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OmpA-like protein influences cell shape and adhesive activity of *Tannerella forsythia*

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SUMMARY

***Tannerella forsythia*, a gram-negative fusiform rod, is implicated in several types of oral anaerobic infections. Most gram-negative bacteria have OmpA-like proteins that are homologous to the OmpA protein in *Escherichia coli*. We identified an OmpA-like protein in *T. forsythia* encoded by the *tf1331* gene as one of the major proteins by mass spectrometric analysis. Two-dimensional, diagonal electrophoresis showed that the OmpA-like protein formed a dimeric or trimeric structure via intermolecular disulfide bonds. A biotin labeling experiment revealed that a portion of the protein was exposed on the cell surface, even though *T. forsythia* possesses an S-layer at the outermost cell surface. Using a *tf1331*-deletion mutant, we showed that the OmpA-like protein affected cell morphology. The length of the mutant cell was reduced almost by half. Cell swelling was observed in more than 40% of the mutant cells. Moreover, the mutant exhibited decreased adhesion to fibronectin, retarded autoaggregation, and reduced cell surface hydrophobicity. These results suggest that the OmpA-like protein in *T. forsythia* plays an important role in cellular integrity and adhesive function.**

INTRODUCTION

Tannerella forsythia was originally isolated from deep periodontal pockets and was named *Bacteroides forsythus* as a new species in the genus *Bacteroides* (Tanner *et al.*, 1986; Tanner & Izard, 2006). After phylogenetic analysis of 16S rRNA sequences, this bacterium was reclassified as *Tannerella forsythensis* (Sakamoto *et al.*, 2002), and then was renamed *T. forsythia* (Maiden *et al.*, 2003). *Tannerella forsythia* is a gram-negative anaerobic fusiform rod showing slow growth and fastidious growth requirements. Recently, *T. forsythia* was recognized as a member of the so-called 'red complex' periodontal pathogens, together with *Porphyromonas gingivalis* and *Treponema denticola* (Socransky *et al.*, 1998; Holt & Ebersole, 2005). As *T. forsythia* is frequently detected not only in severely aggressive periodontal disease (Gersdorf *et al.*, 1993; Haffajee *et al.*, 1998; Yano-Higuchi *et al.*, 2000; Paster *et al.*, 2001) but also in periapical periodontitis (Conrads *et al.*, 1997; Dines *et al.*, 1999; Gonçalves & Mouton, 1999), it is considered to be associated with mixed oral infection under anaerobic conditions. Several studies have reported virulence factors from *T. forsythia* such as trypsin-like protease (Saito *et al.*, 1997), BspA protein (Sharma *et al.*, 1998; Inagaki *et al.*, 2005), sialidase (Ishikura *et al.*, 2003; Thompson *et al.*, 2009), karilysin (Karim

et al., 2010) and S-layer proteins (Higuchi *et al.*, 2000; Yoneda *et al.*, 2003; Sakakibara *et al.*, 2007); however, its detailed pathogenicity remains unclear.

The outer membrane, which is characteristic of gram-negative bacteria, contains major outer membrane protein OmpA or OmpA-like protein (Foulds, 1974; Stocker *et al.*, 1979; Wexler, 2002). OmpA from *Escherichia coli* functions mainly as an anchor of the outer membrane, and contains a binding motif for the peptidoglycan layer in the C-terminal region (De Mot & Vanderleyden, 1994; Nikaido, 2003; Smith *et al.*, 2007). When OmpA is deleted in *E. coli*, the outer membrane structure becomes unstable and the cells tend to be spherical (Sonntag *et al.*, 1978; Nikaido, 1996). Other studies have demonstrated that OmpA-like proteins contribute to attachment to epithelial cells and bacterial survival after phagocytosis by macrophages (Dabo *et al.*, 2003; Lo & Sorensen, 2007; Serino *et al.*, 2007). We have reported the presence of OmpA-like protein in periodontal pathogens. Pgm6/7 proteins in *P. gingivalis*, major outer membrane proteins of approximately 40 kDa, are homologous to *E. coli* OmpA (Murakami *et al.*, 2002, 2004). The Pgm6/7 proteins form heterotrimers and are important in maintaining the outer membrane structure (Nagano *et al.*, 2005; Iwami *et al.*, 2007). In the course of our studies, we have found that anti-Pgm6/7 protein serum detects a major 40-kDa protein in the envelope fraction of *T. forsythia* (Imai *et al.*, 2005). This finding strongly indicates that OmpA-like protein exists in *T. forsythia*. More recently, Veith *et al.* (2009) reported OmpA-like Tf1331 protein through proteomic analysis.

In this study, we reported that the 40-kDa OmpA-like protein in *T. forsythia* was encoded by the *tf1331* gene, and then investigated the effects of the OmpA-like protein on cell morphology and function after construction of a *tf1331*-deletion mutant. We also analyzed oligomeric structure of OmpA-like protein Tf1331 and the relationship between this protein and the S-layer present on the outermost cell surface of *T. forsythia*.

METHODS

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are shown in Table 1. *Tannerella forsythia* ATCC 43037 was originally obtained from A. Tanner (The Forsyth Institute, Boston, MA). All *T. forsythia* strains were grown on Brucella HK agar (Kyokuto Pharmaceutial Industrial, Tokyo, Japan) supplemented with 5% laked rabbit blood, 2.5 μg ml^{-1} hemin, 5 μg ml^{-1} menadione, 0.1 mg ml^{-1} dithiothreitol, 10 μg ml^{-1} *N*-acetylmuramic acid (Sakakibara *et al.*, 2007) (blood agar) and appropriate antibiotics when necessary at 37°C for 7 days under anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2). A TOPO TA cloning kit, which included the plasmid vector pCR4-TOPO, was purchased from Invitrogen (Carlsbad, CA). *Escherichia coli* DH5α carrying pCR4-TOPO and pKD260 was grown in Luria–Bertani medium supplemented with 50 μg ml^{-1} ampicillin and 25 μg ml^{-1} chloramphenicol, respectively.

Table 1 Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics	Source or reference
Tannerella forsythia		
ATCC 43037	Wild-type, type strain	ATCC
Δ*tf1331*	*tf1331*-deletion mutant from ATCC 43037, Cp^r	This study
Escherichia coli		
DH5α	As chemically competent cells	Invitrogen
Plasmids		
pCR4-TOPO	Cloning vector, Ap^r	Invitrogen
pKD260	A derivative of pACYC184 with deletion of a 1.1-kbp *Hin*cII fragment, bearing *cat* gene, Cp^r	Nagano *et al.* (2005)

Cp^r, chloramphenicol resistance; Ap^r, ampicillin resistance; ATCC, American Type Culture Collection.

DNA manipulations

Standard techniques were used for purification and manipulation of DNA. The oligonucleotides used for polymerase chain reaction (PCR) and reverse transcription (RT) -PCR were synthesized by Sigma Genosys (Ishikari, Japan). Standard PCR experiments were performed using high-fidelity Pyrobest DNA polymerase (Takara Bio, Otsu, Japan) with a PCR Thermal Cycler Dice (Takara Bio). DNA sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Constructs for generating mutants were sequenced to rule out unintended base changes.

Total RNA extraction and RT-PCR

Total RNA from bacteria was isolated using a RiboPure kit (Ambion, Austin, TX). SuperScript III RT (Invitrogen) was used to generate cDNA from the total RNA with dNTPs and random decamer primers according to the manufacturer's protocol. The resulting cDNA was used as a template for the following standard PCR.

Construction of deletion mutant

We applied the PCR-based overlap extension method for construction of a deletion mutant in *T. forsythia* as described previously (Sakakibara *et al.*, 2007). DNA fragments that allowed the replacement of the *tf1331* gene with the *cat* gene, encoding chloramphenicol acetyltransferase (CAT), in the *T. forsythia* chromosome were constructed. The primers and their overlapping regions of *cat* are shown in Table 2. Briefly, *cat* was amplified from the ATG start codon to the TAA stop codon with primers AGU01 and AGU02 to generate a 660-base-pair product from pKD260. For construction of the *tf1331* deletion cassette, the flanking sequence upstream of *tf1331* was amplified with MB051 and MB060, which have homology to the 5′ end of the *cat* fragment. The flanking sequence downstream of *tf1331* was amplified with MB070 and MB081, which have homology to the 3′ end of the *cat* fragment. The *cat*, *tf1331*-upstream and *tf1331*-downstream fragments were used as templates for overlap extension PCR to generate a deletion cassette in which *tf1331* was replaced by *cat*.

Table 2 Primers

Primer	Sequence (5′–3′)[1]
AGU01[2]	ATGGAGAAAAAAATCACTGGA
AGU02[2]	TTACGCCCCGCCCTGCCACTC
MB051	TGAGCATTGAAGAAAAACCAGA
MB052	GAATATGCCGAACAGCCTTC
MB060	CCAGTGATTTTTTTCTCCATAATCTATTCATATTTTATTA
MB070	GCAGGGCGGGGCGTAATCAATCGTAAGACCATA
MB081	GACGCCTGAAAGAAACGGTA
MB082	ACTGATCAACCGGCGTAAAC
31F1	GGAGACTTGAACGGCAATGC
31R2	ATACGGCTGAACTGTATCAC
32R1	TTTCGTCTCACCACACATCC
32F2	CATGATCCCGGTCCATACAT
32R2	TGCAAAACTCTGCGAAACAA
33R	CGGTTTGGACATGGTACAGT

[1]Underlining shows overlapping regions of 5′ or 3′ end of *cat*.
[2]The same primers were used by Nagano *et al.* (2005).

The deletion cassette created was ligated into pCR4-TOPO according to the manufacturer's directions and the resulting recombinant plasmids were cloned in *E. coli* DH5α. The plasmid was introduced into competent cells of *T. forsythia* by electroporation. After 24 h of anaerobic incubation in trypticase soy broth (Becton Dickinson, Franklin Lakes, NJ) supplemented with 2.5 mg ml^{-1} yeast extract, 2.5 mg μl^{-1} hemin, 5 mg μl^{-1} menadione, 0.1 mg ml^{-1} dithiothreitol, 10 μg ml^{-1} *N*-acetylmuramic acid, and 5% Fildes extract (Oxoid, Basingstoke, UK), the cells were plated on blood agar supplemented with 20 μg ml^{-1} chloramphenicol and the plates were incubated anaerobically at 37°C for 7 days. Recombination was confirmed by PCR analysis, and the absence of polar effects was further verified by RT-PCR analysis.

Subcellular fractionation

Preparation of whole cell, envelope and soluble fractions from *T. forsythia* strains was performed essentially as described previously (Murakami *et al.*, 2002, 2004). The periplasmic fraction was obtained essentially as described by Fulda *et al.* (1999). Whole cell, envelope and soluble fractions were suspended in 10 mM HEPES (pH 7.4) and stored at −20°C. The periplasmic fraction was suspended in distilled water and stored at −20°C.

SDS–PAGE and Western blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in a 1.0 mm thick 12% gel as described by Lugtenberg *et al.* (1975). The samples were usually solubilized in SDS buffer with or without 2-mercaptoethanol (2-ME) at 100°C for 5 min (Murakami *et al.*, 2002), unless otherwise specified. The gels were stained with Coomassie brilliant blue R-250 (CBB). Western blotting was performed as described previously (Nagano *et al.*, 2005). Proteins in the gel were electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline. Then the membrane was reacted with a specific antiserum against OmpA-like Pgm6/7

proteins (Imai *et al.*, 2005) followed by incubation with peroxidase-conjugated anti-rabbit IgG (ICN Pharmaceuticals, Costa Mesa, CA). After the membrane was washed, signals of Tf1331 were detected with 4-chloro-1-naphthol supplemented with hydrogen peroxide.

Two-dimensional, diagonal SDS–PAGE

Two-dimensional diagonal SDS–PAGE was performed essentially as described previously (Nagano *et al.*, 2005). Cell envelopes were applied to a 12% slab gel under non-reducing conditions as the first dimension. After electrophoresis the gel strip was cut out, placed at the top of the second gel, and overlaid with the SDS buffer containing 2-ME to provide reducing conditions. The gels were stained with CBB. Western blotting was performed as described above.

Protein analysis by mass spectrometry

The CBB-stained protein bands were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Masuda *et al.*, 2006; Sato *et al.*, 2009). After in-gel tryptic digestion, peptides were extracted, concentrated and analyzed using a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems). The identities of the proteins were deduced from MS peaks via the MS/MS methods in MASCOT (http://www.matrixscience.com/). The annotated *T. forsythia* protein sequence database was obtained from the Los Alamos National Laboratory website (http://oralgen.lanl.gov/).

Homology analysis

Homology of *T. forsythia* OmpA with *E. coli* OmpA or *P. gingivalis* Pgm6/7 was analyzed by PSI-BLAST (Altschul *et al.*, 1997). Identity and similarity were evaluated after multiple alignments using the CLUSTAL W program (Thompson *et al.*, 1994).

Biotinylation of surface proteins

Cell surface labeling with biotin was carried out according to the procedure of Dooley & Trust (1988). The *T. forsythia* ATCC 43037 cells suspended in Dulbecco's PBS (pH 7.4) were labeled with EZ-Link NHS-PEG_{12}-biotin (Thermo Scientific, Rockford, IL) at 4°C for 30 min. The reaction was stopped by the addition of glycine. The envelope fraction from biotinylated cells was subjected to SDS–PAGE and blotted onto a nitrocellulose membrane. Biotinylation was detected using peroxidase-conjugated streptavidin.

Measurement of bacterial length

Bacterial images after Gram staining were captured with an Olympus BX51 light microscope using a 60 × objective. The length of the bacterium was measured using IMAGE J software (National Institutes of Health, Bethesda, MD).

Electron microscopy

Bacterial cells, grown for 7 days, were negatively stained on carbon-coated grids with 1% ammonium molybdate (pH 7.0) to examine the bacterial shape. To examine the ultrastructure of the cell surface, thin sections were prepared. In brief, washed cells were fixed with 4% paraformaldehyde and 5% glutaraldehyde in 0.1 M cacodylate buffer at 4°C for 2 h. Samples were post-fixed with 2% osmium tetroxide for 60 min and then with 0.5% uranyl acetate for 20 min. The fixed cells were dehydrated in ethanol and embedded in Quetol-653 resin (Nisshin EM, Tokyo, Japan). Ultrathin sections were stained with 10% uranyl acetate and 1% lead citrate. Stained sections were observed and photographed using a JEM-1210 electron microscope (Jeol, Tokyo, Japan).

Attachment to extracellular matrices

The ability to attach to extracellular matrices was evaluated essentially as described by Narimatsu *et al.* (2004). Ninety-six-well plates coated with fibronectin, laminin, type I collagen and type IV collagen were used (Becton Dickinson). After adjustment of the value for optical density at 660 nm (OD_{660}) to 1.0 in supplemented trypticase soy broth, the cell suspension (100 μl) was plated into wells and incubated at 37°C for 18 h under anaerobic conditions. After non-adherent cells were removed by washing three times with Dulbecco's PBS (pH 7.4), adherent cells were stained with 0.1% crystal violet. The plates were washed with distilled water and dried. To quantify attachment, the OD_{595} was measured after addition of 95% ethanol to the wells. Experiments were

performed using 10 wells for each sample and were repeated three times to verify the results.

Autoaggregation assay

Autoaggregation assay was performed essentially as described previously (Nishiyama *et al.*, 2007). Cells harvested from blood agars were washed three times with 10 mM phosphate buffer (pH 7.0), and then suspended in 10 mM phosphate buffer with various pH values. The OD_{660} value of the cell suspension was adjusted to 1.0. Aliquots (5 ml) in 15-ml culture tubes were allowed to settle for 130 min at room temperature. Autoaggregation was visually evaluated.

Hydrophobicity assay

Hydrophobicity assay was performed according to the procedure of Rosenberg *et al.* (1980). Washed cells were suspended in Dulbecco's PBS (pH 7.4) to the OD_{660} value of 0.5. Aliquots (4 ml) in tubes were vigorously mixed with *n*-hexadecane (0.4 ml) for 60 s and allowed to separate for 10 min. The OD_{660} of the aqueous phase was measured as an index of hydrophobicity. Relative hydrophobicity of the cell surface was calculated by following formula: % hydrophobicity = $[1-(OD_{660}$ after mixing$)/(OD_{660}$ before mixing$)] \times 100$. Experiments were performed using triplicate samples and were repeated twice to verify the results.

Biofilm assay

The basis for this assay has been described previously (Nagano *et al.*, 2010). The *T. forsythia* cells with the OD_{660} adjusted to 1.0 with pre-warmed anaerobic broth were applied onto a polystyrene plastic plate. After an 18-h anaerobic incubation, the plate was washed with Dulbecco's PBS (pH 7.4). Adherent cells were stained with 1% crystal violet, and washed with Dulbecco's PBS. Biofilm-forming activity was evaluated by measurement of the OD_{595} after elution of the crystal violet by ethanol.

Antimicrobial susceptibility

The antibiotics used were chloramphenicol, ampicillin, ofloxacin, tetracycline, erythromycin, bacitracin and vancomycin (Sigma-Aldrich, St. Louis, MO). Assays were performed essentially as described previously (Nagano *et al.*, 2005). The minimum inhibitory concentration (MIC) was evaluated by agar dilution assay, as recommended by the National Committee for Clinical Laboratory Standards (1997). Briefly, serial dilutions of the antibiotics were added to blood agars. After pre-cultured bacteria were grown on blood agar, the washed bacterial suspension (2 μl) was spotted on the antibiotic-containing agar. After 7 days of anaerobic incubation, the susceptibility breakpoints were determined.

Statistical analysis

The Student's *t*-test was used for data analysis. Statistical differences were considered significant at the level of $P < 0.01$.

RESULTS

Identification of major 40-kDa protein as Tf1331 protein

The major 40-kDa protein in the envelope fraction (Fig. 1A) was subjected to MALDI-TOF MS analysis, and was identified as Tf1331 protein encoded by a 1167-base-pair open reading frame. Then the homology of Tf1331 was analyzed by PSI-BLAST, and the identity and similarity of amino acid residues were estimated after multiple alignments by CLUSTAL W (Fig. 2). Tf1331 had homology with OmpA and OmpA-like proteins such as *P. gingivalis* Pgm6 protein (E-value 9×10^{-73}, identity 41.8%, similarity 76.0%), *P. gingivalis* Pgm7 protein (E-value 4×10^{-83}, identity 44.0%, similarity 80.9%) and *E. coli* K12 OmpA (E-value 5×10^{-5}, identity 21.6%, similarity 63.9%). Hence Tf1331 was most homologous to *P. gingivalis* Pgm7 protein, especially in the C-terminal region.

Construction of a deletion mutant

Construction of a deletion mutant from *T. forsythia* wild-type strain ATCC 43037 was performed by the PCR-based overlap extension method, in which the *tf1331* gene was deleted and replaced by *cat*. Replacement of *tf1331* by *cat* was confirmed after the cloning site was amplified by PCR with the use of genomic DNA from the mutant as a template with the

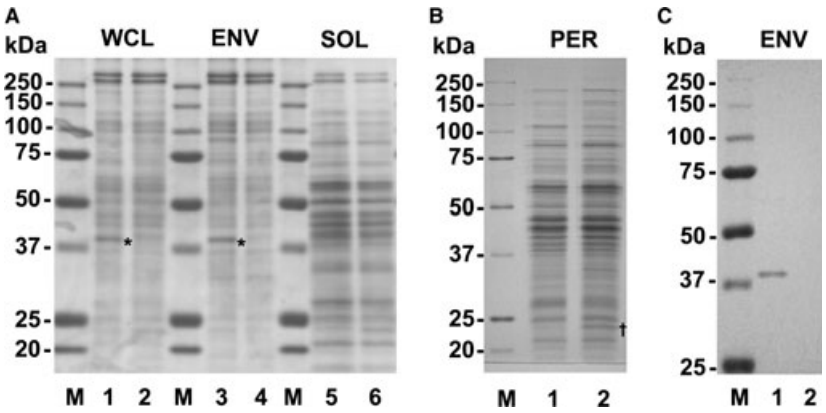

Figure 1 Subcellular localization of Tf1331 protein. (A, B) Bacterial fractions were denatured in sodium dodecyl sulfate (SDS) with 2-mercaptoethanol at 100°C for 5 min and then loaded onto SDS–polyacrylamide gels. The gels were stained with Coomassie brilliant blue after electrophoresis. (A) Lanes: M, molecular mass marker; 1, wild-type whole cell lysate (WCL); 2, Δ*tf1331* WCL; 3, wild-type envelope fraction (ENV); 4, Δ*tf1331* ENV; 5, wild-type soluble fraction (SOL); 6, Δ*tf1331* SOL. *, Tf1331 protein. (B) Lanes: M, molecular mass marker; 1, wild-type periplasmic fraction (PER); 2, Δ*tf1331* PER. †, Chloramphenicol acetyltransferase. (C) Envelope fractions after SDS–polyacrylamide gel electrophoresis were subjected to Western blotting with anti-OmpA serum. Lanes: M, molecular mass marker; 1, wild-type; 2, Δ*tf1331*. *, Tf1331 identified by the anti-OmpA serum.

primer pair MB052 and MB082 designed outside the homologous recombination region. The accuracy of the homologous recombination region was also verified by DNA sequencing. We confirmed that *tf1331* was transcribed in the wild-type, but not in the mutant using RT-PCR, whereas comparable transcription of *tf1332*, an adjacent downstream gene of *tf1331*, was verified in the wild-type and mutant using primer pairs 31F2 and 31R2, and 32F2 and 32R2, respectively. Therefore, no polar effect on the downstream gene was found after deletion of *tf1331*. We also determined that *tf1331* and *tf1332* were not co-transcribed, whereas *tf1332* and *tf1333* were found to be a part of an operon using primer pairs 31F1 and 32R1, and 32F2 and 33R, respectively (data not shown).

SDS–PAGE and Western blot analysis of the mutant

Bacterial fractions from the wild-type and mutant were subjected to SDS–PAGE. The parent strain showed an OmpA-like 40-kDa Tf1331 band, whereas the mutant lost the band in the whole cell and envelope fractions (Fig. 1A). Instead, a 23-kDa band, corresponding to the size of CAT, was found in the periplasmic fraction of the mutant (Fig. 1B). This was verified to be CAT protein by MALDI-TOF MS. No other obvious changes in the electrophoretic pattern were observed. A specific antiserum against the OmpA-like protein, prepared previously as described (Imai *et al.*, 2005) was used to identify Tf1331 by Western blot analysis. The antiserum clearly detected Tf1331 in the parent strain; however, no immunoreactive bands appeared in the mutant strain (Fig. 1C).

Effect of temperature on SDS-PAGE profile

The envelope fraction from the wild-type was solubilized with or without 2-ME at various temperatures and then loaded onto SDS–polyacrylamide gels (Fig. 3A). Under reducing conditions, the apparent molecular mass of Tf1331 was 40 kDa when the protein was heated at 100°C, but 30 kDa when heated below 70°C. In contrast, under non-reducing conditions, the molecular mass was 80 and 120 kDa at 100°C, but 75 and 100 kDa below 70°C. The identity of Tf1331 was confirmed by Western blotting using the anti-OmpA-like protein serum (data not shown). These results indicate that Tf1331 is heat-modifiable.

Oligomeric structure of Tf1331

When the wild-type envelope fraction was denatured in SDS with or without 2-ME at 100°C, Tf1331 was found as a 40-kDa band with 2-ME, but as 120- and 80-kDa protein bands without 2-ME (Fig. 3A). We

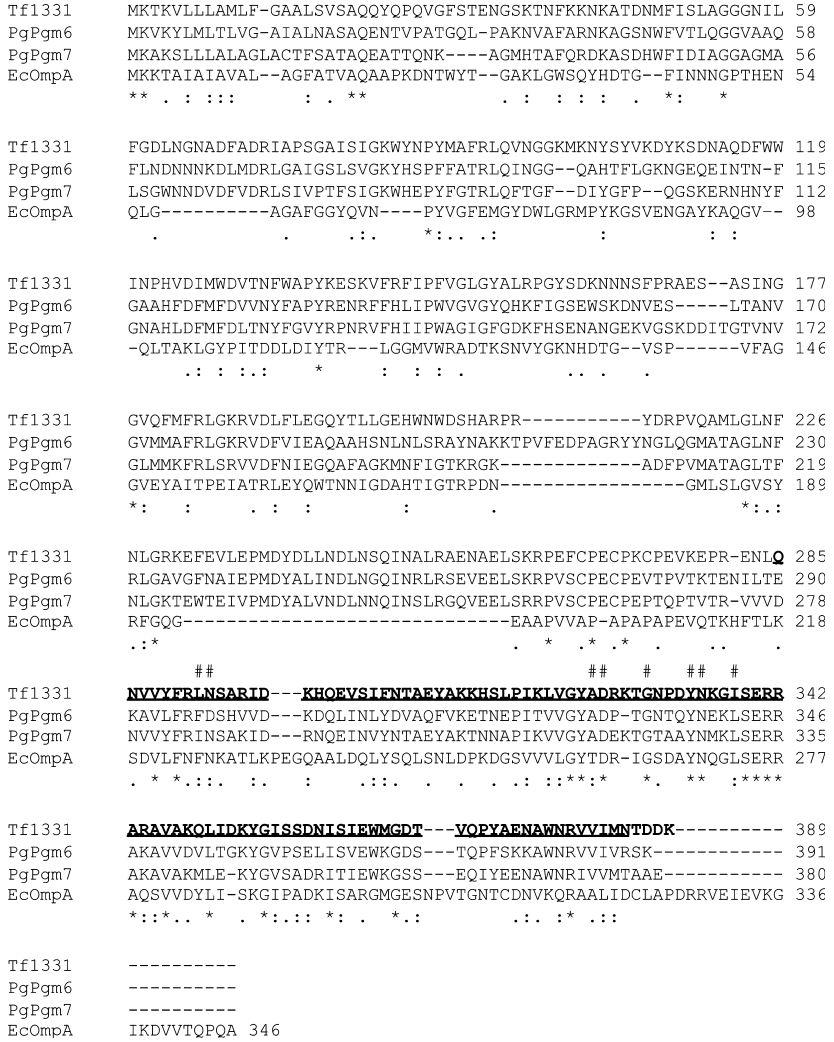

Figure 2 Alignment of OmpA in *Escherichia coli* and OmpA-like proteins in *Porphyromonas gingivalis* and *Tannerella forsythia*. The multiple alignment was made with CLUSTAL W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). Identical amino acids, conservative substitutions and semi-conservative substitutions are indicated by asterisks, colons and dots, respectively. Conserved domain search was carried out with CD Search tool (http://www.ncbi.nih.gov/Structure/cdd/wrpsb.cgi). The characteristic OmpA_C-like domain (cd07185) is shown in underlined bold characters. Conserved ligand binding site is depicted by hash marks above the aligned sequences.

assumed that the 120- and 80-kDa protein bands were trimers and dimers, respectively, based on their sizes. To verify this assumption, two-dimensional diagonal SDS–PAGE and Western blotting using the wild-type envelope fraction were performed. If a protein complex contains disulfide bonds, its components will appear as spots below the diagonal that reflect their individual molecular weights under reducing conditions. Both a CBB-stained gel and a Western blot with the anti-OmpA-like protein serum showed a major 40-kDa spot and several minor spots at the off-diagonal position (Fig. 3B,C). The main 40-kDa spot presumably derived from the trimer band (120 kDa) and dimer band (80 kDa) at their vertical positions. The spots below the 37-kDa marker were identified as Tf1331 by MALDI-TOF MS analysis, suggesting its degradation products.

Surface localization of Tf1331

To confirm localization of proteins, surface-exposed lysine and N-terminal amino groups were labeled with biotin-conjugated NHS reagent. The result revealed a few surface-exposed proteins (270-, 230-, 65-, 60- and

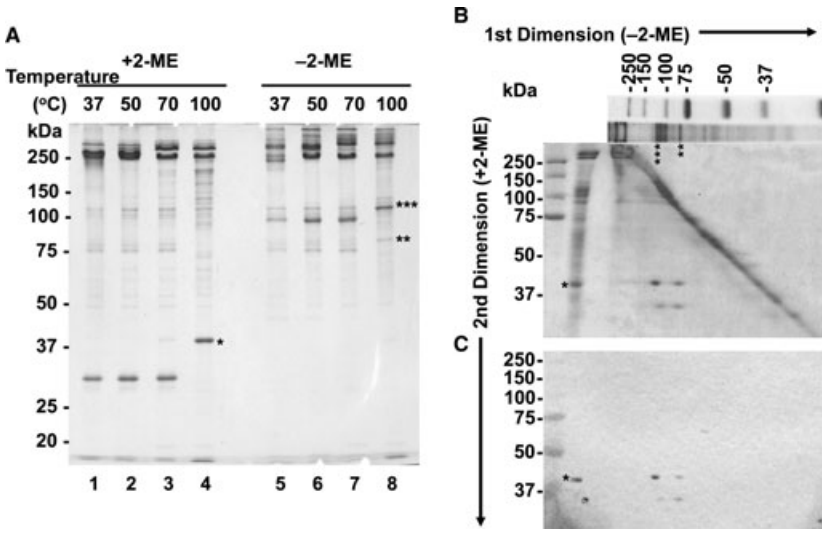

Figure 3 Oligomeric structure of Tf1331. (A) Effects of a reducing agent and temperature on the solubilization of Tf1331. Envelope fraction from the wild-type was solubilized with sodium dodecyl sulfate (SDS) in the presence and absence of 2-mercaptoethanol (2-ME) at various temperatures. The proteins were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and the gel was stained with Coomassie brilliant blue (CBB). Lanes: 1, at 37°C for 20 min with 2-ME; 2, at 50°C for 20 min with 2-ME; 3, at 70°C for 20 min with 2-ME; 4, at 100°C for 5 min with 2-ME; 5, at 37°C for 20 min without 2-ME; 6, at 50°C for 20 min without 2-ME; 7, at 70°C for 20 min without 2-ME; 8, at 100°C for 5 min without 2-ME. The asterisk, double asterisks and triple asterisks show the predicted monomer, dimer and trimer, respectively, that consist of Tf1331. (B,C) Two-dimensional, diagonal SDS–PAGE and Western blotting of *T. forsythia* wild-type envelope fraction. The envelope was denatured in SDS at 100°C for 5 min under non-reducing conditions before the first-dimension electrophoresis. A gel strip was cut from the first gel and placed on a well of the second gel. Then the strip in the well was overlaid with a buffer containing 2-ME. After the second electrophoresis, the gel was stained with CBB (B) or subjected to Western blotting with the anti-OmpA serum (C). The top and left parts of the figure show protein patterns in one dimension of the sample treated without and with 2-ME, respectively, to more easily elucidate the result. The symbols are the same as those indicated in (A).

40-kDa proteins; Fig. 4). No other bands except for the above-mentioned proteins were detected when cell lysate prepared from biotinylated whole cells was used (data not shown). The 270- and 230-kDa proteins were identified as S-layer proteins located outside the outer membrane (Sakakibara *et al.*, 2007). The 40-kDa protein was assigned to be Tf1331 after comparison of SDS–PAGE and Western blots with anti OmpA-like protein serum. Therefore part of the 40-kDa Tf1331 protein was estimated to be surface-exposed despite the fact that S-layer proteins cover the bacterial surface.

Microscopic observation

The lengths of the cells of the wild-type and Δ*tf1331* strains were compared under a light microscope after Gram staining. The mean cell length of the wild-type strain was 4.20 μm (SD 1.36 μm, $n = 266$), whereas that of strain Δ*tf1331* was 2.29 μm (SD 0.59 μm, $n = 252$). The length of the Δ*tf1331* strain was about half that of the wild-type strain with a significant difference ($P < 0.01$; Fig. 5A). The negatively stained Δ*tf1331* strain had an uneven surface as well as shorter cell length (Fig. 5B). Thin-section electron microscopy revealed that the wild-type strain had a regularly arrayed structure at the inner and outer membranes. The Δ*tf1331* strain had a regular structure at the inner membrane; however, its outer membrane was swollen, with a ridge-like protrusion (Fig. 5C). Overall, typical cellular protrusion extended the entire length of the cell along the long axis located on part of the outer membrane. None of the cells ($n = 105$) had swelling in the wild-type strain, in contrast to the 44.2% of the cells ($n = 104$) that were swollen in the Δ*tf1331* strain.

Antimicrobial susceptibility

The MIC of Cp for the wild-type strain was 1.56 μg ml^{-1}, whereas that for the Δ*tf1331* strain was >25 μg ml^{-1}, presumably because of CAT activity.

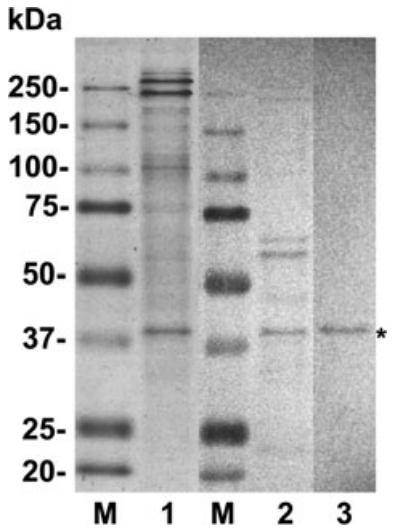

Figure 4 Identification of surface-exposed proteins in *Tannerella forsythia* wild-type cells by using NHS-PEG_{12}-Biotin. Cells were suspended in phosphate-buffered saline (PBS) to an OD_{660} of 3.0. After NHS-PEG_{12}-Biotin was added, the sample was gently shaken at 4°C. Reaction was stopped by washing three times with PBS containing glycine. The envelope fraction was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. Biotinylation was detected using peroxidase-conjugated streptavidin. Lanes: M, molecular mass marker; 1, biotinylated envelope fraction stained with Coomassie brilliant blue; 2, biotinylated proteins detected by avidin-horseradish peroxidase; 3, immunoblot with the anti-OmpA serum. The asterisk shows Tf1331 identified by anti-OmpA.

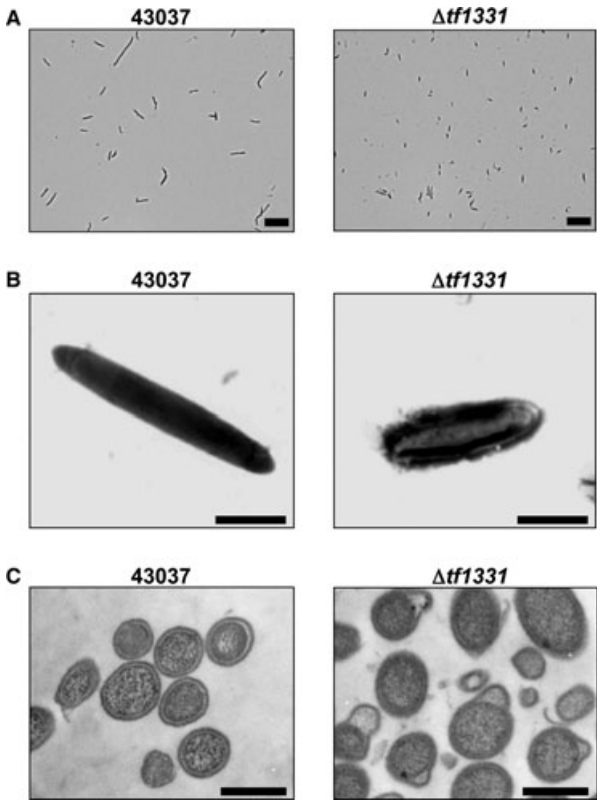

Figure 5 Comparison of cell shape in the wild-type and Δ*tf1331*. (A) Micrographs of gram-stained bacteria. Bars, 10 μm. (B) Electron micrographs of negatively-stained cells. Bars, 1 μm. (C) Electron micrographs of ultrathin-sectioned cells. Bars, 500 nm.

The MICs of ampicillin, ofloxacin and tetracycline were not changed between the wild-type and Δ*tf1331* strains. However the Δ*tf1331* strain showed 8-fold to 16-fold lower MICs for antibiotics with relatively high molecular sizes such as erythromycin, bacitracin and vancomycin (Table 3).

Table 3 Minimum inhibitory concentrations (MIC) of various antibiotics for *Tannerella forsythia* wild-type and Δ*tf1331*

	MIC (μg ml^{-1})						
Strain	CP	ABPC	OFLX	TC	EM	BC	VCM
43037	1.56	0.39	0.78	0.39	0.39	>25	6.25
Δ*tf1331*	>25	0.39	0.78	0.19	0.05	3.13	0.78

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

Attachment to extracellular matrices

We examined bacterial adherence to representative extracellular matrices. Attachment value of the Δ*tf1331* strain to fibronectin was reduced by approximately 50%, but that to laminin, type I collagen and type IV collagen was not changed (Fig. 6A).

Hydrophobicity, autoaggregation and biofilm formation

The hydrophobicity of the Δ*tf1331* strain was significantly reduced (Fig. 6B). We then examined whether the reduced hydrophobicity affected the autoaggregation ability. Autoaggregation in the wild-type strain started from 20 min and finished at approximately 130 min. However, autoaggregation in the Δ*tf1331* strain was not observed until 130 min at pH values of 7.0 and 8.0 (Fig. 6C). After 12 h, both the wild-type and Δ*tf1331* strains showed similar autoaggregation (data not shown). Additionally, the Δ*tf1331* strain showed a reduced biofilm-forming activity (Fig. 6D).

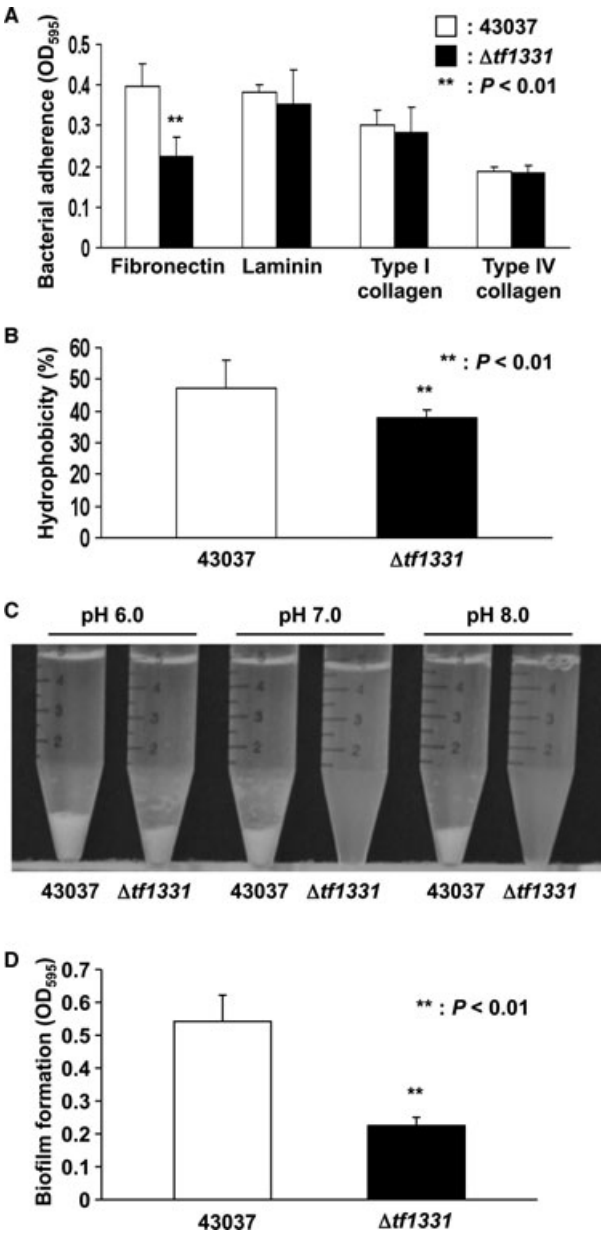

Figure 6 Bacterial adherence, hydrophobicity, and autoaggregation. (A) Quantitative adhesion assay of *Tannerella forsythia* to extracellular matrices. Ninety-six well plates with immobilized fibronectin, laminin, type I collagen and type IV collagen were used. After incubation with *T. forsythia* suspensions, non-adherent cells were removed by washing, followed by crystal violet staining. The means of the optical density at 595 nm (OD_{595}) and SD are shown ($n = 10$). $^{**}P < 0.01$. (B) Hydrophobicity assay. Cells were suspended to OD_{660} in phosphate-buffered saline. An aliquot of cell suspension (4 ml) was mixed with 0.4 ml *n*-hexadecane by vortexing for 60 s and allowed to separate for 10 min. The OD_{660} of the aqueous phase was then measured spectrophotometrically. Bacterial hydrophobicity was determined as $[1-(OD_{660}$ after mixing)$/(OD_{660}$ before mixing)$] \times 100$ (%). Data are means ± SD ($n = 3$). $^{**}P < 0.01$. (C) Autoaggregation activities of the wild-type and Δ*tf1331*. Cells were adjusted to an OD_{660} of 1.0 in phosphate buffer with various pH values. Aliquots (5 ml) in the test tubes were allowed to settle for 130 min. (D) Biofilm formation assay. Adherent cells were quantified by the OD_{595} of eluted crystal violet. The means of the OD_{595} and SD are shown ($n = 4$). $^{**}P < 0.01$.

DISCUSSION

In this study, we identified an OmpA-like protein, Tf1331, present as one of the major membrane proteins from *T. forsythia* ATCC 43037. We also characterized the function of this protein, using a Tf1331-deficient mutant, from morphological and physiological aspects.

Tf1331 is an envelope protein in *T. forsythia*, which shows cross-reaction with the specific antibody against OmpA-like Pgm6/7 proteins in *P. gingivalis* (Imai *et al.*, 2005). A homology search revealed that Tf1331 was more homologous to Pgm6/7 in *P. gingivalis* than OmpA in *E. coli*. In SDS–PAGE, Tf1331 showed an apparent molecular size of 40 kDa under reducing conditions, whereas it appeared as 80- and 120-kDa bands under non-reducing conditions. This phenomenon was confirmed by two-dimensional, diagonal SDS–PAGE. Therefore, we concluded that Tf1331 might form homogeneous dimers or trimers in part. As Tf1331 is predicted to contain three cysteine residues by the genomic sequence database of *T. forsythia* ATCC 43037, disulfide bonds among molecules may contribute to form an oligomeric structure. This finding is not in line with that reported by Veith *et al.* (2009). They claimed that Tf1331 showed only homodimers in mass spectrometric analysis. Similarly, Pgm6/7 in *P. gingivalis* were also shown to form oligomers by disulfide bonds, although they were heterotrimers in this case (Nagano *et al.*, 2005). In contrast, the majority of OmpA in *E. coli* exists as a monomer and only a minority forms a trimeric structure (Sugawara & Nikaido, 1994). Tf1331 was a heat-modifiable protein and its mobility in SDS–PAGE drastically changed with or without a reducing reagent as well (Fig. 3), so every possible precaution against electrophoretic conditions is needed for precise comparison. The heat-modifiability is characteristic of the particular β-barrel conformation of OmpA-like proteins (Garten *et al.*, 1975).

Tannerella forsythia possesses a characteristic S-layer that is located outside the outer membrane of the cell body (Tanner *et al.*, 1986; Sakakibara *et al.*, 2007). We examined whether Tf1331 was exposed to the cell surface, as this is important to determine whether Tf1331 directly interacts with the host cells. We labeled cell surface proteins of *T. forsythia* using a biotin reagent with large molecular weight, which therefore had difficulty permeating through the outer membrane (Dooley & Trust, 1988). As a result, Tf1331 was strongly labeled with the biotin reagent. This indicated that a certain part of Tf1331 was exposed to the cell surface. In general, the N-terminal region of OmpA protein has membrane spanning segments, whereas the C-terminal region is located in the periplasmic space and binds to the peptidoglycan layer (De Mot & Vanderleyden, 1994; Nikaido, 2003). We did not determine the precise membrane spanning region of Tf1331; however, outer loops of the N-terminal region may protrude and be exposed to the outside.

Comparison of gene deletion mutant and parent strains is a highly effective method to clarify the function of proteins in bacteria. Sakakibara *et al.* (2007) successfully constructed a series of S-layer mutants in *T. forsythia* by replacing target genes with *cat*. In this method, the *cat* is expressed using promoter and ribosome binding sites of the gene intended to be deleted. In this study, we again obtained a Tf1331-deficient mutant by replacement of *cat*, reconfirming a feasible approach for mutation in this organism. Replacement of the objective gene with *cat* is excellent to delete genes that code for major proteins such as S-layer proteins and Tf1331 in *T. forsythia*. At present, molecular and genetic manipulation techniques are still limited for *T. forsythia* and development of a gene complementation system in *T. forsythia* is necessary in the future.

Using the constructed mutant, we examined the effect of Tf1331 on cell morphology. Using Gram staining, we found that Tf1331 was involved in the maintenance of cell length. When negatively stained cells were examined by electron microscopy, shorter cell length and an uneven cell surface were observed in the mutant. Moreover, cellular swelling at the outer membrane and S-layer as well as enlarged periplasmic space were detected by examination of ultrathin sections. As C-terminal amino acid sequences in the OmpA protein were reported to form a binding motif for peptidoglycan (De Mot & Vanderleyden, 1994; Nikaido, 2003), the situation may be similar for Tf1331. Therefore, the Tf1331-defective mutant was shortened with an irregular cell surface because of weak binding between peptidoglycan and the outer membrane. We previously reported that spherical cells with a wavy outer membrane were found in OmpA-like, Pgm6/7 protein deletion mutants in *P. gingivalis* (Iwami *et al.*, 2007). The cells of the OmpA-like protein mutant of *Bacteroides fragilis* were also shorter and rounder (Wexler *et al.*, 2009). In contrast, when S-layer proteins were deleted from *T. forsythia*, no morphological change was observed (Sakakibara *et al.*, 2007). Therefore, we consider that the complex structure of OmpA-like protein and peptidoglycan is important for maintaining the cell shape.

The Tf1331 protein-deficient mutant had a growth rate and colony shape similar to the parent strain (data not shown). There were no differences in cellular enzymatic activities between the mutant and the parent, including trypsin-like protease activity, one of the *T. forsythia* virulence factors (Saito *et al.*, 1997). No remarkable differences were observed in oxygen resistance. As mentioned above, deletion of Tf1331 does not affect the essential character for survival.

As *tf1331* was replaced by *cat* in construction of the mutant, the MIC of the mutant for chloramphenicol was higher than that of the parent. However, for vancomycin and bacitracin, which have relatively large molecular weights, the MIC of the mutant became lower. OmpA is a low-efficiency porin that allows non-specific transmembrane diffusion of solutes (Nikaido, 2003). It is thought to be difficult for both vancomycin and bacitracin to permeate into their sites of action because of their sizes. Even if we assume that Tf1331 may act as a slow porin, the effect of Tf1331 deletion on MIC may be negligible for those antibiotics. We speculated that the outer membrane might become somewhat leaky because deletion of Tf1331 caused changes in outer membrane morphology as shown in Fig. 5C. In our previous study, *T. forsythia* became sensitive to vancomycin when S-layer proteins were deleted (Sakakibara *et al.*, 2007). In this case, the antibiotic may easily penetrate into bacterial cells because S-layer proteins have a molecular-sieving function.

To investigate the interaction of the bacterium through Tf1331 with the host, the bacterial adherence to extracellular matrix proteins was examined. Tf1331

was involved in attachment to fibronectin. It has been reported that OmpA-like proteins from *Pasteurella multocida* and *Mannheimia haemolytica* bind to fibronectin (Dabo *et al*., 2003; Lo & Sorensen, 2007). *Campylobacter jejuni* OmpA-like CadF has fibronectine-binding domain FRLS at residues 134–137 (Konkel *et al*., 2005). Similar residues (FRLG) are found in TF1331 at residues 183–186 (Fig. 2) comparing the sequence to CadF. As described above, we speculated that a portion of the N-terminal region of Tf1331 including residues 183–186 might be exposed to the bacterial cell surface. Consequently, this portion may be involved in binding to fibronectin. In *T. forsythia*, the BspA protein has been reported to bind to fibronectin (Sharma *et al*., 1998). Tf1331, in concert with BspA, may contribute to establish initial colonization of *T. forsythia* by attaching to host tissues via fibronectin.

Autoaggregation was examined as an index for biofilm-forming activity. Formation of aggregates was retarded in the Tf1331 protein-deletion mutant near the range of physiological pH of body fluids. The amount of aggregates was also less in the mutant under light microscopic observation (data not shown). When cell surface hydrophobicity was compared, a significant reduction in the mutant was found. These results suggest that the region of Tf1331 exposed to the cell surface is related to hydrophobicity and that hydrophobic bonds at the cell surface affect autoaggregation. However, another explanation for reduced autoaggregation is possible. Physical contact among cells might decrease because of shorter cell length in the mutants. Microplate assay confirmed that Tf1331 played an important role in biofilm formation.

In summary, this study focused on the protein Tf1331 in the outer membrane, which influenced cell shape. We found that this protein was involved in not only attachment to the extracellular matrix, but also biofilm formation. Further investigations on the function of Tf1331 *in vivo* are underway. We believe that the major Tf1331 protein may be a potential target for the prevention and treatment of oral anaerobic infections.

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