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Binding of Tp92 homolog of *Treponema denticola* to fibronectin and epithelial cells

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Treponema denticola is the best studied oral spirochete and numerous studies have shown that it is strongly associated with periodontitis and expresses several putative virulence factors. In this study, we report on a surface protein of *T. denticola*, Td92, which is homologous to Tp92 of *Treponema pallidum*, an agent of syphilis. Immunofluorescence assay and immunogold labeling with anti-Td92 Ab revealed that Td92 had surface-exposed epitopes. And Td92 was capable of binding to fibronectin and KB cells, an oral epithelial cell line. In addition, Td92 could enter the KB cells. These results indicate that Td92 is a fibronectin-binding protein which can bind to and internalize into the host cells, facilitating the virulence of *T. denticola*.

Key words: Treponema denticola, Td92, Surface protein, Adhesin

Introduction

Oral spirochetes are composed of enormously diverse *Treponema* species and most of them are not cultivated. Ten species of oral spirochetes are isolated and characterized so far, and some of them are known to be strongly associated with periodontitis (Ellen *et al*., 2000).

Outer membrane proteins (OMPs) belong to the first molecules that are in contact with host cells and elicit diverse virulence-associated activities like adhesion, cytotoxicity and inflammation. Several OMPs have been found in *Treponema denticola*, the best characterized species of cultivable oral spirochetes. The major surface protein, Msp (53-kDa) exerts multiple functions like pore-forming activity, adhesion, inhibition of agonist-induced Ca^{2+} release from internal stores, and inhibition of collagen phagocytosis in gingival fibroblasts by inducing subcortical actin filament assembly and restricting affinity modulation of β_1 integrins (Edwards *et al*., 2005; Fenno et al., 1998; Mathers et al., 1996; Wang *et al*., 2001). OppA (70-kD) is involved in the binding of soluble host proteins like plasminogen and fibronectin in *T. denticola* (Fenno *et al*., 2000). Chymotrypsin-like protease (CTLP, 95-kDa) in *T. denticola* is a surface protease with adhesion activity and degrades proinflammatory cytokines including IL-1, IL-6, and TNF-α (Miyamoto *et al*., 2006). In *T. pectinovorum*, MompA was identified as the major surface protein, but the function has not been elucidated (Walker *et al*., 1997). Recently, we characterized the major surface proteins (MspA and MspTL) that are conserved in *T. lecithinolyticum* and *T. maltophilum* (Lee *et al*., 2005). These conserved proteins upregulated proinflammatory factors including ICAM-1, IL-6, and IL-8 in THP-1 and periodontal ligament (PDL) cells.

Tp92 is known to be an immunoprotective surface antigen of *Treponema pallidum*, an agent of syphilis, and suggested to be a promising diagnostic antigen (Van Voris et al.,2003; Cameron *et al*., 2000). In this study, we identified Tp92 homolog of *T. denticola*, Td92, and characterized it as an adhesin to adhere to epithelial cells and fibronectin, an extracellular matrix protein.

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Materials and Methods

Bacteria and cells

T. denticola ATCC 33521, *T. lecithinolyticum* ATCC 700332, *T. maltophilum* ATCC 51939 and *T. socranskii* subsp. *socranskii* ATCC 35536 were cultured in an anaerobic atmosphere (10% CO_2, 5% H_2, 85% N_2) using OMIZ-Pat medium as described previously (Wyss *et al*., 1999).

Escherichia coli DH5α-T1 (Invitrogen, Carlsbad, CA) used for TA cloning and *E. coli* M15 (Qiagen, Valencia, CA) used for the expression of Td92. *E. coli* containing the plasmids was cultured aerobically in Luria-Bertani (LB) broth supplemented with 50 μg/ml of ampicillin for the DH5α-T1 or with 100 μg/ml of ampicillin and 25 μg/ml of kanamycin for the M15 strain.

KB cells (ATCC CCL-17), an oral carcinoma epithelial cell line, were cultured in RPMI 1640 medium supplemented with 10% FBS and used for protein binding assay.

Cloning and expression of the *td*92 gene

The *tp*92 homolog of *T. denticola* (ATCC 35405) was identified in the genome sequence of the bacterium using the NCBI BLAST program with the *T. pallidum* subsp. pallidum *tp*92 sequence. The *tp*92 gene homolog of *T. denticola* was amplified from the genomic DNA by PCR and cloned without the sequences encoding the leader peptide. The sequences of the PCR primers were as follows: 5'-AAC TGA GCT CGG ATG GTA TAA TGG AAA ACC TG-3' (underlined sequence: SacI -tagged) and 5'-AAC TCT GCA GCT ATA AAT TGG GTA TAT TGA ATG AA-3' (underlined sequence: PstI- tagged) for *T. denticola*. The primers had restriction sites and an additional four nucleotides to the 5'-end of these restriction sites. PCR was carried on using a standard protocol. The thermal cycle chosen included an initial denaturation step at 94°C for 4 min, 30 cycles of a denaturation step at 94°C for 1 min, an annealing step at 62°C for 1 min, and an extension step at 72°C for 1 min, with a final incubation at 72°C for 5 min. The PCR products were cloned in *E. coli* using the TA cloning vector pCR2.1-TOPO, and the inserts were isolated and cloned in *E. coli* M15 using the expression vector pQE-30 as described previously (Lee et al., 2005). Histidine-tagged recombinant proteins were prepared after induction of E. coli with 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG), as previously described (Lee *et al*., 2005). The recombinant protein was further treated with polymyxin B-agarose according to the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO) to remove endotoxin which could possibly contaminate the Td92. The recombinant protein was designated as Td92 and quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA).

Production of antiserum against the Td92

Polyclonal antibodies against *T. denticola* Tp92 homolog were raised in New Zealand White rabbits by intradermal administration of 500 μg of the purified proteins with Complete Freund's Adjuvant (500 μg, F5881, Sigma Chemical Co., St. Louis, MO, USA) followed by three subsequent boosts of 200 μg of the protein with Incomplete Freund's Adjuvant (500 μg, F5506, Sigma) in two-week intervals. Antiserum was collected one week after the final boost. The antiserum was tested by ELISA and immunoblot using horseradish peroxidase (HRP)-labeled mouse anti-rabbit IgG (R & D Systems, Minneapolis, MN, USA) and showed to contain high titers of specific antibodies against the Td92. The anti-Td92 Ab was further purified using an ImmunoPure (A Plus) IgG purification kit according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

Indirect immunofluorescence microscopy

Treponema spp. grown to an early stationary phase were harvested, resuspended in PBS, and applied to glass slides coated with silane (Sigma) after fixation with 4% paraformaldehyde in PBS. The bacteria were incubated in PBS containing 1% BSA in for 1 h and then reacted with the anti-Td92 Ab (1 : 100) for 1 h. After washing three times with PBS, the bacteria were reacted with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (1:50 dilution in PBS containing 1% BSA). The cells were washed three times with PBS and observed with a fluorescence microscope (Carl ZEISS, Axioskop, Germany) at 1000 × magnification. As a control for nonspecific binding, rabbit preimmunized serum was used instead of anti-Td92 Ab.

Binding assay of Td92 to fibronectin and host cells

Since Td92 was a surface protein, the binding ability of Td92 was evaluated using fibronectin and host cells. In order to see the Td92 binding to immobilized fibronectin, 96-well microtiter plates were coated with 100 μl of fibronectin (10 μg/ml, Sigma) at 4°C overnight. After washing twice with PBST (0.1% Tween20 in PBS), 80 μl of diluted Td92 (0.001 ~ 2.5 μg /well in PBS) was add to each well. After incubation for 1 h at room temperature, the plates were washed three times with PBST. The plates were then incubated with 50 μl of rabbit anti-Td92 antibody at a dilution of 1 : 1000 for 1 h. After washing three times with PBST, the plates were reacted with 50 μl of HRP-conjugated goat anti-rabbit IgG (R & D systems, Minneapolis, MN, USA) at a dilution of 1:1000 for 1 h at room temperature. After washing three times with PBST, 100 μl of tetramethylbenzidine (TMB) solution was added to each well and the colorimetric reaction was stopped by addition of 100 μl of 2 N H_2SO_4. The plates were then read in an ELISA reader at 450 nm.

The binding ability of Td92 to host cells was assessed using KB cells and FITC-labeled Td92. KB cells were seeded with 2×10^5/500 μl in 24-well culture plates. The cells were cultured to 70~80 % confluence and washed with PBS. FITC-labeled Td92 (5 μg) was added to the cells for 1

h. The cells were detached, washed with serum free media, resuspended in 400 µl of PBS, and divided into two portions. One portion (200 µl) was directly subjected to flow cytometry analysis (FACSCalibur; Becton Dickinson, San Jose, CA, USA) to measure total fluorescence. The other was subjected to flow cytometry analysis after adding 0.4% trypan blue solution to quench extracellular fluorescence emissions. Trypan blue quenches to extinguish extracellular fluorescence, allowing analysis of only intracellular fluorescent sources (Innes *et al*., 1999). FITC-labeling of the Td92 was performed with a Fluorescein Labeling Kit-NH2 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instruction.

Immunogold staining of *T. denticola*

T. denticola cells ($OD_{600} = 0.2$; corresponded to 5×10^9 cells/ml) were incubated with 200 µl of blocking solution (PBS containing 1% BSA) for 1 h. The bacteria were then incubated with 100 µl of the anti-Td92 Ab (1 : 50 dilution in PBS containing 1% BSA) for 1 h. After washing three times with PBS, the bacteria were reacted with anti-rabbit IgG conjugated with 10-nm colloidal gold particles (1 : 50 dilution in PBS containing 1% BSA; Sigma) for 1 h. After washing three times with PBS, the bacteria were resuspended in PBS and absorbed onto formvar/carbon-coated 400-mesh copper grids. After washing with PBS and air drying, the bacteria were negative stained with 1% uranyl acetate for 15 s. After washing with PBS and air drying, the bacteria were observed with a transmission electron microscopy (JEOL, JEM-1200EX, Japan) at 80 kV acceleration voltage.

Results

Surface-exposed epitopes of Td92

Tp92 of *T. pallidum* and its homologs in other bacteria are known to be outer membrane proteins. We also observed that Td92 is localized in the outer membrane by immunoblotting of the OM preparation (manuscript accepted in Infection and Immunity). In order to determine whether Td92 is exposed to the bacterial surface, we performed indirect immunofluorescence assay and immunogold labeling of *T. denticola*. As shown in Fig. 1, anti-Td92 Ab reacted with *T. denticola* cells, indicating the Td92 epitopes are surface exposed. The antibody also crossreacted with *T. lecithinolyticum*, *T. maltophilum*, and *T. socranskii* subsp. *socranskii*, suggesting the presence of common surface epitopes of the homologous proteins in these bacteria. Preimmune rabbit serum did not react with the bacteria. Immunogold electron microscopy further showed that gold particles bound to the bacterial surface (Fig. 2).

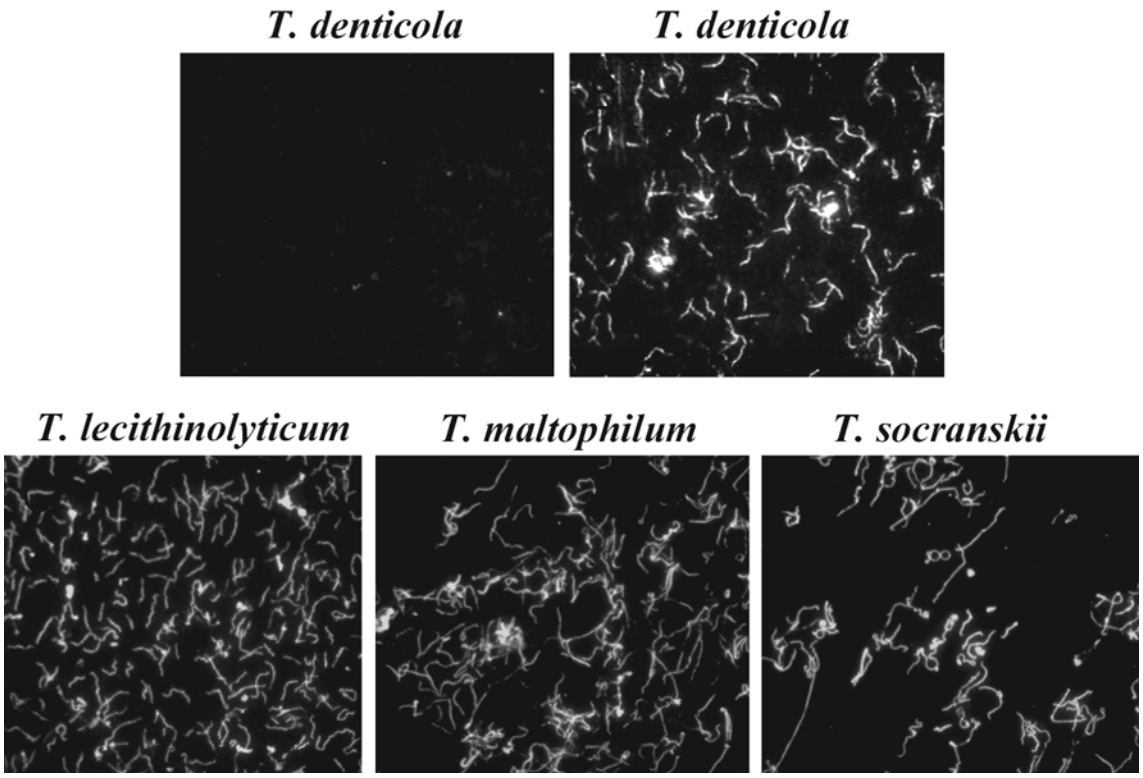

Fig. 1. Immunofluorescence micrographs of oral *Treponema*. Freshly grown *Treponema* species were fixed with 4% paraformaldehyde and reacted with anti-Td92 or rabbit preimmune serum (upper left), followed by reacting with FITC-labeled goat-anti-rabbit IgG. Original magnification × 1000.

Adhesion of Td92 to fibronectin and epithelial cells

Adhesion is the first step for bacteria to colonize host cells. We determined whether Td92 was capable of binding to an extracellualr matrix protein, fibronectin, and host cells. As shown in Fig. 3, Td92 bound immobilized fibronectin in a dose dependent manner.

Flow cytometry analysis showed that Td92 also bound to KB cells, an epithelial cell line (Fig. 4). The incubation of KB cells with FITC-labeled Td92 resulted in the significant increase of fluorescence. After trypan blue quenching, the fluorescence decreased, but a small portion of the fluorescence remained. These results indicate that Td92 is able to bind to and internalize into KB cells.

Discussion

Diverse species of oral spirochetes are found in a single patient or a single diseased site. Therefore it is important to identify and characterize virulence factors common to various species to efficiently develop a strategy for growth inhibition or eradication. Although several OMPs have been identified in oral spirochetes, most of them are species–specific. In attempt to identify more common surface proteins, we identified and characterized Tp92 homologs in oral spirochetes (manuscript accepted in Infection and Immunity). Tp92 homologs were capable of binding to epithelial cells and antiserum (IgG fraction) raised against a recombinant protein of the *T. denticola* Tp92 homolog crossreacted with that of *T. lecithinolyticum*, *T. maltophilum* and *T. socranskii* subsp. *socranskii* by immunodot blot. The Tp92 homologs stimulated IL-1β, TNF-α, IL-6, PGE2, and MMP-9 in host cells like monocytes and fibroblasts which are involved in inflammation and osteoclastogenesis. In this study, we further demonstrated that Td92 had surface exposed epitopes common to four species of oral spirochetes and was capable of internalizing into epithelial cells. *T. denticola* itself is known not to invade epithelial

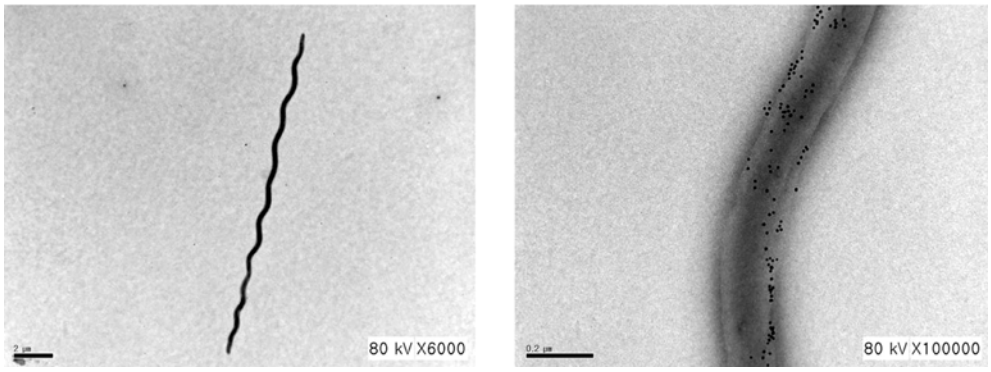

Fig. 2. Transmission electron microscopy of *T. denticola* labeled with gold particles. *T. denticola* was reacted with anti-Td92 Ab and subsequently with anti-rabbit IgG conjugated with 10-nm colloidal gold particles. After negative staining with 1% uranyl acetate, the bacteria were observed by a transmission electron microscopy. A *T. denticola* whole cell (left) and a part of the bacterium showing immunogold labeling (right).

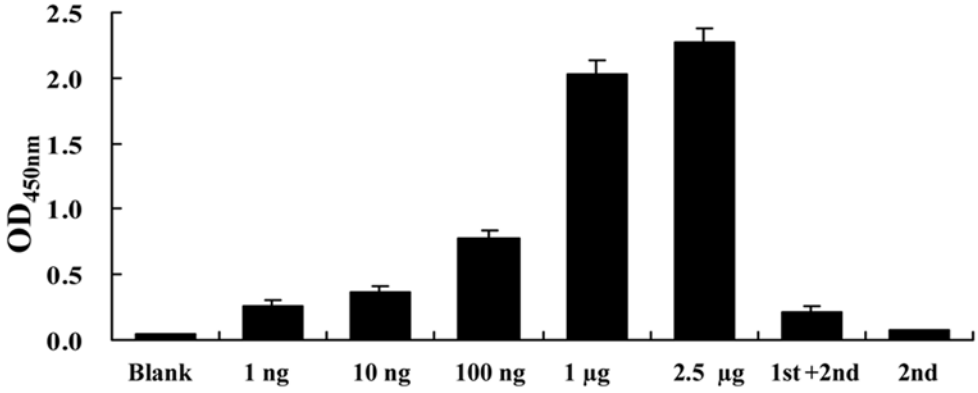

Fig. 3. Binding of Td92 to immobilized fibronectin. Microtiter plates were coated with 10 μg/ml of fibronectin and Td92 at different concentrations was then added for 1 h. After washing with PBST, the bound proteins were reacted with anti-Td92 Ab and subsequently with HRP-conjugated goat anti-rabbit IgG. The detection was performed with the colorimetric substrate TMB for HRP and absorbance was measured at 450 nm in a microplate reader. Data are mean ± SEM values of triplicate wells. 1st + 2nd: Immobilized fibronectin was reacted only with anti-Td92 Ab followed by HRP-conjugated goat anti- rabbit IgG. 2nd: Immobilized fibronectin was reacted only with HRP-conjugated goat anti- rabbit IgG.

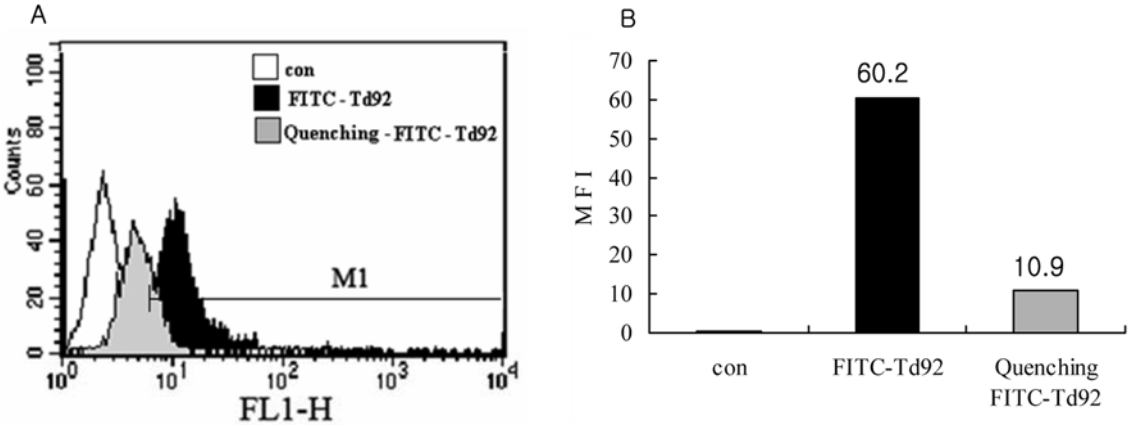

Fig. 4. Binding and internalization of KB cells by Td92. KB cells (2×10^5 cells/500 μl) were cultured in 24-well plates and incubated with FITC-labeled Td92 (5 μg) for 1 h. After the cells were detached and washed, half of the cells were directly subjected to flow cytometry analysis to measure total fluorescence and the other half was analyzed after trypan blue quenching to measure only intracellular fluorescence emission. Experiments were performed three times and the representative histogram (A) and the mean fluorescence intensity (MFI, B) are shown.

cells. However, it secretes CTLP that can degrade junctional complexes and be transported into the cell. CTLP disturbs the actin cytoskeleton in the cell (Chi *et al*., 2003; Yang *et al*., 1998). The major surface protein of *T. denticola*, Msp, is translocated into the epithelial cell membrane, forming pores that depolarize the membrane (Mathers *et al*., 1996). In gingival sulcus, *T. denticola* is in contact with the gingival junctional epithelium. Therefore, binding to and internalization into epithelial cells by Td92 may play an important role for initial colonization and host cell stimulation.

Td92 could also bind to immobilized fibronectin. Fibronectin binding surface proteins have been reported in various pathogenic bacteria and they facilitate bacterial binding to host cells (Chia *et al*., 2000; Fenno et al., 1996 & 2000; Fowler et al., 2000; Hall-Stoodley et al., 2006; Probert *et al*., 2001). Fibronectin exists in a soluble form in body fluids and an insoluble component of cell membranes and the extracellular matrix of most tissues. *T. denticola* Msp bound to immobilized fibronectin (Fenno *et al*.,1996). A 70-kDa surface protein of *T. denticola*, an OppA homolog, bound to soluble fibronectin (Fenno *et al*., 2000), but not to immobilized fibronectin. Although we did not analyze whether Td92 binds to soluble form of fibronectin, Td92 belongs to a fibronectin binding proteins of *T. denticola*.

In summary, Td92 is a fibronectin binding protein with surface-exposed epitopes common to four species of oral spirochetes. It can bind to and internalize into the epithelial cells. These properties suggest that Td92 could facilitate the virulence of *T. denticola*. The study is ongoing to elucidate the signaling pathway of the Td92, which will contribute to clarify the molecular pathogenesis.

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