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Biomolecular Technology of Proteins

Cell surface glycosylation of the periodontal pathogen *Tannerella forsythia*: Investigation of a possible biological role through immunological and biofilm studies

Dissertation

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Zusammenfassung

Metagenomische und kulturunabhängige Methoden zur Analyse des humanen Mikrobioms haben gezeigt, dass viele chronische Erkrankungen nicht von einer einzelnen Art, sondern von polymikrobiellen Gemeinschaften hervorgerufen werden. Dazu zählt auch Parodontitis, eine chronische Entzündungserkrankung des Zahnfleisches, die bei Erwachsenen zum Zahnausfall führen kann. Sie wird durch Veränderungen der Zusammensetzung der polymikrobiellen Artengemeinschaft in der Mundhöhle hervorgerufen, was dazu führt, dass potenziell pathogene Bakterien an Zahl zunehmen und die Pathogenität der gesamten Gemeinschaft durch die Produktion von Virulenzfaktoren verstärken. Diese und andere Substanzen, die aus dem Biofilm freigesetzt werden, verursachen eine Entzündungsreaktion im parodontalen Zahnfleisch, die letztlich zur Zerstörung des Gewebes führt.

Bestimmte Bestandteile der bakteriellen Zelloberfläche können als Virulenzfaktoren fungieren und beeinflussen das Verhalten der Bakterien im Biofilm und deren Interaktion mit oralen Wirtszellen. Eine besondere Rolle kommt dabei glykosylierten Zelloberflächenstrukturen zu, die von vielen Krankheitserregern produziert werden. Allgemein spielt die Proteinglykosylierung als die am häufigsten auftretende posttranslationale Modifikation von Proteinen eine wichtige Rolle in der Zellkommunikation und Immunevasion.

Die Zelloberfläche von *Tannerella forsythia*, einem Gram-negativen Erreger der Parodontitis ist vollständig mit einer glykosylierten Oberflächen (S)-Schicht und einem daran verankerten komplexen *O*-Glykan bedeckt. Die hohe Anzahl, mit der diese Zucker an der Zelloberfläche von *T. forsythia* vorhanden sind, lässt darauf schliessen, dass sie einen Einfluss auf die Interaktion der Bakterien mit ihrer Umwelt haben. Zudem wurden verschiedene Stämme von *T. forsythia* gefunden, die sich in der Zusammensetzung des *O*-Glykans unterscheiden und entweder eine Pseudaminsäure oder eine Legionaminsäure an dessen terminaler Position tragen, wobei der Rest des Glykans weitgehend ident ist. Diese Nonulosonsäuren spielen eine Rolle in der Virulenz und damit beim Überleben des Bakteriums im Wirt und beeinflussen die Biofilmbildung. Während die molekulare Struktur des *T. forsythia* S-Schichtglykans und seine Biosynthese grösstenteils entschlüsselt sind, ist die Frage nach seiner biologischen Funktion weitgehend ungeklärt.

Ziel dieses Projektes war es, die biologische Rolle der *T. forsythia* Oberflächenglykosylierung, insbesondere der Nonulosonsäuren hinsichtlich ihres Einflusses auf

Biofilmbildung, Inter- und Intraspezies-Kommunikation und Wirts-Bakterium-Wechselwirkungen zu charakterisieren. Dazu wurden zwei *T. forsythia* Stämme, die sich in der terminalen Nonulosonsäure unterscheiden sowie eine Reihe definierter Zelloberflächenmutanten dieser beiden Stämme, hinsichtlich ihres planktonischen Wachstums, der Fähigkeit zur Bildung von Monospezies-Biofilmen, ihres Verhaltens in einem Multispezies-Biofilmkonsortium und der Interaktion mit gingivalen Epithelzellen und Monozyten untersucht.

Zuerst wurde mit Hilfe des subgingivalen "Zürich Biofilm Modells", einem *in vitro*-Multispezies-Biofilm-Modell, welches ausgewählte orale Bakterien enthält und die natürliche Situation des bakteriellen Plaques widerspiegelt, untersucht, inwieweit die *Tannerella* Oberflächenglykosylierung eine Rolle in der bakteriellen Interaktion spielt und die bakterielle Zusammensetzung der Biofilme beeinflusst. Desweitern wurde die Immunantwort von humanen oralen Keratinozyten und Monozyten auf *T. forsythia* Stämme und deren Zelloberflächenmutanten charakterisiert.

Dies zeigte, dass in dem Multispezies-Biofilm-Modell weder die glykosylierte S-Schicht in ihrer Gesamtheit noch das intakte *O*-Glykan notwendig ist, damit *T. forsythia* sich in dem Konsortium etablieren kann. Die beiden Stämme *T. forsythia* ATCC 43037 und *T. forsythia* UB4, die sich in der terminalen Nonulosonsäure unterscheiden, zeigten jedoch deutliche Unterschiede im planktonischen Wachstum, der Bildung von Monospezies-Biofilmen und im Wachstum im Multispezies-Modell. Diese Unterschiede im Verhalten der beiden Stämmen setzten sich auch in den immunologischen Studien fort, wo sich IL-1β- und IL-7-Ausschüttung in Monozyten und die IL-8-Sekretion in humanen oralen Keratinozyten in Bezug auf die beiden Stämme unterschieden. Weiters konnten wir eine Funktion der *T. forsythia* ATCC 43037 S-Schicht und insbesondere der terminalen Pseudaminsäure bei der Unterdrückung der Chemokinsekretion durch humane orale Keratinozyten nachweisen, und so unser Verständnis der biologischen Rolle dieser spezialisierten Zelloberflächenstruktur und ihres Einflusses auf die Pathogenität der oralen mikrobiellen Gemeinschaft erweitern.

Abstract

With the advancement of metagenomic and culture-independent bacterial analyses, it has become apparent that many chronic diseases are not caused by a single species but are of polymicrobial nature. Among those is periodontitis, a chronic inflammatory disease and major cause of tooth loss in the adult population. It is induced by a shift in the polymicrobial community colonizing the oral cavity, which enables potentially pathogenic bacteria to increase in numbers and elevate the pathogenicity of the whole biofilm community that constitutes the dental plaque. This occurs through the production of virulence factors and other substances released by the biofilms that cause periodontal tissue inflammation and destruction.

Cell surface components of bacteria are regarded as important factors that influence the bacteria's virulence and biofilm lifestyle, as well as the interaction with host tissues. In this context, the cell surface display of glycosylated appendages as employed by many pathogens is of special interest since protein glycosylation as the most common post-transcriptional protein modification plays an important role in cell-cell recognition and immune evasion.

The Gram-negative periodontopathogen *Tannerella forsythia* covers its cells with a unique surface (S-) layer carrying a complex *O*-glycan. This structure is found in high abundance on the bacterial cell surface and most likely influences the bacterium's lifestyle. Additionally, different strains of *T. forsythia* were found to differ slightly in the composition of the *O*-glycan, carrying either a pseudaminic acid or a legionaminic acid at the terminal position of an otherwise identical glycan. These nonulosonic acids have been implicated in facilitating the bacterium's virulence and survival within the host and impacting biofilm formation. While the molecular properties and biosynthetic pathway of the *T. forsythia* S-layer glycosylation have already been elucidated, its functional characterization still awaits further investigation.

This project was aimed at characterizing the biological role of the *T. forsythia* cell surface glycosylation and in particular the nonulosonic acids with regard to biofilm formation, inter- and intra-species communication and host-bacterium interactions. For this purpose, two *T. forsythia* strains differing in the terminal nonulosonic acid as well as a set of defined cell surface mutants were analyzed with regard to planktonic growth, monospecies biofilm formation, behavior in a multispecies oral biofilm consortium and interaction with gingival epithelial cells and monocytes.

First, the subgingival “Zurich biofilm model”, an *in vitro* multispecies biofilm model which incorporates select oral bacteria and mirrors the natural situation of the multispecies biofilm (dental plaque) was employed to investigate to what extent *Tannerella* surface glycosylation plays a role in bacterial interaction and influences the bacterial composition of the biofilms. Further, the immune response of human oral keratinocytes and monocytes to *T. forsythia* strains and cell surface mutants was characterized.

This study revealed that in the multispecies biofilm model, neither the presence of the glycosylated S-layer as an entity nor the intact *O*-glycan are necessary for *T. forsythia* to establish itself in the consortium. However the two strains, *T. forsythia* ATCC 43037 and *T. forsythia* UB4, differing in the terminal nonulosonic acid, exhibited distinct growth characteristics in planktonic form, in monospecies biofilms and in the multispecies model. The difference between the two strains was sustained in immunological studies where they elicited preferential immune responses for IL-1β and IL-7 in monocytes, and IL-8 in human oral keratinocytes. Further, we could demonstrate a function of the *T. forsythia* ATCC 43037 S-layer and in particular of the terminal pseudaminic acid in dampening the chemokine secretion by human oral keratinocytes, expanding our understanding of the biological role of this specialized cell surface structure and its influence on the pathogenicity of the oral microbial community.

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1. Introduction

Periodontitis is a chronic inflammation of the gingiva and tooth supporting tissues which is induced by the polymicrobial biofilm community that constitutes the oral plaque [1]. With a global prevalence of 10.8% it represents the sixth most prevalent condition and affects around 743 million people worldwide [2]. The disease is characterized by the destruction of the periodontium, the tooth supporting tissue that is the gingiva, periodontal ligament and alveolar bone, through an excessive inflammation, eventually leading to tooth loss [3]. Even though the etiology of periodontitis is not clearly understood - since it involves a range of bacterial, genetic and environmental factors - a group of bacteria have emerged as the causative agents in this process due to their virulent characteristics and strong association with diseased sites [3 ,4]. These include the so called “red complex” bacteria *Tannerella forsythia*, *Porphyromonas gingivalis*, and *Treponema denticola* [4].

Tannerella forsythia, the subject of this study, is a less characterized member of the consortium due to its fastidious growth requirements and the initial lack of tools for genetic manipulation. This bacterium covers its cells with a characteristic surface (S-) layer, which is composed of the two proteins TfsA and TfsB, each modified with a complex, branched dekasaccharide [5 ,6]. Friedrich et *al.* observed, that the composition of this glycan varies in different strains of *T. forsythia* in so far that the terminal sugar, a nonulosonic acid, can be present in the form of one of two stereoisomers, namely pseudaminic acid (Pse) or legionaminic acid (Leg) [7].

While the structure of the *T. forsythia O-glycan* [5] as well as the biosynthetic pathways of both Pse and Leg [7] had already been characterized in our laboratory, the function of this elaborate surface structure for the bacterium’s lifestyle is remaining unclear.

In order to address this question, the goal of this thesis was to functionally characterize two strains of *T. forsythia* differing in the terminal nonulosonic acid on an otherwise identical *O*-glycan, the type strain ATCC 43037 and a clinical isolate UB4 [8], as well as selected cell surface mutants thereof. It should be determined if and to which extent the bacterium requires its surface glycosylation to persist in its natural environment as a member of the multispecies biofilm consortium that constitutes the oral plaque and in its role as periodontopathogen in the interaction with host tissues and immune system, with a special focus on the role of the terminal nonulosonic acids in these processes.

In the following periodontitis, its etiology and the bacterial contribution to disease development and progression with a focus on *T. forsythia* and its glycosylated S-layer and other virulence factors as well as the host response mechanisms in periodontal diseases

will be introduced. Further, the aims of this thesis will be addressed, firstly, the analysis of the two *T. forsythia* strains and their cell surface mutants in an *in vitro* multispecies biofilm model [9], the results of which were published in the journal Molecular Oral Microbiology [10]. Secondly, the interaction of *T. forsythia* wild-type strains and mutants with primary human monocytes and oral keratinocytes as presented in a second publication in Molecular Oral Microbiology [11] will be discussed.

1.1. Periodontitis

1.1.1. Epidemiology

Periodontal disease refers to a chronic inflammation of the gingiva, bone and ligament supporting the teeth and generally starts out as gingivitis, a mild inflammation initiated by the bacteria present in the oral plaque [3]. If left untreated, the disease can progress to chronic periodontitis resulting in the destruction of the tooth-supporting tissues through an excessive inflammatory reaction along with the formation of deep periodontal pockets, a hallmark of the disease, and this can finally lead to tooth loss [12]. Pocket depths as well as clinical attachment loss serve as measures for the diagnosis of periodontitis, which can be classified into generalized or localized chronic periodontitis, depending on the number of teeth affected [12].

Even though it has been established that the main factor influencing disease development and progression is the composition of the resident microbial community [13], a number of risk factors have been found to be associated with periodontal disease. Among them some can be willingly influenced, such as cigarette smoking [14,15] or poor dental hygiene [16], however, epidemiological studies have shown that also systemic diseases like *Diabetes mellitus* [17,18], congenital or acquired host immunodeficiency, for instance in patients with HIV infection [19], or genetic factors [20] influence susceptibility.

1.1.2. Pathophysiology

The pathogenesis of periodontitis is still not fully understood since it depends on a number of genetic, bacterial and environmental factors. Through the use of 16S rRNA- or DNA-hybridization-based molecular as well as immunological tools over 700 different bacterial species have been identified in the human oral cavity, 400 thereof are found in the

periodontal pockets [21]. The oral microbiota coexists with its host in a homeostatic state, but certain factors can cause the community to become dysbiotic [22].

Early observations [1 ,23] as well as, more recently, metagenomic, metatranscriptomic and mechanistic studies showed that disease progression coincides with a drastic remodeling of the microbiome composition which prompted the categorization of the oral bacteria into specific consortia - or "complexes" – according to their association with disease [24-27]. While it is becoming clear that no single pathogenic species but rather the imbalance of the bacterial biofilm consortium as a whole is what is causing periodontal disease, some species have emerged as major periodontal pathogens [28]. Predominant among them are the three so called "red complex" bacteria *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*, which are often isolated together and strongly associated with diseased sites [23]. The "red complex" bacteria are considered keystone pathogens, given that already at a low abundance they can drastically influence the composition of the resident microbiota, leading to the shift from commensalism to dysbiosis [13]. Especially *P. gingivalis* has been extensively studied in its role as keystone pathogen, since it can be cultivated and genetically modified at relative ease and much of our current understanding of the processes promoting inflammation has been deduced from research done on this periodontopathogen.

Already in a healthy state the dental plaque poses a continuous challenge to the adjoining gingival tissue. To cope with this constant stimulation a number of innate defense mediators, such as toll-like receptors (TLRs) [29], β-defensins [30 ,31], lipopolysaccharide binding protein (LPB) [32] and soluble as well as membrane-bound CD14 [33 ,34] are expressed in the gingival epithelium for the recognition and clearance of the colonizing bacteria. Along with these protective innate defense components, host cytokines, chemokines and cell adhesion molecules are required to maintain tissue host-microbe homeostasis [35]. Secretion of pro-inflammatory cytokines like interleukin (IL-) 1β and tumor necrosis factor (TNF) α [36] as well as production of chemokines such as IL-8, which recruits specialized immune cells like neutrophils into the gingival crevice [37], leads to a controlled inflammatory response elicited already by the commensal bacteria in the plaque, as evidenced by studies on germ-free mice [38].

The "red complex" periodontopathogens and other disease-associated bacteria can modulate host responses tipping the balance from a controlled to a destructive inflammation [3] (Fig. 1). On the one hand, *e.g. P. gingivalis* can induce the expression of pro-inflammatory cytokines, such as IL-1, IL-6, IL-8 and TNF-α [39], stimulating the immune response. On the other hand the bacterium can modulate the host immune response through the expression of a set of specialized cell surface cysteine proteinases, the gingipains, through T-cell

receptor cleavage [40], proteolytic processing of components of the complement system [41], activation of protease-activated receptors, and inactivation of pro- and anti-inflammatory cytokines [42-45]. Thus the bacterium manipulates the immune reaction and impairs the host response in a way that is beneficial for the growth of the entire microbial community [46]. Species like *P. gingivalis* enable so called pathobionts, *i.e.* normally harmless commensals [47], to cause a deregulation of the inflammatory response mechanisms leading to tissue destruction and bacterial overgrowth [13].

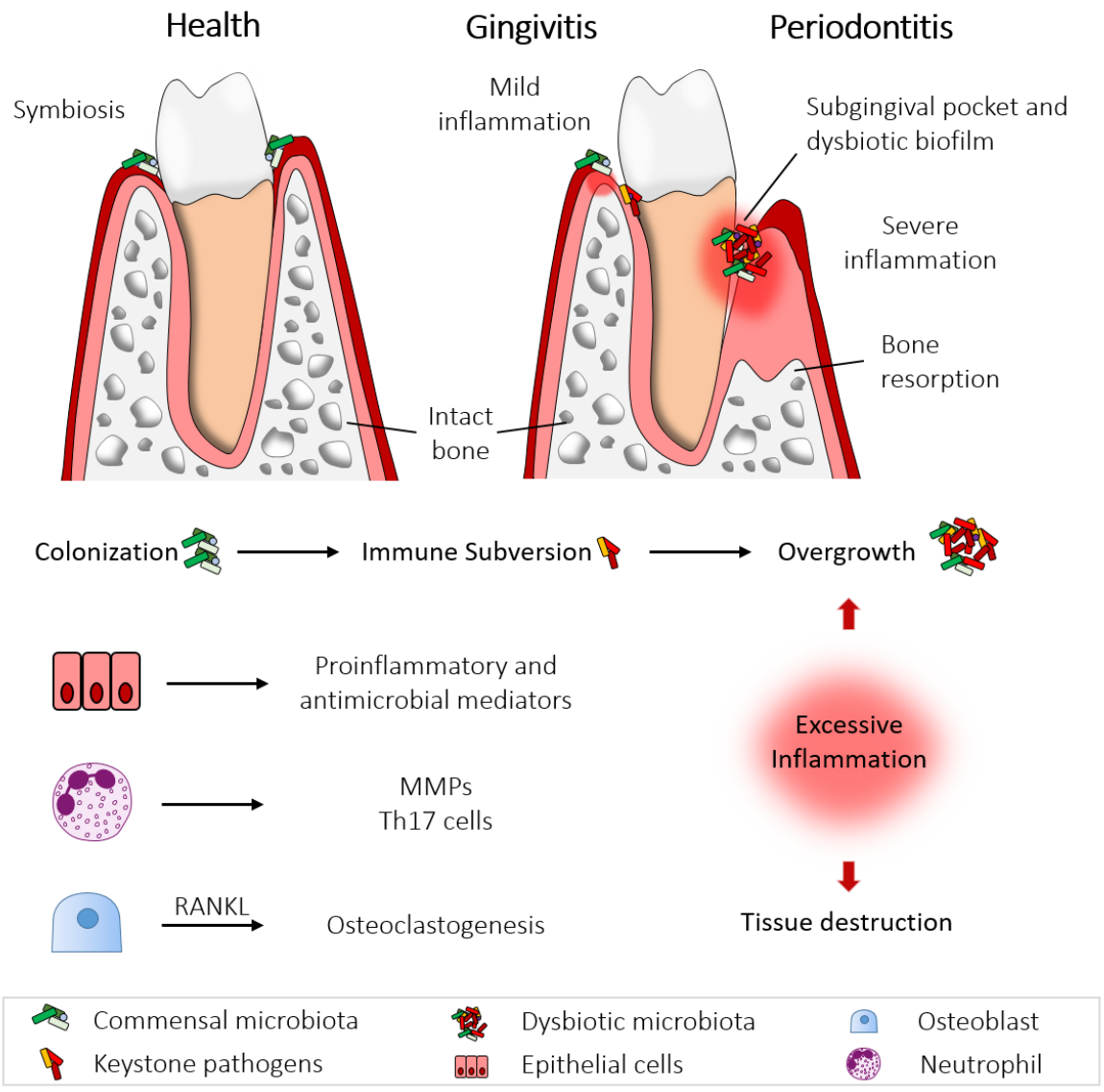

Figure 1: Dysbiosis and immune subversion in periodontitis. Dysbiosis leads to an excessive inflammation, tissue destruction and bacterial overgrowth. The presence of keystone pathogens facilitates this process. Cells of the gingival epithelium produce cytokines, chemokines and antimicrobial mediators. Specialized immune cells such as neutrophils are recruited to the gingival crevice where they exacerbate inflammation and bone resorption by osteoclasts through the action of RANKL and Th17 cells. Figure adapted from [3 ,48].

Destruction of the periodontal tissues is set in motion with an influx of neutrophils, phagocytic cells of the innate immune system that accumulate in the gingival connective tissue and epithelium as well as the periodontal pocket [35]. On site, they can act in multiple ways, directly or indirectly contributing to tissue destruction, through the release of degradative enzymes like matrix metalloproteases (MMPs), cytotoxic substances [49 ,50] and the expression of membrane-bound receptor–activator of nuclear factor-κB ligand (RANKL) [51] as well as through recruitment of IL-17-producing $CD4^+$ T helper (Th) 17 cells [52].

The production of RANKL occurs in a number of cells and can be induced by pro-inflammatory cytokines such as IL-1β and TNF [35]. RANKL binds to its receptor RANK on osteoclast precursors upon which they differentiate into mature osteoclasts, multinucleated macrophage-like cells secreting bone degrading factors [35]. Also IL-17 exerts a pro-osteoclastogenic effect by stimulating RANKL expression by osteoblasts and other stromal cells [53] and additionally facilitates the destruction of connective tissue through the induction of MMPs in fibroblasts and epithelial cells [54]. Together with TNF, IL17 causes a continuing neutrophil recruitment to the site of inflammation exacerbating the inflammatory reaction [55]. For the microbial community tissue breakdown equals the provision of essential nutrients in the form of collagen peptides or heme-containing compounds [56]. This means that excessive inflammation fosters an enrichment of mostly anaerobic pathobionts fueling a “vicious cycle” of destructive inflammation and bacterial colonization [56]

1.1.3. Implications of periodontitis for systemic health

Periodontal pathogens and their products as well as inflammatory mediators are not restricted to the localized inflammation of the gingival tissues; through the ulcerated gingival epithelium they can enter the bloodstream and cause or contribute to systemic conditions [57]. Periodontitis patients have been found to be at higher risk for rheumatoid arthritis (RA) [58 ,59], atherosclerosis, cardiovascular diseases [60 ,61], cancer [62], and adverse pregnancy outcomes [63]. Patients often show an elevated systemic inflammation, which can be reduced through treatment of periodontitis [64].

Periodontal infections can influence systemic health through the following mechanisms. On the one hand, periodontal pathogens can directly infect distant sites, as evidenced for *P. gingivalis* and *T. forsythia* which have been detected in human atherosclerotic tissue [65] as well as in amniotic fluid of pregnant women at risk for premature labor [66] and in the placentas of females with preeclampsia [67]. On the other hand, since numerous oral bacteria are constantly being swallowed with saliva, periodontopathogens might also be able to

cause inflammation indirectly by altering the composition of the gut microbiome which, in turn, adversely affects systemic health. This was evidenced in mice where oral administration of *P. gingivalis* led to a dysbiosis of the gut microbiota and induced systemic inflammation [68]. Last, periodontal infections can indirectly affect a number of systemic inflammatory conditions through the spread of cytokines produced in the periodontium that enter the bloodstream and contribute to systemic inflammation [57].

1.2. *Tannerella forsythia*

1.2.1. Phylogeny and general properties

Tannerella forsythia was first isolated from human periodontal lesions by Anne Tanner and coworkers from the Forsyth Institute in Boston, Massachusetts [69]. Originally termed *Bacteroides forsythus*, the bacterium was reclassified to *T. forsythia*, when 16S rRNA phylogenetic analyses showed that the bacterium belonged to the family of *Porphyromonadaceae* because of it closer relation to *Porphyromonas* than *Bacteroides* within the phylum *Bacteroidetes* [70]. A number of species have been identified in clinical samples clustering within the genus *Tannerella* [71], among them two clones, BU045 and BU063, which, interestingly, were found to be associated with periodontal health [72-74]. To date *T. forsythia* and BU063 are the only cultivable species of the genus *Tannerella* [75], however, within the species of *T. forsythia*, a number of clinical isolates and novel strains exist for which more and more information is becoming available through genome sequencing and mechanistic studies [7 ,8 ,10 ,76].

Tannerella forsythia is a non-motile, Gram-negative, anaerobic bacterium. Its cell morphology varies depending on the growth conditions [77]. Originally described as fusiform rod [69], *T. forsythia* cells can adapt a filamentous morphology as well. For the cultural identification of *T. forsythia* isolates key tests include positive activity for *α*–glucosidase, *β*–glucosidase, sialidase and trypsin-like enzyme, as well as negative indole production and colony morphology and Gram-stain morphology from blood agar medium without *N*-acetylmuramic acid (MurNAc) [78]. *Tannerella forsythia* stands out for its fastidious growth characteristics with an average generation time of up to 20 hours. The bacterium needs external provision of MurNAc for maintenance of cell shape and cell proliferation [77 ,79] and cultivation requires the use of rich medium such as Brain Heart infusion broth with the addition of serum as well as menadione and hemin [80]. *In vivo T. forsythia* relies on the provision of exogenous MurNAc for its growth [79], and therefore might scavenge

peptidoglycan products released during cell wall breakdown of other bacteria in the subgingival multispecies biofilms [81].

Since genetic tools for *T. forsythia* have long been unavailable [82] and due to its demanding growth requirements, the bacterium is generally a less characterized member of the periodontal pathogens. Most of our current understanding of the role of *T. forsythia* in the pathogenesis of periodontitis stems from studies of the reference strain ATCC 43037, which is commercially available from the American Type Culture Collection. However, it should be mentioned that only two years ago, a mismatch between the genome sequence published for this strain and the available *T. forsythia* reference strain was discovered [76]. The published sequence belonging to strain *T. forsythia* 92A2 had been misattributed to *T. forsythia* ATCC 43037 and many studies conducted before this discovery are inadvertently based on the incorrect genome sequence.

1.2.2. Cell envelope

Tannerella forsythia possesses a cell envelope characteristic of Gram-negative bacteria comprising an outer membrane, a periplasmic space, a thin peptidoglycan layer, and an inner membrane (Fig. 2), as observable in transmission electron microscopy of thin-sectioned bacterial cells [5]. Clearly discernible in electron micrographs is the outermost layer, the heavily glycosylated S-layer, which was first described by Kerosuo et *al.* [83] and later investigated in detail in our laboratory [5 ,84], confirming the presence of a two-dimensional crystalline array with square lattice symmetry that completely covers the bacterial cell surface during all stages of the growth cycle. Characteristic of most S-layers, these proteins are of high molecular mass [85]. The two-dimensional crystalline S-layer is anchored to a rough (R)-type lipopolysaccharide (LPS) consisting of the Lipid A portion and a polysaccharide core region built up of one 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo), three mannose and two glucosamine residues [86].

The peptidoglycan of *T. forsythia*, which is required to maintain the bacterium's cell shape [79], is composed of alternating units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues for which the type of cross-linkage and fine structure are not yet fully elucidated. Lacking the biosynthetic enzymes for *de novo* synthesis of these peptidoglycan amino sugars, the bacterium was found to recycle MurNAc via a phosphotransferase (PTS) – independent pathway [87]. Further, orthologues for enzymes of a novel pathway, bypassing *de novo* synthesis of MurNAc and confering fosfomycin

resistance identified in *Pseudomonas putida* [88], are present in the *T. forsythia* genome and might facilitate cell wall recycling by the bacterium [89].

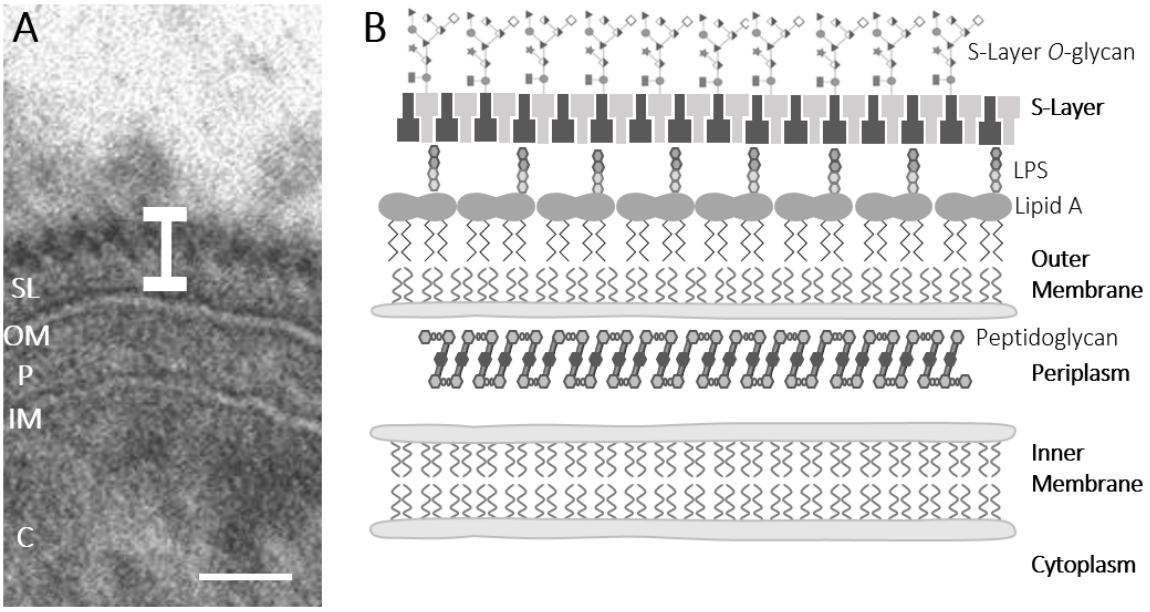

Figure 2: The *T. forsythia* cell envelope. The bacterium's cell envelope as observed through transmission electron microscopy in ultra-thin sections of *T. forsythia* cells (**A**) possesses the typical features of a Gram-negative cell envelope with a unique S-layer as outermost structure (**B**). SL - S-layer, OM - outer membrane, P - periplasm, IM - inner membrane, C - cytoplasm. Figures adapted from [5] (A) and [90] (B).

1.2.3. *O*-Glycosylation of the *T. forsythia* S-layer

Tannerella forsythia was found to possess a rich outer membrane proteome, comprising most prominently the two S-layer proteins TfsA and TfsB [91]. In general the S-layer glycoproteins constitute one of the biggest groups among the prokaryotic glycoproteins and are the building blocks of S-layers formed on the cell surface of *Bacteria* and *Archaea* by self-assembly of monomers into highly structured, two-dimensional protein lattices completely covering the bacterial cell [92]. Given their prominent display at the surface, S-layers stabilize the cellular integrity, serve as protective coating, and facilitate cell recognition, inter- and intraspecies interactions and pathogenic traits in many bacteria [93,94].

The *T. forsythia* S-layer is striking since it is not formed by one, but two intercalated S-layer proteins TfsA and TfsB [5]. Additionally, both S-layer proteins are modified with the same complex glycan that is *O*-glycosidically linked to the protein backbone [6]. The structure of this glycan was elucidated by Posch et *al.* and identified to be a complex oligosaccharide consisting of ten sugar residues that are linked to serine or threonine within a D-(S/T)-(A/I/L/V/M/T) amino acid target sequence [6]. Protein glycosylation occurs in the periplasm via a general *O*-glycosylation system that is conserved among the species of the *Bacteroidetes* phylum of bacteria and does not only include the modification of TfsA and TfsB with the S-layer dekasaccharide, but also of several other proteins of *T. forsythia* such as the surface associated virulence factor BspA [6 ,95-97]. A 40-70 amino acid C-terminal domain (CTD) serves as signal for the translocation of these proteins across the outer membrane via a type IX secretion system (T9SS) whereby this process was found to be uncoupled from post-translational modification [80].

Having calculated molecular masses of approximately 135 and 152 kDa and being heavily glycosylated, TfsA and TfsB are both clearly distinguishable as high molecular-mass glycoprotein bands in SDS-PAGE analysis at 230 and 270 kDa, respectively [6 ,98-100]. The two proteins share 24% sequence homology and are encoded by an operon [100] from which the two genes *tfsA* (Tanf_03370) and *tfsB* (Tanf_03375) are co-transcribed in equimolar ratio in bacteria grown in planktonic culture [5].

Mass spectrometric analysis and NMR spectroscopy revealed the structure of the *T. forsythia* ATCC 43037 *O*-glycan with a total mass of 1621 Da [6] (Fig. 3). The branched dekasaccharide is anchored to the protein backbone via a galactose and further contains glucuronic acid, the core branching sugar residues digitoxose and xylose, two α-L-fucose residues, and a terminal branch containing two *N*-acetylmannosaminuronic (ManNAcA) acid residues as well as a terminal modified pseudaminic acid (Pse5Am7Gra) [6 ,101].

A putative glycosylation gene locus was first described by Honma et *al.* upon discovery of a UDP-*N*-acetylmannosaminuronic acid dehydrogenase WecC (Tanf_1280) [102] located within a large operon that was further analyzed in our laboratory. The genes encoded within this operon encompass a putative flippase, a predicted UDP-*N*-acetylglucosamine 2-epimerase and several glycosyltransferases as well as methyltransferases [6] (V. Friedrich, M.B. Tomek, unpublished data). Similar glycosylation gene clusters have been identified in other species of the *Bacteroidetes* phylum, among others *Bacteroides fragilis*, *Bacteroides uniformis* and *Bacteroides thetaiotaomicron*, documenting the existence of a phylum-wide *O*-glycosylation system [96 ,103].

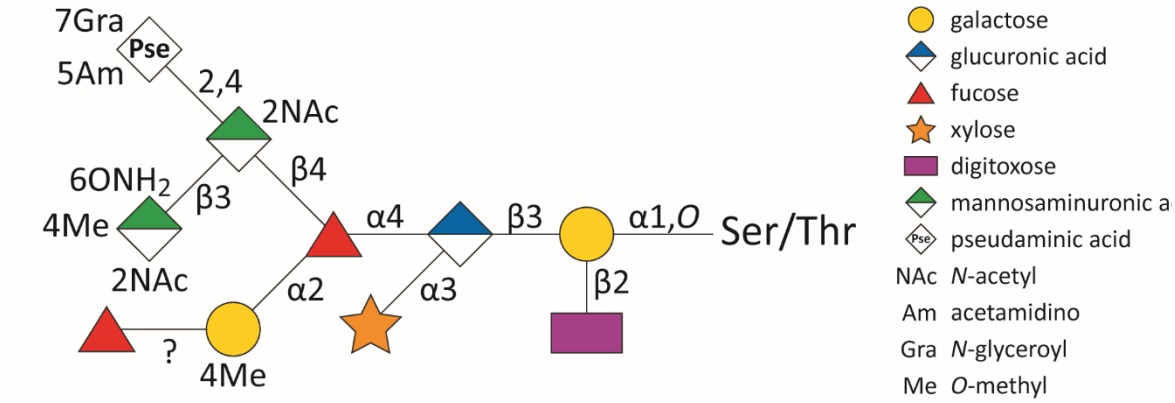

Figure 3: Structure of the *T. forsythia* *O*-glycan. Schematic representation of the *T. forsythia* surface glycan that is *O*-glycosidically linked to Ser/Thr residues of the protein backbone of the S-layer- and other glycoproteins. Figure adapted from [7].

1.2.4. Bacterial nonulosonic acids

Special attention has been given to the terminal nonulosonic acid of the *T. forsythia* *O*-glycan. Nonulosonic acids are a group of acidic nine-carbon (C9) α-keto sugars. In different *T. forsythia* strains, the nonulosonic acid was found to be present as either Pse as in the type strain ATCC 43037 or as Leg as found in strain UB4 (Fig. 4A) [7].

The biosynthetic pathways as well as the structures of Pse and Leg (Fig. 4) share many similarities with sialic acids, which are found in abundance in surface-associated glycans of mammalian cells where they act as important mediators of many physiological and pathological processes [104 ,105]. By influencing the surface hydrophobicity, sialic acids facilitate charge repulsion, for instance preventing clotting of cells in the blood circulation [105], as part of ligands that are specifically recognized by selectins they facilitate cell adhesion and influence interactions between immune and other cells, and as ligands for sialic acid-binding Ig superfamily lectins (Siglecs) they can regulate immune responses [105 ,106]. Additionally sialic acids on the cell surface of host cells can serve as carbon source for bacteria, such as *T. forsythia* which possesses the enzymatic machinery required for sialic acid catabolism [107].

The nonulosonic acid derivatives Pse (5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid) and Leg (5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid), seem to occur exclusively in bacteria and have been found as components of surface associated polysaccharides of a number of pathogenic bacteria [108]. To bacterial nonulosonic acids a prominent role has been attributed in facilitating immune evasion through sialic acid mimicry [109 ,110]. Group B *Streptococcus* capsular polysaccharide, for instance, contains terminal sialic acid-like sugar residues that can be recognized by Siglecs present on monocytes and leukocytes and thereby modulate their functions suppressing the innate immune response [111]. *Campylobacter jejuni* decorates its flagella with multiple *O*-linked Leg and Pse residues [112] the latter of which are recognized by Siglec-10 on bone-marrow derived dendritic cells (BMDCs) of mice promoting the release of the anti-inflammatory cytokine IL-10 indirectly contributing to immune suppression [113]. In the opportunistic pathogen *Pseudomonas aeruginosa* flagellar glycosylation is a prerequisite for the bacterium's virulence by stimulating IL-8 release which is drastically reduced in glycosylation-deficient strains [114]. Flagellar glycosylation with Pse in *C. jejuni*, *Helicobacter pylori* and *Aeromonas spp.* is required for bacterial motility, adherence, and invasion and therefore plays an important role in the physiology and lifestyle of these bacteria [115-117].

In *Tannerella forsythia* bioinformatic analysis and PCR screening of a number of clinical isolates indicated that the respective strains possess the genetic information and, therefore, most likely also the biosynthetic pathways to synthesize one of the two stereoisomers, Pse or Leg, and highlights the prevalence of these sugar residues within the species (Tomek M.B. et *al.*, manuscript in revision).

Friedrich et *al.* functionally characterized the enzymes required for Pse and Leg biosynthesis *in vitro* as well as the glycan structure in the ATCC 43037 reference strain – possessing Pse – and the clinical isolate UB4 – producing Leg – and found that the *O*-glycan of these strains seems to differ only in this terminal sugar residue [7].

Nonulosonic acid biosynthesis was first described in detail by Schoenhofen et *al.* in *H. pylori* for Pse [118] and in *C. jejuni* for Leg [119]. In both pathways nucleotide-activated nonulosonic acid is synthesized in six enzymatic steps from a nucleotide-activated GlcNAc precursor, UDP-GlcNAc for Pse and GDP-GlcNAc for Leg [7]. The enzymes for CMP-Pse production in *T. forsythia* ATCC 43037 (PseB, PseC, PseH, PseG, PseI, PseF) and for CMP-Leg biosynthesis in *T. forsythia* UB4 (LegB, LegC, LegH, LegG, LegI, LegF) are encoded in a dedicated gene locus upstream of the predicted *O*-glycosylation cluster (Fig. 4B) [7]. Apart from *in vitro* characterization of the two biosynthesis pathways, Friedrich et *al.* showed that deletion of the genes encoding the aminotransferase PseC in *T. forsythia* ATCC 43037 and

LegC in *T. forsythia* UB4, respectively, resulted in nonulosonic acid-deficient strains, proving their function *in vivo* [7].

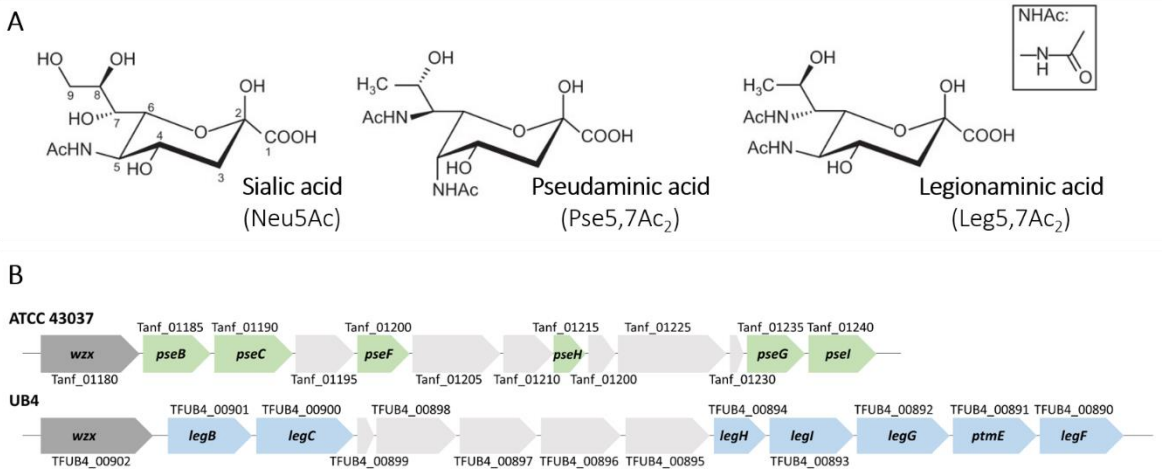

Figure 4. Structure and genomic biosynthesis loci of nonulosonic acids in *T. forsythia*. The structure of sialic acid, Pse and Leg (**A**, figure adapted from [7]) and the genetic loci encoding Pse and Leg biosynthesis genes from *T. forsythia* ATCC 43037 and UB4, respectively, (**B**, figure from [10]) are shown. Pse biosynthesis genes (*pseB*, *pseC*, *pseH*, *pseG*, *pseI* and *pseF*) of the type strain ATCC 43037 are depicted in green. The genes for Leg biosynthesis (*legB*, *legC*, *legH*, *legI*, *legG*, *legF*) and a gene encoding a predicted nucleotidyl transferase (*ptmE*) as present in the strain UB4 are shown in blue.

Tomek et *al.* further showed that in both *T. forsythia* ATCC 43037 and *T. forsythia* UB4 transfer of the respective nonulosonic acid to the terminal *N*-acetylmannosaminuronic acid is catalyzed by a nonulosonic acid derivative transferase highly specific for Pse and Leg, respectively [120]. This occurs after CMP-activation as well as modification of the sugar with an *N*-acetimidoyl group at C-5 and with an *N*-glyceroyl group at C-7 in Pse and a putative *N*-acetyl group at C-5 and a putative *N*-glycolyl group at C-7 in Leg [7 ,120].

While these studies substantially expanded our knowledge of Pse and Leg biosynthesis in *T. forsythia*, the biological function of the terminal nonulosonic acids in the two strains *T. forsythia* ATCC 43037 and UB4 is still little understood. In the project presented here, their role in orchestrating the bacteria's lifestyle was analyzed with regard to biofilm formation and interaction with the host using a set of cell surface mutants, including the nonulosonic acid deficient strains *T. forsythia* ATCC 43037 Δ*pseC* and *T. forsythia* UB4 Δ*legC* described above and the results from these studies will be introduced in section 2.

1.3. Virulence mechanisms of *Tannerella forsythia*

1.3.1. Cell-surface-associated virulence factors

T. forsythia has long been recognized as periodontal pathogen, because it is found in increased levels in periodontal lesions [1,121]. Its antigens were found to elicit a host response in periodontitis patients [122] and infection with *T. forsythia* causes abscess formation in animal models [123-126]. The bacterium was found to produce several virulence factors that mediate its pathogenicity and enable it to interact with the host tissues and immune system in order to persist in the host.

The bacterial cell surface functions as interaction platform with other bacteria of the oral biofilms as well as with host tissues and immune system. In this regard, the *T. forsythia* S-layer plays a pivotal role in orchestrating the bacterium's virulence potential. Purified S-layer proteins were found to exhibit a humoral immune response in patients with aggressive periodontitis [127], induce haemagglutination and act as immunogens in mice [128]. An S-layer deficient mutant, in which both S-layer genes *tfsA* and *tfsB* were deleted, lost its ability to adhere to and invade oral epithelial cells when compared to the *T. forsythia* ATCC 43037 wild-type [129] and was less resistant to high serum concentrations and recognition by factors of the complement system [130].

Using that S-layer deficient mutant, Sekot et *al.* showed that the S-layer functions in suppressing the immune response of U937 macrophages and human gingival fibroblasts since cytokine and chemokine production was significantly elevated in cells infected with the S-layer deficient mutant when compared to cells infected with *T. forsythia* ATCC 43037 wild-type [131]. Further the S-layer is specifically recognized by a macrophage-inducible C-type lectin (Mincle) and modulates cytokine responses of macrophages to the bacterium [132]. A possible contribution to this process by specifically the S-layer *O*-glycosylation was first presented in a study using a UDP-*N*-acetyl-D-mannosaminuronic dehydrogenase deletion mutant (*T. forsythia* ATCC 43037 Δ*wecC*) [133], which causes a truncation of the glycan by three sugars, including the terminal Pse residue [6]. The terminal branch of the *O*-glycan was found to regulate dendritic cell effector function, suppress Th17 responses and neutrophil infiltration into the gingival tissues, thereby facilitating the persistence of the bacterium in the host [133,134]. The availability of deletion mutants of *T. forsythia* ATCC 43037 and *T. forsythia* UB4 lacking only the terminal nonulosonic acid further allowed a dissection of the function of Pse and Leg in the interaction with the host as presented in section 2.

The S-layer proteins and attached glycans are also present on outer membrane vesicles (OMVs) produced by the bacterium [135]. OMVs can be regarded of virulence factors of

Gram-negative bacteria, and carry outer membrane proteins, components of the periplasm, LPS and certain toxins; thus they can execute immunomodulatory actions and facilitate interbacterial and bacteria-host interaction [136]. *Tannerella forsythia* OMVs induce IL-6, IL-8 and monocyte chemoattractant protein 1 (MCP-1) in primary human periodontal ligament fibroblasts and TNF-α and IL-8 in U937 macrophages [135] and the presence of *P. gingivalis* OMVs were found to enhance attachment to and invasion of epithelial cells by *T. forsythia* [137].

The surface-associated glycoprotein *Bacteroides* surface protein A (BspA) is also required for cell adhesion and invasion [137]. Through a leucine rich repeat domain, BspA interacts with components present on host and other bacterial cells [81], binds to fibronectin as well as fibrinogen [97] and mediates interaction with other bacteria such as *Fusobacterium nucleatum* [138]. Secreted BspA stimulates the release of pro-inflammatory cytokines from monocytes [139] and chemokines from gingival epithelial cells (GECs) [140] in a TLR 2 dependent manner. *In vivo* studies performed in mice models showed that the capability of *T. forsythia* to induce alveolar bone loss was significantly reduced in a mutant lacking BspA when compared to the wild-type [125]. In a recent study using recombinantely expressed BspA as well as viable *T. forsythia* ATCC 43037 wild-type cells for infection of apolipoprotein E knock out (ApoE $^{-/-}$) mice, a clear correlation between *T. forsythia* and specifically its virulence factor BspA with the development of atherosclerotic lesions was observed, highlighting a significant role of this protein in the bacterium's pathogenicity [141].

Further, surface lipoproteins [142] as well as the R-type LPS of *T. forsythia* [86] have been found to have a strong immunogenic potential. *T. forsythia* LPS alone as well as in combination with LPS from *P. gingivalis* or *T. denticola* induced the secretion of pro-inflammatory cytokines in an *ex vivo* whole blood model [143] as well as in a macrophage/ epithelial cell co-culture model [144] and a U937 monocytic cell line [86]. A surface lipoprotein fraction of *T. forsythia* containing ester-bound fatty acids stimulated cytokine release and induced apoptosis in monocytes and human gingival fibroblasts through the activation of caspase-8 [142].

1.3.2. Proteolytic enzymes

For *T. forsythia* proteolytic enzymes play an important role in the acquisition of peptides, free amino acids and heme through the degradation of host proteins and are essential mediators of bacterial colonization, tissue breakdown as well as complement inactivation and immune evasion [81 ,145 ,146]. A cysteine protease PrtH, later termed Forsythia detaching

factor (FDF), was among the first proteolytic enzymes characterized in *T. forsythia* [81,147]. FDF is able to induce the detachment of adherent cells most likely by targeting cell-matrix interactions [148] and stimulates IL-8 release in human fibroblasts [149]. Levels of FDF-specific antibodies are significantly increased in the gingival crevicular fluid (GCF) from diseased sites in periodontitis patients [150] pinpointing a prominent role of this factor in pathogenesis.

Additionally a group of proteases characterized by the presence of a CTD terminating with a Lys-Leu-Ile-Lys-Lys motif, hence termed KLIKK proteases, are detectable in the GCF of periodontitis patients [151]. The group of enzymes comprises the metalloproteinases karilysin [152], forsilysin and mirolysin [151] as well as the serine proteases miropsin-1, miropsin-2 [151] and mirolase [153]. Genomic analysis revealed that the genes encoding these proteins are absent in the health-associated *Tannerella* BU063 [73,151], which denotes them as important contributors to *T. forsythia's* virulence potential. The first KLIKK protease to be characterized in detail is the matrix metalloprotease karilysin [152]. Karilysin is able to degrade fibrinogen, fibronectin and elastin and confers resistance to the antibacterial peptide LL-37 [152]. The protease interferes with the classical, alternative and lectin pathways of the complement system by cleavage of the recognition molecules mannose-binding lectin, ficolin-2 and ficolin-3 as well as inactivation of C4 and C5, thereby contributing to serum resistance [154].

Through cleavage of the membrane-bound form of TNF-α on the surface of macrophages, karilysin can modulate the immune response [155]. A second protease, mirolysin, was found to act in synergy with karilysin through the same mode of action of complement inactivation [156].

1.3.3. Glycosidases and other potential virulence mechanisms

Lastly, a high glycosidic activity has been attributed to *T. forsythia*. A number of glycosidases capable of breaking down host oligosaccharides and proteoglycans in order to provide nutrients and facilitate bacterial adhesion to otherwise concealed epitopes have been identified [81]. Among them are the sialidases NanH and SiaH [157,158], α-D-glucosidase and *N*-acetyl-β-D-glucosaminidase [81,159] as well as an α-L-fucosidase TfFuc1 [160]. Especially the scavenging of sialic acid as well as fucose present in mucin and other surface components of host cells has been implicated as an important factor for the survival of *T. forsythia* within the environment of the periodontal pockets [107]. Also TfFuc1, which has recently been characterized in our laboratory, serves foremost in the metabolism of small

oligosaccharides and provision of nutrients and influences bacterial survival and virulence potential [160].

Apart from the above mentioned factors, production of potentially cytotoxic methylglyoxal by *T. forsythia* [161] and the bacterial chaperone GroEL inducing pro-inflammatory cytokine production and bone resorption have been detected as additional putative virulence factors [162].

1.4. Multispecies oral biofilms

1.4.1. Biofilm development

Instead of adopting a purely planktonic lifestyle, most bacteria predominately exist as sessile microbial communities forming biofilms on a diverse range of surfaces [163 ,164]. Biofilm lifestyle requires the interaction with different microbial species and surface structures and ensures bacterial survival by providing protection from environmental challenges such as shear forces [165], dehydration and salinity [166], phagocytosis [167] and other host immune responses [165] as well as antimicrobial agents and antibiotics [168 ,169]. Biofilm communities are highly complex in their composition as well as function and develop and persist in a highly dynamic fashion [163].

In the oral cavity, microbial communities are characterized by their high degree of species diversity and biofilm composition differs in dependence on the site of attachment [21]. The oral cavity offers a variety of ecological niches in which different species can preferentially settle, ranging from the hard surfaces of the teeth to the mucosal surfaces of gingiva, cheeks and tongue, presenting different types of epithelial cells, surface proteins and mucins and being surrounded by saliva or GCF which provide nutrients, remove debris and shuttle bacterial cells to distant sites for *de novo* attachment [4 ,170].

Dental plaque is one of the best studied biofilms and its development occurs in several stages that generally apply to biofilm formation (Fig. 5) [165 ,171]. On the tooth surface, host as well as bacterial molecules such as glycoproteins, phosphoproteins or bacterial glucosyltransferases and glucan are adsorbed and form what is known as enamel pellicle. These surface structures provide a site of attachment for microbes present, for instance, in saliva via weak physicochemical attractive forces [171].

Adhesins present on the cell surface of these so called early colonizers subsequently bind to the pellicle and the so attached bacteria start to proliferate and form microcolonies and

produce an exopolysaccharide matrix [1 ,172]. Bacterial surface components facilitating adhesion include pili, fimbriae and flagella [173 ,174].

The initial colonizers in turn enable late colonizers to coadhere or coaggreate, which they could not do on the pellicle structures alone, and a mature biofilm is formed [175]. The adherent bacteria influence the environment of the biofilm through the consumption of oxygen and production of metabolites and fermentation products that can serve as nutrients and thereby make it more favorable for the subsequent species [171, 176].

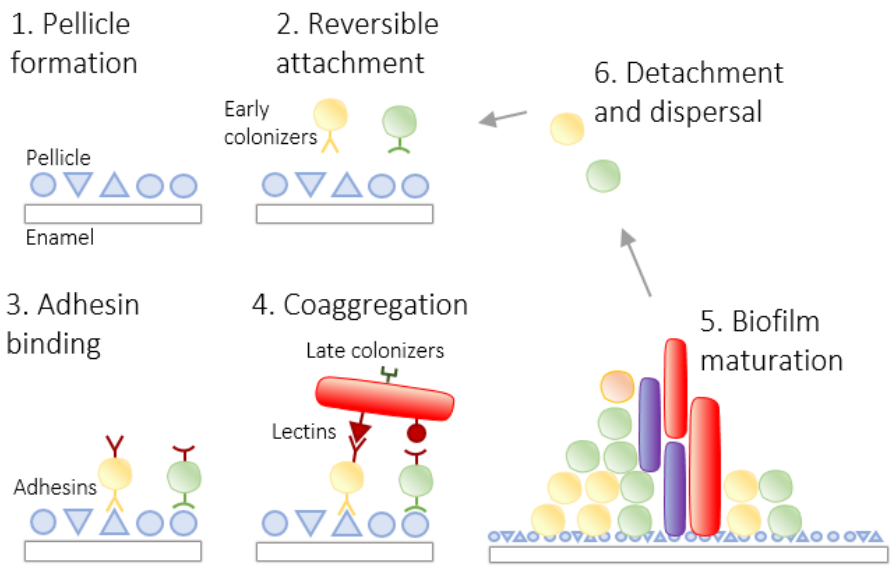

Figure 5. Formation of dental plaque. The different stages of oral biofilm development are shown schematically. After pellicle formation on the tooth surface, early colonizers are initially retained by weak electrostatic interactions. Through the expression of adhesins, bacteria attach to the receptors in the pellicle and start to proliferate, finally allowing late colonizers to coaggregate via lectin-like interactions. Growth and coadhesion leads to biofilm maturation and finally bacteria detach and are dispersed to distant sites for *de novo* attachment. Figure adapted from [171].

Modification of the bacterial habitat results in a drastic change in microbial composition whereby resident bacteria are outcompeted by their successors for which the new environment has become more suitable [4]. This process is termed microbial succession and in the case of the subgingival plaque, this allows the anaerobic pathogens *T. forsythia, P. gingivalis* and *T. denticola* to establish themselves in the microenvironment of the subgingival polymicrobial community [4]. Finally, detachment of bacteria from the biofilm,

translocation to distant sites and re-attachment lead to biofilm dispersal and contribute to the dynamic turn-over of the oral biofilms [177].

1.4.2. Structure of subgingival biofilms

Early observations of bacterial biofilms on natural teeth identified a distinct structural organization of the subgingival plaque with histologically defined zones attributable to a varying species composition [178 ,179]. Biofilms initially develop at the tooth surface benefitting the growth of aerobic or facultative anaerobic bacteria, with lasting periodontal infection and commencing tissue destruction, pocket depth increases and the microenvironment of the biofilm becomes more favourable for anaerobes fuelling the compositional changes towards a dysbiotic microbial community [180].

At the bottom of the periodontal pocket the biofilm adjoins the gingival connective tissue as well as the periodontal ligament and, here, large rod-shaped, filamentous bacteria can be observed [179]. Further up the biofilm is lined by the tooth on one side and the gingival epithelium on the other. While on the tooth surface mostly small bacterial cells embedded in a fibrous biofilm matrix are present, the intermediate biofilm layer is dominated by filamentous and rod-shaped cells, among them *Fusobacterium nucleatum* and *T. forsythia*, and is devoid of a surrounding exopolysaccharide matrix [179 ,181]. The matrix supports adhesion to and colonization of the pellicle and provides a scaffold for the developing biofilm [182]. In supragingival biofilms *Streptococcus mutans* is the main producer of glucans, which form the exopolysaccharide matrix crucial for the development of cariogenic biofilms [183].

At the biofilm surface large filamentous cells surrounded by Gram-negative rods and filaments forming so-called “test tube brushes” and a loose layer of bacteria facing the opposed polymorphonuclear neutrophils (PMNs) and epithelial cells can be observed [179]. In the intermediate and upper layers of subgingival plaque samples, members of the *Cytophaga-Flavobacterium-Bacteroides* cluster as well as *Synergistetes* and *Spirochetes* constitute the predominant phyla [181].

1.4.3. The subgingival microbial consortium

The spatio-temporal distribution of the biofilm bacteria was also observed in an analysis of the microbial composition in numerous plaque samples using a DNA-DNA hybridization technique, which further lead to a categorization of the identified species into five complexes

(or groups) according to their association with either periodontal health or disease (Fig. 6) [1].

Among the commensal bacteria – or members of the green, yellow and purple complexes - predominately streptococci and *Actinomyces* spp. and *Veillonella* spp. being able to adhere to mucins, proline-rich proteins or other components present in the salivary pellicle, resume the role as early colonizers initiating plaque formation [184 ,185]. They enable secondary, often more pathogenic bacteria to co-adhere through the expression of specialized adhesins and other surface components by providing attachment sites and a metabolically compatible environment that favors interactions between species that benefit from each other [186].

These secondary, often disease-associated colonizers include among others *Aggregatibacter actinomycetemcomitans, P. gingivalis, Prevotella intermedia, T. forsythia, F. nucleatum, Campylobacter rectus* and *T. denticola* [187]. They are predominantly found in the microenvironment of the deep periodontal pockets where nutritional and atmospheric conditions favor the growth of these Gram-negative anaerobic species [188 ,189].

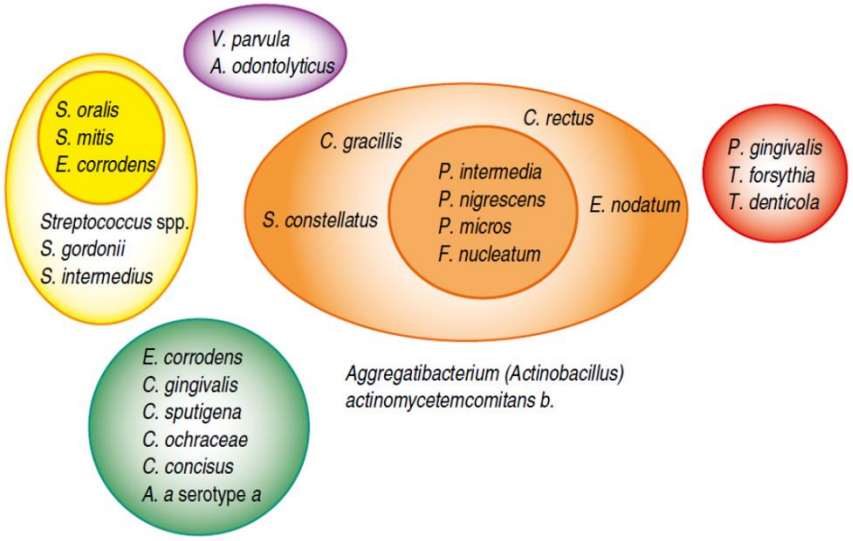

Figure 6. Complexes of subgingival plaque microbiota. Green, yellow and purple complexes are considered to be health-associated. The periodontal pathogens *Tannerella forsythia*, *Porphyromonas gingivalis* and *Treponema denticola* constitute the red complex and succeed the other later colonizers and pathobionts of the orange complex. *A. actinomycetemcomitans* serotype *b* is strongly associated with aggressive periodontitis and does not cluster within the complexes [171]. Figure from [171].

Generally, the periodontal pathogens and other potentially pathogenic Gram-negative anaerobic species can be considered as weak colonizers that can only establish themselves in the biofilms through the cooperation with and adherence to the other species in the

consortium [175 ,190]. Coaggregation and communication between the oral bacteria contributes to biofilm development and architecture and numerous interaction partners have been identified *in vitro* [191]. Among the late colonizers, especially *F. nucleatum* acts as bridging organism given that it coaggregates both with early as well as late colonizers and thereby plays a key role in the succession of genera in the multispecies consortium [192 ,193]. Interaction with its partners, especially the Gram-negative periodontal pathogens, is often facilitated through lectin-carbohydrate interactions [190]. A surface-associated galactose-specific lectin-like adhesin on the surface of *F. nucleatum*, for instance, recognizes galactose moieties in the capsule and LPS of *P. gingivalis* and mediates binding [194] and also the glycosylated S-layer of *T. forsythia* acts as adhesin facilitating attachment between the two species [130]. Also to *P. gingivalis* a bridging function within the microbial community can be attributed due to its close association with the early colonizer *Streptococcus gordonii* [195] as well as *F. nucleatum* [196], *T. forsythia* and other late colonizers [193].

1.4.4. Interspecies interactions in multispecies biofilms

Communication within multispecies communities occurs through physical interactions, metabolite exchange, gene transfer and interbacterial signaling [192]. The latter is facilitated by the universal quorum sensing signaling molecule autoinducer-2 (AI-2) that is produced by several oral bacterial species [197-199] and has been found to promote colonization by the three periodontal pathogens [200]. Interbacterial signaling between *P. gingivalis* and *S. gordonii* [201] as well as *Actinomyces naeslundii* and *Streptococcus oralis* [198] via the AI-2 system has been described as beneficial for mutualistic biofilm development. A model proposed by Kolenbrander et *al.* correlates AI-2 concentrations with species composition as commensals are slowly outcompeted by pathogens when levels of the signaling molecule increase highlighting the role of AI-2 in the development of dysbiotic biofilms [202].

Within the multispecies biofilms, cooperation and competition determine the survival of the individual species. Competitive interactions employed by commensals include the production of H_2O_2 and antimicrobial agents or acidification, and inhibit the growth of secondary colonizers [203].

On the other, hand mutualistic associations between different species often result from metabolic interactions. For instance lactic acid produced by streptococci can be utilized by *A. actino-mycetemcomitans* and veillonellae and promotes their growth [204]. Mutualistic interactions promote survival in the biofilm and affect the community's virulence. In infection studies using murine and rat models, mixed infections with *P. gingivalis*, *T. forsythia* and

T. denticola and *F. nucleatum* [126 ,205 ,206] where found to be more potent in the induction of inflammation and bone resorption when compared to mono-infections. Synergistic effects between the members of the polymicrobial biofilms and especially the periodontal pathogens ensure not only their persistence within the multispecies subgingival biofilms but also influence and potentiate their capability to induce destructive inflammation in their interaction with the host.

1.5. Host-microbe interactions in periodontal tissues

1.5.1. Gingival epithelial tissues and their innate immune defenses

In the oral cavity, potential pathogens face numerous mechanisms dedicated to their clearance in order to maintain a homeostatic balance. The enamel surface of the teeth on the one hand and the oral mucosa on the other act as physical barriers preventing bacterial infiltration into deeper tissues [207 ,208]. In addition to that, saliva and GCF function in host defense being equipped with antimicrobial peptides, mucins, statherins, lysozyme, complement proteins and other inflammatory mediators [209 ,210].

The oral mucosa is formed by the gingival epithelium constituted of tightly connected keratinocytes as well as the underlying connective tissue and periodontal ligament dominated by fibroblasts [211]. In its function as first line of defense against the plaque bacteria, GECs secrete signals in order to attract neutrophils and other specialized immune cells and facilitate their trafficking to the infected sites [35].

The structural integrity of the epithelium is crucial for the protection against invading pathogens and is conferred by strong inter-epithelial adhesion through tight junctions, gap junctions and desmosomes [212]. Infection with subgingival biofilm bacteria leads to disruption and degradation of the epithelium [213], by inducing the down-regulation of components of the adhesion complexes [214] and affecting tissue turn-over and renewal [215].

Apart from functioning as physical barrier, epithelial cells also contribute actively to the innate host defense. The epithelial cells recognize pathogen-associated molecular patterns (PAMPs) through the expression of pattern recognition receptors like TLRs [29] and nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) proteins [216]. TLR 2, 4, 5 and 9 detect bacterial components such as lipoteichoic acids, LPS, flagellin or DNA, and NOD-1 and NOD-2 are activated by components of the bacterial peptidoglycan [216]. TLRs activate different signaling cascades and finally the transcription factors NF-κB, activator protein-1 (AP-1) and interferon-regulatory factor-3 (IRF-3), and thereby stimulate

the production of antimicrobial peptides as well as pro-inflammatory cytokines and chemokines [217]. Apart from immune mediators such as IL-1β, TNF-α and IL-6, also intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function antigen-3 (LFA-3) are expressed in the form of a gradient already in healthy tissues of the junctional epithelium and together with IL-8 serve in channeling PMNs towards the site of infection [23 ,218]. In the tissue also gingival fibroblasts react to the bacterial challenge by the production of the pro-inflammatory cytokines IL-6, IL-8 and prostaglandine E_2 (PGE2) as well as extracellular matrix degrading enzymes such as MMPs [219 ,220].

1.5.2. Cellular effectors of innate immunity

In response to bacterial infection and the subsequent release of chemokines by GECs, PMNs leave the blood vessels and move to the site of infection. Bacterial clearance occurs through recognition via pattern recognition receptors (PRRs), phagocytosis and intracellular killing though the production of reactive oxygen species (ROS) [211]. Additionally, the formation of neutrophil extracellular traps (NETs) through neutrophil lysis and release of chromatin fibers associated with granules containing antimicrobial proteins such as lactoferrin, bactericidal/permeability-increasing protein (BPI), peptidoglycan recognition proteins and extracellular matrix degrading MMP-9 constitutes a mode of action to bind and kill invading microorganisms [221].

On the one hand, neutrophils in periodontal lesions are overpowered by the invading bacteria and their function is impaired, on the other hand the bacterial challenge leads to an overreaction and cellular hyperactivity and an excessive release of ROS, tissue degrading enzymes and pro-inflammatory molecules that damage the surrounding tissue [49]. Through the expression of RANKL, the chemokine monocyte chemoattractant protein 1 (MCP-1) and Th17 recruitment, neutrophils further contribute to bone resorption and tissue degradation, as described above [13].

Bacterial proteins, LPS and MCP-1 attract bloodstream monocytes into the inflamed tissue [222]. Inflammatory monocytes recognize PAMPs and subsequently release pro-inflammatory mediators, predominantly IL-1β and TNF-α, and differentiate into macrophages or dendritic cells on site [223]. Macrophages further contribute to damage repair induced by infection and inflammation by phagocytosis of apoptotic neutrophils and cellular debris [224].

Conversely, monocytes can differentiate into osteoclasts and directly contribute to bone resorption in the presence of *P. gingivalis* LPS within chronic lesions [225].

Macrophages as well as dendritic cells further act as antigen presenting cells (APC) activating mainly T cells, B cells and innate lymphoid cells (ILCs) residing in the gingiva or lymph nodes, respectively [226]. In the context of periodontal disease the most important players of adaptive immunity constitute Th1, Th2 and Th17 as well as regulatory T cells (T_{regs}), with Th1 and Th17 being linked to promotion of bone resorption and Th2 and T_{regs} to controlling it [227].

1.5.3. The complement system in periodontal infection

Apart from these cellular effectors the complement system plays an important role in the response to bacterial infection and, thus, the initiation and progression of periodontitis [228]. The complement system is represented by the serum proteins C1-C9, pattern recognition molecules, convertases and other proteases as well as regulators and receptors interacting with the immune system [229].

Activation of the complement cascade occurs via the classical, lectin or alternative pathway all of which converge in the activation of C3 convertase leading to stimulation of specialized immune cells by C3a and C5a, opsonisation through C3b and phagocytosis or formation of the membrane attack complex (MAC) and direct lysis of the microbial invaders by C5b-C9 [230]. Together with TLRs the humoral innate immune mediators of the complement system facilitate a rapid response to foreign agents such as the bacteria of the subgingival biofilms which, in turn, employ countless mechanisms to avoid detection and ensure persistence by subversion of complement mechanisms [231].

1.5.4. Immune evasion mechanisms

In order to persist in the environment of the subgingival pockets, the resident bacteria have to evade host defense mechanisms on the one hand and elicit inflammation and thereby foster nutrient acquisition on the other. For this purpose, the keystone pathogens have evolved elaborate defense systems that enable them as well as accessory pathogens to ensure a successful existence within their host [3].

One approach to avoid detection by the immune system is the invasion of gingival tissues by the bacteria. Among others the three major periodontal pathogens *T. forsythia*, *P. gingivalis* and *T. denticola* as well as *F. nucleatum* or *A. actinomycetemcomitans* and *P. intermedia* are capable of entering and persisting in epithelial cells [232-234]. Especially *P. gingivalis* has been well studied in its interaction with host tissues.

The bacterium utilizes its major fimbriae as adhesins which directly interact with β1 integrin present on the host cells, and is internalized through a subsequent rearrangement of the actin cytoskeleton [235 ,236]. Once inside the cells, *P. gingivalis*, localized at the perinuclear region [237], interferes with signaling pathways in order to inhibit apoptosis [238] and stimulate cell proliferation and, thereby its own survival [39 ,239]. The presence of *P. gingivalis* may also aid the invasion of GECs by *T. forsythia* [240], while *T. forsythia*, in turn, prevents *P. gingivalis* from entering the cells [241]; still both species where found to be present at the same intracellular location [233]. Attachment to and invasion of GECs by *T. forsythia* has been found to depend on the presence of the S-layer [129], surface associated BspA [242] as well as sialidase NanH [157].

Interestingly, Thurnheer et *al.* showed that the three red complex bacteria in a multispecies biofilm model colonized GECs in a seemingly synergistic manner *in vitro*, while in their absence, especially streptococci, *i.e. S. anginosus* and *S. oralis*, took over as predominant invasive species, suggesting an inhibitory role of the three pathogens on the cell colonization by commensals [213]. In a similar experimental model the three red complex bacteria elicited a rapid IL-8 response by GECs from within a multispecies biofilm but upon longer exposure IL-8 induction drastically decreased in the presence of these three species [243]. Also IL-1β responses by gingival fibroblasts were found to be downregulated by the presence of *P. gingivalis* in the same *in vitro* biofilm model [244].

By such a manipulation of the cytokine and chemokine secretion by GECs in response to the bacterial challenge, species like *P. gingivalis* interfere predominantly with immune cell recruitment. *P. gingivalis* inhibits IL-8 [245] and E-selectin [246] expression to impede neutrophil migration and additionally regulates IFN-γ-inducible protein 10 (IP-10) and other chemoattractants to control Th1 cell migration and keep tissue destruction in check [247].

Another vital part in the evasion of host immune surveillance is the interaction with components of the complement system as well as TLRs. *Phorphyromonas gingivalis* can hinder the activation of the complement system by utilizing its gingipains for the degradation of C3, C4 and C5, thus, blocking formation of the MAC [41 ,247], increase its serum resistance by immobilizing C4b-binding protein (C4BP) on its own cell surface [248], and bind complement receptor 3 (CR3) on macrophages consequently downregulating IL-12 secretion and thereby Th1 differentiation and facilitating its own entry into the cells [249 ,250]. On the other hand, *P. gingivalis* Arg-specific gingipains generate C5a by C5 cleavage and thereby activate C5a receptor (C5aR), locally inducing pro-inflammatory cytokine expression [251]. Through the C5aR-TLR2 crosstalk in macrophages on the other hand C5a generated by *P. gingivalis* also leads to a suppression of IL-12 [252].

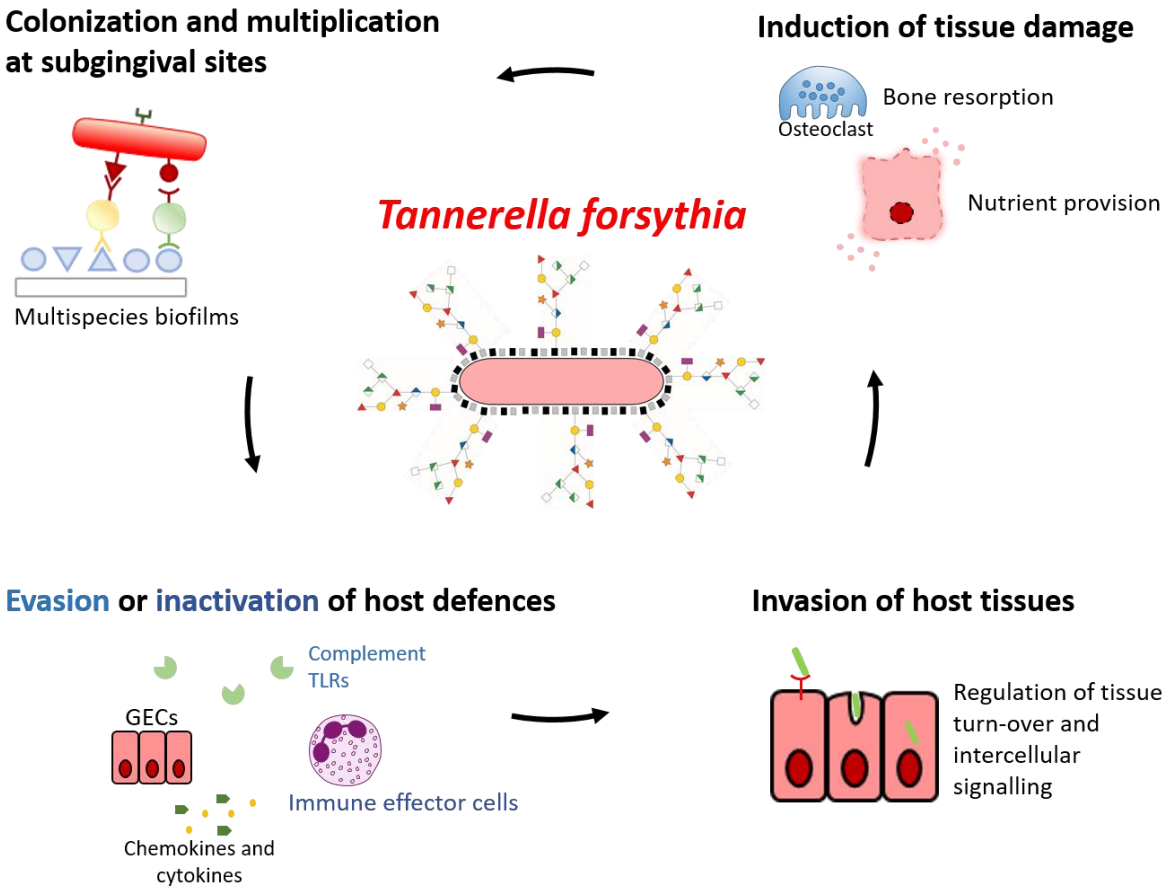

Figure 7. Modes of interaction of periodontal pathogens as exemplified by *T. forsythia*. Periodontal pathogens such as *T. forsythia* have to be able to establish themselves within the multispecies biofilms present in the subgingival pockets through interspecies interactions. By evasion of immune surveillance by complement factors, modulation of the immune response by GECs and immune effector cells and invasion of host tissues pathogens ensure their persistence within the host. Finally, the excessive inflammation caused by the bacteria leads to tissue destruction and through the provision of nutrients this benefits the entire microbial community.

Also *T. forsythia* manipulates the complement system in its favor utilizing its metalloproteases karilysin [154] and mirolysin [156] for degradation of complement components. Most likely mimicking host structures through Pse and Leg incorporation in its glycosylated S-layer, the bacterium also hampers C3b deposition and, consequently, prevents opsonisation [130] and additionally facilitates immune evasion by dampening IL-8, MCP-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion by oral keratinocytes [253] and IL-1β, TNF-α and IL-8 production by U937 macrophages and human gingival fibroblasts [131].

The above mentioned modes of action are just some of the mechanisms of immune subversion and evasion that allow pathogenic bacteria to fuel tissue breakdown while ensuring survival in the host and enable other members of the biofilm to colonize an otherwise unamenable environment.

2. Publications

2.1. Publication I: Behavior of two *T. forsythia* strains and their cell surface mutants in multispecies oral biofilms

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ORIGINAL ARTICLE

WILEY molecular oral microbiology

Behavior of two *Tannerella forsythia* strains and their cell surface mutants in multispecies oral biofilms

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Summary

As a member of subgingival multispecies biofilms, *Tannerella forsythia* is commonly associated with periodontitis. The bacterium has a characteristic cell surface (S-) layer modified with a unique *O*-glycan. Both the S-layer and the *O*-glycan were analyzed in this study for their role in biofilm formation by employing an *in vitro* multispecies biofilm model mimicking the situation in the oral cavity. Different *T. forsythia* strains and mutants with characterized defects in cell surface composition were incorporated into the model, together with nine species of select oral bacteria. The influence of the *T. forsythia* S-layer and attached glycan on the bacterial composition of the biofilms was analyzed quantitatively using colony-forming unit counts and quantitative real-time polymerase chain reaction, as well as qualitatively by fluorescence *in situ* hybridization and confocal laser scanning microscopy. This revealed that changes in the *T. forsythia* cell surface did not affect the quantitative composition of the multispecies consortium, with the exception of *Campylobacter rectus* cell numbers. The localization of *T. forsythia* within the bacterial agglomeration varied depending on changes in the S-layer glycan, and this also affected its aggregation with *Porphyromonas gingivalis*. This suggests a selective role for the glycosylated *T. forsythia* S-layer in the positioning of this species within the biofilm, its co-localization with *P. gingivalis*, and the prevalence of *C. rectus*. These findings might translate into a potential role of *T. forsythia* cell surface structures in the virulence of this species when interacting with host tissues and the immune system, from within or beyond the biofilm.

KEYWORDS

Campylobacter rectus, cell surface, oral biofilm, periodontal disease, S-layer glycosylation, *Tannerella forsythia*

1 | INTRODUCTION

To proliferate and persist in their habitat, bacteria tend to live predominately in biofilms, which are highly complex and dynamic, polymicrobial communities providing protection from shear forces and host immune responses.[1] In the oral cavity, multispecies biofilms constitute what is known as "dental plaque".[2] In a healthy individual, the oral bacteria exist in a natural balance with their host. However, different factors such as smoking, diabetes, genetic predisposition, or poor dental hygiene can cause the community to become dysbiotic,[3,4] enabling potentially pathogenic bacteria to increase in numbers and cause persistent infections, such as periodontitis.

It has been recognized that periodontitis has a polymicrobial biofilm etiology and is primarily characterized by a shift in the microbial composition and promotion of growth of Gram-negative anaerobes; among these are the periodontal pathogens *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*.[5] These so-called "red complex" bacteria are able to subvert host immune responses, modulate the infection process within the subgingival pocket, and promote dysbiosis through the expression of virulence factors.[6] In the case of *P. gingivalis*, interbacterial interaction and adhesion to host cells are facilitated through the production of colonization factors such as hemagglutinins and fimbriae.[7] The latter also induce the expression of pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, IL-8, and tumor necrosis factor-α (TNF-α),[8] stimulating the immune response during infection. *Porphyromonas gingivalis* further possesses a set of specialized cell surface cysteine proteinases, the gingipains. They can modulate the host immune response through T-cell receptor cleavage,[9] proteolytic processing of components of the complement system,[10] activation of protease-activated receptors, and inactivation of pro- and anti-inflammatory cytokines.[11-14] The oral spirochete *T. denticola* is the only motile member of the "red complex" consortium.[15] Through the expression of flagellar, chemotactic, and proteolytic factors, *T. denticola* is able to penetrate and directly interact with the gingival epithelium and underlying connective tissue.[16,17] Here, the principal immunogenic surface antigen of *T. denticola*, the major sheath protein Msp, facilitates actin remodeling and reorganization in host cells and thereby impairs neutrophil chemotaxis and phagocytic activity.[18-20] Through the action of a surface-associated protease dentilisin, *T. denticola* has been shown to modulate host cell immune responses by degradation of IL-1β, IL-6, TNF-α, and monocyte chemoattractant protein 1.[21,22]

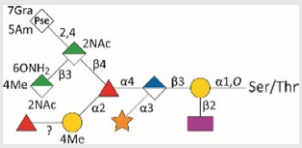
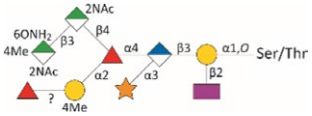
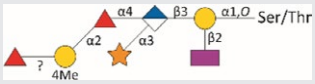
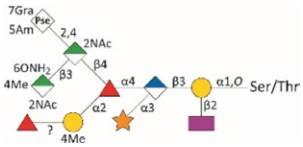
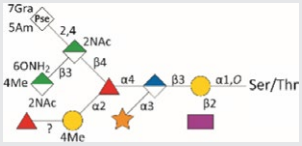
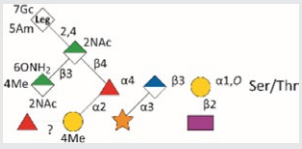
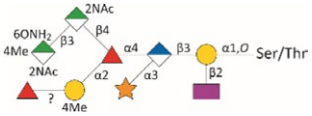
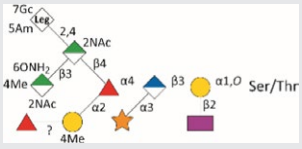
Like *T. denticola*, *T. forsythia* is characterized by its fastidious growth requirements and is, especially through its initial recalcitrance to genetic manipulation, a less characterized member of the "red complex" consortium. It has been shown to express several putative virulence factors;[23] among them is its characteristic two-dimensional (2D) crystalline cell surface (S-) layer.[24,25] *Tannerella forsythia* is the only member of the "red complex" consortium that possesses an S-layer fully covering the bacterial cells; this is formed by self-assembly of the two S-layer proteins TfsA and TfsB,[25] both of which are modified by a unique, complex, branched dekasaccharide that is synthesized by the general protein *O*-glycosylation system of the bacterium[26] (Table 1). This dekasaccharide is *O*-glycosidically bound to multiple serine or threonine residues within a D(S/T)(A/I/L/M/T/V) amino acid target motif present on TfsA and TfsB, but also on several other *T. forsythia* proteins.[26] S-layer protein glycosylation was shown to be completed in the bacterial periplasm before glycoprotein export via a type IX secretion system[27,28] followed by anchoring of the glycoproteins in the cell envelope and equimolar self-assembly into the mature S-layer lattice at the cell surface. Given the nanometer-scaled periodicity of the 2D S-layer lattice, this strategy results in a high-density cell surface display of *O*-glycans. This surface glycosylation affects the physicochemical properties of the bacterial cell surface through the introduction of charged sugar residues (for structure of the *O*-glycan see Table 1) and modulates bacterial cell hydrophobicity. The prominent cellular location and abundance of the *O*-glycan as well as the S-layer matrix itself make them ideal candidates for influencing interbacterial or bacterium–host interactions as may occur in oral biofilms.

In biofilms, the physical properties of the bacterial cell surface come into play, as initial attachment by planktonic bacteria to a substrate is primarily influenced by factors such as surface charge, hydrophobicity or electrostatic interactions, whereas the formation of a stable biofilm is facilitated by specialized surface components such as flagella, fimbriae, or pili and the production of an exopolysaccharide matrix.[29,30] The oral bacterium *Streptococcus sanguis*, for instance, has been shown to largely depend on hydrophobic effect interactions for adhesion to the salivary pellicle.[31,32] *Streptococcus parasanguinis*, another early colonizer of the dental surface, requires glycosylation of the fimbria-associated adhesin Fap1 for the formation of stable biofilms.[33,34] In *Campylobacter* spp., loss of flagellum glycosylation negatively affects the bacterium's ability to form microcolonies and, subsequently, biofilms.[35,36] In *Campylobacter jejuni*, the flagellum is heavily glycosylated by the addition of *O*-linked pseudaminic acid (Pse) and legionaminic acid (Leg),[37,38] a feature that has been shown to orchestrate the bacterium's virulence potential.[36]

We recently found evidence that the *T. forsythia* ATCC 43037 wild-type strain carries a modified Pse residue as a terminal constituent of the S-layer *O*-glycan,[26] whereas in the clinical isolate *T. forsythia* UB4, this residue is present as its stereoisomer, Leg[39] (Table 1, see Supplementary material, Fig. S1). Pse (5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid) as well as Leg (5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid) appear to be unique to bacteria.[39] They belong to the class of nonulosonic acids, acidic nine-carbon (C9) α-keto sugars, which are best represented by the sialic acid family abundantly displayed on the exterior of mammalian cells functioning in cell–cell communication and adhesion.[40]

The *T. forsythia* S-layer has been described to facilitate adhesion to and invasion of gingival epithelial cells,[41] suppress pro-inflammatory cytokine production,[42] and inhibit monospecies biofilm formation;[28] however, without dissecting any potential contribution of the *O*-glycan attached to the S-layer. Honma *et al*. (2007) observed an increase in *T. forsythia* biofilm formation upon deletion of a UDP-*N*-acetyl-D-mannosaminuronic dehydrogenase (WecC) – later found to cause a three-sugar truncation of the *T. forsythia* *O*-glycan[26] (compare with Table 1) – when cells were cultivated in an untreated polystyrene culture dish.[43] In contrast, deficiency in the *O*-glycan's terminal nonulosonic acid in a *T. forsythia* ATCC 43037 Δ*pseC* and a *T. forsythia* UB4 Δ*legC* mutant, respectively, decreased biofilm formation on a mucin-coated surface.[39] Although these data together demonstrate the involvement of both S-layer and attached sugar moieties in monospecies biofilm formation, the question arises to what extent these observations are influenced by the physical properties of the surface provided for cell attachment and, above that, demand an investigation into if and how the described effects translate into a multispecies biofilm that more adequately mirrors the *in vivo* situation. As

TABLE 1 *Tannerella forsythia* strains and their cell surface mutants cultivated in the subgingival "Zurich biofilm model"

Strains	Cell surface and glycan properties	Structure of *O*-glycan
ATCC 43037		
Wild-type	wild-type; S-layer glycan with terminal Pse residue	
Δ*pseC (Tanf_01190)*	S-layer glycan devoid of terminal Pse	
Δ*wecC (Tanf_01280)*	S-layer glycan devoid of trisaccharide branch containing Pse and two ManNAcA residues	
Δ*tfsAB (Tanf_03370; Tanf_03375)*	S-layer deficient mutant; this mutant may expose R-type lipopolysaccharide or *O*-glycans from outer membrane glycoproteins	
Δ*pseC*$_{comp}$ *(Tanf_01190)*	reconstituted mutant Δ*pseC*	
UB4		
Wild-type	wild-type; S-layer glycan with terminal Leg residue	
Δ*legC (TFUB4_00900)*	S-layer glycan devoid of terminal Leg	
Δ*legC*$_{comp}$ *(TFUB4_00900)*	reconstituted mutant Δ*legC*	

galactose; xylose; nonulosonic acid; Gra *N*-glyceroyl; glucuronic acid; digitoxose; NAc *N*-acetyl; Me *O*-methyl; fucose; mannosaminuronic acid; Am acetamidino; Gc glycolyl

part of a multispecies biofilm consortium, *T. forsythia* needs to interact with numerous other bacteria. How these interactions are mediated and whether they depend on the *T. forsythia* S-layer and/or its *O*-glycosylation has yet to be elucidated.

Based on the analysis of planktonic and monospecies biofilm growth, we employed in this study the subgingival "Zurich biofilm model"[44] to investigate how the *T. forsythia* wild-type strains ATCC 43037 and UB4 and defined cell surface mutants thereof perform in a multispecies consortium. Through the incorporation of 10 different species of oral bacteria in the biofilm, this *in vitro* model mimics the natural situation in the oral cavity, whereby several microbial species assemble and grow together in the form of a biofilm, and therefore poses an excellent platform to dissect the role of individual species within the community. In the *in vitro* model, the selected oral bacteria, including the three "red complex" species out of which *T. forsythia* was varied, were co-cultivated to form biofilms on pellicle-coated hydroxylapatite (HA) disks in saliva and serum-containing growth medium.[12,45-49] *Tannerella forsythia* wild-type strains and mutants with different cell surface glycosylation patterns as well as an S-layer-deficient mutant were introduced in order to monitor their biofilm growth as well as the structural behavior of the biofilm communities as a whole.

In particular, biofilms grown under these conditions were analyzed with the following aims: (i) to numerically determine cell numbers of all individual species within the bacterial consortium and the overall biofilm composition by quantitative real-time polymerase chain reaction (qPCR) and colony-forming unit (CFU) counts and (ii) to analyze the localization and distribution of individual species within the microbial structure through fluorescence *in situ* hybridization (FISH) using species-specific probes against the 16S rRNA and confocal laser scanning microscopy (CLSM) analysis. This study is intended to be a first characterization of the behavior of *T. forsythia* strains with varying cell surface composition in a multispecies biofilm setting.

2 | METHODS

2.1 | Bacterial strains

Tannerella forsythia ATCC 43037 (American Type Culture Collection, Manassas, VA) and *T. forsythia* UB4 (obtained from Dr. Ashu Sharma, University of Buffalo, NY, USA) wild-type strains and defined mutants thereof (see below) were grown anaerobically at 37°C for 4-7 days in brain–heart infusion broth (Oxoid, Basingstoke, UK), supplemented with *N*-acetylmuramic acid, horse serum, and 50 μg mL^{-1} gentamicin as described previously,[27] with one passage before biofilm inoculation.

Mutants of *T. forsythia* ATCC 43037 (JUET00000000[50]) and *T. forsythia* UB4 (FMMN01000000[51]) with characterized defects in their cell surface protein glycosylation, affecting the terminal Pse (ATCC 43037) or Leg (UB4) residue, were available in our laboratory from a previous study.[39] Briefly, *T. forsythia* ATCC 43037 Δ*pseC* (coding for a dedicated aminotransferase from the Pse biosynthesis pathway) and *T. forsythia* UB4 Δ*legC* (coding for a dedicated aminotransferase from the Leg biosynthesis pathway) mutants were constructed by chromosomal insertion of a gene knockout cassette consisting of an erythromycin resistance gene flanked by homologous upstream and downstream regions, ~1000 bp, each. The complementation cassette for *T. forsythia* mutants consisted of a chloramphenicol resistance gene flanked by a homologous ~1000-bp upstream region, the gene of interest and a ~1000-bp downstream region. The *T. forsythia* ATCC 43037 Δ*wecC* mutant, which lacks a trisaccharide glycan branch including the Pse residue, was obtained from Dr. Ashu Sharma. In addition to that, the S-layer-deficient mutant *T. forsythia* ATCC 43037 Δ*tfsAB*[41] was included in this study. This mutant lacks S-layer glycans due to the absence of the S-layer, but may expose underlying R-type lipopolysaccharide[52] or even *O*-glycans present on outer membrane glycoproteins that become exposed upon removal of the S-layer.[53] All *T. forsythia* strains and mutants used in this study, together with their cell surface composition, are summarized in Table 1.

2.2 | Monospecies biofilm growth of *T. forsythia*

The monospecies biofilm behavior of all *T. forsythia* strains and mutants included in this study was analyzed in a microtiter plate assay.[39] In brief, bacteria were passaged once before biofilm inoculation at an optical density at 600 nm (OD_{600}) of 0.05 and grown anaerobically for 6 days in 1 mL of half-concentrated brain–heart infusion medium, with supplements as above,[27] in 24-well polystyrene plates (STARLAB) coated with 5 mg mL^{-1} mucin (from bovine submaxillary gland; Sigma-Aldrich, Vienna, Austria) solution (in 0.1 mol L^{-1} sodium acetate buffer pH 4.5). In each experiment, two wells were used to determine the total cells of each strain and mutant, sterile medium served as negative control. For biofilm quantification, medium and planktonic cells were removed and the wells were washed once with 500 μL of PBS. Subsequently, biofilms were resuspended in 1 mL of PBS and the OD_{600} of the biofilm cell suspension was measured. Biofilm values were normalized to the corresponding absorbance (OD_{600}) of the total cells. Data represent mean values ±SD of four independent experiments with three replicates each and were analyzed by the unpaired Student's *t*-test.

2.3 | Multispecies biofilm cultivation

To set-up the "Zurich subgingival biofilm model", *T. forsythia* wild-type strains and defined cell surface mutants thereof (Table 1) were co-cultivated with the following organisms: *Prevotella intermedia* ATCC 25611^T (OMZ278), *Campylobacter rectus* (OMZ388), *Veillonella dispar* ATCC 17748^T (OMZ493), *Fusobacterium nucleatum* (OMZ598), *Streptococcus oralis* SK248 (OMZ607), *Streptococcus anginosus* ATCC 9895 (OMZ871), *Actinomyces oris* (OMZ745), *Porphyromonas gingivalis* (OMZ925), and *Treponema denticola* ATCC 35405 (OMZ661). Each biofilm contained nine standard subgingival species plus one of the eight *T. forsythia* strains and mutants. Biofilm bacteria were maintained as described previously.[44]

For biofilm formation, bacterial cultures at an OD_{600} of 1.0 were mixed at equal volumes and 200 μL of this cell suspension was used to inoculate 1.6 mL of growth medium (60% pooled saliva, 10% fetal bovine serum [Sigma], 30% modified fluid universal medium)[54] for biofilm formation on sintered pellicle-coated HA disks (9 mm in diameter; Clarkson Chromatography Products, South Williamsport, PA) positioned in 24-well polystyrene tissue-culture plates. The medium was changed after 16 and 24 hours and disks were dip-washed in 0.9% NaCl three times a day. After incubating anaerobically at 37°C for 64 hours, biofilms were dip-washed once more and either harvested by vigorous vortexing for 2 minutes in 0.9% NaCl or fixed for 1 hour at 4°C in 4% paraformaldehyde solution (Merck, Darmstadt, Germany) for FISH.

2.4 | Quantitative analysis

Cell numbers were determined by serial dilution plating and CFU counting as well as qPCR on genomic DNA purified from biofilm samples. Cell numbers were taken as a measure for the bacterial growth rate within the biofilm.

For CFU counts, biofilm suspensions were diluted $1:10^4$ and $1:10^5$ in 0.9% NaCl and plated on selective agar plates (Table 2) using a spiral diluter. For the more fastidious strains – i.e. *T. denticola*, *C. rectus*, and *T. forsythia* – cell numbers were determined by qPCR only.

For qPCR, bacterial genomic DNA was extracted from 500 μl of biofilm suspension using the GenEluteTM Bacterial Genomic DNA Kit (Sigma) and qPCR was performed on an ABI Prism SDS 7000 device (Applied Biosystems, Foster City, CA) according to Ammann *et al.*[47]

TABLE 2 Selective agar plates used for colony-forming unit counting

Selective agar plates	Organism
Mitis Salivarius Agar (Difco)+1% sodium tellurite solution	*Streptococcus anginosus*, *Streptococcus oralis*
Columbia Blood Agar (Oxoid)+5% horse blood (Sigma)	*Actinomyces oris*, *Veillonella dispar* total CFU
Fastidious Anaerobe Agar (BAG)+1 mg L^{-1} erythromycin (Sigma), 4 mg L^{-1} vancomycin (Sigma), 1 mg L^{-1} norfloxacin (Sigma)	*Fusobacterium nucleatum*
Columbia Blood Agar+5% horse blood (Sigma), 80 mg L^{-1} phosphomycin (Sigma)	*Prevotella intermedia*, *Porphyromonas gingivalis*

TABLE 3 Combinations of 16S rRNA probes used for fluorescence *in situ* hybridization staining of individual bacterial species

Probes	Target species	FA (%)[a]	NaCl (mmol L^{-1})[b]	Reference
Tfor-997-Cy3/Pging1006-2-prop-Cy5	*Tannerella forsythia/Porphyromonas gingivalis*	40	46	44,75
Tfor-997-Cy3/Pging1006 -Cy5	*T. forsythia/P. gingivalis*	40	46	44,75
Tfor-997-Cy3/TrepG1-679-Cy5	*T. forsythia/Treponema denticola*	40	46	75,76
Tfor-997-Cy3/FUS-664-Cy5	*T. forsythia/Fusobacterium nucleatum*	40	46	55,75
Tfor-997-Cy3/CAMP655-Cy5	*T. forsythia/Campylobacter rectus*	35	70	44,75
Tfor-997-Cy3/Pging1006-2-prop-Cy5	*T. forsythia/P. gingivalis*	40	46	44,75
Tfor-997-Cy3/Pging1006 -Cy5	*T. forsythia/P. gingivalis*	40	46	44,75
Tfor-997-Cy3/TrepG1-679-Cy5	*T. forsythia/T. denticola*	40	46	75,76
Tfor-997-Cy3/FUS-664-Cy5	*T. forsythia/F. nucleatum*	40	46	55,75
Tfor-997-Cy3/CAMP655-Cy5	*T.forsythia/C. rectus*	35	70	44,75

[a]Formamide concentration used in the hybridization buffer.
[b]NaCl concentration in the wash buffer.

Each sample was analyzed using species-specific primers amplifying the 16S rRNA gene.[47] For each species, a standard curve was generated and the sample DNA concentration was calculated from the obtained quantification cycle (Cq) values. The abundance of each organism in the biofilm was calculated using the respective theoretical genome weight.[47] Cell numbers per biofilm were determined in three independent experiments with three technical replicates for each biofilm. Statistical significance was tested by analysis of variance (Tukey's post-hoc test for multiple comparisons, $P \leq .05$) using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA).

2.5 | Structural analysis of biofilms

FISH staining was performed according to the protocol established by Thurnheer *et al.*[55] using the probe combinations listed in Table 3. In brief, after fixation, biofilm samples were pre-hybridized in hybridization buffer (0.9 mol L^{-1} NaCl, 20 mmol L^{-1} Tris–HCl, [pH 7.5], 0.01% sodium dodecyl sulfate, formamide (35%-40%) at 46°C, for 15 minutes, followed by 3 hours of hybridization with specific oligonucleotide probes.[45] Samples were washed in wash buffer (20 mmol L^{-1} Tris–HCl [pH 7.5], 5 mmol L^{-1} ethylene diaminetetraacetic acid, 0.01% sodium dodecyl sulfate, 46-70 mmol L^{-1} NaCl) for 45 minutes at 48°C. For CLSM and image analysis, the samples were counterstained with a mixture of 3 μmol L^{-1} YoPro-1 iodide (Invitrogen, Carlsbad, CA) and 15 μmol L^{-1} Sytox Green (Invitrogen) and embedded in Mowiol[56] for confocal microscopy.

The architecture of the biofilms was analyzed using CLSM. For each of the eight *T. forsythia* strains and mutants, a minimum of three disks carrying fluorescently labeled biofilms was analyzed using a Leica SP-5 microscope (Center of Microscopy and Image Analysis of the University of Zürich). Images were captured using a 100× objective and processed with Imaris 7.4.0 Software (Bitplane, Zürich, Switzerland). Presented CSLM images (Figures 3, 5, 6) are snapshots of the biofilm structures present on the HA disks and the depicted structures represent a comprehensive collection of *T. forsythia* biofilm behavior observed during sampling.

3 | RESULTS

3.1 | Monospecies biofilm formation of *T. forsythia* wild-type strains and mutants

Based on the observations that deficiency in the protein *O*-glycan's terminal nonulosonic acid triggers a decrease in biofilm formation of *T. forsythia* ATCC 43037 Δ*pseC* and *T. forsythia* UB4 Δ*legC* on a mucin-coated surface[39] and that *T. forsythia* ATCC 43037 Δ*wecC* possessing an even more truncated *O*-glycan forms more biofilm on untreated plates,[43] the biofilm formation capacity of all these strains was

compared here in one microtiter plate assay, where the plates were coated with mucin to mimic the native situation on the tooth surface, and biofilm growth was quantified by OD_{600} measurement of biofilm cells and normalized to the corresponding total cell mass for each strain. In our setting, any manipulation of the cell surface decreased the capacity of the bacteria to form biofilms, as was evident in the absence of the S-layer (*T. forsythia* ATCC 43037 Δ*tfsAB*), of the Pse-$(ManNAcA)_2$ *O*-glycan branch (*T. forsythia* ATCC 43037 Δ*wecC*) as well as of the terminal nonulosonic acid alone, i.e. Pse in *T. forsythia* ATCC 43037 Δ*pseC* and Leg in *T. forsythia* UB4 Δ*legC* (Figure 1). More precisely, biofilms of the ATCC 43037 strain reached an average maximum OD_{600} of 0.52 ± 0.05 after 6 days of cultivation, whereas biofilm growth of the Δ*pseC* and Δ*tfsAB* mutants was reduced by 1.6-fold, and in the case of the Δ*wecC* mutant even by five-fold. *Tannerella forsythia* UB4 wild-type

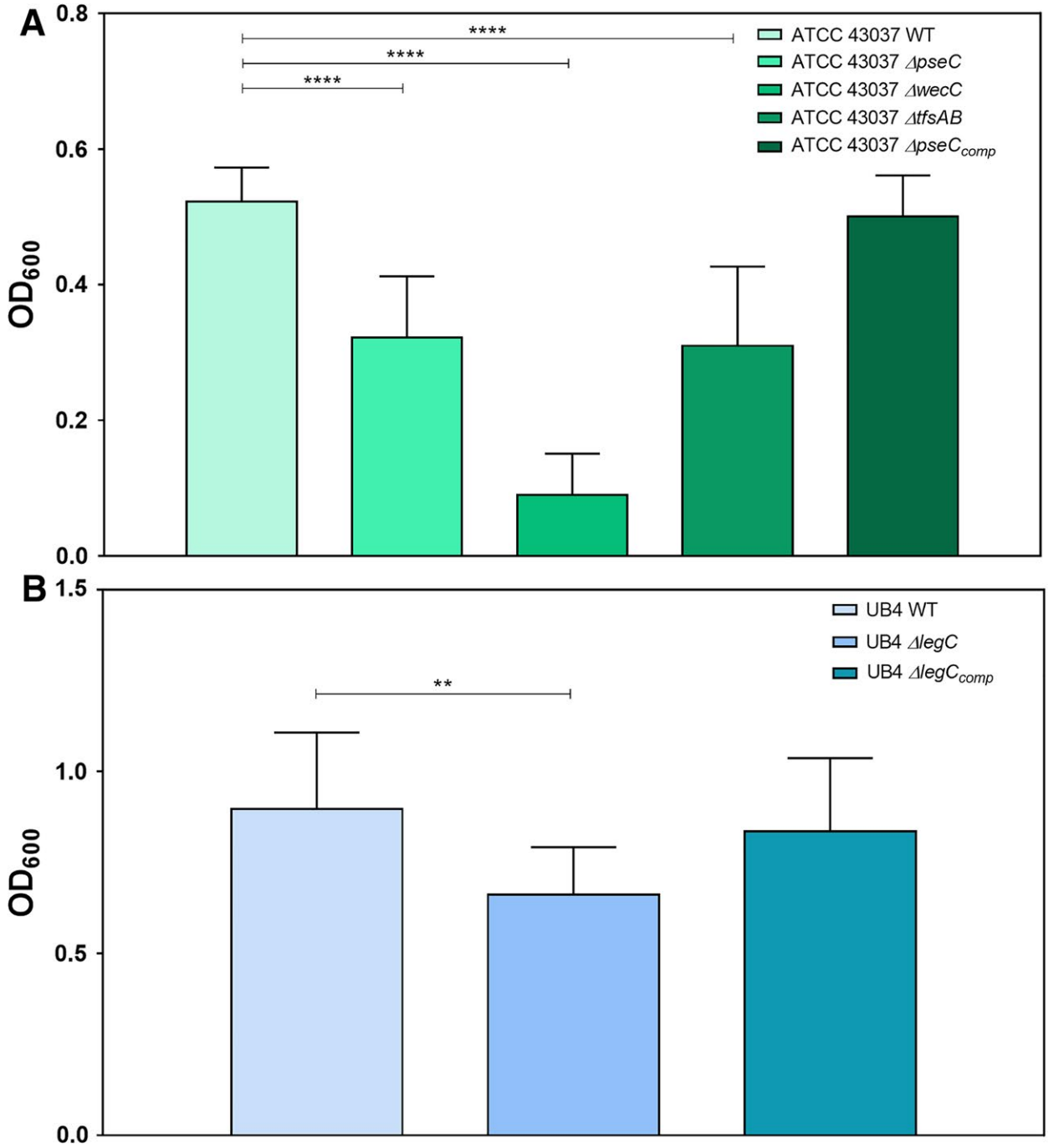


FIGURE 1 Monospecies biofilm formation of *Tannerella forsythia* wild-type and mutant strains. (A) Biofilm formation of *T. forsythia* ATCC 43037 wild-type compared with its mutants ATCC 43037 Δ*pseC*, Δ*wecC*, Δ*tfsAB* and the complemented mutant $\Delta pseC_{comp}$. (B) Biofilm formation of *T. forsythia* UB4 wild-type compared with its mutant UB4 Δ*legC* and the complemented mutant $\Delta legC_{comp}$. Mean values ±SD of four independent experiments with three replicates, each, are shown. Asterisks (**) indicate significant differences between samples as determined by the unpaired Student's *t*-test ($P \leq .01$)

biofilms reached an average maximum OD_{600} of 0.89 ± 0.21 and also here, the biofilm growth was reduced 1.3-fold in the nonulosonic acid-deficient mutant *ΔlegC*. In both nonulosonic acid mutants, the growth behavior in the biofilm was restored to the levels of the respective parent strain, with an average maximum OD_{600} of 0.50 ± 0.06 for $\Delta pseC_{comp}$ and 0.84 ± 0.20 for $\Delta legC_{comp}$ (Figure 1).

All deletion mutants also showed slower planktonic growth in liquid culture, as concluded from the determination of growth curves and doubling times (see Supplementary material, Fig. S2 and Table S1). These obvious growth defects might result from pleiotropic effects due to the genetic manipulation of the strains rather than from changes of the bacterial cell surface, even though during planktonic growth, both complemented strains performed in a similar way to the parent strain in terms of doubling times, with $\Delta pseC_{comp}$ vs ATCC 43037 wild-type revealing doubling times of 14.99 ± 0.83 hours and 14.41 ± 0.54 hours, and $\Delta legC_{comp}$ vs UB4 revealing a slight increase in doubling time (12.28 ± 0.25 hours vs 16.75 ± 3.97 hours) (see Supplementary material, Fig. S2 and Table S1).

3.2 | Determination of total biofilm cell numbers in the presence of *T. forsythia* strains and mutants in the subgingival "Zurich biofilm model"

Total cell numbers in biofilms including nine bacterial species routinely used in the subgingival "Zurich biofilm model" plus one *T. forsythia* wild-type strain (*T. forsythia* ATCC 43037 or UB4) or mutant lacking certain sugar residues (*T. forsythia* ATCC 43037 *ΔpseC*, *T. forsythia* ATCC 43037 *ΔwecC*, *T. forsythia* UB4 *ΔlegC*) or the whole S-layer (*T. forsythia* ATCC 43037 *ΔtfsAB*) were analyzed by quantifying the cell numbers of each of the 10 species.

The total cell number per biofilm was not significantly affected, regardless of which *T. forsythia* strain or mutant had been incorporated into the biofilm (Figure 2). When comparing the total cell number of all biofilm bacteria as determined by strain-specific qPCR and CFU counts, the latter resulted in lower cell numbers, as only viable cells were enumerable. Both methods, however, provided reproducible results for each of the nine disks that were analyzed for each of the eight *T. forsythia* strains and mutants included in this study.

3.3 | Influence of *T. forsythia* wild-type strains on composition and structure of the subgingival "Zurich biofilm model"

First, the multispecies biofilm behavior of the *T. forsythia* wild-type strains ATCC 43037 and UB4 was compared with regard to bacterial growth and localization in the 10-species biofilm.

For quantitative analysis, the cell number of each individual species in the biofilm was determined by qPCR after 64 hours of

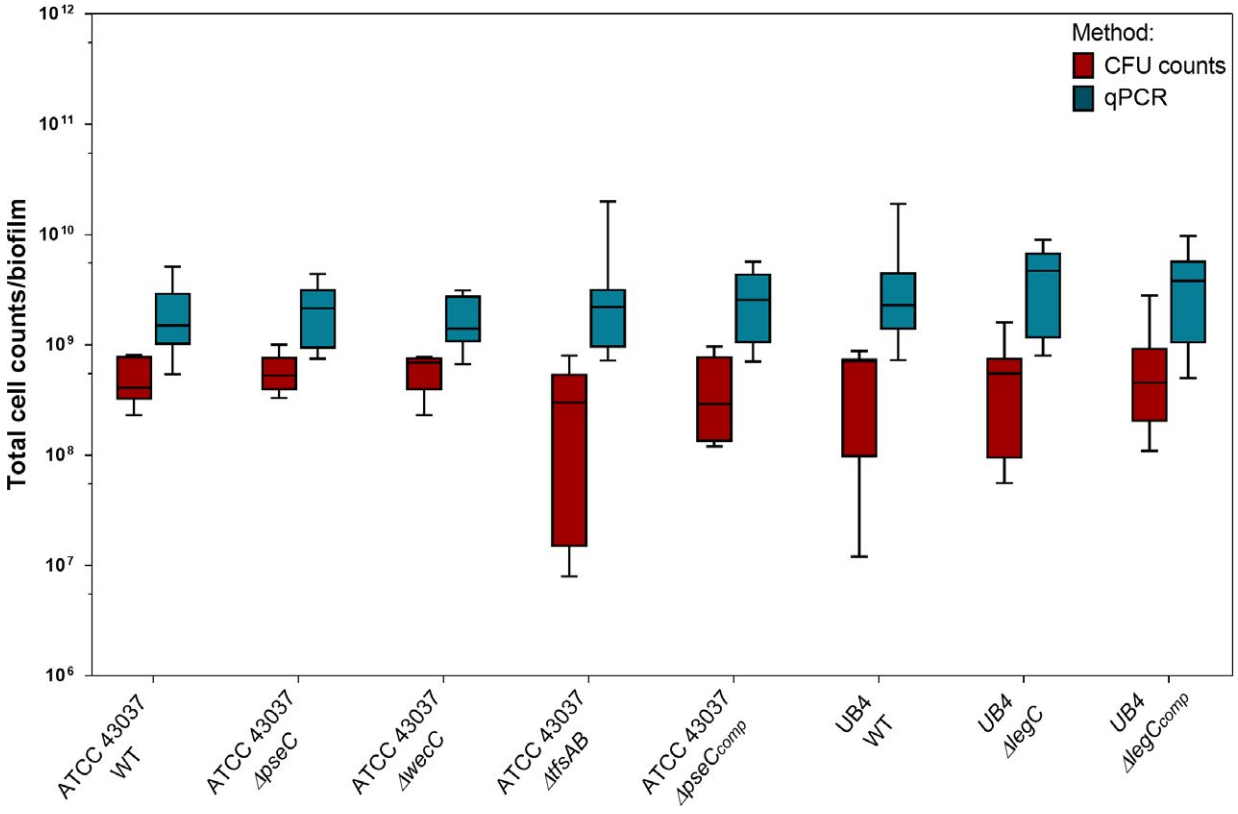


FIGURE 2 Comparison of colony-forming unit (CFU) counting and quantitative polymerase chain reaction (qPCR) for *Tannerella forsythia* wild-type strains and mutants in the subgingival "Zurich biofilm". Total bacteria for 10-species biofilms with different *T. forsythia* strains and mutants enumerated by CFU counts (red boxes) and qPCR (blue boxes) for three independent experiments with three technical replicates, each, are shown (Whiskers boxplots 5th to 95th centile)

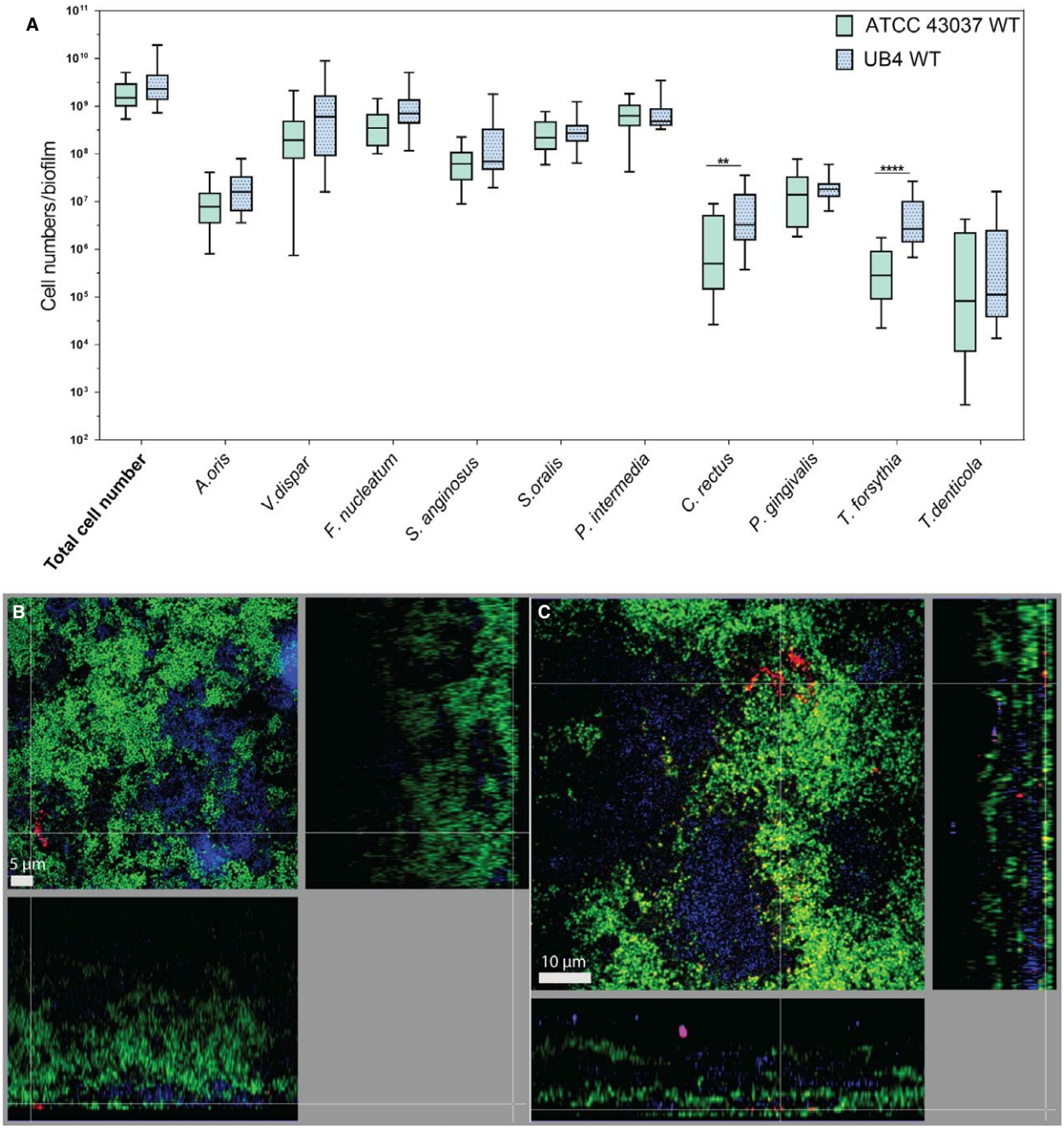


FIGURE 3 Comparison of 10-species biofilms with two *Tannerella forsythia* wild-type strains. (A) Whiskers boxplots (5th to 95th centile) show bacterial numbers determined by quantitative real-time PCR from three independent experiments. Asterisk (*) indicates a statistically significant difference ($P \leq .05$) between groups. The two groups represent biofilms with either *T. forsythia* ATCC 43037 wild-type or *T. forsythia* UB4 wild-type. (B, C) Fluorescence *in situ* hybridization stainings of fixed biofilms showing the localization of ATCC 43037 wild-type (B) and UB4 wild-type (C). Red/yellow: *T. forsythia;* cyan: *Porphyromonas gingivalis*, green: non-hybridized cells (DNA staining YoPro-1+Sytox). Here a representative area for one disk each is shown with a top view in the left panel and a side view with the biofilm–disk interface directed towards the top view; scale bars 5 µm (B) and 10 µm (C)

incubation. The total cell number of all species, except for *C. rectus* OMZ388 (see below), was not affected by the incorporation of the different *T. forsythia* strains, but there was a clear difference in the biofilm growth of the *T. forsythia* strains (Figure 3A). In accordance with the results observed in monospecies biofilms (Figure 1), also in the 10-species consortium, *T. forsythia* UB4 seemed to perform better with mean cell numbers higher by 11.9-fold when compared with the mean cell numbers of *T. forsythia* ATCC 43037 (Figure 3A), as determined by qPCR. This coincided with *C. rectus* OMZ388 to be found at significantly higher levels (3.6-fold) in biofilms containing strain UB4 as determined by analysis of variance ($P \leq .01$).

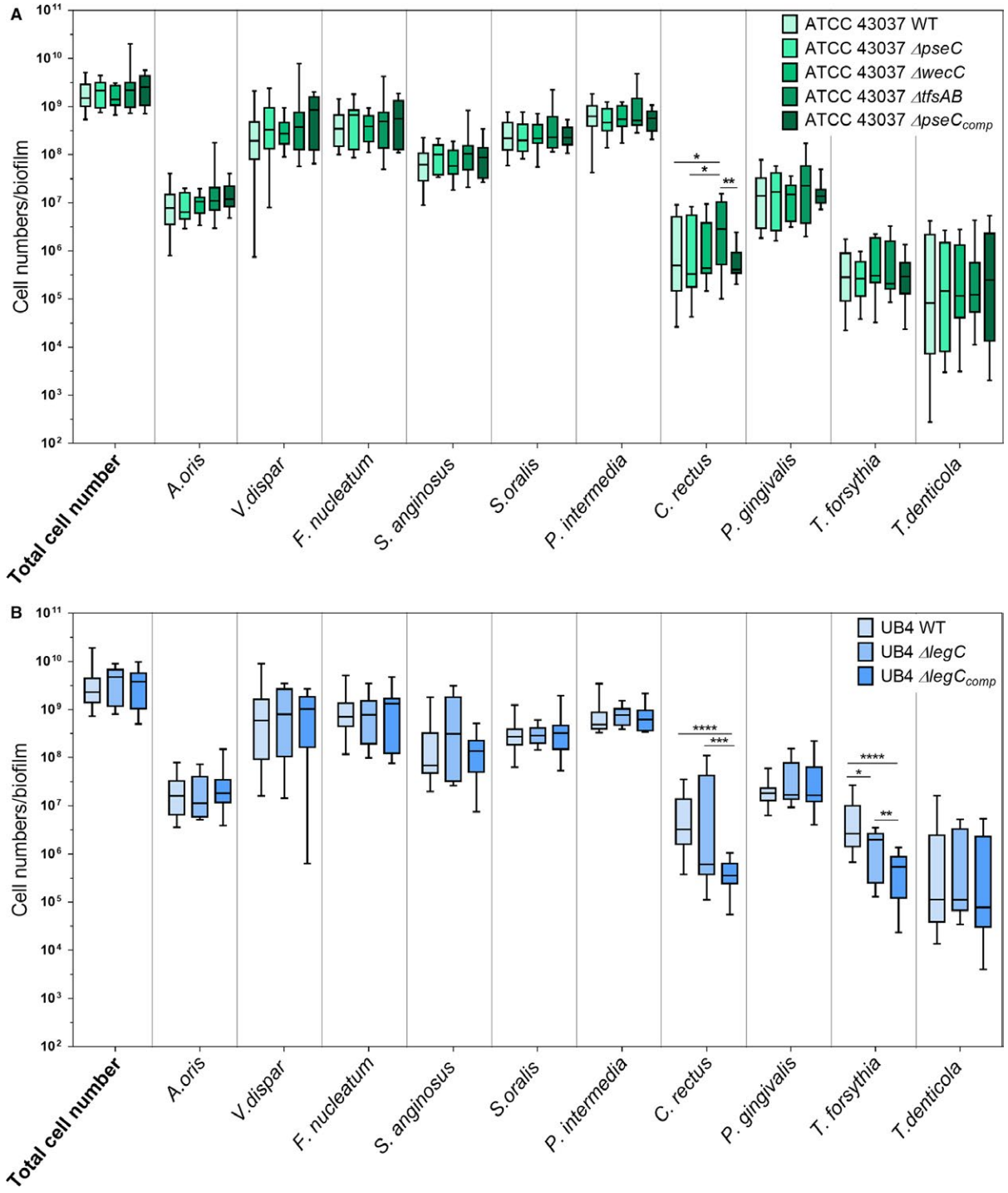


FIGURE 4 Box plots showing cell numbers of all species determined by quantitative real-time PCR for biofilms with *Tannerella forsythia* ATCC 43037 wild-type or mutants (*ΔpseC*, *ΔwecC*, *ΔtfsAB*, $\Delta pseC_{comp}$) (A) and UB4 wild-type or mutants (*ΔlegC*, $\Delta legC_{comp}$), respectively (B). Data derived from three independent experiments were plotted on a logarithmic scale. Asterisk (*) indicates significant differences ($P \leq .05$) between the groups

The influence of the two *T. forsythia* wild-type strains on the biofilm structure and their localization were determined by CLSM. *Tannerella forsythia* ATCC 43037 tended to be primarily localized at the outer biofilm surface in the form of clearly visible cell clusters (Figure 3B). In contrast, *T. forsythia* UB4 was found in the form of microcolonies as well as singly dispersed close to the biofilm surface and in small clusters in deeper layers of the biofilm (Figure 3C).

3.4 | Analysis of *T. forsythia* cell surface mutants in the subgingival biofilm

3.4.1 | Quantitative analysis

In order to assess to what extent the difference in biofilm growth of *T. forsythia* strains ATCC 43037 and UB4 (see above) was influenced

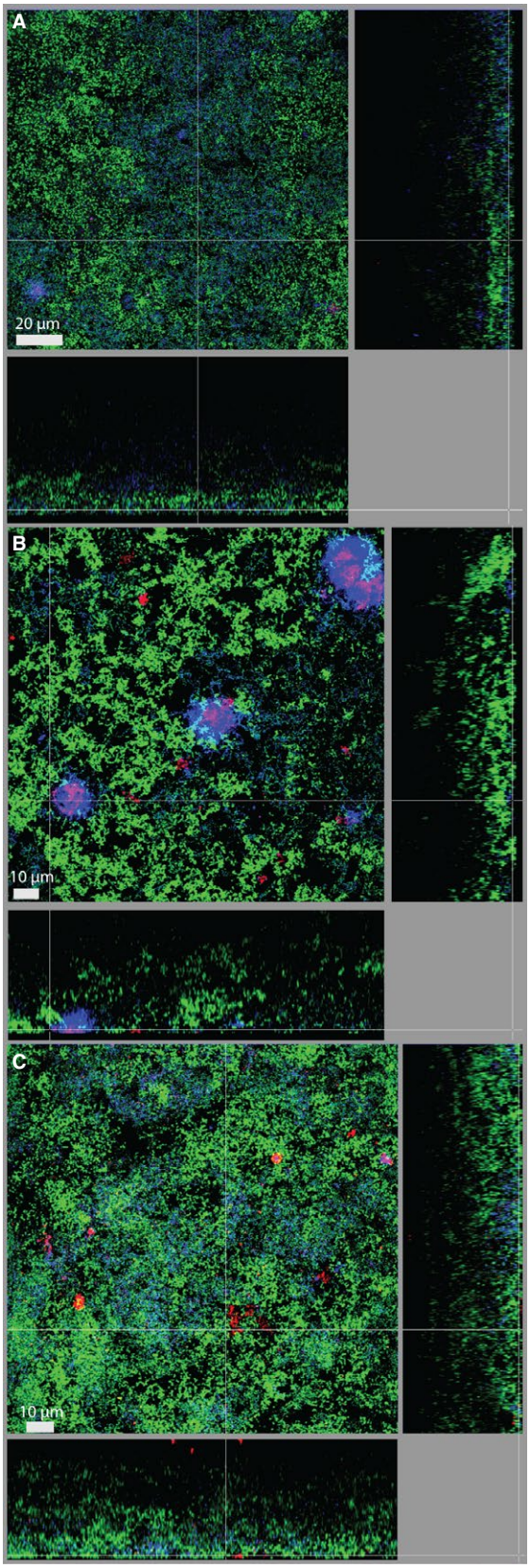


FIGURE 5 Fluorescence *in situ* hybridization staining of biofilms harboring *Tannerella forsythia* ATCC 43037 mutants (A) Δ*pseC*, (B) Δ*wecC*, and (C) Δ*tfsAB*. Red: *T. forsythia*, cyan: *Porphyromonas gingivalis*, green: non-hybridized cells (DNA staining YoPro-1+Sytox). Scale bars 20 µm (A) and 10 µm (B, C)

by their cell surface composition, defined mutants of either strain (Table 1) were incorporated into the biofilm and their cell numbers were again determined via qPCR.

Contrary to their performance in monospecies biofilms (Figure 1) and their slower planktonic growth (see Supplementary material, Fig. S2) in the 10-species consortium, the *T. forsythia* ATCC 43037 mutants behaved in a very similar way to the parent strain, and neither the lack of the terminal Pse residue (Δ*pseC*) nor the lack of the trisaccharide branch (Δ*wecC*) of the S-layer *O*-glycan significantly affected the growth of *T. forsythia* or the other species of the biofilm (Figure 4A). The same was observed for the reconstituted strain ATCC 43037 Δ$pseC_{comp}$. Interestingly, the absence of the S-layer in the Δ*tfsAB* mutant, although not affecting growth of *T. forsythia* itself, led to a strong increase in the growth of *C. rectus* OMZ388 in the biofilm (Figure 4A) when compared with biofilms with *T. forsythia* ATCC 43037 wild-type or Δ*pseC*, indicating that the loss of the S-layer causes a growth benefit for *C. rectus* OMZ388 in these biofilms. As a control, the complemented mutant *T. forsythia* ATCC 43037 Δ$pseC_{comp}$ reverted *C. rectus* OMZ388 cell numbers back to the wild-type level (with a non-significant reduction of *C. rectus* cell numbers).

In contrast to the almost identical performance of *T. forsythia* ATCC 43037 wild-type and mutants in the multispecies biofilm, genetic manipulation of *T. forsythia* UB4 (i.e. *T. forsythia* UB4 Δ*legC* and Δ$legC_{comp}$) resulted in a decrease in cell numbers in multispecies biofilms. The cell number of *T. forsythia* UB4 Δ*legC* was significantly decreased in the biofilm when compared with the parent strain, a fact that had already been observed in monospecies biofilms (Figure 1). Contrary to its behavior in monospecies biofilms, in the multispecies community, the reconstituted strain Δ$legC_{comp}$ could not restore the parent phenotype (Figure 4B).

As described before, at the high levels of *T. forsythia* UB4 wild-type that developed in the biofilm, cell numbers of *C. rectus* OMZ388 were elevated in comparison with biofilms harboring *T. forsythia* ATCC 43037. In biofilms containing the Δ*legC* mutant this effect was less pronounced, with *C. rectus* OMZ388 mean cell numbers being significantly decreased by 1.4-fold when compared with biofilms with UB4 wild-type ($P \leq .001$) (Figure 4B). In the presence of the complemented strain Δ$legC_{comp}$, the growth of *C. rectus* OMZ388 was significantly reduced when compared with biofilms harboring UB4 wild-type or Δ*legC* ($P \leq .001$) (Figure 4B). Given that in monospecies biofilm experiments as well as during planktonic growth, UB4 Δ$legC_{comp}$ was shown to behave in the same way as its parent strain (Figure 1, see Supplementary material, Fig. S2), its impaired growth in the multispecies community suggests that the modification of this gene locus has a pleiotropic effect causing a growth defect in the environment of the multispecies biofilm.

3.4.2 | Evaluation of the biofilm structure by CLSM

Since changes in the cell surface composition of *T. forsythia* did not affect the numeric composition of the 10-species biofilms, FISH staining

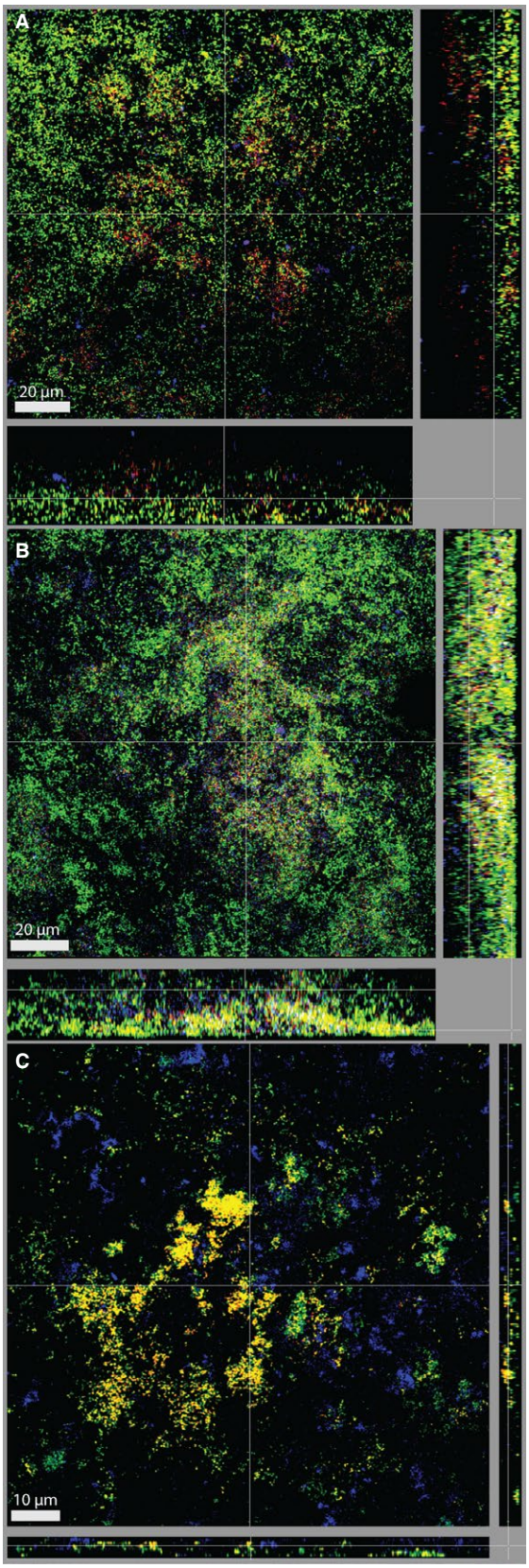


FIGURE 6 Dual fluorescence *in situ* hybridization staining of *Tannerella forsythia* and *Campylobacter rectus* for biofilms harboring ATCC 43037 wild-type (A), UB4 wild-type (B), and ATCC 43037 Δ*tfsAB* (C). Red/yellow: *T. forsythia*, cyan: *C. rectus;* green: non-hybridized cells (DNA staining YoPro-1+Sytox). Scale bars 20 µm (A, B) and 15 µm (C)

and CLSM analysis were performed for a qualitative evaluation of the biofilm structure.

Similar to the *T. forsythia* ATCC 43037 wild-type, both the ATCC 43037 Δ*pseC* and the ATCC 43037 Δ*wecC* mutants were detected at the biofilm surface. Whereas Δ*pseC* was also found singly dispersed and in pronounced cell clusters at the biofilm surface, with only very few cells being detected (Figure 5A), Δ*wecC* formed dense superficial clusters (Figure 5B). The S-layer-deficient mutant *T. forsythia* ATCC 43037 Δ*tfsAB* was observed as small microcolonies scattered along the surface as well as in the form of single cells dispersed in the upper layers of the biofilm (Figure 5C).

Tannerella forsythia ATCC 43037 Δ*wecC* formed clearly distinguishable aggregates with *P. gingivalis* OMZ925, an effect that was not observed for the other *T. forsythia* strains and mutants analyzed. In contrast to biofilms incorporating other *T. forsythia* strains and mutants, in Δ*wecC* biofilms, cells appeared to grow less dense, as seen by YoPro-1+Sytox staining of non-hybridized bacteria. *Porphyromonas gingivalis* OMZ925 appeared to have changed its localization, being detected predominantly at the biofilm surface. This potentially direct interaction of *P. gingivalis* OMZ925 with the Δ*wecC* mutant was followed up in co-aggregation studies (see Supplementary material, Fig. S3B). *Porphyromonas gingivalis* OMZ925 coaggregated with all *T. forsythia* strains at different levels. Significant differences between wild-type and mutant strains or a distinct affinity of *P. gingivalis* OMZ925 for the Δ*wecC* mutant could not be observed in these assays. The truncation of the *O*-glycan, however, was found to affect the autoaggregation of *T. forsythia* (see Supplementary material, Fig. S3A). This could be observed as a strong decrease of the OD_{600} of the cell suspensions and higher percentage of autoaggregation of ATCC 43037 Δ*pseC* (38.1%) compared with *T. forsythia* ATCC 43037 wild-type (1.5%) but also when compared with Δ*wecC* (18.1%), Δ*tfsAB* (4.8%) and $\Delta pseC_{comp}$ (4.7%).

Given the demonstrated growth-promoting effect of *T. forsythia* UB4 wild-type (Figure 3A) and *T. forsythia* ATCC 43037 Δ*tfsAB* (Figure 3A) on *C. rectus* OMZ388, dual FISH stainings were performed to determine a possible coaggregation of these species (Figure 6). In the section of the biofilm shown in the CLSM images, a high number of *T. forsythia* cells was detected for both ATCC 43037 and UB4 wild-type strains. Both were present as single cells throughout the whole biofilm structure as well as in small clusters close to the biofilm surface in the case of the ATCC 43037 wild-type (Figure 6A) and in deeper layers for the UB4 wild-type (Figure 6B), as had been found before (Figure 3B, C). The S-layer mutant Δ*tfsAB* was present in the form of clusters in close proximity to the HA-disc surface in a relatively thin section of the biofilm (Figure 6B). *Campylobacter rectus* OMZ388 cells appeared in the form of irregularly interspersed microcolonies in all layers of the biofilm.

Although it was obvious that, compared with the ATCC 43037 wild-type strain (Figure 6A), *C. rectus* OMZ388 cell numbers in the biofilm were elevated in the presence of the UB4 wild-type strain (Figure 6B) and the ATCC 43037 S-layer mutant Δ*tfsAB* (Figure 6C), co-localization, which would be a prerequisite of a direct interaction

between the bacteria, could not be observed. Also, co-aggregation assays did not show a direct interaction between *C. rectus* OMZ388 and the ATCC 43037 S-layer mutant or UB4 wild-type strain (see Supplementary material, Fig. S3C). Here, as for *P. gingivalis* OMZ925, aggregation was elevated only with the nonulosonic-acid-deficient strains *T. forsythia* ATCC 43037 *ΔpseC* (14.3%) and *T. forsythia* UB4 *ΔlegC* (9.5%) when compared with ATCC 43037 wild-type (4.8%) and UB4 wild-type (4.1%) (see Supplementary material, Fig. S3C).

4 | DISCUSSION

The purpose of this study was to analyze different *T. forsythia* wild-type strains and selected mutants thereof with defined differences in cell surface composition with regard to their behavior in a multispecies biofilm community. First we showed that the monospecies biofilm lifestyle of *T. forsythia* was clearly influenced by its S-layer and attached *O*-glycan. Alteration of the *T. forsythia* cell surface composition significantly reduced the capability of the bacterium to form monospecies biofilms as evidenced previously with the nonulosonic-acid-deficient mutants ATCC 43037 *ΔpseC* and *T. forsythia* UB4 *ΔlegC*.[39] In this study, this effect was confirmed for a mutant with an even more truncated *O*-glycan ATCC 43037 *ΔwecC* as well as for the S-layer-deficient mutant ATCC43037 *ΔtfsAB* (Figure 1). For the nonulosonic-acid-deficient mutants, biofilm formation could be fully restored in the complemented mutants, suggesting a direct correlation between loss of the terminal sugar residue and reduced biofilm formation. These data were derived from biofilm experiments using mucin-coated polystyrene plates and are contradictory to previous findings by others, where on untreated polystyrene, biofilm formation was enhanced for the ATCC 43037 *ΔwecC* mutant.[57] In their natural habitat, mucin provides an initial adhesion site and nutrient source for bacteria and fosters biofilm growth.[58] Mucin coating introduces highly hydrophilic properties to the otherwise hydrophobic polystyrene surface.[59] Bacterial adhesion and interaction is influenced by hydrophobic interactions as well as steric forces and charge effects.[60] The decrease of biofilm formation of strains that lack one (*ΔpseC*, *ΔlegC*) or more (ΔwecC) charged sugar residues on a hydrophilic surface documented in this study vs the previously observed opposite effect on a hydrophobic surface[57] shows that biofilm behavior is decisively influenced by the properties of the surface provided for attachment.

In this study, we investigated polymicrobial biofilms that approximate the native situation in the oral cavity much better than a planktonic or monospecies biofilm culture and, therefore, constitute an ideal system to examine the growth performance of individual species and strains. When introduced into *in vitro* 10-species subgingival biofilms, alteration of the bacterial cell surface composition as present in the defined mutants did not impair the growth behavior of *T. forsythia* in terms of cell numbers per biofilm (Figure 4A). Interestingly, at the wild-type level, *T. forsythia* UB4 occurred in higher numbers than *T. forsythia* ATCC 43037 (Figures 1, 3A, 4A), which may indicate a better adaptation of *T. forsythia* UB4 to the niche.

Comparison of the planktonic growth of both *T. forsythia* ATCC 43037 and UB4 wild-type strains supported these observations, as UB4 was found to have a shorter generation time and grew to a higher OD_{600} before reaching the stationary phase in comparison with strain ATCC 43037 (see Supplementary material, Fig. S2). To our knowledge the data presented here constitute a first description of the different growth characteristics of these two *T. forsythia* strains in biofilm settings as well as in planktonic form. A preliminary bioinformatic analysis of the genomes of different *T. forsythia* isolates available in databases reflects the variability in the genetic make-up for either Leg or Pse biosynthesis.[39] Considering the differences in biofilm behavior of *T. forsythia* ATCC 43037 and UB4 wild-type and that Leg is a better mimic of the biologically important sialic acid than Pse, this might suggest that the presence of either nonulosonic acid could reflect the adaptation of *T. forsythia* strains to different oral microenvironments.

Whereas in the multispecies biofilms the overall cell numbers remained relatively constant, the distribution of *T. forsythia* changed depending on the bacterium's cell surface composition. Neither the S-layer nor its glycosylation seemed to be required for the bacterium to establish itself in the multispecies community. However, changes thereof influenced *T. forsythia*'s autoaggregation, which was enhanced upon truncation of the *O*-glycan in the mutants *T. forsythia* ATCC 43037 *ΔpseC*, *T. forsythia* ATCC 43037 *ΔwecC*, and *T. forsythia* UB4 *ΔlegC* (see Supplementary material, Fig. S3). Alteration of the cell surface might, therefore, change the way that cells interact with each other within the microcolonies and multispecies cell aggregations. As cell surface glycosylation affected biofilm formation on a mucin-coated surface (Figure 1), it is tempting to speculate that the decreased ability to adhere to the heavily sialylated salivary glycoprotein mucin in a monospecies biofilm setting is mirrored in the multispecies consortium in a way that *T. forsythia* cell surface mutants might exhibit an altered capability to adhere to sialic-acid-like structures present on other oral bacteria, such as streptococci or *Campylobacter* species[37,38] and, thereby, vary their localization within the multispecies consortium.

From the other bacterial species in the multispecies biofilm, *C. rectus* OMZ388 seems to be strongly affected by the *T. forsythia* cell surface composition. Upon presence of the *T. forsythia* ATCC 43073 *ΔtfsAB* mutant, which is deficient for the S-layer and, hence, also the attached *O*-glycans, *C. rectus* OMZ388 was increased in its cell numbers per biofilm (Figures 3A, 4A, 6C). Hence, it is conceivable that in the native multispecies situation, the glycosylated S-layer as an entity (ATCC 43037) might have a regulatory role in keeping *C. rectus* cell numbers below a certain threshold. In fact, a previous proteomic analysis of *T. forsythia* biofilms identified the two S-layer proteins TfsA and TfsB to be upregulated in comparison with the planktonic cells,[61] which underlines the importance of the S-layer for the biofilm lifestyle of the bacterium. The causative factors and underlying mechanism for the increased growth of *C. rectus* OMZ388 in biofilms harboring the *T. forsythia* ATCC 43037 Δ*tfsAB* mutant still await further investigation. Structural analysis of these biofilms and coaggregation assays performed so far suggest that the observed growth effect is independent of a direct interaction between the two species (Figure 6, and see Supplementary material, Fig. S3). *Campylobacter rectus* is often associated with periodontal

disease[5,62] where it occurs in elevated numbers in the deep periodontal pockets.[63,64] *Campylobacter* spp. have long been described as of clinical relevance.[65,66] They are Gram-negative, microaerophilic bacteria whose motility is conferred by a single polar, glycosylated flagellum.[67] Even though little has been described about *C. rectus* flagellar glycosylation, the bacterium possesses the genetic make-up for Pse biosynthesis (V. Friedrich, M. L. Braun, S. Bloch, C. Schäffer, unpublished observation) and, interestingly, also covers its cells with a 2D S-layer.[67-69]

For *P. gingivalis* and *T. forsythia*, a direct synergistic interaction has been described previously, albeit for another strain.[70] In the biofilms analyzed in this study, *P. gingivalis* OMZ925 seemed to strongly co-localize with *T. forsythia* ATCC 43037 *ΔwecC* (Figure 4B) but was not affected in its growth by the *T. forsythia* cell surface composition (Figure 4). Coaggregation of *P. gingivalis* OMZ925 with *T. forsythia* did not differ significantly between *T. forsythia* wild-type strains ATCC 43037 and UB4 and their respective mutants and a preferential direct interaction of *P. gingivalis* OMZ925 with *T. forsythia* ATCC 43037 *ΔwecC* could not be observed. *Porphyromonas gingivalis* outer membrane vesicles enhance attachment to and invasion of epithelial cells by *T. forsythia*,[71] co-infection of *T. forsythia* and *P. gingivalis* increases abscess formation in a mouse model,[72] and *T. forsythia* cell extracts have been shown to have a growth-promoting effect on *P. gingivalis*.[73] In support of the synergistic interaction between the two species, Bao *et al*. described, in the very same experimental model as used here, reduced growth of *T. forsythia* in multispecies biofilms containing a *P. gingivalis* Lys-gingipain-deficient strain.[12] However, despite this observation, the molecular mechanism of coaggregation between the two pathogens is still unclear.[74]

In conclusion, the present study shows that the growth of *T. forsythia* in an *in vitro* multispecies biofilm, as represented by the "Zurich biofilm model", does not depend on the bacterium's cell surface composition. Deletion of one or more sugars (*T. forsythia* ATCC 43073 *ΔpseC*, *ΔwecC*, *T. forsythia* UB4 *ΔlegC*) has a disadvantageous effect on neither the biofilm growth of *T. forsythia*, nor on overall cell numbers in the biofilm. *Tannerella forsythia* is able to establish itself in the multispecies consortium even without an S-layer (*T. forsythia* ATCC 43073 *ΔtfsAB)*. These findings suggest that the glycosylated S-layer of *T. forsythia* does not play a crucial role in regulating the bacterium's growth in a multispecies biofilm. Nevertheless, we observed that it affected the bacterium's localization in the biofilm, the interaction with *C. rectus*, for which the glycosylated S-layer has a growth retarding effect, and its co-localization with *P. gingivalis*, which is increased upon a three-sugar truncation of the O-glycan in the *T. forsythia* ATCC 43037 *ΔwecC* mutant. Hence, changes in the S-layer and surface glycosylation of *T. forsythia* might actually contribute to the bacterium's virulence potential by promoting structural arrangements within in the biofilm. Whether this contributes to the immune evasion of the biofilm-associated species needs to be tested in functional interaction assays with host cells.

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to this study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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2.1.1. Supporting material for publication I

SUPPORTING MATERIAL AND METHODS

Planktonic growth of *T. forsythia*

In order to evaluate the growth kinetics of both *T. forsythia* ATCC 43037 and UB4 wild-type strains as well as their respective mutants, bacteria were grown for 3 days in BHI supplemented with *N*-acetylmuramic acid, horse serum and 50 µg/ml gentamycin. From these cultures, 10 ml of fresh BHI was inoculated at a final OD_{600} of 0.05. The OD_{600} was measured in 24 hour intervals for 8 d [1]. Growth curves were obtained from three individual experiments with two technical replicates each.

Coaggregation assay

Coaggregation assays were performed according to Shimotahira *et al.* [1]. In brief, *T. forsythia* wild-type and mutant strains were grown in BHI supplemented with *N*-acetyl-muramic acid, horse serum and gentamycin. *Porphyromonas gingivalis* (OMZ925) was cultivated in BHI (without supplements), *C. rectus* OMZ388 in mFUM supplemented with 0.1% NaFF. All strains were harvested by centrifugation and resuspended in coaggregation buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, and Tris-HCl [pH 8.0]). The suspensions were adjusted to an OD_{600} of 0.5. Coaggregation partner strains were mixed in a 1:1 ratio in a disposable cuvette. Additionally, for each strain the autoaggregation was determined by measuring single bacterial cells only. Suspensions were incubated at room temperature and were measured at 15-min intervals using a spectrophotometer.

Decreases in absorbance indicated cell aggregates precipitating to the bottom of the cuvette. The percentage of aggregation (AI) was calculated as: $\mathrm{AI} = \frac{(\text{initial OD600} - \text{post incubation OD600})}{\text{initial OD600}} \times 100$. Data represent mean values ±SD of five independent experiments with three replicates each for coaggregation *P. gingivalis* and four independent experiments with three replicates each for coaggregation with *C. rectus*. Statistical analysis was performed using the unpaired Student's t-Test. Asterisks indicate significant differences (*, $P < 0.05$).

SUPPORTING TABLES

Table S1. Doubling times of *T. forsythia* wild-type and mutant strains used in this study. Doubling times (mean values ±SD) are shown. Significant differences between strains were determined by the unpaired Student's t-Test ($P \leq 0.05$); * significantly higher *versus T. forsythia* ATCC 43037 wild-type and complemented strain Δ*pseC*$_{comp}$, ¶ significantly higher *versus T. forsythia* ATCC 43037 wild-type, # significantly higher *versus T. forsythia* ATCC 43037 Δ*wecC.*

***T. forsythia* strain**	**Doubling time (h) ±SD**	**Significance**
ATCC43037 WT	14.41 ±1.41	
ATCC43037 Δ*pseC*	25.59 ±9.29	*#
ATCC43037 Δ*wecC*	17.80 ±2.38	¶
ATCC43037 Δ*tfsAB*	20.74 ±5.76	*
ATCC43037 Δ*pseC*$_{comp}$	14.99 ±3.64	
UB4 WT	16.75 ±9.58	
UB4 Δ*legC*	19.07 ±8.40	
UB4 Δ*legC*$_{comp}$	12.28 ±3.75	

SUPPORTING FIGURES

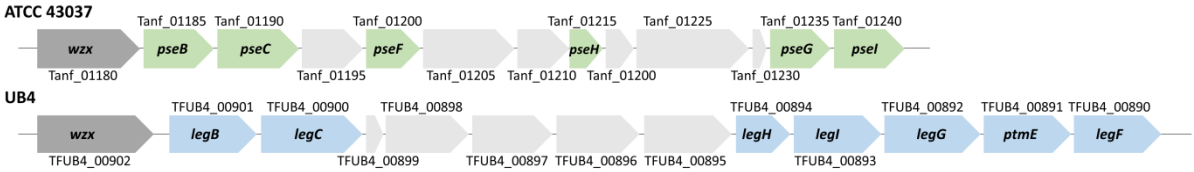

Figure S1. Nonulosonic acid biosynthesis locus of *T. forsythia* ATCC 43037 and *T. forsythia* UB4 wild-type strains. Pse biosynthesis genes (*pseB*, *pseC*, *pseH*, *pseG*, *pseI* and *pseF*) present in the type strain ATCC 43037 are shown in red. Corresponding genes for Leg biosynthesis (*legB*, *legC*, *legH*, *legI*, *legG*, *legF* and a gene encoding a predicted nucleotidyl transferase (*ptmE*) are shown in green for the UB4 strain [2]. NCBI locus tags are shown for each gene.

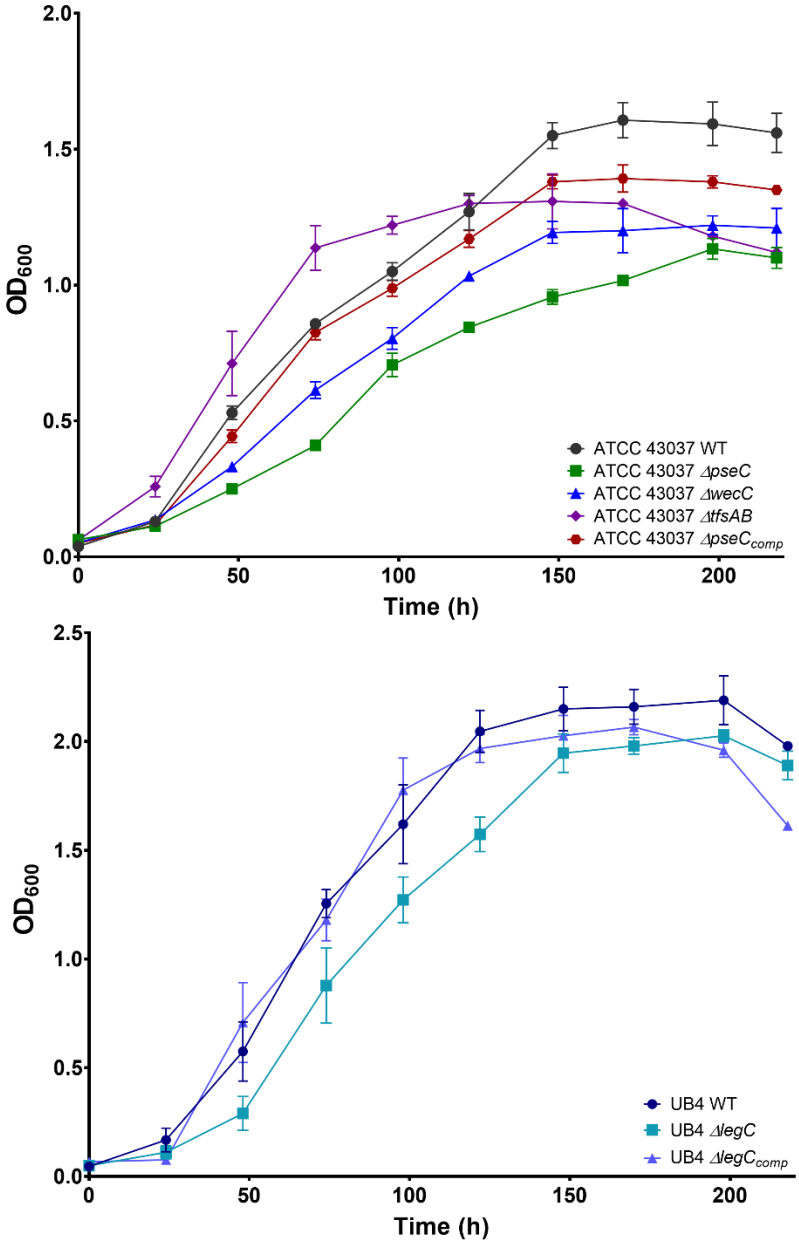

Figure S2. Growth curves of *T. forsythia* wild-type strains and mutants included in this study. Growth of *T. forsythia* strains was monitored by measuring the OD_{600} at 24-hour intervals in three independent experiments with two technical replicates per strain. Data are presented as mean ±SEM. **(A)** Growth curves of ATCC 43037 wild-type, ATCC 43037 Δ*pseC*, Δ*wecC,* Δ*tfsAB* mutants, and complemented strain Δ$pseC_{comp}$ and **(B)** *Tannerella forsythia* UB4 wild-type, UB4 Δ*legC* mutant and complemented strain Δ$legC_{comp}$.

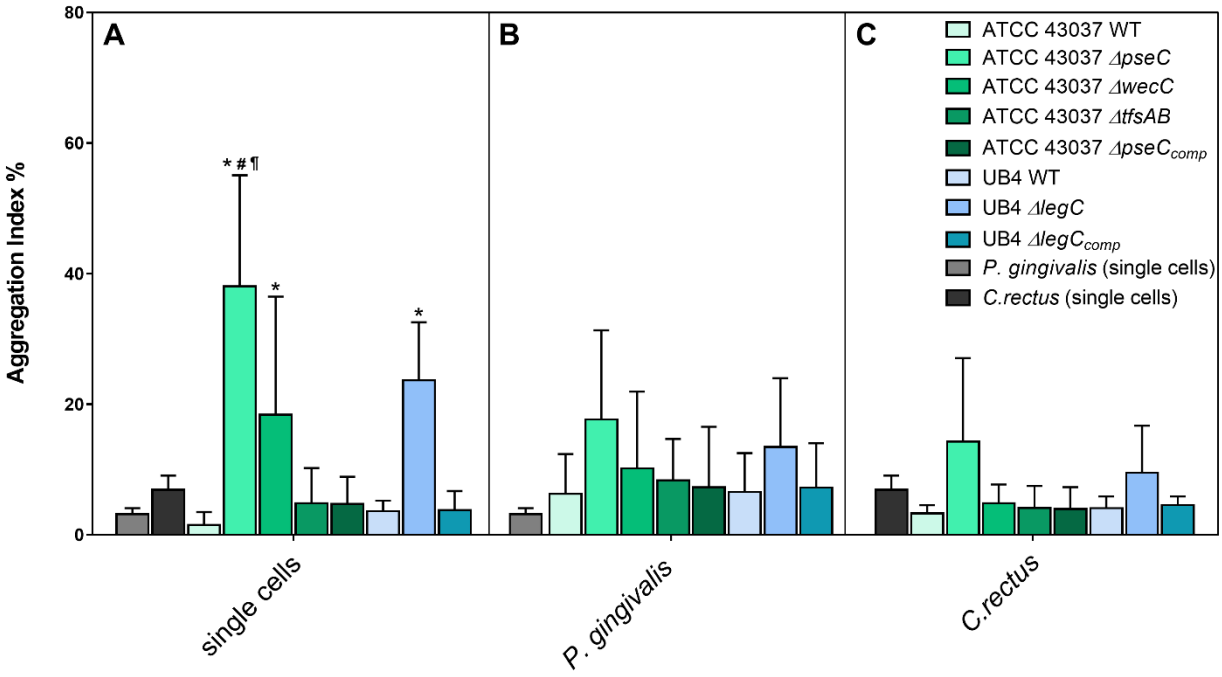

Figure S3. Aggregation of *T. forsythia* wild-type strains and mutants with *P. gingivalis* and *C. rectus*. Each *T. forsythia* strain was mixed with its co-aggregation partner in a 1:1-ratio and the OD_{600} of the suspension was measured in 15-minute intervals. For each strain, the autoaggregation (AI) was calculated as: $\mathrm{AI} = \frac{(\text{initial OD600} - \text{post incubation OD600})}{\text{initial OD600}} \times 100$.

The coaggregation rate (CR) was determined as: $\mathrm{CR} = \frac{(\mathrm{AIx+AIy})}{2} - \frac{\mathrm{AI.x+y}}{(\mathrm{AIx+AIy})/2}$, , where AI_x and AI_y represent the aggregation of one species alone and AI_{x+y} represents the aggregation of the mixture of *T. forsythia* with *P. gingivalis* or *C. rectus*, respectively [3]. Mean values ±SD of five independent experiments for coagggregation with *P. gingivalis* and four independent experiments for coagggregation with *C. rectus* are shown. Statistical significance was tested by ANOVA (Tukey's post-hoc test for multiple comparisons, P≤0.05) using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA); * significantly higher *versus* parental wild-type and complemented strain, ¶ significantly higher *versus T. forsythia* ATCC 43037 Δ*tfsAB*, # significantly higher *versus T. forsythia* ATCC 43037 Δ*wecC*.

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2.2. Publication II: Immune response profiling of primary monocytes and oral keratinocytes to different *Tannerella forsythia* strains and their cell surface mutants

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ORIGINAL ARTICLE

WILEY molecular oral microbiology

Immune response profiling of primary monocytes and oral keratinocytes to different *Tannerella forsythia* strains and their cell surface mutants

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Summary

The oral pathogen *Tannerella forsythia* possesses a unique surface (S-) layer with a complex *O*-glycan containing a bacterial sialic acid mimic in the form of either pseudaminic acid or legionaminic acid at its terminal position. We hypothesize that different *T. forsythia* strains employ these stereoisomeric sugar acids for interacting with the immune system and resident host tissues in the periodontium. Here, we show how *T. forsythia* strains ATCC 43037 and UB4 displaying pseudaminic acid and legionaminic acid, respectively, and selected cell surface mutants of these strains modulate the immune response in monocytes and human oral keratinocytes (HOK) using a multiplex immunoassay. When challenged with *T. forsythia*, monocytes secrete proinflammatory cytokines, chemokines and vascular endothelial growth factor (VEGF) with the release of interleukin-1β (IL-1β) and IL-7 being differentially regulated by the two *T. forsythia* wild-type strains. Truncation of the bacteria's *O*-glycan leads to significant reduction of IL-1β and regulates macrophage inflammatory protein-1. HOK infected with *T. forsythia* produce IL-1Ra, chemokines and VEGF. Although the two wild-type strains elicit preferential immune responses for IL-8, both truncation of the *O*-glycan and deletion of the S-layer result in significantly increased release of IL-8, granulocyte-macrophage colony-stimulating factor and monocyte chemoattractant protein-1. Through immunofluorescence and confocal laser scanning microscopy of infected HOK we additionally show that *T. forsythia* is highly invasive and tends to localize to the perinuclear region. This indicates, that the *T. forsythia* S-layer and attached sugars, particularly pseudaminic acid in ATCC 43037, contribute to dampening the response of epithelial tissues to initial infection and hence play a pivotal role in orchestrating the bacterium's virulence.

KEYWORDS
monocytes, oral keratinocytes, periodontal disease, S-layer glycosylation, *Tannerella forsythia* immune response

1 | INTRODUCTION

Periodontitis is a chronic inflammation of the gingiva and tooth-supporting tissues, including periodontal ligament and alveolar bone, and is induced by the polymicrobial community that is clinically described as oral plaque.[1] Up to 90% of the worldwide population is affected by periodontal disease, with 10%-15% of patients suffering from severe forms, in which, if left untreated, inflammation causes

irreversible tooth loss.[2] Additionally, periodontitis is associated with an increased risk to develop rheumatoid arthritis,[3,4] atherosclerosis, cardiovascular diseases[5,6] and cancer.[7] The pathogenesis of periodontitis is still not fully understood; it involves a complex interplay of bacterial, genetic and environmental factors causing growth promotion of dysbiotic, predominantly Gram-negative, anaerobic bacteria that facilitate the progression of inflammation.[1] A number of potential pathogens promoting the onset of the disease have been identified through high-throughput sequencing, and metagenomic, metatranscriptomic as well as mechanistic studies.[8-10] These include the pathogens *Tannerella forsythia*, *Porphyromonas gingivalis* and *Treponema denticola* constituting the so called "red complex", a group of bacteria clearly associated with periodontal disease and classified as highly virulent.[2,11-13] Through the expression of virulence factors, these bacteria are able to colonize and persist in the host and promote the destruction of gingival tissues.[14-16] In the interaction with the gingival tissues and underlying immune cells, such as monocytes and macrophages which are present in high numbers in periodontal lesions,[17] the "red complex" bacteria induce the secretion of cytokines such as interleukin-1β (IL-1β), IL-6, IL-8 and tumor necrosis factor-α (TNF-α), which contribute to the exacerbation of inflammation.[18-20]

For *T. forsythia*, several virulence factors have been described; among them are components of the cell surface.[21-24] The bacterium possesses a characteristic surface (S-) layer covering the entire cell.[25] This is composed of the two S-layer proteins TfsA and TfsB,[26] both of which are modified with a complex, branched dekasaccharide *O*-glycosidically linked to multiple serine and threonine residues within a D(S/T)(A/I/L/M/T/V) amino acid target motif.[27] Protein glycosylation is a post-transcriptional modification employed by numerous pathogenic bacteria to modulate the host immune response, and glycosylated appendages like pili or flagella play a vital role in orchestrating invasion and infection.[28] Interestingly, the terminal sugar of the *T. forsythia O*-glycan is unique to bacteria and varies in its stereochemistry in different strains of *T. forsythia*. In the *T. forsythia* ATCC 43037 type strain, it is present as a modified pseudaminic acid (Pse, 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-manno-non-2-ulosonic acid), whereas isolate *T. forsythia* UB4 was found to carry a modified legionaminic acid (Leg, 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid) instead.[29] These sugars belong to the structural class of nonulosonic acids, acidic nine-carbon (C9) α-keto sugars, which are best represented by the sialic acid family.[30,31] The two *T. forsythia* strains ATCC 43037 and UB4 vary not only in the terminal nonulosonic acid of the otherwise identical *O*-glycan,[29] but also in displaying different growth characteristics, monospecies biofilm formation and behavior in a multispecies biofilm consortium.[25] Both the *T. forsythia* S-layer and the attached glycan, especially the nonulosonic acids, were found to facilitate monospecies biofilm formation and the localization of *T. forsythia* within a multispecies biofilm.[25,29]

The glycosylated S-layer of *T. forsythia* ATCC 43037 was shown to suppress the production of proinflammatory cytokines in U937 macrophages and human gingival fibroblasts,[32] to modulate the immune response through recognition by the macrophage inducible C-type lectin (Mincle)[33] and facilitate adhesion to and invasion of gingival epithelial cells.[22] Outer membrane vesicles of *T. forsythia* ATCC 43037, naturally carrying the intact glycosylated S-layer, were found to induce expression of TNF-α and IL-8 in U937 macrophages, and IL-6, IL-8 and MCP-1 in primary human periodontal ligament fibroblasts.[34] Taking into account a potential role of the *O*-glycan itself in the interaction with the host, as opposed to the S-layer and attached glycans as an entity, the terminal trisaccharide branch of the *T. forsythia* ATCC 43037 *O*-glycan, containing two *N*-acetylmannosaminuronic acid residues in addition to the terminal Pse residue, has been reported to play a role in the modulation of dendritic cell effector functions, suppressing T helper type 17 (Th 17) responses and ensuring the persistence of the bacterium in the host.[35]

The function of specifically the nonulosonic acids Pse and Leg in the interaction with the host, however, has not yet been elucidated for *T. forsythia*. These sugar acids have been shown to influence the virulence potential of other pathogens such as *Campylobacter jejuni*,[36,37] and because of their similarity to sialic acids, they can generally orchestrate a bacterium's interaction with the host through molecular mimicry and immune evasion. This mechanism might contribute to the development of periodontal inflammation. In the early stage, periodontal inflammation is characterized by an influx of neutrophils. Also monocytes are among the first cells of the immune system to arrive at the site of inflammation and, therefore, play a vital part in orchestrating the acute reaction to bacterial infection.[38] The most abundant cells of the oral mucosa are keratinocytes and fibroblasts, whose primary functions are to protect the underlying tissue against invading pathogens and to initiate a first immune response when pathogens invade.[39]

The aim of this study was (i) to investigate how the two different *T. forsythia* strains ATCC 43037 and UB4 modulate the host immune response, and (ii) to assess a potential role of the bacterial cell surface composition in this process, using a set of defined knockout mutants. The induction of cytokine and chemokine expression upon challenge with these *T. forsythia* wild-type strains and mutants was assayed in human monocytes and human oral keratinocytes, representing two cell types from the first line of defense in the response to acute infection during periodontal inflammation.

2 | METHODS

2.1 | Bacterial strains

In this study, *T. forsythia* wild-type strains ATCC 43037 (type strain, American Type Culture Collection, Manassas, VA) and *T. forsythia* UB4[40] as well as defined mutants thereof were cultured anaerobically in brain-heart infusion (BHI) broth (Oxoid, Basingstoke, UK) and BHI agar plates, supplemented with *N*-acetylmuramic acid and horse serum as described previously.[25] Mutants with characterized defects in protein glycosylation included *T. forsythia* ATCC 43037 Δ*pseC* (deficient in Pse), *T. forsythia* ATCC 43037 Δ*wecC* (deficient in a trisaccharide glycan branch including Pse[27,41]) and *T. forsythia* UB4 Δ*legC* (deficient in Leg[25,29]). Further, an S-layer-deficient mutant *T. forsythia* ATCC 43037 Δ*tfsAB*,[22] lacking both S-layer proteins TfsA and TfsB as well as the attached glycans was included in this study (Figure 1).

2.2 | Monocyte isolation and cell culture

2.2.1 | Monocytes

Peripheral blood mononuclear cells were isolated from buffy-coated blood from a total of six healthy blood donors using Ficoll-Hypaque gradient centrifugation (BD Diagnostics, Franklin Lakes, NJ) followed by monocyte isolation using an EasySep Human monocyte enrichment kit without CD16 depletion according to the manufacturer's protocols (StemCell Technologies, Vancouver, Canada). Monocytes were seeded at 10^5 cells mL^{-1} in 24-well plates in RPMI media supplemented with 10% fetal bovine serum and 1% GlutaMax (all from Gibco-Brl/Life Technologies, Paisley, UK) and cultured at 37°C in 5% CO_2 without active passaging.

2.2.2 | Primary human oral keratinocytes

Human oral keratinocytes (HOK) were purchased from ScienCell Research Laboratories (Carlsbad, CA) and cultured in oral keratinocyte medium (OKM SciencCell, Carlsbad, CA) supplemented with oral keratinocyte growth serum (OKGS) at 37°C in 5% CO_2. Cells were split into 24-well plates when reaching 70%-80% confluency. For infection as well as invasion assays, cells between passage 2 and 7 were used.

2.3 | Infection of human monocytes and HOK with *T. forsythia* wild-types and mutants

Monocytes from three healthy donors, each, were infected with viable *T. forsythia* wild-type strains ATCC 43037 or UB4 for 6 h or 24 h at a multiplicity of infection (MOI) of 10, 100 and 1000, and with the cell surface mutants ATCC 43037 *ΔpseC*, *ΔwecC*, *ΔtfsAB* as well as UB4 *ΔlegC*, respectively, at MOI of 100 for 6 h as indicated in the figure legends. Cell culture supernatants were harvested, centrifuged and protein secretion was analyzed.

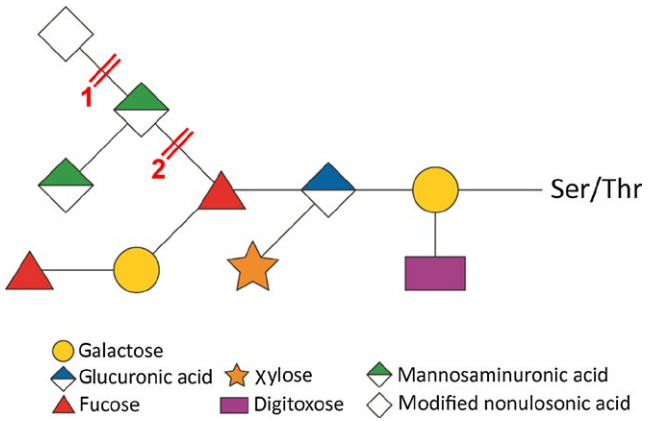

FIGURE 1 Structure of the *Tannerella forsythia* *O*-glycan adapted from Friedrich et al.[29] Red lines indicate the deletion of the terminal nonulosonic acid in the mutants *T. forsythia* ATCC 43037 *ΔpseC* and *T. forsythia* UB4 *ΔlegC* (1) and the three-sugar truncation of the *O*-glycan in *T. forsythia* ATCC 43037 *ΔwecC* (2), respectively

HOK were infected for 6 h with *T. forsythia* wild-type and mutants when reaching 80% confluency with 1×10^7 bacterial cells mL^{-1}, corresponding to MOI of 100. Cell culture supernatants were harvested and centrifuged, and the protein secretion was analyzed.

Additionally, bacterial survival under the given culture conditions was analyzed by serial dilution plating and colony-forming unit (CFU) counting. For this, 5 µL of inoculum, 5 µL of *T. forsythia* cultured in RPMI at 37°C in 5% CO_2 for 6 h and 24 h, respectively, or 5 µl of culture supernatant after 6 h and 24 h of infection were spotted in triplicate from each donor on BHI broth (Oxoid) agar plates, supplemented with *N*-acetylmuramic acid and horse serum and incubated anaerobically at 37°C for 7-10 days before determining CFU counts.

2.4 | Determination of cytokine, chemokine and growth factor levels in cell culture supernatants

Release of cytokines into the cell culture supernatants after infection with *T. forsythia* was measured by IL-1β and IL-8 enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (DuoSet ELISA, R&D Systems, Abingdon, UK). Further, the cellular response of monocytes and HOK to infection with the respective *T. forsythia* strains was analyzed using the Human Cytokine Magnetic 30-Plex Panel (Invitrogen, Carlsbad, CA). Analytes were quantified on a BioRad Luminex 100 plate reader (Bio-Rad Laboratories Inc., Hercules, CA).

2.5 | Determination of IL-1β degradation by *T. forsythia* wild-types ATCC 43037 and UB4

To analyze whether degradation of IL-1β by *T. forsythia* wild-type strains ATCC 43037 and UB4 occurs under the given culture conditions, 10^7 bacterial cells mL^{-1}, corresponding to a cell number equivalent to that used for stimulation of monocytes at MOI of 100, were cultured at 37°C and 5% CO_2 in 300 µL cell-free RPMI spiked with 1000 pg mL^{-1} of IL-1β for 6 h and 24 h, respectively. Spiked medium without bacteria served as control. Interleukin-1β levels after incubation with or without bacteria were determined using an IL-1β ELISA (R&D Systems, Minneapolis, MN, USA).

2.6 | 5-Bromo-2′-deoxyuridine assay

HOK (1.5×10^4 mL^{-1}) were seeded into a 96-well plate and infected with *T. forsythia* wild-type or mutants at a bacterial cell number of 1.5×10^6 mL^{-1}. Cell proliferation was determined using a colorimetric BrdU (5-bromo-2`-deoxyuridine) assay, which was performed according to the manufacturer's protocol (Roche, Stockholm, Sweden).

2.7 | Analysis of keratinocyte invasion by *T. forsythia* wild-types and mutants

HOK were cultured in chamber slides (Sarstedt, Hildesheim, Germany) in OKM supplemented with OKGS and stimulated for 6 h with 3×10^6 bacterial cells, pre-stained with SYTO 17 Red Fluorescent Nucleic Acid stain (Invitrogen). Bacterial cells were harvested by centrifugation,

resuspended in BHI containing 5 μM SYTO17, briefly vortexed and stained for 30 min at 37°C. Stained bacteria were washed in 1× phosphate-buffered saline (PBS) and resuspended in OKM for the infection of HOK. Before fixation, infected HOK were washed in 1 × PBS and treated with OKM containing penicillin/streptomycin for 90 min to kill extracellular bacteria. Subsequently, cells were fixed in ice-cold acetone for 5 min followed by blocking with 10% normal goat serum in PBS for 1 h at room temperature. HOK were stained overnight at 4°C in a wet chamber using a mouse cytokeratin-5 antibody (Abcam, Cambridge, UK) diluted in blocking solution. The secondary antibody goat anti-mouse IgG conjugated with Alexa Fluor® 488 (Invitrogen, Carlsbad, CA) was diluted in blocking buffer and incubated for 1 h at room temperature in a wet chamber in the dark. ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen) was used to mount the slides. HOK without *T. forsythia* infection and staining with secondary antibody alone served as controls. Samples were analyzed using a Leica SP-5 microscope (Vienna Institute of Biotechnology Imaging Center, Universität für Bodenkultur Wien). Images were captured using a 63× objective and processed with FIJI IMAGE Software.[42]

2.8 | Statistical analysis

Statistical differences were analyzed by the unpaired Student's *t*-test, and paired comparisons were performed by the Mann–Whitney *U*-test using GraphPad PRISM version 7.00 for Windows (GraphPad Software, La Jolla, CA). Data are expressed as mean ± SD. Differences were considered to be statistically significant at $P < .05$.

3 | RESULTS

3.1 | *Tannerella forsythia* infection leads to cytokine and chemokine release in monocytes

Infection of human monocytes with *T. forsythia* wild-types ATCC 43037 and UB4 at an MOI of 10, 100 and 1000 for 6 h and 24 h, respectively, resulted in release of IL-1β and IL-8 under all tested conditions (Figure 2A, B). Stimulation at an MOI of 100 showed the most stable results, and for both *T. forsythia* strains, highest cytokine secretion was observed after 24 h of infection; however, at this time-point, bacterial viability of both strains was significantly reduced compared with 6 h as well as compared with the inoculum at t_0 (Figure 2C). Since release of IL-1β was reduced at an MOI of 1000, the ability of the bacteria to degrade IL-1β was assessed (Figure 2D). After 24 h of incubation, levels of spiked IL-1β in cell-free culture medium were reduced when compared with the earlier time-point of 6 h; however, the presence *of T. forsythia* did not affect IL-1β levels at either time-point (Figure 2D).

3.2 | Altered cytokine release after infection of monocytes with *T. forsythia* ATCC 43037 or UB4

Based on the previous results, monocytes were infected with *T. forsythia* wild-types ATCC 43037 and UB4 at an MOI of 100 and incubated for 6 h. We assessed cytokine, chemokine and growth factor release by multiplex analysis. Only 10 out of 30 assayed chemokines, cytokines and growth factors were detected in the culture supernatant, including IL-1β, IL-6, IL-7, IL-8, IL-15, macrophage inflammatory protein 1α (MIP-1α), macrophage inflammatory protein 1β (MIP-1β), monocyte chemoattractant protein 1 (MCP-1), regulated on activation normal T cell expressed and secreted (RANTES) and vascular endothelial growth factor (VEGF) (Figs 2 and 3). All of the released factors were strongly induced when compared with the untreated control, for which the levels of most analytes were too low for detection in the multiplex assay, except for IL-15 and RANTES, where no significant induction was observed upon stimulation with either strain. Increased secretion of IL-1β and IL-6 was detected irrespective of whether infection was with the ATCC 43037 or UB4 wild-type. Furthermore, IL-1β secretion was higher in response to ATCC 43037 than to UB4. The secreted levels of IL-7 were only increased after infection with UB4 wild-type, compared with the untreated control. Chemokine release of MIP-1α, MIP-1β, MCP-1 and IL-8 was increased after infection independent of the *T. forsythia* strain. The same pattern was detected for the release of VEGF, which was significantly elevated upon stimulation with either *T. forsythia* wild-type strain compared with the untreated control (Figure 3).

3.3 | The *T. forsythia* cell surface modulates the immune response in monocytes

Next, we investigated if the bacterial cell surface composition has an impact on the immune response in monocytes. For this purpose, we infected monocytes with defined *T. forsythia* strains and analyzed the release of cytokines, chemokines, and growth factors. Following stimulation with *T. forsythia* wild-type or mutants, monocytes retained their viability, and no significant cytotoxic effects of either strain on the cells was observed, as determined by a lactate dehydrogenase release assay (see Supplementary material, Figure S1).

When comparing the response to infection by the *T. forsythia* ATCC 43037 cell surface mutants and the parent wild-type strain, IL-1β concentrations in the culture supernatants showed a significant decrease upon infection with *T. forsythia* ATCC 43037 *ΔwecC* and were also slightly reduced upon infection with *T. forsythia* ATCC 43037 *ΔpseC* and *T. forsythia* ATCC 43037 *ΔtfsAB* (Figure 4A). On the contrary, deletion of the terminal Leg in *T. forsythia* UB4 *ΔlegC* resulted in slightly, albeit not significantly, increased IL-1β secretion in comparison to UB4 wild-type (Figure 4E).

For the two chemokines MIP-1α and MIP-1β, a similar regulation trend was observed in response to the two *T. forsythia* wild-type strains. For ATCC 43037, their levels were reduced significantly only upon stimulation with *T. forsythia* ATCC 43037 *Δpse* (Figure 4B, C), but increased upon stimulation with *T. forsythia* UB4 *ΔlegC* (Figure 4F, G), compared with the UB4 wild-type. Similarly, the secretion of the chemokine RANTES was significantly upregulated in monocytes stimulated with *T. forsythia ΔlegC* (Figure 4H). In the case of ATCC 43037 wild-type and mutants, RANTES secretion was slightly increased upon stimulation with these strains, but there were no significant differences between groups (Figure 4D).

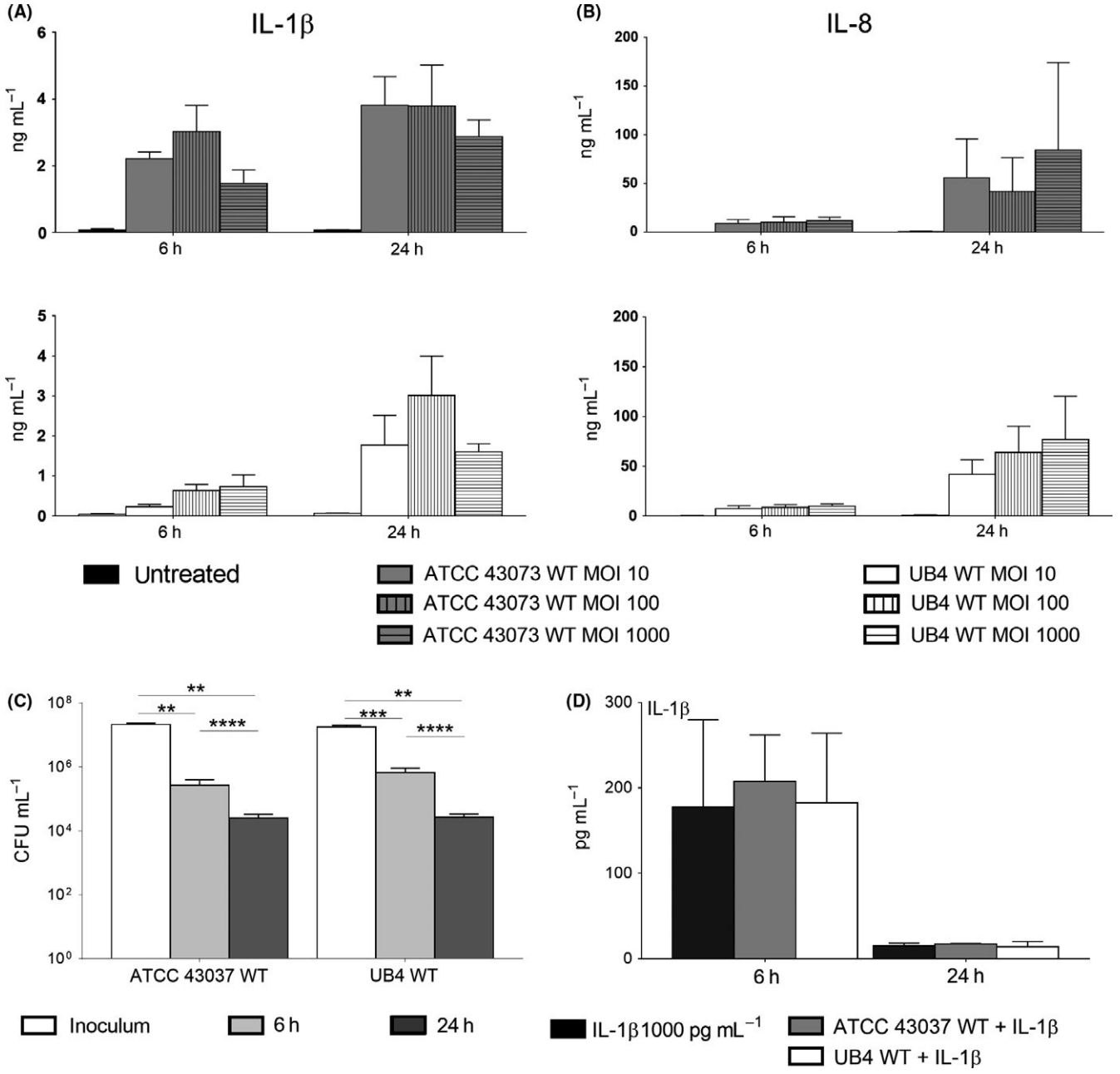

FIGURE 2 Response of human monocytes to infection with *Tannerella forsythia* ATCC 43037 and UB4. (A,B) Monocytes from three healthy donors were infected with *T. forsythia* at an MOI of 10, 100 or 1000 for 6 h and 24 h with three technical replicates, each. Interleukin-1β (IL-1β) (A) and IL-8 (B) concentrations in the culture supernatant as determined by ELISA are shown as mean ± SD. (C) Bacterial viability in RPMI after 6 h and 24 h at 37°C in 5% CO_2 compared with the inoculum at the start of the infection. Bacteria were spotted in triplicate for each donor and cell numbers were determined by CFU counting. Mean values ± SD were plotted on a logarithmic scale. Asterisk (*) indicates significant differences between samples as determined by the unpaired Student's t-test (**$P \leq .01$, ***$P \leq .001$, ****$P \leq .0001$). D, Levels of spiked IL-1β after incubation for 6 h and 24 h in cell-free RPMI at 37°C and 5% CO_2 and in the presence of either *T. forsythia* ATCC 43037 or UB4 wild-type bacteria. IL-1β levels were determined by ELISA are shown as mean values ± SD

For IL-6, MCP-1 and IL-8, stimulation with *T. forsythia* ATCC 43037 *ΔpseC* resulted in a slight reduction of cytokine secretion in comparison with the parental wild-type ATCC 43037 (see Supplementary material, Figure S2A, D, E), whereas in the case of *T. forsythia* UB4 *ΔlegC*, a slight increase of cytokine secretion occurred (see Supplementary material, Figure S2G, J, K).

It is noteworthy, that for all *T. forsythia* strains survival was very low, but increased in the presence of monocytes. Viability of wild-type and respective mutants differed in culture medium and in monocyte culture supernatant (see Supplementary material, Figure S3). In RPMI as well as in cell culture supernatant, the S-layer-deficient strain *ΔtfsAB* showed increased survival; in the presence

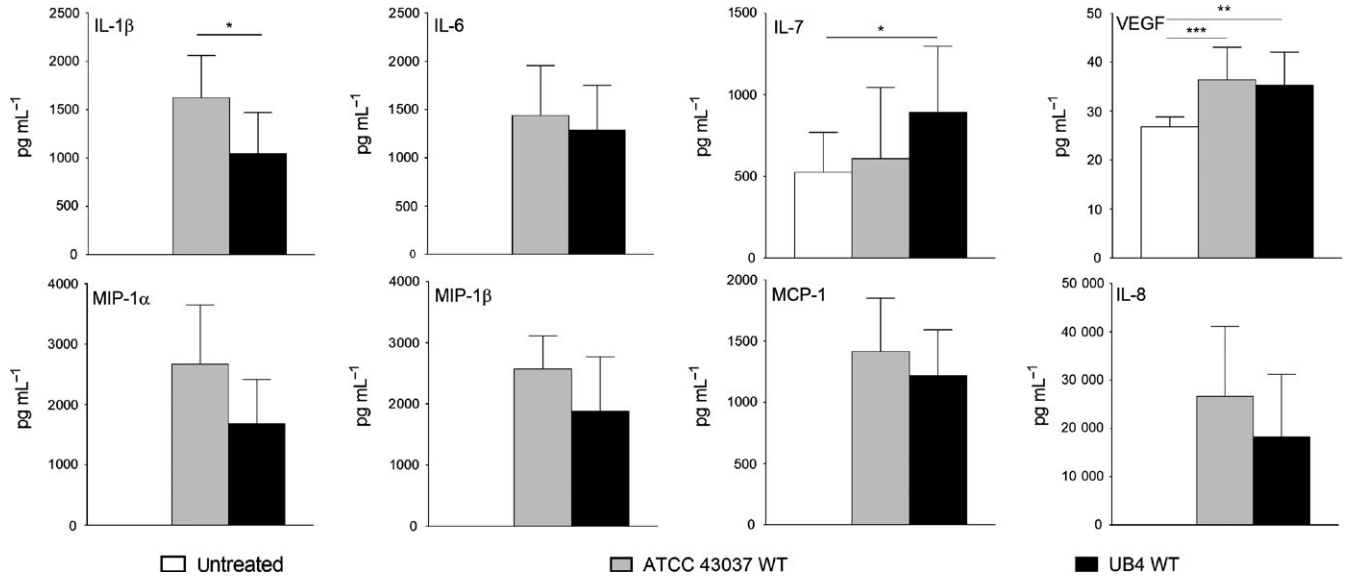

FIGURE 3 Secretion of cytokines, chemokines and growth factors by human monocytes from three healthy donors following infection with viable cells of *Tannerella forsythia* ATCC 43037 and *T. forsythia* UB4, respectively, at an MOI 100. Concentrations in the culture supernatants were measured using a multiplex (30-plex) immunoassay and showed strong induction of interleukin-1β (IL-1β), IL-6. Macrophage inflammatory protein 1α (MIP-1α), MIP-1β, IL-8 and vascular endothelial growth factor (VEGF). IL-7 was induced only in the presence of UB4, whereas stimulation with ATCC 43037 resulted in a higher production of IL-1β than infection with UB4. Data are shown as mean values ± SD. Asterisks indicate statistically significant differences between groups as determined by the unpaired Student's *t*-test (**P* ≤ .05, ***P* ≤ .01, ****P* ≤ .001)

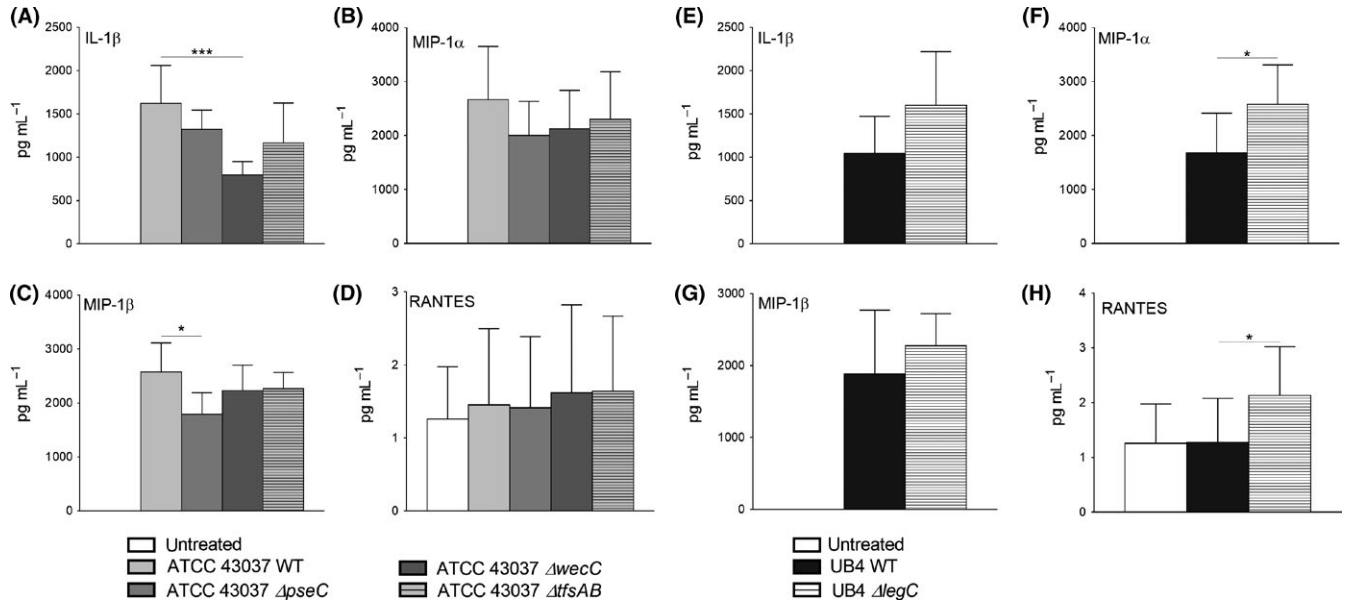

FIGURE 4 Bar graphs showing secretion of cytokine Interleukin-1β (IL-1β) and chemokines macrophage inflammatory protein 1α (MIP-1α) and MIP-1β by human monocytes determined by a 30-plex immunoassay upon infection with *T. forsythia* ATCC 43037 wild-type or mutants (*ΔpseC*, *ΔwecC*, *ΔtfsAB*) (A-D) and UB4 wild-type or mutant (*ΔlegC*), respectively, at MOI 100 (E-F) as mean values ± SD for three healthy donors with three technical replicates each. Asterisks indicate significant differences (**P* ≤ .05, ****P* ≤ .001) between wild-type or mutant strain and an untreated control or mutant strain and respective parental wild-type

of monocytes, UB4 *ΔlegC* was found to have a much higher survival rate than its parental strain (see Supplementary material, Figure S3). For ATCC 43037 *ΔpseC* on the other hand, < 1% of the bacteria survived cultivation in both RPMI and cell culture supernatant (see Supplementary material, Figure S3A).

3.4 | *Tannerella forsythia* cell surface modulates the immune response in HOK

Keratinocytes are the first line of defense, and are the first cells in contact with the potentially invading bacteria of the oral biofilm.

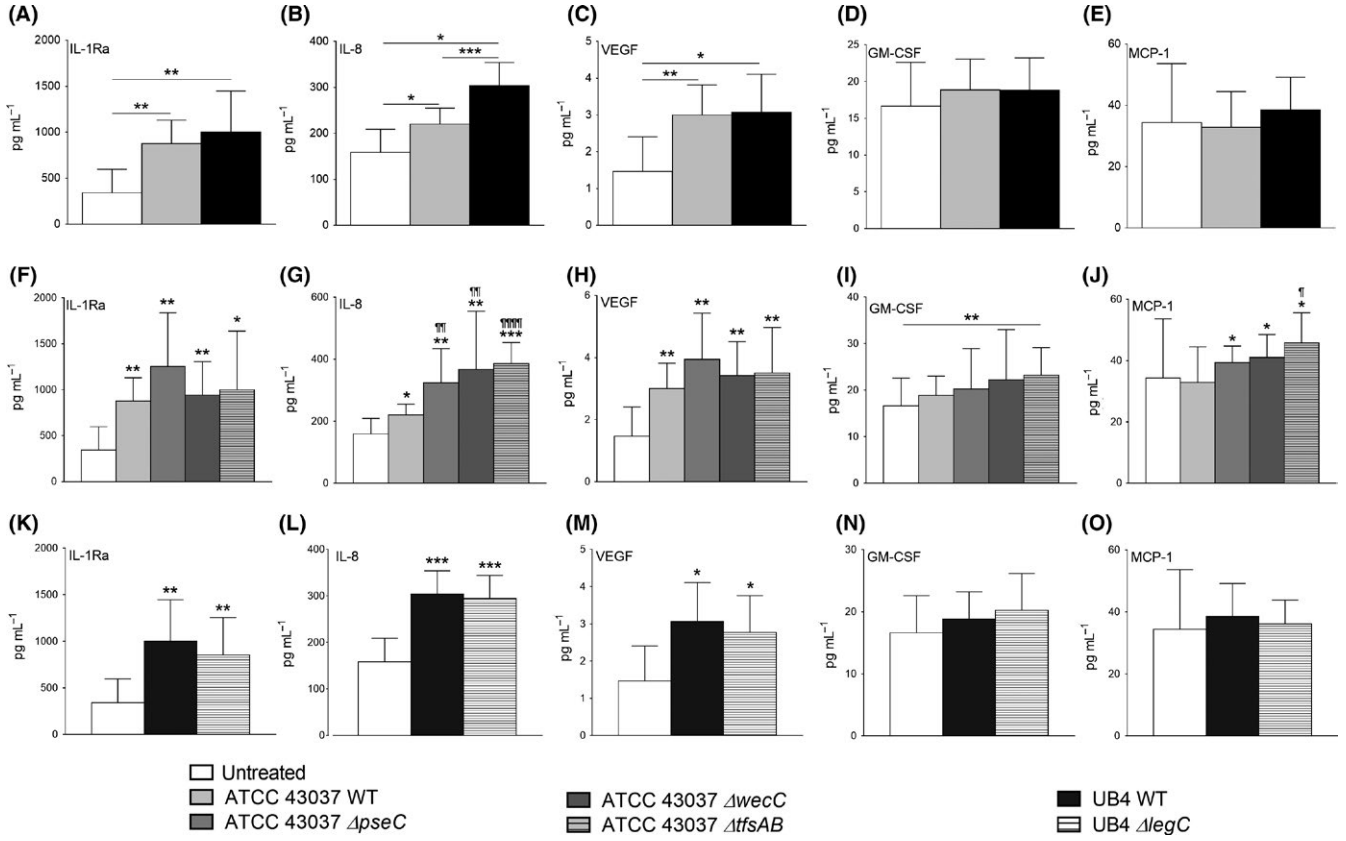

FIGURE 5 Bar graphs showing secretion of cytokines, chemokines and growth factors by human oral keratinocytes following infection with viable cells of *Tannerella forsythia* wild-type strains (A-E), *T. forsythia* ATCC 43037 mutants (Δ*pseC*, Δ*wecC*, Δ*tfsAB*) (F-J) and UB4 mutant (Δ*legC*) (K-O), respectively, at MOI 100. Mean values ± SD of three independent experiments with three replicates, each, are shown. Significant differences between wild-type or mutant strain and an untreated control (*) or mutant strain and respective parental wild-type (¶) are shown. (*/¶ $P \leq .05$, **/¶¶ $P \leq .01$, ***/¶¶¶ $P \leq .001$)

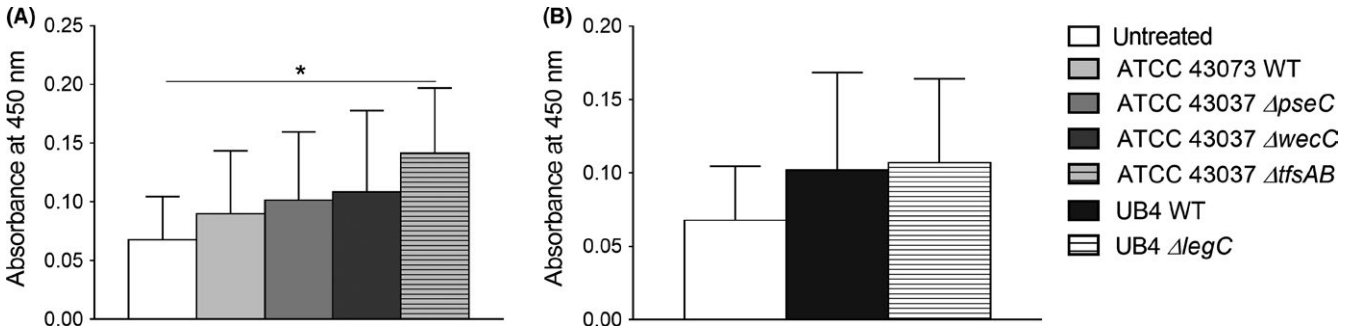

FIGURE 6 Influence of cell surface mutants of *T. forsythia* ATCC 43037 (A) and UB4 (B) on human oral keratinocyte cell proliferation as determined using a BrdU assay. Data from three independent experiments performed in triplicates are shown. Asterisks (**) indicate significant differences ($P \leq .01$)

Therefore, we analyzed the immune response of HOK after infection with *T. forsythia* ATCC 43037 or UB4 wild-type strains and compared the response to infections with selected cell surface mutants. The concentrations of nine out of 30 secreted chemokines and cytokines assayed were quantified in the culture supernatant, including IL-6, IL-1 receptor antagonist (IL-1Ra), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-15, interferon-γ-inducible protein 10 (IP-10), IL-8, MCP-1 and VEGF (Figure 5A-E). We detected higher amounts of IL-1RA, IL-8 and VEGF after stimulation with the wild-type strains (Figure 5A-C), with the *T. forsythia* UB4 wild-type being a more potent inducer of IL-8 secretion in HOK, compared with the *T. forsythia* ATCC 43037 wild-type (Figure 5B). Release of GM-CSF and MCP-1 did not change upon stimulation with either *T. forsythia* wild-type strain (Figure 5D, E).

Stimulation of HOK with bacteria that lacked the terminal modified Pse residue (Δ*pseC*) or a trisaccharide branch (Δ*wecC*) of the

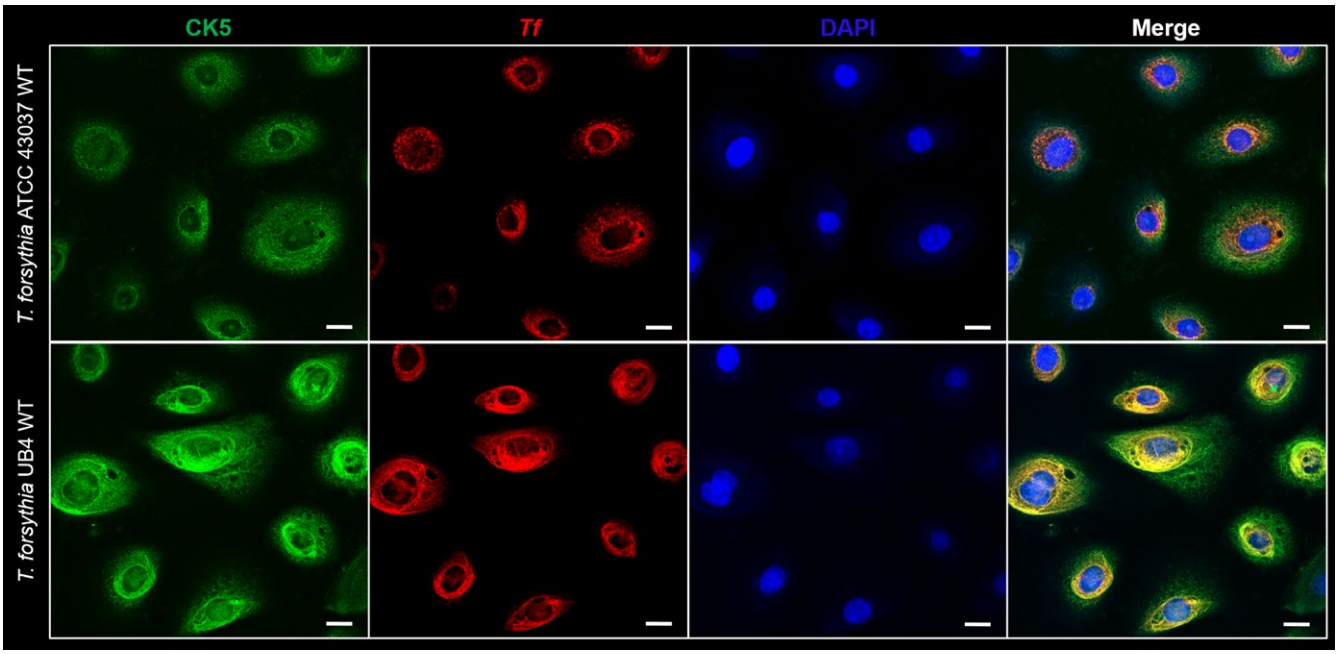

FIGURE 7 Invasion of human oral keratinocytes by *T. forsythia* ATCC 43037 and UB4. *T. forsythia* cells were stained using Syto17 DNA dye (red), human oral keratinocytes were visualized by immunofluorescence using a cytokeratin 5 (CK5) specific primary antibody and a goat anti-mouse IgG secondary antibody conjugated with Alexa Fluor® 488 (green) as well as the nuclear DAPI staining (blue). Results are representative of two independent experiments. Objective 63×; scale bar 20 μm

S-layer *O*-glycan, or were depleted of the S-layer and attached glycan as a whole (Δ*tfsAB*), significantly enhanced production of IL-8, both when compared with the untreated control as well as with cells infected with ATCC 43037 wild-type (Figure 5G). In the case of *T. forsythia* ATCC 43037 Δ*pseC*, IL-1Ra and VEGF levels were also slightly, albeit not significantly, increased, when compared with those observed upon stimulation with the parental wild-type strain (Figure 5F, H). Infection with *T. forsythia* ATCC 43037 cell surface mutants also modulated the secretion of the chemokine MCP-1, which was not observed upon challenge with *T. forsythia* wild-type strain (Figure 5J). The S-layer-deficient mutant Δ*tfsAB* significantly stimulated MCP-1 secretion by HOK, compared with controls or cells infected with ATCC 43037 wild-type. Only this mutant could significantly induce secretion of GM-CSF (Figure 5I, N) and also IP-10 secretion was enhanced in the presence of *T. forsythia* Δ*tfsAB* (see Supplementary material, Figure S4B). No significant differences were observed between cells stimulated with UB4 wild-type and its mutant Δ*legC* (Figure 5K-O, and see Supplementary material, Figure S4C).

3.5 | Influence of *T. forsythia* cell surface mutants on HOK cell proliferation

As we detected differences in the immune responses of HOK to *T. forsythia* we next investigated if the two *T. forsythia* wild-type strains or their cell surface mutants can affect cell proliferation by employing a BrdU-assay. HOK proliferation was increased after infection with ATCC 43037 Δ*tfsAB*, but no other differences in cell proliferation were detectable (Figure 6A, B).

3.6 | *Tannerella forsythia* invades HOK and locates to the perinuclear region

Fluorescence image analysis was employed to investigate, if *T. forsythia* was able to invade HOK and to what extent this depended on the composition of the bacterium's cell surface. After infection of HOK with *T. forsythia*, extracellular bacteria were eliminated through antibiotic treatment, and cell invasion was assessed. At an MOI of 100, *T. forsythia* appeared to be highly invasive and notably localized close to the nucleus within the keratinocytes (Figure 7, and see Supplementary material, Figure S5). When comparing the two wild-type strains ATCC 43037 and UB4 (Figure 7) and their respective mutants (see Supplementary material, Figure S6), all strains seemed to be able to enter HOK independently of their cell surface mutation, exhibiting the same pattern of cellular localization detected predominantly in the perinuclear region. Additionally, the survival of all strains during the SYTO17 staining before infection was analyzed. For both wild-type strains as well as the *T. forsythia* ATCC 43037 Δ*pseC* mutant, 20%-25% of the bacteria survived the staining procedure, whereas for the Δ*wecC* and Δ*tfsAB* mutants, approximately 12% and for Δ*legC*, only 7% viable cells could be recovered (see Supplementary material, Figure S5).

4 | DISCUSSION

The periodontal pathogen *T. forsythia* employs sophisticated strategies to persist in the environment of subgingival biofilms and interact with host tissues and immune system. In this study, we show

that *T. forsythia* modulates the immune response in primary monocytes and oral keratinocytes. In more detail, we detected increased expression of proinflammatory cytokines, chemokines and VEGF in monocytes, whereas HOK showed an increased release of IL-1Ra and chemokines. Furthermore, we detected differences in the release of these factors when comparing the two different wild-type strains *T. forsythia* ATCC 43037 and *T. forsythia* UB4 *per se* and mutants with altered cell surface composition. These results highlight a possible biological role of the bacterium's cell surface composition in the interaction with the host.

A prominent characteristic of *T. forsythia* is its elaborate cell surface structure consisting of the two-dimensional crystalline S-layer with attached, highly complex *O*-glycans.[26] The two *T. forsythia* wild-type strains ATCC 43037 and UB4 differ in the terminal sugar residue of an otherwise identical dekasaccharide, which is present as a modified Pse in *T. forsythia* ATCC 43037 and a modified Leg in *T. forsythia* UB4.[29] Including in this study mutants with selected cell surface defects, we investigated to which extent the molecular mimics of sialic acid—Pse and Leg—as well as the *T. forsythia* S-layer and attached glycan influence the host immune response.

Monocytes, when challenged with *T. forsythia*, produced IL-1β, IL-6, MIP-1α, MIP-1β, MCP-1, IL-8 and VEGF (Figure 3). Although IL-1β secretion was reduced upon infection with the clinical isolate UB4 when compared with the ATCC 43037 reference strain, the former induced secretion of IL-7, as opposed to the latter. On the other hand, challenging HOK with either strain resulted in the secretion of IL-1RA, VEGF and IL-8. The chemokine IL-8 was found in higher levels after infection with UB4 wild-type, when compared with the ATCC 43037 wild-type. Hence, these cell types respond in a different and—in the case of IL-1β, IL-7 and IL-8—strain-specific manner to *T. forsythia* infection.

Both IL-8 and IL-1β have been shown to be induced by viable *T. forsythia* in macrophages and human gingival fibroblasts as well as a macrophage/epithelial cell co-culture model[32,43] and are significantly increased in patients with periodontitis.[44] Interleukin-6 was found to be induced by *T. forsythia* in the same co-culture model[43] as well as in human peripheral blood monocytes, the latter using heat-inactivated instead of viable bacteria.[45] Like monocytes, gingival epithelial cells produce IL-8 in response to infection with biofilms containing the three "red complex" bacteria (i.e. *T. forsythia*, *P. gingivalis* and *Treponema denticola*), exhibiting differential responses in the absence of one or more of these strains.[46] In human gingival fibroblasts, infection with *T. forsythia* ATCC 43037 wild-type alone induced IL-8 secretion.[32] In the present study, IL-8 secretion by HOK was also stimulated upon infection with both *T. forsythia* wild-type strains and, interestingly, UB4 elicited a stronger response than the ATCC 43037 strain.

The chemokines MIP-1α, MIP-1β and MCP-1, which are induced in monocytes upon infection with either strain, have previously been found in elevated levels in gingival tissues from patients with aggressive as well as chronic periodontitis.[47] During inflammation, a local production of these chemokines regulates the infiltration of inflammatory cells, such as macrophages/monocytes and T helper type 1 cells.[48] Infection of HOK with either *T. forsythia* ATCC 43037 or UB4, also induced the release of IL-1Ra, an anti-inflammatory cytokine blocking ligand receptor binding of IL-1β.[49,50] Levels of IL-1Ra are increased in gingival crevicular fluid from periodontitis patients[51] and IL-1Ra levels in saliva have been shown to correlate with the severity of the disease;[52] however, induction of IL-1Ra by *T. forsythia* has not been described.

In contrast, VEGF stimulates migration of endothelial cells, and thereby vascularization, as well as migration of monocytes/macrophages into the inflamed tissue.[53] This is the first report of *T. forsythia* directly inducing VEGF secretion in monocytes. Also in HOK, VEGF was strongly induced by either *T. forsythia* strain. In patients with periodontitis, VEGF has been found in elevated levels in the epithelium and during disease progression, remodeling of the vasculature of the periodontal connective tissues can be observed.[54–56]

In the context of periodontal disease, IL-8 and MCP-1 are among the first factors to be released by the inflamed tissue, with the intent to recruit neutrophils and monocytes to the site of infection.[57] While IL-8 functions in the acute phase of inflammation, MCP-1 secretion is usually prolonged and results in a delayed monocyte recruitment, contributing to a sustained inflammation.[58] The proinflammatory cytokines IL-1β and IL-6 act in the induction of bone resorption and mediate tissue destruction.[59] in addition, IL-7 plays a role in periodontal bone resorption and activates the production of osteoclastogenic cytokines by T cells.[60] Interestingly, a correlation between infection with *T. forsythia* and secretion of IL-7 has not been reported and this might be a trait specific to certain strains of *T. forsythia*, such as UB4 (Figure 3).

Through the analysis of immunofluorescence staining of HOK infected with both *T. forsythia* wild-types we found both strains to be highly invasive and to localize in the perinuclear region of keratinocytes. Bacteria of the subgingival biofilms colonize the gingival epithelium as an important virulence mechanism to avoid host immune surveillance.[61,62] Among them, *P. gingivalis* shows the same cellular localization close to the nucleus, where endoplasmic reticulum, Golgi, mitochondria, endosomes and lysosomes are present.[63] *Tannerella forsythia* has been shown to invade epithelial cells both *in vivo*[64,65] and *in vitro*—the latter in dependence of the surface-associated protein BspA and the S-layer.[22,66,67] Its entry into the cells is proposed to be effected via phosphoinositide 3-kinase activation and clathrin-coated vesicles,[67] which might facilitate trafficking to the perinuclear compartment in the form of endosomes. Given the reduced viability of *T. forsythia* in culture medium, as seen for RPMI and monocyte cell culture supernatant, and during SYTO17 staining (see Supplementary material, Figures S4, S5), invasion of HOK observed here might be a strategy of the bacteria to survive under unfavorable culture conditions. However, the presented observations do not constitute a quantitative assay of cell invasion and bacterial viability within HOK; further, a possible implication of the particular intracellular localization has yet to be elucidated.

As previous studies analyzing the cytokine release induced by *T. forsythia* focused only on the ATCC 43037 type strain, we here provide a first description of the differential immune response to different strains of *T. forsythia*, namely ATCC 43037 and UB4. UB4 was

previously found to have an advantage over *T. forsythia* ATCC 43037 in planktonic as well as biofilm growth and shows a varied localization in a multispecies biofilm, suggesting an altered adaptation to this environmental condition.[25] Here, we additionally show that both strains are highly invasive in HOK and elicit differential immune responses in monocytes as well as in keratinocytes.

In order to analyze whether these effects occur in dependence of the bacterium's cell surface composition, we included a set of defined cell surface mutants in our study. In monocytes, we found that the cell surface composition influences the release of cytokines and chemokines. Truncation of the *O*-glycan in *T. forsythia* ATCC 43037 results in significantly decreased secretion of IL-1β (Δ*wecC*) (Figure 4A) and MIP-1β (Δ*pseC*) (Figure 4C). Previous studies described a dampening effect for the S-layer[32] as well as the terminal trisaccharide branch of the *O*-glycan[35] on the response to *T. forsythia* infection as also evidenced by studies using *T. forsythia* ATCC 43037 S-layer mutant Δ*tfsAB*[23] and Δ*wecC*.[26] In our experimental setup, we observed that cytokine levels upon infection with these strains were lower or equal compared with infection with the parental wild-type strain. The presence of nonulosonic acids—such as Pse and Leg—has been suggested to promote the survival of bacterial pathogens during bloodstream infections[68] and virulence.[37] We observed a drastically decreased survival of *T. forsythia* both in the presence of monocytes and in cell-free culture medium. The S-layer-deficient strain Δ*tfsAB* was more resistant to cultivation in RPMI and cell culture supernatant when compared with its parental strain and the mutants Δ*wecC* and Δ*pseC*; especially the latter displayed a drastically reduced survival under the given culture conditions. For *T. forsythia* UB4 Δ*legC*, on the other hand, a higher number of viable bacteria was recovered from the culture supernatant after infection of monocytes when compared to the parental wild-type (see Supplementary material, Figure S3). Deletion of Leg also resulted in elevated cytokine release, as seen for MIP-1α and RANTES when compared with UB4 wild-type (Figure 4E-H). Being the better mimic of sialic acid in comparison to Pse present in ATCC 43037, this might potentially suggest a more prominent role for Leg than Pse to mask underlying immunogenic surface structures on the *T. forsythia* *O*-glycan in this experimental setup. Bacterial pathogens employ glycosylation of cell surface structures in multiple ways. Flagellar glycosylation in *Camplylobacter jejuni*, for instance, facilitates the bacterium's virulence.[37] Pse residues on the flagella have been found to interact with Siglec-10 on bone-marrow-derived dendritic cells of mice promoting the production of the anti-inflammatory cytokine IL-10, so indirectly contributing to immune suppression.[69] In the opportunistic pathogen *Pseudomonas aeruginosa* on the other hand, flagellar glycosylation orchestrates the bacterium's virulence by stimulating IL-8 release, which is drastically reduced in glycosylation-deficient strains.[70]

In HOK, we could confirm previous findings for the role of *T. forsythia* ATCC 43037 cell surface glycosylation in the immune response[31] and found that *O*-glycan truncation by one (Δ*pseC*) or more sugars (Δ*wecC*) as well as deletion of the complete S-layer (Δ*tfsAB*) resulted in increased release of IL-8 and induction of MCP-1 (Figure 5 G, J). The S-layer-deficient strain was the only one to induce GM-CSF (Figure 5 I). For HOK, these findings support the hypothesis that the S-layer might function in delaying the host immune response.[28]

Due to S-layer deficiency in the Δ*tfsAB* mutant, the R-type lipopolysaccharide of the outer membrane is exposed,[71] which has been found to be a potent inducer of the chemokines IL-8 and RANTES.[43] The strong induction of chemokine production by the Δ*tfsAB* mutant in this experimental setup might stem from this loss of masking of the R-type lipopolysaccharide in the mutant, which in this way can contribute to a stronger immune response, as seen for IL-8, MCP-1 and GM-CSF (Figure 5 G, I, J). For IL-8 and MCP-1 secretion, we showed that the *O*-glycan, specifically the terminal modified Pse residue, plays a role in this process. *Tannerella forsythia* UB4 and its mutant did not differ from each other in their immune response inducing IL-1Ra, IL-8 and VEGF (Figure 5 K, L, M).

GM-CSF influences proliferation of keratinocytes both *in vivo* and *in vitro*,[72,73] so the growth promoting effect of S-layer-deficient *T. forsythia* ATCC 43037 Δ*tfsAB* observed in a BrdU assay (Figure 6) can be explained by the induction of this factor. In periodontal disease, the gingival epithelium constitutes the first line of defense and acts as a physical barrier against invading pathogens. *Porphyromonas gingivalis* invades human epithelial cells, subsequently inhibits apoptosis using the epithelium as a reservoir to persist in the host[74,75] and impedes keratinocyte migration and proliferation during wound healing.[76] It can also dampen the innate immune responses of host cells, such as IL-1β release, when present in biofilms.[77] *Aggregatibacter actinomycetemcomitans* on the other hand, was found to induce apoptotic pathways upon invasion of gingival keratinocytes, promoting tissue destruction and inhibiting epithelial signaling to immune cells in the underlying connective tissue.[75] Also, *T. forsythia* has been shown to be able to invade gingival cells and modulate gene expression in infected tissues[78] and, in this study, we found both *T. forsythia* wild-type strains as well as the cell surface mutants to be able to enter HOK showing the same pattern of perinuclear localization (Figure 7, and see Supplementary material, Figure S5). The fact that the S-layer seems to dampen the GM-CSF response to infection with the bacterium and thereby influences keratinocyte proliferation might suggest a protective role of this structure in the interaction with the gingival epithelium, possibly facilitating persistence of *T. forsythia* within the tissue. However, as it was reported that the S-layer is required for adhesion to and invasion of epithelial cells by *T. forsythia*,[22] it needs to be further investigated if differences in the response to *T. forsythia* ATCC 43037 wild-type and mutants might be influenced by their capability to invade HOK in terms of bacterial cell numbers and in dependence of their cell surface composition.

Changes in the surface glycosylation of *T. forsythia* contribute to the interaction with monocytes as well as keratinocytes, supporting the hypothesis that the bacterium uses this cell surface decoration in the interaction with host tissues and immune system. We found that the two *T. forsythia* strains ATCC 43037 and UB4 differ in the response they elicit in immune cells as well as HOK. Infection of HOK with *T. forsythia* results in the secretion of chemokines involved in the recruitment of specialized immune cells, and IL-1Ra, seemingly controlling inflammation. On the other hand, in monocytes, it causes the induction of proinflammatory cytokines and chemokines, strongly

facilitating inflammation. From our data we cannot infer a clear influence of Pse versus Leg in the differential response elicited by the two *T. forsythia* strains. Observed differences rather stem from other strain-specific traits and the factors influencing UB4 virulence mechanisms still await further investigation. The S-layer and attached glycan of the ATCC 43037 type strain, however, clearly modulate the host response both in monocytes, and–more markedly—in the interaction with HOK. In the interaction with oral keratinocytes we clearly confirm that *T. forsythia* surface glycosylation facilitates a dampening of the primary immune response to infection and thereby might promote persistence in the host, especially in the initial phase of periodontal infection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to this study.

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SUPPORTING INFORMATION

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How to cite this article: Bloch S, Zwicker S, Bostanci N, et al. Immune response profiling of primary monocytes and oral keratinocytes to different *Tannerella forsythia* strains and their cell surface mutants. *Mol Oral Microbiol*. 2018;00:1–13. https://doi.org/10.1111/omi.12208

2.2.1. Supporting material for publication II

SUPPORTING MATERIAL AND METHODS

Lactate dehydrogenase release assay

Lactate dehydrogenase (LDH) activity in cell supernatants from isolated monocytes that were infected with *Tannerella forsythia* wild-type or mutants was measured using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions (Figure S1). LDH release was expressed as the percentage of LDH in the medium relative to the total LDH lysate.

Bacterial viability during stimulation of human monocytes with *T. forsythia* wild-type or mutant strains

Bacterial viability was assessed determining CFU counts for inoculum and bacteria cultivated aerobically for 6 h in RPMI both with and without monocytes. For this, 5 µl of the inoculum, 5 µl of *T. forsythia* cultured at 37°C and 5% CO_2 for 6 h in cell-free RPMI or in RPMI containing 10^5 monocytes ml^{-1}, respectively, were spotted in triplicate from each donor on brain–heart infusion broth (Oxoid, Basingstoke, UK) agar plates, supplemented with *N*-acetylmuramic acid and horse serum and incubated anaerobically at 37°C for 7-10 days before determining CFU counts.

Bacterial viability during SYTO17 staining prior to infection of HOK

Bacterial cells were stained in 5 µM SYTO17 for 30 min at 37°C. Stained bacteria were washed and resuspended in 100 µl 1x PBS and the viability of *T. forsythia* wildtype and mutant strains was assessed by serial dilution plating and spotting of stained as well as untreated bacteria on brain–heart infusion broth (Oxoid, Basingstoke, UK) agar plates, supplemented with *N*-acetylmuramic acid and horse serum. After incubation at 37°C for 7-10 days CFU counts for each strain were determined.

SUPPORTING FIGURES

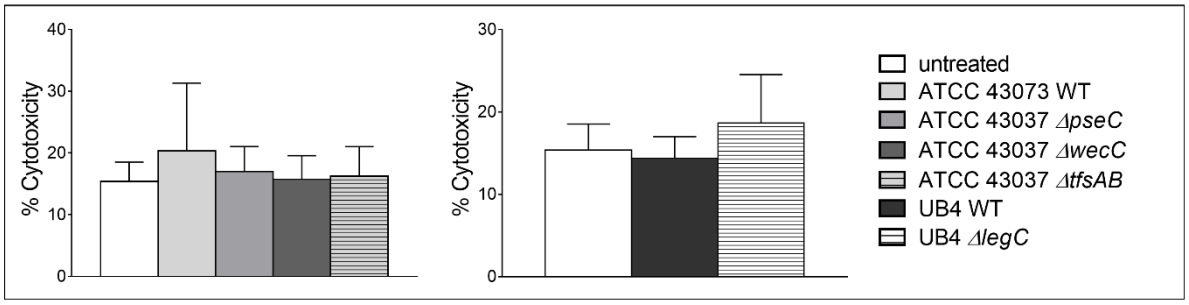

Figure S1. Lactate dehydrogenase release assay. Primary monocytes from three healthy donors were exposed to *T. forsythia* wild-type or mutants for 6 h and cell supernatants were assayed for LDH release to determine potential cytotoxic effects of *Tannerella forsythia* wild-type or mutant strains on the cells. Values are shown as means ± SD (*n*=9).

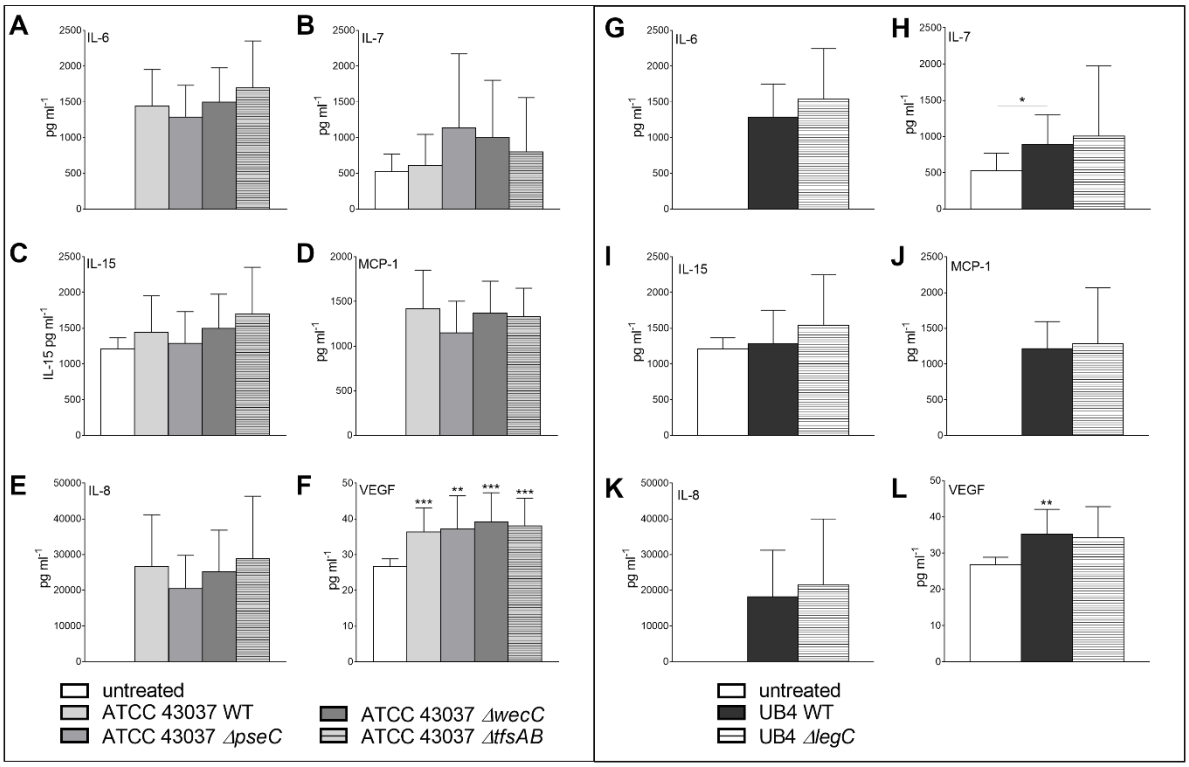

Figure S2. Bar graphs showing secretion of cytokines, chemokines and growth factors by human monocytes from three healthy donors determined by a 30-plex immunoassay upon infection with *T. forsythia* ATCC 43037 wild-type or mutants *(*Δ***pseC***, Δ*wecC*, Δ*tfsAB*) (**A-F**) and UB4 wild-type or mutant (Δ*legC*), respectively (**G-L**), with three technical replicates, each. Data are shown as mean ± SD. Asterisks indicats significant differences between wild-type or mutants and an untreated control or mutant and respective parental wild-type (* *P≤0.05*, ** *P≤0.01, *** P≤0.001*).

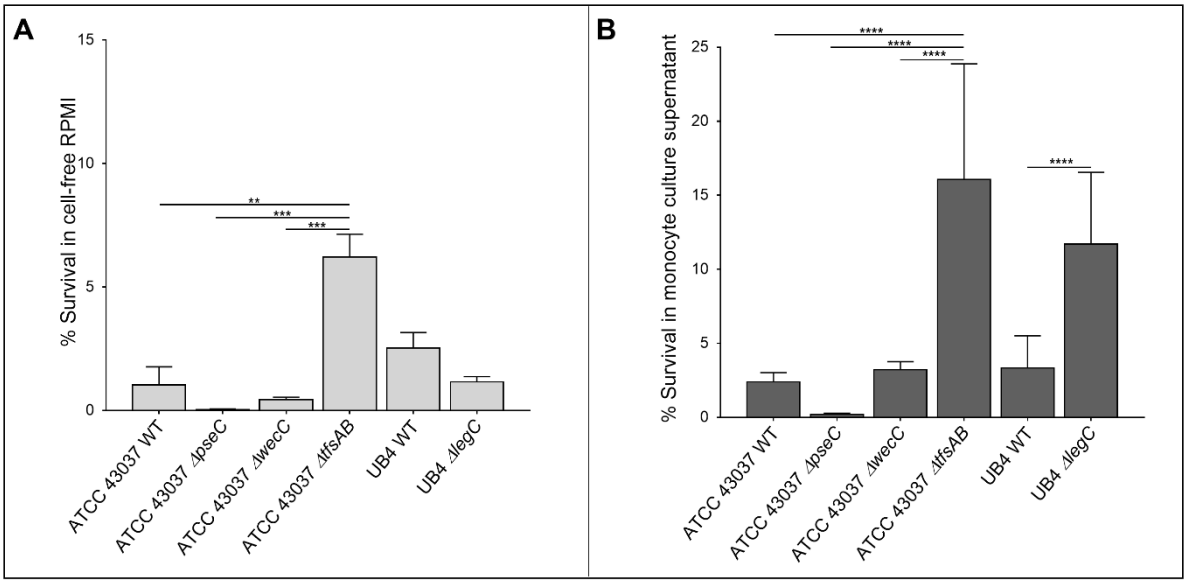

Figure S3. Bar graphs showing survival of *T. forsythia* wild-type and mutant strains normalized to the inoculum after 6 h of cultivation at 37°C and 5% CO_2 in cell-free RPMI (**A**) and after 6 h of stimulation of human monocytes from three healthy donors in RPMI culture supernatant (B). Bacterial cell numbers were determined by spotting bacteria in triplicate for each donor. Bacterial survival was calculated as percentage of the cell number at inoculation for each strain. Statistically significant differences between strains were determined by ANOVA (Tukey's post-hoc test for multiple comparisons (** *P≤0.01*, *** *P≤0.001,* **** *P≤0.0001*).

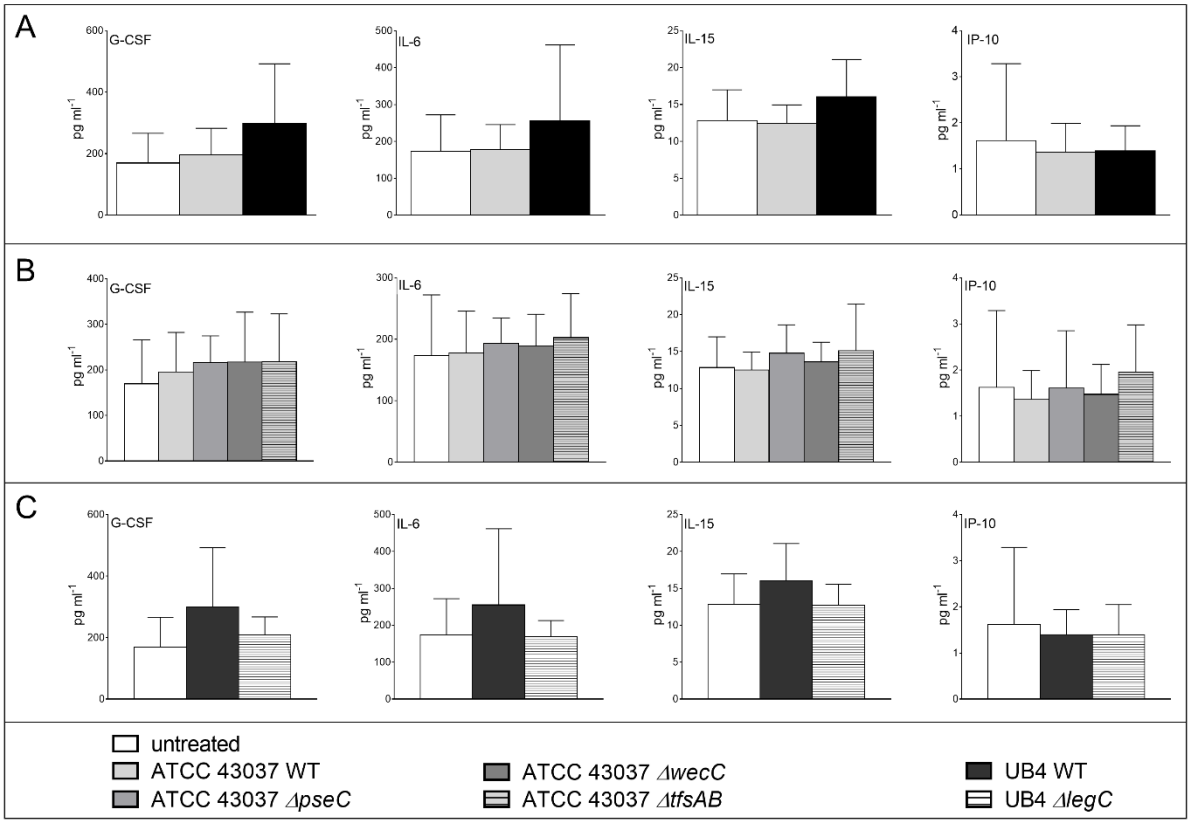

Figure S4. Immune response of human oral keratinocytes to infection with *T. forsythia* ATCC 43037 and UB4 wild-type strains (**A**) and cell surface mutants *T. forsythia* ATCC 43037 Δ*pseC*, Δ*wecC*, Δ*tfsAB* (**B**) and UB4 Δ*legC* (**C**), respectively. Cytokine levels were measured using a 30-plex immunoassay. Data derived from three independent experiments with three technical replicates each are shown as mean ± SD.

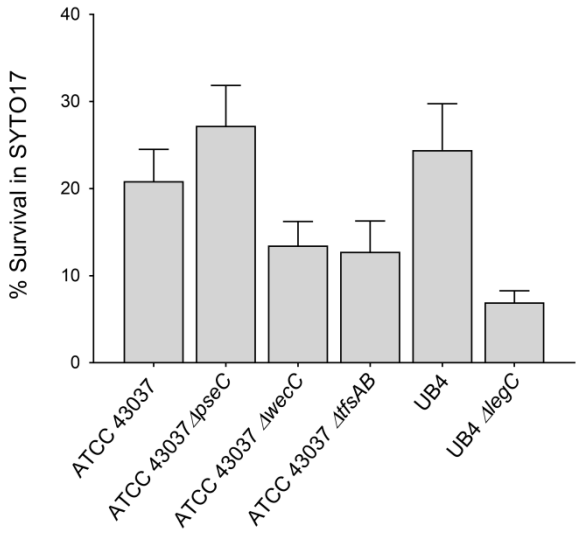

Figure S5. Survival of *T. forsythia* ATCC 43037 and UB4 wild-type strains and cell surface mutants during SYTO17 staining. Data from two experiments with three technical replicates each are shown for each strain. Bacterial survival is plotted as percentage of the cell number of the inoculum, *i.e.* the untreated control for each strain.

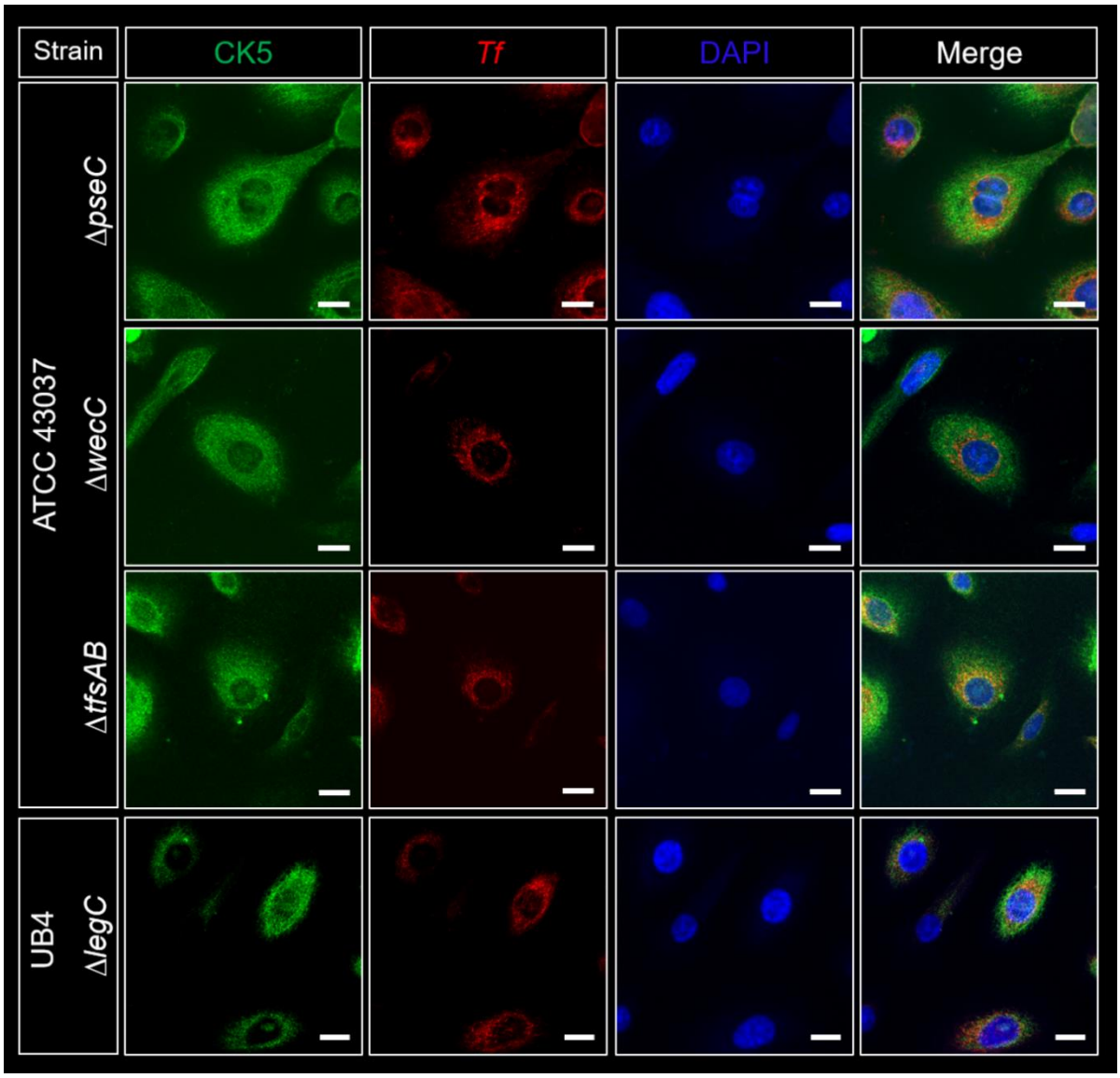

Figure S6. Invasion of human oral keratinocytes (HOKs) by cell surface mutants of *T. forsythia* strains ATCC 43037 and UB4. Bacterial cells were stained using Syto17 DNA dye (red), HOKs were visualized through immunofluorescent staining using a cytokeratin 5 (CK5) specific primary antibody and a goat anti-mouse IgG secondary antibody conjugated with Alexa Fluor® 488 (green) as well as DAPI staining (blue).Objective 63x, scale bar 20 µm.

3. Conclusions of the thesis

The oral bacterium *T. forsythia* has long been recognized as one of the major pathogens in the context of periodontitis, contributing to the development of the excessive inflammation that ultimately destroys the bone supporting tissues of the periodontium [253]. One of the most striking features of this bacterium is the fact, that it decorates its cell with an elaborate S-layer composed of the two proteins TfsA and TfsB both of which carry multiple copies of an *O*-linked dekasaccharide [6]. This structure is characterized by a high complexity and, given that under favorable growth conditions bacteria often cease to produce or assemble their S-layer [94], its biological significance must outweigh the biosynthetic expense. Thus, with the elucidation of the structural composition, the question of the function of the glycosylated S-layer in the bacterium's life-style arose. Therefore, the present study was aimed at the functional characterization of two strains of *T. forsythia* differing in the composition of their *O*-glycan in one terminal sugar only and selected cell surface mutants thereof with regard to their behavior in a multispecies biofilm model and the interaction with the host – with a focus on the terminal nonulosonic acids.

Especially the occurrence of the two sialic acid-like sugars Pse and Leg as terminal residues of the *T. forsythia O*-glycan [6] suggests a role of the glycan in the pathogenicity of the bacterium in the context of immune evasion and molecular mimicry. Nonulosonic acids have been detected as components of flagella [254], LPS [110] or pili [255] in a number of bacteria, potentially mediating pathogenicity. The presence of the *T. forsythia* S-layer as an entity is a prerequisite for the bacterium to adhere to and invade GECs [129] and reduces the immune response by macrophages and gingival fibroblasts [131]. A first insight into the role of the *O*-glycan itself was gained by studies on glycan mutants showing that a three-sugar truncation of the *O*-glycan resulted in increased biofilm formation [256] and impaired Th17 responses and neutrophil recruitment [133].

Friedrich et *al.* discovered a variation of Pse *versus* Leg as constituents of the glycan in different strains of *T. forsythia* and first reported on the role of the terminal nonulosonic acids in monospecies biofilm formation [7]. These monospecies biofilm experiments carried out under different conditions produced contradictory results to data in the literature, with an increase in biofilm formation on untreated polystyrene plates upon glycan truncation [102] and a reduction on mucin coated plates [7 ,10]. Besides, in the oral cavity, *T. forsythia* has to be able to survive in the environment of the multispecies biofilms that constitute the subgingival plaque. Therefore, a subgingival biofilm model mimicking the native situation in the oral cavity [9] was employed in order to analyze if *T. forsythia* utilizes its surface glycosylation to persist in its natural habitat.

Most strikingly, this study showed that the two strains of *T. forsythia* – ATCC 43037 and UB4 – differed considerably in their behavior in the 10-species biofilms. Comparison between the two strains showed, that the latter was detected in much higher cell numbers in the multispecies consortium and both strains exhibited a different localization within the biofilm structure, as determined by confocal laser scanning microscopy (CLSM). *Tannerella forsythia* UB4 additionally displayed an advantageous growth behavior over the *T. forsythia* ATCC 43037 type strain not only in the polymicrobial biofilms, but also in planktonic and monospecies biofilm growth.

Contrary to expectations raised from findings of monospecies biofilm studies, truncation of the *O*-glycan or ablation of the S-layer as a whole did not influence the bacterium's capability to grow in the multispecies biofilms. Nevertheless, mutation of the cell surface influenced how these mutants interacted with each other in terms of autoaggregation as well as in the microcolonies within the biofilms. The presence of the S-layer deficient *T. forsythia* ATCC 43037 Δ*tfsAB* in the multispecies biofilms lead to an increase in *C. rectus* cell numbers suggesting a growth impeding effect of the S-layer on this bacterium, however CSLM analysis and autoaggregation assays revealed this to be independent of a direct contact between the two species. In CSLM however a strong co-localization of *T. forsythia* ATCC 43037 Δ*wecC* with *P. gingivalis* was noticeable. Outside of the multispecies consortium, increased coaggregation between *P. gingivalis* and this mutant compared to the other *T. forsythia* strains analyzed could not be identified. The mechanisms behind this apparent effect of the *O*-glycan composition on the behavior of *P. gingivalis* in the multispecies consortium still awaits further investigation.

Based on these data we conclude that the glycosylated S-layer is not vital for the establishment of the bacterium within the multispecies consortium, but can influence the interaction with other species such as *C. rectus* and *P. gingivalis*. Contrary to a monospecies setting, the nonulosonic acids were not found to influence biofilm formation in the *in vitro* multispecies model and we therefore assumed a more prominent function of these sugars in the interaction with the host tissues and immune system.

The same set of cell surface mutants was subsequently subjected to immunological analyses using human oral keratinocytes (HOK) and human monocytes. *Tannerella forsythia* infection resulted in the secretion of cytokines, chemokines and vascular endothelial growth factor (VEGF) in monocytes and chemokines, interleukin-1 receptor antagonist (IL-1Ra) and VEGF in HOK. Comparison of the immune response to the two *T. forsythia* wild-type strains yielded the interesting observation that the two strains elicit preferential immune responses both in HOK and in monocytes.

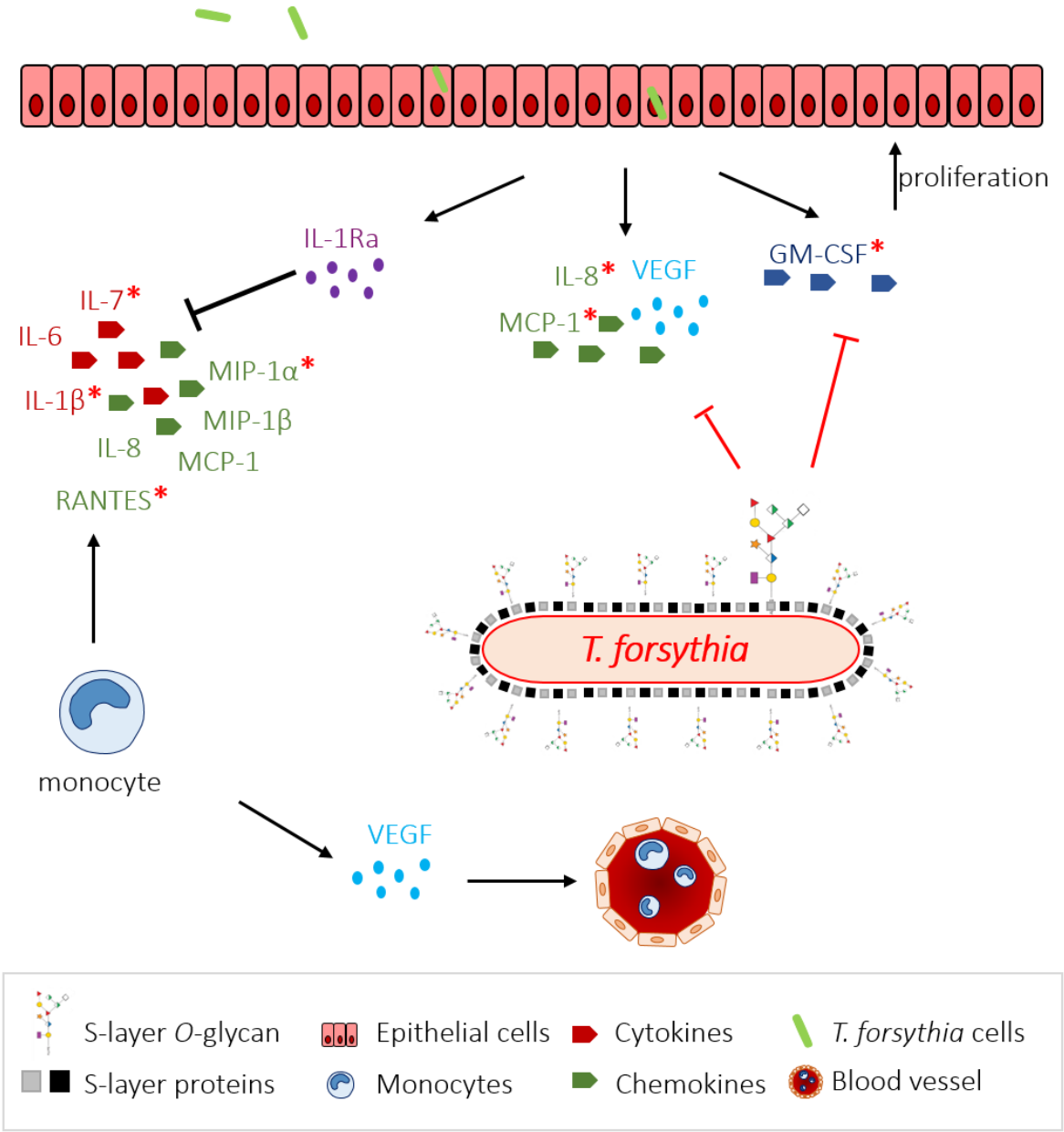

Figure 8. Responses of monocytes and HOK to *T. forsythia* infection. Infection with *T. forsythia* ATCC 43037 and *T. forsythia* UB4 wild-type causes the release of pro-inflammatory cytokines, chemokines and VEGF from monocytes and chemokines, IL-1Ra and VEGF from HOK. In HOK the presence of Pse, the terminal trisaccharide branch and the complete S-layer delays cytokine and chemokine secretion. Red asterisks mark immune modulators that are differentially induced by the two wild-type strains or a cell surface mutant.

Infection with *T. forsythia* UB4 dampened the IL-1β response in monocytes when compared to the *T. forsythia* ATCC 43037 type strain, but contrary to the latter strongly induced IL-7, which had not been observed in response to the species before. Also in HOK challenged with either wild-type, *T. forsythia* UB4 elicited a higher IL-8 release.

Both strains strongly induce VEGF in monocytes as well as keratinocytes, a trait that had not been reported for *T. forsythia* before.

The cell surface composition of the bacterium seemed to be especially relevant in the interaction with HOK. The ablation of the nonulosonic acid, truncation of the *O*-glycan by three sugars and the deletion of the S-layer in *T. forsythia* ATCC 43037 significantly increased the IL-8 release by HOK when compared to the parental wild-type strain. The S-layer deficient mutant further boosted MCP-1 secretion and was the only strain to induce GM-CSF. This was in accordance with previous findings attesting to the S-layer a role in delaying the immune response during *T. forsythia* infection [131], however for the first time we demonstrated a contribution of the terminal Pse in this process.

The data suggest that the surface glycosylation of *T. forsythia* plays a prominent role in the initial phase of infection. By upregulation of IL-1Ra and the suppression of pro-inflammatory immune responses on the one hand *T. forsythia* might not only enable itself to persist in the host, but also other biofilm bacteria could benefit from its presence. On the other hand, release of pro-inflammatory cytokines and chemokines as well as VEGF, which stimulates vascularization and migration of monocytes/macrophages to the site of infection, might foster inflammation and nutrient provision benefitting bacterial overgrowth and tissue destructive processes.

We could further show that all strains of *T. forsythia* that were analyzed in this study are able to invade HOK and localize to the perinuclear region within the cells. In order to analyze if the S-layer and intact *O*-glycan play a part in this process, quantitative analysis of cell invasion would have to be performed. Also uptake of the bacteria by monocytes in dependence of their cell surface composition could be studied to determine if the differential immune response elicited by some of the mutants stems from differences in cellular uptake and bacterial survival.

In summary the study presented here contributes to the understanding of the biological role of the *T. forsythia* surface glycosylation in orchestrating the bacterium's life-style and, specifically, an effect of the nonulosonic acid Pse in delaying the immune response was presented. We conclude that rather than in the interaction within the oral multispecies biofilms, the glycosylated S-layer and nonulosonic acids in particular are vital for the bacterium's interaction with the gingival tissues and possibly the persistence of the bacterium within the host and, thus, potentially orchestrate *T. forsythia*'s virulence potential and pathogenicity.

4. Conference contributions

Presenting author is underlined.

4.1. Oral presentations

- **Development of genetic tools for *Selenomonas sputigena* to unravel mechanisms of dental plaque colonization**
  Bloch, S., Rath, C., Schäffer, C. [DNBT Winter School, Deutschlandsberg, Austria,February 18 - 20, 2015]
- **Biological role and glycobiology of the oral bacterium *Tannerella forsythia***
  Bloch, S., Schäffer, C. [DNBT Spring School, Graz, Austria, April 5-8, 2016]
- **Biological role and glycobiology of the oral bacterium *Tannerella forsythia***
  Bloch, S., Schäffer, C. [BioToP Annual Retreat, Waidhofen/Ybbs, June 27-29, 2016]
- **Biofilm behavior of *Tannerella forsythia* strains and S-layer glycosylation mutants**
  Bloch, S., Thurnheer, T., Murakami, Y., Belibasakis, G.N., Schäffer, C. [12th European Oral Microbiology Workshop, May 25-28, 2017Stockholm, Sweden]
- **Biological role and glycobiology of the oral bacterium *Tannerella forsythia***
  Bloch, S., Belibasakis, G.N., Schäffer, C. [22nd Austrian Carbohydrate Workshop, February 15-16, Vienna]

4.1.1. Biofilm behavior of *Tannerella forsythia* strains and S-layer glycosylation mutants

12th European Oral Microbiology Workshop, Stockholm, Sweden, May 25-28, 2017

Susanne Bloch[1], Thomas Thurnheer[2], Yukitaka Murakami[3], Georgios N. Belibasakis[4], Christina Schäffer[1*]

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The periodontopathogen *Tannerella forsythia* has a characteristic cell surface (S-) layer modified with a unique *O*-glycan. This structure was analyzed for its role in biofilm formation employing an *in vitro* multispecies biofilm model, into which different *T. forsythia* strains and mutants with a modified cell surface composition were incorporated together with nine species of select oral bacteria. The influence of the glycosylated *T. forsythia* S-layer on the bacterial composition of the biofilms was analyzed quantitatively using quantitative real-time PCR as well as qualitatively by fluorescence *in situ* hybridization and confocal laser scanning microscopy. This revealed that changes of the *T. forsythia* cell surface did not affect the quantitative composition of the multispecies consortium, with the exception of *Campylobacter rectus* cell numbers, but the localization of *T. forsythia* within the biofilm and its aggregation with *Porphyromonas gingivalis*, suggesting that the *T. forsythia* S-layer and its *O*-glycan function in the positioning of this species within the biofilm and influence co-localization with *P. gingivalis* and the prevalence of *C. rectus.* This might further pinpoint a pivotal role of *T. forsythia* cell surface structures in the virulence of this species when interacting with host tissues and immune system, from within or beyond the biofilm.

4.2. Poster contributions

- **Development of genetic tools for *Selenomonas sputigena* to unravel mechanisms of dental plaque colonization**

  Bloch, S., Rath, C., Schäffer, C. [PGLondon2015: 2nd International Conference on *Porphyromonas Gingivalis* and Related Species in Oral and Systemic Diseases, June 23-25, 2015 London, UK]

- **Investigation of the biological role of *Tannerella forsythia* cell surface glycosylation**

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

4.2.1. Development of genetic tools for *Selenomonas sputigena* to unravel mechanisms of dental plaque colonization

PGLondon2015: 2nd International Conference on Porphyromonas Gingivalis and Related Species in Oral and Systemic Diseases, London, UK, June 23-25, 2015

Susanne Bloch, Cornelia Rath, Christina Schäffer

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

The oral biofilm is a multispecies community. Oral pathogens utilize protein glycosylation in order to influence their biofilm lifestyle and undermine the host immunity to cause periodontal disease [1], as has been shown for the "red complex" bacterium *Tannerella forsythia*, whose glycosylated surface layer (S-layer) proteins [2] play a key role in adhesion, invasion and modulation of the host immune response. Periodontal pathogenicity however cannot be traced back to select pathogens, but greatly depends on significant interspecies cooperation within the oral biofilm [3] and the contribution of the numerous bacteria present in dental plaque biofilms to disease development is poorly understood.

Among the oral bacteria, *Selenomonas sputigena*, a Gram-negative, anaerobic, mulitflagellated rod [4], is frequently co-isolated with *Tannerella forsythia* and occurs in elevated levels in patients with generalized aggressive periodontitis [5]. The rich glycoproteome of *Selenomonas sputigena* suggests, that this oral species employs various glycobiology-based strategies for oral colonization and survival. Putative glycosylation of its flagella and the prediction of uncommon sugar residues, such as L-fucose make it an interesting candidate to study its glycobiology aspects and possible role in periodontal pathogenesis. Despite their prevalence in the oral biofilm, little is known about the biology and pathogenic potential of *Selenomonas* spp., largely due to the inability to genetically manipulate these microorganisms. Through the establishment of a tractable transformation system for *Selenomonas sputigena* and targeted manipulation of key glycoconjugates, we will be able to study its role within the oral biofilm.

1. Settem, R.P., et al., *Protein-linked glycans in periodontal bacteria: prevalence and role at the immune interface.* Front Microbiol, 2013. **4**: p. 310.
2. Sekot, G., et al., *Potential of the Tannerella forsythia S-layer to delay the immune response.* J Dent Res, 2011. **90**(1): p. 109-114.
3. Hajishengallis, G. and R.J. Lamont, *Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology.* Mol Oral Microbiol, 2012. **27**(6): p. 409-19.
4. Kolenbrander, P.E., R.N. Andersen, and L.V. Moore, *Coaggregation of Fusobacterium nucleatum, Selenomonas flueggei, Selenomonas infelix, Selenomonas noxia, and Selenomonas sputigena with strains from 11 genera of oral bacteria.* Infect Immun, 1989. **57**(10): p. 3194-203.
5. Goncalves, L.F., et al., *Levels of Selenomonas species in generalized aggressive periodontitis.* J Periodontal Res, 2012. **47**(6): p. 711-8.

Development of genetic tools for *Selenomonas sputigena* to unravel mechanisms of dental plaque colonization

Susanne Bloch, Cornelia Rath, Christina Schäffer

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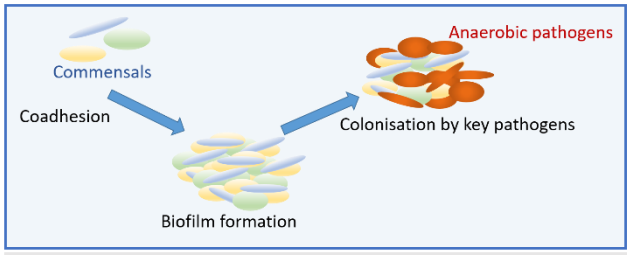
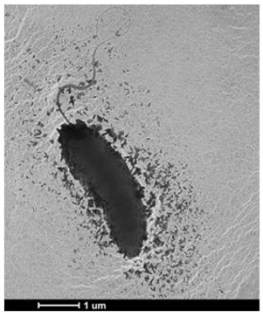
Periodontal pathogenicity cannot only be traced back to select pathogens, but greatly depends on significant interspecies cooperation within the oral biofilm [1]. Physical interactions between microbes in oral biofilms are mediated by specialized cell surface structures, with carbohydrates being the most abundant decoration of bacterial cells. We have chosen *Selenomonas sputigena* ATCC 35185 as candidate strain to investigate the role of glycobiology aspects in the context of periodontal disease and biofilm architecture and to understand the contribution of individual species to the microbial shift from a commensal to a pathogenic oral microflora.



The microbial shift from health to disease requires interactions between numerous oral bacteria

Selenomonas sputigena ATCC 35185



- flagellated, motile, anaerobic rod
- frequently co-isolated with *Tannerella forsythia, a* member of the red-complex bacteria [2]
- role in the architecture and spatial arrangement of periodontal biofilms

TEM picture of *Selenomonas sputigena* showing the presence of a single polar flagellum

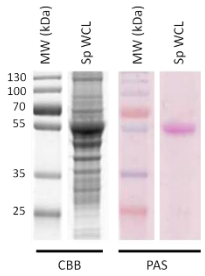
Glycobiology aspects of *Selenomonas sputigena*

The *Selenomonas sputigena* genome provides rich glycobiology information, such as putative glycosylation of its flagellum. Flagella are of interest as bacterial colonization/ communication factors and, especially in glycosylated form, as pathogenicity factors.

Evidence of flagellar glycosylation

Contig	Annotation
Selsp_0327	flagellar protein FliS
Selsp_0329	flagellin-domain protein
Selsp_0330	glycosyltransferase
Selsp_0331	β-1,4 glycosyltranserase
Selsp_0332	O-GlcNAc transferase
Selsp_0333	O-GlcNAc transferase

Flagellar gene cluster of Selspu_ATCC 35185



PAS-Staining of Selspu whole cell lysate (SpWCL)

Positive periodic acid-Schiff (PAS) staining of *S. sputigena* flagellin protein Selsp_0329.

Aims of this project

Establishment of a tractable genetic transformation system

- development of a host-vector system
- targeted mutagenesis

Depletion of key glycoconjugates

- analysis of flagellar gene cluster

Functional studies of glycosylation-deficient mutants

- role of surface glycosylation in biofilm formation and maintenance
- analysis of immunogenic potential

References:

[1] Hajishengallis, G. and R.J. Lamont, *Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology.* Mol Oral Microbiol, 2012. **27**(6): p. 409-19.

[2] Goncalves, L.F. *et al. Levels of Selenomonas species in generalized aggressive periodontitis*. J Periodont Res, 2012. **47**, 711-718

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4.2.2. Investigation of the biological role of *Tannerella forsythia* cell surface glycosylation

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

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With the advancement of metagenomic and culture-independent bacterial analyses, it has become apparent, that many chronic diseases are not caused by a single species but are polymicrobial in nature [1]. Among them is periodontitis, a chronic inflammatory disease and major cause of tooth loss in adults. It is induced by a shift in the polymicrobial community colonizing the oral cavity, which enables potentially pathogenic bacteria to increase in numbers and elevate the pathogenicity of the whole biofilm community [2]. The Gram-negative periodontopathogen *Tannerella forsythia* covers its cells with a unique surface (S-) layer carrying a complex *O*-glycan with several immunologically relevant sugars such as the nonulosonic acids pseudaminic acid and legionaminic acid [3]. The biological role of the *T. forsythia* surface glycosylation and in particular the nonulosonic acids with regard to their influence on biofilm formation was analyzed employing the "Zurich biofilm model"[4]. This *in vitro* multispecies biofilm model with select oral bacteria mirrors the natural situation of the multispecies biofilm that is the dental plaque. By incorporating different *T. forsythia* strains and specific deletion mutants of *Tannerella* spp.with variable surface glycosylation, the effect of the surface characteristics on the bacterial composition of the biofilms was analyzed. Quantitative analysis using colony forming unit (CFU) counts, quantitative real-time PCR (qPCR) and fluorescence *in situ* hybridization (FISH) as well as confocal laser scanning microscopy (CLSM) for the analysis of the structural composition of the biofilm show that in the "Zurich biofilm model", the changes in the surface glycan of *T. forsythia* do not affect the interaction with other bacteria in the multispecies consortium, but the localization of the *Tannerella* spp. differs within the bacterial agglomeration. This suggests, that the *T. forsythia* utilizes the S-layer and its glycan rather for interaction with the host tissues and immune evasion, than for inter-bacterial interaction and communication.

References:

[1] H. M. Ng, L. X. Kin, S. G. Dashper, N. Slakeski, C. A. Butler, and E. C. Reynolds, "Bacterial interactions inpathogenic subgingival plaque," *Microb. Pathog.*, pp. 1–10, 2015.

[2] G. Hajishengallis, "Immunomicrobial pathogenesis of periodontitis: Keystones, pathobionts, and host response," *Trends Immunol.*, vol. 35, no. 1, pp. 3–11, 2014.

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Role of *Tannerella forsythia* cell surface glycosylation in multispecies oral biofilms

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Introduction

The human oral cavity houses approximately 700 species of microorganisms [1] which form polymicrobial biofilms on the tooth surface. In a healthy individual, the microbial consortium exists in a natural balance with its host. However, different factors can cause the bacterial community to become dysbiotic [2], enabling potentially pathogenic bacteria to increase in numbers and cause persistent infections and diseases such as periodontitis. Among them is the Gram-negative periodontopathogen *Tannerella forsythia*, which covers its cells with a unique surface (S-) layer carrying a complex *O*-glycan with several immunologically relevant sugars such as the nonulosonic acids pseudaminic acid or legionaminic acid [3], which seem to be present as a strain-specific feature.

In this study, the biological role of the *T. forsythia* surface glycosylation and in particular the nonulosonic acids with regard to their influence on biofilm formation was characterized employing the "Zurich biofilm model"[4]. This *in vitro* model of a 10-species subgingival biofilm grown on pellicle-coated hydroxyapatite discs mirrors the natural situation in the oral cavity and allows for the quantitative characterization of multispecies biofilms through quantitative real-time PCR (qPCR), as well as the structural analysis of the biofilms through fluorescence *in situ* hybridization (FISH), using species-specific fluorescently labelled DNA probes against 16S rRNA, and confocal laser scanning microscopy (CLSM).

Different *T. forsythia* strains and specific deletion mutants with variable surface glycosylation patterns were incorporated into the oral biofilm model to analyze the influence of the sugar moieties on the structure and composition of the multispecies biofilm.

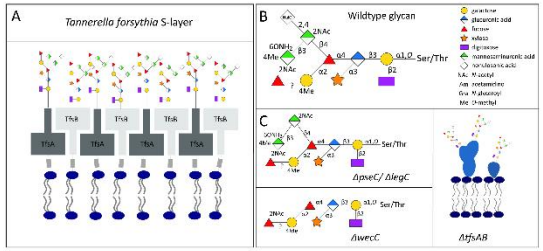

Fig. 1 Surface properties of selected *T. forsythia* wild-type and mutant strains analyzed in the Zurich biofilm model.
(A) Scheme of the *T. forsythia* cell envelope with the glycosylated S-layer proteins TfsA and TfsB
(B) Structure of the T. *forsythia O*-glycan with a terminal nonulosonic acid
(C) *T. forsythia* deletion mutants with truncated glycans (*ΔpseC / ΔlegC / ΔwecC)* and S-layer deficient mutant (*ΔtfsAB*) presumably displaying a few glycoproteins other than the S-layer glycoproteins at the cell surface.

Tannerella spp. and deletion mutants

Strain:	*Cell surface and glycan properties:*
T. forsythia ATCC 43037	wild-type; terminal pseudaminic acid residue (Pse)
T. forsythia ATCC 43037 *ΔpseC*	deletion of terminal Pse*
T. forsythia ATCC 43037 *ΔwecC*	deletion of terminal trisaccharide branch containing Pse and two ManNAcA residues
T. forsythia ATCC 43037 *ΔtfsAB*	deletion of both genes encoding for S-layer proteins TfsA and TfsB
T. forsythia UB4	wild-type; terminal legionaminic acid residue (Leg) (Obtained from Dr. Ashu Sharma, University of Buffalo, USA)
T. forsythia UB4 *ΔlegC*	deletion of terminal Leg*

*reconstituted strains of nonulosonic acid knock-out mutants of $\Delta pseC_{comp}$ and $\Delta legC_{comp}$ were included in the analysis

Results

Influence of *Tannerella* spp. on multispecies oral biofilm composition and structure

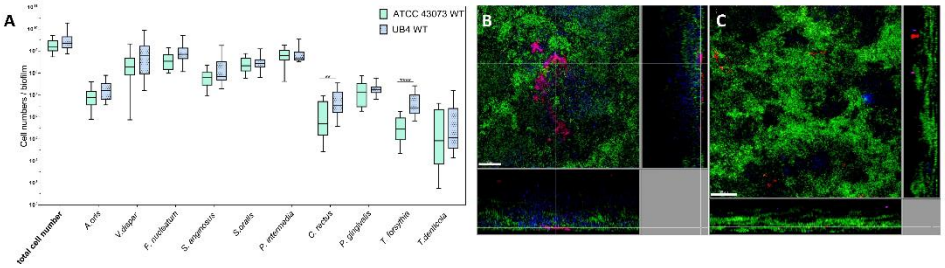

Fig. 2 Comparison of 10-species biofilms including different *Tannerella* spp.
(A) Quantitative analysis of biofilms with either *T. forsythia* ATCC 43073 WT (green boxes) or *T. forsythia* UB4 WT (blue boxes). Cell numbers of each individual species in the subgingival biofilm were determined by qPCR after 64.5 hours of incubation using species-specific primers targeting the 16S ribosomal RNA gene. Whiskers boxplots (5-95 percentile) show total bacteria and all individual taxa from three independent experiments. Asterisks (**) indicate a statistically significant difference ($P \leq 0.01$) between groups.
(B, C) FISH stainings and CLSM analysis of fixed biofilms showing the localisation of ATCC 43073 WT **(B)** and UB4 WT **(C)**. Red:*T. forsythia*, cyan: *P. gingivalis*, green: non-hybridised cells (DNA staining YoPro-1 + Sytox). Here a representative area for one disc each is shown with a top view in the middle panel and side views with the biofilm-disc interface directed towards the top view. Scale bar 20 µm.

Analysis of *T. forsythia* mutants in the subgingival biofilm

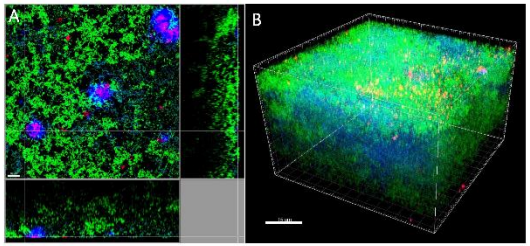

Fig. 3 Influence of S-layer glycan mutants on biofilm structure
(A) FISH staining of a biofilm harbouring ATCC 43073 WT and the *ΔwecC* mutant.
(B) 3D reconstruction of a 10-species biofilm containing the S-layer mutant *ΔtfsAB*.
Scale bar 10 µm (A), 15 µm (B) Red: *T. forsythia*, cyan: *P. gingivalis*, green: non-hybridised cells (DNA staining YoPro-1 + Sytox).

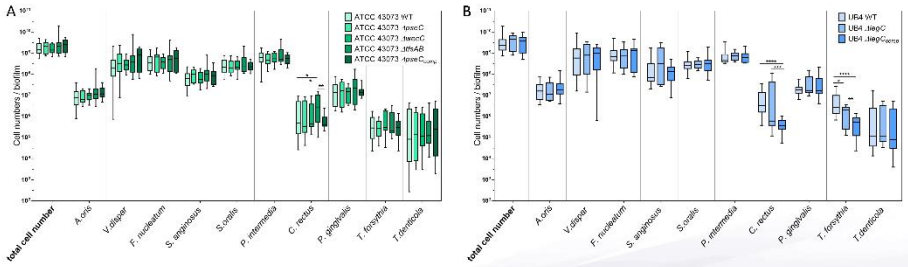

Fig. 4 Determination of biofilm composition
Box plots showing cell numbers of all species determined by quantitative real-time PCR for biofilms with *T. forsythia* ATCC 43073 WT or mutants (*ΔpseC, ΔwecC, Δtfsab, $\Delta pseC_{comp}$*) **(A)** and UB4 WT or mutants (*ΔlegC, $\Delta legC_{comp}$*), respectively **(B)**. Data derived from three independent experiments were plotted on a logarithmic scale. Asterisk (*) indicates significant differences ($P \leq 0.05$) between the groups.

Conclusions

Quantitative analysis:
The two *Tannerella* spp. (ATCC 43037 and UB4) strongly differ in their biofilm behaviour with a 10-fold increase in bacterial numbers for UB4 WT when compared to the ATCC 43073 type strain.
T. forsythia ATCC 43073 WT and mutants behave very similarly in the multispecies biofilms and lacking the terminal nonulosonic acid (*ΔpseC*) or the terminal trisaccharide branch (*ΔwecC*) does not affect the growth of *T. forsythia* or the other species of the consortium. The absence of the S-layer in the mutant *ΔtfsAB*, while not affecting *T. forsythia* itself, strongly promotes growth of *Campylobacter rectus* in the biofilm.
UB4 WT cell numbers were also significantly increased when compared to its respective mutants. Among the other biofilm species here also only *C.rectus* showed an increase in cell numbers in biofilms with UB4 WT and *ΔlegC* when compared to $\Delta legC_{comp}$.

Evaluation of biofilm structure:
While *T. forsythia* ATCC 43073 WT and mutants are predominantly found at the biofilm surface, UB4 strains also appear in the form of small microcolonies in deeper layers of the biofilm. In contrast to biofilms with other *T. forsythia* strains, *ΔwecC* biofilms appear more porous and this mutant forms aggregates with *P. gingivalis*, which has not been observed for the other strains analysed. In these samples, *P. gingivalis* changes its localization and is also detected predominantly at the surface. Devoid of an S-layer, *T. forsythia ΔtfsAB* can be observed in the form of small microcolonies as well as single cells scattered along the upper layers of the biofilm.

Biofilm strains: *Prevotella intermedia* ATCC 25611 T (OMZ278), *Campylobacter rectus* (OMZ388), *Veillonella dispar* ATCC 17748 T (OMZ493), *Fusobacterium nucleatum* (OMZ598), *Streptococcus oralis* SK248 (OMZ 607), *Streptococcus anginosus* ATCC 9895 (OMZ871), *Actinomyces oris* (OMZ 745), *Porphyromonas gingivalis* (OMZ925) and *Treponema denticola* ATCC 35405 (OMZ661).

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6. Index of Figures

7. Abbreviations

AI-2	autoinducer-2
AP-1	activator protein-1
APC	antigen presenting cell
BMDC	Bone-marrow derived dendritic cells
BPI	bactericidal/permeability-increasing protein
BspA	*Bacteroides* surface protein
CMP	cytidine-5'-monophosphate
CTD	C-terminal domain
FDF	Forsythia detaching factor
GCF	gingival crevicular fluid
GDP	guanosine-5'-diphosphate
GEC	gingival epithelial cell
GlcNAc	*N*-acetylglucosamine
GM-CSF	granulocyte-macrophage colony-stimulating factor
HOK	human oral keratinocyte
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-γ
IL	interleukin
IL-1Ra	IL-1 receptor antagonist
ILC	innate lymphoid cell
IP-10	IFN-γ-inducible protein 10
IRF-3	interferon-regulatory factor-3
Kdo	3-deoxy-D-manno-oct-2-ulosonic acid
Leg	legionaminic acid
LFA-3	lymphocyte function antigen-3
LPB	lipopolysaccharide binding protein
LPS	lipopolysaccharide
MAC	membrane attack complex
ManNAcA	*N*-acetylmannosaminuronic
MCP-1	monocyte chemoattractant protein 1
Mincle	macrophage-inducible C-type lectin
MMP	matrix metalloprotease
MurNAc	*N*-acetylmuramic acid
NET	neutrophil extracellular trap
NOD	nucleotide-binding oligomerization domain
OMV	outer membrane vesicle

PAMP	pathogen-associated molecular pattern
PGE2	prostaglandine E2
PMN	polymorphonuclear neutrophil
PRR	pattern recognition receptor
Pse	pseudaminic acid
RA	rheumatoid arthritis
RANKL	receptor–activator of nuclear factor-κB ligand
ROS	reactive oxygen species
Siglec	sialic acid-binding Ig superfamily lectin
TfsA	*Tannerella forsythia* surface layer protein A
TfsB	*Tannerella forsythia* surface layer protein B
TLR	toll-like receptor
TNF	tumor necrosis factor
UDP	uridine 5’-diphosphate
VEGF	vascular endothelial growth factor

8. Curriculum vitae

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EDUCATION

Jan 2015 - current	**International PhD Programme "Biomolecular Technology of Proteins"** University of Natural Resources and Life Sciences (BOKU), Vienna, Austria Department of NanoBiotechnology, *NanoGlycobiology* group under the supervision of Prof. Dr. Christina Schäffer. Project: Investigation of a biological role of *Tannerella forsythia* cell surface glycosylation through immunological and biofilm studies
Oct 2005 – Nov 2011	**University of Vienna** – Vienna, Austria Degree in Molecular Biology, graduated as Mag.ª rer. nat. (equivalent to Master) Diploma project at the Max F. Perutz Laboratories, Vienna, group of Dr. Kristin Tessmar-Raible: Functional characterization of Inner Brain Opsins in Medaka and Zebrafish
Oct 2004 – July 2005	**Philips-Universität Marburg** – Marburg, Germany Studies of Medicine
June 2004	Graduation from the **Gymnasium Leopoldinum Passau**
1995 - 2004	**Gymnasium Leopoldinum Passau** – Passau, Germany

WORK EXPERIENCE AND RESEARCH STAYS ABROAD

April 2017 – July. 2017	**Department of Dental Medicine, Karolinska Institutet,** Stockholm, Sweden Research stay in the laboratory of Prof. Georgios N. Belibasakis: Analysis of immune responses of primary monocytes and oral keratinocytes to different *Tannerella forsythia* strains
Jan 2016 – April 2016	**Institute of Oral Biology, University of Zurich** – Zurich, Switzerland Research stay in the laboratory of Prof. Georgios N. Belibasakis: Analysis of *Tannerella forsythia* strains and cell surface mutants in multispecies biofilms

Feb. 2013 – Dec. 2014	**Research Institute of Molecular Pathology (IMP),** Vienna Research Assistant/ Lab Manager in the Cochella Group: Role of microRNAs in spatio-temporal specificity during development in the nematode *C. elegans*
Nov. 2012 – Dec. 2012	**Department of Cell and Molecular Biology, Karolinska Institutet,** Stockholm, Sweden Research internship in the laboratory of Dr. Andreas Kegel: Role of SMC Protein Complexes in DNA remodelling and topology in *Saccharomyces cerevisiae* and *Drosophila melanogaster*
March 2012- April 2012	**Department of Photochemistry and Molecular Science, Uppsala University,** Uppsala, Sweden Research internship in the laboratory of Prof. Peter Lindblad:The mincCDE gene region in *Nostoc punctiforme*

PUBLICATIONS

Bloch, S.; Zwicker, S.; Bostanci, N.; Sjöling, Å.; Boström, E.A.; Belibasakis, G.N.; Schäffer, C. (2018) Immune response profiling of primary monocytes and oral keratinocytes to different *Tannerella forsythia* strains and their cell surface mutants. *Mol Oral Microbiol.*, in press, doi: 10.1111/omi.12208.

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Fischer, R.M.; Fontinha, B.M.; Kirchmaier, S; Steger, J; **Bloch, S.**; Inoue, D.; Panda, S.; Rumpel, S.; Tessmar-Raible, K. (2013) Co-expression of VAL- and TMT-opsins uncovers ancient photosensory interneurons and motorneurons in the vertebrate brain *Plos Biology*. 11(6):e1001585.

CONFERENCES

12th European Oral Microbiology Workshop (2017), Stockholm, Sweden
Oral presentation

FASEB SRC - Microbial Glycobiology (2016), West Palm Beach, FL, USA
Poster presentation

PG 2015 - 2nd International Conference on *Porphyromonas Gingivalis* and Related Species in Oral and Systemic Diseases (2015), London, UK
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LANGUAGES

German (native speaker)
English (fluent)
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