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

Kiyonobu Honma [a], Angela Ruscitto [a], Andrew M. Frey [b], Graham P. Stafford [b], Ashu Sharma [a, *]

[a] Dept. of Oral Biology, University at Buffalo, Buffalo, NY, United States
[b] Oral & Maxillofacial Pathology, The University of Sheffield, Sheffield, United Kingdom

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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*.

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 [1,2]. 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) [3,4]. 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 [5,6]. 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 [9–13]. 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 [14,15] to release free sialic acid from glycoconjugates that could in turn be utilized by the bacterium [16]. The NanH-dependent release of

* Corresponding author. Department of Oral Biology, School of Dental Medicine University at Buffalo, The State University of New York, 311 Foster Hall, 3435 Main St. Buffalo, New York 14214.
E-mail address: sharmaa@buffalo.edu (A. Sharma).

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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 (K_d ~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% CO_2, 10% H_2, 80% N_2) 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-TGG CTGACCGCTGAAT-′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 OD_{600} 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 (OD_{600} of 0.5) in BF broth and harvested by centrifugation, washed with PBS twice and adjusted to an OD_{600} of 1.0. *T. forsythia* strains were then exposed to a 2- fold diluted series. Assay 2: Plate grown bacteria were suspended to an OD_{600} 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 4^{O}C 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