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An Orthologue of *Bacteroides fragilis* NanH Is the Principal Sialidase in *Tannerella forsythia*▽

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Sialidase activity is a putative virulence factor of the anaerobic periodontal pathogen *Tannerella forsythia*, but it is uncertain which genes encode this activity. Characterization of a putative sialidase, SiaHI, by others, indicated that this protein alone may not be responsible for all of the sialidase activity. We describe a second sialidase in *T. forsythia* (TF0035), an orthologue of *Bacteroides fragilis* NanH, and its expression in *Escherichia coli*. Sialidase activity of the expressed NanH was confirmed by using 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid as a substrate. Biochemical characterization of the recombinant *T. forsythia* NanH indicated that it was active over a broad pH range, with optimum activity at pH 5.5. This enzyme has high affinity for 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (K_m of 32.9 ± 10.3 μM) and rapidly releases 4-methylumbelliferone (V_{max} of 170.8 ± 11.8 nmol of 4-methylumbelliferone min^{-1} mg of $protein^{-1}$). *E. coli* lysates containing recombinant *T. forsythia* NanH cleave sialic acid from a range of substrates, with a preference for α2-3 glycosidic linkages. The genes adjacent to *nanH* encode proteins apparently involved in the metabolism of sialic acid, indicating that the NanH sialidase is likely to be involved in nutrient acquisition.

The fastidious gram-negative, anaerobic, bacterium *Tannerella forsythia* is one of the main etiological agents of periodontitis—a multifactorial, destructive gum disease that causes tooth loss (44). Although a complete genome sequence is available for this bacterium (http://www.stdgen.lanl.gov/), relatively little is known about the biology of this species. It has been frequently isolated with *Porphyromonas gingivalis* from diseased sites in patients with chronic periodontitis, but its precise mode of action in periodontal disease has not been established (11, 13, 43). Nevertheless, a number of putative virulence factors have been identified, including a slime (S) layer (22, 34); an α-D-glucosidase and a *N*-acetyl-β-glucosaminidase (18); proteases (15, 35); and a cell surface-associated protein, BspA (39).

Another putative virulence factor of *T. forsythia* is its sialidase activity. Sialidases (neuraminidases, EC 3.2.1.18) are glycohydrolases, which release the terminal sialic acid residues from sialoglycoconjugates. Sialic acids are 9-carbon α-keto acids, which are common sugars at the terminal residue of glycoproteins and glycolipids. Such sialic acid-containing glycoconjugates are widely distributed on eukaryotic cells and secreted glycoproteins (3, 40). The sialic acid residues contribute to a range of important biological functions, including cellular interactions and stabilizing the conformation of glycoproteins and cellular membranes; these residues also expose or mask receptors for ligands, antibodies, or enzymes and contribute to the function and stability of glycoproteins in serum (3, 40). Sialidases are implicated in the pathogenicity of some bacteria, including *Clostridium perfringens* (33), *Streptococcus pneumoniae* (46), *Pasteurella multocida* (42), and *Pseudomonas aeruginosa* (41). They can modify the host's ability to respond to bacterial infection by increasing the susceptibility of immunoglobulin molecules to proteolytic degradation (28). Sialidases can also facilitate colonization by exposing cryptic receptors for bacterial adhesion (9). They may also provide bacteria with a nutritional carbon source (9, 38).

Several studies have demonstrated sialidase activity in *T. forsythia* isolates, and this is used as a diagnostic tool for identification of the species (5, 26). Although a *T. forsythia* sialidase gene, *siaHI*, has been reported (19), it is unclear whether this alone is responsible for sialidase activity in *T. forsythia*. A sialidase unrelated to *T. forsythia* SiaHI has been described in the related bacterium, *Bacteroides fragilis* (49), and it may be important for nutrient acquisition, supporting the growth of this bacterium in vivo (14). We report that an orthologue of *B. fragilis* NanH is the principal sialidase in *T. forsythia*. We describe the biochemical properties of this sialidase and discuss its association with genes encoding proteins that may affect sialic acid uptake and metabolism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in the present study are described in Table 1. *T. forsythia* ATCC 43037 was routinely grown on fastidious anaerobe agar (LabM, United Kingdom) containing 0.001% *N*-acetylmuramic acid and 5% defibrinated horse blood (TCS Biosciences, United Kingdom) at 37°C, under anaerobic conditions (80% N_2, 10% H_2, and 10% CO_2). *Escherichia coli* strains were grown on Luria-Bertani (LB) agar or LB broth (Melford, United Kingdom) under aerobic conditions at 37°C. For transformant selection and plasmid maintenance in *E. coli*, media were supplemented with 30 or 50 μg of kanamycin/ml. For Topo cloning LB agar was also supplemented with 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)/ml and 100 μg of IPTG (isopropyl-β-D-thiogalactopyranoside)/ml to select appropriate transformants.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
ATCC 43037	*T. forsythia*	45
DH5α	*E. coli* K-12 cloning host	Invitrogen
α-Select	*E. coli* K-12 cloning host	Bioline
BL21(DE3)	*E. coli* B expression host	Novagen
KCL116	BL21(DE3)/pET30	This study
KCL117	BL21(DE3)/pET30::*nanH*	This study
KCL120	BL21(DE3)/pET30::*siaHI*	This study
Plasmids		
pCR4-Topo	PCR cloning vector; Km^r Amp^r	Invitrogen
pET30a, -b, and -c	pET30 expression vectors a, b, and c containing an inducible T7 promoter; Km^r	Novagen
pKCL175	pCR4-Topo containing *nanH* from *T. forsythia*, amplified using primers P265 and P266	This study
pKCL191	pET30c containing *nanH* from pKCL175 cloned into the BamHI site	This study
pKCL202	pET30b containing *siaHI* from *T. forsythia*, amplified using the primers P291 and P292	This study

[a] Amp^r, ampicillin resistance; Km^r, kanamycin resistance.

Genetic modification of bacterial strains. The genetic manipulation of bacterial strains was carried out as described previously (36). Genomic DNA was isolated from *T. forsythia* cells by using Genelute (Sigma Aldrich, United Kingdom) according to the manufacturer's instructions.

PCR primers used to amplify *nanH* and *siaHI* (TF0035 and TF2207, respectively; see Fig. 1 for a representation of the binding sites)—P265 (GGATCC**A AGGAG**ATATACATATGAAAAAGTTTTTTTGGAT), P266 (GGATCCAAA AGAAAAGACAAACGA), P291 (GGCTGATATCGGATCC**AAGGAG**ATAT ACATATGACAAAAAAAAGCAGTAT), and P292 (GCTCGAATTCGGAT CCGATACTCATGACTTTTTCTCTAA)—were designed by using the Vector NTI Suite (v10; Invitrogen, United Kingdom) and synthesized by MWG Biotech (Germany). Included on the 5′ end of selected primers were BamHI restriction enzyme recognition sites (underlined) and primers P265 and P291 also included a ribosome-binding site (boldfaced). To enable ligation-independent cloning, primers P291 and P292 were designed to include 15 nucleotides with identity at the 5′ end to the 15 nucleotides flanking the desired insertion point in pET30. The primers were used to amplify *nanH* and *siaHI* from *T. forsythia* genomic DNA using Bio-X-Act DNA polymerase (Bioline, United Kingdom). Amplifications were carried out using the following cycle parameters: 1 cycle at 95°C for 2 min; 30 cycles of 95°C for 0.5 min, 57°C for 0.5 min, and 72°C for 2.5 min; and a final extension cycle of 72°C for 10 min.

Cloning was carried out as described in Table 1. Briefly, amplified *nanH* was initially cloned into pCR4-Topo (Invitrogen), creating pKCL175, and subsequently transferred into pET30c as a BamHI fragment to create pKCL191. Amplified *siaHI* DNA was cloned directly into the pET30b vector by using an In-Fusion Dry-Down PCR cloning kit (Clontech/Takara Bio, France) according to the manufacturer's instructions, to create pKCL202. The authenticity and orientation of the inserts was confirmed by DNA sequencing with T7 promoter and T7 terminator primers (TAATACGACTCACTATAGGG and CTAGTTA TTGCTCAGCGGT, respectively).

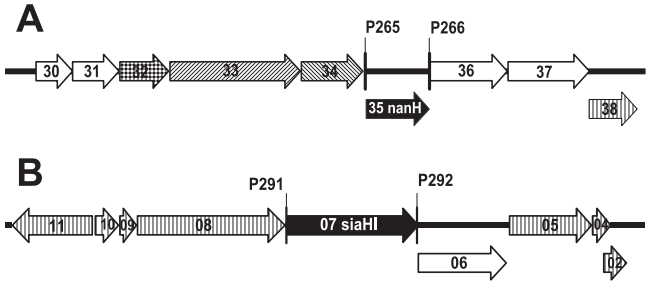

FIG. 1. *T. forsythia* genetic loci containing genes encoding putative sialidases. The genes encoding the putative sialidases in *T. forsythia* are represented by the black arrows. Surrounding these are genes encoding putative outer membrane proteins (diagonal hatching, panel A only), a transport protein (cross-hatching, panel A only), enzymes (no hatching), or hypothetical proteins of unknown function (vertical hatching). The numbers correspond to gene numbers TF00xx (panel A, TF0030 to TF0038) or TF22xx (panel B, TF2211 to TF2202). The binding sites of the primers used to amplify *nanH* (A) and *siaHI* (B) are indicated by vertical lines.

Expression of putative sialidases from *T. forsythia* in *E. coli*. Expression of the cloned proteins was induced in the exponential phase of growth (optical density at 600 nm of 0.5 to 0.8) by the addition of 1 mM IPTG and further incubation for 2 or 18 h at 37°C. Bacterial lysates were prepared by using a BugBuster (Novagen, United Kingdom) according to the manufacturer's protocol. The total protein concentration of the cell lysates was determined by using a bicinchoninic acid assay (Sigma). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5% stacking gel, 10% resolving gel) (21) and visualized by Coomassie brilliant blue staining.

Biochemical assays of sialidase activity. The fluorogenic substrate, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4-MU-NeuNAc; Sigma) was used to assay sialidase activity. Qualitative screening of sialidase activity in intact bacteria or lysates was done by using a rapid filter paper assay (5). Samples were applied to filter paper presoaked with 4-MU-NeuNAc (279 μM) in 0.1 M sodium acetate buffer (pH 4.6) and examined under long-wavelength UV light (365 nm) after 15 min at 37°C. Sialidase activity was indicated by blue-white fluorescence.

Quantitative sialidase assays involved measuring 4-methylumbelliferone (4-MU) fluorescence, released from 4-MU-NeuNAc by sialidase activity, in a Fluoroskan Ascent FL fluorimeter (Labsystems Thermo, United Kingdom) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm (6, 7). To determine the optimal pH for sialidase activity, assays were carried out with 50 μM 4-MU-NeuNAc in 70 mM sodium citrate buffer (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or 6.0), 70 mM sodium phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, or 8.0), 70 mM potassium phosphate buffer (pH 6.5, 7.0, or 7.5), or 70 mM Tris-HCl (pH 7.5, 8.0, 8.5, or 8.9) and appropriately diluted cell lysates, such that the rate of release of 4-MU from 4-MU-NeuNAc was linear with respect to time. Assays were stopped after 30 min of incubation at 37°C by the addition of an equal volume of 0.5 M sodium carbonate buffer (pH 10.5) (6). The kinetic characteristics of the sialidases were determined with assays carried out with 70 mM sodium citrate buffer (pH 5.5) and 0.05 to 2 mM 4-MU-NeuNAc (6).

The substrate specificity of the sialidases was determined using sialyl-α2,3-lactose, sialyl-α2,6-lactose, porcine gastric mucin, fetuin, bovine submaxillary mucin, and 4-MU-NeuNAc (Sigma) and colominic acid (containing α2,8 linked *N*-acetylneuraminic acid; MP Biomedicals, United Kingdom) in 50 mM sodium citrate buffer (pH 5.5). Sialic acid release was determined by using high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), essentially as described previously (6, 7). Briefly, HPAEC-PAD was performed by using a Dionex DX-500 chromatography system (Dionex, United Kingdom) and a Carbopac PA1 column (250 by 4 mm). Sialic acids were eluted from the column by using a linear gradient of sodium acetate (20 to 150 mM over 40 min) in 100 mM sodium hydroxide at a flow rate of 1 ml/min. *N*-Acetylneuraminic acid and *N*-glycolylneuraminic acid concentrations in assays were determined by comparison of peak areas with those of the authentic sialic acids (Sigma) as standards over a concentration range of 0 to 50 μM.

RESULTS

***T. forsythia* contains an orthologue of *B. fragilis nanH*.** Although a *T. forsythia* sialidase, SiaHI (TF2207; see Fig. 1B), has been described (19), it is not apparent whether this alone is sufficient to account for all of the sialidase activity in this bacterium. Sialidase activity not directly attributable to SiaHI was observed upon nondenaturing gel electrophoresis of *T. forsythia* proteins (19). Also, SiaHI is an unusual sialidase since it does not contain motifs associated with other characterized sialidases (31). For example, the SiaHI sequence does not include any Asp box motifs (29). It also has no significant similarity to sialidases from related species, such as *Bacteroides fragilis* (1). Therefore, further analysis of the available *T. forsythia* genome sequence was initiated.

The *B. fragilis* NanH sequence (accession number BAA05853) was used as a template to identify a putative orthologue in the *T. forsythia* genome (available in the OralGen database [http://www.stdgen.lanl.gov/]) by BLAST (2). This putative orthologue, encoded by TF0035, is 65% identical to *B. fragilis* NanH over the full length of the 540-amino-acid sequence. It also contains motifs typically associated with sialidases (31), including four perfect Asp boxes (SxDxGxTW; amino acids 236 to 243, 315 to 322, 375 to 382, and 482 to 489), a further imperfect Asp box (amino acids 422 to 429, TRDLGKSW), and the conserved FRIP region (amino acids 198 to 201, FRIP), which is typically located at the N terminus of sialidases (31). Therefore, we propose that gene TF0035 is renamed *nanH*. The original annotation of TF0035 included 13 amino acids (MPFFGLSCKNTCR) at the N terminus that had no significant similarity to *B. fragilis* NanH. An alternative start codon follows the sequence encoding these amino acids, in the same reading frame, and we propose that this is the true N terminus of the TF0035 preprotein. The N terminus of this "truncated" TF0035 protein contains a 20-amino-acid signal sequence (MKKFFWIIGLFASMQMTRAA), indicating that this putative NanH sialidase is most likely secreted and/or surface localized. There are approximately 170 amino acids between the signal sequence and the sialidase catalytic domain containing the Asp boxes and the FRIP region. This additional domain shares no similarity with known proteins; in particular, it is distinct from the lectin domain present in other large sialidases (48).

The genetic context of the *T. forsythia nanH* gene is illuminating (Fig. 1A). It is located in an apparent operon, which includes genes encoding an *N*-acetylneuraminate lyase (TF0030; NanA), an *N*-acetylglucosamine epimerase (TF0031; NanE), a transport protein of the major facilitator superfamily (TF0032; MFS), and two proteins with similarity to RagAB-like TonB-dependent receptors (TF0033 and TF0034). This operon also encodes a β-hexosaminidase (TF0036) and a putative sialate 9-*O*-acetylesterase (TF0037).

In contrast, the previously described *T. forsythia* sialidase, SiaHI, is encoded within a putative operon (Fig. 1B) that does not contain genes encoding proteins explicitly involved in glycoprotein degradation or sialic acid metabolism. Genome annotation (http://www.stdgen.lanl.gov/) and BLAST searches indicate that six genes in this putative *siaHI* operon encode hypothetical proteins of unknown function and one, TF2206, encodes a putative enzyme with some similarity to sugar phosphate isomerase/epimerases. Surprisingly, although SiaHI does not contain any motifs associated with typical sialidases, it does have a NAD-binding Rossman fold and C-terminal alpha/beta domains of the oxidoreductase family (pfam01408 and pfam02894 [24]) and is therefore more characteristic of a dehydrogenase (COG0673). This is not apparently consistent with SiaHI being a sialidase.

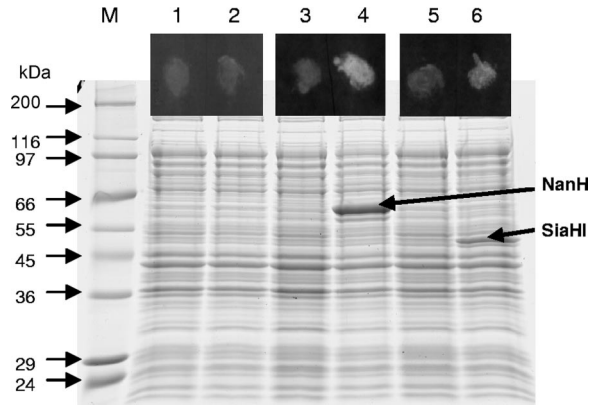

FIG. 2. Sialidase activity of *T. forsythia* NanH and SiaHI expressed in *E. coli*. Strains KCL116 (*E. coli* pET30; lanes 1 and 2), KCL117 [*E. coli* BL21(DE3)/pET30::*nanH*; lanes 3 and 4], and KCL120 [*E. coli* BL21(DE3)/pET30::*siaHI*; lanes 5 and 6] were grown in LB until mid-exponential phase and for a further 2 h with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) induction with 1 mM IPTG. Cells were assayed for sialidase activity in a filter paper spot assay using the fluorogenic substrate 4-MU-NeuNAc (spots in the black boxes above the corresponding lanes). Cell lysates were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis, and proteins were visualized by Coomassie blue staining.

Sialidase activity of *T. forsythia* NanH and SiaHI. Since the genetic manipulation of *T. forsythia* is still in development (44), we selected a heterologous expression strategy to confirm that the *T. forsythia nanH* gene encodes a sialidase. TF0035 (*nanH*) and, for comparison, the previously described *siaHI* (TF2207) were cloned into pET30 and expressed in *E. coli* BL21(DE3). The protein profiles of the IPTG-induced KCL117 (NanH) and KCL120 (SiaHI) cultures contained an additional protein not apparent in the control cultures (Fig. 2). These proteins, approximately 60 and 50 kDa, respectively, for NanH and SiaHI, are consistent with the sizes predicted from the amino acid sequence: the predicted molecular mass of NanH is 59.7 kDa (57.4 kDa without the signal sequence) and that of SiaHI is 51.0 kDa (48.7 kDa without the signal sequence). The fluorescence observed in a qualitative assay of whole cells expressing recombinant sialidase, using the fluorogenic sialidase substrate 4-MU-NeuNAc, indicated that, when expressed in *E. coli*, both *T. forsythia* NanH and SiaHI have sialidase activity (Fig. 2).

Biochemical characteristics of *T. forsythia* NanH. Quantitative 4-MU-NeuNAc sialidase assays of *E. coli* whole-cell lysates containing *T. forsythia* NanH (strain KCL117) indicated that this sialidase is active over a wide pH range (between pH 4.5 and 8.0 at least 40% of the maximum activity was observed), with an optimum pH of 5.5 (Fig. 3). Further assays carried out at pH 5.5 confirmed that NanH is a sialidase with a high affinity for 4-MU-NeuNAc (K_m = 32.9 ± 10.3 μM) and a rapid reaction rate (V_{max} = 170.8 ± 11.8 nmol of 4-methylumbelliferone min^{-1} mg of $protein^{-1}$). In stark contrast, we were unable to

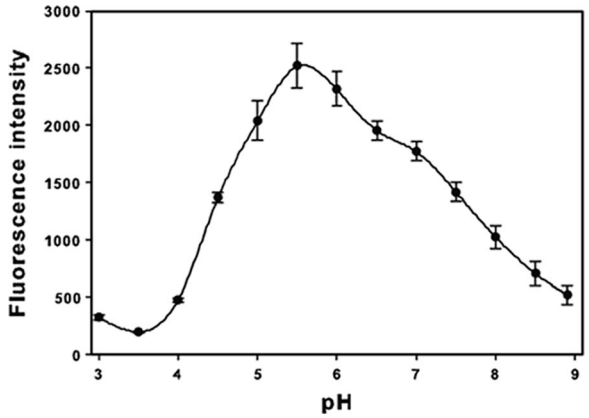

FIG. 3. pH optimum of *T. forsythia* NanH. Strain KCL117 [*E. coli* BL21(DE3)/pET30::*nanH*] was grown in LB until mid-exponential phase and for a further 18 h after induction with 1 mM IPTG. Cell lysates were prepared as described in *Materials and Methods* and assayed using 4-MU-NeuNAc in sodium citrate buffer (pH 3.0 to 6.0), sodium phosphate buffer (pH 6.0 to 8.0), potassium phosphate buffer (pH 6.5 to 7.5), and Tris-HCl (pH 7.5 to 8.9). The release of 4-MU was determined as fluorescence intensity (excitation and emission wavelengths of 380 and 460 nm, respectively). The data shown are the means from at least three independent experiments. Error bars represent ± the standard error of the mean.

detect any sialidase activity (<0.2 nmol of 4-methylumbelliferone min^{-1} mg of $protein^{-1}$) in lysates containing SiaHI using such quantitative assays over the full pH range tested.

The specificity of sialidases is influenced by the nature of the glycosidic linkage to the next sugar. In sialylated oligosaccharides, sialic acid can be linked by its C-2-OH to the C-3, C-4, or C-6 (α2,3, α2,4, or α2,6) of the next sugar, which is typically galactose, *N*-acetylgalactosamine, or *N*-acetylglucosamine. If the linked sugar is another sialic acid, an α2,8-linkage is possible (40). Another factor that influences sialidase specificity is O acetylation of C-7 to C-9 of the sialic acid (9). Therefore, to determine the specificity of the *T. forsythia* NanH sialidase, we treated a range of commercially available substrates with KCL117 lysate and determined sialic acid release (Table 2). *T. forsythia* NanH favors substrates with α2,3 (sialyl-α2,3-lactose) linkages, and α2,6 (sialyl-α2,6-lactose) are preferred to α2,8 linkages (colominic acid). We observed no sialidase activity with more complex substrates (fetuin, bovine submaxillary mucin, and porcine gastric mucin), perhaps reflecting the extent of O acetylation of the sialic acids in these molecules. For example, 80% of the sialic acids in bovine submaxillary mucin are O acetylated (47). Consistent with the lack of release of 4-MU from 4-MU-NeuNAc in quantitative assays (see above), we were unable to detect any release of sialic acids when any of the other commercial substrates were treated with lysates containing SiaHI.

DISCUSSION

The previously described SiaHI protein of *T. forsythia* (19) does not account for all of the sialidase activity of this putative periodontal pathogen. This bacterium has an orthologue of *B. fragilis* NanH, TF0035 (renamed NanH), which has sialidase activity when expressed in *E. coli*. Unlike *T. forsythia* SiaHI, *T. forsythia* NanH contains all of the attributes classically associated with sialidases. The NanH enzyme contains four or five Asp box motifs, which typically occur four to five times in bacterial sialidases (29, 30). The conserved FRIP motif, which may be involved in substrate binding (32), is also present in *T. forsythia* NanH. Although characterized sialidases from other bacterial species differ in size and structure, they each have Asp box and FRIP motifs. To our knowledge, the *T. forsythia* SiaHI enzyme is the only reported bacterial sialidase that does not. BLAST searches indicate that, in addition to *B. fragilis*, putative orthologues of *T. forsythia* NanH with >39% identity over the full length of the protein are present in other sequenced *Bacteroides* and *Parabacteroides* species, *Capnocytophaga canimorsus* (23), and *Akkermansia muciniphilla* (10; data not shown). Therefore, this subclass of sialidases is thus far only apparent in a small group of bacteria. These NanH orthologues each contain the novel 170-amino-acid region between the signal sequence and the sialidase region. However, the function of this additional domain in this group of sialidases is not apparent.

Most characterized bacterial sialidases are secreted or are cell bound (9). The signal sequence present in *T. forsythia* NanH indicates that this protein is also likely to be secreted, hydrolyzing the terminal sialic acid residues on glycoconjugates outside the cell. The habitat of *T. forsythia*, the periodontal pocket, contains an abundance of sialylated glycoproteins found in saliva and gingival crevicular fluid, such as fibronectin, transferrin, and immunoglobulins (27). A number of periodontal bacteria are known to exploit host sialylated glycoproteins as a nutrient source. For example, immunoglobulin G, which is abundant in gingival crevicular fluid, can support the growth of *P. gingivalis*, *Prevotella oralis*, and a number of other periodontal pathogens (20). The NanH of *B. fragilis* is important for growth in vivo: sialidase-deficient mutants of *B. fragilis* have impaired growth in a rat pouch model (14). Although it is likely that a primary role of the *T. forsythia* NanH is nutritional, bacterial sialidases have additional effects. Sialidase treatment of immunoglobulins makes them more susceptible to proteolytic degradation (28); sialidases may also reveal cryptic receptors and/or adhesion sites for bacteria (9). It is therefore likely that the NanH sialidase could contribute to the pathogenicity of *T. forsythia* in a number of different ways.

TABLE 2. Substrate specificity of *T. forsythia* NanH

Substrate	Total sialic acid concn (μM)	Sialyl linkage(s)	Relative rate of cleavage (%)[a]
4-MU-NeuNAc	10		100
Sialyl-α2,3-lactose	20	α2,3	35.6
Sialyl-α2,6-lactose	20	α2,6	17.4
Colominic acid (poly α2,8 Neu5Ac)	100	α2,8	3.3
Bovine submaxillary mucin (NeuNAc)	100	α2,6	0
Bovine submaxillary mucin (NeuNGc)	100	α2,6	0
Pig gastric mucin	100	α2,3	0
Fetuin	100	α2,3 and α2,6	0

[a] The cleavage rates shown are relative to that obtained with 4-MU-NeuNAc as the substrate (121.7 nmol min^{-1} mg of $protein^{-1}$).

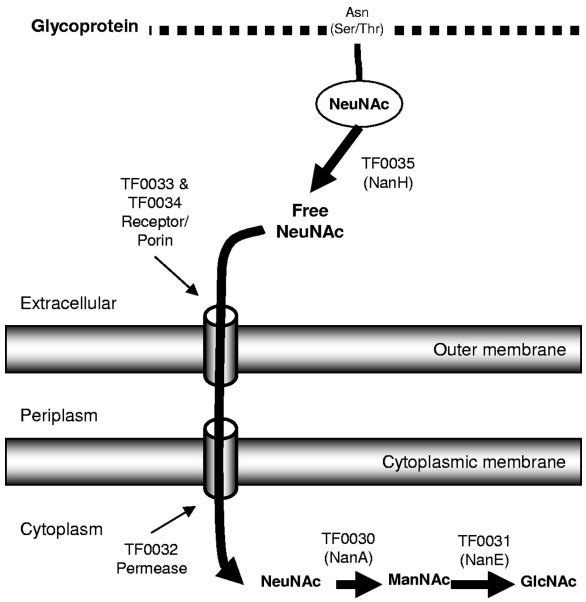

FIG. 4. Schematic representation of the proposed pathway for sialic acid metabolism in *T. forsythia* highlighting the initiating role of NanH. The removal of sialic acid from the terminus of glycoproteins by NanH allows it to pass through the outer membrane; we propose that this occurs with the help of the proteins encoded by TF0033 and TF0034. The free sialic acid is most likely further transported into the bacterium by the MFS permease encoded by TF0032. TF0030 and TF0031 encode the initial enzymes for sialic acid catabolism, NanA and NanE.

T. forsythia NanH has a preference for α2-3-linked sialyl oligosaccharides, like many sialidases from bacteria, including *Salmonella enterica* serovar Typhimurium NanH (17), *Streptococcus oralis* sialidase (6), and *P. multocida* NanH (25). Consistent with the properties reported for other bacterial sialidases (9), the *T. forsythia* NanH is active over a broad pH range, with optimum activity observed at pH 5.5. This activity at differing pHs may ensure that NanH remains active in vivo, withstanding fluctuations in pH. For example, NanH is active at pH 7.0, the average pH in periodontal pockets (12), with ca. 70% maximum sialidase activity. Our results also indicate that NanH should be active even at the higher pH detected by others in crevicular fluid at inflamed sites (up to pH 8.66) (4). Comparison of the kinetic properties of *T. forsythia* NanH to other bacterial sialidases is difficult, since a range of substrates and different assay conditions have been used in other studies. Nevertheless, the low K_m indicates that the *T. forsythia* NanH has a high affinity for 4-MU-NeuNAc.

Although we observed no sialic acid release from mucin molecules, it is known that the sialic acids of such glycoproteins may be heavily O acetylated (16), which adversely affects bacterial sialidase activity (8). One of the enzymes encoded by the putative *nanH* operon is a sialate 9-*O*-acetylesterase (TF0037, EC 3.1.1.53), which may remove acetyl groups from sialic acids (8). Therefore, it is apparent that in vivo *T. forsythia* NanH and TF0037 may act in unison to facilitate removal of sialic acid from glycoconjugates in saliva or gingival crevice fluid. Another enzyme in the putative *nanH* operon (TF0036) is a putative *N*-acetyl-β-hexosaminidase (NahA, EC 3.2.1.52), which is distinct from a previously characterized *T. forsythia N*-acetyl-β-glucosaminidase (18), sharing only 18% amino acid identity. Such enzymes can be involved in the release of terminal *N*-acetylglucosamine or *N*-acetylgalactosamine from glycoproteins (37). Therefore, the proteins encoded by the *nanH* operon are not only likely to be involved in sialic acid metabolism but rather are dedicated to glycoprotein degradation in general. The remaining genes in the *nanH* operon encode proteins likely to be involved in the uptake and metabolism of sialic acid (Fig. 1 and 4).

In contrast, the operon encoding the reported SiaHI sialidase does not obviously include genes encoding proteins which are required for sialic acid catabolism. Furthermore, although our qualitative screen for sialidase activity apparently confirmed the report of Ishikura et al. (19) since we observed fluorescence when *E. coli* expressing recombinant *SiaHI* was tested with 4-MU-NeuNAc, we were unable to detect any sialidase activity by quantitative assays of any sample containing SiaHI. Since SiaHI has none of the characteristics of typical sialidases but rather has similarity to NAD-dependent dehydrogenases, it is possible that the primary role of this enzyme is other than its supposed function as a sialidase.

The sialidase activity long associated with *T. forsythia* can now be attributed, at least in part, to the orthologue of *B. fragilis* NanH we describe here. Although the biological role of the alternative sialidase SiaHI remains unclear, it is probable that *T. forsythia* NanH is a sialidase involved in nutrient acquisition.

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