Loss of adherence ability to human gingival epithelial cells in S-layer protein-deficient mutants of *Tannerella forsythensis*

Junpei Sakakibara,^{1,2} Keiji Nagano,^{1†} Yukitaka Murakami,¹
Naoya Higuchi,³ Hiroshi Nakamura,³ Kazuo Shimozato²
and Fuminobu Yoshimura¹

Correspondence

Yukitaka Murakami
yukimu@dpc.agu.ac.jp

¹Department of Microbiology, School of Dentistry, Aichi-Gakuin University, Nagoya, Aichi 464-8650, Japan

²Department of Oral and Maxillofacial Surgery II, School of Dentistry, Aichi-Gakuin University, Nagoya, Aichi 464-8650, Japan

³Department of Endodontics, School of Dentistry, Aichi-Gakuin University, Nagoya, Aichi 464-8650, Japan

Tannerella forsythensis, one of the important pathogens in periodontal disease, has a typical surface layer (S-layer) consisting of regularly arrayed subunits outside the outer membrane. The S-layer in *T. forsythensis* is suggested to be associated with haemagglutinating activity, adhesion and invasion of host cells; however, its precise functions have been unknown. ORFs encoding the major S-layer proteins (230 and 270 kDa) of *T. forsythensis* ATCC 43037, *tfsA* and *tfsB*, respectively, following the names in a recent report [Lee, S.-W., Sabet, M., Um, H. S., Yang, L., Kim, H. C. & Zhu, W. (2006). *Gene* **371**, 102–111] were determined. To verify the function of the S-layer proteins, three mutants with *tfsA*, *tfsB*, or both deleted were successfully constructed by a PCR-based overlapping method. S-layer proteins were completely lost in the double mutant. The single-deletion mutants appeared to lose one of the 230 and 270 kDa proteins. Thin-section microscopy clearly revealed that the 230 and 270 kDa proteins composed the S-layer. Although the S-layer proteins may be weakly related to haemagglutinating activity, these proteins were highly responsible for adherence to human gingival epithelial cells (Ca9-22) and KB cells. These results suggest that the S-layer proteins in *T. forsythensis* play an important role in the initiation stage of oral infection including periodontal disease.

Received 1 July 2006

Revised 11 November 2006

Accepted 16 November 2006

INTRODUCTION

Tannerella forsythensis (formerly named *Bacteroides forsythus*) is a slow-growing, fastidious Gram-negative fusiform anaerobe that was originally isolated from advanced periodontal disease in the human oral cavity (Tanner *et al.*, 1986; Sakamoto *et al.*, 2002). *T. forsythensis*, *Porphyromonas gingivalis* and *Treponema denticola* form the so-called 'red complex', which is thought to be important in the pathogenesis and progression of destructive forms of periodontitis (Socransky *et al.*, 1998; Holt & Ebersol, 2005). Mixed infections by *P. gingivalis* and *T. forsythensis* showed a synergic effect on enhanced abscess formation in a murine model (Takemoto *et al.*, 1997; Yoneda *et al.*, 2001).

Recent studies have suggested an aetiological role of this organism in several forms of periodontal disease (Gersdorf *et al.*, 1993; Takemoto *et al.*, 1997; Haffajee *et al.*, 1998; Yano-Higuchi *et al.*, 2000), periradicular lesion (Gonçalves & Mouton, 1999; Siqueira & Rôças, 2003) and systemic disease (Noack *et al.*, 2001; Hajishengallis *et al.*, 2002).

Surface layers (S-layers) are regularly ordered proteins found as the outermost cell envelope components of many bacteria, representing approximately 10–15 % (w/w) of the total cellular protein of the bacterial cells (Boot & Pouwels, 1996). The majority of S-layers are composed of a single protein or glycoprotein with a molecular mass of 40–200 kDa (Sleytr & Messner, 1988; Sleytr *et al.*, 1993; Sleytr & Beveridge, 1999; Sára & Sleytr, 2000). S-layers are considered to function as protective barriers, as molecular sieves, and in adhesion to cells, surface recognition, and maintenance of cell shape and envelope rigidity (Sleytr & Messner, 1988; Sleytr *et al.*, 1993; Sleytr & Beveridge, 1999; Sára & Sleytr, 2000).

†Present address: Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720-3202, USA.

Abbreviations: CAT, chloramphenicol acetyltransferase; CBB, Coomassie brilliant blue; Cp, chloramphenicol; TIGR, The Institute for Genomic Research; TRITC, tetramethyl rhodamine isothiocyanate.

T. forsythensis has been reported to have an S-layer consisting of regularly arrayed subunits outside the outer membrane revealed by electron microscopy (Tanner *et al.*, 1986; Kerosuo, 1988). Several studies reported that major high molecular mass proteins larger than 200 kDa were characteristic of *T. forsythensis* (Kerosuo, 1988; Gersdorf *et al.*, 1993). We have shown by immunoelectron microscopy that the S-layer is composed of 230 and 270 kDa major proteins (Higuchi *et al.*, 2000; Moriguchi *et al.*, 2003). These S-layer proteins were shown to be specifically recognized by sera from patients with periodontitis (Yoneda *et al.*, 2003). Recently, the S-layer protein was isolated and reported to be associated with haemagglutination, cell adhesion and invasion (Sabet *et al.*, 2003).

In the 'red complex' bacteria, complete genome sequences of *P. gingivalis* and *Treponema denticola* have been determined (Nelson *et al.*, 2003; Seshadri *et al.*, 2004). A genome sequencing project for *Tannerella forsythensis* is also in progress at The Institute for Genomic Research (TIGR). From information on N-terminal and internal amino acid sequences of the 230 and 270 kDa proteins, we attempted to identify S-layer protein genes (*tfsA* and *tfsB*, respectively, following the names in a recent paper by Lee *et al.*, 2006) utilizing unfinished genome sequences, and then obtain deletion mutants to elucidate the function of the S-layer proteins. In this paper, we also describe the role of the S-layer proteins in haemagglutination and adherence to host cells, determined using S-layer gene deletion mutants.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *T. forsythensis* ATCC 43037 was originally obtained from A. Tanner (The Forsyth Institute, Boston, MA, USA). All *T. forsythensis* were

grown on Brucella HK agar (Kyokuto Pharmaceutical Industrial) supplemented with 5% (v/v) laked rabbit blood, 2.5 µg haemin ml⁻¹, 5 µg menadione ml⁻¹, 0.1 mg DTT ml⁻¹, 10 µg *N*-acetylmuramic acid ml⁻¹ (Wyss, 1989; Braham & Moncla, 1992) (blood agar) and appropriate antibiotics when necessary at 37 °C for 7 days under anaerobic conditions [10% (v/v) CO₂, 10% (v/v) H₂ and 80% (v/v) N₂]. A Zero Blunt TOPO PCR cloning kit, which included the plasmid vector pCR-Blunt II-TOPO, was purchased from Invitrogen, and *Escherichia coli* TOP10 carrying pCR-Blunt II-TOPO was grown in Luria-Bertani medium supplemented with 25 µg kanamycin ml⁻¹. *E. coli* DH5α carrying pKD260 was grown in Luria-Bertani medium supplemented with 25 µg chloramphenicol (Cp) ml⁻¹ as described previously (Ueda & Yoshimura, 2003).

Construction of deletion mutants. We applied the PCR-based overlap extension method (Horton *et al.*, 1993) described for deletion mutants in *P. gingivalis* (Nagano *et al.*, 2005). DNA fragments that allowed the replacement of *tfsA*, *tfsB*, or both genes with the *cat* gene in the *T. forsythensis* chromosome were constructed. The primers and their annealing sites are shown in Table 2 and Fig. 1(a), respectively. Briefly, the *cat* gene, encoding chloramphenicol acetyltransferase (CAT), was amplified from the ATG start codon to the TAA stop codon with primers AGU-01 and AGU-02 to generate a 660 bp product from pKD260. For construction of the *tfsA*-deletion cassette, the flanking sequence upstream of *tfsA* was amplified with primers AGU-71 and AGU-72, which have homology to the 5' end of the *cat* fragment. The flanking sequence downstream of *tfsA* was amplified with AGU-73 and AGU-74, which have homology to the 3' end of the *cat* fragment. The *cat*, *tfsA*-upstream and *tfsA*-downstream fragments were used as templates for overlap extension PCR to generate a deletion cassette in which *tfsA* was replaced by *cat*.

The deletion cassettes of *tfsB* and *tfsA/tfsB* were generated by similar procedures. Each deletion cassette created was ligated into pCR-Blunt II-TOPO and the resulting recombinant plasmids were transformed into competent cells of *E. coli* TOP10 according to the manufacturer's directions (Invitrogen). Constructs to be introduced for making mutants were sequenced before electroporation to rule out unintended base changes. Electroporation of *T. forsythensis* was performed essentially as described by Fletcher *et al.* (1995), except that the organism was grown on the plates for 7 days, scraped, washed and

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics*	Source or reference
<i>Tannerella forsythensis</i>		
ATCC 43037	Wild-type, type strain ATCC†	
Δ230	<i>tfsA</i> -deletion mutant from ATCC 43037, Cp ^r	This study
Δ270	<i>tfsB</i> -deletion mutant from ATCC 43037, Cp ^r	This study
Δ230-270	<i>tfsA</i> - and <i>tfsB</i> -deletion mutant from ATCC 43037, Cp ^r	This study
<i>Escherichia coli</i>		
TOP10	As chemically competent cells, F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) <i>φ80lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>deoR</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
Plasmids		
pCR-Blunt II-TOPO	Cloning vector, linearized with DNA topoisomerase I bound to the 3' end of each DNA strand, Km ^r	Invitrogen
pKD260	A derivative of pACYC184 with deletion of a 1.1 kbp <i>HincII</i> fragment, bearing <i>cat</i> gene, Cp ^r	Nagano <i>et al.</i> (2005)

*Cp^r, chloramphenicol resistance; Km^r, kanamycin resistance.

†ATCC, American Type Culture Collection.

Table 2. Primers

Primer-binding sites are shown in Fig. 1.

Primer	Sequence (5'–3')*
AGU-01†	ATGGAGAAAAAATCACTGGA
AGU-02†	TTACGCCCCGCCCTGCCACTC
AGU-71	CGTTCGGCGATCAATAAAGT
AGU-72	<u>CCAGTGATTTTTTCTCCATAATCAAATAATTAAATATTA</u> ACTAGTAAAT
AGU-73	<u>GCAGGGCGGGGCGTAATAAGGTATTCATTA</u> AAATGGAAAGGAA
AGU-74	GGTGTGAGCATTCGGATT
AGU-75	TCCATGCAGGAGATACGTTG
AGU-76	<u>CCAGTGATTTTTTCTCCATTTT</u> TAATATTAATATTCAATTCGTATGGG
AGU-77	<u>GCAGGGCGGGGCGTAAATGTTGAAATAATCATTTGATTCAATATTAAG</u>
AGU-78	CCGAACATCGTGAGGAATCT
AGU-79	TGATGAGCAAGAGCGTCTGT
AGU-83	ACCGTTCGATAAGGATGTCG
Cat-39F	ATCCCAATGGCATCGTAAAG
Cat-486R	GGGCGAAGAAGTTGTCCATA

*Underlines show overlapping regions of 5' or 3' end of *cat*.

†The same primers were used by Nagano *et al.* (2005).

suspended to obtain competent cells (10^{11} cells ml^{-1}). The plasmid constructs (5 μg) were linearized by digestion with endonucleases *Kpn*I and *Xba*I and introduced into electrocompetent cells of *T. forsythensis* (10^{10} cells). After 12 h anaerobic incubation in Trypticase soy broth (Becton Dickinson) supplemented with 2.5 mg yeast extract ml^{-1} , 2.5 μg haemin ml^{-1} , 5 μg menadione ml^{-1} , 0.1 mg DTT ml^{-1} , 10 μg *N*-acetylmuramic acid ml^{-1} and 5% (v/v) Fildes extract (Oxoid), the pulsed cells were plated on blood agar supplemented with 12 μg Cp ml^{-1} and the plates were incubated anaerobically at 37 °C for 7 days. Possible recombinants were verified by PCR.

Membrane preparation. Separation of whole envelopes from *T. forsythensis* strains was performed essentially as described previously (Murakami *et al.*, 2002b, 2004). Briefly, bacterial cells were washed with 10 mM HEPES/NaOH (pH 7.4) containing 0.15 M NaCl and then resuspended with 10 mM HEPES/NaOH (pH 7.4) containing 0.1 mM *N*- α -*p*-tosyl-L-lysine chloromethyl ketone, 0.2 mM PMSF and 0.1 mM leupeptin (HEPES buffer). The cells were disrupted by sonication and the remaining undisrupted bacterial cells were removed by centrifugation at 1000 *g* for 10 min. The envelope was collected as a pellet by centrifugation at 100 000 *g* for 60 min at 4 °C. The pellet was washed once by resuspending it in HEPES buffer and recentrifuged. The protein content of the membrane preparation was estimated by the method of Bradford (1976).

SDS-PAGE and Western blotting. SDS-PAGE was performed in a 1.0 mm thick 10% (w/v) gel as described by Lugtenberg *et al.* (1975). The samples were usually solubilized in SDS buffer with 2-mercaptoethanol at 100 °C for 5 min (Hongo *et al.*, 1999). The gels were stained with Coomassie brilliant blue R-250 (CBB). Western blotting was performed as described previously (Nishikawa & Yoshimura, 2001). Proteins in the SDS-polyacrylamide gel were electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 1% (w/v) BSA (fraction V) in Tris-buffered saline [TBS, 20 mM Tris/HCl (pH 7.5) containing 0.5 M NaCl]. Then the membrane was reacted with the antiserum followed by incubation with peroxidase-conjugated anti-rabbit IgG (MP

Biomedicals). After the membrane was washed, signals of the S-layer proteins were detected with 0.01% (w/v) 4-chloro-1-naphthol in TBS supplemented with hydrogen peroxide.

N-terminal or internal amino acid sequence analysis. The S-layer proteins (230 and 270 kDa) from *T. forsythensis* ATCC 43037 were excised from CBB-stained SDS-PAGE gels. N-terminal amino acid sequences or internal amino acid sequences after digestion with *Staphylococcus aureus* V8 protease were analysed as described previously (Murakami *et al.*, 2002b; Imai *et al.*, 2005).

Preparation of specific antisera. Synthetic peptides derived from less-related amino acid sequences selected in the 230 and 270 kDa proteins (CR⁵¹⁰LDPKNKKDYSPVMGR⁵²⁶ and R¹⁰VKPYDNAKPTLKFK²⁴C-NH₄, respectively, numbered from the N-terminal amino acid estimated in ORFs of contig 5077) were purchased from the Peptide Institute. After peptide conjugation to thyroglobulin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce Biotechnology), as described in the company's instructions, each peptide was emulsified with complete Freund's adjuvant and injected into rabbits subcutaneously four times at 2-week intervals. Specific antisera that reacted to both the 230 and 270 kDa proteins of *T. forsythensis* were prepared as described previously (Higuchi *et al.*, 2000).

Haemagglutination assay. Bacterial cells were washed once with 10 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl (PBS) and resuspended in the same buffer (OD₆₀₀ 1.0). The haemagglutinating activity was determined at room temperature (20–22 °C) in a round-bottomed microtitre plate (Murakami *et al.*, 2002a). The bacterial sample (50 μl) was diluted in twofold series in PBS, and 50 μl of a 2% (v/v) suspension of chicken or human erythrocytes was added. The mixtures were left standing for 90 min. The titre was expressed as the reciprocal of the highest dilution showing positive agglutination.

For the screening of haemagglutination inhibition, 25 μl of the cell suspension and 50 μl of the erythrocyte suspension were sequentially added to 25 μl of the solution containing the inhibitor to be tested (Murakami *et al.*, 2002a). The concentration of the cell suspension was

Fig. 1. Construction of *tfsA*- and *tfsB*-deletion mutants from *T. forsythensis* ATCC 43037. (a) Gene arrangement in the chromosome and the procedure for construction of allele-exchange gene deletion, PCR primers and their annealing sites. (b) Predicted lengths of the PCR products in each genotype. Sizes of the DNA fragments are shown as base pairs in parentheses. (c) Verification of the mutants by gel electrophoresis of PCR products. (1) After amplification by PCR with primers AGU-79 and AGU-83, shown at the top of panel (b) as 79 and 83, respectively, the PCR products were subjected to electrophoresis. (2) After amplification by PCR with primers AGU-79 and Cat-486R. (3) After amplification by PCR with primers Cat-39F and AGU-83. Sequences of primers used are shown in Table 2. Dotted arrows, *tfsA* and its transcriptional direction; diagonally striped arrows, *tfsB*; grey arrows, *cat*; small arrows, primers and directions for chain elongation; wavy line, overlap regions on *cat*; underlined numbers, simplified primer designation (see Table 1 for details).

adjusted to twice the minimal concentration at which distinct haemagglutination occurred. Haemagglutination was recorded after 90 min. The reaction mixture that did not contain the substances was used as a positive control of haemagglutination.

Cell cultures. Cell lines Ca9-22 (JCRB0625, epithelial cell-like gingival carcinoma cell line) and KB (JCRB9027) were obtained from Health Science Research Resources Bank, Osaka, Japan. KB cells are now known to be HeLa cells (Masters, 2002). The cells were grown at 37 °C for 2–3 days in 5% (v/v) CO₂ in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% (v/v) fetal calf serum (Invitrogen).

Bacterial adherence assay by confocal laser scanning microscopy. Ca9-22 and KB cells were subcultured into Lab-Tek II-chamber slides (Nalge Nunc International) and grown for 24 h before adherence study. Ca9-22 or KB cells grown to near confluence of 10⁵ cells per well were washed with PBS to remove unattached cells from the chamber slides. Then *T. forsythensis* cells, which were washed with PBS and resuspended in DMEM at a concentration of 10⁷ cells ml⁻¹, were added at a m.o.i. of 50. After incubation under 5% CO₂ at 37 °C for 3 h, chamber slides were washed three times with PBS, each followed by gentle shaking for 15 min at room temperature, and fixed with 4% (v/v) paraformaldehyde at 4 °C overnight. After the fixed cells had been washed three times with TBS, they were permeabilized with TBS containing 0.05% (v/v) Triton X-100 at 37 °C for 30 min. They were washed again and then blocked with TBS containing 1% (w/v) BSA at 37 °C for 30 min. Bacterial cells on chamber slides were labelled with anti-*T. forsythensis* whole-cell antiserum (1:1000 dilution) for 60 min at 37 °C and washed five times with TBS containing 0.05% Tween 20. Then bacterial cells were incubated with the FITC-conjugated goat IgG fraction to the rabbit IgG F(ab')₂ secondary antibody (1:1000 dilution, ICN Pharmaceuticals). Actin filaments in Ca9-22 or KB cells were simultaneously stained with tetramethylrhodamine isothiocyanate (TRITC)-labelled phalloidin (1 µg ml⁻¹, Sigma-Aldrich) for 60 min at 37 °C in the dark. After five washes with TBS containing 0.05% (v/v) Tween 20, chamber slides were mounted onto a slide containing 10 µl SlowFade Light Antifade (Invitrogen). Immunostained slides were kept in the dark until analysed. Adherent bacteria on the cell surfaces were examined by the laser scanning system Radiance 2100 (Bio-Rad) mounted on an Axiovert 200 inverted microscope (Carl Zeiss) equipped with a ×63 objective.

Quantitative bacterial adherence assay. For quantitative bacterial adherence assay, Ca9-22 and KB cells were subcultured into 24-well culture plates (Corning) and grown to near confluence of 5 × 10⁵ cells per well for 24 h. The cells were washed with PBS to remove unattached cells from the wells. Then *T. forsythensis* cell suspension at a concentration of 10⁷ cells ml⁻¹ was added at a m.o.i. of 100. After incubation under 5% CO₂ at 37 °C for 3 h, non-adherent bacteria were removed by washing three times with PBS. After the cells had been disrupted by the addition of sterile distilled water (100 µl) and incubated at 37 °C for 10 min, serial dilutions of the disrupted mixture were plated on blood agar plates and incubated anaerobically at 37 °C for 7 days. The numbers of viable adherent bacteria were determined by plating followed by counting the colonies that appeared. All assays were performed in triplicate.

Electron microscopy. *T. forsythensis* whole cells were fixed at 4 °C for 2 h with 4% (w/v) paraformaldehyde and 5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer. Samples were washed and post-fixed with 4% (w/v) osmium tetroxide for 90 min and then 0.5% (w/v) uranyl acetate for 20 min. The fixed cells were dehydrated in ethanol and embedded in L-R white resin (London Resin). Ultrathin sections were stained with uranyl acetate and lead citrate. Cells were also negatively stained on carbon-coated grids with 2% (w/v) uranyl acetate. Stained samples were observed using a transmission electron microscope (JEM-1210, JEOL).

Antimicrobial susceptibility. All antibiotics (chloramphenicol, ampicillin, tetracycline, cephalothin, erythromycin, ofloxacin, bacitracin and vancomycin) were purchased from Sigma-Aldrich. Assays

were performed essentially as described previously (Nagano *et al.*, 2005). MIC was evaluated by agar dilution assay, as recommended by the National Committee for Clinical Laboratory Standards (1997). Briefly, serial dilutions of the corresponding antibiotics were added to blood agars. After precultured bacteria were grown on blood agar, and washed bacterial suspension (2 µl) was spotted on the antibiotic-containing agar. After 7 days of anaerobic incubation, the susceptibility breakpoints were determined.

RESULTS

Identification of S-layer protein genes

Partial amino acid sequences of electrophoretically purified S-layer proteins were identified (Table 3). The sequences were compared with the unfinished genome of *T. forsythensis* obtained from the TIGR website (<http://www.tigr.org>) using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>), and two ORFs encoding the S-layer proteins were found. Two genes, *tfsA* encoding a 230 kDa protein and *tfsB* encoding a 270 kDa protein, were tandemly located from 2833655 bp to 2837191 bp and 2837292 bp to 2841383 bp in contig 5077, respectively. In the case of *tsfA*, the stop codon (TAG) was found at 2835326 bp to 2835328 bp in the unfinished genome of *T. forsythensis*. Since the internal amino acid sequences (230K fragments 1, 2 and 3 in Table 3), determined using partial digests of pure 230 kDa protein, were distributed before and after the stop codon at the corresponding DNA region in contig 5077, we noticed that the stop codon was incorrectly assigned due to the incomplete sequence. The calculated molecular masses of TsfA and TsfB based on the ORFs are about 120 kDa and 140 kDa, respectively, much smaller than the apparent molecular masses of 230 kDa and 270 kDa, respectively. Therefore, we speculate that the S-layer proteins might be post-translationally modified.

Construction of deletion mutants

Construction of three deletion mutants from *T. forsythensis* wild-type strain ATCC 43037 was performed by the PCR-based overlap extension method, in which ORFs for *tfsA*

and/or *tfsB* were deleted and replaced by *cat*, the gene encoding CAT. The overall design for construction of the mutants is shown in Fig. 1. Transformants were detected at a frequency of 10⁻⁷ to 10⁻⁶ per pulsed cell, and the number of transformants obtained was 200–2000 per µg plasmid DNA. To confirm the replacement of ORFs for 270 (*tfsA*) and/or 230 kDa proteins (*tfsB*) by *cat*, the cloning sites were amplified by PCR with the use of chromosomes from each mutant as a template with several primers as shown in Fig. 1(b). All the amplified products showed the predicted fragments (Fig. 1c). All the constructed mutants showed no difference from the parent strain in colony morphology or growth on blood agar plates.

SDS-PAGE and Western blotting

Bacterial envelopes were subjected to SDS-PAGE. The parent strain showed major 230 and 270 kDa protein bands, whereas the single mutants had lost one of these bands and the double mutant both bands (Fig. 2). Specific antisera to the 230 and 270 kDa proteins, prepared using conjugated synthetic peptides as antigens, successfully discriminated the two proteins by Western blot analysis. Anti-230 kDa protein antiserum detected the 230 kDa protein in the parent and Δ270, and anti-270 kDa protein antiserum exclusively detected the 270 kDa protein in the parent and Δ230, as predicted. No immunoreactive bands appeared in the double mutant, confirming that the 230 and 270 kDa proteins were products of *tfsA* and *tfsB*, respectively. The single-deletion mutants appeared to have lost the corresponding 230 or 270 kDa protein. Immunoreactive bands at around 100 kDa in Fig. 2(c) were derived from the 270 kDa protein because none of these bands was detected in Δ270 and Δ230-270.

Electron microscopy

Thin-section microscopy revealed that the parent had a regularly arrayed structure at the outermost cell surface. In contrast, the double mutant had completely lost the structure, and the single mutants retained smaller amounts of it (Fig. 3). These results clearly indicated that the S-layer

Table 3. N-terminal and internal amino acid sequences of the S-layer proteins

S-layer protein*	Apparent mol. mass (kDa)	Amino acid sequence	Nucleotide position in contig 5077† (bp)
230K	230	RPFYGDVAVKYLPEGMGKGAY	2833727–2833786
230K fragment 1	100	YYLVLDNDNGRYGYTPEVI	2835689–2835742
230K fragment 2	25	GSSISKIHLINREFDDIRI	2835074–2835130
230K fragment 3	20	FSQKVSAGQVNPFEQPLMAE	2834414–2834473
270K	270	Unidentified	
270K fragment 1	200	VEYLDGISPVTAMLIDSSA	2838279–2838335
270K fragment 2	40	QQQLRVKPYDNAKPTLKFKE	2837391–2837450
270K fragment 3	25	QQQLRVKPYDNAKPTLKFKE	2837391–2837450

*Fragments were obtained from partial digestion of the S-layer protein with *Staphylococcus aureus* V8 protease.
†Preliminary sequence data of contig 5077 in the *T. forsythensis* genome were obtained from TIGR website at <http://www.tigr.org>.

Fig. 2. Typical protein patterns and Western blots of envelope fractions. Bacterial envelopes were denatured in SDS with 2-mercaptoethanol at 100 °C for 5 min and then loaded onto SDS-polyacrylamide gels. The gels were stained with CBB (a) and subjected to Western blot analysis with anti-230 (b) or -270 kDa protein-specific serum (c). Fifty micrograms of each protein was applied to each lane of the gels. See Methods for details of the sera.

was composed of the 230 and 270 kDa proteins. Even after deletion of the S-layer genes, the fusiform shape was not changed. Negative staining microscopy also showed that the S-layer-deficient mutants maintained almost the same cell shape as the wild-type (data not shown).

Haemagglutinating activity

Compared to the parent, $\Delta 230$ and $\Delta 270$ showed two- to fourfold lower haemagglutination, whereas the double mutant retained almost the same activity (Table 4). Similar results were obtained when erythrocytes from different volunteers or chicken were used, and experiments

were independently repeated. Haemagglutination inhibition assay using specific antibodies to the 270 and/or 230 kDa proteins revealed that the contribution of the S-layer proteins to haemagglutination was much less, because inhibition was almost the same as with pre-immune control serum (data not shown).

Bacterial adhesion to host cells

Bacteria located at the surface of host cells were observed by confocal laser scanning microscopy. *T. forsythensis* were labelled with FITC (green), and skeletal actin in the host cell was stained with TRITC-phalloidin (red). The parent

Fig. 3. Electron micrographs of the thin-sectioned *T. forsythensis* cells. The cells were stained with uranyl acetate and lead citrate. Micrographs in which spindle-shaped cells were cross-sectioned are selected for S-layer observation. (a) The parent ATCC 43037; (b) $\Delta 230$; (c) $\Delta 270$; (d) $\Delta 230-270$. Bars, 100 nm.

Table 4. Comparison of haemagglutination of *T. forsythensis* wild-type and the S-layer-deficient mutants

Strain	Haemagglutinating titre	
	Human erythrocytes	Chicken erythrocytes
ATCC 43037	16	16
$\Delta 230$	8	4
$\Delta 270$	4	2
$\Delta 230-270$	16	8

adhered to Ca9-22 cells in a dispersed fashion, without forming large aggregates, whereas marked decreases of bacterial adherence were observed in the three mutants (Fig. 4a). Since there was no distinctive difference among observed fields, a set of representative images of the parent and the mutants is shown. Similarly, the parent, but not the mutants, had the ability to adhere to KB cells (Fig. 4b); however, careful staining procedures were necessary to avoid detaching KB cells from the chamber slide surface.

Quantitative adhesion assay by colony counting clearly revealed that adherence of *T. forsythensis* to both Ca9-22 and KB cells was significantly reduced when either of the S-layer proteins was deleted (Fig. 5). Thus, the observations by confocal laser microscopy were quantitatively confirmed by viable-cell counting data.

Antimicrobial susceptibility

The MIC of Cp for *T. forsythensis* ATCC 43037 was $3 \mu\text{g ml}^{-1}$, while those for the three mutants were $>25 \mu\text{g ml}^{-1}$, probably due to the strong CAT expression (Table 5). Strong CAT activity was detected only in the mutants where *cat* had been introduced (data not shown). The mutants showed fourfold lower MICs to vancomycin ($0.78 \mu\text{g ml}^{-1}$) than the wild-type ($3.13 \mu\text{g ml}^{-1}$). However, MICs of other antibiotics (chloramphenicol, ampicillin, tetracycline, cephalothin, erythromycin, ofloxacin and bacitracin) did not differ significantly between the parent and the three mutants. Most MIC tests were repeated three times.

DISCUSSION

T. forsythensis is an important bacterium in periodontitis. Most studies have been focused on detection of this bacterium from periodontal disease; relatively few have dealt with potential virulence factors at the molecular level. Several *T. forsythensis* antigens have been cloned (Saito *et al.*, 1997; Sharma *et al.*, 1998, 2005; Hughes *et al.*, 2003; Ishikura *et al.*, 2003; Inagaki *et al.*, 2005). Honma *et al.* (2001) reported the generation of specific gene knockout mutants of *T. forsythensis* for the first time using a suicide plasmid. We followed their procedure to obtain deletion mutants; however, no mutants were obtained. Therefore, we employed PCR-based overlapping methods, which were successful in producing deletion mutants in *P. gingivalis*

(a) Ca9-22 cells

43037

$\Delta 230$

$\Delta 270$

$\Delta 230-270$

(b) KB cells

43037

$\Delta 230$

$\Delta 270$

$\Delta 230-270$

Fig. 4. Confocal laser scanning photomicrographs showing adherence of *T. forsythensis* to Ca9-22 and KB cells. The bacteria were stained with FITC (green) and the cytoskeleton of the epithelial cells was visualized with TRITC (red). (a) Ca9-22 cells; (b) KB cells. Bars, 10 μm .

Fig. 5. Quantitative adhesion assay of *T. forsythensis* to Ca9-22 and KB cells. *T. forsythensis* suspensions were added to monolayers of the epithelial cells in 24-well plates at a m.o.i. of 100 and allowed to adhere for 3 h. After the monolayers had been washed with PBS three times, the cells were lysed with distilled water. The numbers of viable, adherent *T. forsythensis* cells were determined by plating followed by counting the colonies that appeared. Values represent the means $\pm$ SD of the triplicate determinations from a typical experiment.

(Nagano *et al.*, 2005). The gene to be deleted by this method should at least have a strong promoter in front of it to express the *cat* gene adequately. Since the S-layer proteins were expressed in a large amount in *T. forsythensis*, the *cat* gene would be strongly transcribed by the promoter of *tfsA* and *tfsB*. Consequently, Cp-resistant mutants could be selected. To the best of our knowledge, this is the first report of gene deletion being systematically performed in *T. forsythensis* studies. This method would be useful to determine the functions of other putative virulence factors in *T. forsythensis*.

Very recently, Lee *et al.* (2006) reported S-layer genes from *T. forsythensis*. Sequences of the genes reported (accession no. AY23857) completely matched those we analysed. Although we initially speculated that the S-layer proteins

(230 and 270 kDa) might be single gene products due to cross-immunoreactivity in a previous paper (Higuchi *et al.*, 2000), these proteins turned out to be encoded by two genes, *tfsA* and *tfsB*, when the unfinished genome sequence became available soon after. *tfsA* and *tfsB* form an operon structure according to Northern blot and RT-PCR analyses (Lee *et al.*, 2006). Both gene products, which are reported to exhibit 24 % similarity to each other (Lee *et al.*, 2006), seemed to be expressed in almost the same amount, judged from intensities of the protein bands in CBB-stained gels (Higuchi *et al.*, 2000). It was hypothesized that S-layer genes of *T. forsythensis* might belong to a novel class, since two large proteins were encoded by two separate genes (Lee *et al.*, 2006). Generally, S-layers are composed of a single protein or glycoprotein subunit (Sára & Sleytr, 2000). Although several lactobacilli possess multiple S-layer protein genes, these genes can be differentially or simultaneously expressed depending on growth conditions (Åvall-Jääskeläinen & Palva, 2005). Signal sequences for TfsA and TfsB (230 and 270 kDa, respectively) were estimated as having 22 and 28 amino acids (although unintentionally depicted as 19 and 30 amino acids, respectively, in the figures), respectively, in a previous report (Lee *et al.*, 2006). The signal peptide for TfsA was assigned as MNKKVFTLLAASLMLFMTAFMADA (24 amino acids) because we successfully determined the N-terminal sequence of the 230 kDa protein (Table 3). However, the N-terminal sequence of TfsB was unidentified, presumably because of N-terminal blockage. The predicted N-terminal amino acid of the mature TfsB protein is presumably glutamine, 5 amino acids ahead of the N-terminal amino acid of the two digested peptides (270K fragments 2 and 3 in Table 3), since it is known to be convertible to pyroglutamic acid, an undetected amino acid derivative in the sequence reaction (Murakami *et al.*, 2002b). As another possibility, glycosylation might cause N-terminal blockage. Therefore, we estimated here that the signal sequence was MIMIMNKKIFTLLAGAFMGFAAVSSVTA (28 amino acids). We have shown by electron microscopy using the deletion mutants that the 230 and 270 kDa proteins compose the S-layer; however, the precise molecular arrangement of these proteins in the S-layer remains unclear. Lee *et al.* (2006) also reported that the S-layer proteins from *T. forsythensis* were glycoproteins. We confirmed this using a commercial glycoprotein staining kit after SDS-PAGE (data not shown). However, the exact contents and compositions of sugars in the S-layer proteins are unknown.

Previous studies indicated that *T. forsythensis* cells (Munemasa *et al.*, 2000), especially the S-layer (Sabet *et al.*, 2003), had haemagglutinating and adherence activities. We therefore examined whether the S-layer proteins on the *T. forsythensis* cell surface might contain haemagglutinin, using S-layer protein deletion mutants. Our results indicated that lack of the S-layer had a minor effect on haemagglutination. Moreover, haemagglutination inhibition by the S-layer-specific antisera raised against the

Table 5. MICs of various antibiotics for *T. forsythensis* wild-type and the S-layer-deficient mutants

Abbreviations: CP, chloramphenicol; ABPC, ampicillin; TC, tetracycline; CFX, cephalothin; EM, erythromycin; OFLX, ofloxacin; BC, bacitracin; VCM, vancomycin.

Strain	MIC ($\mu\text{g ml}^{-1}$)							
	CP	ABPC	TC	CFX	EM	OFLX	BC	VCM
ATCC 43037	3.13	0.78	0.78	0.78	1.56	3.13	25	3.13
Δ230	>25	0.78	0.78	0.78	1.56	3.13	25	0.78
Δ270	>25	0.78	0.78	0.78	1.56	3.13	25	0.78
Δ230-270	>25	0.78	0.78	0.78	1.56	3.13	25	0.78

230 and/or 270 kDa proteins was almost the same as that by the control pre-immune serum. Thus the S-layer seemed to have a marginal association with the haemagglutinating activity. Since thin-section microscopy revealed that the cell surfaces of the single mutants ($\Delta 230$ and $\Delta 270$) were partially covered with substances like the S-layer, an abnormal state of the cell surface could hinder haemagglutinating activity. On the other hand, in the double mutant ($\Delta 230$ -270), which lacked the S-layer completely, the putative haemagglutinin might be fully exposed and function. We have previously reported *N*-acetylneuraminylactose-sensitive haemagglutinin on the envelope, although its gene has not been identified yet (Murakami *et al.*, 2002a). We could not find any reliable report on other bacteria whose S-layer protein had haemagglutinating activity.

In contrast, the S-layer proteins in *T. forsythensis* were clearly responsible for adhesion to the host cells. The S-layer protein-deletion mutants almost completely lost adherence. Although only a few bacteria were internalized into the host cells even in the wild-type strain (data not shown), we speculated that invasion of the host cells, which may occur after adhesion, would be lower in the deletion mutants. It has been reported that *Lactobacillus* S-layers are involved in mediating adhesion to different host tissues (Åvall-Jääskeläinen & Palva, 2005), whereas the S-layer from *Campylobacter rectus* had little effect on adhesion to HEP-2 epithelial cells (Wang *et al.*, 2000). To examine the pathogenicity of the S-layer in *T. forsythensis*, a murine abscess lesion model, as described essentially for *P. gingivalis* (Kumagai *et al.*, 2001), was used; however, the parent and all three mutants caused abscesses to similar degrees and we could not detect any difference between the parent and the mutant strains (data not shown). Mixed infection with other bacteria in the murine abscess model may be needed to detect differences of virulence among them (Yoneda *et al.*, 2001).

The S-layer is an important interface between the bacterial cell and its environment (Sleytr *et al.*, 1993). We used several representative antibiotics to examine whether the S-layer might contribute to antimicrobial susceptibility. MICs for the wild-type were similar to those described by Takemoto *et al.* (1997). Most of the MICs for the mutants were not different from that for the wild-type. The mutants showed higher MICs to Cp because of *cat* gene expression. On the other hand, MICs to vancomycin were lower in the mutants. The reason is unclear; however, S-layers in prokaryotes are considered to function as molecular sieves and as ion traps (Sleytr & Messner, 1988; Sleytr *et al.*, 1993; Sleytr & Beveridge, 1999; Sára & Sleytr, 2000). Regarding another function, the *T. forsythensis* S-layer proteins did not play a role in cell shape determination and maintenance as judged from electron microscopy. Not only thin-sectioned cells but also negatively stained cells showed similar cell shapes for wild-type and the S-layer-deficient strains. Other possible functions of S-layer proteins, molecules related to S-layer synthesis, and molecules for anchoring S-layer

proteins to the outer membrane will be elucidated in the near future.

In this study, we have shown a systematic method for construction of gene-deletion mutants in *T. forsythensis*. Although *T. forsythensis* S-layer proteins seemed to have a marginal relationship to haemagglutinating activity, these proteins were responsible for adhesion to Ca9-22 gingival epithelial cells and KB cells. In conclusion, S-layer proteins in *T. forsythensis* may play an important role in the early stage of oral infections, including periodontal disease.

ADDENDUM

After the completion of this study, we learned that a similar approach was taken by Lee *et al.* (2006).

ACKNOWLEDGEMENTS

Part of this work is taken from the thesis submitted by Junpei Sakakibara to the Graduate Faculty, School of Dentistry, Aichi-Gakuin University, in partial fulfilment of the requirements for his doctoral degree. We thank T. Hirose and the late Y. Abe (Center for Instrumental Analysis, Hokkaido University) for analysing amino acid sequences. Preliminary sequence data were obtained from the TIGR website at <http://www.tigr.org>. This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (15591957 to F.Y. and 15591958 to Y.M.) and the AGU High-Tech Research Center Project for Private Universities: matching fund subsidy from MEXT (the Ministry of Education, Culture, Sports, Science and Technology, Japan), 2003–2007.

REFERENCES

- Åvall-Jääskeläinen, S. & Palva, A. (2005). *Lactobacillus* surface layers and their applications. *FEMS Microbiol Rev* **29**, 511–529.
- Boot, H. T. & Pouwels, P. H. (1996). Expression, secretion and antigenic variation of bacterial S-layer proteins. *Mol Microbiol* **21**, 1117–1123.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Braham, P. H. & Moncla, B. J. (1992). Rapid presumptive identification and further characterization of *Bacteroides forsythus*. *J Clin Microbiol* **30**, 649–654.
- Fletcher, H. M., Schenkein, H. A., Morgan, R. M., Bailey, K. A., Berry, C. R. & Macrina, F. L. (1995). Virulence of a *Porphyromonas gingivalis* W83 mutant defective in the *prfH* gene. *Infect Immun* **63**, 1521–1528.
- Gersdorf, H., Meissner, A., Pelz, K., Krekeler, G. & Göbel, U. B. (1993). Identification of *Bacteroides forsythus* in subgingival plaque from patients with advanced periodontitis. *J Clin Microbiol* **31**, 941–946.
- Gonçalves, R. B. & Mouton, C. (1999). Molecular detection of *Bacteroides forsythus* in infected root canals. *J Endod* **25**, 336–340.
- Haffajee, A. D., Cugini, M. A., Tanner, A., Pollack, R. P., Smith, C., Kent, R. L., Jr & Socransky, S. S. (1998). Subgingival microbiota in healthy, well-maintained elder and periodontitis subjects. *J Clin Periodontol* **25**, 346–353.

- Hajishengallis, G., Sharma, A., Russell, M. W. & Genco, R. J. (2002). Interactions of oral pathogens with Toll-like receptors: possible role in atherosclerosis. *Ann Periodontol* 7, 72–78.
- Higuchi, N., Murakami, Y., Moriguchi, K., Ohno, N., Nakamura, H. & Yoshimura, F. (2000). Localization of major, high molecular weight proteins in *Bacteroides forsythus*. *Microbiol Immunol* 44, 777–780.
- Holt, S. C. & Ebersol, J. L. (2005). *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the “red complex”, a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol* 2000 38, 72–122.
- Hongo, H., Osano, E., Ozeki, M., Onoe, T., Watanabe, K., Honda, O., Tani, H., Nakamura, H. & Yoshimura, F. (1999). Characterization of an outer membrane protein gene, *pgmA*, and its gene product from *Porphyromonas gingivalis*. *Microbiol Immunol* 43, 937–946.
- Honma, K., Kuramitsu, H. K., Genco, R. J. & Sharma, A. (2001). Development of a gene inactivation system for *Bacteroides forsythus*: construction and characterization of a BspA mutant. *Infect Immun* 69, 4686–4690.
- Horton, R. M., Ho, S. N., Pullen, J. K., Hunt, H. D., Cai, Z. & Pease, L. R. (1993). Gene splicing by overlap extension. *Methods Enzymol* 217, 270–279.
- Hughes, C. V., Malki, G., Loo, C. Y., Tanner, A. C. R. & Ganeshkumar, N. (2003). Cloning and expression of α -D-glucosidase and N-acetyl- β -glucosaminidase from the periodontal pathogen, *Tannerella forsythensis* (*Bacteroides forsythus*). *Oral Microbiol Immunol* 18, 309–312.
- Imai, M., Murakami, Y., Nagano, K., Nakamura, H. & Yoshimura, F. (2005). Major outer membrane proteins from *Porphyromonas gingivalis*: strain variation, distribution, and clinical significance in periradicular lesions. *Eur J Oral Sci* 113, 391–399.
- Inagaki, S., Kuramitsu, H. K. & Sharma, A. (2005). Contact-dependent regulation of a *Tannerella forsythia* virulence factor, BspA, in biofilms. *FEMS Microbiol Lett* 249, 291–296.
- Ishikura, H., Arakawa, S., Nakajima, T., Tsuchida, N. & Ishikawa, I. (2003). Cloning of the *Tannerella forsythensis* (*Bacteroides forsythus*) *siaHI* gene and purification of the sialidase enzyme. *J Med Microbiol* 52, 1101–1107.
- Kerosuo, E. (1988). Ultrastructure of the cell envelope of *Bacteroides forsythus* strain ATCC 43037^T. *Oral Microbiol Immunol* 3, 134–137.
- Kumagai, Y., Konishi, K., Gomi, T., Yagishita, H., Yajima, A. & Yoshikawa, M. (2001). Enzymatic properties of dipeptidyl aminopeptidase IV produced by the periodontal pathogen *Porphyromonas gingivalis* and its participation in virulence. *Infect Immun* 68, 716–724.
- Lee, S.-W., Sabet, M., Um, H. S., Yang, L., Kim, H. C. & Zhu, W. (2006). Identification and characterization of the genes encoding a unique surface (S-) layer of *Tannerella forsythia*. *Gene* 371, 102–111.
- Lugtenberg, B., Meijers, J., Peters, R., van der Hoek, P. & van Alphen, L. (1975). Electrophoretic resolution of the “major outer membrane protein” of *Escherichia coli* K12 into four bands. *FEBS Lett* 58, 254–258.
- Masters, J. R. (2002). HeLa cells 50 years on: the good, the bad and the ugly. *Nature Rev Cancer* 2, 315–319.
- Moriguchi, K., Higuchi, N., Murakami, Y., Yoshimura, F., Nakamura, H. & Ohno, N. (2003). A morphological and immunolabeling study of freeze-substituted *Bacteroides forsythus*. *Biotechnol Histochem* 78, 129–133.
- Munemasa, T., Takemoto, T., Dahlén, G., Hino, T., Shiba, H., Ogawa, T. & Kurihara, H. (2000). Adherence of *Bacteroides forsythus* to host cells. *Microbios* 101, 115–126.
- Murakami, Y., Higuchi, N., Nakamura, H., Yoshimura, F. & Oppenheim, F. G. (2002a). *Bacteroides forsythus* hemagglutinin is inhibited by N-acetylneuraminylactose. *Oral Microbiol Immunol* 17, 125–128.
- Murakami, Y., Imai, M., Nakamura, H. & Yoshimura, F. (2002b). Separation of the outer membrane and identification of major outer membrane proteins from *Porphyromonas gingivalis*. *Eur J Oral Sci* 110, 157–162.
- Murakami, Y., Imai, M., Mukai, Y., Ichihara, S., Nakamura, H. & Yoshimura, F. (2004). Effects of various culture environments on expression of major outer membrane proteins from *Porphyromonas gingivalis*. *FEMS Microbiol Lett* 230, 159–165.
- Nagano, K., Read, E. K., Murakami, Y., Masuda, T., Noguchi, T. & Yoshimura, F. (2005). Trimeric structure of major outer membrane proteins homologous to OmpA in *Porphyromonas gingivalis*. *J Bacteriol* 187, 902–911.
- National Committee for Clinical Laboratory Standards (1997). *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria*, 5th edn, vol. 21. Approved standard M11-A5. Wayne, PA: National Committee for Clinical Laboratory Standards.
- Nelson, K. E., Fleischmann, R. D., DeBoy, R. T. & 20 other authors (2003). Complete genome sequence of the oral pathogenic bacterium *Porphyromonas gingivalis* strain W83. *J Bacteriol* 185, 5591–5601.
- Nishikawa, K. & Yoshimura, F. (2001). The response regulator FimR is essential for fimbrial production of the oral anaerobe *Porphyromonas gingivalis*. *Anaerobe* 7, 255–262.
- Noack, B., Genco, R. J., Trevisan, M., Grossi, S., Zambon, J. J. & De Nardin, E. (2001). Periodontal infections contribute to elevated systemic C-reactive protein level. *J Periodontol* 72, 1221–1227.
- Sabet, M., Lee, S.-W., Nauman, R. K., Sims, T. & Um, H.-S. (2003). The surface (S-) layer is a virulence factor of *Bacteroides forsythus*. *Microbiology* 149, 3617–3627.
- Saito, T., Ishihara, K., Kato, T. & Okuda, K. (1997). Cloning, expression, and sequencing of a protease gene from *Bacteroides forsythus* ATCC 43037 in *Escherichia coli*. *Infect Immun* 65, 4888–4891.
- Sakamoto, M., Suzuki, M., Umeda, M., Ishikawa, I. & Benno, Y. (2002). Reclassification of *Bacteroides forsythus* (Tanner *et al.*, 1986) as *Tannerella forsythensis* corrig., gen. nov., comb. nov. *Int J Syst Evol Microbiol* 52, 841–849.
- Sára, M. & Sleytr, U. B. (2000). S-layer proteins. *J Bacteriol* 182, 859–868.
- Seshadri, R., Myers, G. S., Tettelin, H., Eisen, J. A., Heidelberg, J. F., Dodson, R. J., Davidsen, T. M., DeBoy, R. T., Fouts, D. E. & other authors (2004). Comparison of the genome of the oral pathogen *Treponema denticola* with other spirochete genomes. *Proc Natl Acad Sci U S A* 101, 5646–5651.
- Sharma, A., Sojar, H. T., Glurich, I., Honma, K., Kuramitsu, H. K. & Genco, R. J. (1998). Cloning, expression, and sequencing of a cell surface antigen containing a leucine-rich repeat motif from *Bacteroides forsythus* ATCC 43037. *Infect Immun* 66, 5703–5710.
- Sharma, A., Inagaki, S., Honma, K., Sfintescu, C., Baker, P. J. & Evans, R. T. (2005). *Tannerella forsythia*-induced alveolar bone loss in mice involves leucine-rich-repeat BspA protein. *J Dent Res* 84, 462–467.
- Siqueira, J. F., Jr & Rôças, I. N. (2003). *Bacteroides forsythus* in primary endodontic infections as detected by nested PCR. *J Endod* 29, 390–393.
- Sleytr, U. B. & Beveridge, T. J. (1999). Bacterial S-layers. *Trends Microbiol* 7, 253–260.
- Sleytr, U. B. & Messner, P. (1988). Crystalline surface layers in prokaryotes. *J Bacteriol* 170, 2891–2897.

- Sleytr, U. B., Messner, P., Pum, D. & Sára, M. (1993). Crystalline bacterial cell surface layers. *Mol Microbiol* **10**, 911–916.
- Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C. & Kent, R. J., Jr (1998). Microbial complexes in subgingival plaque. *J Clin Periodontol* **25**, 134–144.
- Takemoto, T., Kurihara, H. & Dahlén, G. (1997). Characterization of *Bacteroides forsythus* isolates. *J Clin Microbiol* **35**, 1378–1381.
- Tanner, A. C. R., Listgarten, M. A., Ebersole, J. L. & Strzempko, M. N. (1986). *Bacteroides forsythus* sp. nov., a slow-growing, fusiform *Bacteroides* sp. from the human oral cavity. *Int J Syst Bacteriol* **36**, 213–221.
- Ueda, O. & Yoshimura, F. (2003). Transposon-induced norfloxacin-sensitive mutants of *Bacteroides thetaiotaomicron*. *Microbiol Immunol* **47**, 17–25.
- Wang, B., Kraig, E. & Kolodrubetz, D. (2000). Use of defined mutants to assess the role of the *Campylobacter rectus* S-layer in bacterium-epithelial cell interaction. *Infect Immun* **68**, 1465–1473.
- Wyss, C. (1989). Dependence of proliferation of *Bacteroides forsythus* on exogenous *N*-acetylmuramic acid. *Infect Immun* **57**, 1757–1759.
- Yano-Higuchi, K., Takamatsu, N., He, T., Umeda, M. & Ishikawa, I. (2000). Prevalence of *Bacteroides forsythus*, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* in subgingival microflora of Japanese patients with adult and rapidly progressive periodontitis. *J Clin Periodontol* **27**, 597–602.
- Yoneda, M., Hirofuji, T., Anan, H., Matsumoto, A., Hamachi, T., Nakayama, K. & Maeda, K. (2001). Mixed infection of *Porphyromonas gingivalis* and *Bacteroides forsythus* in a murine abscess model: involvement of gingipains in a synergistic effect. *J Periodont Res* **36**, 237–243.
- Yoneda, M., Hirofuji, T., Motooka, N., Nozoe, K., Shigenaga, K., Anan, H., Miura, M., Kabashima, H., Matsumoto, A. & Maeda, K. (2003). Humoral immune responses to S-layer-like proteins of *Bacteroides forsythus*. *Clin Diagn Lab Immunol* **10**, 383–387.

Edited by: P. E. Kolenbrander