Virulence and glycobiology of the periodontal pathogen *Tannerella forsythia*

Dissertation

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Zusammenfassung

Tannerella forsythia ist ein orales, pathogenes Bakterium, das mit der Entwicklung von Parodontitis in Zusammenhang steht. Diese Erkrankung stellt eine der Hauptursachen von Zahnausfall beim Menschen dar und ist durch eine unkontrollierte Entzündungsreaktion und die Zerstörung des Zahnfleisches und des Alveolarknochens charakterisiert. Darüber hinaus gibt es Hinweise auf eine Verbindung zwischen Parodontitis und zahlreichen systemischen Krankheiten.

Obwohl *T. forsythia* ein relativ wenig erforschter Organismus ist, so gibt es mittlerweile dennoch einige Studien, die diverse Virulenzfaktoren identifiziert haben. Diese Mechanismen erlauben es dem Bakterium, den Wirt zu besiedeln, vorhandene Nährstoffe zu nutzen und das Immunsystem zu unterwandern. Dabei wird die Physiologie des Krankheitserregers maßgeblich von glykobiologischen Faktoren bestimmt. Beispiele für solche Virulenzfaktoren sind die glykosylierten S-Schicht-Proteine, die NanH Sialidase und das glykosylierte Oberflächenprotein BspA.

Der Inhalt dieser Arbeit sind zwei unterschiedliche Projekte zur Erforschung weiterer glykobiologischer Aspekte, die mit der Pathogenität des Organismus im Zusammenhang stehen.

Nach der erfolgreichen Klonierung und Expression eines im Genom von *T. forsythia* als α-L-Fukosidase annotierten Gens konnte das resultierende Enzym als α1,2-L-Fukosidase charakterisiert werden. In der Gruppe der "Glykosidase Hydrolase Familie 29" stellt dies den ersten Fall einer solchen Bindungsspezifität dar. Das Enzym ist in der Lage, terminale Fukose-Einheiten von komplexen Glykanen abzuspalten und zeigte Aktivität mit der Blutgruppe H. Die vorhergesagte Lokalisation der Glykosidase ist das Periplasma, wo sie potenziell auch auf α1,6-Fukose-Einheiten als Teil von kurzen linearen Oligosac-

chariden wirken könnte. Im Zusammenspiel mit der für den Krankheitserreger wichtigen NanH Sialidase könnte das Enzym auch an Folgereaktionen im Abbau von wirtseigenen Glykoproteinen beteiligt sein.

Im Rahmen der Erforschung des Lipidoms von *T. forsythia* wurden zwei bisher unbekannte Phosphodihydroceramide (PDHC) charakterisiert. Diese enthalten *myo*-Inosit und stellen einen der Hauptbestandteile der Lipide des Bakteriums dar. In *Porphyromonas gingivalis*, wie auch einigen anderen mit Menschen assoziierten Bakterien und *T. forsythia* selbst wurden bereits ähnliche PDHCs, die Glyzerin und Ethanolamin beinhalten, nachgewiesen. In diesen Studien stimulierten diese Lipide entzündungsfördernde Reaktionen in gingivalen Fibroblasten, hemmten die Differenzierung und Funktion von Osteoblasten, steigerten die Autoimmunität, förderten Apoptose und akkumulierten in Geweben, die sonst nicht von den Bakterien kolonisiert werden. In dieser Arbeit wurde mittels GC-MS, ESI-MS und NMR die Struktur der beiden neuartigen Inosit-PDHCs bestimmt. Eine bioinformatische Analyse des Genoms zeigte, dass *T. forsythia* nicht über die nötigen Gene für die Biosynthese von Inosit verfügt. Die Analyse der Lipide nach der Zugabe von radioaktiven und stabilen Isotopen (14C Kohlenstoff und deuteriertem *myo*-Inosit) in das Nährmedium erbrachte den Beweis, dass *T. forsythia* exogenes Inosit für die Synthese dieser Lipide verwendet.

Abstract

Tannerella forsythia (T. forsythia) is an oral pathogen that lives in deep periodontal pockets and is strongly associated to periodontitis, a set of periodontal diseases characterized by a deregulated inflammatory response and destruction of the tissues supporting the teeth, such as the gum and the alveolar bone. Periodontitis has also been linked to systemic chronic diseases.

Although *T. forsythia* is a poorly studied bacterium, recent publications have reported various virulence factors which the pathogen uses to invade the host, exploit the resources available and evade the immune system. Many studies involve different glycobiology aspects of the bacterium as these are proving to be determining factors for the pathogen's lifestyle. Some known virulence factors include the glycosylated S-layer proteins, the NanH sialidase and the glycosylated surface BspA protein.

This thesis presents two different projects which were aimed at investigating further glycobiology aspects of *T. forsythia* that could potentially be involved in its pathogenicity.

Cloning and expression of an α-L-fucosidase annotated in the genome of *T. forsythia* led to its characterization as an α1,2-L-fucosidase, the first enzyme to have this specificity within the Glycoside Hydrolase family 29. The enzyme cleaves terminal fucose residues off complex glycans and was seen to be active on blood group H. The glycosidase is predicted to be periplasmic where it could also be active on α1,6 linked fucose residues on short linear oligosaccharides. The enzyme possibly functions downstream from the pathogen's important NanH sialidase in the breakdown of salivary and other host glycoproteins.

Investigation into the lipidome of *T. forsythia* revealed the presence of two novel phosphodihydroceramides (PDHC) containing *myo*-inositol which are major lipid com-

ponents in the bacterium. Similar PDHCs containing glycerol and ethanolamine have been described previously for *Porphyromonas gingivalis* and other common human bacteria, including *T. forsythia*, and were seen to stimulate proinflammatory responses in gingival fibroblasts, inhibit osteoblast differentiation and function, enhance autoimmunity, promote apoptosis, and accumulate in host tissues distant from the sites normally colonized by the bacteria. Here the structure of these novel inositol-PDHCs was determined by GC-MS, ESI-MS and NMR. A bioinformatic analysis of the genome revealed that *T. forsythia* does not have the genes for *de novo* synthesis of inositol. By means of radioactive and stable isotope incorporation using carbon-14 and deuterium labeled *myo*-inositol, added to the growth medium, it could be demonstrated that *T. forsythia* uptakes and incorporates exogenous inositol for the synthesis of these major lipids.

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Abbreviations

S-layer surface-layer

FKP L-fucokinase/GDP-fucose pyrophosphorylase

Am acetimidyl

Ac acetyl

CDP cytidine diphosphate

Da dalton

DAG diacylglycerol

Dig digitoxose

DHC dihydroceramide

ESI-MS electrospray ionization mass spectrometry

FAS fatty acid synthase

Fuc fucose

Gal galactose

GC-MS gas-chromatography coupled to mass spectrometry

GCF gingival crevicular fluid

Glc glucose

GlcA glucuronic acid

Gc glycolyl

GH glycoside hydrolase

GDP guanosine diphosphate

IPC inositol phosphorylceramide

LRR leucine rich repeat

LPS lipopolysaccharide

ManNAc mannosaminuronic acid

Me methyl

NMR nuclear magnetic resonance

OMV outer membrane vesicles

p pyranosidic

PI phosphatidylinositol

PDHC phosphoryl DHC

PUL polysaccharide utilization loci

Pse pseudaminic acid

RNA ribonucleic acid

R-LPS rough type LPS

SEM scanning electron microscopy

SPT serine-palmitoyltransferase

Sus starch utilization system

TEM transmission electron microscopy

UDP uridine diphosphate

WT wild-type

Xyl xylose

1. Introduction

Tannerella forsythia (*T. forsythia*) is a Gram-negative, filamented, anaerobic oral pathogen, member of the so called "red complex" of bacteria which is associated to a set of inflammatory diseases named periodontitis, affecting millions of people worldwide (Vos *et al.* 2013; Socransky *et al.* 1998; Marsh *et al.* 2009). The effects on the periodontium include swelling and bleeding of the gum, loss of the alveolar bone around the teeth, and in more severe cases, loss of teeth. Periodontitis has also been linked to systemic inflammation and to an increased risk of stroke, heart attacks and atherosclerosis, amongst others (Cullinan *et al.* 2009).

The aim of this thesis was to investigate certain aspects of this bacterium which could aid in invasion of the host and establishment of infection. The work is divided into two separate studies on different types of potential virulence factors related to the bacterium's glycobiology. The first work package led to a publication in the journal *Virulence* and describes the characterization of a putative α-L-fucosidase in *T. forsythia* (Megson *et al.* 2015a). The second work package deals with novel phospho-*myo*-inositol-dihydroceramide lipids found in the lipidome of this bacterium and was published in *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* (Megson *et al.* 2015b).

The following sections aim to provide information for a general understanding of the pathogen's niche and of periodontal disease, down to more specific information about *T. forsythia* and some of its important glycobiology aspects. This is then continued by an introduction into virulence, including the known virulence factors in *T. forsythia*, and by background information leading up to the two publications that will later on be discussed, allowing the reader to better assess the contribution of this thesis to the field.

1.1. The niche of *Tannerella forsythia*

The healthy human mouth contains a number of different organisms, including bacteria, predominantly, but also yeast, mycoplasma, protozoa and viruses (Marsh *et al.* 2009). There are up to 700 different bacterial species known to colonize the oral cavity, of which around 400 can be found associated to the dental tissues (Stathopoulou *et al.* 2010). The mouth is the only direct accessible site of the body that has hard non-shedding surfaces, the teeth, which allow for colonization of bacteria in the form of biofilms, named dental plaque (Marsh *et al.* 2009). This is in contrast to mucosal surfaces, which bacteria find harder to colonize because of factors such as desquamation. Additionally, areas between adjacent teeth and in the gingival crevice offer protection from unfavourable conditions. Both sites are anaerobic and the gingival crevice is bathed in nutritionally rich crevicular fluid containing many serum components.

Saliva covers all surfaces of the mouth and has a crucial role in maintaining homeostasis as it protects from mechanical stress and infection and acts as a buffer to maintain the mean pH between 6.75 and 7.25, amongst other functions (Marsh *et al.* 2009). The main constituents of saliva are proteins and glycoproteins such as salivary mucin, one of the most abundant components, which influence the resident microflora by determining the attachment of microorganisms, providing them with a nutrient source and aggregating bacteria to facilitate clearance. Saliva also contains nitrogenous compounds such as urea and amino acids, which oral microorganisms require for growth. The amount of free carbohydrates in saliva is low and therefore most oral microorganisms produce glycosidases to degrade glycan chains from host glycoproteins. Many bacteria are also proteolytic possessing a repertoire of proteases for peptide and protein degradation. These enzymatic activities interact synergistically between species to breakdown host glycoproteins.

Properties of each microenvironment in the mouth will determine which microorganisms can reside there. In turn, the microorganisms that are able to establish themselves will change the conditions of their surroundings, for example by consuming oxygen or

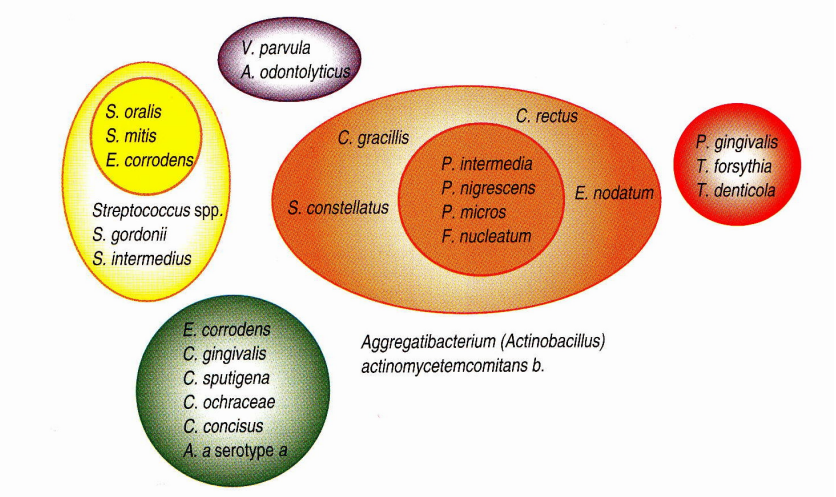

Figure 1.1.: Grouping of bacteria according to their association with health and periodontal disease. The "red complex" bacteria are isolated together in deep periodontal pockets in patients with advanced periodontitis and therefore are strongly associated with pathogenesis. These bacteria are usually preceded by bacteria from the "orange" complex. "Yellow", "green" and "purple" complexes are associated with healthy sites. *Aggregatibacter* (formely *Actinobacillus*) *actinomycetemcomitans* serotype *b* did not fall within a complex but is usually associated with disease. [Taken from (Marsh *et al.* 2009)]

by changing the pH of the environment, which then allows growth of secondary colonizers and the formation of complexes of organisms that coexist and are usually isolated together (Figure 1.1). The dental plaque that is formed in this process is associated with both health and disease. Bacterial genera associated with health include *Streptococcus*, *Actinomyces*, and many *Capnocytophaga* species. If there is a perturbation in the well-balanced resident microflora at a healthy site, the composition of the multispecies plaque changes according to the new environment created. *Prevotella* and *Fusobacterium* species, although still present at healthy sites, usually precede a group of bacteria named the "red complex" that includes *P. gingivalis*, *Treponema denticola* and *T. forsythia* and is associated to disease.

The perturbations that occur in the oral cavity are usually small but prolonged changes that can affect the competitiveness of individual species. An example of this is how the mean pH value of the mouth changes as disease progresses from 6.90-7.25 to around 7.8

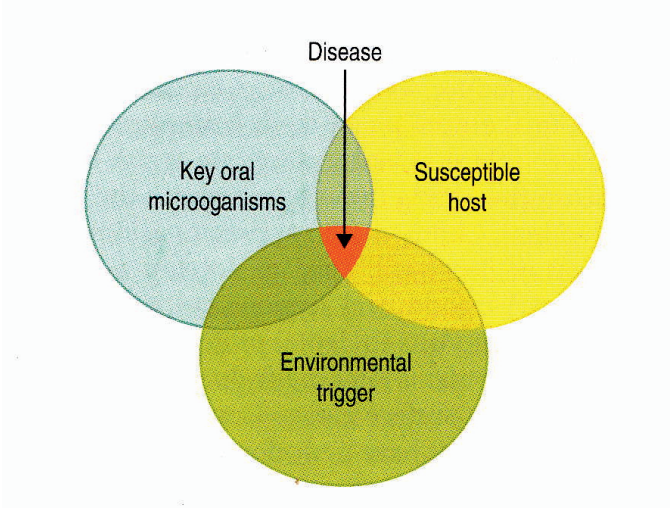

Figure 1.2.: Contributing factors to the development of oral disease. When the host is predisposed or susceptible, presence of key oral microorganisms and an environmental stimulus can trigger the onset of disease. [Taken from (Marsh *et al.* 2009)]

in periodontal disease. The growth of some putative pathogens has indeed been found to be favoured by an alkaline surrounding (Chew *et al.* 2012). Furthermore, some proteases and glycosidases isolated from oral pathogens were seen to have a higher activity in the neutral to alkaline pH range (Takahashi *et al.* 1990; Megson *et al.* 2015a). Temperature can also rise from 36°C to 39°C at sites of inflammation, which invariably shifts the population further towards more resilient bacteria, those with the ability to adapt to changing conditions. Development of disease is ultimately triggered by different factors coming together: key oral bacteria present can thrive due to some environmental stimulus (*e.g.*, high sugar diet leads to acidification of the mouth) in a situation of host-susceptibility (*e.g.*, immunosupression) (Figure 1.2). Disease can progress in two different directions. Acidification of the oral cavity leads to the development of acidogenic bacteria and as a result, dental caries. On the other hand, periodontal disease is associated with the development of pathogenic Gram-negative anaerobes, such as *P. gingivalis* and *T. forsythia*, which are mostly proteolytic (Figure 1.3). Metabolism of amino acids, peptides, proteins and urea from the saliva leads to a rise in the pH and as a consequence results in a more alkaline environment.

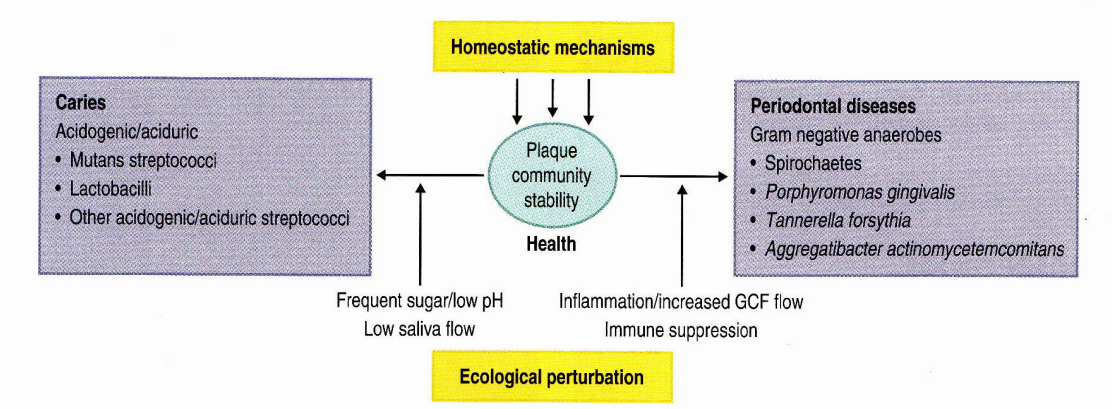

Figure 1.3.: Shifts in the bacterial population in the mouth can lead to either caries or periodontal disease. An environmental stimulus perturbs the community that resides in the healthy mouth. Perturbations such as frequent drops in the pH lead to an increase of the acidogenic fermentative bacteria that cause caries. On the other hand, immune suppression or debilitation in the host can lead to growth of pathogenic Gram-negative anaerobes which are mostly proteolytic and lead to periodontal diseases. [Taken from (Marsh *et al.* 2009)]

1.2. Periodontal diseases

Periodontal diseases affect the supporting tissue of the teeth, that is to say the gingiva and the underlining alveolar bone. The tissues are attacked not only by the organisms causing the infection but also, and principally, by the host's deregulated inflammatory response (Marsh *et al.* 2009). The main types of periodontal disease that will be discussed here are gingivitis and chronic periodontitis.

Gingivitis is a reversible inflammatory response that is transient when good oral hygiene is practiced. The bacterial population changes and there is a general increase in plaque mass and diversity of the organisms present as new species begin to coexist with those which are health-associated. It can be understood as a transitory phase leading to periodontitis.

The term periodontitis implies advanced periodontal disease and affects not only the gum but also the alveolar bone causing possible tooth loss and other irreversible tissue destruction (Figure 1.4) (Hajishengallis 2015). Studies have implicated clusters of bacteria that play a role in the development of periodontitis giving the disease a polymi-

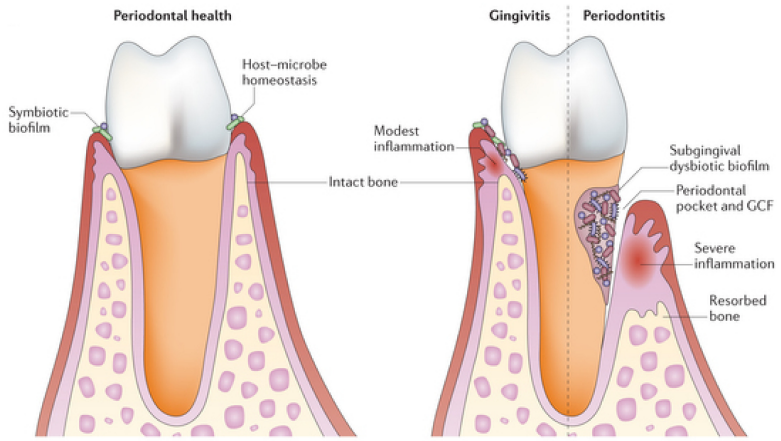

Figure 1.4.: The periodontal pocket seen in health and in gingivitis and periodontitis. Bacterial biofilms are associated with both health, where bacteria live in homeostasis and symbiosis, and disease, where the bacterial population shifts away from that of health and causes inflammation. If left untreated, gingivitis can lead to periodontitis, causing destruction of the tissues underlining the periodontal pocket, the gingiva and alveolar bone. GCF, gingival crevicular fluid. [Taken from (Hajishengallis 2015)]

crobial aetiology (Socransky *et al.* 1998). In deep periodontal pockets in advanced forms of the disease, the "red complex" bacteria are often isolated together, and so these microorganisms are strongly associated with pathogenesis (Figure 1.1).

Apart from oral health issues, emerging evidence shows a relationship between periodontal disease, cardiovascular disease and other systemic chronic diseases involving inflammation, such as aspiration pneumonia and rheumatoid arthritis (Hajishengallis 2015). It is also thought that there exists a link with osteoporosis, type 2 diabetes and adverse pregnancy outcomes (Cullinan *et al.* 2009). The link to cardiovascular disease, for example, is supported by several studies (Ford *et al.* 2005; Nichols *et al.* 2011). Analysis of atherosclerotic plaques revealed that the oral bacterium *P. gingivalis* was present in 100% of cases and *T. forsythia* and *Fusobacterium nucleatum* in up to 80% of cases, demonstrating that these species are able to enter the blood and accumulate at sites distant from that of infection and suggesting a possible role in the inflammatory process leading to atherosclerosis (Ford *et al.* 2005). In addition, dihydroceramide lipids

isolated from *P. gingivalis*, *T. forsythia* and *Pr. intermedia* were seen to have a proinflammatory effect *in vitro* and have been found in blood and different host vasculature and brain tissues, supporting a possible relationship between oral disease and systemic inflammation (Nichols *et al.* 2011).

1.3. *Tannerella forsythia* and its glycobiology

T. forsythia is a Gram-negative, filamented and non-motile bacterium (Figure 1.5) that was originally isolated from the oral cavity of patients with advanced periodontal disease (Tanner *et al.* 1979). It is implicated in the pathogenesis of periodontitis alongside other members of the "red complex" of bacteria and was discovered and named after *Anne Tanner* at The Forsyth Institute (Cambridge, MA, USA) (Tanner *et al.* 1979). The bacterium was originally classified in the *Bacteroides* genus and named *Bacteroides forsythus*. After phylogenetic studies comparing 16S rRNA of the *Bacteroides* subgroup in the *Cytophaga-Flavobacter-Bacteroides* (CFB) phylum, it did not fall into the *Bacteroides* cluster but rather belonged to the *Porphyromonas* cluster (Paster *et al.* 1994) (Figure 1.6). It was later reclassified into the family *Porphyromonadaceae* to its own genus, *Tannerella*, with the species name *Tannerella forsythensis*, later changed to *Tannerella forsythia* (Sakamoto *et al.* 2002; Maiden *et al.* 2003).

The closest relative to *T. forsythia* is the uncultivated bacterium *Tannerella* BU063, which is not pathogenic and is health associated (Beall *et al.* 2014). The organism lacks quite a few virulence factors present in *T. forsythia* and therefore could help in the understanding of the relationship between bacterium and host and aid in the identification of new virulence factors.

In 2005, an incorrect genome sequence (derived from strain ATCC BAA-2717, also referred to as *T. forsythia* 92A2) was attributed to the type strain *T. forsythia* ATCC 43037 and has only just recently been corrected by our group with a draft sequence produced from shotgun sequencing (Chen *et al.* 2005; Friedrich *et al.* 2015b). Whilst the genomic content is very similar in these two sequences, the error led to inconsistencies in PCR-amplified products and some important genomic regions, which deviated largely

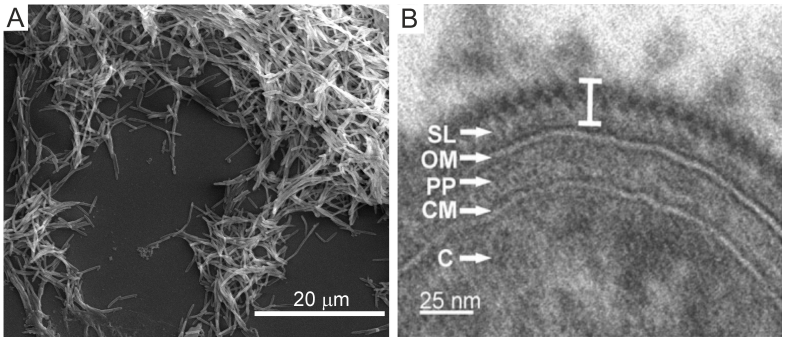

Figure 1.5.: SEM and TEM of *T. forsythia*. A. Scanning electron micrograph of *T. forsythia* biofilm showing a filamented morphology. [Z. Megson, BIMat/NanoGlycobiology, BOKU Wien] B. Transmission electron micrograph of a negative stained ultra-thin cross-section of *T. forsythia* showing the structure of the Gram-negative cell envelope, composed of a cytoplasmic membrane (CM), a periplasm (PP) and an outer membrane (OM) which is additionally covered by an S-layer (SL). Other abbreviations: C, Cytoplasm. [Taken from (Sekot *et al.* 2012)]

from the available sequence, could not be amplified at all. The new draft sequence has a predicted total of 2753 genes, consisting of 2491 coding sequences, 210 pseudogenes, 44 tRNAs, 7 rRNAs, and 1 noncoding RNA (ncRNA) (Friedrich *et al.* 2015b). Publications up to now have referred to annotated genes in the old incorrect genome sequence and are denoted with the locus tags "*BFO*" (NCBI) or "*TF*" (Los Alamos National Laboratory) whilst upcoming publications will refer to genes in the new published genome sequence with the locus tag "*Tanf*".

Despite the implications of *T. forsythia* in periodontitis and systemic disease, relatively little is known about its physiology and it is still a poorly studied organism. Its fastidious growth requirements are a contributing factor to this and genetic manipulation in *T. forsythia* is challenging as genetic tools and protocols are not readily available. Up to date, many of the studies on this oral pathogen involve its glycobiology as this aspect is proving to be a pivotal factor in determining its lifestyle and pathogenicity. In this context, *T. forsythia* also represents the first known pathogenic organism to have a glycosylated surface (S-)layer and therefore, offers the first opportunity to assess the virulence potential of S-layer glycosylation (Posch *et al.* 2011).

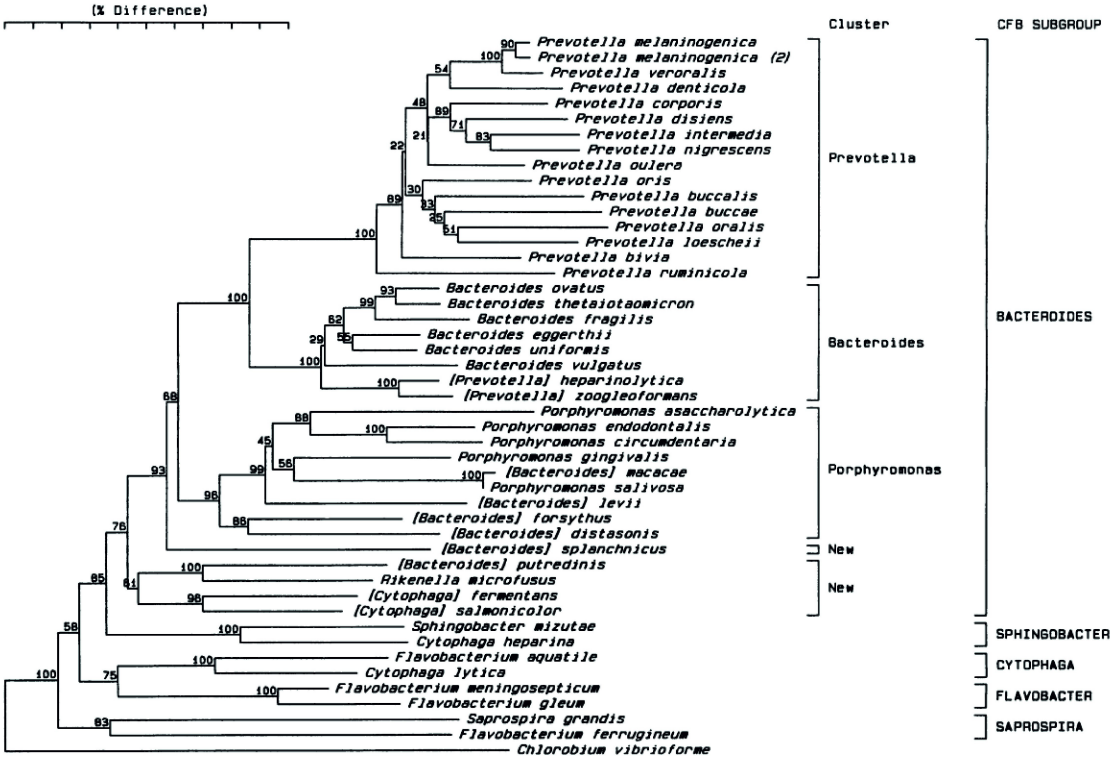

Figure 1.6.: Phylogenetic tree for the *Bacteroides* subgroup of the *Cytophaga-Flavobacter-Bacteroides* (CFB) phylum. After phylogenetic studies comparing 16S rRNA, *B. forsythus* did not fall into the *Bacteroides* cluster but rather belonged to the *Porphyromonas* cluster. [Taken from (Paster *et al.* 1994)]

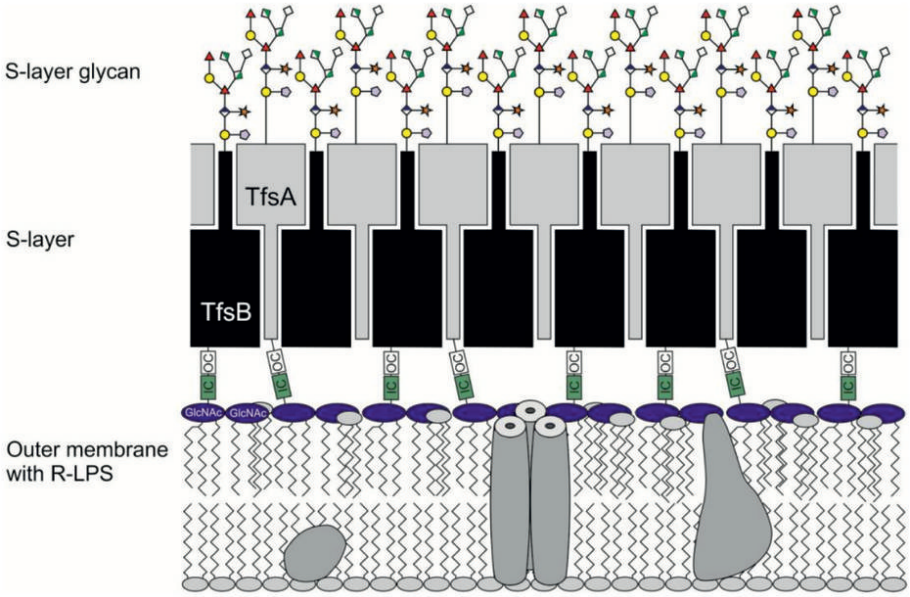

Figure 1.7.: Proposed structure for the outer membrane and S-layer of *T. forsythia*. A rough-type (R) LPS forms the outermost layer of the outer membrane and serves as an anchor for the S-layer which is composed of two coassembling proteins, TfsA and TfsB, which are glycosylated with the same *O*-glycan. [Taken from (Posch *et al.* 2012)]

T. forsythia has a typical Gram-negative cell envelope composed of a cytoplasmic membrane, a periplasm and a rough-type lipopolysaccharide (R-LPS)-containing outer membrane (Posch *et al.* 2012). The cell is then additionally covered by a unique protein S-layer which forms a 2D crystalline lattice with square symmetry and is composed of two different co-assembling proteins (Figure 1.5B and 1.7).

These two high molecular mass proteins, named TfsA (230 kDa) and TfsB (270 KDa), correspond to approximately 10% of the whole cellular protein and are *O*-glycosylated via an *O*-glycosylation system with a unique glycan of the structure

4-Me*O*-β-Man*p*NAcCONH$_2$-(1$\rightarrow$3)-[Pse5Am7Gc-(2$\rightarrow$4)-]-β-Man*p*NAcA-(1$\rightarrow$4)-[4-Me*O*-α-Gal*p*-(1$\rightarrow$2)-]-α-Fuc*p*-(1$\rightarrow$4)-[-α-Xyl*p*-(1$\rightarrow$3)-]-β-Glc*p*A-(1$\rightarrow$3)-[-β-Dig*p*-(1$\rightarrow$2)-]-α-Gal*p* (Figure 1.8) linked to serine and threonine residues within the amino-acid motif D(S/T)(A/I/L/M/T/V) (Posch *et al.* 2011; Fletcher *et al.* 2009). Other proteins are also seen to be glycosylated with the same glycan indicating the presence of a general protein *O*-

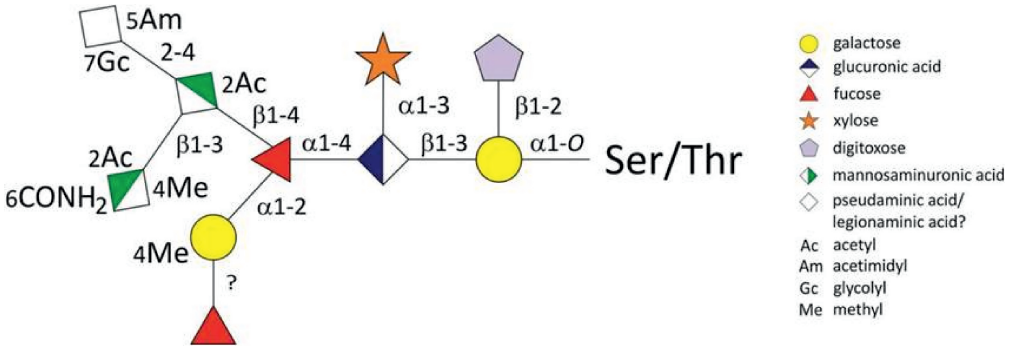

Figure 1.8.: *O*-glycan attached to the S-layer glycoproteins of *T. forsythia.*

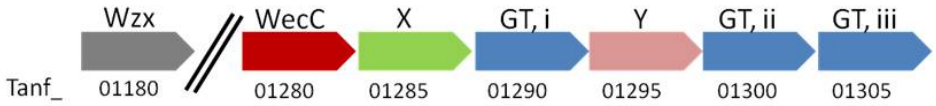

Figure 1.9.: Part of the glycosylation locus in *T. forsythia.* Abbreviations: Tanf, protein/gene tag; Wzx, predicted flippase; X, predicted UDP-*N*-acetylglucosamine 2-epimerase; GT i-iii, predicted glycosyltransferases; Y, predicted methyltransferase. [Modified from (Posch *et al.* 2011), according to the new genome sequence (Friedrich *et al.* 2015b)]

glycosylation system. The dekasaccharide has the unique core branching sugar residues xylose and digitoxose, and displays a terminal pseudamininc acid derivative and two fucose residues, one of which is terminal and is present in substoichiometric amounts, linked to a methylated galactose in an unknown glycosidic linkage. The decoration of the cell surface with sialic acid derivatives and fucose mimics host glycoconjugates and might be crucial for evading the immune response.

The *tfsA* (Tanf_03370) and *tfsB* (Tanf_03375) genes are transcribed from a single promotor and the glycosylation machinery lays within the locus Tanf_01280-Tanf_01305 containing a predicted WecC (Tanf_01280), a predicted UDP-*N*-acetylglucosamine 2-epimerase (Tanf_01285), three predicted glycosyltransferases (Tanf_01290, Tanf_01300, and Tanf_01305) and a predicted methyltransferase (Tanf_01295). *T. forsythia* also encodes for a flippase upstream of the predicted glycosylation locus (Tanf_01180), which could be crucial for transport of the glycan through the membrane for transfer onto the acceptor proteins (Figure 1.9) (Posch *et al.* 2011).

Many efforts are now being made in the *Schäffer* research group to identify and characterize the different enzymes that make up the glycosylation system in *T. forsythia.* Newly developed genetic tools for the creation of knock-out mutants have been essential for this investigation. The acquired knowledge will ultimately help in the study of the role of the S-layer and its glycosylation in infection and disease. This virulence potential is discussed further in the following section.

1.4. Virulence

There is a subtle difference of meaning between pathogenicity and virulence. Pathogenicity is understood as the ability of an organism to cause disease, whereas virulence indicates the degree of pathogenicity, and is often expressed by case-fatality rates or the efficiency or speed at which the host tissues are invaded. In turn, the attributes of an organism that are responsible for the onset of disease are named virulence factors, and it is this latter concept around which the work presented here will be centred.

In bacteria, horizontal gene transfer allows the acquisition of virulence factors in clusters of genes termed pathogenicity islands, which can encode for various functions such as toxins, secretion systems and antibiotic resistance (Beall *et al.* 2014; Hentschel *et al.* 2001). Horizontal gene transfer by conjugation or transduction, amongst other mechanisms, speeds up the evolutionary process greatly and is the primary reason for many novel attributes acquired by bacteria (*e.g.*, the ability to degrade a new substrate) (Hentschel *et al.* 2001). Bacterial pathogens can also lose genes that are not needed due to their ever growing relationship with the host, leading to better adaptability to the surroundings and exploitation of resources, often resulting in a competitive advantage (Keen 2012). This process is one that forever distinguishes them from their independent-living relatives. Moreover, bacteria could have evolved over time in this way to give rise to obligate pathogens or could rapidly and reversibly change their behaviour by factors triggered by mobile genetic elements such as bacteriophages and plasmids. One of the clearest examples of this is when common human bacteria that are usually

commensal, such as *E. coli*, are transformed into virulent organisms after being infected by bacteriophages encoding virulence factors (Keen 2012).

A successful human pathogen must be able to efficiently use the resources available for growth and persistence while also evading the host's defences, either by immune evasion by molecular mimicry or antigenic variation, or by debilitating or modulating the innate and acquired immune system. Other factors enabling attachment to host surfaces and establishment within a multispecies biofilm are also major contributors to disease. The "anti-immunology" aspect of an infection requires special attention (Finlay *et al.* 2006). Although suppressing the host's immune response is the most obvious way for a pathogen to persist, some pathogens benefit from activating inflammation (*e.g.*, provides additional host cells to promote infection, increases the blood irrigation and nutrition, causes tissue destruction), as long as there are mechanisms to keep the response generalized in a way that no specific immune response develops (*e.g.*, altering cytokine profiles, blocking lectins and receptors which would otherwise lead to a Th1 response, blocking T-cell proliferation). Periodontitis follows gingivitis and, therefore, the organisms that are involved are thought to benefit from, and maintain, chronic inflammation.

T. forsythia meets the criteria for an oral pathogen for several reasons: it can be repeatedly isolated in patients with periodontal disease and is a risk factor for attachment loss (loss of supporting structures around the teeth) (Grossi *et al.* 1994), it is capable of causing disease in animal models (abscesses and alveolar bone loss), and its antigens can elicit an immune response (Sharma 2010). Some virulence factors have already been identified, whilst others are only postulated, and will be discussed below. The uncultivated bacterium *Tannerella* BU063 represents a possible "control organism" for the study of virulence factors in this bacterium (Beall *et al.* 2014) as it is the closest relative to *T. forsythia* but is not pathogenic.

1.4.1. Virulence factors in *Tannerella forsythia*

The known virulence factors in *T. forsythia* up to date can be classified into surface components, hydrolytic enzymes, and outer membrane vesicles, which combine many of the other factors.

Surface components

T. forsythia possesses an S-layer composed of two high-molecular mass co-assembling proteins, which are highly glycosylated with a unique glycan composed of 10 sugar residues, some of which are quite rare or have rare modifications (see above; Figures 7 and 8). S-layers are thought to provide the organism with a protective shield and act as a sieve for molecules, and also aid in attachment, biofilm formation and in the interaction with the surrounding environment (Messner *et al.* 2010). The S-layer proteins in *T. forsythia* are strongly antigenic and *T. forsythia* S-layer-deficient mutants present a loss of adherence to human gingival epithelial cells (Posch *et al.* 2012). Additionally, the S-layer-deficient mutants were seen to have less serum resistance than the wild-type and their aggregation with other oral bacteria was impaired (Shimotahira *et al.* 2013). Upon stimulation of macrophages and gingival fibroblasts with the wild-type and the S-layer-deficient mutant, the mutant induced significantly higher levels of proinflammatory mediators, suggesting that the S-layer attenuates the host response, at least at the early stage of infection (Sekot *et al.* 2011). The glycan carried by the S-layer could play a crucial role in this immune evasion, for example by molecular mimicry, as there are indications that the glycan suppresses a Th17-mediated response. The $\Delta wecC$ mutant, which is missing the three branched terminal sugar residues on the S-layer glycan (compare with Figure 1.8), was also seen to be less virulent than the wild-type (Settem *et al.* 2013). In addition, the glycan could be an important ligand for lectins on host surfaces or participate in the co-aggregation of bacteria in the multispecies biofilm (Liu *et al.* 2009; Stafford *et al.* 2012).

Another surface component that is thought to be linked to virulence is the protein BspA (*Bacteroides* surface protein A), which is also glycosylated (Sharma *et al.* 1998).

This protein is surface associated or secreted and belongs to the leucine-rich repeat (LRR) family. Its sequence contains two regions with tandem repeats of an LRR motif. LRR proteins have been identified in other oral bacteria such as *P. gingivalis*, *Tr. denticola* and *Pr. intermedia* (Ikegami *et al.* 2004; Capestany *et al.* 2006; Lewis *et al.* 2008) and have a number of different functions in eukaryotic and prokaryotic cells linked to protein-protein interactions and signalling. Specific roles for some bacterial LRR proteins include promoting adherence and entry into cells (Mengaud *et al.* 1996a; Mengaud *et al.* 1996b), triggering release of proinflammatory cytokines from macrophages (Mansell *et al.* 2000) and co-aggregation and biofilm development (Ikegami *et al.* 2004; Capestany *et al.* 2006). Studies revealed that the entry of *T. forsythia* into host cells is dependent on host phosphatidylinositol 3-kinase signalling, and that purified BspA protein causes activation of this lipid kinase (Mishima *et al.* 2011). BspA has also been shown to bind to components of the extracellular matrix (fibronectin, fibrinogen) (Sharma *et al.* 1998) and to other LRR-containing proteins from other bacteria (*Tr. denticola, F. nucleatum*) causing co-aggregation (Sharma *et al.* 2005b; Ikegami *et al.* 2004). The virulence of the BspA protein was proved *in vivo* when the BspA-defective mutant of *T. forsythia* was less efficient in inducing alveolar bone loss in mice, as compared to the wild-type (Sharma *et al.* 2005a).

Like most Gram-negative bacteria, *T. forsythia* has outer membrane proteins that contain OmpA domains (Veith *et al.* 2009). Known functions for these OmpA-like proteins include being an anchor for the peptidoglycan to the outer membrane and contributing to the attachment to epithelial cells and to bacterial survival after phagocytosis by macrophages (Abe *et al.* 2011). Many are porin-like proteins or form proton channels (Hosking *et al.* 2006). In *T. forsythia*, one of the major OmpA-like proteins was found to be the product of the gene *tanf*_10935 and is exposed on the cell surface (Veith *et al.* 2009; Abe *et al.* 2011; Friedrich *et al.* 2015a). The Δ*tanf*_10935 mutant lost its characteristic morphology and presented a decreased adhesion to fibronectin and retarded autoaggregation, indicating a role in adhesion (Abe *et al.* 2011).

Lipids and surface-lipid associated proteins can also be key virulence factors. Proteins modified with lipids to form lipoproteins facilitates the anchoring of hydrophilic proteins to cellular membranes and helps interaction and penetration of foreign membranes (Kovacs-Simon *et al.* 2011). In the bacterial pathogens *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Borrelia burgdorferi* and *Neisseria meningitidis*, lipoproteins have been shown to play key roles in adhesion to host cells, modulation of inflammatory processes, and translocation of virulence factors into host cells (Kovacs-Simon *et al.* 2011). In *T. forsythia*, a total lipoprotein fraction was shown to activate host cells to release proinflammatory cytokines and to induce apoptosis and, therefore, it was postulated that they could play a role in the pathogenesis of disease (Hasebe *et al.* 2004).

Lipopolysaccharides (LPS), which are found in large quantities at the outermost layer of the outer membrane, are important molecules for both pathogen and host. For the host, the well conserved lipid portion of LPS, lipid A, is a ligand for Toll-like receptor (TLR) 2 and TLR 4 and activates the immune system (Finlay *et al.* 2006). On the other hand, the variable carbohydrate structure (O-antigen) forming the outer part of LPS allows a pathogen to survive as a species, as different strains can present different O-antigens which allows for reinfection. There are many more potential roles for LPS during infection. Another member of the "red complex", *P. gingivalis*, contains a lipid A structure that has been shown to function both as an agonist and an antagonist of TLR 2 and 4. The LPS of *Helicobater pylori* can bind to the DC_SIGN lectin of dendritic cells and block a Th1 immune response (Finlay *et al.* 2006). Not much is known about the role of LPS in *T. forsythia*. LPS in this pathogen is a complex, rough-type LPS composed of a core region made up of one 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residue, three mannose residues and two glucosamine residues (Posch *et al.* 2013). A phosphoethanolamine residue and a possible galactose-phosphate residue are also present. It has been shown to have a proinflammatory effect, although this response was not very potent in the absence of serum cofactors. The LPS in *T. forsythia* is proposed to anchor the S-layer to the outer-membrane (Sekot *et al.* 2012; Posch *et al.*

2012). Structural studies into the lipid A of *T. forsythia* were not completed and much remains to be investigated on the inflammatory potential of LPS in this oral pathogen.

Recently, novel phosphorylated dihydroceramide (DHC) lipids were identified in the periodontal pathogen *P. gingivalis* to be potential virulence factors (Nichols *et al.* 2004). These include both low-mass and high-mass forms of phosphoglycerol DHC and phosphoethanolamine DHC lipids. They were found to stimulate pro-inflammatory responses in gingival fibroblasts (Nichols *et al.* 2004), inhibit osteoblast differentiation and function (Wang *et al.* 2010), enhance autoimmunity (Nichols *et al.* 2009), promote apoptosis (Minamino *et al.* 2003), and accumulate in diseased gingival tissue and other host tissues distant from the sites normally colonized by the bacteria (Nichols *et al.* 2006; Nichols *et al.* 2011). This led to the hypothesis that they might form the link between periodontal disease and systemic chronic diseases involving inflammation. These lipids were since found also in other common human bacteria including *T. forsythia* (Nichols *et al.* 2011). The following thesis deals in part with the identification of yet another class of phosphorylated DHCs in this organism which contains phospho-*myo*-inositol as a polar head-group (Megson *et al.* 2015b). This rare and important polar head group is often involved in protein-lipid interactions on cell surfaces (Michell 2008) and therefore further increases the interaction possibilities and widens the spectra of effects this type of lipid could have on the host. Moreover, the true virulence factor concerning these inositol-containing lipids could be reflected in the efficient uptake and utilization of the inositol from the host.

Hydrolytic enzymes

T. forsythia is considered asaccharolytic and therefore requires peptides and amino acids for growth, which can be obtained from degraded host proteins. Quite a few different proteases have been described for *T. forsythia* as possible virulence factors. The ability to cleave larger proteins in *T. forsythia* was identified for a protease termed PrtH (Saito *et al.* 1997). This enzyme was seen to be active on milk proteins and synthetic peptides and cause the hemolysis of horse blood. Based on inhibition studies, PrtH was identified

as a cysteine proteinase. The protease could be found in the spent medium after culture and was able to cause the detachment of adherent cells. The studies suggest that PrtH is involved in the destruction of host tissues and is heavily associated with periodontitis and loss of attachment.

Proteolytic activity also serves *T. forsythia* for evading the first branch of the host's immune response, the complement system. *P. gingivalis* is resistant to the bacteriolytic activity of human serum thanks to its gingipain proteases, which degrade components of the complement (Popadiak *et al.* 2007). *T. forsythia* possesses a similar mechanism by encoding for a metalloprotease, karilysin, which is secreted and is able to cleave elastin, fibrinogen and fibronectin and is an effective inhibitor of all complement pathways (Jusko *et al.* 2012). The karilysin protease is more closely related to mammalian metalloproteases than bacterial ones, indicating that it might have been a product of the rare phenomenon of horizontal gene transfer between bacteria and humans (Jusko *et al.* 2012).

T. forsythia also has other putative secretory proteases, named KLIKK proteases because of the presence of the CTD domain (-Lys-Leu-IIe-Lys-Lys motif) allowing for secretion. The enzymes showed different degrees of proteolytic activity on collagen, gelatin, elastin, casein, fibrinogen, hemoglobin, and the antimicrobial peptide LL-37, suggesting a protective role against host-bacteriolytic activity and involvement in host tissue destruction (Ksiazek *et al.* 2015b; Ksiazek *et al.* 2015a).

Although *T. forsythia* is generally considered asaccharolytic and therefore not capable of breaking down carbohydrates for energy, the organism possesses genes for various different glycosidases, some of which have been proved experimentally including two exo-α-sialidases, SiaHI and NanH (H. Thompson *et al.* 2009; Ishikura *et al.* 2003), an α -D-glucosidase (SusB), an *N*-acetyl-β-D-glucosaminidase (HexA) (Hughes *et al.* 2003) and an α-L-fucosidase (TfFuc1), characterized in the work presented in this thesis (Megson *et al.* 2015a). In addition there are other predicted glycosidases in the genome, such as β-hexosaminidases (Roy *et al.* 2012), galactosidases and mannosidases. This repertoire of glycosidases presumably contributes to the breakdown of the complex oligosaccharides

and proteoglycans of the periodontium, ultimately effecting its integrity (Sharma 2010). Thus, glycan-interaction based processes, such as movement of leukocytes to the site of infection, could potentially be hindered, while protein epitopes for bacterial adhesion are created, further promoting disease progression (Prakobphol *et al.* 1999). Recent evidence suggests that for several periodontal pathogens, but particularly for the "red complex" organism *T. forsythia*, sialic acid-presenting host molecules play an important role *in vivo* (Stafford *et al.* 2012). Mutants lacking the main *T. forsythia* sialidase, NanH, were observed to have hindered attachment and invasion of human oral epithelial cells (Honma *et al.* 2011). The enzyme was also seen to play an important role in biofilm growth on surfaces coated with salivary glycoproteins (Roy *et al.* 2011). This indicates that cleavage of the sialic acid residues from host glycoproteins might expose adhesion sites and is a growth factor in biofilm formation. The *nanH* gene is located in a large cluster that contains all the genes required for sialic acid catabolism, which indicates that the cleaved sialic acid can additionally be taken up and utilized (Stafford *et al.* 2012). A β-hexosaminidase enzyme is also found in this operon and is thought to contribute to the adhesion process by cleaving the exposed β-linked glucosamine or galactosamine residues after removal of sialic acid (Roy *et al.* 2012). Additionally, in a separate study, transcriptome analysis of the oral microbiome also showed up-regulation of the sialidase NanH mRNA in dental plaque (Duran-Pinedo *et al.* 2014).

The recent characterization of the protein NanS, a sialate-*O*-acetylesterase found in the sialic acid catabolic operon of *T. forsythia*, strengthens the importance of this pathogen's siallobiology (Phansopa *et al.* 2015). This enzyme was shown to cleave acetyl groups off the sialic acid Neu5,9Ac_2, which is not efficiently cleaved by most sialidases due to its additional acetyl group, as compared to the more common sialic acid in humans, Neu5Ac. When incubated together with the NanH sialidase, NanS increased the release of sialic acid residues from salivary glycoproteins, representing yet another mechanism that *T. forsythia* uses to exploit available resources.

Outer membrane vesicles

Bacterial outer membrane vesicles (OMV) are integral parts of biofilm matrices and are thought to be "bacterial warfare" agents, selectively carrying specific cargo (Flemming *et al.* 2007; Haurat *et al.* 2011). They have been shown to play roles in establishing colonization, transport virulent factors into host cells and modulate the host's defence and response (Ellis *et al.* 2010). Just recently, a compositional proteomic analysis of the OMV in *T. forsythia* revealed that they were enriched in glycoproteins and different identified virulence factors (Friedrich *et al.* 2015b). Out of the 175 proteins studied, 80 proteins contained the *Bacteroidales* phylum-wide *O*-glycosylation motif and 18 were confirmed to be glycosylated, representing a larger ratio of glycosylated proteins than what normally occurs in the bacterium. The BspA protein was found in high abundance as well as the S-layer proteins and many components of the outer membrane. The sialidase NanH and SiaH1 alongside peptidases and other proteins with hydrolytic activity were also found and some proteins were identified as homologues to known virulence factors in other organisms, such as the protease HtrA from *H. pylori*, involved in E-cadherin cleavage, facilitating epithelium invasion (Hoy *et al.* 2010).

1.4.2. The health-associated *Tannerella* BU063

When comparing the virulent strain *T. forsythia* to the health-associated *Tannerella* BU063, a lot of these potential virulence factors are present in the former but absent in the latter, supporting that they do indeed contribute to virulence in this organism (Beall *et al.* 2014). The PrtH protease, the karilysin metalloprotease and other KLIKK proteases, the BspA-cell surface protein and the NanH silaidase and its operon, are all good examples of this. Other attributes which are not found in the health-associated strain include a number of genes for arginine biosynthetic enzymes, a glutaminase, two copies of a putative operon involved in 1,4-dihydroxy-2-napthoate biosynthesis (an intermediate in production of the quinone electron acceptor) and the F0F1 type of rotary ATPases. Other genes are found in the health-associated strain but not in *T. forsythia*, representing an adaptation to a pathogenic lifestyle. Examples of this are

two *de novo* synthesizing enzymes required to convert UDP-*N*-acetyl-D-glucosamine to UDP-*N*-acetylmuramic acid for peptidoglycan synthesis. *N*-Acetylmuramic acid or its precursors can be presumably scavenged by *T. forsythia* from the host in its natural habitat (Wyss 1989). Similarly, *T. forsythia*, unlike the harmless *Tannerella* sp. BU063, does not have any gene homologs to *ino1*, involved in inositol synthesis, but can import inositol from the medium for synthesis of major lipid components (Megson *et al.* 2015b).

1.5. Background information for the published work

The two published papers, which are presented in the publication section of this thesis, deal with two new potential virulence factors in the oral pathogen *T. forsythia*.

The first publication, "Characterization of an α-L-fucosidase from the periodontal pathogen *Tannerella forsythia*" (Megson *et al.* 2015a), was inspired by the work published by the *Stafford* group on the sialidases in *T. forsythia* and deals with the cloning, expression and characterization of an α-L-fucosidase encoded in the pathogen's genome. In this work, the enzyme, thought initially to be localized in the outer membrane, was seen to have an α1,2 fucose specificity for terminal fucose residues on unsubstituted galactoses (blood group H) and also an α1,6 specificity on small linear disaccharides. The enzyme was active on the bovine submaxillary mucin after cleavage of the terminal sialic acid residues with the recombinant NanH sialidase of *T. forsythia*, indicating that it possibly works synergistically and downstream from NanH. Following biofilm experiments and invasion/adhesion assays on epithelial cells using a fucosidase knock-out mutant of *T. forsythia*, none of these extracellular roles for the enzyme could be confirmed. The enzyme is therefore most likely in the periplasm where it can exert its full potential as both an α1,2- and α1,6- fucosidase.

The second publication, "Inositol-phosphodihydroceramides in the periodontal pathogen *Tannerella forsythia*: structural analysis and incorporation of exogenous *myo*-inositol" (Megson *et al.* 2015b), describes the isolation and structure of novel dihydroceramide lipids that contain *myo*-inositol as a polar head group, as elucidated by GC-MS, ESI-MS

and NMR, and which represent major lipid components in *T. forsythia*. The importance of the finding lays not only in the structure, but also in how *T. forsythia* acquires and incorporates inositol into its biosynthetic pathways. It is postulated that the pathogen acquires the important metabolite exclusively from the medium, which could represent an adaption to a pathogenic lifestyle.

In order to provide context for the reader, relevant information for both of these publications can be found in the following sections.

A co-authored review entitled "Glycobiology aspects of the periodontal pathogen *Tannerella forsythia*" includes some of the aspects discussed here and can be found at the end of the publication section.

1.5.1. Publication: *Characterization of an α-L-fucosidase from the periodontal pathogen* Tannerella forsythia

Background: Host glycoproteins and bacterial glycosidases

Oligosaccharides attached to proteins and lipids on cell surfaces often have important roles in cell-cell interactions. Both sialic acid (*O*- and *N*- derivatives of neuraminic acid, *e.g.*, *N*-acetylneuraminic acid) residues and fucose residues are common sugars found on the non-reducing ends of these glycans, both in the animal host and in their residing microflora. Bacteria decorate their cell surfaces with these residues to enable colonization and persistence in their niche. In the human host, the residues are found on blood group glycoconjugates (ABO, H antigen and Lewis antigens (Figure 1.10)), on serum proteins and on mucins, amongst others. Some are also present due to diet, for example on milk oligosaccharides (2'-fucosyllactose; 3'-fucosyllactose) or fucogalacto-xyloglucan, which presents α1-2 fucose residues.

Fucose is found in α1,2-, α1,3- and α1,4- linkages in terminal positions and in an α1,6 linkage in the case of core-glycan fucose residues on *N*-linked glycans (Ma *et al.* 2006). It has generally been shown that terminal fucose residues play important roles in the interaction of mammalian cells with pathogenic bacteria and surface fucosylation enables bacteria to evade the immune system. For instance, the pathogen *H. pylori* is known to

establish infection by binding and expressing certain fucosylated blood groups such as Le^b and Le^x and also utilizes fucose from the host in order to do this (Boren *et al.* 1993). The intestinal symbiont *B. fragilis* incorporates fucose into capsular polysaccharides and *O*-saccharides on proteins and can GDP-activate exogenous fucose which is then used in its biosynthetic pathways (Coyne *et al.* 2005). This gives the bacterium a survival advantage in the intestinal ecosystem.

As *T. forsythia* is an oral pathogen, special mention must be made of mucins, one of the most abundant glycoproteins found in the mouth. Mucins are high-molecular-mass glycoproteins that are ubiquitous in mucous secretions and in body fluids. They are produced by epithelial tissues and they can be either embedded in the cell membrane or secreted, for example to become one of the main components of saliva. They serve many functions, including lubrication, hydration and protection from mechanical stress and microbial infection. Membrane-bound mucins also play roles in cell-cell and cell-matrix interactions and signalling, and present adherence motifs for microorganisms whilst also providing them with a source of nutrition. The central region of these proteins is rich in serine and threonine residues, which become heavily *O*-glycosylated, leading to glycosylation accounting for up to 80% of their molecular weight. They can also be *N*-glycosylated, albeit to a much lower extent. The *O*-saccharides are based on eight known core structures (Figure 1.10) and can vary in length from disaccharides to large oligosaccharides of approximately 20 monosaccharide residues and exhibit a high degree of diversity. The core structures can be extended with different sugar residues and can present different blood-group epitopes that are often fucosylated or sialylated at terminal positions (Figure 1.10) (Tailford *et al.* 2015). Bovine submaxillary mucin, used in the work presented here for surface coatings and for *in vitro* growth assays of *T. forsythia* in biofilm (Roy *et al.* 2011), presents predominantly blood group H at its fucosylated epitopes (Chai *et al.* 1992a; Chai *et al.* 1992b). Blood group A and the $Lewis^{X/Y}$ antigens were also present, although these findings could vary depending on the source of mucin.

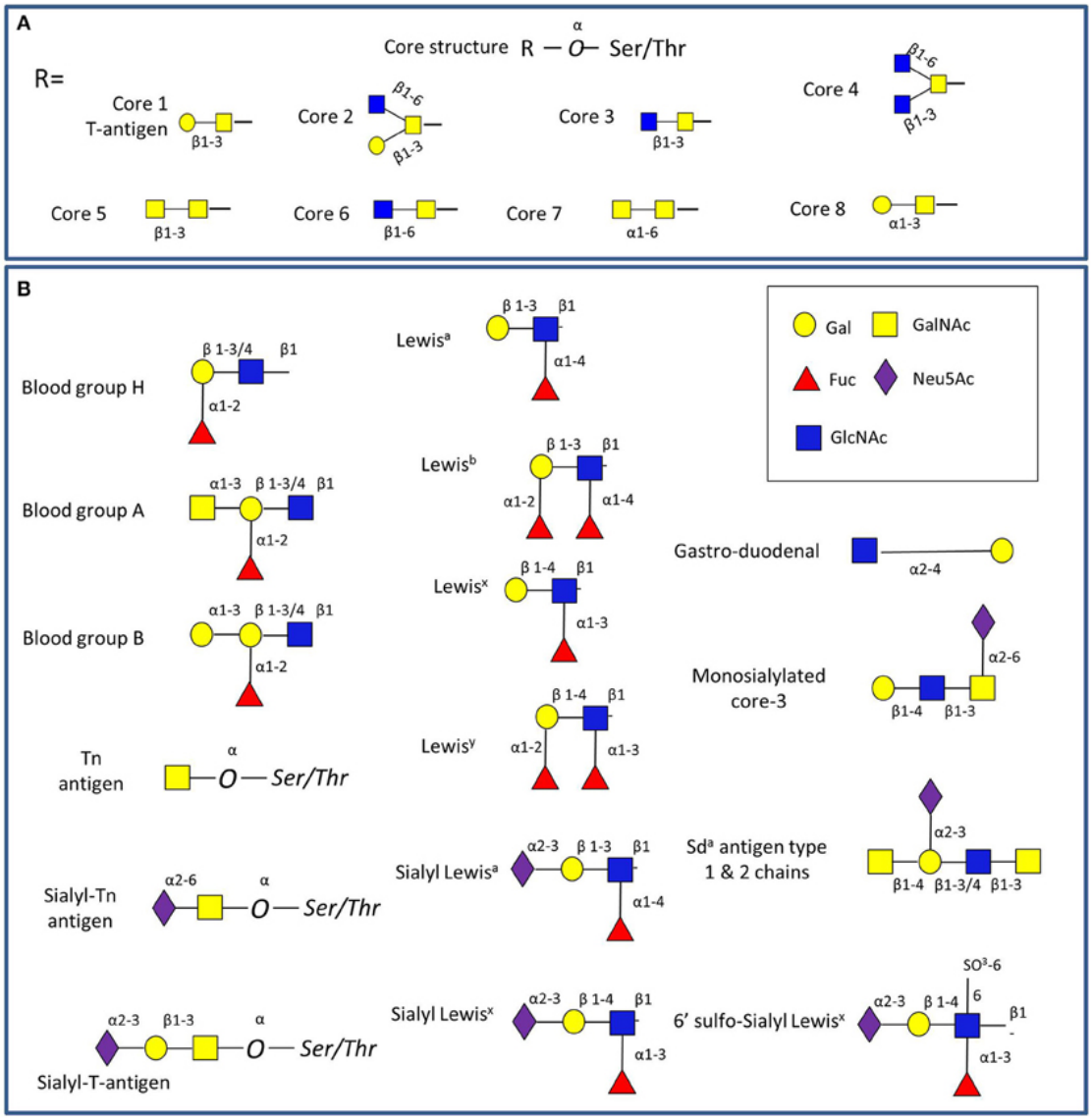

Figure 1.10.: Representation of the core structures (A) and epitopes (B) on mucin *O*-glycans. Many epitopes present blood groups and Lewis antigens. On bovine submaxillary mucin, predominantly blood group H is found, although blood group A and the Lewis$^{X/Y}$ antigens can also be present. The structures are depicted according to the symbolic nomenclature of the Consortium for Functional Glycomics. [Taken from (Tailford *et al.* 2015)]

The fucosylated mucin glycans are adherence targets for many bacteria in the intestine, including *Campylobacter jejuni* which binds the H(O) antigen (Ruiz-Palacios *et al.* 2003). In addition, the ability to utilize available fucose as a nutrient or in surface presentation provides many bacteria with a nutritional advantage and contributes to survival in a highly competitive ecosystem, such as the human body. In this context, many bacterial pathogens express fucosidases amongst other glycosidases which play roles in scavenging sugar residues off mucins and other host glycoproteins and are involved in the invasion of host tissues (Dwarakanath *et al.* 1995).

Glycosidases or glycoside hydrolases (GH) are hydrolytic enzymes that catalyse the hydrolysis of glycosidic linkages. They are classified according to their protein sequence and fold into more than 100 different families in the Carbohydrate Active enZyme database (CAZy: www.cazy.org). As the catalytic mechanism tends to be conserved within a family, the classification helps to make predications on both type of glycosidase and the molecular mechanism of a yet uncharacterized enzyme. There are two main mechanisms for catalytic activity, the retaining mechanism and the inverting mechanism, depending on whether the configuration of the bond being hydrolysed is then inverted or not at the end of the reaction. Fucosidases are found in two GH families, family 29 and 95. Up to now, all fucosidases in the GH family 95 are exclusively α1,2-L-fucosidases that hydrolyze Fucα1-2Gal linkages attached at the non-reducing ends of oligosaccharides by an inverting mechanism. GH family 29 are retaining enzymes and contains both α1,3/1,4-L-fucosidases and enzymes of a broader specificity (fucosidase and galactosidase dual specificity in *B. thetaiotaomicron*). It is notable that the vast majority of GH members have not been characterized (Tailford *et al.* 2015).

The genome of *T. forsythia* encodes 3 putative α-L-fucosidases, Tanf_06770 (BFO_2737 in the previous genome sequence) and Tanf_09440 (previously BFO_1182), both classified in the glycosyl hydrolase family GH29, and Tanf_07610 (previously BFO_3101), classified in the family GH95. While all three enzymes possess a glycosyl hydrolase domain and are classified by CAZy according to their mechanism of action, Tanf_09440

and Tanf_07610 were not strictly annotated as α-L-fucosidases but as an F5/8 type C domain protein and a putative lipoprotein, respectively.

The interplay between host glycoproteins, surface glycosylation and glycoside hydrolase activity, plays a crucial role in the attachment of *T. forsythia (*Stafford et al. *2012)*. Mutants of *T. forsythia* lacking the main sialidase, NanH, have hindered attachment and invasion of human oral epithelial cells indicating that recognition and cleavage of the sialic acid residue off host glycoproteins triggers adhesion. The enzyme was also seen to play an important role in biofilm growth on surfaces coated with salivary mucins (Roy *et al.* 2011). The *nanH* gene is located in a large cluster that contains all the genes required for sialic acid catabolism, which indicates that the cleaved sialic acid can additionally be taken up and utilized (H. Thompson *et al.* 2009). A model for the role of sialic acid was postulated where the presence of these residues on host surfaces and on other bacteria, which in turn express sialidases, is a key factor for the adherence and interaction between each other to from a multispecies biofilm (Figure 1.11) (Stafford *et al.* 2012). During the course of this thesis, we investigated whether the annotated fucosidase in *T. forsythia*, Tanf_06770, could process fucose residues on host glycoproteins and also contribute to the adhesion, invasion and biofilm formation of this oral pathogen. The Tanf_06770 protein is a predicted periplasmic protein but it was found in outer membrane preparations of *T. forsythia* (Veith *et al.* 2009), making it a good candidate to modulate surface interactions.

The investigated fucosidase in *T. forsythia* could also be important in the cleavage of fucose for use as a nutrition source or be a necessary enzyme for the stepwise breakdown of host glycoproteins or a key enzyme in the recycling of fucose into the pathogen's glycosylation pathway.

Whether cleaved sugars can be used in *T. forsythia* as a nutrition source is unclear. Different sugars would have to be broken down into metabolites which can then feed into the glycolysis pathway, the pentose phosphate pathway or the Entner-Doudoroff pathway. Although the pathogen is classified as asaccharolytic because of its inability to ferment sugars, as no detectable pH decrease was observed when grown in media supple-

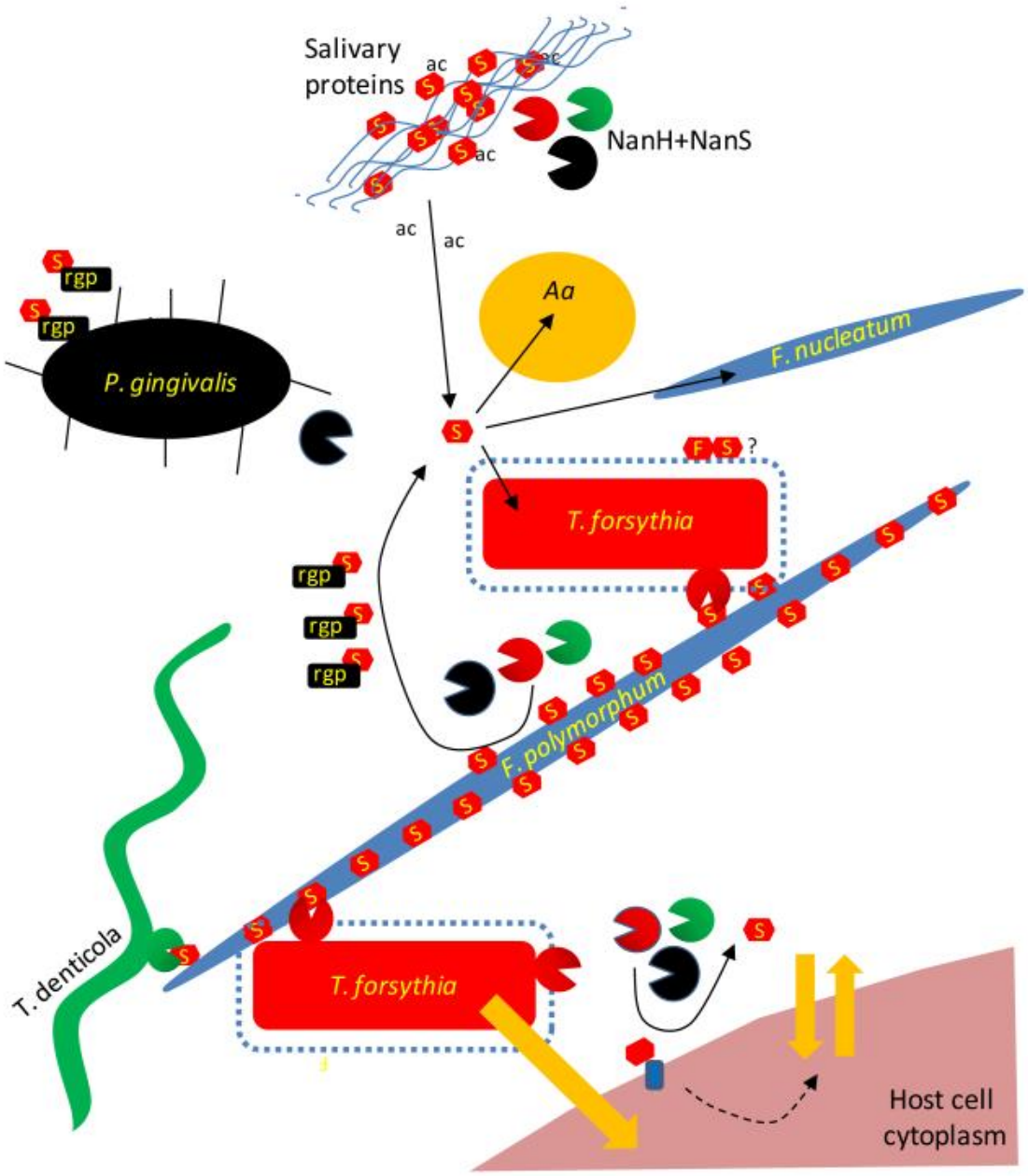

Figure 1.11.: Model for the role of surface sialic acid residues on the host and on bacterial oral pathogens in the development of a multispecies biofilm. Sialic acid residues are cleaved off by bacterial membrane-bound or excreted sialidases (*e.g.*, NanH form *T. forsythia*) which exposes adhesion sites leading to bacterial attachment to host-surfaces and bacterial co-aggregation. Cleaved sialic acid residues can, in turn, be internalized by the bacteria and utilized. Key: yellow arrows: signalling cascades, Ac: acetyl group, "s" in a hexagon: sialic acid residue, Aa: *Aggregatibacter actninomycetemconcomitans*, rgp: arginine gingipain (protease). [Taken from (Stafford *et al.* 2012)]

mented with carbohydrates, enzymatic activities for glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, involved in the pentose phosphate pathway, have been detected (Sakamoto *et al.* 2002). The genome additionally encodes enzymes involved in glycolysis, but key enzymes from the Entner-Doudoroff pathway are not present.

The genomes of *Bacteroidetes* that form part of the gut microbiota contain polysaccharide utilization loci (PULs) that code for the enzymes required for the breakdown of complex carbohydrates through a Sus (starch utilization system)-like system (Martens *et al.* 2009). These loci usually contain membrane-associated carbohydrate-binding proteins and lipoproteins and both endoglycosidases and all exoglycosidases needed to sequentially break down a particular glycan. Many of the glycosidases are actually located in the periplasm in a way that the majority of the glycan degradation occurs in a "selfish manner", meaning that it is compartmentalized so that resources are not used by other competing bacteria (Cuskin *et al.* 2015). Sequestered oligosaccharides in the periplasm are degraded into their sugar components before final transport into the cytoplasm (see Figure 1.12). It is interesting to note here, that, whilst various fucosidases in the gastrointestinal tract are found to be secreted by the resident bacteria, α1,6 fucosidase activity could not be detected (Dallas *et al.* 2012), indicating that the degradation of core fucoses probably occurs in this manner, inside the cell. Other glycosidases act on the outside of the cell and represent a possible synergistic interaction between the microbiota, complementing each other's glycosidase repertoire. Other similar loci to PULs exist in bacteria and are significantly less complete, for example in *Cellovibrio japonicus*, which is found in soil and degrades plant xyloglucans (Larsbrink *et al.* 2014). *C. japonicus* has a locus composed of three periplasmic GH (including a fucosidase) and a TonB-dependent receptor, whilst the other enzymes needed (*e.g.*, endoglycosidases) are encoded elsewhere and are not necessarily clustered together in the genome (Figure 1.12).

The annotated fucosidase in *T. forsythia*, Tanf_06770, does not form part of a Sus-like operon, yet the genome does encode a fucose permease elsewhere. For the metabolism

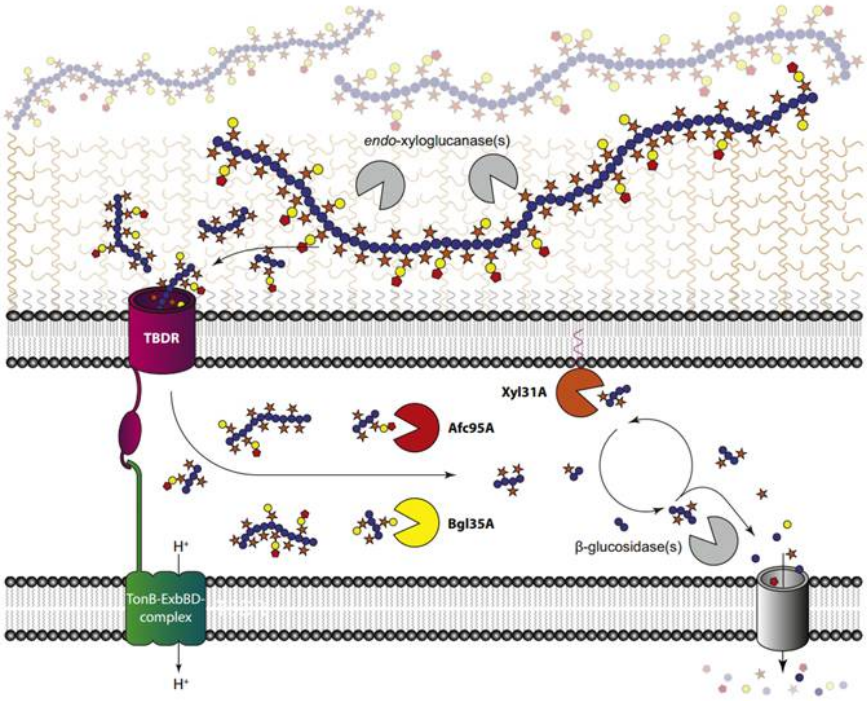

Figure 1.12.: Simplified Sus-like system in *Cellvibrio japonicus* for (fucogalacto)xyloglucan degradation. Endo-xyloglucanases are excreted to breakdown the bulk substrate. Xyloglucan oligosaccharides are then imported into the periplasm by a TonB-dependent receptor. Different exoglycosidases (Afc95A, Bgl35A, Xyl31A) gradually strip the galactose, fucose, xylose and glucose residues from the oligosaccharides, which are then transported into the cell via permeases for utilization. (Taken from (Larsbrink *et al.* 2014)]

of fucose, bacteria typically have a catabolic operon that encodes a regulator (*fucR*), a permease (*fucP*), an isomerase (*fucI*), a kinase (*fucK*) and an aldolase (*fucA*) to convert the imported fucose to lactaldehyde and dihydroxyacetone which can then enter the glycolytic pathway (Stahl *et al.* 2011). *T. forsythia* has no classical fucose catabolism locus in its genome, nor does it have the pathway described for *Xanthomonas campestris* and postulated for *C. jejuni* (see Figure 1.13). *Bacteroides* species can also GDP-activate the imported fucose and recycle it into their glycosylation pathway thanks to a bifunctional L-fucokinase/GDP-fucose pyrophosphorylase enzyme (FKP) (Coyne *et al.* 2005) (Figure 1.13). The S-layer glycan in *T. forsythia* has two fucose residues. Therefore, the pathogen could be recycling the cleaved fucose for use in the glycosylation of the S-layer and other glycosylated proteins. However, an FKP enzyme is not encoded in the genome and therefore, if activation of internalized residues occurs in *T. forsythia*, it would probably be accomplished by an alternative pathway.

1.5.2. Publication: *Inositol-phosphodihydroceramides in the periodontal pathogen* Tannerella forsythia *: structural analysis and incorporation of exogenous* myo*-inositol*

Background: Bacterial sphingolipids

Sphingolipids are integral components of eukaryotic cell membranes and are rarely found amongst bacteria (Heung *et al.* 2006). They have been shown to have important roles in cell signalling and in mammalian cells they regulate processes such as the cell stress response, proliferation, apoptosis and angiogenesis. In fungi they are implicated in the heat stress response, endocytosis and fungal pathogenesis, amongst others. Importantly, they play a major role in microbial pathogenesis in maintaining the balance between the microbe and the host. Bacteria and viruses, which in most cases do not produce sphingolipids, are able to utilize host sphingolipids to promote their virulence, for example by incorporating them into their membranes for immune evasion. On the other hand, microbial sphingolipids which are incorporated into the host tissues can interfere with the hosts signalling pathways and elicit autoimmune responses (Heung *et al.* 2006).

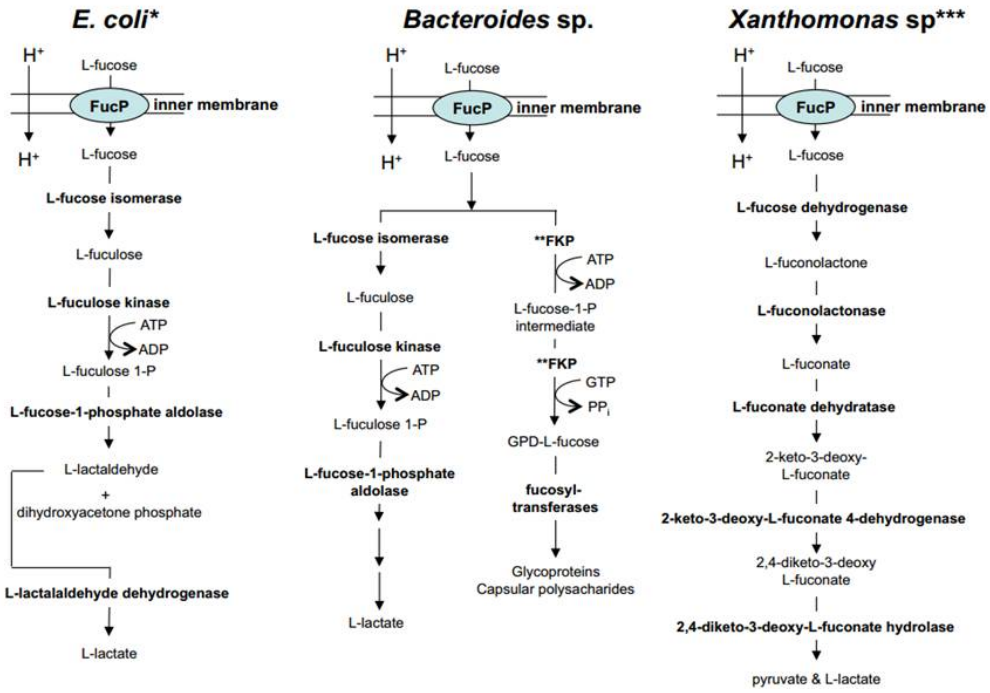

Figure 1.13.: Different fucose catabolic pathways described for *E. coli*, *Bacteroides* sp. and *Xanthomonas* sp. Enzymes are in bold. **FKP: bifunctional L-fucokinase/GDP-fucose pyrophosphorylase. Other asterisks are adaptations from other publications (please see original article (Stahl *et al.* 2011)). Abbreviations: FucP, fucose permease (fucose proton symporter of the major facilitator superfamily); FucK, fuculose kinase. [Taken from (Stahl *et al.* 2011)]

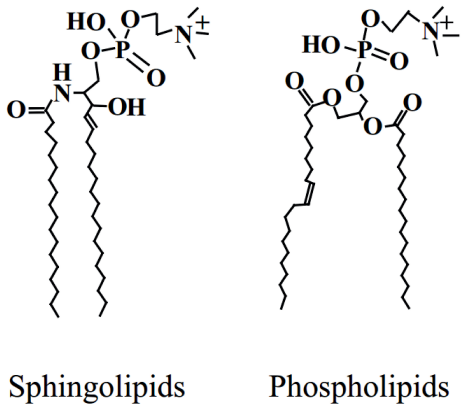

Figure 1.14.: Example of a sphingolipid and a phospholipid with phosphatidylcholine as a polar head group. Sphingolipids have an amino alcohol base backbone whilst phospholipids have a glycerol backbone. [Taken from (An *et al.* 2011)]

Sphingolipids are a family of lipids which contain a sphingoid base (amino alcohol base plus hydrocarbon chain) as a backbone, also referred to as "long-chain base". This distinguishes them from phospholipids, which have a glycerol-based backbone (Figure 1.14). The variability of sphingolipids is large and is reflected in the structural diversity of the sphingoid base (length, degree of saturation, branching and various substitutions), in the *N*-acylation to different fatty acids, and in the modifications at the hydroxyl group on carbon 1 with phosphate and other polar head groups to form more complex phosphosphingolipids and glycosphingolipids (Pruett *et al.* 2008).

Because of the differences in structure and chemistry of phospholipids and sphingolipids, they are thought to from separate domains, which can be seen in model membranes (Figure 1.15) (An *et al.* 2011). In eukaryotes, the sphingolipid domains are enriched in cholesterol and other proteins and are tightly packed due to the interaction between the sphingolipid carbon chains and the cholesterol and because of extensive hydrogen bonding formed by the amide linkage and hydroxyl groups. This high degree of order in these domains is thought to allow for membrane signalling. It has been suggested that bacteria containing sphingolipids have similar membrane structures, and although not able to synthesise cholesterol themselves, bacteria which reside in animal hosts are

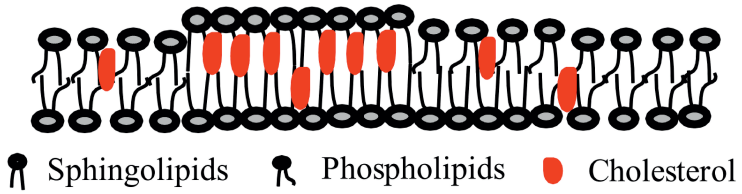

Figure 1.15.: Phospholipids and sphingolipids form separate domains in model membranes. Sphingolipid domains are enriched in cholesterol. [Taken from (An *et al.* 2011)]

able to incorporate exogenous cholesterol into their membranes, as seen for *B. fragilis* where both sphingolipids and addition of cholesterol to the medium were found to be needed for an efficient stress response (An *et al.* 2011).

The synthesis of sphingolipids is highly conserved amongst eukaryotes and begins with the condensation of serine and the activated fatty acyl palmitoyl-coenzymeA by serine palmitoyltransferase (SPT) (Figure 1.16) (Pruett *et al.* 2008). The resulting lipid is then reduced, which leads to dihydrosphingosine. From here, mammals synthesise complex sphingolipids (dihydroceramides and ceramides), whereas plants and fungi synthesise phytoceramide (dihydroceramides with an additional hydroxy group at the C4 position), which is then used to form the abundant inositolphosphoryl ceramide (IPC); alternatively, some fungi also produce glucosylceramide. Protozoa produce both mammalian-like and plant/fungal-like sphingolipids. Normally, bacterial membranes contain only glycerol-based phospholipids and sphingolipids occur in only a few bacterial genera: *Sphingobacterium*, *Sphingomonas*, *Bacteroides* and *Bdellovibrio* (Heung *et al.* 2006). There is a very high representation of sphingolipid-containing bacteria in the phylum *Bacteroidetes*, including gut *Bacteroides*, oral cavity associated *P. gingivalis*, gut and oral *Prevotella*, and *T. forsythia* (An *et al.* 2011; Nichols *et al.* 2011). Not much is known about sphingolipid synthesis in bacteria but it is thought to proceed in much the same way, as SPT homologues have been reported in several sphingolipid-containing bacteria and are well conserved in *Bacteroides* and related species (An *et al.* 2011).

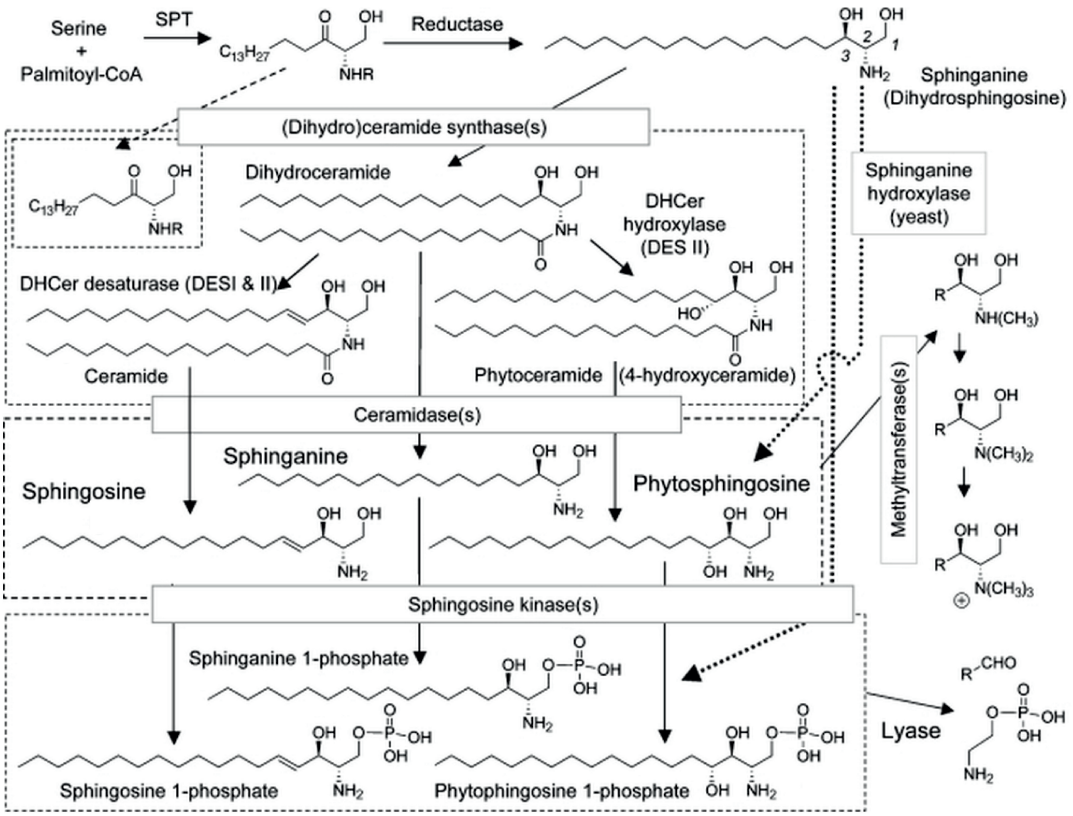

Figure 1.16.: Biosynthesis of sphingolipids in eukaryotes. Synthesis begins with the condensation of serine and the activated fatty acyl palmitoyl-coenzymeA by serine palmitoyltransferase (SPT) to form sphinganine (dihydrosphingosine). From here, different sphingolipids can be produced by different enzymes, as indicated. The sphingoid base can vary in length, degree of saturation and substitutions (hydroxyl and methyl groups). Fatty acids can be *N*-acylated to form dihydroceramides and ceramides. Polar head groups can be added to the carbon 1 of the sphingoid base to form phosphosphingolipids. [Taken from (Pruett *et al.* 2008)]

Figure 1.17.: *Iso*-branching and *anteiso*-branching of sphingoid bases. Branching can also occur in fatty acids. [Taken from (Pruett *et al.* 2008)]

An important factor determining the variability of the sphingolipids is the fatty acid synthesis as activated fatty-acyl groups are needed for both the synthesis of the sphingoid base and for the fatty acid that is *N*-acylated to form ceramides or dihydroceramides. Straight chain fatty acid synthesis in eukaryotes and bacteria occurs from acetyl-coA (primer) and malonyl-coA (chain extender-C2 subunits) by the repetition of six subsequent reaction steps until a 16-carbon fatty acid, palmitic acid, is formed. Palmitic acid is the most common fatty acid found in organisms, the first produced during fatty acid synthesis and the precursor for longer fatty acids. These synthesis steps occur by the action of the fatty acid synthase (FAS) I in animals, which is one large dimeric protein capable of performing all the steps required for palmitic acid synthesis, or FAS II, mainly in prokaryotes, plants and fungi, which is constituted by multiple enzymes that act as a complex. Type I fatty acid synthases are generally considered to be more efficient and the product is largely the single fatty acid palmitate and other fatty acids that differ in chain lengths by two -CH_2- units (28 Da difference). In contrast, type II fatty acid synthases can produce many different products including fatty acids of different chain lengths which can differ in a single -CH_2- unit (14 Da), *iso*- and *anteiso*-methyl-branched (Figure 1.17) (Kaneda 1991), and hydroxy fatty acids. The branching and differences in chain lengths can be used as an indication of bacterial lipids in mass spectrometric analysis of animal tissues. Branched-chain sphingoid bases have been found in human tissues and in bovine milk and kidney, but might only become associated with them by microorganisms that are a part of the normal or pathogenic microflora (Pruett *et al.* 2008). For example, branched dihydroceramide lipids from *P. gingivalis*, were recovered in human brain tissues and atherosclerotic lesions (Nichols *et al.* 2011) and will be discussed below.

It is worth noting that, whilst bacteria with straight chain fatty acids usually require monounsaturated chains for control of membrane fluidity and growth, unsaturated fatty acids are non-essential for bacteria with the branched chain system (Kaneda 1991).

Several mechanisms are known for the addition of polar head groups to lipids. Addition of groups to glycerolipids can involve the activation of diacylglycerol (DAG) with

CDP, for example for the synthesis of phosphatidylinositol (PI) (Morii *et al.* 2010). In most bacteria and archaea, the synthesis of PI needs not only activation of DAG but also the additional phosphorylation of inositol (Morii *et al.* 2014). Addition of the polar head group can also occur by activation of the polar head group with CDP instead of activation of the lipid, as used for the synthesis of phosphatidylethanolamine and phosphatidylcholine and also for PI in certain bacteria such as *Rhodothermus marinus* (Jorge *et al.* 2015). In sphingolipids, the synthesis of glucosylceramide and galactosylceramide occurs from the activated polar head groups, UDP-glucose and UDP-galactose, or by transfer of a polar head group from a glycerolipid to the ceramide (or dihydroceramide). For example, the generation of sphingomyelin occurs through the transfer of a phosphocholine headgroup from phosphatidylcholine to the ceramide, yielding the products DAG and sphingomyelin (Gault *et al.* 2010). The fungus-specific enzyme IPC-synthase-1 also synthesises IPC from phytoceramide by transfer of the polar head group from a glycerolipid (Luberto *et al.* 2001).

Some bacteria from the phylum *Bacteroidetes* are known to produce, amongst others, dihydroceramide (DHC) lipids containing 3-hydroxy (3-OH) *iso*-branched (*iso*) 17:0 linked to either an 18 or 19 carbon saturated long-chain base (Kato *et al.* 1995). This was described for the free DHCs of *B. fragilis* and for the novel DHCs in *P. gingivalis* (Kato *et al.* 1995; Nichols *et al.* 2004). This latter study in the oral pathogen *P. gingivalis,* describes the structures of lipids which include both low-mass and high-mass forms of phosphoglycerol DHC and phosphoethanolamine DHC, which showed a series of proinflammatory effects (Nichols *et al.* 2009; Minamino *et al.* 2003; Wang *et al.* 2010). In the low-mass forms, the phosphorylated polar head groups are linked to a core lipid structure consisting of either a 17-, 18-, or 19-carbon base in amide linkage to *iso*-17:0(3-OH) fatty acid. The high-mass forms, seen for the phosphoglycerol DHC lipids, present an additional *iso*-15:0 fatty acid in ester linkage to the hydroxyl group of the *iso*-17:0(3-OH) fatty acid (Figure 1.18). These lipids were also found in other common human bacteria, in *Bacteroides*, *Parabacteroides* and *Prevotella* species of intestinal bacteria and in the oral pathogens *T. forsythia* and *Pr. intermedia* and were

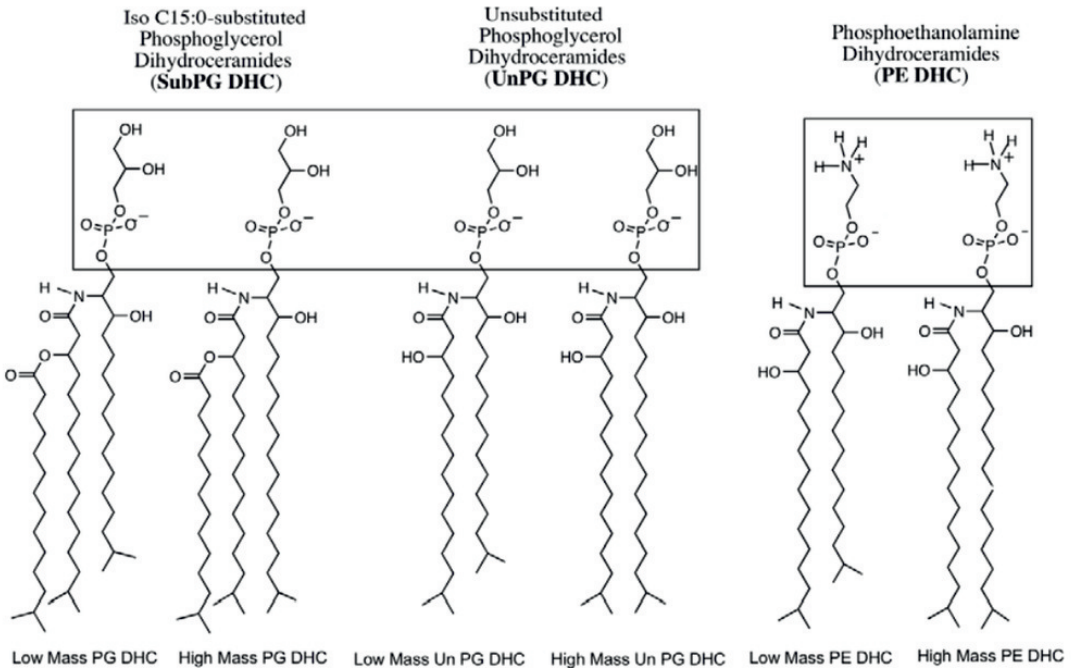

Figure 1.18.: Bacterial phosphorylated dihydroceramides (DHCs) found in *P. gingivalis*. Low-mass and high-mass forms (containing an additional 15:0 fatty acid substitution) of phosphoglycerol DHC and phosphoethanolamine DHC. [Taken from (Nichols *et al.* 2011)]

seen to accumulate in host tissues (Nichols *et al.* 2011). Apart from the study into these specific lipids in *T. forsythia* and studies on fatty acid composition involved in the reclassification of *T. forsythia* into its own genus (Sakamoto *et al.* 2002), no further knowledge on the lipidome of this pathogen has been acquired.

During the course of this thesis, two new DHC lipids where found in *T. forsythia*, one low-mass form and one high-mass form, containing *myo*-inositol as a polar head group. The study focuses on the structural analysis of the DHCs and on how the organism acquires the metabolite inositol for the synthesis of these novel lipids. Inositol-containing lipids are main constituents in all eukaryotic cell membranes and are also found in archaea but are found in relatively few bacteria. In eukaryotes they play important roles in protein-lipid interactions on cell surfaces and cell signalling (Michell 2008). Several Actinobacteria contain inositol in their lipids, including a few pathogenic genera such

as *Mycobacterium* where they have been shown to have important roles in macrophage infection and immuno-modulatory activities (Morita *et al.* 2011).

There are two main mechanisms for acquiring inositol in pathogenic microorganisms, synthesizing it *de novo* from glucose-6-phosphate and importing it from the host (Reynolds 2009). *De novo* generation of inositol occurs by a universal mechanism that is well conserved and depends on the action of two enzymes, inositol-3-phosphate synthase and inositol monophosphatase, which dephosphorylates inositol-3-phosphate to form inositol. Import of exogenous inositol occurs via Na^+- and H^+- linked transporters, but no transporters have been experimentally verified in bacteria that use inositol in their biosynthetic pathways. Some transporters have, however, been identified in bacteria which use inositol as a carbon source (*e.g.*, *Bacillus subtilis*).

Most pathogenic microorganisms that are known to use inositol have both pathways for acquiring the metabolite (Reynolds 2009). This includes the protozoa *Trypanosoma brucei* and *Leishmania* spp., the yeast *Candida albicans* and bacteria from the genus *Mycobacterium*. Many rely on the synthesis *de novo* to maintain the normal wild-type phenotype as the uptake of the exogenous metabolite cannot compensate for the synthesis pathway, suggesting that they may lack dedicated inositol transporters for efficient uptake. In this thesis, we show that *T. forsythia*, despite not having the genes for inositol synthesis *de novo*, can efficiently import exogenous inositol to sustain the synthesis of major lipid components.

2. Publications

2.1. Characterization of an α-L-fucosidase from the peridontal pathogen *Tannerella forsythia*

RESEARCH PAPER

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Characterization of an α-L-fucosidase from the periodontal pathogen *Tannerella forsythia*

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Keywords: α(1, 2) fucosidase, enzyme activity, enzyme specificity, oral pathogen, periodontitis, tannerella forsythia

Abbreviations: Amp, ampicillin; BHI, brain heart infusion medium; CBB, Coomassie brilliant blue G 250; DFJ, deoxyfuconojirimycin; Erm, erythromycin; TfFuc1, *T. forsythia* ATCC 43037 fucosidase-1 encoded by the *bfo_2737 g*ene, equally *Tffuc1*; FDH, fucose dehydrogenase; HPAEC, high-performance anion-exchange chromatography with pulsed amperometric detection; LC-ESI-MS, liquid chromatography-electrospray ionisation-mass spectrometry; rTfFuc-1, recombinant TfFuc1 enzyme; NAM, *N*-acetylmuramic acid; PBS, phosphate-buffered saline; pNP-fucose; 4-nitrophenyl-α-L-fucopyranoside; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; *T. forsythia*, *Tannerella forsythia* ATCC 43037; WT, wild-type bacterium.

The periodontal pathogen *Tannerella forsythia* expresses several glycosidases which are linked to specific growth requirements and are involved in the invasion of host tissues. α-L-Fucosyl residues are exposed on various host glycoconjugates and, thus, the α-L-fucosidases predicted in the *T. forsythia* ATCC 43037 genome could potentially serve roles in host-pathogen interactions. We describe the molecular cloning and characterization of the putative fucosidase TfFuc1 (encoded by the *bfo_2737 = Tffuc1* gene), previously reported to be present in an outer membrane preparation. In terms of sequence, this 51-kDa protein is a member of the glycosyl hydrolase family GH29. Using an artificial substrate, p-nitrophenyl-α-fucose (K_M 670 μM), the enzyme was determined to have a pH optimum of 9.0 and to be competitively inhibited by fucose and deoxyfuconojirimycin. TfFuc1 was shown here to be a unique α(1,2)-fucosidase that also possesses α(1,6) specificity on small unbranched substrates. It is active on mucin after sialidase-catalyzed removal of terminal sialic acid residues and also removes fucose from blood group H. Following knock-out of the *Tffuc1* gene and analyzing biofilm formation and cell invasion/adhesion of the mutant in comparison to the wild-type, it is most likely that the enzyme does not act extracellularly. Biochemically interesting as the first fucosidase in *T. forsythia* to be characterized, the biological role of TfFuc1 may well be in the metabolism of short oligosaccharides in the periplasm, thereby indirectly contributing to the virulence of this organism. TfFuc1 is the first glycosyl hydrolase in the GH29 family reported to be a specific α(1,2)-fucosidase.

Introduction

Tannerella forsythia is a Gram-negative anaerobic oral pathogen, a member of the so-called "red complex" of bacteria that causes a set of inflammatory diseases named periodontitis, affecting millions of people worldwide.[1-3] The effects on the periodontium include loss of the alveolar bone around the teeth, swelling and bleeding of the gum and, in more severe cases, loss of teeth. Periodontitis has also been linked to systemic inflammation and to an increased risk of stroke, heart attacks and atherosclerosis, among others.[4]

Like other bacteria residing in human hosts, *T. forsythia* has adapted to better suit its niche with cell surface glycosylation thought to be key to this adaptation.[5] As previously described, *T. forsythia* cells are completely covered by a unique surface (S-) layer formed by co-assembly of 2 different proteins both of which are highly *O*-glycosylated with an equally unique glycan.[6-8] Mutant strains lacking either the S-layer or glycan assembly and maturation genes, display phenotypes involving altered human cell attachment to host cells, biofilm formation, and disease progression.[9-11] In addition, the structure of the glycan partially imitates that of host glycoproteins, having a terminal sialic acid-like residue (precisely, a modified pseudaminic acid residue) and a terminal fucose, with the latter shown to be present in substoichiometric amounts and linked to a methylated galactose in an unknown glycosidic linkage.[8] The glycobiology of this pathogen, including its repertoire of glycosidases, seems to be key to its physiology and, potentially, its pathogenicity.[5,12]

Recent evidence suggests that for several periodontal pathogens, but particularly for the "red complex" organism *T. forsythia*,

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sialic acid-containing host molecules play an important role *in vivo*.[13] Two different sialidases have been found in *T. forsythia*, SiaHI and NanH. In the case of SiaHI, its function is unclear. It is not a canonical sialidase (*i.e.,* not in the GH35 family), a *siaH1* mutant has no discernible phenotype, and experiments point to it being a periplasmic protein without any role in extracellular interactions.[14] These same studies also indicated that mutants lacking the main *T. forsythia* sialidase NanH had hindered attachment and invasion of human oral epithelial cells. The enzyme was also seen to play an important role in biofilm growth on surfaces coated with salivary glycoproteins.[15] The *nanH* gene is located in a large cluster that contains all the genes required for sialic acid catabolism, which indicates that the cleaved sialic acid can additionally be taken up and utilized.[12,13] This gene cluster also contains a β-hexosaminidase that may cleave sub-terminal residues after sialidase action and may also play a role *in vivo*.[15] Additionally, in a separate study, transcriptome analysis of the oral microbiome also showed up-regulation of the sialidase *nanH* mRNA in dental plaque.[16]

α-L-Fucosyl residues are, like sialic acid, frequently located at a terminal position on various host glycoconjugates including blood groups, milk oligosaccharides, gastric and submaxillary mucin, and serum glycoproteins.[17,18] Therefore, fucosidases in *T. forsythia* could potentially play similar roles to sialidases.[19] It has generally been shown that terminal fucose residues play important roles in mammalian cell-cell communication and also in their interaction with pathogenic bacteria; for instance, *Campylobacter jejuni* and *Helicobacter pylori* are known to bind certain fucosylated blood groups (e.g., 0-antigen) in order to mediate infection.[19-21] In addition, the ability to utilize available fucose provides many bacteria with a nutritional advantage and contributes to survival in a highly competitive ecosystem, such as the human body.[22,23]

Characterization of α-L-fucosidases in *T. forsythia* could aid in the elucidation of the structure-function relationship of fucosylated host and bacterial surfaces in the virulence of oral pathogens. The genome of *T. forsythia* encodes 3 putative α-L-fucosidases, BFO_2737 and BFO_1182, both classified in the CAZy (Carbohydrate Active enZymes; http://www.cazy.org/) glycosyl hydrolase family GH29, and BFO_3101, classified in family GH95. While all 3 enzymes possess a glycosyl hydrolase domain and are classified by CAZy according to their mechanism of action, BFO_1182 and BFO_3101 are not strictly annotated as α-L-fucosidases but as a F5/8 type C domain protein and a putative lipoprotein, respectively.

Here, we describe the molecular cloning and characterization of BFO_2737, which we named TfFuc1. This protein has previously been reported to form part of an outer membrane preparation of *T. forsythia*[24] and, thus, was a good candidate to be involved in host-pathogen interactions. TfFuc1 is a 446-amino acid protein with a theoretical pI and molecular mass of 6.9 and 50.8 kDa, respectively. It is the first fucosidase in this organism to be characterized to date. The enzyme was shown here to be an α(1,2)-fucosidase and also possesses an α(1,6) specificity on small unbranched substrates. It is a predicted periplasmic protein, possibly playing a role in the breakdown of small oligosaccharides. It is, to the best of our knowledge, the first glycosyl hydrolase in its family (GH29) reported to be a specific α(1,2)-fucosidase.

Results

Enzymatic characterization of rTfFuc1

The *Tffuc1* gene was cloned into pET22-b(+) vector and expressed in *E. coli* as a C-terminally His_6-tagged protein, which enabled purification via nickel affinity chromatography (**Fig. 1**). The enzymatic activity was then tested using the standard colorimetric α-fucosidase substrate 4-nitrophenyl-α-L-fucopyranoside (pNP-fucose) at 22°C in a range of different pH values and in the presence of $MgCl_2$, KCl and NaCl, in order to establish its pH optimum and cation dependence, respectively. By stopping the reaction with the addition of an alkaline buffer at pH 11.4, it was ensured that all wells where at the same pH for consequent absorbance readings (**Fig. 2**). The activity of the enzyme was seen to start to plateau at the neutral to alkaline pH range and was considered most active at pH 9.0, assayed in glycine buffer, and not at pH 9.25 where the activity suddenly peaks and then rapidly decreases thereafter. The activity remained largely unaffected by the presence of cations at the 2 concentrations tested (results not shown). The K_M and V_{max} catalytic constants at 22°C, calculated from the activity of the enzyme at different pNP-fucose concentrations, were 670 μM and 20.4 μmol/min (U) per mg of protein, respectively (**Table 1**). The determined catalytic constants for rTffuc1 are in the range of those reported for other fucosidases/ glycosylhydrolases when tested on their corresponding pNP-substrates.[25-27]

Substrate linkage specificity of rTfFuc1

To determine the enzyme linkage specificity, rTfFuc1 was incubated with a set of different fucosylated substrates of defined structure representing a range of fucose linkages available on host glycoproteins and on oral surfaces (**Fig. 3**). The reaction products obtained after overnight incubation were analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC) where the release of fucose was confirmed by comparison with the retention time of the standard monosaccharide and of a substrate standard after overnight incubation at 37°C. The enzyme was seen to be active on both α(1,2) fucose containing substrates, 2-fucosyllactose and H-trisaccharide, and on the α(1,6) fucose disaccharide α-L-Fuc-(1,6)-β-D-GlcNAc, although this latter reaction did not reach completion, indicating weak specificity for this linkage. The α(1,3) and α(1,4) linkages were not cleaved on 3-fucosyllactose and the Lewis A trisaccharide, respectively. The enzyme was also inactive on the substrate α-L-Fuc-(1,4)-β-D-Gal, added as a second disaccharide control to prove that the α(1,6) activity was not due to differences in substrate length (**Fig. 4**).

In order to obtain accurate activity values on the cleaved substrates, the K-FUCOSE kit from Megazyme was used, coupled to the enzymatic reaction with rTfFuc1. First, FDH, which also has an alkaline pH optimum, and $NADP^+$ were added to the substrate solution reaction mixture in order to convert any free fucose

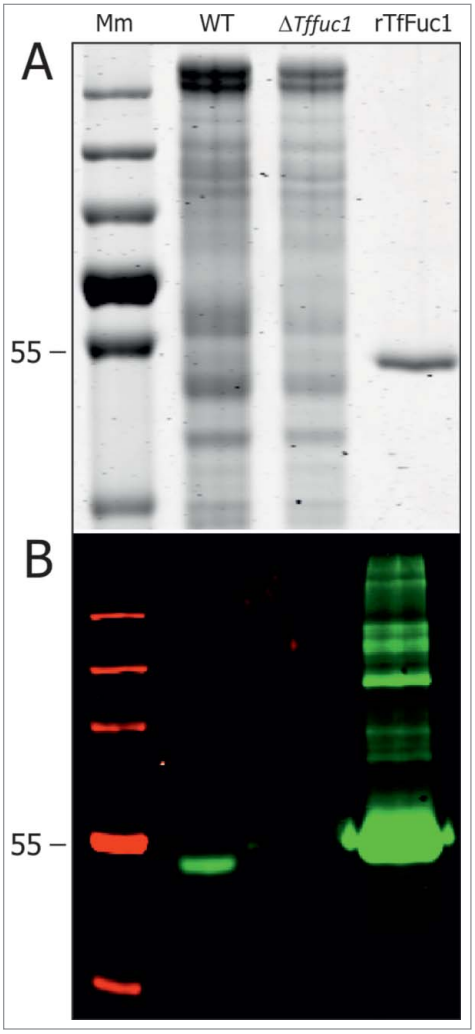

Figure 1. SDS PAGE (**A**) and Western immunoblot (**B**) of total cell extracts from *T. forsythia* WT (lane 2) and Δ*Tffuc1* strains (lane 3) and of the His_6-tagged rTfFuc1 as purified from *E. coli* (lane 4), used for activity studies and to raise a polyclonal anti-TfFuc1 antiserum. Western immunoblotting using the anti-TfFuc1 antiserum recognized the protein (~51 kDa) specifically in the WT strain (lane 2) and indicated absence of the protein in the Δ*Tffuc1* strain (lane 3), proving that the enzyme was effectively knocked-out. In the preparation of rTfFuc 1 (B, lane 4), the polyclonal antiserum recognizes also minor contaminating *E. coli* proteins not visible on the SDS-PAGE gel (A, lane 4). Mm; PageRuler Plus prestained protein ladder (Thermo Scientific).

already present in the sample to L-fucono-1,5-lactone by the reduction of $NADP^+$ to NADPH ($\varepsilon_{340} = 6.022\ mM^{-1}cm^{-1}$). rTfFuc1 was then added to the mixture and the reaction was monitored by following the increase in Abs_{340}. The activity was calculated from where the formation of NADPH was linear over time. The enzyme was most active on 2-fucosyllactose and H-trisaccharide with specific activities of 0.8 U/mg and 0.6 U/mg, respectively. The activity on the α(1,6) disaccharide was significantly lower at 0.35 U/mg (**Table 2**).

The activity of the enzyme on the various substrates could be calculated approximately (as some loss of material occurred during sample preparation) from the HPLC experiments after 1-h incubation periods (results not shown) and was found to be markedly lower than that observed with the K-FUCOSE kit, indicating that free fucose, which is consumed in the latter, could be inhibiting the enzymatic activity significantly. In order to determine the extent of such an effect, measurement of K_M and V_{max} values were repeated with pNP-fucose in the presence of either 0.25 mM L-fucose or 0.1 μM deoxyfuconojirimycin (DFJ), which is a strong fucosidase inhibitor.[28] The enzyme was competitively inhibited by both fucose and DFJ as the V_{max} remained largely unaffected but the K_M value increased from 0.67 mM to 16.5 mM and 28.3 mM, respectively (**Table 1**).

Further, the ability of the enzyme to cleave fucose residues off more complex natural glycans and those on branched sugar residues was assayed (compare with **Fig. 3**). As expected, the enzyme was unable to cleave the α(1,3) fucose linkage on GalFGalF-pep, included in the assays, as a trace amount of activity could be observed when using 3-fucosyllactose, as measured by the K-FUCOSE kit. The enzyme was also not able to cleave the core α-1,6 fucose linkage on $GnGnF^6$-pep nor the branched α(1,2)-linked fucose on the A antigen. The non-branched α(1,2) fucose linkage present on the Eastern oyster substrate, however, was cleaved off the substrate GalF, seen by the loss of a fucose residue in the MS spectra of the substrate. The major m/z 1703 glycan ($[M+H]^+$) was approximately 50% digested to a defucosylated species of m/z 1557 after overnight incubation with the enzyme (**Fig. 5**). The enzyme is, therefore, able to cleave off fucose residues which are α(1,2) linked on more complex glycans only when in a terminal unbranched position and is unable to cleave core α(1,6) fucose. This data supports that the enzyme acts as an α(1,2) fucosidase.

rTfFuc1 activity on bovine submaxillary mucin

rTfFuc1 was incubated with mucin from bovine submaxillary glands and the release of fucose was measured with the K-FUCOSE kit. Incubation was performed also in combination with rNanH from *T. forsythia* and activities were calculated from the slope of ΔAbs_{340} where it was linear over time. During the assayed incubation period of 10 min, no activity could be detected when rTfFuc1 was incubated alone with mucin. The ΔAbs_{340} lead to an irregular data set with a very low r^2 value. However, fucose release was detectable when the incubation was performed in conjunction with the rNanH sialidase, presenting a slow but steady increase in the Abs_{340}. The activity was calculated over a period of 300 s where the data points fitted a linear regression with an r^2 of 0.98 (**Fig. 6**). The experiment was repeated several times and yielded an activity of 24 ± 4 mU/mg. rTfFuc1 might cleave fucose off mucin over longer periods of time, but the data shows a significantly higher activity when sialic acid residues are first removed from mucin, indicating that the fucosidase TfFuc1 could work downstream from the sialidase in *T. forsythia* and presumably cooperate with other glycosidases in the degradation of complex glycans.

Table 1. rfFuc1 activity on 4-nitrophenyl-α-L-fucopyranoside (pNP-fucose). Catalytic constants K_M and V_{max} and the inhibitory effect of DFJ and L-fucose were measured using the colorimetric substrate pNP-fucose within a concentration range from 0.01 to 50 mM at 22°C in glycine buffer at pH 9.0

Substrate*	Inhibitor	K_M (mM)	V_{max} (U/mg)**
pNp-fucose	None	0.67 (±0 .2)	20.4 (±0 .8)
pNp-fucose	0.1 μM DFJ***	28.3 (±3 .7)	28.1 (±2 .4)
pNp-fucose	0.25 mM Fucose	16.5 (±4 .7)	28.6 (±4 .4)

*4-nitrophenyl-α-L-fucopyranoside.
**μmol/min/mg of enzyme.
***deoxyfuconojirimycin.

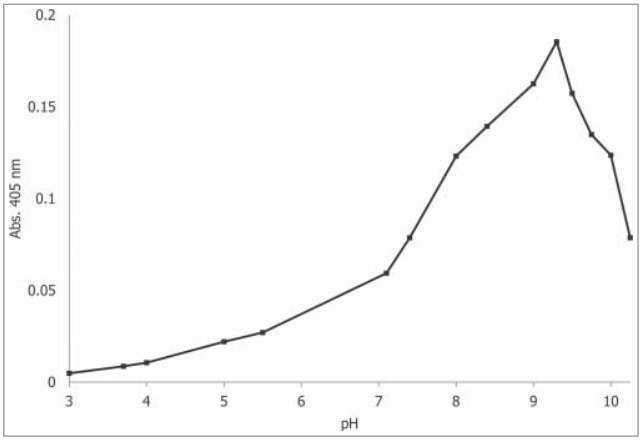

Figure 2. pH profile of rTfFuc1 using 4-nitrophenyl-α-L-fucopyranoside (pNP-fucose) as a substrate. Activity was measured as the increase in Abs_{405} due to the released 4-nitrophenol product. Citrate/phosphate buffer (0.1 M) was used to assay the pH range from 3–8, 50 mM glycine buffer was used for the pH range from 8.0–10.25.

Cellular localization of TfFuc1

TfFuc1 was previously reported to be present in the outer membrane fraction of *T. forsythia*.[24] In an effort to investigate its presence on the surface of *T. forsythia* cells, TfFuc1-specific polyclonal antiserum was raised against the recombinant enzyme in mice and used for Western immunoblotting of cellular fractions separated by SDS-PAGE. Protein visualization by CBB staining showed good separation between the fractions, as the S-layer bands were very prominent in the outer membrane fraction but not in the inner-membrane and non-membrane associated fractions. Western immunoblotting showed that all the detectable TfFuc1 fucosidase was found in the non-membrane associated fraction comprising both the cytoplasmic and periplasmic content (**Fig.** 7), arguing against surface localization of the TfFuc1 enzyme.

Discussion

Colonization of the periodontal pocket by the pathogenic late colonizer *T. forsythia* depends largely on pre-existing bacteria that have already tipped the oral balance away from health and toward disease.[29] Factors such as a pH shift from neutral to alkaline and slight raises in the temperature due to the host inflammatory response could be contributing factors favoring the process.[30] In a situation of oral disease, the number of different bacteria living in the gingival crevice decreases markedly due to putative pathogenic bacteria being more competitive in such an environment.[31] It is in these conditions that *T. forsythia* seems to thrive and becomes one of the key players in severe cases of periodontitis.

The NanH sialidase in *T. forsythia* has been well established to play important roles in adherence to sialylated glycoprotein-coated surfaces and epithelial cells in addition to triggering biofilm growth and being up-regulated in dental plaque.[14-16] As the other important terminal sugar on host glycoproteins is fucose, here, we performed an initial characterization of a putative α-L-fucosidase encoded in the *T. forsythia* genome, product of the gene *Tffuc1*, previously reported to be part of the outer membrane proteome.[24]

By producing the enzyme recombinantly in *E. coli* we were able to show that the enzyme is active across a broad pH range from 7.0–9.0, having an unusually high pH optimum of 9.0. It presents a unique α(1,2)-linkage specificity on terminal non-branched fucose residues, being also active on small non-branched α(1,6) fucosylated substrates. While both these linkages are cleaved at a considerable rate in the case of small linear substrates, the α(1,6) specificity is not detected on core fucoses on more complex glycopeptides. The α(1,2) linkage specificity was apparent on both small linear substrates, such as 2-fucosyllactose and H-trisaccharide, and on more complex glycans only when fucose occupied a terminal position, but not on a branched substrate where the fucose residue is linked to a fully substituted sugar. The enzyme seems to be, to the best of our knowledge, the first fucosidase in its GH family (GH29) to have a specific α(1,2) activity. The broad, high pH activity profile of this fucosidase ties in with its physiological niche which is known to have a pH that rises as periodontal disease progresses.[30] The possession of such enzymes with higher activities in alkaline surroundings could contribute to competiveness and virulence of *T. forsythia* in a diseased environment.

During the course of this study, it became clear that one of the issues possibly underlining our observations was the enzyme's cellular localization. Even though TfFuc1 was found previously to be present in the outer membrane proteome of the pathogen,[24] localization of the enzyme on the surface of *T. forsythia* cells by fluorescent immunolabelling was not successful (data not shown; see Experimental Procedures in the Supplementary Information) and cell fractionation also showed the detectable protein to be found in the non-membrane associated fraction (**Fig.** 7). This would be in agreement with bioinformatics interrogation indicating that, while it has a Sec-dependent secretion signal (as predicted by SignalP 4.1 Server), it is not predicted to reside in the outer membrane or be secreted, nor does it contain a T9SS C-terminal secretion signal.[32] The difference in location of TfFuc1 between the present study and the outer membrane proteomics experiment[24] can be reconciled when considering that

in the latter study, cross-contamination of individual proteins between cellular fractions was not investigated.

Incubation of the recombinant fucosidase with bovine submaxillary mucin showed no detectable release of fucose over an incubation period of 10 min. Activity on this complex substrate could only be detected when the incubation was performed in combination with the recombinant NanH sialidase from *T. forsythia*. It is, therefore, conceivable that TfFuc1 could play an accompanying role to the sialidase in the interaction between *T. forsythia* and host glycoproteins, but given its periplasmic location, this could merely reflect the need for removal of terminal sialic acid residues for the enzyme to work, either indicating that it most likely acts on internalised fucosyl substrates after sialic acid has already been removed by the action of sialidases or, less likely, that it acts in concert with sialidases externally.

This notion that the *T. forsythia* fucosidase TfFuc1 plays an internal role was corroborated when we tested the effect of the Δ*Tffuc1* mutation on the ability of *T. forsythia* to interact with and invade human oral epithelial cells using an antibiotic protection assay on the oral epithelial cell line H357.[33] We found no significant differences in the ability of the Δ*Tffuc1* to invade these human cells as compared to the WT strain (**Fig. S1**), indicating that TfFuc1 has no effect on epithelial cell-invasion under the conditions tested. In addition, the mutant did not show hindered biofilm formation when cultured on bovine submaxillary mucin, contrary to the *T. forsythia NanH* sialidase mutant,[15] but showed a slightly increased biofilm formation (**Fig. S2**).

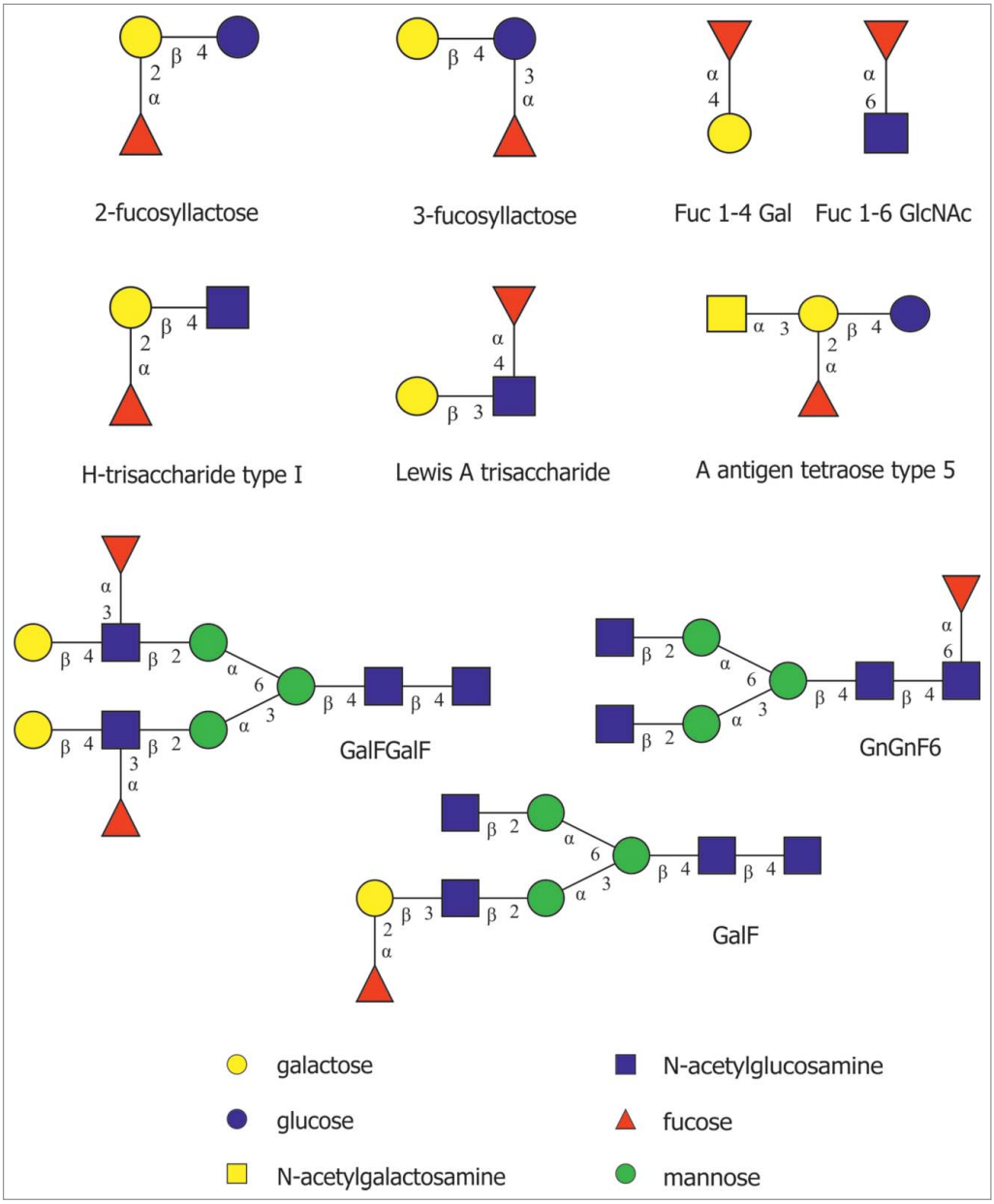

Figure 3. Fucosylated substrates used in this study. The structures are depicted according to the symbolic nomenclature of the Consortium for Functional Glycomics (http://www.functionalglycomics.org/static/consortium/Nomenclatures.html).

Our data supports the idea of a periplasmic fucosidase involved in the final breakdown of small substrates that have been internalized, possibly owing to the action of exoglycosidases and endoglycosidases which break-down larger glycans on the outside of the cell.[34,35] TfFuc1 would possibly then be able to exert its full potential freeing both α(1,2) and α(1,6) fucoses on small linear substrates. These findings are also in agreement with the hypothesis that the fucosidase acts downstream of the sialidases, which have been shown to act on whole glycoproteins on bacterial and host surfaces.[14,15,36] The sialic acid would,

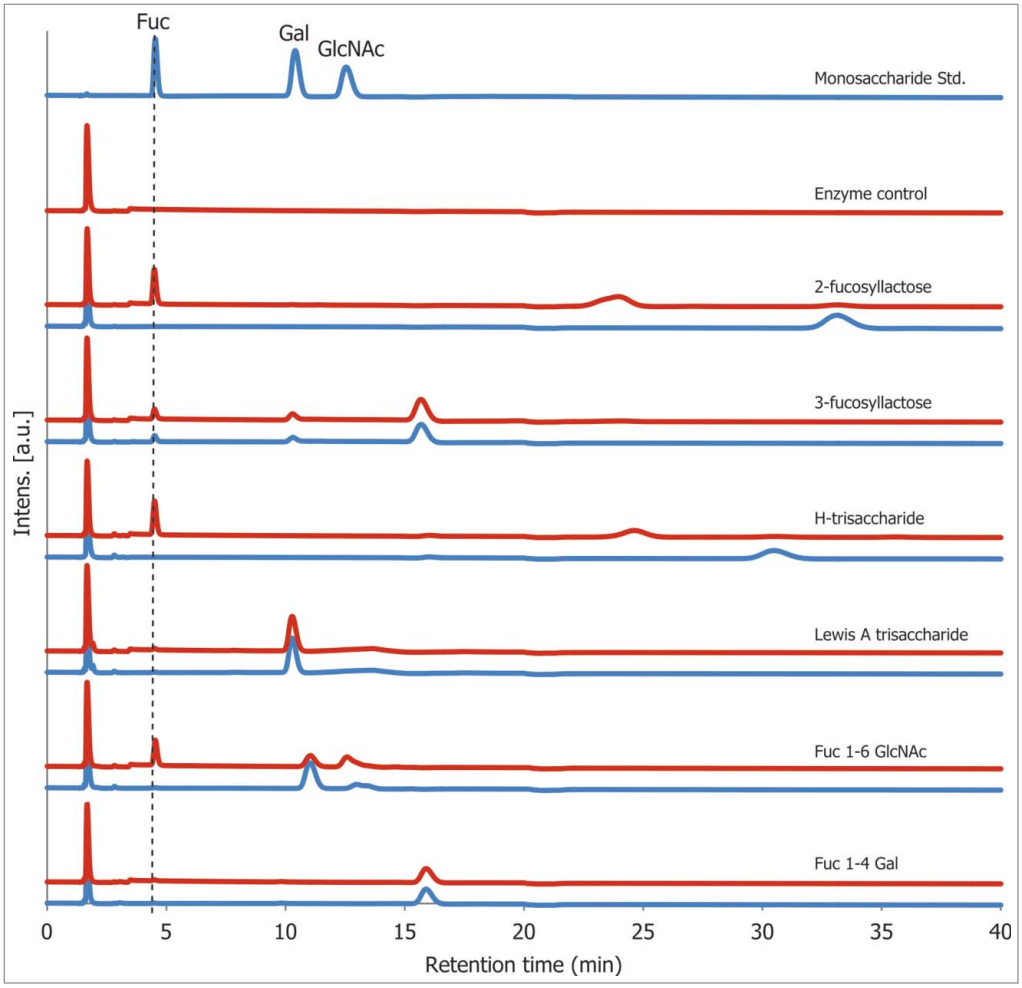

Figure 4. rTfFuc1 activity on standard fucosylated substrates after overnight incubation as determined by HPAEC. Blue lines represent samples which were incubated in absence of rTfFuc1 (substrate standard) and red lines represent samples incubated in the presence of rTfFuc1. Cleavage of the substrates was determined by the appearance of a fucose peak, as determined by the retention time of the standard monosaccharide.

therefore, already be missing as smaller parts of the glycan are transported inside the cell.

T. forsythia has no straightforwardly identifiable fucose catabolism locus in its genome, nor does it have the bifunctional L-fucokinase/GDP-fucose pyrophosphorylase required normally for *Bacteroidetes* to recycle the fucose into its glycans.[23] In an effort to see the effect on the latter scenario, the fucose containing S-layer glycan from both the WT and the Δ*Tffuc1* strains were compared by LC-ESI-MS with no obvious change under the growth conditions used (Z.A. Megson, L. Neumann, F. Altmann, C. Schäffer, unpublished data). However, the microheterogeneity of the S-layer glycan regarding the terminal fucose residue complicates interpretation of MS data. Therefore, it remains unclear whether the released fucose in the periplasm can be used as a nutrient source or is recycled by the bacteria into its glycosylation pathway, and, thus, is subject of further studies.

Overall, our data suggest that TfFuc1 is a unique α-L-(1,2)-fucosidase which could potentially contribute to fucose utilization in *T. forsythia*. In order to better elucidate this role and rule out any redundancy in the system, 2 further annotated fucosidases in *T. forsythia*, BFO_1182 and BFO_3101, together with the annotated fucose permease, BFO_0307, are now being investigated to elucidate the role of fucose in the physiology of *T. forsythia*.

Table 2. rTfFuc1 activity on standard fucosylated substrates. Cleavage was determined by HPAEC after overnight incubations with rTfFuc1 (see Fig. 4, with the exception of A antigen tetraose) and specific activities were calculated using the K-FUCOSE kit

Substrate	Structure	Fucose linkage	Enzyme cleaves	Activity (U/mg)*
2-Fucosyllactose	α-L-Fuc-1,2-β-D-Gal-1,4-D-Glc	α(1,2)	Yes	0.8
3-Fucosyllactose	β-D-Gal-1,4(α-L-Fuc-1,3)-D-Glc	α(1,3)	No	—
H-trisaccharide	α-L-Fuc-1,2-β-D-Gal-1,3-β-D-GlcNAc	α(1,2)	Yes	0.6
Lewis A trisaccharide	β-D-Gal-1,3(α-L-Fuc-1,4)-β-D-GlcNAc	α(1,4)	No	—
Fuc(1,6)GlcNAc	α-L-Fuc-1,6-β-D-GlcNAc	α(1,6)	Yes	0.35
Fuc(1,4)Gal	α-L-Fuc-1,4-β-D-Gal	α(1,4)	No	—
A antigen tetraose	β-GalNAc-1,3(α-L-Fuc-1,2)-β-D-Gal-1,4-D-Glc	α(1,2)	No	—

*μmol/min/mg of enzyme.

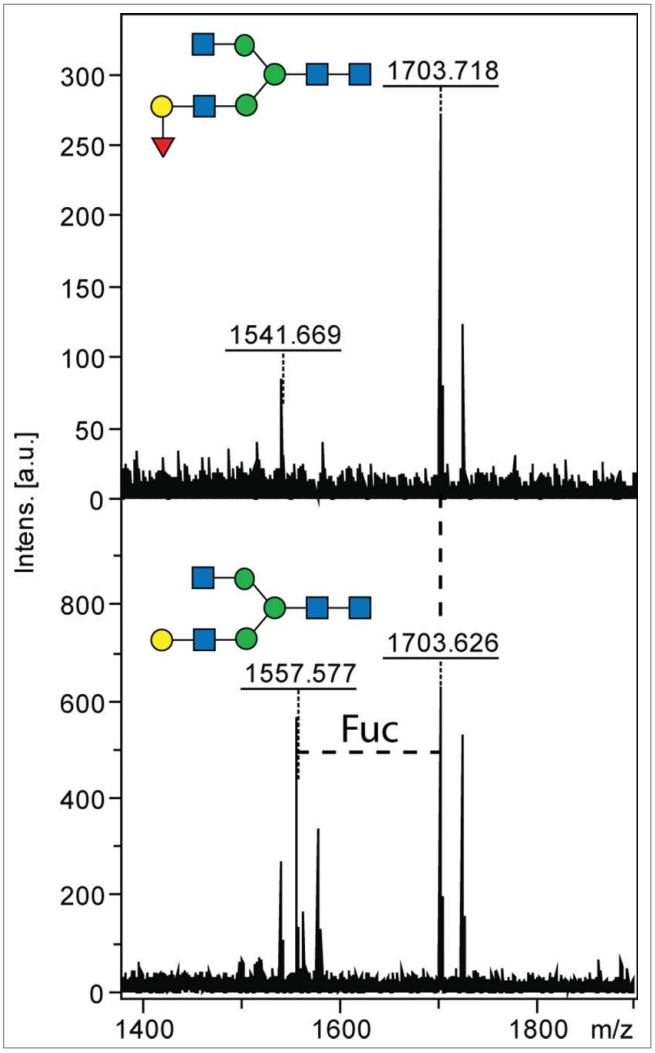

Figure 5. Cleavage of natural α(1,2) fucosylated glycans by rTfFuc1. Cleavage of fucose from a large *N*-glycan substrate was monitored by MALDI-TOF MS spectra after overnight incubation; the conversion of the *m/z* 1703 glycan (GalF) to one of *m/z* 1557 (Δ*m/z* 146) is indicative of the loss of fucose. The structures of the substrate and product are depicted according to the symbolic nomenclature of the Consortium for Functional Glycomics.

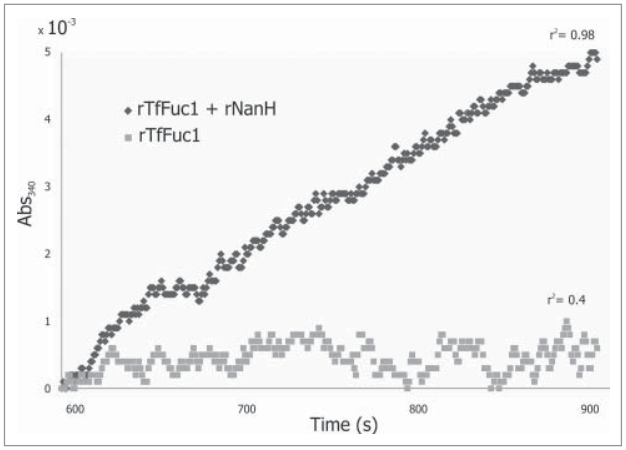

Figure 6. rTfFuc1 was incubated with mucin from bovine submaxillary glands and the release of fucose was measured with the K-FUCOSE kit. When incubations were performed in conjunction with the rNanH sialidase, a slow steady increase in the Abs_{340} was observed. The activity was calculated over a period of 300 s where the data points fitted a linear regression with an r^2 of 0.98. No activity could be detected when rTfFuc1 was incubated alone with the mucin. The ΔAbs_{340} lead to an irregular data set with a very low r^2 value of 0.4.

Experimental Procedures

Bacterial strains, medium and culture conditions

T. forsythia wild-type (WT) strain ATCC 43037 (American Type Culture Collection) and the knockout mutant Δ*Tffuc1* were grown anaerobically at 37°C for 4–7 d in brain heart infusion (BHI) broth or 0.8% (w/v) BHI agar, supplemented with *N*-acetylmuramic acid (NAM), horse serum and gentamycin as described previously.[32] *Escherichia coli* DH5α and BL21 (DE3) (Invitrogen) were cultivated in selective Luria Bertani (LB) medium (agar and broth) supplemented with 100 μg/ml ampicillin (Amp). All strains and plasmids used in the course of this study are summarised in **Table 3**.

Molecular methods

All enzymes were purchased from Fermentas. Genomic DNA of *T. forsythia* WT strain ATCC 43037 was isolated from 2 ml of bacterial suspension as described previously and used as the DNA template in all PCRs, unless otherwise specified.[37] The GeneJET™ Gel Extraction Kit (Fermentas) was used to purify DNA fragments from agarose gels and to purify digested plasmids and oligonucleotides. Plasmid DNA from transformed cells was isolated with the GeneJET™ Plasmid Miniprep kit (Fermentas). Agarose gel electrophoresis was performed as described elsewhere.[38] Primers for PCR and DNA sequencing were purchased from Invitrogen (**Table 4**). PCR was performed using the Phusion® High-Fidelity DNA Polymerase (Fermentas) and a My CyclerTM (Bio-Rad) thermal cycler. Transformation of chemically competent *E. coli* DH5α and BL21 (DE) cells was performed according to the manufacturer's protocol (Invitrogen). *E. coli* transformants were screened by PCR using RedTaq ReadyMix PCR mix (Sigma-Aldrich) and recombinant clones were analyzed by restriction mapping. Expression vector and knockout cassette were sequenced (Microsynth) prior to transformation.

Recombinant production of His_6-tagged TfFuc1

The *Tffuc1* gene was amplified from the chromosome of *T. forsythia* ATCC 43037 with a fused C-terminal His_6-tag by PCR using primer pair 1/2 (**Table 4**). The His_6-tagged amplification product was digested using restriction enzymes NdeI/XhoI and cloned into NdeI/XhoI-linearized pET22-b (Novagen). The corresponding plasmid was transformed into *E. coli* BL21 (DE3) cells for protein expression. Freshly transformed cells were grown in 2 400-ml Erlenmeyer flasks to an OD_{600} of 0.4–0.5 in the presence of 100 μg/ml of Amp at which point protein expression

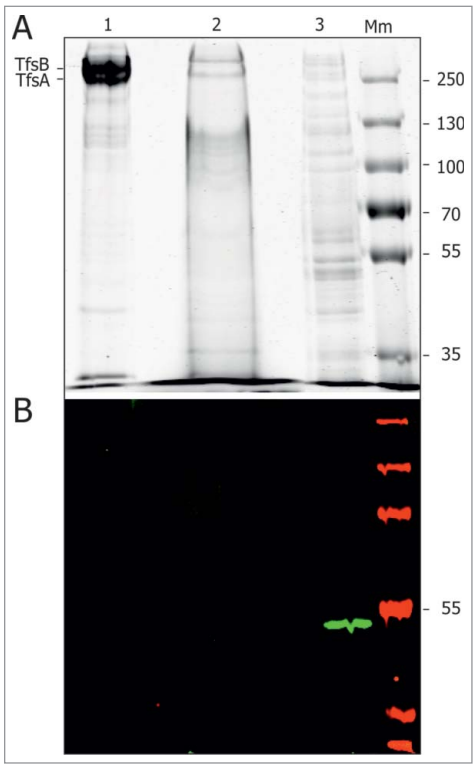

Figure 7. Presence of TfFuc1 in cell fractions of *T. forsythia* WT. (**A**) SDS-PAGE analysis of the outer membrane fraction (OM) (1), membrane fraction (2) and non-membrane associated fraction (3) showed good separation between the fractions, as the S-layer bands were very prominent in the OM but not in the membrane and non-membrane associated fractions. Protein loaded was 20 μg of the OM and non-membrane associated fractions and 400 μg of the membrane fraction. Protein visualization was by CBB. (**B**) Western immunoblot using anti-TfFuc1 antiserum showed the TfFuc1 fucosidase in the non-membrane associated fraction comprising both the cytoplasmic and periplasmic content. Mm; PageRuler Plus prestained protein ladder (Thermo Scientific).

was induced with a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and cultures were shaken (200 rpm) overnight at 18°C. Cells were harvested by centrifugation (6500 g, 20 min, 4°C).

Collected bacterial cells were lysed by sonication in buffer A (50 mM phosphate buffer pH 8, 0.3 M NaCl) containing 5 mM imidazole and cleared lysates after ultracentrifugation at 150000 g for 30 min at 4°C were incubated with 1 ml of Ni-NTA beads (Qiagen) for 1 h at 4°C, shaking slightly. The beads were placed in a chromatography column and the His_6-tagged protein was purified using an imidazole gradient in buffer A; 25 mM imidazole (10 ml), 50 mM imidazole (10 ml), followed by 5 elution steps with 500 μl of 250 mM imidazole in buffer A. Eluted fractions containing the purified recombinant protein, rTfFuc1, as determined by SDS-PAGE analysis, were pooled and dialysed overnight at 4°C against 3 l of 10 mM phosphate buffer, pH 8.0. The volume was then reduced 5-fold using a concentration centrifuge yielding a protein concentration of 0.35 mg/ml (as determined by Nanodrop) in 50 mM phosphate buffer.

Construction of a *T. forsythia* Δ*Tffuc1* knockout strain

Disruption of the *Tffuc1* gene in *T. forsythia* was performed by gene knockout, as described previously.[32] The *Tffuc1* gene is not part of an operon, thus, downstream effects due to the chosen mutation strategy are not expected to occur. Briefly, the flanking genomic regions (1000 bp) up-stream and down-stream of *Tffuc1* were amplified using primer pairs 3/4 and 5/6, respectively (**Table 4**). The two resulting fragments were joined with the erythromycin resistance gene *ermF-ermAM* (amplified using primer pair 7/8) by overlap extension PCR and sub-cloned into the blunt-end cloning vector pJET1.2 (Thermo Scientific), resulting in pJET1.2/*Tffuc1*_ko. Approximately 5 μg of the knockout cassette was transferred by electroporation into 100 μl of competent *T. forsythia* cells. Cells were regenerated in BHI medium for 24 h before plating on BHI agar plates containing erythromycin (10 μg/ml) as a selection marker. Single colonies were picked and used for inoculation of liquid BHI medium. Genomic DNA of the new Δ*Tffuc1* mutants were isolated as mentioned above and the absence of the *Tffuc1* gene and the correct integration of the erythromycin resistance gene (upstream and downstream) was evaluated by PCR using primer pairs 1/2, 9/10, and 11/12, respectively (**Table 4**). Absence of the enzyme in the Δ*Tffuc1* strain was also confirmed by Western immunoblotting of the total cell extract separated by SDS-PAGE using TfFuc1-specific polyclonal antiserum (**Fig. 1**).

General and analytical methods

SDS-PAGE was carried out according to a standard protocol using a Protean II electrophoresis apparatus (Bio-Rad).[39] Protein bands were visualized with Coomassie brilliant blue G 250

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant characteristic(s)	Source
Escherichia coli DH5α	F^-φ80dlacZ M15 (lacZYA-argF)U169 deoR recA1 endA1 hsdR17 (rK^-mK^-) phoA supE44 thi-1 gyrA96 relA1$^-$	Invitrogen
Escherichia coli BL21 (DE)	F^-, ompT, hsdS (rB^-mB^-), gal, dcm (DE3)	Invitrogen
Tannerella forsythia ATCC 43037	Wild-type isolate	American Type Culture Collection, USA
T. forsythia ΔTffuc1	*T. forsythia* knockout of the *Tffuc1* gene; Erm^r	This study
pET-22b (+)	Expression vector with a His_6-tag, Amp^r	Novagen
pJET1.2-*Tffuc1*_ko	pJET1.2 carrying the *Tffuc1_ermF*-AM knockout cassette	This study

Table 4. Oligonucleotide primers used for PCR amplification reactions[a]

Primers	Sequence (5′ → 3′)[a]
1	gcggCATATGAAAACAAGAACATTACTTCTTTGTG
2	gctaCTCGAGGTTTAGAGGCAATTCATTGGCAAATG
3	GACCAAGCTGCAGGCCATCATCGATGTGCTCAAC
A	*GAAGCTATCGGGGGTACCTCCCCCGGG*-AGAATAATTTTTGTTTATTACTAAAAAATAACG
5	*GCTTCGGGGATCCTCTAGCCCCCGGG*-CAGAAATATCTTTATGAAACATCCTATTGATGGGGTG
6	GCTCAGCCAGCCGATAGTTACTTTTTTCGTTATGTGTTCCC
7[b]	CGTTATTTTTTAGTAATAAACAAAAATTATTCT-CCCGGGGGAGGTACCCCCGATAGCTTC
8[b]	CACCCCATCAATAGGATGTTTCATAAAGATATTTCTG-CCCGGGGGCTAGAGGATCCCCGAAGC
9	CACGATGAACGTGTCGGTCATTAAC
10	GAAGCTATCGGGGGTACCTCCCCCGGG
11	GCTTCGGGGATCCTCTAGCCCCCGGG
12	GCACATATTTAGTAACCCGATAGCC

[a.] Artificial restriction sites are underlined. Lowercase letters indicate artificially introduced bases to improve restriction enzyme cutting.
In italics are the overlap sequences complementary to *ermF-ermAM*. In bold are the overlap sequences complementary to the *BFO_2737* flanking regions.
[b.] Primer sequences were taken from Honma et al.[11]

(CBB) staining reagent. For Western immunoblotting of proteins onto a nitrocellulose membrane (Peqlab), a Mini Trans-Blot Cell (Bio-Rad) was used. Detection of the His_6-tag fused to rTfFuc1 and detection of TfFuc1 was done with the Li-Cor Odyssey Infrared Imaging System using an anti-His_6 mouse antibody (Roche) or TfFuc1-specific polyclonal antiserum raised in mice (EF-BIO), respectively, both in combination with goat anti-mouse IgGIR Dye 800CW conjugate (Li-Cor).

Enzymatic characterization of rTfFuc1

Enzymatic characterization of rTfFuc1 was performed essentially as described elsewhere.[40] 0.17 μM of purified, recombinant enzyme was incubated with 5 mM of the colorimetric substrate 4-nitrophenyl-α-L-fucopyranoside (pNP-fucose) (Sigma) at a range of different pH values (3.0–10.25) and cation concentrations in 96-well plates at 22°C in a total volume of 40 μl. The enzymatic reaction was stopped after 3 min by addition of 260 μl of phosphate buffer, pH 11.4. Citrate/phosphate buffer (0.1 M) was used to assay the pH range from 3.0–8.0,[41] 50 mM glycine buffer was used for a pH range from 8.0–10.0. The effect of $MgCl_2$ (5 mM, 10 mM), KCl (5 mM, 10 mM) and NaCl (50 mM, 150 mM) on the enzyme's activity was assayed in the same way in 50 mM glycine buffer, pH 9.

A 4-nitrophenol standard curve was made by measuring the absorbance at 405 nm (Abs_{405}) of 0, 4, 8, 12, 16, 20 and 24 nmol of 4-nitrophenol per well in 300 μl of phosphate buffer, pH 11.4. The K_M and V_{max} catalytic constants were calculated at pH 9.0 in 50 mM glycine buffer at 22°C in the presence of 0.01 to 50 mM pNP-fucose. The inhibitory effect of fucose and deoxyfuconojirimycin (DFJ) on the K_M and V_{max} of the enzyme were assayed in the same way in the presence of 0.25 mM fucose and 0.1 μM DFJ, respectively.[28] Readings were performed using an Infinite 200 plate reader (TECAN) and catalytic constants were calculated with the Sigma Plot 12, Systat Software.

Substrate specificity of rTfFuc1

For the determination of enzyme linkage specificity, a set of commercially available fucosylated substrates (2-fucosyllactose and 3-fucosyllactose from Dextra laboratories; H-Trisaccharide, Lewis A trisaccharide, Fuc-α-(1,4)-Gal and Fuc-α-(1,6)-GlcNAc, all from Carbosynth) (**Fig. 3**) were incubated with the enzyme and reaction mixtures were analyzed by HPAEC using an ICS3000 chromatographic system (Dionex, Thermo Fisher) on a CarboPac PA-1 column. Incubations were made overnight at 37°C in a total volume of 100 μl by mixing 0.34 μM of enzyme with 0.5 mM of substrate in 50 mM glycine buffer, pH 9.0. In order to minimize the effect of the buffer, the reaction volume was then diluted with 400 μl of Milli-Q water and the enzyme was removed using an Amicon 3 kDa cut-off spin column (Millipore). Twenty-five microliters of this flow through was then applied to the CarboPacPA-1 column using full-loop injection.

In order to obtain reliable activity values, the K-FUCOSE kit (Megazyme) was adapted to suit requirements. In a total volume of 250 μl of 50 mM glycine buffer at pH 9.0, the substrates 2-fucosyllactose, H-trisaccharide and Fuc-α(1,6)-GlcNAc, were incubated separately at a concentration of 0.5 mM with 1.83 μl of fucose dehydrogenase (FDH) and 9.15 μl of $NADP^+$ (both as supplied) in a cuvette at 37°C for 10 min. When the reaction had reached a constant absorbance at 340 nm (Abs_{340}), rTfFuc1 was added to the mixture at a concentration of 0.34 μM and the formation of NADPH was followed by continuous measurement of the increase in Abs_{340}. The activity of the enzyme on each substrate was calculated according to the supplier's specifications from ΔAbs_{340}/min where the formation of NADPH was linear over time. The experiment was repeated with different enzyme dilutions (1:10; 1:100) to prove the reliability of the method.

The ability of the enzyme to cleave fucose residues off more complex natural glycans and those on branched sugar residues was assayed on the substrates A antigen tetraose type 5 (Carbosynth), $GnGnF^6$-peptide, GalFGalF, and an *N*-glycan derived from *Crassostrea virginica* (Eastern oyster) haemocyte treated with chicken liver α-*N*-acetylgalactosaminidase and bovine β-galactosidase (both from Sigma) to reveal the underlying H epitope presenting a terminal α(1,2) fucose on an unsubstituted β(1,3)-galactose (*i.e.,* histo blood group antigen H), referred to here as GalF[42] (**Fig. 3**). Activity on the A antigen substrate was assayed using the K-FUCOSE kit as described above. For all other substrates, incubations were performed overnight at 37°C and analyzed by MALDI-TOF MS using an Autoflex Speed instrument (Bruker) in positive ion mode with 6-aza-2-thiothymine (ATT) as matrix. Spectra were processed with the manufacturer's software (Bruker Flexanalysis 3.3.80) using the SNAP algorithm with a signal/noise threshold of 6 for MS (unsmoothed) and then manually interpreted.

rTfFuc1 activity on mucin from bovine submaxillary glands

rTfFuc1 was incubated with bovine submaxillary mucin (Sigma) in combination with the His-tagged recombinant NanH sialidase (rNanH) from *T. forsythia*.[15] Incubations were performed at 37°C in 50 mM glycine buffer, pH 9.0, at a final concentration of 0.1 μM of either enzyme, 0.2 mg/ml of mucin and $NADP^+$ and FDH as described above, in a total volume of 250 μl. rTfFuc1 (and rNanH) was added to the mixture after an initial 5-min incubation period. The release of fucose (ΔAbs_{340}) was followed over 10 min and calculated according to the supplier's specifications from ΔAbs_{340}/min where the formation of NADPH was linear over time.

Presence of TfFuc1 in *T. forsythia* membrane, outer membrane and non-membrane preparations

Cells were harvested by centrifugation from a 4-day-old 100-ml *T. forsythia* culture. Separation of cellular fractions was performed as described previously.[24] Briefly, cells were washed once in Tris (2-amino-2-hydroxymethy-L-propane-1,3-diol)-buffer, pH 7.5, sonicated, and cell debris were removed by centrifugation. The collected supernatant was ultracentrifuged (100000 g, 4°C, 40 min) to separate the whole membrane fraction (pellet) from the membrane non-associated fraction (cytoplasm and periplasm, supernatant). The pellet was resuspended in 2% (w/v) N-lauroylsarcosine (Sigma) in Tris buffer and mixed. After incubation (2 h, 25°C), the outer membrane fraction (OM) was collected by centrifugation (100000 g, 4°C, 40 min) and the pellet was resuspended in Tris buffer. The protein content was determined in each fraction by the Bradford method (Bio-Rad).[43] A total of 20 μg of protein from the OM and non-membrane associated fractions and 400 μg of the membrane fraction was loaded onto an SDS-PAGE gel and ran as described above. The presence of TfFuc1 in each fraction was determined by Western immunoblotting.

Microtiter assays of Hoechst-stained biofilms

T. forsythia WT and Δ*Tffuc1* strains were compared in respect to the biofilm formation[44] on mucin-coated polystyrene microtiter plates in dependency of the strength of the BHI liquid medium. Biofilm was stained with Hoechst 33258 Fluorescent Stain (Thermo Scientific). Details are described in the Supplementary Information.

Attachment and invasion assays

For both attachment and invasion assays, *T. forsythia* WT and Δ*Tffuc1* strains were incubated with the oral epithelial cell line H357 (CCL17; American Type Culture Collection) at a multiplicity of infection of 1:100, as described previously.[45] Details are described in the Supplementary Information.

Disclosure and Potential Conflict of Interest

No potential conflicts of interests were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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2.2. Inositol-phosphodihydroceramides in the periodontal pathogen *Tannerella forsythia*: Structural analysis and incorporation of exogenous *myo*-inositol

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Inositol-phosphodihydroceramides in the periodontal pathogen *Tannerella forsythia*: Structural analysis and incorporation of exogenous *myo*-inositol

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ABSTRACT

Background: Unique phosphodihydroceramides containing phosphoethanolamine and glycerol have been previously described in *Porphyromonas gingivalis*. Importantly, they were shown to possess pro-inflammatory properties. Other common human bacteria were screened for the presence of these lipids, and they were found, amongst others, in the oral pathogen *Tannerella forsythia*. To date, no detailed study into the lipids of this organism has been performed.
Methods: Lipids were extracted, separated and purified by HPTLC, and analyzed using GC-MS, ESI–MS and NMR. Of special interest was how *T. forsythia* acquires the metabolic precursors for the lipids studied here. This was assayed by radioactive and stable isotope incorporation using carbon-14 and deuterium labeled *myo*-inositol, added to the growth medium.
Results: T. forsythia synthesizes two phosphodihydroceramides (Tf GL1, Tf GL2) which are constituted by phospho-*myo*-inositol linked to either a 17-, 18-, or 19-carbon sphinganine, *N*-linked to either a branched 17:0(3-OH) or a linear 16:0(3-OH) fatty acid which, in Tf GL2, is, in turn, ester-substituted with a branched 15:0 fatty acid. *T. forsythia* lacks the enzymatic machinery required for *myo*-inositol synthesis but was found to internalize inositol from the medium for the synthesis of both Tf GL1 and Tf GL2.
Conclusion: The study describes two novel glycolipids in *T. forsythia* which could be essential in this organism. Their synthesis could be reliant on an external source of *myo*-inositol.
General significance: The effects of these unique lipids on the immune system and their role in bacterial virulence could be relevant in the search for new drug targets.

1. Introduction

Tannerella forsythia is a Gram-negative anaerobic oral pathogen, a member of the so-called "red complex" of bacteria that is strongly associated with periodontitis, affecting millions of people worldwide [1–3]. The effects on the periodontium include loss of the alveolar bone around the teeth, swelling and bleeding of the gums and, in more severe cases, loss of teeth. Apart from oral health issues, emerging evidence shows a relationship between periodontal disease, cardiovascular disease and other systemic chronic diseases involving inflammation [4].

Research into bacterial phospholipid synthesis pathways has received attention related to both the study of host-pathogen interactions and the continuously growing interest in new drug targets.

Abbreviations: BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; CID, collision-induced dissociation; COSY, correlation spectroscopy; DHC, dihydroceramide; ESI–MS, electrospray ionization mass spectrometry; FA, fatty acid; GC–MS, gas chromatography coupled with mass spectrometry; HMBC, heteronuclear multiple-bond correlation spectroscopy; HPTLC, high-performance thin layer chromatography; HSQC–DEPT, heteronuclear single quantum coherence–distortionless enhancement by polarization transfer; MALDI–MS, matrix-assisted laser desorption/ionization mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; PDHC, phosphorylated dihydroceramide; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PTFE, polytetrafluoroethylene; TMS, tetramethylsilane; TOCSY, total correlation spectroscopy.

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In particular, the study of bacterial sphingolipids has recently come into the spotlight as these compounds seem to play an important role in the host-microbe balance and could, therefore, be key to the pathogenesis of diseases [5,6]. In mammalian cells, sphingolipids have been seen to act as signaling molecules and regulators of important processes [7]. Most prokaryotic cells do not contain sphingolipids. However, they have been shown to be present in some bacteria, particularly in anaerobes, including *Sphingobacterium*, *Sphingomonas*, *Bacteroides*, and *Bdellovibrio stolpii*, some of which are pathogens or parasites able to synthesize their own membrane sphingolipids [8]. Bacterial pathogens which cannot produce sphingolipids, can utilize host sphingolipids to promote their own virulence [5]. A host sphingolipid acquired by a pathogen can be used to evade the immune system and, in turn, microbial sphingolipids incorporated into host membranes could interfere with intercellular signaling and, therefore, impair an immune response to the pathogen or cause an autoimmune response leading to the destruction of host tissues through molecular mimicry [5,9].

Recently, novel phosphorylated dihydroceramide (DHC) lipids were identified in the periodontal pathogen *Porphyromonas gingivalis* [10]. These include both low-mass and high-mass forms of phosphoglycerol DHC and phosphoethanolamine DHC lipids. The phosphorylated polar head groups are linked to a core lipid structure consisting of either a 17-, 18-, or 19-carbon base in amide linkage to *iso*-17:0(3-OH) fatty acid (FA). The phosphoglycerol DHC lipids present forms with an additional *iso*-15:0 FA in ester linkage to the hydroxyl group of the *iso*-17:0(3-OH) FA. Other bacteria produce lipids that are similar to these but still markedly distinct. *Sphingobacterium* species synthesize phosphoethanolamine, phosphomannose and phospho-*myo*-inositol DHCs with the long-chain base in amide linkage to either *iso*-15:0 or *iso*-15:0(2-OH) FAs [11]. Neither phosphoglycerol DHCs nor additional ester-linked FAs were reported. In turn, phosphoglycerol ceramide has been found in *Bacteroides melaningenicus* but with different FA substitutions, containing a variety of saturated and monounsaturated FAs [12], suggesting that Nichols et al. [10] found novel lipids that are distinct from those produced by other organisms. These unusual DHCs have since also been isolated from some *Bacteroides*, *Parabacteroides* and *Prevotella* species of intestinal bacteria and from the oral pathogens *T. forsythia* and *Prevotella intermedia* [9]. In addition, they have been found to stimulate pro-inflammatory responses in gingival fibroblasts, enhance autoimmunity, promote apoptosis, and accumulate in diseased gingival tissue and other host tissues distant from the sites normally colonized by the bacteria, hence leading to the hypothesis that they might form the link between periodontal disease and systemic chronic diseases involving inflammation [9,13–15].

In this study, we report novel classes of phosphorylated DHCs which are major cellular lipid components in *T. forsythia* and have a similar lipid portion to the DHCs described by Nichols et al. [10] but contain phospho-*myo*-inositol as a polar head-group, instead of phosphoglycerol or phosphoethanolamine. We describe the chemical structure of these glycosphingolipids as elucidated by ESI–MS, GC–MS and NMR, and demonstrate how *T. forsythia* can internalize radiolabeled *myo*-inositol from the growth medium and incorporate it into its inositol-containing lipids. The potential relevance of these newly identified unique DHC sphingolipids lies in their structure and presumable function, as inositol-containing sphingolipids, rarely found in bacteria, can serve as powerful signal transduction molecules and, therefore, could have an important role in the pathogen–host interaction [6,16].

2. Materials and methods

2.1. Bacterial strains, medium and culture conditions

T. forsythia strain ATCC 43037 (American Type Culture Collection, Manassas, VA, USA) was grown anaerobically at 37 °C for 4–7 days in either 1-L or 10-mL flasks in brain heart infusion medium (BHI) supplemented with *N*-acetylmuramic acid, horse serum and gentamicin, as described previously [17].

For radioisotope labeling experiments, *T. forsythia* was grown for 4 days as described above in a 10-mL batch culture in the presence of 1 μCi of [^{14}C(U)] *myo*-inositol (Biotrend, Cologne, Germany), added to the media in 10 μL of ethanol:water (9:1).

For stable isotope labeling experiments, *T. forsythia* was grown for 4 days as described above in a 250-mL batch culture in the additional presence of 400 μM deuterium labeled *myo*-inositol-1,2,3,4,5,6-D6 (EQ Laboratories GmbH, Augsburg, Germany) to give a 1:1 ratio between deuterated and endogenous inositol.

2.2. General and analytical methods

All solvents were purchased reagent-grade from Roth or Sigma-Aldrich (both Vienna, Austria), unless stated otherwise.

Lipids were analyzed by HPTLC on nano-silica gel glass plates (Sigma-Aldrich) or HPTLC-silica 60 on aluminum sheets (Merck, Darmstadt, Germany) developed in chloroform:methanol:water (65:25:4), referred to here as solvent system A.

For lipid analysis by mass spectrometry (ESI–MS), lipids were either visualized after HPTLC separation under 365-nm UV-light after staining with a primuline (Sigma-Aldrich) solution [18] and then extracted from the matrix, or purified from the silica plates as described in Section 2.4.

Other lipid visualization techniques used include the multipurpose Hanessian's stain for total lipid visualization [19] and carbohydrate staining with thymol [20].

Lipids of interest were scratched from the HPTLC plate and the scratched silica matrix was placed in a glass Pasteur pipette packed with glass-wool and the lipids were eluted with chloroform:methanol (1:1). The sample was then filtered through a 0.2-μm PTFE filter (Roth) and dried under a flow of nitrogen.

2.3. Lipid extraction

One gram of lyophilized *T. forsythia* wild-type biomass was washed extensively with ethanol and acetone prior to lipid extraction in order to remove any lipid contaminants coming from the rich growth medium [21].

Lipid extraction proceeded stepwise following the method established by Bligh and Dyer [22]. Eight mL of water were added to the biomass followed by 30 mL of chloroform:methanol (1:2) and sonicated for 15 min using a probe Branson sonifier (output 4, duty cycle 40%). Subsequently, 10 mL of chloroform were added followed by 10 mL of water, performing sonication for 5 min after each addition. Phase separation was then accelerated using centrifugation (25 min, 10,000 ×*g*) and the bottom organic phase was separated and filtered through a 0.45-μm PTFE filter followed by a 0.2-μm PTFE filter. The procedure was repeated twice on the solid interphase, and the organic phases were pooled and dried yielding 30 mg of total lipids which were redissolved in chloroform:methanol (8:2) to a concentration of 6 mg/mL.

2.4. Isolation of Tf GL1 and Tf GL2 glycolipids

The glycolipids of interest, named Tf GL1 and Tf GL2, were purified separately from several HPTLC-plates by applying the sample over the full length of the silica plate (50 μg of total lipid extract per cm of baseline). The TLC plates were developed in solvent A, air-dried, and the silica gel was scratched from the glass plate as a band from 1.5 mm below to 1.5 mm above the respective R_f value. The scratched plates were then treated with Hanessian's stain in order to ensure that Tf GL1 and Tf GL2 had been selectively removed. The lipids were then re-eluted from the combined silica matrix, as described above, filtered and dried under a flow of nitrogen. Lipids were stored at −20 °C until further analysis.

2.5. GC–MS compositional analysis

The composition of Tf GL1 and Tf GL2 was determined after methanolysis (2 M HCl/methanol, 85 °C, 2 h) and acetylation (85 °C, 10 min) by GC–MS. FAs were additionally recovered from the chloroform phase after hydrolysis (4 M HCl, 100 °C, 4 h) and neutralization (5 M NaOH, 100 °C, 30 min) and detected by GC–MS as methyl esters (diazomethane, 10 min, 22 °C). In order to determine the position of the hydroxy groups, after evaporation to dryness, hydroxyl groups of methyl ester-FAs were incubated with BSTFA (4 h, 65 °C) and measured as TMS derivatives. The identification of the detected FAs and carbon chain branching (branched or linear) was achieved based on comparison with external standards of 15:0, 16:0(3-OH) (Larodan AB, Solna, Sweden) and 17:0(3-OH) FAs (Biotrend).

For the determination of the absolute configuration of the hydroxy FAs 16:0(3-OH), 17:0(3-OH) and the most abundant 18:0-sphinganine, Tf GL2 was treated with 48% aqueous HF (4 °C, 48 h) in order to cleave the 1-P-*myo*-inositol moiety. The remaining ceramide (200 μg) was subjected to methanolysis (2 M HCl/MeOH, 85 °C, 4 h), peracetylated and analyzed by GC–MS by use of D-*erythro*-sphinganine C18 and L-*threo*-sphinganine C18 (Sigma-Aldrich) as authentic standards. The obtained sample was further hydrolyzed (4 M HCl, 100 °C, 30 min) and the OH-FAs analyzed by GC–MS after L-phenylethylamide derivatization [23].

GC–MS measurements were performed on an Agilent Technologies 7890A gas chromatograph equipped with a dimethylpolysiloxane column (Agilent Technologies, Santa Clara, CA, USA; HP Ultra 1, 12 m × 0.2 mm × 0.33 μm film thickness) and a 5975C series MSD detector with electron impact ionization (EI) mode under autotune condition at 70 eV. The temperature program was 70 °C for 1.5 min, then 60 °C/min to 110 °C followed by 5 °C/min to 320 °C, held for 10 min.

2.6. ESI–MS analysis

Mass spectrometric measurements of purified lipid samples (see *2.4*) were performed on an Esquire 3000^{plus} 3D-quadrupole ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) in the negative-ion mode electrospray ionization (ESI–MS). Mass calibration was done with a commercial mixture of perfluorinated trialkyltriazines (ES Tuning Mix, Agilent Technologies, Santa Clara, CA, USA). All analytes were dissolved in methanol hypergrade LC–MS quality (Merck) to a concentration of ~1 mg/mL. Direct infusion experiments were carried out using a Cole Parmer model 74900 syringe pump (Cole Parmer Instruments, Vernon Hills, IL, USA) at a flow rate of 2 μL/min. Full MS-scans were acquired in the range *m/z* 100–1300 with the target mass set to *m/z* 1200. Further experimental conditions include: drying gas temperature, 200 °C; capillary voltage, −4 kV; skimmer voltage, 40 V; octapole and lens voltages, according to the target mass set. Helium was used as a buffer gas for full scans. Mass spectra were averaged during a data acquisition time of 0.5 to 1 min and one analytical scan consisted of three successive micro scans resulting in 20 and 40 analytical scans, respectively, for the final mass spectrum. For all low-energy CID-MS^2 experiments the precursor ion mass was selected at an isolation and activation width of 4 Da. The fragmentation amplitude for dissociating the precursor ions was set to 0.45–0.5 V and the corresponding CID-spectra were collected for at least one minute.

2.7. Nuclear magnetic resonance spectroscopy of Tf GL2

Prior to NMR analyses, purified Tf GL2, 1-P-*myo*-inositol (Sigma-Aldrich), L-*threo*-sphinganine C18 (Sigma-Aldrich) and D-*erythro*-sphinganine C18 (Sigma-Aldrich) were dissolved in deuterated methanol (Deutero GmbH, Kastellaun, Germany) and placed in 5-mm NMR tubes.

All 1D (^{1}H, ^{13}C and ^{31}P) and 2D NMR ^{1}H,^{1}H COSY, TOCSY as well as ^{1}H,^{13}C HSQC, ^{1}H,^{13}C HMBC (only for Tf GL2), and ^{1}H,^{31}P HMQC experiments performed on Tf GL2 and 1-P-*myo*-inositol were recorded at 27 °C on a Bruker DRX Avance III 700 MHz spectrometer (operating frequencies of 700.75 MHz for ^{1}H NMR, 176.2 MHz for ^{13}C NMR and 283.7 MHz for ^{31}P) and standard Bruker software (TopSpin ver. 3.2). COSY and TOCSY experiments were recorded using data sets (t1 by t2) of 4096 by 512 points, with 6 scans for COSY, and 16 for TOCSY. The TOCSY experiments were carried out in the phase-sensitive mode with mixing times of 120 ms. The ^{1}H,^{13}C correlations measured in the ^{1}H-detected mode *via* HSQC-DEPT with proton decoupling in the ^{13}C domain and HMBC spectra were acquired using data sets of 4096 by 512 points and 64 scans (HSQC) and 80 scans (HMBC) for each t1 value. HMBC spectra were adjusted to *J* coupling constant value of 145 Hz and long range proton carbon coupling constant of 10 Hz. Chemical shifts were reported relative to internal methanol (δ_H 3.34, δ_C 49.86) [24] or external phosphoric acid (δ_P 0.0) [25].

2.8. Incorporation of radiolabeled myo-inositol into T. forsythia lipids

The study of radiolabeled inositol incorporation was based on procedures described previously with substantial modification [26–28]. *T. forsythia* was grown in the presence of 1 μCi of [^{14}C(U)] *myo*-inositol (specific radioactivity 200–250 mCi/mmoL) as described in Section 2.1. After 4 days of growth, the biomass was pelleted and lipids were extracted using the Bligh–Dyer method [22]. 80 μL of water were added to the wet biomass followed by 300 μL of chloroform:methanol (1:2), vortexed for 5 min and left shaking for an additional 10 min. Subsequently, 100 μL of chloroform were added followed by 100 μL of water, vortexing for 5 min and left shaking for 10 min, after each addition. After phase separation, the top aqueous phase was carefully removed. The bottom organic phase was then backwashed three times by addition of a 100 μL of water. The radioactivity in each wash fraction and in the organic phase was determined using a liquid scintillation analyzer Tri-Carb 2910 TR (Perkin Elmer, Waltham, MA, USA). The organic phase containing the extracted lipids was dried under a flow of nitrogen, redissolved in chloroform:methanol (8:2), spotted onto a HPTLC silica-gel aluminum-back plate (Merck) and then chromatographed in solvent A. The plate was then subjected to autoradiography using Kodak BioMax XAR film (Sigma-Aldrich) for three weeks at −80 °C.

2.9. Determination of free D_6-labeled and endogenous inositol in the culture medium

The determination of free deuterated and natural inositol in the medium was performed by RP–PGC–TOFMS, using known amounts of pure *myo*-inositol (Sigma-Aldrich) as standards. The medium samples were prepared by centrifugal filtration (60 min, 10 000 rpm, 4 °C; Amicon Ultra, 3 kDa MW cut-off) and diluted 1:100 in LC–MS grade H_2O (Sigma Aldrich) prior to injection. A detailed description of the chromatographic separation is provided elsewhere [29]. The chromatography column was coupled on-line to a time-of-flight mass spectrometer (Agilent 6230 LC–TOFMS) *via* a Dual AJS ESI spray chamber. ESI was performed in negative mode (45 psig nebulizer pressure, 350 °C sheath gas temperature, 11 L/min sheath gas flow, 250 °C drying gas temperature, 12 L/min drying gas flow, 110 V fragmentor voltage, 60 V skimmer voltage, 3500 V capillary voltage). MS data was acquired in the mass range 50-1000 *m/z* at an acquisition rate of 3 spectra per second. Both unlabeled and D_6-labeled *myo*-inositol were detected as formic acid adducts ($[M + COO^-]^-$, 225.0616 m/z and 231.0993 m/z, respectively) at a retention time of 3.9 min. After peak integration (Agilent MassHunter Quantitative Analysis 7.0, extraction window ±5 ppm), the peak areas were used to calculate the ratio between D_6-labeled and unlabeled inositol.

3. Results

3.1. Isolation of Tf GL1 and TF GL2

HPTLC of the Bligh–Dyer extracts from *T. forsythia* cells followed by a first screening of the primuline-stained bands using MALDI–MS (data not shown) allowed for the identification of the most abundant lipids present (phosphatidylethanolamine, monogalactosyl diacylglycerol, phosphatidic acid and cardiolipin) as well as of the phosphorylated DHC lipids previously reported by Nichols et al. [9]. In addition, we detected two novel DHC lipids which could be resolved with the solvent system A. The less abundant, more polar lipid, designated as Tf GL1, migrated with an R_f value of 0.10 and the second, more abundant lipid, designated here as Tf GL2, migrated slightly in front of Tf GL1 with an R_f value of 0.15 (Fig. 1A).

The Tf GL1 and Tf GL2 lipids were subsequently purified from several HPTLC-plates by applying the sample over the full length of the silica plate and scratching the silica from the glass plates at the corresponding R_f values. Fig. 1B shows purified Tf GL1 and Tf GL2 fractions after elution from the silica gel. Both *T. forsythia* lipids stained positive for carbohydrates as assayed with thymol. Two mg of purified Tf GL2 and 400 μg of purified Tf GL1 were recovered from the total lipid extract, representing ~20% and ~5% of the total lipid fraction, respectively.

3.2. GC–MS compositional analysis of purified Tf GL1 and Tf GL2 glycolipids

Compositional analyses of Tf GL1 and Tf GL2 revealed the presence of inositol, linear 16:0(3-OH) FA, branched 17:0(3-OH) FA as well as sphinganine with a long-chain-length of 17, 18 and 19 carbon atoms. Tf GL2 contained additionally a branched 15:0 FA. The most prominent signals belonged to 17:0(3-OH) FA and 18:0 sphinganine. GC–MS spectra of the sample after methanolysis also contained low intensity signals for glycerol, 16:0, 18:1 and 18:0 FA. However, further analyses by ESI–MS and NMR (see Sections 3.3. and 3.4. below) did not provide evidence that the aforementioned compounds were a structural part of Tf GL1 and Tf GL2. The GC–MS analyses performed on the dephosphorylated Tf GL2 revealed that the 18:0 sphinganine possessed an *erythro* configuration and both hydroxy FAs were R configured.

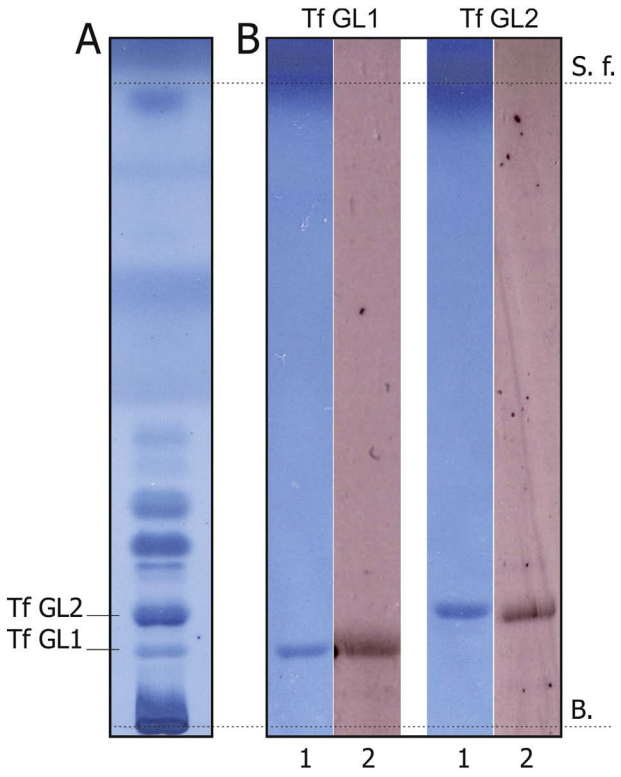

Fig. 1. HPTLC of *T. forsythia* lipids developed with the solvent system chloroform: methanol:water (65:25:4). Visualization was performed with the Hanessian's stain for lipids (blue) and with thymol for carbohydrates (pink). Abbreviations: B., baseline; S. f., solvent front. A. Total lipid extract obtained using the Bligh–Dyer extraction method. 30 μg of sample were spotted at the baseline. TF GL1 and Tf GL2 ran at R_f values of 0.10 and 0.15, respectively. B. Purified Tf GL1 and Tf GL2 appeared as single bands in HPTLC and stained positive for carbohydrates. Lane 1: 3 μg of sample, lane 2: 10 μg of sample.

3.3. ESI–MS analyses of purified Tf GL1 and TF GL2 glycolipids

Negative-ion ESI–MS spectra of purified Tf GL1 and Tf GL2 (Fig. 2) revealed five $[M–H]^-$ ions for each lipid sample corresponding to the deprotonated lipid species of M_1–M_5, where M stands for the neutral mass of a given molecular species. Tf GL1 consisted mainly of the deprotonated ions of M_1, *m/z* 768; M_2, *m/z* 782; M_3, *m/z* 796; M_4, *m/z* 810; and M_5, *m/z* 824. The corresponding signals for Tf GL2 were M_1, *m/z* 992; M_2, *m/z* 1006; M_3, *m/z* 1020; M_4, *m/z* 1034; and M_5, *m/z* 1048. The spacing of 14 Da between the M_1–M_5 species is indicative of variations in FA chain length by single [$-CH_2-$] groups, which is typical for bacteria.

Other ions present were the deprotonated sodium chloride adducts of M_3 and M_4. Both samples revealed a markedly similar quantitative distribution of M_1–M_5, with M_4 being the most predominant. The mass difference between the ions of Tf GL1 and Tf GL2 was 224 Da, which could correspond to a missing 15:0 FA in the Tf GL1 structure as compared to Tf GL2, indicating that Tf GL1 and Tf GL2 could be low- and high-mass forms of a single lipid class.

3.3.1. CID–MS^2 of Tf GL1 and Tf GL2

CID–MS^2 of Tf GL1 revealed major product ions for all four of the $[M_{2-5}–H]^-$ precursor ions (Fig. 3, Table 1). The resulting spectrum for each parent ion included peaks corresponding to the loss of inositol ± H_2O, $[M–H – (162 / 180)]^-$, loss of an amide-linked 16:0(3-OH) or 17:0(3-OH) FA, $[M–H – (254 \text{ or } 268)]^-$, and loss of the inositol and partial loss of the latter FA forming a McLafferty ion, $[M–H – (212 \text{ or } 226)]^-$ [30].

CID–MS^2 of $[M_3–H]^-$ for Tf GL1 proved the parent ion to be a mixture of two separate species of equal mass as both *m/z* 570 ($[M–H – 226]^-$) and *m/z* 584 ($[M–H – 212]^-$) McLafferty ions were present amongst the product ions indicating the presence of both amide-linked 16:0(3-OH) and amide-linked 17:0(3-OH) FAs. In addition, complete loss of this amide-linked hydroxy-FA revealed two separate ions (*m/z* 528 and 542) indicating that carbon chain length variations occurred also in the remaining core-lipid structure.

CID–MS^2 of Tf GL2 also revealed product ions for all four of the $[M_{2-5}–H]^-$ precursor ions (Fig. 4, Table 1). The detected product ions corresponded to the loss of inositol ± H_2O, $[M–H – (162 / 180)]^-$, but did not result in the previous McLafferty ions but in a loss of an ester-bonded 15:0 FA, forming a double bond in the remaining structure, $[M–H – 242]^-$, partially accompanied by the additional loss of the inositol ± H_2O, $[M–H – (404 / 422)]^-$. Loss of the ester-bonded 15:0 FA was followed by a full loss of the amide linked 16:0(3-OH) or 17:0(3-OH) FA $[M–H – (478 \text{ or } 492)]^-$, which are 3-hydroxy FAs as determined by GC–MS and the McLafferty ions formed in the CID–MS^2 of Tf GL1.

The lower molecular-mass species, Tf GL1, clearly showed two diagnostic low mass product ions for phospho-inositol at *m/z* 241 and 259 (which could not be found in Tf GL2 due to the low mass cut-off of the 3D-ion trap MS utilized) and presented peaks at *m/z* 528, 542 and 556 which also appeared as minor product ions in the higher molecular-mass species. These ions could be assigned to a sphinganine (dihydroceramide)-phospho-inositol structure where the sphinganine presents variations in the chain length assigned as 17:0 sphinganine, 18:0 sphinganine and 19:0 sphinganine, respectively.

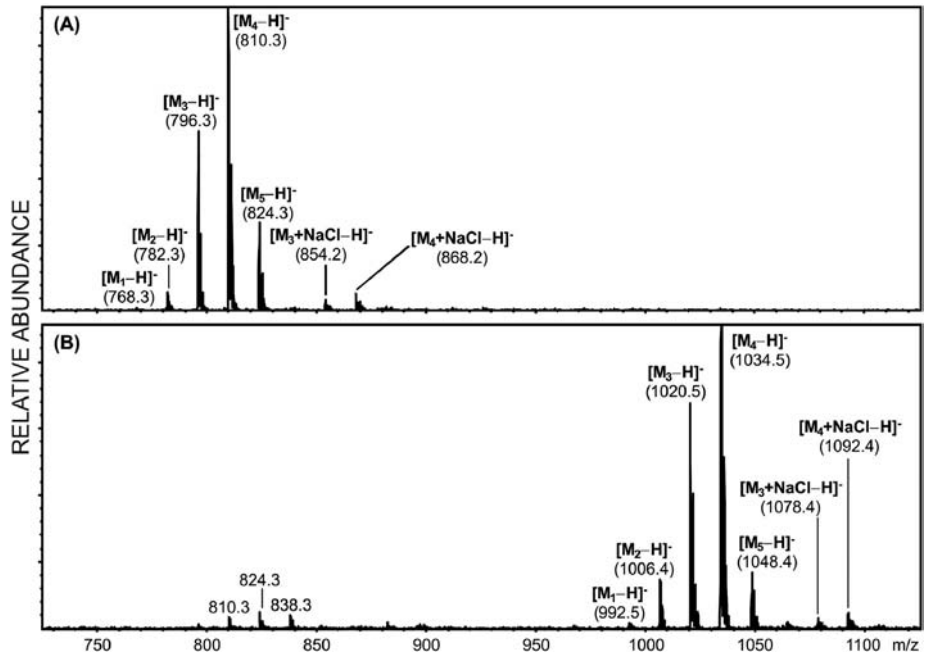

Fig. 2. High-mass region of the negative-ion ESI-mass spectra ($[M–H]^-$, where M stands for the neutral mass of a given molecular species) of Tf GL1 (A) and Tf GL2 (B).

All resulting major precursor and product ions are summarized in Table 1, along with how the variations in carbon chain lengths of both the sphinganine and the amide-linked 3-hydroxy FAs result in the lipid species M_2 to M_5. The most abundant M_4 for both Tf GL1 and Tf GL2 corresponded to the species having 18:0 sphinganine and the amide-linked 17:0(3-OH) FA, in good agreement with the GC–MS data. Fig. 5 shows the structure of Tf GL1 and Tf GL2, as elucidated from the combined GC–MS and ESI–MS data, and the described fragmentation pathways in CID–MS^2.

3.4. NMR analyses of Tf GL2

The chemical nature of the more abundant Tf GL2, composed of phospho (P)-inositol linked to *erythro*-sphinganine, further substituted by amide-linked hydroxy FAs and an ester-linked 15:0 FA, could be confirmed by NMR analyses (Fig. 6, Table 2). The P-inositol was identified as 1-P-*myo*-inositol based on the chemical shifts of the authentic standard (atoms 1–6; Fig. 6). The ^1H spectrum of Tf GL2 showed, in addition to the signals of 1-P-myo-inositol, the downfield shifted multiplet at 5.27 ppm (due to the strong deshielding effect), signals from the α-methylene groups of the amide- and ester-bound FAs (2.54, 2.35 ppm), signals resonating in the region from 1.68 to 1.61 ppm (γ-methylene group of the amide bound FA and methylene group at position 4 of the sphinganine base), and the groups of signals from 1.41 to 1.27 ppm and from 0.95 to 0.88 ppm corresponding to $-CH_2-$ and terminal methyl groups of the alkyl chains, respectively. Scalar correlation of δ_H 5.27 to δ_C 172.98 and 175.78 in the HMBC spectrum enabled assignment of the signal to the

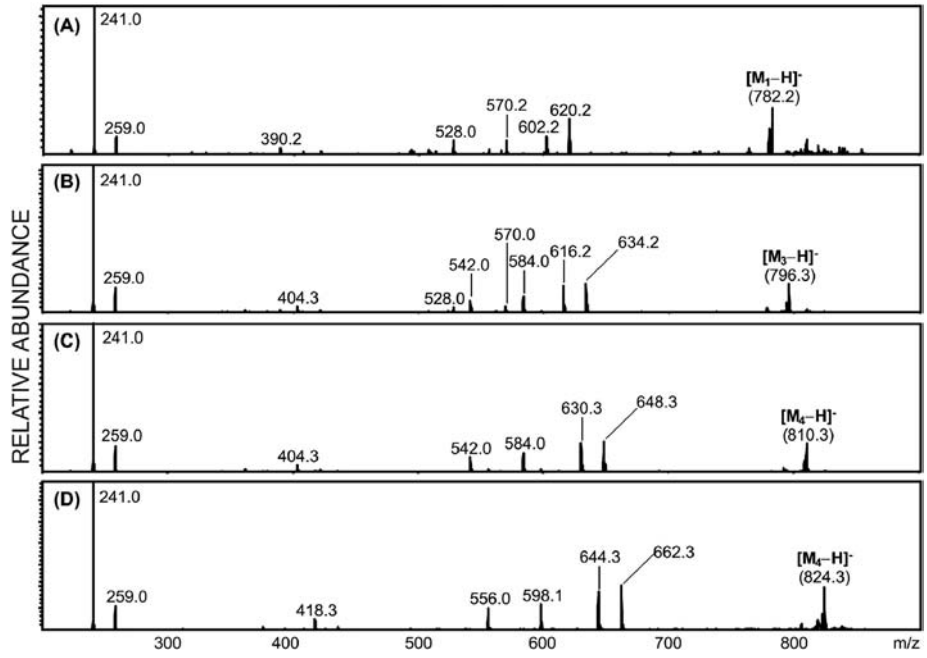

Fig. 3. Negative-ion ESI low-energy CID–MS^2 of the Tf GL1 $[M–H]^-$ precursor ions: *m/z* 782 (A), *m/z* 796 (B), *m/z* 810 (C) and *m/z* 824 (D).

Table 1
Combined GC–MS and ESI–MS data. Major losses and product ions of the $[M–H]^-$ precursor ions of the sphingo-phosphoinositols of Tf GL1 (A) and the corresponding *O*-acyl-sphingo-phosphoinositols of Tf GL2 (B). An asterisk (*) indicates those sphingolipid species containing additionally two diagnostic low-mass product ions for phosphoinositol at *m/z* 259 and at *m/z* 241 which are not detected for the *O*-acyl-sphingo-phosphoinositols (Tf GL2) due to the low mass cut-off of the 3D-ion trap instrument utilized for the experiments. Abbreviations: FA, fatty acid; Sph, sphinganine.

Sphingolipid species	$[M–H]^-$	−162/−180 (loss of inositol)	−242 (loss of the *O*-linked 15:0 FA)	−(242 + 162/+180) (loss of inositol plus the *O*-linked 15:0 FA)	Partial loss of the *N*-linked OH–FA (McLafferty rearrangement) including additional loss of inositol	Loss of the *N*-linked OH–FA
A						
*C_{33};Sph: 17:0; (N)HO–FA: 16:0 linear	782	620/602	–	–	570 (−180:390)	528
*C_{34};Sph: 17:0; (N)HO–FA: 17:0 branched	796	634/616	–	–	570 (−180:390)	528
*C_{34};Sph: 18:0; (N)HO–FA: 16:0 linear	796	634/616	–	–	584 (−180:404)	542
*C_{35};Sph: 18:0; (N)HO–FA: 17:0 branched	810	648/630	–	–	584 (−180:404)	542
*C_{36};Sph: 19:0; (N)HO–FA: 17:0 branched	824	662/644	–	–	598 (−180:418)	556
B						
C_{33};Sph: 17:0; (N)HO − FA: 16:0 linear, (O)FA: 15:0 branched	1006	844/826	764	602/584	–	528
C_{34};Sph: 17:0; (N)HO–FA: 17:0 branched, (O)FA: 15:0 branched	1020	858/840	778	616/598	–	528
C_{35};Sph: 18:0; (N)HO–FA: 17:0 branched, (O)FA: 15:0 branched	1034	872/854	792	630/612	–	542
C_{36};Sph: 19:0; (N)HO–FA: 17:0 branched, (O)FA: 15:0 branched	1048	886/868	806	644/626	–	556

β-hydroxy group of the amide linked FA, being further *O*-acylated. The ^{31}P signal at 1.84 ppm correlated with the signals at 3.92 ppm (position 1 of inositol) and 4.26 and 3.97 ppm (position 1 of the sphinganine base) as seen in the $^{1}H,^{31}P$ HMQC experiment, identifying the position of the phospho-ester linkage. A HSQC-DEPT experiment (Fig. 6, Table 2) provided the direct correlation of all assigned ^{1}H with ^{13}C signals. Comparing ^{1}H spectra of the Tf GL2 with the authentic standards of the *erythro* and *threo* forms of sphinganine (that differ in the $J_{2,3}$ coupling constants, being ~6 Hz for *erythro* and ~2 Hz for *threo*, respectively [31]), enabled the identification of an *erythro*-sphinganine in Tf GL2. Taken together, NMR analysis of Tf GL2 confirmed the structure to be composed of 1-P-*myo*-inositol phosphoester-linked to an *erythro*-sphinganine base which is substituted *via* the amide group to an R-hydroxy FA, in turn ester-substituted by a second FA.

3.5. Uptake of the glycolipid metabolite myo-inositol from the medium

A BLASTP search against the proteome of *T. forsythia* ATCC 43037 did not identify proteins homologous to the two enzymes involved in inositol synthesis, *i.e.* inositol-3-phosphate synthase and inositol-monophosphatase. This suggests that *T. forsythia* utilizes exogenous *myo*-inositol for the synthesis of Tf GL1 and Tf GL2. In order to prove the uptake of the glycolipid precursor *myo*-inositol from the medium, *T. forsythia* was grown in the presence of [^{14}C(U)] *myo*-inositol. Lipids were extracted using the Bligh–Dyer method and the organic phase was backwashed several times with water to insure complete removal of non-incorporated radiolabeled inositol from the organic phase. The latter fraction (containing about 0.2% of the radioactive precursor initially added) was spotted onto a HPTLC plate and separated using solvent system A. After a 3-week exposure time, two bands were clearly

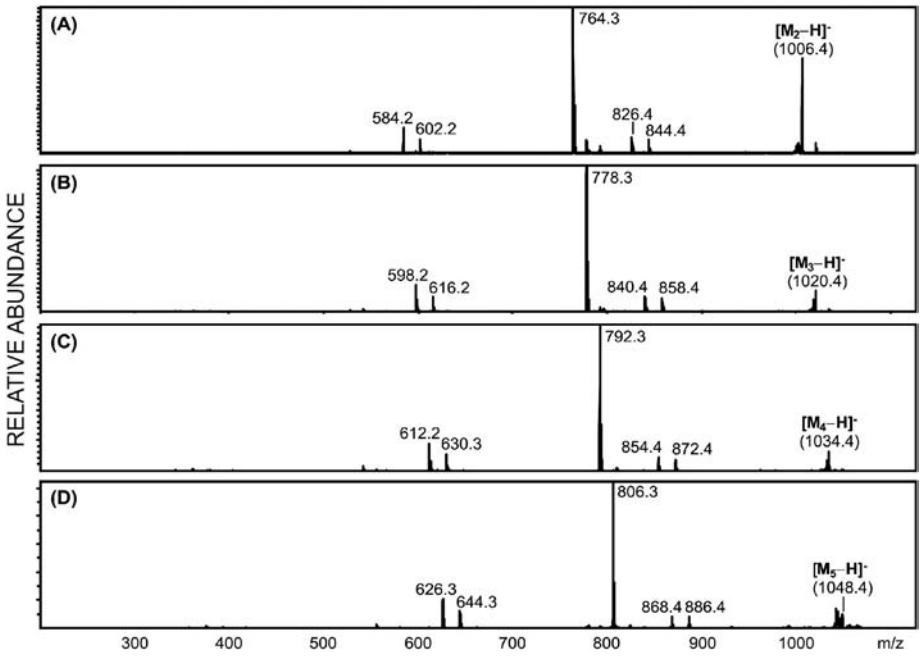

Fig. 4. Negative-ion ESI low-energy CID-MS^2 of the Tf GL2 $[M–H]^-$ precursor ions: *m/z* 1006 (A), *m/z* 1020 (B), *m/z* 1034 (C) and *m/z* 1048 (D).

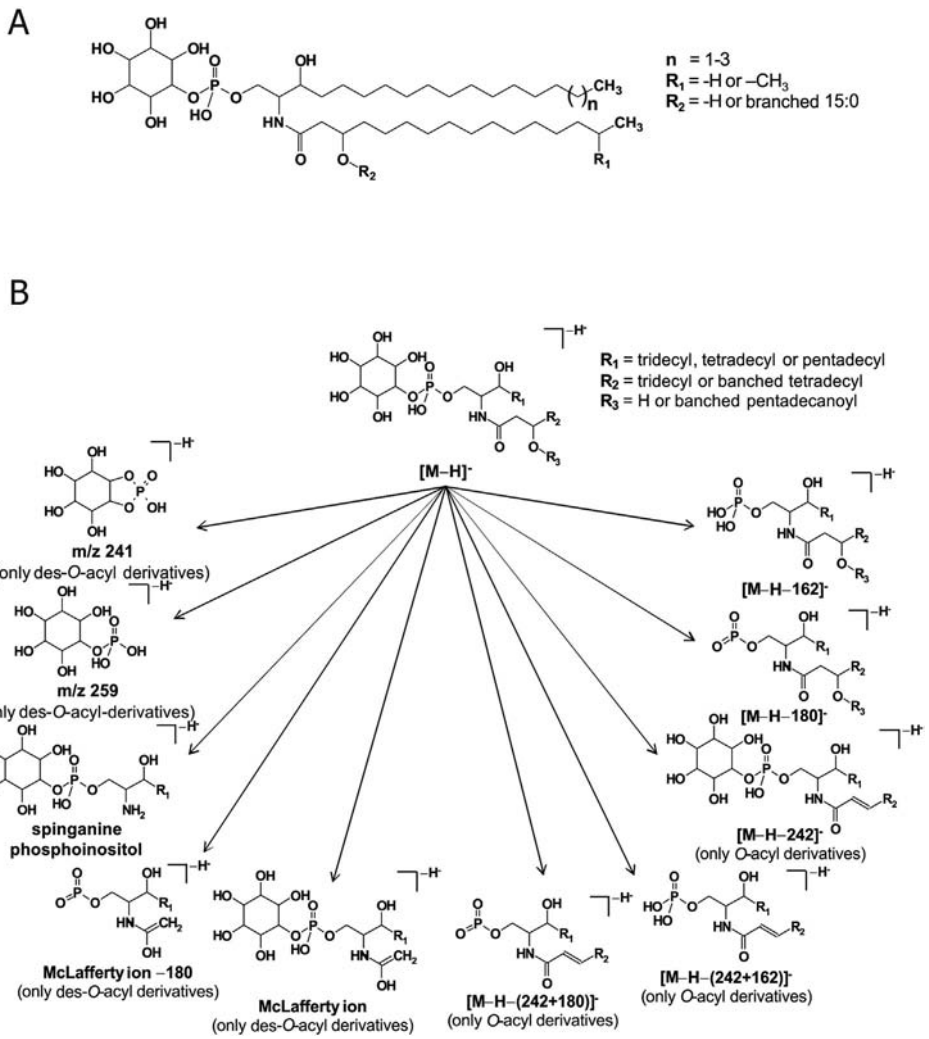

Fig. 5. Structure (A) and general fragmentation pathways of $[M–H]^-$ precursor ions of *O*-acyl-sphingo-phosphoinositols (Tf GL2) and *O*-deacyl-sphingo-phosphoinositols (Tf GL1) (B).

visible at the exact R_f values of 0.10 and 0.15 corresponding to Tf GL1 and Tf GL2, respectively (Fig. 7). This indicated that *T. forsythia* is able to import inositol from the medium and incorporate it into its lipids. No other bands were detected, indicating that [^{14}C(U)] *myo*-inositol was solely incorporated into Tf GL1 and Tf GL2.

3.6. Tf GL2 labeling with D_6-labeled myo-inositol

Quantification of free inositol in the medium revealed its presence at an approximate concentration of 400 μM, as estimated by using an external *myo*-inositol standard. D_6-labeled *myo*-inositol was then added to the medium before inoculation to give a ratio between deuterated and unlabeled inositol of close to 1:1. The ratio at the beginning of the culture and after harvesting the biomass was experimentally determined as 1.05 and 1.03, respectively. Tf GL2 was then purified and subjected to ESI–MS analysis. The ratio between deuterated and unlabeled Tf GL2 was found to be 1.11 (Fig. 8), demonstrating that the isotopic distribution of D_6-labeled inositol in Tf GL2 was practically equal to that in the medium. This provides evidence that the incorporated inositol is exclusively derived from the medium.

4. Discussion

As a close relative of *Bacteroides*, the ability of *T. forsythia* to synthesize sphingolipids, an attribute rarely found amongst bacteria, is phylogenetically well justified [6]. Although the lipidome of this bacterium is poorly characterized, some of its sphingolipids are unique phosphorylated DHCs, prevalent amongst a set of common human bacteria and biologically relevant due to their putative role in eliciting an immune response [9,10,13].

In this study, we report on two new phosphorylated DHCs (Tf GL1 and Tf GL2) which are major cellular lipid components of *T. forsythia*, structurally similar to those already described [10], but containing *myo*-inositol as a polar head-group. The structure of these unusual glycolipids was elucidated by a combination of ESI–MS, GC–MS and NMR. Tf GL1 and Tf GL2 consist of phospho-*myo*-inositol linked to a core lipid structure consisting of either a 17-, 18-, or 19-carbon sphinganine base in amide linkage to either branched 17:0(3-OH) or linear 16:0(3-OH) FA which, in the higher molecular weight structure of Tf GL2, is, in turn, substituted with an additional branched 15:0 FA in ester linkage. The lipid core described here is similar to that described by Nichols et al. [10]. These authors reported only 17:0(3-OH) FA to be present in amide linkage to sphinganine. In addition, the 17:0(3-OH) FA, 15:0 FA and 17- and 19-carbon sphinganine were determined to be *iso*-branched (the 18-carbon sphinganine was reported to be linear), as determined by comparison with the synthetic standards.

It has been postulated that there exists a relationship between periodontal disease and other systemic chronic diseases where inflammation seems to be the common link [4]. The novel DHCs published by Nichols et al. [10] were seen to be present in host tissues distant from the sites of infection and evoke an inflammatory response *in vitro* [9]. Their FA substitutions are thought responsible for this observation as

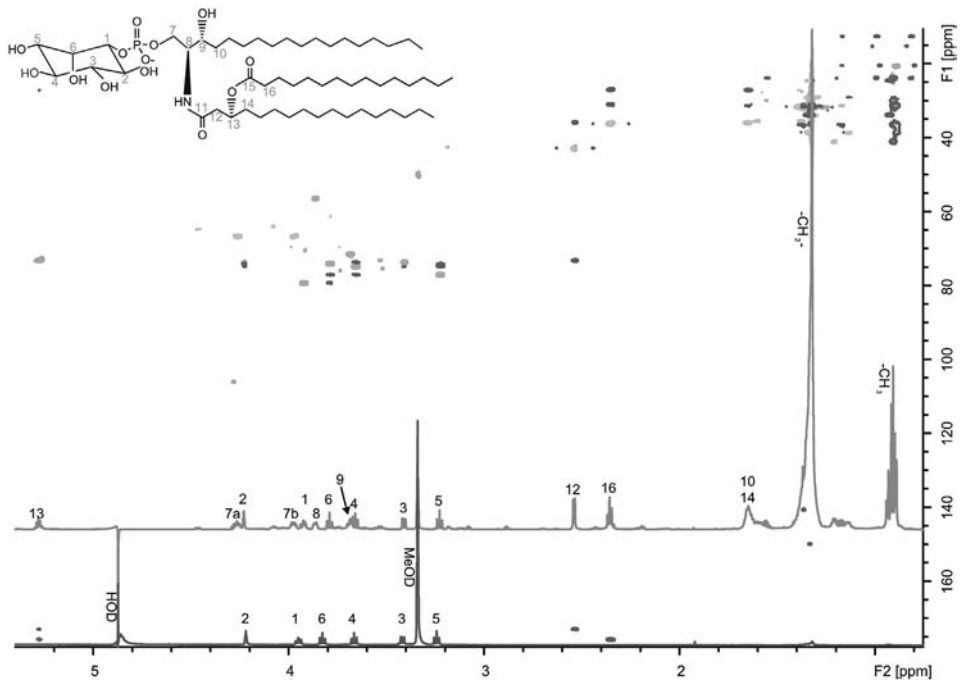

Fig. 6. Overlay of the ^{1}H NMR spectra of 1-P-*myo*-inositol (black) and Tf GL2 (grey), along with the overlay of the 2D experiments HSQC-DEPT (grey dots) and HMBC (black dots). Numbers indicate the position in the structure of Tf GL2 (top, left). *Erythro*-sphinganine is drawn here in its D (8S, 9R) absolute configuration, however D- or L-configuration could not be distinguished.

the lipid portion is what differs structurally from other DHCs. The finding that these core lipid structures also exist with a rare and important polar head group like inositol, often involved in protein–lipid interactions on cell surfaces [32], further increases the interaction possibilities and widens the spectra of effects this type of lipid could have on the immune system, especially when considering that inositol-containing lipids and sphingolipids, although rare in microorganisms, seem more prevalent amongst pathogenic bacteria [5,6,33]. Confirmed functional information on inositol-containing structures is known for just a few organisms [32]. The use of *myo*-inositol for the synthesis of

Table 2
^{1}H,^{13}C and ^{31}P NMR assignments of 1-P-*myo*-inositol and Tf GL2 and scalar correlations seen in the HMBC spectrum. Numbers in bold indicate the position in the structure of Tf GL2 as shown in Fig. 6. Multiplicity abbreviations: a, b, hydrogen atoms; d, doublet; t, triplet; m, multiplet; dd, double doublet.

	^{1}H (ppm)	^{13}C (ppm)	^{31}P (ppm)	HMBC (ppm)
1-*P*-*myo*-Inositol				
1	m 3.95	78.21	2.22	
2	t 4.22	73.95	–	
3	m 3.42	73.68	–	
4	t 3.66	74.72	–	
5	t 3.23	77.11	–	
6	t 3.82	74.28	–	
Tf GL2				
1	m 3.92	79.33	1.84	
2	t 4.23	73.93	–	
3	dd 3.41	73.68	–	
4	t 3.65	74.83	–	
5	t 3.22	77.03	–	
6	t 3.78	74.05	–	
7a	m 4.26	66.69	1.84	
7b	m 3.97			
8	m 3.86	56.43	–	**8** 3.86/**11** 172.98
9	m 3.68	71.45	–	
10a, b	1.68–1.61	27.02	–	
11	–	172.98	–	172.98
12	d 2.54	42.94	–	**12** 2.54/**13** 73.19
				12 2.54/**11** 172.98
13	m 5.27	73.19	–	**13** 5.27/**11** 172.98
				13.5.27/**15** 175.78
14a, b	1.68–1.64	27.02	–	**14** 1.66/**15** 175.78 (faint signal)
15	–	175.78	–	175.78
16a, b	t 2.35	36.12	–	**16** 2.35/**15** 175.78 (strong signal)

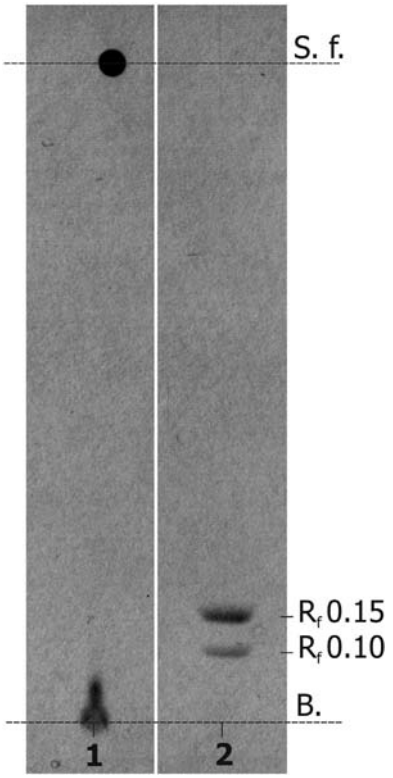

Fig. 7. Autoradiography after HPTLC analysis of a total lipid extract of *T. forsythia* grown in the presence of [^{14}C(U)] *myo*-inositol. Lane 1 shows free [^{14}C(U)] *myo*-inositol which does not migrate in the solvent system used. Lane 2 shows the total lipid extract containing two lipid bands with incorporated [^{14}C(U)] *myo*-inositol with R_f values of 0.10 and 0.15 corresponding to Tf GL1 and Tf GL2, respectively. Abbreviations: B., baseline; S. f., solvent front; R_f, retardation factor.

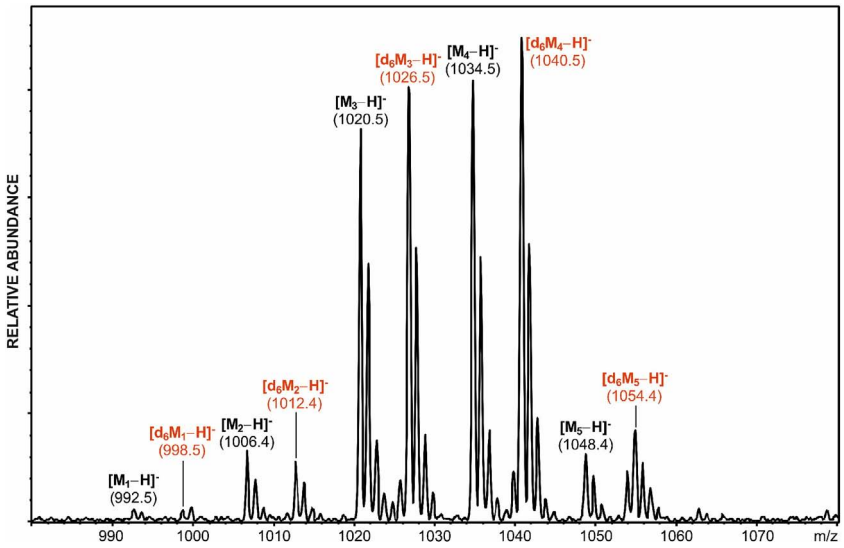

Fig. 8. High-mass region of the negative-ion ESI-mass spectra of Tf GL2 isolated from *T. forsythia* grown in medium containing deuterium labeled and unlabeled inositol at a ratio of close to 1:1. Peaks labeled with $[d_6M–H]^-$ correspond to ions derived from deuterated inositol labeled Tf GL2, as indicated from the mass difference of 6 Da from the corresponding Tf GL2 $[M–H]^-$ ions.

phosphatidylinositol (PI) and other lipids is essential in eukaryotes, including pathogenic fungi and protozoa, but is rare amongst bacteria and has been described only for a small group of actinobacteria (including mycobacteria, *Streptomyces* and corynebacteria), *Bacteroidetes* (*Sphingobacterium*) and in myxobacteria and *Treponema* [33,34]. Further occurrence of inositol in bacteria includes the use of this metabolite in *Mycobacteria* for the synthesis of mycothiol, or in other bacteria to produce di-*myo*-inositol-phosphate, or it can be used as a precursor for various aminoglycoside/aminocyclitol antibiotics [34–37]. A number of bacteria can internalize *myo*-inositol and use it as a nutrient source but cannot incorporate it into biosynthetic pathways [33,38,39].

Not only is it unique to find *T. forsythia* with inositol containing lipids but it is of particular interest how the pathogen acquires this basic molecule for the synthesis of those. For the synthesis of PI, organisms either synthesize *myo*-inositol from glucose-6-phosphate or import it from the host [33]. There is a universal mechanism for the synthesis of inositol which requires the combined action of two enzymes, inositol-3-phosphate synthase (named Ino1 in yeast) and inositol-monophosphatase, both of which are highly conserved proteins between the different domains of life [27,33]. Genes homologous to *ino1* have been found in related *Bacteroides* sp. and in the uncultured, health-associated *Tannerella* sp. BU063 [40], although the physiological significance of inositol synthesis in these bacteria is not clear [34]. In contrast, no genes predicted to be involved in inositol synthesis could be identified in the genome of *T. forsythia* [41]. By adding radiolabeled *myo*-inositol to the medium of *T. forsythia* during growth, we were able to demonstrate that the organism is able to internalize this compound and incorporate it into its lipids. Moreover, labeling experiments with deuterated inositol indicated that the source of this metabolite is solely exogenous.

In some bacteria, such as in mycobacteria, PI is further built up with other sugar residues, such as mannose, to form phosphatidyl-*myo*-inositol mannosides (PIMs) [42]. In our study, no other lipids were seen to incorporate labeled *myo*-inositol, and so further derivatization of the inositol-containing lipids to form other major lipid components is not expected to occur in *T. forsythia*.

Like other bacteria residing in human hosts, *T. forsythia* has adapted to better suit its niche and has optimized its physiology to better use the nutrients and resources available, minimizing the need of *de novo* synthesis of vital components. Furthermore, this ability in *T. forsythia* may be a key to pathogenicity. For example, when comparing this virulent strain to the health-associated *Tannerella* sp. BU063, the latter presents two *de novo* synthesizing enzymes required to convert UDP-*N*-acetyl-D-glucosamine to UDP-*N*-acetylmuramic acid, enzymes which lack in *T. forsythia* [40]. *N*-Acetylmuramic acid can be presumably scavenged by *T. forsythia* from the host in its natural habitat but has to be added to the media for growth under laboratory conditions [43]. Similarly, *T. forsythia*, unlike the harmless *Tannerella sp.* BU063, does not have any gene homologs to *ino1*, involved in inositol synthesis, but can import inositol from the medium for synthesis of major lipid components. Human serum, contained for example in the gingival crevice, contains 61 ± 12 μM of *myo*-inositol which the pathogen could exploit for this purpose [33] and hence, taken together, we hypothesize that the uptake of inositol could be a contributing factor to virulence.

In mycobacteria, internalized inositol is first phosphorylated in order to be used as a substrate for the formation of phosphatidylinositol phosphate (PIP), formed as an intermediate during the formation of PI. This led to a newly proposed mechanism for synthesis of inositol-containing lipids in bacteria, different to that in eukaryotes [27]. Here, exogenous inositol is imported *via* an inositol or a sugar transporter and then phosphorylated to form *myo*-inositol-1-phosphate which can be coupled to CDP-diacylglycerol to form the intermediate PIP, by a CDP-alcohol phosphatidyltransferase. An added dephosphorylation step would then be needed to convert PIP to PI. As in mycobacteria [16,35], no obvious transporter genes or candidates for an inositol kinase could be identified in *T. forsythia*. Jorge et al. also described a new biosynthetic pathway restricted to bacteria which does not rely on CTP-activated glycerolipids but uses instead activated inositol in the form of CDP-inositol [44]. This pathway was elucidated for *Rhodothermus marinus* (phylum *Bacteroidetes*) and implies the inositol-3-phosphate synthase gene to be located within an operon-like structure together with a L-*myo*-inositol-1-phosphate cytidylyltransferase (IPCT) and a bacterial dialkylether glycerophosphoinositol synthase (BEPIS). This operon is found in a number of different bacterial genera and a BEPIS homologue can also be found in *T. forsythia* (Tanf_07760), but the other members of the operon are not present; especially the IPCT would be needed for synthesis of activated CDP-inositol from the imported metabolite. Therefore, in this context, the physiological importance of this putative BEPIS is currently not clear.

In order to import inositol from the medium in sufficient quantity to support the synthesis of such major lipid components, representing ~25% of its membrane lipids, we would expect a designated inositol transporter to be present. The major transporter of *myo*-inositol in *Bacillus subtilis* strains and in *Lactobacillus casei* BL23 was found to be

encoded by the *iol*T gene which forms part of an operon for inositol catabolism, as inositol in these organisms can be used as a nutrient source [38,39]. As done for *Mycobacterium tuberculosis* [35], *iol*T sequences were used in a BLASTP search against the proteome of *T. forsythia* ATCC 43037. Only one significant hit could be found, Tanf_07060, classified as a major facilitator superfamily (MFS) transporter of the sugar porter (SP) family. However, this gene lies within a possible operon for the uptake and use of xylose. Therefore, the capacity of this protein to act as an inositol transporter has to be verified experimentally which is now subject of further studies. Identification of the responsible gene and creation of the corresponding knock-out mutant might give insight into how essential the utilization of inositol is in this pathogen.

5. Conclusion

The study performed here on the lipids of *T. forsythia*, describes the novel structures of two classes of phospho-*myo*-inositol-DHCs. Considering that these lipids make up such a large portion of the membrane, they are possibly essential in this oral pathogen which most likely obtains all of the *myo*-inositol needed from the surrounding media. The effects of these lipids on the host immune system and its implication in the virulence of this pathogen are yet to be evaluated but could help get functional information on inositol-containing lipids and could be relevant in the search for new drug targets.

Conflict of interest

None of the authors have any conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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2.3. Glycobiology aspects of the periodontal pathogen *Tannerella forsythia*

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Review

Glycobiology Aspects of the Periodontal Pathogen *Tannerella forsythia*

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Abstract: Glycobiology is important for the periodontal pathogen *Tannerella forsythia*, affecting the bacterium's cellular integrity, its life-style, and virulence potential. The bacterium possesses a unique Gram-negative cell envelope with a glycosylated surface (S-) layer as outermost decoration that is proposed to be anchored via a rough lipopolysaccharide. The S-layer glycan has the structure 4-Me*O*-β-Man*p*NAcCONH$_2$-(1→3)-[Pse5Am7Gc-(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* and is linked to distinct serine and threonine residues within the D(S/T)(A/I/L/M/T/V) amino acid motif. Also several other *Tannerella* proteins are modified with the S-layer oligosaccharide, indicating the presence of a general *O*-glycosylation system. Protein *O*-glycosylation impacts the life-style of *T. forsythia* since truncated S-layer glycans present in a defined mutant favor biofilm formation. While the S-layer has also been shown to be a virulence factor and to delay the bacterium's recognition by the innate immune system of the host, the contribution of glycosylation to modulating host immunity is currently unraveling. Recently, it was shown that *Tannerella* surface glycosylation has a role in restraining the Th17-mediated neutrophil infiltration in

the gingival tissues. Related to its asaccharolytic physiology, *T. forsythia* expresses a robust enzymatic repertoire, including several glycosidases, such as sialidases, which are linked to specific growth requirements and are involved in triggering host tissue destruction. This review compiles the current knowledge on the glycobiology of *T. forsythia*.

Keywords: Biofilm; general *O*-glycosylation system; Gram-negative oral pathogen; glycosidases; S-layer glycoproteins; *Tannerella forsythia*; virulence

1. Introduction to *Tannerella forsythia*

1.1. Occurrence of T. forsythia

It has been estimated that nearly 700 bacterial taxa, phylotypes and species can colonize the oral cavity of humans [1]. Many of them trigger periodontal diseases which are multifactorial infections implicating interactions with host tissues and cells. These may lead to destruction of the periodontal structures, including the tooth-supporting tissues, alveolar bone, and periodontal ligament [2]. Frequently, the trigger for the initiation of periodontal diseases is the presence of complex microbial biofilms that colonize the sulcular regions between the tooth surface and the gingival margin [3,4]. Recently, the link between oral microbial communities with the change from health to disease was investigated, leading 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 [5–7]. The ‘red complex’, which has been classified as a late colonizer in multispecies biofilm development, comprises species that are considered periodontal pathogens [4]; these are *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia.*

1.2. Description of T. forsythia

1.2.1. Taxonomic Affiliation

T. forsythia was first isolated in the mid-1970s from subjects with progressing advanced periodontitis and described as ‘fusiform *Bacteroides*’ by Tanner *et al.* [8]. Initially, its taxonomic affiliation was unclear because it did not resemble described species of oral or enteric Gram-negative anaerobic rods [9]. 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 [10]. Subsequently, *B. forsythus* was affiliated to the genus *Tannerella* [11]. Here it was first formally classified to *Tannerella forsythensis* but then reclassified to *T. forsythia* [12].

Recent taxonomic analyses have revealed that *Bacteroides*, *Porphyromonas* and *Tannerella* are all contained in the order *Bacteroidales* and that *Porphyromonas* and *Tannerella* are phylogenetically even more closely related, as both are affiliated to the *Porphyromonadaceae* family [13].

A preliminary rapid identification of human-derived *T. forsythia* strains can be based on the following eight criteria [14]: positive activity for (i) α-glucosidase; (ii) β-glucosidase; (iii) sialidase;

(iv) trypsin-like enzyme; (v) negative indole production; (vi) requirement for *N*-acetylmuramic acid; (vii) colonial morphology; and (viii) Gram-stain morphology from blood agar medium deficient in *N*-acetylmuramic acid.

The full genome sequence of *T. forsythia* ATCC 43037 is available through the Oral Pathogen Sequence Databases at Los Alamos National Laboratory Bioscience Division [15]. The genome consists of 3,405,543 base pairs with 3,034 predicted open reading frames.

1.2.2. *T. forsythia* as a Periodontal Pathogen

T. forsythia meets the criteria for periodontal pathogens postulated by Socransky [16] and Socransky *et al.* [17], (i) because this bacterium is present in increased levels in periodontitis [17]; (ii) there is evidence for host response to its antigens [18–20]; (iii) it is able to cause disease in animal models [21–23]; and (iv) it expresses virulence factors that can potentially contribute to the disease process [24]. Among the characterized *T. forsythia* virulence factors related to the field of glycobiology are a sialidase [25], an α-D-glucosidase and an *N*-acetyl-β-glucosaminidase [26], as well as the glycosylated surface (S-) layer [27–29].

Despite the growing evidence implicating this bacterium in the pathogenesis of periodontitis, it is up to now a rather poorly studied organism, a fact that can be attributed to its fastidious growth requirements as well as to the lack of molecular tools for genetic manipulation [24].

Recent evidence suggests that glycobiology is important for defining the life of the periodontal pathogen *T. forsythia*. This review compiles the current state of knowledge about the glycobiology aspects of *T. forsythia*, with relevance for the bacterium's cellular integrity, its life-style and metabolism as well as its virulence potential.

2. Cellular Integrity

2.1. S-Layer Glycosylation of T. forsythia

2.1.1. S-Layers in General

Many bacteria from all phylogenetic lineages are covered by regularly arrayed superficial layers, termed S-layers. The protomeric units of S-layers usually consist of high-molecular-mass proteins or glycoproteins [30,31]. Particularly in Gram-negative bacteria it has been difficult to isolate these structures in a form which truly represents what might occur in the living organism, although some attempts have been made [32–34]. Different functions, such as protective covering, molecular sieve and ion trap, phage receptor, providing an adhesion and surface recognition mechanism, as well as involvement in mediation of virulence, have been attributed to S-layers [30,31].

2.1.2. S-Layer Ultrastructure of *T. forsythia*

About 25 years ago, Kerosuo [35] reported for the first time on the ultrastructure of the *T. forsythia* ATCC 43037^T S-layer. Later, Sabet *et al.* [27] published their findings regarding the isolation, purification, and initial studies on the virulence potential of the S-layer from *T. forsythia* strains. SDS-PAGE analysis revealed the presence of two high molecular-mass, glyco-positive protein bands [36–38],

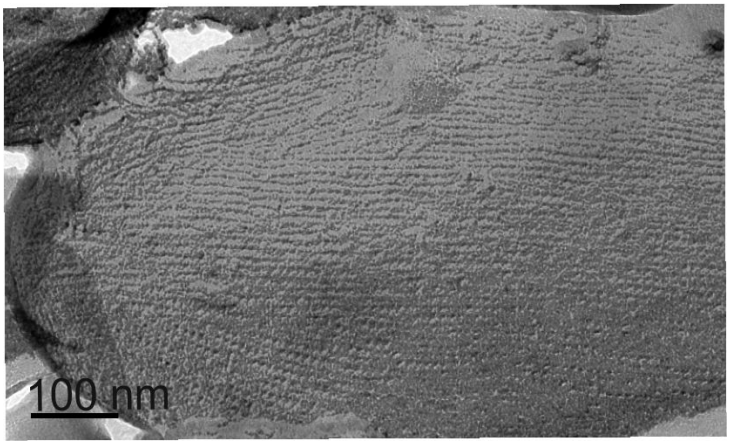
which were later confirmed by our research group to be the components of the S-layer [39]. The ~135-kDa *Tannerella* S-layer protein TfsA and the ~152-kDa S-layer protein TfsB are encoded by the genes *tfsA* (TF2661-2662) and *tfsB* (TF2663), respectively, which are co-transcribed from a single promoter [38]. The two S-layer proteins share 24% amino acid similarity. They do not show overall homology to any other S-layer protein sequence deposited in databases, except for their *C*-terminal regions, which have profound similarity to putative S-layer glycoproteins of the phylogenetically closely related bacterium *Bacteroides distasonis* [40]. The *Tannerella* S-layer proteins exhibit C-terminal sequence similarity to the CTD (C-Terminal Domain) family proteins of *Porphyromonas gingivalis* which supports the assumption of a novel CTD *Bacteroidales* secretion pathway [41,42].

Our interest in the S-layer of *T. forsythia* was aroused by the facts that (i) it represents the first glycosylated S-layer of a Gram-negative organism and (ii) it is structurally unique due to the simultaneous presence of two S-layer proteins [38]. Interestingly, in periodontal lesions, another S-layer-carrying bacterium, namely *Campylobacter rectus* is found. This organism is thought to be capable of inducing pro-inflammatory cytokines and its S-layer may temper this response to facilitate the survival of *C. rectus* at the site of infection [43].

Recently, Sekot *et al.* [39] extended the previous structural characterization of the *T. forsythia* S-layer [35] by a combined ultrastructural/immunological approach, showing that the two S-layer glycoproteins TfsA-GP and TfsB-GP are intercalated to form a monolayer on the cell surface of *T. forsythia*. The lattice spacing for the square S-layer lattice was determined to be 10.1 ± 0.7 nm (Figure 1) in freeze-etching, freeze-drying, and negative staining experiments, and a thickness of the layer of approximately 22 nm was found in ultrathin sections.

In that study, sheared flagella with intact hook regions have been identified by freeze-etching [39], which somehow challenges the description of *T. forsythia* as a non-motile species [44]. However, their role in bacterial motility and a possible impact on co-aggregation of *T. forsythia* with other species in the biofilm is still unclear. In this context it should also be mentioned that according to a recent partial annotation of the open reading frames of the *T. forsythia* genome, homologous genes for hook and other pilus/flagella forming units have not been identified [45].

Figure 1. Freeze-etched and platinum-carbon shadowed preparation of a *T. forsythia* cell showing the square S-Layer lattice with a lattice spacing of approximately 10 nm × 10 nm.



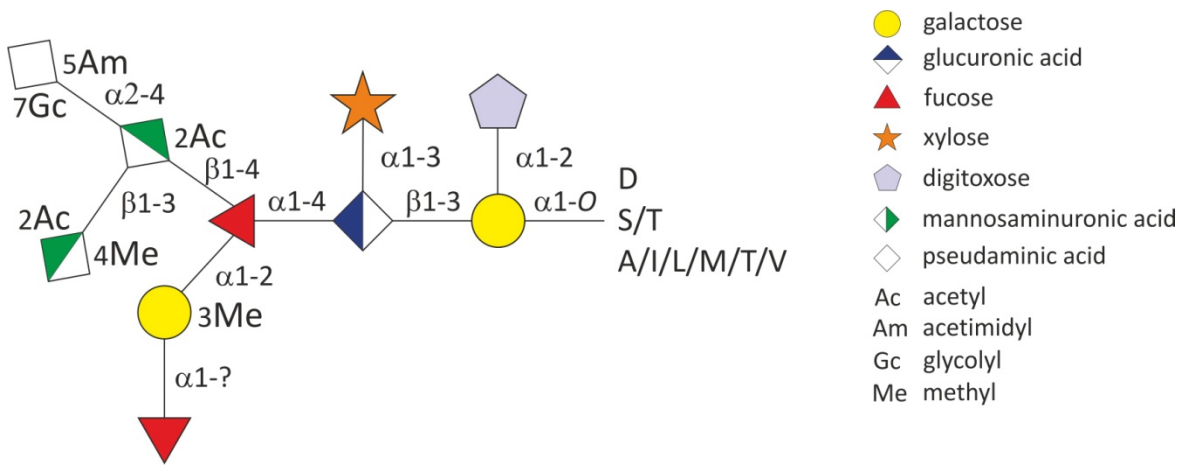
2.1.3. Glycosylation of the *T. forsythia* S-layer Proteins TfsA and TfsB

As mentioned above, Lee *et al.* [38] inferred from SDS-PAGE that the two *T. forsythia* S-layer proteins are glycosylated. However, no further compositional or structural details were provided. In our laboratory we focused the efforts on the characterization of the glycoproteome of *T. forsythia*.

By SDS-PAGE, glycosylation of the two S-layer proteins of *T. forsythia*, the 153-kDa TfsA-GP and the 180-kDa TfsB-GP (calculated molecular masses), could be confirmed. In-gel reductive β-elimination of purified S-layer glycoproteins was employed to release the glycans from the protein backbone [29]. Mass spectrometric analysis revealed a dominant glycan structure of 1,621 Da. Upon CID fragmentation analysis a hetero-oligomer consisting of eight different sugar residues was observed. Mass increments for one pentose, one deoxyhexose, three uronic acids (modified or free), one methylhexose, and one reduced hexose in addition to one, yet non-described, 361-Da sugar residue were identified. Further, some of the oligosaccharides were substituted by one dideoxyhexose and one additional deoxyhexose. Interestingly, the glycans released from either S-layer glycoprotein showed identical glycan mass profiles, indicating the presence of a uniform glycosylation pattern.

NMR spectroscopy of the glycan samples was used to identify the methyl-hexose as methyl-galactose and the two *N*-acteylhexosaminuronic acid residues as *N*-acetylmannosaminuronic acid and as *O*-methyl-*N*-acetylmannosaminuronic acid, respectively. The dideoxyhexose that was already observed in the MS/MS fragmentation profile was identified as a digitoxose residue. The identity of the 361-Da sugar residue, however, remained unclear even after high resolution MS/MS and NMR analysis of the intact *O*-glycan. In a screen of nucleotide-activated sugars from the bacterial cytoplasm large amounts of CMP-activated substance matching the 361-Da unit were observed. This was indicative for the presence of an α-keto sugar. Combined NMR analyses of the purified DMB (1,2-diamino-4,5-methylene dioxybenzene dihydrochloride)-labelled 361-Da sugar residue and of the complete oligosaccharide revealed the presence of a non-2-ulosonic acid carrying two substituents on the amino functions at carbons 5 and 7. Comparison of all recorded NMR spectroscopic data of the *O*-glycan with those of the isolated $C_{14}H_{25}O_9N_3$ compound showed good concordance. The configuration of the non-2-ulosonic acid has been proven by the few detectable coupling constants and NOEs and is in better accordance with those of a pseudaminic acid residue than with those of legionaminic acid. The whole $C_{14}H_{25}O_9N_3$ compound can hence be considered as Pse5Am7Gc, where Am is an amidinyl and Gc a glycolyl group [29]. The structure of the highly complex decaglycan of *T. forsythia* including the so far determined glycosidic linkages is given in Figure 2. The heterosaccharide is *O*-glycosidically linked via the reducing-end α-galactose to serine and threonine residues of both the TfsA and TfsB S-layer proteins. Interestingly, all identified glycosylation sites match the D(S/T)(A/I/L/M/T/V) three-amino acid motif that was recently described for the general protein *O*-glycosylation system in *B. fragilis* [46].

Figure 2. Schematic drawing of the structure of the abundant *O*-glycan in *T. forsythia*. Modified from [29].



2.2. General O-Glycosylation System of T. forsythia

2.2.1. *O*-Glycoproteins of *T. forsythia*

Carbohydrate-stained SDS-PAGE gels of *T. forsythia* whole cell lysates indicated, besides the S-layer glycoproteins, the presence of several other glycosylated proteins in the molecular mass range between 60 and 250 kDa [29]. Some of these bands were also reactive with the fucose-specific *Aleuria aurantia* lectin, which readily detects the S-layer glycan. Upon closer examination, all bands were shown to carry the same glycosylation profile as the S-layer glycoprotein bands, with slight variations detected in their relative ratios and in *O*-methylation of the mannosaminuronamide residue. This demonstrates that the S-layer *O*-glycosylation system is also involved in the glycosylation of other *T. forsythia* proteins [29].

Among these proteins are the predicted outer-membrane proteins TF2339 and its paralog TF1259, as well as the predicted lipoproteins TF1056 and TF0091. The former two proteins exhibit similarity to the CTD family of *P. gingivalis* [41,42]; the latter show similarity to TonB-dependent receptor associated proteins [47]. Interestingly, all of these glycoproteins are antigenic upon probing with an antiserum raised against a *T. forsythia* outer membrane preparation [45]. The finding that several abundant proteins in *T. forsythia* are modified with the S-layer glycan is fueled by the recent identification of a rich outer membrane glycoproteome in *T. forsythia* [45].

2.2.2. Theoretical analysis of protein *O*-glycosylation in *T. forsythia*

Initial information about glycosylation in *T. forsythia* was provided by the description of a so-called exopolysaccharide operon [48], of which WecC (TF2055) coding for a predicted UDP-*N*-acetylmannosaminuronic acid dehydrogenase is part of. Closer inspection of that genomic region revealed the presence of a 6.8-kb gene locus of *T. forsythia* spanning TF2055-TF2049, encoding in addition to WecC (TF2055), a predicted UDP-*N*-acetylglucosamine 2-epimerase (NeuC, TF2054), three predicted glycosyltransferases (TF2053; TF2050; TF2049), a predicted acetyltransferase (TF2052) and one ORF with yet unassigned function (TF2051) [29].

Since deletion of TF2055 caused truncation of S-layer protein glycans [48] by lacking the 809-Da Pse-containing trisaccharide side branch [29], it is evident that this genomic region carries crucial information for proper *O*-glycan assembly (see Sections *3.2.* and *6.2.*). Interestingly, similar glycosylation loci are also present in other phylogenetically related species, including, for instance, *Bacteroides fragilis* NCTC 9343, *Bacteroides thetaiotaomicron* VPI 5492, *Bacteroides uniformis* ATCC 8492, *Porphyromonas gingivalis* ATCC 33277, and *Parabacteroides distasonis* ATCC 8503.

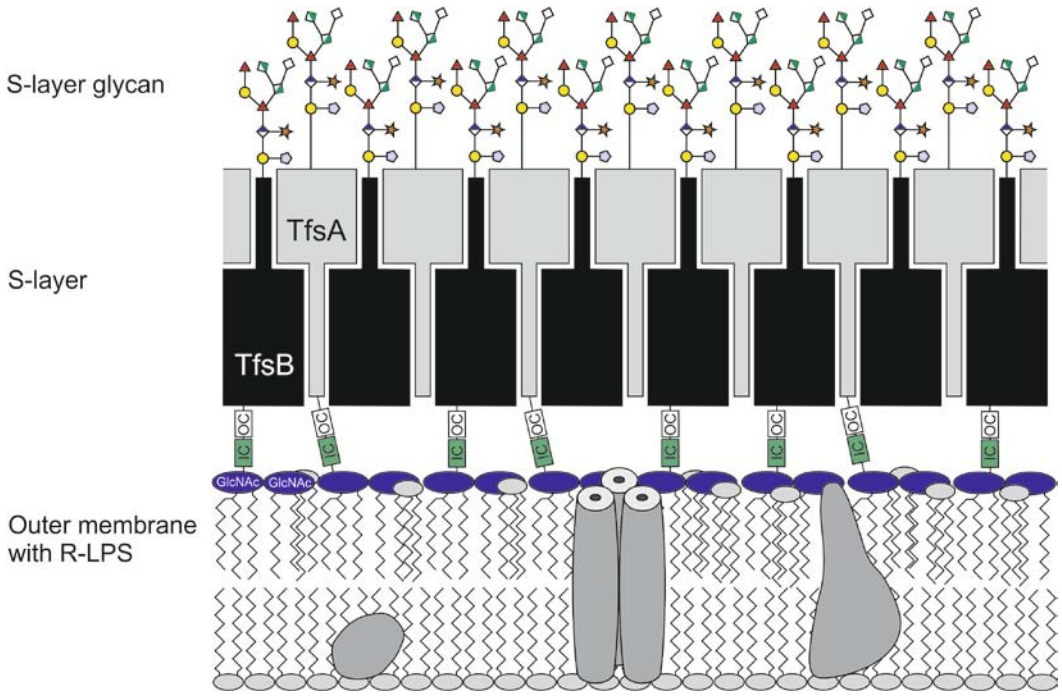
2.3. Lipopolysaccharide (LPS) of T. forsythia

LPS is an intrinsic feature of Gram-negative bacteria where it is located in the outer leaflet of the outer membrane [49]. So far, structure-function studies on the LPS from *T. forsythia* do not exist but are considered essential to further understand the bacterium's pathogenesis.

Currently, we are analyzing the LPS from *T. forsythia* and first results indicate that in the wild-type organism, an R-form LPS is present (G. Posch, O. Andrukhov, B. Lindner, P. Messner, E. Vinogradov, O. Holst, C. Schäffer, manuscript in preparation). Currently it is unknown, if LPS biosynthesis and the S-layer glycosylation pathway have cross-points. In a recent comparison of the LPS isolated from *T. forsythia* wild-type and Δ*wecC* cells by SDS-PAGE, no detectable effect of WecC on the chemical nature of LPS was observed [50].

In this context it is interesting to note that in *Aeromonas salmonicida*, an R-form LPS is assumed to serve as an anchor with defined length for attaching the S-layer protein to the outer membrane [51] (Figure 3). Considering that both *T. forsythia* S-layer proteins TfsA and TfsB have been classified as CTD-proteins [45], it should be noted that in a recent study with *P. gingivalis* it has been shown that LPS attaches to this class of proteins. It was suggested that LPS deacetylation is part of the co-ordinated secretion of LPS and CTD-proteins by a novel secretion and attachment system [42].

Figure 3. Theoretical model of the cell envelope architecture of *T. forsythia*. IC (inner core) and OC (outer core) represent partial structures of the rough LPS. Not to scale.



3. Virulence Potential of the Glycosylated S-Layer of *T. forsythia*

3.1. Virulence and Glycosylation in General

Virulence is the relative ability of an organism to cause disease or to interfere with a metabolic or physiological function of its host. Thus, virulence factors can have a multitude of functions, including the ability to induce microbe-host interactions such as attachment, to invade the host, to grow in the confines of a host cell, and to evade or even interfere with host defense mechanisms [4].

Cell surface glycosylation and, in that context, specifically glycosylation of proteins, might serve specific functions in infection and interaction with host tissues [52] as well as modulation of immune responses during pathogenesis [53,54]. Via interaction with components of the immune system, the glycan rather than the amino acid residues represent immuno-dominant epitopes that are recognized by blocking antibodies or modulate the immune response [52].

3.2. Immunological Data of T. forsythia

The S-layer glycoproteins TfsA-GP and TfsB-GP of *T. forsythia* are strongly antigenic, mediate hemagglutination as well as adherence to- and invasion of KB cells [38,55]. While the levels of IgG antibody against the S-layer of *T. forsythia* are low in healthy individuals, they are significantly elevated in adult and early-onset periodontitis patients. These results do not only indicate that this major surface protein is antigenic in humans, but also suggest that an increased interaction between host adaptive immune mechanisms and this pathogen occurs during periodontal disease progression [37].

Data on the virulence potential of the *T. forsythia* S-layer were also supported in our laboratory by investigating the immune responses of human macrophages and gingival fibroblasts upon stimulation with wild-type *T. forsythia* and an S-layer-deficient mutant [28]. This mutant induced significantly higher levels of the proinflammatory mediators IL-1β, TNF-α, and IL-8 compared with wild-type cells, especially at the early phase of response. This suggests that the S-layer attenuates the host immune response to this pathogen by evading its recognition by the innate immune system of the host [28]. It will be interesting to see, whether this finding can be confirmed in an animal model.

In a very recent study, first insights into the impact of *Tannerella* cell surface glycosylation on the modulation of the host immunity could be obtained [50]. By comparing the immunological effects evoked with *T. forsythia* wild-type and a Δ*wecC* mutant (see Sections 2.2.2. and 6.2.), in which the terminal sugar motif consisting of the two subterminal Man*p*NAcA and 4-Me*O*-β-Man*p*NAcCONH$_2$ residues and the terminal Pse5Am7Gc residue are missing [29], it became evident that the mutant is less virulent in a periodontitis model [50]. There are indications that the glycan decoration on *Tannerella* cells has a role in suppressing Th17-mediated neutrophil infiltration in the gingival tissue, allowing pathogen persistence in the host and induction of disease [50].

4. Cultivation and growth of *T. forsythia*

T. forsythia can be cultivated under anaerobic conditions at 37 °C in tryptic soy broth, supplemented with yeast extract (5 g/L), phytone peptone (5 g/L), cysteine (0.2 g/L), horse serum (20 mL/L), hemin (2.5 μg/mL), menadione (2 g/mL), and *N*-acetylmuramic acid (10 μg/mL) [28].

N-acetylmuramic acid, which is the monomeric form of the bacterial cell wall component, is an important growth-stimulating factor for *T. forsythia*, both in broth and plate culture [56]. Grown on agar media in presence of *N*-acetylmuramic acid, *T. forsythia* cells appear as regularly-shaped, short, Gram-negative rods, while in the absence of *N*-acetylmuramic acid growth is retarded and *T. forsythia* cells appear large, filamentous and pleomorphic with tapered (fusiform) ends [9,14]. Since *T. forsythia* lacks a metabolic pathway to synthesize *N*-acetylmuramic acid, the bacterium may possess unique systems to scavenge peptidoglycan degradation products released during cell-wall recycling of oral biofilm bacteria [24], or it derives this compound from sialylated glycoproteins like salivary mucins and fibronectin present in the oral cavity [57].

Because of its unique growth requirements [5] and the fact that it is quite difficult to grow, the precise role of *T. forsythia* in the severe bone and tissue destruction at sites from which it can be isolated remains to be determined.

5. Glycosidic Activity of *T. forsythia*

Possibly linked to its asaccharolytic nature, *T. forsythia* possesses genes for at least eight different glycosidases, including sialidases, an α-glucosidase, a β-glucosidase, a fucosidase, an arabinosidase, a glucosaminidase, a galactosidase and a mannosidase; which are able to process terminal glycosidic linkages of the complex oligosaccharides and proteoglycans of the periodontium [26]. This degradation creates a pool of accessible sugars for uptake and nutrition of oral bacteria and affects the functional integrity of the periodontium. Thus, glycan-interaction based processes, such as movement of leukocytes to the site of infection, could potentially be hindered, while protein epitopes for bacterial adhesion are created, further promoting disease progression [24,58]. In this context, an *N*-acetylneuraminyllactose-sensitive hemagglutinin has been identified in *T. forsythia* [59], which may mediate bacterial binding to host cell-surface sugars exposed by bacterial glycosidases [24].

One class of enzymes that is active on these host molecules are sialidases which represent a family of glycosylhydrolases that cleave α-ketosidic linkages between sialic acid and the glycosyl residues of host glycoproteins, glycolipids or colominic acid. Recent evidence suggests that for several periodontal pathogens, but particularly for the ‘red complex’ organism *T. forsythia*, sialic acid-containing host molecules play an important role *in vivo* [60]. Two different sialidases have been found to be expressed in *T. forsythia*, SiaHI and NanH [61]. Previously, Ishikura *et al.* [25] cloned the *siaHI* gene from *T. forsythia* ATCC 43037. The enzyme is found in a variety of cells, including viruses, bacteria, protozoa, fungi, and metazoans. In the case of *T. forsythia*, no definite function has yet been attributed to this enzyme, although experiments point to it being a periplasmic protein that plays no role in extracellular interactions [61]. These same studies also indicated that mutants of the NanH sialidase, the higher expressed sialidase in *T. forsythia*, had hindered attachment and invasion capabilities on epithelial cells. The enzyme was later on seen to play an important role in biofilm growth on surfaces coated with salivary glycoproteins [62]. Furthermore, sialic acid, glycolylsialic acid, and sialyllactose, all of which are common sugar moieties on a range of important host glycoproteins, were seen to stimulate growth of the pathogen only when cultured as a biofilm. The *nanH* gene is located in a large cluster that expands over a 16-kb section of its genome. This cluster contains all the genes required for

sialic acid catabolism, which indicates that the cleaved sialic acid can additionally be up-taken and utilized [62].

The sialic acid catabolism cluster shows strong sequence and genome organization similarity to sialic acid loci of related gastrointestinal anaerobes and represents a new route differing from the *Escherichia coli* paradigm pathway for sialic acid use [60]. Taken together, these data indicate that sialic acid is a key growth factor for *T. forsythia* and may be the key to its physiology *in vivo*.

6. Biofilm Life-Style of *T. forsythia*

6.1. General Remarks

In its native environment of the oral cavity, *T. forsythia* is present in a biofilm, which, in turn, is crucial for the virulence potential of the bacterium. Thus, the knowledge of factors triggering biofilm formation might reveal valuable strategies for interfering with periodontal disease. Despite *T. forsythia* being a late colonizer intercalating with other species from the oral microflora in dental plaque biofilms [63], which is consistent with a polymicrobial disease etiology [24], investigation of monospecies biofilms can also shed light onto factors affecting this specific life style.

6.2. Biofilm Life-Style of T. forsythia *and Glycosylation*

Several studies evaluating the presence of *T. forsythia* in subgingival plaque have demonstrated a significantly higher frequency in diseased subjects compared to healthy controls [9]. Moreover, *T. forsythia* was frequently associated with colonization by *P. gingivalis* and was elevated in groups of older patients [64]. Since biofilms formed by periodontal bacteria are considered important in disease progression and pose difficulties in treatment, the investigation of the underlying mechanism of the *T. forsythia* biofilm formation has been initiated [48]. This was carried out by screening random insertion mutants of *T. forsythia* for alterations in biofilm development. The approach led to the identification of a Δ*wecC* mutant [48], with *wecC* encoded in the predicted S-layer glycosylation gene locus [29]. This mutant showed increased cell surface hydrophobicity, which would promote bacterial attachment and/or aggregation [48] and increased ability to form biofilms as compared to the parent strain.

The truncated S-layer oligosaccharide isolated from the *T. forsythia* Δ*wecC* mutant represents a partial structure of the above described S-layer oligosaccharide, in which the acidic branch is missing (see Section *3.2*) [29]. Considering a pK value of sialic acids of ~2.6, it is evident that under physiological conditions of the basic saliva environment in the oral cavity, the acid function of Pse5Am7Gc is dissociated and, thus contributes to charge repulsive forces which impair biofilm formation. The negative correlation between *wecC* transcription and biofilm formation [48] supports this assumption. However, since no truncated S-layer glycans could be detected on *T. forsythia* wild-type cells when grown under biofilm conditions, it is conceivable to assume that the specific glycosylation status of the cell surface is a means to balance the tendency for biofilm-formation at a certain level *in vivo*.

In this context it is interesting to note that, while *wecC* is down-regulated, the *T. forsythia* S-layer genes *tfsA* and *tfsB* as well as the genes encoding the glycoproteins TF1259 and TF2339 are up-regulated in biofilm formation [65]. This is a further indication of the S-layer protein *O*-glycosylation system to be linked with the biofilm life-style of *T. forsythia*.

7. Conclusions

T. forsythia is a Gram-negative oral pathogen for which sialic acid is a key growth factor that may be crucial for its physiology *in vivo.* Understanding the biology of oral pathogens and their virulence factors is a prerequisite for the maintenance of both general and oral health. Especially those factors that are associated with the bacterial cell envelope and/or exposed to the environment are prime candidates for mediating virulence through their direct involvement in pathogen-host interactions. Given that *T. forsythia* emerged as a crucial periodontal pathogen [4,9,24,28,60], several approaches were undertaken to characterize its cell surface properties including S-layer topography and glycosylation [29,35,38,39]. Considering the fact that this bacterium can affect systemic health, *T. forsythia* and especially its mechanisms governing pathogenicity deserve detailed investigation.

Recently, detailed microscopic, biochemical, and molecular analyses from our laboratory revealed that the outer membrane of *T. forsythia* is covered with a so far unique S-layer. The monolayer has a width of approximately 22 nm and is built up by the co-assembly of the glycosylated S-layer proteins TfsA and TfsB [39]. The *O*-glycosidically linked *T. forsythia* S-layer oligosaccharide is an overall highly diverse structure containing several rare sugar residues [29] contradicting the so far valid building plan of bacterial S-layer glycans [66,67], and, thus, being reminiscent of archaeal S-layer glycans [68]. It is tempting to speculate that the terminal Pse5Am7Gc residue participates in the bacterium-host cross-talk, although the relevance of the modification of the pseudaminic acid remains yet unclear. This notion is supported by the fact that members of this class of sialic acid-like sugars have been found in many Gram-negative bacterial species as constituents of important cell surface glycoconjugates, such as LPS [69], capsules [70], pili [71], and flagella [72,73], all of which are important mediators of pathogenicity, possibly influencing bacterial adhesion, invasion, and immune evasion [74]. It seems plausible that the glycans are recognized by lectin-like receptors that may facilitate adhesion to and invasion of specific host cells [55]. In this context it is interesting to note that a novel sialic acid utilization and uptake system has been described for *T. forsythia* [56]. This opens the possibility of using the sialic acid pathway for biosynthesis of Pse5Am7Gc. On the other hand, it is also possible that the pseudaminic acid might be prone to at least partial degradation by the sialidase system [60].

Crucial for the overall virulence potential of *T. forsythia* is its specific biofilm life-style. Thus, the knowledge of factors triggering biofilm formation might reveal valuable strategies for interfering with periodontal disease. Surprisingly, increased biofilm formation could be correlated with the presence of truncated S-layer glycans on *T. forsythia*, in which the “acidic” branch of the decasaccharide is missing. In the context of biofilm formation, it is interesting to note that besides the *T. forsythia* S-layer glycoproteins, two other glycoproteins that have been identified recently [29], are up-regulated in biofilm formation [65]. This is a further indication that the S-layer protein *O*-glycosylation system is linked with the biofilm life-style of *T. forsythia*. The finding that several abundant proteins in *T. forsythia* are modified with the S-layer glycan (as shown in Figure 2) is supported by the recent identification of a rich outer membrane glycoproteome in *T. forsythia* [45]. Our data corroborate and extend this study by Veith *et al.* [45], in which all but one protein (TF0091) have already been identified as glycoproteins.

Thus, the periodontal pathogen *T. forsythia* possesses a general protein *O*-glycosylation pathway that modifies proteins of yet undefined function at multiple sites with a complex oligosaccharide within the D(S/T)(A/I/L/M/T/V) amino acid motif. The underlying glycosylation machinery as well as the glycosylation 'sequon' seems to be conserved within *Bacteroidales* species [46]. The role of protein *O*-glycosylation in underpinning the pathogenic strategy of *T. forsythia* and in its interaction with other bacteria from the oral microflora will be the subject of future studies.

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Conflict of Interest

The authors declare no conflict of interests.

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3. Concluding remarks

The work presented in this thesis was aimed at investigating two new potential virulence factors in the oral pathogen *T. forsythia* related to the bacterium's glycobiology.

Research into the annotated fucosidase Tanf_06770, named TfFuc1, and believed initially to be an outer membrane protein, led to its characterization as a periplasmic α1,2-L-fucosidase as it was active both on small oligosaccharides and larger more complex glycans and glycoproteins presenting the blood group H epitope. The activity on glycoproteins, studied here using bovine submaxillary mucin, showed that the release of fucose residues was greatly enhanced when terminal sialic acid residues were first removed, indicating that it probably works downstream from the *T. forsythia* NanH sialidase that was seen to have extracellular roles in adhesion and biofilm formation. TfFuc1 also showed an α1,6 activity on small linear substrates but not on core α1,6 linkages, which is the occurring form of α1,6 fucose residues on host glycans. Nevertheless, linear α1,6 fucoses could occur through the degradation of larger glycans into oligosaccharides that are transported into the periplasm (Martens *et al.* 2009) and, therefore, supports the cellular location and function proposed, that TfFuc1 is located in the periplasmic space and contributes to the degradation of host glycoproteins. As no extracellular function in adhesion and biofilm formation could be attributed to this enzyme and no fucose catabolism operon could be found in the genome, it remains to be investigated what role the released fucose plays in the physiology of *T. forsythia*. Whether the pathogen can GDP-activate the released fucose for incorporation into its glycosylation pathway remains a key point in this investigation. With the exception of mammals, most organisms can only synthesise GDP-fucose from GDP-mannose, yet many *Bacteroides* species residing in mammals and presenting fucose on their surface

glycoconjugates can directly GDP-active this sugar (Coyne *et al.* 2005). The enzyme known to do this, the bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (FKP), cannot be found in the genome of *T. forsythia* and, therefore, if this pathogen can recycle fucose into its glycosylation pathway, another unique enzyme or set of enzymes could be present.

The other two annotated proteins in the genome (Tanf_09440, Tanf_07610) with a glycosyl hydrolase domain classified in the fucosidase GH families 29 and 95, remain to be investigated, as these could be extracellular or membrane bound fucosidases with active roles in adhesion and biofilm, as originally hypothesised for TfFuc1, or could simply help to rule out any redundancy in the current investigation. Glycosyl hydrolases are poorly studied enzymes, as reflected by the fact that the majority of GH members have not yet been characterized. The work presented here represents the first fucosidase with this specificity in the GH family 29.

A study of the major lipids of *T. forsythia* revealed two new phosphorylated dihydroceramides (DHCs) (Tf GL1 and Tf GL2) which are major cellular lipid components of *T. forsythia* and contain *myo*-inositol as a polar head-group. The structure of these unusual glycolipids was elucidated by a combination of ESI-MS, GC-MS, and NMR. Tf GL1 and Tf GL2 consist of phospho-*myo*-inositol linked to a core lipid structure consisting of either a 17-, 18-, or 19-carbon sphinganine base in amide linkage to either a branched 17:0(3-OH) or a linear 16:0(3-OH) fatty acid which, in the higher-molecular-mass structure of Tf GL2, is, in turn, substituted with an additional branched 15:0 fatty acid in ester linkage. The lipid core described here is similar to that described by Nichols *et al.* for the DHCs of *P. gingivalis* which contain phosphoethanolamine or phosphoglycerol as a polar head group, although these authors reported only 17:0(3-OH) fatty acid to be present in amide linkage to sphinganine (Nichols *et al.* 2004). In addition, in this latter study, the 17:0(3-OH) fatty acid, 15:0 fatty acid and 17- and 19-carbon sphinganine were determined to be *iso*-branched and the 18-carbon sphinganine was reported to be linear.

Sphingolipids in general are thought to be particularly important for pathogenic bacteria to persist in the host (An *et al.* 2011; Heung *et al.* 2006). However, the DHCs in

the oral pathogen *P. gingivalis,* which were also found in other common human bacteria (Nichols *et al.* 2011), did not receive attention due to their function in the bacteria, but rather because it was hypothesised that they could form the link between oral disease and systemic diseases involving chronic inflammation. This was supported by findings showing accumulation of these lipids in host tissues distant from the sites normally colonized by the bacteria (Nichols *et al.* 2011), together with other data showing their stimulation of proinflammatory responses in gingival fibroblasts (Nichols *et al.* 2004), inhibition of osteoblast differentiation and function (Wang *et al.* 2010), enhancement of autoimmunity, and promotion of apoptosis (Nichols *et al.* 2009).

Whilst inositol-containing lipids are present in all eukaryotes, they are rarely found amongst bacteria. The effects of these lipids on the host's immune system and their implication in the virulence of this pathogen are yet to be evaluated. Whilst the lipid portion of these DHCs could be expected to have very similar proinflammatory effects as those described for the DHCs in *P. gingivalis*, inositol, when found as a polar head group in the lipids of other organisms, plays a pivotal role in protein-lipid interactions and cell signalling (Michell 2008) and has been seen in human pathogenic microorganisms to be an important mediator of infection and immunomodulation (Reynolds 2009).

As *T. forsythia* does not have the genes allowing for the *de novo* synthesis of inositol, we demonstrated that *T. forsythia* can efficiently import exogenous inositol for the synthesis of these major lipid components. Inositol containing lipids have been found in various pathogenic microorganisms that usually have both pathways for achieving inositol, *de novo* synthesis of the metabolite and uptake of exogenous inositol (Reynolds 2009). In these cases, the uptake of inositol from the medium can often not compensate for a dysfunctional synthetic pathway. As the uptake of inositol in *T. forsythia* supports the synthesis of lipids that make up approximately 25% of its membrane lipids, without the need of an additional inositol synthesis pathway, we expect a designated inositol transporter to be present. Once identified, this transporter could be relevant in the search for new drug targets. In addition, the health-associated *Tannerella* BU063, in contrast to *T. forsythia* ATCC 43037, encodes for the gene *ino1* in its genome, involved

in inositol synthesis, indicating that, whilst it is not clear whether these inositol-DHCs are a virulence factor in the pathogen, this efficient uptake and loss of the gene *ino1* could represent an adaptation to a pathogenic lifestyle.

This thesis contributes to the field of virulence and glycobiology of the poorly studied oral pathogen *T. forsythia*. Although periodontitis can be treated, it is a recurring chronic disease and has been linked to serious systemic diseases such as atherosclerosis and to an increased risk of strokes and heart attacks. Research into its vast repertoire of mechanisms to persist in the host and cause infection will ultimately lead to a better understanding of its physiology and aid in the search for new drug targets. A major challenge in this field is the polymicrobial aetiology of the disease. *T. forsythia* is most often studied as an isolated species under laboratory conditions that do not replicate the complexity of the mouth. Achieving setups that will imitate, to some extent, how the many species involved in periodontitis interact, is a hurdle difficult to overcome and, once achieved, will open the door to new possibilities in the study of virulence factors in this organism.

4. Conference contributions

Presenting author is underlined.

4.1. Oral presentations

- **S-layer glycan assembly in *Tannerella forsythia* and the pathogen-host cross-talk.** Megson, Z. A.; Posch, G.; Sekot, G.; Friedrich, V.; Pabst, M.; Altmann, F.; Guan, Z.; Schäffer, C.; Messner, P. [EMBO practical course: Modern biophysical methods for protein-ligand interactions, Oulu, Finland, October 17-21, 2011]

- **Isolating the lipid linked oligosaccharides in *Tannerella forsythia*.** Megson, Z. A.; Mach, L.; Messner, P.; and Schäffer, C. [BioToP Annual Retreat, Semmering, Austria, January 30 - February 1, 2012]

- **Characterization of the putative α-L-fucosidase TF0421 in *Tannerella forsythia*.** Megson, Z. A.; Koerdt, A.; Schuster, H.; Messner, P.; and Schäffer, C. [BioToP Annual Retreat, Retz, Austria, April 8 - 10, 2013]

- **Identification and characterization of an α-L-fucosidase in *Tannerella forsythia*.** Megson, Z. A.; Koerdt, A.; Schuster, H.; Messner, P.; and Schäffer, C. [DNBT Winter School, Lutzmannsburg, Austria, February 25 - 26, 2014]

- **Identification and characterization of an α-L-fucosidase in *Tannerella forsythia*.** Megson, Z. A.; Koerdt, A.; Schuster, H.; Messner, P.; and Schäffer, C. [BioToP Annual Retreat, Rust, Austria, April 28 - 30, 2014]

- **Characterization of an α-L-fucosidase in *Tannerella forsythia* and its role in biofilm formation.** Megson, Z. A.; Koerdt, A.; Schuster, H.; Messner, P.; and Schäffer, C. [FASEB SRC Microbial Glycobiology, Itasca, Illinois (USA), June 8 - 13, 2014]

- **Inositol uptake and incorporation into novel inositol-containing lipids.** Megson, Z. A.; Pittenauer, E.; Duda, K.A.; Engel, R.; Mach, L.; Allmaier, G.; Holst, O.; Messner, P.; Schäffer, C. [DNBT Winter School, Deutschlandsberg, Austria, February 18 - 20, 2015]

4.2. Poster contributions

- **S-layer glycan assembly in *Tannerella forsythia* and the pathogen-host cross-talk.** Megson, Z. A.; Posch, G.; Sekot, G.; Friedrich, V.; Pabst, M.; Altmann, F.; Guan, Z.; Schäffer, C.; Messner, P. [EMBO practical course: Modern biophysical methods for protein-ligand interactions, Oulu, Finland, October 17-21, 2011]

- **Characterization of an α-L-fucosidase in *Tannerella forsythia* and its role in biofilm formation.** Megson, Z. A.; Koerdt, A.; Schuster, H.; Messner, P.; and Schäffer, C. [Biofilms 6, Vienna, Austria, May 11-13, 2014]

- **Characterization of an α-L-fucosidase in *Tannerella forsythia* and its role in biofilm formation.** Megson, Z. A.; Koerdt, A.; Schuster, H.; Messner, P.; and Schäffer, C. [FASEB SRC Microbial glycobiology, Itasca, Illinois (USA), June 8 - 13, 2014]

4.2.1. S-layer glycan assembly in *Tannerella forsythia* and the pathogen-host cross-talk

EMBO practical course: Modern Biophysical Methods for protein-ligand interactions, Oulu, Finland, October 17-21, 2011 Abstract

Megson, Z.[1]; Posch, G.[1]; Sekot, G.[1]; Friedrich, V.[1]; Pabst, M.[2]; Altmann, F.[2]; Guan, Z.[3]; Schäffer, C.[1]; Messner, P.[1]

[1] Department of NanoBiotechnology and [2] Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria [3] Department of Biochemistry, Duke University Medical Center, Durham, NC, USA

Periodontitis, an inflammatory disease caused by the "red complex" bacteria of which *Tannerella forsythia* (*Tf*) is a member of, is characterized by an immune imbalance in the periodontal tissue [1]. Infection is thought to be made possible by the pathogen's ability to exploit the host's molecules for nutrition and adherence whilst sustaining immune evasion. Many of the bacteria's cell surface structures have a clear impact on this pathogen-host cross-talk and therefore are important in mediating infection [2]. For this reason, knowledge on the self-assembly of the *Tf* S-layer and the glycosylation of the integrating proteins is of uppermost importance in this field of research [3, 4]. One of the goals following this line of investigation is the isolation and characterization of *Tf* lipid carriers which transport the S-layer glycan from the cytoplasm across the membrane to the target proteins. On a different scope, different enzymes contribute to adding and modifying sugar residues on the S-layer glycan in order to change the surface properties of the cell envelope. Here we investigate a protein observed to effect biofilm formation [5], probably by controlling the presence of a side chain on the *Tf* S-layer glycan. *Tf* also contains a hypothetical gene in its genome that encodes a putative α-L-fucosidase. This protein could potentially cleave off fucose residues from glycoproteins in the mouth and saliva in order to use them as an energy source and for incorporation into the S-layer glycans to aid in immune evasion [6]. Both genes are currently being cloned and ex-

pressed in order to evaluate their function on the *Tf* envelope and on fucose containing eukaryotic glycans.

The work is supported by the Austria Science Fund, projects BioToP W1244 (to PM & CS), P20605-B12 and P21954-B20 (to CS) and P20745-B4 (to PM)

1. Sharma, A., Virulence mechanisms of *Tannerella forsythia*. Periodontology 2000, 2010. 54(1): p. 106-116.

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S-layer glycan assembly in *Tannerella forsythia* and the pathogen-host crosstalk

Megson, Z.[1]; Posch, G.[1]; Sekot, G.[1]; Friedrich, V.[1]; Pabst, M.[2]; Altmann, F.[2]; Guan, Z.[3]; Schäffer, C.[1]; Messner, P.[1]
[1]Department of NanoBiotechnology and [2]Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria
[3]Department of Biochemistry, Duke University Medical Center, Durham, NC, USA

Introduction

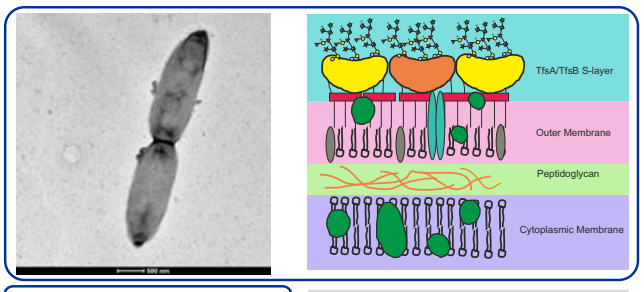

Fig. 1. *Tannerella forsythia*. **Top, left**. Negative staining of a specimen. **Top, right.** Cell wall architecture. *Tf* presents a unique S-layer composed of two different proteins glycosylated with the same glycan chain. **Bottom, left**. The S-layer glycan is composed of 10 sugars, including terminal fucoses and several acidic residues.

Tannerella forsythia (*Tf*) is a member of the red complex bacteria, a group of bacteria associated with periodontal disease. This Gram-negative, anaerobic, filamentous bacterium is poorly understood but is of great interest as it is one of the first opportunities to assess the virulence potential of the surface-layer (S-layer) glycosylation [1]. The structurally unique S-layer of *Tf* is composed of two different glycoproteins, both presenting the same glycan on the surface [2] (Fig. 1). In order to contribute to the understanding of the S-layer biosynthesis, we are aiming at isolating and identifying the lipid-linked oligosaccharides (LLOs) carrying this glycan of interest.

Isolation of LLOs

LLOs are phosphopolyisoprenoid alcohols that transport a polar glycan across the hydrophobic membrane. Fig.1 shows the *Tf* S-layer glycan that should be isolated on *Tf* lipid carriers. Lipid extraction of a 5 liter cell culture of *Tf* is performed following a modified Blight and Dyer method for polar lipids (Fig. 2) [3] and the total extract is then separated on a Silica 60 column according to polarity. Fractions are collected and further separated by thin layer chromatography (TLC). Staining for lipids (with primulin), carbohydrates (thymol), and phosphate (molybdenum blue) show triple positive spots possibly containing our lipid carrier (Fig. 3).

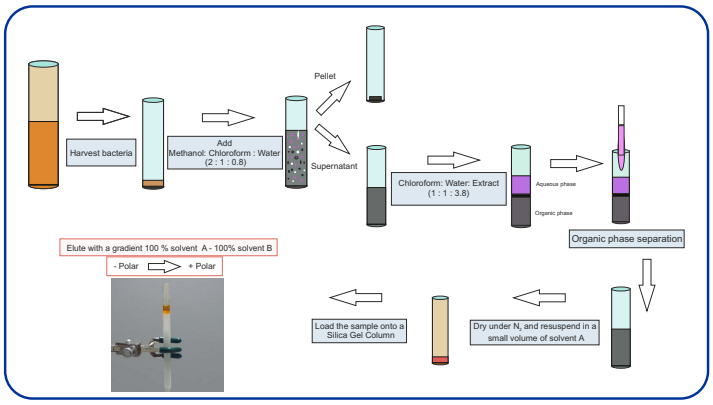

Fig. 2. Lipid extraction method following a modified Blight and Dyer procedure for polar lipids.

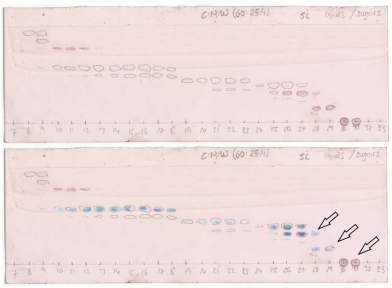

Fig. 3. TLC of each fraction of the total lipid extract after separation on a Si60 column. **Top:** In pencil, lipids stained with primulin. In red, sugars stained with Thymol. **Bottom:** Merged image of the previous TLC plate and the staining with Molybdenum blue for phosphate detection. Triple positives are the three more polar spots at the end of the separation.

Mass Spectrometry

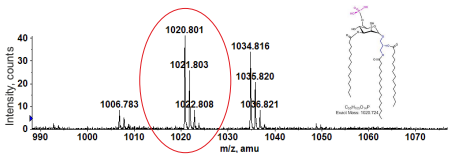

Fig. 4. MS spectrum of the most polar fraction of the lipid extract (last spot on the TLC). Encircled is the peak that corresponds to the glycolipid isolated in this fraction. Its structure and formula are depicted on the right. Polarity predictions indicate that the molecule would be much more hydrophobic than that predicted for the glycolipid we are trying to isolate.

Mass spectrometry of the carbohydrate fraction (released by strong acid hydrolysis) or the whole glycolipid contained in the triple positive spots show only single monosaccharide components, the lipids of which could be part of another biosynthetic pathway (Fig. 4). Using ALOGPS 2.1 (available online) to infer the different molecules' polarity, the most polar lipid in the extraction is more hydrophobic than that predicted for the LLO of the S-Layer glycan in *Tf*, indicating that the lipid carrying the glycan of interest is most probably lost in the water/methanol fraction during extraction.

Conclusions and Outlook

The work presented here is the initial step towards isolating the LLOs carrying the glycan destined for export to the S-layer glycoproteins in *Tannerella forsythia*. The glycan chain confers a large degree of polarity to the lipid carrier, thus making it difficult to separate it from other cell components. Finding the proper extraction procedure is a key step in this process and requires adequate solvent polarity. Here, the LLOs are most probably lost in the aqueous fraction and therefore cannot be detected in the total lipid pool. In future experiments, the polarity of the organic phase must be increased and better, more sensitive methods of detection should be tested. Identifying the LLO in *Tf* will allow for further advances in understanding the S-layer biosynthetic pathway.

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[1]Sabet, M., et al., *The surface (S-) layer is a virulence factor of Bacteroides forsythus.* Microbiology, 2003. **149**(12): p. 3617-3627. doi.10.1099/mic.0.26535-0
[2]Posch, G., et al., *Characterization and scope of S-layer protein O-glycosylation in Tannerella forsythia.* J Biol Chem, 2011. doi. 10.1074/jbc.M111.284893
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Acknowledgements:
The work is supported by the Austria Science Fund, projects BioToP W1244 (to PM & CS), P20605-B12 and P21954-B20 (to CS) and P20745-B4 (to PM)

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4.2.2. Characterization of an α-L-fucosidase in *Tannerella forsythia* and its role in biofilm formation

Biofilms 6, Vienna, Austria, May 11-13, 2014 Abstract

Zoë A. Megson, Andrea Koerdt, Heinrich Schuster, Paul Messner and Christina Schäffer.

Department of NanoBiotechnology, NanoGlycobiology unit, Universität für Bodenkultur Wien, Muthgasse 11, 1190 Vienna, Austria

Tannerella forsythia (*Tf*) is a Gram-negative, filamented, anaerobic oral pathogen, member of the so called red complex of bacteria which cause a set of inflammatory diseases named periodontitis, affecting millions of people worldwide. The effects on the periodontium include loss of the alveolar bone around the teeth, swelling and bleeding of the gum and in more severe cases, loss of teeth. Periodontitis has also been linked to systemic inflammation and to an increased risk of stroke, heart attacks and atherosclerosis, amongst others. *Tf* possesses a unique cell surface (S)-layer composed of two different co-assembling glycoproteins [1]. Mutant strains of the S-layer proteins and the glycans they carry give insight into the importance of this S-layer in attachment, biofilm formation and disease progression. In addition, the structure of the glycan somewhat imitates that of host glycoproteins, having terminal sialic acid derivatives and fucose [2]. The importance of this finding resonates also in the robust repertoire of glycosidases which *Tf* and other oral bacteria express. Sialic acid residues present on both bacterial outer leaflets and host surfaces are cleaved off by host and bacterial neuraminidases to uncover sialic acid-masked epitopes for adhesion, triggering a multispecies biofilm formation [3]. In *Tf* in particular, this behaviour was observed in NanH sialidase mutants which had hindered attachment and growth on surfaces coated with salivary glycoproteins, giving this enzyme a crucial role in biofilm formation [4]. As the other key terminal sugar on host glycoproteins is fucose, we propose that the annotated α-L-fucosidase in *Tf* has a similar purpose. Here we present the characterization of the recombinant protein expressed in *E. coli*, by elucidating its specificity using various standard substrates and

blood group oligosaccharides. We also discuss initial biofilm and attachment assays using the knock-out mutant, Δ*fuc*. Complex salivary glycoproteins are considered the major source of nutrients for the oral supragingival microbiota and so the *in vitro* model glycoproteins used here are mucin from bovine submaxillary glands and fetuin from fetal bovine serum, as a non-fucosylated control in our biofilm assays.

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

1. Sekot, G., et al., Analysis of the cell surface layer ultrastructure of the oral pathogen *Tannerella forsythia*. Archives of Microbiology, 2012. 194(6): p. 525-539.
2. Posch, G., et al., Characterization and scope of S-layer protein *O*-glycosylation in *Tannerella forsythia*. J Biol Chem, 2011.
3. Stafford, G., et al., Sialic acid, periodontal pathogens and *Tannerella forsythia*: stick around and enjoy the feast! Molecular Oral Microbiology, 2012. 27(1): p. 11-22.
4. Roy, S., et al., Role of sialidase in glycoprotein utilization by *Tannerella forsythia*. Microbiology (Reading, England), 2011. 157(Pt 11): p. 3195-3202.

Characterization of an α-L-fucosidase in *Tannerella forsythia* and its role in biofilm formation

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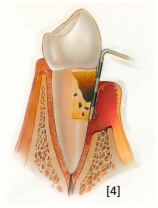
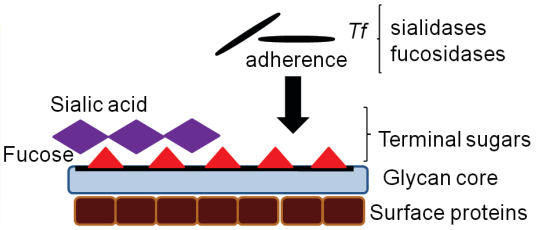
Introduction





The oral pathogen *Tannerella forsythia (Tf)* is one of the principal causes of periodontitis, affecting millions of people worldwide. It possesses a repertoire of glycosidases that allow for colonization of the oral cavity by using host glycoproteins for adherence and nutrition [1] [2].

The NanH sialidase in *Tf* is well described but up to now there has been no characterization of its annotated α-L-fucosidase, TF0421. Here we describe the enzyme's linkage specificity and cellular location and evaluate its role in biofilm formation.

Enzyme characterization

A

Fucose linkage	Linked to	Enzyme cleaves	Activity (U/mg)	
α 1-2	Gal	Yes	0.8 / 0.6	gal-glc / gal-glcNAc
α 1-3	Glc , GlcNAc	No	-	
α 1-4	Gal, GlcNAc	No	-	
α 1-6	GlcNAc	Yes	0.34	

The recombinant fucosidase was tested on various fucose-containing substrates of well defined structure. The cleavage products were analyzed by high performance anion exchange chromatography. The enzyme was seen to be most active on the α 1-2 linkage and, to a lesser degree, on the α 1-6 linkage.

B

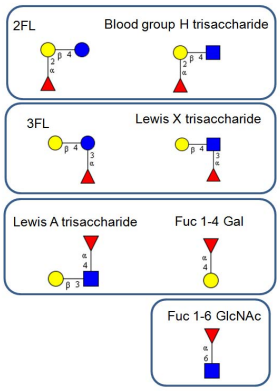
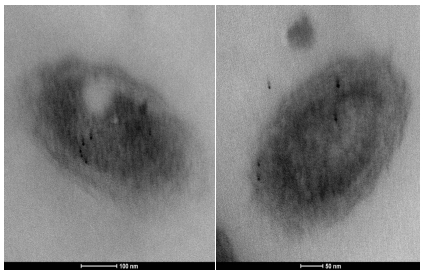


Fig.2. A) Enzyme activity on different fucose linkages linked to different sugar residues. **B**) Seven different substrates of well defined structure were tested representing the different fucose linkages to be found in the oral cavity. **C**) Immunogold labelling confirmed the cellular location of the enzyme to be in the cytoplasm and periplasm on *Tf*.

Although the enzyme is predicted to be cytoplasmic/periplasmic, it had been found present in an outer membrane fraction [3]. By immunogold labelling we were able to refute this recent finding and confirm the predicted cellular location.

C



Biofilm formation

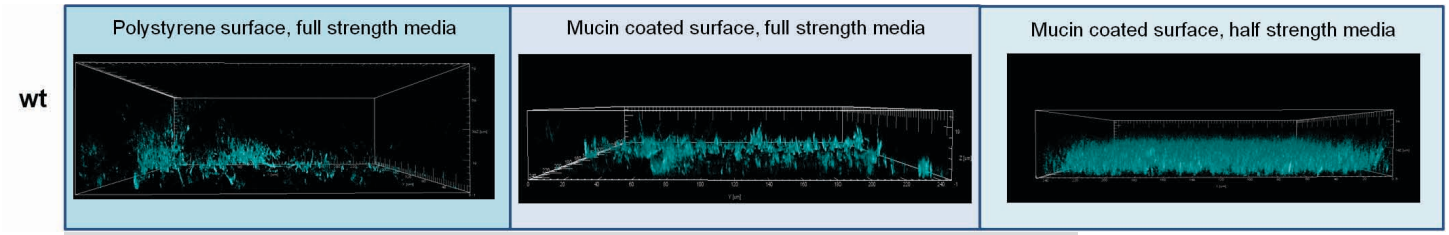


Fig. 3. Confocal Laser Scanning Microscopy (CLSM) of Hoechst-stained biofilm (wild-type *Tf*) on two different surfaces and in two different media strengths.

Confocal Laser Scanning Microscopy (CLSM) of Hoechst-stained biofilm shows very nicely how 1) *Tf* needs a surface displaying adhesion sites (e.g. a mucin coated surface [2]) in order to allow for biofilm formation and 2) decreasing the strength of the media leads to a significant increase in biofilm formation, indicating that starvation is the driving force for this change in phenotype **(see figure 3)**. The same can be seen in a more quantitative manner by reading the overall fluorescence intensity of the biofilm and correcting the value by the growth of the strain in each condition **(see figure 4)**. The *Δfuc* mutant, lacking fucosidase activity, presents increased biofilm formation in full strength media in respect to the wild-type. When the strength of the media was halved, the difference between these strains was much more pronounced **(see figure 5)**. Considering the above, it can be concluded that the fucosidase confers *Tf* a competitive advantage which is not needed in rich media but becomes especially important when nutrients are scarce, a regular occurrence in the nutrient-limited environment of the oral cavity.

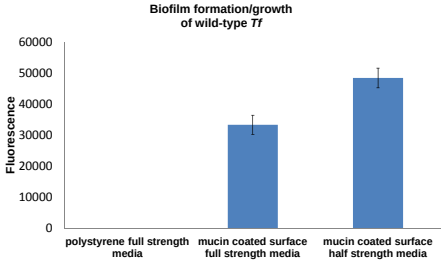


Fig. 4. Biofilm formation of wild-type *Tf* corrected by the growth in each condition.

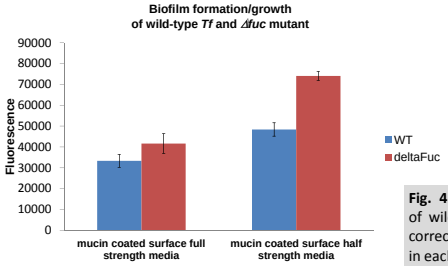


Fig. 4. Biofilm formation of wild-type and *Δfuc Tf* corrected by the growth in each condition.

Conclusions and outlook

TF0421 is a cytoplasmic/periplasmic fucosidase in the oral pathogen *Tannerella forsythia* that possesses unique linkage specificity cleaving α1-(2,6) terminal fucoses. Biofilm assays using wild type *Tf* and a mutant lacking fucosidase activity showed that the fucosidase gives *Tf* a competitive advantage when nutrients are scarce which translates into increased biofilm formation.

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[1] Stafford, G., Roy, S., Honma, K., and Sharma, A. (2012) *Mol Orlal Microbiol* **27**, 11-22
[2] Roy, S., Honma, K., Douglas, C. W., Sharma, A., and Stafford, G. P. (2011) *Microbiology* **157**, 3195-3202
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[4] Image taken from drug discovery.com

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4.2.3. Characterization of an α-L-fucosidase in *Tannerella forsythia* and its role in biofilm formation

FASEB SRC Microbial Glycobiology, Itasca, Illinois (USA), June 8 - 13, 2014 Abstract

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Characterization of an α-L-fucosidase in *Tannerella forsythia* and its Role in Biofilm Formation

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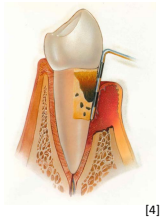
Introduction



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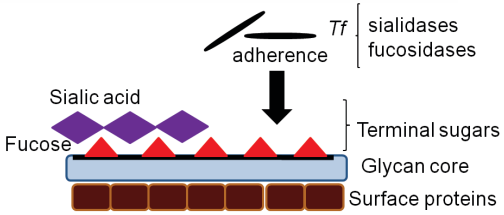


The oral pathogen *Tannerella forsythia (Tf)* is one of the principal causes of periodontitis, affecting millions of people worldwide. It possesses a repertoire of glycosidases that allow for colonization of the oral cavity by using host glycoproteins for adherence and nutrition [1] [2].
The NanH sialidase in *Tf* is well described but up to now there has been no characterization of its annotated α-L-fucosidase, TF0421. Here we describe the enzyme's linkage specificity and cellular location and evaluate its role in biofilm formation.

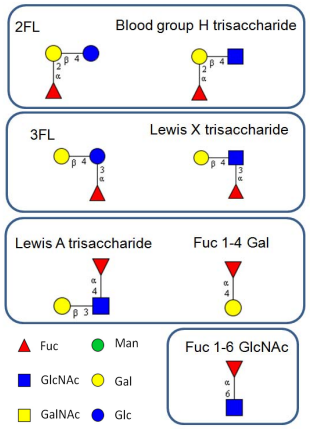
Enzyme characterization

The recombinant fucosidase was tested on various fucose-containing substrates of well defined structure. The cleavage products were analyzed by high performance anion exchange chromatography. The enzyme was seen to be most active on the α 1-2 linkage and, to a lesser degree, on the α 1-6 linkage. The ability of the enzyme to cleave fucose residues off more complex natural glycans and those on branched sugar residues was assayed on substrates GnGnF^6-peptide, A-antigen tetrasaccharide **(see figure 2C)** and on bovine submaxillary mucin. The enzyme cleaved only terminal fucoses residues (presumably α 1-2 linked) from the latter.

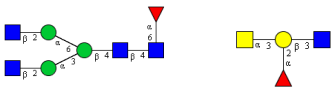
A

Fucose linkage	Linked to	Enzyme cleaves	Activity (U/mg)	
α 1-2	Gal	Yes	0.8 / 0.6	Gal-Glc / Gal-GlcNAc
α 1-3	Glc , GlcNAc	No	-	
α 1-4	Gal, GlcNAc	No	-	
α 1-6	GlcNAc	Yes	0.34	

B



C



Biofilm formation

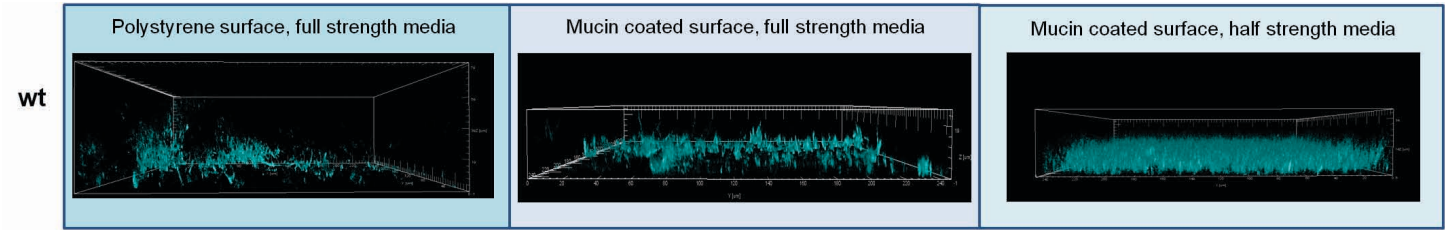


Fig. 3. Confocal Laser Scanning Microscopy (CLSM) of Hoechst-stained biofilm (wild-type *Tf*) on two different surfaces and in two different media strengths.

Although the enzyme is predicted to be cytoplasmic/ periplasmic, it had been found present in an outer membrane fraction [3]. By immunogold labeling we were able to refute this recent finding and confirm the predicted cellular location.

D

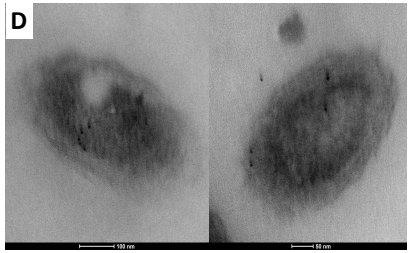


Fig.2. A) Enzyme activity on different fucose linkages linked to different sugar residues. **B**) Seven different substrates of well defined structure were tested representing the different fucose linkages to be found in the oral cavity. **C)** GnGnF^6 and A- antigen tetrasaccharide. **D**) Immunogold labeling confirmed the cellular location of the enzyme to be in the cytoplasm and periplasm on *Tf*.

Confocal Laser Scanning Microscopy (CLSM) of Hoechst-stained biofilm shows how 1) *Tf* needs a surface displaying adhesion sites (e.g. a mucin coated surface [2]) in order to allow for biofilm formation and 2) decreasing the strength of the media leads to a significant increase in biofilm formation, indicating that starvation is the driving force for this change in phenotype **(see figure 3)**. The same can be seen in a more quantitative manner by reading the overall fluorescence intensity of the biofilm and correcting the value by growth of the strain in each condition **(see figure 4)**. The *Δfuc* mutant, lacking fucosidase activity, presents increased biofilm formation in full strength media with respect to the wild-type. When the strength of the media was halved, the difference between these strains was much more pronounced **(see figure 5)**. Considering the above, it can be concluded that the fucosidase confers *Tf* a competitive advantage which is not needed in rich media but becomes especially important when nutrients are scarce, a regular occurrence in the nutrient-limited environment of the oral cavity.

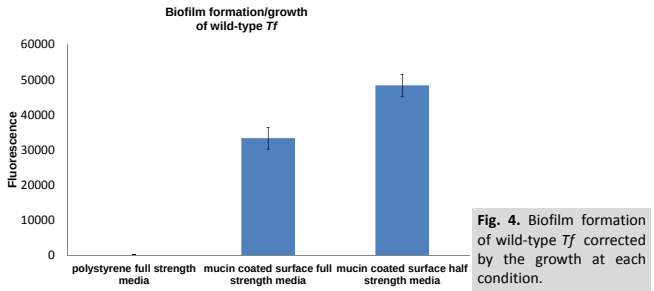


Fig. 4. Biofilm formation of wild-type *Tf* corrected by the growth at each condition.

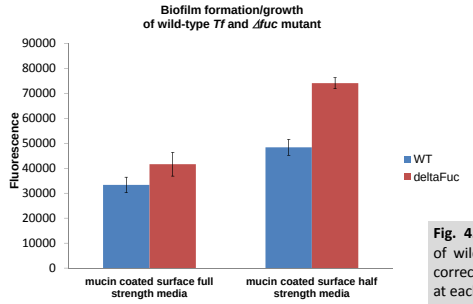


Fig. 4. Biofilm formation of wild-type and *Δfuc Tf* corrected by the growth at each condition.

Conclusions

TF0421 is a cytoplasmic/periplasmic fucosidase for the oral pathogen *Tannerella forsythia* that possesses unique linkage specificity cleaving α 1-(2,6) terminal fucoses. Biofilm assays using wild type *Tf* and a mutant lacking fucosidase activity showed that the fucosidase gives *Tf* a competitive advantage when nutrients are scarce which translates into increased biofilm formation in the mutant.

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[4] Image taken from drug discovery.com

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A. Appendix

A.1. Supplementary information for the publication *Characterization of an α-L-fucosidase from the periodontal pathogen* Tannerella forsythia

A.1.1. Experimental Procedures

Attachment and invasion assays

The H357 oral epithelial cell line (CCL17; American Type Culture Collection) was maintained in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum and L-glutamine. The cultures were incubated at 37°C under 5% CO2. H357 cells were grown to near-confluence (90 to 95%) for the assays. For both attachment and invasion assays, *T. forsythia* WT and Δ*Tffuc1* strains were incubated with H357 cells at a multiplicity of infection of 100, as described previously.[1] Briefly, for attachment assays, epithelial cell monolayers incubated with bacteria for 1.5 h were washed three times with sterile PBS, epithelial cell-associated bacteria were retrieved by lysing monolayers by addition of distilled water before physical disruption by scraping, and bacteria were plated for counting. For invasion assays, epithelial cell monolayers incubated with bacteria were washed with DMEM and then treated with metronidazole (200 µg/ml) for 1 h to kill external bacteria. The monolayers were then washed with PBS and were lysed as above to allow intracellular bacteria counting.

Microtiter assays of Hoechst-stained biofilms

Tannerella forsythia WT and Δ*Tffuc1* biofilms were grown essentially as previously described in both full-strength and half-strength liquid BHI medium.[2] Before starting the

bacterial cell culture, a 0.5-mg/ml solution of mucin from bovine submaxillary gland (Sigma) was prepared in 0.1 M sodium citrate buffer, pH 4.5, containing 0.1 M NaCl, added to non-treated polystyrene 24 well-plates (500 µl/well; Thermo Scientific) and incubated overnight at 37°C. On the next day, the mucin solution was removed completely and strains were inoculated at an OD_{600} 0.05 in full-strength BHI medium supplemented with horse serum and *N*-acetylmuramic acid or in medium diluted 1:2 with phosphate-buffered saline (PBS), yielding half-strength medium. In both cases, the medium was also supplemented with gentamycin (50 µg/ml) and gentamycin/erythromycin (50 µg/ml, 10 µg/ml) for WT and Δ*Tffuc1*, respectively. Triplicates were made for each condition and a fourth well served for measurement of total growth (OD_{600}). The plates were incubated anaerobically for 48 h at 37°C; subsequently, the supernatant was removed and the biofilm was washed once with 800 µl PBS before adding 1 ml/well of 10 µg/ml Hoechst 33258 Fluorescent Stain (Thermo Scientific) in PBS.[3] The biofilm was then removed from the bottom of the well using vigorous pipetting and the plates were left in the dark on a shaker for 45 min. 100 µl from each well was placed in a black 96-well-plate with a flat optical bottom (Thermo Scientific) for fluorescence intensity readings using excitation/emission filters of 360(35)/485(20) in an Infinite 200 plate reader (TECAN). The fluorescence intensity was corrected by the total OD_{600} reached by each strain.

Immunofluorescence microscopy of *T. forsythia* cells using TfFuc1-specific polyclonal antiserum

The surface localization of TfFuc1 was investigated by immunofluorescence staining of *T. forsythia* WT as described elsewhere.[4] Cells were grown until the late-exponential phase (OD_{600} 1.0), washed with PBS and adsorbed on a glass slide for 2 h. All procedures were performed at 22°C, if not stated otherwise. Cells were washed twice with PBS and fixed for 30 min at -20°C in 70% (v/v) ethanol in PBS. After two washing steps with PBS, cells were incubated in blocking buffer [10%, (w/v) bovine serum albumin (Sigma) in PBS] for 1 h, followed by incubation in blocking buffer containing TfFuc1-specific polyclonal antiserum or pre-immune serum (1:10-dilution) for 1 h, washed once

with blocking buffer and twice with PBS, and subsequently incubated in blocking buffer containing goat anti-mouse IgG (1:100) conjugated to FITC (Sigma-Aldrich) for 2 h. The cells were washed once with blocking buffer and twice with PBS to remove unbound antibodies. One drop of 50% (v/v) glycerol in PBS was added onto the cells and a coverslip was mounted. Confocal laser scanning microscopy analysis was performed using a Leica TCS SP5 II system. Images were taken with a 63.0 x 1.40 oil-immersion objective. Immunofluorescence-stained bacterial cells were excited at 488 nm using an argon laser and detected at an emission bandwidth of 500-595 nm. Images were acquired and processed with Leica LAS AF software.

A.1.2. Results and Discussion

Attachment and invasion assays.

The effect of the *Tffuc1* mutation on the ability of *T. forsythia* to interact with and invade human oral epithelial cells was tested using an antibiotic protection assay on the oral epithelial cell line H357. Results are shown as percentage of the bacterial cells recovered compared to a viability control run in parallel during the course of the whole experiment. The data shows no significant difference in invasion between WT and Δ*fuc* strains (Figure A.1). In addition, we compared the ability of the strains to attach to the epithelial cell line and again, no significant difference could be highlighted between the two (not shown), thus, indicating that TfFuc1 most likely does not play a role in direct human-pathogen interactions.

Microtiter assays of Hoechst-stained biofilms

T. forsythia WT and the Δ*Tffuc1* mutant were cultured anaerobically for 2 d in full-strength and half-strength medium in 24-well microtiter plates coated with mucin,[2] which is needed to promote biofilm formation as the presence of terminal sialic acid is an important factor allowing for *T. forsythia* surface attachment and biofilm growth.[5] Biofilm formation was evaluated by measuring the fluorescence intensity of the Hoechst-stained biofilm (Figure A.2). As the different strains presented a different growth in the

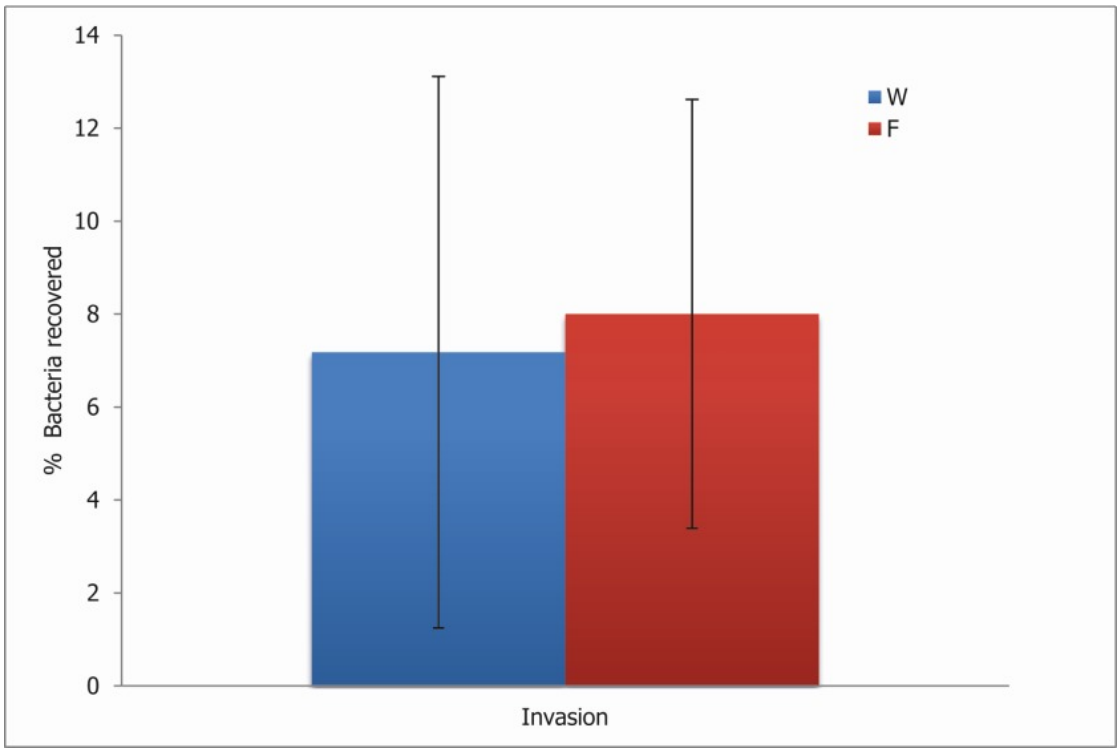

Figure A.1.: Cell invasion assays. The effect of the *Tffuc1* mutation on the ability of *T. forsythia* to interact and invade human oral epithelial cells was tested on the oral epithelial cell line H357. The combined results from three experimental repeats are shown as a percentage of the bacterial cells recovered compared to a viability control. W, *T. forsythia* wild-type; F, *T. forsythia* Δ*Tffuc1*.

different conditions tested, the fluorescence intensity was corrected by the total OD_{600} reached in each experiment. The WT strain increased its biofilm formation 1.4-fold as the strength of the medium was decreased to half by diluting with PBS. This indicates that biofilm formation is quite sensitive to changes of the medium strength, potentially indicating a degree of starvation (Figure A.2). In all experiments, the Δ*Tffuc1* had a higher biofilm formation phenotype than the WT strain, but how large the difference was depended largely on the strength of the growth medium. In full-strength BHI medium, the increased biofilm formation for the Δ*fuc* mutant was 1.2-fold that of the WT. In the case of half-strength medium, the difference became more prominent as Δ*fuc* presented a 1.5-fold increase (Figure A.2). These results could indirectly indicate a difference between the strains in their ability to utilize and scavenge components in the medium but it is currently not clear whether the observed phenotype reflects solely the loss of *Tffuc1* as no gene complementation assay was performed. We, therefore, conclude that the loss of the gene *Tffuc1* does not lead to a decreased biofilm formation indicating no direct role of the protein in the interaction of *T. forsythia* with the mucin-coated surface.

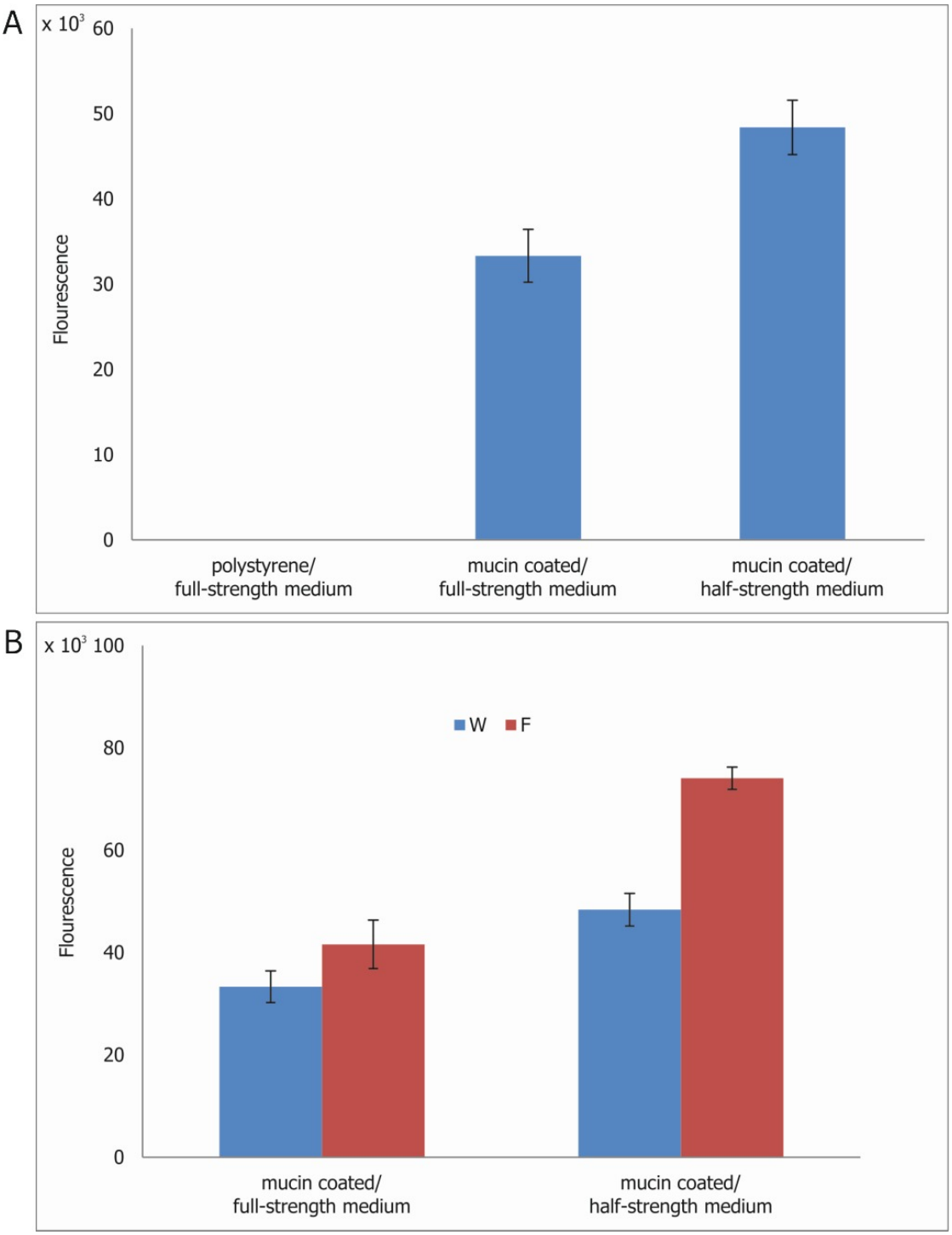

Figure A.2.: Microtiter assays of Hoechst-stained biofilms. A. The WT strain increased its biofilm formation on a mucin-coated surface as the strength of the medium was decreased to half that of the original broth. B. The difference in biofilm formation between the WT and the Δ*Tffuc1* depends largely on the strength of the medium. W, T. forsythia wild-type; F, T. forsythia Δ*Tffuc1*.

A.1.3. References

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5. Roy S, Honma K, Douglas CW, Sharma A, Stafford GP. Role of sialidase in glycoprotein utilization by *Tannerella forsythia*. Microbiology 2011; 157:31953-202.

B. Appendix

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Academic Background

Oct. 2009 - Sep. 2010 **University Master degree in Nanotechnology**, *Universitat Autònoma de Barcelona*, Campus de Bellaterra, Spain.
Supervisor: Victor Puntes (ICREA Research Professor)- Institut Català de Nanotecnologia Dissertation: "Immune-modulation effect by gold nanoparticle constructs". Passed with distinction.

Oct. 2005 - Jun. 2009 **Bachelor of Science in Biotechnology**, *Universitat Autònoma de Barcelona*, Campus de Bellaterra, Spain.
Internship for bachelor thesis at the Institut Català de Nanotecnologia Jun. 2008 – Jun. 2009 Project: "Gold Nanoparticle synthesis and functionalization with oligonucleotides".

Research stays abroad

2010 **Unité Interactions Hôte Pathogènes (CRSSA)**, *2 month-secundment (placement) Oct. 2010 – Nov. 2010, Grenoble, France, supervised by Dr. Jean Nicolas Tournier*, Project: Immune-modulation effect by gold nanoparticle constructs.

2013 **Forschungszentrum Borstel**, *4.5 months in the laboratory of Structural Biochemistry, Borstel, Germany, supervised by Prof. Otto Holst*, Project: Isolation of lipid-linked oligosaccharides in *Tannerella forsythia*.

2014 **School of Clinical Dentistry Sheffield**, *1.5 months in the lab of Integrated BioSciences, University of Sheffield, Sheffield, UK, supervised by Prof. Graham Stafford*, Project: Invasion of human oral epithelial cells by fucosidase mutants of *Tannerella forsythia*.

Sci-Publications

- Ojea-Jimenez, I., Comenge, J., Garcia-Fernandez, L., Megson, Z.A., Casals, E., Puntes, V.F. (2013) Engineered inorganic nanoparticles for drug delivery applications. Curr Drug Metab. 14, 518-30

- Posch, G., Sekot, G., Friedrich, V., Megson, Z.A., Koerdt, A., Messner, P., Schäffer, C. (2012), Glycobiology Aspects of the Periodontal Pathogen *Tannerella forsythia*. Biomolecules 2, 467-482
- Megson, Z.A., Koerdt, A., Schuster, H., Ludwig, R., Janesch, B., Frey, A., Naylor, K., Wilson, I., Stafford, G., Messner, P., Schäffer, C. (2015) Characterization of an α-L-fucosidase from the periodontal pathogen *Tannerella forsythia*, Virulence, 6, 282-292.
- Megson, Z.A., Pittenauer, E., Duda, K.A., Engel, R., Ortmayr, K., Koellensperger, G., Mach, L., Allmaier, G., Holst, O., Messner, P., Schäffer, C. (2015) Inositol-phosphodihydroceramides in the periodontal pathogen *Tannerella forsythia*: structural analysis and incorporation of exogenous *myo*-inositol, Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 11, 1417-1427

Teaching experience within the institution and language courses

- Part-supervision of master students (2): Manfred Taschner, Heinrich Schuster
- Part-supervision of bachelor student (1): Kristina Fintzen
- German Levels A1-B2 at VHS Wien / Deutschinstitut Wien

Skills and expertise

Field of expertise	Biochemistry, Microbiology, Glycobiology, Molecular biology
Languages	English (native), Catalan (native), Spanish (fluent), German (good)
Computer literacy	MS office (Word, Power Point, Excel), Corel Draw, Sci-Ed Clone Manager
Personal interests	Racket sport, handcrafts, music