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 (pHI-1) was screened from a total of 455 recombinant clones of a genomic DNA library using the 2'-(4-methylumbelliferyl)- α -D-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, pHI-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^T and recombinant *E. coli*.

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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 colominic 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

Abbreviation: MUNeuNAc, 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid.

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

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 43037^T 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) containing 5 µg haemin ml⁻¹ (Wako), 1 µg ml⁻¹ menadione, 1% L-cysteine, 15 µg N-acetylneuraminic acid ml⁻¹ (Sigma) and 10% sheep blood (Nippon Bio-Test Laboratories), with or without 1.5% agar. *E. coli* HB101 (Takara Shuzo) containing plasmid pBluescript SKII (Stratagene) 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 43037^T 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 43037^T was isolated, size-fractionated (4–10 kbp) using a QIAquick gel extraction kit (Qiagen) after partial digestion with *Sau3AI* (New England Biolabs) and ligated to the *Bam*HI site of pBluescript SKII (+). This ligation reaction was used to transform *E. coli* HB101 and transformants were incubated aerobically on LB-ampicillin agar plates. Colonies were lifted from the agar plates onto a membrane (Hybond-N+; Amersham) and transferred to another agar plate. The plates were incubated overnight at 37 °C. Colonies were transferred from this agar plate to a nylon membrane and soaked in a 100 µM solution of the fluorogenic neuraminidase substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNeNAc sodium salt; Sigma) (Myers *et al.*, 1980) in 0.17 M sodium acetate buffer (pH 5.4) and incubated at 37 °C for 10–15 min. Colonies on the membrane were examined for sialidase activity by detecting the release of 4-methylumbelliferone from MUNeNAc as fluorescence under long-wavelength UV light (450 nm) (Moncla & Braham, 1989).

Nucleotide sequencing of *T. forsythensis* sialidase gene. Nucleotide sequencing of the sialidase-positive clone (pHI-1) was performed using the dideoxynucleotide chain termination method of Sanger *et al.* (1977), with a template of double-stranded plasmid DNA and site-specific synthetic oligonucleotide primers (Nisshin Seifun Corp.). Template plasmid DNA was prepared with a QIA plasmid isolation kit (Qiagen). The sequences of both DNA strands were determined and all sequences overlapped with adjacent sequences. Nucleotide sequences were assembled and analysed using DNASIS software (Hitachi).

Construction of deletion plasmids. In order to identify the smallest fragment that encoded sialidase, deletion plasmids were constructed (see Fig. 2a) and activity was measured using a fluorescent assay method

(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 pHI-1. We used the following synthetic oligonucleotide primers: R1 (sense primer, 5'-CCGAATTC AAAATTGTCATGACA-3'; *Eco*RI site underlined), R2 (sense primer, 5'-CCGAATTC AAAATTGTCCGGACA-3'; ATG start codon changed to CGG) and F1 (antisense primer, 5'-GGCTCGAGTCAT CACTTTTCTC-3'; *Xho*I 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 *Eco*RI and *Xho*I, 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 ÄKTA explorer 10S 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 : 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 Na₂HPO₄, 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 Bhowm & 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^T was digested with *Bbs*I (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 pHI-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 43037^T sialidase gene

A clone bank of *T. forsythensis* ATCC 43037^T 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 pHI-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 pHI-1 was determined (Fig. 2a). Four ORFs were identified in the inserted DNA of pHI-1. In order to determine which ORF encodes sialidase, several deletion derivatives of pHI-1 were constructed. The sialidase activity of each derivative was assessed using a filter paper spot assay

Fig. 1. Restriction map of clone pHI-1. The map shows the initiation site and direction of transcription of ORF1 (*siaHI*), as determined by sequence analysis of inserted DNA in pHI-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 (pHI-1:1) and reverse direction (pHI-1:2); *PstI*–*PstI* (pHI-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 ORF1 (primers R2 and F1). Similar results were obtained in several repetitions of this experiment. Sequences were confirmed using an ABI 370 DNA sequencer.

(Fig. 2b). The deletion derivatives pHI-1:1 and pHI-1:2 contained the 1.4-kbp *BamHI*–*HindIII* fragment of pHI-1 in pBluescript SKII (+) and pBluescript KSII (+), respectively. Clone pHI-1:3 contained the 1.5-kbp *PstI*–*PstI* fragment (Fig. 2a). After incubation with the fluorogenic substrate, *E. coli* carrying the deletion derivative pHI-1:1 exhibited blue fluorescence with an intensity comparable with that observed for pHI-1, whereas neither pHI-1:2 nor pHI-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 (initial ATG codon unchanged), whereas the R2 product contained a mutant ORF1 (first ATG codon changed to CGG). Transformant pBR1 (which contained the R1 PCR

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 43037^T sialidase

Nucleotide sequence analysis of pH1-1 : 1 revealed a potential translation initiation site at base position 313. An ORF of 1395 nucleotides (*Bam*HI–*Hind*III 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 43037^T 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

Fig. 3. (a)–(b) Fractionation of sialidase from *T. forsythensis* (a) and recombinant *E. coli* (pHI-1 : 1) (b) through a cation-exchange Resource S column. Sonicated extracts were loaded onto the column and flow-through (FT) and eluted adsorbates were assayed for sialidase. Sialidase activity was detected at 150–250 mM NaCl in both samples (double-headed arrows). (c)–(d) Fractionation of sialidase from *T. forsythensis* (c) and recombinant *E. coli* (pHI-1 : 1) (d) through an anion-exchange Resource Q column. Sialidase-positive fractions from the Resource Q column were fractionated through the Resource S column. Sialidase activity was detected (double-headed arrows) in the flow-through fraction in both samples.

enzymes from *T. forsythensis* ATCC 43037^T 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 sialidase assay using MUNeUNAc. As shown in Fig. 4(b), the purified protein preparation from pH1-1:1 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 pH1-1, 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 43037^T and the three clinical isolates (Fig. 5a). On the other hand, chromosomal DNA from *E. coli* HB101 containing plasmid pBlue-script 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

Fig. 4. Comparison of the molecular mass of purified enzyme from *T. forsythensis* and recombinant *E. coli* (pH1-1:1) 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 (pH1-1:1); 2, purified sample of *T. forsythensis* ATCC 43037^T.

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 pH1-1 was labelled using an ECL labelling 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, 2.3-kbp *BbsI*–*BbsI* fragment used as a probe; 2, *T. forsythensis* ATCC 43037^T; 3–5, three clinical isolates of *T. forsythensis*.

in their orientations and lengths. The ORF of the sialidase gene was identified as follows. (i) Only pHI-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 pHI-1. (iii) A stop codon existed upstream from the ATG codon (at position 313) of pHI-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^T and sialidase-positive clone pHI-1:1 by cation- and anion-exchange column chromatography. The enzymes from *T. forsythensis* ATCC 43037^T 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 the sialidase assay using MUNeUNAc (Fig. 4b). Furthermore, as shown in Fig. 3, the enzymes from *T. forsythensis* and the *E. coli* clone (pHI-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 pHI-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 pHI1:1 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 (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 periodontopathogenic 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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