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Full Length Research Paper

Monitoring of a novel bacterium, *Lactobacillus thermotolerans,* in chicken intestine by fluorescence *in situ* hybridization (FISH)

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We successfully established fluorescence *in situ* hybridization (FISH) method for specific detection and enumeration of a novel bacterium, *Lactobacillus thermotolerans*, in chicken feces. The specific FISH probes were designed based on the *L. thermotolerans* 16S *rRNA* gene sequences, and these sequences were compared to those of all available 16S *rRNA* genes in the GenBank database. The assay, targeting 16S *rRNA* gene, was evaluated using DNA from a pure culture of *L. thermotolerans*, DNA from the closely related bacteria *Lactobacillus mucosae* DSM 13345[T] and *Lactobacillus fermentum* JCM 1173[T], and DNA from other lactic acid bacteria in quantitative experiments. The assay was then applied to two individual chicken trials. In trial 1, the cell population of *L. thermotolerans* ranged from 1.6×10^6 to 3.4×10^8 cells/g feces and from 2.6×10^7 to 3.6×10^8 cells/g cecal content. In trial 2, *L. thermotolerans* had also almost similar concentration (2.0×10^6 to 3.4×10^8 cells/g feces and 2.7×10^7 to 2.9×10^8 cells/g cecal content). We were not able to detect any bacterial cells at day one in both the trials. The results suggest that the newly developed FISH technique might be used for monitoring *L. thermotolerans* in the chicken intestine despite of its low sensitivity.

Key words: *Lactobacillus thermotolerans*, fluorescence *in situ* hybridization (FISH), probiotic, chickens.

INTRODUCTION

Lactobacillus thermotolerans, a novel thermotolerant species, was isolated from chicken feces collected in Thailand (Niamsup et al., 2003). The bacterium is characterized as facultatively anaerobic, Gram-positive, catalase-negative, non-motile and non-spore-forming rods. Lactic acid bacteria have been used for centuries in the preparation and processing of foods and beverages, and are nowadays used in numerous fermentation processes. As well as silage manufacture, they are used in the manufacture of fermented dairy products, in the production and preservation of sausages and meat, in the fermentation of fruits and vegetables.

Over the last two decades, probiotics, which includes *Lactobacillus* cultures, have been used as an alternative to antibiotics in animal production. However, inconsistent results of using probiotics in animal production have been a constraint to the promotion of their uses. This may be due to differences in microbial species or strains of micro-organisms used or the methods of preparing the supplement (Jin et al., 1998). For the evaluation of the usefulness of probiotics strain, a reliable detection method is required.

Fluorescence *in situ* hybridization (FISH) with specific 16S rRNA-based oligonucleotide probes has proven to be a useful tool for the identification of single cells within complex ecosystems (Harmsen et al., 2000; Licht et al.,

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Abbreviations: FISH, Fluorescence *in situ* hybridization; **DAPI,** 4', 6'-diamidino-2- phenylindole.

Table 1. Specificity test of the newly designed FISH probe for *L. thermotolerans* in FISH assay of pure culture of various species.

Organism	Strain[a]	Hybridization
Target strain		
L. thermotolerans	JCM 11425^T	+
Negative controls		
Lactobacillus aviarius subsp. *Araffinosus*	JCM 1044	-
L. aviarius subsp. *Aviaries*	JCM 5666	-
Lactobacillus crispatus	JCM 5810	-
Lactobacillus gallinarum	JCM 2011	-
Lactobacillus acidophilus	JCM 1132	-
Lactobacillus casei	JCM 1134	-
Lactobacillus helveticus	JCM 1120^T	-
L. mucosae	JCM 12515^T	-
L. fermentum	JCM 1173^T	-
Enterococcus cecorum	JCM 8724	-
Lactobacillus lactis subsp. *Lactis*	JCM 1158	-
Lactobacillus agilis	JCM 1187^T	-
Lactobacillus brevis	JCM 1059^T	-
Lactobacillus ruminis	JCM 1152^T	-
L. salivarius	JCM 1150^T	-
Lactobacillus animalis	JCM 5670^T	-
Lactobacillus equinum	JCM 7876	-
Lactobacillus pentosus	JCM 1588	-

[a]Strains are from the Japan Collection of Microorganisms (JCM).

1996). In the last decade, FISH became the method of choice for the direct detection and identification of microorganisms in natural environments (Amann and Schleifer, 1995). Moreover, FISH is a recognized tool for the specific and sensitive identification of target organisms within complex microbial communities (Amann and Schleifer, 1995).

Therefore, in this study, the detection and enumeration of *L. thermotolerans* in chicken intestine was investigated first time by FISH using newly designed 16S rDNA targeted oligonucleotide probe.

MATERIALS AND METHODS

Bacterial strains and culture conditions

L. thermotolerans (JCM 11425^T) and 18 reference strains were used in this study (Table 1). *L. thermotolerans* was grown anaerobically in deMan, Rogosa and Sharpe (MRS) medium (Difco) for 13 h at 42°C and the reference strains were grown in the same medium for 13 h at 37°C.

Animal management and sampling

Ten layer chicks (Hyline, one day old) were obtained from Hokuren Federation of Agricultural Cooperatives, Hokkaido, Japan. They were divided into two groups (trials 1 and 2, as replicate), kept in an individual room of a wooden box and reared for a period of three weeks at Animal Experimental House of Hokkaido University. Chicks were fed *ad libitum* a commercial diet containing crude protein 200 g/kg and energy 2950 kcal/ kg. Water was available all the time of the experiment. Fresh feces were collected from individual chicks in both groups at days one, seven, 14 and 21 using sterile spatula. After collection, the feces were placed in falcon tubes, which were kept on ice until further use. Chicks were sacrificed at day 21 and their cecal were collected. Then the cecum was opened longitudinally and collected the contents.

Fecal sample (2 g) diluted 10 times with 1× phosphate-buffered saline (PBS) buffer and centrifuged at low speed (200 × g, 4°C, 5 min). Then the pellet was washed with 1× PBS buffer three times and the supernatant was pooled with previous supernatant. After that, the supernatant was centrifuged at high speed (10000 × *g*, 4°C, 2 min) followed by washing with 1× PBS buffer three times. Then 3× PBS buffer was added with the pellet and treated with lysozyme (1 mg/ml) and added fixative solution. Then the cell was ready for FISH.

Design and specificity of the FISH probe

The specific probe was designed based on 16S rDNA of *L. thermotolerans* sequences and have been checked for its specificity in all 16S rDNA sequences available in GenBank. The probe sequences were (*L. thermotolerans*) 5-'CCGTCCGCCAC-TCGTTGGGA-3', which was labeled with Cy3. Figure 1 shows the selected oligonucleotide sequences, their target sites and alignments which have two or more mismatch with target sites. We also tested the specificity of the probes directly in a FISH

Probe S-S-Lthe-113	3′GGCAGGCGGTGAGCAACCCT5′ 5′CCGTCCGCCACTCGTTGGGA3′
Tm =76.5°C Target	
Lactobacillus thermotolerans JCM 11423 (AF 308146)	....
L. thermotolerans JCM 11424 (AF 308147)	....
L. thermotolerans JCM 11425^T (AF 317702	....
L. thermotolerans JCM 11426 (AF 317703)	....
L. thermotolerans JCM 11427 (AF 317704)	....
L. ingluviei KR3 LMG 20380^T	....
L. mucosae (DSM 13345^T)	..............C...T.
L fermentum (JCM 1173^T)	..................CG
L. reuteri (JCM 1112^T)	GT...CA.............
L. vaginalis (JCM 9505^T)	.....CA..G..........

Figure 1. Selected oligonucleotide sequences, their target sites and alignments which have two or more mismatch with target sites.

assay using pure culture of *L. thermotolerans* and reference strains (Table 1).

In situ hybridization

Bacterial cells (10^{10}/ml) were treated with lysozyme at a concentration of 1 mg/ml at 37°C for 15 min and fixed in 4% paraformaldehyde in phosphate buffer (pH 7.2) for 16 h. *In situ* hybridizations were performed as described by Amann et al. (1990) with some modifications. In 8 µl of hybridization buffer, the solution contains 20 mM Tris-HCl (pH 7.2), 0.9 M NaCl, 0.1% (w/v) and 20 to 60% formamide with 1 µl of probe solution at 46°C for 16 h in an equilibrated sealed moisture chamber. Then a stringent washing step was performed at 48°C for 20 min in 50 ml of pre-warmed washing buffer (20 mM Tris-HCl (pH 7.2) containing 0.9 M NaCl). The washing buffer was removed by rinsing the slides with cold distilled water followed by drying and stained with 4',6'-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. The slides were then rinsed briefly in distilled water, allowed to air dry, and mounted with Vectashield (Vector Laboratory), and the fluorescent cells in the samples were visualized with an Olympus BX50 epifluorescence microscope (Olympus Optical Company, Ltd. Tokyo, Japan) with filter set specific for DAPI and Cy3. We counted the cells manually, using 10 random microscopic fields per sample.

Total count of bacterial population by DAPI

The total cell number in pure culture (*L. thermotolerans* cells/ml) and in feces were counted after staining with DAPI solution (Wako Pure Chemicals Industries, Ltd. Osaka, Japan) for 5 min at room temperature. The cell was examined under an Olympus BX50 epifluorescence microscope (Olympus Optical Company, Ltd. Tokyo, Japan). DAPI signal was captured in 10 random microscopic fields.

RESULTS

Probe design and specificity

To detect, enumerate and visualize *L. thermotolerans* in chicken feces and cecum epithelium, a specific 16S rDNA-targeted probe was designed based on 16S rDNA of *L. thermotolerans* sequences and has been checked for its specificity in all 16S rDNA sequences available in GenBank. The discrimination of *L. thermotolerans* with reference strains were made by direct FISH assay (Table 1). Table 1 shows that after hybridization, there were no hybridized cells in negative control. Moreover, the specificity was also checked against pure culture of *L. thermotolerans* (Figure 2). Figure 2 shows that after hybridization with pure culture, we were able to capture almost all the cells from DAPI staining. In particular, we did not find any hybridized cells in reference strains except few signals in *Lactobacillus fermentum* and *Lactobacillus mucosae*, the most closely related strains using 20% formamide. To overcome this problem, we repeated the hybridization trials using 20, 30, 40, 50 and 60% formamide. After such hybridization, we could not find any signal of *L. fermentum* and *L. mucosae* at 60% formamide. Thus, hybridization conditions for the discrimination of reference strains from target strain were established.

Enumeration of bacteria in fecal and cecal sample

Cell population of *L. thermotolerans* in chicken feces and cecum of individual chickens in two trials monitored by FISH is shown in Figure 3. In trial 1, the cell population of *L. thermotolerans* ranged from 1.6×10^6 to 3.4×10^8 cells/g feces and from 2.6×10^7 to 3.6×10^8 cells/g cecal content. In trial 2, *L. thermotolerans* had also almost similar concentration (2.0×10^6 to 3.4×10^8 cells/g feces and 2.7×10^7 to 2.9×10^8 cells/g cecal content). We were not able to detect any cells at day one in both trials. Table 2 shows the mean counts of *L. thermotolerans* of two trials. In general, chicks had an increasing tendency of *L.*

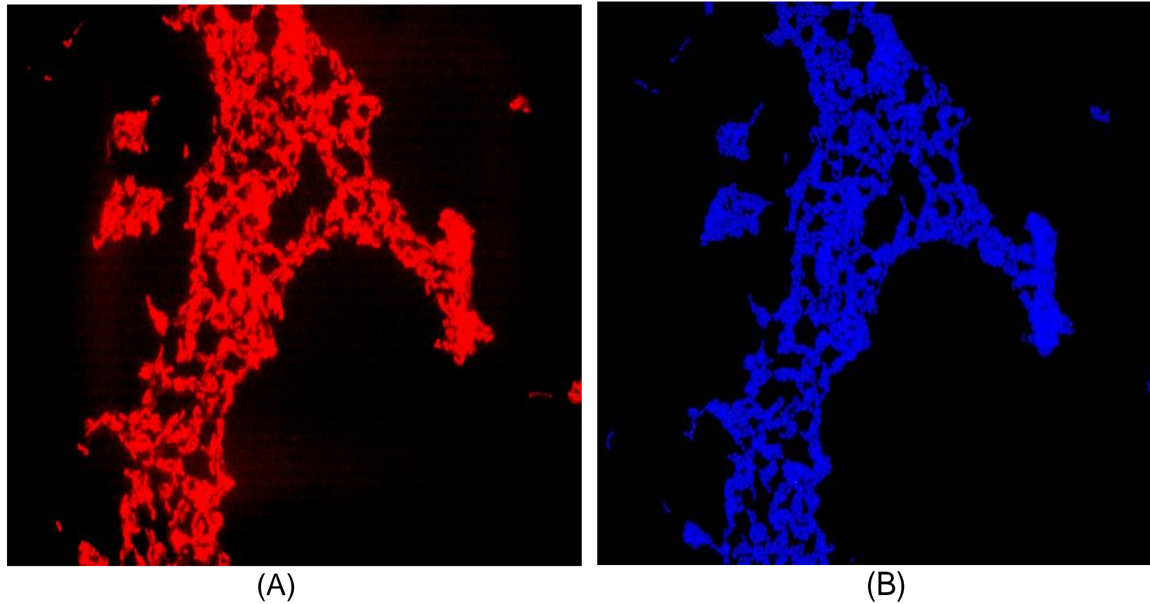

Figure 2. Checking of probe specificity. FISH image using Cy3 labeled newly designed probe for *L. thermotolerans.* (A) and DAPI stained cells of *L. thermotolerans* (B).

thermotolerans cells from days 7 to 21. The total cell populations were around 10^{10} to 10^{11} cells/g feces or cecal content as determined by DAPI staining. Thus, in percentage, *L. thermotolerans* ranged between 0.0011 to 0.0685% for trial 1 and 0.0068 to 0.10% for trial 2 in fecal and cecal samples against total cells in this study.

DISCUSSION

Molecular methods have facilitated culture-independent studies of gastrointestinal (GI) tract microbes. The value of molecular methods for studying GI tract microbes is therefore immense. Recent advances in rRNA-based molecular techniques make it possible to identify different bacterial populations in environmental samples without prior cultivation (Kreader, 1995; McCartney et al., 1996). FISH has been extensively used in ecological studies of bacteria (Rice et al., 1996). This technique has been used to quantify bifidobacteria in the human gut (Langendijk et al., 1995). Depending on the probes chosen, FISH can be used to detect bacteria on different phylogenetic levels. Specific oligonucleotide probes and primers have been designed for many bacterial species which are known to be present in the intestinal tract (Harmsen et al., 1997; Hertel et al., 1993). However, considering the importance of FISH technique, we designed a new probe for the enumeration of *L. thermotolerans* in chicken feces in order for better understanding of the ecology of the chicken intestine.

Specificity of the new probe was checked by direct FISH assay using pure culture of *L. thermotolerans* and some strains that are normal member of the chicken microbiota (Figure 1 and Table 1). We did not find any signal in reference strain after direct FISH assay (Table 1). This suggests that the probe is specific to *L. thermotolerans.* Moreover, Figure 2 shows that the newly designed probe is highly specific to target strain that can capture almost all the DAPI stained cells. However, to overcome some positive signal from the most closely relatives, *L. fermentum* and *L. mucosae*, we did the repeated trials to make the discrimination with the target strain.

To evaluate our newly established FISH assay, we applied this method to real chicken samples in different time points in two different replicate trials. We successfully enumerated the cell population of *L. thermotolerans* in feces sample on days seven, 14, 21 and in cecum sample on day 21 in both trials (Table 2). However, we did not obtain any signal of *L. thermotolerans* on day one in both trials (Table 2 and Figure 3). This may be due to the fact of low density of cells in the samples and FISH counts were often just above detection limit of $10^{5.8}$ (Harmsen et al., 2002). The cell population of *L. thermotolerans* had an increasing tendency from days 7 to 21 (Figure 3), which corresponds to the monitoring of a single strain, *Lactobacillus salivarius,* in chicken feces (Pham, 2003). The cell population in cecum was slightly higher than that in previous study (Fuller, 1971) as determined by culture method. Moreover, the cell population monitored by FISH technique was little bit higher (10^6 to 10^8 versus 10^6 to 10^7) compared to that of our real time monitoring (Selim et al., 2005). This may be due to the counting error of

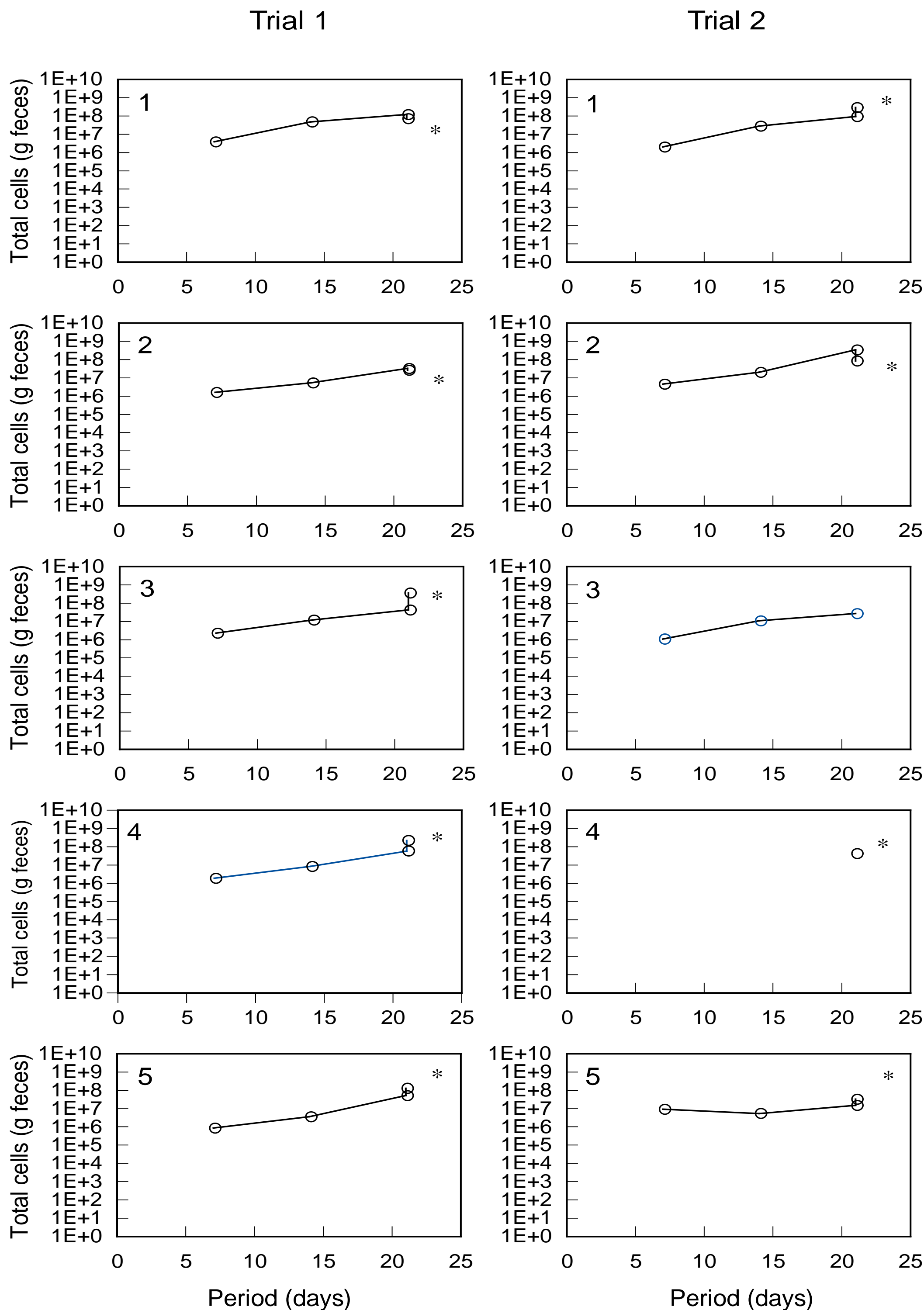

Figure 3. Changes in the total number of *L. thermotolerans* cells at different time points for individual chickens (*cells per g cecal content), as detected by FISH. The results of trials 1 and 2 are shown. Each trial was conducted using five chicks.

FISH technique.

FISH technique is comparatively cheaper to that of other molecular technique such as real time-polymerase chain reaction (RT-PCR) which involves huge investment.

Table 2. Mean counts of total cells and total *L. thermotolerans* in chicken feces and cecum as determined by DAPI (total cells) and by FISH (*L. thermotolerans*).

Trial 1	Cells/g feces)				
	Day 1 (n = 5)	**Day 7 (n = 5)**	**Day 14 (n = 5)**	**Day 21 (n = 5)**	**Cecum (n = 5)**
Total cells	$2.3 (2.7) \times 10^{10}$	$1.5 (1.0) \times 10^{11}$	$2.4(1.2) \times 10^{10}$	$8.9(7.4) \times 10^{10}$	$8.0 (12) \times 10^{11}$
L. thermotolerans[a]	ND	$1.7 (1.5) \times 10^{6}$	$1.5 (1.8) \times 10^{7}$	$6.1 (3.2) \times 10^{7}$	$1.6 (1.3) \times 10^{8}$
Percentage (%)[b]		1.1×10^{-3}	6.2×10^{-2}	6.8×10^{-2}	2.0×10^{-2}
Trial 2					
Total cells	$1.3 (1.5) \times 10^{10}$	$5.4 (7.1) \times 10^{10}$	$1.8(0.7) \times 10^{10}$	$1.5(1.8) \times 10^{11}$	$2.8(4.2) \times 10^{11}$
L. thermotolerans[a]	ND	$3.7 (1.9) \times 10^{6}$	$1.3 (1.1) \times 10^{7}$	$1.5 (1.7) \times 10^{8}$	$9.4 (11) \times 10^{7}$
Percentage (%)[b]		6.8×10^{-3}	7.2×10^{-2}	1.0×10^{-1}	3.3×10^{-2}

ND, Not detected; [a]values in the parentheses indicate the standard deviation; [b]percentage of *L. thermotolerans* relative to the total number of cells.

In this study, we could easily monitor *L. thermotolerans* in chicken feces. In spite of low sensitivity, applying FISH with the described probe has provided new information on the composition of *L. thermotolerans* in fecal and cecal chicken sample. However, this bacterium may be a potential candidate for poultry probiotic as it exists at a good number in the intestine.

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