



# Transcellular penetration of *Treponema phagedenis* isolated from papillomatous digital dermatitis in polarized normal human epidermal keratinocytes *in vitro*

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**ABSTRACT.** Papillomatous digital dermatitis (PDD) is a polymicrobial infection causing lameness in dairy cattle. Culture-independent analysis has shown that *Treponema phagedenis* is present consistently and predominantly in the lesions. However, the pathogenesis of PDD, especially the tissue penetration pathway, has not been examined. In the present study, we investigated whether *T. phagedenis* strains isolated from PDD produce proteolytic enzyme (s) for disruption of the epithelial cell barrier and have the ability to translocate in polarized normal human epidermal keratinocytes (NHEK) *in vitro*. Ten strains of *T. phagedenis* isolated from lesions did not show proteolytic activity on modified skim milk agar, although a human strain of *T. denticola* used as a control showed such activity. The integrity of tight junctions was monitored by measurement of transepithelial electrical resistance (TER). The TER values after inoculation of the *T. phagedenis* strains examined did not change during the experimental period; however, apical to basolateral translocation of *T. phagedenis* was confirmed after 24 hr by microscopy and *Treponema*-specific PCR. We further confirmed that translocation of *T. phagedenis* was accelerated by co-inoculation with live *T. denticola*, but not with heat-killed organisms. Furthermore, tight junction ZO-1 protein was not lost intensity after inoculation with *T. phagedenis* and the organism was observed in NHEK cells using a fluorescence microscope. These results suggest that *T. phagedenis* strains may translocate via a transcellular route *in vitro* and that the invasion is accelerated by other bacteria, such as *T. denticola*, producing proteolytic activity.

**KEY WORDS:** dairy cattle, normal human epidermal keratinocytes cell, papillomatous digital dermatitis, translocation, *Treponema phagedenis*

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Papillomatous digital dermatitis (PDD) is a contagious foot disease of dairy cattle and its high prevalence worldwide can be attributed to the spread of modern rearing systems [12, 13, 26]. PDD often leads to lameness due to severe pain, resulting in decreased body weight and milk production, and thereby serious economic loss and animal welfare problems [25]. Although its etiology has not been fully clarified, culture-independent approaches such as cloning and sequencing of microbial 16S rRNA genes have demonstrated the presence of multiple *Treponema* species consistently and predominantly in PDD lesions [14, 24]. Therefore, these treponemes are considered to be the most important agents in the pathogenesis of the disease [17]. In our previous case-control-study for comparison of the microbial populations in Japan using about 1,500 clones in 5 PDD lesions of affected cattle and 4 skin samples from healthy cattle [27], we demonstrated that *T. phagedenis* represented the highest population in the PDD lesions. Moreover, multiple bacteria other than *Treponema* species were also detected in the lesions, suggesting that PDD is a

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polymicrobial infection [28]. These observations were further confirmed by next-generation sequencing analyses [15].

PDD begins as a superficial type of dermatitis with an erosive lesion and later forms a hyperkeratotic papillomatous lesion with long hair-like projections [29]. It remains unclear how the papillomatous lesion is formed by the infecting bacteria. *Treponema* species, Gram-negative, strictly anaerobic, spiral shaped and motile bacteria have been detected and/or isolated from PDD lesions [10, 18, 24]. Since a considerable number of spirochetes are found in both the superficial layer of the epidermis and in the deeper layer [11], it is considered that these organisms have a propensity for tissue invasion and play an important role in inflammation and lesion formation. However, the pathogenetic role of these organisms has not been fully examined due to their fastidious culture requirements *in vitro*.

*T. denticola*, which is associated with periodontal disease, has a variety of virulence factors. The organism produces dentilisin, a chymotrypsin-like protease, which is thought to facilitate penetration of the epithelial cell layer by disruption of tight junction proteins [6]. The bacterial translocation pathway *in vitro* has been investigated using polarized epithelial cells combined with measurement of transepithelial electrical resistance (TER) using a membrane insert system to indicate the integrity of tight junctions [4, 19]. Although we have isolated *T. phagedenis* from PDD lesions, its role in pathogenesis—especially the mechanism of tissue penetration—has not been examined. In the present study, we tested whether *T. phagedenis* strains isolated from PDD produce proteolytic enzyme (s) and have the ability to translocate in polarized epidermal keratinocytes *in vitro*.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

A total of 10 *T. phagedenis* strains (HT201, YG3903R, CH9, HG42, IZ6-2, HD27-4, HD22R11, HD26R, HD21R-R7 and HD26-67) isolated from PDD lesions of dairy cattle (Holstein) in Japan were used in this study. *T. phagedenis* ATCC27087 isolated from a human genital organ and *T. denticola* JCM8225 isolated from the human oral cavity were used as controls. All of the bacterial strains were grown on PDDTp agar plates developed for the cultivation of *T. phagedenis*, containing GAM agar (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.8% Brain Heart Infusion broth (Nissui Pharmaceutical), 0.8% Brucella broth (Becton Dickinson and Co., Tokyo, Japan), 10% defibrinated horse blood (Nippon Biotest Laboratories, Tokyo, Japan) and 10% heat inactivated fetal bovine serum (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C for 10–14 days under anaerobic conditions using Aneropack (Mitsubishi Gas Chemical, Tokyo, Japan). All of the strains examined were suspended in Brucella broth containing 10% (v/v) glycerol (Kanto Kagaku, Tokyo, Japan), and stored at –80°C until testing.

### Human epidermal keratinocyte cell culture

Normal Human Epidermal Keratinocytes (NHEKs) (DS Pharma Biomedical, Osaka, Japan) were cultured in serum-free medium for NHEKs supplemented with bovine pituitary extract (BPE) (DS Pharma Biomedical) in accordance with the manufacturer's instructions at 37°C under a 5% CO<sub>2</sub> humidified atmosphere. The cells were used for measurement of tight junction integrity within 3 passages from the stock culture.

### Detection of proteolytic activity from *T. phagedenis* strains

Detection of proteolytic activity was examined by a casein hydrolysis test [22]. One loopful of 10 *T. phagedenis* strains isolated from PDD lesions was inoculated onto a modified skim milk agar (mSMA) plate, containing GAM agar (Nissui Pharmaceutical) supplemented with 0.8% Brain Heart Infusion broth (Nissui Pharmaceutical), 0.8% Brucella broth (Becton Dickinson and Co.), 10% skim milk (Becton Dickinson and Co.) and 10% heat inactivated fetal bovine serum (GIBCO, Thermo Fisher Scientific), and then incubated at 37°C for 7–10 days under anaerobic conditions as described above. Proteolytic activity was detected as a transparent zone around the inoculated bacterial cells. The type strains, *T. phagedenis* ATCC27087 and *T. denticola* JCM8225, were used as controls.

### Analysis of epithelial barrier integrity

NHEK cells were suspended in serum free medium as described above and seeded onto 0.33-cm<sup>2</sup> cell culture inserts with a pore size of 3.0 µm (Merck Millipore, Darmstadt, Germany) in 24-well microplates at a concentration of approximately  $1 \times 10^5$  cells. A 0.8-ml volume of cell culture medium was added into each well of the microplate for maintaining the filters. The microplates with cell culture inserts were maintained at 37°C in a 5% CO<sub>2</sub> humidified atmosphere and the cell medium was changed twice a week. The integrity of tight junctions was monitored by measurement of transepithelial electrical resistance (TER) using a Millicell Electrical Resistance System (Merck Millipore). TER was measured as ohms ( $\Omega$ )  $\times$  cm<sup>2</sup>, and fluid resistance was subtracted. When the TER value reached maximal polarity ( $>40 \Omega/\text{cm}^2$ ) after 6–7 days of incubation, the monolayers were washed three times with 10 mM phosphate-buffered saline (PBS) (pH 7.4). Two *T. phagedenis* strains, HT201 and YG3903R, chosen randomly from the PDD isolates and a control strain of *T. phagedenis*, ATCC27087, were used for this purpose. Approximately  $2 \times 10^8$  cells of *T. phagedenis* suspended in 200 µl of cell culture medium were inoculated into the apical side of the monolayer. Since *T. denticola* JCM8225 showed protease-like activity on mSMA plates and it has been reported to destroy tight junction proteins, it was used as a positive control. Furthermore, *T. denticola* JCM8225 or *T. phagedenis* YG3903R at a concentration of  $2 \times 10^8$  cells/ml was killed by heating at 100°C for 10 min and harvested by centrifugation for 5 min at  $10,000 \times g$ . A mixture of each strain, alive or dead, as well as each single strain suspended in 200 µl of cell culture medium, were inoculated into the apical compartment. The TER values were then measured at 0, 24, and 48 hr after inoculation of the bacterial samples examined. Cell culture inserts that had not

been inoculated with bacteria were used as a negative control. The TER value for the cell-free control well was subtracted from the values obtained to remove any background effect.

#### *Effect of co-inoculation of T. phagedenis and T. denticola on penetration of NHEK cells*

We further examined if *T. denticola* producing proteolytic activity accelerated the translocation of *T. phagedenis* when both species were inoculated at the same time. In the case of co-inoculation, *T. denticola* JCM8225 was used as live or heat-killed cells as described above. Approximately  $2 \times 10^8$  cells of live *T. phagedenis* YG3903R and live or heat-killed *T. denticola* JCM8225 cells suspended in 100  $\mu$ l of cell culture medium were mixed and then the mixtures were inoculated into the apical side of the cell culture insert. The TER values were then measured at 0, 24 and 48 hr after co-inoculation. The bacterial cells in the medium on the basolateral side were harvested by centrifugation for 5 min at  $10,000 \times g$  after each incubation time. The number of *T. phagedenis* cells translocated was counted using a flow cytometer with fluorescence-labeled anti-*T. phagedenis* IgG antibody as described below, since *T. phagedenis* does not form single colonies on agar plates.

#### *Detection of treponemes in the basolateral compartment of the cell culture insert*

To determine whether the PDD strains of *T. phagedenis* translocated across the monolayer of NHEK cells on a transmembrane of the cell culture insert, the bacterial cells in the medium on the basolateral side were harvested by centrifugation for 5 min at  $10,000 \times g$  after a predetermined inoculation time. The precipitate was resuspended in 80  $\mu$ l of sterilized distilled water and 30  $\mu$ l of the suspension was observed by microscopy for the presence of spirochete-like bacteria after staining with crystal violet. The bacterial DNA from 50  $\mu$ l of the suspension was extracted by alkaline and heat treatments [3]. Bacterial 16S rRNA genes from the samples were amplified using a universal primer set for amplification of *Treponema* species (Forward, 5'-TTACGTGCCAGCAGCCGCGTAAC-3'; Reverse, 5'-GTCRYMGGCAGTTCCGCCWGAGTC-3'), which had been designed to target common regions in the 16S rRNA sequence (657 bp) of *Treponema* sp. [1]. PCR was performed in a final reaction volume of 20  $\mu$ l. Each reaction contained 20 pM each primer (forward and reverse), 200  $\mu$ M each deoxynucleoside triphosphate, 0.5 U of *Taq* DNA polymerase (Qiagen, Tokyo, Japan),  $1 \times$  PCR buffer and 2  $\mu$ l of extracted DNA. The thermal cycling conditions included 30 cycles of denaturation at 95°C for 30 sec, annealing at 69.5°C for 30 sec and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The specific amplification of each target gene was confirmed by 1% agarose gel electrophoresis.

#### *Immunization of rabbit for Treponema phagedenis antibody production*

Polyclonal antiserum against *T. phagedenis* YG3903R strain was used to immunize 2-month-old New Zealand white rabbits as described in our previous study [16]. In brief, the rabbits were inoculated intracutaneously with each emulsified mixture of bacterial suspension and Freund's incomplete adjuvant (Nacalai Tesque, Kyoto, Japan) on three different days with 2-week intervals. Two weeks after the third immunization, whole blood was collected from the immunized rabbits. The collected serum was inactivated by incubation at 56°C for 30 min in a water bath and was stored in aliquots at -20°C until use. The protocol was approved by the committee for animal experimentation at the University of Miyazaki (acceptance number; 2007-024-4).

#### *Purification and fluorescence labeling of IG*

IgG immunoglobulins were precipitated from polyclonal antisera against *T. phagedenis* YG3903R by affinity chromatography using Recombinant Protein G Agarose (Thermo Fisher Scientific) (loading buffer: 10 mM sodium phosphate, pH 7.0, 150 mM NaCl; elution buffer: 100 mM glycine HCl, pH 2.6). Elution fractions were neutralized with 100 mM Tris-HCl, pH 7.0 and dialyzed against PBS. To avoid any cross-reaction, rabbit anti-*T. phagedenis* YG3903R antiserum was absorbed with *T. denticola* JCM8225 by incubation at 37°C for 1 hr. The purified IgG preparation against *T. phagedenis* YG3903R was labeled with Alexa Fluor 647 using an Alexa Fluor Protein Labeling Kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions.

#### *Fluorescence microscopy*

To observe internalized *T. phagedenis* in NHEK cells, an immunofluorescence procedure was performed [23]. Trypsinized  $1 \times 10^5$  NHEK cells were placed into each well of a chamber slide (Nalgene Nunc, Rochester, NY, USA) and incubated at 37°C for 3 days under 5% CO<sub>2</sub> in humidified atmosphere. A total of 500  $\mu$ l of *T. phagedenis* strain CH9 (approximately  $1 \times 10^8$  cell/ml) in antibiotic-free EMEM, supplemented with 10% FBS was inoculated into the cell monolayers, which were prewashed twice with PBS. The cell monolayers were incubated at 37°C for 24 hr in a 5% CO<sub>2</sub> humidified atmosphere. Then, the cell monolayers were fixed using 4% paraformaldehyde in PBS and permeabilized using 0.1% Triton X-100 (Nacalai Tesque) with 0.5% bovine serum albumin (Sigma-Aldrich, Tokyo, Japan). *T. phagedenis* that adhered to and invaded NHEK cells were labeled with anti-*T. phagedenis* serum (1:200) at 37°C for 1 hr, and goat anti-rabbit antibodies conjugated with fluorescein isothiocyanate (FITC) (7.5  $\mu$ g/ml) (Invitrogen, Carlsbad, CA, USA) were added. Rhodamine phalloidin (1 U/ml) (Invitrogen) was added to detect the actin of NHEK cells. NHEK cells were visualized by three-dimensional analysis using a fluorescence microscope BIORIVO BZ-9000 (KEYENCE, Osaka, Japan).

#### *Counting of fluorescence-labeled bacteria*

The bacterial cells in the medium on the basolateral side after 48 hr of incubation were collected and harvested by centrifugation as described above. The precipitate was resuspended in 1 ml of sterilized water and bacterial cells were labeled with 2  $\mu$ l of FITC-conjugated IgG immunoglobulin at 37°C for 1 hr. The organisms were harvested by centrifugation for 5 min at  $10,000 \times g$

and suspended in 1 ml of sterilized water. This process was repeated three times. To calculate the numbers of cells labeled with the FITC-conjugated IgG, a 10-fold serial dilution of the *T. phagedenis* YG3903R suspension was prepared as a standard and bacterial cell at each dilution were labeled with FITC-conjugated IgG immunoglobulin as described above. These labeled bacteria were analyzed using a flow cytometer (Cell Analyzer BD FACS Canto™ II: BD Bioscience, Billerica, MD, USA). The count of *T. phagedenis* cells was calculated based on the fluorescence intensity of the standards.

#### Immunofluorescence staining of tight junction protein ZO-1

Immunofluorescence analysis of ZO-1 was performed as described [6]. Briefly, the NHEK cell monolayers with or without inoculation of *T. phagedenis* YG3903R or *T. denticola* JCM8225 were washed once with cold PBS+ (containing 0.5 mM MgCl<sub>2</sub> and 0.9 mM CaCl<sub>2</sub>) and fixed with 4% formaldehyde at 4°C for 20 min. The cells were washed three times with PBS+ and incubated with 5% FBS in PBS+ at 37°C for 1 hr, then labeled with rabbit anti-ZO-1 polyclonal antibody (1:100 dilution in PBS+) (Cosmo Bio Inc., Tokyo, Japan) at 37°C for 1 hr. The monolayers were washed four times with PBS+ and developed with goat anti-rabbit antibody conjugated with FITC (1:300 dilution in PBS+) (Cosmo Bio Inc.) at 37°C for 1 hr, followed by four washes with PBS+. The monolayers were post-fixed with 4% formaldehyde at room temperature for 15 min. After fixation, the cells were washed with PBS+ to remove excess formaldehyde and stop the fixing reaction. The stained cells were visualized using a BIOREVO BZ-9000 fluorescence microscope (KEYENCE, Osaka, Japan).

#### Statistical analysis

The results were expressed as the mean ± standard deviation for every experiment, and the experiments were performed in triplicate. Statistical analysis was performed using Student's *t* test. Statistical significance was defined as  $P < 0.05$ .

## RESULTS

#### Proteolytic activity among *T. phagedenis* strains

After incubation for 7–10 days under anaerobic conditions, *T. denticola* JCM8225 showed considerable proteolytic activity and formed distinct transparent zones around bacterial cells inoculated on mSMA plates. However, no transparent zone was observed among any of the PDD-derived *T. phagedenis* strains examined, or the type strain (Fig. 1).

#### Epithelial cell integrity

Three *T. phagedenis* strains, two PDD strains and a type strain, and *T. denticola* JCM8225 were examined for their translocation ability and possible route in polarized NHEK cell monolayers using a membrane insert system. TER across polarized monolayers of the NHEK cells was not affected by either of the PDD strains of *T. phagedenis* examined, as well as the type strain, during the experimental period (Fig. 2A). In contrast, TER decreased significantly ( $P < 0.05$ ) to  $76.8 \pm 4.2\%$  and  $73.0 \pm 5.5\%$  at 24 and 48 hr after inoculation of *T. denticola* JCM8225, respectively.

Migration of treponemes from the apical side to the basolateral side was confirmed at 6, 12, 24 and 48 hr after inoculation by microscopic observation and by *Treponema*-specific PCR using the basolateral medium (Fig. 2B). PCR amplicons of *T. phagedenis* strains HT201 and ATCC27087 were detected within 12 hr after cell inoculation, whereas *T. phagedenis* strain YG3903R was detected after 24 hr of the inoculation. The amplicons of *T. denticola* JCM8225 were detected from the basolateral medium during 6–48 hr after inoculation. In line with the PCR results, spirochete-like bacteria were observed from the basolateral side by microscopy (Fig. 2C). However, neither PCR amplicons nor bacterial cells were observed after inoculation of heat-killed cells (data not shown).

#### Internalization of *T. phagedenis* in NHEK cells

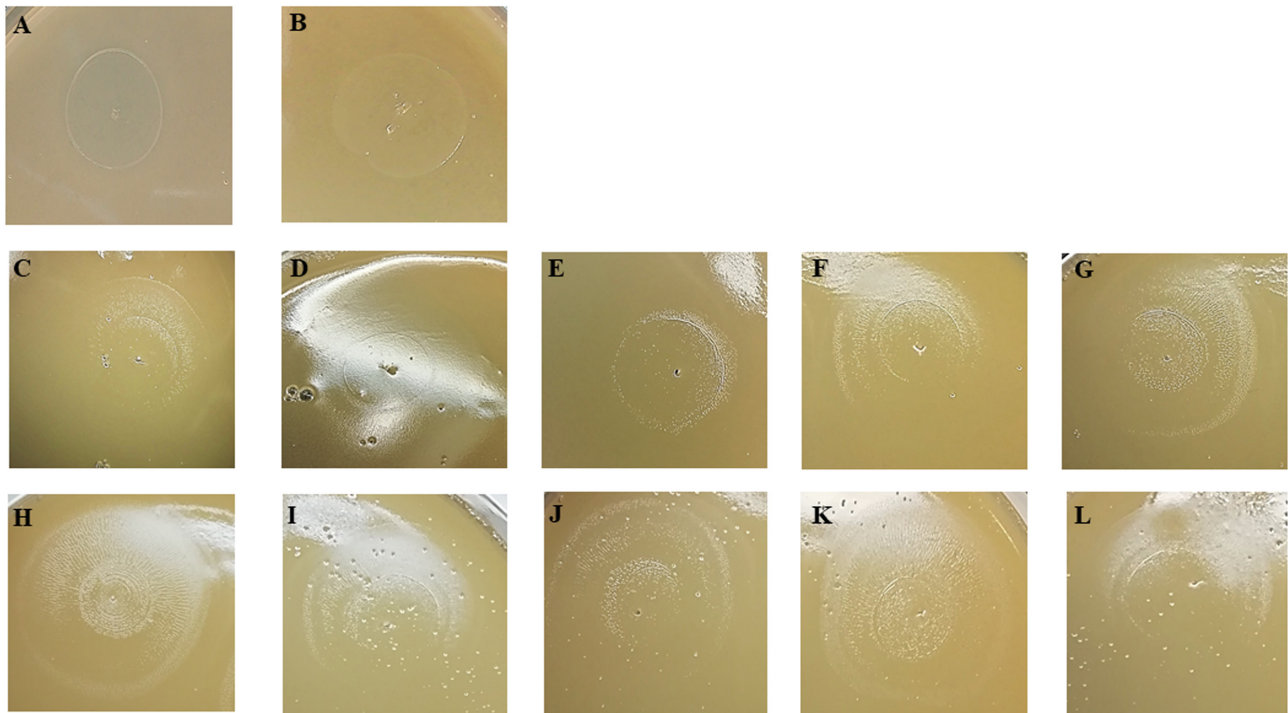
The monolayers were infected with CH9 strain for 24 hr to investigate the internalization of *T. phagedenis* in NHEK cells, and cell internalization was visualized using a fluorescence microscope. The presence of *T. phagedenis* inoculated in NHEK cells was observed in the cross-section of the X-, Y- and Z-axes (Fig. 3).

#### Immunofluorescence staining of ZO-1 protein

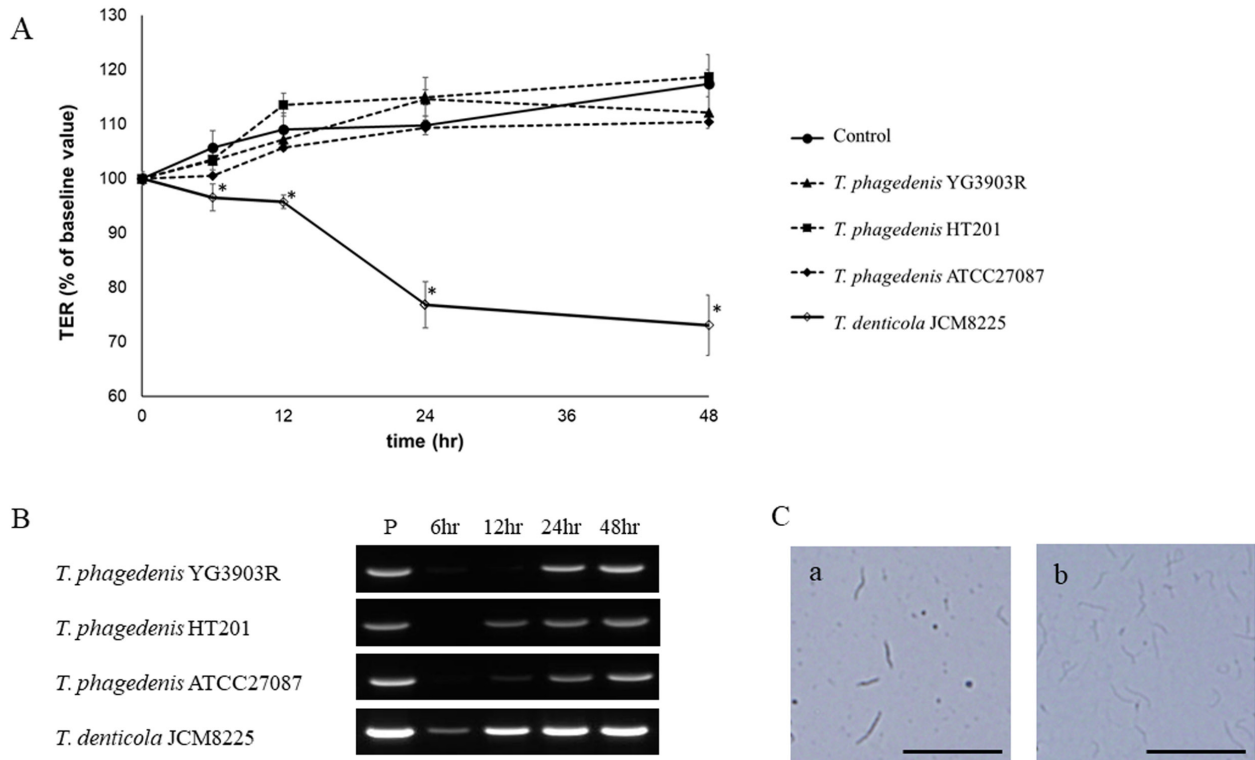
To further confirm the translocation route of *T. phagedenis* in the NHEK cells, the tight junction protein ZO-1 was subject to immunofluorescence staining after penetration of inoculated bacteria. When *T. phagedenis* YG3903R was inoculated, ZO-1 proteins surrounding NHEK cells were stained strongly, as was the case for the control without bacterial inoculation (Fig. 4A and 4B). In contrast, NHEK cells inoculated with *T. denticola* JCM 8225 showed decreased intensity of ZO-1 (Fig. 4C), suggesting disruption of ZO-1 protein at the tight junction.

#### Co-inoculation of two *Treponema* species with NHEK cells

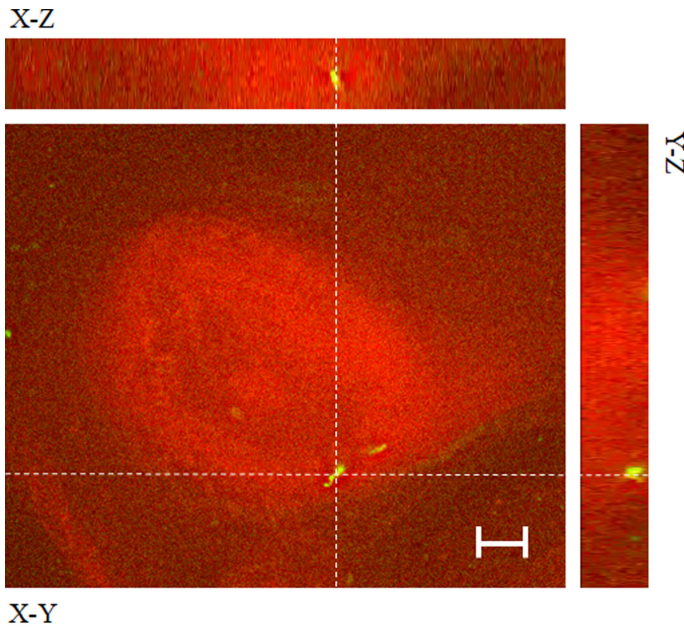
Although single inoculation of the live *T. phagedenis* strain examined did not change the TER values during the experimental period, as shown in Fig. 2, inoculation of *T. phagedenis* together with live *T. denticola* resulted in a significant decrease ( $P < 0.05$ ) of TER after 48 hr of incubation, as well as after single inoculation of live *T. denticola* (Fig. 5). However, the TER values did not vary according to the combination of live *T. phagedenis* and heat-killed *T. denticola* during the experimental period, as was seen for single inoculation of live *T. phagedenis* (Fig. 5). Furthermore, counting of fluorescence-labeled *T. phagedenis* cells demonstrated



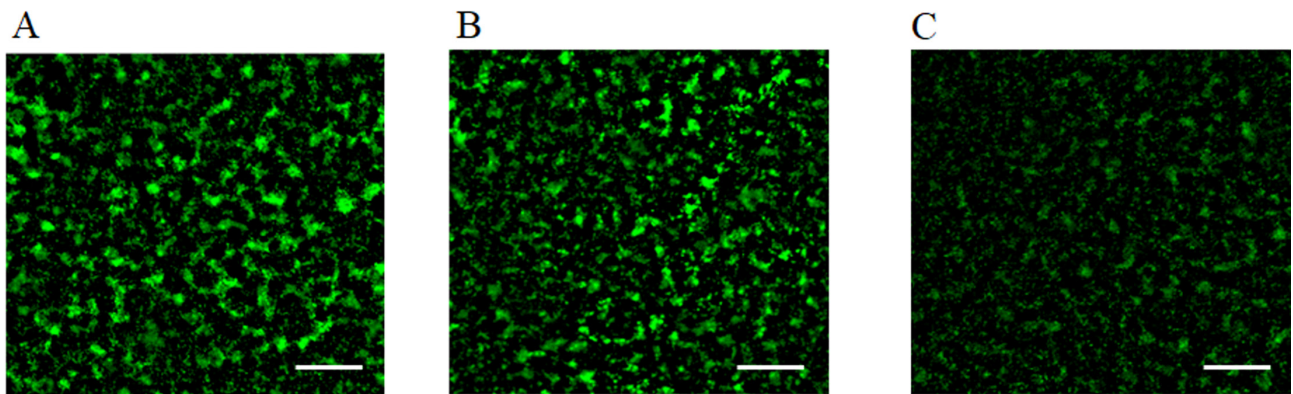
**Fig. 1.** Casein hydrolysis by *Treponema* isolates on modified skim milk agar plates; *T. denticola* JCM8225 (A), *T. phagedenis* ATCC27087 (B), *T. phagedenis* papillomatous digital dermatitis isolated strain YG3903R, HT201, CH9, HD26R, HD26-67, HD27-4, HD22R-11, IZ6-2, HG42, HD21R-R7 (C–L), respectively.



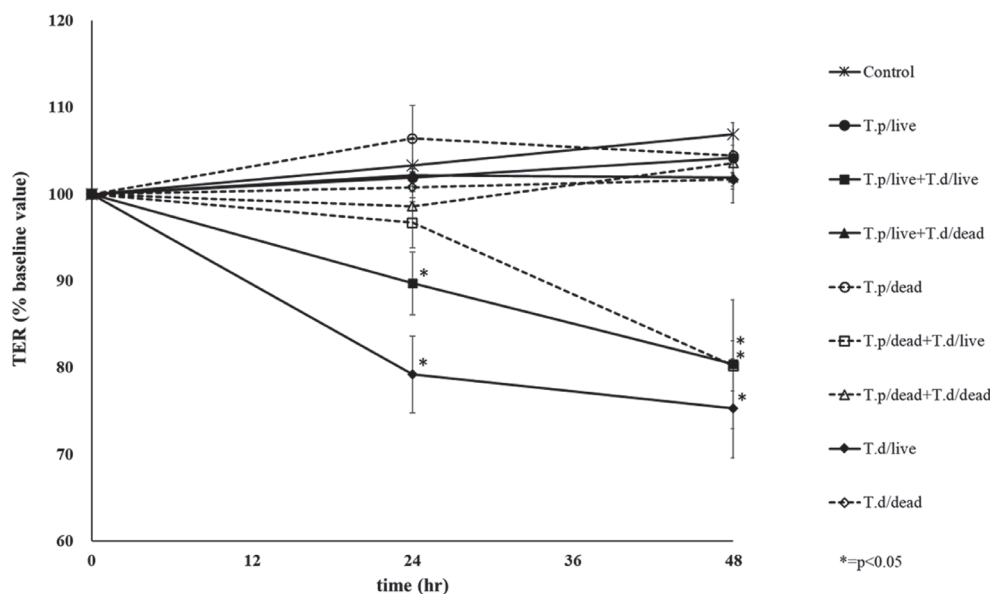
**Fig. 2.** Transepithelial electrical resistance (TER) in normal human epidermal keratinocytes cells infected with *Treponema* sp. strains. (A) TER was determined at 0, 6, 12, 24, and 48 hr post-infection. The values are expressed as the percentage of TER immediately before bacterial inoculation. The values are the mean (n=3) ± standard deviation. \*,  $P < 0.05$ . (B) Detection of *Treponema* sp. strains that migrated from the apical to the basolateral side using *Treponema*-specific PCR with DNA extracted from the basolateral medium. P, positive control for *Treponema*-specific PCR. (C) Microscopy observation of xsp. strains that migrated from the apical to basolateral side 24 hr after inoculation. (a) *T. phagedenis* YG3903R strain. (b) *T. denticola* JCM8225 strain. Bar=20  $\mu\text{m}$ .



**Fig. 3.** Three-dimensional images of normal human epidermal keratinocytes cell monolayers infected with *Treponema phagedenis* strain CH9 at 24 hr after inoculation using a fluorescence microscope (Z-axis scans, XY plane). Cytoskeletal actin is stained red with rhodamine phalloidin. Papillomatous digital dermatitis isolate *T. phagedenis* strain CH9 is stained green using an anti-*T. pallidum* antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. The scale bar represents 5  $\mu\text{m}$ .



**Fig. 4.** Immunofluorescence analysis of ZO-1 protein of normal human epidermal keratinocytes (NHEK) cells after inoculation of *Treponema* sp. (A) control NHEK cells without *Treponema* sp. (B) Exposure of NHEK cells to  $2 \times 10^8$  cell/ml of *T. phagedenis* strain YG3903R for 24 hr. (C) Exposure of NHEK cells to  $2 \times 10^8$  cell/ml of *T. denticola* strain JCM8225 for 24 hr image magnifying was taken at 40 $\times$ . The scale bars represent 50  $\mu\text{m}$ .



**Fig. 5.** Transepithelial electrical resistance (TER) in normal human epidermal keratinocytes cells infected with live and dead *Treponema* sp. strains: *T. p*; *T. phagedenis* YG3903R strain, *T. d*; *Treponema denticola* JCM8225 strain. TER was determined at 0, 24, and 48 hr post-infection. The values are expressed as the percentage of TER immediately before bacterial inoculation. \*,  $P < 0.05$ . Bars, standard errors.

that the number recovered from the basolateral medium was significantly increased by inoculation with live *T. denticola* ( $P < 0.05$ ) (Fig. 6) relative to single inoculation of *T. phagedenis* or co-inoculation with heat-killed *T. denticola*.

## DISCUSSION

PDD is known to be a polymicrobial disease, and a variety of bacterial species have been found in the lesions by culture-dependent and -independent analyses [7, 9]. Many studies have demonstrated that multiple *Treponema* species are consistently present in the lesions [2, 11, 21]. Therefore, these treponemes are considered crucial agents in the pathogenesis of the disease. However, since it has not been proved that these organisms satisfy Koch's postulates, the disease etiology is not yet fully understood.

We have demonstrated that *T. phagedenis* is one of the species with the highest populations in PDD lesions in Japan, and have developed an effective and simple two-step culture technique for the organism [28]. Since immunohistochemical examinations showed that *T. phagedenis* was present in both the superficial and deeper layers of the epidermis [8], we considered that the organism might have an ability to invade the epidermal cell barrier. Electron microscopy of *T. phagedenis* strain HT201 showed that it had eight axial flagella attached to each end, conferring motility. The motility of treponemes may allow them to move toward anaerobic environments and optimal sites in the host, thus improving their survival. However, in the present study, the casein hydrolysis test revealed no proteolytic activity among the *T. phagedenis* strains examined. Similarly, in our previous study of the biochemical characteristics of *T. phagedenis*, none of the strains examined showed trypsin and chymotrypsin activities [28]. These results suggest that *T. phagedenis* may invade dermal tissue from wounds in the foot skin, regardless of whether it possesses proteolytic enzyme(s) and/or exploits the proteases produced by other co-infecting bacteria.

The possible pathways used by *T. phagedenis* to penetrate the dermal epithelial layer are considered to be transcellular and/or intercellular. The ability of *T. phagedenis* strains to translocate across the epithelial cell barrier was examined by measuring TER in polarized NHEK cell monolayers. The NHEK cell line is able to form tight junction tissue layers and prevent ions from permeating between the apical and basolateral compartments when forming a confluent cell sheet. To determine how *T. phagedenis* is able to invade dermal tissue *in vitro*, we used a NHEK cell line because it is difficult to keep bovine epidermal cells established by primary culture. TER is used frequently as an index of tight junction permeability and monolayer integrity. When bacteria use the intercellular route from the apical to basolateral cell surface, the tight junction is disrupted and TER decreases [5]. The present study showed that *T. phagedenis* penetrated from the apical to basolateral area of NHEK cells on the transmembrane without any variation in the TER value during the observation period (Fig. 2). Furthermore, the presence of *T. phagedenis* in the NHEK cells was also confirmed by fluorescence microscopy (Fig. 3), suggested that the organism invaded via a transcellular pathway. We inoculated  $2 \times 10^8$  cells of *T. phagedenis* to the cell culture with high multiplicity of infection (MOI). Since *Treponema* species are strict anaerobes which are difficult to cultivate and maintain in culture media under microaerobic conditions for tissue culture, the high MOI is required to ensure that bacteria used in this study could infect the cell culture.

The inoculated *T. phagedenis* strains were confirmed in the basolateral compartment of the cell culture insert by microscopic observation and PCR. To confirm whether the membrane insert system was working, we employed *T. denticola*, which is able to penetrate cells by intercellular passage due to production of the protease, dentilisin [6]. As expected, the TER index decreased after inoculation with the live *T. denticola* bacteria, and the organisms were detected by microscopy and PCR in the basolateral medium. In contrast, the TER index did not vary after inoculation of the heat-killed bacteria, and neither the bacterium nor the PCR amplicon was detected in the basolateral compartment of the cell culture insert. These suggests that this system for assay of epithelial barrier integrity worked well.

Tight junctions act as a barrier between cells to prevent leakage of a variety of molecules [6]. ZO-1 has been demonstrated to interact with the transmembrane protein occludin, a second tight junction-specific membrane-associated guanylate kinase homolog, ZO-2 and F-actin [20]. Since *T. phagedenis* penetrates NHEK cells by the transcellular route, we investigated whether tight junction proteins such as ZO-1 are disrupted. For this purpose, tight junction protein ZO-1 of NHEK cells was subject to immunofluorescence staining before and after inoculation of *T. phagedenis*, and this revealed no change in the fluorescence intensity. However, inoculation of *T. denticola* decreased the staining of ZO-1 protein (Fig. 4), suggesting loss of the ZO-1 protein

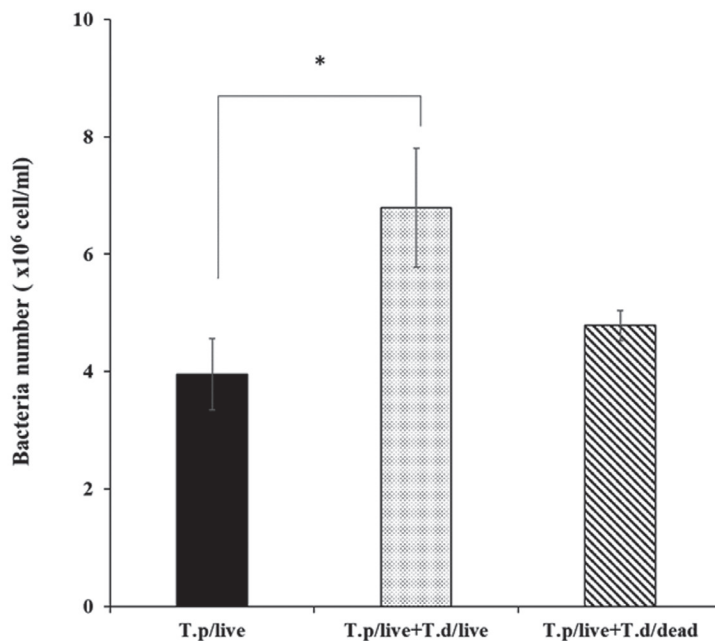


Fig. 6. The number of *Treponema phagedenis* strain YG3903R (*Tp*) cells that migrated from the apical to the basolateral side: *Td*; *Treponema denticola* strain JCM8225. The number was determined at 48 hr post-infection. \* $P < 0.05$ . Bars, standard errors.

at the tight junction. These findings further supported the possibility of transcellular translocation by *T. phagedenis*.

Next, we further investigated whether the translocation of *T. phagedenis* was accelerated by co-inoculation with live *T. denticola*, which is able to disrupt the tight junctions of dermal epithelial cells through proteolytic activity. As expected, the number of *T. phagedenis* bacteria on the basolateral side increased after co-inoculation with live *T. denticola*, but not with heat-killed *T. denticola*. These findings may explain why multiple treponemes exist in the same PDD lesion. If such a bacterial community is able to compensate for the lack of pathogenicity of bacteria present in PDD lesions, it would confer a survival advantage on the bacteria, thus causing illness and/or sustaining the infection. These present findings may provide a new insight for better understanding the pathogenetic mechanism of polymicrobial infections. However, since a variety of bacteria exist in the lesion and may be associated with PDD pathogenesis, the pathophysiological mechanism of the disease may be complex and difficult to elucidate. To better understand the pathology of PDD as a polymicrobial infection, further approaches will be required.

These observations demonstrate that *T. phagedenis* strains isolated from PDD lesions have the potential to pass through dermal tissue transcellularly *in vitro*. Furthermore, co-infection with *T. denticola* producing proteolytic activity accelerated the penetration of *T. phagedenis* across dermal epithelial cells. The present findings may help to explain how *T. phagedenis* is able to reach the deeper tissue in PDD lesions.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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