Peptidoglycan Salvage Enables the Periodontal Pathogen *Tannerella forsythia* to Survive within the Oral Microbial Community

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Abstract

Tannerella forsythia is an anaerobic, fusiform Gram-negative oral pathogen strongly associated with periodontitis, a multibacterial inflammatory disease that leads to the destruction of the teeth-supporting tissue, ultimately causing tooth loss. To survive in the oral habitat, *T. forsythia* depends on cohabiting bacteria for the provision of nutrients. For axenic growth under laboratory conditions, it specifically relies on the external supply of *N*-acetylmuramic acid (MurNAc), which is an essential constituent of the peptidoglycan (PGN) of bacterial cell walls. *T. forsythia* comprises a typical Gram-negative PGN; however, as evidenced by genome sequence analysis, the organism lacks common enzymes required for the *de novo* synthesis of precursors of PGN, which rationalizes its MurNAc auxotrophy. Only recently insights were obtained into how *T. forsythia* gains access to MurNAc in its oral habitat, enabling synthesis of the own PGN cell wall. This re-

port summarizes *T. forsythia*'s strategies to survive in the oral habitat by means of PGN salvage pathways, including recovery of exogenous MurNAc and PGN-derived fragments but also polymeric PGN, which are all derived from cohabiting bacteria either via cell wall turnover or decay of cells. Salvage of polymeric PGN presumably requires the removal of peptides from PGN by an unknown amidase, concomitantly with the translocation of the polymer across the outer membrane. Two recently identified exo-lytic *N*-acetylmuramidases (Tf_NamZ1 and Tf_NamZ2) specifically cleave the peptide-free, exogenous (nutrition source) PGN in the periplasm and release the MurNAc and disaccharide substrates for the transporters Tf_MurT and Tf_AmpG, respectively, whereas the peptide-containing, endogenous (the self-cell wall) PGN stays unattached. This review also outlines how *T. forsythia* synthesizes the PGN precursors UDP-MurNAc and UDP-*N*-acetylglucosamine (UDP-GlcNAc), involving homologs of the *Pseudomonas* sp. recycling enzymes AmgK/MurU and a monofunctional uridylyl transferase (named Tf_GlmU*), respectively.

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Introduction

Tannerella forsythia, originally designated *Bacteroides forsythius*, is an anaerobic Gram-negative bacterium taxonomically grouped to the *Porphyromonadaceae* within the *Bacteroidetes* phylum [Tanner et al., 1986; Maiden et al., 2003]. It was first described by Anne Tanner at the Boston Forsyth Institute as a “slow growing fusiform *Bacteroides*” and was isolated from apical plaque samples from patients suffering from advanced periodontitis [Tanner et al., 1979; Tanner et al., 1986]. Remarkably, the bacterium could not be obtained in pure culture, but it grew in the presence of a helper bacterium, such as *Fusobacterium nucleatum*, even when the two bacteria were separated by a semipermeable membrane [Tanner et al., 1986; Dzink et al., 1987]. This indicated that survival of *T. forsythia* depends on an essential growth factor that is released from the helper bacterium. This mysterious compound was subsequently identified by Chris Wyss at the University of Zürich as *N*-acetylmuramic acid (MurNAc), which supported axenic growth of *T. forsythia* [Wyss, 1989]. Together with *N*-acetylglucosamine (GlcNAc), MurNAc is an essential carbohydrate constituent of the peptidoglycan (PGN) of the bacterial cell wall. The glycan backbone of PGN is built from these two carbohydrates, which are connected via β -1,4 glycosidic bonds in an alternate arrangement and multiple of these glycan chains are cross-linked via short peptides linked to MurNAc [Weidel and Pelzer, 1964; Walter and Mayer,

2019]. Thereby a flexible mesh-like structure is generated, the PGN sacculus, that allows the bacterial cell to withstand intracellular osmotic pressure (turgor), thus protecting from lysis [Vollmer and Seligman, 2010]. *T. forsythia* possesses a typical Gram-negative PGN [Mayer et al., 2019b]. However, genome analysis of *T. forsythia* revealed that the bacterium lacks orthologs of *murA/murB* and *glmS/glmM/glmU* (shown in Fig. 1; proteins missing in *T. forsythia* but commonly present in bacteria are depicted in gray). These genes encode generally essential enzymes for the *de novo* biosynthesis of the PGN precursors UDP-MurNAc and UDP-GlcNAc [Chen et al., 2005; Friedrich et al., 2015]. Specifically, due to the lack of *murA/murB*, *T. forsythia* is unable to synthesize UDP-MurNAc from UDP-GlcNAc, rationalizing the MurNAc auxotrophy of *T. forsythia*. Moreover, due to the lack of *glmS/glmM/glmU*, *T. forsythia* is also unable to synthesize UDP-GlcNAc from fructose 6-phosphate (fructose 6P) (Fig. 1).

Since *T. forsythia* apparently fails to *de novo* synthesize UDP-MurNAc and UDP-GlcNAc, it depends on the salvage of MurNAc and, presumably, also of GlcNAc from the environment to synthesize its cell wall. It is conceivable to assume that the polymicrobial flora in the oral cavity, harboring more than 700 bacterial species [Lamont et al., 2018], provides to *T. forsythia* distinct options to access various sources of MurNAc and GlcNAc. Indeed, the ability to establish itself in a polymicrobial biofilm consortium, commonly known as dental plaque, is supposed

Fig. 1. Overview of the PGN/MurNAc salvage and biosynthetic pathways of *T. forsythia* crucial for its survival within polymicrobial biofilms. *T. forsythia* lacks homologs of GlmS, GlmM, a bifunctional GlmU, MurA, and MurB (light gray), which are generally essential enzymes in bacteria, generating UDP-GlcNAc and UDP-MurNAc required for PGN biosynthesis. The later steps of PGN biosynthesis via MurCDEF, MurI, Alr, Ddl, MraY, and MurG are present in *T. forsythia* (brown). Hence, *T. forsythia* depends on the salvage of external MurNAc-sources. Exogenous MurNAc (for symbols refer to legend) and GlcNAc-MurNAc/GlcNAc-anhMurNAc disaccharides are internalized via the inner membrane (IM) transporters Tf_MurT (MurT) and Tf_AmpG (AmpG), respectively. Translocation across the outer membrane (OM) is supposedly facilitated via SusC-like TonB-dependent uptake machineries that work in complex with SusD-like glycan-binding lipoproteins. Uptake of both, (anhydro-)muropeptides and longer PGN chains, presumably occurs by concomitant removal of the stem peptides by a so far unknown PGN amidase, thereby releasing peptide-free disaccharides and PGN-glycans (poly-GlcNAc-MurNAc chains) into the periplasm. The removal of the peptide side chains from the PGN upon entry in the cell likely allows *T. forsythia* to distinguish between exogenous (nutrition source) and endogenous (the

self-cell wall) PGN within the periplasm. Peptide-free poly-GlcNAc-MurNAc chains are readily degraded in the periplasm by the recently identified paralogous exo-*N*-acetylmuramidases Tf_NamZ1 and Tf_NamZ2, that act on peptide-free PGN-glycans releasing GlcNAc-MurNAc disaccharides and MurNAc, respectively. Subsequently, these cleavage products are translocated across the IM by the transporters Tf_AmpG and Tf_MurT, respectively. PGN-derived disaccharides may be cleaved by an *N*-acetylglucosaminidase (Tf_NagZ) (cf. Table 1) within the cytoplasm. MurNAc can either be shuttled into the PGN synthesis pathway via the Tf_AmgK/Tf_MurU enzymes of *T. forsythia* (blue) thereby generating UDP-MurNAc, or enters the PGN catabolic pathway via Tf_MurK/Tf_MurQ (green), leading to formation of GlcNAc 6P, which is further catabolized by Tf_NagA and Tf_NagB. Tf_AmgK may phosphorylate GlcNAc, besides MurNAc, as shown for the *Pseudomonas* homolog [Gisin et al., 2013]. In this work, we report on the discovery of a monofunctional GlcN 1P uridylyl transferase (GlmU*) that converts GlcNAc 1P to UDP-GlcNAc. Thus, *T. forsythia* is able to bypass the missing PGN *de novo* biosynthetic enzymes MurA and MurB (gray) by using salvage pathways for exogenous PGN and fragments thereof (red and blue).

(For figure see next page.)

to be a prerequisite for the survival of *T. forsythia* [Bloch et al., 2019]. Formation of multispecies dental plaque biofilms follows a clear spatiotemporal scheme and its progression plays a crucial role in periodontitis. Periodontitis is a multifactorial inflammatory disease, characterized by the irreversible destruction of the teeth-supporting tissue and leading to tooth loss if untreated [Holt and Ebersole, 2005]. A distinction is drawn between the different colonizers and stages of maturation of dental plaque biofilms, according to which the so-called “early colonizers,” which are mainly Gram-positive streptococci, precondition the

oral surfaces for the subsequent adherence and growth of so called “bridge bacteria” [Socransky et al., 1998; Kolenbrander, 2000]. During biofilm maturation, these “bridge bacteria,” including *Fusobacterium nucleatum*, create a suitable environment for the so-called “late colonizers” [Diaz et al., 2000; 2002]. Among these are the periodontal pathogens *T. forsythia*, the related *Bacteroidetes* species *Porphyromonas gingivalis*, and the spirochete *Treponema denticola*, which together build up the so-called “red complex” [Paster et al., 2006]. The late colonizing red complex consortium is associated with severe forms of

periodontitis [Holt and Ebersole, 2005]. Given the fastidious growth requirements of *T. forsythia* in a laboratory monoculture, the question arises, which natural sources the bacterium can exploit to ensure its survival in the oral cavity. In this review, we describe the most recent advances in the understanding of the salvage pathways used by *T. forsythia* within oral microbial communities to acquire the essential growth factor MurNAc and how this is metabolized to allow PGN biosynthesis.

PGN Type and Cell Envelope Structure of *T. forsythia*

As a Gram-negative bacterium, *T. forsythia* contains a thin, moderately crosslinked PGN layer located within the periplasm, embedded between the inner membrane and the lipopolysaccharide (LPS)-containing outer membrane. A recent compositional analysis of the PGN of *T. forsythia* revealed the typical carbohydrate building blocks GlcNAc and MurNAc as well as the 1,6-anhydro-form of MurNAc (anhMurNAc), constituting the terminal ends of the PGN chains [Mayer et al., 2019b]. *T. forsythia* PGN was assigned to the classical A1 γ -type PGN of Gram-negative bacteria with *meso*-diaminopimelic acid (*m*DAP) involved in direct crosslinks [Schleifer and Kandler, 1972; Mayer et al., 2019b]. The LPS of *T. forsythia* is of the rough type and contains the membrane anchored lipid A, inner and outer core – but lacks a distal O-specific antigen [Raetz and Whitfield, 2002; Posch et al., 2013]. A characteristic of the cell envelope of *T. forsythia* is the additional coverage of the outer membrane by a two-dimensional crystalline surface (S)-layer, consisting of self-assembling glycoproteins (Tf_TfsA and Tf_TfsB) [Lee et al., 2006; Posch et al., 2011b; Sekot et al., 2012]. A glycosylated S-layer is rare among Gram-negative bacteria and characterizes different *T. forsythia* strains, containing either a pseudaminic acid or legionaminic acid derivative at the non-reducing terminal end of a complex S-layer oligosaccharide. These sialic acid-like nonulosonic acids (9-carbon backbone 2-keto acid sugars) were shown to influence pathogenicity of the bacterium [Posch et al., 2011a; Friedrich et al., 2017; Bloch et al., 2019] and their synthesis was shown to depend on either UDP-GlcNAc or GDP-GlcNAc, respectively, as precursors [Schoenhofen et al., 2006; Schoenhofen et al., 2009; Friedrich et al., 2017]. Since *T. forsythia* constantly builds up its complex cell envelope during growth, the organism relies on the supply of a suitable amino sugar precursors for an efficient synthesis of its complex cell wall glycoconjugates, including LPS, S-layer glycopro-

teins, and importantly, it relies on the supply of UDP-GlcNAc and UDP-MurNAc for efficient synthesis of its PGN.

PGN Biosynthesis in *T. forsythia* in Comparison to Other Bacteria and Characterization of GlmU*

Given the presence of multiple amino sugars in its different cell envelope structures, *T. forsythia* surprisingly lacks generally essential proteins of the amino sugar biosynthetic pathway. The bacterium lacks a glucosamine 6-phosphate synthase (glutamine-fructose 6P amidotransferase; GlmS), which converts the fructose 6-phosphate intermediate of glycolysis to glucosamine 6-phosphate (GlcN 6P) [Badet et al., 1987]. This enzyme is the initial amino sugar biosynthetic enzyme of most bacteria, indicating that *T. forsythia* is unable to *de novo* synthesize GlcN 6P and, hence, amino sugars derived from it. This enzyme is also the main regulator of amino sugar metabolism in other bacteria. To adjust the cellular level of GlcN 6P to the growth requirements, *glmS* expression is controlled via a GlmS-riboswitch in the Gram-positive bacterium *Bacillus subtilis* and via small regulatory RNAs and the sensor protein RapZ in the Gram-negative bacterium *Escherichia coli* [Klein and Ferre-D'Amare, 2006; Kalamorz et al., 2007]. Since *T. forsythia* lacks GlmS, the regulation of amino sugar metabolism has to be regulated in a different way in this organism. The presence of the subsequent enzyme of the general amino sugar anabolic pathway, which isomerizes GlcN 6P to glucosamine α -1-phosphate (GlcN 1P) [Mengin-Lecreulx and van Heijenoort, 1996], is uncertain in *T. forsythia*. A glucosamine-phosphate isomerase (Tf_GlmM; BFO_1882; Tanf_04345) annotated in the genome of *T. forsythia* reveals higher similarity to phosphogluco-/phosphomannomutases (e.g., 30% amino acid identity with AlgC of *Pseudomonas putida*) than to GlmM-like phosphoglucosamine mutases. In most bacteria, GlcN 1P is further converted to UDP-GlcNAc by a bifunctional *N*-acetyltransferase/uridylyl transferase (GlmU), which comprises an N-terminal GlcN 1P acetyltransferase domain and a C-terminal GlcNAc 1P uridylyl transferase domain [Mengin-Lecreulx and van Heijenoort, 1994; Gehring et al., 1996]. *T. forsythia* lacks a bifunctional UDP-GlcNAc synthase [Hottmann et al., 2018; Ruscitto and Sharma, 2018]. We found, however, that the organism possesses a monofunctional GlcNAc 1P uridylyl transferase (designated Tf_GlmU*; BFO_1878; Tanf_04325), which is a homolog of a monofunctional Cc_GlmU* (43% overall amino acid

Fig. 2. Purity and activity of recombinant Tf_GlmU* analyzed by SDS-PAGE and by UDP-GlcNAc formation using a radioactive enzyme assay. Left: Coomassie stained 12% SDS-PAGE gel; lane 1, protein standard mixture with molecular masses as indicated; lane 2, purified, recombinant GlmU*. Right: Radioactive assay of recombinant GlmU*. [^{32}P]-GlcNAc α -1P (GlcNAc 1P) was generated from GlcNAc and [^{32}P]-ATP using an anomeric MurNAc/GlcNAc kinase (AmgK from *P. putida* KT2440) [Gisin et al., 2013; Renner-Schneck et al., 2015]. Furthermore, [^{32}P]-GlcNAc α -1P (GlcNAc 1P) was incubated with 5 μg of Tf_GlmU* and 50 mM UTP (control with water instead of enzyme) and reactions were spotted on a TLC plate immediately (0) and after 3 h (3) of incubation at 37°C.

sequence identity to Ccan_15070) that has been identified in the oral pathogen *Capnocytophaga canimorsus* [Renzi et al., 2015]. *C. canimorsus* is auxotrophic for GlcNAc (not MurNAc), but is able to salvage this amino sugar from salivary mucins present in the oral cavity. As an adaption to the availability of GlcNAc in the oral bacterium's settling, the dog mouth, *C. canimorsus* apparently has lost its capability to *de novo* synthesize UDP-GlcNAc, hence, its genome has lost the genes encoding GlmM and the bifunctional GlmU [Renzi et al., 2015].

To determine the function of Tf_GlmU*, the 309-amino acid recombinant enzyme containing a C-terminal His₆-tag was expressed in *E. coli* BL21 (DE3) cells. The protein was purified using nickel-affinity chromatography, followed by size exclusion chromatography (using HiLoad 16/60 Superdex 200 column, GE Healthcare) to near homogeneity (shown in Fig. 2). With a radioactive uridylyl transferase assay as described earlier [Gisin et al., 2013; Renner-Schneck et al., 2015], the Tf_GlmU*-reac-

tion was analyzed using [^{32}P]-GlcNAc α -1-phosphate (GlcNAc 1P) as a substrate (shown in Fig. 2; also see material and methods). After the addition of GlmU* and UTP, we observed the direct conversion of GlcNAc 1P to UDP-GlcNAc, which was not detectable in the negative control. For an unequivocal product determination, a reaction mixture with non-labeled GlcNAc 1P was also analyzed by electrospray-ionization time-of-flight (ESI-TOF) mass spectrometry as described earlier [Gisin et al., 2013] (shown in Fig. 3). The substrate GlcNAc 1P appeared with a mass of $(\text{M}-\text{H})^- = 300.063 \text{ m/z}$ in negative ion mode, which is in accordance with the theoretical mass of GlcNAc 1P $(\text{M}-\text{H})^- = 300.049 \text{ m/z}$ (Fig. 3; in blue). After incubation with Tf_GlmU*, the substrate was partly converted into a product with $(\text{M}-\text{H})^- = 606.100 \text{ m/z}$, which is in agreement with the expected mass of UDP-GlcNAc $(\text{M}-\text{H})^- = 606.074 \text{ m/z}$ (Fig. 3b; in orange)).

The biosynthesis of PGN is a conserved process within bacteria that can be divided into three major steps occurring in different cell compartments. In the first step, the soluble precursor molecules UDP-MurNAc and UDP-MurNAc-pentapeptide are synthesized from UDP-GlcNAc in the cytoplasm (shown in Fig. 1). In the second step, the undecaprenyl-phosphate-anchored PGN precursors lipid I and lipid II are synthesized at the cytoplasmic membrane, which, in the last step, are assembled to polymeric, crosslinked PGN in the periplasm. UDP-MurNAc, the first dedicated precursor of PGN, is synthesized from UDP-GlcNAc *via* the enzymes MurA and MurB, representing the known route for MurNAc synthesis in nature. In the first reaction, MurA catalyzes the transfer of an enolpyruvate from phosphoenolpyruvate to the 3'-hydroxyl group of UDP-GlcNAc. The thereby generated UDP-GlcNAc-enolpyruvate is subsequently reduced by MurB using one reduction equivalent of NADPH to generate UDP-MurNAc [Benson et al., 1993]. In *T. forsythia*, these first committed steps in the biosynthesis of PGN are not possible due to the lack of the initiating enzymes MurA/MurB. All the following reactions leading to UDP-MurNAc-pentapeptide (MurC-F, Ddl, MurI, Alr) and the further reactions leading to lipid II (MraY, MurG), are present in *T. forsythia* (shown in Fig. 1). These reactions are responsible for the sequential ATP-dependent ligation of single amino acids. MurC (BFO_0400; Tanf_11285) transfers L-alanine to UDP-MurNAc, and MurD (BFO_0403; Tanf_11300) and MurE (BFO_0405; Tanf_11310) further add D-glutamic acid (generated by the L-/D-glutamate racemase MurI; BFO_1448; Tanf_09790) and mDAP, yielding UDP-MurNAc-tripeptide. Finally, MurF (BFO_1349; Tanf_06315) is re-

Fig. 3. Analysis of uridylyl transferase activity of recombinant Tf_GlmU* by LC-MS. The reaction mixture containing GlcNAc 1P in Tris-HCl buffer (pH 7.6) was analyzed prior (**a**) and after addition of UTP and purified recombinant Tf_GlmU* of *T. forsythia* (**b**). Shown are the base peak chromatograms (BPCs; grey) and extract-

ion chromatograms (EICs) for GlcNAc 1P (blue) and UDP-GlcNAc (orange). The measured mass-to-charge ratios of the compounds, indicated in the figure, are in agreement with the theoretical m/z values of $(M-H)^- = 300.049$ m/z for GlcNAc 1P and of $(M-H)^- = 606.074$ m/z for UDP-GlcNAc.

quired for the addition of the dipeptide D-Ala-D-Ala, which is generated by ligation of two D-alanine molecules by the ligase Ddl (BFO_3290; Tanf_02600), yielding UDP-MurNAc-pentapeptide [Smith, 2006]. In the second step, the membrane-anchored precursors lipid I and lipid II are synthesized by MraY (BFO_0404; Tanf_11305) and MurG (BFO_0401; Tanf_11290), respectively (shown in Fig. 1). The prenyl sugar transferase MraY firstly transfers phospho-MurNAc-pentapeptide to the lipid carrier undecaprenyl phosphate, generating undecaprenyl pyrophosphoryl-MurNAc-pentapeptide, named lipid I [Al-Dabbagh et al., 2008]. Subsequently, lipid II is generated by the action of the glycosyltransferase MurG that transfers a GlcNAc moiety from UDP-GlcNAc onto lipid I [Bouhss et al., 2004]. In the final third step, lipid II is translocated to the outer leaflet of the cytoplasmic membrane, which is mediated by MurJ-like flippases belonging to the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily, allowing the assembly of PGN by PGN-synthases [Typas et al., 2012; Sham et al., 2014; Cho et al., 2016]. Surprisingly, *T. forsythia* lacks an obvious MurJ homolog, and it is thus unclear how lipid II is translocated in this organism. Importantly, however, an undecaprenyl-phosphate carrier system is present, as *T. forsythia* contains both an undecaprenyl diphosphate synthase (UppS; BFO_1444; Tanf_09810) and an undecaprenyl diphosphatase (UppP/BacA, BFO_1764; Tanf_03865). Additionally, homologs of FtsW

(BFO_0400; Tanf_11295) and RodA (BFO_3355; Tanf_08125) can be found on the *T. forsythia* genome, as well as Rod-complex and divisome components and penicillin-binding protein PGN synthases.

MurNAc Uptake and Metabolism in *T. forsythia*: Evidence for an AmgK-MurU Salvage Route

Since *T. forsythia* cannot *de novo* synthesize UDP-MurNAc due to the lack of *murA* and *murB* orthologs (Fig. 1), it relies on the salvage of exogenous MurNAc or alternative sources of MurNAc. As mentioned above, *T. forsythia*'s dependency on external MurNAc was first reported by Wyss in 1989, who observed that exogenous MurNAc is a crucial growth factor and required for the maintenance of a rod-shaped (fusiform) cell morphology [Wyss, 1989]. We confirmed the MurNAc-dependency of *T. forsythia* and the transition from healthy rod-shaped to fusiform cells upon MurNAc starvation [Hottmann et al., 2018]. This observation clearly showed that *T. forsythia* must utilize an uptake system for MurNAc (and other MurNAc-sources) and a biosynthesis pathway leading to UDP-MurNAc that substitutes for MurA and MurB.

The ability to recover components of their own cell wall is a common feature of bacteria. During growth of bacteria, the cell wall undergoes a permanent turnover, and the released breakdown products are recovered in a

process known as PGN recycling [Mayer et al., 2019a]. In Gram-negative bacteria, PGN recycling involves the cleavage of the PGN sacculus by lytic transglycosylases generating anhydromuropeptides (GlcNAc-anhMurNAc-peptides) which are efficiently recovered *via* the AmpG transporter [Mayer et al., 2019a]. Some bacteria can grow on PGN components provided as nutrients. For instance, in *E. coli*, the uptake of MurNAc is mediated by a phosphotransferase system (PTS) transporter that imports and simultaneously phosphorylates MuNAc, yielding MurNAc 6P [Dahl et al., 2004]. The typical PTS transports and phosphorylates sugars via the concerted action of the cytoplasmic enzymes EI (enzyme I) and HPr (histidine protein), and the EII complex, which is composed of the sugar-specific domains EIIA and EIIB, and the transmembrane domain EIIC [Siebold et al., 2001]. MurNAc 6P is the substrate of MurQ, a key enzyme of cell wall recycling, *i.e.*, degradation of anhydromuropeptides, and MurNAc metabolism, which converts MurNAc 6P to GlcNAc 6P [Jaeger et al., 2005; Borisova et al., 2016]. Genome analysis of *T. forsythia* revealed the presence of an ortholog of *murQ* but not of *murP* [Ruscitto et al., 2016]. In view of the genetic context, *Tf_murQ* is part of a three-gene cluster encoding a putative sodium solute transporter (*Tf_MurT*; BFO_0041; Tanf_08375), a putative sugar kinase (*Tf_MurK*; BFO_0042; Tanf_08380), and the putative MurNAc 6P lactyl ether hydrolase *MurQ* (*Tf_MurQ*; BFO_0044; Tanf_08385). The *Tf_MurT* transporter was characterized as a PTS-independent MurNAc transporter, the first known MurNAc uptake system of this kind, and *Tf_MurK* and *Tf_MurQ* were characterized as a specific MurNAc kinase and a *bona fide* MurNAc 6P lactyl ether hydrolase, respectively [Ruscitto et al., 2016; Hottmann et al. 2018]. The clustered organization of the *murTKQ* genes seems quite common among organisms from the *Bacteroidetes* phylum [Ruscitto et al., 2016; Ruscitto and Sharma, 2018].

For the metabolization of sugars their phosphorylation is mandatory. As MurNAc is not phosphorylated in *T. forsythia* by the transporter *MurT*, the concomitance of a sugar kinase (*Tf_MurK*) also encoded within the *murTKQ* cluster is consequential [Ruscitto et al., 2016; Hottmann et al., 2018]. Biochemical investigations of kinase activity of *MurK* revealed stringent specificity for MurNAc phosphorylation at the position C6, thereby generating MurNAc 6P, the substrate for the etherase *MurQ* and catabolism [Hottmann et al., 2018]. The phosphorylation position of MurNAc represents a metabolic branching point in *T. forsythia*. Intriguingly, the deletion of *Tf_murK* (ΔTf_murK) results in the accumulation of

two metabolites, MurNAc and the PGN precursor molecule UDP-MurNAc-pentapeptide [Hottmann et al., 2018]. The accumulation of the latter provided that MurNAc can also be channeled into the anabolic PGN synthesis route as evidenced by a growth advantage of a *T. forsythia* $\Delta murK$ mutant in MurNAc-depleted growth medium [Hottmann et al., 2018].

Pseudomonas spp. possess an alternative route for the synthesis of MurNAc that bypasses the *de novo* biosynthesis of UDP-MurNAc and recovers MurNAc directly as UDP-MurNAc [Gisin et al., 2013]. This pathway includes, first, the phosphatase *MupP*, which dephosphorylates MurNAc 6P forming MurNAc [Borisova et al., 2017]. Next, the anomeric kinase *AmgK* phosphorylates the amino sugars GlcNAc and MurNAc forming GlcNAc α -1P and MurNAc α -1P, respectively. Subsequently, the α -1-phosphorylated sugars can be used by *GlmU* and the uridylyl transferase *MurU* for the generation of UDP-GlcNAc and UDP-MurNAc, respectively. Biochemical characterization of *MurU* of *P. putida* revealed stringent specificity for the uridylation of MurNAc α -1P and not GlcNAc α -1P [Renner-Schneck et al., 2015]. Similar functions might be provided by the orthologs *Tf_amgK* and *Tf_murU* found in *T. forsythia* (unpublished data). Most likely, the *Tf_AmgK/Tf_MurU* pathway is the principal route for PGN synthesis in *T. forsythia* and replaces the missing *MurA/MurB* pathway. The deficit of genes is a phenomenon reported from other bacteria that thrive in biofilms. For so far unknown reason, this might provide an increased fitness [Koskiniemi et al., 2012].

***T. forsythia* Salvages PGN Turnover Products from Cohabiting Bacteria**

T. forsythia grows on MurNAc, yet anhydromuropeptides (GlcNAc-AnhMurNAc-peptides) and muropeptides (GlcNAc-MurNAc-peptides) are more commonly found in the natural environment [Fujimoto and Fukase, 2011]. Anhydromuropeptides constitute the general PGN turnover products of bacteria, while muropeptides represent PGN degradation products resulting from the activity of lysozyme-like muramidases [Mayer et al., 2019a]. Thus, it is reasonable to assume that *T. forsythia* salvages anhydromuropeptides and muropeptides which both likely occur in oral biofilms. Anhydromuropeptides are generated in large amounts during cell wall turnover, whereby the PGN layer is steadily cleaved by autolytic enzymes (autolysins), namely lytic transglycosylases and endopeptidases to enable bacterial growth [Höltje, 1995;

Table 1. Occurrence of selected recycling/turnover enzymes in *T. forsythia* and cohabiting bacteria

PGN recycling & turnover enzyme (Uniprot ID)	Reference strain	<i>T. forsythia</i> ^a	<i>F. nucleatum</i> ^a	<i>T. denticola</i> ^a	<i>P. gingivalis</i> ^a
MurQ (P76535)	<i>E. coli</i> K12	46% ^{b, c}	nd	nd	47% ^f
AmpG (P0AE16)	<i>E. coli</i> K12	24% ^{b, c}	nd	nd	nd
NagZ (P75949)	<i>E. coli</i> K12	28% ^{b, c}	nd	33% ^d	31% ^f
AnmK (P77570)	<i>E. coli</i> K12	nd	nd	nd	nd
AmgK (Q9I5U1)	<i>P. aeruginosa</i> PA01	25% ^b , 26% ^c	nd	nd	nd
NamZ (P40407)	<i>B. subtilis</i> 168	47, 36, 38% (three orthologs) ^{b, c}	nd	46% ^e	37% ^f

^a BlastP analysis was performed using the non-redundant protein sequences database for searching of orthologs in the genome of *T. forsythia* 92A2 (taxid: 203275) and ATCC 43037 (accession number JUET000000000 [Friedrich et al., 2015]), *F. nucleatum* (taxid: 851), *T. denticola* (taxid: 158) and *P. gingivalis* (taxid: 837). Identity percentages relative to the indicated query protein are shown for orthologs from ^b *T. forsythia* strain 9 A2A, ^c *T. forsythia* strain ATCC 43037, ^d *T. denticola* strain ATCC 35405, ^e *T. denticola* strain SP3, ^f *P. gingivalis* strain W83. nd, orthologs not detected.

van Heijenoort, 2011; Mayer et al., 2019a]. In *E. coli*, per generation, about 50% of the PGN of the cell wall is cleaved by autolysins releasing anhydromuropeptides into the periplasm, from where they are efficiently internalized (recycled) by the inner membrane permease AmpG [Park and Uehara, 2008; Mayer et al., 2019a]. While in *T. forsythia*, a AmpG transporter homolog (BFO_0039; Tanf_08365) was identified, several cohabiting bacteria of *T. forsythia* (shown in Table 1), such as *F. nucleatum*, *T. denticola*, and *P. gingivalis*, lack orthologs of the *E. coli* AmpG permease. This indicates that *T. forsythia* might benefit from PGN turnover products released by these bacteria within the oral biofilm. Although Tf_AmpG shows rather low (24%) sequence identity to *E. coli* AmpG [Ruscitto et al., 2017], its function as an anhydromuropeptide permease was initially proposed on the basis the ability of Tf_ampG to complement an *E. coli* ampDampG mutant using a reporter assay [Ruscitto et al., 2017]. Subsequently, we showed that *T. forsythia* grows on anhydromuropeptides derived from *E. coli* PGN cleaved with a lytic transglycosylase, while a *T. forsythia* ΔampG deletion mutant cannot [Mayer et al., 2020]. Intriguingly, we also revealed Tf_AmpG to be responsible for growth in the presence of muropeptides derived from muramidase-cleaved PGN of either *F. nucleatum* or *E. coli* [Mayer et al., 2020]. Surprisingly, growth on muropeptides was clearly favoured over that on anhydromuropeptides, as evidenced by a final OD about twice as high with muropeptides as compared to identical amounts of anhydromuropeptides [Mayer et al., 2020]. Further, we could show that peptide-free disaccharides, i.e. GlcNAc-anhMurNAc and GlcNAc-MurNAc generated by pre-

treatment of the (anhydro-)muropeptides with a recombinant *E. coli* amidase (AmiD), yielded identical growth rates compared with the peptide-containing (anhydro-)muropeptides. Thus, apparently, an endogenous amidase in *T. forsythia* is able to remove the peptide portions from the (anhydro-)muropeptides, yielding disaccharides. Intriguingly, the *T. forsythia* ΔampG mutant, in contrast to the *T. forsythia* wild-type, did not grow in medium supplemented with the peptide-free disaccharides GlcNAc-MurNAc and GlcNAc-anhMurNAc. This distinguishes the *T. forsythia* AmpG-ortholog from *E. coli* AmpG, as the specificity of the latter is restricted to anhydromuropeptides and GlcNAc-anhMurNAc but does not accept GlcNAc-MurNAc [Cheng and Park, 2002].

PGN Serves as MurNAc-Source for *T. forsythia*

An exciting recent finding was that *T. forsythia* could also use intact PGN as a growth factor [Mayer et al., 2020]. PGN from the cohabiting bacteria *P. gingivalis* and *F. nucleatum*, from *E. coli*, as well as from *T. forsythia*, served as a growth factor for *T. forsythia*, replacing MurNAc or (anhydro-)muropeptides. In contrast, the *T. forsythia* ΔampG mutant was severely impaired in growth when cultured with intact PGN instead of MurNAc, indicating that transport *via* AmpG is critical to sustain growth on PGN [Mayer et al., 2020]. This observation implies that *T. forsythia* is able to degrade PGN mainly to GlcNAc-MurNAc or GlcNAc-anhMurNAc disaccharides rather than to MurNAc. In our group, we recently identified and characterized a novel *exo-N*-acetylmuramidase in *B. sub-*

tilis named NamZ [Müller et al., 2021]. This enzyme releases MurNAc from glycoside substrates carrying a MurNAc at the non-reducing end, such as the chromogenic reporter substrate *para*-nitrophenyl-MurNAc (pNP-MurNAc), the natural PGN-derived disaccharide MurNAc-GlcNAc as well as peptide-free PGN chains terminating with a MurNAc residue. NamZ was found to constitute the founding member of a novel family of exo-acting PGN β -*N*-acetylmuramidases [Müller et al., 2021]. In the light of MurNAc-auxotrophy of *T. forsythia*, we reasoned that the presence of enzymes exhibiting exo- β -*N*-acetylmuramidase activity could be beneficial. Intriguingly, the genome of *T. forsythia* encodes three orthologs of *B. subtilis* NamZ (Tf_NamZ1: BFO_0040; Tanf_08370; Tf_NamZ2: BFO_2140; Tanf_00660, and Tf_NamZ3: BFO_2092; Tanf_00855; see Table 1). Furthermore, NamZ orthologs were also found in *P. gingivalis* as well as in *T. denticola* (Table 1), consistent with the finding that the NamZ family glycosidases (CAZy family GH171; www.cazy.org/GH171) are prominently found within the members of the *Fibrobacteres-Chlorobi-Bacteroidetes* FCB superphylum [Müller et al., 2021]. The *T. forsythia* NamZ2 was shown to have a similar function to NamZ of *B. subtilis*. This enzyme cleaves peptide-free PGN glycans and MurNAc-glycosides with a MurNAc at the reducing end, and generates MurNAc, which can be transported into the cell *via* MurT (shown in Fig. 1). The other NamZ ortholog, Tf_NamZ1, was identified as a GlcNAc-MurNAc disaccharide-releasing exolytic β -*N*-acetylmuramidase. This enzyme also releases MurNAc by hydrolysis of MurNAc-glycosides, albeit less efficiently than NamZ2; its main function is the cleavage of peptide-free PGN yielding GlcNAc-MurNAc disaccharides (as well as GlcNAc-anhMurNAc disaccharides, which commonly resides at the reducing ends of the glycans). The *namZ1* gene is located directly adjacent to *ampG* on the *T. forsythia* genome, which encodes the transporter of GlcNAc-MurNAc and GlcNAc-anhMurNAc [Mayer et al., 2020]. Intriguingly, the subsequent gene downstream of *ampG*, *lytB*, displays significant similarity to SpoIID-domain containing lytic transglycosylases [Morlot et al., 2010]. Moreover, *T. forsythia* possesses at least three further putative lytic transglycosylases belonging to glycosyl hydrolase family 23 (www.cazy.org). These enzyme may be involved in trimming peptide-free, exogenous PGN or be involved in PGN recycling in *T. forsythia*. In summary, the exo-*N*-acetylmuramidases Tf_NamZ1 and Tf_NamZ2 presumably degrade peptide-free PGN fragments in the periplasm to yield GlcNAc-MurNAc (besides GlcNAc-anhMurNAc) and MurNAc, which are the sub-

strates of the *T. forsythia* AmpG and MurT transporters, respectively. Based on our findings, we suggest that PGN is internalized by *T. forsythia* through the outer membrane, possibly *via* a SusCD-like uptake complex (shown in Fig. 1). Sus complexes are common uptake complexes within the polysaccharide utilization loci (PUL) of *Bacteroidetes* bacteria, consisting of TonB-dependent outer membrane transporter (SusC) and cell surface glycan-binding proteins (SusD) [Grondin et al., 2017; Lapebie et al., 2019]. An amidase, as mentioned earlier, is required to remove the peptides from PGN upon internalization. Peptide-free exogenous PGN is then processed by NamZ1 and NamZ2 to yield GlcNAc-MurNAc/GlcNAc-anhMurNAc and MurNAc (shown in Fig. 1) and subsequently these fragments are taken up *via* Tf_AmpG and Tf_MurT and are further processed intracellularly by shuttling to the UDP-MurNAc biosynthesis pathway, *via* Tf_AmgK and Tf_MurU, or through the catabolic pathway *via* Tf_MurK and Tf_MurQ (shown in Fig. 1).

Conclusion

The MurNAc auxotrophic oral pathogen *T. forsythia* compensates for its fastidious growth requirements in the oral cavity by salvaging of MurNAc, and MurNAc-containing PGN fragments such as GlcNAc-MurNAc and GlcNAc-anhydroMurNAc disaccharides as well as (anhydro-)muropeptides, which are common cell wall turnover products, presumably released into the environment by cohabiting bacteria. Intriguingly, we revealed that many of the cohabiting bacteria lack an ortholog of AmpG, and thus cannot recover the GlcNAc-MurNAc and GlcNAc-anhydroMurNAc disaccharides released from their PGN cell wall during turnover. Sometimes they even lack further important PGN recycling enzymes (MurQ, AnmK, AmgK; see Table 1). Thus, these bacteria likely provide *T. forsythia* with essential PGN turnover fragments. Moreover, *T. forsythia* is able to salvage intact PGN, which is processed by two members of a newly identified family of exo- β -*N*-acetylmuramidases (Tf_NamZ1 and Tf_NamZ2) within the periplasm. Since these enzymes require peptide-free PGN as substrates, we suggest that the initial removal of the peptide stems from incoming PGN by an unknown amidase is critical to degrade exogenous PGN within the periplasm. This might be a strategy of *T. forsythia* to distinguish between exogenous PGN (nutritional source) and endogenous PGN (*i.e.*, its own cell wall), because the exo- β -*N*-acetylmuramidases specifically cleave peptide-free exo-

genous PGN and leave the structural PGN unattached. Follow-up studies will identify the proposed amidase and clarify whether exogenous PGN is transported through the outer membrane via SusCD-like complexes. These complexes are clustered within so-called PULs and frequently used by *Bacteroidetes* to forage polysaccharides. However, a PGN-specific PUL has so far not been identified in the *T. forsythia* genome. Moreover, the roles of AmgK and MurU in PGN biosynthesis remains to be clarified. The understanding of cell wall and amino sugar metabolism in *T. forsythia* and the elucidation of the different MurNAc-containing sources and routes of uptake and metabolism used by this bacterium to survive within the oral microbial community will help to understand its physiological demands and set the basis to develop new approaches for the treatment of periodontitis.

Materials and Methods/Experimental Procedures

Plasmid Construction, Expression, and Purification of GlmU*

To determine the function of Tf_GlmU*, the encoding gene was amplified from genomic DNA of *T. forsythia* ATCC 43037 using the primer pair GlmU*fw_ TACGAACATATGAAACCAACATTATTTGTGCTGGCGGC/GlmU*rev_ GCGCTCGAGACCGAACAGCTGAGCGGGATATTC (introduced restriction sites are shown in bold) and cloned in vector pET29b for the expression as a C-terminally His₆-tagged-fusion protein. The PCR product and vector pET29 were digested with *Nde*I and *Xho*I (NEB, Frankfurt, Germany) and ligated using T4 DNA ligase (Thermo Fisher Scientific, Waltham, MA, USA). The resulting plasmid (pET29_GlmU*) was transformed into *E. coli* BL21(DE3) cells for overproduction.

Radioactive Uridyl Transferase Assay

The Tf_GlmU* reaction was analyzed with a radioactive uridylyl transferase assay using [³²P]-GlcNAc α-1-phosphate as a substrate, which was prepared from GlcNAc and γ-[³²P]-ATP using AmgK from *P. putida* as described earlier [Gisin et al., 2013; Renner-Schneck et al., 2015]. For the radioactive uridylyl transferase assay, 5 μL of the AmgK reaction mixture were incubated with 50 mM UTP and 1 μg of Tf_GlmU* in 100 mM Tris-HCl buffer (pH 7.6) and spotted immediately and after 3 h of incubation at 37°C on a TLC plate (silica gel 60 F₂₅₄; Merck, Darmstadt, Germany). After separation in *n*-butyl alcohol:methanol:25% (wt/vol) ammonium hydroxide:water (5:4:2:1), the TLC plate was analyzed using a Typhoon Trio Biomolecular Imager (GE Healthcare).

LC-MS Uridyl Transferase Assay

A non-radioactive, LC-MS uridylyl transferase assay was performed as described above but using unlabeled GlcNAc α-1P as the substrate, which was prepared according to a published protocol [Gisin et al., 2013]. 25 mM of GlcNAc α-1P was used in a 100-μL reaction mixture containing 100 mM Tris-HCl (pH 7.6), 50 mM UTP, 1 U baker's yeast inorganic pyrophosphatase and 5 μg of Tf_GlmU*. The reaction was incubated for 3 h at 37°C. The reac-

tion mixture was analyzed before and after the addition of GlmU* and 50 mM UTP, using HPLC connected to an electrospray-ionization time-of-flight (ESI-TOF) mass spectrometer (Micro-TOF II; Bruker) [Gisin et al., 2013].

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Statement of Ethics

Ethics approval was not required, as the work includes only an in vitro characterization of a carbohydrate metabolic enzyme.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

I.H. performed all experiments. I.H., M.B., C.S. and C.M. conceived and wrote the manuscript.

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