Cloning of the *Tannerella forsythensis* (Bacteroides forsythus) siaHI gene and purification of the sialidase enzyme

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"Tannerella forsythensis* (previously named *Bacteroides forsythus*) is a Gram-negative, anaerobic, fusiform bacterium that is a primary or secondary aetiological agent in periodontal disease in humans. *T. forsythensis* expresses several putative virulence factors, including a sialidase; however, there has been no molecular genetic characterization of this enzyme. A sialidase clone (pH1-1) was screened from a total of 455 recombinant clones of a genomic DNA library using the 2'- (4-methylumbelliferyl)-α-β-N-acetylneuraminic acid (MUNeuAc) filter-paper spot assay. The sialidase gene ORF (*siaHI*) consists of a 1395 bp coding sequence and encodes a protein with 465 amino acids with an overall molecular mass of 52 kDa. The sialidase does not have sequence similarity to any other bacterial sialidase. The entire sialidase ORF was expressed in *Escherichia coli*. Furthermore, the sialidase was purified from the type strain of *T. forsythensis* and from a recombinant clone, pH1-1:1, and was analysed using a non-denaturing gel, revealing that the enzyme preparations were respectively separated as two major bands and as a single band. Southern blot hybridization analysis revealed similar patterns of *siaHI*-hybridizing bands among clinical isolates of *T. forsythensis* from periodontitis patients. This is the first study on the cloning and expression of a *T. forsythensis* sialidase gene and the purification of the SiaHI enzyme from *T. forsythensis* ATCC 43037 7 and recombinant *E. coli*.

Abbreviation: MUNeuNAc, 2’-(4-methylumbelliferyl)-α-β-N-acetylneuraminic acid.

The GenBank/EMBL/DDBJ accession number for the *siaHI* gene sequence of *T. forsythensis* ATCC 43037 is AY069941.

INTRODUCTION

*Tannerella forsythensis* (Bacteroides forsythus), a Gram-negative, anaerobic, fusiform bacterium, has been implicated as one of the periodontal pathogens associated with periodontitis. Several studies have shown a strong correlation of *T. forsythensis* with the severity of periodontitis in adult patients (Grossi et al., 1994, 1995; Tanner et al., 1985, 1986). Identification of virulence factors of this bacterium could aid in the development of strategies for prevention and treatment of periodontitis. However, due to difficulties in culturing this organism from periodontitis patients, its role in the initiation and progression of periodontitis has been difficult to determine. Only a few putative virulence factors have been identified in this bacterium, based on their in vitro properties, including a sialidase, a trypsin-like protease, N-benzoyl-Val–Gly–Arg–p-nitroanilide-specific protease, a cell surface-associated BspA protein and a cell-death-inducing factor (Arakawa et al., 2000; Holt & Bramanti, 1991; Saito et al., 1997; Sharma et al., 1998).

Several bacterial enzymes, including phospholipases and proteases, have been suggested as potential virulence determinants in prematurity (McGregor et al., 1990). Among these, there has recently been interest in the sialidases of pathogenic bacteria and their potential role in pathogenesis. Sialidases are enzymes that cleave α-ketosidic linkages between sialic acid and the glycosyl residues of glycoproteins, glycolipids or colomonic acids. Sialidase activity has been found in viruses, bacteria, protozoa, fungi and metazoans (Berry et al., 1988). The sialidase of the Gram-negative bacillus *Erysipelothrix rhusiopathiae* has been shown to be involved in infection and tissue destruction (Gyles, 1986). Anaerobic Gram-negative rods belonging to the genera *Prevotella* and *Bacteroides* were suggested to be the main sources of sialidase activity in vaginal fluid (Briselden et al., 1992). *Bacteroides* isolates obtained from pathological specimens were found to have significantly higher levels of sialidase activity than isolates from non-pathological specimens (Grossi et al., 1994). Sialidases have been demonstrated...
to modify a variety of host-associated activities that result in a modification of the host’s ability to respond to bacterial infection and could be an important virulence factor of *T. forsythensis*. However, little is known about how this putative *T. forsythensis* virulence factor may contribute to its pathogenesis in vivo. In order to define the molecular nature of the *T. forsythensis* sialidase and as a first step to determining its specific role in the virulence of this bacterium, the sialidase gene was isolated, its nucleotide sequence was determined, recombinant sialidase was expressed in *Escherichia coli* and sialidases were purified from both *T. forsythensis* and the *E. coli* clone.

**METHODS**

**Bacterial strains, media and growth conditions.** In this study, we used *T. forsythensis* ATCC 43037T and three clinical isolates (42-11, 29-41 and 33-22) that were isolated in our periodontal clinic from periodontitis patients, with their consent. These bacteria were grown anaerobically in heart infusion broth (Difco) and ligated to the BamHI site of pBluescript SKII (Strategene) was grown in Luria–Bertani (LB) medium (Difco) with 100 µg ampicillin ml⁻¹.

**Isolation of chromosomal DNA.** After culturing as described above, *T. forsythensis* ATCC 43037T and the clinical isolates were harvested by centrifugation (5000 g at 4 °C for 20 min). Chromosomal DNA was isolated using the method of Marmur (1961).

**Construction of a *T. forsythensis* genomic library and screening for sialidase-producing clones.** Chromosomal DNA of *T. forsythensis* ATCC 43037T was isolated, size-fractionated (4–10 kbp) using a fragment that encoded sialidase, deletion plasmids were constructed (Moncla & Braham, 1989). Sialidase activity by detecting the release of 4-methylumbelliferone from 37-8 ATCC 43037T was isolated, size-fractionated (4–10 kbp) using a fragment that encoded sialidase, deletion plasmids were constructed.

**Construction of deletion plasmids.** In order to identify the smallest fragment that encoded sialidase, deletion plasmids were constructed (Myers et al., 1980). The digested fragments were separated on 1-0 % agarose gels and purified from these agarose gels using a QIAquick gel extraction kit (Qiagen). The fragments were ligated with unique sites of pBluescript SKII (+) and pBluescript KSII (+), digested with the appropriate enzymes. The deletion plasmids thus obtained were examined qualitatively for sialidase activity as outlined above.

**In vitro mutagenesis.** The predicted sialidase structural gene, containing the first ATG codon of ORF1 (translation start codon), was amplified by PCR from pH1-1. We used the following synthetic oligonucleotide primers: R1 (sense primer, 5'-CCGAATTCTAAATTGTGATGACA-3'; EcoRI site underlined), R2 (sense primer, 5'-CCGAATTCTAAATTGTGATGACA-3'; ATG start codon changed to CCGG) and F1 (antisense primer, 5'-GGCTCGAGTCATCATGTTTTCT-3'; XhoI site underlined) (see Fig. 2b). Pwo DNA polymerase (Roche Diagnostics), known for its high proofreading activity (Roggentin et al., 1989), was used in order to prevent unwanted mutations. After digestion with EcoRI and XhoI, these products were ligated to the appropriate site of pBluescript SKII (+) and used to transform *E. coli* HB101. Transformants were screened for sialidase activity as outlined above.

**Chromatography.** The AKTA explorer 105 protein purification system (Amersham Pharmacia Biotech) was employed for column chromatography to regulate buffer flow and to monitor elution status and absorbance at 254 and 280 nm was recorded automatically. The sialidase-positive clone (pHI-1) and *T. forsythensis* were cultured in 5 l broth and grown to early stationary phase. Bacterial cells were harvested by centrifugation at 5000 g for 30 min at 4 °C. Cells were disrupted by sonication, centrifuged to remove cellular debris and finally filtered using a 0.22 µm filter. At the first step, 15 ml of the resultant crude extract was diluted with 35 ml phosphate buffer (10-1 mM NaH2PO4, 1-76 mM KH2PO4) and loaded onto a cation-exchange Resource S column (Amersham) equilibrated with 0.1 M MES/NaOH (pH 6.0) buffer at a flow rate of 2 ml min⁻¹. Adsorbed components were eluted with a 300 ml linear gradient of NaCl from 0 to 1 M. Active fractions with sialidase activity were collected and dialysed against 50 mM Tris/HCl (pH 8.0) buffer using a Hitrap column (Amersham). Thereafter, the concentrate was subjected to an anion-exchange Resource Q column (Amersham) equilibrated with 50 mM Tris/HCl (pH 8.0) buffer. Active sialidase fractions were collected and concentrated and NaCl was removed using a vivaspin centrifugal concentrator (exclusion molecular mass 10 kDa; Sartorius). Protein concentrations were measured using Protein Assay reagent (Bio-Rad) as an equivalent concentration of BSA.

**Non-denaturing PAGE.** Since the purified preparations stagnated in the stacking gel during SDS-PAGE, the purity and activity of the purified enzymes were determined by non-denaturing PAGE, which was performed according to the method of Brawn & Bennett (1983). All buffers and solutions were made fresh, stored at 4 °C and used within a week to obtain the most consistent results. Non-denaturing gels (10 %) were electrophoresed at 4 °C for 4–5 h. The gel was then washed with 0.17 M sodium acetate buffer (pH 5.4) for 5 min and sialidase activity was examined with the fluorogenic substrate as described above. In order to visualize sialidase activity, a CCD image of the gel was taken under long-wavelength UV light (450 nm) and another gel loaded under the same conditions was stained with 0-2 % Coomassie brilliant blue R-250 in 50 % methanol and destained for 1 h (two or three times) at room temperature in order to visualize protein bands.

**Southern blot analysis.** DNA from clinical isolates and *T. forsythensis* ATCC 43037 was digested with *BstEII* (New England Biolabs). Following electrophoresis on 1 % agarose gels, the separated DNA fragments were transferred onto a Hybond-N⁺ membrane (Amersham) using a capillary transfer technique (Southern, 1975). Transferred DNA frag-
ments were cross-linked to the membrane using a UV cross-linker (FUNA; Funakoshi). The 2.3-kbp BbsI–BbsI DNA fragment of pH1-1 was labelled with peroxidase using an ECL non-radioactive labelling kit (Amersham) and hybridized at high stringency to the membrane, in accordance with the manufacturer’s instructions.

RESULTS

Cloning of T. forsythensis ATCC 43037T sialidase gene

A clone bank of T. forsythensis ATCC 43037T DNA fragments inserted in the cloning vector pBluescript SKII (+) was constructed using E. coli HB101, a strain with no endogenous sialidase activity (Vimr & Troy, 1985). Of a total of 455 colonies, one was found to be positive for sialidase activity, as indicated by results of a filter-paper spot assay. This colony was purified twice more using the single colony isolation method. It was then retested and found to be positive for sialidase activity. This clone, designated pH1-1, was used for subsequent analysis (Fig. 1).

Identification of an ORF encoding sialidase

The nucleotide sequence of each strand of the 3.2-kbp insert of pH1-1 was determined (Fig. 2a). Four ORFs were identified in the inserted DNA of pH1-1. In order to determine which ORF encodes sialidase, several deletion derivatives of pH1-1 were constructed. The sialidase activity of each derivative was assayed using a filter paper spot assay (Fig. 2b). The deletion derivatives pH1-1:1 and pH1-1:2 contained the 1.4-kbp BamHI–HindIII fragment of pH1-1 in pBluescript SKII (+) and pBluescript KSII (+), respectively. Clone pH1-1:3 contained the 1.5-kbp PstI–PstI fragment (Fig. 2a). After incubation with the fluorogenic substrate, E. coli carrying the deletion derivative pH1-1:1 exhibited blue fluorescence with an intensity comparable with that observed for pH1-1, whereas neither pH1-1:2 nor pH1-1:3 produced a fluorescence signal (Fig. 2a). These results suggest that ORF1, contained in the BamHI–HindIII fragment, conferred sialidase activity on the E. coli host.

In order to confirm that translation starts from the first ATG of ORF1, in vitro mutagenesis was performed. The region containing ORF1 was amplified by PCR with the primers R1, R2 and F1. The mean length of the PCR products of these primers was 1.4 kbp (range 313–1710 bp). Sequencing confirmed that the R1 PCR product contained the wild-type ORF1 (first ATG codon unchanged), whereas the R2 product contained a mutant ORF (first ATG codon changed to CGG). Transformant pBR1 (which contained the R1 PCR

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**Fig. 1.** Restriction map of clone pH1-1. The map shows the initiation site and direction of transcription of ORF1 (siaHI), as determined by sequence analysis of inserted DNA in pH1-1. The 3/2-kbp Sau3AI fragment was cloned into the unique BamHI site within the multiple cloning region of pBluescript SKII (+).

**Fig. 2.** (a) Construction of deletion mutants and their sialidase activity. In order to identify the smallest fragment that encoded sialidase, deletion mutants were constructed and sialidase activity was assayed using a filter-paper spot assay. Deletion mutants were made in pBluescript SKII (+) and contained the following fragments: BamHI–HindIII in the forward direction (pH1-1:1) and reverse direction (pH1-1:2); PstI–PstI (pH1-1:3). The arrow indicates the direction of transcription in each ORF. Similar results were obtained in several repetitions of this experiment. (b) In vitro mutagenesis. Arrows indicate PCR primers used to amplify the DNA and create EcoRI and XhoI sites. R2 was designed to replace the initiation codon (first ATG codon) with a CGG codon. pBR1 contained the wild-type ORF1 (primers R1 and F1) and pBR2 contained the mutant ORF (primers R2 and F1). Similar results were obtained in several repetitions of this experiment. Sequences were confirmed using an ABI 370 DNA sequencer.
product) exhibited sialidase activity, whereas transformant pBR2 (which contained the R2 PCR product) did not (Fig. 2b). These results suggest that ORF1 of pH1-1 encodes *T. forsythensis* sialidase. We chose the name siaHI for the gene corresponding to ORF1.

**Nucleotide and predicted amino acid sequences of *T. forsythensis* ATCC 43037T sialidase**

Nucleotide sequence analysis of pH1-1 revealed a potential translation initiation site at base position 313. An ORF of 1395 nucleotides (BamHI–HindIII fragment of pH1-1) would encode a protein of 465 amino acids, with a calculated molecular mass of 52 kDa and a deduced pI of 6.60. A comparison of the properties of other bacterial sialidases with those of the siaHI product, even with PSI BLAST searches, suggested that the enzyme may represent a novel protein that has not previously been identified. For example, a similarity search of the EMBL and DDBJ databases using the FASTA and BLAST programs revealed that there was only 24 % sequence identity (overlap of 379 amino acids) between the predicted amino acid sequences of *T. forsythensis* sialidase and ORF5 (oxidoreductase) of the *Streptococcus pneumoniae* neuraminidase gene (nanB; accession no. U43526). Moreover, the siaHI product exhibited no structural similarity to Asp box motifs (−Ser−X−Asp−X−Gly−X−Thr−Trp−) or RIP motifs (−Arg−Ile−Pro−), which are highly conserved among sialidases from phylogenetically unrelated organisms but not the active site of these enzymes (Roggentin *et al.*, 1989). Therefore, it appears that the siaHI sialidase is not a classical one that has been reported previously but a novel protein unrelated to any known sialidases.

**Purification of *T. forsythensis* sialidase**

As a first step towards purification, a cation-exchange column was employed to purify the sialidase from sonicated extracts from *T. forsythensis* ATCC 43037T and the sialidase-positive recombinant clone pH1-1:1. The major sialidase activity was detected in two fractions that eluted at NaCl concentrations of 150–250 mM in both samples (Fig. 3a, b). The concentrates were then subjected to an anion-exchange column. Active fractions having sialidase activity were eluted with the flow-through fraction in both samples (Fig. 3c, d) and were concentrated and NaCl was removed. In order to check the purity of these fractions, samples of 1 mg (BSA equivalent) were analysed by SDS-PAGE (7.5–12.0 %). However, the purified samples stagnated in the stacking gel and did not migrate to the running gel; therefore, these samples were subjected to non-denaturing PAGE. The

![Figure 3](image)
enzymes from *T. forsythensis* ATCC 43037T and pHI-1 : 1 were respectively separated as two major bands and as a single band (Fig. 4a), and the sialidase activity of these protein bands was confirmed by sialidase assay using MUNeuNAc. As shown in Fig. 4(b), the purified protein preparation from pHI-1 : 1 showed sialidase activity and the size of this band was identical to that of the lower band from *T. forsythensis*. Furthermore, the upper band from *T. forsythensis* also demonstrated sialidase activity.

**Determination of the prevalence of the *siaHI* gene among clinical isolates of *T. forsythensis***

In order to determine whether *siaHI* was present in clinically isolates of *T. forsythensis*, genomic DNA was prepared from three *T. forsythensis* isolates and subjected to hybridization at high stringency with a DNA probe representing the BbsI–BbsI DNA fragment of pHI-1, a fragment (bases 165 to 2487) that contains the *siaHI* structural gene. This DNA probe hybridized strongly with a 2.3-kbp BbsI fragment of chromosomal DNA from *T. forsythensis* ATCC 43037T and the three clinical isolates (Fig. 5a). On the other hand, chromosomal DNA from *E. coli* HB101 containing plasmid pBluescript SKII (+) or (−) did not react with this probe in a separate experiment (data not shown). The three clinical isolates all exhibited sialidase activity using the filter-paper assay, but the presence of two distinct proteins was not determined. These results suggest that this sialidase gene is present in all three *T. forsythensis* clinical isolates (42-11, 29-41 and 33-22).

**DISCUSSION**

The present communication describes the identification of the gene *siaHI*, encoding a sialidase, from *T. forsythensis*, which has been implicated as a major aetiological agent in periodontitis and a key pathogen for severe forms of chronic periodontitis. Sialidases have been demonstrated to function in a variety of host-associated activities that result in a modification of the host’s ability to respond to bacterial infection (Holt & Bramanti, 1991). Furthermore, the sialidase might be used by proteolytic bacteria to remove glycans from glycoproteins, thus rendering them more susceptible to proteolysis (Moncla et al., 1990). Sialidase production is also used to meet a nutritional requirement of the organism: without growth, there is usually no disease (Byers et al., 1999). *T. forsythensis*, similarly to *Porphyromonas gingivalis*, which is also strongly associated with periodontitis, has long been reported to produce sialidase (Moncla et al., 1990). However, whether sialidase production is involved in the pathogenesis of *T. forsythensis* and causes such periodontal disease remains to be clarified.

There were four potential ORFs in the sequence of the sialidase-encoding genomic DNA fragment, which differed

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**Fig. 4.** Comparison of the molecular mass of purified enzyme from *T. forsythensis* and recombinant *E. coli* (pHI-1 : 1) by non-denaturing PAGE. Separate samples were fractionated by electrophoresis in a 10% polyacrylamide non-denaturing gel with TG buffer (adjusted to pH 8.0) at 4 °C and visualized with Coomassie blue R-250 protein staining (a) or stained for sialidase activity as outlined in Methods (b). Lanes: 1, purified fraction of a sialidase-positive *E. coli* clone (pHI-1 : 1); 2, purified sample of *T. forsythensis* ATCC 43037T.

**Fig. 5.** Southern blot analysis. (a) DNA from *T. forsythensis* was digested with BbsI, separated on 1.0% agarose gel and transferred to Hybond-N+ membrane. The 2.3-kbp BbsI–BbsI fragment of pHI-1 was labelled using an ECL labeling kit and used as the probe and hybridized bands were detected according to the manufacturer’s instructions (Amersham). (b) Ethidium-bromide-stained gel. Lanes: 1, 23-kbp BbsI–BbsI fragment used as a probe; 2, *T. forsythensis* ATCC 43037T; 3–5, three clinical isolates of *T. forsythensis*. 

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in their orientations and lengths. The ORF of the sialidase gene was identified as follows. (i) Only pH1-1:1 of the deletion mutants that contained the four potential ORFs expressed sialidase activity. (ii) There was no other potential ORF that encoded a 52 kDa protein in pH1-1. (iii) A stop codon existed upstream from the ATG codon at position 313 of pH1-1:1. (iv) Although there was no cluster that consisted of a ribosome-binding site and the corresponding ATG codon, protein was produced by this structural gene. In other words, this ORF was translated in E. coli. (v) Mutation of the corresponding ATG (Met) residue of this ORF led to loss of enzyme activity. These results suggested that the 1·4 kbp sequence encoded sialidase. The overall G+C content of ORF1 was 52·5 mol%, which is slightly higher than that of the T. forsythensis prtH genes (43·7 mol%) (Saito et al., 1997). Since analysis of the 5′ end of this sequence revealed that there was a potential ribosome-binding site but no promoter-like sequences, it is possible that the siaHI gene is a part of an operon and is transcribed from a promoter further upstream. Ongoing DNA sequence studies and RNA transcriptional analyses should help to elucidate the transcriptional control of the siaHI gene.

The enzyme was purified from sonicated extracts of T. forsythensis ATCC 43037” and sialidase-positive clone pH1-1:1 by cation- and anion-exchange column chromatography. The enzymes from T. forsythensis ATCC 43037” and pH1-1:1 were respectively separated as two major bands and as a single band (Fig. 4a) and the sialidase activity of these protein bands was confirmed by the sialidase assay using MUNeuNAc (Fig. 4b). Furthermore, as shown in Fig. 3, the enzymes from T. forsythensis and the E. coli clone (pH1-1:1) did not behave identically during chromatography (Fig. 3c, d). These results could be explained as follows: (i) modification of the enzyme might occur in T. forsythensis and not in E. coli, (ii) other sialidases might exist in this bacterium. Streptococcus pneumoniae expresses two distinct sialidases (neuraminidases), NanA (Berry et al., 1988), which is cell-associated, and NanB (Berry et al., 1996). These results revealed that the purified sample from pH1-1:1 might be identical to that from the original bacterium T. forsythensis. Obviously, further studies on the sizes of purified preparations using gel filtration, its location in the organism and the biological significance of T. forsythensis sialidase are required. Analysis of the crystal structure of purified sialidase should be carried out to elucidate the structure of this enzyme in nature and to elaborate selective potent inhibitors against the enzyme in a rational way.

In order to determine the prevalence and heterogeneity of the sialidase gene in this species, a flanking region of pH11-1:1 was detected in chromosomal DNA from clinical isolates by Southern blotting. Similar patterns of siaHI-hybridizing bands were obtained for all three clinical isolates of T. forsythensis from periodontitis patients. These results showed that siaHI is conserved among clinical isolates of T. forsythensis from periodontitis patients. These results showed that siaHI is conserved among clinical isolates of T. forsythensis from periodontitis patients (all of which were found to produce sialidase; data not shown), suggesting that the sialidase encoded by siaHI could be a potential virulence factor in the pathogenesis of T. forsythensis in periodontal disease. Taken together, the available findings suggest that the sialidase produced by T. forsythensis strains, including clinical isolates from periodontitis patients, might be encoded by the novel sialidase gene siaHI.

The present results represent the first successful cloning of a sialidase gene (siaHI) from T. forsythensis. The evidence that we have cloned the structural sialidase gene consists of the observed enzyme expression in E. coli and the results of Southern blot analysis. A gene inactivation system has been developed for T. forsythensis (Honma et al., 2001) and isolation of the siaHI gene will make it possible to construct T. forsythensis mutants that are defective in this gene for testing in appropriate animal models. In this manner, it should be possible to define the existence of a second sialidase and the potential role of siaHI in the virulence of this periodontopathic bacterium. Clinically, the siaHI gene and the recombinant protein (SiaHI) could be utilized as a probe or antigen for detecting the virulence factor: sialidase or anti-sialidase antibody developed in patients from specimens such as subgingival plaque and saliva, and the correlation between the detection of this enzyme and clinical symptoms also should be evaluated.

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