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# Sialic acid transporter NanT participates in Tannerella forsythia biofilm formation and survival on epithelial cells

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Running title: Role of *T. forsythia* NanT in biofilm formation

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- Tannerella forsythia requires exogenous peptidoglycan amino sugar N-acetylmuramic acid (NAM) for growth.
- During biofilm growth the bacterium can metabolize sialic acid (Neu5Ac) instead of NAM.
- Bacterial sialidase cleaves Neu5Ac from glycoconjugates on cohabiting bacteria, salivary proteins and epithelial cells.
- NanT transporter is critical for sialic acid acquisition during biofilm growth and survival on epithelial cells.

Abstract

Tannerella forsythia is a periodontal pathogen implicated in periodontitis. This gram-negative pathogen depends on exogenous peptidoglycan amino sugar N-acetylmuramic acid (NAM) for growth. In the biofilm state the bacterium can utilize sialic acid (Neu5Ac) instead of NAM to sustain its growth. Thus, the sialic acid utilization system of the bacterium plays a critical role in the growth and survival of the organism in the absence of NAM. We sought the function of a T. forsythia gene annotated as nanT coding for an inner-membrane sugar transporter located on a sialic acid utilization genetic cluster. To determine the function of this putative sialic acid transporter, an isogenic nanT-deletion mutant generated by allelic replacement strategy was evaluated for biofilm formation on NAM or Neu5Ac, and survival on KB epithelial cells. Moreover, since T. forsythia forms synergistic biofilms with Fusobacterium nucleatum, co-biofilm formation activity in mixed culture and sialic acid uptake in culture were also assessed. The data showed that the nanT-inactivated mutant of T. forsythia was attenuated in its ability to uptake sialic acid. The mutant formed weaker biofilms compared to the wild-type strain in the presence of sialic acid and as co-biofilms with F. nucleatum. Moreover, compared to the wild-type T. forsythia nanT-inactivated mutant showed reduced survival when incubated on KB epithelial cells. Taken together, the data presented here demonstrate that NanT-mediated sialic transportation is essential for sialic acid utilization during biofilm growth and survival of the organism on epithelial cells and implies sialic acid might be key for its survival both in subgingival biofilms and during infection of human epithelial cells in vivo.

Keywords

Oral bacteria; Biofilms; Sialic acid transport; Epithelial cells

## 1. Introduction

Tannerella forsythia is a gram-negative fusiform anaerobe implicated in periodontitis, a common form of inflammatory disease that leads to tooth loss in adults [1] and [2]. This bacterium expresses a number of virulence factors that allow the bacterium to colonize, survive, and trigger inflammation of the tooth-supporting tissues [3]. The bacterium lacks the key enzymes required in bacteria for the synthesis of peptidoglycan amino sugars N-acetylmuramic acid (NAM) and N-acetyglucosamine (NAG) [3] and [4]. Recent work in our laboratories have shown that the bacterium targets host glycoconjugates through its ability to express sialidase that release the terminal sialic acid residues from salivary glycoproteins and cell surface glycoproteins including the oral mucosa. This ability to target host sialic acids can play important roles in a wide range of biological functions, including cell–cell interactions, immunomodulation, and pathogen recognition [5] and [6]. Sialic acid has also recently been shown to decorate the surface of the human oral opportunistic pathogen Fusobacterium nucleatum [7] to which T. forsythia has been shown to aggregate and form synergistic mixed biofilms [8].

Several bacteria have evolved the ability to use sialic acid either as a nutrient, or to decorate their surface molecules such as lipopolysaccharides (LPS), lipooligosacharide (LOS), or capsule to escape from the host immune defense system [9], [10], [11], [12] and [13]. In addition, bacteria can metabolically shuttle sialic acid into the peptidoglycan synthesis pathway [13], which might be essential for T. forsythia. T. forsythia produces sialidase enzymes NanH and SiaH1 [14] and [15] to release free sialic acid from glycoconjugates that could in turn be utilized by the bacterium [16]. The NanH-dependent release of sialic acid on epithelial cell glycoconjugates has been shown to facilitate T. forsythia adhesion to and invasion into epithelial cells [17]. In addition, we showed that the sialic acid-specific transport system in T. forsythia plays a role in biofilm formation [18]. This sialic acid-specific utilization system includes a novel outer membrane sialic acid–transporter complex NanOU and an inner membrane transporter NanT. NanO is a TonB-dependent outer membrane permease and NanU is an extracellular high affinity neuraminate binding (Kd ~400 nM) protein [19].

Previous investigation from our laboratories showed that sialic acid could serve as an important metabolite for the biofilm growth of T. forsythia [18]. This was based on the results demonstrating that sialic acid (Neu5Ac) in the absence of NAM supported the biofilm growth of the organism. In addition, early attachment and biofilm growth of T. forsythia on sialoglycoprotein substrates was significantly reduced when sialidase activity was blocked with inhibitors or deleted by gene deletion.

In this study, we generated T. forsythia mutant deficient in the gene, annotated as nanT (Oralgen at Human Oral Microbiome Database), coding for a putative cytoplasmic membrane sialic acid transporter. The nanT-deletion mutant exhibited reduced ability to uptake sialic acid, to form biofilms in the presence of sialic acid, and survive on epithelial cells. Based on these data we suggest NanT in biofilm growth plays a role in the transport of sialic acid, as a precursor for conversion to glycolytic and/or peptidoglycan sugars in T. forsythia.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

T. forsythia strains were grown anaerobically (10% CO2, 10% H2, 80% N2) in BF broth or on BF agar plates with or without appropriate antibiotics [20]. F. nucleatum polymorphum ATCC 10953 was grown anaerobically at 37 °C in Trypticase soy broth (TSB) or on TSB blood agar plates supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml) and menadione (1 μg/ml). E. coli strains were grown in Luria–Bertani (LB) medium aerobically at 37 °C. E. coli strain DH5α (Life Technologies) was used as a host for cloning and plasmid purification.

### 2.2. Construction of nanT-inactivated mutant

T. forsythia gene sequences were retrieved from the Oral Pathogen Sequence Database (Oralgen) and gene designations correspond to identification (ID) numbers deposited in that database (which we now know to be the sequence of T. forsythia 92A.2). The nanT-gene was deleted in T. forsythia ATCC 43037 (WT) by a previously described allelic replacement strategy [17]. Briefly, a DNA fragment containing the ermF gene flanked by upstream and downstream DNA regions of nanT (Oralgen designation TF0032) (NanT homolog) was electroporated into T. forsythia ATCC 43037 and transformants were selected on agar containing erythromycin plates. The DNA fragment containing TF0032 with flanking sequences was amplified by PCR using primers #1 (′ 5-TGGCTGACCGCTGAAT-′ 3) and #2 (′ 5 – GGCATTCACACCGTTC-′ 3) from T. forsythia 43037 genomic DNA and subcloned into pGEM-T TA cloning vector (Promega, Madison, WI). Resulting plasmid pGEM-TfnanT was then used as template to amplify the linearized vector with primers TfnanT INF1R (′ 5-CGTCCGATTTTAATCCATA- ′ 3) and TfnanT INF 2F ( ′ 5 TGGTTACCGACGCTGTTCTCCGA-′ 3).

The ermFAM fragment (1597 bp) containing 15-basepair overlap region was amplified from pVA2198 [21] with primers TfnanT ermF INF F (5′ - GATTAAAATCGGACGatgacaaaaaagaaattgccc-3′ ; overlap region is shown in capital letters) and TfnanT ermFAM R ( ′ 5-CAGCGTCGGTAACCAttatttcctcccgttaaataat-3′ ; overlap region is shown in capital letters). The generated ermFAM fragment was cloned into linearized pGEM-TfnanT vector by Infusion cloning system (Clonetech, Mountain view, CA). The ermFAM fragment flanked by with 5′ - and 3′ - NanT regions was amplified by PCR with primers #1 and #2. The PCR product was transformed into T. forsythia 43037 by electroporation as previously described [17]. Transformants were plated onto BF agar plates containing 5 mg/ml erythromycin and incubated anaerobically at 37 °C for 14 days. Following incubation, 14 erythromycin-resistant colonies were isolated, which were then screened by PCR and DNA sequencing. The transformants, named TFM32, which was confirmed to have a nanT deletion, was used for further analyses.

2.3. Sialic acid uptake assays

Two independent assays were used to assess sialic acid uptake in T. forsythia. Assay 1: Bacteria were incubated with sialic acid and the depletion of sialic acid in the medium due to bacterial uptake was monitored by a method described previously [22]. Briefly, agar plate grown T. forsythia cells were washed in PBS and resuspended to OD600 of 2.0. Neu5Ac was added (final concentration of 200 μM) to a 100 μl bacterial suspension in a 1.5 ml microfuge tube in triplicates, and cells were incubated for 2 h at 37 °C anaerobic. Bacteria were pelleted by centrifugation in a microfuge at 13, 000 g for 3 min, and supernatant (50 μl) from each tube was collected and placed into a fresh 1.5 ml microfuge tube for estimating Neu5Ac as follows. 25ul of periodate solution (25 mM periodate in 60 mM $H_2SO_4$) was added to the supernatant and incubated for 30 min at 37 °C. 20ul of 2% sodium arsenite solution was added to each tube, followed by the addition of 100 mM thiobarbituric acid and incubation of the mixture at 95 °C for 5 min. After this step, samples were cooled on ice and 500ul acidified butanol

(butan-1-ol, 5% v/v 600 mM HCl). Tubes were vortexed, centrifuged at 10, 000 g for 1 min and supernatants were transferred to fresh tubes and color was read at 549 nm.Briefly, T. forsythia strains were grown to mid-log phase (OD600 of 0.5) in BF broth and harvested by centrifugation, washed with PBS twice and adjusted to an OD600 of 1.0. T. forsythia strains were then exposed to a 2- fold diluted series. Assay 2: Plate grown bacteria were suspended to an OD600 of 0.3. Cells were washed 2X with PBS, pelleted as above, resuspended in 500ul of 1 mM sodium periodate in PBS and incubate at 4 °C for 30 min. Cells were then pelleted, washed 1x with 1000 ul PBS and resuspended in 100 uM aminoxy-biotin in PBS and 10 mM aniline, followed by rocking at 4OC for 90 min. Bacterial were washed 2X with PBS and stained by incubating with streptavidin conjugated Texas red solution (500ul of 2ug/ml for 30 min at 37 °C). After washing 2x with PBS, bacteria were mounted on a slide under a coverslip with mounting medium that contains DAPI and visualized with a fluorescent microscope. The phase contrast and fluorescence images were recorded using Zeiss Axio Observer and Axio Imager wide-field fluorescence microscope (Carl Zeiss) at the University at Buffalo Confocal and Imaging Core Facility. Texas-red signal was collected with the filter set ex560/40 and em630/75, and DAPI signal with the filter set ex365/50 and em445/50. DIC filter was used for phase contrast. Phase contrast and fluorescence images were obtained from the same area and matched using Axio Vision software (release 4.8). All image data were taken at 600x magnification.

2.4. Sialidase activity assay

To estimate sialidase activities of T. forsythia strains, the fluorogenic substrate, 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (4-MU-NeuNA) (Sigma) was used to assay the sialidase activity as described previously [17]. Briefly, 50 μl cell lysates prepared from equal number of cells from the wild-type or the mutant strains were added to 50 μl of 0.1 M sodium acetate buffer with 2% Triton X-100 (pH 4.5) in microtiter wells. Following incubation of the mixture at room temperature for 5 min, 100 μl of 50 μM 4-MU-NeuNAc in 0.1 M sodium acetate buffer (pH 4.5) was added, and the mixtures were incubated at 37 °C for 30 min. Fluorescence was examined under long-wavelength UV light (365 nm) after 15 min at 37 °C. White fluorescence indicated the presence of sialidase activity. The fluorescence intensity was estimated with a microplate reader (Flex Station 3, Molecular Devices).

2.5. Biofilm formation assay

To determine the role of NanT-dependent sialic acid transport in biofilm formation single and dual-species biofilm formation assays were carried out as described previously [23]. Briefly, bacterial cultures were grown and adjusted to OD at 600 nm (OD600) of 0.05 in TSB. For biofilm growth in the presence of sugars, bacterial strains were inoculated at a final OD600 of 0.05 into the TSB medium containing either NAM or sialic acid (Neu5Ac) at the concentrations indicated in the figures in 24- or 48-well plates. Controls included TSB alone with bacteria. After incubation for 3 days, planktonic cell growth was measured at 600 nm and the wells were washed two times with distilled water followed by

staining of biofilms with crystal violet (CV: 0.1% for 15 min at room temperature). Bound crystal violet dye was solubilized in 20% acetic acid and absorbance was read at 600 nm. For assessing biofilm growth on glycoconjugate substrates, wells were first coated with fetuin or asialofetuin at concentration as described before. Bacteria were then added to wells in TSB diluted to OD600 of 0.05 and biofilm mass was determined after 3 days with crystal violet staining as above. For mixed biofilms, each bacterial culture was adjusted to OD600 of 0.025 in TSB without any other additives, dispensed into the wells and incubated as above. Biofilm mass after 2–3 days of incubation was determined as above.

### 2.6. T. forsythia survival on epithelial cells

KB cell binding assay was carried out to evaluate the effect of NanT transporter deficiency on bacterial survivability by modification of a protocol described previously [24]. Briefly, KB cells (HeLa derived epithelial cell line CCL17 from American Type Culture Collection, Manassas, VA from ATCC) were maintained in DMEM medium supplemented with 10% FBS at 37 °C under 5% CO2. Prior to an experiment, KB cells were plated into a 48-well cell culture plate to obtain confluent monolayers in each well after 48 h. KB cells monolayers were washed twice with DMEM and T. forsythia strains suspended in DMEM were added to monolayers at an MOI of 25. In parallel, bacteria were incubated in wells without the monolayers as control. KB-bacteria or bacteria alone were incubated for 2, 6 or 18 h in a CO2 incubator. After incubation, wells were washed with DMEM twice and KB cells were lysed with distilled water to release associated bacteria (surface attached outside and internalized bacteria). The numbers of input and KB cell associated bacteria at different time points were calculated by CFU counting. Survival rates of bacterial strains were expressed as percentages of input bacteria recovered from KB cells. All samples were made quadruplicate and experiments were repeated three times.

### 2.7. Statistical analysis

Data were analyzed on the Graph Pad Prism software (Graph Pad, San Diego, CA). Comparisons between groups were made using a Student's t-test between two groups or ANOVA for multiple group comparisons as appropriate. Statistical significance was defined as $P < 0.05$.

## 3. Results

### 3.1. Deletion of nanT in T. forsythia results in reduced sialic acid uptake

In this study we sought the functional role of a predicted MFS (major facilitator superfamily) transporter coded by an open reading frame TF0032 in T. forsythia. TF0032 is located in a genetic cluster predicted to be involved in sialic acid utilization in T. forsythia [18]. To confirm the functional role of this predicted transporter, deletion mutants were constructed and characterized. We utilized double-crossover recombination strategy that resulted in the replacement of TF0032 ORF from the translational start to stop codons with the ermF gene, keeping the upstream and downstream regions (putative transcriptional terminators) intact and eliminating the risk of polar effects in the mutant. The sequence analysis of DNA sequences flanking ermF insertion site confirmed that these sequences were indeed kept intact in the mutant (data not shown). In

addition, RT-PCR analyses confirmed that the expression of the genes downstream from the NanT site were not affected (namely TF0036 (beta-hexosaminidase), TF0037 (putative sialate-esterase and TF0038 (putative sialate mutarotase). Moreover, functional activity of NanH sialidase (TF0035), located downstream of NanT, in several transformants that we analyzed did not differ from that of the wild-type strain. As shown, the fluorescent intensity of one of the randomly selected mutants, TFM32, did not differ significantly from that of the wild-type ( Table 1). A NanH deficient mutant TFM35 [17] as expected showed minimal (background) to no fluorescence under similar conditions. Thus, in all probability the insertion of the ErmF cassette did not cause any polar effects. In order to characterize the role of TF0032, one mutant with correct integration, named TFM32, was selected for further study. Growth phenotype of the mutant strain was analyzed in a broth supplemented with NAM. The data showed no growth retardation of the TFM32 mutant compared to the parental strain under these conditions (Fig. 1). As previously noted [18], Neu5Ac did not support the planktonic growth of either the parental strain or the mutant (Fig. 1).

Table 1.
Sialidase activity of T. forsythia ATCC 43037 (Tf WT) and NanT-deletion mutant TFM32.

| Strain | Fluorescence intensity[a] (mean ± SD) |
|---|---|
| Tf WT | 512 ± 55 |
| TFM32 | 542 ± 63; not significant versus WT |
| TFM35 | 43 ± 5 |

[a]Bacteria at equal density (OD600 of 0.1) were mixed with 1% Triton X 100 containing fluorogenic substrate 4-MU-NeuAc in the wells of a black well plate and fluorescence fluorescent intensities of each strain from triplicate wells are shown. A NanH deletion mutant TFM35 used as a negative control. As shown, no significant difference in the fluorescent intensities of the wild-type and TFM32 is observed.

Two independent assays were utilized to compare the sialic uptake between the wild-type and TFM32. Firstly, T. forsythia wild-type or the mutant cells were suspended in sialic acid containing medium and sialic acid in the medium was assayed with colorimetric assay as a function of time. The results showed increasing depletion of sialic acid with time in case of the wild-type bacteria ( Fig. 2). The mutant TFM32 was significantly reduced in its ability to uptake sialic acid. Since the mutant is not completely attenuated in its ability to take up sialic acid suggests that sialic acid may enter the cytoplasm via other non-specific transporters as well. In parallel to the depletion assay above, bacteria were stained for sialic to confirm uptake. Here sialic acid within the cells was detected by aniline catalyzed aminoxy-biotin tagging followed by staining with Texas-red conjugated streptavidin as described in the Methods. The results showed Texas

red staining on the DAPI labeled wild-type cells but not on the nanT-inactivated mutant cells ( Fig. 2B).

### 3.2. NanT dependent sialic acid uptake is important in biofilm formation

Both the wild-type strain and the nanT-deficient mutant TFM32 strain formed comparable biofilm in the presence of NAM ( Fig. 3). Biofilm levels of the T. forsythia wild-type and TFM32 were not significantly different in the presence of 10 mM or 5 mM NAM coated plate ( Fig. 3A). However, in the presence of sialic acid significant differences in the biofilms levels were noted between the wild-type and the TFM32 strains (Fig. 3A). As previously shown [18], the wild-type strain formed biofilms in the presence of sialic acid in the absence of amino-sugar NAM. Under these conditions, however, the NanT mutant TFM32 formed weaker biofilms compared to the wild-type strain at various concentration of Neu5Ac with growth at lower concentrations of sialic acid showing a more pronounced difference between Wild-type and nanT mutant, indicating NanT might function to facilitate uptake at low concentrations of sialic acid ( Fig. 3A). These data were reproducible with two other nanT-deficient mutants obtained above (data not shown). In order to further validate the role of NanT in biofilm formation, sialoglycoproteins and Fusobacteria were used as exogenous sources of sialic acid. T. forsythia can release sialic acid from these glycoconjugates through the mediation of NanH sialidase as shown previously [17], [18] and [25], while several Fusobacteria spp., express sialic acid on their surface as recently been shown [7]. We confirmed this in the F. nucleatum ATCC 10953 strain by aniline based staining method (data not shown). The wild-type strain formed significantly higher biofilm on the sialloglycoprotein fetuin as compared to asialofetuin, in which the glycan group terminates in N-acetylgalactosamine residues (i.e. no sialic acid is present). Importantly, compared to the wild-type strain the mutant formed significantly less biofilm on fetuin, and moreover, the difference in the biofilm growth between fetuin and asialofetuin was not significantly different at various glycoprotein concentrations tested ( Fig. 3B). In the co-biofilm setting, the wild-type strain T. forsythia formed robust biofilm with F. nucleatum polymorphum 10953 as shown previously [8]. Compared to the wild-type T. forsythia-F. nucleatum biofilm the NanT mutant - F. nucleatum co-biofilms showed a modest but a statistically significant reduction ( Fig. 3C). It is likely that in addition to sialic acid T. forsythia might be scavenging on F. nucleatum for peptidoglycan and amino sugars, which could minimize the contribution of sialic acid transport by NanT transport.

### 3.3. T. forsythia forages epithelial cell sialic acid

Next we asked the question if sialoglycans on or within epithelial cells are important sources of sialic acid for T. forsythia and hence support survival on or inside epithelial cells following attachment. The reason for this interrogation was to confirm if the bacterium in addition to utilizing oral epithelium as a substratum for attachment and colonization also obtains sialic acid from

epithelial cells for growth and survival. This is critical since peptidoglycan sugars NAM and NAG required by the bacterium are not synthesized by the human host and could only be derived from cohabiting bacteria in vivo. Moreover, these sugars are not available once T. forsythia enters the epithelial cell, which T. forsythia is known to do [17], [26] and [27]. Our previous studies have shown that T. forsythia can release terminal sialic acid residues from epithelial cell host glycoproteins and expose beta-linked glucosamine or galactosamine, which may also be important adhesive molecules on epithelial cell surface [17], [25] and [28]. Here we compared the survival rate of T. forsythia strains after they were allowed to attach and enter epithelial cells and viable cell counts measured at different time points. Survival rates were calculated as percent of input bacteria recovered for each strain at different time points of incubation. The data is summarized in Fig. 4A. Similar levels of wild-type and mutant strains were recovered 2 h post-infection of KB cells. These data indicate that the both strains attach to epithelial cells and potentially gain entry with equal efficiencies. Interestingly, at 6 and 18 h post-infection significant differences were noted between the survival rates of the two strains. As shown (Fig. 4A), higher levels of the wild-type cells were recovered compared to the mutant at 6 and 18 h post-infection. Bacteria incubated alone in the absence of KB cells showed no significant differences in their survival rates (Fig. 4B). This also indicated that the aerotolerance ability of the two strains was similar. To rule out differences in the oxidative stress defenses of the strains to ROS (reactive oxygen species) from epithelial cells as the likely cause of the differences in the survivability of the strains observed, the survivability of the strains against oxidative stress was tested. We determined bacterial survival against hydrogen peroxide at concentrations of 0.05 and 0.25 mM (these concentrations were selected based on our previous study [20]). The results showed that both the wild-type and mutant strains were similar in their peroxide sensitivity (data not shown). Taken together these data show that sialic acid uptake is critical in the bacterium's survival during its incubation phase with epithelial cells.

## 4. Discussion

In this study we confirmed the identity and proved the functional role of a predicted sialic acid specific MFS transporter, NanT, in T. forsythia. Further, we demonstrated that NanT transporter plays an essential role in providing the bacterium with sialic acid during its biofilm growth and interaction with a co-habiting bacterium and biofilm partner, F. nucleatum. We also demonstrated that sialic acid transport via NanT is essential in extending the survival of T. forsythia on and within epithelial cells.

Bacteria can utilize sialic as a carbon and nitrogen source, shuttle this sugar into the peptidoglycan pathway, or decorate their surfaces with this sugar to escape from the host immune surveillance [9], [10], [12], [13] and [29]. In T. forsythia, investigations from our laboratories showed that sialic acid serves as a metabolite for the biofilm growth of the organism in the absence of NAM [18]. T. forsythia's ability to target host sialoglycoproteins to release and utilize sialic is evident from in silico and experimental evidence gathered by our laboratories

[16], [17], [18], [25] and [28]. We identified a major sialidase, NanH, expressed by the bacterium that releases sialic acid from soluble and epithelial cell associated glycoconjugates. Recently, an outer membrane high affinity neuraminate binding protein, NanU, from T. forsythia has been characterized biochemically. The NanU protein with a Ton-B dependent transporter, NanO, are thought to be involved in sialic acid uptake in T. forsythia. In silico, sialic acid catabolic pathway is evident from the presence of orthologs of N-acetylneuraminate lyase (NanA TF0030) required to initiate sialic acid conversion into N-acetylmannosamine (ManNAc) and N-acetylmannosamine-6-phosphate 2-epimerase (NanE, TF0031) to convert ManNAc into GlcNAc (N-acetylglucosamine) for subsequent glycolysis or peptidoglycan synthesis (model pathway summarized in Fig. 5). The ability of the bacterium to use sialic acid from host and cohabiting partners likely plays a role in the survival of T. forsythia in the harsh oral environment. NanT-dependent sialic acid transport likely plays a role in mixed biofilm formation and virulence of the organism. Previous studies have shown that T. forsythia and F. nucleatum form synergistic biofilms in vitro and as mixed infection they induce synergistic alveolar bone loss in a mouse periodontitis model [30].

The fate of the sialic acid once it is transported into the T. forsythia cytoplasm remains to be determined. Since T. forsythia is unable to synthesis its own peptidoglycan precursors in the absence of exogenous NAM, we predict that sialic acid is utilized for the synthesis of peptidoglycan sugars. However, it could additionally serve as a nutrient source for the organism. These pathways are currently under investigation in our laboratories.

Importantly our data provide the first evidence that utilization of sialic acid by T. forsythia actually appears to be essential not only for biofilm formation in the presence of sialylated glycoproteins, but also potentially in the intracellular phase of its lifestyle. On the surface of cells the organism has access to several sialloglycoproteins such as membrane inserted mucins, while intracellularly, free sialic acids and recycled sialloglycoproteins are present in the cytoplasm and lysosomes respectively [31]. This finding that NanT potentially plays a role in T. forsythia persistence on and potentially inside the epithelial cells indicates that sialic acid is important for the bacterium's proliferation in the oral cavity. The ability of the bacterium to reside inside and on the surface of epithelial cells might provide a means to persist in the mouth and evade the immune system, and eventually cause disease.

In conclusion, we confirmed that in T. forsythia NanT functions as a sialic acid transporter and it plays a role in biofilm formation and survival of the organism on sialic acid containing glycoproteins, while also showing for the first time that sialic acid might be an intracellular source of nutrition during intracellular colonization of human cells by an oral bacterium.

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**FIGURE LEGENDS**

Fig. 1 Growth rates of the wild-type (ATCC 43037) and NanT mutant TFM32 in TSB broth with NAM (N-acetylmuramic acid) or sialic acid (Neu5Ac). Growth in each condition was assessed by inoculating wells of a 96-well plate with corresponding strain and growth was monitored by measuring absorbance at 600 nm at 1-day intervals for 6 days. The data points are means of triplicate wells from one of three independent experiments with similar results. Abbreviations: Tf WT, T. forsythia ATCC 43037; TFM32, nanT-deletion mutant.

Fig. 2. Sialic acid uptake by T. forsythia strains. (A) T. forsythia strains were incubated with 200 mM Neu5Ac. At 3 and 9 h post-incubation Neu5Ac remaining in the medium was assayed by a colorimetric assay described in materials and methods. Means and standard deviations of three independent replicates are shown. The experiment was conducted on two separate occasions with essentially identical results. (B) Sialic acid within Tannerella cells was detected by aniline catalyzed aminoxy-biotin tagging of sialic acid and Texas-red conjugated streptavidin staining as described in the Methods. Bacterial nucleic acids for localization was performed with DAPI staining (blue). Images were obtained with Axio Imager fluorescence microscope at X600 magnification. Majority of the wild-type cells are observed as doubly stained (Texas red and DAPI) cells whereas no or a few TFM32 doubly stained cells are observed. Image for each strain is representative of several microscopic fields from an experiment that was repeated three times with similar results.

Fig. 3. Sialic acid dependent biofilm growth of bacteria. (A) Growth in free sialic acid. Bacterial at OD600 of 0.05 in trypticase soy broth supplemented with NAM or sialic acid (Neu5Ac) at the concentrations indicated were dispensed in wells (quadruple replicates) of a 24-well plate. Control wells included bacteria in TSB with no sugar additive. After anaerobic incubation for 3 days wells were washed two times with distilled water and biofilms were stained with crystal violet. Bound crystal violet dye was solubilized in 20% acetic acid and absorbance was read at 600 nm; *, $P < 0.05$ (Tf WT versus TFM32) (B) Biofilm growth on glycoconjugate substrates. Bacteria in TSB diluted to OD600 0.05 were added to fetuin or asialofetuin coated wells (quadruple replicates) and biofilm mass was determined after 3 days with crystal violet staining as above; *, $P < 0.05$ (Tf WT versus TFM32). (C) Co-biofilms formation. T. forsythia and F. nucleatum were adjusted to OD600 of 0.025 in TSB without any other additives, mixed, and dispensed into the wells of a 24-well plate (quadruple replicates), incubated for two days and biofilm mass was determined as above; *, $P < 0.05$ (Tf WT + F. nucleatum co-biofilm versus TFM32 + F. nucleatum co-biofilm. Mean and

standard deviation values are shown in each graph. The experiments in A, B, and C were conducted three times with similar results.

Fig. 4. Survival rate of T. forsythia strains on KB cells. A. KB cell monolayers were infected with bacteria at an MOI of 25 and KB cell-bacteria were incubated for 2, 8, and 18 h. At each time point monolayers were washed twice with PBS, KB cells were lysed with distilled water and number of associated bacteria was enumerated by CFU counting. B. Bacteria were incubated in wells without the KB cells. The survival rate at each time point was calculated as the percentage of live bacteria recovered of the total input bacteria (time zero).

Fig. 5. Model depicting the role of sialic acid uptake system of T. forsythia

A.

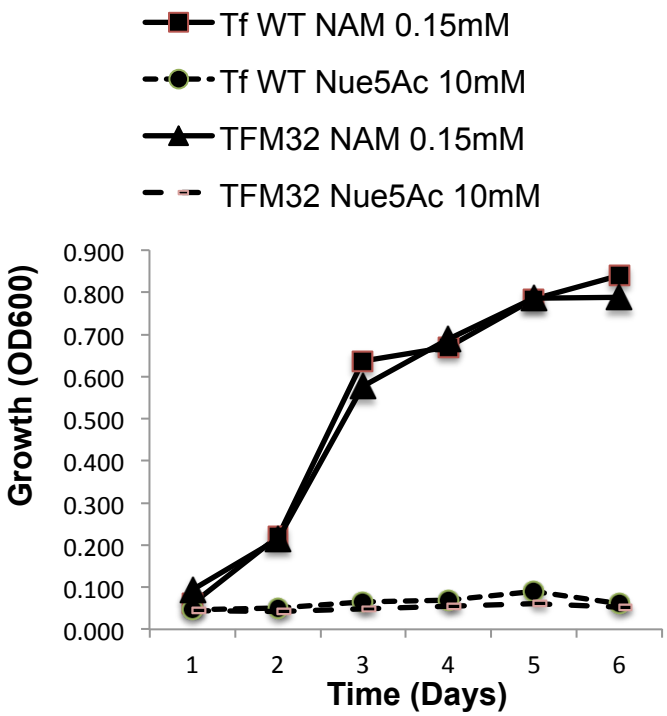
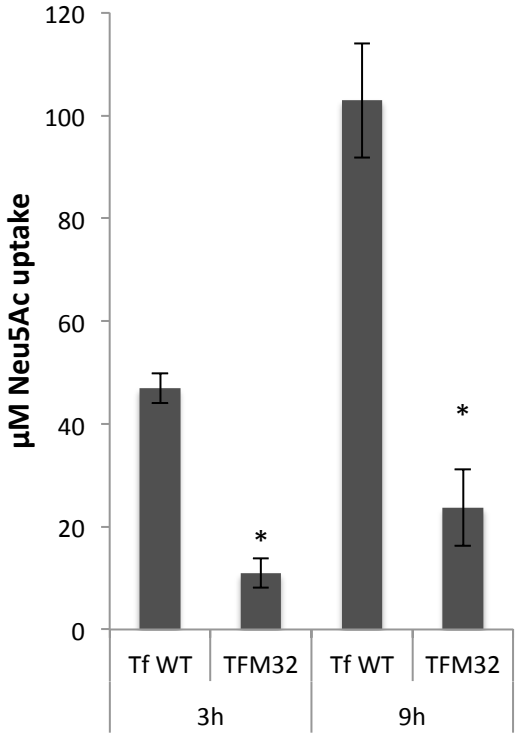

Tf WT NAM 0.15mM
Tf WT Nue5Ac 10mM
TFM32 NAM 0.15mM
TFM32 Nue5Ac 10mM
Growth (OD600)
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
1
2
3
4
5
6
Time (Days)

Fig. 1

A.


120
100
80
60
40
20
0
µM Neu5Ac uptake
*
*
Tf WT
TFM32
Tf WT
TFM32
3h
9h

B.

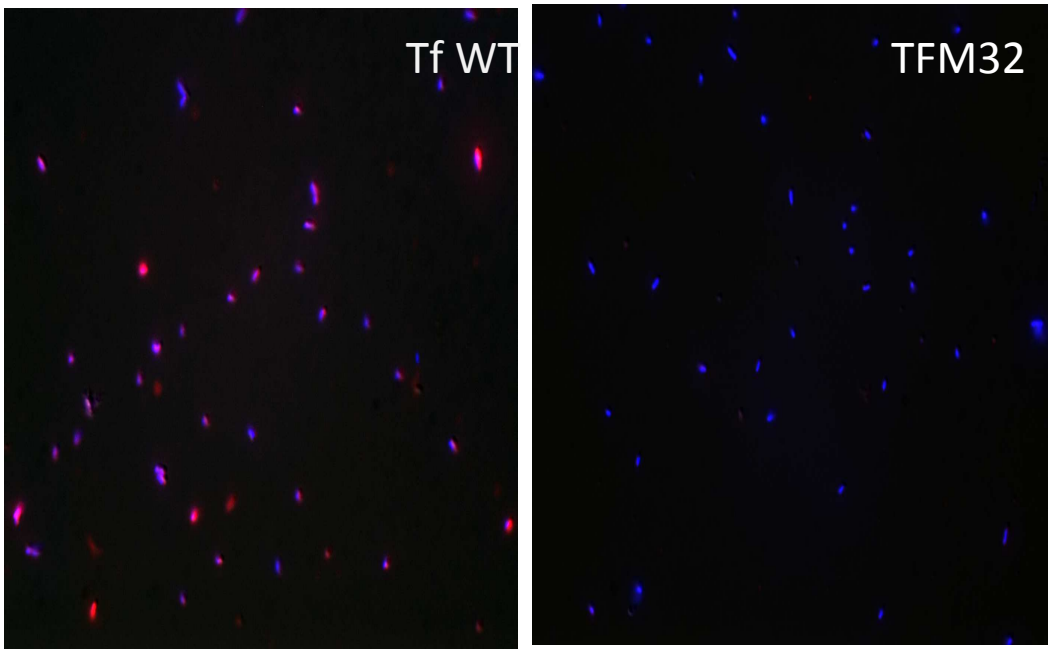

Tf WT
TFM32

Fig. 2

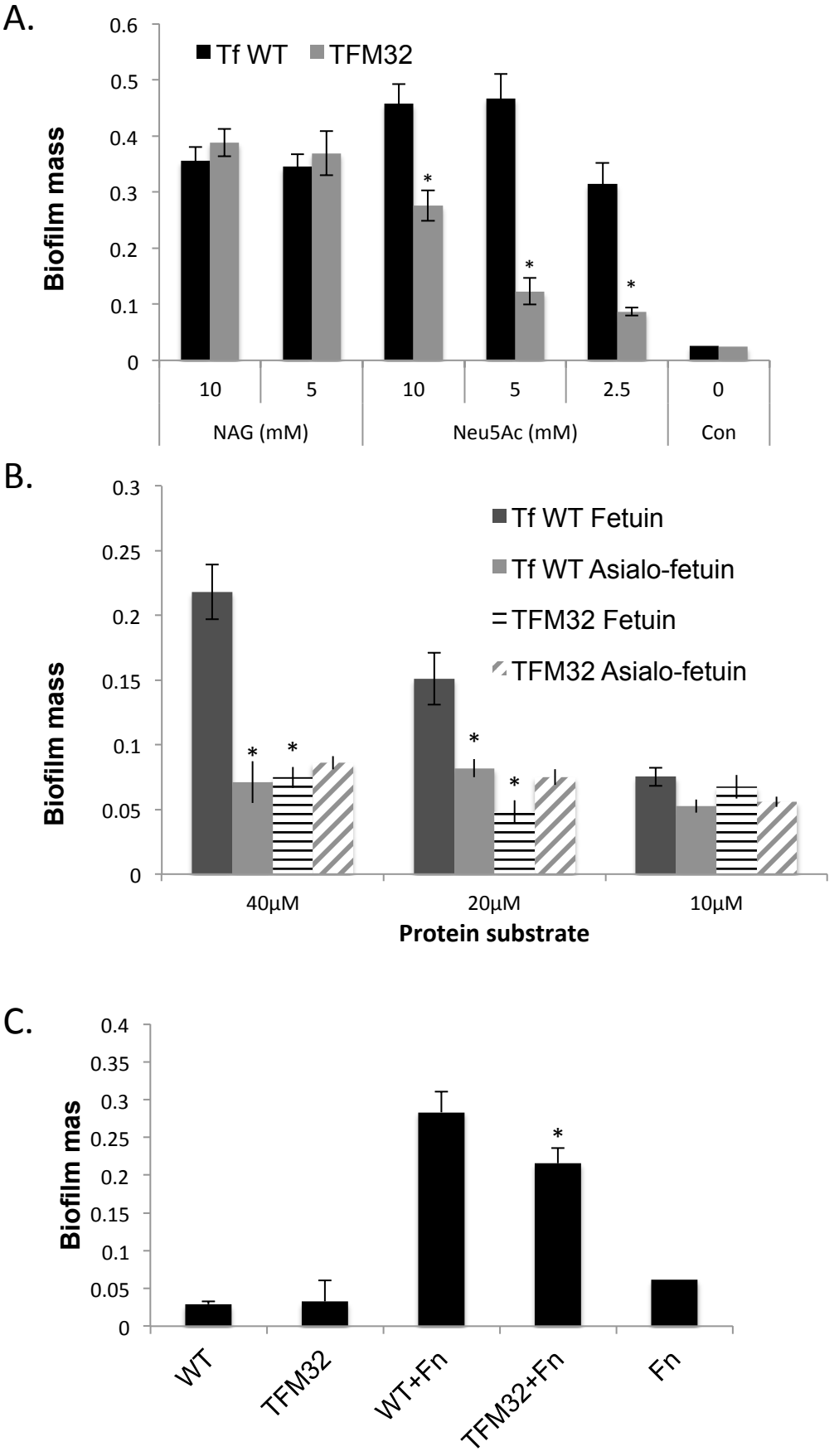

A.
Biofilm mass
0.6
0.5
0.4
0.3
0.2
0.1
0
Tf WT
TFM32
*
*
*
10
5
10
5
2.5
0
NAG (mM)
Neu5Ac (mM)
Con
B.
Biofilm mass
0.3
0.25
0.2
0.15
0.1
0.05
0
Tf WT Fetuin
Tf WT Asialo-fetuin
TFM32 Fetuin
TFM32 Asialo-fetuin
*
*
*
*
40µM
20µM
10µM
Protein substrate
C.
Biofilm mas
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
WT
TFM32
WT+Fn
TFM32+Fn
Fn

Fig. 3

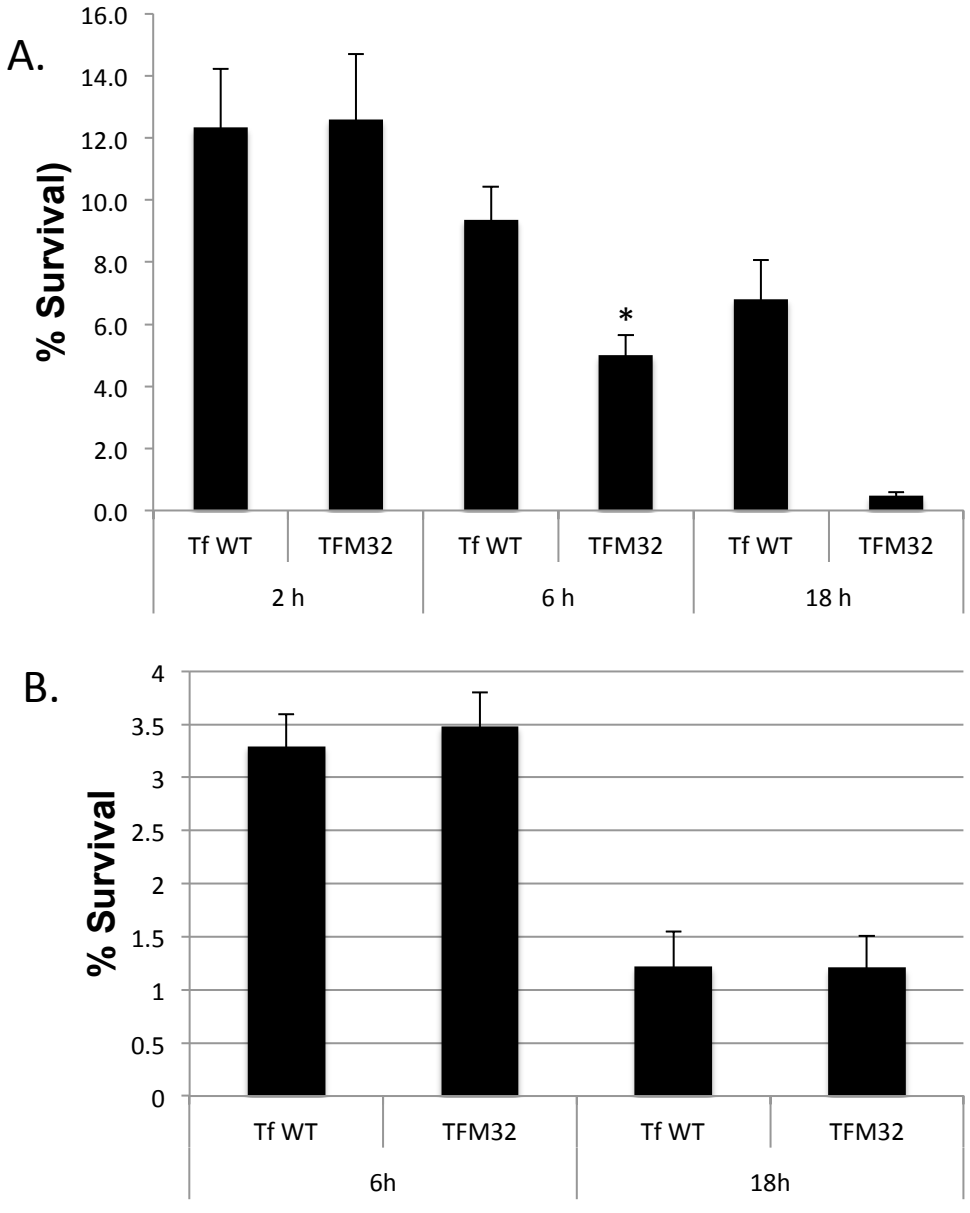

A.
% Survival)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
Tf WT
TFM32
Tf WT
TFM32
Tf WT
TFM32
2 h
6 h
18 h
B.
% Survival
4
3.5
3
2.5
2
1.5
1
0.5
0
Tf WT
TFM32
Tf WT
TFM32
6h
18h

Fig. 4

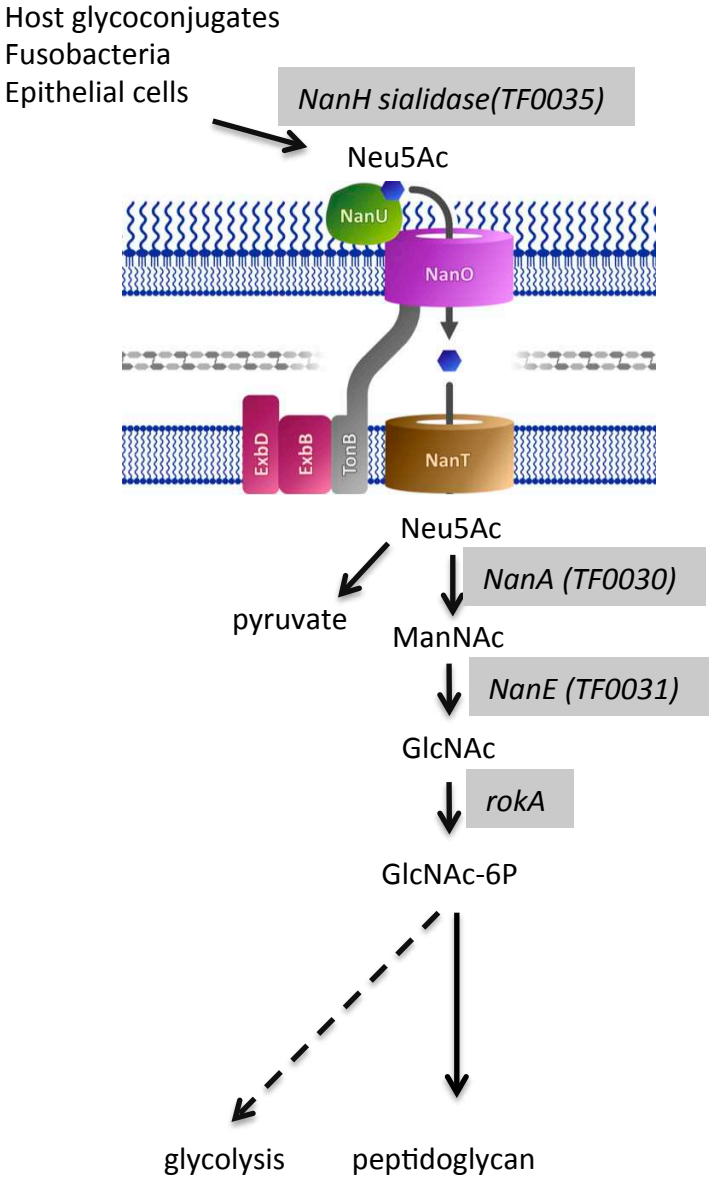

Host glycoconjugates
Fusobacteria
Epithelial cells
NanH sialidase(TF0035)
Neu5Ac
NanU
NanO
ExbD
ExbB
TonB
NanT
Neu5Ac
pyruvate
NanA (TF0030)
ManNAc
NanE (TF0031)
GlcNAc
rokA
GlcNAc-6P
glycolysis
peptidoglycan

Fig. 5