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# Nonulosonic acids in clinical isolates of the periodontal pathogen *Tannerella forsythia*


Eingereicht von

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## Abbreviations

| | |
|---|---|
| 30S | small ribosomal subunit |
| 0348 | *Tannerella forsythia* clinical isolate 0348 |
| 0408 | *Tannerella forsythia* clinical isolate 0408 |
| 0471 | *Tannerella forsythia* clinical isolate 0471 |
| 1046 | *Tannerella forsythia* clinical isolate 1046 |
| 1055 | *Tannerella forsythia* clinical isolate 1055 |
| 1056 | *Tannerella forsythia* clinical isolate 1056 |
| UB 4 | *Tannerella forsythia* clinical isolate UB 4 |
| UB 20 | *Tannerella forsythia* clinical isolate UB 20 |
| °C | degree Celsius |
| 2D | 2-dimensional |
| Ala | alanine |
| Asn | asparagine |
| APS | ammoniumpersulfate |
| ATCC | American Type Culture Collection |
| BHI | brain heart infusion |
| C | carbon |
| *C. jejuni* | *Campylobacter jejuni* |
| $CH_3COOH$ | acetic acid |
| CMP | cytidine monophosphate |
| Da | Dalton |
| dNTP’s | nucleoside-triphosphates |
| DNA | desoxyribonucleic acid |
| *et al.* | *et alii* (and others) |
| EDTA | ethylenediaminetetraacetic acid |

| | |
|---|---|
| g | gram |
| *g* | gravity |
| H | hydrogen |
| *H. pylori* | *Helicobacter pylori* |
| HAc | acetic acid |
| Ile | isoleucine |
| IgG | immunoglobulin G |
| kDa | kilo-Dalton |
| kb | kilo-bases |
| Leu | leucine |
| L | liters |
| Leg | legionaminic acid |
| LegB | 4, 6-dehydratase |
| LegC | aminotransferase |
| LegF | CMP-Leg-synthetase |
| LPS | lipopolysaccharide |
| Met | methionine |
| M | molar |
| mA | milli-Ampères |
| mM | milli-molar |
| Me | methyl |
| mg | milli-gram |
| MilliQ-water | ultrapure water |
| µm | micrometer |
| N, $N_2$ | nitrogen |
| NY | New York |
| NanH | sialidase enzyme from *T. forsythia* |
| DNBT | Department of Nanobiotechnology |

| | |
|---|---|
| nm | nanometer |
| NMR | nuclear magnetic resonance spectroscopy |
| O, $O_2$ | oxygen |
| OD, $OD_{600}$ | optical density (at wavelength 600 nanometer) |
| PAA | polyacrylamide |
| PAS | periodic acid Schiffs' staining |
| PBS | phosphate-buffered saline |
| PBST | phosphate-buffered saline with Tween |
| PCR | polymerase chain reaction |
| PhD | Doctor of philosophy |
| PrtH | protease from *T. forsythia* |
| Pse | pseudaminic acid |
| PseB | 4, 6-dehydratase 5-epimerase |
| PseC | aminotransferase |
| PseF | CMP-Pse-synthetase |
| Pse5Am7Gc | 5-acetimidol-7-N-glycolylpseudaminic acid |
| PVDF | polyvinylidene fluoride |
| rpm | rounds per minute |
| rRNA | ribosomal ribonucleic acid |
| Ser | serine |
| S-layer | surface layer |
| SDS | sodiumdodecylsulfate |
| SDS-PAGE | sodium dodecylsulfate polyacrylamide gel electrophoresis |
| Thr | threonine |
| TRIS | tris(hydrooxymethyl)aminomethane |
| TEMED | tetramethylethylene diamine |
| TfsA | S-layer protein A from *T. forsythia* |

| | |
|---|---|
| TfsB | S-layer protein B from *T. forsythia* |
| Th17 | T-helper cells of the mammalian immune system |
| Trp | tryptophane |
| TIGR | The Institute for Genomic Research |
| *T. forsythia, T.f.* | *Tannerella forsythia* |
| Tm | melting temperature |
| Tyr | tyrosine |
| UDP | uridine diphosphate |
| V | Volt |
| Val | valine |
| (v/v) | volume per volume |
| (w/v) | weight per volume |
| wt | wild type |

# Contents

# Abstract

*Tannerella forsythia* is a Gram negative, anaerobic oral pathogen that colonizes subgingival plaque biofilms of the human oral cavity and can cause severe periodontal diseases [Tanner and Izard 2006]. The bacterium possesses a unique cell envelope profile; its outer cell surface is covered with a 2-dimensional crystalline surface (S-) layer, which is composed of two glycoproteins named TfsA and TfsB [Sekot, Posch *et al.* 2012]. These two S-layer proteins are decorated with a unique oligo-saccharide that carries a modified nonulosonic acid as a terminal sugar residue [Posch, Pabst *et al.* 2011]. This sugar is of special interest because of its documented involvement in mediation of bacterial virulence. Interestingly, also several other proteins of *T. forsythia* ATCC 43037 – most of which are predicted outer membrane or cell surface proteins - were shown to be modified with the S-layer glycan; this has led to the proposal of a general protein *O*-glycosylation system to be present in *T. forsythia* [Posch, Pabst *et al.* 2011; Coyne, Fletcher *et al.* 2013].

Considering that the glycosylated S-layer of *T. forsythia* ATCC 43037 has been shown to act as a virulence factor of the organism [Settem, Honma *et al.* 2013], in this master thesis, eight clinical isolates of *T. forsythia* should be analyzed for their S-layer glycosylation profile in order to determine the extent of variations of protein *O*-glycans in this bacterial species. This should further allow the assessment of whether or not certain glycosylation details are associated with the virulence potential of *T. forsythia*. All clinical *T. forsythia* isolates used in the course of this study were obtained from periodontitis patients following ethical guidelines and provided by cooperation partners affiliated to dental clinics.

The specific aims of this study were 1) to optimize the growth conditions for the clinical isolates, 2) to identify the S-layer proteins and analyze their *O*-glycan structures with a focus on the nonulosonic acid and 3) to determine the bacteria's genetic equipment for nonulosonic acid biosynthesis. The *T. forsythia* type strain ATCC 43037 was used as a reference for detecting the TfsA and TfsB glycoprotein bands on SDS-PAGE gels as well as for glycan analysis by mass spectrometry. Some of the isolates revealed only either TfsB or TfsA to be present, suggesting that these isolates have lost their second S-layer protein either while developing in the dental pockets, during passaging in liquid culture in the laboratory or because of an inherent natural lack of one S-layer protein. In the course of this master thesis, we found that *T. forsythia* isolates differ in the capability to synthesize pseudaminic acid and legionaminic acid respectively, as terminal *O*-glycan residue. To determine whether the biosynthetic pathways for building up pseudaminic acid or legionaminic acid or both are present, conserved primers coding for crucial enzymes of the respective genomic loci were constructed and used to screen the genome of the clinical isolates for nonulosonic acid biosynthesis enzymes. PCR amplification data from the re-sequenced *T. forsythia* type strain ATCC 43037 [Friedrich, Pabinger *et al.* 2015], from *T. forsythia* strain FDC 92A2 (deposited at Genbank) and from six clinical isolates demonstrated that six out of the eight investigated strains have the genetic information for synthesizing Leg, while only two have genes for Pse. These data were confirmed by MS analyses of the β-eliminated S-layer glycans of the respective organisms.

# Zusammenfassung

*Tannerella forsythia* ist ein Gram-negatives, anaerobes orales Pathogen, das in Form von subgingivalen Biofilmen die Mundhöhle des Menschen besiedelt und schwere Parodontitis verursachen kann [Tanner and Izard 2006]. Das Bakterium besitzt ein einzigartiges Zellwandprofil; seine Zelloberfläche ist von einer 2-dimensionalen kristallinen Schicht (S-Schicht), bestehend aus zwei Glykoproteinen mit den Namen TfsA und TfsB bedeckt [Sekot, Posch *et al.* 2012]. Diese beiden S-Schichtproteine sind mit einem einzigartigen Oligosaccharid verknüpft, welches eine modifizierte Nonulosonsäure als terminalen Zuckerrest trägt [Posch, Pabst *et al.* 2011]. Dieser Zucker ist aufgrund seiner dokumentierten Vermittlung bakterieller Virulenz von großem Interesse. Interessanterweise hat sich gezeigt, dass auch andere Proteine von *T. forsythia* ATCC 43037, die meisten davon sind in der äußeren Membran oder an der Zelloberfläche lokalisiert, mit diesem S-Schichtglykan modifiziert sind; dies legt das Vorhandensein eines generellen Protein-*O*-Glykosylierungssystems in *T. forsythia* nahe [Posch, Pabst *et al.* 2011; Coyne, Fletcher *et al.* 2013].

In Anbetracht dessen, dass sich die glykosylierte S-Schicht von *T. forsythia* ATCC 43037 als Virulenzfaktor des Organismus erwiesen hat [Settem, Honma *et al.* 2013], sollen in dieser Masterarbeit acht klinische Isolate von *T. forsythia* analysiert werden, um ihr S-Schicht Glykosylierungsprofil zu untersuchen, um weiters das Ausmaß der Variationen der Protein *O*-Glykane in dieser Bakterienspezies zu untersuchen. Dies soll eine Abschätzung erlauben, ob gewisse Glykosylierungsdetails mit dem Virulenzpotenzial von *T. forsythia* assoziiert sind. Sämtliche klinische Isolate, welche im Verlauf dieser Studie verwendet wurden, sind aus Parodontitis-Patienten unter Einhaltung sämtlicher ethnischer Richtlinien isoliert und von Kooperationspartnern aus Zahnkliniken bereitgestellt worden.

Die spezifischen Ziele dieser Masterarbeit umfassten 1) das Optimieren der Kultivierungsbedingungen für die klinischen Isolate, 2) die Identifizierung der S-Schichtproteine und Analyse ihrer *O*-Glykanstrukturen mit dem Fokus auf den Nonulosonsäure-Rest und 3) die Ermittlung der genetischen Ausstattung der Bakterien für die Biosynthese von Nonulosonsäuren. Der *T. forsythia* Wildtyp-Stamm ATCC 43037 wurde als Referenz für die Detektion der TfsA und TfsB Glykoproteinbanden auf SDS-PAGE Gelen und für die Glykananalyse mittels Massenspektrometrie, verwendet. Manche der Isolate deuteten nur entweder auf Präsenz von TfsA oder TfsB hin, dadurch entstand die Annahme, dass diese Stämme das jeweils zweite S-Schichtprotein entweder während der Entwicklung in den Zahntaschen, oder während des Passagierens in Flüssigkultur im Labor, verloren haben. Das Fehlen des zweiten S-Schichtproteins von Natur aus, kann ebenso nicht ausgeschlossen werden. Im Verlauf dieser Arbeit wurde gezeigt, dass die *T. forsythia* Isolate sich in ihrer Fähigkeit Pseudaminsäure und respektive Legionaminsäure als terminalen *O*-Glykanrest zu synthetisieren, unterscheiden. Um festzustellen, ob Biosynthesewege für Pseudaminsäure beziehungsweise Legionaminsäure in den Genomen der Isolate vorherrschen, sind spezifische Primer, kodierend für wichtige Enzyme im jeweiligen Pseudaminsäure oder Legionaminsäure Biosyntheselokus verwendet worden. Die Amplifizierung der jeweiligen Gene durch PCR des re-sequenzierten *T. forsythia* Wildtyp-Stammes [Friedrich, Pabinger *et al.* 2015], von FDC 92A2 (hinterlegt bei Genbank) und von sechs klinischen Isolaten zeigte, dass sechs der insgesamt acht untersuchten Stämme die genetische Information für die Biosynthese von Legionaminsäure tragen, während zwei der Stämme Information für Pseudaminsäure innehaben. Diese Entdeckungen wurden mithilfe von Massenspektrometrie der β-eliminierten Glykane der jeweiligen Organismen bestätigt.

# 1 Introduction

This master thesis deals with the investigation of eight clinical isolates of the periodontal pathogen *Tannerella forsythia* with regards to the variation in nonulosonic acid biosynthesis as integral part of the bacterium's general protein *O*-glycosylation system. The isolates were taken from patients suffering from advanced forms of periodontitis accompanied by the typical clinical measurements and were kindly provided by Dr. Ashu Sharma from the Department of Oral Biology, University of Buffalo, NY, USA, and from Dr. Georgios Belibasakis from the Center of Dental Medicine at the University of Zurich, Switzerland, respectively. Both collaborators are greatly acknowledged for their gifts.

This master thesis deals with the 1) optimization of the growth conditions for the clinical isolates, 2) identification of the S-layer protein(s) and analysis of their *O*-glycan signature with a focus on the terminal nonulosonic acid and 3) determination of the bacteria's genetic equipment for nonulosonic acid biosynthesis.

The following chapter will provide an overview of the Gram-negative bacterium *T. forsythia*, its virulence factors and periodontal diseases in general. It will further explain the bacterial complexes naturally occurring in the oral cavity and how health can turn into disease governed by a switch in polymicrobial community interactions of the oral microbiota. Furthermore, brief information is given about S-layer proteins and their glycosylation, particularly with regard to the nonulosonate (*i.e.*, sialic acid-like sugar) present in *T. forsythia* (S-layer) *O*-glycans.

## 1.1 Periodontal diseases

Periodontal diseases are multifactorial infections elicited by a complex of bacterial species that interact with host tissues and cells, causing the release of a broad array of inflammatory cytokines, chemokines

and mediators, some of which lead to destruction of the periodontal structures, including the tooth supporting tissues, alveolar bone, and periodontal ligament [Kortsik, Elmer *et al.* 2003]. The trigger for the initiation of disease is the presence of complex microbial biofilms that colonize the sulcular regions between the tooth surface and the gingival margin through specific adherence interactions and accumulation due to architectural changes in the sulcus (*i.e.*, attachment loss and pocket formation) [Smalley, Birss *et al.* 2002].

It has been estimated that around 700 bacterial taxa, phylotypes and species, showing a distinct structural organization in the biofilms, can colonize the oral cavity of humans, although it remains unclear how this multitude of bacteria compete, coexist and synergize to initiate the chronic disease process [Partridge, Hillyard *et al.* 1985; Hajishengallis and Lamont 2012]. So far, no single species has been implicated as the primary pathogen [Socransky, Haffajee *et al.* 1998]. Therefore, it is strongly believed that periodontitis has a polymicrobial disease etiology. Investigations into the link between oral microbial communities with the change from health to disease led to a classification of the microbiota into bacterial consortia (‘complexes‘) that occur together and are associated with the sequence of colonization on the tooth surface as well as with disease severity [Wyss 1989; Haffajee and Socransky 1994; Socransky and Haffajee 2005]. The “red complex” bacteria, which are late colonizers in biofilm development, comprise species that are considered periodontal pathogens, namely *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*. It was suggested that the “red complex” presents a portion of the climax community in the biofilms at sites expressing progressing periodontitis [Holt and Ebersole 2005]. The characteristics of microbiological progression from periodontal health to gingivitis (*e.g.*, chronic inflammation of the gingival tissue without tissue destruction), and eventually to periodontal disease are vast and complicated [Moore, Holdeman *et al.* 1982] and for that reason are undergoing many investigations [Hajishengallis and Lamont 2012; Belibasakis and Mylonakis 2015] today.

Recent studies have indicated that the “red complex” bacteria may contribute to serious systemic diseases, such as cardiovascular diseases, rheumatoid arthritis and diabetes [Manjunath,

Praveen *et al.* 2011], which makes it even more necessary to unravel the molecular basis of the virulence mechanisms of the periodontal pathogens.

## 1.2 *Tannerella forsythia*

*T. forsythia* is a Gram-negative, anaerobic, filament-shaped, non-pigmented bacterium inhabiting the human oral cavity [Tanner and Izard 2006]. It is a member of the *Cytophaga-Bacteroides* family of bacteria and lives in a biofilm lifestyle in the subgingival plaque [Posch, Pabst *et al.* 2011]. *T. forsythia* lacks surface fimbriae, a capsule and is non-motile but exclusively possesses a unique surface layer (S-layer) glycoprotein lattice as outermost cell surface decoration. S-layers are water-insoluble proteins intrinsically capable of self-assembling into 2D crystalline lattices on bacterial cell surfaces, and are believed to provide selective advantages to bacteria such as resistance to predation or immune clearance [Sára and Sleytr 2000]. Interestingly, *T. forsythia* is the only Gram-negative bacterium that is known to possess a glycosylated S-layer [Lee, Sabet *et al.* 2006]. *T. forsythia* was first isolated at 'The Forsyth Institute' from subjects with progressing advanced periodontitis in the mid-1970s and was described as fusiform *Bacteroides*. It was initially a taxonomic enigma because it did not resemble described species of oral enteric, Gram-negative anaerobic rods, particularly with regard to its cell morphology and slow and fastidious growth requirements [Tanner, Haffer *et al.* 1979].

The phylogeny of oral *Bacteroides* species in the *Cytophaga–Flavobacterium–Bacteroides* family was reorganized after *Bacteroides forsythus* had been described and eventually clarified in the phylogenetic studies comparing 16S rRNA sequence data [Paster, Dewhirst *et al.* 1994]. Subsequently, *B. forsythus* was affiliated to the genus *Tannerella* [Sakamoto, Suzuki *et al.* 2002]. Here it was first formally classified to *Tannerella forsythensis* but then reclassified to *Tannerella forsythia* [Maiden, Cohee *et al.* 2003]. Because of *T. forsythia's* unique growth requirements (hemin, menadione, L-cysteine, and N-acetylmuramic acid) [Wyss 1989] and the fact that it is somewhat difficult to grow, its precise role in the severe bone and tissue destruction at sites from which it can be isolated, remains to be determined.

*T. forsythia* grows on media supplemented with vitamin K (menadione) and N-acetylmuramic acid [Tanner, Maiden *et al.* 1998], which is a major component of bacterial peptidoglycan. *T. forsythia* is indeed an anaerobe, but it also has been suggested that it is, similar to *Bacteroides fragilis*, able to grow in the presence of low levels of oxygen [Baughn and Malamy 2004].

## 1.3 Bacterial complexes and the "red complex" consortium'

Certain periodontal bacteria are often found together in subgingival plaque samples. Cluster analysis and community ordination techniques have been used [Socransky, Haffajee *et al.* 1998] to further define these bacterial relationships and to determine whether there were correlations between certain clusters and clinical parameters of disease. Results of these studies demonstrated that the bacteria could be sorted into five major groups that were given color designations. The designated "red complex" (*Treponema denticola*, *Porphyromonas gingivalis*, and *T. forsythia*) and the "orange complex" (*Prevotella intermedia*, *Prevotella nigrescens*, *Peptostreptococcus micros*, *Fusobacterium nucleatum* subspecies, *Eubacterium nodatum*, *Streptococcus constellatus*, and three *Campylobacter* species) were generally found together, and evidence showed that colonization by the "red complex" was preceded by colonization by "orange complex" species (**Figure 1**). Both complexes could be associated with clinical parameters of disease supporting the polymicrobial nature of periodontitis [Socransky, Haffajee *et al.* 1998]. Based on the strong virulence of the" red complex" bacteria, at least one of them will be found in periodontal lesions, what declares further investigation of these organisms as a necessary goal.

Multiple studies evaluating the presence of *T. forsythia* in subgingival plaque have demonstrated a significantly higher frequency in diseased subjects compared to healthy controls [Haffajee, Cugini *et al.* 1998; Ximenez-Fyvie, Haffajee *et al.* 2000].

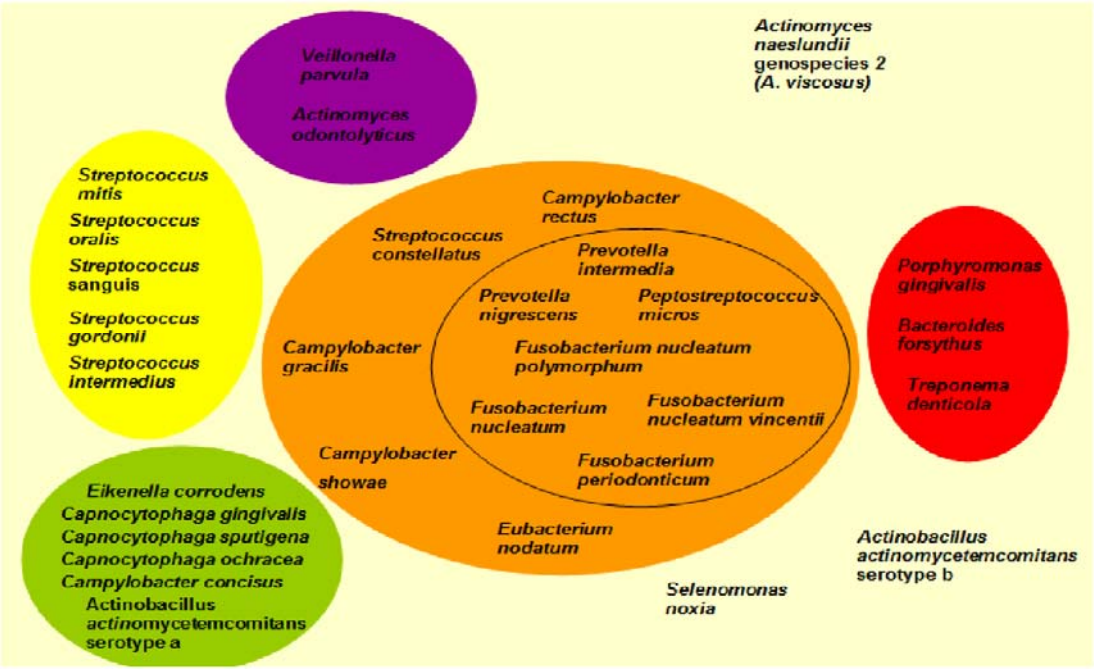


**Figure 1.** "Red complex" bacteria inhabiting the human oral cavity besides four other bacterial complexes [Socransky, Haffajee *et al.* 1998]

## 1.4 Virulence of *Tannerella forsythia*

The term 'virulence' is defined as the relative ability of an organism to cause disease or to interfere with a metabolic or physiological function of its host. The word derives from the Latin word "*virulentus*", full of poison [Wain 1952]. Poulin and Combes have defined the concept of virulence in terms of the type of molecules being produced by a microbe [Poulin and Combes 1999]. As such, virulence is defined by virulence factors, which are components of a microbe that, when present, harm the host, but when absent (*e.g.*, upon mutation) impair this ability. An underlying loss-of-function mutation, however, does not affect the ability of the microbe to grow and reproduce [Casadevall and Pirofski 2000]. Thus, virulence factors can have a multitude of functions, such as the ability to induce microbe-host interactions (attachment), to invade the host, to grow in the confines of a host cell and to evade or interfere with host defenses.

Several virulence factors have been identified in *T. forsythia*. These include a trypsin-like protease [Grenier and Mayrand 1995] and PrtH proteases [Sabet, Lee *et al.* 2003], sialidases [Braham and Moncla 1992; Ishikura, Arakawa *et al.* 2003] such as NanH [Thompson, Homer *et al.* 2009], a

leucine-rich repeat cell-surface-associated and secreted protein BspA [Sharma, Sojar *et al.* 1998], an apoptosis-inducing activity [Nakajima, Tomi *et al.* 2006], α-D-glucosidase and N-acetyl-β-glucosaminidase [Hughes, Malki *et al.* 2003], a hemagglutinin [Murakami, Higuchi *et al.* 2002], components of the *T. forsythia* S-layer [Sabet, Lee *et al.* 2003; Sakakibara, Nagano *et al.* 2007] and methylglyoxal production [Maiden, Pham *et al.* 2004]. Other virulence factors that can potentially contribute to the disease process are cell surface proteolytic enzymes, glycosidases, envelope lipo-proteins, the bacterial lipopolysaccharide (LPS) [Posch, Pabst *et al.* 2013], and a recently identified fucosidase [Megson, Koerdt *et al.* 2015]. Grossi and coworkers have reported that members of the *Bacteroides* species isolated from pathologic oral tissues produce significantly more sialidase activity than *Bacteroides* isolated from non-pathologic tissues [Grossi, Zambon *et al.* 1994].

## 1.5 S-Layer proteins

Surface (S-) layer proteins are the most frequently observed cell surface decoration of bacterial cells, with representatives found in all major phylogenetic branches of bacteria [Sára and Sleytr 2000]. S-layers are 2-D crystalline arrays composed of regularly aligned protein or glycoprotein subunits. The highly structured S-layer is assumed to provide to the bacteria a protective coat against external host or natural environmental forces, acting as a molecular sieve and ion trap, as well as providing an adhesion and surface recognition mechanism [Sleytr, Messner *et al.* 1993].

In most *Archaea*, S-layers represent the only wall component outside the cytoplasmic membrane as can be seen in **Figure 2** (a, b). Only a few *Archaea* possess a rigid wall layer (c) (*e.g,*. pseudomurein in methanogenic *Archaea*) as intermediate layer between the cytoplasmic membrane and the S-layer [Claus and König 2010; Albers and Meyer 2011]. In Gram-positive *Bacteria*, S-layers are attached to the rigid peptidoglycan-containing layer via a secondary cell wall polymer (d), while in the more complex Gram-negative bacterial cell envelope, the S-layer is postulated to adhere to the LPS of the outer membrane (e).

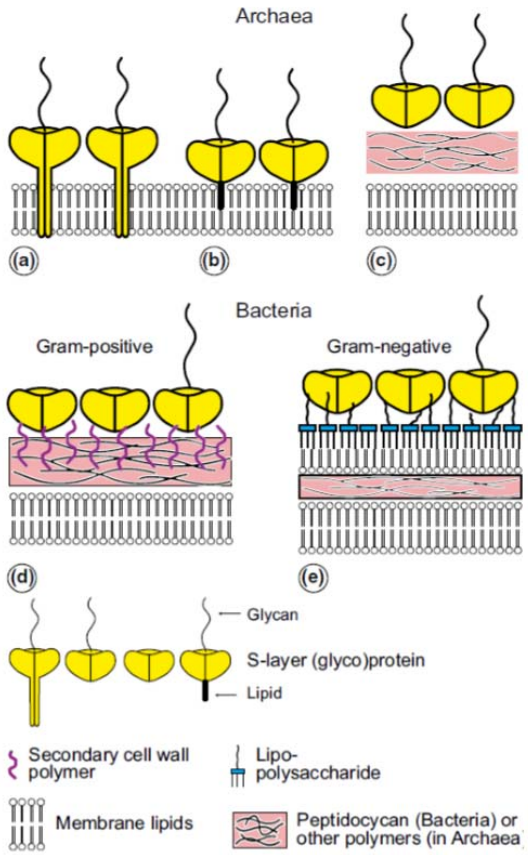


**Figure 2.** Schematic illustration of bacterial S-layers on different bacterial cell envelope profiles; taken from [Sleytr, Schuster *et al.* 2014]

*T. forsythia* was recently shown to be covered by a 22-nm thick, square glycoprotein lattice (**Figure 3**) with a spacing between the morphological units of ~ 10 nm [Sekot, Posch *et al.* 2011]. The glycoproteins building up the *T. forsythia* S-layer are the TfsA and TfsB proteins encoded by the *tfsA* and *tfsB* gene, respectively, which migrate as high molecular-mass bands (in the range of 230-270 kDa) on SDS-PAGE gels [Lee, Sabet *et al.* 2006].

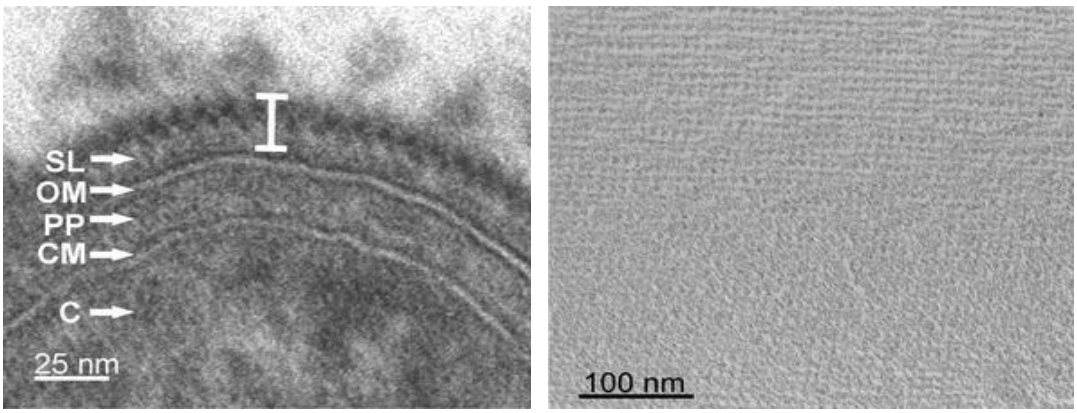


**Figure 3.** Cross sectioned *T. forsythia* cell (left) showing the S-layer as outermost cell envelope layer and freeze-etched preparation of a *T. forsythia* (right) cell showing the square S-layer lattice. Taken from Sekot *et al.*, 2012

Recent *in vitro* studies suggested that the *T. forsythia* S-layer could play a role in dampening the immune response during infection [Settem, Honma *et al.* 2013]. A *T. forsythia* S-layer deficient mutant was shown to cause increased secretion of inflammatory cytokines in a monocytic cell line and human gingival fibroblasts *in vitro* compared to the wild type strain [Sekot, Posch *et al.* 2011]. However, IgG antibody to the S-layer of *T. forsythia* was low in healthy individuals and significantly elevated in adult and early-onset periodontitis patients. These results suggest that the S-layer proteins are antigenic in humans and that an increased interaction between host adaptive immune mechanisms and this pathogen occurs during periodontal disease progression [Yoneda, Hirofuji *et al.* 2003].

## 1.6 S-layer glycosylation

S-layer proteins were the first glycoproteins detected in prokaryotes and still are among the best studied examples of glycosylated prokaryotic proteins [Sleytr and Thorne 1976]. In general, protein glycosylation is a very heterogeneous group of posttranslational modifications that has been found in essentially all living organisms, ranging from the domain of *Archaea* to that of *Eukarya* [Spiro 2002]. Glycosylation is the most abundant polypeptide modification in nature. Glycans can be covalently attached to the amide nitrogen of Asn residues (*N*-glycosylation), to the hydroxyl oxygen of, typically, Ser, Thr residues or, predominantly in bacteria, Tyr (*O*-glycosylation), and, in rare cases, to the indole C2 carbon of Trp through a C-C linkage (known as *C*-mannosylation [Furmanek and Hofsteenge 2000]).

The S-layer proteins of *T. forsythia* carry a unique complex *O*-glycosidically linked oligo-saccharide as presented in **Figure 4**, which is also found on other proteins within the organism, with slight variations detected in their relative ratios in O-methylation of the mannosaminuronamide residue [Posch, Sekot *et al.* 2012]. This demonstrates the presence of a general protein *O*-glycosylation system in *T. forsythia*.

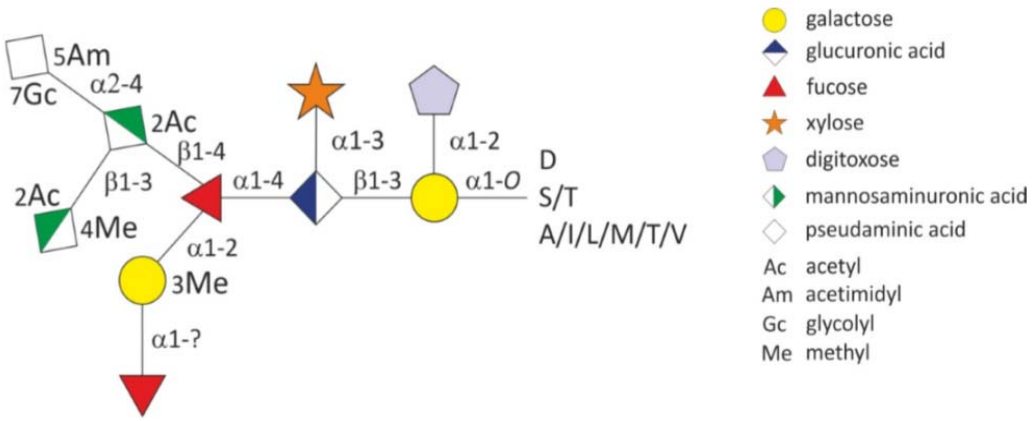


**Figure 4.** Schematic representation of the abundant protein *O*-glycan structure in *T. forsythia* (taken from [Posch, Sekot *et al.* 2012])

The 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* is *O*-glycosidically linked via the reducing-end α-galactose, to distinct serine and threonine residues of the S-layer proteins TfsA and TfsB within the specific amino acid motif D (S/T) (A/I/L/M/T/V). The branching fucose unit labelled with a question mark occurs with a natural variability, meaning that it is not always found on the *O*- glycans. Additionally, the linkage configuration between this fucose and the galactose is currently unknown.

**Figure 5** shows the outermost cell envelope architecture of *T. forsythia* starting, from inside out, with the outer membrane, into which many LPS molecules are embedded. LPS is an intrinsic feature of Gram-negative bacteria where it is located in the outer leaflet of the outer membrane [Raetz and Whitfield 2002]. It is evident that LPS is an immunostimulatory agent counteracting the mammalian immune system. It has been discovered that *T. forsythia* wild type ATCC 43037 cells carry a rough-type LPS [Posch, Pabst *et al.* 2013]. *In vivo*, the virulence of the LPS cannot be decoupled from the virulence of the S-layer proteins, because it is proposed that the rough-type LPS serves as an anchor for attaching the *T. forsythia* S-layer proteins to the outer membrane [Posch, Sekot *et al.*, 2012]. The S-layer as outermost cell surface decoration is built up from the two intercalating S-layer proteins TfsA and TfsB, with each of them modified at multiple sites with the S-layer *O*-glycan.

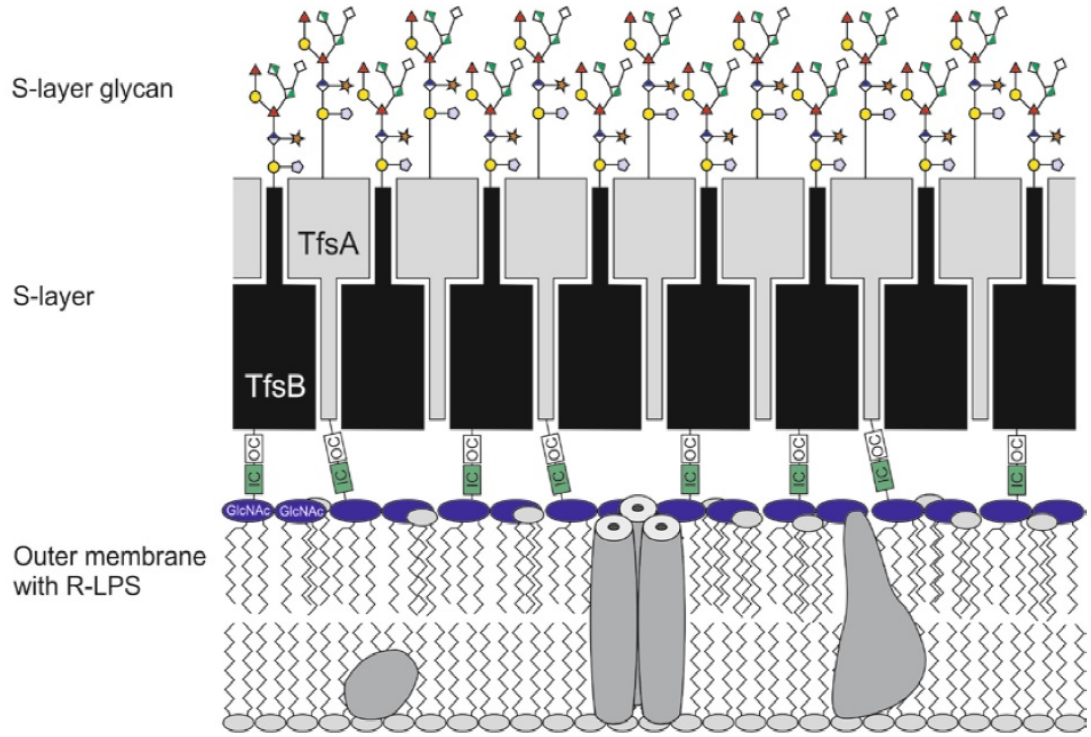


**Figure 5.** Schematic representation of the cell envelope of *T. forsythia;* taken from [Posch, Sekot *et al.* 2012]

*O*-Glycan biosynthesis in *T. forsythia* ATCC 43037 was found to be encoded in a genomic glycosylation locus [Posch, Pabst *et al.* 2011]. This locus was recently revisited in our laboratory and revealed to be ~30-kb in size and to encode, among others, a flippase, which might be crucial for catalyzing the transport of the glycan moiety to the periplasmic space. According to recent evidence in our laboratory, six enzymes that form the biosynthetic pathway from UDP-GlcNAc to CMP-pseudaminic acid are encoded in close vicinity of that particular locus (Valentin Friedrich *et al.*, manuscript in preparation). These enzymes include the dehydratase/epimerase PseB (Tanf_01185), the aminotransferase PseC (Tanf_01190), the *N*-acetyltransferase PseH (Tanf_01215), the UDP-sugar hydrolase PseG (Tanf_01235), the pseudaminic acid synthase PseI (Tanf_01240) and the CMP-pseudaminic acid synthetase PseF (Tanf_01200) (**Figure 6**, Valentin Friedrich, unpublished data). In another strain of *T. forsythia*, ATCC BAA 2717, the pseudaminic acid biosynthesis locus is replaced

by genes of the legionaminic acid pathway. These include the dehydratase LegB (BFO1074), the aminotransferase LegC (BFO1073), the nucleotidyltransferase ptmE (BFO1064), the hydrolyzing epimerase LegG (BFO1065), the legionaminic acid synthase LegI (BFO1066) and the CMP-legionaminic acid synthetase LegF (BFO1063) (**Figure 7**, Valentin Friedrich, unpublished data).

Both clusters also incorporate a number of predicted glycosyltransferases, the function of which is currently investigated in our laboratory.

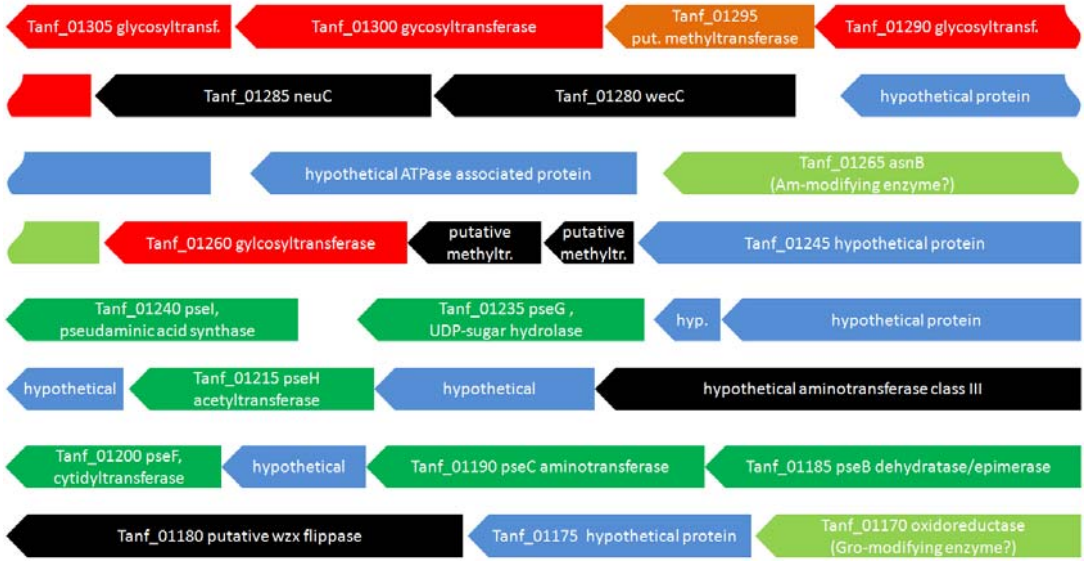


**Figure 6.** Genomic *O*-glycan biosynthesis locus with pseudaminic acid biosynthesis enzymes (indicated in green) of *T. forsythia* ATCC 43037

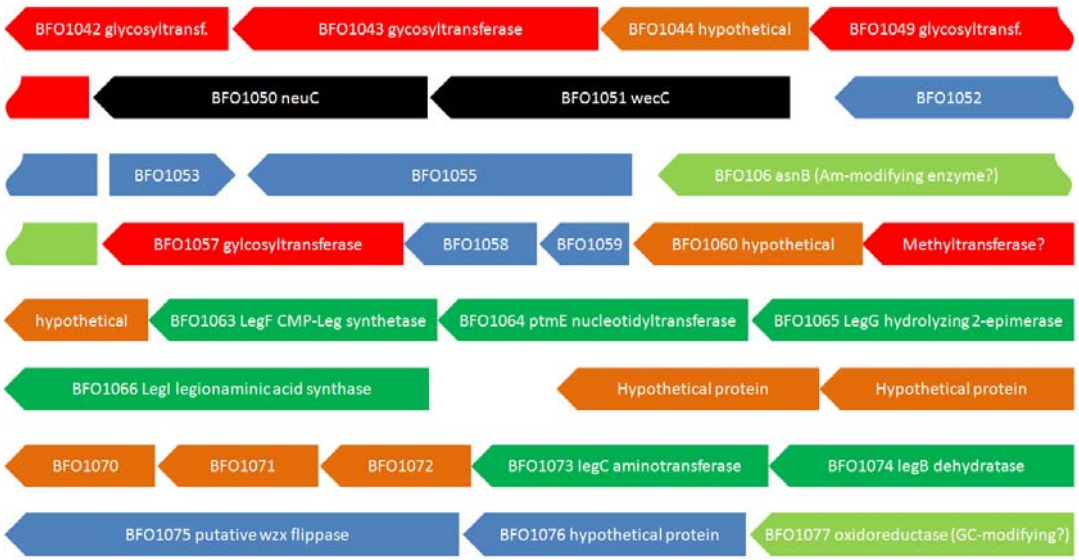


**Figure 7.** Genomic *O*-glycan biosynthesis locus with legionaminic acid biosynthesis enzymes (indicated in green) of *T. forsythia* ATCC BAA-2717 (FDA 92A2)

### 1.6.1 Immunogenicity of the *T. forsythia* S-layer glycan

An S-layer-deficient *T. forsythia* mutant induced significantly higher levels of proinflammatory mediators compared with wild type *T. forsythia*, especially at the early phase of response. These data provided by Sekot *et al.* suggest that the S-layer of *T. forsythia* is an important virulence factor that attenuates the host immune response to this pathogen by evading the bacterium's recognition by the innate immune system [Sekot, Posch *et al.* 2011]. By comparing the immunological effects evoked by the *T. forsythia* wild type and a Δ*wecC* mutant [Honma, Inagaki *et al*, 2007], in which a branching trisaccharide motif consisting of the two subterminal Man*p*NAcA and 4-Me*O*-β-Man*p*NAcCONH$_2$ residues and the terminal nonulosonate Pse5Am7Gc are missing [Posch, Pabst *et al.* 2011], it became evident that the mutant is less virulent in a periodontitis mouse model [Settem, Honma *et al* 2013]. There are indications that the glycan decoration on *T. forsythia* cells has a role in suppressing Th17-mediated neutrophil infiltration in the gingival tissue, allowing pathogen persistence in the host and induction of disease [Settem, Honma *et al.* 2013].

## 1.7 Sialic acids

Sialic acids, namely *N*- and *O*-acyl derivatives of 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-ulosonic acid (neuraminic acid, **Figure 8**), are α-keto acids with a 9-carbon backbone [Schoenhofen, McNally *et al.* 2006], that are widely expressed on the surfaces of all cells in all animals of vertebrates and also in certain pathogenic or symbiotic bacteria that associate with them. Given their remarkable diversity in structure, glycosidic linkage, and underlying glycan chains, as well as their exposed location, it is not surprising that sialic acids have numerous roles in many aspects of immunity [Varki, Cummings *et al.* 2009].

The most widespread derivatives of neuraminic acid, which itself does not occur naturally, is the N-acetylated form, known as N-acetylneuraminic acid or sialic acid (**Figure 9**).

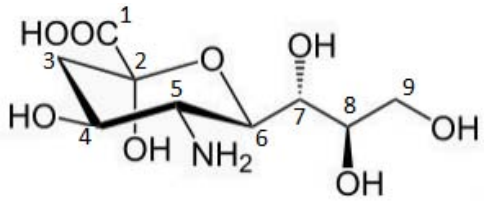


**Figure 8.** Neuraminic acid

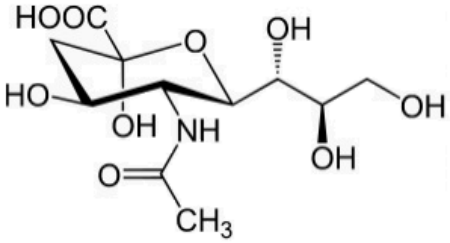


**Figure 9.** *N*-acetylneuraminic acid

Sialic acid and its derivatives (**Figure 10**), falling all into the category of nonulosonic acids, occupy the terminal position within glycan molecules on the surfaces of many vertebrate and non-vertebrate cells, where they function in diverse pivotal cellular processes such as intercellular adhesion and cell signaling.

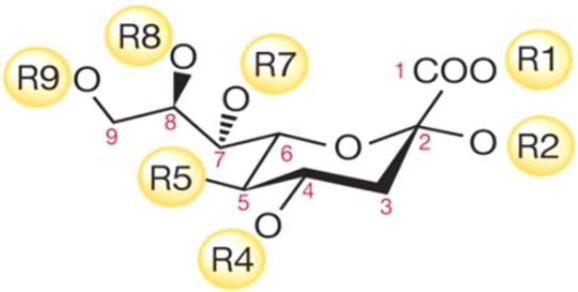


**Figure 10.** Diversity of sialic acids. The 9-carbon backbone common to all known sialic acids is shown in the α-configuration. Variations can occur at the yellow carbon positions indicated, where a hydrogen rest is replaced. Taken from [Varki, Cummings *et al.* 2009]

Also pathogenic bacteria have evolved to use this sugar residue beneficially as integral component of their fimbriae, flagella, pili, LPS or other cell surface glycoconjugates in at least two different ways; they can coat themselves in sialic acid, providing resistance to components of the host's innate immune response (molecular mimicry), or they can use it as a nutrient for carbon, nitrogen or cell wall synthesis. Sialic acid itself is either synthesized *de novo* by these bacteria or scavenged directly from the host [Severi, Hood *et al.* 2007]. With regard to bacteria, the nonulosonic

acids of *Helicobacter pylori* and *Campylobacter jejuni* have been intensively investigated [Schoenhofen, McNally *et al.* 2006; Schoenhofen, Vinogradov *et al.* 2009].

### 1.7.1 Nonulosonic acids

Also in the *T. forsythia* protein *O*-glycans, nonulosonic acids (sialic acid-like sugars) are present as terminal residues [Posch, Sekot *et al.*]. In the course of the interpretation of ESI-mass spectra of β-eliminated S-layer glycans of *T. forsythia* ATCC 43037 wild-type, an uncommon sugar residue of 361.15 kDa in mass was identified. NMR spectrometry revealed that this residue was in better accordance with a pseudaminic acid than with its stereoisomer legionaminic acid.

During this master thesis it became evident that besides pseudaminic acid (**Figure 11**) there are isolates of *T. forsythia* which possess the respective genes for crucial enzymes for building up legionaminic acid (**Figure 12**) instead (see below).

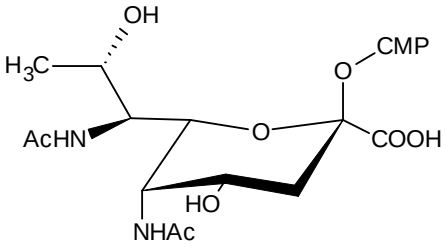


**Figure 11.** CMP-pseudaminic acid

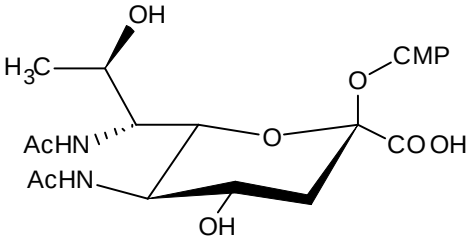


**Figure 12.** CMP-legionaminic acid

### 1.7.2 Virulence potential of pseudaminic and legionaminic acid

Pseudaminic acid is found on multiple sites in flagellar proteins, and disruption of the genes responsible for the biosynthesis of this sugar was shown to have adverse effects on bacterial motility, adherence and invasion [Guerry, Ewing *et al* 2006]. Further investigation into the glycome of these pathogenic bacteria regarding glycosylation with pseudaminic acid is still necessary [Morrison and Imperiali 2014]. Legionaminic acid was first identified in a repeating unit homopolymer in the *O*-

polysaccharide of LPS in *Legionella pneumophila*, which is the causative agent of Legionnaires' disease [Knirel, Rietschel *et al.* 1994]. Work on the *O*-antigen of *Vibrio fischeri* has illustrated the importance of legionaminic acid in colonization of the natural host by this bacterium. The disruption of this *O*-antigen through a gene knockout of the *waaL* ligase responsible for the assembly of the *O*-antigen onto the LPS, resulted in a motility defect of the bacterium. Further studies indicated that this *O*-antigen null strain has a significantly weakened ability to colonize its natural host organism and cannot compete with the wild type in co-colonization assays [Post, Yu *et al.* 2012].

In fact, more than 20 % of the 1000 microbial genomes examined to date appear to contain the biosynthetic genes for nonulosonic acid biosynthesis pathways, making this class of sugar far more widespread than originally believed [Lewis, Desa *et al.* 2009]. These findings corroborate the need to further investigate the nonulosonic acids of the *T. forsythia O*-glycan, because it has been hypothesized that bacterial pathogens utilize these sugars as a molecular mimic of sialic acid, which is, as described above, prominently presented on mammalian cells and an important factor in immune system regulation and adhesion [Tsvetkov, Shashkov *et al.* 2001]. Comparable functions might assist *T. forsythia* to colonize the dental pockets. Furthermore, the ever-increasing resistance toward present-day antibiotics has resulted in the search for novel agents to address this challenge. Highly modified carbohydrates like nonulosonic acids are related to pathogenicity of bacteria and as they do not appear in eukaryotes could be considered as targets for novel drugs. It should be mentioned that when nonulosonic acid biosynthesis of pathogenic bacteria is hindered, their pathogenicity is decreasing [Morrison and Imperiali 2014].

### 1.7.3 Biosynthesis of pseudaminic and legionaminic acid

Elucidation of the biosynthetic pathways of the *de novo* biosynthesis of pseudaminic and legionaminic acid in *Helicobacter pylori* and *Campylobacter jejuni* was done by Schoenhofen *et al*. [Schoenhofen, McNally *et al.* 2006; Schoenhofen, Vinogradov *et al.* 2009]. Several enzymes, including dehydratases, epimerases, aminotransferases, N-acetyltransferases, sugar-hydrolases, synthases and synthetases catalyze a reaction cascade leading to the final, CMP-activated nonulosonate.

Similar investigation of the biosynthetic pathways for elucidation of the *de novo* synthesis of nonulosonate sugars in *T. forsythia* is now under way in our group in collaboration with Ian Schoenhofen. Based on the evidence of the presence of pseudaminic acid on the protein *O*-glycans of the *T. forsythia* wild type ATCC 43037 strain [Posch, Sekot *et al.* 2012] and of legionaminic acid in the FDA 92A2 strain (Valentin Friedrich *et al*., manuscript in preparation), in this master thesis, it should be investigated, if in different clinical isolated of *T. forsythia*, there is evidence for the presence of the respective genetic locus for building up either pseudaminic or legionaminicacid. PCR screening with primers for the crucial enzymes PseB, PseC, PseF, LegB, LegC and LegF of these nonulosonic acid biosynthetic pathways (compare with **Figure 13**) were used for screening the genome of the type type strain ATCC 434037, strain ATCC BAA-2717 (FDC 92A2) and the clinical isolates of *T. forsythia* for the presence of these enzymes.

## 1.8 Clearing up a mismatch of *T. forsythia* strains ATCC 43037 and FDC 92A2

A very recent genome announcement published by Friedrich *et al.* [Friedrich, Pabinger *et al.* 2015] unraveled a confusing situation affecting two *T. forsythia* strains mentioned in this master thesis, which are the type strain *T. forsythia* ATCC 43037 and strain ATCC BAA-2717. Based on the identification of pseudaminic acid in the *T. forsythia O*-glycans [Posch, Sekot *et al.* 2012], it was attempted by Valentin Friedrich during his PhD thesis to amplify the genes involved in the biosynthesis of this nonulosonic acid. However, close inspection of the publicly available genome sequence of *T. forsythia* for primer design to amplify the putative enzymes (PseB-PseF, compare with **Figure 13**) involved in pseudaminic acid biosynthesis indicated that the genes better matched the enzymes required for biosynthesis of legionaminic acid.

The type strain of *T. forsythia* (ATCC 43037, FDC 338) was originally isolated by Tanner *et al.* and deposited with ATCC in the year 1986. The genome sequence of a different strain, FDC 92A2, was obtained by TIGR, annotated by the Los Alamos National Laboratory, made publicly available in

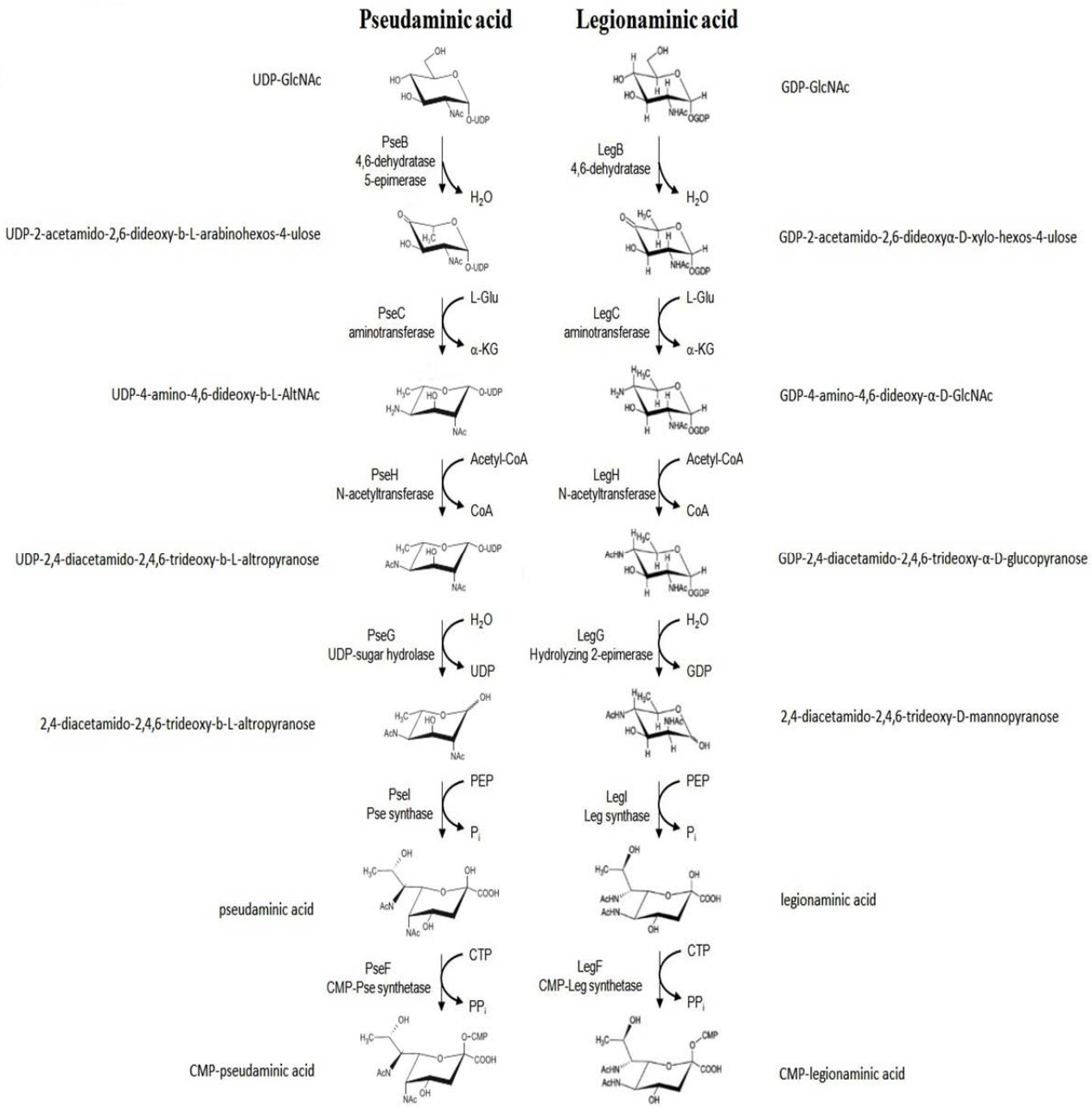


**Figure 13.** CMP-pseudaminic acid biosynthetic pathway in *Helicobacter pylori* and *Campylobacter jejuni* and CMP-legionaminic acid biosynthetic pathway in *C. jejuni*. Names of enzymes and products are indicated in the figure, taken from [Schoenhofen, McNally *et al.* 2006; Schoenhofen, Vinogradov *et al.* 2009]

2005, released through the NCBI in 2013 as GenBank accession number CP003191. Regrettably, the genome of strain FDC 92A2 was erroneously attributed to the type strain ATCC 43037 in CP003191. After repeated cases of finding mismatches between the sequence of amplified PCR products from ATCC 43037 and the CP003191 genome, decision to perform shotgun sequencing of ATCC43037 and discovery of the strain attribution problem was done. Recently, *T. forsythia* strain FDC 92A2 was made available through ATCC under the deposit number ATCC BAA-2717, and the strain attribution of CP003191 was corrected at the NCBI as of 10 April 2015 [Friedrich, Pabinger *et al.* 2015].

Despite the time-consuming procedure of unraveling the mismatch between available *T. forsythia* strain and deposited genome sequence, this finding let us predict that *T. forsythia* possesses a certain degree of variation in nonulosonic acid biosynthesis. This has laid the basis for detailed investigations of nonulosonic acid biosynthesis in *T. forsythia*.

# 2 Aims of the study

## 2.1 Growth of clinical isolates of *T. forsythia*

The first goal of this master thesis was to revive and cultivate different clinical *T. forsythia* isolates provided by our collaboration partners, some of which had been stored at -70°C for several years. Isolates 0348, 0408, 0471, 1046, 1055, and 1056 were obtained from Dr. Georgios Belibasakis, Center of Dental Medicine at the University of Zurich, Switzerland, and isolates UB 4 and UB 20 were obtained from Dr. Ashu Sharma, University of Buffalo, NY, USA. As *T. forsythia* wild-type ATCC 43037 is a slow growing organism with fastidious nutrient demands [Tanner and Izard 2006], growing of the clinical isolates was a challenge at the beginning of the study. To ensure growth, the isolates should be thawed first and then stroken out on BHI-containing-agar plates and subsequently been grown anaerobically at 37°C. Eight clinical isolates named 0348, 0408, 0471, 1046, 1055, 1056, UB 4 and UB 20 should reach amounts of biomass sufficient for biochemical and MS glycan analysis while being compared with the known growth pattern and glycosylation profile of *T. forsythia* ATCC 43037 wild type strain. Strain ATCC BAA-2717 (FDA 92A2) should be grown in parallel with the isolates and handled like the isolates, because as described above, this strain was only recently deposited and thus, no further information except for its genome sequence had been known until now.

## 2.2 Investigation of the presence of S-layer proteins in the clinical isolates

As soon as there would be enough biomass of the *T. forsythia* isolates to be harvested, bacterial cell pellets should be prepared for SDS-PAGE analysis. The S-layer should be identified on Coomassie Brilliant Blue-stained gels and corresponding bands should be excised from the gel followed by tryptic digest and reductive β-elimination of the glycans for detailed MS analysis.

## 2.3 Identification of the genomic loci for the *de novo* synthesis of nonulosonic acids

Based on the elucidation of the biosynthesis pathways for building up nonulosonic acids in *H. pylori* and *C. jejuni* [Schoenhofen, McNally *et al.* 2006; Schoenhofen, Vinogradov *et al.* 2009], this master thesis should deliver information, if similar pathways are present in *T. forsythia* strains. Using PCR analysis with well-defined primer pairs, the genome of the *T. forsythia* strains should be screened for the genes coding for crucial enzymes of pseudaminic and legionaminic biosynthesis, respectively.

## 2.4 Analysis of the *T. forsythia O*-glycan signature

As a last objective, the β-eliminated glycans of the *T. forsythia* clinical isolates and strain ATCC BAA-2717 should be analyzed for their sugar composition by mass spectrometry. Results from the study of clinical isolates taken from patients suffering from severe periodontal disease should contribute to broadening our understanding of the glycosylation capacity of *T. forsythia.* As the glycosylation system of *T. forsythia* ATCC 43037 wild type strain was shown to be a virulence factor [Settem, Honma *et al.* 2013], specifically the elucidation of the glycan composition of the different clinical isolates should enable the assessment of the overall potential and variability of nonulosonic acid biosynthesis in this periodontal pathogen. This may eventually pinpoint new glycobiology-based targets for designing strategies to combat periodontal diseases in the future.

# 3 Materials and Methods

## 3.1 Water

Water used for all experiments was distilled water, deionized by reverse osmosis. For DNA-isolation and PCR experiments, Milli-Q-water was used. Distilled water was purified by several filter units and membranes by Milli-Q Integral 10 (Millipore). Particles larger than 0.22 µm were retained to obtain ultrapure water needed for the experiments.

## 3.2 Chemicals

The chemicals needed for the experiments within the frame of this master thesis were purchased as indicated in **Table 1**. In the table, all chemicals are categorized according to substance class, application fields and provider.

**Table 1.** List of chemicals used in this study

| **Media components:** | |
|---|---|
| BHI | Oxoid |
| Yeast extract | Fluka |
| Cysteine | Sigma Aldrich |
| Hemin | Fluka |
| Menadione | Sigma Aldrich |
| N-acetylmuramic acid | Carbosynth |
| Horse serum | Life Technologies |
| Agar-agar | Gerbu |
| Fastidious anaerobe agar | Lab M Limited |
| Horse blood, defibrinated | Thermo Scientific |

| | |
|---|---|
| **Salts:** | |
| NaCl | Sigma Aldrich |
| $MgCl_2$ | Gerbu |
| $NaBH_4$ | Sigma Aldrich |
| $NaIO_4$ | Merck |
| $K_2S_2O_5$ | Fluka |
| $KH_2PO_4$ | Gerbu |
| $Na_2HPO4$ | Gerbu |
| KCl | Merck |
| | |
| **Sugars/Sugar alcohols:** | |
| Glucose | Gerbu |
| Agarose | Sigma Aldrich |
| Polyoxyethylensobiton monolaurate* polyethyleneglycol sorbiton monolaurate (Tween) | Sigma Aldrich |
| | |
| **PCR:** | |
| 5x Phusion buffer | Thermo Scientific |
| Phusion polymerase | Thermo Scientific |
| 5 x Herculase II reaction buffer | Agilent |
| Herculase II phusion polymerase | Agilent |
| dNTP's 10 mM | Thermo Scientific |
| RedTaq polymerase ‘ready to use’ mix | Sigma Aldrich |
| | |
| **Acids/bases:** | |
| Trizma base | Sigma Aldrich |
| $CH_3COOH$ | Roth |
| HCl | Roth |
| NaOH | Fluka |
| $H_5IO_6$ | Merck |

| | |
|---|---|
| **Organic solvents:** | |
| Ethanol | Roth |
| Methanol | Roth |
| Isopropanol | Roth |
| Acetonitrile | Sigma Aldrich |
| Dimethylsulfoxid | Sigma Aldrich |
| | |
| **Dyes:** | |
| Coomassie Brilliant Blue G250 | Serva |
| SYBR Safe | Invitrogen |
| | |
| **Ladders:** | |
| GeneRuler 1 kb plus (DNA ladder) | Fermentas |
| PageRuler plus pre-stained (Protein ladder) | Thermo Scientific |
| | |
| **Aromatic compounds:** | |
| Tris saturated Phenol | Invitrogen |
| | |
| **SDS - gels:** | |
| Polyacrylamide 30 % (PAA) | Sigma Aldrich |
| Ammoniumperoxodisulfate 10 % (APS) | Roth |
| Tetraethylendiamine (TEMED) | Sigma Aldrich |
| Sodiumdodecylsulfate (SDS) | Gerbu |
| | |
| **Antibodies:** | |
| Rabbit –anti TfsA | Polyclonal antibodies from rabbit (not purified serum), Baxter |
| Rabbit –anti TfsB | |
| Goat-anti-rabbit antibody | Fluorescently labelled with IRdye 800CN, LI-COR Bioscience |
| | |

| **Other materials:** | |
|---|---|
| Ethylenediaminetetraacetic acid (EDTA) | Roth |
| β-mercaptoethanol | Roth |
| Skim milk powder | Gerbu |
| Chloroform | Sigma Aldrich |
| Glycerol | Merck |

## 3.3 Equipment

All laboratory equipment that was used during the course of this study is listed in **Table 2** and is categorized according to method or device and respective manufacturer.

**Table 2.** List of equipment used in this study

| **Kits:** | |
|---|---|
| Miniprep gene JET Gel extraction Kit | Fermentas |
| | |
| **Centrifuges:** | |
| Centrifuge | Beckman coulter Avanti J-26XP Rotor: JLA-16-250 |
| Ultracentrifuge | Beckman coulter optima L-100XP Rotor: 70Ti |
| Desk centrifuge | Eppendorf centrifuge 5415R |
| | |
| **Photometer:** | |
| for *T. forsythia* OD-measurement | Ultraspec 10 cell densitometer, Amersham Biosciences |
| for plastic cuvettes | Spectrophotometer U-2001, Hitachi |
| NanoDrop | Spectrophotometer ND1000, Peqlab |
| | |
| **Laminar work bench:** | |
| Biosafety level 2 lab microbiology | Hera safe, Thermo scientific |

| | |
|---|---|
| **Incubator:** | |
| Biosafety level 2 lab microbiology | Heraeus oven, Thermo scientific |
| | |
| **PCR:** | |
| Power pac | Power pac 300, Bio-Rad |
| Genetic biosafety level 1 lab microbiology | iCycler, Bio-Rad |
| | |
| **Genetics software:** | |
| Clone manager | Scientific & Educational software |
| | |
| **Electrophoresis:** | |
| Power pac | Mini Protean electrophoresis apparatus Bio-Rad |
| Gel preparation system | Bio-Rad |
| | |
| **Scanner:** | |
| SDS-gels, Western blots | Odyssey Infrared Imaging System LI-COR Biosciences |
| PAS-stained gels | LiDE 110, Canon |
| | |
| **Western blot:** | |
| Polyvinylidene difluoride (PVDF) membrane (Imobilan FL-Transfer membrane, Milliproe) Mini Trans-Blor cell | Bio-Rad |
| Power supply | Power Pac 300, Bio-Rad |
| | |
| **Glycan-mass spectrometry** | |
| Analysis | PGC-ESI-MS/MS ( 30 % collision energy using CID with argon gas) |
| Detection | ESI-Q-TOF Global Ultima from Micromass (Waters, USA) |
| Evaluation | Bruker's Data Analysis 4.0 |

| **Other equipment:** | |
|---|---|
| Vortex | Vortex genie 2, Scientific Industries |
| Anaerobic jar | Oxoid |
| $O_2$ absorption pack | AnaeroGen, Oxoid |
| $O_2$ indicator | Anaerobic indicator BR0055B, Oxoid |
| Speedvac | Concentrator Savant ISS110, Thermo Scientific |
| Transilluminator | Biovision 3026WL/26MX, Peqlab |
| Column for β-elimination | 25-mg Hypersep Hypercarb SPE-cartridge (Thermo Scientific) |
| Autoclave | CertoClave, CertoClave Sterilizer GmbH |
| pH-meter | MP220, Mettler Toledo |
| Analytical balances | R160P; Sartorius research |
| Laboratory balances | PJ400, Mettler Toledo |

## 3.4 Media and buffers

**Brain heart infusion [Mahtout, Curt *et al.*] medium for liquid *T. forsythia* cultures:**

| | |
|---|---|
| BHI powder | 37 g/L |
| Yeast extract | 10 g/L |
| Cysteine | 1 g/L |
| Menadione (2 mg/mL) | 2 mg/L |
| Hemin (1 mg/mL) | 5 mg/L |

These components were measured and filled up to the desired volume with distilled water. Appropriate mixing was performed using a magnetic stirrer until full homogeneity was achieved. The

medium was autoclaved for 15 min and cooled down. Right before usage, the following components were added under sterile working conditions:

| | |
|---|---|
| N-acetylmuramic acid (10 mg/mL) | 20 mg/L |
| Horse serum (1x) | 50 mL/L |

**Brain heart infusion medium [Mahtout, Curt *et al.*] for *T. forsythia* plate cultures:**

To grow *T. forsythia* on plates, solid medium containing agar was used.

| | |
|---|---|
| BHI powder | 37 g/L |
| Yeast extract | 5 g/L |
| Cysteine | 1 g/L |
| Menadione (2 mg/mL) | 1 mg/L |
| Hemin (1 mg/mL) | 5 mg/L |
| Agar Agar | 8 g/L |

The components were measured and filled up with water to a desired volume. After autoclaving at 121°C for 15 min, the following components were added:

| | |
|---|---|
| N-acetylmuramic acid (10 mg/mL) | 20 mg/L |
| Horse serum (1x) | 50 mL/L |

Instantly the medium was transferred aseptically into plates at ~50-60°C (25 mL per plate). Further cooling down solidified the medium.

**Fastidious anaerobe agar for *T. forsythia* plate cultures:**

46 g of fastidious anaerobe agar powder was dispersed in 1 L distilled water and autoclaved at 121°C for 15 min. After cooling down 50 mL horse blood was added and instantly the medium was transferred aseptically into plates at 50-60°C (25 mL/plate). Further cooling generated plates ready for use.

**50 x TAE buffer** (stock solution)**:**

| | |
|---|---|
| Trizma base | 242 g |
| 0,5 M EDTA | 100 mL |
| Acetic acid | 57.1 mL |
| Aqua dest. | 700 mL |

All components were mixed together. A pH value of 8.0 was adjusted with 37 % HCl and the bottle was filled up to 1000 mL with distilled water.

**1 x TAE buffer** (for agarose gel electrophoresis)**:**

50 x TAE buffer was diluted 1:50 with distilled water for electrophoresis purposes.

**10 x Agarose gel electrophoresis loading buffer:**

| | |
|---|---|
| Bromphenol Blue | 0.25 % (w/v) |
| Glycerol | 30 % (w/v) |
| Xylencyanol | 0.25 % (w/v) |

**1 x Laemmli buffer:**

| | |
|---|---|
| Sodiumdodecylsulfate | 2 % |
| ß-mercaptoethanol | 1 % |
| Glycerol | 10 % |
| Tris/HCl | 62.5 mM |

All components were mixed together and the pH was adjusted to 6.8.

**10 x SDS running buffer** (stock solution)**:**

| | |
|---|---|
| Trizma base | 30 g |
| Sodiumdodecylsulfate | 5 g |
| Glycerol | 144 g |

The components were measured and transferred into a bottle, which was finally mixed and filled up to 1000 mL with distilled water.

**1 x SDS running buffer:**

A fresh 1:10-dilution was made of the 10 x SDS running buffer stock solution prior to running an SDS-gel.

**Coomassie Brilliant Blue solution:**

| | |
|---|---|
| Coomassie Brilliant Blue G250 | 0.05 % (w/v) |
| Methanol | 40 % |
| HAc | 10 % |

**Schiff's reagent:**

| | |
|---|---|
| para-Rosanilin | 5 g dissolved in 150 mL 1 M HCl |
| $K_2S_2O_5$ | 5 g dissolved in 850 mL distilled water |

The two solutions were mixed and stirred overnight in the dark. On the next day, 5 g of active carbon was added to the mixture, which was stirred again for 10 min followed by filtration into a new glass bottle.

**Blotting buffer for Western blots:**

| | |
|---|---|
| Trizma base | 9.1 g |
| Glycine | 43.2 g |
| Methanol | 600 mL |
| Sodiumdodecylsulfate | 3 g |

These components were mixed, then filled up to 3000 mL with distilled water.

**10 x PBS (phosphate buffered saline):**

| | |
|---|---|
| NaCl | 80 g |
| KCl | 2 g |
| $Na_2HPO_4$ | 14.2 g |
| $KH_2PO_4$ | 2.4 g |

**1 x PBS (phosphate buffered saline):**

Right before usage the 10 x PBS solution was diluted 1:10 for blotting purposes.

**1 x PBST (phosphate buffered saline with Tween):**

10 x PBS was diluted 1:10 and 0.1 % (v/v) Tween was added.

**STE-buffer:**

| | |
|---|---|
| NaCl | 100 mM |
| Tris/HCl | 10 mM |
| EDTA | 1 mM |

The pH was adjusted to pH 8.0.

**TE-buffer:**

| | |
|---|---|
| Tris/HCl | 10 mM |
| EDTA | 1 mM |

The pH was adjusted to pH 8.0.

**Agarose gel (0.9 %):**

| | |
|---|---|
| Agarose | 3.6 g |
| 1 x TAE buffer | 400 mL |

Agarose was dissolved by heating the liquid in the microwave with shaking the flask every minute for a few seconds to avoid boiling retardation. After cooling down the agarose solution to approximately 50°C, the agarose was carefully poured into the scaffold of 1-cm thickness. Instantly 0.5 µL of SYBR Safe intercalating reagent was dissolved in it and evenly spread with a pipette tip. The gel was then left to solidify at room temperature for 30-40 min.

**SDS polyacrylamide gel:**

**Table 3.** Pipetting scheme for SDS-gel preparation

| Volume | Stacking gel 4 % | Runninggel 7.5 % | Running gel 8 % | Running gel 10 % | Running gel 12 % |
|---|---|---|---|---|---|
| **Aqua dest. (mL)** | 3.4 | 7.4 | 7.3 | 6.2 | 5.2 |
| **30 % PAA (mL)** | 0.8 | 3.8 | 4 | 5 | 6 |
| **1.5 M Tris/HCl,pH 8.8 (mL)** | - | 3.8 | 3.8 | 3.8 | 3.8 |
| **0.5 M Tris/HCl, pH 6.8 (mL)** | 1.4 | - | - | - | - |
| **10 % SDS (µL)** | 60 | 150 | 150 | 150 | 150 |
| **10 % APS (µL)** | 60 | 150 | 150 | 150 | 150 |
| **TEMED (µL)** | 12 | 14 | 14 | 14 | 14 |

First, the thin glass slides of the BioRad® SDS-system were assembled and locked with the appropriate scaffold (**Figure 14**). The arrangement was now sat down on a thick rubber band to prevent the buffer from running out of the spacer between the glass slides. The components for the running and stacking gel (**Table 3**) were pipetted separately in a small beaker glass finishing with the linking catalysts for PAA, which were 10 % APS and TEMED right before usage. The spacer was now filled to three-fourths with the running gel of choice using a disposable transfer pipette and then coated with isopropanol to avoid the building of air bubbles on the top of the stacking gel. After 30 minutes at room temperature, the stacking gel was prepared and all of the isopropanol was discarded. The stacking gel was filled into the last free quarter of the spacer and a suitable comb was put into the spacer displacing just a little bit of stacking gel to be sure everything is filled up with gel. For polymerization the gel was again incubated at room temperature for 30 minutes.

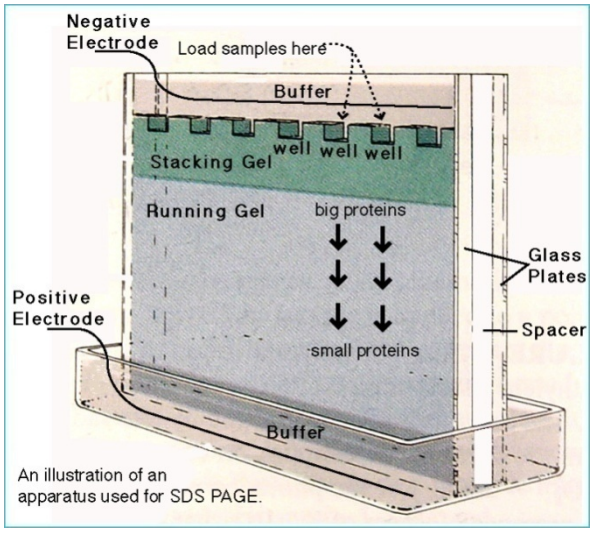


**Figure 14.** Illustration of an apparatus used for SDS-PAGE (taken from https://ww2.chemistry.gatech.edu/~lw26/bCourse_Information/4581/techniques/gel_elect/page_protein.html)

## 3.5 *T. forsythia* isolates

**Table 4.** List of *T. forsythia* strains used in this study.

| **Organisms and isolates** | |
|---|---|
| *Tannerella forsythia* ATCC 43037 | American Type Culture Collection |
| *Tannerella forsythia* ATCC BAA-2717 (strain FDC 92A2) | American Type Culture Collection |
| *Tannerella forsythia* clinical isolate UB 4 | provided by A. Sharma, University of Buffalo, Department of Oral Biology, isolated from anonymous patient |
| *Tannerella forsythia* clinical isolate UB 20 | provided by A. Sharma |
| *Tannerella forsythia* clinical isolate 0471 | provided by G. Belibasakis, University of Zurich, Center of Dental Medicine, isolated from anonymous patient |
| *Tannerella forsythia* clinical isolate 0408 | provided by G. Belibasakis |
| *Tannerella forsythia* clinical isolate 0348 | provided by G. Belibasakis |
| *Tannerella forsythia* clinical isolate 1046 | provided by G. Belibasakis |
| *Tannerella forsythia* clinical isolate 1055 | provided by G. Belibasakis |
| *Tannerella forsythia* clinical isolate 1056 | provided by G. Belibasakis |

## 3.6 Cultivation of *T. forsythia* strains

*T. forsythia* ATCC 43037 and the clinical isolates (see **Table 4**) were cultivated anaerobically in liquid BHI broth (**Figure 15**) and on BHI agar plates, respectively (for medium composition see 3.4). The *T. forsythia* type strain ATCC 43037 and isolates 0348, 0408, 0471, 1046, 1055 and 1056 were thawed out of cryo stocks and streaked out aseptically with an inoculation loop on freshly made BHI agar plates, supplemented with N-acetylmuramic acid and horse serum, in a laminar working bench. The plates were then put into an anaerobic jar (**Figure 16**) with a sachet, which generates an anaerobic atmosphere by irreversibly binding oxygen. Further addition of an anaerobic indicator, containing sensitive resazurin indicated oxygen free conditions visualized as the resazurin color changed from pink to white after the anaerobic jar was locked. The anaerobic jar was then incubated at 37°C for 7 days.

**Figure 15.** Lockable glass vials used for anaerobic growth of *T. forsythia* in liquid BHI culture (taken from www.thermoscientific.com)

**Figure 16.** Anaerobic jar used for anaerobic incubation of *T. forsythia* on BHI plates (taken from www.thermoscientific.com)

Isolates UB 4, UB 20 and ATCC BAA-2717 (FDA 92A2) were provided by collaboration partners as solid stab cultures in 2 mL vials. Some biomass was taken out with an inoculation loop and was transferred into 10 mL lockable vials (**Figure 15**) containing autoclaved liquid BHI medium, supplemented with N-acetylmuramic acid and horse serum. The vials were flushed with $N_2$ and then immediately locked and incubated anaerobically at 37°C for 5 days.

The anaerobic jar was opened after 7 days and biomass from colonies was picked with an inoculation loop, then was transferred into liquid BHI medium and was treated in the same way as described above. If bacterial growth on the BHI plates was not satisfying after 7 days, the plates were incubated in an anaerobic jar for additional 5 days. If then there was still no growth visible, biomass was again taken from cryostock and stroken out on fresh BHI and fastidious anaerobe agar plates supplemented with horse blood. The BHI plates were handled as described before. The blood agar plates were incubated in an anaerobic chamber (see **Figure 17**) for 7 days.

**Figure 17.** Anaerobic incubator used in the course of this study (taken from www.800ezmicro.com)

After 7 days, newly formed biomass was taken from blood agar plates with an inoculation loop and was transferred into liquid BHI medium, supplemented with *N*-acetylmuramic acid and horse serum, incubation was carried out for 5 days at 37°C.

As *T. forsythia* is a slow growing organism with a generation time of ~20 h (as opposed to *E. coli* with a generation time of 20 min) [Tanner and Izard 2006], it took five days until first cells were visible in the form of a sediment at the bottom of the incubation vial. As soon as a solid pellet had been formed, the biomass was harvested. After re-suspending the pellet in BHI medium, $OD_{600}$ was measured. Only some well growing strains (i.e., ATCC 43037, UB 4, UB 20, 1055, 0471) reached an $OD_{600}$ of 1.5, unsatisfactorily growing strains (ATCC BAA-2717, 0408, 1046, 1056) not more than $OD_{600}$ of 0.5. Ideally, harvest and passage of the cells was at $OD_{600}$~ 1.0. 1.5 mL of the harvested cell suspension was transferred into an Eppendorf tube for protein analysis by SDS-PAGE. Another 1.5 - 2

mL samples were taken to isolate genomic DNA of the respective strains or clinical isolates of *T. forsythia*. Moreover, cryo stocks of the well growing strains were prepared. Subsequently, 250 µL of the cell suspension was transferred into fresh BHI medium and was incubated for another 5 days after flushing with $N_2$ for 10 seconds.

### 3.6.1 Preparation of cryo stocks

Once *T. forsythia* has reached an $OD_{600}$ of ~0.5-1.0, cryo stocks were prepared by taking 875 µL of suspended BHI culture and mixing it with 125 µL of glycerol in a cryogenic vial. All these steps were performed using liquid nitrogen for cooling to guarantee a fast freezing of the samples to avoid destroying of the cell surface structures during crystallization. After freezing the samples they were stored in the -80°C freezer until further use.

## 3.7 Protein analysis and characterization

### 3.7.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a widespread method for the separation of proteins based on their molecular weight. Proteins are separated according to their molecular weight based on their differential migration through a polyacrylamide gel matrix under the influence of an applied electrical field. For any kind of molecule, movement through an electrical field is determined by charge, molecular radius and by the applied electrical field. In native proteins, charge is determined by the sum of the positive and negative amino acids and the molecular radius is dependent of the protein's tertiary structure. Proteins with the same molecular weight would migrate at different speeds in an electrical field depending on their charge and shape. In this study, SDS-PAGE according to U.K. Laemmli [Laemmli 1970] separates proteins in an electrical field based just on their molecular weight. To achieve this, tertiary structures are destroyed by reducing the protein to a linear molecule by suspending the harvested cell pellet, containing all proteins, in Laemmli buffer. In this buffer, SDS is a strong negatively charged detergent and along with the reducing β-mercaptoethanol breaks down the protein-protein disulphide bonds and disrupts the tertiary structures of proteins. **Figure 18** shows how folded proteins break down to linear

molecules where SDS coats them with a uniform negative charge, meaning that the charge of the protein is now approximately proportional to its molecular weight.

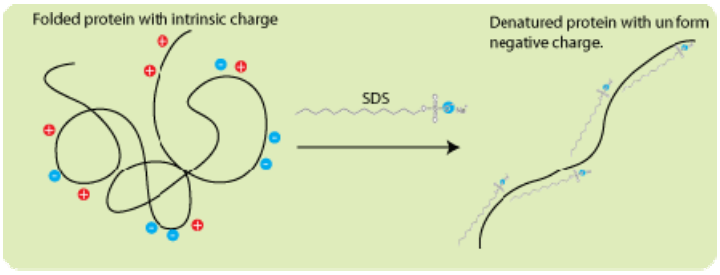


**Figure 18.** Impact of SDS on folded proteins with intrinsic charge, converting them to uniformly negative charged linear molecules (taken from http://bitesizebio.s3.amazonaws.com/content/uploads/2008/05/sds-protein-denaturation.gif)

The polyacrylamide gel, used for separating the proteins, is a matrix formed from monomers of acrylamide and bis-acrylamide (**Figure 19**). It is chemically inert and thus won't interact with the protein as they pass through. The polymerization reaction is initiated by TEMED, which induces free radical formation from ammonium persulphate (APS). These free radicals transfer electrons to the acrylamide and bis-acrylamide monomers and cause them to react with each other. Bis-acrylamide cross-links the acrylamide chains to form a porous gel matrix. The pore size and separation properties

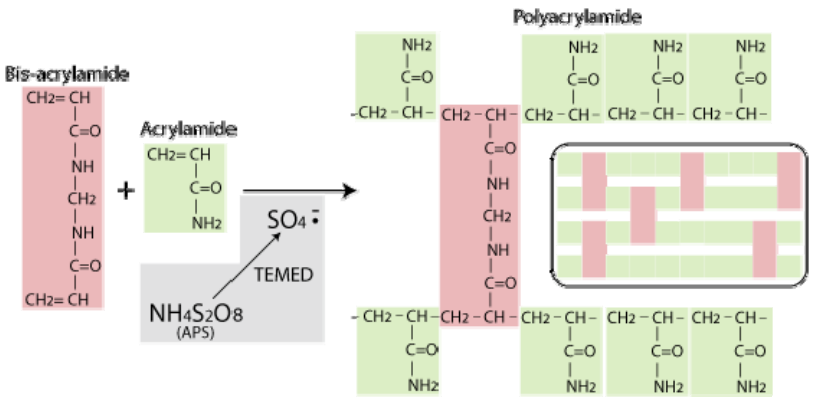


**Figure 19.** Polymerization starts as TEMED induces ammoniumpersulphate molecules to form free radicals, which transfer electrons to acrylamide and bis-acrylamide, causing them to react with each other forming the gel matrix (taken from http://bitesizebio.com/10248/agarose-gels-do-not-polymerise/)

are depending on the amount of crosslinking which can in turn be controlled by the variation of the ratio of acrylamide to bis-acrylamide.

The discontinuous Laemmli [Laemmli 1970] system is set up with a stacking gel at pH 6.8, buffered by Tris-HCl and a running gel buffered to pH 8.8 by Tris-HCl. The running buffer has a pH of 8.3 and surrounds the system (for preparation of gels and buffers, see 3.4). The concentration of acrylamide is lower in the stacking gel than in the running gel leading to bigger pores in the stacking gel. When the power is turned on and proteins start migrating, the stacking gel ensures the arrival of all of the proteins on top of the running gel at the same time and proteins of the same molecular weight will migrate through the gel as tight bands. Upon entering the running gel, the proteins are separated because higher molecular weight proteins move slower through the pores of the acrylamide gel than lower molecular weight proteins. The weight of separated proteins can be roughly estimated by comparison with a protein ladder and after staining the protein bands (**Figure 20**).

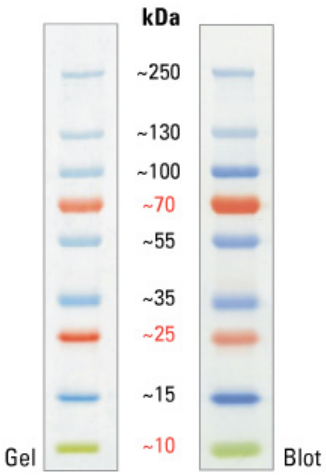


**Figure 20.** PageRuler Plus pre-stained protein ladder for SDS-PAGE and Western blotting (taken from: http://www.wegene-china.com/Upload/26619-ladder-002.jpg)

In this study, proteins out of whole cell lysates were analyzed by SDS-PAGE at an $OD_{600}$ of 0.5-1.0, depending on the strain. 1.5 mL of the cell suspension were transferred in an Eppendorf tube and centrifuged at 13000 x *g* for 5 min. The supernatant was discarded and the pellet was re-suspended with a certain amount of 1x Laemmli buffer. The amount of buffer was calculated as follows: volume of Laemmli buffer (µL) = measured $OD_{600}$ x empiric factor of 40 x volume of cell suspension (mL).

After re-suspension in 1x Laemmli buffer, the sample was either stored at -20°C or directly used for SDS-PAGE. Before application to the gel the sample was boiled for 5-10 min and right after the boiling spun down at 10000 x *g* for 1 min. Then the sample was applied to the SDS-PAGE gel. Between 1-20 µL of sample supernatant was loaded to the gel. Additionally 5 µL of Page Ruler plus pre-stained protein ladder was loaded. After running the gel for about 60 min at 200 V, the gel was either stained with Coomassie Brilliant Blue (CBB) or with Periodic acid Schiff's staining (PAS).

#### 3.7.1.2 Coomassie Brilliant Blue staining

After the separation of proteins by SDS-PAGE, bands on the gel were stained with Coomassie Brilliant Blue G250 solution for approximately 20-40 min, depending on how old the used staining solution was. After staining, the gel was destained using 10 % acetic acid until only the blue stained protein bands were remaining on the cleared gel. The gel was stored in tap water until it was scanned.

### 3.7.2 Periodic acid Schiff's staining (PAS)

This staining method was used to detect the glycan portions of the two S- layer proteins of *T. forsythia*. Periodic acid oxidizes neighboring diols of sugar residues breaking up the bond between two adjacent carbons not involved in the glycosidic linkage and creating a pair of new aldehydes at the two free tips of each broken monosaccharide ring. These aldehydes then react with the Schiff reagent to give a purple-magenta color [Doerner and White 1990].

Selected SDS-PAGE gels were coated with fixing solution (50 % methanol, 5 % acetic acid) in a plastic shell for 1 h. Three subsequent washing steps with 3 % acetic acid were followed by incubation in oxidation solution (1 % periodic acid in 3 % acetic acid) for 20 min. After that, again three washing steps with 3 % acetic acid were made. Now, the gel was incubated with Schiff's reagent for 15 min in the dark. Reduction was then performed using a 0.5 % sodium-metabisulfite solution for 10 min. Again, four washing steps with 3 % acetic acid followed and the gel was stored in distilled water until scanning.

### 3.7.3 Pro-Q-Emerald glycostaining

Another more sensitive method was used for detecting glycoproteins in this study, called Pro-Q-Emerald glycostaining. Pro-Q Emerald glycoprotein stain reacts with periodic acid-oxidized carbohydrate groups, generating a bright green-fluorescent signal on glycoproteins. The stain permits detection of less than 5-18 ng of glycoprotein per band, depending on the nature and the degree of protein glycosylation, making it roughly 8-16-fold more sensitive than the standard colorimetric periodic acid-Schiff base method using para-Rosaniline (acidic fuchsin dye) [Hart, Schulenberg *et al.* 2003].

A representative SDS-PAGE gel with protein bands of well growing *T. forsythia* ATCC 43037, ATCC BAA-2717 strain and the clinical isolates of *T. forsythia* was fixed for 45 min. Subsequently, the gel was washed twice with washing solution for 15 min. Subsequently, the oxidation of sugars was performed with oxidation solution for 45 min. Three washing steps with wash solution followed for 15min, each. The gel was stained using freshly made staining solution for 2 h. After two final washing steps in wash solution for 15min, again the gel was ready to be analyzed under an UV-illuminator at 300 nm.

### 3.7.4 Western Blot

Western blotting is a commonly used method for the detection of proteins and other macromolecules immobilized on nitrocellulose or PVDF membranes. The method enables the identification and quantification of a specific protein from preceding sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was incubated in a solution containing primary antibody and the resultant antigen-antibody complex is detected by using appropriately labeled ligands. The used method is based on the enzyme-linked immunodetection of antigen-specific antibodies using anti-IgG secondary antibodies conjugated with either horseradish peroxidase or alkaline phosphatase. Visualization of the

antibody-antigen complex was achieved through the use of an enhanced chemiluminescent method [Claus and König 2010].

In this study, the protocol from the company LI-COR (2003) together with a Mini Tran-Blot cell from Biorad (**Figure 21**) was used. Here, a nitrocellulose membrane activated with methanol was equilibrated together with fiber pads, filter papers and the SDS-gel with the separated proteins, in blotting buffer for 15 min. In the meantime, the gel holder cassette was prepared. A pre-soaked fiber pad was placed on the black side of the holder cassette and now filter paper and the SDS-gel were put together (for detail of the arrangement see **Figure 22**).


**Figure 21.** Western blotting system components (taken from: http://www.bio-rad.com/webroot/web/images/lsr/solutions/technologies/protein_electrophoresis_blotting_and_imaging/western_blotting/technology_detail/pet22_mini_transblot_cell.jpg)

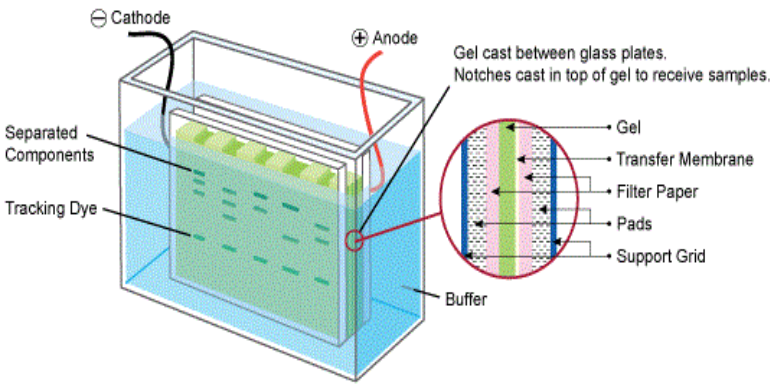


**Figure 22.** Arrangement of the gel holder cassette during Western blotting (taken from:http://www.leinco.com/includes/templates/LeincoCustom/images/WesternBlotSetup.gif)

The nitrocellulose membrane was placed directly on the gel and another filter paper was laid on the membrane. Potential air bubbles, which could have occurred during assembly were expelled with a roller. Last, another fiber pad was placed onto the second filter paper and the gel holder cassette was closed with a lockable hinge. Then the closed cassette was put into the buffer tank which was subsequently filled with blotting buffer. An ice cooling unit and a magnetic stirrer should avoid heating up the system too much. The blotting procedure was then started after the power was turned on at 350 mA for 75 min. After the run was done the membrane was rinsed with PBS and shaken for 1 h at 60 rpm in 5 % skim milk solution in PBS to block the membrane in order to avoid unspecific binding of the detection antibodies. Right after the blocking step, the membrane was incubated with the primary antibody which was a polyclonal antibody against either TfsA (rabbit-anti TfsA, 1:1000 in PBST) or TfsB S-layer protein (rabbit-anti TfsB, 1:1000 in PBST). Three washing steps with PBST lasting for 5 min, each, followed to remove unbound primary antibodies. Furthermore the membrane was incubated with the secondary antibody (goat anti-rabbit, fluorescently labelled, 1:1000 in PBST) for 1 h. After treatment with secondary antibody the membrane finally was again washed three times with PBST for 5 min and once with PBS for another 5 min. The membrane was stored in PBS until it was scanned using the Odyssey infrared imaging system (LI-COR).

### 3.7.5 β-Elimination of glycoprotein glycans

For analysis of the glycans bound to the S-layer proteins TfsA & TfsB of the *T. forsythia* ATCC 43037; ATCC BAA-2717 strains and clinical isolates, reductive in-gel β-elimination of the glycans [Zauner, Kozak *et al.* 2012] was performed upon separation of the glycoproteins on SDS-PAGE gels. First, representative SDS-PAGE gels were run using high amounts of sample to obtain sufficient material for further analyses. Once the bands of separated TfsA and TfsB glycoprotein bands from *T. forsythia* type strain ATCC 43037 as reference and the clinical isolates were excised from the gel, the gel bands were minced into pieces, put into Eppendorf tubes and washed with 500 µL of 0.5 M NaOH and incubated at room temperature for 5 min. After the washing step, the solution was discarded. 100 µL of 1 M $NaBH_4$ in 0.5 M NaOH were freshly prepared and added to the samples so

that the gel pieces were covered well. The mixture of gel pieces and sodium borohydride was now incubated overnight at 50°C and the Eppendorf tubes were weighed down with a small metal heat block just to make sure that they do not pop open because of the pressure of built-up elementary hydrogen. On the next day, the $NaBH_4$ supernatant, containing the glycans of interest, was transferred into a fresh Eppendorf tube. Together with the supernatant of two washing steps (2 x 500 µL MilliQ-water) it was loaded onto a HyperSep Hypercarb SPE 25-mg cartridge, equilibrated with 60 % acetonitrile, in order to remove the excess of salt. After three washing steps with 500 µL MilliQ-water, the glycans were eluted using 60 % acetonitrile. After evaporating the acetonitrile for a few hours using the SpeedVac, the glycans were ready for further analysis.

### 3.7.6 LC ESI-MS/MS

**Liquid Chromatography:** Separation of the sample molecules was first carried out by liquid chromatography where the analysts are differentially partitioned between the mobile phase (eluent) and the stationary phase (column). Samples were introduced to the ESI-source via a porous graphitic carbon (PGC) column (100 x 0.32 mm, 5 µm particle size, Thermo Scientific). A gradient from 2 % to 42 % of 100 %- acetonitrile was developed over 20 min at a flow rate of 6 µL/min using an Ultimate 3000 capillary flow LC (Dionex).

**Ion Source:** Production of gas phase-ions of the glycan samples was done by electron spray ionization (ESI) [Fenn, Mann *et al.* 1989]. ESI allows very high sensitivity to be reached and is easy to couple to high-performance liquid chromatography HPLC or capillary electrophoresis. ESI is produced by applying a strong electric field, under atmospheric pressure, to a liquid passing through a capillary tube with a weak flux (normally 1–10 µL/min). In the ESI source ions are formed which will be guided into the MS. Depending on the polarity positive or negative charged ions can be formed. **Figure 23** shows an ESI source and a drop at the tip of the capillary, as observed with binoculars while increasing the voltage. Left: at low voltage, the drop is almost spherical. Centre: at about 1 or 2 kV, but below the onset potential, the drop elongates under the pressure of the charges accumulating

at the tip. Right: at onset voltage, the pressure is higher than the surface tension, the shape of the drop changes at once to a Taylor cone and small droplets are released [Cole 1997]. The droplets divide and explode, producing the spray.

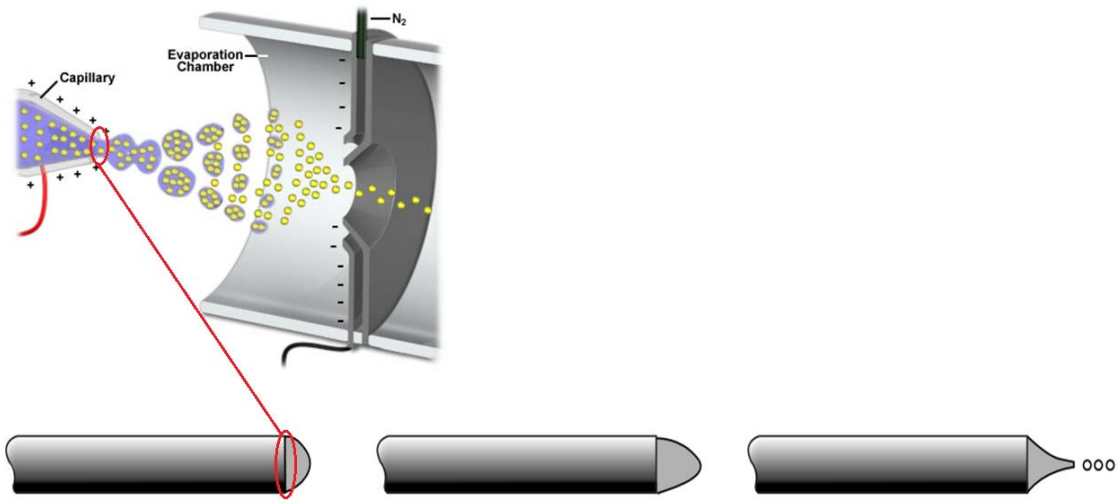


**Figure 23.** Electrospray ionization. As solvent evaporates until equal charges converge so close that they repel each other until the Rayleigh limit is reached, thereby provoking Coulomb explosion generating smaller droplets for further evaporation (slightly modified pictures taken from https://nationalmaglab.org/images/users/icr/techniques/ionization_esi.jpg & Hoffmann, Mass spectrometry; principles and applications)

**Mass analyzer:** During the course of this study, a Bruker amaZon ion trap was used for mass analysis. Ion trap mass spectrometers work on the basis of storing ions in a 'trap', and manipulating the ions by using applied DC and RF fields. The amplitude of the applied voltages enables the analyzer to trap ions of specified mass to charge ratios within the analyzing device. Non-selected ions are given a trajectory by the electrostatic field that causes them to exit the trap. By filling the trap with an inert gas fragmentation of selected ions is possible (compare with **Figure 24**).

This is useful when structural information is required. The system is also able to perform multiple isolation and fragmentation stages (MSn) with very good sensitivity (taken from http://www.ecs.umass.edu/eve/background/methods/chemical/Openlit/Chromacademy%20LCMS%20Intro.pdf)

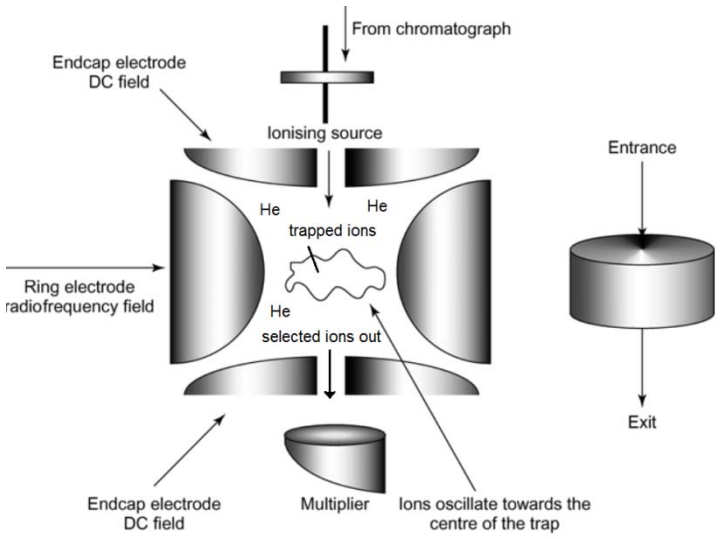


**Figure 24.** Ion trap filled with helium used to select ionized sample molecules (slightly modified taken from https://www.medicinescomplete.com/mc/clarke/2010/Clkmass_spectrometryF007_default.png)

**Detection and data dependent acquisition:** The ions pass through the mass analyzer and are then detected and transformed into a usable signal by a detector. Detectors are able to generate from the incident ions an electric current that is proportional to their abundance. The most widely used ion detector in mass spectrometry is the electron multiplier (EM). In this detector, ions from the analyzer are accelerated to a high velocity in order to enhance detection efficiency. This is achieved by holding an electrode called a conversion dynode at a high potential from ±3 to ±30kV, opposite to the charge on the detected ions. A positive or negative ion striking the conversion dynode causes the emission of several secondary particles. These secondary particles can include positive ions, negative ions, electrons and neutrals. When positive ions strike the negative high-voltage conversion dynode, the secondary particles of interest are negative ions and electrons. When negative ions strike the positive high-voltage conversion dynode, the secondary particles of interest are positive ions. These secondary particles are converted to electrons at the first dynode. These are then amplified by a cascade effect in the electron multiplier to produce a current [de Hoffmann and Stroobant 2007].

A powerful software control system enables the instrument to perform data dependent acquisition (DDA), switching from the MS to MS/MS mode and then returning to the MS mode using

data dependent criteria. This method obviates the need to analyze the sample in MS mode to identify the target precursor ions and then re-run the sample in MS/MS mode to acquire the MS/MS data from each of these precursors. This is particularly valuable in the analysis of unknown samples using on-line chromatography where the target precursor ions and their retention times may be different for each sample (taken from http://scs.illinois.edu/massSpec/ion/ei.php).

Qualitative evaluation was done using Bruker's Data Analysis 4.0, where all the spectra within the relevant elution range were summed up to generate an average spectrum. The glycan nature of the main peaks was confirmed by the identification of specific reporter ions in MS/MS spectra, which was done by the help of Glycoworkbench 2.0.

## 3.8 Genetic methods and investigations

### 3.8.1 Extraction of genomic DNA

DNA extraction was performed from *T. forsythia* cells following the "extremely rapid genomic DNA extraction from bacteria and yeasts" protocol [Cheng and Jiang 2006]. 1 mL of the cell suspension was centrifuged at 8000 x *g* for 2 min. The supernatant was discarded and the cells were washed twice with 400 µL of STE-buffer. Again, a centrifugation step at 8000 x *g* for 2 min was performed and then, the cell pellet was resuspended in 200 µL of TE buffer. Addition of 100 µL of Tris-saturated phenol (pH 8.0) was performed under extreme caution. The cells were lysed by being vortexed for 60 s. Another centrifugation step at 13000 x *g* for 5 min at 4°C separated the aqueous phase from the organic phenolic phase. 160 µL of the aqueous phase was transferred to a fresh 1.5 mL Eppendorf tube. The following addition of 40 µL TE buffer and 100 µL of chloroform was done twice, everytime transferring 160 µL of aqueous phase into a fresh Eppendorf tube. 2.5 µL of RNAse was added followed by incubation for 10 min at 37°C to digest RNA. Final addition of 100 µL of chloroform and thorough mixing followed by centrifugation at 13000 x *g* for 5 min at 4°C delivered an aqueous phase containing purified DNA, which was either used directly for screening PCR or stored at -20°C until further use.

### 3.8.2 Spectrophotometric determination of DNA concentration

Purified DNA was analyzed for concentration and quality using the NanoDrop spectrophotometer, using 2 µL of sample. The Nanodrop software is based on the Beer-Lambert equation to correlate the absorbance with the concentration according to: A = E * b * c. Where A is the absorbance represented in absorbance units (A), E is the wavelength-dependent molar absorptivity coefficient (or extinction coefficient) with units of liter/mol-cm, b is the path length in cm, and c is the analyte concentration in mol/liter or molarity (M). The ratio of absorbance at 260 and 280 nm was used to assess the purity of DNA. A ratio of about 1.8 is generally accepted as pure for DNA. If the ratio was lower, protein, phenol or other contaminants that absorb strongly at or near 280 nm might disturb the measurement.

### 3.8.3 Agarose gel electrophoresis

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying, and purifying 0.5- to 25-kb DNA fragments [Zauner, Kozak *et al.* 2012]. First, an agarose gel of 0.9 % agarose in 1x TAE buffer was prepared by pouring a preheated agarose solution into adequate scaffolds. SYBR green intercalating reagent hast been used in 1:50000-dilution to make DNA bands visible. A comb has been put into the liquid gel to enable sample loading. Solidifying of the gel took about 30 min and then the gel was put into a tank and was fully coated with 1x TAE buffer. Application of 10 µL samples into the gel wells was done and a DNA ladder (standard; **Figure 25**) was used to compare separated bands later on. Separation was performed at 80 V for 35 min. Subsequently, the agarose gel was analyzed under UV light at 254 nm.

### 3.8.4 DNA extraction from agarose gels

DNA extraction from agarose gels was done using the GeneJET gel extraction kit from Fermentas according to the manufacturer's instructions. Separated DNA bands were excised from the agarose gel under UV light with a sharp scalpel. The excised bands were transferred into Eppendorf tubes and binding buffer was added until the gel pieces were fully covered with it. Dissolving of all the pieces

was done in a 50°C oven for about 10 min. Thereafter, the solution was transferred onto the GeneJET gel extraction kit column followed by centrifugation for one minute at maximum speed. This step bound the DNA specifically to the gel extraction column material. Impurities were spun down with the flow-through which was then discarded. Subsequently, 700 µL of wash buffer was added to the column and again a centrifugation step for 1 minute at maximum speed was performed. Finally, 50 µL of elution buffer was added exactly onto the membrane and a last centrifugation step washed the bound DNA away from the membrane into the elution buffer which was then ready for further experiments.

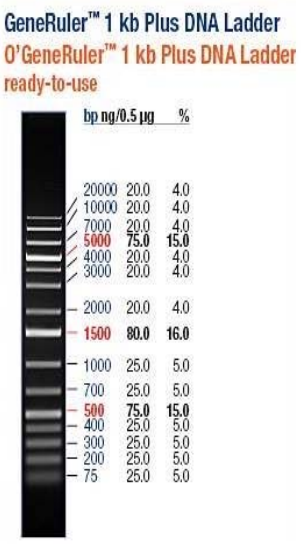


**Figure 25.** GeneRuler 1 kb Plus DNA Ladder (taken from https://beta-static.fishersci.com/images/F100447~wl.jpg)

### 3.8.5 16S rRNA-amplification for identification of *T. forsythia*

The 16S rRNA gene is used for phylogenetic studies [Weisburg, Barns *et al.* 1991] as it is highly conserved between different species of bacteria and archaea [Coenye and Vandamme 2003]. In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for identification of bacteria. To identify *T. forsythia* bacteria, specific primer pairs were used to screen the genomes of *T. forsythia* ATCC 43037 and isolates UB 4, UB 20, 0471, 1055 and 1056. For details in terms of the pipetting

scheme and PCR-conditions see **Tables 5** and **6** respectively, moreover the primers used for screening the 16S rRNA gene loci and their respective sequences are listed in **Table 7**.

PCR-mix using Herculase II fusion polymerase:

**Table 5:** PCR-pipetting scheme for DNA amplification using herculase II phusion polymerase

| Solutions | Volume (µL) |
|---|---|
| MilliQ water | 34.3 |
| 5 x Herculase II reaction buffer | 10.0 |
| 10 mM dNTP | 1.3 |
| 10 µM forward primer | 1.3 |
| 10 µM reverse primer | 1.3 |
| Template | 1.0 |
| Herculase II phusion polymerase | 1.0 |
| Final volume | 50.0 |

PCR-conditions using Herculase II fusion polymerase:

**Table 6**: PCR-conditions for DNA amplification with Herculase II phusion polymerase

| Cycle steps | Time | Temperature (°C) | Cycles |
|---|---|---|---|
| Initial denaturation | 2 min | 95 | 1 |
| Denaturation | 20 s | 95 | 30 |
| Annealing | 30 s | 50 | 30 |
| Extension | 30 s/kbp | 72 | 30 |
| Final extension | 3 min | 72 | 1 |
| storage | infinite | 4 | 1 |

**Table 7:** Primer list and primer sequences for 16S rRNA amplification

| Primer | Length (bp) | $T_m$ °C | Sequence 5'-3' |
|---|---|---|---|
| 013-Fuso-16S-f-universal | 20 | 61 | AGAGTTTGATCCTGGCTCAG |
| 013-Fuso-16S-r-universal | 23 | 72 | CTTAACTGCCCCCGGGCGTGTTC |

### 3.8.6 Screening PCR to detect the genes encoding the TfsA and TfsB S-layer proteins

To screen the genomes of the *T. forsythia* type strain ATCC 43037, BAA-2717 (strain 92A2) and clinical isolates UB 4, UB 20, 0348, 0471, 1055 and 1056 for the presence of the respective genes coding for the two S-layer proteins TfsA and TfsB, PCR amplifications were conducted using specific primer pairs **(**compare **Table 10)**. The S-layer is important for *T. forsythia* because it provides a selection advantage to the bacterium in the natural habitat [Sekot, Posch *et al.* 2011]. In addition, the *T. forsythia* S-layer has been shown to promote epithelial cell adherence and invasion [Sabet, Lee *et al.* 2003; Sakakibara, Nagano *et al.* 2007]. To determine the presence of genetic information for building up S-layer proteins was therefore inevitable especially because the work included isolate strains, which have never been investigated before. Details of the pipetting scheme and PCR conditions are listed in **Tables 8** and **9**.

PCR-mix using Phusion polymerase:

**Table 8**: Pipetting scheme for DNA amplification with PCR using phusion polymerase

| Solutions | Volume (µL) |
|---|---|
| MilliQ water | 32.5 |
| 5 x Phusion reaction buffer | 10.0 |
| 10 mM dNTP | 1 |
| 10 µM forward primer | 2.5 |
| 10 µM reverse primer | 2.5 |

| | |
|---|---|
| Template | 1.0 |
| Phusion polymerase | 0.5 |
| Final volume | 50.0 |

PCR-conditions using Phusion polymerase:

**Table 9:** PCR-conditions for DNA amplification with phusion polymerase

| Cycle steps | Time | Temperature (°C) | Cycles |
|---|---|---|---|
| Initial denaturation | 30 s | 98 | 1 |
| Denaturation | 10 s | 98 | 30 |
| Annealing | 30 s | 55 | 30 |
| Extension | 120 s (15 s/kbp) | 72 | 30 |
| Final extension | 8 min | 72 | 1 |
| storage | infinite | 4 | 1 |

**Table 10**: primer list and primer sequences for screening of tfsA and tfsB loci

| Primer | Length (bp) | $T_m$ °C | Sequence 5'-3' |
|---|---|---|---|
| TfsA for | 27 | 75 | GGGCCATGGATGCGCGACCCTTTTACG |
| TfsA rev | 36 | 77 | CTTACGTCACTTTCGACATCATTTTCGCCGGCGGGG |
| TfsB for | 36 | 78 | GGGGGTCTCCCATGGCACAAATAGCACTGGAGCAAC |
| TfsB rev | 32 | 80 | CGACATTTTCGCTACCACTTCCGCCGGCGGGG |

### 3.8.7 Screening PCR to check for the presence of genetic loci for *de novo* biosynthesis of nonulosonic acids

Polymerase chain reaction was used for amplification of genetic target sequences coding for enzymes involved in the *de novo* biosynthetic pathway for building up nonulosonic acids. According to the

genomic *O*-glycan biosynthesis locus of the type strain shown in **Figure 6,** primers aimed at the pseudaminic acid pathway were used to amplify pseB, pseC and pseF. The legionaminic acid biosynthesis locus found in ATCC BAA-2717 (strain 92A2) is significantly different from the type strain and is shown in **Figure 7**. In this case, primers for screening purposes were targeted at loci coding for LegB, LegC and LegF.

*T. forsythia* ATCC 43037, ATCC BAA 2717 (strain 92A2) and the genomes of clinical isolates 0348, 0471, 1046, 1055, 1056, UB 4 and UB 20 were screened with primers listed in **Table 13** on the presence of genes for *de novo* biosynthesis of nonulosonic sugars. Details of the pipetting scheme and PCR conditions are listed in **Tables 11** and **12**.

PCR-mix using RedTaq polymerase:

**Table 11**: Pipetting scheme for DNA amplification with PCR using RedTaq polymerase

| Solutions | Volume (µL) |
|---|---|
| MilliQ water | 11.0 |
| 10 µM forward primer | 0.5 |
| 10 µM reverse primer | 0.5 |
| Template | 0.5 |
| Final volume | 25 |

PCR-conditions using RedTaq polymerase:

**Table 12**: PCR-conditions for DNA amplification with RedTaq polymerase

| Cycle steps | Time | Temperature (°C) | Cycles |
|---|---|---|---|
| Initial denaturation | 60 s | 94 | 1 |
| Denaturation | 60 s | 94 | 30 |
| Annealing | 120 s | 55 | 30 |

| | | | |
|---|---|---|---|
| Extension | 180 s (60 s/kbp) | 72 | 30 |
| Final extension | 8 min | 72 | 1 |
| storage | infinite | 4 | 1 |

**Table 13**: **P**rimer list and primer sequences for *pseB*, *pseC*, *pseF*, *legB*, *legC* and *legF* amplification

| Primer | Length (bp) | $T_m$ °C | Sequence 5'-3' |
|---|---|---|---|
| TF2065 ndeI for (legF) | 35 | 67 | GCCGCATATGATAAATACGGATAAAAAGATTTTGG |
| TF2065 XhoI rev (legF) | 43 | 69 | CAAATAATGCTTATATTACCTCGTTAGATATAGGAGCTCTACG |
| TF2074 NdeI for (legC) | 35 | 67 | GCATCATATGGAGAGCAGCTATAAGAAAATAACAG |
| TF 2074 XhoI rev (legC) | 35 | 69 | CCATTGATCATAACAAACGTATACACGT |
| TF2075-C-for (legB) | 28 | 63 | TGCACATATGCAAACAATACTAGTTACC |
| TF2075-C-rev (legB) | 31 | 72 | ATTTAGGCCCTATATGTTGCACGAGCTCCGG |
| pseB_C_NcoI_for | 40 | 68 | AATCACCATGGTAAATAATAAATCCATATTGATTACCGGG |
| pseB_C_XhoI_rev | 61 | 76 | CTTTTATACAACTAGGAGTAAAATTGCGTGTGGTAGTGGTAGTGGTAATTGAGCTCACTAA |
| pseC_C_NcoI_for | 33 | 69 | AATCACCATGGTAACAAGACCGATACCATATGG |
| pseC_C_XhoI_rev | 59 | 74 | CTCTTTCACTAATTAAAAAAATAGTTTGTGGTAGTGGTAGTGGTAATCGAGCTCACTAA |
| pseF_C_NcoI_for | 33 | 68 | AATCACCATGGAAATATTGGCTATTACTCAGGC |
| pseF_C_XhoI_rev | 60 | 72 | CAGTTATTTATTACTAAATTTTTATTCTGTGGTAGTGGTAGTGGTAATTGAGCTCACTAA |

# 4 Results and Discussion

## 4.1 Cultivation and selection of clinical isolates for further investigations

The provided clinical isolates UB 4, UB 20, 0408, 0348, 1046, 1055, 1056 and 0471 were taken out of the cryogenic stocks and then incubated anaerobically in 10 mL of BHI medium supplemented with 500 µL of horse serum and 20 µL of *N*-acetylmuramic acid at 37°C. As *T. forsythia* is a slow growing organism [Tanner and Izard 2006], *in vitro* cultivation in media took a few days before growth was visible. In case growth could be accomplished, after two to three days, sedimenting cells formed at the bottom of the vial. Periodic measurement of$OD_{600}$ was taken as an evidence for increase in biomass.

Considering that the *T. forsythia* isolates differed markedly in the duration of their lag-phases, cells were passaged as soon as an $OD_{600}$of 1.0 had been reached. While *T. forsythia* ATCC 43037 type strain had lag phase times of ~8 h, slow growing isolates 0348, 0471, 1056, 0408 and 1046 exhibited lag phases that lasted for 48 h until first growth was visible. Inoculation of 250 µL was done in 10 mL of medium and growth was prolonged until an $OD_{600}$ of ~0.25 had been reached. Subsequently, 1-mL samples were taken for further biochemical and genetic characterization. For a back-up, cryogenic stocks were made of the well growing isolates by mixing 875 µL of cells in media with 125 µL of glycerol followed by storage of the cultures at -80°C.

## 4.2 Protein-chemical description

The isolates were first analyzed by SDS-PAGE to screen for glycosylated *T. forsythia* S-layer proteins [Posch, Pabst *et al.* 2011].

### 4.2.1 SDS-PAGE gel pattern of the type strain, BAA-2717 and clinical isolates

An SDS-PAGE gel where the type strain of *T. forsythia* ATCC 43037, BAA-2717 (strain 92A2) and clinical isolates UB 4, UB 20, 1055, 0471 and 0348 are shown together is provided in **Figure 26**.

The type strain (lane 1) of *T. forsythia* ATCC 43037 was used as a reference for detecting the TfsA and TfsB S-layer glycoproteins. The SDS-PAGE protein pattern of ATCC 43037 typically showed bands migrating at 230 kDa and 270 kDa of mass, which corresponds to the glycosylated S-layer proteins TfsA and TfsB, respectively.

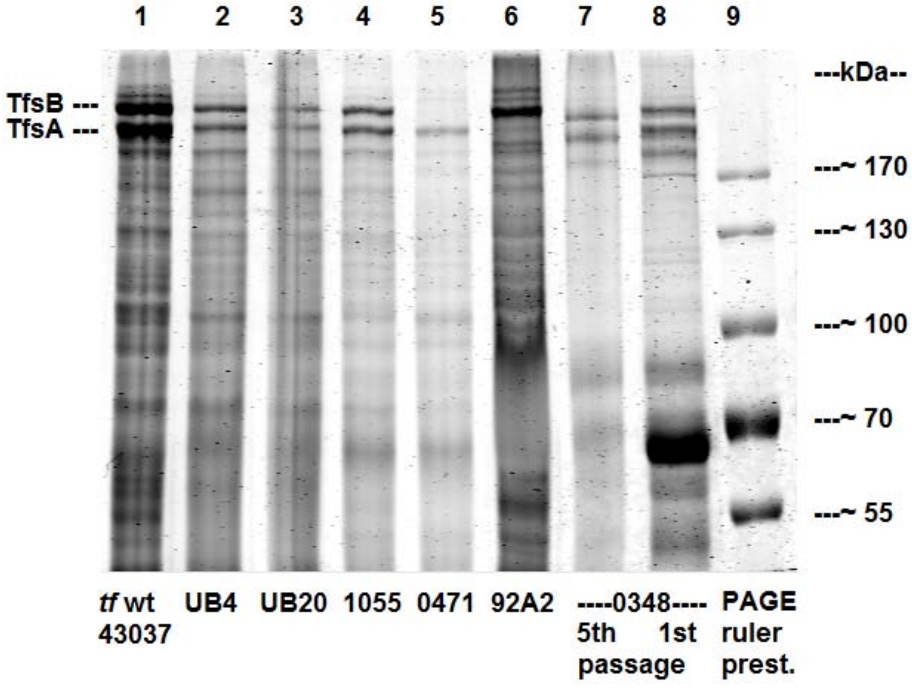


**Figure 26.** CBB-stained 7.5 % SDS-PAGE gel with *T. forsythia* type strains ATCC 43037 and FDA 92A2 and clinical isolates UB 4, UB 20, 1055, 0471, 92A2 and 0348. Page ruler pre-stained protein ladder was used in lane 9

Isolate UB 4 (lane 2) grew well from the start when cultivating it under given *Tannerella* cultivation conditions (compare with 3.6), showing immediate growth and well-formed pellets after about 5 days of incubation. Isolate UB 4 clearly revealed both TfsA and TfsB S-layer proteins on the gel. After a

few cultivation attempts out of the stab culture provided by Dr. Ashu Sharma, cultivation on agar plates followed by cultivation in liquid BHI-medium was successful. Cultivation as well as passaging of isolate UB 20 (lane 3) turned out to be reproducible under the standard conditions applied. It grew well after each passage and managed to build up an acceptable pellet to work on with after 5-6 days of growth.

Clinical isolate 1055 (lane 4) needed 5-7 passages to get used to liquid BHI medium. Fortunately this isolate formed a usable pellet of biomass but never reached an $OD_{600}$ above 0.75. Both TfsA and TfsB S-layer proteins were visible on an SDS-PAGE gel. This isolate might be a promising candidate to be included in further studies of *T. forsythia.*

Clinical isolate 0471 (lane 5) of *T. forsythia* started to grow slowly with a lag phase of 48 h in liquid BHI culture when biomass was taken out of cryo stock. After 3 passages growth started to get well enough to generate enough biomass to work with.

As described above (see chapter 1.8), the genome of strain ATCC BAA 2717 (synonymous to 92A2) was erroneously attributed to the type strain ATCC 43037. After clarifying this situation by [Friedrich, Pabinger *et al.* 2015] and the fact that actually everyone in the field of *T. forsythia* research was working with the genome information of a strain that had never been investigated experimentally, it was analyzed together with the clinical isolates within the frame of this master thesis. The strain (lane 6) grew satisfyingly after 3 passages and formed a little pellet.

In clinical isolate 0348, both TfsA and TfsB could be visualized by SDS-PAGE, where in lane 8, after the first passage the S-layer bands were migrating regularly at 230 and 270 kDa respectively. In lane 7, the same strain suddenly showed slightly down-shifted bands after 5 passages. It could be assumed that loss of a charged group caused this downshift. However this observation has not been further investigated because strain 0348 was very challenging to grow in liquid BHI culture and even after 6-8 passages only a small pellet of bacterial biomass to work on was formed. It was hardly enough to perform further. This strain cannot be recommended for future work approaches.

Many approaches like cultivating on BHI and blood agar plates have been made to cultivate isolate 1046 in a suitable manner but no approach was successful. 1046 (**Figure 27**) grew slowly with lag phases of 3-4 days and only with little pellet formation in liquid BHI medium and biomass was barely enough to take samples. Samples for SDS-PAGE never showed the desired S-layer proteins on the CCB- stained gel and, therefore, this isolate was not further analyzed.

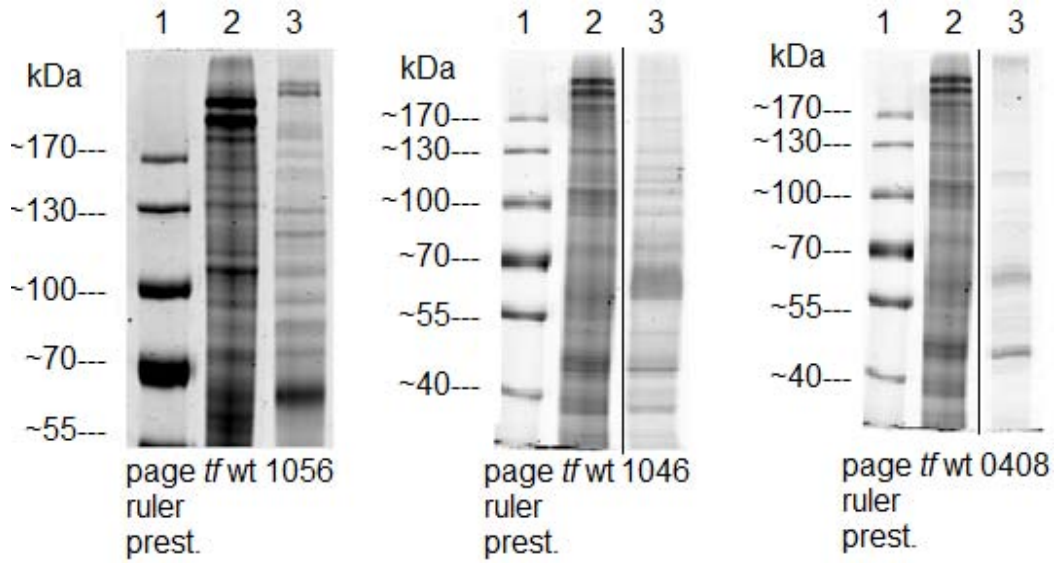


**Figure 27.** SDS-PAGE pattern of clinical isolates 1056, 1046 and 0408

Clinical isolate 1056 (**Figure 27**) initially appeared to be a promising candidate for further investigations, because it grew relatively well and fast on BHI plates and in liquid BHI medium with proper pellet formation to work on. However, according to SDS-PAGE analysis biomass production was very low; thus no S-layer proteins for further investigations could be visualized. There is currently no explanation so far why this strain is not expressing the S-layer proteins under given *Tannerella* cultivation conditions (see chapter 3.6), because the strain possesses the genetic information for synthesizing S-layer proteins TfsA & TfsB (compare with chapter 4.3.1.3).

Clinical isolate 0408 (**Figure 27**) showed severely retarded growth on BHI plates and when transferred into liquid BHI medium growth still was not satisfying with lag phases of 3-4 days. However the strain managed to build up a very small incompact pellet which was difficult to analyze. As several cultivation attempts on blood agar plates and in liquid BHI medium supplemented with 500 µL of horse blood did not change the situation and still only negligible growth occurred, the strain was not analyzed further.

## 4.3 Genetic description

### 4.3.1 Polymerase chain reactions

#### 4.3.1.1 16S rRNA - PCR reaction

To check that the clinical isolates UB 4, UB 20, 0471, 1055 and 1056 were indeed *T. forsythia* bacteria, 16S rRNA PCR analysis was done with the type strain ATCC 43037 serving as a positive reference. 16S ribosomal RNA appears as a component in the 30S small subunit of prokaryotic ribosomes and the genes coding for the 16S rRNA are highly conserved throughout organisms.

Suitable primers (compare with **Table 7**) for the genetic locus coding for *T. forsythia* 16S rRNA were chosen and PCR was performed. The amplified gene product was 923 bp in length and was calculated with 'Clone manager' software. The 30S small subunit of prokaryotic ribosomes is highly conserved within organisms due to the slow rates of evolution of this region of the gene. The 923-bp product of the 16S rRNA - PCR reaction (see **Figure 28**) was then sent for DNA sequencing (Microsynth), confirming that all isolates selected for further investigations are *T. forsythia* bacteria.

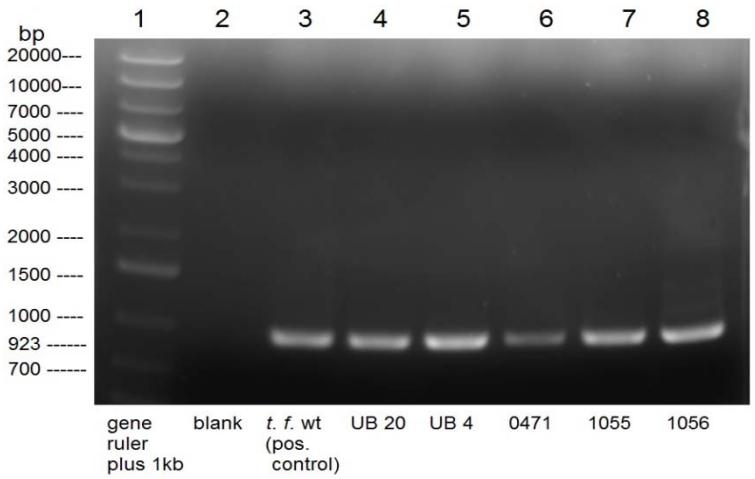


**Figure 28.** Agarose gel (0.9 %) showing the amplified 16S rRNA gene of selected *T. forsythia* clinical isolates

#### 4.3.1.2 Screening the genomes of *T. forsythia* strains for the presence of genes of nonulosonic biosynthesis pathways

The complex *T. forsythia* S-layer *O*-glycan structure of the ATCC 43037 type strain has been described by our group previously (compare with **Figure 4**) [Posch, Pabst *et al.* 2011]. At the prominent, terminal position, pseudaminic acid (Pse) was found to be present on the S-layer glycan of this strain in a combined NMR and MS approach.

Using carefully designed primer pairs (compare with **Table 13**) targeted at individual genes encoding enzymes of nonulosonic biosynthesis pathways (*i.e.,* Pse and Leg, respectively, compare **Figures 6** and **7**) the genomes of the type strains and of the clinical isolates were screened (for combination of primers see **Table 14**). *T. forsythia* type strain ATCC 43037 served as a positive control for Pse [Posch, Pabst *et al.* 2011] and the clinical isolates UB 4, UB 20, 0348, 1055, 1046, 0408, 0471, 1056 as well as strain ATCC BAA-2717 (92A2) were subjected to screening and further analysis.

**Table 14**: Primer pairs for amplification of putative nonulosonic biosynthesis genes and product formation for unraveling the structure of the *T. forsythia*. Obtained PCR products of the respective combinations are highlighted in bold.

| | pseB for/rev | pseC for/rev | pseF for/rev | legB for/rev | legCfor/rev | legF for/ rev |
|---|---|---|---|---|---|---|
| ***T.f.* wt** | **product** | **product** | **product** | no product | no product | no product |
| **UB4** | no product | no product | no product | **product** | **product** | **product** |
| **UB20** | no product | no product | no product | **product** | **product** | **product** |
| **1055** | no product | no product | no product | **product** | **product** | **product** |
| 1046 | no product | no product | no product | no product | no product | no product |
| 0408 | no product | no product | no product | no product | no product | no product |
| **0471** | no product | no product | no product | **product** | **product** | **product** |
| **1056** | no product | no product | no product | **product** | **product** | **product** |
| **0348** | **product** | **product** | **product** | no product | no product | no product |
| **BAA-2717 (92A2)** | no product | no product | no product | **product** | **product** | **product** |

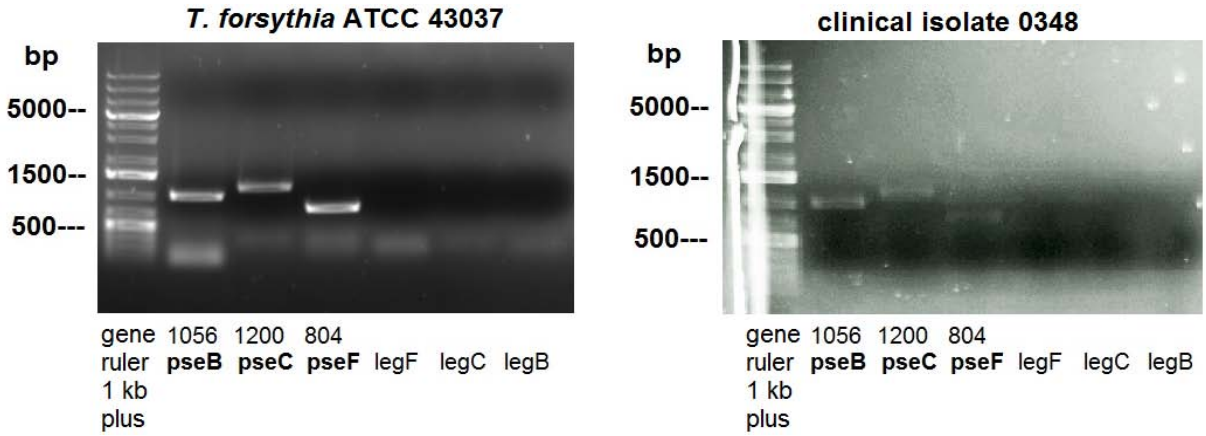


**Figure 29.** Agarose gels (0.9 %) of *T. forsythia* ATCC 43037 type strain and isolate 0348, both respective genomes generated products of amplified genes during screening PCR with selected primers for *pseB, pseC* and *pseF* but no products were obtained with primers aiming at *legB, legC* and *legF*

**Figure 29** indicates the presence of *pseB*, *pseC* and *pseF* indicative of pseudaminic acid in *T. forsythia* ATCC 43037 and clinical isolate 0348. In **Table 14**, the primer pairs used for the screening are listed and the respective products formed are written in boldface. *T. forsythia* ATCC BAA-2717 and clinical isolates UB 4, UB 20, 1055, 0471 and 1056 delivered products when screened with primer pairs (listed

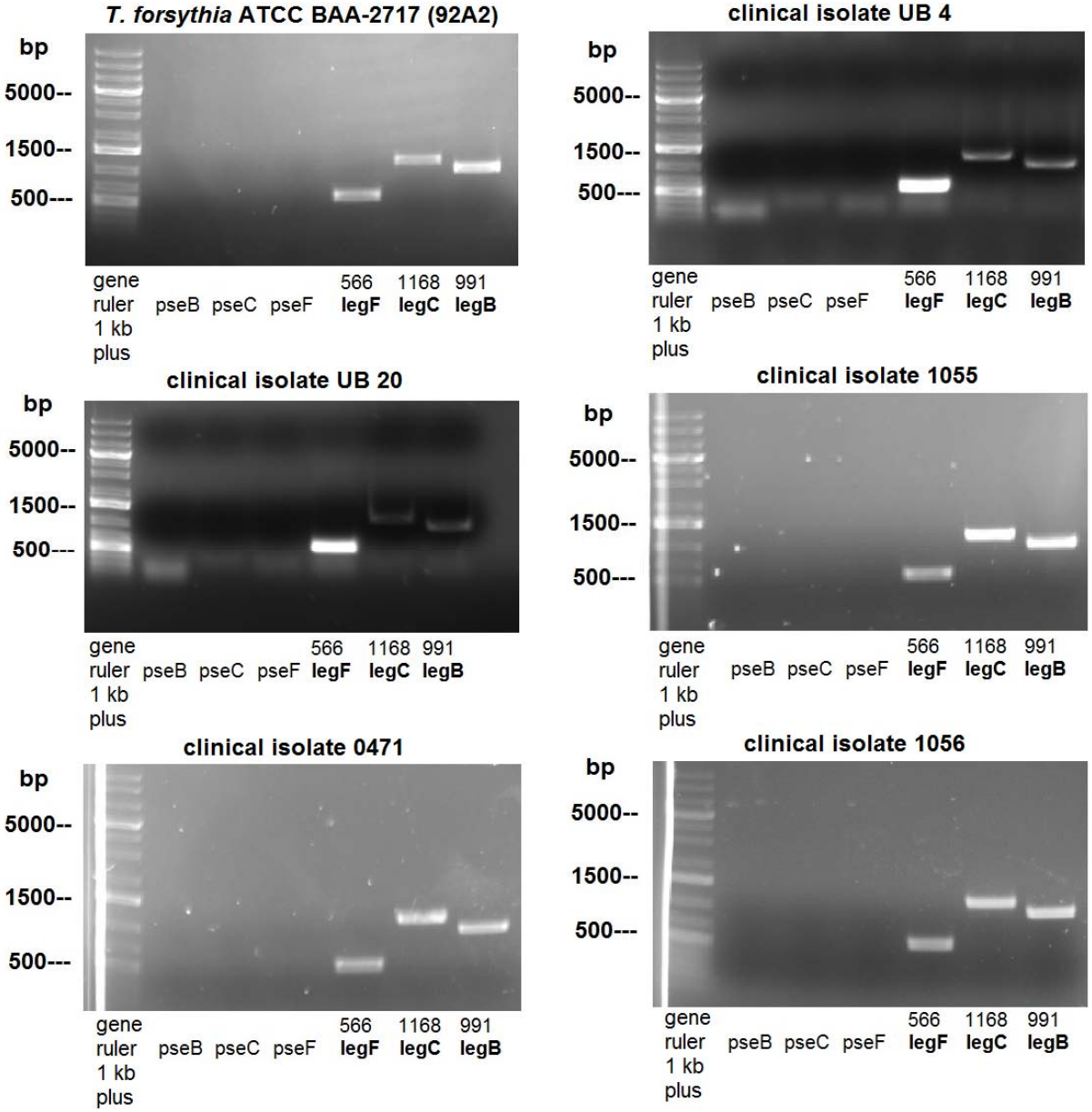


**Figure 30.** Agarose gels (0.9 %) of *T. forsythia* ATCC BAA-2717, isolates UB 4, UB 20, 1055, 0471 and 1056. All respective genomes generated products of amplified genes during screening PCR with selected primers for *legB*, *legC* and *legF* but generated no products with primers aiming at *pseB*, *pseC* and *pseF*

in **Table 14**) for *legB*, *legC* and *legF* indicative of legionaminic acid. The result of the screening is provided by **Figure 30**. The clinical isolates 0408 and 1046 have also been screened using the same primer pairs (see **Table 14**) but no amplification products could be obtained (**Figure 31)**.

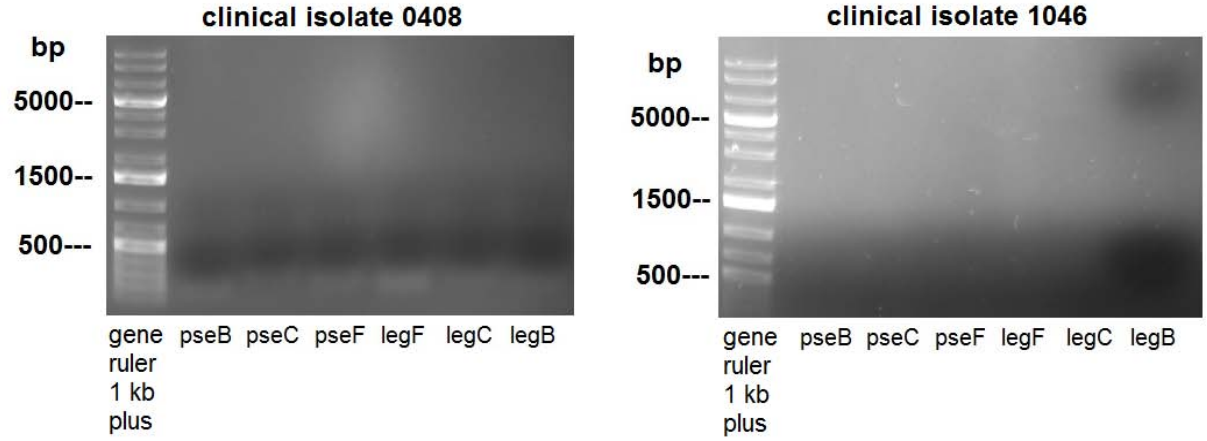


**Figure 31.** Agarose gels (0.9 %) of clinical isolates 0408 and 1046. In both strains neither the primers aiming at crucial enzymes appearing during the pseudaminic acid biosynthesis nor primers targeting enzymes appearing in the legionaminic acid biosynthesis pathway delivered a product during PCR screening

The screening of the genomes of the selected strains and clinical isolates revealed that two strains, including the ATCC 43037 type strain and clinical isolate 0348 possess genes of the *de novo* biosynthetic pathway for pseudaminic acid (compare with **Figure 6**). Six strains, including the ATCC BAA-2717 strain and clinical isolates UB 4, UB 20, 1055, 0471 and 1056 show the presence of genes coding for the respective enzymes of the legionaminic acid biosynthesis pathway (see **Figure 7**). Clinical isolates 0408 and 1046 could not be satisfyingly analyzed due to insufficient growth.

It became evident that none of the investigated *T. forsythia* strains showed both pathways for building up pseudaminic acid and legionaminic acid. Thus, coexistence of the two nonulosonic acid biosynthetic pathways could be excluded for *T. forsythia*, at least for the investigated strains.

#### 4.3.1.3 Screening PCR to detect the genes encoding the TfsA and TfsB S-layer proteins

As not all of the isolates expressed the two S-layer proteins TfsA or TfsB proteins according to SDS-PAGE evidence (see **Figures 26** and **27**), it should be analyzed whether or not the isolates had the principal genetic potential of expressing respective S-layer genes. Our data shows that all *T. forsythia* isolates included in this study possessed both S-layer genes, *tfsA* and *tfsB*, as evidenced by the amplification products of 4035 and 3490 bp, respectively (**Figure 32**), conforming with the calculation using the 'Clone manager' software.

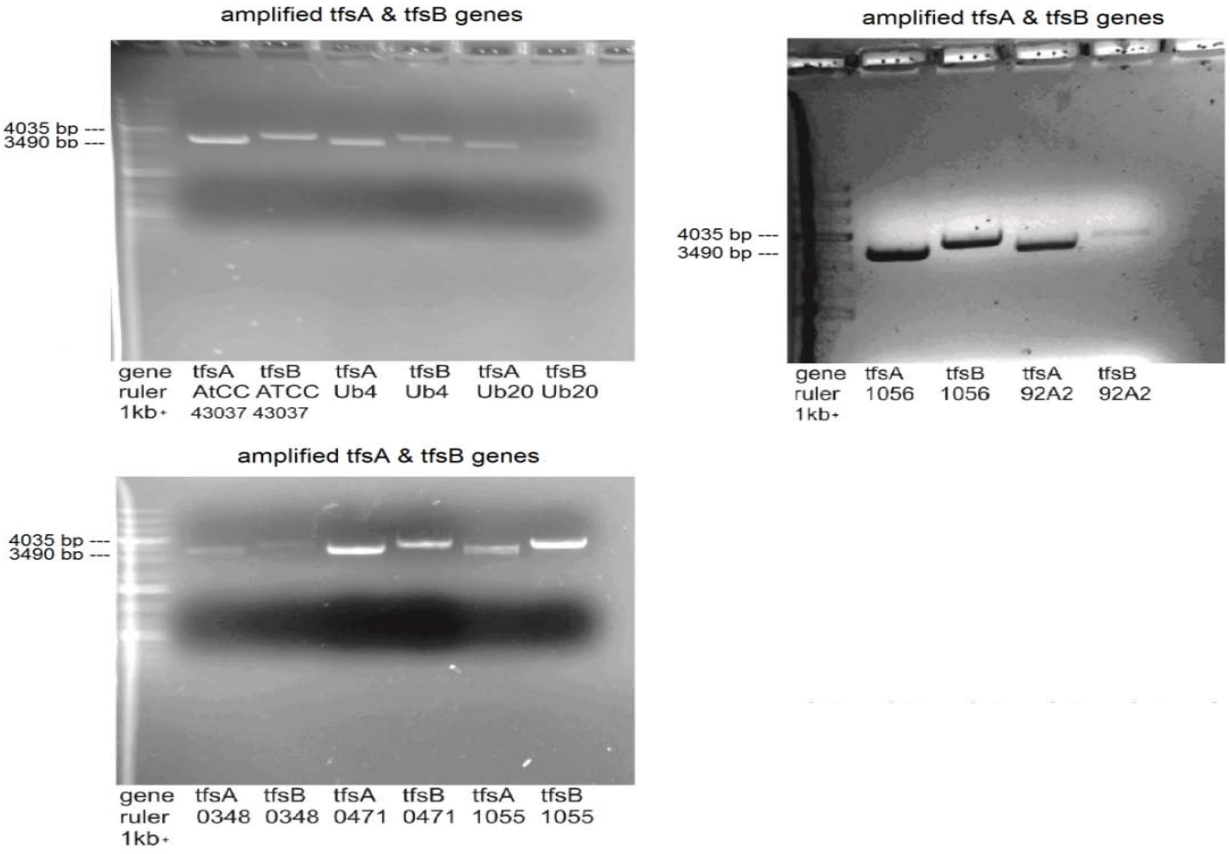


**Figure 32.** PCR screen for the presence of the S-layer genes *tfsA* and *tfsB* in clinical isolates of *T. forsythia*

## 4.4 Glycanstaining

### 4.4.1 PAS staining

To visualize glycoproteins, especially the S-layer glycoproteins, upon separation by SDS-PAGE, the PAS staining reaction was used. The highly glycosylated S-layer proteins could be made easily visible by this staining method. The method was applied early in the course of this study after the first clinical isolates were growing to immediately investigate the presence of carbohydrates on S-layer proteins. *T. forsythia* ATCC 43037, clinical isolates UB 20, UB 4, 0471 and 1055 were investigated by PAS staining and ATCC 43037, UB 20 and UB 4 delivered positive results, clearly visible as pink bands, as shown in **Figure 33**. As expected the highly glycosylated S-layer proteins were identifiable by strongly stained bands at 230 and 270 kb respectively. It should be mentioned that isolates 0471 and 1055 only showed very low amounts of stained protein due to the lack of biomass at the beginning of the study.

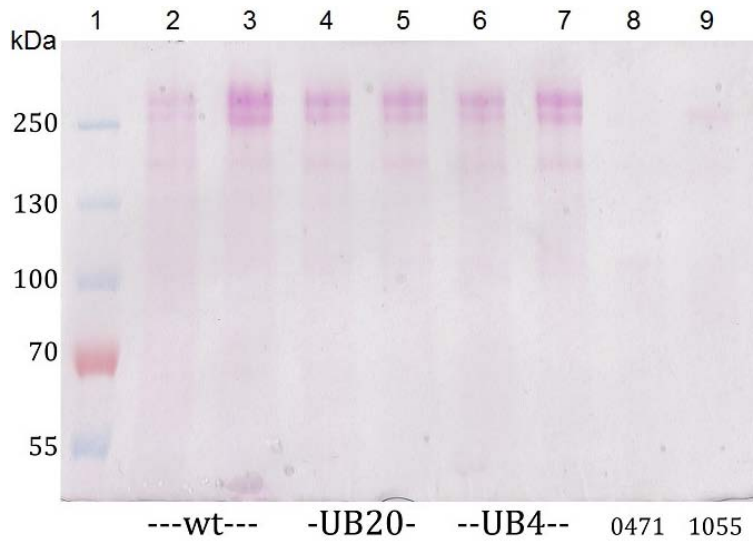


**Figure 33.** PAS stained SDS-gel of *T. forsythia* wt, isolate UB 20 and isolate UB 4

### 4.4.2 Pro-Q-Emerald glycostaining

Further, the *T. forsythia* ATCC 43037, ATCC BAA-2717 and isolates UB 4, UB 20, 1055, 0471 and 0348 were stained according to the Pro-Q-Emerald supplier's manual. The bright green fluorescent signal on glycoproteins, especially on S-layer proteins, was visible throughout the investigated strains as shown by **Figure 34**. This sensitive carbohydrate staining method delivered useful results and the presence of glycosylated S-layer proteins on ATCC 43037 type strain, isolates UB 4, UB 20, 1055 and 0348 could be confirmed. Clinical isolate 0471 only expressed S-layer protein TfsA, as mentioned earlier, thereby TfsA could be stained. In contrast to 0471, ATCC BAA-2717 only expressed TfsB, which could be affirmed by the stained TfsB S-layer glycoprotein.

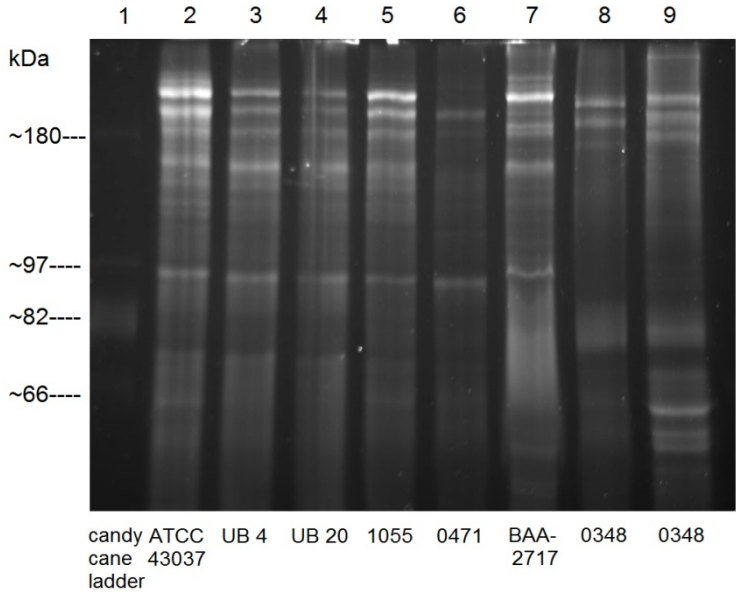


**Figure 34**. Pro-Q-Emerald glyco-stained SDS-gel of *T. forsythia* wt and isolates UB 4, UB 20, 1055, 0471, 0348 and strain BAA-2717 (92A2)

## 4.5 Western Blot

The S-layer proteins TfsA and TfsB were detected with anti-TfsA and anti-TfsB antibodies using the Western immuno-blotting technique. Before blotting, two gels loaded with the same amount of the particular isolate were run on SDS-PAGE gels at 200 V for 90 min.

PCR revealed that *T. forsythia* ATCC 43037, ATCC BAA-2717 and clinical isolates UB 4, UB 20, 1055, 1056, 0348 and 0471 possessed the genetic information for expressing the two S-layer proteins. SDS-PAGE displayed that certain isolates express both S-layer proteins equally strong, other isolates only showed one of the S-layer proteins on the CBB-stained gel. Western immunoblotting is a sensitive technique for detection of also small amounts of protein. Thus, it was clarified whether ATCC BAA-2717 and clinical isolate 0471, indeed expressed only one S-layer protein upon prolonged passaging.

Antibodies against S-layer protein TfsA are known to bind properly and were detected with an Odyssey infrared imaging scanner. While TfsA bands were clearly visible on *T. forsythia* ATCC 43037, isolates UB 4, UB 20, 1055 and 0471, strain BAA 2717 (92A2) delivered no band for TsfA, confirming the lack of its expression (**Figure 35**).

Anti-TfsB antibody gave a signal in *T. forsythia* ATCC 43037, isolates UB 4, UB 20, 1055, and strain BAA-2717 (92A2). Isolate 0471 never showed a protein band on SDS-PAGE gels for TfsB and, here, Western blotting also failed in detecting the respective S-layer protein band (**Figure 36**).

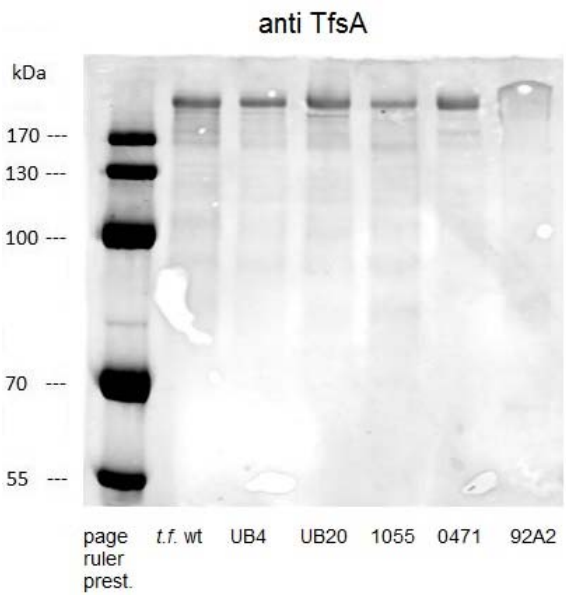


**Figure 35.** Westernblot of *T. forsythia* strains upon probing with anti-TfsA antibody

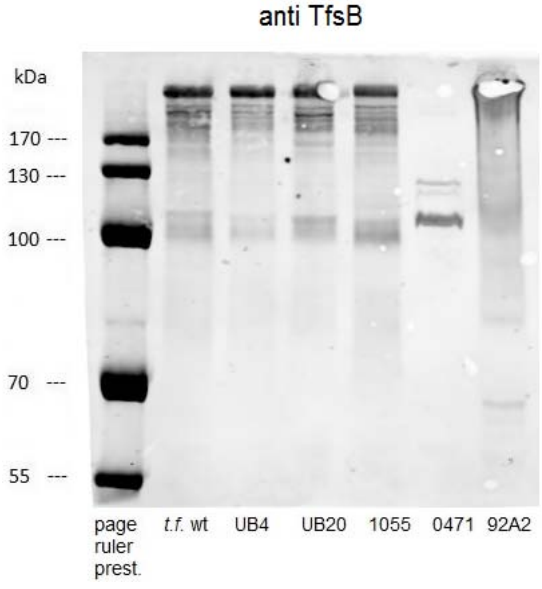


**Figure 36.** Westernblot of *T. forsythia* strains upon probing with anti-TfsB antibody

## 4.6 MS analysis of S-layer *O*-glycans of *T. forsythia* isolates after β-elimination

Based on the clear evidence of S-layer protein glycosylation and the presence of Pse and Leg biosynthesis genes in the genomes of strains ATCC 43037, isolate 0348 and ATCC BAA-2717, UB 4, UB 20, 1055, 1056, 0471 and 0348, respectively, (see above), analysis of the β-eliminated S-layer glycans was done after purifying them over a HyperSepHypercarb SPE 25 mg cartridge with following elution in Milli-Q water. Subsequent liquid chromatography, electrospray ionization and mass spectrometry of the samples delivered the following spectra and results.

For facilitated interpretation, the already investigated *T. forsythia* S-layer glycan structure (**Figure 4**) [Posch, Pabst *et al.* 2011] for the ATCC 43037 wild-type strain and the MS fragmentation pattern (**Figure 37**) was used for comparison with the obtained results.

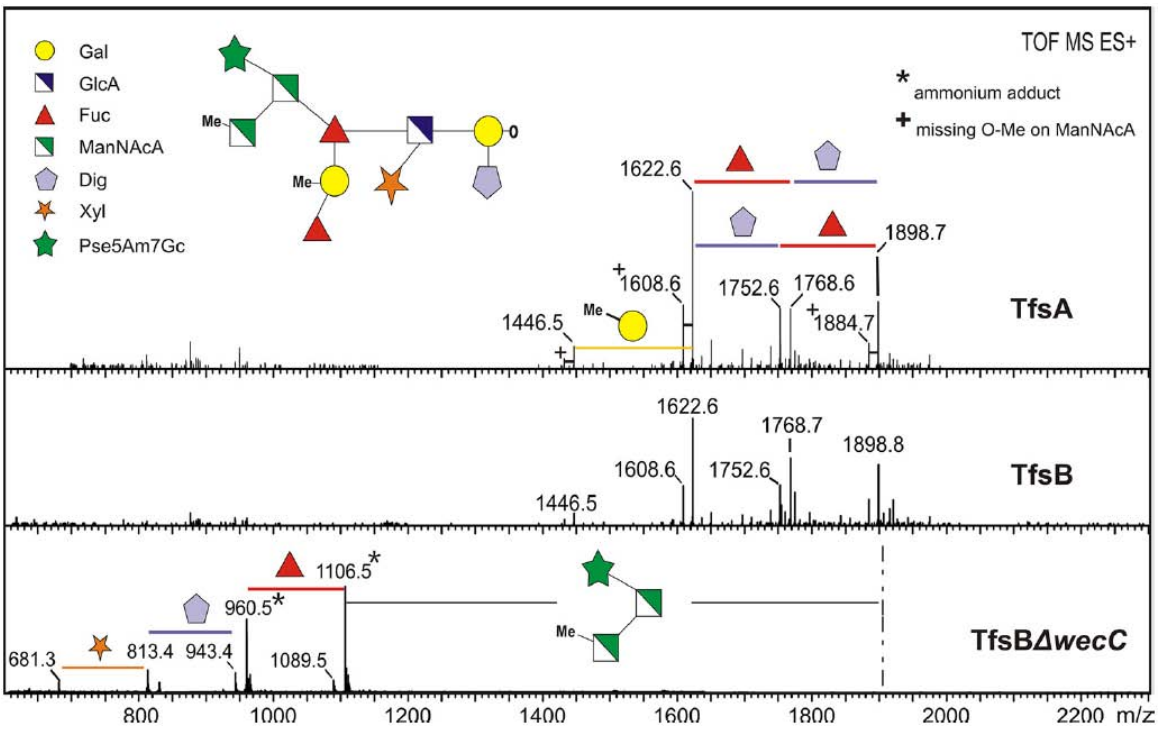


**Figure 37.** MS-glycan fragmentation of *T. forsythia* ATCC 43037 type strain serving as a control in the course of this study; taken from [Posch, Pabst *et al.* 2011]

A slight difference to the structure published for ATCC 43037 by Posch *et al.* (2011) was found out during the PCR experiments in this thesis and is included in **Figure 38**. According to our data, the nonulosonic acid present in the individual S-layer glycans of the different isolates can either be pseudaminic or legionaminic acid.

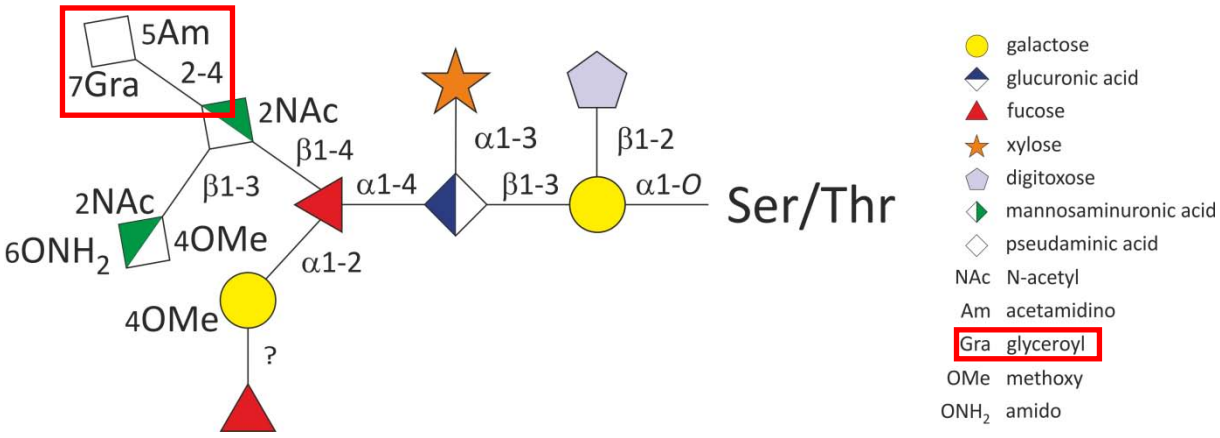


**Figure 38.** Refined S-layer glycan structure of *T. forsythia* ATCC 43037 type strain according to latest data (compare with **Figure 4** and [Posch, Pabst *et al.* 2011])

### 4.6.1 Glycan composition of *T. forsythia* type strain ATCC 43037

The S-layer oligosaccharide is *O*-glycosidically linked via the reducing-end residue α-galactose to serine and threonine residues of both the TfsA and TfsB S-layer proteins. The wild-type ATCC 43037 glycan consists of eight or nine common and one uncommon sugar residue, sometimes a fucose residue labelled with a question mark is missing with a natural variability.

Posch and coworkers found that their MS and NMR data was in better accordance with pseudaminic acid than with the stereoisomer legionaminic acid to be the nonulosonic acid compound found on the glycan [Posch, Pabst *et al.* 2011]. Within this master thesis, the findings of PCR analysis of the respective locus confirmed the presence of pseudaminic acid in the ATCC 43037 type strain of *T. forsythia.* Through an error in nomenclature, the nonulosonic compound was found to be Pse5Am7Gra (compare with **Figure 39**) and not Pse5Am7Gc, indicating that this sugar compound is

modified on C7 with a glyceroyl group instead of a glycolyl group. So the overall updated composition of the glycan is 4-Me*O*-β-Man*p*NAcCONH2-(1→3)-[Pse5Am7Gra-(2→4)-]-β-Man*p*NAcA-(1→4)-[4-Me*O*-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*. The molecular formula of the entire ATCC 43037 glycan is $C_{73}H_{120}N_6O_{51}$ with a mass of 1897.74 Da, which corresponds to the published mass of 1897.7 [Posch, Pabst *et al.* 2011].

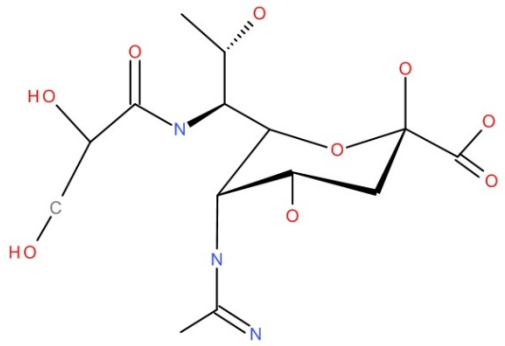


**Figure 39.** Structure of Pse5Am7Gra (pseudaminic acid with acetamidino- on the C5 and glyceroyl-modification on the C7) as it appears on the *T. forsythia* ATCC 43037 wild-type glycan

### 4.6.2 Glycan composition of *T. forsythia* clinical isolate UB 4

For evaluation of MS data of clinical isolate UB 4, fragmentation panels 1 and 4 are presented (**Figure 40**). Analysis of the peak at 863.3 Da (m/z) showed that the intact glycan is missing 27 Da in comparison to the ATCC 43037 wild type glycan analyzed by [Posch, Pabst *et al.* 2011]. This difference could derive from the loss of the acetamidino-modification on C5 of the legionaminic acid and the loss of both 4-O-Me modifications, where one is situated on the outer arm of a terminal N-acetyl-mannosaminuronic acid. Additionally, a fucose broke away from the glycan during fragmentation. The mass of the wild-type ATCC 43037 glycan was affirmed to be 1897.7 Da. During this fragmentation, the charge was 2 fold. The peak at 863.3 Da had to be doubled to back-calculate the charge and gave 1726.6 Da. The fucose contributed for 146.06 Da and both 4-O-Me modifications were 28 Da and assumed as the mass defect. The acetamidino-modification gave another 1 Da of mass

and finally there were two hydrogen adducts in the spectrum which contributed another 2 Da. Calculation of 1726.6 intact glycan peak + 146.06 Da fucose + 28 Da of 2 times 4-O-Me - 1 Da of missing acetamidino - modification – 2 Da of the hydrogen adducts gave back 1897.66 Da which is a difference of -0.04 to the wild type glycan mass.

The peak at 842.2 was doubled to incorporate the charge into the calculations and gave 1684.4 for the molecule ion. To calculate back to the known mass of the wild type ATCC 43037 glycan, this peak was analyzed as follows: 1684.4 Da + 146.06 Da fucose + 2 fold 4-O-Me + 42 Da *N*-acetyl-group - 2 Da of the hydrogen adducts gave a mass of 1898.46 Da which is +0.76 Da higher than the mass of the wild type glycan.

Fragmentation panel 4 of the sample showed different fractioning of the glycan during MS analysis. Molecule peaks at *m/z* 1106.3 Da and 960.3 Da were predominant. The mass at 1106.3 is interpreted in the following way: the ammonium adduct charge accounted for a single charged molecule which was multiplied with the mass of the molecule peak of 1106.3. Then, the mass of the ammonium adduct (18 Da) had to be subtracted, mass of a methoxy group (14 Da, situated on the outer arm of a terminal ManNAc residue) added, mass of Leg (361.15 Da) added and finally masses of 2 molecules of N-acetyl-mannosaminuronic acid (2 x 217.07 Da) had to be added. The final mass gave 1897.59, which is the same mass as the wild type glycan displays.

The peak at 960.3 was interpreted in the same way as the one at 1106.3, with the only difference that an additional fucose has broken away from the molecule ion. Addition of the mass of fucose (146.06 Da) and calculation as above delivers a mass of 1897.65 Da, which is again in good agreement with the wild type glycan.

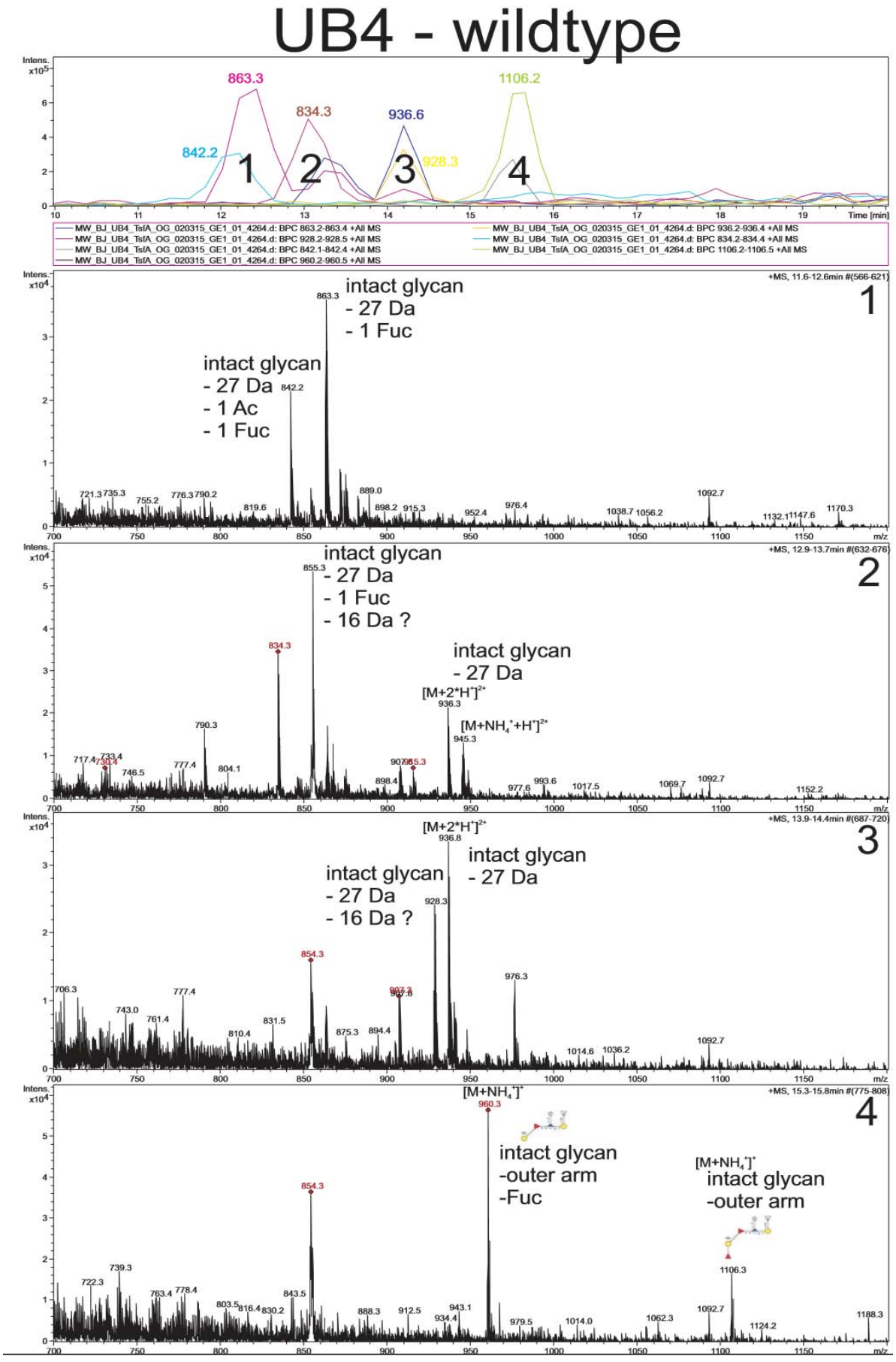


**Figure 40.** MS-spectrum for clinical isolate UB 4

### 4.6.3 Glycan composition of *T. forsythia* clinical isolate UB 20

The glycan of clinical isolate UB 20 showed very near comparable fragmentation patterns as isolate UB 4. For analysis, panels 1 and 4 were taken (see **Figure 41**). Masses of -27 Da and some additional sugars were missing on the formed molecule ions. These masses could be explained in the same way they were analyzed with isolate UB4 and could derive from an acetamidino-modification on the final nonulosonate and derive from missing methoxy-groups. Since the fragmentation results were comparable, isolate UB 20 is not explained further. All in all it looks like these isolates are very similar.

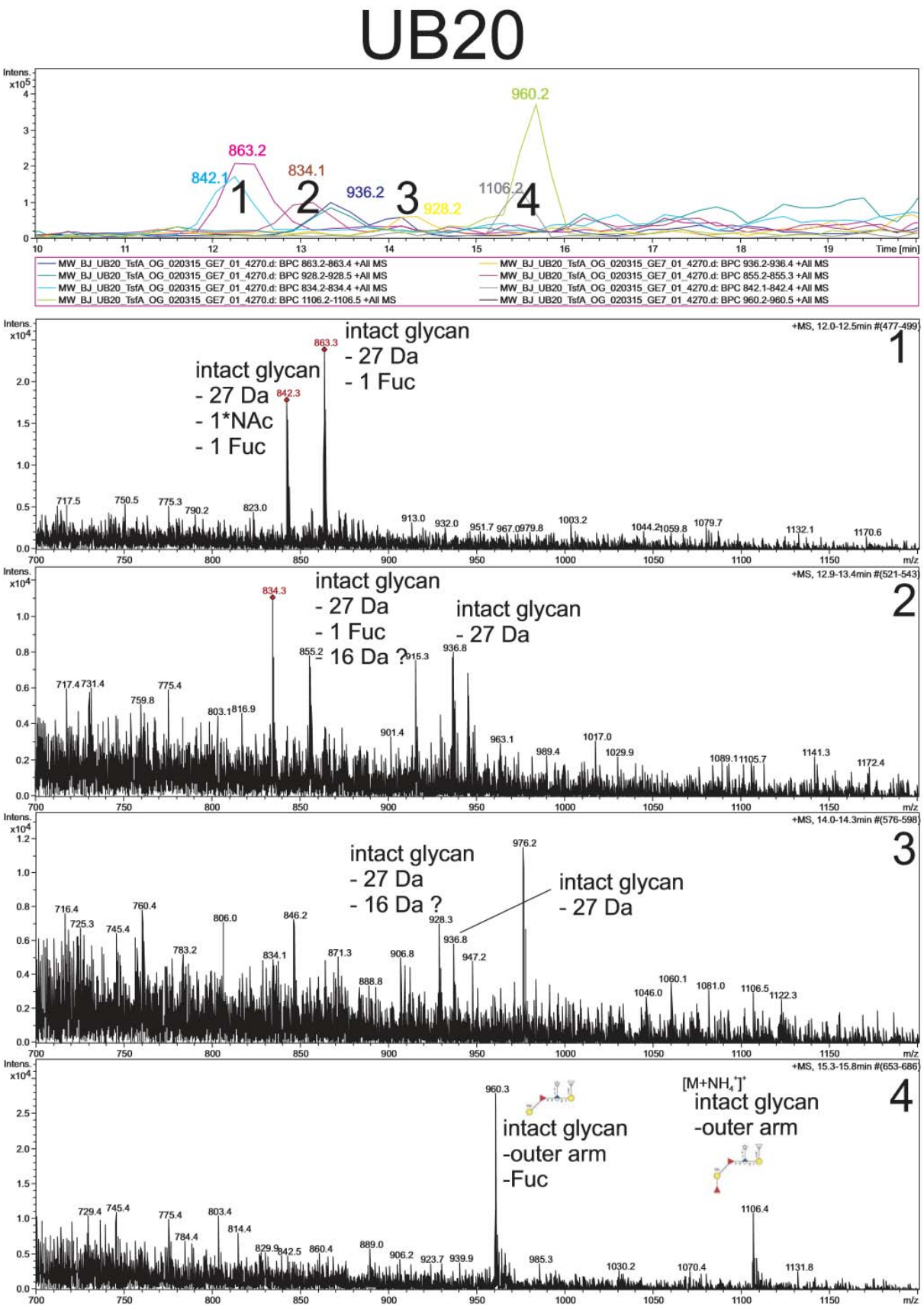


**Figure 41.** MS-spectrum for clinical isolate UB 20

### 4.6.4 Glycan composition of *T. forsythia* clinical isolate 1055

The mass of the molecule ion of the 1055 glycan could not be assigned with the MS experiment. There were just a few assumptions that have been made during analysis of fragmentation panels 1 of 1055 analysis (**Figure 42**). First, the mass difference to the ATCC 43037 *T. forsythia* type strain glycan was -57 Da in mass. After consideration of the charge of the hydrogen and ammonium adducts calculation was done as follows: the peak at 929.8 Da was multiplied by 2 and the adducts were subtracted, 1859.6 Da (assumed molecule ion) – 1 Da (hydrogen) – 18 Da (ammonium) gave 1840.6 Da. The increment of the mass was still - 57 Da that could not be explained just by adding up the mass of a common sugar of the glycan. So, this mass must stand for a modification on the glycan. Both O-Me modifications and a glycolyl- instead of a glyceroyl modification on the legionaminic acid could count for that difference of -57 Da: 2x -14 (methoxy-groups) -30 (glycolyl-modification on legionaminic acid) +1 (N-acetyl instead of N-acetamidino or amido instead of carboxy group) = -57.

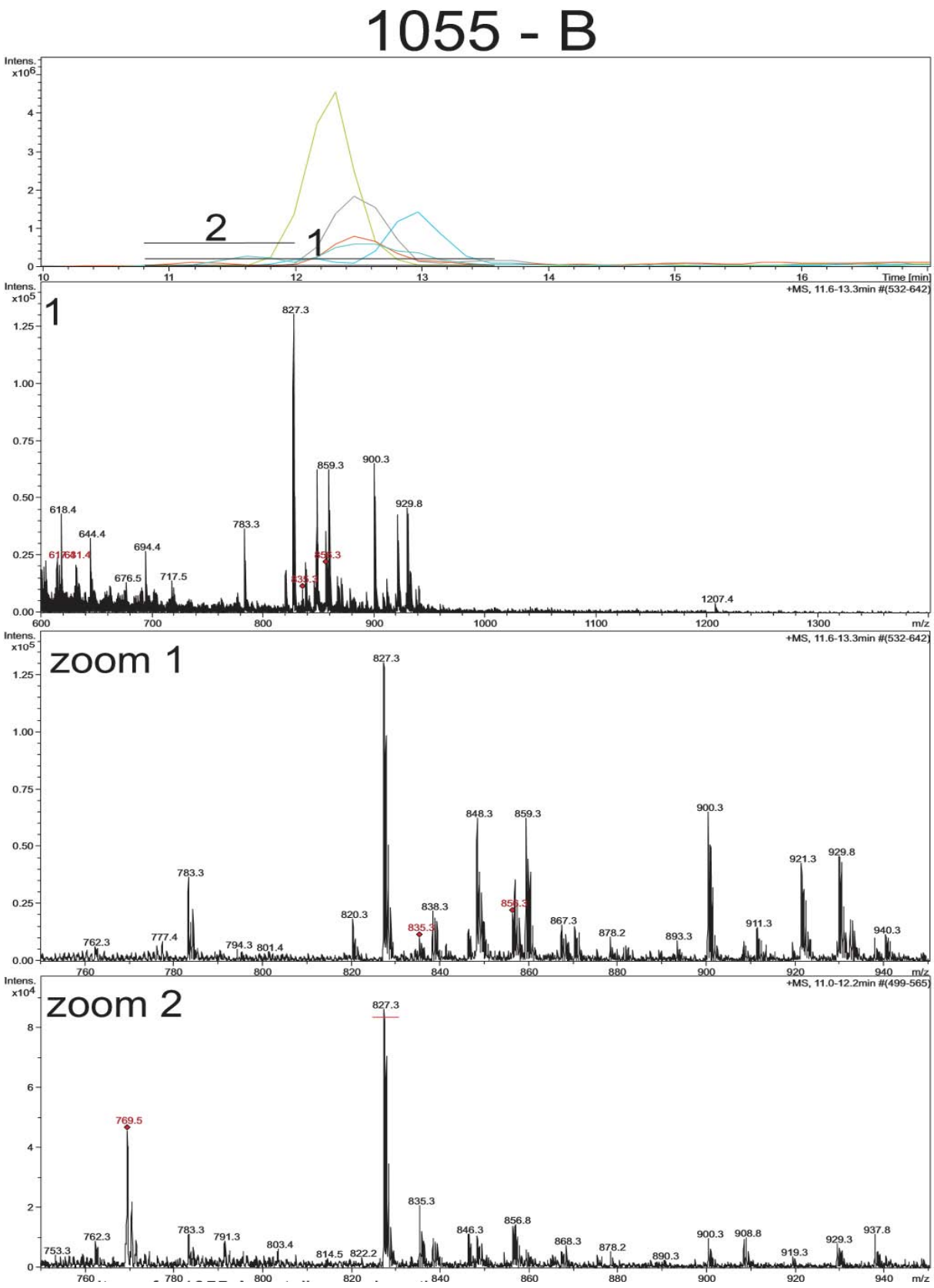


**Figure 42.** MS-spectrum for clinical isolate 1055

### 4.6.5 Glycan composition of *T. forsythia* BAA-2717 (92A2)

The glycan fragmentation pattern of this strain showed the same glycan profile as clinical isolate 1055. Thus, there is again no distinct sugar that could account for the mass differences in the MS-fragmentation spectrum (**Figure 43**). Moreover bad signal intensity was prevalent during mass spectrometry. The same assumptions as for isolate 1055 have been made and it is surely important that this strain undergoes further investigation in the future, but unfortunately that was not possible during the course of this study.

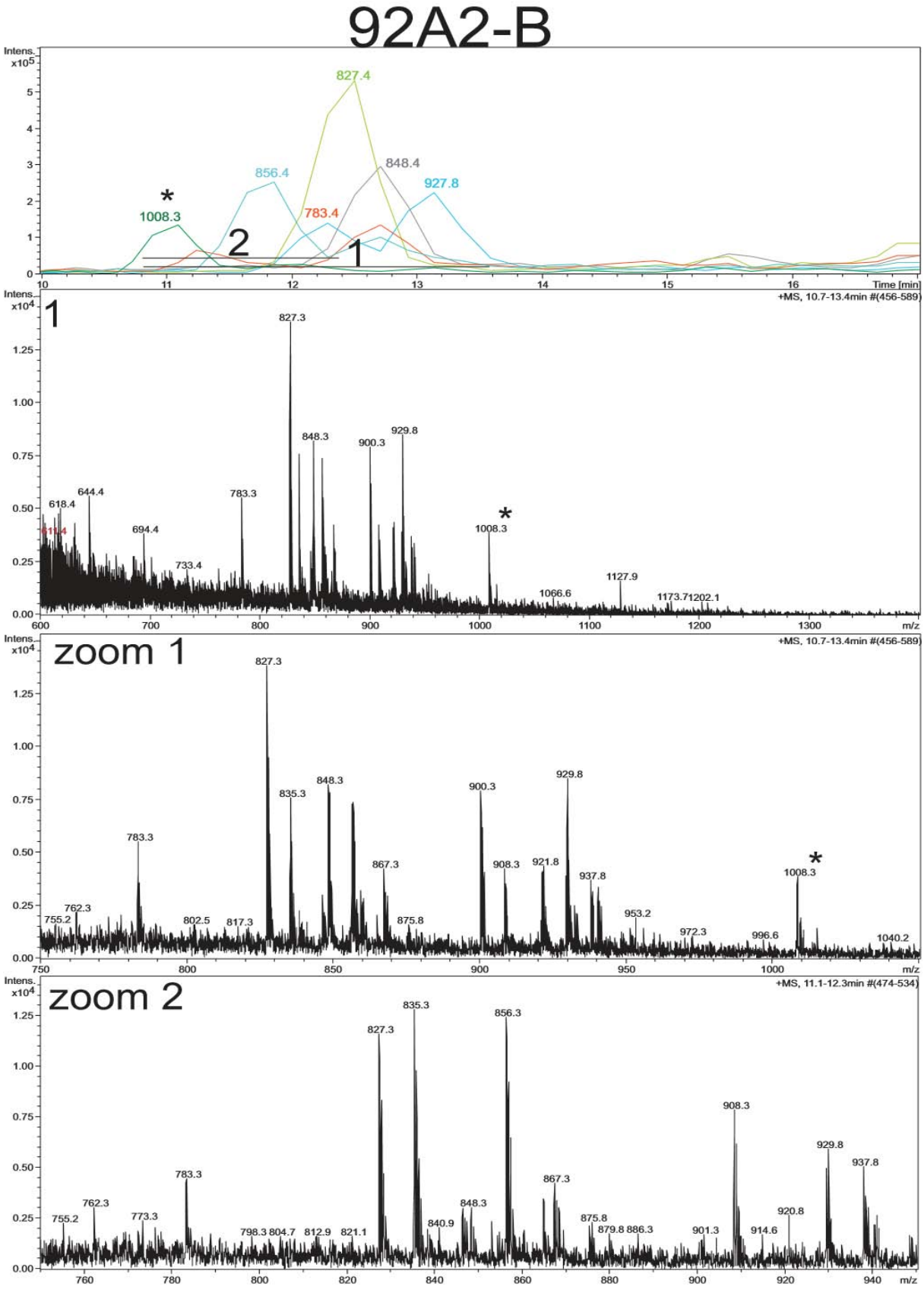


**Figure 43.** MS-spectrum for clinical isolate *T. forsythia* ATCC BAA-2717 (92A2)

### 4.6.6 Glycan composition of *T. forsythia* clinical isolate 0471

Only one S-layer could be analyzed, because isolate 0471 was always expressing only TfsA (compare with chapters 4.2.1 & 4.5). Here, a difference of -87 Da (**Figure 44**) to the *T. forsythia* ATCC 43037 type strain was observed as a peak at 906.33 Da and was assumed to be the molecule ion of the glycan structure, because the mass of the complete glycan could not be assigned to any structure. Incorporation of charge doubles the mass to 1812.66 Da. The charge came from 2 hydrogen molecules which were subtracted to make 1810.66 Da. An idea was that the missing 87 Da came from complete lack of any of the modifications which means there was no acetamidino modification on C5 and no glyceroyl- modification on C7 of the legionaminic acid. These assumptions would contribute for 45 Da, resulting in a mass of 1855.66 Da. The remaining 42 Da could come from a missing N-acetyl-group somewhere on the glycan. Adding up these 42 Da made 1897.66 Da which is exactly the mass of the *T. forsythia* ATCC 43037 wild type glycan. The profile of other detected glycan peaks (with fucose, digitoxose and other increments visible) indicated that the glycan is very similar to ATCC 43037 or isolate UB 4 glycans.

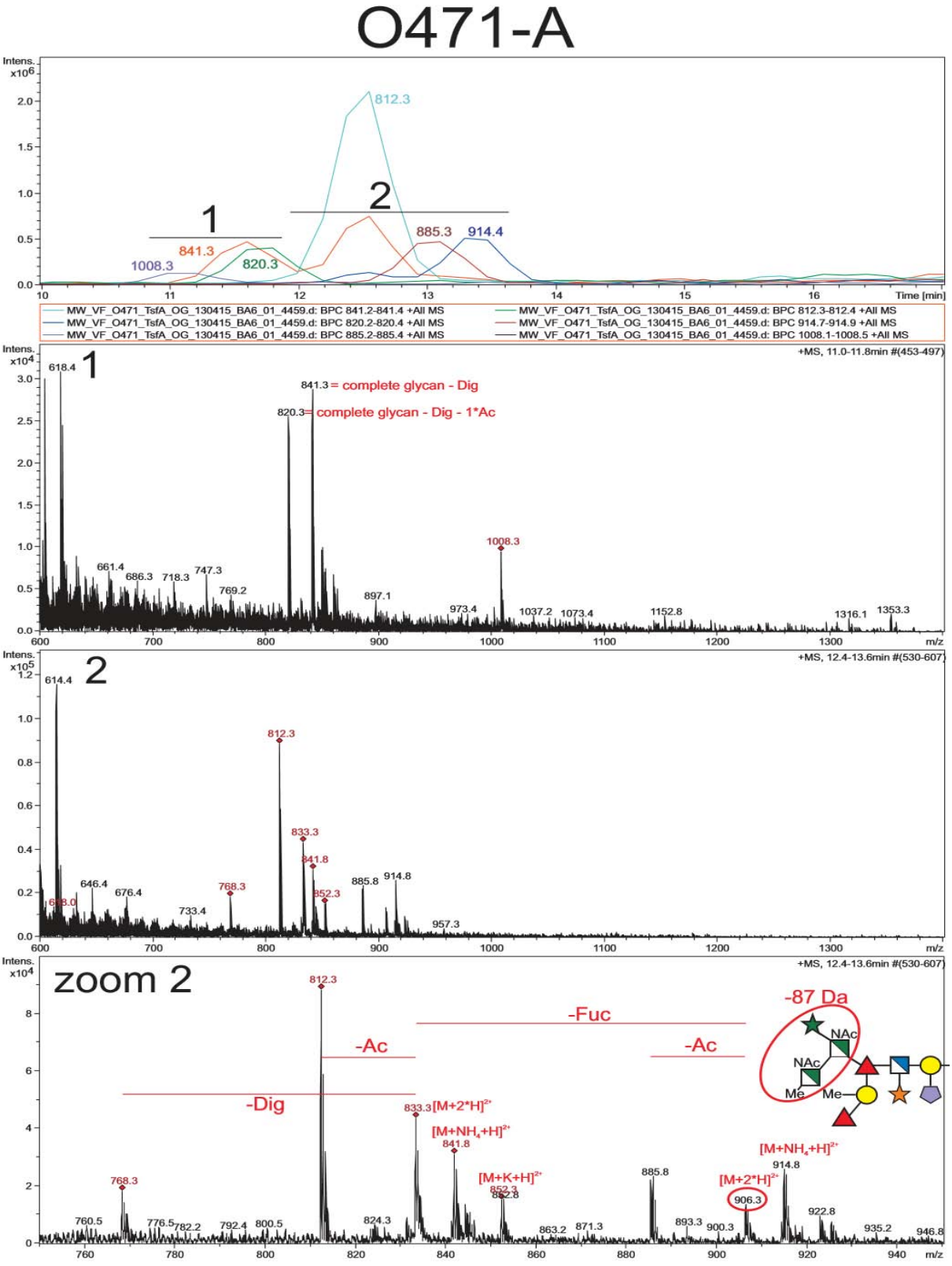


**Figure 44.** MS-spectrum for clinical isolate 0471

# 5 Conclusions

The investigated clinical isolates of *T. forsythia* exhibited different ability to grow on BHI-medium, whereas isolates 0408 and 1046 never exhibited satisfying growth neither when grown on BHI- and blood-agar plates nor when grown in liquid BHI-culture. After initial efforts these isolates were not used for further investigation.

Isolates UB 4, UB 20, 1055, 0471 showed reasonable growth. UB 4, UB 20 and 1055 presented both of the S-layer proteins TfsA and TfsB when run on CBB- stained SDS-gels and thus, were used for further investigations. 0471 expressed always just the S-layer protein TfsA, but could as well be considered as a solid candidate for future experiments. In contrast, the ATCC BAA-2717 (FDC 92A2) strain expressed only S-layer protein TfsB when grown on BHI- and blood agar plates and also in BHI- liquid culture, the strain is currently undergoing further investigations in our lab.

The genomes of the ATCC 43037 type strain of *T. forsythia,* ATCC BAA-2717 and clinical isolates UB 4, UB 20, 0348, 0471, 1055, 1056, 0408 and 1046 were screened by PCR for the presence of genetic loci for building up pseudaminic and/or legionaminic acid. Amplification by PCR showed that *T. forsythia* wild-type ATCC 43037 and clinical isolate 0348 have the genes for pseudaminic acid biosynthesis. ATCC BAA-2717 (FDC 92A2) and clinical isolates UB 4, UB 20, 0348, 0471, 1055, 1056 showed those for building up legionaminic acid. Neither one of the investigated clinical isolates including ATCC BAA-2717 (FDC 92A2) nor the *T. forsythia* ATCC 43037 type strain showed the genes for both pseudaminic and legionaminic acid. At least within the investigated strains no coexistence of both biosynthetic pathways could be demonstrated. It remains a subject for future studies conducted with more clinical isolates of *T. forsythia* to confirm this finding.

Glycan analysis was carried out with investigated *T. forsythia* isolates UB 4, UB 20, 1055, 0471, ATCC BAA-2717 (FDC 92A2) and type strain ATCC 43037. The obtained MS spectra of β-eliminated glycans were compared with the known fragmentation pattern of *T. forsythia* ATCC 43037 wild-type. The truncated glycan molecule ions were assumed and interpretation was difficult because there is evidence for various modifications on the terminal nonulosonic acid. These modifications within the clinical isolates are versatile and appear to be on the outer arm of the nonulosonic acid part of the glycans consisting of two N-acetylmannosaminuronic residues and either pseudaminic or legionaminic acid. A future perspective would be to perform more β-eliminations to exactly elucidate the structures of the unique glycans from the investigated clinical isolates of *T. forsythia*.

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# Appendix

## Poster presentation

Matthias Braun, Valentin Friedrich, Bettina Janesch, Paul Messner and Christina Schäffer**.** Characterisation of clinical isolates of *Tannerella forsythia.* Winter school of the Department of NanoBiotechnology, February 18-20, 2015, Deutschlandsberg, Austria

# Characterisation of clinical isolates of *Tannerella forsythia*

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## Introduction

***Tannerella forsythia*** **is a Gram negative, anaerobic oral pathogen that colonizes the oral dental cavity and can cause severe periodontitis. Its cell surface is covered with a 2-dimensional glycosylated S-layer, composed of two unique glycoproteins called TfsA and TfsB. The T. forsythia protein glycosylation sytem was shown to be a virulence factor. To extend the glycobiology knowledge on *T. forsythia*, clinical isolates of patients suffering from periodontitis are being investigated .**

## Aim

The *T. forsythia ATCC 43037* glycan is composed of several uncommon sugar residues, among them a modified sialic acid, which can either be pseudaminic or legionaminic acid.

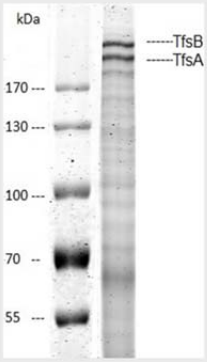


Figure 1: S–layer protein bands of TfsB and TfsA at 270 and 230 kDa.

Based on the demonstrated influence of S-layer glycosylation on the pathogenicity of *T. forsythia*, the aim of this study was to investigate S-layer glycosylation of clinical *T. forsythia* isolates.

The main objective was to optimize growth of several clinical isolates, identify the S-layer proteins, isolate the glycan and have a closer look on the nonu´-losonate of the S-layer glycan.

The second objective was to identify, if biosynthetic pathways for building up pseudaminic or legionaminic or both are encoded in the genomes of the clinical isolates.

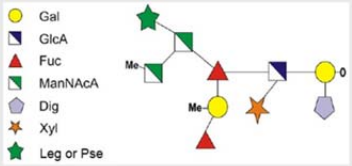


Figure 2: Scheme of the S-layer glycan structure of *T. forsythia* (1)

## Potential of sugar residues

Nonulosonic acids such as pseudaminic (Pse) or legionaminic acid (Leg) are known to act as bacterial virulence factors and play roles as recognition molecules for cell – cell interaction.

Such sialic acid-like residues can be part of a protection mechanism for microbes, shielding themselves against environmental hazards or to evade the host immune system.

## Materials and Methods

Clinical isolates were provided by collaboration partners from the University of Zurich, Switzerland, and the University of Buffalo, NY, USA, and were originally derived from anonymous periodontitis patients.

*T. forsythia* isolates were cultivated anaerobically at 37°C in brain-heart infusion media both in liquid culture and on plates supplemented by yeast extract, cysteine, menadione, hemin, N-acetylmuramic acid and horse serum.

Cells were harvested at $OD_{600}$ ~ 0.75 - 1.0 and proteins were further analyzed on 8% SDS-PAGE gels run at 200 V for 90 min and stained with Coomassie Brilliant Blue.

Genetic loci were analyzed using conserved primer pairs specific for distinct genes from the biosynthsis pathway of either nonulosonic acid and primer products were amplified by PCR.

Separation of amplification products was done on 0.9% agarose gels at 90 V for 25 min.

## Results

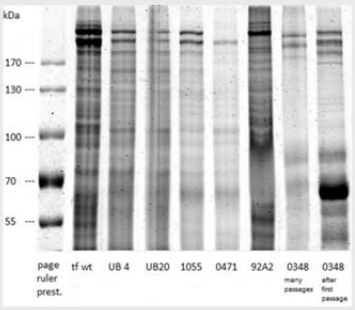


Figure 3: SDS PAGE of *T. Forsythia* ATCC 43037 (*wt*) and some well growing clinical isolates

*T. forsythia wt* was used as a reference for detecting the TfsA and TfsB glycoprotein bands. Some of the isolates revealed only either TfsB or TfsA, implicating that they have lost their second S-layer protein either while developing in the dental pockets or during passaging.

Interestingly, isolate 0348 in the last lane shows wildtype-like TfsB and TfsA bands only after the first passage in liquid brain heart infusion(BHI) media.
In lane 8, 0348 presents slightly down-shifted bands, which suggests loss of a smaller charged molecule after many passages in liquid culture (BHI) and indicates a change in protein expression pattern over time

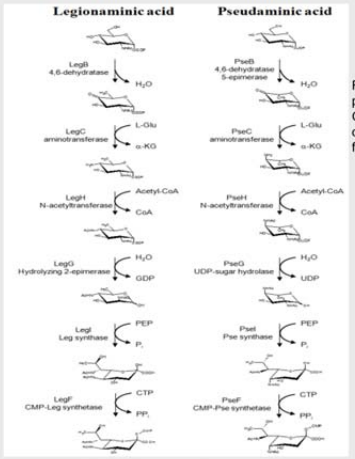


Figure 4: Biosynthetic pathways for legionaminic and pseudaminic acid. The pathways involve the synthesis of GDP and UDP building blocks respectively. The synthesis of the final nonulosonate is subsequently per-formed by further enzymatic steps (2).

To identify whether the biosynthetic pathways for Leg, Pse or both are present, various primers for the genetic loci of the genes encoding the enzymes PseB, PseC, PseF, LegF, LegC and LegB were used to search the genome of the clinical isolates for their presence.
Amplification by PCR showed that *Tf* wt ATCC 43037 and a total of six investigated clinical isolates indicate a ratio of Pse:Leg as 2:5. Neither one of the investigated isolates nor the *Tf* wt showed both of the genetic loci for building up the final nonulosonate.

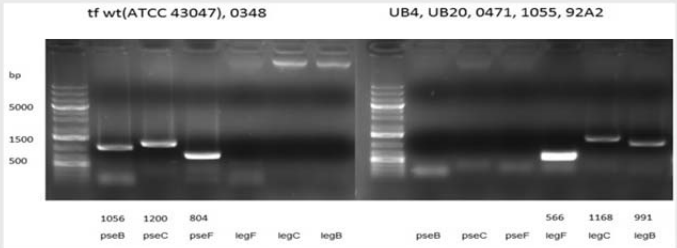


Figure 5: PCR analysis of the genetic loci coding for Pse and Leg showed amplified genes for *pseB, C* and *F* in tf wt 43037 and isolate 0348 but are negative for Leg. Isolates UB4, UB20, 0471, 1055, 92A2 displayed amplified genes for *legF, C* and *B* but no genetic locus for pseudaminic acid synthesis.

## Conclusions and Outlook

Initial glycobiology analyses of six clinical isolates of *T. forsythia* in addition to the type culture strain relvealed that this bacterium is capable of synthesizing either Pse or Leg – both known to act as important virulence determinats. Future studies will focus on how these nonulosonates are involved in the development of periodontitis and how they influence the host immune response.

Next steps will involve the eludication of the proteinglycans of the clinical isolates and the analysis of their biofilm life-style.

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## Manuscript in preparation

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*Glycobiology, manuscript in preparation*

# Nonulosonic acids in the oral pathogen *Tannerella forsythia*

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Key words: pseudaminic acid / legionaminic acid / biosynthesis pathway/ biofilm / protein *O*-glycan/ periodontitis

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## *Curriculum vitae*

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### Persönliche Angaben

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### Ausbildung

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| seit Nov. 2011 | Masterstudium Biotechnologie<br>Universität für Bodenkultur Wien<br><br>Diplomarbeit:<br>„Nonulosonic acids in clinical isolates of the periodontal pathogen *Tannerella forsythia*“ |
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| 2009 | Ausbildung zum staatlich geprüften Tennisinstruktor<br>Bundessportakademie Graz |
| 2005 | Wehrdienst in der Maria Theresien Kaserne<br>Ausbildung zum Gardewachsoldaten |
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|---|---|
| seit 2005 | Angestellter bei Sportstättenverein Marswiese<br>Verbuchung der Leistungen und Kassaführung |
| seit 2008 | selbstständige Tätigkeit als Tennistrainer<br>Organisation und Durchführung von Tenniscamps |

## Praktika

| | |
|---|---|
| 09/2007 | Agentur für Gesundheit und Ernährungssicherheit<br>Abteilung für Rückstandsanaytik |
| 07/2008 | Baxter AG<br>Abteilung 'Cell culture fermentation' |
| 08/2015 | Medizinische Universität Wien, Bernhard Gottlieb Universitätszahnklinik |
| 09/2015 | Medizinische Universität Wien, Zentrum für Pathophysiologie, Infektiologie u. Immunologie<br>Institut für Pathophysiologie und Allergieforschung |

## Sprachen

| | |
|---|---|
| Deutsch | Muttersprache |
| Englisch | fließend in Wort und Schrift |
| Französisch | Grundkenntnisse |
| Italienisch | Grundkenntnisse |

## EDV

| | |
|---|---|
| Microsoft Office | sehr gut |
| Molekularbiologische Software (Clonemanager) | sehr gut |

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| | |
|---|---|
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| Rekombinante Proteinexpression | in *Escherichia coli* |
| Proteinreinigung | Affinitätschromatografie |
| Biochemische Methoden | SDS-PAGE<br>Western Blot<br>ELISA |

## Freizeit

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|---|---|
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