Role of *Tannerella forsythia* NanH sialidase in epithelial cell attachment

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**ABSTRACT**

*Tannerella forsythia* is a Gram-negative oral anaerobe which contributes to the development of periodontitis, an inflammatory disease of the tooth-supporting tissues leading to tooth loss. The mechanisms by which this bacterium colonizes the oral cavity are poorly understood. The bacterium has been shown to express two distinct sialidases, namely SiaHI and NanH, with latter being the major sialidase. Bacterial sialidases can play roles in pathogenesis by cleaving sialic acids on host glycoproteins, destroying their integrity and/or unmasking hidden epitopes on host surfaces for colonization. In the present study, we investigated the roles of SiaHI and NanH sialidases by generating and characterizing specific deletion mutants. Our results showed that the NanH deficiency resulted in total loss of sialidase activity associated with the outer-membrane and secreted fractions. On the other hand, the SiaHI deficiency resulted in only a slight reduction in the total sialidase activity, with no significant differences in the levels of sialidase activity in the outer-membrane or secreted fractions compared to that in the wild-type strain. The results demonstrated that NanH is both surface localized and secreted. The NanH-deficient mutant, but not the SiaHI-deficient mutant, was significantly attenuated in epithelial cell binding and invasion abilities compared to the wild-type strain. Moreover, the NanH-deficient mutant alone was impaired in cleaving surface sialic acids on epithelial cells. Thus, our study suggests that NanH sialidase might play roles in bacterial colonization by exposing sialic acid hidden epitopes on epithelial cells.

**INTRODUCTION**

*Tannerella forsythia* is a fastidious Gram-negative anaerobe which grows in the subgingival crevice, a space between the tooth and gum tissue. It is implicated in the development of periodontitis (38), a chronic inflammatory disease resulting in the destruction of connective tissue supporting teeth as well as bone, leading to tooth loss. A number of putative virulence factors have been identified in *T. forsythia* (33); such as a cell surface-associated leucine-rich-repeat BspA protein (34), a surface S-layer (28, 30), an α-D-glucosidase and a N-acetyl-β-glucosaminidase (14); proteases (9, 29), apoptosis inducing activity (23), and sialidases (3, 22). Sialidases are glycohydrolases which release the terminal sialic acid residues from sialoglycoconjugates. Sialic acids are commonly present as terminal sugars on glycoproteins and glycolipids on the surface of a wide variety of eukaryotic cells and secreted glycoproteins (1, 42). The sialic acid residues modulate a variety of biological functions, such as mediating...
cell-cell interactions via carbohydrate-lectin interactions, stabilizing conformation of the
glycoproteins or masking receptors for ligand binding (1, 42). With respect to bacterially
expressed sialidases, they have been shown to provide pathogenic advantages to bacteria by
countering the host’s innate and adaptive immune responses (6, 25), inducing chemokine
release from epithelial cells (19), exposing cryptitopes by unmasking sialic acid masked
epitopes for adhesion (6, 40), promoting biofilm formation (36) and degrading host glycoproteins
to obtain nutrients (2, 5, 18, 31, 40).

Previous studies have shown expression of sialidase in T. forsythia isolates, which has also
been used for diagnostic identification of the species. T. forsythia has been reported to express
two different sialidases encoded by the siaHI (17) and nanH genes (39). The NanH sialidase
appears to be the major sialidase expressed by the bacterium. A recent study has
demonstrated that sialic acid released from the sialylated glycoconjugates by the NanH
sialidase action may serve as a growth factor for biofilm growth (26). These previous studies
utilized recombinant expression and gene complementation in Escherichia coli for the
characterization of sialidases and the sialic acid uptake system of T. forsythia. In the present
study we generated and characterized siaHI and nanH deletion mutants of T. forsythia to define
the roles of the respective sialidases in host-cell interactions. T. forsythia has been identified
within the buccal epithelium of patients (27) and has been demonstrated to attach and invade
epithelial cells in vitro (16, 28, 30). Since oral bacteria can exploit glycoconjugates and
glycolipids on buccal and gingival epithelial cell for mediating attachment, we predicted that T.
forsythia sialidases would play roles in such interactions. For this purpose we tested siaHI and
nanH deletion mutants for their ability to attach and invade epithelial cells. Our studies
demonstrated that NanH activity plays an important role in the bacterial attachment and invasion
into epithelial cells, likely through unmasking sialic acid hidden bacterial binding epitopes on
epithelial cells.

MATERIALS AND METHODS

Bacterial strains and culture conditions

T. forsythia strains were grown anaerobically (5% CO₂, 10% H₂, 85% N₂) in BF broth or on BF
agar plates (12) with or without appropriate antibiotics. E. coli strains were grown in LB media
aerobically at 37°C. E. coli DH5α (Invitrogen, Carlsbad, CA) was used as a host for cloning and
plasmid purification and BL21 (DE3) (EMD Bioscience, Gibbstown, NJ) was used as a host for
expression and purification of the recombinant proteins. KB epithelial cell line (CCL17, American Type Culture Collection, Manassas, VA) was maintained in Dulbecco’s modified Eagle medium (Gibco, Buffalo, NY) supplemented with 10% fetal bovine serum and 50 µg/ml of gentamycin. The cultures were incubated at 37°C under 5% CO₂. KB cells were grown to near-confluence (90-95%) for the assays. KB cells were originally thought to be derived from an oral epidermal carcinoma. However, DNA fingerprinting later confirmed that this cell line is derived from HeLa cell line (human cervical epithelial carcinoma) due to cross-contamination (American Type Culture Collection, Manassas, VA). The human gingival epithelial cell line, OBA-9, was obtained from Dr. Denis Kinane (University of Pennsylvania, PA) and maintained at 37°C, 5% CO₂ in keratinocyte serum-free media supplemented with 10 µg/ml of insulin, 5 µg/ml of transferrin, 10 µM 2-mercaptoethanol, 10 µM of 2-aminoethanol, 10 nM of sodium selenite, 50 µg/ml of bovine pituitary extract, 100 units/ml of penicillin/streptomycin and 50 ng/ml Amphotericin B (Lonza Inc., Allendale, NJ).

Construction of SiaHI and NanH mutants
siaHI (TF2207) and nanH (TF0035) sequences were retrieved from the Oral Pathogen Sequence Database at Los Alamos under the previous name of the organism as T. forsythensis (http://www.oralgen.lanl.gov/). Insertional mutants for each of the genes were constructed by an allelic replacement strategy described previously (12). Briefly, DNA fragments containing the ermF gene flanked by upstream and downstream regions of either TF2207 (siaHI) or TF0035 (nanH) were electroporated into T. forsythia ATCC43037 cells and transformants were selected on agar-erythromycin plates. The primer sequences used in the study are shown in Table 1. Briefly, for constructing the siaHI-deletion mutant, a DNA fragment containing TF2207 with flanking sequences was initially amplified by PCR using primers #1 and #2 from T. forsythia 43037 genomic DNA. This PCR product was then used as a template to amplify the upstream and downstream fragments of TF2207 with primer sets #1 and #3 and #2 and #5, respectively. The ermF fragment (797 bp) was amplified from pVA2198 (8) with primers #4 and #6. Primers #3, #4, #5 and #6 contained overlapping sequences for ermF and TF2207 to allow generation of a fusion fragment by the PCR overlap strategy. Purified PCR products of TF2207 with flanking regions and ermF (797 bp) were used as templates for the overlap PCR (13) using primers #1 and #2. Likewise, a fusion construct was generated by PCR overlap strategy for constructing a nanH-deletion mutant. The primers sets #1N and #3N and #2N and #5N were used to amplify upstream and downstream fragments flanking nanH (TF0035), respectively, and
the primers #3N, #4N, #5N, and #6N containing overlap sequences of *ermF* and TF0035 were used for constructing the fusion fragment. The fusion product for each gene was then transformed into *T. forsythia* 43037 by electroporation as previously described (12). Transformants were plated onto BF agar plates containing 5 µg/ml of erythromycin and incubated at 37°C anaerobically for 14 days. Following incubation, erythromycin resistant colonies were screened by PCR and Southern blotting. One representative transformant corresponding to each gene deletion, confirmed by PCR and Southern blotting, was selected and further used for analyses.

**RNA isolation and reverse transcription (RT) PCR**

Total bacterial RNA was isolated from 2-ml cultures (OD$_{600}$=0.4) by the RiboPure total RNA isolation kit (Ambion Inc. Austin, TX) followed by DNase I treatment (1 U/µg RNA for 1 h at 37°C). DNase was inactivated by treatment with the DNase Deactivating resin (Ambion). The total RNA from each of the wild type and mutant strains was isolated from the mid log phase culture. The cDNA was synthesized from 1 µg total RNA by utilizing Super Script III reverse transcriptase (Invitrogen, Carlsbad, CA) with random hexamers (Promega, Madison, WI) according to the supplier's recommendations. 20% of the cDNA preparation or of a preparation without addition of reverse transcriptase was subjected to PCR using gene specific primers. *T. forsythia* 16s rRNA specific primers were utilized for normalization. Primer sequences are listed in Table 1.

**Sialidase activity**

The total cell lysate, surface-associated (membrane fraction) and secreted (spent medium) sialidase activities of wild-type, SiaHI- and NanH-deletion mutants were compared. The fluorogenic substrate, 2'-(4-methylumbelliferyl)-α-D-NeuAc2en (4-MU-NeuNA; Sigma) was used to assay sialidase activity as described previously (15). Qualitative screening of sialidase activity in intact bacteria, total cell lysate, outer membrane fraction or spent medium filtered through 0.2 µm filter was performed in microtiter plate wells. The *T. forsythia* membrane fraction was prepared as follows. Cells were suspended in Tris-buffer (50 mM Tris pH 7.3, 0.15M NaCl, 5 mM MgCl$_2$) and untrasonicated on ice for 5 min (50% power and a duty cycle of 5). Undisrupted cells and debris were removed by centrifugation (10,000 g, 4 °C, 30 min) and the supernatant collected. The supernatant was then untracentrifuged (80,000 g, 4 °C, 1.5 h) and the pellet was washed twice with Tris-buffer, and finally resuspended in Tris-buffer and stored at −70 °C. Briefly, for activity assay 50 µl of test sample was added to 50 µl of 0.1 M sodium acetate buffer containing 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 incubated at 37°C for 15-30 min. The fluorescence intensity was then examined under long-wavelength UV light (365 nm) after 15 min at 37°C. Blue-white fluorescence indicated the presence of sialidase activity. Quantitative sialidase assays were performed after stopping the reaction with 100 µl 100 mM Tris-HCl, pH 10.0. The fluorescent intensities were measured in a fluorimeter (Labsystems Thermo, Waltham, MA) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm every 10 min starting from 0-1 h. The specific activity of the T. forsythia cell associated or secreted sialidase was determined by extrapolating fluorescence intensities to a standard curve generated with a commercial purified *Clostridium perfringens* sialidase (Sigma, St Louis, MO) with known enzyme activity.

Expression and purification of recombinant NanH.

The NanH-encoding ORF (TF0035) was PCR amplified from genomic DNA using primers nanH-F (engineered BamHI site) and nanH-R (engineered EcoRI site). These primers amplify the NanH ORF from the start codon as previously proposed (39). The PCR fragment digested with BamHI and EcoRI was cloned into pGEX-4T expression vector (GE Life Sciences) at the same sites. The recombinant plasmid clone with the correct in-frame insert sequence, designated pGEX-nanH, allowing expression of NanH as a fusion protein with N-terminal glutathione-S-transferase was transformed into *E. coli* BL21. An *E. coli* transformant clone grown to an optical density of 0.2 at 600 nm was induced with IPTG (0.1 mM final concentration) and culture was incubated for an additional 3 h with vigorous shaking to express the fusion protein. The cells were harvested by centrifugation at 5,000 × g for 30 min, suspended in PBS and subjected to cell disruption by ultrasonication. The cell lysates were centrifuged at 10,000 × g for 10 min at 4°C to separate the soluble fraction from the debris. The soluble fraction was used for analyzing sialidase activity and also subjected to SDS-PAGE to detect the fusion GST-NanH protein. The recombinant fusion protein was purified from *E. coli* lysates by affinity purification on glutathione-agarose (Sigma) media according to a previously described protocol (35). The specific activity of the purified fractions was determined by comparison with *Clostridium perfringens* sialidase (Sigma). Fusion protein samples with sialidase activities in the range of 10-50 units/mg protein were routinely purified.

Attachment and Invasion Assays

For both attachment and invasion assays, *T. forsythia* cells were used at a multiplicity of infection of 100. Epithelial cell monolayers (KB or OBA-9) were incubated for either 1 h or 4 h
for attachment or invasion assays, respectively, as described previously (16). Briefly, for attachment assays monolayers incubated with bacteria were washed three times with Dulbecco modified Eagle medium (DMEM), epithelial cell associated bacteria were retrieved by lysing monolayers in distilled water and bacteria were plated for counting. For invasion assays, epithelial cell monolayers incubated with bacteria were washed with DMEM and then treated with gentamicin (300 µg/ml) and metronidazole (200 µg/ml) to kill external bacteria. The monolayers were then washed with DMEM and were lysed by water to allow for intracellular bacteria counting. For inhibition assays, bacteria were pretreated with sialidase inhibitor 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en) at a concentration of 10 mM for 30 min.

Lectin Staining

10^5 KB or OBA-9 cells were seeded on poly-D-lysine coated slides. Infection was carried out with 4×10^8 bacteria for 2 h. Uninfected cells were alternatively treated with recombinant Gst-NanH (0.2 units/ml sialidase). Cells were fixed with 3% paraformaldehyde for 15 min. Biotinylated maackia amurensis lectin (MAL) (Vector Laboratories) was incubated with cells at 2.5 µg/ml, respectively, for 1 h. After washing with PBS, cells were treated with 1 µg/ml DyLight 488 conjugated streptavidin (Vector Laboratories, Burlingame, CA) and nuclei were then stained with 4',6-diamidino-2-phenylindole (DAPI) fluorescent stain (Vector Laboratories). Fluorescence was determined on mounted slides with Vectashield (Vector Laboratories).

Statistical analysis.

Strain comparisons for sialidase expression and attachment assays were analyzed using the Student’s T-test using Prism 5.0 software (GraphPad Software, San Diego, CA)

RESULTS

NanH- and SiaHI-deficient mutants show significant loss of *T. forsythia*-associated sialidase activity.

*T. forsythia* expresses two distinct sialidases, namely SiaHI (TF2207) (17) and NanH (TF0035) (39). While SiaHI shows no significant sequence similarity to any of the known bacterial sialidases, NanH (TF0035) shows characteristic motifs prototypical of sialidases. NanH is 65% identical to *Bacteroides fragilis* NanH and is the major sialidase in *T. forsythia*. In addition, analysis of the *nanH* associated operon indicated that the operon contains genes with putative functions in glycoprotein degradation, sialic acid release and assimilation; the genes include N-
acetylneuraminate lyase (TF0030; NanA), an N-acetylglucosamine epimerase (TF0031; NanE), a transport protein of the major facilitator superfamily (TF0032; MFS), a β-hexosaminidase (TF0036), a putative 9-O-acetylesterase (TF0037), and two proteins with similarity to RagAB-like TonB-dependent receptors (TF0033 and TF0034). To determine the roles of SiaHI and NanH sialidases, we constructed specific gene deletion mutants for each and characterized the mutants for their epithelial cell binding and invasion abilities. We wanted to determine if sialidases were involved in exposing sialic acid masked cryptic epitopes on epithelial cells for bacterial interactions. For this purpose, siaHI and nanH-deletion mutants were constructed by allelic replacement strategy with the erythromycin resistance marker, ermF. The double-cross over strategy utilized in the study results in the replacement of sialidase genes (siaHI or nanH) from their translational start to stop codons with that of the ermF gene, thus expressing the erythromycin resistance marker from the corresponding sialidase gene promoters and maintaining the integrity of the operons. The expected integration events were confirmed by sequencing the PCR generated fragments encompassing the integration sites in the respective transformants (data not shown). A representative mutant for each of the siaHI and nanH genes, named TFM2207 (siaHI) and TFM0035 (nanH), were further characterized. Specifically, with regard to the nanH deletion, since the nanH gene is located downstream of the putative sialic acid uptake locus, the disruption is likely not to effect uptake of free sialic acid as well. Conclusive evidence to rule out any polar effects was obtained by RT-PCR and electrophoretic analysis. The results showed similar intense PCR products for TF2206 (ORF downstream of siaHI) in the siaHI mutant and TF0036 (ORF downstream of nanH) in nanH mutant as in the wild-type strain (data not shown). In comparison to the wild-type strain, although siaHI and nanH mutants showed a slight growth lag in the early log phase, they reached full growth similar to the wild-type strain by late log phase (absorbance of 1.2 ± 0.1 at 600 nm; Fig. 1A) in nutrient rich BF broth (34). An initial slight lag in mutants during early log phase is likely due to the metabolic burden from expression of the antibiotic resistance (EmR) marker. Thus, the deletions of siaHI or nanH did not affect the overall fitness of the respective mutants in broth cultures.

The loss of sialidase activity in mutants was demonstrated by enzymatic assays. Sialidase activity was determined in total cell extracts, outer membrane fractions and culture supernatants. The cells and spent culture supernatants from mid-log phase (absorbance of 0.6 at 600nm) were harvested. Cell pellets were either lysed in 2% Triton-X 100 to estimate total enzyme activity or were used to prepare outer-membrane fractions as described previously (12). The culture supernatants were concentrated with a Centricon-30 device as per the manufacturer’s recommendations. Equal amounts of protein fractions from respective strains...
were assayed for sialidase activity. The results showed that the total sialidase activity in siaHI-mutant TFM2207 and nanH-mutant TFM0035 were diminished approximately 10% and 90% of the activity observed in the wild-type strain, respectively (Fig. 1B). Moreover, sialidase activity was detected in the outer-membrane and secreted fractions from the wild-type but not in the TFM0035 strain; TFM2207 did not show significant loss of activity in either fraction (Fig. 1B). These results indicated that NanH, in accordance with in silico structure predictions, is membrane associated as well as secreted, and SiaHI is likely periplasmic.

**Expression of enzymatically active NanH sialidase in *E. coli***. NanH was expressed as a fusion protein with glutathione S-transferase (GST) by pGEX-4T expression plasmid in *E. coli* BL21. Consistent with the predicted molecular size of the GST-NanH fusion protein, a band migrating at approximately 85 kDa (27.5 kDa GST plus predicted molecular mass of 59.7 kDa for NanH) on 10% SDS-PAGE gels was observed in *E. coli* cell lysates with recombinant plasmid following IPTG induction (Fig. 2A). *E. coli* with control plasmid showed an expected 27.5 kDa GST band following IPTG induction. Moreover, the soluble fraction obtained from GST-NanH expressing (BL21/pGEX-nanH) but not GST alone expressing clone (BL21/pGEX-4T) showed strong sialidase activity (Fig. 2B) following IPTG induction. These results thus demonstrated that NanH when expressed in *E. coli* as a fusion partner with GST is enzymatically active. The recombinant GST-NanH fusion protein was purified from lysates using glutathione-agarose affinity media to near homogeneity, indicated by a major band of approximately 85 kDa on a 4-12% gradient SDS-PAGE gel (Fig. 2C). We routinely obtained enzymatically active GST-NanH fusion protein samples with specific activities in the range of 10-50 U/mg protein. These samples were utilized as the source for NanH activity for subsequent investigations.

**NanH-deletion results in reduced bacterial attachment to epithelial cells**. Epithelial cells are decorated with a variety of sialic acid containing glycoconjugates and conceivably the sialic acid residues may provide bacteria a carbon and nitrogen source and/or may modulate bacterial attachment. With respect to the role of sialic acids in bacterial interactions with epithelial cells, terminal sialic acids might serve as ligands for bacterial lectins or alternatively mask hidden epitopes for bacterial binding. We utilized a KB cell model system described previously for studying the role of sialidases in bacterial interaction with epithelial cells (16). KB cells are a derivative of HeLa cells (human cervical epithelium), and both of these cell types have been extensively used for studying oral pathogen-epithelial cell interactions (10, 28). In addition, we used OBA-9 cell line as a representative of human gingival epithelial cell model (20). Our
results showed that the wild-type *T. forsythia* ATCC 43037 strain and its sialidase-defective mutants TFM2207 (*siaHI*) and TFM0035 (*nanH*) adhered to and invaded both KB as well as OBA-9 cells to different degrees. In comparison to the wild-type and TFM2207 (*siaHI*), there was significant reduction in the total attachment of TFM0035 (*nanH*) mutant to KB (Fig. 3A) as well as OBA-9 cells (Fig. 3B). However, no significant differences were observed between the attachment levels of wild-type and TFM2207 to either KB or OBA-9 cells. In addition, significant reductions in the invasion abilities of TFM0035 in comparison to that of the wild-type or TFM2207 strains with respect to KB (Fig. 3C) as well as OBA-9 cells (Fig. 3D) was evident.

Sialidase activity associated with NanH mediates bacterial attachment. As described above, the deletion of *nanH* and not *siaHI*, resulted in the impairment of bacterial attachment to epithelial cells. To confirm if deficiency of sialidase activity in TFM0035 (*nanH*) was responsible for the observed phenotype, we complemented TFM0035 in trans with purified recombinant sialidase since gene complementation in *T. forsythia* has been so far unsuccessful. For trans complementation, rGST-NanH (0.02 units/ml) along with bacteria was added to the KB cell monolayers and attachment abilities were determined. The results demonstrated that complementation with sialidase significantly enhanced the attachment ability of TFM0035 as compared to the attachment ability of the non-complemented controls (Fig. 4). To further confirm the involvement of sialidase activity of NanH in bacterial attachment, we utilized a broad spectrum sialidase inhibitor Neu5Ac2en. Neu5Ac2en caused dose-dependent inhibition of *T. forsythia*-associated sialidase activity (Fig. 5A). For inhibition assays, bacteria were pretreated with Neu5Ac2en (10 mM) for 30 min at 37°C and the inhibitor (10 mM) was added to the culture medium. The results showed that this treatment significantly reduced the association of the wild-type strain with both KB and OBA-9 cells compared to the corresponding non-treated controls (Fig. 5B). Moreover, the inhibitor treatment did not reduce the total association of the NanH mutant strain TFM0035 further to either KB or OBA-9 cells. Taken together, these results confirm that sialidase activity associated with NanH plays a significant role in epithelial cell attachment by *T. forsythia*.

*T. forsythia* desialylates epithelial cells surfaces. Previous studies have shown that NanH cleaves sialic acid from a variety of glycoconjugates, with a preference for α2-3 glycosidic linkages. In order to validate that *T. forsythia* indeed cleaves cell surface sialic acids by its NanH activity, we visualized epithelial cell surfaces following infection with different strains by fluorescence microscopy using *maackia amurensis* lectin (MAL), which recognizes α2-3 linked sialic acids. The treatment of epithelial cells with rGST-NanH sialidase (0.02 units/ml)
significantly reduced the lectin binding compared to that with the non-treated controls (Fig. 6), demonstrating loss of cell surface α2-3 linked terminal sialic acids by NanH sialidase. Furthermore, α2-3 linked terminal sialic acids were also conspicuously absent from the epithelial cell surfaces treated with the wild-type T. forsythia strain compared to cells infected with TFM0035 (Fig. 6). These results thus demonstrated that T. forsythia NanH cleaves α2-3 linked terminal sialic acids from epithelial cell surfaces.

**DISCUSSION**

*T. forsythia* grows subgingivally under anaerobic conditions where it likely utilizes host proteinaceous components for growth. While this suggests predominantly an asaccharolytic physiology, the bacterium produces a variety of glycosidases which can provide a rich source of sugars. The roles of these glycosidases including recently identified SiaHI and NanH sialidases in bacterial physiology or pathogenesis have not been fully investigated.

Bacterial sialidases have been shown to be important in providing free sialic acid as a nutritional carbon and nitrogen source from glycoconjugates (43) as well as in contributing to pathogenesis by exposing epitopes for bacterial adherence through cleaving terminal sugars on host glycoconjugates (6). During the colonization process, *Capnocytophaga canimorsus* surface-localized sialidase has also been shown to initiate host deglycosylation promoting bacterial growth and persistence in vivo (21). *Pseudomonas aeruginosa* sialidase has been shown to promote biofilm formation in the lungs of cystic fibrosis patients (37). In addition, *Streptococcus pneumoniae* surface-anchored sialidase has been shown to promote endothelial cell attachment and invasion (41). *S. pneumoniae* sialidases have also been further shown to initiate deglycosylation of host proteins, including IgA1 and human secretory component for growth and persistence in the airway (4). In addition, bacterial sialidases have also been shown to be involved in defense against host immune surveillance. In this regard, deglycosylation of human serum glycoproteins by the combined action of *S. pneumoniae* sialidases and exoglycosidases has been shown to increase resistance to complement-dependent killing and phagocytosis of bacteria (7).

The current study was undertaken specifically to determine the roles of sialidases produced by *T. forsythia*. We were interested in determining the role of *T. forsythia* sialidases in adhesion to and invasion into epithelial cells. Epithelial cells are decorated by a wide variety of glycoconjugates (31), which in many cases have terminal sialic acid residues. These terminal sialic acid residues on host surfaces can serve as ligands for bacterial colonization, maintain net
negative charge repulsive for bacteria or mask epitopes (cryptitopes) that would otherwise facilitate bacteria adherence. Additionally, removal of these terminal sialic acids may expose other sugars for binding or utilization by bacteria. Thus, we hypothesized that *T. forsythia* sialidases might be involved in exposing new cryptitopes for bacterial binding. Our study demonstrated a role for *T. forsythia* surface associated and secreted NanH sialidase in attachment and invasion of epithelial cells. We observed that in comparison to the wild-type and SiaHI-deficient strains, a NanH-deficient mutant TFM0035 was significantly attenuated in binding and invasion ability. Furthermore, inactivation of sialidase activity with an inhibitor significantly blocked bacterial attachment to epithelial cells. Since complementation in trans with recombinant enzyme did not restore the epithelial cell binding ability of the NanH mutant to the wild-type levels, it remains to be seen if functions other than the sialic acid removal might also contribute to the attachment; such as NanH protein directly interacting as an adhesin with epithelial cell. A role for SiaHI could not be demonstrated in the current study. Our results utilizing a SiaHI-deficient mutant suggested that this sialidase is periplasmic in nature and thus may not be accessible for interactions. It is likely that this enzyme may be involved in scavenging sialic acid from internalized glycoconjugates in the periplasm. We concluded that NanH is the major sialidase utilized by the bacterium for mediating epithelial cell interactions.

We and others have shown that other components including a leucine-rich repeat surface protein BspA (16) as well as other surface-layer proteins (28, 30) are involved in bacterial binding to and invasion into epithelial cells. Since bacterial attachment and invasion is a multi-step process, it is reasonable to predict that NanH sialidase along with other attachment and invasion factors are coordinately regulated. In this process, unmasking of binding epitopes for *T. forsythia* attachment factors by sialidase activity may be an initial step. Since sialic acid serves as a growth factor only in biofilms and not planktonic growth (26), NanH-deficient mutant TFM0035 showed no significant growth defects and reached full growth like the wild-type in broth cultures. Based on these studies, we predict that the NanH deficient mutant TFM0035 might be defective in biofilm growth on human glycoconjugated coated surfaces, such as human salivary mucins. We are currently exploring the effects of sialidase deletion on biofilm growth. The role of SiaHI sialidase could not be assessed since the siaHI deletion did not result in any change in bacterial growth or bacterial ability to attach/invade epithelial cells in vitro. Moreover, since in silico structural predictions suggest NanH to be surface-localized and SiaHI to be periplasmic, suggests that these enzymes might be performing different functions in the pathogenesis and physiology of *T. forsythia*. In addition to the roles of NanH sialidase in epithelial cell interactions (this study) and providing sialic acid as a biofilm growth promoting
factor (26), it is possible that NanH-dependent release of sialic acid could be important for sialylation of surface macromolecules such as LPS or outer membrane proteins important for host immune evasion. For example, *Neisseria gonorrhoeae* (24) and *Haemophilus influenzae* (32) sialylated bacterial components have been suggested to allow pathogens to disguise themselves from host immune systems through molecular mimicry and avoid killing by the host immune complement pathway and phagocytes (11).

In the oral cavity, *T. forsythia* comes in direct contact with sialylated glycoproteins present in saliva, mucin-coated barriers formed on teeth and mucosa as well as gingival epithelial cell surfaces. *T. forsythia* encodes several other glycosidases, such as; α-D-glucosidase (TF0094, *SusB*), N-acetyl β-D glucosaminidase (TF2925, hexA), β-glucosidase (TF0014, *bglX*), α-fucosidase (TF0421, *fucO*), α-arabinofuranosidase (TF0229 abfA), β-galactosidase (TF0229, TF1468, TF2386), and α-1,2-mannosidase (TF0617, TF1335). In conjunction with NanH and SiaHI these glycosidases might hydrolyze terminal glycosidic linkages of complex oligosaccharides, proteoglycans and oligosaccharides associated with the host glycoproteins, affecting their functional integrity and could promote disease progression and/or help provide nutrient sources for coexisting bacteria residing in the oral cavity. It is also tempting to speculate that NanH and SiaHI sialidases together with these glycosidases could be involved in the maintenance of bacterial communities within the oral cavity by providing sugar metabolites as nutrients or providing new cryptitopes to other bacteria. In this regard, the cohabiting bacterium *Fusobacterium nucleatum* which binds galactose residues can benefit from the NanH activity of *T. forsythia*. Plausibly, NanH might expose penultimate galactose residues on oral mucosal surfaces by removing terminal sialic acid residues for *F. nucleatum* binding.

In summary, we describe a role for NanH sialidase in epithelial cell attachment by *T. forsythia* and speculate that sialidase inhibitors might be utilized to block *T. forsythia* pathogenesis and modulate periodontitis.

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REFERENCES


FIGURE LEGENDS

1 Fig 1. Characterization of sialidase mutants. (A). Growth of strains in BHI broth supplemented with fetal bovine serum and N-acetyl muramic acid as determined by OD_{600}. (B). Sialidase activity of bacterial strains measured with fluorescent substrate 4-methylumbelliferyl-N-acetyl-α-D-neuraminic acid. Sialidase activity associated with total cell-extract, membrane and secreted fractions was determined for each strain. Equal amounts of protein fractions (25-100 µg) for each strain were assayed. Bars represent fluorescence values (mean and standard deviation) of triplicate readings. Data are representative of more than three independent experiments with similar results.

2 Fig. 2. Expression of T. forsythia NanH in E. coli expressed as GST fusion protein. (A). SDS-PAGE of E. coli clones harboring the control (BL21/pGEX-4T; lanes 1 and 2) or recombinant (BL21/pGEX-nanH) plasmid in the presence (lanes 2 and 4) or absence of IPTG (lanes 1 and 3). E. coli cultures grown to an absorbance of 0.2 at 600 nm were incubated for additional 3 hrs in the presence or absence of IPTG. Cell lysates (50 µg protein for each) were separated on 10% gels and stained with Coomassie (B) Sialidase activity assayed in lysates of corresponding clones in microtiter wells (BL21/pGEX-4T: 1 (uninduced) & 2 (induced); BL21/pGEX-nanH: 3 (uninduced) & 4 (induced); 10 µg protein lysate for each) using 4-MU-NeuAc as substrate. Fluorescence was visualized on a UV-transilluminator. (C) SDS-PAGE of purified rGST-NanH protein on a 4-12% gradient polyacrylamide gel. Lane M, protein markers; lane 1, rGst-NanH.

3 Fig. 3. (A & B) Attachment of bacteria to epithelial cells. Epithelial cell monolayers (KB and OBA-9) were incubated for 1 h with bacteria in CO₂ incubator at 37°C. Monolayers were then washed, lysed in distilled water and total associated bacteria were plated onto agar plates for counting. Attachment levels (mean and standard deviation of triplicate readings) were expressed as the percentage of attached bacteria relative to the total number of input bacteria. (A) KB cells. Data representative of three independent experiments show significant reduction in the attachment of nanH mutant strain TFM0035 compared to the wild-type strain ATCC 43037. The mean attachment levels for TFM0035 were 23.5, 29.4, and 22.5 % of that of the wild-type strain while the TFM2207 attachment levels were not significantly different from that of the wild-type strain in three independent experiments. (B) OBA-9 cells. Reduced attachment of TFM0035 compared to the wild-type strain was also observed for OBA-9 cells; mean TFM0035 attachment levels were 37.2 and 30.7% of that of the wild-type strain in two independent experiments whereas TFM2207 attachment levels were not significantly different from that of...
the wild-type strain. (C & D) Invasion of epithelial cells. Strains were incubated with epithelial
cell monolayers for 4 h followed by metronidazole treatment for 1 h to kill outside bacteria. The
invaded bacteria were then counted after epithelial cell lysis and plating on agar plates. (C) KB
cells. Data representative of three independent experiments shows significantly reduced
invasion (mean and standard deviations) by TFM0035 compared to the wild-type strain; mean
invasion levels of TFM0035 were 46.5, 42.1, and 38.6 % of that of the wild-type strain. In none
of the independent experiments siaH mutant TFM2207 showed reduced invasion compared to
the wild-type strain. (B) OBA-9 cells. Reduced invasion of TFM0035 compared to the wild-type
strain was also observed for OBA-9 cells; TFM0035 invasion levels were 49.5 and 42.8% of that
of the wild–type strain and TFM2207 invasion levels were not significantly different from that of
the wild-type strain in two independent experiments.

Fig. 4. Trans-complementation of mutants with recombinant sialidase. Epithelial cell
monolayers were incubated for 1 h in a CO\textsubscript{2} incubator at 37\degree C with different strains in the
presence or absence of recombinant sialidase and bacterial attachment for each strain was
determined. Data represented is one of three independent experiments with similar results.

Fig. 5. Effect of sialidase inhibitor Neu5Ac2en on epithelial cell attachment. (A) Dose-dependent
inhibition of \textit{T. forsythia}-associated sialidase activity by Neu5Ac2en. TritonX-100 extracted fractions
from \textit{T. forsythia} wild-type cells were treated with increasing concentrations of sialidase inhibitor for
30 min at 37\degree C. Sialidase activity was then measured with fluorescent substrate 4-
methylumbelliferyl-N-acetyl-\alpha-D-neuraminic acid. Bars represent fluorescence values (means
and standard deviations) of triplicate readings. Data are representative of more than three
independent experiments with similar results. (B) Inhibition of \textit{T. forsythia} wild-type attachment to
epithelial cells (KB or OBA-9) by sialidase inhibitor. \textit{T. forsythia} wild-type cells were pre-treated with
10 mM Neu5Ac2en for 30 min prior to incubation with epithelial cell monolayers. Monolayers with
bacteria were incubated for 1 h and bacterial attachment levels were determined as previously
described. Data are representative of three (KB cells) or two (OBA-9 cells) independent
experiments with similar results.

Fig. 6. \textit{T. forsythia} cleaves epithelial cell surface sialic acids. Surface carbohydrates of
exthelial cells were analyzed by lectin binding after 2 h treatment with rNanH, \textit{T. forsythia}
43037 (WT), or TFM0035 (\textit{nanH}). Cells were fixed with 3% paraformaldehyde for 30 min and
then reacted for 1 h with biotinylated maackia amurensis lectin, specific for \alpha2-3 linked terminal
sialic acids. Lectin binding was visualized by staining with fluorescent (DyLight 488) labeled
streptavidin. Nuclei were stained with DAPI.
Table 1. Primers used in this study

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<th>Description</th>
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<td>PCR primer for TF2207</td>
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Fig. 1
Fig. 3
Fig. 4

Total Associated Bacteria (%)

MannH

TF43037 (WT)

TFM2207 (staH)

TFM0035 (nanH)

0.0 0.5 1.0 1.5 2.0 2.5

P<0.05
Fig. 5

Fluorescence units (x10^2)

Neu5Ac2en (mM)

P < 0.01

P < 0.05

No Inhibitor
Neu5Ac2en (10 mM)

P < 0.05

P < 0.05

TF43037 TFM0035
TF43037 TFM0035

KB

OBA-9
Fig. 6