responsible for the biosynthesis of the nucleotide-activated carbohydrate unit UDP-diNAcBac (including the enzymes PglD, PglC, PglB-acetyltransferase domain) the transfer of the first unit onto the und-P lipid carrier (PglB-phospho-glycosyltransferase domain) and the addition of two sequential galactose units (PglA, PglE) to complete the trisaccharide precursor prior to flipping the glycan into the periplasmic space (PglF). In the final step, the transfer of the glycan onto serine residues of the acceptor protein is performed by the OTase (PglO) in an *en bloc* transfer reaction (Hartley *et al.* 2011). *Neisseria meningitidis* employs a highly homologous glycosylation system adding either an *O*-linked di- or trisaccharide onto its pili (Power *et al.* 2003; Gault *et al.* 2015).

To date a general protein *O*-glycosylation system has likewise been described to be present in the opportunistic human pathogen *Acinetobacter baumanii* (Lees-Miller *et al*. 2013; Harding *et al*. 2015), the human and plant pathogen *Burkholderia cepacia* complex (Bcc) common to cystic fibrosis patients (Lithgow *et al.* 2014) and in one of the most lethal plant pathogens, *Ralstonia solanacearum* (Elhenawy *et al.* 2016).

3.6 The protein *O*-glycosylation gene cluster in *Tannerella forsythia*

The protein *O*-glycosylation gene cluster of *T. forsythia* ATCC 43037 spans an area of 27-kb and comprises five glycosyltransferases, three putative glycan modifying methyltransferases and all six genes responsible for the biosynthesis of the CMP-activated pseudaminic acid. The first gene of the cluster is a *wzx* flippase-like gene (*Tanf_01180*; *TF2076*) which is followed by genes encoding the synthesis of CMP-pseudaminic acid (*pseB, pseC, pseF, pseH, pseG, pseI*). Downstream, glycosyltransferases (named GtfS, GtfM, GtfI, GtfL, GtfE), methyltransferases (named MtfJ, MtfO, MtfY) and further carbohydrate-modifying enzymes (AsnB, WecC, WecB) are encoded (Figure 5). Individual single gene knock-outs targeted at all five glycosyltransferase genes within the gene cluster, experienced step-wise truncations of the glycan (Figure 6), as SDS-PAGE, Western immunoblots and mass spectrometry analyses revealed (Figure 7).

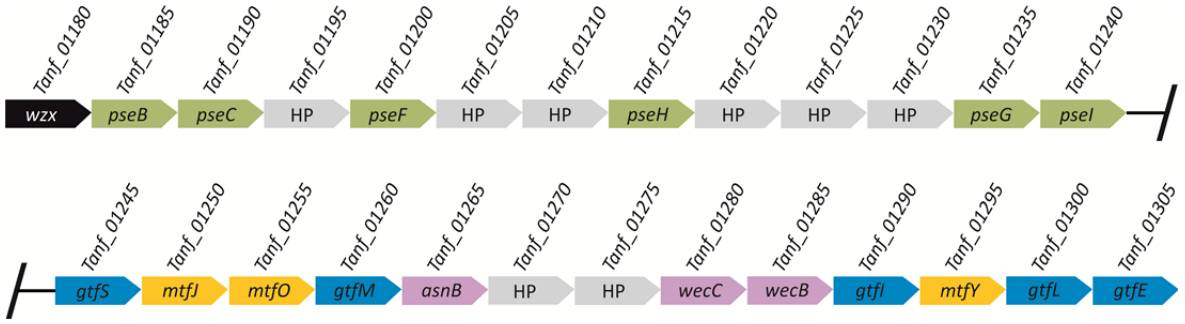


Figure 5 The 27-kb protein *O*-glycosylation gene cluster of *T. forsythia* ATCC 43037. *wzx* (black), flippase; *pseBCFHGI* (green), pseudaminic acid biosynthesis genes; *gtfSMILE* (blue), glycosyltransferase genes; *mtfJOY* (yellow), methyltransferase genes; *asnB* (put. asparagine synthetase B), *wecC* (UDP-*N*-acetyl-D-mannosamine dehydrogenase) and *wecB* (UDP-*N*-acetylglucosamine 2-epimerase) (purple); hypothetical proteins, HP (grey) (**M.B. Tomek, unpublished data**).

Western immunoblots probed with anti-TfsA anti-serum and anti-TfsB antiserum confirmed the identity of the S-layer glycoproteins and showed a clear down-shift of both S-layer glycoproteins in glycosyltransferase deficient strains, while reconstituted strains regained their native migration profile when compared to the wild-type strain (Figure 7B). In a *gtfS* (Δ*Tanf_01245*; Δ*TF2062*) knock-out mutant, the terminal pseudaminic acid unit of the *O*-glycan (compare with Figure 2) is absent, suggesting that the encoded protein is an α-2,4 pseudaminic acid transferase (**Tomek *et al.* 2017**). A mutant strain deficient in *gtfM* (Δ*Tanf_01260;* Δ*TF2060*), lacks a mannosaminuronic acid unit in addition to the terminal pseudaminic acid, suggesting a β-1,3 *N*-acetylmannosaminuronic acid transferase activity. The glycan is further truncated when knocking-out *gtfI* (Δ*Tanf_01290*; Δ*TF2053*). Here, the three-sugar containing branch composed of pseudaminic acid and two *N*-acetyl mannosaminuronic acid residues, is absent. Results from a *gtfL* (Δ*Tanf_01300*; Δ*TF2050*) deficient strain are ambiguous, as a 146.0 *m/z* cannot be assigned to the known *O*-glycan structure. A *gtfE* (Δ*Tanf_01305*; Δ*TF2049*) knock-out mutant results in a glycan comprised of the five carbohydrate core glycan structure. Therefore, the protein putatively transfers the branching α-1,2 galactose unit (**M.B. Tomek, unpublished data**) (Figure 7C). Pseudaminic acid is generated from UDP-*N*-acetylglucosamine in a five-step biosynthetic pathway. Deletion of the essential aminotransferase PseC (Δ*pseC*, Δ*Tanf_01190*; Δ*TF2074*) results in an incomplete synthesis of pseudaminic acid, which is, thus, absent on the *O*-glycan (Friedrich *et al.* 2017).

To date, a *T. forsythia O*-OTase transferring the glycan onto the acceptor proteins could not be identified and genes responsible for the assembly of the five-carbohydrate core glycan remain elusive, too.

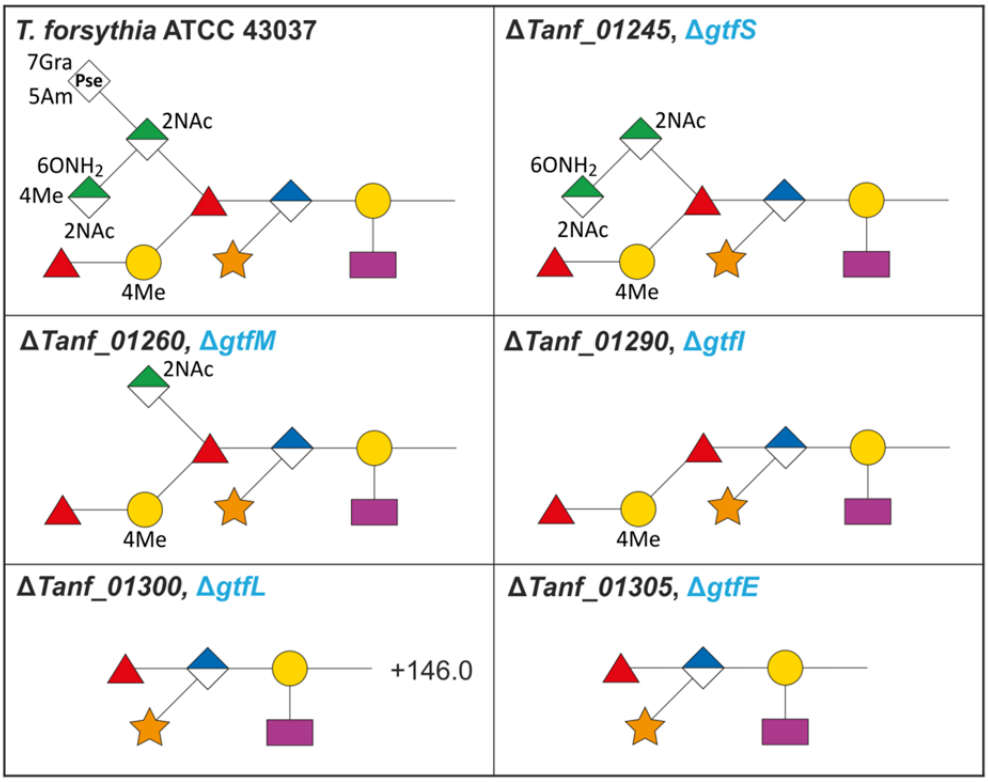


Figure 6 Schematic drawing of the protein *O*-glycan in *T. forsythia* ATCC 43037 wild-type and the truncated glycan form from glycosyltransferase deficient mutants (Δ*gtfSMILE*) (**M.B. Tomek, unpublished data**).

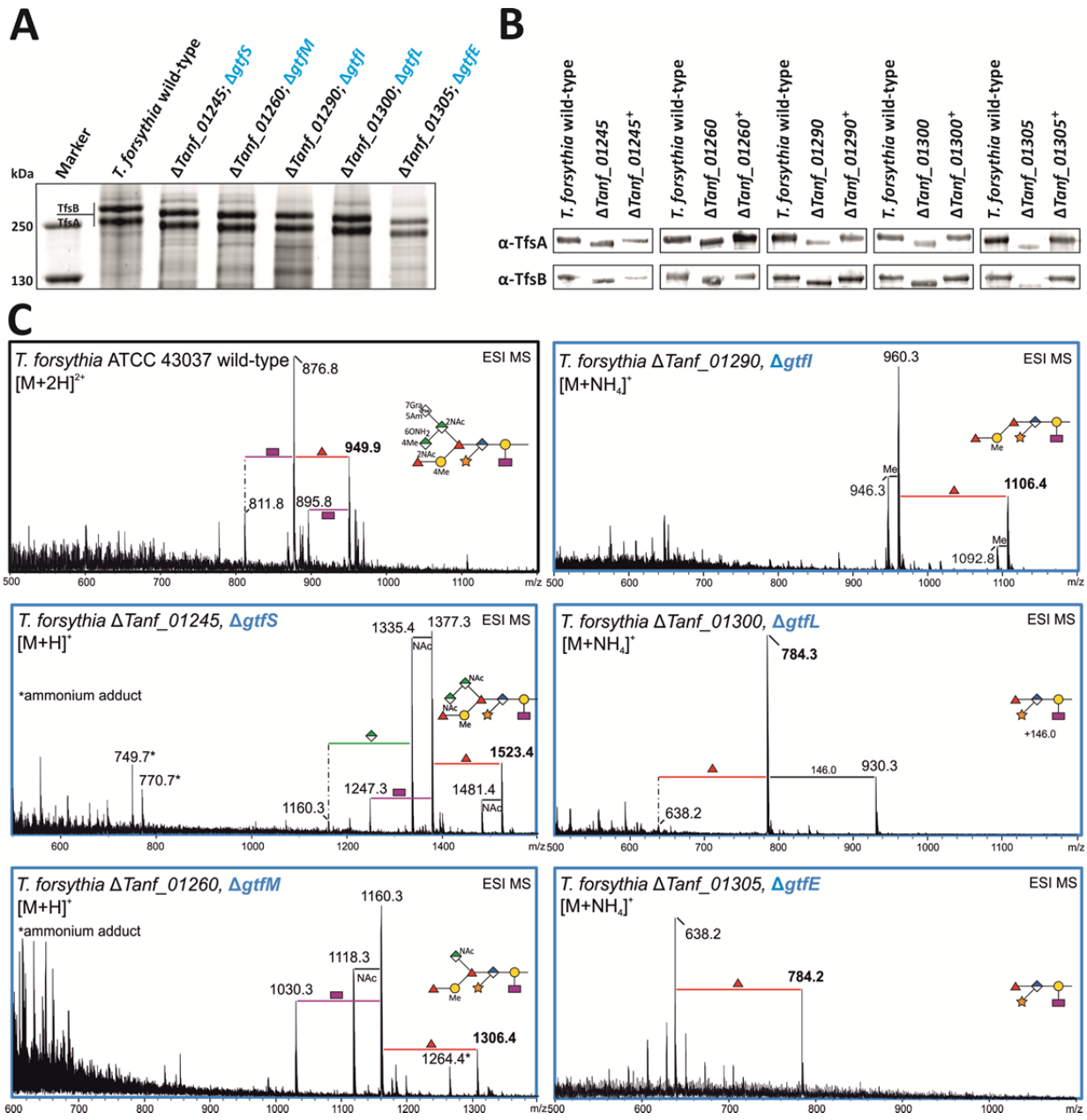


Figure 7 (**A**) Coomassie Brilliant Blue staining of crude cell extracts from *T. forsythia* ATCC 43037 wild-type and glycosyltransferase deficient mutants (Δ*gtfSMILE*) after separation on a 7.5% SDS-PA gel. The S-layer glycoproteins (labelled TfsA and TfsB) are indicated and the down-shift resulting from the truncated glycan can be observed in the mutants. The S-layer glycoprotein bands were further processed for MS analyses. PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific) was used as a protein molecular weight marker. (**B**) Western immunoblots probed with anti-TfsA anti-serum and anti-TfsB antiserum confirmed the identity of the S-layer glycoproteins in all analysed *T. forsythia* species. All glycosyltransferase deficient mutants (Δ*gtfSMILE*) experienced down-shifts resulting from the truncated glycan, whereas the reconstituted strains (denoted with a plus sign) migrated at the same height compared to the wild-type strain on a 7.5% SDS-PA gel. The migration profiles of the reconstituted strains indicate the full mature glycan form, thus, proving successful recombinations. (**C**) Deconvoluted ESI-IT MS sum spectra of β-eliminated TfsB *O*-glycans from *T. forsythia* wild-type and mutant strains. The glycan structures of the highest mass peaks (written in bold) are shown as symbolic representations. Mass peaks from the subsequent fragmentation pattern were assigned according to the loss of carbohydrate units and modifications. Relative peak intensities of occurring peaks are given on the *y* axis (**M.B. Tomek, unpublished data**).

Methylation of bacterial glycans is a rarely reported event. Although it has been discussed as a stop-signal for glycan chain elongation, clear determination of its biological function is still missing, especially when occurring at multiple sites of the glycan. Only a few data can be found in the literature concerning bacterial glycoproteins. *Mycobacterium tuberculosis* synthesizes *O*-methylated rhamnose units which can be di- or trimethylated. Further, a methylated glucuronic acid was described to be present in *Mycobacterium habana* TMC5135. Some *Rhizobium* strains have *O*-methylated fucoses, *O*-methylated rhamnose and *N*-acetylglucosamine residues have been identified, too (Staudacher 2012).

Knowledge of corresponding methyltransferases acting on sugar residues is scarce. Methyltransferases are known in *Mycobacterium smegmatis* where they sequentially methylate rhamnose at the C-3 (Mtf1), C-4 (Rmt4) and C-2 position (Rmt2) (Jeevarajah *et al.* 2002). Methylation of a galacturonosyl residue of a teichuronic-type polysaccharide at the C-2 position was identified in *Rhizobium meliloti* (Ruiz and Ugalde 1998). It remains to be investigated, if methylation is performed as a final modification of the synthesized glycan utilizing S-adenosylmethionine or if glycosyltransferases are able to transfer a methyl sugar from its nucleotide-activated form onto a growing oligosaccharide.

Three putative methyltransferases (MtfJ, MtfO, MtfY) are encoded within the protein *O*-glycosylation gene cluster of *T. forsythia,* while only two methyl groups are present on the bacterium´s protein *O*-glycan (compare with Figure 2). Single gene knock-outs were generated to further investigate these methyltransferases. Both mutant strains, deficient of the methyltransferase genes *mtfJ* (Δ*Tanf_01250*) or *mtfO* (Δ*Tanf_01255*), respectively, experienced the loss of the 4-*O*-methyl group at the terminal *N*-acetylmannosaminuronic acid residue, as MS analyses revealed. A *mtfY* knock-out mutant (Δ*Tanf_01295*) however, lacked the 4-*O*-methyl group on the proximal galactose residue (Figure 8) (**M.B. Tomek, unpublished data**).

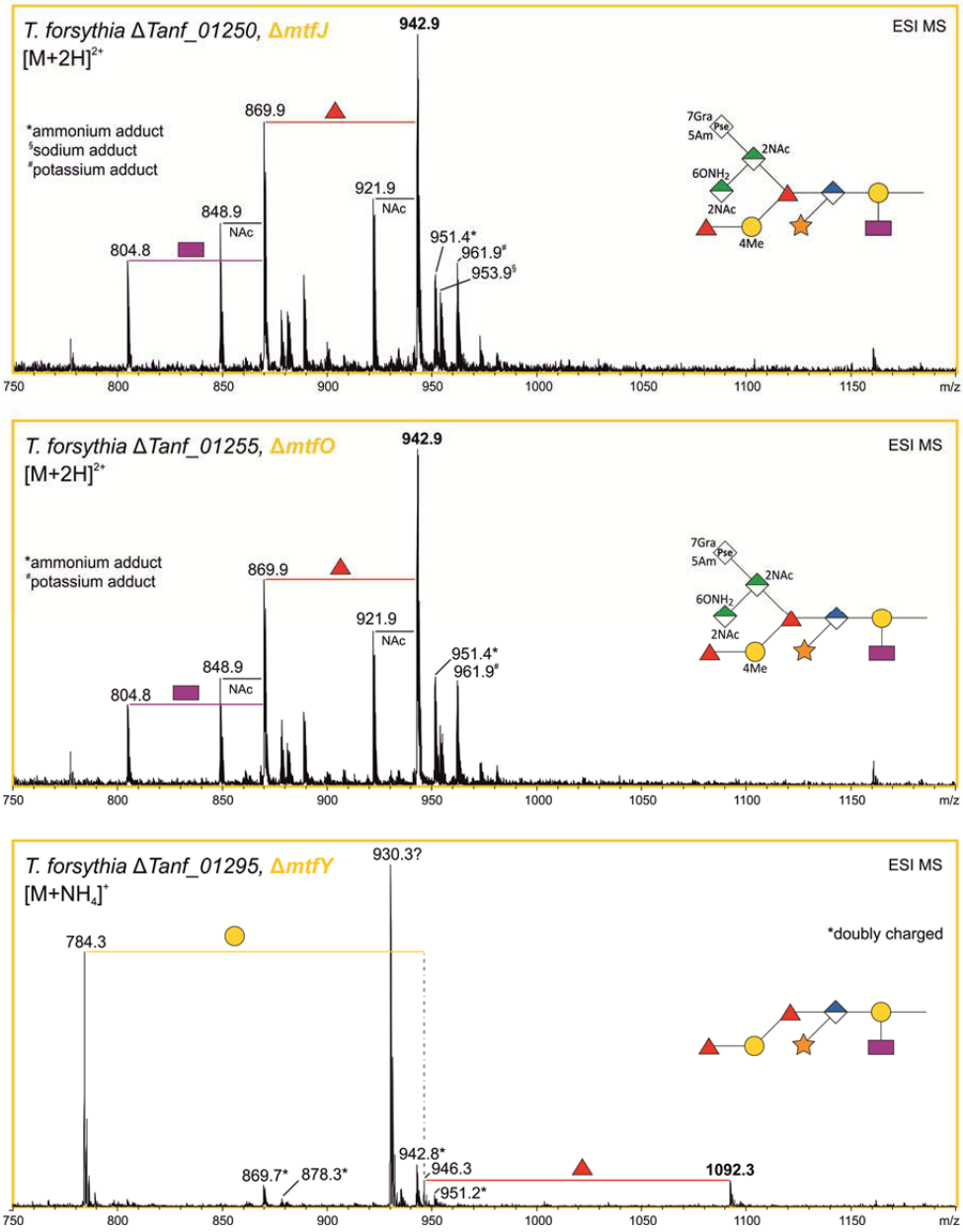


Figure 8 Deconvoluted ESI-IT MS sum spectra of β-eliminated TfsB *O*-glycans from three *T. forsythia* ATCC 43037 methyltransferase knock-out mutants. The glycan structure of the highest mass peak (written in bold) is shown as symbolic representation. Mass peaks from the subsequent fragmentation pattern were assigned according to the loss of carbohydrate units and modifications. Relative peak intensities of occurring peaks are given on the *y* axis (**M.B. Tomek, unpublished data**).

3.7 Publication 1: A pseudaminic acid or a legionaminic acid derivative transferase is strain-specifically implicated in the general protein *O*-glycosylation system of the periodontal pathogen *Tannerella forsythia*

M.B. Tomek, B. Janesch, D. Maresch, M. Windwarder, F. Altmann, P. Messner and C. Schäffer. *Glycobiology*, in press, doi: 10.1093/glycob/cwx019

Journal: Glycobiology

DOI: 10.1093/glycob/cwx019

Title: A pseudaminic acid or a legionaminic acid derivative transferase is strain-specifically implicated in the general protein *O*-glycosylation system of the periodontal pathogen *Tannerella forsythia*

Oxford Open Licence

GLYCO-2016-00160.R1

A pseudaminic acid or a legionaminic acid derivative transferase is strain-specifically implicated in the general protein *O*-glycosylation system of the periodontal pathogen *Tannerella forsythia*

[1,§]Markus B. Tomek, [1,§,x]Bettina Janesch, [2]Daniel Maresch, [2,y]Markus Windwarder, [2]Friedrich Altmann, [1]Paul Messner and [1]Christina Schäffer*

[1]Department of NanoBiotechnology, *NanoGlycobiology* unit, Universität für Bodenkultur Wien, Muthgasse 11, A-1190 Vienna, Austria.

[2]Department of Chemistry, Universität für Bodenkultur Wien, Muthgasse 18, A-1190 Vienna, Austria.

[§]These authors contributed equally to this work

Key words: *Bacteroidetes* / glyco-engineering / glycosyltransferase / nonulosonic acids / periodontitis

Supplementary data: Supplementary Figures 1-3

[x]Present address: Ryerson University Biomedical Research Facilities, 661 University Avenue, Toronto, Ontario, M5G 0A3, Canada

[y]Present address: Shire, Uferstraße 15, A-2304 Orth, Austria

* To whom correspondence should be addressed: Christina Schäffer: Tel: +43-1-47654-80203; Fax: +43-1-4789112; e-mail: christina.schaeffer@boku.ac.at

Abstract

The occurrence of nonulosonic acids in bacteria is wide-spread and linked to pathogenicity. However, knowledge of cognate nonulosonic acid transferases is scarce. In the periodontopathogen *Tannerella forsythia*, several proposed virulence factors carry strain-specifically either a pseudaminic or a legionaminic acid derivative as terminal sugar on an otherwise structurally identical, protein-bound oligosaccharide. This study aims to shed light on the transfer of either nonulosonic acid derivative on a proximal *N*-acetyl mannosaminuronic acid residue within the *O*-glycan structure, exemplified with the bacterium's abundant S-layer glycoproteins. Bioinformatic analyses provided the candidate genes *Tanf_01245* (strain ATCC 43037) and *TFUB4_00887* (strain UB4), encoding a putative pseudaminic and a legionaminic acid derivative transferase, respectively. These transferases have identical C-termini and contain motifs typical of glycosyltransferases (DXD) and bacterial sialyltransferases (D/E-D/E-G and HP). They share homology to type B glycosyltransferases and TagB, an enzyme catalyzing glycerol transfer to an *N*-acetylmannosamine residue in teichoic acid biosynthesis. Analysis of a cellular pool of nucleotide-activated sugars confirmed the presence of the CMP-activated nonulosonic acid derivatives, which are most likely serving as substrates for the corresponding transferase. Single gene knock-out mutants targeted at either transferase were analyzed for S-layer *O*-glycan composition by ESI-MS, confirming the loss of the nonulosonic acid derivative. Cross-complementation of the mutants with the non-native nonulosonic acid transferase was not successful indicating high stringency of the enzymes. This study identified plausible candidates for a pseudaminic and a legionaminic acid derivative transferase; these may serve as valuable tools for engineering of novel sialoglycoconjugates.

Introduction

Glycosylation as the most frequent modification of proteins (Faridmoayer and Feldman 2010, Messner 1997, Messner et al. 2013, Nothaft and Szymanski 2010, Ohtsubo and Marth 2006, Schäffer and Messner 2017, Tytgat and Lebeer 2014, Varki 2006) is well known to modify protein properties, endowing them with a wide repertoire of glycan-mediated functions. These can be as diverse as enabling site-directed delivery of glycoconjugates as well as mediating signaling events and modulating cell adhesion processes (Taylor and Drickamer 2003, Varki 1993). In the context of bacterial physiology, glycosylation can trigger the colonization of a specific host (region). Due to the increasing discovery of glycoconjugates in pathogenic bacteria, their investigation in a biomedical context is of great relevance. Especially bacterial cell surface glycans which represent the immediate contact zone of bacteria with the environment/host, seem to be prone to act as specific ligands for cell-cell or cell-bacterium interactions, or to serve as virulence factors based on molecular mimicry of host glycans (Varki et al. 2009).

Nonulosonic acids are particularly important at the interface of bacterial pathogenicity and human physiology (Morrison and Imperiali 2014, Varki 2008). They share a nine-carbon carbohydrate monomer as a common core structure, with additional structural variations mostly occurring at the C-5 and C-7 leading to over 50 derivatives identified so far across archaea, bacteria and eukaryotes (Angata and Varki 2002, Knirel et al. 2003). Sialic acids (Sia; neuraminic acid) carrying an *N*-acetyl group at the C-5 are the most abundant naturally occurring and best studied nonulosonic acids. Two exclusively bacterial derivatives, present in pathogens, are pseudaminic acid (5,7-bis(acetylamino)-3,5,7,9-tetradeoxy-L-*glycero*-α-L-*manno*-non-2-ulopyranosonic acid; Pse) and legionaminic acid (5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid; Leg) (Zunk and Kiefel 2014).

The exact biological roles of pseudaminic acid and legionaminic acid and their derivatives are not yet fully understood. The modification of flagellins with these nonulosonic acids in *Campylobacter* and *Helicobacter* species confers bacterial virulence in facilitating bacterial host interactions (Schirm et al. 2003, Thibault et al. 2001). In two *Campylobacter jejuni* strains, the modification of flagellin subunits with Pse5Ac7Ac was found to be necessary for the assembly of a functional flagellum and, consequently, bacterial motility (Goon et al. 2003). Further, the structural similarity of pseudaminic acid and legionaminic acid to eukaryotic sialic acid indicates molecular mimicry as a basic strategy these pathogens may employ to evade the host immune response (Knirel et al. 2003, Vimr et al. 2004). However, it should be noted here that that Pse and Leg have different stereochemistry, making Leg a potentially better mimic of host neuraminic acids than Pse.

The complete five-step biosynthesis pathway of cytidine monophosphate-activated pseudaminic acid (CMP-Pse) representing the biologically active form of pseudaminic acid for its incorporation into glycoconjugates has been characterized in detail in *Helicobacter pylori* (Schoenhofen et al. 2006), the pathway leading to CMP-Leg has been elucidated in *C. jejuni* (Schoenhofen et al. 2009). However, the subsequent and necessary transfer step of the nonulosonic acid from its nucleotide activator onto the acceptor - which might be the glycosylation site on a target polypeptide or a sugar residue within an oligosaccharide - by a dedicated nonulosonic acid transferase remains elusive. So far, the only report of a candidate pseudaminic acid transferase found in the literature concerns the motility-associated factor Maf1 predicted to be involved in the transfer of pseudaminic acid onto the flagellin of *Aeromonas caviae* (Parker et al. 2012). This was concluded from pull-down experiments between Maf1 and the flagellin, without provision of direct evidence of enzymatic function (Parker et al. 2014). With regards to legionaminic acid transferases, no predictions of such enzymes are presently available neither in the literature nor in databases. Interestingly,

selected sialyltransferases, *i.e.,* porcine ST3Gal1, *Pasteurella multocida* sialyltransferase, *Photobacterium* α2,6-sialyltransferase, and *Neisseria meningitidis* MC58 α2,3-sialyltransferase, were shown to accept CMP-Leg5Ac7Ac as a donor substrate to replace Sia as terminal sugar (Watson et al. 2011, 2015).

Tannerella forsythia provides the unique situation of a bacterium that strain-specifically displays either a pseudaminic or a legionaminic acid derivative as terminal sugar on an otherwise structurally very similar, protein-bound oligosaccharide (Friedrich et al. 2016, Posch et al. 2011). *Tannerella forsythia* is a Gram-negative bacterium that is recognized as a key periodontal pathogen (Holt and Ebersole 2005, Socransky et al. 1998) following the polymicrobial synergy and dysbiosis model of periodontal disease etiology (Hajishengallis 2014, Hajishengallis and Lamont 2012). The molecular basis of its pathogenicity is only slowly unravelling. Among several identified virulence factors (Sharma 2010, Veith et al. 2009), such as the outer membrane protein BspA (Onishi et al. 2008), KLIKK-proteases (Ksiazek et al. 2015) and outer membrane vesicles (Friedrich et al. 2015a, Veith et al. 2015), are the two glycosylated cell surface (S-) layer proteins TfsA and TfsB which self-assemble into a 2D crystalline array on the bacterial cell surface, completely covering the outer membrane (Posch et al. 2012, Sabet et al. 2003, Sekot et al. 2011). We have shown for the *T. forsythia* ATCC 43037 type strain that the S-layer proteins as well as several other cell surface and outer membrane proteins of *T. forsythia* are modified at multiple sites at the conserved D(S/T)(A/I/L/V/M/T) motif with the same *O*-linked dekasaccharide (Fletcher et al. 2009, Posch et al. 2011) which displays a pseudaminic acid with an acetamidino (Am) group at C-5 and a glyceric acid at C-7 (Gra) - Pse5Am7Gra - as terminal, non-reducing end residue.

The biosynthesis of CMP-Pse in *T. forsythia* ATCC 43037 was only recently shown to be encoded by a dedicated gene locus (Friedrich et al. 2016) present in immediate vicinity to the general protein *O*-glycosylation gene cluster of *T. forsythia* ATCC 43037 (Posch et al.

2011). In fact, we expressed and confirmed the activity of all five necessary enzymes from the CMP-Pse biosynthetic pathway in that strain (Friedrich et al. 2016), which proceeds in analogy to what has been described for *H. pylori* (Schoenhofen et al. 2006). Since candidates for the enzymes modifying the pseudaminic acid in *T. forsythia* ATCC 43037 have not been identified so far, a crucial point still to be answered concerns the biosynthetic stage at which the modifications are transferred onto the nonulosonic acid.

On the genome of *T. forsythia* UB4 (Genbank accession number FMMN00000000; (Stafford et al. 2016)) the genes encoding the biosynthetic enzymes for CMP-Leg are replacing those for CMP-Pse in strain ATCC 43037. This was confirmed in step-wise *in vitro* assays using the recombinant enzymes (Friedrich et al. 2016), based on the knowledge of the CMP-Leg biosynthetic pathway in *C. jejuni* (Schoenhofen et al. 2009). In terms of basic sugar composition and glycan structure, the *O*-glycan of strain UB4 was found to be identical to that of strain ATCC 43037 (Posch et al. 2011) apart from a mass defect of 29 Da on the terminal Leg residue, likely reflecting different non-carbohydrate substituents as compared to the Gra and Am modifications of the ATCC 43037 pseudaminic acid residue, as well as a missing methyl group on the proximal *N*-acetyl mannosaminuronic acid residue (-14 Da) (Friedrich et al. 2016).

Considering that the *T. forsythia* S-layer is classified as a virulence factor (Sharma 2010), it might well be that the involvement of the S-layer in the cell adhesion and invasion capability of the bacterium (Sakakibara et al. 2007) as well as in the delay of the host immune response against the bacterium (Honma et al. 2007, Sekot et al. 2011) is impacted by the bacterium's nonulosonic acids.

In this study, we investigated a putative pseudaminic acid derivative transferase (Tanf_01245) from the oral pathogen *T. forsythia* ATCC 43037 and a putative legionaminic acid derivative transferase (TFUB4_00887) from the clinical isolate *T. forsythia* UB4, using

the abundant *T. forsythia* S-layer glycoproteins as a model system (Posch et al. 2011). Specifically, this included (i) construction of *T. forsythia* deletion mutants targeted at the candidate nonulosonic acid derivative transferases and subsequent determination of the effect of the gene deletion on S-layer *O*-glycan composition by mass spectrometry; (ii) analysis of the mutants for their cellular pool of nucleotide-activated sugars in order to exclude interference of the candidate transferases with the biosynthetic pathways of the nonulosonic acid precursor and to unravel if the nonulosonic acid modifications are present already at the CMP-bound state; and (iii) a cross-complementation experiment of the nonulosonic acid derivative transferase mutants with the non-native *T. forsythia* enzyme to learn about the specificity of the enzymes, considering that the core structures of pseudaminic acid and legionaminic acid are stereoisomers and that these residues would be transferred onto the same sugar acceptor residue within the S-layer *O*-glycan structure.

This is the first report on plausible enzyme candidates for the transfer of a pseudaminic acid (Pse5Am7Gra) and a legionaminic acid derivative as a terminal residue onto a glycoprotein glycan.

Results

Bioinformatic analyses of candidate genes for the transfer of nonulosonic acids in *T. forsythia*

Recent findings from our laboratory revealed the presence of a functional biosynthetic pathway for either CMP-Pse or CMP-Leg in different *T. forsythia* strains (ATCC 43037, UB4 and 92A2, respectively) (Friedrich et al. 2016), elaborated in the course of the general *T. forsythia* protein *O*-glycosylation system (Markus Tomek, Valentin Friedrich,

Christina Schäffer, unpublished data). To investigate the subsequent transfer step of the activated nonulosonic acid derivative on a proximal *N*-acetyl mannosaminuronic acid residue as present in *T. forsythia* protein *O*-glycans, we were focusing here on two genes encoding putative nonulosonic acid derivative transferases. The candidate pseudaminic acid derivative transferase gene *Tanf_01245* is located immediately down-stream of the CMP-Pse biosynthesis locus on the *T. forsythia* ATCC 43037 genome and the candidate Leg-derivative transferase gene *TFUB4_00887* is found on the *T. forsythia* UB4 genome down-stream of the CMP-Leg biosynthesis gene locus, separated only by a putative methyltransferase (Friedrich et al. 2016). The predicted transferases Tanf_01245 (445 amino acids; calculated molecular weight, 51.9 kDa) and TFUB4_00887 (442 amino acids; calculated molecular weight, 51.3 kDa) share 81% amino acid sequence identity. Precisely, the sequences of the 241-amino acid long C-terminal domains are identical in both proteins (Figure 1A). A DXD motif starting at position D205 (strain ATCC 43037) and D202 (strain UB4), respectively, is present at the beginning of the conserved C-terminal domain (Figure 1A). This short motif is found in many families of glycosyltransferases, which add a range of different sugars to other sugars, phosphates and proteins. All DXD-containing glycosyltransferases use nucleoside diphosphate sugars as donors and require divalent cations, typically manganese (Breton et al. 2005). Usually, however, DXD-motifs are absent in sialyltransferases (Brockhausen 2014), which do not require divalent metal ions for enzymatic activity. Two recently identified functional motifs (D/E-D/E-G and HP) found in *Neisseria meningitidis* (*Nm*B-polyST) and *Pasteurella multocida* (*Pm*ST1), are highly conserved in bacterial sialyltransferases and important to enzyme catalysis and CMP-Neu5Ac binding (Freiberger et al. 2007). Both motifs are present in the putative nonulosonic acid transferases studied here (Figure 1A). Further bioinformatic analyses (Basic Local Alignment Search Tool - BLAST at http://blast.ncbi.nlm.nih.gov/Blast.cgi) suggested a glycosyltransferase B-type

superfamily domain using NCBI´s conserved domain database (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, version CDD v3.15) or a putative conserved TagB superfamily domain (version CDD v3.14) (Marchler-Bauer et al. 2015), spanning the same amino acid residues, to be present in both proteins (Figure 1B). The TagB protein has been implicated in the priming step of poly(glycerol phosphate) wall teichoic acid synthesis in *Bacillus subtilis* (Swoboda et al. 2010). There, TagB adds a single glycerol phosphate residue to the non-reducing end of undekaprenyl-phosphate-linked *N*-acetylmannosamine-β(1,4)-*N*-acetylglucosamine-1-phosphate, which reveals analogy in basic sugar structure with the *N*-acetylmannosaminuronic acid residue present as acceptor in the *T. forsythia* *O*-glycan. Secondary structure predictions performed at http://bioinf.sce.carleton.ca/PCISS/start.php indicate for *T. forsythia* ATCC 43037

Tanf_01245 and *T. forsythia* UB4 TFUB4_00887, an α-helix content of 34.38% and 32.58%, a β-sheet content of 20.90% and 22.40%, respectively, and 45% turns, each. No transmembrane regions are predicted for the nonulosonic acid derivative transferases using the prediction server TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

To obtain insight in the prevalence of the predicted nonulosonic acid derivative transferases in *T. forsythia* strains, we included in the multiple sequence alignment further sequences of homologous proteins from other publically available genomes of *T. forsythia* strains, including Tanf_01245 (strain ATCC 43037), TFUB4_00887 (strain UB4), TFUB20_01003 (strain UB20), BFO_1060 (strain FDC 92A2), TFKS16_1100 (KS16), TFUB22_00887 (strain UB22), and TF3313_0988 (strain 3313), revealing 246 identical C-terminal amino acid residues in all compared sequences. Based on differences at the amino acid level in the N-terminal regions, all aligned proteins except TF3313_0988 matched either the Tanf_01245 sequence from strain ATCC 43037 or the TFUB4_00887 sequence from

strain UB4 (Figure 1A). This result is also reflected by analyzing the phylogenetic relationship of nonulosonic acid transferases (Jones et al. 1992, Kumar et al. 2016) (Figure 1C).

The migration behavior of S-layer glycoproteins on SDS-PAGE is affected by knocking-out the putative Pse and Leg transferases

The glycosylated S-layer proteins TfsA and TfsB are the most abundant and best characterized glycoproteins in *T. forsythia* strains (Posch et al. 2011, Sekot et al. 2012) and, thus, were chosen as model glycoproteins for investigating the putative nonulosonic acid derivative transferases.

To assess an overall involvement of Tanf_01245 (candidate Pse derivative transferase) and TFUB4_00887 (candidate Leg derivative transferase) in S-layer protein glycosylation, the SDS-PAGE pattern of the respective gene deletion mutants *T. forsythia* ATCC 43037 Δ*Tanf_01245* and *T. forsythia* UB4 Δ*TFUB4_00887* was compared to that of the corresponding parent strains and the reconstituted strains (*T. forsythia* ATCC 43037 Δ*Tanf_01245*$^{+}$ and *T. forsythia* UB4 *TFUB4_00887*$^{+}$). Upon protein staining of crude cell extracts with CBB (Figure 2A), the S-layer glycoproteins of both strain ATCC 43037 and UB4 appeared as prominent high-molecular mass bands, with TfsA migrating at ~230 kDa (calculated MW, 135 kDa) and TfsB at ~270 kDa (calculated MW, 152 kDa), as expected (Posch et al. 2011). A clear down-shift of both S-layer glycoproteins (~20 kDa, each) was visible in ATCC 43037 Δ*Tanf_01245*, and the native migration behavior could be restored in the reconstituted strain ATCC 43037 Δ*Tanf_01245*$^{+}$ (Figure 2A). The same result was obtained in an analogous experiment performed with *T. forsythia* UB4, where the parent strain, UB4 Δ*TFUB4_00887* and the reconstituted strain were compared (Figure 2A). Western

immunoblots using polyclonal antibodies directed against the S-layer proteins TfsA (Figure 2B) and TfsB (Figure 2C) confirmed the identity of the said proteins in either *T. forsythia* strain, and simultaneously pinpointed a role of Tanf_01245 and TFUB4_00887 in the general protein *O*-glycosylation pathway of ATCC 43037 and UB4, respectively.

O-Glycans of *T. forsythia* Pse and Leg glycosyltransferase knock-out mutants are devoid of the terminal nonulosonic acid

Knocking-out *Tanf_01245* resulted in a loss of the terminal Pse5Am7Gra residue on the *O*-glycans of *T. forsythia* ATCC 43037 as revealed by electrospray-ion trap-mass spectrometry (ESI-IT-MS) analyses of *β*-eliminated S-layer glycans. Deconvoluted mass spectra showed the highest mass peak at *m*/*z*=1523.4 $[M+H]^+$, which conforms with the *m/z* value of the *T. forsythia O*-glycan lacking the Pse5Am7Gra residue (361.2 Da) and, additionally, one methyl group (14.0 Da); the latter was determined to be missing at the branching *N*-acetylmannosaminuronic acid residue (Figure 3A). Additional peaks, with a typical fragmentation pattern of a glycan moiety were assigned and showed the subsequent loss of sugar residues and side chain modifications of the mutant *O*-glycan. The doubly charged complete wild-type *O*-glycan exhibited an *m*/*z* signal of 949.9 $[M+2H]^{2+}$, which corresponds to *m*/*z*=1898.8 when calculating a singly charged form thereof. In the reconstituted strain ATCC 43037 Δ*Tanf_01245*$^+$, Pse5Am7Gra transfer could be fully restored, as evidenced by the detection of a glycan with *m*/*z*=949.7 $[M+2H]^{2+}$, which corresponds to the mass of the wild-type glycan (Figure 3A).

The wild-type *O*–glycan of *T. forsythia* UB4, where the Pse derivative is replaced by a Leg residue with calculated acetyl (Ac) and glycolyl (Gc) modifications (Friedrich et al. 2016), exhibits *m*/*z*=936.8 $[M+2H]^{2+}$, which corresponds to an *m*/*z*=1872.6 of the $[M+H]^+$ ion. In the UB4 Δ*TFUB4_00887* mutant, the Leg derivative (350.2 Da) as well as one methyl

group (-14.0 Da) modifying the *N*-acetylmannosaminuronic acid residue, was no longer present as evidenced by the prominent peak with *m*/*z*=1523.4 $[M+H]^+$ (Figure 3B). As reported recently (Friedrich et al. 2016), also in this analysis, *O*-glycans of both the UB4 wild-type and the UB4 Δ*TFUB4_00887* mutant another glycan species with additional +16 Da at the position of the digitoxose could be observed, indicating the presence of a deoxyhexose instead of a dideoxyhexose in some forms of the glycan. Still, in the reconstituted strain *T. forsythia* UB4 Δ*TFUB4_00887*$^+$, the production of the Leg derivative was fully restored, resulting in *m*/*z*=936.8 $[M+2H]^{2+}$, which conforms with that of the *T. forsythia* UB4 wild-type glycan (Figure 3B).

These data corroborated the involvement of Tanf_01245 and TFUB4_00887 in the transfer of the Pse and Leg derivative, respectively, during *T. forsythia O*-glycan assembly. Furthermore it is indicated that methylation of the branching *N*-acetylmannosaminuronic acid residue occurs only after transfer of the respective nonulosonic acid derivative to the other, terminal *N*-acetylmannosaminuronic acid residue of *T. forsythia* ATCC 43037 *O*-glycan.

CMP activation and modification of nonulosonic acids occur prior to their transfer onto the *O*-glycan

Considering that the nonulosonic acids present in the *T. forsythia O*-glycans carry modifications, *i.e.*, the proven Am (at C-5) and Gra (at C-7) modifications of the Pse residue in ATCC 43037 and the calculated Ac and Gc modifications (based on MS data) of the Leg residue in UB4 (Friedrich et al. 2016, Posch et al. 2011) and that the modifying enzymes are still unknown, we wanted to rule out that the candidate transferases (Tanf_01245 and TFUB4_00887) would be involved in the modification instead of the predicted transfer process – which would also result in a loss of the nonulosonic acid on the *O*-glycans if the modifications were a prerequisite for the transfer to occur. Thus, the cellular pool of

nucleotide-activated sugars was analyzed for the *T. forsythia* ATCC 43037 Δ*Tanf_01245* and *T. forsythia* UB4 Δ*TFUB4_00887* mutants and compared to that of the parent strains. Besides known UDP- and GDP-activated sugars, CMP-Pse5Am7Gra with *m*/*z*=683.2 (Posch et al. 2011) representing the fully modified Pse precursor, was clearly visible in the pool of nucleotide-activated sugars prepared from cell extracts of both the ATCC 43037 wild-type and the ATCC 43037 Δ*Tanf_01245* mutant (Figure 4A). As a negative control, the ATCC 43037 Δ*pseC* mutant available from a recent study (Friedrich et al. 2016) where the *m*/*z*=683.2 compound is missing as a result of abolishing Pse biosynthesis, was used (Figure 4A), confirming the validity of the analysis.

Legionaminic acid modification prior to transfer to the *O*-glycan was confirmed for *T. forsythia* UB4 as well, where a unique peak at *m*/*z*=654.3 could be detected in the cellular pool of nucleotide-activated sugars of both the *T. forsythia* UB4 wild-type and the UB4 Δ*TFUB4_00887* mutant (Figure 4B). This compound eluted just after CMP-Pse5Am7Gra on a porous graphitic carbon (PGC) column and showed the typical *m*/*z*=322.0 fragment peak of CMP upon collision induced decay (Supplementary Figure S1), supporting its identity as a CMP-activated Leg derivative. In analogy to the data presented for *T. forsythia* ATCC 43037 (Figure 4A), the said *m*/*z*=654.3 peak was absent in a UB4 Δ*legC* mutant which is deficient in CMP-Leg biosynthesis (Friedrich et al. 2016).

Tannerella forsythia strains cross-complemented with the non-native putative nonulosonic acid derivative transferase remain without nonulosonic acid derivative attachment to the protein *O*-glycan

Considering the high amino acid sequence similarity (81%) of the two candidate transferases under investigation and the stereoisomeric character of the Pse and Leg backbones, cross-complementation experiments of the *T. forsythia* mutants with proven deficiency in the

respective nonulosonic acid derivative in the *O*-glycan structure (Figures 3A and 3B) with the non-native putative nonulosonic acid transferase were performed. Specifically, the *T. forsythia* ATCC 43037 Δ*Tanf_01245* mutant was complemented with the *TFUB4_00887* gene, and *vice versa*, *i.e.,* the UB4 Δ*TFUB4_00887* mutant was complemented with the ATCC derived *Tanf_01245* gene (Supplementary Figures S 2A and S 3A).

The cross-complemented strains *T. forsythia* ATCC 43037 $\Delta Tanf\_01245^{+TFUB4\_00887}$ and *T. forsythia* UB4 $\Delta TFUB4\_00887^{+Tanf\_01245}$ were first analyzed on CBB-stained SDS-PAGE gels and Western immunoblots targeted at the TfsA and TfsB S-layer glycoproteins. However, neither of the cross-complemented strains showed the up-shift of the S-layer glycoproteins to the wild-type level (Figure 2). This result was confirmed by mass spectrometric analyses, in which both cross-complemented strains exhibited an *m*/*z* signal of 1523.3 $[M+H]^+$ as maximum glycan mass. This mass was clearly indicative of the presence of *T. forsythia O*-glycans without the terminal nonulosonic acid and without a methyl group (Figure 3).

In conclusion, these data suggest that the candidate transferases Tanf_01245 and TFUB4_00887 have high stringency for their CMP-substrate.

Discussion

Pathogenic bacteria frequently decorate their cell envelope with nonulosonic acids (Lewis et al. 2009, Morrison and Imperiali 2014). Major roles have been attributed to these sugar acids in biology and disease, including involvement in bacterial biofilm formation and motility. For instance, Leg glycan modification in *C. jejuni* was shown to be important for colonization of chickens (Howard et al. 2009), nonulosonic acid modification of the LPS of *Vibrio vulnificus* was shown to promote survival in the host bloodstream (Lubin et al. 2015), and there are

various reports on the requirement of Pse modification for flagellar filament assembly in *Campylobacter* strains (Goon et al. 2003) and in *Treponema denticola* (Kurniyati et al. 2017). Further, it is speculated that pathogens may use nonulosonic acids other than sialic acids in molecular mimicry (Varki and Gagneux 2012), with a direct interaction between Pse on *C. jejuni* flagella and Siglec-10 receptor having been experimentally demonstrated (Stephenson et al. 2014).

Biosynthetic pathways for Sia, Pse and Leg can be predicted with a wide distribution among archaea and bacteria, including the bacterial phylum *Bacteroidetes* to which *T. forsythia* is affiliated (Lewis et al. 2009). While the biosynthetic pathways leading from UDP-GlcNAc to CMP-Pse and that from GDP-GlcNAc to CMP-Leg have been elucidated in detail in different bacteria (Schoenhofen et al. 2006, 2009), and, recently, also in *T. forsythia* (Friedrich et al. 2016), it is still unknown how the indispensable subsequent transfer step of Pse and Leg or derivatives thereof from the activated state onto an acceptor saccharide or polypeptide is elaborated. Considering the increasing documentation of the occurrence of Pse and Leg in pathogens, it is surprising that reports on putative Pse- or Leg-transferases in the literature are scarce (Parker et al. 2012, Watson et al. 2015).

This study was designed to shed light on putative nonulosonic acid derivative transferases encoded in the genome of different strains of *T. forsythia*, a periodontal pathogen which was shown to decorate its cell surface with either a Pse or a Leg derivative present as a terminal residue on otherwise structurally identical and abundant S-layer glycoprotein *O*-glycans (Friedrich et al. 2016, Posch et al. 2011). Specifically, we investigated the candidate CMP-Pse5Am7Gra:ManNAcA transferase Tanf_01245 from *T. forsythia* ATCC 43037 and the candidate CMP-Leg derivative:ManNAcA transferase TFUB4_00887 from *T. forsythia* UB4 (which is also present in *T. forsythia* FDC 92A2) (for glycan structure see (Friedrich et al. 2016)). The identification of biosynthetic pathway genes for either CMP-

Pse or CMP-Leg in conjunction with a gene encoding a putative nonulosonic acid transferase in immediate vicinity on the genome of different strains of the same bacterium is interesting from an evolutionary point of view, since it raises the question about what has shaped the genome content of different *T. forsythia* strains and how this may contribute to the pathogenesis of periodontal diseases.

The high similarity of the two candidate transferases at primary sequence level was not unexpected, since their nonulosonic acid substrates are stereoisomers, albeit with different non-carbohydrate modifications (5Am7Gra in Pse *versus* calculated AcGc in Leg). A multiple sequence alignment revealed an identical, 245-amino acid comprising, C-terminal protein region of Tanf_01245 and TFUB4_00887, and a GT-B or TagB superfamily domain (*vide infra*) is predicted (Figure 1). The prediction of two domains on the amino acid sequence level is in agreement with the two-domain presentation of known 3-D structures from crystallized glycosyltransferases with both GT-A and GT-B folds (Breton et al. 2005, Lairson et al. 2008). A membrane associated third-fold family has been identified based on the crystal structure and kinetic data from an α-2,3/2,8-sialyltransferase (CstII) in *C. jejuni* (Chiu et al. 2004). In agreement with the experimental evidence provided in the present study, bioinformatic analysis predicts a GT-fold for the putative *T. forsythia* nonulosonic acid transferases. However, it cannot be ruled out that a novel GT-fold might be involved in nonulosonic acid transfer, which makes these enzymes exciting objects to study.

Interestingly, a DXD motif as well as a D/E-D/E-G and HP motif, common to bacterial sialyltransferases, were identified in all investigated nonulosonic acid transferases (Figure 1A). Glycosyltransferases from the A-type family share this common DXD motif, which interacts primarily with the phosphate group of the respective nucleotide donor (Breton et al. 2005, Lairson et al. 2008). Yet, a DXD motif is only a decent indication of a glycosyltransferase, since it is not necessarily present in this class of enzymes as exemplified

by a CMP-Sia utilizing enzyme from *C. jejuni* (Chiu et al. 2004). A significant support for a transferase function of both enzymes, Tanf_01245 and TFUB4_00887, is derived from homology searches predicting a TagB superfamily domain or a glycosyltransferase B-type superfamily domain located at the rather N-terminal part of the proteins (Figure 1B). TagB is a glycerophosphate transferase from the wall teichoic acid biosynthesis in *Bacillus subtilis* 168 where it catalyzes the transfer of a single phosphoglycerol unit from CDP-glycerol onto the C-4 hydroxyl of a ManNAc residue (Swoboda et al. 2010). Even though glycerol and nonulosonic acids are clearly different substrates, two remarkable analogies are evident; first, the ManNAc residue from the teichoic acid backbone has the same basic structure as the ManNAcA residue, which is the acceptor saccharide for the nonulosonic acids in *T. forsythia* *O*-glycan biosynthesis, and secondly, an 1,4-linkage as formed upon TagB catalysis is also implemented in the terminal transfer of the Pse and Leg derivative onto the ManNAcA residue within the known *O*-glycan structure (for glycan structure see (Friedrich et al. 2016)).

Thus, it is conceivable to assume that the conserved C-terminal protein region of Tanf_01245 and TFUB4_00887, encompassing the DXD, the D/E-D/E-G and HP motifs, is involved in the binding of the CMP activator of both the Pse and Leg derivative, while the TagB domain might specifically bind the nonulosonic acid portion of CMP-Pse5Am7Gra and the CMP-Leg derivative, respectively (Figure 1A).

Experimentally, we have successfully proven the requirement of Tanf_01245 from *T. forsythia* ATCC 43037 and TFUB4_00887 from *T. forsythia* UB4 for the full saccharide assembly of the *T. forsythia* protein *O*-glycans. Deletion of *Tanf_01245* and *TFUB4_00887*, respectively, resulted in a Pse5Am7Gra and Leg derivative deficient phenotype as revealed by LC-ESI-MS of *β*-eliminated *O*-glycans from TfsB (Figure 3) and TfsA (not shown). To exclude that polar effects would cause the loss of the respective nonulosonic acid, we showed

that reconstituted strains regained the Pse5Am7Gra and Leg derivative transfer activity yielding the native dekasaccharide form of the glycan (Figure 3).

Since the biosynthetic enzymes for the non-carbohydrate modifications on the *T. forsythia* nonulosonic acids are currently unknown, it was important to demonstrate that these have not been unintentionally targeted in the deletion mutants and that, consequently, indeed the fully modified sugar acid would be available in CMP-activated form. For instance, deletion of genes involved in the biosynthesis of the acetamidino group in *Methanococcus maripaludis* resulted in flagellins with a truncated glycan that did not only miss the acetamidino group present on an inner sugar (a modified ManNAc residue) but also the terminal sugar of the flagellin glycan (Jones et al. 2012). This indicates that the loss of non-carbohydrate substituents within a glycan might influence downstream enzymes. ESI-IT MS analyses of the cellular pools of nucleotide activated sugars from the Δ*Tanf_01245* and Δ*TFUB4_00887* mutant strains in comparison to the ATCC 43037 and UB4 parent strains demonstrated the presence of the mature Pse and Leg derivative, respectively, at *m/z*=683.2 in both ATCC 43037 samples and *m/z*=654.3 in both UB4 samples (Figure 4). Further, the presence of CMP was proven by the appearance of a typical *m/z*=322.0 fragment in the MS/MS spectrum (Supplementary data, Figure 4B), which was especially important for the *T. forsythia* UB4 strain, as the structure of whose Leg derivative has not been fully elucidated so far.

To learn about the substrate specificity of the predicted *T. forsythia* Pse5Am7Gra and Leg derivative transferase, cross-complementation experiments were performed. Complementation of *T. forsythia* ATCC 43037 Δ*Tanf_01245* and *T. forsythia* UB4 Δ*TFUB4_00887* with the non-native enzyme could not restore the native *O*-glycan phenotype (Figure 3), despite identity of the underlying *O*-glycan structure. Considering that even cross-glycosylation experiments between different species of the *Bacteroidetes*, namely between

T. forsythia ATCC 43037 and *Bacteroides fragilis* have been successful (Posch et al. 2013), it is conceivable to assume that Tanf_01245 from *T. forsythia* ATCC 43037 and TFUB4_00887 from *T. forsythia* UB4 possess high stringency for the CMP-activated nonulosonic acid substrate. Whether stringency relates to the stereoisomery or the modifications of the nonulosonic acids or both remains to be investigated.

While we have provided strong evidence of the discovery of two new nonulosonic acid transferases in different strains of the periodontal pathogen *T. forsythia*, the development of a dedicated *in vitro* assay to unequivocally prove the enzymatic activity of the investigated *T. forsythia* enzymes is currently under way. Given the power of current glycoengineering approaches the present study may contribute to the design of novel nonulosonic acid-based glycoconjugates of potential therapeutic relevance in the future.

Materials and Methods

Bacterial strains and growth conditions

Tannerella forsythia ATCC 43037 type-strain (American Type Culture Collection - ATCC, Manassas, VA) (Friedrich et al. 2015b) and *T. forsythia* UB4 (clinical isolate kindly provided by Dr. Ashu Sharma, State University of New York at Buffalo, NY) and defined mutants (Table I) were grown anaerobically as described previously (Tomek et al. 2014). Briefly, Brain-Heart-Infusion (BHI) liquid medium (Oxoid, Basingstoke, UK) with a concentration of 37.0 g/l was supplemented with 10.0 g/l of yeast extract (Oxoid), 1.0 g/l L-cysteine (Sigma, Vienna, Austria), 5.0 µg/ml hemin (Sigma), 2.0 µg/ml menadione (Sigma), 20 µg/ml *N*-acetylmuramic acid (Carbosynth, Compton, UK) and 5% (v/v) horse serum (Thermo Fisher Scientific, Vienna, Austria). For cultivation of *T. forsythia* wild-type and mutant strains on

BHI agar plates (0.8% w/v), incubation was performed in anaerobic jars (AnaeroJar; Oxoid) at 37°C. Media were supplemented with 50 µg/ml gentamycin, 5 µg/ml erythromycin or 10 µg/ml chloramphenicol, when appropriate.

Escherichia coli strains (Table I) were grown under standard conditions in lysogeny broth (LB) medium supplemented with 100 µg/ml ampicillin, when appropriate.

Construction of knock-out strains deficient in pseudaminic or legionaminic acid transferase activity

Vectors were constructed to knock-out candidate genes for a Pse5Am7Gra transferase in *T. forsythia* ATCC 43037 (gene *Tanf_01245)* and for a Leg derivative in *T. forsythia* UB4 (gene *TFUB4_00887*) (Table I). A detailed description of the cloning procedure and the transformation of knock-out cassettes into *T. forsythia* is published elsewhere (Tomek et al. 2014). For PCR amplifications, Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) was used according to the manufacturer's instructions. Oligonucleotides (Thermo Fisher Scientific) used in this study are listed in Table II. Extraction of genomic DNA was performed according to a published protocol (Cheng and Jiang 2006).

The knock-out vectors for the construction of a *T. forsythia* ATCC 43037 Δ*Tanf_01245* and a *T. forsythia* UB4 Δ*TFUB4_00887* mutant consisted of approximately 1-kbp up- and down-stream homology regions and an erythromycin resistance marker (*ermF*) cloned in between. Primer pairs 474/475 and 476/477, respectively, were used to amplify the up- and down-stream homology regions from genomic DNA of *T. forsythia* ATCC 43037, and primer pairs 120/121 and 122/123 were used to amplify those from genomic DNA of *T. forsythia* UB4. The antibiotic resistance gene *ermF* (805 bp) was amplified from pJET/TF0955ko (Tomek et al. 2014), either without the promotor region using primers 460 and 461 or including the native promotor using primers 1 and 2. Subsequently, each knock-

out cassette was blunt-end cloned into the cloning vector pJET1.2, creating the final knock-out vectors pJET1.2/Δ*Tanf_01245* and pJET1.2/Δ*TFUB4_00887*. Transformed and viable clones on selective plates containing erythromycin were further tested for correct integration of the knock-out cassette by screening PCR (Supplementary Figures S2 and S3).

To reconstitute the transferase function in the *T. forsythia* ATCC 43037 Δ*Tanf_01245* and *T. forsythia* UB4 Δ*TFUB4_00887*mutants, the genes of interest were re-integrated by homologous recombination including chloramphenicol (*cat*) as an alternate resistance gene for selection purposes (Supplementary Figures S2 and S3). The up-stream region, including the approximately 1-kbp up-stream homology region and the 1338-bp *Tanf_01245* gene, was amplified with primer pair 540/130 from genomic DNA of *T. forsythia* ATCC 43037. The 650-bp *cat* gene (without the native promotor) was amplified using pEXALV as a template (Zarschler, K., Janesch, B., et al. 2009) and primers 126/77 prior to addition to the up-stream region by way of overlap-extension PCR. The construct was blunt-end ligated into the cloning vector pJET1.2. The approximately 1-kbp down-stream homology region (541/542) was cloned into the above mentioned pJET1.2 construct using the restriction sites *Kpn*I and *Sph*I, assembling the final construct pJET1.2/Δ*Tanf_01245*$^{+}$. After control digestion (not shown), electrocompetent *T. forsythia* Δ*Tanf_01245* cells were transformed with the final construct.

Analogously, up-stream and *TFUB4_00887* gene primers 120/130, *cat* primers 126/127 and down-stream primers 128/140 (with *Kpn*I and *Nde*I restriction sites) were used to construct the reconstitution cassette pJET1.2/Δ*TFUB4_00887*$^{+}$. Subsequent steps were performed as described for the *Tanf_01245*-based construct, yielding the final construct pJET1.2/Δ*TFUB4_00887*$^{+}$.

Cross-complementation of nonulosonic acid transferase deficient mutants

Based on the reconstitution cassette pJET1.2/Δ*Tanf_01245*$^{+}$, a cross-complementation cassette was constructed by replacing the native gene *Tanf_01245* with *TFUB4_00887*. The native up-stream region was amplified with primers 543 (including an *Xho*I restriction site) and 575. The UB4 *TFUB4_00887* gene was amplified using primer pair 576/133(*BsrG*I) and cloned to the up-stream region by overlap-extension-PCR. Via the restriction sites *Xho*I and *BsrG*I, the native gene was replaced by the non-native gene, assembling the final cross-complementation cassette pJET1.2/Δ*Tanf_01245*$^{+TFUB4\_00887}$.

Analogously, the cross-complementation cassette pJET1.2/Δ*TFUB4_00887*$^{+Tanf\_01245}$ for the UB4 strain, was constructed. Briefly, primers 134(*Xho*I)/153 were used for the amplification of the up-stream homology region and 152/133(*BsrG*I) for the amplification of the *Tanf_01245* gene. Clones were tested for correct integration on genomic level after transformation and selection (Supplementary Figures S2 and S3).

SDS-PAGE and Western immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (7.5%) were prepared according to a standard protocol (Laemmli 1970). Crude cell extracts of *T. forsythia* ATCC 43037, UB4 and defined mutants thereof were run in a Mini Protean electrophoresis apparatus (Bio-Rad, Vienna, Austria) and proteins were visualized with colloidal Coomassie Brilliant Blue R-250 (CBB). For Western immunoblot analyses, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a Mini Trans-Blot Cell (Bio-Rad). Polyclonal antisera raised in rabbits against the recombinant S-layer proteins TfsA (α-TfsA) and TfsB (α-TfsB) (Sekot et al. 2012) were used as primary antibodies followed by a monoclonal goat anti-rabbit secondary antibody labelled with IRDye 800CW (LI-COR

Biosciences, Lincoln, NE). S-layer protein bands were visualized at 800 nm using an Odyssey Infrared Imaging System (LI-COR Biosciences).

S-layer *O*-glycan preparation and liquid chromatography ESI-IT MS

The preparation and release of *O*-glycans from the glycosylated S-layer proteins TfsA and TfsB by in-gel reductive β-elimination was performed as described previously (Posch et al. 2011, Tomek et al. 2014). Removal of excess salt via PGC cartridges was performed according to published protocols (Pabst and Altmann 2008, Stadlmann et al. 2008) and the final purification of borohydride reduced *O*-glycans prior to ESI-IT-MS analyses was completed using preparative PGC-HPLC (Posch et al. 2011). The glycan mixture was analyzed using a Dionex Ultimate 3000 system directly linked to an ion trap instrument (amaZon speed EDT, Bruker, Germany) equipped with the standard ESI source in the positive ion, DDA mode (=switching to MSMS mode for eluting peaks). MS-scans were recorded (range, 450-1650 *m*/*z*; icc target was set to 100,000; maximum accumulation time, 200 ms) and the highest peaks were selected for fragmentation. Instrument calibration was performed using ESIcalibration mixture (Agilent, Vienna, Austria). For separation of the glycans, a Thermo Hypercarb separation column (5 µm particle size; 100 x 0.360 mm) was used. A gradient from 99% solvent A and 1% solvent B (solvent A, 65 mM ammonium formate buffer (pH 3.0); B, 100% acetonitrile) to 21% B in 20 min was applied, followed by a 10-min gradient from 21% B to 50% B, at a flow rate of 6 µl/min. Data were evaluated using the DataAnalysis 4.0 software (Bruker).

Extraction, purification and analysis of nucleotide-activated sugars

Cellular pools of nucleotide-activated sugars were extracted and purified as described before (Posch et al. 2011). Briefly, two ml of bacterial solution were harvested by centrifugation,

washed in PBS (phosphate-buffered saline) and lysed by ultrasonication in 62 mM sodium fluoride buffer before loading onto a Hypersep Hypercarb 10 mg column (Thermo Scientific, Vienna, Austria). Elution of GDP-, UDP- and CMP-activated sugars was done with 50% acetonitrile in 50 mM ammoniumformate buffer pH 9.0. The glycan mixture was analyzed using a Dionex Ultimate 3000 system directly linked to an ion trap instrument (amaZon speed EDT) equipped with the standard ESI source in the negative ion, DDA mode (=switching to MSMS mode for eluting peaks). MS-scans were recorded (range, 200-900; *m*/*z*; icc target was set to 15000; maximum accumulation time, 200 ms; target mass was set to 600 *m/z*) and the five highest peaks were selected for fragmentation. Instrument calibration was performed using ESIcalibration mixture (Agilent). For separation of the analytes, a Thermo Hypercarb separation column (5 µm particle size, 100 x 0.360 mm) was used. A gradient from 99% solvent A and 1% solvent B (solvent A, 0.3 % formic acid adjusted to pH 9.0 with ammonia solution, B, 100% acetonitrile) to 25% B in 20 min was applied, followed by a 10-min gradient from 25% B to 50% B, at a flow rate of 6 µl/min. Data were evaluated using the DataAnalysis 4.0 software (Bruker).

Acknowledgments

The authors would like to thank Dr. Ashu Sharma (State University of New York at Buffalo, NY) and Dr. Graham Stafford (University of Sheffield, UK) for kindly providing the clinical isolate *T. forsythia* UB4.

Funding

This work was supported by the Austrian Science Fund FWF, projects P24317-B22 (to C.S.), P24305-B20 (to P.M.), and the Doctoral Programme “Biomolecular Technology of Proteins” W1224.

Abbreviations

Ac, *N*-acetyl or acetamido; Am, *N*-acetimidoyl or acetamidino; Amp, ampicillin; BHI, brain heart infusion; Cat, chloramphenicol; CBB, Coomassie Brilliant Blue G250; CMP, cytidine-5'-monophosphate; Erm, erythromycin; ESI-IT MS, electrospray ionization ion-trap mass spectrometry; ESI-TOF MS, electrospray ionization time-of-flight mass spectrometry; Gc, *N*-glycolyl; GDP, guanosine-5'-diphosphate; GT, glycosyltransferase; Gra, *N*-glyceroyl or *N*-2,3-dihydroxypropionyl or glycerate group; LB, lysogeny broth; LC, liquid chromatography; Leg, legionaminic acid (Leg5,7Ac_2),5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*- nonulosonic acid; ManNAc, *N*-acetylmannosamine; ManNAcA, *N*-acetylmannosaminuronic acid; ManNAc$CONH_2$, *N*-acetylmannosaminuronamide; MS, mass spectrometry; NeuAc, *N*- acetylneuraminic acid; PBS, phosphate-buffered-saline; PGC, porous graphitized carbon; Pse, pseudaminic acid (Pse5,7Ac_2), 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*- nonulosonic acid; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate- polyacrylamide gel electrophoresis; Sia, sialic acid (Neu5Ac), 5-acetamido-3,5-dideoxy-D- *glycero*-D-*galacto*-nonulosonic acid; UDP, uridine 5'-diphosphate.

References

Angata T, Varki A. 2002. Chemical diversity in the sialic acids and related α-keto acids: an evolutionary perspective. *Chemical Reviews*, 102:439-470.

Breton C, Šnajdrová L, Jeanneau C, Koca J, Imberty A. 2005. Structures and mechanisms of glycosyltransferases. *Glycobiology*, 16:29R-37R.

Brockhausen I. 2014. Crossroads between bacterial and mammalian glycosyltransferases. *Front Immunol*, 5:492.

Cheng HR, Jiang N. 2006. Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnol Lett*, 28:55-59.

Chiu CPC, Watts AG, Lairson LL, Gilbert M, Lim D, Wakarchuk WW, Withers SG, Strynadka NCJ. 2004. Structural analysis of the sialyltransferase CstII from *Campylobacter jejuni* in complex with a substrate analog. *Nat Struct Mol Biol*, 11:163-170.

Faridmoayer A, Feldman MF. 2010. Bacterial protein glycosylation. In: Mander L, Liu H-W editors. *Comprehensive Natural Products II: Chemistry and Biology* Kidlington (UK): Elsevier Ltd. p. 351-380.

Fletcher CM, Coyne MJ, Villa OF, Chatzidaki-Livanis M, Comstock LE. 2009. A general *O*-glycosylation system important to the physiology of a major human intestinal symbiont *Bacteroides fragilis*. *Cell*, 137:321-331.

Freiberger F, Claus H, Günzel A, Oltmann-Norden I, Vionnet J, Mühlenhoff M, Vogel U, Vann WF, Gerardy-Schahn R, Stummeyer K. 2007. Biochemical characterization of a *Neisseria meningitidis* polysialyltransferase reveals novel functional motifs in bacterial sialyltransferases. *Mol Microbiol*, 65:1258-1275.

Friedrich V, Gruber C, Nimeth I, Pabinger S, Sekot G, Posch G, Altmann F, Messner P, Andrukhov O, Schäffer C. 2015a. Outer membrane vesicles of *Tannerella forsythia*: biogenesis, composition, and virulence. *Mol Oral Microbiol*, 30:451-473.

Friedrich V, Janesch B, Windwarder M, Maresch D, Braun ML, Megson ZA, Vinogradov E, Goneau MF, Sharma A, Altmann F, Messner P, Schoenhofen IC, Schäffer C. 2016. *Tannerella forsythia* strains display different cell-surface nonulosonic acids: biosynthetic pathway characterization and first insight into biological implications. *Glycobiology*, in press.

Friedrich V, Pabinger S, Chen T, Messner P, Dewhirst FE, Schäffer C. 2015b. Draft genome sequence of *Tannerella forsythia* type strain ATCC 43037. *Genome Announc*, 3:e00660-00615.

Goon S, Kelly JF, Logan SM, Ewing CP, Guerry P. 2003. Pseudaminic acid, the major modification on *Campylobacter flagellin*, is synthesized via the Cj1293 gene. *Mol Microbiol*, 50:659-671.

Hajishengallis G. 2014. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. *Trends Immunol*, 35:3-11.

Hajishengallis G, Lamont RJ. 2012. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol Oral Microbiol*, 27:409-419.

Holt SC, Ebersole JL. 2005. *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol 2000*, 38:72-122.

Honma K, Inagaki S, Okuda K, Kuramitsu HK, Sharma A. 2007. Role of a *Tannerella forsythia* exopolysaccharide synthesis operon in biofilm development. *Microb Pathog*, 42:156-166.

Howard SL, Jagannathan A, Soo EC, Hui JP, Aubry AJ, Ahmed I, Karlyshev A, Kelly JF, Jones MA, Stevens MP, Logan SM, Wren BW. 2009. *Campylobacter jejuni* glycosylation island important in cell charge, legionaminic acid biosynthesis, and colonization of chickens. *Infect Immun*, 77:2544-2556.

Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices from protein sequences. *Comput Appl Biosci*, 8:275-282.

Jones GM, Wu J, Ding Y, Uchida K, Aizawa S, Robotham A, Logan SM, Kelly J, Jarrell KF. 2012. Identification of genes involved in the acetamidino group modification of the flagellin *N*-linked glycan of *Methanococcus maripaludis*. *J Bacteriol*, 194:2693-2702.

Knirel YA, Shashkov AS, Tsvetkov YE, Jansson PE, Zähringer U. 2003. 5,7-diamino-3,5,7,9-tetradeoxynon-2-ulosonic acids in bacterial glycopolymers: chemistry and biochemistry. *Adv Carbohydr Chem Biochem*, 58:371-417.

Ksiazek M, Mizgalska D, Eick S, Thøgersen IB, Enghild JJ, Potempa J. 2015. KLIKK proteases of *Tannerella forsythia*: putative virulence factors with a unique domain structure. *Front Microbiol*, 6:312.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol*, 33:1870-1874.

Kurniyati K, Kelly JF, Vinogradov E, Robotham A, Tu Y, Wang J, Liu J, Logan SM, Li C. 2017. A novel glycan modifies the flagellar filament proteins of the oral bacterium *Treponema denticola*. *Mol Microbiol*, 103:67-85.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227:680-685.

Lairson LL, Henrissat B, Davies GJ, Withers SG. 2008. Glycosyltransferases: structures, functions, and mechanisms. *Annu Rev Biochem*, 77:521-555.

Lewis AL, Desa N, Hansen EE, Knirel YA, Gordon JI, Gagneux P, Nizet V, Varki A. 2009.

Innovations in host and microbial sialic acid biosynthesis revealed by phylogenomic prediction of nonulosonic acid structure. *Proc Natl Acad Sci U S A*, 106:13552-13557.

Lubin JB, Lewis WG, Gilbert NM, Weimer CM, Almagro-Moreno S, Boyd EF, Lewis AL. 2015. Host-like carbohydrates promote bloodstream survival of *Vibrio vulnificus in vivo*. *Infect Immun*, 83:3126-3136.

Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu SN, Chitsaz F, Geer LY, Geer RC, He J, Gwadz M, Hurwitz DI, Lanczycki CJ, Lu F, Marchler GH, Song JS, Thanki N, Wang ZX, Yamashita RA, Zhang DC, Zheng CJ, Bryant SH. 2015. CDD: NCBI's conserved domain database. *Nucleic Acids Research*, 43:D222-D226.

Messner P. 1997. Bacterial glycoproteins. *Glycoconj J*, 14:3-11.

Messner P, Schäffer C, Kosma P. 2013. Bacterial cell-envelope glycoconjugates. *Adv Carbohydr Chem Biochem*, 69:209-272.

Morrison MJ, Imperiali B. 2014. The renaissance of bacillosamine and its derivatives: pathway characterization and implications in pathogenicity. *Biochemistry*, 53:624-638.

Nothaft H, Szymanski CM. 2010. Protein glycosylation in bacteria: sweeter than ever. *Nat Rev Microbiol*, 8:765-778.

Ohtsubo K, Marth JD. 2006. Glycosylation in cellular mechanisms of health and disease. *Cell*, 126:855-867.

Onishi S, Honma K, Liang S, Stathopoulou P, Kinane D, Hajishengallis G, Sharma A. 2008. Toll-like receptor 2-mediated interleukin-8 expression in gingival epithelial cells by the *Tannerella forsythia* leucine-rich repeat protein BspA. *Infect Immun*, 76:198-205.

Pabst M, Altmann F. 2008. Influence of electrosorption, solvent, temperature, and ion polarity on the performance of LC-ESI-MS using graphitic carbon for acidic oligosaccharides. *Anal Chem*, 80:7534-7542.

Parker JL, Day-Williams MJ, Tomas JM, Stafford GP, Shaw JG. 2012. Identification of a putative glycosyltransferase responsible for the transfer of pseudaminic acid onto the polar flagellin of *Aeromonas caviae* Sch3N. *Microbiologyopen*, 1:149-160.

Parker JL, Lowry RC, Couto NAS, Wright PC, Stafford GP, Shaw JG. 2014. Maf-dependent bacterial flagellin glycosylation occurs before chaperone binding and flagellar T3SS export. *Mol Microbiol*, 92:258-272.

Posch G, Pabst M, Brecker L, Altmann F, Messner P, Schäffer C. 2011. Characterization and scope of S-layer protein *O*-glycosylation in *Tannerella forsythia*. *J Biol Chem*, 286:38714-38724.

Posch G, Pabst M, Neumann L, Coyne MJ, Altmann F, Messner P, Comstock LE, Schäffer C. 2013. "Cross-glycosylation" of proteins in *Bacteroidales* species. *Glycobiology*, 23:568-577.

Posch G, Sekot G, Friedrich V, Megson ZA, Koerdt A, Messner P, Schäffer C. 2012. Glycobiology aspects of the periodontal pathogen *Tannerella forsythia*. *Biomolecules*, 2:467-482.

Sabet M, Lee SW, Nauman RK, Sims T, Um H-S. 2003. The surface (S-) layer is a virulence factor of *Bacteroides forsythus*. *Microbiology*, 149:3617-3627.

Sakakibara J, Nagano K, Murakami Y, Higuchi N, Nakamura H, Shimozato K, Yoshimura F. 2007. Loss of adherence ability to human gingival epithelial cells in S-layer protein-deficient mutants of *Tannerella forsythensis*. *Microbiology*, 153:866-876.

Schäffer C, Messner P. 2017. Emerging facets of prokaryotic glycosylation. *FEMS Microbiol Rev*, 41:49-91.

Schirm M, Soo EC, Aubry AJ, Austin J, Thibault P, Logan SM. 2003. Structural, genetic and functional characterization of the flagellin glycosylation process in *Helicobacter pylori*. *Mol Microbiol*, 48:1579-1592.

Schoenhofen IC, McNally DJ, Brisson JR, Logan SM. 2006. Elucidation of the CMP-pseudaminic acid pathway in *Helicobacter pylori*: synthesis from UDP-*N*-acetylglucosamine by a single enzymatic reaction. *Glycobiology*, 16:8C-14C.

Schoenhofen IC, Vinogradov E, Whitfield DM, Brisson JR, Logan SM. 2009. The CMP-legionaminic acid pathway in *Campylobacter*: biosynthesis involving novel GDP-linked precursors. *Glycobiology*, 19:715-725.

Sekot G, Posch G, Messner P, Matejka M, Rausch-Fan X, Andrukhov O, Schäffer C. 2011. Potential of the *Tannerella forsythia* S-layer to delay the immune response. *J Dent Res*, 90:109-114.

Sekot G, Posch G, Oh YJ, Zayni S, Mayer HF, Pum D, Messner P, Hinterdorfer P, Schäffer C. 2012. Analysis of the cell surface layer ultrastructure of the oral pathogen *Tannerella forsythia*. *Arch Microbiol*, 194:525-539.

Sharma A. 2010. Virulence mechanisms of *Tannerella forsythia*. *Periodontol 2000*, 54:106-116.

Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. 1998. Microbial complexes in subgingival plaque. *J Clin Periodontol*, 25:134-144.

Stadlmann J, Pabst M, Kolarich D, Kunert R, Altmann F. 2008. Analysis of immunoglobulin glycosylation by LC-ESI-MS of glycopeptides and oligosaccharides. *Proteomics*, 8:2858-2871.

Stafford GP, Chaudhuri RR, Haraszthy V, Friedrich V, Schäffer C, Ruscitto A, Honma K, Sharma A. 2016. Draft genome sequences of three clinical isolates of *Tannerella forsythia* isolated from subgingival plaque from periodontitis patients in the United States. *Genome Announc*, 4.

Stephenson HN, Mills DC, Jones H, Milioris E, Copland A, Dorrell N, Wren BW, Crocker PR, Escors D, Bajaj-Elliott M. 2014. Pseudaminic acid on *Campylobacter jejuni* flagella

modulates dendritic cell IL-10 expression via Siglec-10 receptor: a novel flagellin-host interaction. J Infect Dis, 210:148714-148798.

Swoboda JG, Campbell J, Meredith TC, Walker S. 2010. Wall teichoic acid function, biosynthesis, and inhibition. *Chembiochem*, 11:35-45.

Tanner ACR, Listgarten MA, Ebersole JL, Strezempko MN. 1986. *Bacteroides forsythus* sp. nov., a slow-growing, fusiform *Bacteroides* sp. from the human oral cavity. *International Journal of Systematic Bacteriology*, 36:213-221.

Taylor ME, Drickamer K. 2003. Introduction to Glycobiology Oxford University Press: Oxford and New York.

Thibault P, Logan SM, Kelly JF, Brisson JR, Ewing CP, Trust TJ, Guerry P. 2001. Identification of the carbohydrate moieties and glycosylation motifs in *Campylobacter jejuni* flagellin. *J Biol Chem*, 276:34862-34870.

Tomek MB, Neumann L, Nimeth I, Koerdt A, Andesner P, Messner P, Mach L, Potempa JS, Schäffer C. 2014. The S-layer proteins of *Tannerella forsythia* are secreted via a type IX secretion system that is decoupled from protein *O*-glycosylation. *Mol Oral Microbiol*, 29:307-320.

Tytgat HLP, Lebeer S. 2014. The sweet tooth of bacteria: common themes in bacterial glycoconjugates. *Microbiol Mol Biol Rev*, 78:372-417.

Varki A. 1993. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology*, 3:97-130.

Varki A. 2006. Nothing in glycobiology makes sense, except in the light of evolution. *Cell*, 126:841-845.

Varki A. 2008. Sialic acids in human health and disease. *Trends Mol Med*, 14:351-360.

Varki A, Cummings RD, Esko JD, Hudson FH, Stanley P, Bertozzi CR, Hart GW, Etzler ME. 2009. *Essentials of Glycobiology*. 2nd ed. Cold Spring Harbor Laboratory Press:Cold Spring Harbor (NY).

Varki A, Gagneux P. 2012. Multifarious roles of sialic acids in immunity. *Ann N Y Acad Sci*, 1253:16-36.

Veith PD, Chen YY, Chen D, O'Brien-Simpson NM, Cecil JD, Holden JA, Lenzo JC, Reynolds EC. 2015. *Tannerella forsythia* outer membrane vesicles are enriched with substrates of the type IX secretion system and TonB-dependent receptors. *J Proteome Res*, 14:5355-5366.

Veith PD, O'Brien-Simpson NM, Tan Y, Djatmiko DC, Dashper SG, Reynolds EC. 2009. Outer membrane proteome and antigens of *Tannerella forsythia*. *J Proteome Res*, 8:4279-4292.

Vimr ER, Kalivoda KA, Deszo EL, Steenbergen SM. 2004. Diversity of microbial sialic acid metabolism. *Microbiol Mol Biol Rev*, 68:132-153.

Watson DC, Leclerc S, Wakarchuk WW, Young NM. 2011. Enzymatic synthesis and properties of glycoconjugates with legionaminic acid as a replacement for neuraminic acid. *Glycobiology*, 21:99-108.

Watson DC, Wakarchuk WW, Leclerc S, Schur MJ, Schoenhofen IC, Young NM, Gilbert M. 2015. Sialyltransferases with enhanced legionaminic acid transferase activity for the preparation of analogs of sialoglycoconjugates. *Glycobiology*, 25:767-773.

Zarschler K, Janesch B, Zayni S, Schäffer C, Messner P. 2009. Construction of a gene knockout system for application in *Paenibacillus alvei* CCM 2051^{T}, exemplified by the S-layer glycan biosynthesis initiation enzyme WsfP. *Appl Environ Microbiol*, 75:3077-3085.

Zunk M, Kiefel MJ. 2014. The occurrence and biological significance of the α-keto-sugars pseudaminic acid and legionaminic acid within pathogenic bacteria. *RSC Adv.*, 4:3413-3421.

Figures

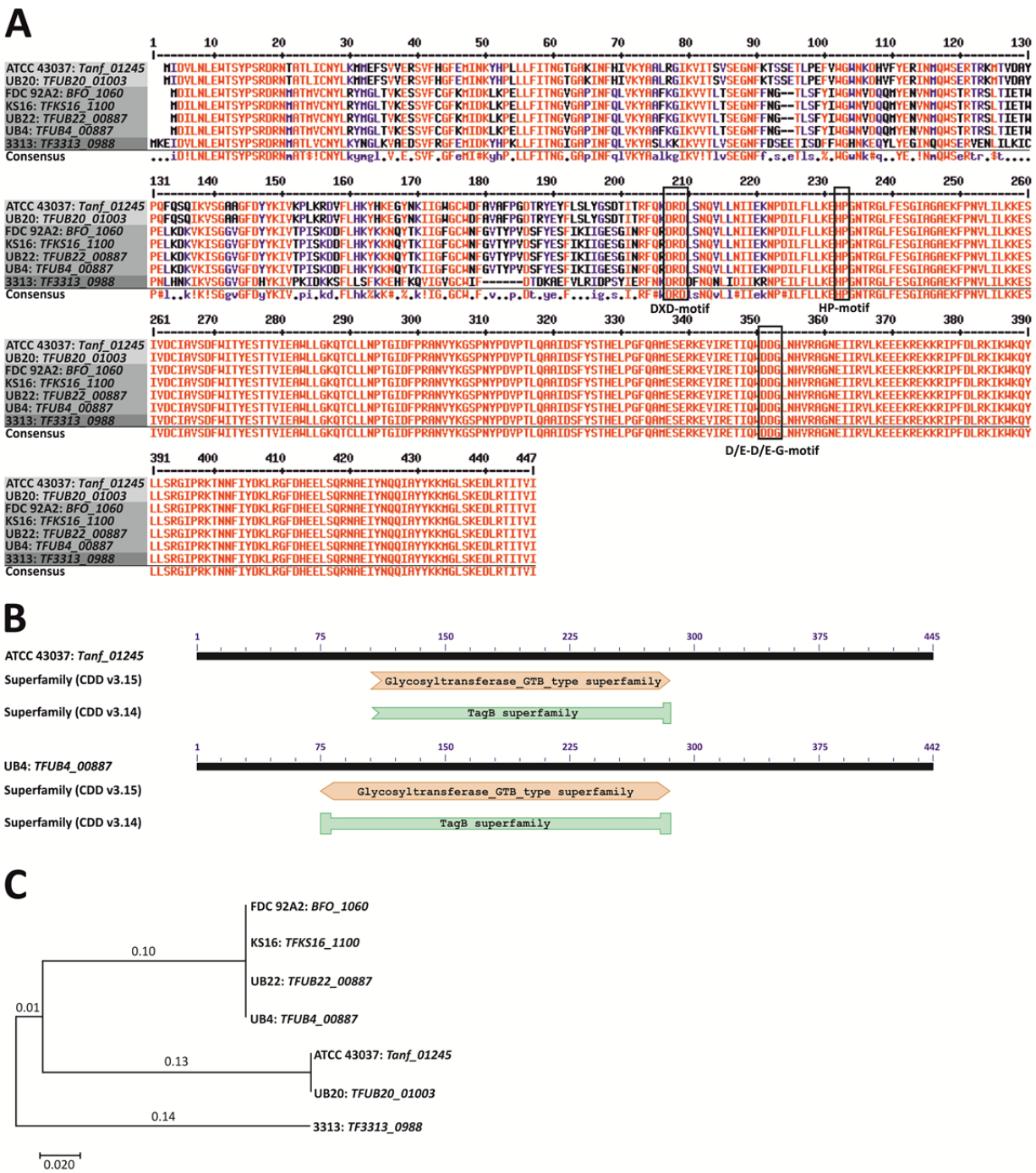


Fig. 1. Bioinformatic and molecular phylogenetic analyses of putative nonulosonic acid derivative transferases from different *T. forsythia* strains. (A) Amino acid sequence alignment of Tanf_01245 (strain ATCC 43037), TFUB4_00887 (strain UB4), TFUB20_01003 (strain UB20), BFO_1060 (strain FDC 92A2), TFKS16_1100 (KS16), TFUB22_00887 (strain UB22), and TF3313_0988 (strain 3313) illustrates a high sequence

identity (81%) for all compared sequences except for that from strain 3313, and conservation of the C-terminal domain. Conserved motifs (DXD, D/E-D/E-G and HP) are indicated within black boxes. (Alignment was done with the software Multalin at http://multalin.toulouse.inra.fr). **(B)** A conserved TagB superfamily domain (version CDD v3.14) or a glycosyltransferase B-type (version CDD v3.15) superfamily domain was identified in all candidate nonulosonic acid transferases using the NCBI´s conserved domain database (CDD, https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer et al. 2015). **(C)** The evolutionary history of nonulosonic acid transferases from different *T. forsythia* strains was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al. 1992). Four investigated strains (FDC 92A2, KS16, UB22 and UB4) have identical amino acid sequences and group together in the phylogenetic tree, thus representing strains with legionaminic acid transferases, while two strains, including the ATCC type strain (ATCC 43037) and strain UB20 are representing strains with pseudaminic acid transferases. Interestingly, strain 3313 does not group in neither of the nonulosonic acid transferases and thus has an unknown glycosyltransferase activity. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

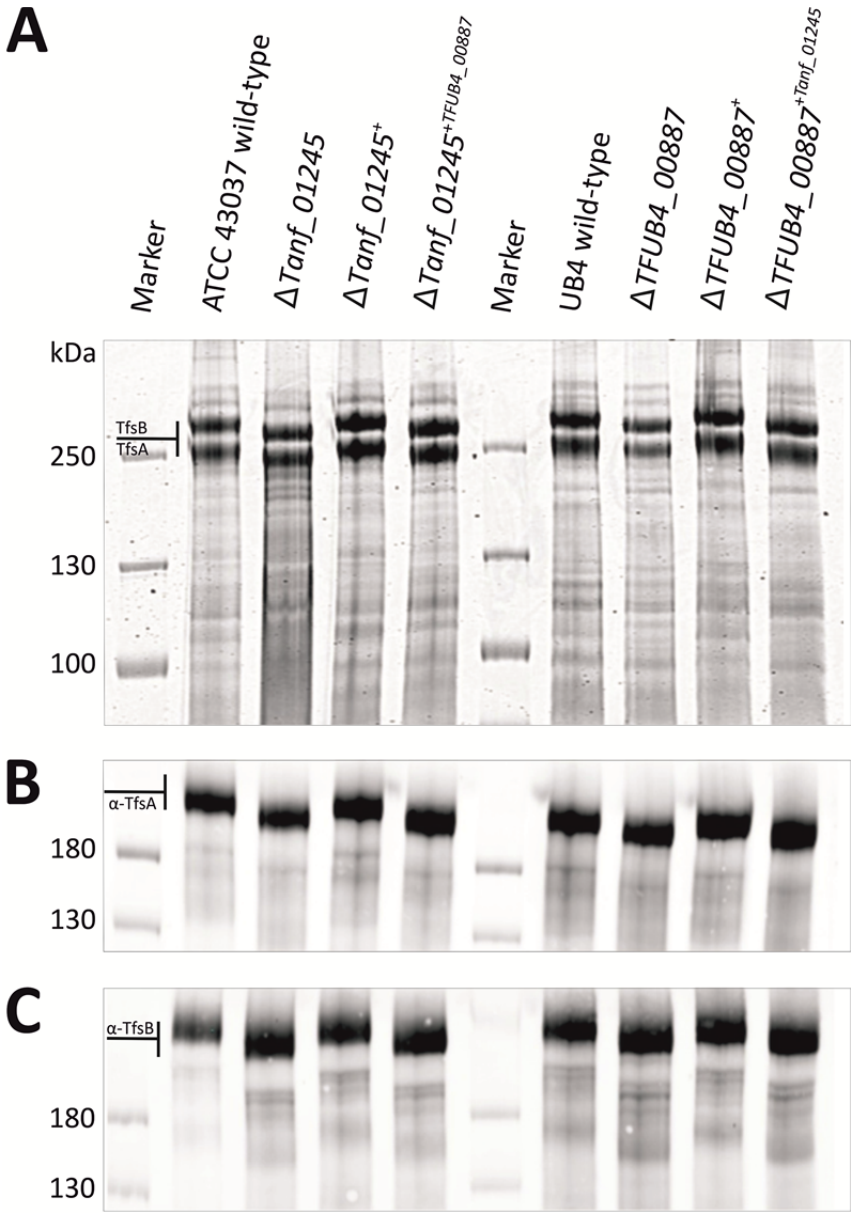


Fig. 2. SDS-PAGE and Western immunoblot analyses of *T. forsythia* ATCC 43037 and *T. forsythia* UB4 wild-type and mutants. (A) CBB staining of crude cell extracts from *T. forsythia* ATCC 43037 wild-type, Δ*Tanf_01245* mutant, reconstituted mutant Δ*Tanf_01245*$^{+}$ and cross-complemented mutant Δ*Tanf_01245*$^{+TFUB4\_00887}$ after separation on a 7.5% SDS-PA gel. The S-layer glycoproteins (labelled TfsA and TfsB) are indicated and the down-shift resulting from the loss of the Pse5Am7Gra residue can be observed in the deletion mutant and in the cross-complemented mutant, while in the reconstituted strain the bands are up-shifted again to wild-type level. The same

migration profiles could be observed for *T. forsythia* UB4 wild-type, Δ*TFUB4_00887* mutant, reconstituted mutant Δ*TFUB4_00887*$^{+}$ and cross-complemented mutant Δ*TFUB4_00887*$^{+Tanf\_01245}$. PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific) was used as a protein molecular weight marker. The S- layer glycoprotein bands were further processed for MS analyses. Western immunoblots probed with anti-TfsA antiserum **(B)** and anti-TfsB antiserum **(C)** confirmed the identity of the S-layer glycoproteins in all analyzed *T. forsythia* species. PageRuler Prestained Protein Ladder (Thermo Fisher Scientific) was used as a molecular weight marker.

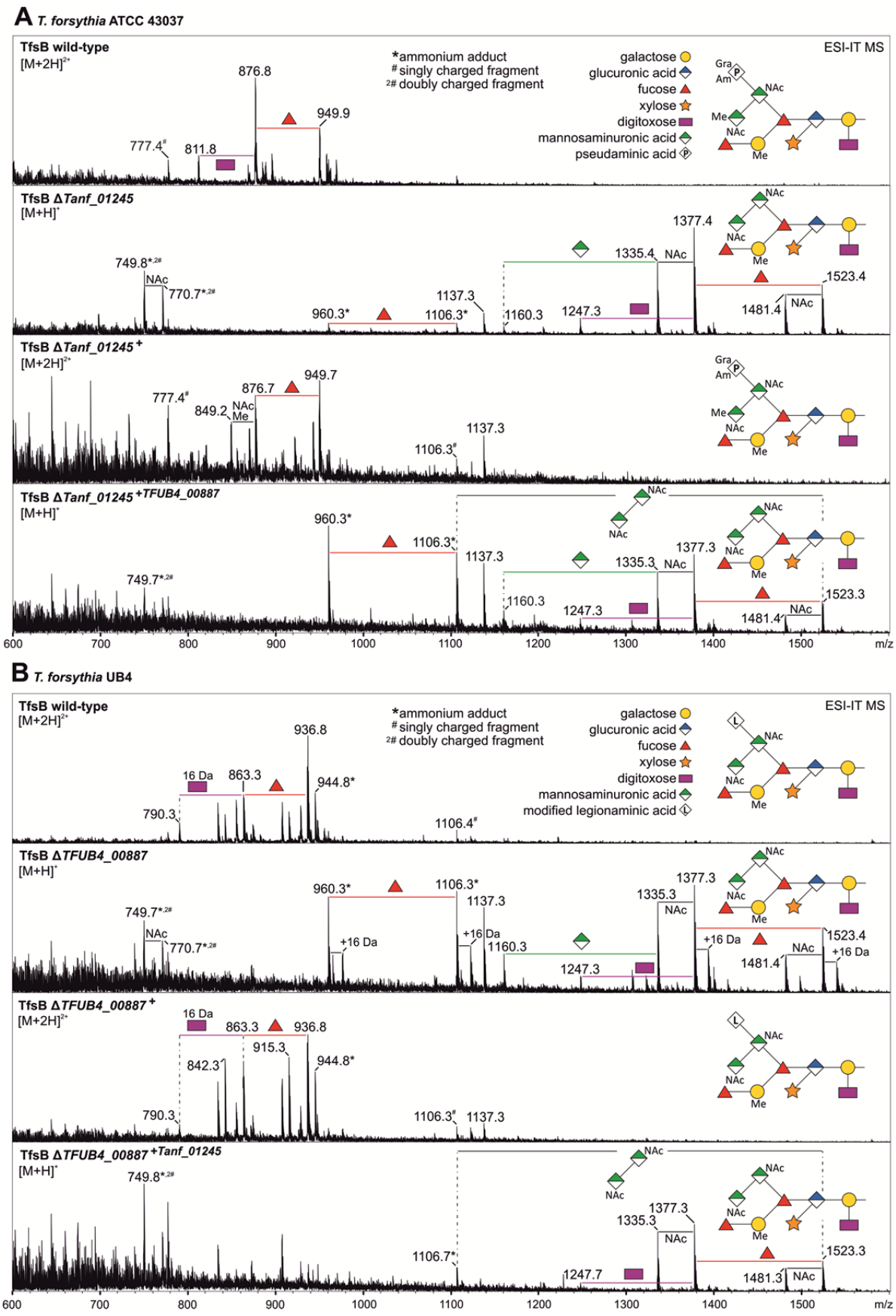

A T. forsythia ATCC 43037
TfsB wild-type
[M+2H]2+
*ammonium adduct
#singly charged fragment
2#doubly charged fragment
galactose
glucuronic acid
fucose
xylose
digitoxose
mannosaminuronic acid
pseudaminic acid
ESI-IT MS
876.8
949.9
777.4#
811.8
TfsB ΔTanf_01245
[M+H]+
1377.4
1335.4
NAc
749.8*,2#
770.7*,2#
1523.4
1137.3
960.3*
1106.3*
1160.3
1247.3
1481.4
TfsB ΔTanf_01245+
[M+2H]2+
949.7
876.7
849.2
Me
1106.3#
TfsB ΔTanf_01245+TFUB4_00887
[M+H]+
749.7*,2#
B T. forsythia UB4
TfsB wild-type
[M+2H]2+
936.8
16 Da
863.3
944.8*
790.3
1106.4#
modified legionaminic acid
TfsB ΔTFUB4_00887
[M+H]+
+16 Da
TfsB ΔTFUB4_00887+
[M+2H]2+
915.3
842.3
TfsB ΔTFUB4_00887+Tanf_01245
[M+H]+
600
700
800
900
1000
1100
1200
1300
1400
1500
m/z

Fig. 3. Deconvoluted ESI-IT MS sum spectra of β-eliminated TfsB *O*-glycans from *T. forsythia* parent and mutant strains. (A) Comparison of the spectra from *T. forsythia* ATCC 43037 wild-type, Δ*Tanf_01245* mutant, reconstituted mutant Δ*Tanf_01245*$^{+}$ and cross-complemented mutant Δ*Tanf_01245*$^{+TFUB4\_00887}$. **(B)** Comparison of the spectra from *T. forsythia* UB4 wild-type, Δ*TFUB4_00887* mutant, reconstituted mutant Δ*TFUB4_00887*$^{+}$ and cross-complemented mutant Δ*TFUB4_00887*$^{+Tanf\_01245}$. Another glycan species with additional +16 Da at the position of the digitoxose was observed, indicative of the presence of a deoxyhexose instead of a dideoxyhexose in some forms of the glycan.

The glycan structures of the highest mass peaks are shown as symbolic representations. Mass peaks from the subsequent fragmentation pattern were assigned according to the loss of carbohydrate units and modifications. Relative peak intensities of occurring peaks are given on the *y* axis.

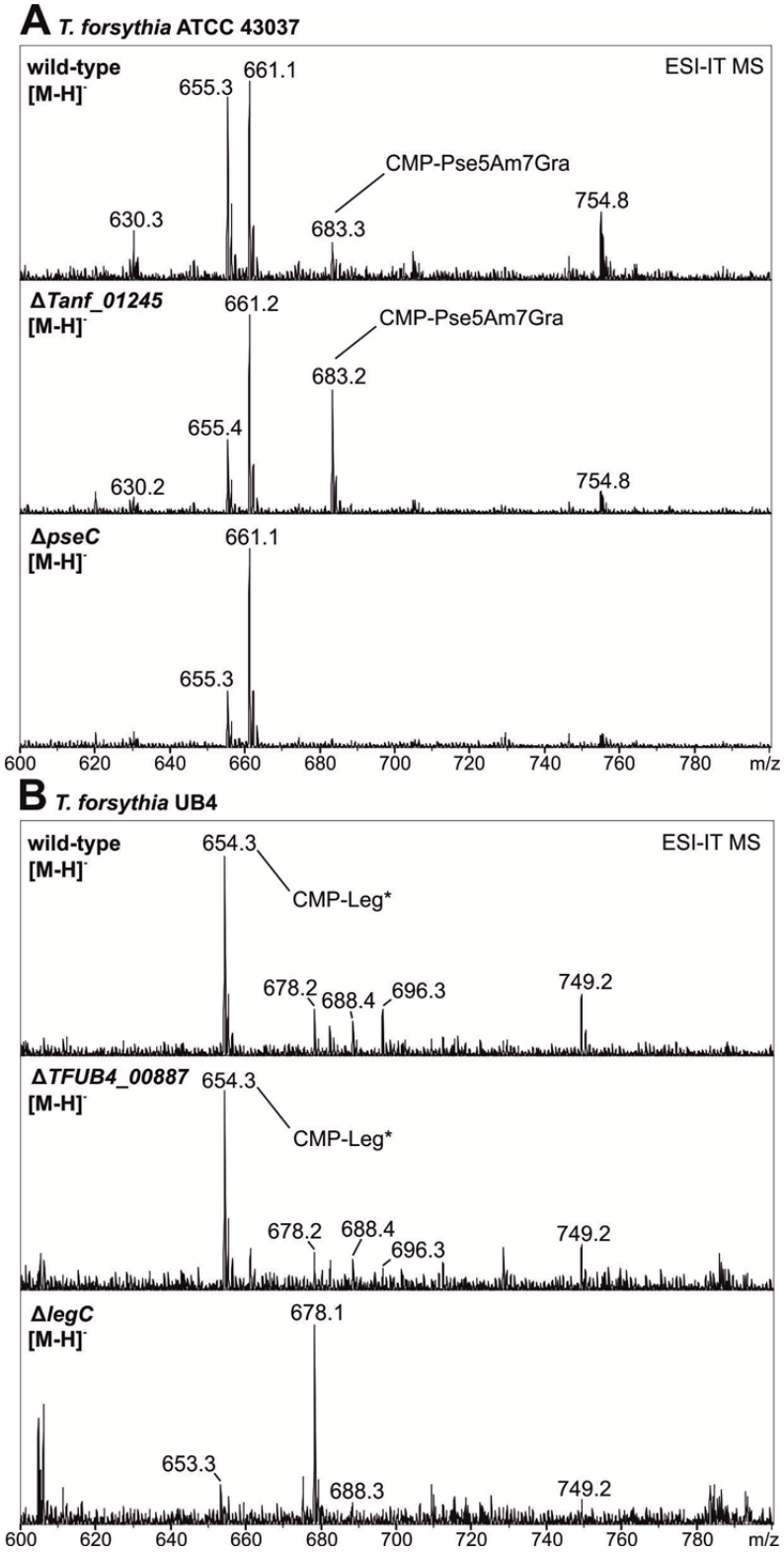


Fig. 4. ESI-IT MS analysis of cellular nucleotide sugar pools from *T. forsythia* strains. (A) CMP-activated Pse5Am7Gra (*m*/*z* 683.3) was detected in the *T. forsythia* ATCC 43037 wild-type and in the Δ*Tanf_01245* mutant, whereas this mass was absent in a

Pse biosynthesis deficient strain (Δ*pseC*) which served as a negative control. **(B)** In *T. forsythia* UB4 wild-type and in the Δ*TFUB4_00887* mutant, a *m*/*z* 654.3 peak was identified, which was attributed to a CMP-activated Leg derivative (CMP-Leg*). This mass is consistent with having Ac and Gc modifications on Leg, based on calculation. Notably, this peak was absent in the Leg- biosynthesis deficient strain (Δ*legC*) which served as a negative control. Relative peak intensities are given on the *y* axis.

Table I. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and use or description	Source or reference
Escherichia coli strain		
DH5α	F^{-} Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1; cloning strain	Invitrogen, Austria
Tannerella forsythia strains		
ATCC 43037	type strain, wild-type	ATCC; (Friedrich *et al.* 2015b)
ATCC 43037 Δ*Tanf_01245*	Δ*Tanf_01245*::*ermF*; knock-out strain of *Tanf_01245*	This work
ATCC 43037 Δ*Tanf_01245*$^{+}$	Δ*Tanf_01245*::*Tanf_01245 cat*; reconstituted knock-out strain	This work
ATCC 43037 Δ*Tanf_01245*$^{+TFUB4\_00887}$	Δ*Tanf_01245*:: *TFUB4_00887cat*; cross-complemented knock-out strain	This work
ATCC 43037 Δ*pseC*	Δ*pseC*::*ermF*; knock-out strain of *Tanf_01190*	(Friedrich *et al.* 2016)
UB4	clinical isolate; wild-type strain	(Stafford et al. 2016)
UB4 Δ*TFUB4_00887*	Δ*TFUB4_00887*::*ermF*; knock-out strain of *TFUB4_00887*	This work
UB4 Δ*TFUB4_00887*$^{+}$	Δ*TFUB4_00887*::*cat*; reconstituted knock-out strain	This work
UB4 Δ*TFUB4_00887*$^{+Tanf\_01245}$	Δ*TFUB4_00887*::*Tanf_01245 cat*; cross-complemented knock-out strain	This work
UB4 Δ*legC*	Δ*legC*::*ermF*; knock-out strain of *TFUB4_00900*	(Friedrich *et al.* 2016)
FDC 92A2	wild-type strain	ATCC; (Tanner *et al.* 1986a)
Plasmids		
pJET1.2/blunt	cloning vector; amp^{R}	Thermo Scientific, Austria
pJET/ΔTF0955ko	vector for amplification of the erythromycin resistance gene	(Tomek *et al.* 2014)
pEXALV	vector for amplification of the chloramphenicol resistance gene	(Zarschler *et al.* 2009)
pJET1.2/Δ*Tanf_01245*	*Tanf_01245* knock-out cassette; amp^{R} $ermF^{R}$	This work
pJET1.2/Δ*Tanf_01245*$^{+}$	cassette for reconstitution of Δ*Tanf_01245*; amp^{R} cat^{R}	This work
pJET1.2/Δ*Tanf_01245*$^{+TFUB4\_00887}$	cassette for cross-complementation of Δ*Tanf_01245* with *TFUB4_00887*; amp^{R} cat^{R}	This work
pJET1.2/Δ*TFUB4_00887*	*TFUB4_00887* knock-out cassette; amp^{R} $ermF^{R}$	This work
pJET1.2/Δ*TFUB4_00887*$^{+}$	cassette for reconstitution of Δ*TFUB4_00887*; amp^{R} cat^{R}	This work
pJET1.2/Δ*TFUB4_00887*$^{+Tanf\_01245}$	cassette for cross-complementation of Δ*TFUB4_00887* with *Tanf_01245*; amp^{R} cat^{R}	This work

Table II. Oligonucleotide primers used for PCR amplification reactions. Nucleotides used for overlap extension PCRs are written in bold, artificial restriction sites are underscored. Lowercase letters indicate artificially introduced bases to improve restriction enzyme digestion

Primers	Sequence (5´-3´)
1[a]	GGTACCCCCGATAGCTTCCGCTATTGC
2	CTACGAAGGATGAAATTTTTCAGGG
3[a]	GCAATAGCGGAAGCTATCGGGGGTACC
4	CCCTGAAAAATTTCATCCTTCGTAG
48	GTCAGATAGGCCTAATGACTGGC
76	TTATAAAAGCCAGTCATTAGGCCTATCTGAC
77[b]	aatcaGCATGC GGTACCTTATAAAAGCCAGTCATTAGGCCTATCTGAC
118	ATGGCTACAATGGTCTGTAATTATCTTC
119	TTATATTACTGTTATTGTTCGTAGATCC
120	CCATGATAATCTCGACTTCGG
121	**GCAATAGCGGAAGCTATCGGGGGTACC**ATTCCTATCTCTTGAAGGATAGG
122	**CCCTGAAAAATTTCATCCTTCGTAG**GTATAGAGGTACAATGGATATAGGGC
123	GCACCCATTTATCTAAATAATCTTC
124	GGCCCTCAACCTTTTCTGGC
125	CCTATCCTTTAGGTATCTATATG
126[b]	**CGAACAATAACAGTAATATAA**TGTACAATGAACTTTAATAAAATTGATTTAGAC
127	aatcaCATATGGGTACCTTATAAAAGCCAGTCATTAGGCCTATCTGAC
128	aatcaGGTACCGTATAGAGGTACAATGGATATAGGGC
130	**CCAATTGTCTAAATCAATTTTATTAAAGTTCAT**TGTACATTATATTACTGTTATTGTTCGTAGATCC
133	aatcaTGTACATTATATTACTGTTATTGTTCGTAGATCCTC
134	aatcaCTCGAGCCATGATAATCTCGACTTCGG
140	aatcaCATATGGCACCCATTTATCTAAATAATCTTC
152	**CTAAATTTCTTTCATAATAATTCTTTGTATA**ATGATTGATGTTTTAAATTTAGAATGGACC
153	**GGTCCATTCTAAATTTAAAACATCAATCAT**TATACAAAGAATTATTATGAAAGAAATTTAG
460[c]	ATGACAAAAAAGAAATTGCCCGTTCGTTTTAC
461[c]	CTACGAAGGATGAAATTTTTCAGGGACAAC
474[d]	**CGGGCAATTTCTTTTTTGTCAT**TTCTTAATGTAATCTAAGTCCAACCG
475[d]	TTGTAGCAGAACTATCAGCCAATCAC
476[d]	**GTTGTCCCTGAAAAATTTCATCCTTCGTAG**GTATAGAGGTACAATGGATATAG
477[d]	CCCAGACTCTTCTTTAACAAGAAACC
512	TGCAGGCTGCAATTGATTCC
513	GATCCCACGTGAAAGCAAATA
524	GTAAAACGAACGGGCAATTTCTTTTTTGTCAT
525	CCCTGAAAAATTTCATCCTTCGTAG
530	CGTATGATATTTGCAGTCTTG
531	GTAATAACCATATCTGCCTCTGGAAC
540	CAACAATTGTAGCAGAACTATCAGCC
541	aatcaGGTACCTATAAGTATAGAGGTACAATGGATATAGG
542	aatcaGCATGCGGTATCTATATGAAGTACGCACC
543	ctgaCTCGAGCAACAATTGTAGCAGAACTATC
565	CTAAATCAATTTTATTAAAGTTCAT
575	**TCTAAATTAAGAATATCCAT**TTCTTAATGTAATCTAAGTCCAACCGCATTCC
576	**GACTTAGATTACATTAAGAA**ATGGATATTCTTAATTTAGAATGGACTTCC

[a](Friedrich et al. 2016), [b](Zarschler *et al.* 2009), [c](Tomek *et al.* 2014); [d] Sequence (3´-5´)

Supplementary data to

A pseudaminic acid or a legionaminic acid derivative transferase is strain-specifically implicated in the general protein *O*-glycosylation system of the periodontal pathogen *Tannerella forsythia*

[1,§]Markus B. Tomek, [1,§,x]Bettina Janesch, [2]Daniel Maresch, [2,y]Markus Windwarder, [2]Friedrich Altmann, [1]Paul Messner and [1]Christina Schäffer*

[1]Department of NanoBiotechnology, *NanoGlycobiology* unit, Universität für Bodenkultur Wien, Muthgasse 11, A-1190 Vienna, Austria.

[2]Department of Chemistry, Universität für Bodenkultur Wien, Muthgasse 18, A-1190 Vienna, Austria.

[§]These authors contributed equally to this work

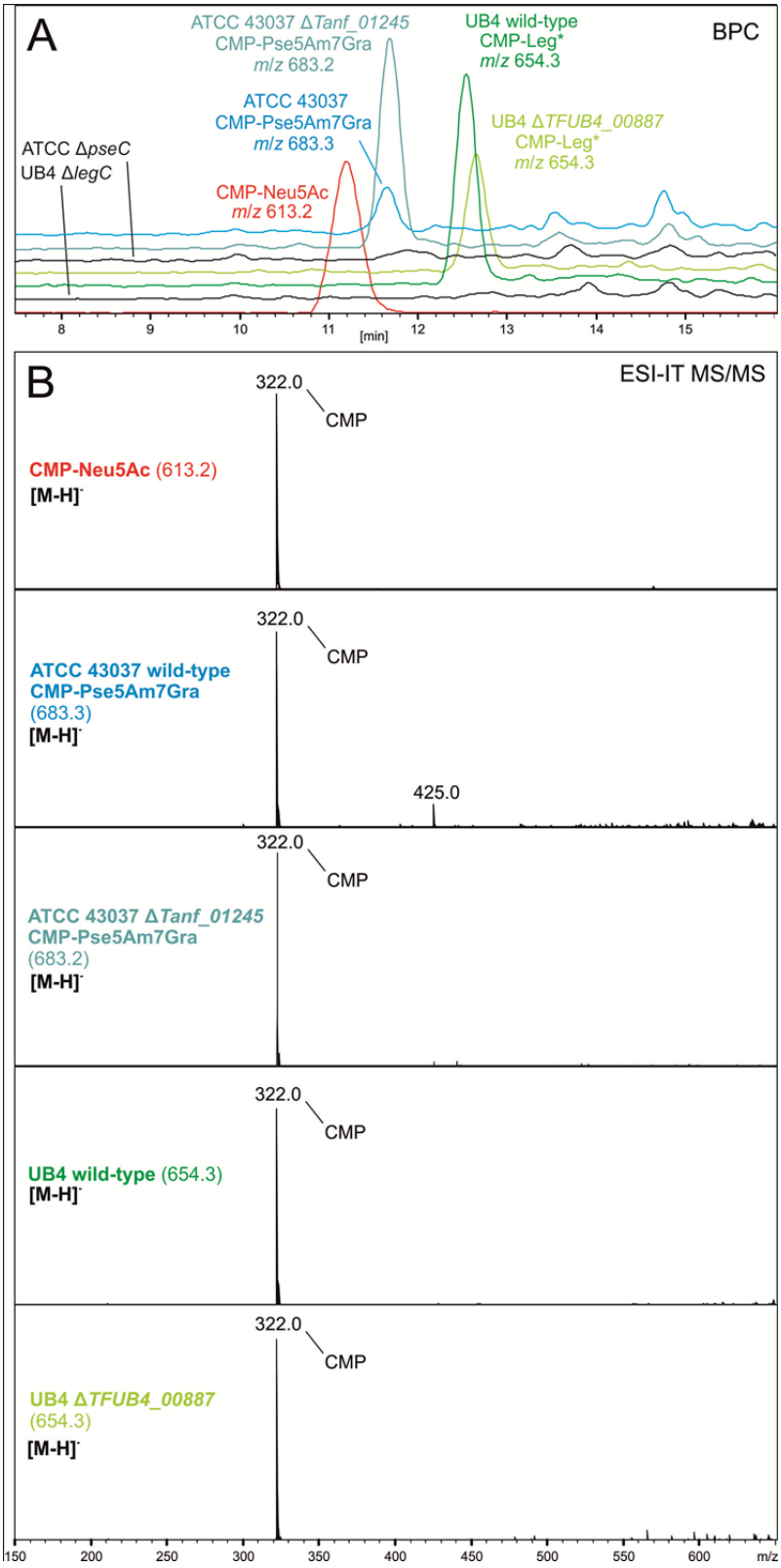
Key words: *Bacteroidetes* / glyco-engineering / glycosyltransferase / nonulosonic acids / periodontitis

Supplementary data: Supplementary Figures 1-3

[x]Present address: Ryerson University Biomedical Research Facilities, 661 University Avenue, Toronto, Ontario, M5G 0A3, Canada

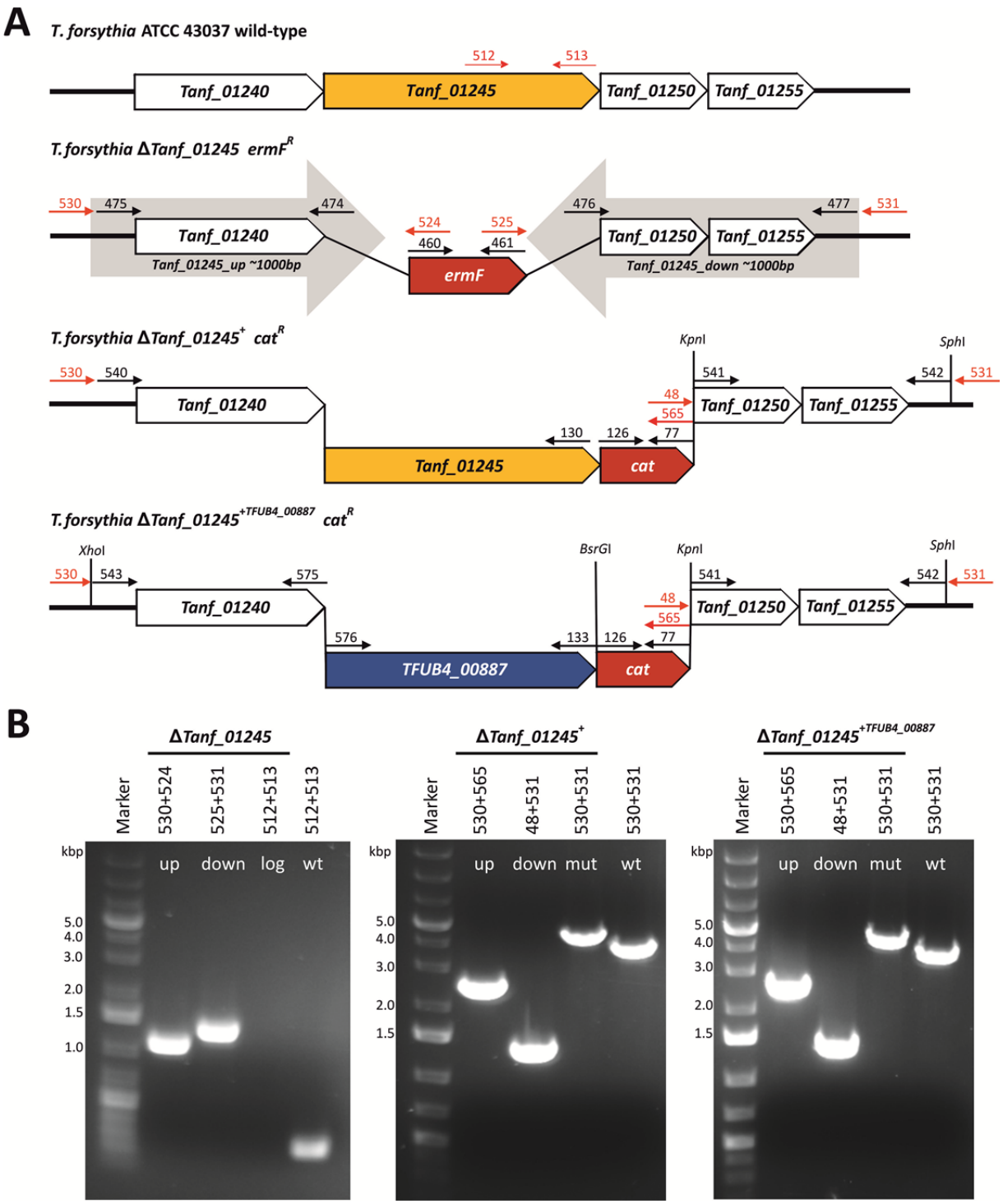
[y]Present address: Shire, Uferstraße 15, 2304 Orth, Austria

* To whom correspondence should be addressed: Christina Schäffer: Tel: +43-1-47654-80203; Fax: +43-1-4789112; e-mail: christina.schaeffer@boku.ac.at



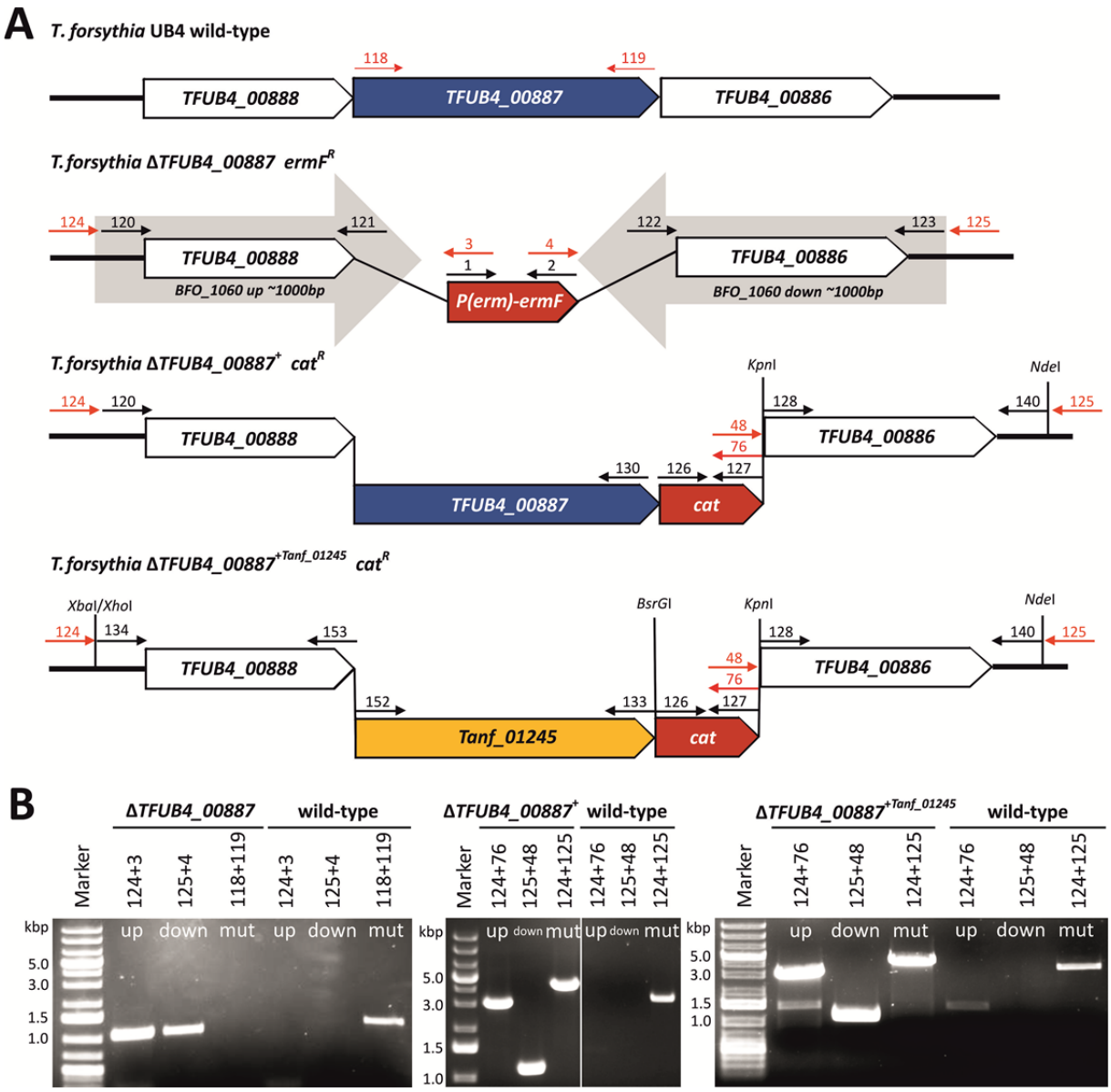
Supp. Fig. 1. ESI-MS analyses of CMP-activated sugars from *T. forsythia* ATCC 43037 and *T. forsythia* UB4. (**A**) Overlay of the PGC column elution profile of CMP-Neu5Ac serving as standard (*m/z* 613.2; red) and the known CMP-Pse5Am7Gra unit

from ATCC 43037 (*m*/*z* 683.3; dark blue) and the Δ*Tanf_01245* mutant (*m*/*z* 683.2; light blue) in comparison to the uniquely found UB4 mass of *m*/*z*=654.3, present in the parent strain (dark green) and the Δ*TFUB4_00887* mutant (light green), shown in a base peak chromatogram (BPC). (**B**) Confirmation of the presence of the CMP-activator in all of the analyzed samples by CID-fragmentation, where all CMP-activated sugars show the typical *m*/*z*=322.0 peak originating from CMP. The sugar part did not ionize well and, therefore, could not be detected. Relative intensities of occurring peaks are given on the *y* axis.



Supp. Fig. 2. Strategy for the generation of *T. forsythia* ATCC 43037 mutants at the *Tanf_01245* locus and confirmation by PCR. (**A**) The genomic organization of the *Tanf_01245* locus is shown for the parent strain *T. forsythia* ATCC 43037, the Δ*Tanf_01245* mutant, the reconstituted mutant Δ*Tanf_01245*$^{+}$ and the cross-complemented mutant Δ*Tanf_01245*$^{+TFUB4\_00887}$. Black colored arrows represent primers used for PCR

amplification of genes and homologous regions, red colored primers represent those used to screen for correct integration of the knock-out, reconstitution and cross-complementation cassettes; restriction sites used for cloning are indicated (not drawn to scale). (**B**) Agarose gel electrophoresis (left) confirms the deletion of *Tanf_01245* using the up-stream primers 530/524 (1105 bp) and down-stream primers 525/531 (1314 bp) on genomic DNA of *T. forsythia* ATCC 43037 Δ*Tanf_01245* mutant with integrated *ermF* cassette. Primers 512/513 yield in a 239-bp PCR fragment when using *T. forsythia* wild-type genomic DNA, whereas this fragment is absent on genomic DNA of the Δ*Tanf_01245* mutant confirming the loss of the gene (log). The agarose gel electrophoresis (middle) confirms the reconstitution of the deleted *Tanf_01245* gene using up-stream primers 530/565 (2442 bp) and down-stream primers 48/531 (1337 bp). Screening primers 530/531 yield in a 4374-bp PCR product on genomic DNA of the reconstituted mutant Δ*Tanf_01245*$^{+}$ with integrated *cat* resistance gene, whereas the same primer pair results in a 3700-bp product on genomic DNA of the *T. forsythia* wild-type. Agarose gel electrophoresis (right) confirms the cross-complementation with *TFUB4_00887* using up-stream primers 530/565 (2433 bp) and down-stream primers 48/531 (1337 bp) on genomic DNA of Δ*Tanf_01245*$^{+TFUB4\_00887}$. Screening primers 530/531 yield in a 4365-bp PCR product on genomic DNA of the cross-complemented mutant Δ*Tanf_01245*$^{+TFUB4\_00887}$, whereas the same primer pair results in a 3700-bp product on genomic DNA of the *T. forsythia* wild-type. O´Gene Ruler 1 kb Plus DNA Ladder (Thermo Fisher Scientific) was used as a gene ladder.



Supp. Fig. 3. Strategy for the generation of *T. forsythia* UB4 mutants at the *TFUB4_00887* locus and confirmation by PCR. (**A**) The genomic organization of the *TFUB4_00887* locus is shown for the parent strain, the UB4 Δ*TFUB4_00887* mutant, the reconstituted UB4 Δ*TFUB4_00887*$^{+}$ mutant and the cross-complemented UB4 Δ*TFUB4_00887*$^{+Tanf\_01245}$ strain. Black colored arrows represent primers used for PCR amplification of genes and homologous regions, red colored represent those used to screen for correct integration of the knock-out, reconstitution and cross-complementation cassettes; restriction sites used for cloning are indicated (not drawn to scale). (**B**) Agarose gel electrophoresis (left) confirms the deletion of *TFUB4_00887* using the up-stream

primers 124/3 (1079 bp) and down-stream primers 4/125 (1090 bp) on genomic DNA of *T. forsythia* UB4 Δ*TFUB4_00887*mutant with integrated *ermF* cassette and on UB4 wild-type genomic DNA. Primers 118/119 yield in a 1278-bp PCR fragment when using *T. forsythia* UB4 wild-type genomic DNA, whereas this fragment is absent on genomic DNA of the Δ*TFUB4_00887* mutant (mut), confirming the loss of the gene. The agarose gel electrophoresis (middle) confirms the reconstitution of the deleted *TFUB4_00887* gene using up-stream primers 124/76 (2987 bp) and down-stream primers 48/125 (1102 bp). Screening primers 124/125 yield in a 4048-bp PCR product on genomic DNA of the reconstituted mutant Δ*TFUB4_00887*$^{+}$ with integrated *cat* resistance gene, whereas the same primer pair results in a 3395-bp product on genomic DNA of the *T. forsythia* UB4 wild-type. Agarose gel electrophoresis (right) confirms the cross-complementation with *Tanf_01245* using up-stream primers 124/76 (3047 bp) and down-stream primers 48/125 (1102 bp) on genomic DNA of Δ*TFUB4_00887*$^{+Tanf\_01245}$. Screening primers 124/125 yield in a 4118-bp PCR product on genomic DNA of the cross-complemented mutant Δ*TFUB4_00887*$^{+Tanf\_01245}$, whereas the same primer pair results in a 3395-bp product on genomic DNA of the *T. forsythia* UB4 wild-type. O´Gene Ruler 1 kb Plus DNA Ladder (Thermo Fisher Scientific) was used as a gene ladder.

4

Cell surface glycoprotein translocation in *Tannerella forsythia*

4.1 Protein secretion systems in Gram-negative bacteria

4.2 The type IX secretion system in *Bacteroidetes*

Protein secretion plays a central role in modulating the interactions of bacteria with their environment and, thus, affects the development, proliferation and the overall lifestyle of an organism (Wickner and Schekman 2005). While exoproteins of Gram-positive (monoderm) bacteria have to translocate only across the cytoplasmic (inner) membrane (Desvaux *et al.* 2006; Schneewind and Missiakas 2012), Gram-negative (diderm) bacteria have to translocate across the cytoplasmic membrane as well as the outer membrane (Pugsley 1993; Costa *et al.* 2015). Once translocated across the outermost membrane, the final destination of a secreted protein can vary. The release into the extracellular milieu or the covalent or non-covalent association to cell-wall or outer membrane components can occur. Assembly into macromolecular structures on the cell surface such as flagella, pili or S-layers are key purposes of secreted proteins (Desvaux *et al.* 2009; Chagnot *et al.* 2013). The terminology of protein trafficking in bacteria is depicted in Figure 9. Focusing on Gram-negative bacteria, to which also *T. forsythia* belongs, known translocation systems are briefly described in this chapter (Figure 10).

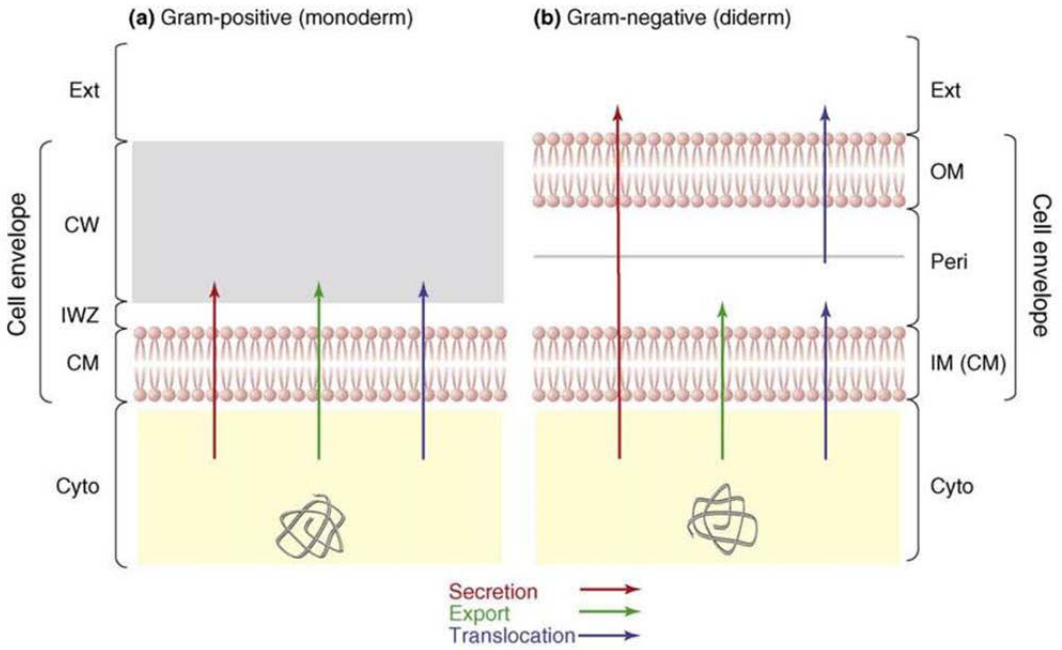


Figure 9 Protein translocation, export and secretion in Gram-positive (a) and Gram-negative (b) bacteria. Ext, extracellular milieu; CW, cell wall; IWZ, inner wall zone; CM, cytoplasmic membrane; Cyto, cytoplasm; OM, outer membrane; Peri, periplasm; IM, inner membrane. [Taken from (Desvaux *et al.* 2009)].

4.1 Protein secretion systems in Gram-negative bacteria

A general discrimination of secretion systems can be made based upon their structural features, either spanning a single or a double membrane (Pugsley 1993; Costa et al. 2015). Five double-membrane-spanning systems are known to date, classified as type I secretion system (T1SS), T2SS, T3SS, T4SS and T6SS (Desvaux et al. 2009; Chagnot et al. 2013). The T5SS only spans the outer membrane and the T7SS belongs to mycobacteria, having a Gram-negative-like cell envelope, and has not been observed in Gram-negative bacteria, so far. The outer membrane-localized T8SS is proposed to be involved in the secretion and assembly of curli, which are extracellular amyloid-like protein fibres (Barnhart and Chapman 2006). Systems only spanning the outer membrane (T5SS, T7SS, T8SS), require a separate export across the inner membrane. The evolutionary conserved and universally distributed Sec (secretion) translocation system, the Tat (twin-arginine translocation) and holin (hole forming) translocation systems can mediate the export of proteins into the periplasm, thereby discriminating about the status of protein folding (Natale *et al.* 2008; Fröbel *et al.* 2012; Lycklama á Nijeholt and Driessen 2012). Underlying all exported proteins are signal peptides targeting the protein to the desired translocation system. However, exceptions to this general rule may also arise.

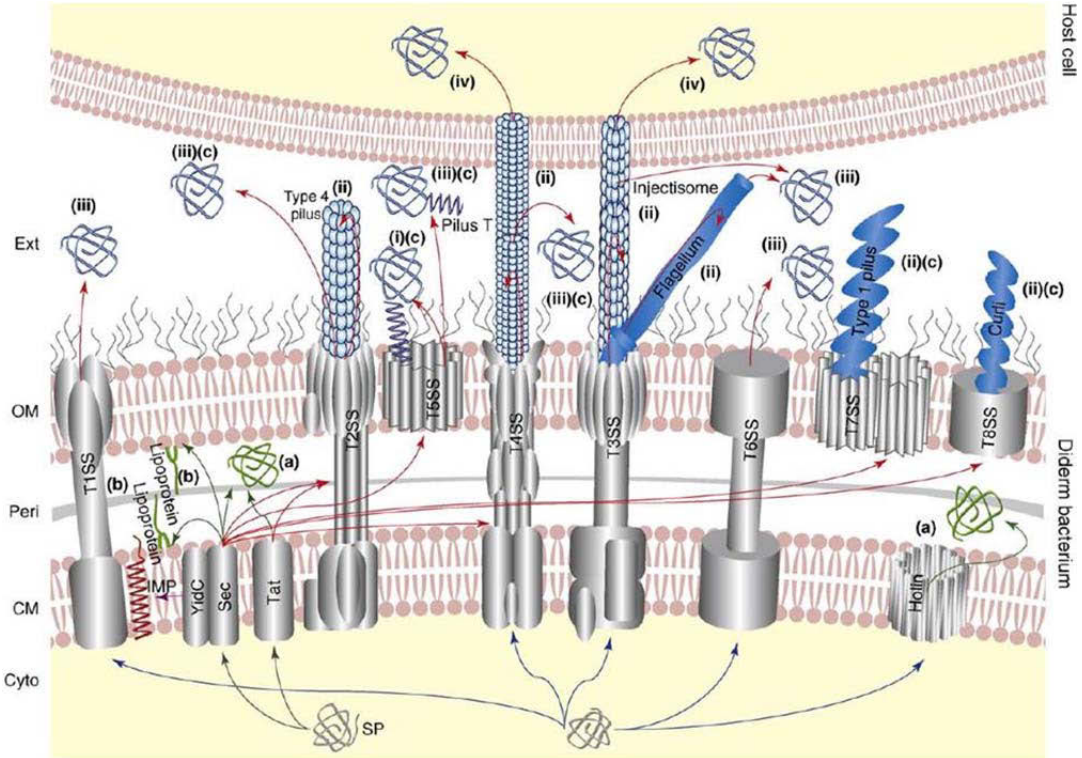


Figure 10 Schematic presentation of translocation systems in Gram-negative (diderm) bacteria. (a) Protein is released into the periplasm; (b) lipoproteins are anchored onto CM or OM; (c) proteins undergo a second translocation event. Cyto, cytoplasm; CM, cytoplasmic membrane; Peri, periplasm; OM, outer membrane; Ext, extracellular milieu; SP, signal peptide. [Taken from (Desvaux *et al.* 2009)].

The double-membrane-spanning T1SS mediates the secretion of a wide variety of protein substrates comprised of between 78 and 8682 amino acids from the cytoplasm into the extracellular milieu. These include, for instance, hemolysin A (HlyA) from *Escherichia coli*, metalloproteases (PrtA, PrtB, PrtC, PrtG) from the plant pathogen *Erwinia chrysanthemi* (initially described as *Dickeya dadantii*) and the S-layer protein RsaA from *Caulobacter crescentus*. Targeting the T1SS is mediated by a C-terminal signal sequence which is not cleaved upon translocation (Delepelaire 2004).

Colonization factors and pilus proteins are translocated via a T2SS. Initial bacterial attachment and adherence processes are important in the early stages of biofilm development (Conrad 2012). Type 4 pili are associated with bacterial motility and are best studied in the opportunistic pathogen *Pseudomonas aeruginosa* (Burrows 2012). *Vibrio cholerae* secretes its cholera toxin (AB_5) and the colonization factor GbpA (*N*-acetylglucosamine-binding protein A) into the extracellular milieu via a T2SS mechanism. GbpA promotes efficient colonization due to chitin and mucin binding of human epithelial cells (Kirn *et al.* 2005). Components of the T2SS are located in the inner and outer membrane and enable folding of multimeric proteins in the periplasm. The exact secretion motif on the proteins recognized by the T2SS is still a biological puzzle. Some studies point towards a conformational signal gathering from several residues from different locations in the polypeptide chain (Sandkvist 2001).

The T3SS was initially discovered in pathogenic bacteria, although plant, fly or nematode mutualists also employ this secretion system. The delivery of protein effectors across eukaryotic cellular membranes is the principal function of this protein secretion system. Hypersensitive response and pathogenicity pilus (Hrp) and flagellum formation may also be exerted via the T3SS, mediating bacterial adhesion and colonization of cells. The injectisome of the T3SS is composed of several rings, spanning the bacterial inner and outer membrane, which are connected to a needle (*Yersinia pestis*), a filament (*Salmonella typhimurium*) or a pilus (*Pseudomonas syringe*) (Mota and Cornelis 2005; Cornelis 2006). Two hypotheses, resulting from experimental studies of Yop (*Yersinia* outer membrane) proteins in *Yersinia enterocolitica*, are under debate, explaining the recognition mechanism for T3SS substrates. First, a non-cleavable N-terminal signal peptide was observed (Michiels *et al.* 1990), and second, a secretion signal present on the mRNA was detected (Anderson and Schneewind 1997).

Transport of nucleic acids and proteins into prokaryotic and eukaryotic cells is allowed by the T4SS, and ,thus, it is ancestrally related to bacterial conjugation systems transporting DNA. A secretion channel and an extracellular T-pilus form the T4SS (Christie and Cascales 2005; Low *et al.* 2014), transporting a wide variety of virulence factors of human pathogens, *e.g., Helicobacter pylori*, *Bordetella pertussis, P. aeruginosa* and *Legionella pneumophila* (Wallden *et al.* 2010). In the context of bacterial antibiotic resistances, horizontal gene transfer is often mediated by the T4SS. Substrate recognition and recruitment is generally conferred by means of C-terminal located translocation signals, which, however, show little sequence conservation (Christie and Cascales 2005).

The outer membrane spanning T5SS (five subclasses are denoted type Va-e) requires the Sec translocon to transfer an unfolded polypeptide across the inner membrane. Uniquely, the substrate and its secretion pore are fused together forming a single peptide, which can drive its own secretion across the outer membrane. This exclusive feature explains the common term autotransporter system (Leo *et al.* 2012). Mainly virulence factors are secreted by the T5SS, but also participation of the T5SS in cell-cell adhesion processes and biofilm formations are reported (Henderson *et al.* 2004; Benz and Schmidt 2011). Upon translocation, the substrate may be cleaved by its peptidase domain and is released into the extracellular environment, or remains anchored to the outer membrane.

In about a quarter of all *Proteobacteria*, genes coding for the T6SS are present. The T6SS consists of a membrane complex with homologous components to the T4SS, and a tail complex, which is evolutionary related to contractible bacteriophage tails. The tail complex is anchored to the inner membrane and contains a tail sheath, an inner tube and a baseplate including a sharp spike. Effectors are loaded into the inner tube followed by a contraction of the sheath. The released energy can thrust the sharp spike and the loaded tube across membranes into the target cell (Filloux *et al.* 2008; Basler 2015). Proteins secreted by the T6SS belong to the Hcp (haemolysin A co-regulated protein) family, which are best studied in *Vibrio cholerae* and *Pseudomonas aeruginosa*, as well as the VgrG (valine-glycine repeat protein G) family present in *V. cholerae* (Filloux et al. 2008; Chen et al. 2015). However, it is under debate, if these protein families are part of the translocation system. Cytotoxic non-VgrG effectors can attack glycan bonds and bacterial peptidoglycan, other activities include muramidases and peptidases, lipases as well as actin modifications (Ho *et al.* 2014).

The T7SS is involved in the outer membrane secretion and assembly of pili (type 1 pili, P pili), fimbriae or capsules (F1). The secretion system belongs to the chaperon-usher pathway in diderm-LPS bacteria (Desvaux *et al.* 2009; Zav'yalov *et al.* 2010). Secretion across the inner membrane is mediated by the Sec translocon, before subunits polymerize and assemble into pili structures using a dedicated periplasmic chaperone and an assembly platform located at the outer membrane termed "the usher" (Thanassi *et al.* 2012). Mono- or polyadhesive pili are involved in bacterial adhesion processes and interbacterial interactions, studied in uropathogenic *E. coli* (P and type 1 pili) and *Y. pestis* (F1 capsule) (Thanassi *et al.* 2012). The T7SS, present in *M. tuberculosis* and in Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Listeria monocytogenes*), has a different structure and function than the T7SS from diderm bacteria (Abdallah *et al.* 2007; Houben *et al.* 2014)

The extracellular nucleation-precipitation pathway involved in the secretion and assembly of curli is proposed as the T8SS. Containing a Sec translocon signal sequence, protein export into the periplasm is achieved via this route. After attachment to the cell wall and secretion across the outer membrane, nucleation performed by the nucleator protein CsgB (minor curli subunit protein B) and polymerization of the major curli subunit CsgA into functional curli fibres occur (Van Gerven *et al.* 2015). Curli are amyloid fibres involved in bacterial adhesion processes and the formation of protective biofilms. Involvement in host cell-bacteria interactions during infection has also been reported (Barnhart and Chapman 2006; Epstein and Chapman 2008; Goyal *et al.* 2014).

4.2 The type IX secretion system in *Bacteroidetes*

The most recent addition to the list of protein secretion systems in Gram-negative bacteria is the Por (porphyrin accumulation on the cell surface) secretion system (PorSS), now classified as the T9SS (Chagnot *et al.* 2013; McBride and Zhu 2013). First studied in *P. gingivalis* and *Flavobacterium johnsoniae*, the secretion system seems to be restricted to members of the phylum *Bacteroidetes* (McBride and Zhu 2013; Veith *et al.* 2013; Nakayama 2015). Deletion mutations in *P. gingivalis* initially identified 13 genes (*i.e. porK*, *porL*, *porM*, *porN*, *porP*, *porQ*, *porT*, *porU*, *porV*, *porW*, *porX*, *porY* and *sov*) to be involved in the secretion of gingipains (Potempa *et al.* 2003; Sato *et al.* 2010). The exact biological roles of these genes remain still unclear *porU*, however, could be identified as the gene encoding the essential C-terminal signal peptidase, PorU (Glew *et al.* 2012). Interactions between the signal peptidase and PorV (LptO/PG27) as well as Sov were also reported (Saiki and Konishi 2010; Glew *et al.* 2014; Saiki and Konishi 2014). Currently, crystallographic data are only available for the membrane protein PorM,

however, only the periplasmic part of this protein could be solved to a resolution of 2.85 Å (Stathopulos *et al.* 2015). The formation of a 50-nm ring-shaped structure was observed between the two lipoproteins PorK and PorN. The interaction consists of 32-36 subunits of each protein and may form a part of the secretion channel (Gorasia *et al.* 2016). PorT was predicted as an integral outer-membrane spanning protein channel involved in gingipain secretion (Nguyen *et al.* 2009). Components of the T9SS sharing high sequence identity (>30%) between *P. gingivalis* and *T. forsythia* are listed in Table 1.

Table 1 Predicted or experimentally verified T9SS components (**M.B. Tomek, unpublished data**).

	Locus Tag		**Protein ID**	**Description**	**Sequence identity** [%]
T. forsythia ATCC 43037	*T. forsythia* (prev.)	*P. gingivalis* ATCC 33277			
Tanf_00060	TF1742	PGN_1877	PorW	T9SS protein W	38
Tanf_02260	TF2304	PGN_1296	-	Lipoprotein	48
Tanf_02355	TF2326	PGN_1677	PorP	T9SS protein P	37
Tanf_02360	TF2327	PGN_1676	PorK	T9SS protein K	62
Tanf_02365	TF2328	PGN_1675	PorL	T9SS protein L	41
Tanf_02370	TF2329	PGN_1674	PorM	T9SS protein M	46
Tanf_02375	TF2330	PGN_1673	PorN	T9SS protein N	40
Tanf_02580	TF0955	PGN_0026	PorU	C-terminal signal peptidase	36
Tanf_04220	TF2852	PGN_0023	PorV/LptO	T9SS protein V	54
Tanf_04410	TF2901	PGN_0832	Sov	Gliding motility protein	51
Tanf_10520	TF0188	PGN_0778	PorT	T9SS protein T	52
Tanf_12330	TF1930	PGN_1019	PorX	Chemotaxis protein CheY	67
Tanf_12435	TF1952	PGN_0509	PorZ	T9SS protein Z	31
Tanf_12465	TF1959	PGN_0645	PorQ	T9SS protein Q	41
Tanf_13050	TF0671	PGN_2001	PorY	Sensor histidine kinase	48

Central to substrates secreted via the T9SS are C-terminal signal sequences present on the target proteins. Putative C-terminal domain (CTD) proteins have been found in 87 organisms so far, all belonging to the phylum *Bacteroidetes*. The CTD shows conservation over a length of ~60 amino acids and contains three sequence motifs (B, D and E) resulting in the formation of a conserved C-terminal β-sandwich domain (Nguyen *et al.* 2007; Veith *et al.* 2009; 2013; de Diego *et al.* 2016). In *T. forsythia,* 37 proteins are predicted to possess a CTD, out of which 19 have been verified experimentally (Veith *et al.* 2013; **Tomek *et al.* 2014**). The C-terminal domains of selected CTD-proteins present in *T. forsythia* are shown in Figure 11. The importance of the CTD as recognition signal for the T9SS was elucidated in earlier studies, as well as the necessity of CTD-cleavage prior

to attachment of the protein onto the outer membrane or LPS-structures (Chen *et al.* 2011; Shoji *et al.* 2011; Glew *et al.* 2012). Latest research suggests a sortase-like mechanism for cleavage and subsequent modification or attachment processes (Gorasia *et al.* 2015).

Extracellular and surface proteins are secreted across the T9SS, many of which have been characterized as virulence factors (Sharma 2010; Nakayama 2015). CTD proteins from *T. forsythia* include both S-layer proteins TfsA and TfsB (Sabet *et al.* 2003; Sekot *et al.* 2011; **Tomek *et al.* 2014**), and the leucine-rich repeat BspA, which activates the Toll-like receptor 2 in cooperation with Toll-like receptor 1 (Onishi *et al.* 2008). The first proteins found to be secreted using the T9SS were the gingipains-group proteins, Kgp (lysine gingipain), RgpA (arginine gingipain A) and RgpB (arginine gingipain B) from the oral pathogen *P. gingivalis*. Additionally, a peptidylarginine deiminase (PPAD) associated with rheumatoid arthritis (Maresz *et al.* 2013) and the hemin binding protein 35 (hbp35) (McGraw *et al.* 1999; Shoji *et al.* 2011) have been experimentally verified as important virulence factors, belonging to the CTD containing protein family. Cells of *Flavobacterium johnsoniae* neither possess flagella nor pili, but can crawl rapidly over surfaces using a novel motility machine. This process is called gliding motility and involves two motility adhesion proteins, called SprB (surface protein B) and RemA (redundant motility gene A). Both are secreted via the T9SS to the cell surface and promote forward movement and rotation of the cell (Kharade and McBride 2015; McBride and Nakane 2015).

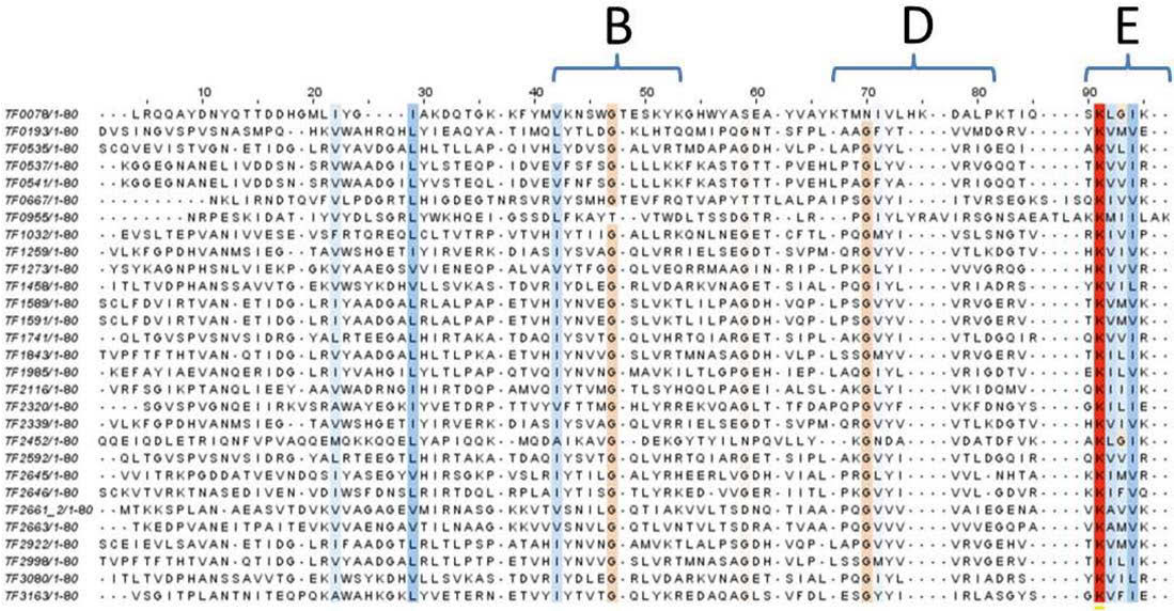


Figure 11 The C-terminal domains of CTD-containing proteins of *T. forsythia* are aligned and the conserved motifs are indicated (Veith *et al.* 2013). The alignment was prepared using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/); the figure was prepared using Jalview (http://www.jalview.org/) (**M.B. Tomek, unpublished data**).

Genes involved in the assembly of the T9SS are a subset of gliding motility genes, indicating interplay between protein secretion and gliding motility. Comparative genome analyses between 37 members of the phylum *Bacteroidetes* revealed a widespread occurrence of predicted gliding motility genes (McBride and Zhu 2013). Recently, interplay between protein secretion, gliding motility and biofilm formation was investigated in *Capnocytophaga ochracea*. Strains deficient in T9SS had an altered biofilm formation resulting in a lower biomass and thickness as compared with the wild-type biofilm (Kita *et al.* 2016). Altered biofilm formation in T9SS deficient mutants has also been observed in *T. forsythia* (**Tomek *et al.* 2014**). A further species with experimentally proven functional T9SS is the soil inhabiting cellulolytic bacterium *Cytophaga hutchinsonii*. Defects in cellulose degradation and protein secretion have been observed in a T9SS deficient mutant (*porU*) (Wang *et al.* 2014).

Due to lack of structural and functional knowledge of individual components of the T9SS, a full functional comparison of the T9SS to other Gram-negative protein translocation systems is currently not possible. However, novel experimental data contributing to the determination of the biological functions of the T9SS are steadily arriving. On a genomic level, the T9SS is found exclusively within members of the phylum *Bacteroidetes*. Unique to the secretion system are conserved CTDs acting as cleavable signal sequences for the translocation of the proteins across the outer membrane. The individual contributions of T9SS associated proteins are still poorly defined and further research is needed to clarify the exact mode of protein translocation and post-secretory processes. A proposed model for T9SS secretion and maturation, exemplified by the S-layer protein TfsB from *T. forsythia*, is shown in Figure 12.

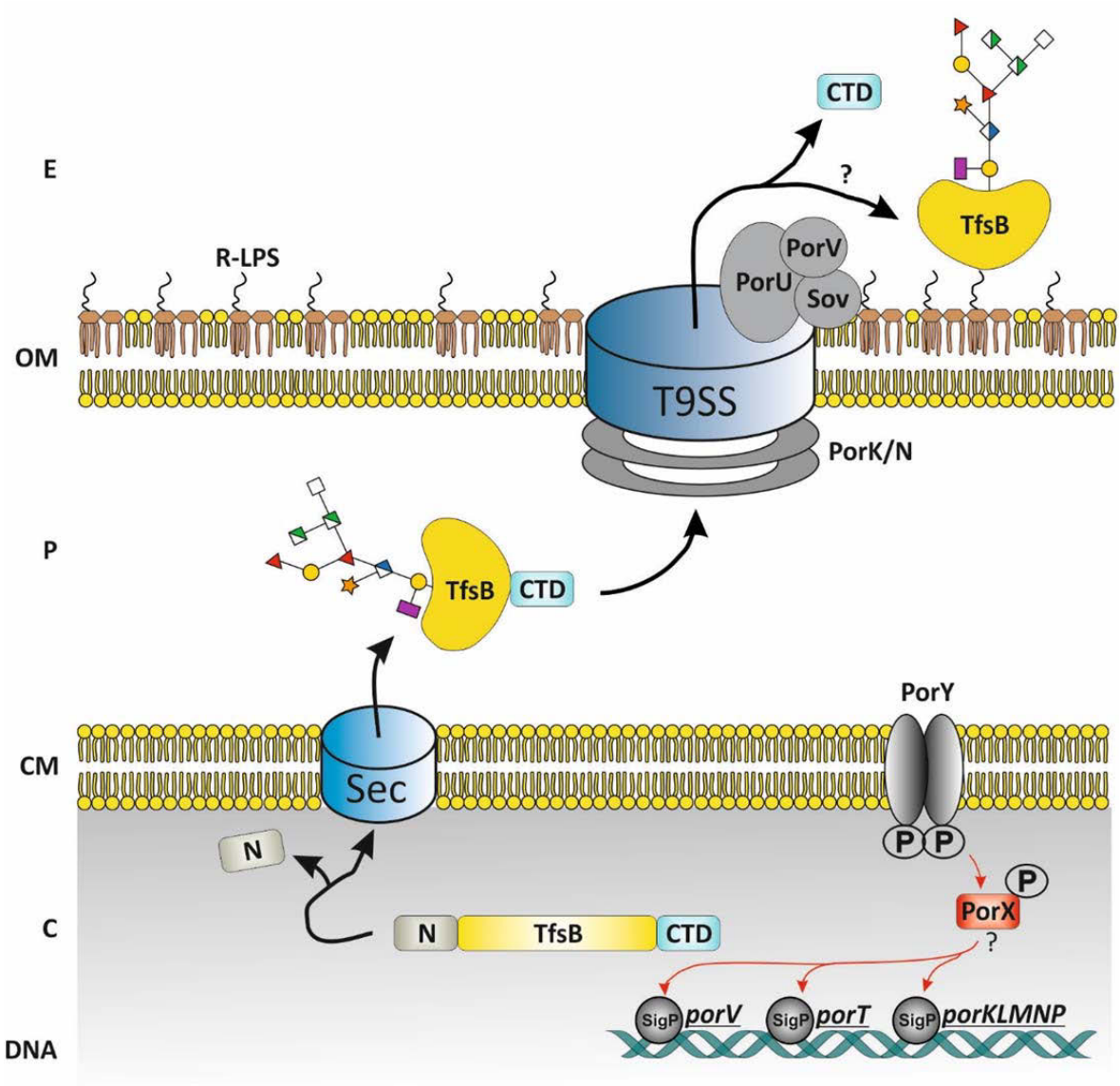


Figure 12 Schematic model of the proposed mechanism of the T9SS exemplified with the *T. forsythia* S-layer protein TfsB. The N-terminal signal sequence targets the translated TfsB protein via the Sec system across the inner membrane, upon cleaving off the signal sequence. Subsequently, the S-layer protein is glycosylated in the periplasm before the CTD is recognized by the T9SS. Upon translocation across the outer membrane the CTD is cleaved off by the C-terminal signal peptidase (PorU). Subsequently, involving PorV and Sov, the S-layer protein is presumably anchored onto the R-type LPS present in the outer membrane (Posch *et al.* 2013a). PorY is a histidine kinase and can regulate, together with the response regulator PorX, transcription of components of the T9SS via the extracytoplasmic function (ECF) sigma factor (SigP). In addition, PorX can also bind to the cytoplasmic domain of PorL (not shown) (Kadowaki *et al.* 2016; Vincent *et al.* 2016). E, extracellular milieu; OM, outer membrane; P, periplasm; CM, cytoplasmic membrane; C, cytoplasm; R-LPS, rough-type lipopolysaccharide; N, N-terminal signal sequence; CTD, C-terminal domain (**M.B. Tomek, unpublished data**).

4.3 Publication 2: S-layer glycoproteins of *Tannerella forsythia* are secreted via a type IX secretion system that is decoupled from protein *O*-glycosylation.

Tomek, M.B., Neumann, L., Nimeth, I., Koerdt, A., Andesner, P., Mach, L., Messner, P., Potempa, J.S., Schäffer, C. (2014) *Mol. Oral Microbiol.*, 29:307-320. doi: 10.1111/omi.12062

molecular oral microbiology

molecular oral microbiology

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

M.B. Tomek[1], L. Neumann[2], I. Nimeth[1], A. Koerdt[1], P. Andesner[1], P. Messner[1], L. Mach[3], J.S. Potempa[4,5] and C. Schäffer[1]

1 Department of NanoBiotechnology, NanoGlycobiology Unit, Universität für Bodenkultur Wien, Vienna, Austria
2 Department of Chemistry, Universität für Bodenkultur Wien, Vienna, Austria
3 Department of Applied Genetics and Cell Biology, Universität für Bodenkultur Wien, Vienna, Austria
4 Oral Health and Systemic Disease Group, University of Louisville, Louisville, KY, USA
5 Department of Microbiology, Jagiellonian University, Krakow, Poland

Correspondence: Christina Schäffer, Department of NanoBiotechnology, NanoGlycobiology Unit, Universität für Bodenkultur Wien, Muthgasse 11, 1190 Vienna, Austria Tel.: +43 1 47654 2203; fax: +43 1 4789112; E-mail: christina.schaeffer@boku.ac.at

Keywords: C-terminal domain; C-terminal signal peptidase; protein secretion; S-layer glycosylation; T9SS
Accepted 7 June 2014
DOI: 10.1111/omi.12062

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 two-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 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 being 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 fewer voids compared with 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 by the two S-layer proteins. In addition, T9SS protein translocation is decoupled from *O*-glycan attachment in *T. forsythia*.

INTRODUCTION

Protein translocation across the cell envelope of Gram-negative bacteria has been intensively studied for decades and 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 before 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 Manα1-2Manα1-phosphate A-LPS antibody; however, the mode of attachment is not yet fully understood (Saiki & Konishi, 2014), and neither is 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 have yet been assigned (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 & Konishi, 2014). Sov was found in the OM fraction of *P. gingivalis* and was shown to be involved in the secretion of gingipains (Saiki & 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 the development and progression of periodontal diseases (Socransky & Haffajee, 1992; Socransky *et al.*, 1998; Holt & 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 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 Toll-like receptor 2 in cooperation with Toll-like receptor 1 (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 two-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 type 17 responses, thereby ensuring the persistence of the pathogen in the host (Settem *et al.*, 2013).

The identification of proteins that 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, Manassas, VA) and defined T9SS mutants (see below) were grown in 37 g l^{-1} of brain–heart infusion (BHI) liquid media (Oxoid, Basingstoke, UK), containing 5 g l^{-1} yeast extract (Oxoid), 0.5 g l^{-1} L-cysteine (Sigma, Vienna, Austria), 2.5 μg ml^{-1} hemin (Sigma), 2.0 μg ml^{-1} menadione (Sigma), 10 μg ml^{-1} *N*-acetylmuramic acid (Carbosynth, Compton, UK) and 5% (volume/volume) horse serum (Life Technologies, Vienna, 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% weight/volume (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 and 5 μg ml^{-1}, respectively, when appropriate.

Escherichia coli strains were grown under standard conditions in Luria–Bertani medium supplemented with 100 μg ml^{-1} ampicillin, when appropriate.

Porphyromonas gingivalis W83 is used as a reference strain for comparison with predicted components of the T9SS in *T. forsythia* ATCC 43037.

DNA isolation and polymerase chain reaction amplification

Genomic DNA was isolated from 2 ml of bacterial suspension as described previously (Cheng & Jiang, 2006). Plasmid DNA was purified using the GeneJET Plasmid MiniPrep Kit (Thermo Scientific, Vienna, Austria). Polymerase chain reaction (PCR) fragments were amplified either by Phusion High-Fidelity DNA Polymerase (Thermo Scientific) or by Herculase II Phusion Polymerase (Agilent Technologies, Waldbronn, Germany) according to the manufacturers' protocols. 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.

Table 1 Oligonucleotides used in this study

#	Sequence (5′ → 3′)
413	TCCACGGAGTAATTCCTCAGAGCGATTG
414	*GAAGCTATGGGGGTACCTCCCCCGGG*AG TTGATTTTTCCAACTGCCGTAGGCCGGGGTC
415[a]	CCCGGGGGAGGTACCCCCGATAGCTTC
416[a]	CCCGGGGGCTAGAGGATCCCCGAAGC
417	*GCTTCGGGGATCCTCTAGCCCCCGGG* CTGCTTTATTGCCGATGATGGAAGCAGCAG
418	AGCCGTTCGCTTCAAGAGCATTCGTCTCGTC
426	GTGTCGGACGCCTTGGGGCTGCTCTG
429	CAGACATTATCAGGCCTTTC
436	TAGCTTCTGCTTGGGCGCAGGTTGAC
437	*GAAGCTATGGGGGTACCTCCCCCGGG*CTT CCTGATTGTCTATTGTCTGTTCTTATC
438	*GCTTCGGGGATCCTCTAGCCCCCGGG*ACT CCAACGACAACAATACATCAG
439	GCAGCAGACGTCCGTAAACCTGATTC
448	CTTTCGAATGACGATCAAACGATTCTG
449	GAGGCTGCTGAGGCGGATACATC

Oligonucleotides used for overlap extension polymerase chain reaction are written in italics.
[a]Honma *et al.* (2007).

Construction of T9SS-deficient mutants (TFΔ*0955*, TFΔ*2327*)

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

The gene knockout cassette for constructing the TFΔ*0955* mutant consisted of a 2188-base-pair (bp) erythromycin resistance gene *ermF-ermAM* (primer pair 415/416) flanked by homologous upstream and downstream regions (Fig. 1A). Genomic DNA from *T. forsythia* Δ*wecC* (obtained from A. Sharma, State University of New York at Buffalo, NY) was used as a template. The 1035-bp upstream and 1027-bp downstream 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 knockout construct was 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 knockout cassette via PCR amplification using the primer pair 426/429.

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

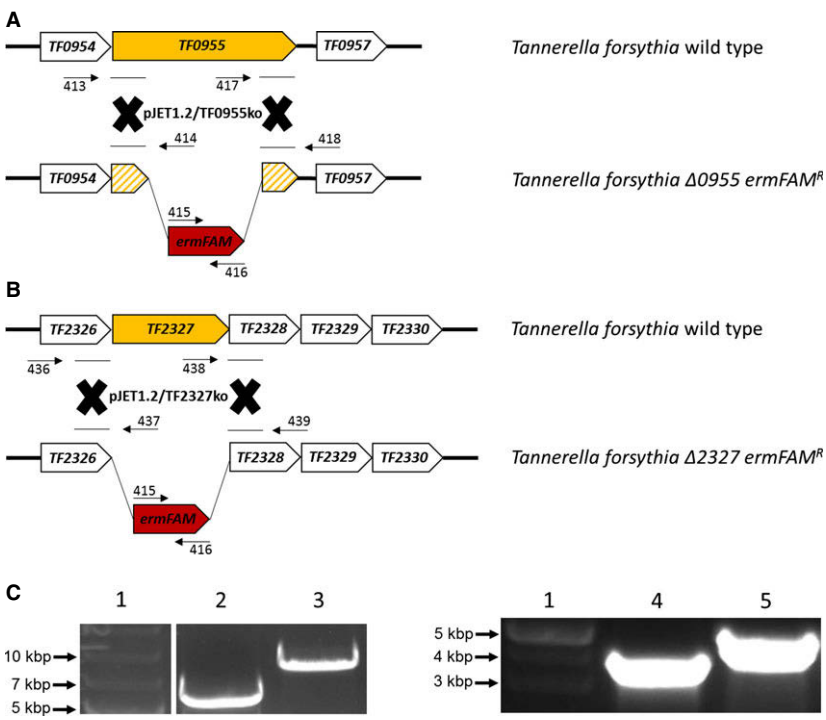


Figure 1 Strategy for TF*0955* gene disruption and TF*2327* gene deletion at the *Tannerella forsythia* wild-type locus and confirmation by polymerase chain reaction (PCR) (not drawn to scale). (A) Genomic organization of the TF*0955* gene showing the upstream and downstream homology regions used for homologous recombination and insertion of the selectable *ermF-AM* cassette disrupting TF*0955*. Primers (compare with Table 1) are indicated with arrows. (B) TF*2327* 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 positions 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 5490-bp PCR product (lane 2) and with integrated *ermF-AM* cassette, yielding a 7678-bp PCR product (lane 3); *T. forsythia* wild-type (primer pair 448/449) yielding a 3529-bp PCR product (lane 4) and with integrated *ermF-AM* cassette, yielding a 4277-bp PCR product (lane 5).

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.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western immunoblotting

Crude cell extracts of *T. forsythia* wild-type and mutant cells (TFΔ*0955*, TFΔ*2327*) were run on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels in a Mini Protean electrophoresis apparatus (Bio-Rad, Vienna, 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, Lincoln, NE). 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 reagent following a standard protocol (Doerner & White, 1990). For semi-quantification of S-layer proteins present in *T. forsythia* wild-type and TFΔ*0955* and TFΔ*2327* mutant cells, equal amounts of total cell protein were loaded on the SDS–PAGE gels in two different dilutions, each, followed by Western immunoblotting. The integrated intensity of the detected bands was determined using the LI-COR Odyssey Application software 3.0.21.

S-layer *O*-glycan preparation and liquid chromatography 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–PAGE gels and *O*-glycans were released from the protein backbone by in-gel reductive β-elimination followed by removal of excess salt using a 25-mg HyperSep Hypercarb SPE cartridge (Thermo Scientific). Borohydride-reduced *O*-glycans were analyzed by porous graphitic carbon-electrospray ionization-tandem mass spectrometry (ESI-MS/MS) as described recently (Hypercarb, 0.32 × 150 mm, particle size 5 μm) (Stadlmann *et al.*, 2008). Detection was performed using an ESI-Q-TOF Global Ultima from Micromass (Waters, Milford, MA). Data were evaluated using MassLynx 4.0 software (Waters). The MS/MS experiments were performed at 30% collision energy using collision-induced dissociation with argon gas. Slices from the SDS–PAGE gel without sample application were treated in the same way as described for the *O*-glycan samples and the MS spectra acquired 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 digestion 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 carried out using the ProteinScape 3 software tool (Bruker-Daltonik, Leipzig, Germany) with Mascot 2.3.02 algorithm for peptide identification or the X!tandem algorithm (The Global Proteome Machine Organization; www.thegpm.org).

Scanning electron microscopy

Tannerella forsythia cells with an initial optical density of OD_{600} 0.07 were grown anaerobically on sterile glass slides at 37°C for 3 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 (20–100% 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, Tulln, Austria). At one side of the glass slide, one drop of silver solution was applied before sputter-coating with gold (EM SDC005; Leica, Wetzlar, Germany). Biofilms were imaged using an Inspect S50 scanning electron microscope (FEI, Eindhoven, the Netherlands).

Ultra-thin sectioning and transmission electron microscopy

Ultrathin-sectioning of *T. forsythia* wild-type and TFΔ*0955* and TFΔ*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).

Ultrathin-sectioned samples were investigated in a Tecnai G^2 20 Twin transmission electron microscope (FEI), operating at 80 kV. Pictures were taken with an FEI Eagle 4k CCD camera (4096 $\times$ 4096 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

Tannerella 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 was transferred into micro dishes (35-mm height; Ibidi, Munich, 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 μg 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) 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 TF*0955* or TF*2327*, 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 & Zhu, 2013). In the case of the ΔTF*0955* mutant, no polar effects were expected, because the downstream gene TF*0957* is separated by 106 non-coding nucleotides (Fig. 1A). In contrast, the other targeted gene, TF*2327*, is predicted to constitute an operon (BioCyc) (Caspi *et al.*, 2012) together with the immediate downstream genes TF*2328*, TF*2329* and TF*2330*, implicating potential polar effects causing the inactivation of the said genes in the mutant (Fig. 1B). However, as these latter genes code for predicted components of the T9SS, their inactivation would not have adversely affected the usability of the ΔTF*2327* 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 ΔTF*0955* (primer pair 426/429) and ΔTF*2327* (primer pair 448/449) conforming with the calculated size of 7678 bp (Fig. 1C, lane 3) and 4277 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 with 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 two-dimensional crystalline S-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). Hence, 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 in the range of 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 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 periodic acid-Schiff reagent (Fig. 2B, lane 2). In addition, in both the ΔTF*0955* (Fig. 2B, lane 3) and the ΔTF*2327* (Fig. 2B, lane 4) mutants two prominent protein bands that were carbohydrate-positive were detected; however, these bands were clearly downshifted compared with the wild-type (Fig. 2A,B, 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. 2C) 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 ΔTF*0955* and the ΔTF*2327* mutants. However, the integrated intensity of either S-layer protein expressed in the mutant cells was only about one-third compared with that of the wild-type cells, possibly as the result of feedback inhibition, as the protein secretion was blocked. Integrated intensity values were 34.5 and 35.5 for TfsB from ΔTF*0955* and ΔTF*2327*, respectively, as opposed to 94.7 from wild-type, and 21.3 and 22.0 for TfsA from ΔTF*0955* and ΔTF*2327*, 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 ΔTF*0955* and ΔTF*2327* 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*.,

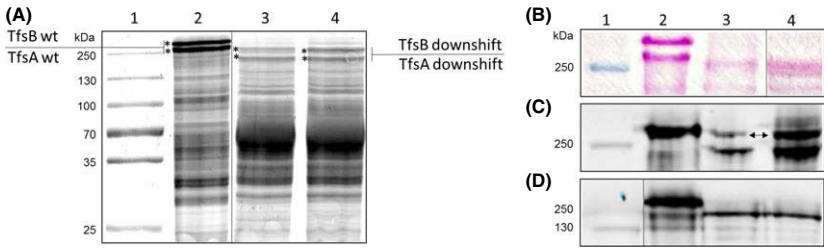


Figure 2 (A) Coomassie Brilliant Blue R-250 stained SDS gel (10%) of crude cell extracts from *Tannerella forsythia* wild-type (lane 2), Δ*TF2327* (lane 3) and Δ*TF0955* (lane 4). The prominent S-layer proteins (identity confirmed by proteomics analyses) are significantly downshifted in both T9SS-deficient mutants, which is also evident from the periodic acid-Schiff 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 mass spectrometric analysis (Table 2, Fig. 3).

Table 2 Tryptic peptides derived from the C-terminus of TfsA and TfsB as detected in *Tannerella forsythia* wild-type and T9SS mutants

Protein	Unique peptides	log(*e*)[1]	Score[2]	Sequence[3]
TfsA				
1083-SRGAYTDWIAEAETWNVKMTKKSPL**ANAEASVTDVKVVAGAGEVMIRNASGK**				
KVTVSNILGQTIAKVVLTSDNQTIAAPQGVVVVAIEGENAVKAVVK-1179				
TfsA wild-type	9	−2.8		visr^{1084} GAYTDWIAEAETWNVK ^{1099}mtkk
TfsA Δ*2327*	3	−3.1		kmtk^{1103} KSPLANAEASVTDVK ^{1117}vvag
	5	−2.4		mtkk^{1104} SPLANAEASVTDVK ^{1117}vvag
	2	−1.5		asgk^{1134} KVTVSNILGQTIAK ^{1147}vvlt
TfsA Δ*0955*	1	−1.7		kmtk^{1103} KSPLANAEASVTDVK ^{1117}vvag
TfsB				
1268-RSDVLQAIADAEIFNVEKTKEDPV**ANEITPAITEVKVVAENGAVTILNAAGKK**				
VVVSNVLGQTLVNTVLTSDRATVAAPQGVVVVVVEGQPAVKAMVK-1364				
TfsB wild-type	39	−3.2		visr^{1268} SDVLQAIADAEIFNVEK ^{1284}tked
TfsB Δ*2327*	1	−5.1		nvek^{1285} TKEDPVANEITPAITEVK ^{1302}vvae
	13	−3.2		tevk^{1303} VVAENGAVTILNAAGK ^{1318}kvvv
	2		58.2	agkk^{1318} VVVSNVLGQTLVNTVLTSDR ^{1339}atva
	5		58.4	tsdr^{1339} ATVAAPQGVVVVVVEGQPAVK ^{1361}amvk
TfsB Δ*0955*	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

The CTD predicted for TfsA and TfsB according to 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.

[1]log(*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.

[2]Mascot Score.

[3]Sequence: the sequence of the assigned peptide sequence. The immediate N- and C-terminal sequences of the identified peptides are also shown.

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 ΔTF*0955* and ΔTF*2327* 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 ΔTF*2327*, and 16 and 36% for ΔTF*0955*. 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). These data imply 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 cytoplasmic membrane 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 the 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 ΔTF*2327*, ΔTF*0955* 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* decasaccharide (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 was 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-time-of-flight-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–PAGE gel slices without sample application and Coomassie Brilliant Blue R-250 staining, no glycans were detected. Full assembly of the *T. forsythia* *O*-glycan and its transfer onto the proteins occur before T9SS-mediated translocation across the OM and, therefore CTD protein glycosylation and CTD protein secretion are independent pro-

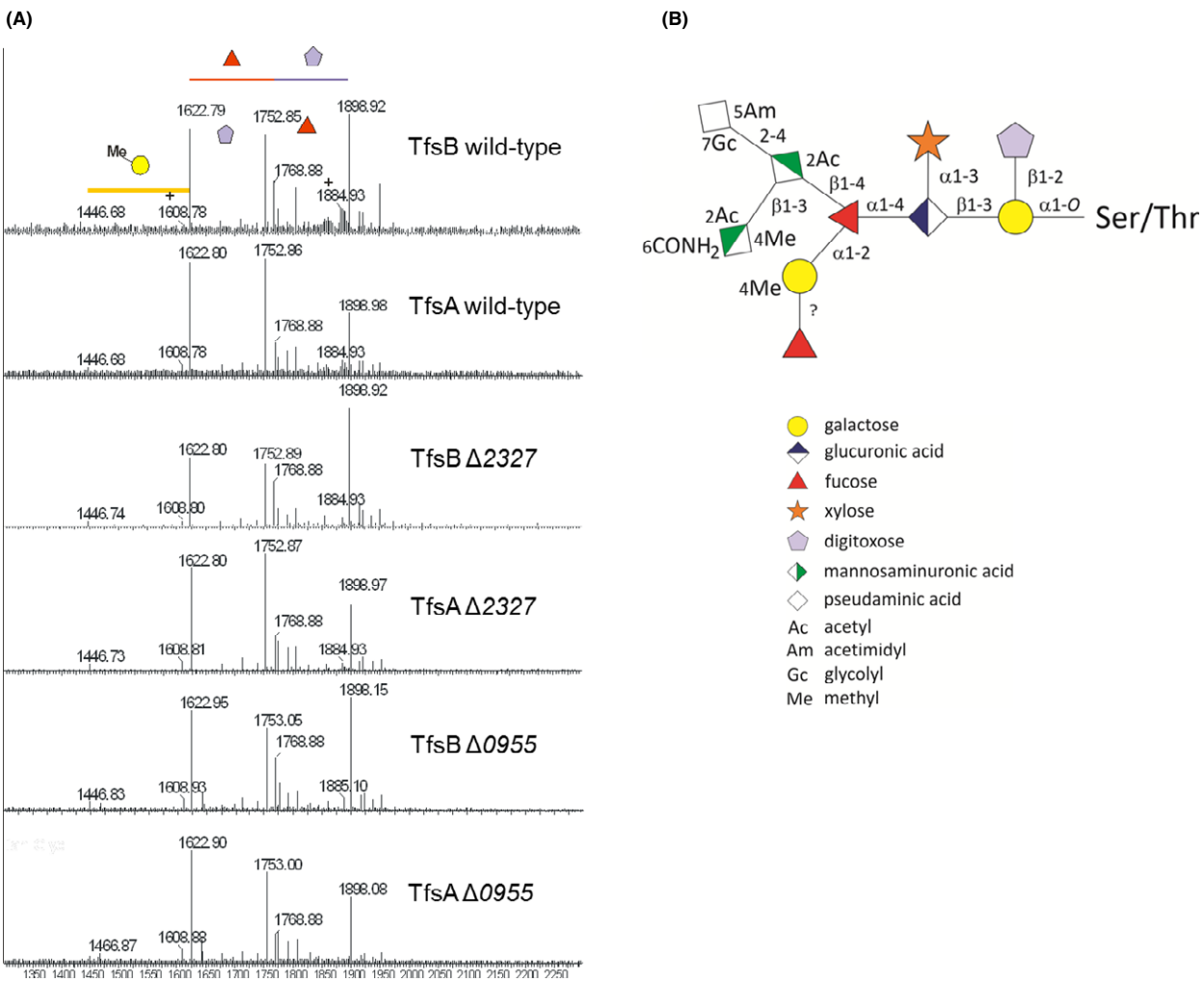


Figure 3 (A) Deconvoluted electrospray ionization-time of flight-mass spectrometry spectrum of TfsA and TfsB *O*-glycans in *Tannerella forsythia* wild-type and mutant strains. Identical spectra were recorded for the *O*-glycan derived from the Δ*TF0955* and Δ*TF2327* mutants and the wild-type. (B) For clarification, the complete S-layer glycan structure is shown. Ser, serine; Thr, threonine.

cesses. With respect to the still missing explanation of the downshifted S-layer glycoprotein bands from the ΔTF*2327* and ΔTF*0955* mutants on SDS–PAGE gels (Fig. 2), it is tempting to speculate that either fewer 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 the 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 two-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 × 10 nm and a thickness of about 22 nm (Sekot *et al.*, 2012). To visualize, if 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 ΔTF*2327* and ΔTF*0955* 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 mutants, 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 that are known to enable nutrition delivery to the base of the biofilm (Fig. 5A). The biofilm of

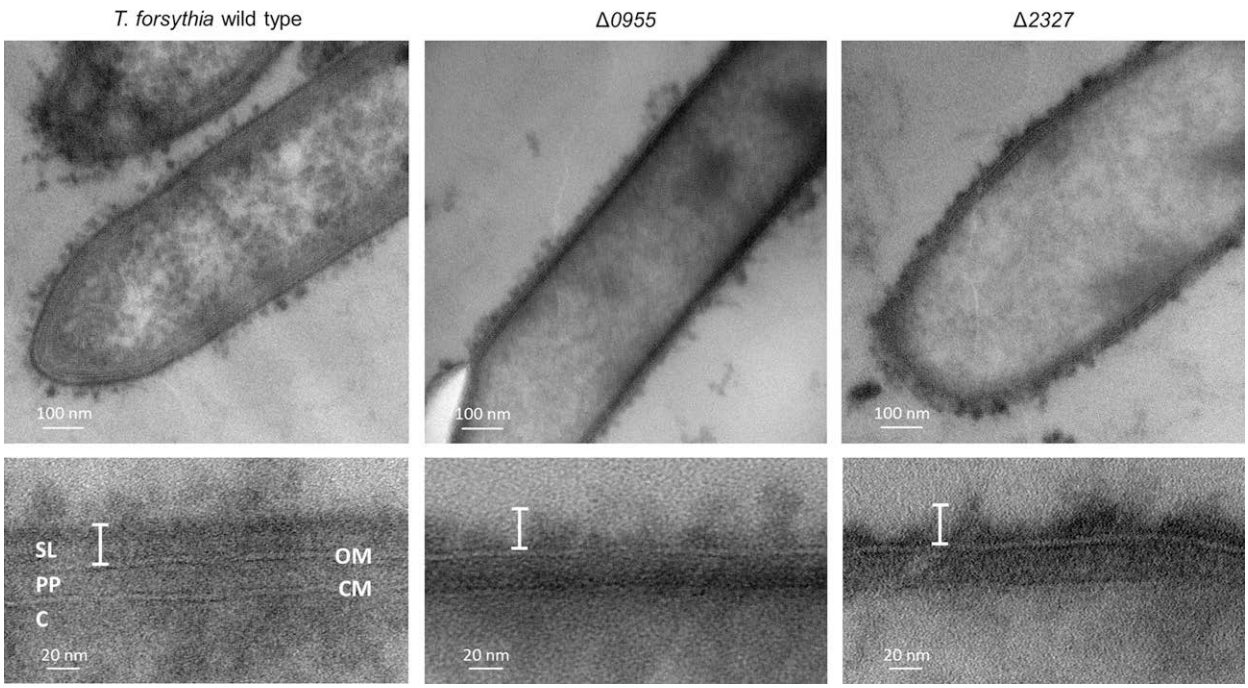


Figure 4 Transmission electron micrographs showing ultra-thin cross-sectioned cells of *Tannerella forsythia* and T9SS mutants. The *T. forsythia* wild-type displays a serrated S-layer, whereas no S-layer is present on *T. forsythia* Δ*TF0955* and Δ*TF2327* cells. SL, S-layer; OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane; C, cytoplasm.

the ΔTF*0955* and ΔTF*2327* 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 with the wild-type biofilm with fewer 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 with the wild-type was confirmed by confocal laser scanning microscopy 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 ΔTF*2327* 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 thataiotaomicron*. 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

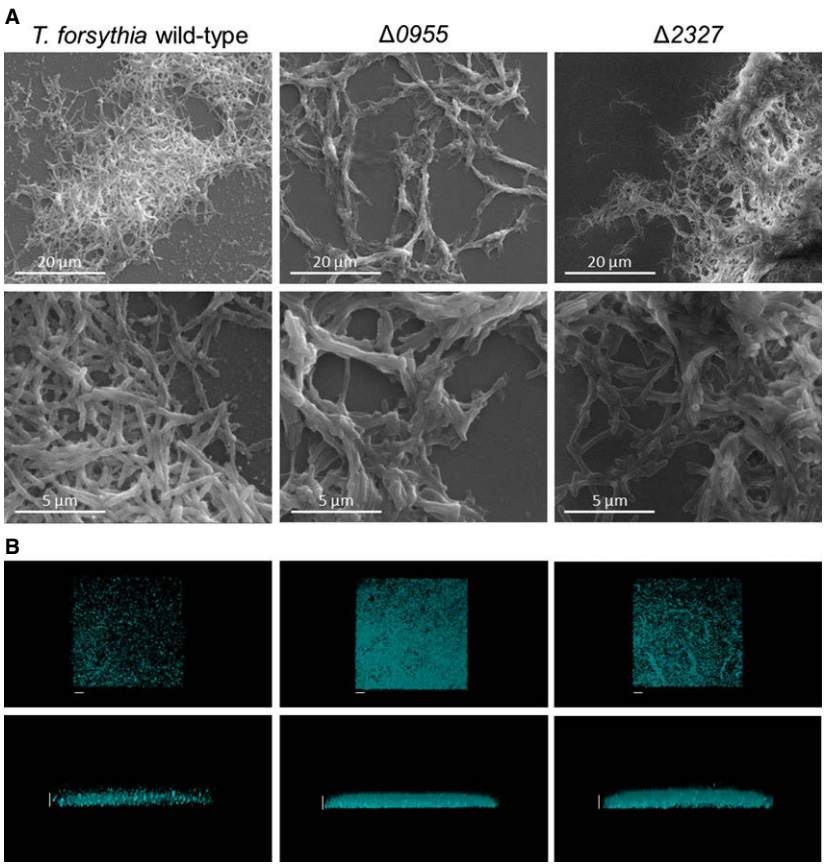


Figure 5 (A) Scanning electron micrographs showing altered biofilm and cell morphology in T9SS-deficient mutants in comparison to the wild-type. Biofilm was grown anaerobically on glass for 3 days; (B) Confocal laser scanning micrographs of *Tannerella forsythia* wild-type and mutant biofilms. Mutant biofilms revealed denser growth and a height comparable to that of the wild-type biofilm. Scale bar, 20 μm.

porK (TF*2327*) *and porU* (TF*0955*) 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 two-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 predicted to function as the C-terminal signal peptidase required for cleaving the CTD from proteins destined 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 – ΔTF*0955* and ΔTF*2327* – 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 extracellular S-layer (Fig. 2). A similar effect was seen for a *P. gingivalis lptO* (PG0027) knock-out mutant missing its electron-dense surface layer (Saiki & 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 scanning electron microscopy and confocal laser scanning microscopy analysis (Fig. 5). These abnormalities are most likely a combined effect of the blocked secretion of CTD proteins in the *T. forsythia* ΔTF*0955* and ΔTF*2327* 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 proteins 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-containing 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 because of 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 in 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 (TFΔ*0955*, TFΔ*2327*), which, consequently, also lacked the S-layer. Further, we could show that protein translocation and *O*-glycosylation are decoupled from each other.

ACKNOWLEDGEMENTS

We thank Andrea Scheberl for excellent technical assistance with ultra-thin sectioning. Financial support

came from the Austrian Science Fund FWF, project P24317-B22 (to C.S.) and the PhD program 'BioToP – Biomolecular Technology of Proteins' (Austrian Science Fund, FWF project W1224). J.P. acknowledges support by grants from US National Institutes of Health (DE 09761), the European Commission (FP7-PEOPLE-2011-ITN-290246 'RAPID' and FP7-HEALTH-F3-2012-306029 'TRIGGER'), and the National Science Center (2012/04/A/NZ1/00051, NCN, Krakow, Poland). The Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University is a beneficiary of structural funds from the European Union (POIG.02.01.00-12-064/08).

REFERENCES

Amos, L.A., Henderson, R. and Unwin, P.N. (1982) Three-dimensional structure determination by electron microscopy of two-dimensional crystals. *Prog Biophys Mol Biol* **39**: 183–231.

Caspi, R., Altman, T., Dreher, K. *et al.* (2012) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res* **40**: D742–D753.

Chen, Y.Y., Peng, B., Yang, Q. *et al.* (2011) The outer membrane protein LptO is essential for the O-deacylation of LPS and the co-ordinated secretion and attachment of A-LPS and CTD proteins in *Porphyromonas gingivalis*. *Mol Microbiol* **79**: 1380–1401.

Cheng, H.R. and Jiang, N. (2006) Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnol Lett* **28**: 55–59.

Coyne, M.J., Fletcher, C.M., Chatzidaki-Livanis, M., Posch, G., Schäffer, C. and Comstock, L.E. (2013) Phylum-wide general protein *O*-glycosylation system of the *Bacteroidetes*. *Mol Microbiol* **88**: 772–783.

Crowther, R.A., Henderson, R. and Smith, J.M. (1996) MRC image processing programs. *J Struct Biol* **116**: 9–16.

Dashper, S.G., Seers, C.A., Tan, K.H. and Reynolds, E.C. (2011) Virulence factors of the oral spirochete *Treponema denticola*. *J Dent Res* **90**: 691–703.

Desvaux, M., Hebraud, M., Talon, R. and Henderson, I.R. (2009) Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue. *Trends Microbiol* **17**: 139–145.

Doerner, K.C. and White, B.A. (1990) Detection of glycoproteins separated by nondenaturing polyacrylamide gel electrophoresis using the periodic acid-Schiff stain. *Anal Biochem* **187**: 147–150.

Glew, M.D., Veith, P.D., Peng, B. *et al.* (2012) PG0026 is the C-terminal signal peptidase of a novel secretion system of *Porphyromonas gingivalis*. *J Biol Chem* **287**: 24605–24617.

Holt, S.C. and Ebersole, J.L. (2005) *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol 2000* **38**: 72–122.

Honma, K., Inagaki, S., Okuda, K., Kuramitsu, H.K. and Sharma, A. (2007) Role of a *Tannerella forsythia* exopolysaccharide synthesis operon in biofilm development. *Microb Pathog* **42**: 156–166.

Ishiguro, I., Saiki, K. and Konishi, K. (2009) PG27 is a novel membrane protein essential for a *Porphyromonas gingivalis* protease secretion system. *FEMS Microbiol Lett* **292**: 261–267.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Lee, S.W., Sabet, M., Um, H.S., Yang, J., Kim, H.C. and Zhu, W. (2006) Identification and characterization of the genes encoding a unique surface (S-) layer of *Tannerella forsythia*. *Gene* **371**: 102–111.

McBride, M.J. and Zhu, Y. (2013) Gliding motility and Por secretion system genes are widespread among members of the phylum *Bacteroidetes*. *J Bacteriol* **195**: 270–278.

Messner, P., Hollaus, F. and Sleytr, U.B. (1984) Paracrystalline cell wall surface layer of different *Bacillus stearothermophilus* strains. *Int J Syst Bacteriol* **34**: 202–210.

Nguyen, K.A., Travis, J. and Potempa, J. (2007) Does the importance of the C-terminal residues in the maturation of RgpB from *Porphyromonas gingivalis* reveal a novel mechanism for protein export in a subgroup of Gram-negative bacteria? *J Bacteriol* **189**: 833–843.

Onishi, S., Honma, K., Liang, S. *et al.* (2008) Toll-like receptor 2-mediated interleukin-8 expression in gingival epithelial cells by the *Tannerella forsythia* leucine-rich repeat protein BspA. *Infect Immun* **76**: 198–205.

Posch, G., Pabst, M., Brecker, L., Altmann, F., Messner, P. and Schäffer, C. (2011) Characterization and scope of S-layer protein *O*-glycosylation in *Tannerella forsythia*. *J Biol Chem* **286**: 38714–38724.

Posch, G., Andrukhov, O., Vinogradov, E. *et al.* (2013) Structure and immunogenicity of the rough-type lipopolysaccharide from the periodontal pathogen *Tannerella forsythia*. *Clin Vaccine Immunol* **20**: 945–953.

Pugsley, A.P. (1993) The complete general secretory pathway in gram-negative bacteria. *Microbiol Rev* **57**: 50–108.

Sabet, M., Lee, S.W., Nauman, R.K., Sims, T. and Um, H.S. (2003) The surface (S-) layer is a virulence factor of *Bacteroides forsythus*. *Microbiology* **149**: 3617–3627.

Saiki, K. and Konishi, K. (2014) *Porphyromonas gingivalis* C-terminal signal peptidase PG0026 and HagA interact with outer membrane protein PG27/LptO. *Mol Oral Microbiol* **29**: 32–44.

Sato, K. (2011) Por secretion system of *Porphyromonas gingivalis*. *J Oral Biosci* **53**: 187–196.

Sato, K., Naito, M., Yukitake, H. *et al.* (2010) A protein secretion system linked to bacteroidete gliding motility and pathogenesis. *Proc Natl Acad Sci USA* **107**: 276–281.

Sato, K., Yukitake, H., Narita, Y., Shoji, M., Naito, M. and Nakayama, K. (2013) Identification of *Porphyromonas gingivalis* proteins secreted by the Por secretion system. *FEMS Microbiol Lett* **338**: 68–76.

Schneider, C.A., Rasband, W.S. and Eliceiri, K.W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**: 671–675.

Seers, C.A., Slakeski, N., Veith, P.D. *et al.* (2006) The RgpB C-terminal domain has a role in attachment of RgpB to the outer membrane and belongs to a novel C-terminal-domain family found in *Porphyromonas gingivalis*. *J Bacteriol* **188**: 6376–6386.

Sekot, G., Posch, G., Messner, P. *et al.* (2011) Potential of the *Tannerella forsythia* S-layer to delay the immune response. *J Dent Res* **90**: 109–114.

Sekot, G., Posch, G., Oh, Y.J. *et al.* (2012) Analysis of the cell surface layer ultrastructure of the oral pathogen *Tannerella forsythia*. *Arch Microbiol* **194**: 525–539.

Settem, R.P., Honma, K., Nakajima, T. *et al.* (2013) A bacterial glycan core linked to surface (S)-layer proteins modulates host immunity through Th17 suppression. *Mucosal Immunol* **6**: 415–426.

Sharma, A. (2010) Virulence mechanisms of *Tannerella forsythia*. *Periodontol 2000* **54**: 106–116.

Shoji, M., Sato, K., Yukitake, H. *et al.* (2011) Por secretion system-dependent secretion and glycosylation of *Porphyromonas gingivalis* hemin-binding protein 35. *PLoS ONE* **6**: e21372.

Sleytr, U.B., Messner, P., Pum, D. and Sára, M. (1993) Crystalline bacterial cell surface layers. *Mol Microbiol* **10**: 911–916.

Socransky, S.S. and Haffajee, A.D. (1992) The bacterial etiology of destructive periodontal disease: current concepts. *J Periodontol* **63**: 322–331.

Socransky, S.S., Haffajee, A.D., Cugini, M.A., Smith, C. and Kent, R.L. Jr (1998) Microbial complexes in subgingival plaque. *J Clin Periodontol* **25**: 134–144.

Stadlmann, J., Pabst, M., Kolarich, D., Kunert, R. and Altmann, F. (2008) Analysis of immunoglobulin glycosylation by LC-ESI-MS of glycopeptides and oligosaccharides. *Proteomics* **8**: 2858–2871.

Tseng, T.T., Tyler, B.M. and Setubal, J.C. (2009) Protein secretion systems in bacterial–host associations, and their description in the Gene Ontology. *BMC Microbiol* **9** (Suppl. 1): S2.

Veith, P.D., O'Brien-Simpson, N.M., Tan, Y., Djatmiko, D.C., Dashper, S.G. and Reynolds, E.C. (2009) Outer membrane proteome and antigens of *Tannerella forsythia*. *J Proteome Res* **8**: 4279–4292.

Veith, P.D., Nor Muhammad, N.A., Dashper, S.G. *et al.* (2013) Protein substrates of a novel secretion system are numerous in the *Bacteroidetes* phylum and have in common a cleavable C-terminal secretion signal, extensive post-translational modification, and cell-surface attachment. *J Proteome Res* **12**: 4449–4461.

Veith, P.D., Chen, Y.Y., Gorasia, D.G. *et al.* (2014) *Porphyromonas gingivalis* outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors. *J Proteome Res* **13**: 2420–2432.

Wacker, M., Linton, D., Hitchen, P.G. *et al.* (2002) N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. *Science* **298**: 1790–1793.

5

Conclusions of the thesis

The modification of proteins with complex glycans is pivotal to the successful lifestyle of bacterial pathogens. The investigation of glycoprotein glycan biosynthesis and the translocation of glycoproteins across the cell membranes in the oral pathogen *T. forsythia* were the main focuses of the work presented in this thesis.

Research into the predicted protein *O*-glycosylation gene cluster (Posch *et al.* 2011) led to the identification of a 27-kb gene cluster consisting of five glycosyltransferases, three glycan modifying methyltransferases, necessary genes coding for the six-step biosynthesis of the CMP-activated pseudaminic acid as well as a putative Wzx-like flippase located at the 3´-end of the gene cluster. Although the identified glycosylation gene cluster does not encode all genes required for the synthesis of the complete *O*-glycan present on various *T. forsythia* proteins, single gene knock-out mutants allowed to elucidate the step-wise assembly of the glycan by five glycosyltransferases and the modification of the glycan by three methyltransferases using a mass spectrometric approach. Further, a novel α-2,4 glycosyltransferase (Tanf_01245) transferring a modified pseudaminic acid (Pse5Am7Gra) could be identified in the type strain (ATCC 43037) and analysed in detail. Likewise a legionaminic acid transferase (TFUB4_00887) was identified and analysed in another *T. forsythia* strain, UB4 (Tomek *et al.* 2017).

Glycosyltransferase deficient mutants generated in the frame of this thesis also identified transferases linking either the terminal β-1,3 *N*-acetylmannosaminuronic acid (Tanf_01260) or the internal β-1,4 *N*-acetylmannosaminuronic acid (Tanf_01290) onto the growing glycan chain. An α-1,2 galactosyl transferase (Tanf_01305) was identified adding the branching galactose unit. Taken together, all glycosyltransferases located within the general protein *O*-glycosylation gene cluster are required for the assembly of the glycan, but are not sufficient for assembling the underlining five carbohydrate core structure of the glycan (**M.B. Tomek, unpublished data**). Corresponding carbohydrate-active enzymes obviously localize outside of the gene cluster elsewhere on the genome and need to be identified in future research. Mutant strains with truncated glycans, generated in the course of elucidating the biosynthesis pathway of the protein *O*-glycan, are valuable for the investigation of the biological function attributed to each carbohydrate unit present on the glycan. First insights resulted from a pseudaminic acid biosynthesis deficient mutant lacking the terminal carbohydrate residue. This mutant exhibited significantly reduced biofilm formation on mucin-coated surfaces as compared to the wild-type strain, indicating a possible influence of the glycan on diverse biological processes (Friedrich *et al.* 2017).

The identification of methyltransferases modifying either the branching galactose residue or the terminal *N*-acetylmannosaminuronic acid is a further outcome of this thesis, in order to characterize the enzymes and implicated biological effects of this rare and largely unknown glycan modification in upcoming experiments (**M.B. Tomek, unpublished data**).

Secretion systems in Gram-negative bacteria have been drug targets for a long time, as protein secretion systems fulfil a wide variety of essential functions for pathogens. Specifically designed drugs can block these machineries and, thus, can either attenuate the virulence of pathogenic bacteria or kill them (Durand *et al.* 2009; Baron 2010; Heras *et al.* 2015). Given the fact that antibiotic resistances in human pathogens relentlessly increase, new antibacterial targets are of great interest to ensure, in combination with regulatory changes in antibiotic use, therapeutic options against infectious diseases (Ruer *et al.* 2015). The proof of the presence of a novel secretion system (type IX secretion system) in *T. forsythia* (**Tomek *et al.* 2014**) may be the initiating discovery in the development of a novel antibacterial therapy against this worldwide occurring human pathogen. Further, the fact that this secretion system is limited to the phylum *Bacteroidetes* might be of great interest with regard to treatment strategies (McBride and Zhu 2013; Veith *et al.* 2013; Nakayama 2015). Although 3D structures and biological functions of components constituting the T9SS are only slowly being discovered in *P. gingivalis* (Gorasia *et al.* 2016; Heath *et al.* 2016; Lasica *et al.* 2016; Vincent *et al.* in press), the prerequisite of C-terminal domains, acting as signal sequence for proteins intended to cross the outer cell membrane, could be verified for *T. forsythia* in course of this thesis (**Tomek *et al.* 2014**).

In summary, the thesis contributes to the understanding of the molecular basis of the biosynthesis of a complex bacterial *O*-glycan and the translocation of (glyco-)proteins across the outer membrane in the oral pathogen *T. forsythia*. This included

- the description of its 27-kb *O*-glycosylation gene cluster.
- the identification and experimental verification of five glycosyltransferases and three methyltransferases involved in assembling and modifying the *O*-glycan.
- the detailed characterization of a so far unknown pseudaminic acid and legionaminic acid derivative transferase.
- the proof of presence of a type IX secretion system and the importance of the C-terminal domain as a signal sequence for protein cell surface translocation.
- and the independence of protein glycosylation and translocation when employing a T9SS.

6

Conference contributions

6.1 Oral presentations [1]

- Characterization of TF0955 - A putative protease from the oral pathogen *Tannerella forsythia*
  Tomek, M.B. [30th Winter School on Proteases and Their Inhibitors, Tiers, Italy, February 27-March 3, 2013]

- Glycoprofiling of the oral pathogen *Tannerella forsythia* for identifying novel strategies in pathogenicity
  Tomek, M.B. [BioToP Annual Retreat, Retz, Austria, April 8-10, 2013]

- Glycoprotein biosynthesis in the oral pathogen *Tannerella forsythia*
  Tomek, M.B. [DNBT Winter School, Lutzmannsburg, Austria, February 25-26, 2014)

- Glycoprotein biosynthesis in the oral pathogen *Tannerella forsythia* [2]
  Tomek, M.B., Neumann, L., Potempa, J., Messner, P., Schäffer, C. [6th Annual ÖGMBT Meeting, Vienna, Austria, September 15-18, 2014]

- *Tannerella forsythia* – A sweet periodontal pathogen
  Tomek, M.B., Friedrich, V., Megson, ZA., Posch, G., Koerdt, A., Nimeth, I., Andesner, P., Altmann, F., Messner, M., Schäffer, C. [Society for Glycobiology Meeting 2014, Honolulu, Hawaii, November 16-19, 2014]

- Biosynthesis and translocation of glycoproteins in the oral pathogen *Tannerella forsythia*
  Tomek, M.B., Windwarder, M., Neumann, L., Nimeth, I., Altmann, F., Messner, P., Potempa, J., Schäffer, C. [DNBT Winter School, Deutschlandsberg, Austria, February 18-20, 2015]

- Biosynthesis and translocation of glycoproteins in the oral pathogen *Tannerella forsythia*
  Tomek, M.B. [Proteolysis – from the bench to the bed; 40 years of scientific breakthrough, Kraków, Poland, May 15-16, 2015]

- Biosynthesis and translocation of glycoproteins in the oral pathogen *Tannerella forsythia*
  Tomek, M.B., Windwarder, M., Neumann, L., Nimeth, I., Altmann, F., Messner, P., Potempa, J., Schäffer, C. [BioToP Annual Retreat, Puchberg, June 29-July 1, 2015]

[1] Presenting author is underlined.
[2] Awarded with the "Austrian Young Glycoscientist Best Talk Award".

- Nonulosonic acids in the periodontal pathogen *Tannerella forsythia*
  Tomek, M.B., Friedrich, V. [DNBT Spring School, Graz, Austria, April 5-8, 2016]

- Biosynthesis and translocation of glycoproteins in the oral pathogen *Tannerella forsythia*
  Tomek, M.B., [Joint DK Meeting, Semmering, Austria, September 29-30, 2016]

6.2 Poster presentations

- Glycoprofiling of the oral pathogen *Tannerella forsythia*
  Tomek, M.B., Posch, G., Friedrich, V., Megson, Z., Koerdt, A., Schäffer, C. [12th European Training Course on Carbohydrates, Groningen, Netherlands, June 3-7, 2012]

- Glycoprotein biosynthesis in the oral pathogen *Tannerella forsythia*
  Tomek, M.B., Neumann, L., Potempa, J., Messner, P., Schäffer, C [FASEB Science Research Conference on Microbial Glycobiology, Itasca, IL, USA, June 8-13, 2014]

- Predicted *O*-glycosylation locus and glycoprotein biosynthesis in *Tannerella forsythia* [1]
  Nimeth, I., **Tomek, M.B.**, Neumann, L., Messner, P., Schäffer, C. [6th Baltic Meeting on Microbial Carbohydrates, Gdansk, Poland, September 7-14, 2014]

- Biosynthesis and translocation of glycoproteins in the oral pathogen *Tannerella forsythia*
  Tomek, M.B., Windwarder, M., Neumann, L., Nimeth, I., Altmann, F., Messner, P., Potempa, J., Schäffer,C. [2nd International Conference on *Porphyromonas gingivalis* and related species in oral and systemic diseases, London, United Kingdom, June 23-25, 2015]

- The *O*-linked protein glycosylation cluster of the oral pathogen *Tannerella forsythia* [2]
  Tomek, M.B., Maresch, D., Friedrich, V., Windwarder, M., Janesch, B., Neumann, L., Nimeth, I., Posch, G., Altmann, F., Schäffer, C. [FASEB Science Research Conference on Microbial Glycobiology, West Palm Beach, FL, USA, June 12-17, 2016]

- Pseudaminic and legionaminic acid transferases from the oral pathogen *Tannerella forsythia*
  Tomek, M.B., Janesch, B., Maresch, D., Windwarder, M., Altmann, F., Schäffer, C. [GlycoT16 - 10th International Symposium on Glycosyltransferases, Toronto, Canada, June 19-21, 2016]

[1] Awarded with a poster prize.
[2] do.

Glycoprofiling of the oral pathogen *Tannerella forsythia*

[12th European Training Course on Carbohydrates, Groningen, Netherlands, June 3-7, 2012]

Markus Tomek, Gerald Posch, Valentin Friedrich, Zoë Megson, Andrea Koerdt, Christina Schäffer
Department of NanoBiotechnology, *NanoGlycobiology*, Universität für Bodenkultur Wien,
Muthgasse 11, A-1190 Vienna, Austria

The anaerobic, biofilm-forming oral pathogen *Tannerella forsythia* is one of the main etiological agents of periodontitis. *T. forsythia* is the only Gram-negative bacterium known so far to possess a 2D crystalline glycoprotein layer (S-layer) as outermost cell envelope layer.[1] This S-layer is composed of two intercalating S-layer proteins[2] that are modified with a unique *O*-glycosidically linked oligosaccharide with the structure 4-Me-β-Man*p*NAcCONH(2)-(1→3)-[Pse5Am7Gc-(2→4)-]-β-Man*p*NAcA-(1→4)-[4-Me-α-Gal*p*-(1→2)-]-α-Fuc*p*-(1→4)-[-α-Xyl*p*-(1→3)-]-β-Glc*p*A-(1→3)-[-β-Dig*p*-(1→2)-]-α-Gal*p*. [1] The *T. forsythia* S-layer is a virulence factor and contributes to delaying the host immune defence. The outer membrane of the bacterium is dominated by additional antigenic, high molecular-mass glycoproteins that are all modified with the "S-layer *O*-glycan". Some of these as well as the S-layer glycoproteins are up-regulated in the biofilm lifestyle of *T. forsythia*.[3,4]

Because of the increasing frequencies with which bacterial glycosylation systems are seen in pathogenic species, they have come under enhanced scrutiny; especially the "sweet" cell envelope of pathogens contributes to their cross-talk with the hosts. Consequently, for the study of virulence mechanisms and the associated development of therapeutic strategies, a molecular understanding of the glycans displayed in the cell envelope of pathogens is necessary.

Assessment of the influence of these glycoproteins on the immune response and investigating the "glyco-impact" on the life style of *T. forsythia* by analysing biofilm formation at the molecular level may reveal novel pathogenic strategies in Gram-negative organisms. This may constitute new targets for interfering with the pathogen's ability to establish infection in periodontal disease and for developing of novel diagnostic tools.

This work is supported by the Austrian Science Fund, projects P20605-B20 and P24317-B22 (to C.S.), and the doctoral programme BioTop, project W1224.

[1]Posch G. *et al*. (2011) *J Biol Chem* 286:38714-24
[2]Sekot G. *et al*. (2012) *Arch Microbiol* 6:525-539
[3]Pham T.K. *et al*. (2010) *Proteomics* 10:3130-41
[4]Veith P.D. *et al*. (2009) *J Proteome Res* 8:4279-92

Glycoprofiling of the oral pathogen *Tannerella forsythia*

Markus Tomek[1]*, Gerald Posch[1], Valentin Friedrich[1], Zoe Megson[1], Andrea Koerdt[1] and Christina Schäffer[1]
[1]Department of NanoBiotechnology, *NanoGlycobiology*, University of Natural Resources and Life Sciences Vienna
*markus.tomek@boku.ac.at

Introduction

The anaerobic oral biofilm-forming pathogen *Tannerella forsythia* is one of the main etiological agents of periodontitis. Periodontitis is one of the most common inflammatory diseases amongst the adult population, and, in its chronic form, it is the principal cause of tooth loss. *T. forsythia* infection may also exert a long-term impact on systemic health, such as cardiovascular disease or arteriosclerosis.

T. forsythia is the only Gram-negative bacterium currently known to possess a 2D crystalline glycoprotein layer (S-layer) as outermost cell envelope layer[1] [Fig. 1A, B]. This unique S-layer is a virulence factor and contributes to delaying the host immune defense.[2]

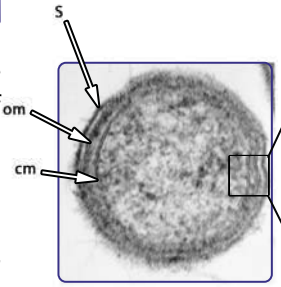


Fig. 1A. Transmission electron micrograph of an ultra-thin sectioned cell of *T. forsythia* showing the cell wall architecture.
cm, cytoplasmic membrane, **om**, outer membrane, **S**, S-layer

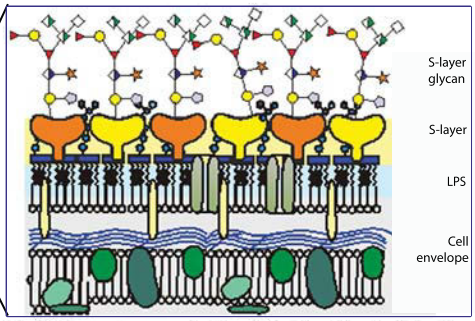


Fig. 1B. Scheme of a cross section through the *T. forsythia* cell wall showing the S-layer with the glycan chains protruding into the exterior.

O-Glycosylation in *T. forsythia*

T. forsythia S-layer is composed of two intercalating S-layer glycoproteins[2,3] that are both modified with a uniquely composed oligosaccharide [Fig. 2].

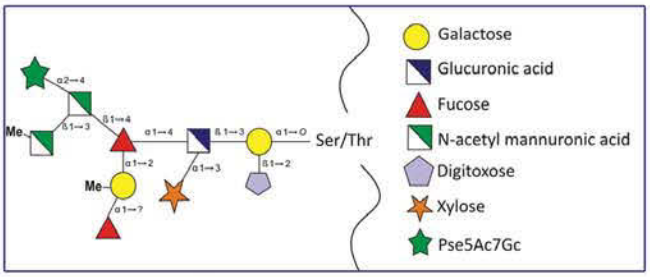


Fig. 2. The oligosaccharide is *O*-glycosidically linked to either serine or threonine present within the three-amino acid motif (D)(S/T)(A/I/L/M/T/V)[1] via α-D-Galp. Note the rare modified pseudaminic acid as terminal residue which might mediate adhesion to and invasion of specific host cells.

The outer membrane is dominated by additional antigenic, high molecular-mass glycoproteins which are modified with that specific oligosaccharide.[1]
Proteomics identified several of them, eg. the two S-layer glycoproteins TfsA and TfsB, as well as the hypothetical proteins TF1259 and TF2339, being up-regulated in the biofilm life-style of *T. forsythia*.[4,5]

The finding that several abundant proteins in *T. forsythia* are modified with the S-layer glycan[1] reveal the presence of a protein *O*-glycosylation system that is essential for creating a rich (outer membrane) glycoproteome. This can pinpoint a possible relevance for the virulence and pathogenic strategy of this bacterium.

Bacterial glycosylation systems and especially the "sweet" cell envelope contribute to the bacterium-host cross-talk. This may effect, for instance, bacterial adherence to specific tissues or triggering of a host immune response.

Characterizing the virulence potential of glycoproteins

For the study of virulence mechanisms and the associated development of therapeutic strategies, a molecular understanding of the glycans displayed in the cell envelope of pathogens is necessary.

We have evidence [Fig. 3] that several *T. forsythia* proteins (the two S-layer glycoproteins TfsA (TF2261-2262) and TfsB (TF2663), TF2339 and its paralog TF1259, TF1056 and TF0091) are modified with this specific S-layer *O*-glycan. We hypothesize that the S-layer glycosylation system of *T. forsythia* affects a wide number of different target proteins and, thus, is of high relevance for the bacterium.

Assessment of the influence of these glycoproteins on the immune response and investigating the "glyco-impact" on the life style of *T. forsythia* by analyzing biofilm formation at the molecular level may reveal novel pathogenic strategies in Gram-negative organisms.

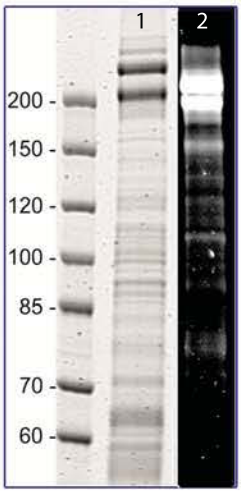


Fig. 3. SDS-PA gels stained for proteins (1) and glycoproteins (2) from crude *T. forsythia* extracts.
It is noteworthy that not only the S-layer proteins give a positive signal in the glyco-stained gel but also multiple other proteins appear to be glycosylated.

References
[1]Posch G, Pabst M, Brecker L, Altmann F, Messner P, Schäffer C (2011) *J Biol Chem* 286:38714-24
[2]Sekot G, Posch G, Messner P, Matejka M, Rausch-Fan X, Andrukhov O, Schäffer C (2011) *J Dent Res* 90(1):109-114
[3]Sekot G, Posch G, Oh JY, Zaynj S, Mayer HF, Pum D, Messner P, Hinterdorfer P, Schäffer C (2012) *Arch Microbiol* 194(6):525-39
[4]Veith PD, O'Brien-Simpson NM, Tan Y, Djatmiko DC, Dashper SG, Reynolds EC (2009) *J Proteome Res* 8:4279-92
[5]Pham TK, Roy S, Noirel J, Douglas I, Wright PC, Stafford GP (2010) *Proteomics* 10:3130-41

This work is supported by the Austrian Science Fund

Glycoprotein biosynthesis in the oral pathogen *Tannerella forsythia*

[FASEB Science Research Conference on Microbial Glycobiology, Itasca, IL, USA, June 8-13, 2014]

Markus Tomek (1), Laura Neumann (2), Jan Potempa (3,4), Paul Messner (1), Christina Schäffer (1)
(1) Department of NanoBiotechnology, NanoGlycobiology unit, Universität für Bodenkultur Wien, Muthgasse 11, 1190 Vienna, Austria
(2) Department of Chemistry, Division of Glycobiology, Universität für Bodenkultur Wien, Muthgasse 18, 1190 Vienna, Austria
(3) Oral Health and Systemic Diseases Group, School of Dentistry, University of Louisville, 501 South Preston St., Louisville, KY 40202, USA
(4) Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland

The anaerobic, biofilm-forming oral pathogen *Tannerella forsythia* is one of the main etiological agents of periodontitis. *T. forsythia* is the only Gram-negative bacterium known so far to possess a 2D crystalline glycoprotein surface layer (S-layer) as outermost cell envelope layer. The two S-layer proteins as well as additional antigenic, high molecular-mass glycoproteins, mostly residing in the outer membrane, are modified with a unique *O*-glycosidically linked oligosaccharide (1).

Bacteroidetes-specific C-terminal domain sequences (2) are necessary for successful translocation across the outer membrane mediated by a recently discovered translocation system from the phylogenetically related bacterium *Porphyromonas gingivalis* (por Secretion System) (3,4). A porSS-deficient mutant strain still shows the complete glycan attached to the S-layer proteins. This supports the general assumption that glycan biosynthesis and its transfer to the *Bacteroidetes O*-glycosylation motif take place in the cytoplasm and the periplasm, respectively. Further, the por secretion system itself is not involved in the synthesis of the glycan.

Currently, it is unknown if the oligosaccharides are transferred via a lipid carrier *en bloc* by an oligosaccharyltransferase or if monosaccharides are sequentially added via glycosyltransferases onto the target protein. To gain insight into the *O*-glycan biosynthesis of *T. forsythia*, three independent knock-out mutants of putative glycosyltransferases located within a predicted *O*-glycosylation locus (1), are currently being generated. The structure of β-eliminated glycans will be analysed by MS to unravel a sequential order of glycosyltransferase activities.

This work was supported by the Austria Science Fund, project P24317-B22 (to CS) and the Doctoral Programme BioToP-W1244.

1. Posch, G., Pabst, M., Brecker, L., Altmann, F., Messner, P., and Schäffer, C. (2011) *J Biol Chem* 286, 38714-38724
2. Veith, P. D., Nor Muhammad, N. A., Dashper, S. G., Likic, V. A., Gorasia, D. G., Chen, D., Byrne, S. J., Catmull, D. V., and Reynolds, E. C. (2013) *J Proteome Res* 12, 4449-4461
3. Glew, M. D., Veith, P. D., Peng, B., Chen, Y. Y., Gorasia, D. G., Yang, Q., Slakeski, N., Chen, D., Moore, C., Crawford, S., and Reynolds, E. C. (2012) *J Biol Chem* 287, 24605-17
4. Zhou, X. Y., Gao, J. L., Hunter, N., Potempa, J., and Nguyen, K. A. (2013) *Mol Microbiol* 89, 903-917

Glycoprotein Biosynthesis in the Oral Pathogen *Tannerella forsythia*

*Markus Tomek[1], Laura Neumann[2], Jan Potempa[3], Paul Messner[1], Christina Schäffer[1]

1 Department of NanoBiotechnology, *Nanoglycobiology* unit, Universität für Bodenkultur Wien, Muthgasse 11, 1190 Vienna, Austria
2 Department of Chemistry, Division of Glycobiology, Universität für Bodenkultur Wien, Muthgasse 18, 1190 Vienna, Austria
3 Oral Health and Systemic Disease Group, School of Dentistry, University of Louisville, 501 S Preston St., Louisville, KY 40202, USA

*markus.tomek@boku.ac.at

Overview

The anaerobic, biofilm-forming oral pathogen *Tannerella forsythia* is one of the main etiological agents of periodontitis. *T. forsythia* is the only Gram-negative bacterium known so far to possess a 2D crystalline glycoprotein surface (S-) layer as outermost cell envelope. The two S-layer proteins as well as additional antigenic, high molecular-mass glycoproteins, mostly residing in the outer membrane, are modified with a unique *O*-glycosidically linked oligosaccharide. (1)

Bacteroidetes-specific C-terminal domain (CTD) sequences (2) are necessary for successful translocation across the outer membrane mediated by a recently discovered translocation system in the phylogenetically related bacterium *Porphyromonas gingivalis* (Type IX Secretion System) (3). In *T. forsythia*, 37 proteins are predicted to possess a CTD, out of which 19 have been verified experimentally (2), including major virulence factors of this oral pathogen (eg. TfsA, TfsB, BspA) (4).

To verify the functionality of the T9SS in *T. forsythia*, we have deleted either TF2327 or TF0955 in the *T. forsythia* genome **(Fig. 1)**, which are orthologs of PorK and PG0022 (C-terminal signal peptidase), respectively, 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.

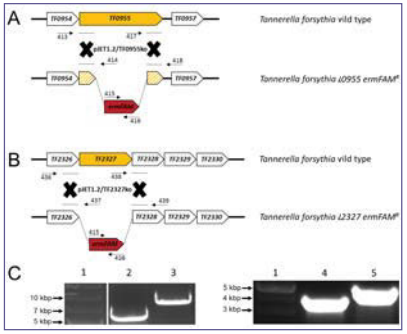


Figure 1. Strategy for TF0955 gene disruption and TF2327 gene deletion at the *T. forsythia* wild-type locus and confirmation by PCR (not drawn to scale). (A) Genomic organization of the TF0955 gene showing the up- and down-stream homology regions used for homologous recombination and insertion of the selectable *ermFAM* cassette disrupting TF0955. Primers are indicated with arrows. (B) TF2327 gene deletion and insertion of the selectable *ermFAM* 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 *ermFAM* 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 *ermFAM* 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 *ermFAM* cassette, yielding a 4,277-bp PCR product (lane 5).

Processing of the CTD during translocation across T9SS

Proteomics 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 **(Fig. 4)**.

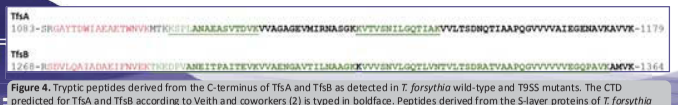


Figure 4. Tryptic peptides derived from the C-terminus of TfsA and TfsB as detected in *T. forsythia* wild-type and T9SS mutants. The CTD predicted for TfsA and TfsB according to Veith and coworkers (2) is typed in boldface. Peptides derived from the S-layer proteins of *T. forsythia* wild-type are written in red, those derived exclusively from the TfsA and TfsB proteins of the T9SS deficient mutants are written in green.

References

(1) Posch, G., Pabst, M., Brecker, L., Altmann, F., Messner, P., and Schaffer, C. (2011) *J Biol Chem* **286**, 38714-38724
(2) Veith, P. D., Nor Muhammad, N. A., Dashper, S. G., Likic, V. A., Gorasia, D. G., Chen, D., Byrne, S. J., Catmull, D. V., and Reynolds, E. C. (2013) *J Proteome Res* **12**, 4449-4461
(3) Glew, M. D., Veith, P. D., Peng, B., Chen, Y. Y., Gorasia, D. G., Yang, Q., Slakeski, N., Chen, D., Moore, C., Crawford, S., and Reynolds, E. C. (2012) *J Biol Chem* **287**, 24605-24617
(4) Zhou, X. Y., Gao, J. L., Hunter, N., Potempa, J., and Nguyen, K. A. (2013) *Mol Microbiol* **89**, 903-917

T9SS deficient strains are lacking the crystalline S-layer...

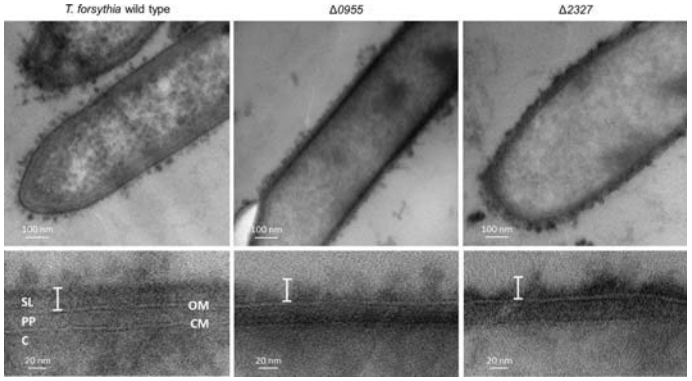


Figure 2. TEM micrographs showing ultra-thin cross-sectioned cells of *T. forsythia* and T9SS mutants. *T. forsythia* wild-type displays a serrated S-layer, whereas no S-layer is present on *T. forsythia* Δ*TF0955* and Δ*TF2327* cells. SL, S-layer; OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane; C, cytoplasm.

Biofilm formation in the absence of fully processed CTD proteins

T. forsythia wild-type strain forms 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. 5)**.

The biofilm of the ΔTF*0955* and ΔTF*2327* 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.

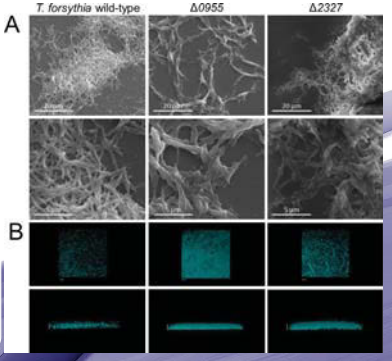


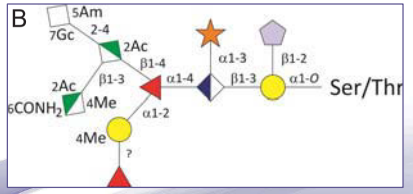
Figure 5. (A) SEM micrographs showing altered biofilm and cell morphology in T9SS deficient mutants in comparison to the wild-type. Biofilm was grown anaerobically on glass for two days; (B) CLSM micrographs of *T. forsythia* wild-type and mutant biofilms. Mutant biofilms revealed denser growth and a height comparable to that of the wild-type biofilm. Size bar, 20 µm.

... but both S-layer proteins have their native glycan attached!

Intercalating high molecular weight glycoproteins TfsA and TfsB form a self-assembled monolayer on the cell surface of *T. forsythia*. The 22 nm thick S-layer as outermost part on the cell surface can be seen in ultra-thin cross-sections of whole cell preparations from *T. forsythia* wild-type **(Fig.2)**. Inactivation of TF2327 and TF0955 blocked secretion of the S-layer proteins TfsA and TfsB and therefore no assembly of the composite S-layer on the bacterial cell surface can be seen in ultra-thin cross-sections **(Fig. 2)**.

O-glycans were released from the protein backbones of TfsA and TfsB by in-gel reductive β-elimination. Mass analyses presented the wild-type glycan with the highest mass peak at 1898 **(Fig. 3)** which is in agreement with the known structure of the *T. forsythia* dekasaccharide (1). Significantly, S-layer glycans from the T9SS deficient *T. forsythia* mutants showed the same pattern and masses on deconvoluted ESI-TOF-MS spectra, proving the complete glycan to be attached to the S-layer proteins **(Fig. 3)**.

The assembly of the glycan and the transfer onto the glycoproteins must therefore be finished in the periplasm before translocation across the outer membrane can occur.



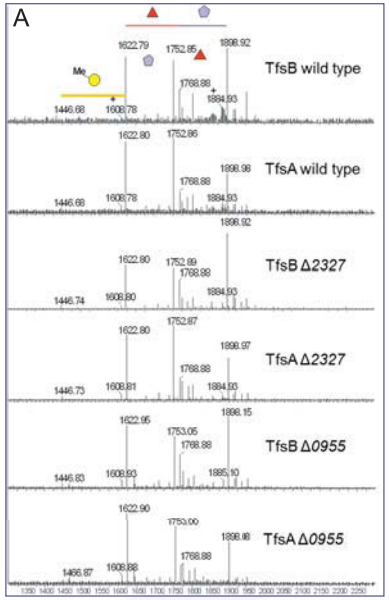


Figure 3. (A) Deconvoluted ESI-TOF-MS spectrum of TfsA and TfsB *O*-glycans in *T. forsythia* wild-type and mutant strains. Identical spectra were recorded for the *O*-glycan derived from the Δ*TF0955* and Δ*TF2327* mutants and the wild-type. (B) For clarification, the complete S-layer glycan structure 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

Conclusions

- Two *T. forsythia* gene knock-out strains targeting the type IX secretion system (put. C-terminal signal peptidase, PorK ortholog) were generated.
- C-terminal domains (CTD) were not processed in T9SS deficient mutants.
- The S-layer proteins TfsA and TfsB, both carrying a CTD were blocked for successful translocation in T9SS deficient strains and therefore could not assemble the crystalline S-layer.
- Periplasmic S-layer proteins had their native *O*-glycan attached.
- Absence of CTD proteins on the bacterial surface lead to a much denser biofilm and in the occurence of fibers and bundles of cells rather than a well-connected meshwork as seen in the wild-type strain.

Taken together we could demonstrate the functionality of the T9SS and the requirement of the C-terminal domain 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*.

This work is supported by the Austrian Science Fund

Predicted *O*-glycosylation locus and glycoprotein biosynthesis in *Tannerella forsythia*

[6th Baltic Meeting on Microbial Carbohydrates, Gdansk, Poland, September 7-14, 2014]

Irene Nimeth[1], Markus Tomek[1], Laura Neumann[2], Paul Messner[1], Christina Schäffer[1]
1. Department of NanoBiotechnology, NanoGlycobiology unit, Universität für Bodenkultur Wien, Muthgasse 11, 1190 Vienna, Austria
2. Department of Chemistry, Division of Glycobiology, Universität für Bodenkultur Wien, Muthgasse 18, 1190 Vienna, Austria

Tannerella forsythia is a Gram-negative, anaerobic periodontal pathogen inhabiting subgingival plaque biofilms, which is completely covered by a 2 D crystalline cell surface (S) layer, composed of two glycoproteins. There is evidence that a unique oligosaccharide that is *O*-glycosidically linked to either S-layer protein is important for the virulence of the bacterium (1, 2). The elucidation of the S-layer glycan and the prediction of a general protein *O*-glycosylation gene locus in *T. forsythia* by our group allowed the construction of mutants affecting distinct genes encoded in that locus (3). Three independent glycosyltransferases knock-out mutants predicted to be involved in protein *O*-glycosylation were constructed and initial analysis confirmed the synthesis of truncated *O*-glycans in all three mutants. Detailed analyses with respect to glycan composition, phenotype, and effect on biofilm formation are currently in progress. For some proteins from the *Bacteroidetes*, specific C-terminal domain sequences (4) are necessary for the successful translocation across the outer membrane mediated by a novel type 9 secretion system (T9SS). Recently, we have shown that a *T. forsythia* T9SS-deficient mutant has the complete glycan attached to the S-layer proteins. This supports the general assumption that glycan biosynthesis and its transfer to the *Bacteroidetes O*-glycosylation motif take place in the cytoplasm and the periplasm, respectively, and that these processes are decoupled from outer membrane translocation (5).

This work was supported by the FWF project P24317-B22 (to CS) and the FWF PhD Programme W1224 “Biomolecular Technology of Proteins”.

1. Sekot G *et al.* (2011*) J Dent Res* 90, 109-114
2. Settem RP *et al.* (2013) *Mucosal Immunol* 6, 415-426
3. Posch G *et al.* (2011) *J Biol Chem* 286, 38714-38724
4. Veith PD *et al.* (2013) *J Proteome Res* 12, 4449-4461
5. Tomek MB *et al.* (2014) *Mol Oral Microbiol*, doi: 10.1111/omi.12062

Predicted *O*-glycosylation locus and glycoprotein biosynthesis in *Tannerella forsythia*

Irene Nimeth[1], Markus Tomek[1], Laura Neumann[2], Paul Messner[1], Christina Schäffer[1]

1 Department of NanoBiotechnology, *Nanoglycobiology* unit, Universität für Bodenkultur Wien, Muthgasse 11, 1190 Vienna, Austria
2 Department of Chemistry, Division of Glycobiology, Universität für Bodenkultur Wien, Muthgasse 18, 1190 Vienna, Austria

Introduction

Tannerella forsythia is a Gram-negative, anaerobic periodontal pathogen inhabiting subgingival plaque biofilms. It is completely covered by a 2-D crystalline cell surface (S-) layer, composed of two glycoproteins (TfsA, TfsB). There is evidence that a unique oligosaccharide that is *O*-glycosidically linked to either S-layer protein is important for the virulence of the bacterium (1, 2).

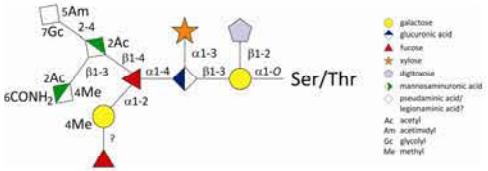


Figure 1. complete *Tannerella forsythia* S-layer glycan structure

The elucidation of the S-layer glycan **(Figure 1)** and the prediction of a general protein *O*-glycosylation gene locus in *T. forsythia* by our group allowed the construction of mutants affected in distinct genes encoded in that locus **(Figure 2)** (3). Three independent glycosyltransferase knock-out mutants predicted to be involved in protein *O*-glycosylation were constructed and initial analysis confirmed the synthesis of truncated *O*-glycans in all three mutants. Detailed analyses with respect to glycan composition, phenotype, and effect on biofilm formation are currently in progress.

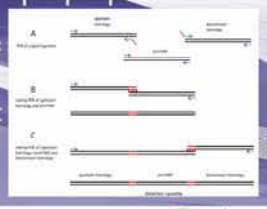
For some proteins from the *Bacteroidetes*, specific C-terminal domain sequences (CTD) (4) are necessary for the successful translocation across the outer membrane mediated by a novel type 9 secretion system (T9SS). We have shown that a *T. forsythia* T9SS-deficient mutant has the complete glycan attached to the S-layer proteins **(Figures 6 + 7)**. This supports the general assumption that glycan biosynthesis and its transfer to the *Bacteroidetes* *O*-glycosylation motif takes place in the cytoplasm and the periplasm, respectively, and that these processes are decoupled from outer membrane translocation (5).

O-glycosylation locus conserved within *Bacteroidetes* species

As Posch *et al.* (3) have postulated the predicted *O*-glycosylation locus in *T. forsythia* (6.8 kb) is clustering highly homologous genes in different *Bacteroidetes* species. This phylum-wide conservation shows the importance of this locus.

Figure 2. Predicted *O*-glycosylation gene locus in *T. forsythia* (A), *B. fragilis* NCTC 9343 (B), *B. thetaiotaomicron* VPI 5482 (C), *B. uniformis* ATCC 8492 (D), *P. gingivalis* ATCC 33277 (E), and *P. distasonis* ATCC 8503(F). Wzx (gray), flippase; X (green), UDP-GlcNAc 2-epimerase; Y (pink), acetyltransferase; GTi, GTii, GTiii and GTiv (blue), glycosyltransferases; Cpbp (blue), possibly capsule polysaccharide biosynthesis protein (1).

Generation of knock-out mutants



The knock-out cassette is based on resistance against erythromycin. The resistance is conferred via the *ermF* gene (001b) which is transcribed by *Bacteroides* sp. Upstream and downstream flanking homology regions are around 1 kb in size, making the full knock-out cassette around 2.8 kb. This knock-out cassette can than be transferred into electrocompetent *Tannerella* cells.

Figure 3. Construction of the knock-out-cassette by 2-step overlap extension PCR

Glycosyltransferase-deficient mutants

In order to investigate the stepwise assembly of the S-layer glycans we generated three independent glycosyltransferase deficient mutants (Δ*Tf2049*, Δ*Tf2050*, Δ*Tf2053*). The deleted genes are located in the predicted *O*-glycosylation locus **(Figure 2)** and therefore are proposed to be essential for the S-layer glycan biosynthesis.

As expected the mutants show truncated *O*-glycans **(Figure 4 + 5)**.

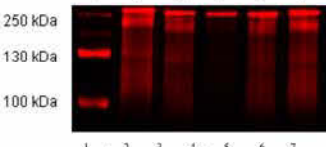


Figure 4. Western blot using αTfsA
lane 1: DNA-ladder page ruler plus prestained;
lane 2: *T.forsythia* wild-type;
lane 3: Δ*TfwecC* lacking the last trisaccharide branch;
lane 4: Δ*Tf2053* glycosyltransferase deficient mutant;
lane 5: Δ*Tf2050* glycosyltransferase deficient mutant;
lane 6: Δ*TfpseC* lacking the very last sugar residue pseudaminic acid;
lane 7: Δ*Tf2049* glycosyltransferase deficient mutant

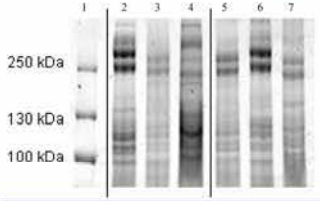


Figure 5. Coomassie Brilliant blue stained SDS-PAGE
lane 1: DNA-ladder page ruler plus prestained;
lane 2: *T.forsythia* wild-type;
lane 3: Δ*TfwecC* lacking the last trisaccharide branch;
lane 4: Δ*Tf2053* glycosyltransferase deficient mutant;
lane 5: Δ*Tf2050* glycosyltransferase deficient mutant;
lane 6: Δ*TfpseC* lacking the very last sugar residue pseudaminic acid;
lane 7: Δ*Tf2049* glycosyltransferase deficient mutant

T9SS-deficient mutants

To verify the functionality of the T9SS in *T. forsythia* we have deleted either Tf2327 or Tf0955, which are orthologs of PorK and the C terminal signal peptidase, respectively, and are essential for the translocation of proteins via T9SS in *Porphyromonas gingivalis*.

Inactivation of these genes blocked secretion of the glycosylated S-layer proteins TfsA and TfsB. Accordingly no assembly of the S-layer on the bacterial cell suface was observed **(Figure 6)**.

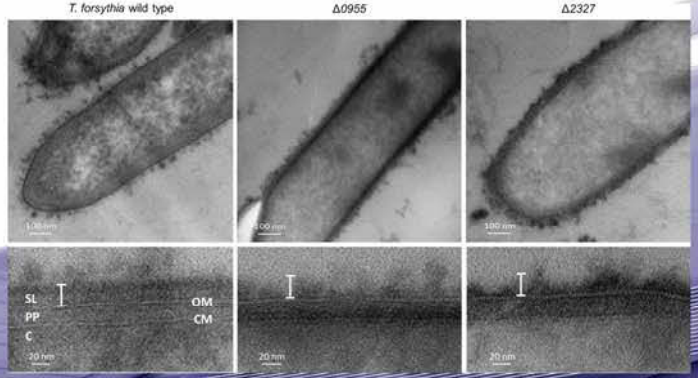
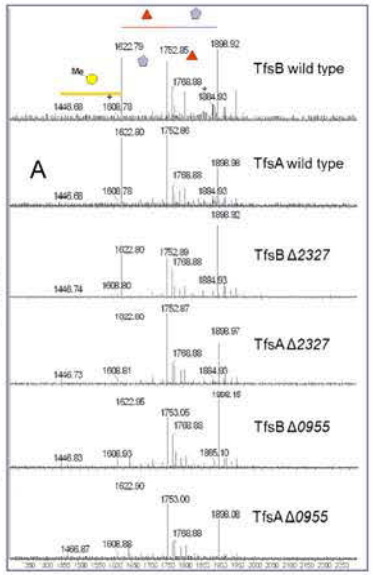


Figure 6. TEM micrographs showing ultra-thin cross-sectioned cells of *T. forsythia* and T9SS mutants. *T. forsythia* wild-type displays a serrated S-layer, whereas no S-layer is present on *T. forsythia* Δ*Tf0955* and Δ*Tf2327* cells. SL, S-layer; OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane; C, cytoplasm.

The *O*-glycans of TfsA and TfsB were released from the protein backbones by an in-gel β-elimination. Mass analysis by ESI-TOF-MS confirmed the presence of the whole glycan in *T. forsythia* wild-type with the highest mass peak at 1898, as postulated by Posch *et al.* (3) **(Figure 1)**.

The glycans of the S-layer deficient mutants show the same mass patterns indicating the complete glycan to be attached to the S-layer proteins.

Figure 7. 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 Δ*Tf0955* and Δ*Tf2327* mutants and the wild-type.



Proteomics data implicate 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 **(Figure 8)**.

Figure 8. Tryptic peptides derived from the C-terminus of TfsA and TfsB as detected in *T. forsythia* wild-type and T9SS mutants. 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.

TfsA
1083-SRGAYTDWIAEADTKVVKMCKKPLANAEASVTDVKVVAGAGEVMIRNASGKKVTVSNILQQTIAKVVLTSDNQTIAAPQGVVVVAIEGENAVKAVVK-1179

TfsB
1268-RGDVLQAIADAEIFMVENTKKDPVANEITPAITEVKVVAEHGAVTILNAAGKKVVVSNVLQQTLVNTVLTSDRATVAAPQGVVVVVEGQPAVEAMVK-1364

Conclusion and outlook

- Two *T. forsythia* the type 9 secretion system mutants were generated (PorK and C-terminal signal peptidase knock out mutants).
- Contrary to *T. forsythia* wild-type both mutants could not translocate the S-layer proteins from the periplasm to the bacterial cell suface.
- As a consequence, the S-layer proteins (TfsA, TfsB) of the two mutants carry the full CTD, whereas the wild-type CTD of the S-layer proteins are cut-off.
- The S-layer protein glycans are fully present in all three strains.

➡ The assembly of the glycan and the transfer onto the proteins must be finished in the periplasm before the translocation accross the outer membrane takes place.

- Already three glycosyltransferase deficient mutants have been generated.
- Detailed analyses with respect to glycan composition, phenotype, and effect on biofilm formation are currently in progress.
- Several more genes probably involved in glycan biosynthesis are going to be deleted.

➡ The aim is to fully elucidate glycan biosynthesis of the two S-layer proteins.

1. Sekot G, Posch G, Messner P, Matejka M, Rausch-Fan X, Andrukhov O, Schäffer C (2011) *J Dent Res* 90, 109-114
2. Settem RP, Honma K, Nakajima T, Phansopa C, Roy S, Stafford GP, Sharma A (2013) *Mucosal Immunol* 6, 415-426
3. Posch G, Pabst M, Brecker L, Altmann F, Messner P, Schäffer C (2011) *J Biol Chem* 286, 38714 38724
4. Veith PD, Nor Muhammad NA, Dashper SG, Likic VA, Gorasia DG, Chen D, Byrne, SJ, Catmull DV, Reynolds EC (2013) *J Proteome Res* 12, 4449-4461
5. Tomek MB, Neumann L, Nimeth I, Koerdt A, Andesner P, Messner P, Mach L, Potempa JS, Schäffer C *Mol Oral Microbiol*, accepted doi: 10.1111/omi.12062

This work is supported by the Austrian Science Fund

Biosynthesis and translocation of glycoproteins in the oral pathogen *Tannerella forsythia*

[2nd International Conference on Porphyromonas gingivalis *and related species in oral and systemic diseases, London, United Kingdom, June 23-25, 2015]*

Markus B. Tomek[1], Markus Windwarder[2], Laura Neumann[2], Irene Nimeth[1], Friedrich Altmann[2], Paul Messner[1], Jan Potempa[3,4] and Christina Schäffer[1]

1 Department of NanoBiotechnology, NanoGlycobiology unit, Universität für Bodenkultur Wien, Muthgasse 11, 1190 Vienna, Austria
2 Department of Chemistry, Universität für Bodenkultur Wien, Muthgasse 18, 1190 Vienna, Austria
3 Department of Oral Immunology and Infectious Diseases, University of Louisville, Louisville, KY 40202, USA
4 Department of Microbiology, Jagiellonian University, 30-387 Kraków, Poland

The anaerobic, biofilm-forming oral pathogen *Tannerella forsythia* is one of the major etiological agents of periodontitis. *T. forsythia* is the only Gram-negative bacterium known so far to possess a 2D crystalline glycoprotein surface layer (S-layer) as outermost cell envelope decoration. The two S-layer proteins (TfsA, TfsB) as well as additional antigenic, high molecular-mass glycoproteins, mostly residing in the outer membrane, are modified with a unique *O*-glycosidically linked oligosaccharide (1).

The biosynthesis of the oligosaccharide is encoded by a putative glycosylation gene cluster consisting of four glycosyltransferases, several modifying enzymes and hypothetical proteins as well as a flippase (1). The individual contributions of the respective carbohydrate-active enzymes to S-layer glycan biosynthesis are currently under investigation in our laboratory.

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) (2, 3). The genome sequence of the periodontal pathogen *T. forsythia* predicts the presence of the components for a T9SS in conjunction with a suite of CTD proteins. 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 analysed with respect to secretion, assembly and glycosylation of the S-layer proteins as well as proteolytic processing of the CTD and biofilm formation (4).

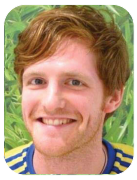
In either mutant, TfsA and TfsB were incapable of translocation, as evidenced by the absence of the S-layer in transmission electron microscopy of ultrathin-sectioned bacterial cells. Despite being entrapped within the periplasm, mass spectrometry analysis revealed that the S-layer proteins were modified with the complete, mature glycan found on the secreted proteins, indicating that protein translocation and glycosylation are two independent processes. This study as also that of Narita *et al.* (5), demonstrates the functionality of the T9SS and the requirement of CTD for the outer membrane passage of extracellular proteins in *T. forsythia*, exemplified by the two S-layer proteins. In addition, T9SS protein trans-location is decoupled from *O*-glycan attachment in *T. forsythia* (4).

This work was supported by the Austria Science Fund, project P24317-B22 (to CS) and the Doctoral Programme BioToP-W1244.

1. Posch, G. *et al.* (2011) *J Biol Chem* 286, 38714-38724
2. Veith, P. D. *et al*. (2013) *J Proteome Res* 12, 4449-4461
3. Glew, M. D. *et al.* (2012) *J Biol Chem* 287, 24605-24617
4. Tomek, M. B. *et al.* (2014) *Mol Oral Microbiol* 29, 307-320
5. Narita, Y. *et al.* (2014) *Microbiology* 160, 2295-2303

Biosynthesis and translocation of glycoproteins in the oral pathogen *Tannerella forsythia*

*Markus B. Tomek[1], Markus Windwarder[2], Laura Neumann[2], Irene Nimeth[1], Friedrich Altmann[2], Paul Messner[1], Jan Potempa[3], Christina Schäffer[1]



*markus.tomek@boku.ac.at

1 Department of NanoBiotechnology, *Nanoglycobiology* unit, Universität für Bodenkultur Wien, Muthgasse 11, 1190 Vienna, Austria
2 Department of Chemistry, Division of Glycobiology, Universität für Bodenkultur Wien, Muthgasse 18, 1190 Vienna, Austria
3 Department of Oral Immunology and Infectious Diseases, University of Louisville, Louisville, KY 40202, USA and Department of Microbiology, Jagiellonian University, 30-387 Krakow, Poland

Overview

The anaerobic, biofilm-forming oral pathogen *Tannerella forsythia* is one of the main etiological agents of periodontitis. *T. forsythia* is the only Gram-negative bacterium known so far to possess a 2D crystalline glycoprotein surface (S-) layer as outermost cell envelope. The two S-layer proteins as well as additional antigenic, high molecular-mass glycoproteins, mostly residing in the outer membrane, are modified with a unique *O*-glycosidically linked oligosaccharide **(Fig. 1A)**. (1)

Aim 1

Bacteroidetes-specific C-terminal domain (CTD) sequences (2) are necessary for successful translocation of proteins across the outer membrane mediated by a recently discovered translocation system in the phylogenetically related bacterium *Porphyromonas gingivalis* (Type IX Secretion System) (3). In *T. forsythia*, 37 proteins are predicted to possess a CTD, 19 of which have been verified experimentally (4), including major virulence factors of this oral pathogen (eg. TfsA, TfsB, BspA).

To verify the functionality of the T9SS in *T. forsythia*, we have deleted either TF2327 or TF0955 in the *T. forsythia* genome, which are orthologs of PorK and PG0022 (C-terminal signal peptidase), respectively, 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.

Aim 2

To elucidate the biosynthesis of the *T. forsythia* glycan, five putative glycosyltransferases within a predicted *O*-glycosylation gene locus (1) were knocked-out **(Fig. 1B)**. The resulting effects on the structure of the glycan are currently being analyzed.

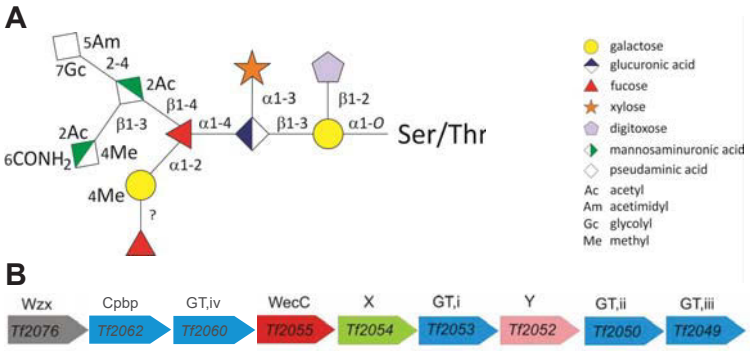


Figure 1. (A) Schematic drawing of the structure of the abundant *O*-glycan in *T. forsythia*. Modified from (1). **(B)** Predicted *O*-glycosylation gene locus in *T. forsythia.* Wzx (gray), flippase; Cpbp (blue), put. capsule polysaccharide biosynthesis protein; WecC (red), UDP-*N*-acetylmannosaminuronic acid dehydrogenase; X (green), UDP-*N*-acetylglucosamine 2-epimerase; Y (pink), acetyltransferase; GTi, GTii, GTiii and GTiv (blue), glycosyltransferases. Modified from (1).

Glycosyltransferase knock-out mutants

Five carbohydrate-active genes located within an *O*-glycosylation cluster **(Fig. 1B)** were targeted to generate single gene knock-out mutants:

TF2049=BFO_1042=Tanf_01305
TF2050=BFO_1043=Tanf_01300
TF2053=BFO_1049=Tanf_01290
TF2060=BFO_1057=Tanf_01260
TF2062=BFO_1060=part of Tanf_01245

First results show clear downshifts visible on Coomassie-stained SDS-PAGE gels of both S-layer proteins TfsA and TfsB in comparison to the wild-type strain, indicating a truncated version of the glycan **(Fig. 5)**. Periodic acid Schiff- (PAS) stainings confirmed carbohydrates still being attached to both S-layer protein backbones (data not shown).

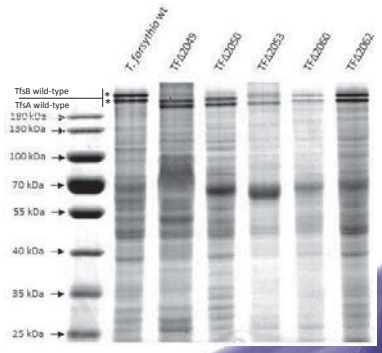


Figure 5. Coomassie Brilliant Blue R-250 stained SDS-PAGE gel (10%) of crude cell extracts from *T. forsythia* wild-type and mutants. Page Ruler Prestained Protein Ladder (Fermentas); wt, wild-type

T9SS deficient strains are lacking the crystalline S-layer...

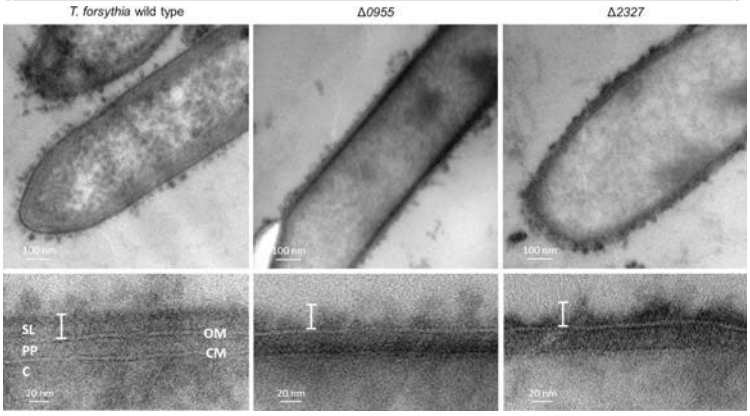


Figure 2. TEM micrographs showing ultra-thin cross-sectioned cells of *T. forsythia* and T9SS mutants. *T. forsythia* wild-type displays a serrated S-layer, whereas no S-layer is present on *T. forsythia* ΔTF0955 and ΔTF2327 cells. SL, S-layer; OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane; C, cytoplasm.

... but S-layer proteins have their native glycan attached!

Intercalating high molecular weight glycoproteins TfsA and TfsB form a self-assembled monolayer on the cell surface of *T. forsythia*. The 22 nm thick S-layer as outermost part on the cell surface can be seen in ultra-thin cross-sections of whole cell preparations from *T. forsythia* wild-type **(Fig.2)**. Inactivation of TF2327 and TF0955 blocked secretion of the S-layer proteins TfsA and TfsB and therefore no assembly of the composite S-layer on the bacterial cell surface can be seen in ultra-thin cross-sections **(Fig. 2)**.

O-glycans were released from the protein backbones of TfsA and TfsB by in-gel reductive β-elimination. Mass analyses presented the wild-type glycan with the highest mass peak at 1898 **(Fig. 3)** which is in agreement with the known structure of the *T. forsythia* decasaccharide (1). Significantly, S-layer glycans from the T9SS deficient *T. forsythia* mutants showed the same pattern and masses on deconvoluted ESI-TOF-MS spectra, indicating the complete glycan to be attached to the S-layer proteins **(Fig. 3)**.

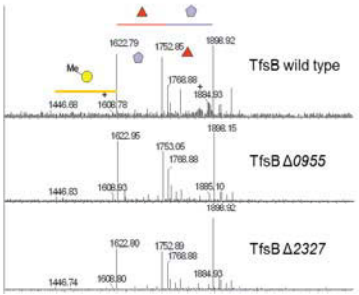


Figure 3. Deconvoluted ESI-TOF-MS spectrum of TfsB *O*-glycans in *T. forsythia* wild-type and mutant strains. Identical spectra were recorded for the *O*-glycan derived from the ΔTF0955 and ΔTF2327 mutants and the wild-type.

Proteomics data implicate 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 **(Fig. 4)**.

Figure 4. 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 *et al.* (4) 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.

Conclusions

- The S-layer glycoproteins TfsA and TfsB, both carrying a CTD, were blocked for successful translocation in T9SS deficient strains and therefore could not assemble the crystalline S-layer.
- C-terminal domains (CTD) were not processed in T9SS deficient mutants.
- Periplasmic S-layer proteins had their native *O*-glycan attached.

Taken together we could demonstrate the functionality of the T9SS and the requirement of the C-terminal domain for the outer membrane passage of extracellular proteins in *T. forsythia*, exemplified by the two S-layer proteins. In addition, T9SS protein translocation is decoupled from *O*-glycan attachment in *T. forsythia*.

Original publication
Tomek, M., Neumann, L., Nimeth, I., Koerdt, A., Andesner, P., Mach, L., Messner, P., Potempa, J. S., Schäffer, C. (2014) *Mol. Oral Microbiol.*, **29**, 307-320

References
(1) Posch, G., Pabst, M., Brecker, L., Altmann, F., Messner, P., and Schaffer, C. (2011) *J Biol Chem* **286**, 38714-38724
(2) Nguyen, K. A., Travis, J. and Potempa, J. (2006) *J. Bacteriol.* **189**(3), 833-843
(3) Glew, M. D., Veith, P. D., Peng, B., Chen, Y. Y., Gorasia, D. G., Yang, Q., Slakeski, N., Chen, D., Moore, C., Crawford, S., and Reynolds, E. C. (2012) *J Biol Chem* **287**, 24605-24617
(4) Veith, P. D., Nor Muhammad, N. A., Dashper, S. G., Likic, V. A., Gorasia, D. G., Chen, D., Byrne, S. J., Catmull, D. V., and Reynolds, E. C. (2013) *J Proteome Res* **12**, 4449-4461

This work is supported by the Austrian Science Fund

The O-linked protein glycosylation cluster of the oral pathogen *Tannerella forsythia*

[FASEB Science Research Conference on Microbial Glycobiology, West Palm Beach, FL, USA, June 12-17, 2016]

Markus B. Tomek (1), Daniel Maresch (2), Markus Windwarder (2), Bettina Janesch (1), Laura Neumann (2), Irene Nimeth (1), Gerald Posch (1,3), Friedrich Altmann (2), Paul Messner (1), Christina Schäffer (1)
(1) Department of NanoBiotechnology, NanoGlycobiology unit, Universität für Bodenkultur Wien, Muthgasse 11, 1190 Vienna, Austria (2) Department of Chemistry, Division of Glycobiology, Universität für Bodenkultur Wien, Muthgasse 18, 1190 Vienna, Austria (3) present address: GlaxoSmithKline AG, Switzerland

The Gram-negative anaerobe *Tannerella forsythia* is recognized as an important pathogen implicated in the development and progression of periodontal diseases. It is considered a keystone pathogen and follows the polymicrobial synergy and dysbiosis model of periodontal disease aetiology (1). Among described virulence factors such as BspA, KLIKK-proteases and outer membrane vesicles are the two surface (S-) layer proteins TfsA and TfsB (2). The *O*-glycosylated surface layer protein TfsA serves as a model protein in this study (3). The glycan carries a complex decasaccharide including a modified pseudaminic acid (Pse5Am7Gra) as non-reducing end sugar residue, potentially implicated in the delay of the host immune response by molecular mimicry or by cell-host interactions (3,4). Little is known about the biosynthesis of the glycan. Within a predicted *O*-glycosylation cluster, glycosyltransferases, modifying enzymes and nucleotide sugar biosynthesis genes are encoded. Experimental data, however, are so far only presented for WecC, an UDP-*N*-acetylmannosaminuronic acid dehydrogenase. In a *wecC* knock-out mutant (5), the terminal three-sugar branch, consisting of Pse5Am7Gra and two modified mannosaminuronic acids, is absent. This truncated glycan modulates dendritic cell effector functions in suppression of T-helper 17 cell responses (6).

This study provides an extended view of the *O*-glycosylation gene cluster in *T. forsythia*, including the genomic information of the recently re-sequenced genome. All five predicted glycosyltransferases located within the cluster were individually knocked-out. The consequences for the glycan biosynthesis within these mutant strains were examined, using a mass spectrometric approach.

This work was supported by the Austrian Science Fund, project P24317-B22 (to CS) and the Doctoral Programme BioToP (W1244).

1. Hajishengallis, G., Lamont, R. (2012) *Mol Oral Microbiol*, 27:409-419
2. Sharma, A. (2010) *Periodontol* 2000, 54:106-116
3. Sekot, G. *et al.* (2011) *J Dent Res*, 90:109-114
4. Posch, G. *et al*. (2011) *J Biol Chem*, 286:38714-38724
5. Honma, K. *et al*. (2007) *Microb Pathog*, 42:156-166
6. Settem, RP. *et al.* (2013) *Mucosal Immunol*, 2:415-426

A general protein *O*-glycosylation gene cluster in the oral pathogen *Tannerella forsythia*

*Markus B. Tomek[1], Daniel Maresch[2], Valentin Friedrich[1], Markus Windwarder[2], Bettina Janesch[1], Laura Neumann[2], Irene Nimeth[1], Gerald Posch[1], Friedrich Altmann[2], Christina Schäffer[1]

1) Department of NanoBiotechnology, *NanoGlycobiology* unit and 2) Department of Chemistry, Division of Glycobiology, BOKU - Universität für Bodenkultur Wien, Vienna, Austria

*Markus.Tomek@boku.ac.at

Introduction

The Gram-negative anaerobe *Tannerella forsythia* is recognized as an important oral pathogen implicated in the development and progression of periodontal diseases, following the polymicrobial synergy and dysbiosis model of periodontal disease etiology (1). Among described virulence factors are the two cell surface (S-) layer proteins TfsA and TfsB (2), which are modified with an *O*-linked decasaccharide (3) **(Fig. 1)**.

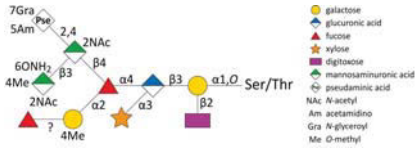


Figure 1. Schematic drawing of the *O*-glycan present in *T. forsythia*.

This study provides an extended view of the general protein *O*-glycosylation gene cluster in *T. forsythia*, including genomic information of the recently re-sequenced type strain (ATCC 43037) (4). For all five predicted glycosyltransferases as well as one essential gene from the pseudaminic acid biosynthesis pathway, knock-out mutants were constructed **(Fig. 2)**. The effect of gene deletions on the S-layer *O*-glycan composition was determined using mass spectrometry.

Materials and methods

Homologous recombination was used for the generation of gene knock-outs and for the integration of an erythromycin resistance gene for selection purposes. Upon confirmation of the loss of the gene of interest and the correct integration of the resistance gene on the genome, crude cell extracts from defined mutants were separated by SDS-PAGE and proteins were stained with Coomassie Brilliant Blue **(Fig. 3)**. Both high molecular weight S-layer proteins (TfsA-wt: 230 kDa; TfsB-wt: 270 kDa) were used as model proteins.

O-glycans from the S-layer protein TfsA were excised from an SDS-PAGE gel and released by in-gel reductive β-elimination from the protein backbone. Final purification was completed with preparative porous graphitized carbon (PGC)-HPLC (3) prior to electrospray-ionization mass spectrometry (ESI-MS) analyses.

Occurring masses are represented in deconvoluted sum spectra. Glycan structures of the highest mass peaks are represented by symbols. Molecular ion peaks from the subsequent fragmentation pattern are assigned according to the loss of carbohydrate units and modifications. Relative intensities of occurring peaks are given on the *y*-axis **(Fig. 4)**.

Results: Genes of the cluster

The protein *O*-glycosylation gene cluster spans an area of 27 kb and includes five glycosyltransferases, three putative glycan modifying methyltransferases and all six genes responsible for the biosynthesis of pseudaminic acid. The first gene is a *wzx* flippase-like gene (*Tanf_01180*) which is followed by genes synthesizing CMP-pseudaminic acid (*pseBCFHGI*). Downstream, glycosyltransferases (GT,i-v), methyltransferases (MT,i-iii) and further modifying enzymes (*asnB*, *wecC*, *wecB*) are encoded **(Fig. 2)**.

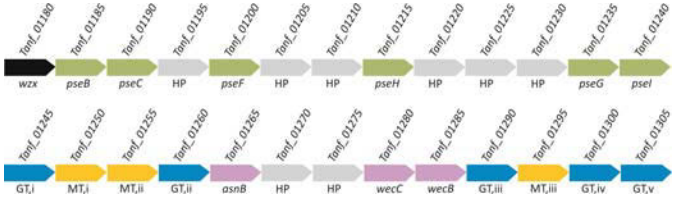


Figure 2. Schematic drawing of the general protein *O*-glycosylation cluster in *T. forsythia*. Genes are not drawn to scale.

Results: Carbohydrate-active deficient strains

In the **ΔGT,i** (Δ*Tanf_01245*) knock-out mutant, the terminal pseudaminic acid unit is absent, suggesting that the encoded protein is an α-2,4 pseudaminic acid transferase (MB. Tomek *et al.*, submitted). A mutant strain deficient in **ΔGT,ii** (Δ*Tanf_01260*), lacks a mannosaminuronic acid unit in addition to the terminal pseudaminic acid, suggesting a β-1,3 *N*-acetylmannosaminuronic acid transferase activity. The glycan is further truncated when knocking-out **ΔGT,iii** (Δ*Tanf_01290*). Here, the three-sugar containing branch composed of pseudaminic acid and two *N*-acetyl mannosaminuronic acids is absent. Results from a **ΔGT,iv** (Δ*Tanf_01300*) deficient strain are ambiguous, as a mass of 146.0 *m/z* cannot be assigned to the known *O*-glycan structure.

A **ΔGT,v** (Δ*Tanf_01305*) knock-out mutant consists of the five carbohydrate units assembling the core glycan structure. Therefore, the protein putatively transfers the branching α-1,2 galactose unit.

Pseudaminic acid is generated from UDP-*N*-acetylglucosamine in a five-step biosynthetic pathway. These enzymatically characterized proteins (V. Friedrich *et al.*, submitted) are encoded within the general protein *O*-glycosylation gene cluster in *T. forsythia*. Deletion of the essential aminotransferase PseC (Δ*pseC*, Δ*Tanf_01190*) results in an incomplete synthesis of pseudaminic acid, which is, thus, absent on the *O*-glycan.

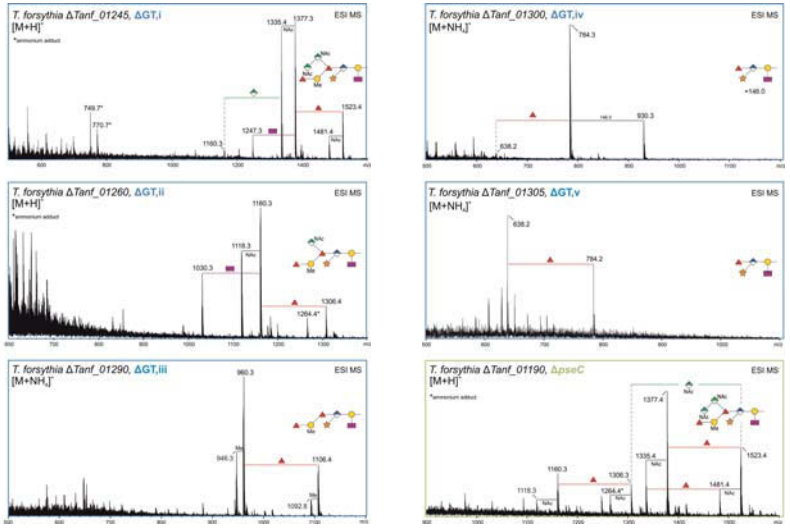


Figure 4. Deconvoluted ESI-MS sum spectra of TfsA *O*-glycans in *T. forsythia* mutant strains with deleted glycosyltransferases (GT,i-v) or the essential pseudaminic acid biosynthesis pathway gene, *pseC*.

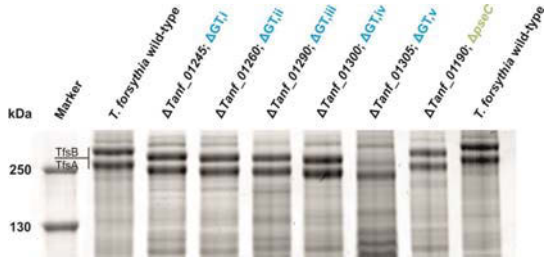


Figure 3. Coomassie Brilliant Blue R-250 stained SDS-PAGE gel of crude cell extracts of *T. forsythia* wild-type and mutant strains showing downshifted S-layer proteins TfsA and TfsB.

Conclusions

- The 27 kb general protein *O*-glycosylation gene cluster comprises a flippase, five genes for the biosynthesis of pseudaminic acid, five glycosyltransferases, three methyltransferases and three further carbohydrate-modifying enzymes.
- Single gene knock-out mutants of an essential pseudaminic acid biosynthesis gene (*pseC*) and all five glycosyltransferases (GT,i – GT,iv) encoded within the cluster were generated. All knock-out mutants experienced step-wise truncations of the *O*-glycan, as SDS-PAGE and MS analyses revealed.
- Carbohydrate-active enzymes within the cluster only encode for genes responsible for the assembly of the two branches of the glycan. The assembly of the five carbohydrate-containing core structure is encoded elsewhere on the genome.
- An oligosaccharyltransferase transferring *O*-glycans onto their appropriate proteins remains to be identified.

Literature cited

(1) Hajishengallis, G. and Lamont, R. (2012) *Mol Oral Microbiol* **27**, 409-419
(2) Sharma, A. (2010) *Periodontol 2000* **54**, 106-116
(3) Posch, G., Pabst, M., Brecker, L., Altmann, F., Messner, P. and Schäffer, C. (2011) *J Biol Chem* **286**, 38714-38724
(4) Friedrich, V., Pabinger, S., Chen, T., Messner, P., Dewhirst, FE. and Schäffer, C. (2015) *Genome Announce* **3**

This work is supported by the Austrian Science Fund P24317-B22 (to CS) and the doctoral programme W1224 (BioToP)

Putative pseudaminic and legionaminic acid transferases from the oral pathogen *Tannerella forsythia*

[GlycoT16 – 10th International Symposium on Glycosyltransferases, Toronto, Canada, June 19-21, 2016]

MB Tomek, B Janesch, D Maresch, M Windwarder, F Altmann, C Schäffer
Department of NanoBiotechnology, Universität für Bodenkultur Wien, Vienna, Austria

The occurrence of nonulosonic acids in bacteria is more wide-spread than originally anticipated and frequently linked to bacterial pathogenicity. In the periodontal pathogen *Tannerella forsythia*, several proposed virulence factors carry strain-specifically either a pseudaminic acid or a legionaminic acid derivative as a terminal sugar on a protein-bound oligosaccharide. This study aims to shed light on the transfer of either nonulosonic acid on a proximal *N*-acetyl-mannosaminuronic acid residue constituting the final step in *T. forsythia O*-glycan assembly. Bioinformatic analyses provided candidate genes *Tanf_01245* (ATCC 43037) and *BFO_1060* (FDC 92A2) encoding a putative pseudaminic acid and a legionaminic acid transferase, respectively. The transferases show a two-domain structure, with the N-terminal domain containing a predicted nucleosidediphosphate binding site and the C-terminal domain exhibiting homology to the TagB enzyme catalysing glycerol transfer to an *N*-acetylmannosamine residue in teichoic acid biosynthesis. Single gene knock-out mutants targeted at either transferase were analysed for their S-layer protein *O*-glycan composition by ESI-MS confirming the absence of the terminal nonulosonic acid. Analysis of a cellular pool of nucleotide-activated sugars confirmed the presence of CMP-activated nonulosonic derivatives conforming with the mass of a modified pseudaminic acid and legionaminic acid, respectively, as found in the mature *O*-glycans and, thus, likely serving as substrates for the corresponding transferase. Cross-complementation studies of the mutants were not successful indicating high stringency of the enzymes. This study identified plausible candidates for a pseudaminic acid and a legionaminic acid transferase; these may serve as a basis for the engineering of novel sialoglycoconjugates.

Pseudaminic and legionaminic acid transferases from the oral pathogen *Tannerella forsythia*

*Markus B. Tomek[1], Bettina Janesch[1], Daniel Maresch[2], Markus Windwarder[2], Friedrich Altmann[2], Christina Schäffer[1]

1) Department of NanoBiotechnology, *NanoGlycobiology* unit and 2) Department of Chemistry, BOKU - Universität für Bodenkultur Wien, Vienna, Austria

*Markus.Tomek@boku.ac.at

Introduction

The Gram-negative anaerobe *Tannerella forsythia* is recognized as an important oral pathogen implicated in the development and progression of periodontal diseases (1). Several proposed virulence factors (2,3) carry strain-specifically either a pseudaminic acid or a legionaminic acid derivative as a terminal sugar on a protein-bound oligosaccharide (V. Friedrich *et al.*, unpublished) **(Fig. 1)**. Among proposed virulence factors of *T. forsythia* are the abundant surface (S)-layer proteins TfsA and TfsB (3).

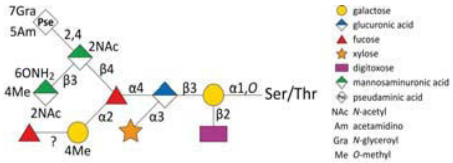


Figure 1. Schematic drawing of the *O*-glycan present in *T. forsythia* ATCC 43037.

This study aims to shed light on the transfer of either nonulosonic acid on a proximal *N*-acetyl mannosaminuronic acid residue constituting the final step in *T. forsythia O*-glycan assembly. Both candidate genes *Tanf_01245* (ATCC 43037) and *BFO_1060* (FDC 92A2, UB4) encoding a putative pseudaminic acid and legionaminic acid transferase, respectively, were knocked-out. The effect of gene deletions on the *O*-glycan composition was determined using mass spectrometry.

Materials and methods

Single gene knock-out mutants targeted at either transferase were constructed using homologous recombination. Crude cell extracts from defined mutants were either stained with Coomassie Brilliant Blue (CBB) or were detected using immunoblotting with α-TfsA or α-TfsB antibodies **(Fig. 2)**.

O-glycans from surface (S)-layer protein TfsB were released by in-gel reductive β-elimination. Nucleotide-activated sugar (NAS) pools were extracted and purified using Hypersep Hypercarb columns. Final purification was completed with preparative porous graphitized carbon (PGC)-HPLC (3) prior to electrospray-ionization mass spectrometry (ESI-MS). Analyses were performed in positive (*O*-glycans) or negative (NAS) mode on an amaZon speed EDT ion-trap instrument.

Occurring masses are represented in deconvoluted sum spectra **(Fig. 3 and 4)**. Glycan structures of the highest mass peaks are represented by symbols. Molecular ion peaks from the subsequent fragmentation pattern are assigned according to the loss of carbohydrate units and modifications. Relative intensities of occurring peaks are given on the *y*-axis.

Results

Glycoproteins are downshifted in transferase deficient mutants

Bioinformatic analyses show a two-domain structure for both transferases, containing a predicted nucleosidediphosphate binding site (DXD-motif) and exhibiting homology to the TagB enzyme catalyzing glycerol transfer to an *N*-acetylmannosamine residue in teichoic acid biosynthesis.

A 20 kDa down-shift of both S-layer proteins occurs in a Δ*Tanf_01245* mutant, whereas the native migration behavior could be regained in the reconstituted strain Δ*Tanf_01245*$^{+}$. An equivalent migration behavior of S-layer proteins can be observed for the UB4 strain **(Fig. 2A)**. Western immunoblots directed against TfsA **(Fig. 2B)** and TfsB **(Fig. 2C)** show the corresponding migration behavior as described.

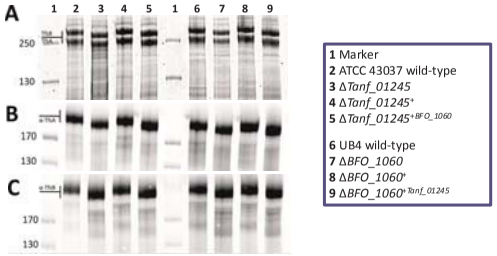


Figure 2. Migration behavior of the glycoproteins TfsA and TfsB in wild-type and mutant strains. **(A)** Protein stain with CBB; Western immunoblots directed against TfsA **(B)** and TfsB **(C)**.

Terminal nonulosonic acids are absent in transferase knock-out strains

Deletion of *Tanf_01245* results in the loss of the terminal modified pseudaminic acid in strain ATCC 43037. The highest mass peak at a *m/z* of 1523.4 $[M+H]^+$ can be assigned to a nonasaccharide lacking the modified pseudaminic acid unit (361.2) and a methyl group (14.0) which is absent at the branching mannosaminuronic acid. The reconstituted strain Δ*Tanf_01245*$^{+}$ recovered its pseudaminic acid transferase activity, resulting in the native glycan mass of *m/z* of 949.7 $[M+2H]^{2+}$ **(Fig. 3A)**.

T. forsythia UB4 carries a legionaminic acid with yet unknown modifications as terminal carbohydrate unit on its glycan that is otherwise almost identical to that of strain ATCC 43037. The wild-type glycan exhibits a *m/z* of 936.8 $[M+2H]^{2+}$. A Δ*BFO_1060* knock-out mutant is deficient in the modified legionaminic acid (335.2), as well as a methyl group (14.0), resulting in a highest mass peak with a *m/z* of 1523.4 $[M+H]^+$. The reconstituted strain *T. forsythia* Δ*BFO_1060*$^{+}$ regains the fully modified legionaminic acid, resulting in the same *m/z* signal of 936.8 $[M+2H]^{2+}$, as displayed by the UB4 wild-type glycan **(Fig. 3B)**.

Cross-complementation experiments were made in both directions. Results show that both enzymes, despite of high amino acid sequence homology (81%), are obviously too stringent for their respective substrates, resulting in a failure to restore the native phenotype.

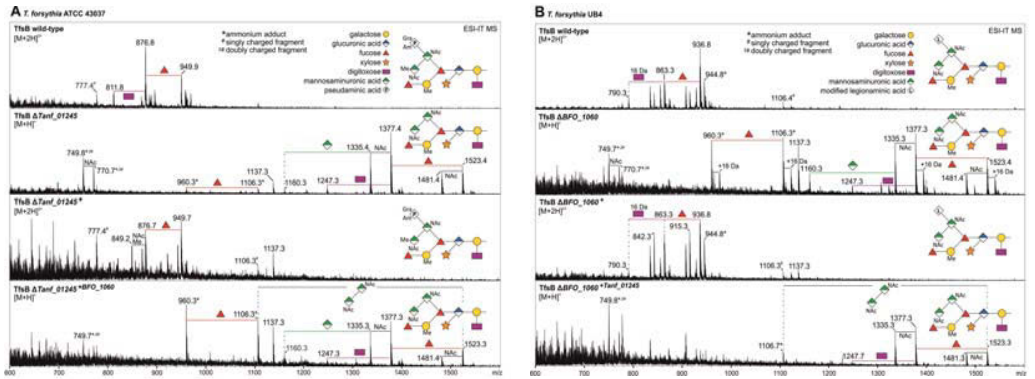


Figure 3. Deconvoluted sum spectra of TfsB *O*-glycans from *T. forsythia* wild-type, transferase knock-out, reconstituted and cross-complemented strains **(A)** *T. forsythia* ATCC 43037, **(B)** *T. forsythia* UB4.

CMP-activation and modification of nonulosonic acids occur prior to their transfer

CMP-activated nonulosonic derivatives are present in nucleotide activated sugar pools of wild-type and transferase deficient mutants. Masses found are conform with a modified pseudaminic acid (Pse5Am7Gra, 683 *m/z*) and a legionaminic acid with unknown modifications (Leg*, 654 *m/z*), respectively **(Fig. 4)**.
Thus, these compounds likely serve as substrates for the corresponding transferase.

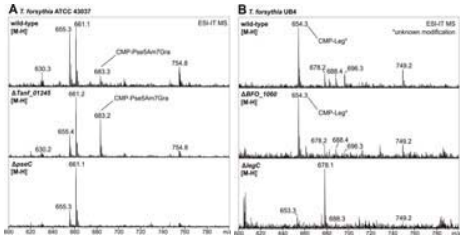


Figure 4. Deconvoluted sum spectra of nucleotide-activated sugar pools from wild-type, transferase knock-out and nonulosonic acid biosynthesis deficient strains (Δ*pseC*, Δ*legC*).
(A) *T. forsythia* ATCC 43037 **(B)** *T. forsythia* UB4.

Conclusions

- A putative pseudaminic acid transferase (Tanf_01245) from the oral pathogen *T. forsythia* ATCC 43037 and a putative legionaminic acid transferase (BFO_1060) from the clinical isolate *T. forsythia* UB4 were identified and separately knocked-out.
- Both transferase deficient mutant strains resulted in 20 kDa downshifted S-layer glycoproteins TfsA and TfsB.
- MS analyses demonstrated that the terminal sugar residue, pseudaminic acid or legionaminic acid, respectively, was absent when knocking-out the respective transferase gene.
- Donor substrates (CMP-Pse5Am7Gra and CMP-Leg) for their respective transferase are present in wild-type and transferase knock-out strains of *T. forsythia*.

Literature cited
(1) Hajishengallis, G. and Lamont, R. (2012) *Mol Oral Microbiol* **27**, 409-419
(2) Sharma, A. (2010) *Periodontol 2000* **54**, 106-116
(3) Posch, G. *et al.* (2011) *J Biol Chem* **286**, 38714-38724

This work is supported by the Austrian Science Fund P24317-B22 (to CS) and the doctoral programme W1224 (BioToP)

7

References

Abdallah AM, Gey van Pittius NC, DiGiuseppe Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CMJE, Appelmelk BJ, Bitter W. 2007. Type VII secretion - *mycobacteria* show the way. *Nat Rev Micro*, 5:883-891.

Anderson DM, Schneewind O. 1997. A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. *Science*, 278:1140-1143.

Angata T, Varki A. 2002. Chemical diversity in the sialic acids and related α-keto acids: An evolutionary perspective. *Chem Rev*, 102:439-470.

Anyaogu DC, Mortensen UH. 2015. Manipulating the glycosylation pathway in bacterial and lower eukaryotes for production of therapeutic proteins. *Curr Opin Biotechnol*, 36:122-128.

Baker JL, Çelik E, DeLisa MP. 2013. Expanding the glycoengineering toolbox: the rise of bacterial *N*-linked protein glycosylation. *Trends Biotechnol*, 31:313-323.

Banerjee A, Ghosh SK. 2003. The role of pilin glycan in neisserial pathogenesis. *Mol Cell Biochem*, 253:179-190.

Barnhart MM, Chapman MR. 2006. Curli biogenesis and function. *Ann Rev Microbiol*, 60:131-147.

Baron C. 2010. Antivirulence drugs to target bacterial secretion systems. *Curr Opin Microbiol*, 13:100-105.

Basler M. 2015. Type VI secretion system: secretion by a contractile nanomachine. *Philos Trans R Soc Lond B Biol Sci*, 370.

Beall CJ, Campbell AG, Dayeh DM, Griffen AL, Podar M, Leys EJ. 2014. Single cell genomics of uncultured, health-associated *Tannerella* BU063 (Oral Taxon 286) and comparison to the closely related pathogen *Tannerella forsythia*. *PLoS One*, 9:e89398.

Benz I, Schmidt MA. 2002. Never say never again: protein glycosylation in pathogenic bacteria. *Mol Microbiol*, 45:267-276.

Benz I, Schmidt MA. 2011. Structures and functions of autotransporter proteins in microbial pathogens. *Int J Med Microbiol*, 301:461-468.

Beveridge TJ, Pouwels PH, Sára M, Kotiranta A, Lounatmaa K, Kari K, Kerosuo E, Haapasalo M, Egelseer EM, Schocher I, Sleytr UB, Morelli L, Callegari M-L, Nomellini JF, Bingle WH, Smit J, Leibovitz E, Lemaire M, Miras I, Salamitou S, Béguin P, Ohayon H, Gounon P, Matuschek M, Sahm K, Bahl H, Grogono-Thomas R, Dworkin J, Blaser MJ, Woodland RM, Newell DG, Kessel M, Koval SF. 1997. V. Functions of S-layers. *FEMS Microbiol Rev*, 20:99-149.

Breton C. 2005. Structures and mechanisms of glycosyltransferases. *Glycobiology*, 16:29R-37R.

Breton C, Fournel-Gigleux S, Palcic MM. 2012. Recent structures, evolution and mechanisms of glycosyltransferases. *Curr Opin Struct Biol*, 22:540-549.

Bryzek D, Ksiazek M, Bielecka E, Karim AY, Potempa B, Staniec D, Koziel J, Potempa J. 2014. A pathogenic trace of *Tannerella forsythia* – shedding of soluble fully active tumor necrosis factor α from the macrophage surface by karilysin. *Mol Oral Microbiol*, 29:294-306.

Burrows LL. 2012. *Pseudomonas aeruginosa* twitching motility: Type IV pili in action. *Ann Rev Microbiol*, 66:493-520.

Chagnot C, Zorgani MA, Astruc T, Desvaux M. 2013. Proteinaceous determinants of surface colonization in bacteria: bacterial adhesion and biofilm formation from a protein secretion perspective. *Front Microbiol*, 4:303.

Chen L, Zou Y, She P, Wu Y. 2015. Composition, function, and regulation of T6SS in *Pseudomonas aeruginosa*. *Microbiol Res*, 172:19-25.

Chen YY, Peng B, Yang Q, Glew MD, Veith PD, Cross KJ, Goldie KN, Chen D, O'Brien-Simpson N, Dashper SG, Reynolds EC. 2011. The outer membrane protein LptO is essential for the *O*-deacylation of LPS and the co-ordinated secretion and attachment of A-LPS and CTD proteins in *Porphyromonas gingivalis*. *Mol Microbiol*, 79:1380-1401.

Chiu CPC, Watts AG, Lairson LL, Gilbert M, Lim D, Wakarchuk WW, Withers SG, Strynadka NCJ. 2004. Structural analysis of the sialyltransferase CstII from *Campylobacter jejuni* in complex with a substrate analog. *Nat Struct Mol Biol*, 11:163-170.

Christie PJ, Cascales E. 2005. Structural and dynamic properties of bacterial type IV secretion systems (review). *Mol Membr Biol*, 22:51-61.

Colombo AP, Boches SK, Cotton SL, Goodson JM, Kent R, Haffajee AD, Socransky SS, Hasturk H, Van Dyke TE, Dewhirst F, Paster BJ. 2009. Comparisons of subgingival microbial profiles of refractory periodontitis, severe periodontitis, and periodontal health using the human oral microbe identification microarray. *J Periodontol*, 80:1421-1432.

Comstock LE, Kasper DL. 2006. Bacterial glycans: key mediators of diverse host immune responses. *Cell*, 126:847-850.

Conrad JC. 2012. Physics of bacterial near-surface motility using flagella and type IV pili: implications for biofilm formation. *Res Microbiol*, 163:619-629.

Cornelis GR. 2006. The type III secretion injectisome. *Nat Rev Microbiol*, 4:811-825.

Costa TRD, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, Waksman G. 2015. Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Micro*, 13:343-359.

Coutinho PM, Deleury E, Davies GJ, Henrissat B. 2003. An evolving hierarchical family classification for glycosyltransferases. *J Mol Biol*, 328:307-317.

Coyne MJ, Fletcher CM, Chatzidaki-Livanis M, Posch G, Schäffer C, Comstock LE. 2013. Phylum-wide general protein *O*-glycosylation system of the *Bacteroidetes*. *Mol Microbiol*, 88:772-783.

Darveau RP. 2010. Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol*, 8:481-490.

de Diego I, Ksiazek M, Mizgalska D, Koneru L, Golik P, Szmigielski B, Nowak M, Nowakowska Z, Potempa B, Houston JA, Enghild JJ, Thogersen IB, Gao J, Kwan AH, Trewhella J, Dubin G, Gomis-Ruth FX, Nguyen KA, Potempa J. 2016. The outer-membrane export signal of *Porphyromonas gingivalis* type IX secretion system (T9SS) is a conserved C-terminal beta-sandwich domain. *Sci Rep*, 6:23123.

Delepelaire P. 2004. Type I secretion in gram-negative bacteria. *Biochim Biophys Acta - Mol Cell Res*, 1694:149-161.

Desvaux M, Dumas E, Chafsey I, Hébraud M. 2006. Protein cell surface display in Gram-positive bacteria: from single protein to macromolecular protein structure. *FEMS Microbiol Lett*, 256:1-15.

Desvaux M, Hebraud M, Talon R, Henderson IR. 2009. Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue. *Trends Microbiol*, 17:139-145.

Durand E, Verger D, Rego AT, Chandran V, Meng G, Fronzes R, Waksman G. 2009. Structural biology of bacterial secretion systems in gram-negative pathogens-potential for new drug targets. *Infect Disord Drug Targets*, 9:518-547.

Dye BA. 2012. Global periodontal disease epidemiology. *Periodontol 2000*, 58:10-25.

Egge-Jacobsen W, Salomonsson EN, Aas FE, Forslund AL, Winther-Larsen HC, Maier J, Macellaro A, Kuoppa K, Oyston PC, Titball RW, Thomas RM, Forsberg A, Prior JL, Koomey M. 2011. *O*-linked glycosylation of the PilA pilin protein of *Francisella tularensis*: identification of the endogenous protein-targeting oligosaccharyltransferase and characterization of the native oligosaccharide. *J Bacteriol*, 193:5487-5497.

Elhenawy W, Scott NE, Tondo ML, Orellano EG, Foster LJ, Feldman MF. 2016. Protein *O*-linked glycosylation in the plant pathogen *Ralstonia solanacearum*. *Glycobiology*, 26:301-311.

Epstein EA, Chapman MR. 2008. Polymerizing the fibre between bacteria and host cells: the biogenesis of functional amyloid fibres. *Cell Microbiol*, 10:1413-1420.

Espitia C, Servin-Gonzalez L, Mancilla R. 2010. New insights into protein *O*-mannosylation in *actinomycetes*. *Mol Biosyst*, 6:775-781.

Fagan RP, Fairweather NF. 2014. Biogenesis and functions of bacterial S-layers. *Nat Rev Micro*, 12:211-222.

Faridmoayer A, Feldman MF. 2010. Bacterial protein glycosylation. In: Mander L, Liu H-W editors. *Comprehensive Natural Products II*. Kidlington (UK): Elsevier, pp. 351-380.

Filloux A, Hachani A, Bleves S. 2008. The bacterial type VI secretion machine: yet another player for protein transport across membranes. *Microbiology*, 154:1570-1583.

Fletcher CM, Coyne MJ, Villa OF, Chatzidaki-Livanis M, Comstock LE. 2009. A general *O*-glycosylation system important to the physiology of a major human intestinal symbiont. *Cell*, 137:321-331.

Fodor AA, DeSantis TZ, Wylie KM, Badger JH, Ye Y, Hepburn T, Hu P, Sodergren E, Liolios K, Huot-Creasy H, Birren BW, Earl AM. 2012. The "most wanted" taxa from the human microbiome for whole genome sequencing. *PLoS One*, 7:e41294.

Friedrich V, Gruber C, Nimeth I, Pabinger S, Sekot G, Posch G, Altmann F, Messner P, Andrukhov O, Schäffer C. 2015a. Outer membrane vesicles of *Tannerella forsythia*: biogenesis, composition, and virulence. *Mol Oral Microbiol*, 30:451-473.

Friedrich V, Janesch B, Windwarder M, Maresch D, Braun ML, Megson ZA, Vinogradov E, Goneau M-F, Sharma A, Altmann F, Messner P, Schoenhofen IC, Schäffer C. 2016. *Tannerella forsythia* strains display different cell-surface nonulosonic acids: biosynthetic pathway characterization and first insight into biological implications. *Glycobiology*, in press.

Friedrich V, Janesch B, Windwarder M, Maresch D, Braun ML, Megson ZA, Vinogradov E, Goneau MF, Sharma A, Altmann F, Messner P, Schoenhofen IC, Schäffer C. 2017. *Tannerella forsythia* strains display different cell-surface nonulosonic acids: biosynthetic pathway characterization and first insight into biological implications. *Glycobiology, Epub ahead of print*.

Friedrich V, Pabinger S, Chen T, Messner P, Dewhirst FE, Schäffer C. 2015b. Draft genome sequence of *Tannerella forsythia* type strain ATCC 43037. *Genome Announc*, 3:e00660-00615.

Friedrich V, Pabinger S, Chen T, Messner P, Dewhirst FE, Schäffer C. 2015c. Draft genome sequence of *Tannerella forsythia* type strain ATCC 43037. *Genome Announc*, 3(3):e00660-15.

Fröbel J, Rose P, Müller M. 2012. Twin-arginine-dependent translocation of folded proteins. *Philos Trans R Soc Lond B Biol Sci*, 367:1029-1046.

Gault J, Ferber M, Machata S, Imhaus AF, Malosse C, Charles-Orszag A, Millien C, Bouvier G, Bardiaux B, Pehau-Arnaudet G, Klinge K, Podglajen I, Ploy MC, Seifert HS, Nilges M, Chamot-Rooke J, Dumenil G. 2015. *Neisseria meningitidis* type IV pili composed of sequence invariable pilins are masked by multisite glycosylation. *PLoS Pathog*, 11:e1005162.

Glew MD, Veith PD, Chen D, Seers CA, Chen YY, Reynolds EC. 2014. Blue native-PAGE analysis of membrane protein complexes in *Porphyromonas gingivalis*. *J Proteomics*, 110:72-92.

Glew MD, Veith PD, Peng B, Chen YY, Gorasia DG, Yang Q, Slakeski N, Chen D, Moore C, Crawford S, Reynolds EC. 2012. PG0026 is the C-terminal signal peptidase of a novel secretion system of *Porphyromonas gingivalis*. *J Biol Chem*, 287:24605-24617.

Gloster TM. 2014. Advances in understanding glycosyltransferases from a structural perspective. *Curr Opin Struct Biol*, 28:131-141.

Goon S, Kelly JF, Logan SM, Ewing CP, Guerry P. 2003. Pseudaminic acid, the major modification on *Campylobacter* flagellin, is synthesized via the Cj1293 gene. *Mol Microbiol*, 50:659-671.

Gorasia DG, Veith PD, Chen D, Seers CA, Mitchell HA, Chen YY, Glew MD, Dashper SG, Reynolds EC. 2015. *Porphyromonas gingivalis* type IX secretion substrates are cleaved and modified by a sortase-like mechanism. *PLoS Pathog*, 11:e1005152.

Gorasia DG, Veith PD, Hanssen EG, Glew MD, Sato K, Yukitake H, Nakayama K, Reynolds EC. 2016. Structural insights into the PorK and PorN components of the *Porphyromonas gingivalis* type IX secretion system. *PLoS Pathog*, 12:e1005820.

Gosling PT, Gemmell E, Carter CL, Bird PS, Seymour GJ. 2005. Immunohistological analysis of *Tannerella forsythia*-induced lesions in a murine model. *Oral Microbiol Immunol*, 20:25-30.

Goyal P, Krasteva PV, Van Gerven N, Gubellini F, Van den Broeck I, Troupiotis-Tsailaki A, Jonckheere W, Pehau-Arnaudet G, Pinkner JS, Chapman MR, Hultgren SJ, Howorka S,

Fronzes R, Remaut H. 2014. Structural and mechanistic insights into the bacterial amyloid secretion channel CsgG. *Nature*, 516:250-253.
Greenfield LK, Whitfield C. 2012. Synthesis of lipopolysaccharide O-antigens by ABC transporter-dependent pathways. *Carbohydr Res*, 356:12-24.
Hajishengallis G. 2014. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. *Trends Immunol*, 35:3-11.
Hajishengallis G. 2015. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol*, 15:30-44.
Hajishengallis G, Darveau RP, Curtis MA. 2012. The keystone-pathogen hypothesis. *Nat Rev Microbiol*, 10:717-725.
Hajishengallis G, Lamont RJ. 2012. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol Oral Microbiol*, 27:409-419.
Hamlet SM, Taiyeb-Ali TB, Cullinan MP, Westerman B, Palmer JE, Seymour GJ. 2007. *Tannerella forsythensis prtH* genotype and association with periodontal status. *J Periodontol*, 78:344-350.
Harding CM, Nasr MA, Kinsella RL, Scott NE, Foster LJ, Weber BS, Fiester SE, Actis LA, Tracy EN, Munson RS, Jr., Feldman MF. 2015. *Acinetobacter* strains carry two functional oligosaccharyltransferases, one devoted exclusively to type IV pilin, and the other one dedicated to *O*-glycosylation of multiple proteins. *Mol Microbiol*, 96:1023-1041.
Hartley MD, Morrison MJ, Aas FE, Borud B, Koomey M, Imperiali B. 2011. Biochemical characterization of the *O*-linked glycosylation pathway in *Neisseria gonorrhoeae* responsible for biosynthesis of protein glycans containing N,N'-diacetylbacillosamine. *Biochemistry*, 50:4936-4948.
Hasebe A, Yoshimura A, Into T, Kataoka H, Tanaka S, Arakawa S, Ishikura H, Golenbock DT, Sugaya T, Tsuchida N, Kawanami M, Hara Y, Shibata K. 2004. Biological activities of *Bacteroides forsythus* lipoproteins and their possible pathological roles in periodontal disease. *Infect Immun*, 72:1318-1325.
Heath JE, Seers CA, Veith PD, Butler CA, Nor Muhammad NA, Chen YY, Slakeski N, Peng B, Zhang L, Dashper SG, Cross KJ, Cleal SM, Moore C, Reynolds EC. 2016. PG1058 is a novel multidomain protein component of the bacterial type IX secretion system. *PLoS One*, 11:e0164313.
Henderson IR, Navarro-Garcia F, Desvaux M, Fernandez RC, Ala'Aldeen D. 2004. Type V protein secretion pathway: the autotransporter story. *Microbiol Mol Biol Rev*, 68:692-744.
Heras B, Scanlon MJ, Martin JL. 2015. Targeting virulence not viability in the search for future antibacterials. *Br J Clin Pharmacol*, 79:208-215.
Herrmann JL, O'Gaora P, Gallagher A, Thole JE, Young DB. 1996. Bacterial glycoproteins: a link between glycosylation and proteolytic cleavage of a 19 kDa antigen from *Mycobacterium tuberculosis*. *EMBO J*, 15:3547-3554.
Highfield J. 2009. Diagnosis and classification of periodontal disease. *Aust Dent J*, 54:S11-S26.
Hitchen PG, Dell A. 2006. Bacterial glycoproteomics. *Microbiology*, 152:1575-1580.
Ho BT, Dong TG, Mekalanos JJ. 2014. A view to a kill: the bacterial type VI secretion system. *Cell Host Microbe*, 15:9-21.
Holt SC, Bramanti TE. 1991. Factors in virulence expression and their role in periodontal disease pathogenesis. *Crit Rev Oral Biol Med*, 2:177-281.
Holt SC, Ebersole JL. 2005. *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol 2000*, 38:72-122.
Honma K, Inagaki S, Okuda K, Kuramitsu HK, Sharma A. 2007. Role of a *Tannerella forsythia* exopolysaccharide synthesis operon in biofilm development. *Microb Pathog*, 42:156-166.
Honma K, Mishima E, Sharma A. 2011. Role of *Tannerella forsythia* NanH sialidase in epithelial cell attachment. *Infect Immun*, 79:393-401.

Horn C, Namane A, Pescher P, Riviere M, Romain F, Puzo G, Barzu O, Marchal G. 1999. Decreased capacity of recombinant 45/47-kDa molecules (Apa) of *Mycobacterium tuberculosis* to stimulate T lymphocyte responses related to changes in their mannosylation pattern. *J Biol Chem*, 274:32023-32030.

Houben ENG, Korotkov KV, Bitter W. 2014. Take five — Type VII secretion systems of *Mycobacteria*. *Biochim Biophys Acta - Mol Cell Res*, 1843:1707-1716.

Igura M, Maita N, Kamishikiryo J, Yamada M, Obita T, Maenaka K, Kohda D. 2008. Structure-guided identification of a new catalytic motif of oligosaccharyltransferase. *EMBO J*, 27:234-243.

Iwashkiw JA, Seper A, Weber BS, Scott NE, Vinogradov E, Stratilo C, Reiz B, Cordwell SJ, Whittal R, Schild S, Feldman MF. 2012. Identification of a general *O*-linked protein glycosylation system in *Acinetobacter baumannii* and its role in virulence and biofilm formation. *PLoS Pathog*, 8:e1002758.

Iwashkiw JA, Vozza NF, Kinsella RL, Feldman MF. 2013. Pour some sugar on it: the expanding world of bacterial protein *O*-linked glycosylation. *Mol Microbiol*, 89:14-28.

Jeevarajah D, Patterson JH, McConville MJ, Billman-Jacobe H. 2002. Modification of glycopeptidolipids by an *O*-methyltransferase of *Mycobacterium smegmatis*. *Microbiology*, 148:3079-3087.

Jung YJ, Choi YJ, An SJ, Lee HR, Jun HK, Choi BK. in press. *Tannerella forsythia* GroEL induces inflammatory bone resorption and synergizes with IL-17. *Mol Oral Microbiol, Epub ahead of print*.

Jusko M, Potempa J, Karim AY, Ksiazek M, Riesbeck K, Garred P, Eick S, Blom AM. 2012. A metalloproteinase karilysin present in the majority of *Tannerella forsythia* isolates inhibits all pathways of the complement system. *J Immunol*, 188:2338-2349.

Kadowaki T, Yukitake H, Naito M, Sato K, Kikuchi Y, Kondo Y, Shoji M, Nakayama K. 2016. A two-component system regulates gene expression of the type IX secretion component proteins via an ECF sigma factor. *Sci Rep*, 6:23288.

Kassebaum NJ, Bernabe E, Dahiya M, Bhandari B, Murray CJ, Marcenes W. 2014. Global burden of severe periodontitis in 1990-2010: a systematic review and meta-regression. *J Dent Res*, 93:1045-1053.

Kelly J, Jarrell H, Millar L, Tessier L, Fiori LM, Lau PC, Allan B, Szymanski CM. 2006. Biosynthesis of the *N*-linked glycan in *Campylobacter jejuni* and addition onto protein through block transfer. *J Bacteriol*, 188:2427-2434.

Kerosuo E. 1988. Ultrastructure of the cell envelope of *Bacteroides forsythus* strain ATCC 43037^{T}. *Oral Microbiol Immunol*, 3:134-137.

Kharade SS, McBride MJ. 2015. *Flavobacterium johnsoniae* PorV is required for secretion of a subset of proteins targeted to the type IX secretion system. *J Bacteriol*, 197:147-158.

Kirn TJ, Jude BA, Taylor RK. 2005. A colonization factor links *Vibrio cholerae* environmental survival and human infection. *Nature*, 438:863-866.

Kita D, Shibata S, Kikuchi Y, Kokubu E, Nakayama K, Saito A, Ishihara K. 2016. Involvement of the type IX secretion system in *Capnocytophaga ochracea* gliding motility and biofilm formation. *Appl Environ Microbiol*, 82:1756-1766.

Knirel YA, Shashkov AS, Tsvetkov YE, Jansson P-E, Zähringer U. 2003. 5,7-diamino-3,5,7,9-tetradeoxynon-2-ulosonic acids in bacterial glycopolymers: Chemistry and biochemistry. *Adv Carbohydr Chem Biochem*, 58:371-417.

Kobe B, Kajava AV. 2001. The leucine-rich repeat as a protein recognition motif. *Curr Opin Struct Biol*, 11:725-732.

Kolenbrander PE, Palmer Jr RJ, Periasamy S, Jakubovics NS. 2010. Oral multispecies biofilm development and the key role of cell–cell distance. *Nat Rev Micro*, 8:471-480.

Koneru L, Ksiazek M, Waligorska I, Straczek A, Lukasik M, Madej M, Thogersen IB, Enghild JJ, Potempa J. in press. Mirolysin, a LysargiNase from *Tannerella forsythia*, proteolytically inactivates the human cathelicidin, LL-37. *Biol Chem, Epub ahead of print*.

Ksiazek M, Karim AY, Bryzek D, Enghild JJ, Thogersen IB, Koziel J, Potempa J. 2015a. Mirolase, a novel subtilisin-like serine protease from the periodontopathogen *Tannerella forsythia*. *Biol Chem*, 396:261-275.

Ksiazek M, Mizgalska D, Eick S, Thogersen IB, Enghild JJ, Potempa J. 2015b. KLIKK proteases of Tannerella forsythia: putative virulence factors with a unique domain structure. *Front Microbiol*, 6:312.

Ku SC, Schulz BL, Power PM, Jennings MP. 2009. The pilin *O*-glycosylation pathway of pathogenic *Neisseria* is a general system that glycosylates AniA, an outer membrane nitrite reductase. *Biochem Biophys Res Commun*, 378:84-89.

Kugelberg E, Gollan B, Tang CM. 2008. Mechanisms in *Neisseria meningitidis* for resistance against complement-mediated killing. *Vaccine*, 26 Suppl 8:I34-39.

Kuo C, Takahashi N, Swanson AF, Ozeki Y, Hakomori S. 1996. An *N*-linked high-mannose type oligosaccharide, expressed at the major outer membrane protein of *Chlamydia trachomatis*, mediates attachment and infectivity of the microorganism to HeLa cells. *J Clin Invest*, 98:2813-2818.

Lairson LL, Henrissat B, Davies GJ, Withers SG. 2008. Glycosyltransferases: structures, functions, and mechanisms. *Annu Rev Biochem*, 77:521-555.

Lamont RJ, Hajishengallis G. 2015. Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol Med*, 21:172-183.

Lasica AM, Goulas T, Mizgalska D, Zhou X, de Diego I, Ksiazek M, Madej M, Guo Y, Guevara T, Nowak M, Potempa B, Goel A, Sztukowska M, Prabhakar AT, Bzowska M, Widziolek M, Thogersen IB, Enghild JJ, Simonian M, Kulczyk AW, Nguyen KA, Potempa J, Gomis-Ruth FX. 2016. Structural and functional probing of PorZ, an essential bacterial surface component of the type-IX secretion system of human oral-microbiomic *Porphyromonas gingivalis*. *Sci Rep*, 6:37708.

Lee SW, Sabet M, Um HS, Yang J, Kim HC, Zhu W. 2006. Identification and characterization of the genes encoding a unique surface (S-) layer of *Tannerella forsythia*. *Gene*, 371:102-111.

Lees-Miller RG, Iwashkiw JA, Scott NE, Seper A, Vinogradov E, Schild S, Feldman MF. 2013. A common pathway for *O*-linked protein-glycosylation and synthesis of capsule in *Acinetobacter baumannii*. *Mol Microbiol*, 89:816-830.

Leo JC, Grin I, Linke D. 2012. Type V secretion: mechanism(s) of autotransport through the bacterial outer membrane. *Philos Trans R Soc Lond B Biol Sci*, 367:1088-1101.

Lindenthal C, Elsinghorst EA. 2001. Enterotoxigenic *Escherichia coli* TibA glycoprotein adheres to human intestine epithelial cells. *Infect Immun*, 69:52-57.

Lithgow KV, Scott NE, Iwashkiw JA, Thomson EL, Foster LJ, Feldman MF, Dennis JJ. 2014. A general protein *O*-glycosylation system within the *Burkholderia cepacia* complex is involved in motility and virulence. *Mol Microbiol*, 92:116-137.

Logan SM. 2006. Flagellar glycosylation - a new component of the motility repertoire? *Microbiology*, 152:1249-1262.

Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. 2014. The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res*, 42:D490-495.

Lovering AL, de Castro LH, Lim D, Strynadka NC. 2007. Structural insight into the transglycosylation step of bacterial cell-wall biosynthesis. *Science*, 315:1402-1405.

Low HH, Gubellini F, Rivera-Calzada A, Braun N, Connery S, Dujeancourt A, Lu F, Redzej A, Fronzes R, Orlova EV, Waksman G. 2014. Structure of a type IV secretion system. *Nature*, 508:550-553.

Lycklama á Nijeholt JA, Driessen AJM. 2012. The bacterial Sec-translocase: structure and mechanism. *Philos Trans R Soc Lond B Biol Sci*, 367:1016-1028.

Maiden MF, Pham C, Kashket S. 2004. Glucose toxicity effect and accumulation of methylglyoxal by the periodontal anaerobe *Bacteroides forsythus*. *Anaerobe*, 10:27-32.

Maresz KJ, Hellvard A, Sroka A, Adamowicz K, Bielecka E, Koziel J, Gawron K, Mizgalska D, Marcinska KA, Benedyk M, Pyrc K, Quirke AM, Jonsson R, Alzabin S, Venables PJ, Nguyen

KA, Mydel P, Potempa J. 2013. *Porphyromonas gingivalis* facilitates the development and progression of destructive arthritis through its unique bacterial peptidylarginine deiminase (PAD). *PLoS Pathog*, 9:e1003627.

McBride MJ, Nakane D. 2015. *Flavobacterium* gliding motility and the type IX secretion system. *Curr Opin Microbiol*, 28:72-77.

McBride MJ, Zhu Y. 2013. Gliding motility and Por secretion system genes are widespread among members of the phylum *bacteroidetes*. *J Bacteriol*, 195:270-278.

McGraw WT, Potempa J, Farley D, Travis J. 1999. Purification, characterization, and sequence analysis of a potential virulence factor from *Porphyromonas gingivalis*, peptidylarginine deiminase. *Infect Immun*, 67:3248-3256.

Megson ZA, Koerdt A, Schuster H, Ludwig R, Janesch B, Frey A, Naylor K, Wilson IB, Stafford GP, Messner P, Schäffer C. 2015. Characterization of an α-L-fucosidase from the periodontal pathogen *Tannerella forsythia*. *Virulence*, 6:282-292.

Merritt JH, Ollis AA, Fisher AC, DeLisa MP. 2013. Glycans-by-design: engineering bacteria for the biosynthesis of complex glycans and glycoconjugates. *Biotechnol Bioeng*, 110:1550-1564.

Mescher MF, Strominger JL. 1976. Purification and characterization of a prokaryotic glucoprotein from the cell envelope of *Halobacterium salinarium*. *J Biol Chem*, 251:2005-2014.

Messner P. 1997. Bacterial glycoproteins. *Glycoconj J*, 14:3-11.

Messner P, Schäffer C, Egelseer E-M, Sleytr UB. 2010. Occurrence, structure, chemistry, genetics, morphogenesis, and functions of S-Layers. In: König H, Claus H, Varma A editors. *Prokaryotic Cell Wall Compounds: Structure and Biochemistry*. Berlin, Heidelberg: Springer, pp. 53-109.

Messner P, Schäffer C, Kosma P. 2013. Bacterial cell-envelope glycoconjugates. *Adv Carbohydr Chem Biochem*, 69:209-272.

Messner P, Steiner K, Zarschler K, Schäffer C. 2008. S-layer nanoglycobiology of bacteria. *Carbohydr Res*, 343:1934-1951.

Michiels T, Wattiau P, Brasseur R, Ruysschaert JM, Cornelis G. 1990. Secretion of Yop proteins by *Yersiniae*. *Infect Immun*, 58:2840-2849.

Moens S, Vanderleyden J. 1997. Glycoproteins in prokaryotes. *Arch Microbiol*, 168:169-175.

Mota LJ, Cornelis GR. 2005. The bacterial injection kit: type III secretion systems. *Ann Med*, 37:234-249.

Nakajima T, Tomi N, Fukuyo Y, Ishikura H, Ohno Y, Arvind R, Arai T, Ishikawa I, Arakawa S. 2006. Isolation and identification of a cytopathic activity in *Tannerella forsythia*. *Biochem Biophys Res Commun*, 351:133-139.

Nakayama K. 2015. *Porphyromonas gingivalis* and related bacteria: from colonial pigmentation to the type IX secretion system and gliding motility. *J Periodontal Res*, 50:1-8.

Natale P, Brüser T, Driessen AJM. 2008. Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane—Distinct translocases and mechanisms. *Biochim Biophys Acta (BBA) - Biomembranes*, 1778:1735-1756.

Nguyen KA, Travis J, Potempa J. 2007. Does the importance of the C-terminal residues in the maturation of RgpB from *Porphyromonas gingivalis* reveal a novel mechanism for protein export in a subgroup of Gram-Negative bacteria? *J Bacteriol*, 189:833-843.

Nguyen KA, Zylicz J, Szczesny P, Sroka A, Hunter N, Potempa J. 2009. Verification of a topology model of PorT as an integral outer-membrane protein in *Porphyromonas gingivalis*. *Microbiology*, 155:328-337.

Nothaft H, Szymanski CM. 2010. Protein glycosylation in bacteria: sweeter than ever. *Nat Rev Microbiol*, 8:765-778.

Nothaft H, Szymanski CM. 2013. Bacterial protein *N*-glycosylation: new perspectives and applications. *J Biol Chem*, 288:6912-6920.

Onishi S, Honma K, Liang S, Stathopoulou P, Kinane D, Hajishengallis G, Sharma A. 2008. Toll-like receptor 2-mediated interleukin-8 expression in gingival epithelial cells by the *Tannerella forsythia* leucine-rich repeat protein BspA. *Infect Immun*, 76:198-205.

Otomo-Corgel J, Pucher JJ, Rethman MP, Reynolds MA. 2012. State of the science: chronic periodontitis and systemic health. *J Evid Based Dent Pract*, 12:20-28.

Petersen PE, Ogawa H. 2005. Strengthening the prevention of periodontal disease: the WHO approach. *J Periodontol*, 76:2187-2193.

Posch G, Andrukhov O, Vinogradov E, Lindner B, Messner P, Holst O, Schäffer C. 2013a. Structure and immunogenicity of the rough-type lipopolysaccharide from the periodontal pathogen *Tannerella forsythia*. *Clin Vaccine Immunol*, 20:945-953.

Posch G, Pabst M, Brecker L, Altmann F, Messner P, Schäffer C. 2011. Characterization and scope of S-layer protein *O*-glycosylation in *Tannerella forsythia*. *J Biol Chem*, 286:38714-38724.

Posch G, Pabst M, Neumann L, Coyne MJ, Altmann F, Messner P, Comstock LE, Schäffer C. 2013b. "Cross-glycosylation" of proteins in *Bacteroidales* species. *Glycobiology*, 23:568-577.

Potempa J, Pike RN. 2009. Corruption of innate immunity by bacterial proteases. *J Innate Immun*, 1:70-87.

Potempa J, Sroka A, Imamura T, Travis J. 2003. Gingipains, the major cysteine proteinases and virulence factors of *Porphyromonas gingivalis*: structure, function and assembly of multidomain protein complexes. *Curr Protein Pept Sci*, 4:397-407.

Power PM, Roddam LF, Rutter K, Fitzpatrick SZ, Srikhanta YN, Jennings MP. 2003. Genetic characterization of pilin glycosylation and phase variation in *Neisseria meningitidis*. *Mol Microbiol*, 49:833-847.

Price NL, Goyette-Desjardins G, Nothaft H, Valguarnera E, Szymanski CM, Segura M, Feldman MF. 2016. Glycoengineered outer membrane vesicles: A novel platform for bacterial vaccines. *Sci Rep*, 6:24931.

Pugsley AP. 1993. The complete general secretory pathway in gram-negative bacteria. *Microbiol Rev*, 57:50-108.

Ristl R, Steiner K, Zarschler K, Zayni S, Messner P, Schäffer C. 2011. The S-layer glycome-adding to the sugar coat of bacteria. *Int J Microbiol*, 2011.

Roy S, Honma K, Douglas CWI, Sharma A, Stafford GP. 2011. Role of sialidase in glycoprotein utilization by *Tannerella forsythia*. *Microbiology*, 157:3195-3202.

Ruer S, Pinotsis N, Steadman D, Waksman G, Remaut H. 2015. Virulence-targeted antibacterials: Concept, promise, and susceptibility to resistance mechanisms. *Chem Biol Drug Des*, 86:379-399.

Ruiz OA, Ugalde RA. 1998. Partial characterization and photolabeling of a *Rhizobium meliloti* polysaccharide methyltransferase with S-adenosylmethionine. *Int Microbiol*, 1:225-230.

Sabet M, Lee SW, Nauman RK, Sims T, Um HS. 2003. The surface (S-) layer is a virulence factor of *Bacteroides forsythus*. *Microbiology*, 149:3617-3627.

Saiki K, Konishi K. 2010. The role of Sov protein in the secretion of gingipain protease virulence factors of *Porphyromonas gingivalis*. *FEMS Microbiol Lett*, 302:166-174.

Saiki K, Konishi K. 2014. *Porphyromonas gingivalis* C-terminal signal peptidase PG0026 and HagA interact with outer membrane protein PG27/LptO. *Mol Oral Microbiol*, 29:32-44.

Sakakibara J, Nagano K, Murakami Y, Higuchi N, Nakamura H, Shimozato K, Yoshimura F. 2007. Loss of adherence ability to human gingival epithelial cells in S-layer protein-deficient mutants of *Tannerella forsythensis*. *Microbiology*, 153:866-876.

Sandkvist M. 2001. Biology of type II secretion. *Mol Microbiol*, 40:271-283.

Sato K, Naito M, Yukitake H, Hirakawa H, Shoji M, McBride MJ, Rhodes RG, Nakayama K. 2010. A protein secretion system linked to bacteroidete gliding motility and pathogenesis. *Proc Natl Acad Sci U S A*, 107:276-281.

Schäffer C, Graninger M, Messner P. 2001. Prokaryotic glycosylation. *Proteomics*, 1:248-261.

Schäffer C, Messner P. 2001. Glycobiology of surface layer proteins. *Biochimie*, 83:591-599.

Schäffer C, Messner P. 2017. Emerging facets of prokaryotic glycosylation. *FEMS Microbiol Rev*, 41:49-91.

Schmidt MA, Riley LW, Benz I. 2003. Sweet new world: glycoproteins in bacterial pathogens. *Trends Microbiol*, 11:554-561.

Schneewind O, Missiakas DM. 2012. Protein secretion and surface display in Gram-positive bacteria. *Philos Trans R Soc Lond B Biol Sci*, 367:1123-1139.

Schuman B, Evans SV, Fyles TM. 2013. Geometric attributes of retaining glycosyltransferase enzymes favor an orthogonal mechanism. *PLoS One*, 8:e71077.

Sekot G, Posch G, Messner P, Matejka M, Rausch-Fan X, Andrukhov O, Schäffer C. 2011. Potential of the *Tannerella forsythia* S-layer to delay the immune response. *J Dent Res*, 90:109-114.

Sekot G, Posch G, Oh YJ, Zayni S, Mayer HF, Pum D, Messner P, Hinterdorfer P, Schäffer C. 2012. Analysis of the cell surface layer ultrastructure of the oral pathogen *Tannerella forsythia*. *Arch Microbiol*, 194:525-539.

Settem RP, Honma K, Nakajima T, Phansopa C, Roy S, Stafford GP, Sharma A. 2013a. A bacterial glycan core linked to surface (S)-layer proteins modulates host immunity through Th17 suppression. *Mucosal Immunol*, 6:415-426.

Settem RP, Honma K, Sharma A. 2014. Neutrophil mobilization by surface-glycan altered Th17-skewing bacteria mitigates periodontal pathogen persistence and associated alveolar bone loss. *PLoS One*, 9:e108030.

Settem RP, Honma K, Stafford GP, Sharma A. 2013b. Protein-linked glycans in periodontal bacteria: prevalence and role at the immune interface. *Front Microbiol*, 4:310.

Sharma A. 2010. Virulence mechanisms of *Tannerella forsythia*. *Periodontol 2000*, 54:106-116.

Sharma A, Inagaki S, Honma K, Sfintescu C, Baker PJ, Evans RT. 2005. *Tannerella forsythia*-induced alveolar bone loss in mice involves leucine-rich-repeat BspA protein. *J Dent Res*, 84:462-467.

Sharma A, Sojar HT, Glurich I, Honma K, Kuramitsu HK, Genco RJ. 1998. Cloning, expression, and sequencing of a cell surface antigen containing a leucine-rich repeat motif from *Bacteroides forsythus* ATCC 43037. *Infect Immun*, 66:5703-5710.

Shoji M, Sato K, Yukitake H, Kondo Y, Narita Y, Kadowaki T, Naito M, Nakayama K. 2011. Por secretion system-dependent secretion and glycosylation of *Porphyromonas gingivalis* hemin-binding protein 35. *PLoS One*, 6:e21372.

Sleytr UB, Messner P. 1983. Crystalline surface layers on bacteria. *Annu Rev Microbiol*, 37:311-339.

Sleytr UB, Thorne KJ. 1976. Chemical characterization of the regularly arranged surface layers of *Clostridium thermosaccharolyticum* and *Clostridium thermohydrosulfuricum*. *J Bacteriol*, 126:377-383.

Socransky SS. 1979. Criteria for the infectious agents in dental caries and periodontal disease. *J Clin Periodontol*, 6:16-21.

Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. 1998. Microbial complexes in subgingival plaque. *J Clin Periodontol*, 25:134-144.

Spiro RG. 2002. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology*, 12:43R-56R.

Stafford GP, Chaudhuri RR, Haraszthy V, Friedrich V, Schäffer C, Ruscitto A, Honma K, Sharma A. 2016. Draft genome sequences of three clinical isolates of *Tannerella forsythia* isolated from subgingival plaque from periodontitis patients in the United States. *Genome Announc*, 4(6):e01286-16.

Stathopulos J, Cambillau C, Cascales E, Roussel A, Leone P. 2015. Crystallization and preliminary X-ray analysis of the C-terminal fragment of PorM, a subunit of the *Porphyromonas gingivalis* type IX secretion system. *Acta Crystallogr F Struct Biol Commun*, 71:71-74.

Staudacher E. 2012. Methylation-an uncommon modification of glycans. *Biol Chem*, 393:675-685.

Szafranski SP, Wos-Oxley ML, Vilchez-Vargas R, Jauregui R, Plumeier I, Klawonn F, Tomasch J, Meisinger C, Kuhnisch J, Sztajer H, Pieper DH, Wagner-Dobler I. 2015. High-resolution taxonomic profiling of the subgingival microbiome for biomarker discovery and periodontitis diagnosis. *Appl Environ Microbiol*, 81:1047-1058.

Szymanski CM, Logan SM, Linton D, Wren BW. 2003. *Campylobacter* - a tale of two protein glycosylation systems. *Trends Microbiol*, 11:233-238.

Szymanski CM, Wren BW. 2005. Protein glycosylation in bacterial mucosal pathogens. *Nat Rev Microbiol*, 3:225-237.

Szymanski CM, Yao R, Ewing CP, Trust TJ, Guerry P. 1999. Evidence for a system of general protein glycosylation in *Campylobacter jejuni*. *Mol Microbiol*, 32:1022-1030.

Tanner AC, Haffer C, Bratthall GT, Visconti RA, Socransky SS. 1979. A study of the bacteria associated with advancing periodontitis in man. *J Clin Periodontol*, 6:278-307.

Tanner AC, Izard J. 2006. *Tannerella forsythia*, a periodontal pathogen entering the genomic era. *Periodontol 2000*, 42:88-113.

Tanner ACR, Listgarten MA, Ebersole JL, Strezempko MN. 1986a. *Bacteroides forsythus* sp. nov., a slow-growing, fusiform *Bacteroides* sp. from the human oral cavity. *International Journal of Systematic Bacteriology*, 36:213-221.

Tanner ACR, Listgarten MA, Ebersole JL, Strzempko MN. 1986b. *Bacteroides forsythus* sp. nov., a slow-growing, fusiform *Bacteroides* sp. from the human oral cavity. *Int J Syst Evol Microbiol*, 36:213-221.

Teng YT, Taylor GW, Scannapieco F, Kinane DF, Curtis M, Beck JD, Kogon S. 2002. Periodontal health and systemic disorders. *J Can Dent Assoc*, 68:188-192.

Thanassi DG, Bliska JB, Christie PJ. 2012. Surface organelles assembled by secretion systems of Gram-negative bacteria: diversity in structure and function. *FEMS Microbiol Rev*, 36:1046-1082.

Tomek MB, Janesch B, Maresch D, Windwarder M, Altmann F, Messner P, Schäffer C. 2017. A pseudaminic acid or a legionaminic acid derivative transferase is strain-specifically implicated in the general protein *O*-glycosylation system of the periodontal pathogen *Tannerella forsythia*. *Glycobiology, Epub ahead of print*.

Tomek MB, Neumann L, Nimeth I, Koerdt A, Andesner P, Messner P, Mach L, Potempa JS, Schäffer C. 2014. The S-layer proteins of *Tannerella forsythia* are secreted via a type IX secretion system that is decoupled from protein *O*-glycosylation. *Mol Oral Microbiol*, 29:307-320.

Tytgat HL, de Vos WM. in press. Sugar coating the envelope: Glycoconjugates for microbe-host crosstalk. *Trends Microbiol, Epub ahead of print*.

Tytgat HL, Lebeer S. 2014. The sweet tooth of bacteria: common themes in bacterial glycoconjugates. *Microbiol Mol Biol Rev*, 78:372-417.

Upreti RK, Kumar M, Shankar V. 2003. Bacterial glycoproteins: functions, biosynthesis and applications. *Proteomics*, 3:363-379.

Valguarnera E, Kinsella RL, Feldman MF. 2016. Sugar and spice make bacteria not nice: Protein glycosylation and its influence in pathogenesis. *J Mol Biol*, 428:3206-3220.

Van Gerven N, Klein RD, Hultgren SJ, Remaut H. 2015. Bacterial amyloid formation: Structural insights into curli biogensis. *Trends Microbiol*, 23:693-706.

van Sorge NM, Bleumink NM, van Vliet SJ, Saeland E, van der Pol WL, van Kooyk Y, van Putten JP. 2009. *N*-glycosylated proteins and distinct lipooligosaccharide glycoforms of *Campylobacter jejuni* target the human C-type lectin receptor MGL. *Cell Microbiol*, 11:1768-1781.

Varki A. 1992. Diversity in the sialic acids. *Glycobiology*, 2:25-40.

Varki A. 1993. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology*, 3:97-130.

Varki A. 2008. Sialic acids in human health and disease. *Trends Mol Med*, 14:351-360.

Varki A. 2016. Biological roles of glycans. *Glycobiology*, 27:3-49.

Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME, (editors). 2009. *Essentials of Glycobiology*, 2nd edition. Cold Spring Harbor (NY):Cold Spring Harbor Laboratory Press.

Varki A, Gagneux P. 2012. Multifarious roles of sialic acids in immunity. *Ann N Y Acad Sci*, 1253:16-36.

Vartoukian SR, Moazzez RV, Paster BJ, Dewhirst FE, Wade WG. 2016. First cultivation of health-associated *Tannerella* sp. HOT-286 (BU063). *J Dent Res*, 95:1308-1313.

Veith PD, Chen YY, Chen D, O'Brien-Simpson NM, Cecil JD, Holden JA, Lenzo JC, Reynolds EC. 2015. *Tannerella forsythia* outer membrane vesicles are enriched with substrates of the type IX secretion system and TonB-dependent receptors. *J Proteome Res*, 14:5355-5366.

Veith PD, Nor Muhammad NA, Dashper SG, Likic VA, Gorasia DG, Chen D, Byrne SJ, Catmull DV, Reynolds EC. 2013. Protein substrates of a novel secretion system are numerous in the *Bacteroidetes* phylum and have in common a cleavable C-terminal secretion signal, extensive post-translational modification, and cell-surface attachment. *J Proteome Res*, 12:4449-4461.

Veith PD, O'Brien-Simpson NM, Tan Y, Djatmiko DC, Dashper SG, Reynolds EC. 2009. Outer membrane proteome and antigens of *Tannerella forsythia*. *J Proteome Res*, 8:4279-4292.

Vik A, Aas FE, Anonsen JH, Bilsborough S, Schneider A, Egge-Jacobsen W, Koomey M. 2009. Broad spectrum *O*-linked protein glycosylation in the human pathogen *Neisseria gonorrhoeae*. *Proc Natl Acad Sci U S A*, 106:4447-4452.

Vincent MS, Canestrari MJ, Leone P, Stathopoulos J, Ize B, Zoued A, Cambillau C, Kellenberger C, Roussel A, Cascales E. in press. Characterization of the *Porphyromonas gingivalis* type IX secretion trans-envelope PorKLMNP core complex. *J Biol Chem, Epub ahead of print*.

Vincent MS, Durand E, Cascales E. 2016. The PorX response regulator of the *Porphyromonas gingivalis* PorXY two-component system does not directly regulate the type IX secretion genes but binds the PorL subunit. *Front Cell Infect Microbiol*, 6:96.

Wacker M, Linton D, Hitchen PG, Nita-Lazar M, Haslam SM, North SJ, Panico M, Morris HR, Dell A, Wren BW, Aebi M. 2002. *N*-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. *Science*, 298:1790-1793.

Wallden K, Rivera-Calzada A, Waksman G. 2010. Type IV secretion systems: versatility and diversity in function. *Cell Microbiol*, 12:1203-1212.

Wang Y, Wang Z, Cao J, Guan Z, Lu X. 2014. FLP-FRT-based method to obtain unmarked deletions of *CHU_3237* (*porU*) and large genomic fragments of *Cytophaga hutchinsonii*. *Appl Environ Microbiol*, 80:6037-6045.

Wickner W, Schekman R. 2005. Protein translocation across biological membranes. *Science*, 310:1452-1456.

Woolard MD, Frelinger JA. 2008. Outsmarting the host: bacteria modulating the immune response. *Immunol Res*, 41:188-202.

Zarschler K, Janesch B, Zayni S, Schäffer C, Messner P. 2009. Construction of a gene knockout system for application in *Paenibacillus alvei* CCM 2051T, exemplified by the S-layer glycan biosynthesis initiation enzyme WsfP. *Appl Environ Microbiol*, 75:3077-3085.

Zav'yalov V, Zavialov A, Zav'yalova G, Korpela T. 2010. Adhesive organelles of Gram-negative pathogens assembled with the classical chaperone/usher machinery: structure and function from a clinical standpoint. *FEMS Microbiol Rev*, 34:317-378.

Zunk M, Kiefel MJ. 2014. The occurrence and biological significance of the α-keto-sugars pseudaminic acid and legionaminic acid within pathogenic bacteria. *RSC Adv.*, 4:3413-3421.

8

Curriculum vitae

Personal information

First name	Markus Bernhard
Family name	TOMEK
Date of birth	1983, November 18th
Nationality	Austrian
Academic degree	Dipl.-Ing. Bakk. techn.
Affiliation	University of Natural Resources and Life Sciences Vienna, Austria
E-mail	markus.tomek@boku.ac.at

Academic credentials

2012, March - present	**PhD** at the Department of NanoBiotechnology, University of Natural Resources and Life Sciences, Vienna Member of the international PhD program "BioToP – Biomolecular Technology of Proteins"
2011, June	**Master degree** (Diplom-Ingenieur) in „**Biotechnology**" at the University of Natural Resources and Life Sciences, Vienna
2009, February	**Bachelor degree** (Bakk. techn.) in „**Food- and Biotechnology**" at the University of Natural Resources and Life Sciences, Vienna

Research stays abroad

2014	**Jagiellonian University**, Kraków, Poland – Department of Microbiology (Prof. Jan Potempa), 2.5 months
2014	**University of Louisville**, Kentucky, USA – Department of Oral Immunology and Infectious Diseases (Prof. Jan Potempa), 4 months
2010/2011	**Karolinska Institute**, Stockholm, Sweden – Department of Microbiology, Tumor and Cell Biology (Prof. Adnane Achour), Master Thesis, 7 months
2008	**KTH – Royal Institute of Technology**, Stockholm, Sweden, (Erasmus Programme), 6 months
2007	**Pibulsongram Rajabhat University**, Phitsanulok, Thailand – Faculty of Food and Agricultural Technology (Dr. Khongsak Srikaeo), Bachelor Thesis, 3 months

SCI publications

- **Tomek, M.B.**, Janesch, B., Maresch, D., Windwarder, M., Altmann, F., Messner, P. and Schäffer, C. (2017) A pseudaminic acid or a legionaminic acid derivative transferase is strain-specifically implicated in the general protein *O*-glycosylation system of the periodontal pathogen *Tannerella forsythia*. *Glycobiology*, in press, doi: 10.1093/glycob/cwx019
- **Tomek, M.B.**, Neumann, L., Nimeth, I., Koerdt, A., Andesner, P., Mach, L., Messner, P., Potempa, J.S., Schäffer, C. (2014) S-layer glycoproteins of *Tannerella forsythia* are secreted via a type IX secretion system that is decoupled from protein *O*-glycosylation. *Mol Oral Microbiol*, 29:307-320. doi: 10.1111/omi.12062
- Allerbring, EB., Duru, AD., Uchtenhagen, H., Madhurantakam, C., **Tomek, M.B.**, Grimm, S., Mazumdar, PA., Friemann, R., Uhlin, M., Sandalova, T., Nygren, PÅ., Achour, A. (2012) Unexpected T-cell recognition of an altered peptide ligand is driven by reversed thermodynamics. *Eur J Immunol*. 11:2990-3000. doi: 10.1002/eji.201242588

Education related industrial work experience

2008, Oct - 2009, Nov	**Medical University Vienna** Laboratory assistant at the Institute of Hygiene and Applied Immunology
2005, July	**Umweltbundesamt GmbH Wien** Analytical chemistry internship
2002, July	**Hagold Hefe GmbH** Microbiology and quality assurance internship

Awards and teaching experience

- Poster prize (FASEB Science Research Conference on Microbial Glycobiology, 2016, West Palm Beach, Florida, USA)
- Austrian Young Glycoscientist Best Talk Award (6th Annual ÖGMBT Meeting 2014, Vienna)

Supervision of Master students: Irene Nimeth, Kristina Fuchs

Supervision of Bachelor students: Vanessa Traxler, Johanna Bacher, Carmen Negrau, Lena Flörl, Anna-Sophia Kampl