Tannerella forsythia and its virulence factors

Miharija, David

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UNIVERSITY OF RIJEKA

DEPARTMENT OF BIOTECHNOLOGY

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Sažetak

Tannerella forsythia je gram-negativna bakterija koja naseljava ljudsku gingivu te je pripadnica crvenog kompleksa, bakterijskog konzorcija povezanog s razvojem parodontalnih i drugih bolesti. *T. forsythia* doprinosi razvoju parodontalnih bolesti potičući disbiozu u oralnoj mikrobioti i suzbijanjem imunološkog odgovora domaćina, u čemu sudjeluje veliki broj faktora virulencije. Glavni faktori virulencije *T. forsythia* mogu se podijeliti na proteaze, glikozidaze te proteine povezane sa staničnom membranom i staničnom stjenkom. Većina proteaza *T. forsythia* razgrađuje tkivo domaćina i potiskuje imunološku signalizaciju interleukinima. Glikozidaze razgrađuju izvanstanične polisaharide i proteoglikane, čime izmjenjuju gingivalni okoliš i prikupljaju hranjive tvari. Protein vanjske membrane BspA sudjeluje u vezanju bakterijskih stanica za tkivo i interakcijama s drugim bakterijama zubnog plaka. Serpin *T. forsythia*, poznat i kao miropin, sudjeluje u inhibiciji serinskih proteaza neutrofila. S-sloj *T. forsithya* jedinstven je zato što je sastavljen od dvaju glikoziliranih komponenti TfsA i TfsB te sudjeluje u formiranju biofilma, invaziji tkiva i imunosupresiji. Mnogi od ovih faktora virulencije prenose se do stanica domaćina u vezikulama vanjske membrane. Ovi faktori virulencije pružaju moguće mete za buduće terapije parodontalnih bolesti.

Ključne riječi: *Tannerella forsythia*, parodontitis, faktori virulencije

Abstract

Tannerella forsythia is a gram negative bacteria inhabiting human gingival tissue. It is a member of a bacterial consortium known as the red complex. This consortium is connected to the development of periodontal disease and several other conditions. *T. forsythia* contributes to periodontal disease development by inducing dysbiosis in oral microbial community and suppressing the host immune response. It achieves this via a number of virulence factors. Major *T. forsythia* virulence factors can be divided into proteases, glycosidases and cell membrane and cell wall associated proteins. Most *T. forsythia* proteases partake in virulence via degradation of host tissue and suppression of immune signalling via interleukins. *T. forsythia* glycosidases are taught to degrade extracellular polysaccharides and proteoglycans, thereby reshaping the gingival environment and providing nutrients. The outer membrane protein BspA is likely involved in adhesion of bacterial cells to tissue surface and interactions with other periodontal bacteria. *T. forsythia* serpin, known as miropin, is taught to partake in inhibition of neutrophil serine proteases. The S-layer of *T. forsithya*, uniquely composed of two glycosylated components TfsA and TfsB, regulates biofilm formation, tissue invasion and immunosuppression. Outer membrane vesicles deliver these virulence factors to host cells. These virulence factors provide possible targets for future therapies of periodontal diseases.

Key words: *Tannerella forsythia*, periodontitis, virulence factors

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1. Introduction

Tannerella forsythia is a species of gram-negative, obligatorily anaerobic and asaccharolytic bacterium (1). It possesses filamentous cellular morphology with pointed ends, with dimensions of 0.30-0.50 µm in diameter and 1-30 µm in length, and is non-motile (Figure 1.) (2,3). Many cells possess a central swelling up to 3 µm in diameter while others are spherical in shape (3). Its cell wall consists of a very thin peptidoglycan layer, an outer membrane and a glycosylated S-layer (3–5). Genome size for most strains ranges between 3,2-3,4 Mbp with median GC content of 46,0 mol% (3,6). Uniquely, *T. forsythia* lacks the metabolic pathway required to synthesize the bacterial peptidoglycan component N-acetylmuramic acid (4).

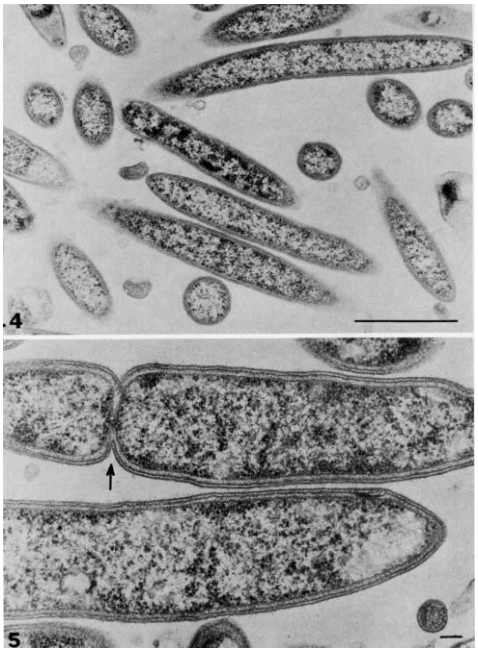

Figure 1. Transmission electron microscopy images of T. forsythia cells (top) and T. forsythia membrane septum formation during cell division (bottom). Figure adapted from (3).

T. forsythia was first described by Anne Tanner at the Boston Forsyth Institute from gingival plaques of periodontitis patients (3). Originally, it was known as *Bacteroides forsythius* and taught to belong to *Cytophaga–Bacteroides* family. However, taxonomic studies involving 16S rRNA

sequence analysis revealed it to be a member of the *Porphyromonadaceae* family, after which it was reclassified as *T. forsythiensis* (2,7). Finally, it was reclassified again as *T. forsythia* in 2005. (8). It is the sole member of the *Tannerella* genus, with uncultivated phylotypes BU045, BU063, 97 and 997 considered its closest relatives (4).

T. forsythia has proven exceptionally difficult to study, mostly due to the extensive list of conditions required for its growth *in vitro*. Growth on standard forms of agar media typically results in small, isolated colonies (3). To grow successfully, *T. forsythia* should be provided with media containing exogenous N-acetylmuramic acid (3,7,9). In addition, *T. forsythia* appears to require an unidentified growth factor found in blood serum. This condition can be met by utilising agar containing whole blood (3,7). Cultures can also potentially grow in Levinthal's agar and Trypticase soy or mycoplasma broth containing 8-12% gelatin (3). Cultures grow optimally at 37 °C in anaerobic conditions (3). An atmosphere consisting of 90% N_2 and 10% mixture of CO_2 and H_2 should be provided (3). Visible colonies appear after approximately 4 to 6 days (3). Growth can be stimulated by presence of *Fusobacterium nucleatum* (3,7,9).

T. forsythia is primarily found in human subgingival plaque and was shown to have been present in humans at least since the Neolithic period (10). *T. forsythia* is a member of a bacterial consortium known as the red complex. Alongside *T. forsythia*, the group also contains the gram-negative species *Porphyromonas gingivalis* and *Treponema denticola* (11). It was first described by Socransky et. al. alongside the yellow, green, purple and orange complex (Figure 2.) (11,12). These complexes exist within the larger polymicrobial biofilm consortium known as dental plaque which contains over 700 species of bacteria (5).

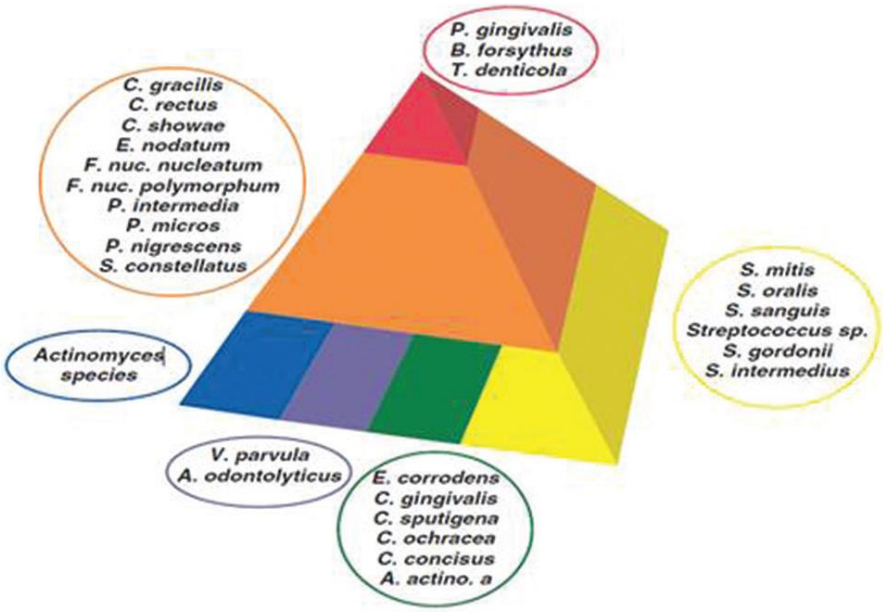

Figure 2. Subgingival plaque complexes and their member bacteria. These include red, orange, yellow, purple and green complexes. The species closer to the base of the pyramid colonise the tooth surface during the earlier stages of the disease. Figure adapted from (11).

The red complex bacteria are strongly associated with the medical progression of periodontal disease (11). Periodontal diseases are etiologically multifactorial infections characterised by chronic inflammation of the gums and periodontal pockets, as well as alveolar bone resorption. Common symptoms consist of bad breath, receding and bleeding gums, loss of dental attachment, endodontic abscesses, and in severe cases, loss of teeth. The initial stages of periodontal disease are commonly referred to as gingivitis, while later complications are referred to as periodontitis (13,14). Estimates indicate that 10-15% of the global adult population suffers from severe periodontitis (15). Interestingly, research has indicated that overweight individuals possess higher levels of *T. forsythia* growth in dental plaque, indicating a potential correlation between excess body weight and periodontal disease (10,11).

The red complex bacteria were previously taught to be primary contributors to the pathogenesis and progression of periodontal disease. However, the red complex bacteria have been discovered in periodontium of healthy individuals (16). Moreover, the microbial diversity of dental plaque has been

shown to be dramatically larger than previously taught, with over 700 species of bacteria identified (5). These newer studies led to the proposal of a new model called "polymicrobial synergy and dysbiosis" model (Figure 3.). According to this model, periodontal disease appears due to synergic interactions between various "keystone pathogens". These pathogens modulate the host immune response and stimulate the pathogenicity of the "accessory pathogens" such as red complex bacteria, resulting in the disruption of microbial ecology and dysbiosis (17). The proliferation of pathogenic strains and host's self-mutilating immune response lead to tissue damage and loss of dental attachment (1,17). Some of the factors known to correlate with dysbiosis include smoking, diabetes, genetic predisposition or poor dental hygiene (2).

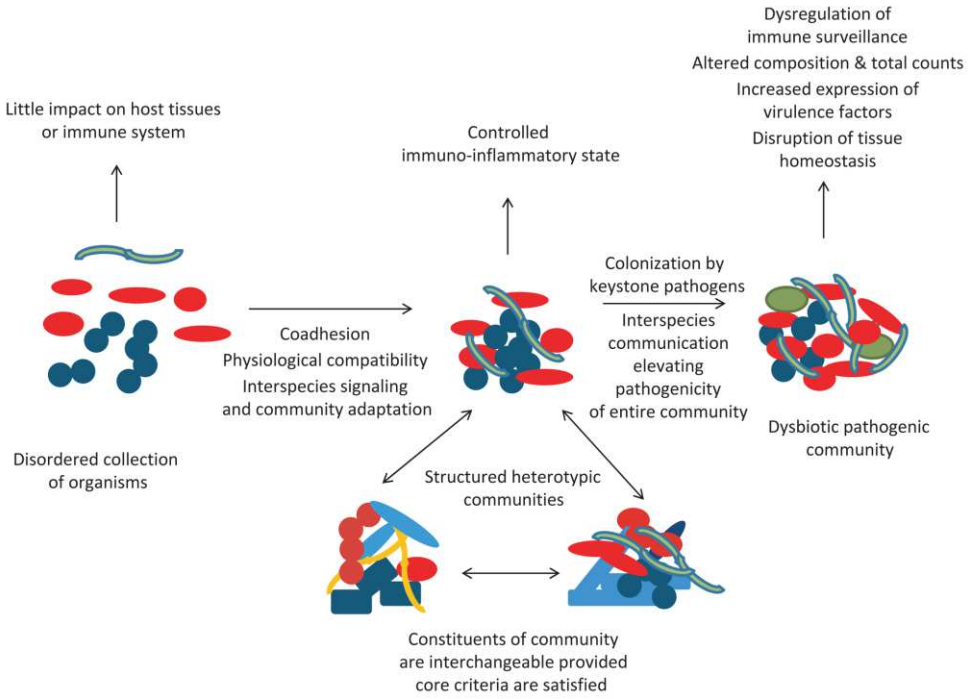

Figure 3. Schematic representation of the polymicrobial synergy and dysbiosis model. In physiological conditions, colonising bacteria organise themselves into structured heterotypic communities in homeostatic equilibrium with the host. The colonisation of periodontium by keystone pathogens disrupts this balance by elevating the overall pathogenicity, leading to dysbiosis and disease. Figure adapted from (17).

T. forsythia is rarely found in the absence of other members of the oral microbial community. In particular, red complex bacteria *Porphyromonas*

gingivalis and an orange complex bacterium *Fusobacterium nucleatum* have been shown to strongly correlate with presence of *T. forsythia*. It has been shown that *T. forsythia* can promote the growth of *P. gingivalis* by reducing fumarate to succinate (1,4). *P. gingivalis*, in turn degrades the host peptides which can then be utilised by *T. forsythia* (1,4). Additionally, coexistence of *P. gingivalis*, *T. denticola* and *T. forsythia* has been reported to cause a synergic effect on alveolar bone loss and abscess formation in rats (4). These and other studies suggest that *T. forsythia* survival and growth *in vivo* is dependent on other periodontal plaque bacteria that modulate the oral physiological conditions.

In addition to periodontal disease, *T. forsythia* has been implicated in several other conditions. While *T. forsythia* has been isolated from pregnant women, it has not been directly linked to adverse pregnancy outcomes (18). Most interestingly, a study by Peters et. al. has demonstrated a strong link between *T. forsythia* and esophageal adenocarcinoma (1,19). On the global scale, esophageal adenocarcinoma is the less common of the two major forms of esophageal carcinoma, the other being oral squamous cell carcinoma. However, it is the most common form of esophageal carcinoma in the western countries. It has a favourable prognosis in case of early diagnosis. However, due to most cases being detected in later stages of the disease, it has a 5-year survival rate comparable to liver or lung cancer (20). Therefore, the connection between esophageal adenocarcinoma and *T. forsythia* presents a possibility for development of preventative diagnostic tests for this disease.

2. Aim of the thesis

Despite difficulties regarding culture growth, a number of molecules synthesized by *Tannerella forsythia* that were isolated and described have been demonstrated to function as virulence factors. The aim of this thesis is to give an overview of major *T. forsythia* virulence factors. These include proteases, glycosidases and protein components of cell membrane and cell wall. We will look at each of these groups individually in three dedicated chapters.

3. Proteases

Since *Tannerella forsythia* is asaccharolytic, it acquires the majority of its carbon from host peptides and free amino acids (AA). Hence, *T. forsythia* employs a number of protease enzymes that are excreted either bound to outer membrane or into extracellular space (1). Some proteases, like *T. forsythia* trypsin-like protease described by Grenier, assist in protein metabolism by degrading small peptides (21). However, the majority of these molecules degrade large host proteins into smaller peptides or individual AAs. In addition, some *T. forsythia* proteases also partake in reshaping of the oral environment to better accommodate dysbiotic organisms via damaging of the periodontal tissue, cleavage of molecules related to innate and adaptive immunity, modification of host membrane proteins to assist in colonisation and activation of clotting pathway components (4).

3.1. Mirolase

Mirolase is a novel subtilisin-like calcium-dependant serine protease found in *T. forsythia* (13,22). Mirolase is a member of KLIKK protease family, which is characterised by C-terminal KLIKK AA sequence (23). Its AA sequence consists of a signal domain, an N-terminal prodomain, a catalytic domain containing a characteristic subtilisin-like Asp-His-Ser catalytic triad, and a C-terminal extension consisting of a 30 residue long variable region and a highly conserved C-terminal domain (Figure 4.) (22). Mirolase C-terminal domain appears to share some similarity with C-terminal domain of proteins secreted by novel type 9 secretion system (T9SS) (22). The optimum pH for mirolase activity is 9,5, with activity present in range 7 to 10 (22).

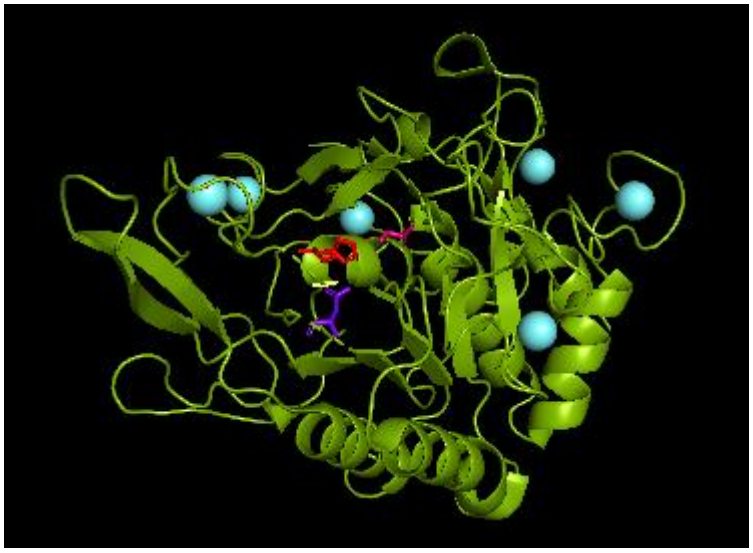

Figure 4. Molecular structure of mirolase. The six calcium ions forming coordinate complexes are marked in light blue. The catalytic triad is marked in purple (asparagine), red (histidine) and serine (pink). Figure adapted from PDB 8EHE.

Like other subtilisin-like proteases, full-length mirolase is transcribed as a 85 kDa zymogen (22). The enzyme is activated through several stages of autoproteolysis. Firstly, the N-terminal prodomain and a 10 kDa C-terminal domain segment are cleaved. The N-terminal prodomain forms a noncovalent complex with the 60 kDa form of mirolase and sterically inhibits it. Gradually, the N-terminal prodomain degrades, leading to an increase of enzyme activity. A final cleavage of 20 kDa C-terminal extension leaves a mature 40 kDa mirolase enzyme (22).

Mirolase activity is calcium dependant. The optimal Ca^{2+} concentration appears to be between 100 µM and 1 mM. Interestingly, in most of the tested bacteria, cytoplasmic Ca^{2+} is maintained between 60 µM and 300 µM. It appears likely that *T. forsythia* could maintain Ca^{2+} levels at the lower end of this range, thereby keeping the enzyme inactive inside of the cytoplasm (22).

The primary function of mirolase is inhibition of the complement system (13). Jusko et. al. demonstrated that the inhibition of classical and lectin pathway is achieved by degradation of mannose binding lectin, ficolin-2, ficolin-3 and C4, while the alternative pathway is inhibited via degradation

of C5 (13). The same study demonstrated the capability of mirolase to generate heightened levels of C5a in plasma and promote the recruitment of neutrophils, which could contribute to vasodilatation, vascular permeability and tissue damage (13). Moreover, mirolase complement inhibition functions synergistically with *T. forsythia* protease karilysin and with *Prevotella intermedia* gingipain enzymes (13). Interestingly, in the presence of normal human serum, mirolase increases levels of C1q (13). C1q could be selectively targeting commensal oral bacteria, thus promoting dysbiosis (13). Finally, mirolase degrades fibrinogen and LL-37, both of which play a critical role in blood clothing and regulation of periodontal homeostasis respectively (22). This research demonstrates that mirolase is a vital tool in destruction of periodontal homeostasis and dysbiosis induction.

3.2. *Tannerella forsythia* DPP IV-like protease

Dipeptidyl peptidase IV (DPP IV), also known as dipeptidyl aminopeptidase IV and CD-26, is a serine protease expressed on surface of epithelial cells, some endothelial cells, leukocyte subtypes and fibroblasts. Analogous proteins are expressed by many bacteria, including *P. gingivalis*. It is 766 AAs long. It can exist as a membrane bound homodimer or as a free floating enzyme. DPP IV cleaves X-Pro and X-Ala dipeptide sequence on the penultimate position near the N-terminus. Its active site appears to be located in the N-terminal domain. Its primary function is degradation of collagen, immunoregulation and regulation of fibrotic response (24,25).

It was shown that in periodontal pockets suffering from progressive periodontitis, *T. forsythia bfor_1659* gene is upregulated (24). *bfor_1659* codes for BFOR_1659 protein, which was demonstrated to be an 82 kDa serine protease. Interestingly, Yost and Duran-Pinedo demonstrated that BFOR_1659 has a 62% and 44% sequence similarity with *P. gingivalis* and human DPP IV respectively. The similarity was especially prominent in the

N-terminal domain with all crucial functional elements, such as S1 and S2 pockets and surrounding residues, highly conserved (24).

Yost and Duran-Pinedo also demonstrated the ability of BFOR_1659 to degrade gelatin, type I collagen and type III collagen, indicating that it shares a similar function to DPP IV (24). Interestingly, the same study indicated that BFOR_1659 is excreted into extracellular space (24). The mechanism of excretion remains unknown (24). Taken together, this research shows that BFOR_1659 is excreted by *T. forsythia* in virulence to damage host tissue by degrading collagen.

3.3. PrtH protease

PrtH protease is a cysteine protease encoded by *T. forsythia prtH* gene (26). The initial *prtH* gene was described as encoding for a 423 AA long protein with molecular weight of 48 kDa (26). A separate study identified a 536 residues long protein named *forsythia* detachment factor encoded by *fdf* gene (26). Later, it was discovered that not only does *fdf* gene contain the p*rtH*, but that the translational start site of the *prtH* gene was incorrectly predicted (26). Therefore, both *forsythia* detachment factor and PrtH refer to the same protein.

Using comparative sequence and structural analysis, Pei and Grishin determined that PrtH and its close relatives in C84 protease family, such as *P. gingivalis* gingipain proteases, possess a caspase-like fold characteristic of CD protein clan (26). This includes highly conserved Rossmann-fold like core, with sequence conservation especially high around catalytic histidine and cysteine residues (26). At the C-terminus, two consecutive α-strands and a β-strand antiparallel to central β-sheet strands, a distinct feature of gingipains and caspases, was also present (26). This data indicates that PrtH and its homologues in protease family C84 belong to CD clan (26).

PrtH is likely involved in virulence of *T. forsythia*. Interestingly, it appears that 85% of adults with periodontitis carried a *prtH* positive genotype

compared to only 10% of *T. forsythia* infected healthy people (16). Studies also showed that the presence of *prtH* positive strain is associated with heightened levels of *T. forsythia* and that long-term dental attachment loss is associated with significantly higher PrtH levels (16). This research indicates that *T. forsythia* pathogenic strain outcompetes the commensal strain during the dysbiosis.

The function of PrtH appears to be the disintegration of the periodontal tissue via induction of periodontal cell detachment (1,4). PrtH may play a role in metabolism of large host proteins and glycoproteins (16). PrtH also appears to possess haemolytic activity (16). It has been demonstrated that PrtH can increase mitochondrial oxidative membrane potential in detached cells, thereby increasing Il-8 production and neutrophil accumulation (4). A possible cytopathic activity which arrested cells in G2 phase was also demonstrated, although its connection to PrtH is uncertain (4). In summary, PrtH functions as a virulence factor by damaging periodontal tissue.

3.4. Karilysin

T. forsythia Karilysin is a KLIKK metalloprotease structurally related to human matrix metalloproteases, particularly with MMP-13 (23). It was first identified as a 472 AA long secretory protein encoded by *tf_0367* gene (27). The structure of karilysin consists of a signal peptide, an N-terminal prodomain, a matrix metalloprotease-like catalytic domain and a C-terminal extension (23,27). It posesses a C-terminal domain which likely marks the protein for excreteion via the T9SS (23). It possesses a 3-histidine zinc-binding motif and a conserved methionine curve characteristic for matrix metalloproteases (27).

Karilysin is expressed in a zymogenic form proKly (27). The activation is regulated via the interactions between the N-terminal prodomain and a zinc ion (Figure 5.) (27). The zinc ion is released through an undescribed mechanism, beginning the maturation process (27). It matures in a three step autoproteolysis. The first step, the cleavage at Asn14-Tyr15, is crucial

and since the mutation in this region leads to loss of nearly all enzyme activity. The second and third steps cleave the C-terminal extension of the proKly at Pro371-Phe372 and Leu180-Tyr181, resulting in a 38 kDa and 18 kDa protein respectively (27). Interestingly, it was demonstrated that the 38 kDa is the most active form of the enzyme and that the cleavage of the 38 kDa was significantly slower than other steps (27). This raises a possibility of 38 kDa form being the mature enzyme. However, 18 kDa form appears to possess the highest structural similarity with MPP-13 (27). Therefore, it is likely that karilysin is excreted via the same method.

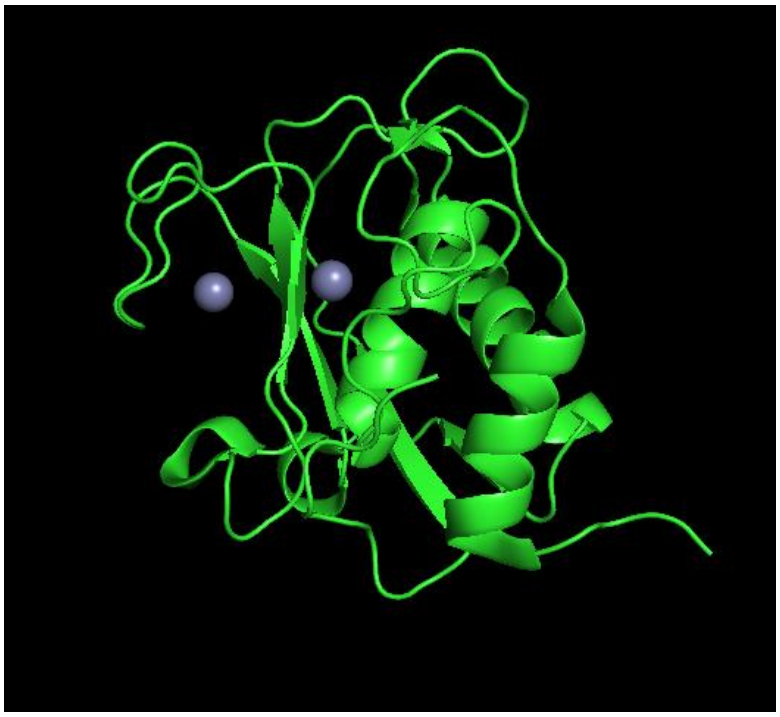

Figure 5. Molecular structure of proKly. The two zinc ions forming coordinate complexes are marked in grey. Figure adapted from PDB 2XS4.

Karilysin is capable of inhibiting the complement system. Jusko et. al. demonstrated that *T. forsythia* strains expressing karilysin were less vulnerable to opsonisation and bactericidal activity of serum (28). The mechanism of inhibition is similar to mirolase, with the lectin pathway inhibited via degradation of mannose binding lectin, ficolins and C4 (28). Karilysin also degrades the C5 protein which leads to an increase of C5a

peptide levels in blood, thereby inducing neutrophil recruitment to the periodontium (28).

Karilysin is capable of modulating the immune response via tumour necrosis factor-α (TNF-α). Bryzek et. al. demonstrated Karilysin to be capable of increasing TNF-α levels by cleaving the membrane form of TNF-α (29). Curiously, despite being partially digested by karilysin, the released TNF-α was able to induce apoptosis and stimulate autocrine pathway pro-inflammatory gene expression (29).

Finally, karilysin has been demonstrated to inhibit the LL-37. LL-37 is an important innate immune response protein with bactericidal activity and capable of inhibiting bacterial lipopolysaccharide (LPS) by forming a LPS/LL-37 complex (30). Koziel et. al. showed that karilysin effectively degraded LL-37 (30). Based on these findings, it has been suggested that karilysin serves a function of protecting members of subgingival plaque sensitive to LL-37 and to promote LPS-mediated local inflammation (30). Taken together, these findings show that *Tannerella forsythia* karilysin plays an important role as a virulence factor in periodontitis.

4. Glycosidases

Glycosidases are enzymes capable of degrading terminal glycoside bonds of large oligosaccharides (4). *Tannerella forsythia* is an asaccharolytic pathogen, meaning it lacks the ability to break down sugars for energy, relying on breakdown of large peptides and lipids (1). Despite this fact, a large number of glycosidases are expressed by *T. forsythia* (4). Some, like the putative SiaH1 sialidase, have a poorly understood function and likely play a minor role in virulence while others, such as alpha-D-glucosidase SusB or TfFuc1 fucosidase, play a role in metabolism of host glycans (4,31). Others are taught to function as virulence factors, degrading gingival host oligosaccharides and proteoglycans (4). They are also taught to contribute to dysbiosis of the periodontium by providing virulent strains of various bacteria with nutrients and exposing protein epitopes for adherence and colonisation (4).

4.1. *nan* gene cluster sialidases

Sialic acid is a monosaccharide in the nonulosonic acid family (32). It is commonly found on the terminal position of human surface glycans (32). It is typically connected to the rest of polysaccharide via α2,3 or α2,6 glycosidic bond on its C2 atom (32). In bacterial infections, it typically functions as a nutrient source for pathogens, as well as playing an important role in bacterial surface adhesion and bacterial toxin attachment (32).

Sialidases are a group of glycosidases capable of hydrolysing glycoside bonds between terminal sialic acid residues and other carbohydrates (32). Most bacterial sialidases are members of GH33 family (32). Their structures include several conserved AA residues such as catalytic tyrosine together with an acid/base catalyst, an arginine triad responsible for controlling the carboxylate moiety of sialic acid, structural Asp-boxes distributed around the six blades of a conserved beta-propeller structure, bacterial Phe–Arg-Ile–Pro motif and carbohydrate-binding modules 32 and 40, which are involved in binding of carbohydrates with sialidases (32).

The majority of *Tannerella forsythia* sialidase activity appears to be dependent on a 16 kbp gene cluster called *nan* gene cluster (33). *nan* cluster appears to contains a unique set of genes required for sialic acid harvesting and utilisation. This includes *nanA*, *nanE*, *nanT*, *nanO*, *nanU* *nanS*, *hexA* and *nanH*. *nanA* and *nanE, which* encode for enzymes neuraminate lyase and N-acetylmannosamine epimerase respectively, partake in sialic acid catabolism (33). Interestingly, *T. forsythia* was shown to be able to utilise sialic acid as a substitute for N-acetylmuramic acid in biofilm culture (33). *nanA* is hypothesised to convert sialic acid into N-acetylmuramic acid, although further research is required to determine the exact mechanism *in vivo* (33). *nanT*, *nanO* and *nanU* encode for components of sialic acid membrane uptake system, with NanO and NanU proteins forming an outer membrane TonB-dependant NanOU uptake system, while *nanT* encodes for a Major facilitator permease protein found on the inner membrane (Figure 6.) (33,34).

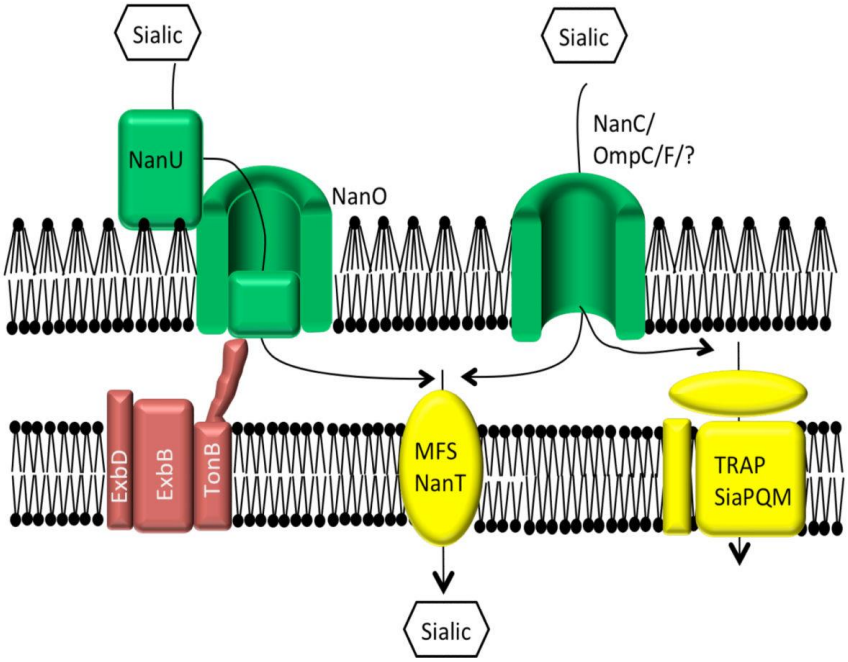

Figure 6. Several sialic acid uptake mechanisms of periodontal bacteria. In case of Tannerella forsythia, The TonB-dependant NanOU type system transports sialic acid into the periplasm while the NanT MFS permease transports it from periplasm into the cytoplasm, Figure adapted from (33)

nanS, *hexA*, *nanH* appear to encode for enzymes with sialidase activity. NanH is a secreted sialidase enzyme in the GH33 family with a preference for α2,3 bond over α2,6 and a pH optimum between 5,5 and 6 (32). It possesses a C-terminal catalytic domain and an N-terminal carbohydrate-binding module specific for sialoglycans (Figure 7.) (32). Its spatial conformation is very stable, with enzyme completely denaturing at 55 °C (32). The primary functions of this sialidase appears to be cleavage of glycosidic bonds between human membrane glycan and terminal sialic acid residues, as well as mediating cell attachment invasion and biofilm formation via their carbohydrate-binding module domains (32).

Figure 7. Molecular structure of NanH. The N-terminal carbohydrate domain is marked in orange while the C-terminal catalytic domain is marked in blue. Figure adapted from PDB 7QYJ.

Interestingly, it has been demonstrated that NanH does not efficiently cleave glycosidic bonds of double O-acetylated sialic acid (32). This is notable, considering that up to 80% of human mucin glycans contain acetylated Neu5Ac, Neu5,4Ac_2 and Neu5,9Ac_2 forms of sialic acid (35). NanS enzyme, also known as sialate-O-acetylesterase, has been demonstrated to efficiently remove the O-9 acetyl group, but not the O-4 acetyl group (35). It has also been demonstrated to increase the sialic acid release from glycans *in vitro* when incubated with NanH (35). This implies that NanS may play a key role in *T. forsythia* exploitation of host glycan-associated sialic acid.

F. nucleatum subsp polymorphum was also shown to possess an operon that is potentially involved in LPS sialylation (33). Since *T. forsythia* and *F. nucleatum* are known to form communal biofilms in oral plaque, this raises the possibility of *T. forsythia* harvesting *F. nucleatum* surface sialic acid residues. This hypothesis is further supported by research showing decreased *F. polymorphum* biofilm formation with *nanH* deficient *T. forsythia* mutant and that sialidase treatment causes alteration of *F. polymorphum* LPS banding pattern (33).

Finally, *hexA* encodes for a β-glucosaminidase enzyme. It is taught that it might assist in cleaving of sub-terminal glycan residues after sialic acid removal. Further research is required to better understand it's function in T. forsythia metabolism (31,33). In conclusion, *nan* gene cluster seems to play an important role in not only virulence, but also in bacterial metabolism.

4.2. β-glucanase (TfGlcA)

BFO_0186-BFO_0188 is a *T. forsythia* locus encoding for a TonB-dependant receptor with an associated SusD lipoprotein, which partake in the uptake of extracellular glycan across the membrane, and a BFO_0186 encoded β-glucanase with β-1,6 and β-1,3 specificity belonging to GH16_laminarinase_like glycosyl hydrolase family (36). This operon is

located downstream of another 2-gene operon encoding for stress-responsive extracytoplasmic function sigma factor (BFO_0190) and an anti-sigma factor (BFO_0188), indicating the possibility that the expression of BFO_0186-BFO_0188 might be externally influenced (36). Interestingly, Honma et. al. showed that the *tfGlcA* was upregulated and that colony growth was significantly larger in WT *T. forsythia* and *F. nucleatum* cobiofilm than in TfGlcA deficient *T. forsythia* and *F. nucleatum* cobiofilms (36). This research indicates that *F. nucleatum* is capable of stimulating *T. forsythia* β-glucanase activity. Since *T. forsythia* is known to be asaccharolytic, free glucose is likely utilised by *F. nucleatum*.

Interestingly, *in vitro* studies demonstrated *T. forsythia* is capable of accumulating high levels of toxic glucose metabolism product methylglyoxal (MGO) via enzyme methylglyoxal synthase in presence of glucose, likely due to an imbalance between rate of MGO synthesis and degradation (4,10). MGO levels were found to be increased in crevicular fluid of individuals with periodontitis (4). Due to its high electrophilic reactivity with arginine, lysine and, in particular, cysteine, MGO can cause protein cross linking and a loss of function (4). The products of these modifications are advanced glycation end-products which can promote inflammation by binding to advanced glycation end-product receptors (10). Other studies indicate that higher MGO levels are correlated with *T. forsythia* loads in inflamed periodontal tissue and that free MGO can damage tissue by promoting apoptosis of gingival fibroblasts (10).

In vitro studies have demonstrated that free glucose promotes *F. nucleatum* growth while inhibiting *T. forsythia* growth, likely due to an increase in methylglyoxal production (36). This research, combined with the fact that the growth of *T. forsythia* and *F. nucleatum* was increased in cobiofilms, indicates that *in vivo* consumption of free glucose by *F. nucleatum* reduces MGO to levels not harmful to the bacterium while maintaining its virulent properties (36). The exact mechanism of this regulation is not well understood, although it is likely regulated at protein level via cell-to-cell

contact, since the presence of *F. nucleatum* did not affect the expression of BFO_0190 and BFO_0188 sigma factors (36). Taken together, this research demonstrates that TfGlcA β-glucanase serves as a virulence factor which plays a role in dysbiosis of the oral plaque.

5. Cell membrane and cell wall-associated proteins

Being a gram negative bacteria, in addition to cellular membrane, *T. forsythia* also possesses a cellular wall consisting of a thin peptidoglycan layer, the outer membrane and an S-layer (3–5). The outer membrane possesses a 6 nm thick outer layer and a 1 nm thick inner layer, which are separated by 2 nm (3). The inner membrane possesses two layers, each 2 nm thick, separated by 1 nm of space (3). The 16 nm thick periplasm contains a thin A1γ-type peptidoglycan layer with meso-diaminopimelic acid forming crosslinks (3,5). The 22 nm thick S-layer consists of serrated subunits (3,4). Like the rest of gram-negative bacteria, *T. forsythia* outer membrane possesses a lipopolysaccharide (LPS) that induces an inflammatory response by interacting with toll-like receptor-4 (37).

5.1. Peptidoglycan recovery and harvesting pathway

Unlike most bacteria, *T. forsythia* is unable to synthesize N-acetylmuramic acid, a key component of bacterial peptidoglycan. *T. forsythia* seems to have lost a gene encoding for a bifunctional UDP-GlcNAc synthase and glucosamine 6-phosphate synthase, the latter of which is an initial amino sugar biosynthetic enzyme (5). Glucosamine 6-phosphate synthase converts fructose 6-phosphate to glucosamine 6-phosphate, while UDP-GlcNAc synthase synthesizes UDP-GlcNAc from fructose 6-phosphate (5). It also lacks UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), UDP-N-acetylenolpyruvoylglucosamine reductase (MurB) and several other enzymes involved in N-acetylmuramic acid synthesis (10). Without these enzymes, *T. forsythia* lacks the ability to *de novo* synthesize N-acetylmuramic acid and cell wall.

T. forsythia is known to possess several mechanisms for bypassing its inability to synthesize N-acetylmuramic acid. During cell growth and the turnover of own cell wall, enzymes called lytic transglycosylases release disaccharidic anhydromuropeptides in a process known as peptidoglycan

salvage (5). Next, periplasmic N-acetylmuramidases NamZ1 and NamZ2 specifically cleave the exogenous peptidoglycan and release N-acetylmuramic acid (5). The transporters MurT and AmpG transport N-acetylmuramic acid and anhydromuropeptides respectively from the periplasm into the cytoplasm where they are metabolised (Figure 8.) (5). Interestingly, it appears that many of the bacterial species cohabiting with *T. forsythia*, such as *F. nucleatum*, *T. denticola*, and *P. gingivalis*, lack the *tf_AmpG* ortholog, indicating that *T. forsythia* might utilise anhydromuropeptides from their cell wall turnover (5). Finally, *T. forsythia* was demonstrated to be able to utilise whole peptidoglycan as a N-acetylmuramic acid substitute via cleavage by NamZ1 and NamZ2 activity (5). In particular, *T. forsythia* is known to utilise peptidoglycan of F*. nucleatum* cell walls (5).

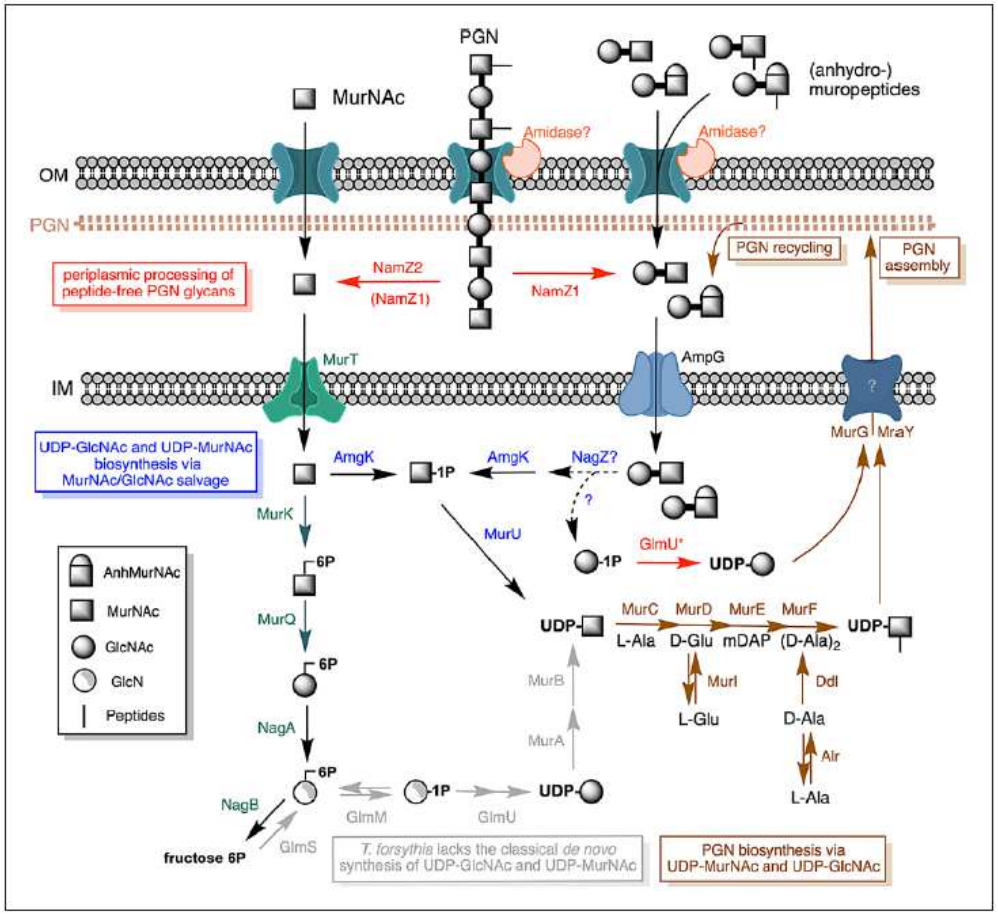

Figure 8. Peptidoglycan salvage pathway of Tannerella forsythia. Figure adapted from (5).

Interestingly, it is possible that *T. forsythia* N-acetylmuramic acid scavenging might play a role in virulence. Dipeptides D-γ-glutamyl-meso-DAP and MurNAc-L-Ala-D-isoGln, released by cell wall turnover and cell division, are known to act as ligands of NOD1 and NOD2 pathogen recognition receptors respectively (10). It is possible that *T. forsythia* N-acetylmuramic acid scavenging can reduce NOD1 and NOD2 activation by reducing muropeptide levels in its environment, thereby delaying the immune response (10).

5.2. Type 9 secretion system (T9SS)

T9SS is a transmembrane glycoprotein translocon unique to the outer membrane of *Bacterioidetes* bacteria (38). Its most common function in pathogenic bacteria is secretion of virulence factors and other proteins from the periplasm into the extracellular space, while many commensal bacteria utilise it for a type of movement called gliding motility (38). All proteins transported by T9SS possess a conserved C-terminal domain that marks them for secretion (38). 18 genes in total are responsible for proper function of T9SS (38). With the exception of porP-porK-porL-porM-porN operon, genes encoding for T9SS are scattered through the genome. It is composed of cytoplasmic, inner membrane associated, periplasmic and outer membrane associated proteins (38).

There are two steps in protein secretion by T9SS. The cargo proteins are initially guided by a classical signal peptide to the inner membrane translocon. There, type I signal peptidase cleaves the signal peptide and the protein enters the periplasm. In the periplasm, transported proteins fold into a stable conformation, likely via the assistance of chaperons. The C-terminal domain then guides the protein to the outer membrane component of the T9SS. Upon secretion protease with sortase-like Activity removes the C-terminal domain and the new C-terminus is attached to an anionic LPS. The secreted protein can either be free floating or attached to the cell membrane via the LPS (38).

5.3. BspA

BspA is an outer membrane associated protein, although it is also present in the secreted form (4). It belongs to a family of proteins characterised by leucine rich repeat (LRR) motifs (4). These 23 AA long motifs are common in eukaryotic and prokaryotic proteins involved in protein-protein interactions (4). The N-terminal region is composed of smaller D1 and D2 regions, each containing 14 and 6 tandem repeats of LRR motif respectively (4). The repeating LRRs result in a series of α-helices and β-sheets all parallel to a single axis. Due to this, BspA molecule has a non-globular horseshoe-like shape (Figure 9.) (4). The parallel β-sheets cover the inner circumference of the horseshoe while the α-helices cover the outer circumference (4). The C-terminal portion contains four immunoglobulin-like domains known as Big_2 and a conserved C-terminal domain (4). At least one of the Big_2 is predicted to assist in structural stabilisation of the N-terminal curve (4). The C-terminal domain is taught to function as a signal for *T. forsythia* glycosylation system and assist in transit of the protein to the outer membrane (4). The protein is mostly O-glycosylated (39).

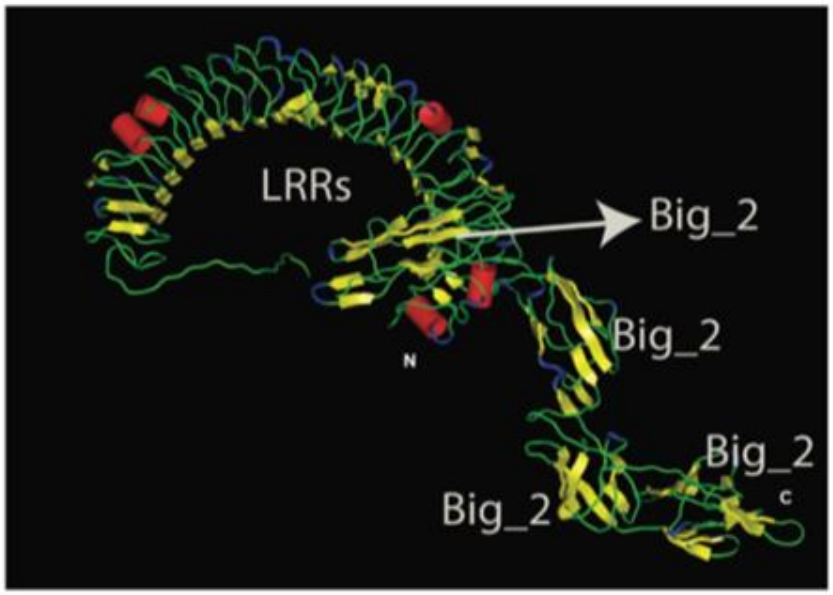

Figure 9. Molecular structure of BspA. The repeating LRR domains form a N-terminal horseshoe-like structure. One of the four Big_2 C-terminal immunoglobulin-like domains interacts with the horseshoe-like domain. Figure adapted from (4).

LRR proteins are a large family of proteins involved in protein-protein interactions and signal transduction, but the exact function appears to vary depending on the AA sequence of the LRR motif (4). It has been demonstrated that BspA- defective mutant strain showed significantly lower potency to induce alveolar bone loss in mice compared to wild type strain (4). Therefore, it is likely that BspA partakes *T. forsythia* virulence via protein-protein interactions. To date, a large amount of protein-BspA interactions have been described. BspA LRR domain has been shown to stimulate TLR2/1 receptor heterodimer, leading to IL-8 release from gingival epithelial cells and bone-resorbing proinflammatory cytokine release from monocytes (4). BspA was also shown to bind to extracellular fibronectin and fibrinogen, thereby assisting in adhesion, proliferation and tissue invasion of *T. forsythia* (1,4). BspA has been demonstrated to mediate interactions and coaggregation with other periodontal bacteria such as *T. denticola*, *F. nucleatum* and *P. gingivalis* (4). Interestingly, LRR motif was shown to activate gp340 pathogen recognition receptor (4). Although its primary function appears to be protection of epithelial tissue form bacterial invasion and pro-inflammatory cytokines, it is also found in saliva where it acts as a salivary agglutinin (4). However, further research is required to confirm if *T. forsythia* BspA interacts with salivary gp340 receptor. In summary, BspA is an LRR family protein involved in large number of protein-protein mediated virulence interactions.

5.4. Miropin

Serpins, or serine protease inhibitors, are a large family of proteins found in eukaryotic organisms, but are especially common in mammals (15). In humans they are known to have various functions such as immune response regulators, chaperones, storage proteins or hormone transporters (15). Prokaryotic serpins are, on the other hand, relatively rare and poorly understood (15). One of the few characterised prokaryotic serpins is the novel *T. forsythia* serpin known as miropin. It is predicted to be an outer

membrane associated lipoprotein exposed to extracellular environment, which is supported by presence of lipoprotein signal peptide and a threonine residue at P1 position (15). The inhibitory core region of serpins, composed of three β-sheets (A-C), eight to nine α-helices, and an exposed reactive centre loop (RCL), is highly conserved in miropin (15). Interestingly, unlike other serpins, miropin retains some serine protease inhibition in aggregated form (15).

Serpin mechanism of action, unique among protease inhibitors, irreversibly inhibits the enzyme via the creation of an ester covalent bond with catalytic serine or tyrosine residue. After the serine protease cleaves the RCL at P1-P1' site and the ester bond is created, the enzyme rapidly changes from stressed to relaxed structure, where the RCL migrates and inserts into the β-sheet A, the structure of the protease changes and it loses its catalytic function (Figure 10.) (15,40). Interestingly, Ksiazek et. al. demonstrated that, alongside P1-P1' site, miropin RCL can be cleaved at several additional sites, of which P5-P4 (Glu-Met), P6-P5 (Val-Glu) and P2-P1 (Lys-Thr) sites are the most notable (15). In addition, it appears that miropin inhibits a number of different serine proteases, in contrast to other serpins which are enzyme specific (15). The association constant of miropin appears to be sufficient for *in vivo* inhibition (15). However, miropin's serpin index, representing the number of serpin molecules required to inhibit 1 enzyme molecule, equals ~3, while this value for most other serpins it is ~1 (15). Taken together, this research presents miropin as an effective serine protease inhibitor capable of inhibiting a large amount of enzymes. This presents it as a potential virulence factor playing a role in *T. forsythia* survival, since in the gingival environment contains a large amount of both bacterial and host proteases (15). Compared to other bacteria, serine protease inhibitors are relatively common in commensal species, further indicating a protective function of miropin (15). Interestingly, miropin appears to be more closely related to human SCCA1 serpin than to other bacterial serpins, indicating a potential horizontal gene transfer origin (15).

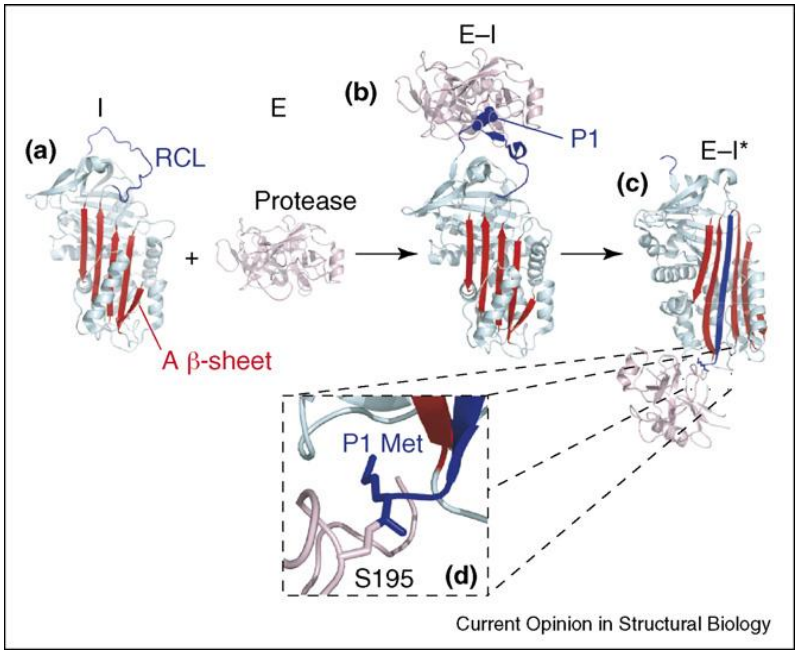

Figure 10. Serpin mechanism of action. (a) A typical serpin possesses a reactive centre loop (RCL; blue) and a series of β-sheets, of which β-sheet A (red) is the most important. (b) The RCL is cleaved by a serine protease (pink) and the covalent bond is formed. (c) The cleaved RCL changes conformation and inserts itself as a component of β-sheet A, consequently changing the conformation of serine protease and inactivating it. (d) The covalent bond forms between the catalytic Ser residue and the P1 Met residue of the RCL. Figure adapted from (40).

5.5. The S-layer

The S-layer of *T. forsythia* is unique among gram-negative bacteria. Not only is it composed of two proteins instead of just one, but these proteins are also glycosylated (2). These two glycoproteins, known as TfsA and TfsB, are the most abundant *T. forsythia* glycoproteins (2). They are encoded by *tfsA* and *tfsB* genes and have a molecular weight of 220 and 210 kDa respectively (2). They require the T9SS to be secreted across the outer membrane (41).

Both of these proteins are O-glycosylated at Ser or Thr residues within the (D)(S/T)(A/I/L/V/M/T) motif with a unique decasaccharide glycan (2,41). The structure of this glycan is composed of a conserved pentasaccharide core, on which fucose, O-methylated galactose and two N-acetylmannosaminuronic acid residues are subsequently added (2). Finally, the terminal N-acetylmannosaminuronic acid is linked to a nonulosonic acid

residue (Figure 11.A) (2). Nonulosonic acids are 9-carbon α-keto acids (2). Of these, modified sialic acid-like sugars pseudoaminic acid and legionaminic acid are found as terminal sugars of *T. forsythia* glycan (Figure 11.B) (2).

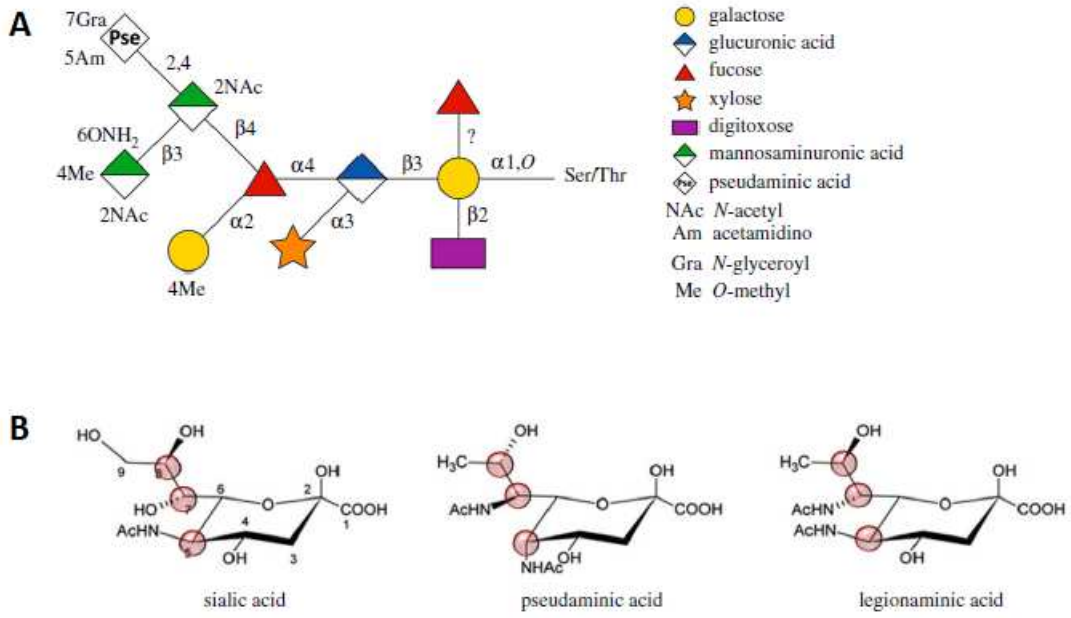

Figure 11. (A) T. forsythia characteristic decasaccharide glycan. The nonulosonic acid in this glycan is shown as pseudoaminic acid. (B) Three most common nonulosonic acids present in T. forsythia. Figure adapted from (2).

The function of TfsA and TfsB glycoproteins appears to be immune evasion. The structural similarity of pseudoaminic acid and legionaminic acid to sialic acid, which is commonly found on the surface of human cells, indicates that the S-layer glycoproteome might play a role in immune suppression via molecular mimicry (2). This possibility is confirmed by research demonstrating that S-layer of *T. forsythia* suppresses Th17 response and production of IL-1b, TNF-a and IL-8 in macrophages and human gingival fibroblasts, while the truncation of O-glycan and S-layer deletion lead to significant increase in IL-8 production and Th17 cell differentiation (2,39). The S-layer was also shown to inhibit the deposition of complement factor C3b (2).

TfsA and TfsB glycoproteins appear to be implicated in biofilm formation and tissue invasion (2). TfsA and TfsB glycans could function as ligands for *F. nucleatum* lectin-like receptors, and *T. forsythia* strains possessing legionaminic acid were found to be much more prevalent in multispecies biofilms than those containing pseudoaminic acid (2,4). Interestingly, the S-layer ablation and O-glycan truncation did not influence *T. forsythia* ability to grow in a multispecies biofilm. Instead, it lead to decrease of *Campylobacter rectus* numbers and increase in *T. forsythia* and *P. gingivalis* coaggregation (2). This indicates that the S-layer glycans might play a regulatory role in gingival plaque formation.

5.6. Outer membrane vesicles

Outer membrane vesicles (OMVs) are spherical particles usually 20–250 nm in diameter that are secreted by gram-negative bacteria (Figure 12.) (39). They are formed via budding from the outer membrane (39). Alongside outer membrane derived components such as LPS, phospholipids and outer membrane proteins, they can contain periplasmic proteins (39). They have been demonstrated to transport virulence factors into host cells, modulate the immune response and partake in colony establishment (39).

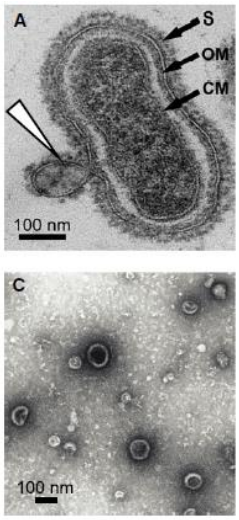

Figure 12. Transmission electron microscopy images of (top) budding of OMVs from T. forsythia membrane and (bottom) mature T. forsythia OMVs. Figure adapted from (39).

T. forsythia has been demonstrated to produce OMVs ~100 nm in diameter (39). Interestingly, *T. forsythia* OMVs appear to be completely covered with intact S-layer (39). They were determined to contain 175 different proteins, most of which are periplasmic and outer membrane-associated proteins (39).

Major *T. forsythia* virulence factors that were detected in OMVs include S-layer glycoproteins, BspA, *nan* gene cluster proteins NanO, NanU and NanH, sialidase SiaHI, β-N acetylglucosaminidase HexA, miropin and KLIKK proteases forsylin and miropsi-2 (39).

T. forsythia OMVs have been demonstrated to induce macrophage and human periodontal ligament fibroblast mediated release of proinflammatory cytokines, which were further enhanced in presence of soluble form of CD14 receptor and at higher OMV concentrations (39). Further research is required to understand the exact mechanism of *T. forsythia* OMV virulence.

6. Discussion

Despite all research regarding *T. forsythia* and its virulence factors, it remains an understudied organism. Nevertheless, several potential avenues for research can be identified. *T. forsythia*, just like the rest of the red complex, partakes in dysbiosis of the periodontal plaque in coordination with a rich and complex microbial community (17). The most thoroughly studied red complex bacteria, *P. gingivalis*, is taught to cause dysbiosis by impairing the innate immunity which promotes the growth of pathogenic microbiota (17). Therefore, it is possible that *T. forsythia* possesses a similar mechanism, with KLIKK proteases, miropin and the S-layer sialic acid residues acting in synergy to inhibit innate immune response. However, the functions of PrtH protease and BspA, involving degradation of collagen and induction of IL-8 proinflammatory cytokine release respectively, contradict this. A possible explanation could be that *prtH* and *bspA* genes are downregulated during inflammation suppression and become upregulated when microbial community becomes dysbiotic. Further research is needed to confirm this hypothesis.

Curiously, despite indications that *T. forsythia* is connected with esophageal adenocarcinoma, none of the described virulence factors were found to possess cancerogenic properties. In fact, research regarding esophageal adenocarcinoma etiology points to the chronic inflammation caused by periodontal infection as a likely culprit (1,14). Bacterial infection related inflammation can influence induction, progression, invasion, and metastasis stages of cancerogenesis (14). Therefore, it is likely that *T. forsythia* indirectly partakes in development of esophageal cancer by promoting inflammation and tissue damage in later stages of periodontal disease.

Interestingly, host matrix metalloproteases, particularly MMP2 and MMP9, have been implicated in pathogenesis of esophageal cancer. *P. gingivalis* can impair expression of MMP2 and MMP9 inhibitors TIMP-1 and TIMP-2, as well as promote MMP2 and MMP9 release by stimulating Protease Activated

Receptor (PAR) via gingipain proteases. Unregulated matrix metalloproteases cleave type IV collagen, thereby damaging extracellular matrix and allowing cancer cells to metastasize (1). This indicates *T. forsythia* matrix metalloproteases, like karilysin, could also partake in esophageal adenocarcinoma pathogenesis. Gingipain-like PrtH protease could also be involved. Further studies are required to confirm this possibility.

Among the rest of the periodontal bacteria, *T. forsythia* has a closest mutualistic bond with *F. nucleatum*. *T. forsythia* is capable of harvesting *F. nucleatum* peptidoglycan fragments and LPS sialic acid residues. In turn, *T. forsythia* provides *F. nucleatum* with a carbon source in form of free glucose via β-glucanase cleavage, thereby avoiding accumulation of toxic MGO product (10). *F. nucleatum* is considered a 'bridge bacterium' because of its role in coaggregation of early and late coloniser bacteria. This, combined with the fact that *T. forsythia* plays a role in dysbiosis, indicates that a similar 'bridging' role in *T. forsythia*. Future research should focus on examining this possibility.

Finally, it remains to be seen if the better understanding of *T. forsythia* virulence will lead to new drug development. *T. forsythia*'s dependence on exogenous N-acetylmuramic acid via the peptidoglycan recovery and harvesting pathway renders it vulnerable to drugs targeting this pathway, such as N-acetylmuramic acid analogues. The dysbiotic activity of *T. forsythia* could be reduced via inhibitors of KLIKK proteases or miropin antagonists. The large amount of periodontal bacteria involved in periodontal dysbiosis likely means that no drug specifically targeting *T. forsythia* virulence factors will be sufficient to prevent or treat periodontal disease by itself. Still, *Tannerella forsythia* plays an important role in pathogenesis of these conditions and better understanding of its virulence remains an important step in development of new drugs and therapies.

7. Conclusion

Tannerella forsythia is a periodontal bacterium implicated in several diseases. Despite difficulties regarding cultivation of bacteria and, consequently, low number of studies dedicated to its research, numerous virulence factors have been identified. KLIKK proteases, miropin and the S-layer sialic acid residues impair host immune response, DPP IV-like protease, PrtH protease and BspA damage host tissue, TfGlcA β-glucanase and peptidoglycan recovery pathway regulate the mutualistic relationship with *F. nucleatum* and T9SS and OMVs help in excretion of other virulence factors. Still, the lack of research means potentially a large number of virulence factors remains undescribed. Further research could assist in development of new drugs and therapies for treating periodontal disease.

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LANGUAGE SKILLS

MOTHER TONGUE(S): Croatian

Other language(s):

English

Listening	Reading	Spoken production	Spoken interaction	Writing
C2	C2	C2	C2	C2

Levels: A1 and A2: Basic user; B1 and B2: Independent user; C1 and C2: Proficient user

DIGITAL SKILLS

Microsoft Office (Outlook, Excel, Word, PowerPoint) | PyMOL, Avogadro, ChemAxon, Marvin, VMD, GAMESS, MacMOLPit

ADDITIONAL INFORMATION

Driving Licence

Driving Licence: AM 27/04/2023 – 26/08/2032

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