Simulated gastrointestinal environment affects sustainability of adhesion factors in *Lactobacillus reuteri* DSM 20016T.

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**Abstract**

Many species/strains of *Lactobacillus* colonize in the gastrointestinal tracts of animals including human, and some of them are used as probiotics. *Lactobacillus reuteri* is frequently isolated from the gut of wide range of animals, and thus as a model of the intestinal microbiota, investigations on mechanism of their colonization are underway. While previous studies have described some *L. reuteri* adhesins, comparative analysis of those proteins have rarely been pursued. In the current study, *L. reuteri* DSM 20016T was recruited and its potential adhesion factors were evaluated. After *L. reuteri* DSM 20016T was exposed in simulated gastrointestinal conditions, the remaining adhesins were detected. As a result, the adhesins were relatively resistant to simulated gastric juice but were sensitive to simulated intestinal fluid. RT-qPCR was used to examine changes in the expression of the proteins, and results indicated that the expression of the adhesins tended to be promoted in comparison with their expression during normal culturing. In conclusion, the *in vitro* assay of this study suggested that cell-surface adhesins of *L. reuteri* could be damaged by digestive process but the bacterium may be capable of reproducing those proteins thereafter.

**Key words:** *Lactobacillus reuteri*, adhesin

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**Introduction**

Many species/strains of *Lactobacillus* reside in the gastrointestinal tracts of animals and are found primarily in the upper gastrointestinal tract to the small intestine¹. In addition, some *Lactobacillus* spp. from the gut are used as probiotics. Taken orally, these probiotics are not permanently but temporary remain in the gastrointestinal tract for a while². Lactobacilli have developed unique colonization factors depending on their own niches. Several research groups have previously identified and described adhesins, the proteins potentially allow bacteria to colonize at the intestinal mucosa. For instance, a ABC transporter-associated protein, MapA, is capable of binding to Caco-2 cells and FbpA mediates adhesion to extracellular matrix³,⁴. In other cases, some housekeeping proteins could localize outside of the bacterial cells and involved in the attachment to the host cells⁵⁻⁷. A single strain possesses several adhesins, thus even if one of those adhesins is inactivated the strain will not necessary lose all of its ability to adhere to host⁸. Various factors presumably play an integral role in allowing lactobacilli to colonize, thus considerable effort should be made to discern those factors.

Previously, a comprehensive analysis by Frese *et al*. showed some of its mechanisms of host specialization and colonization⁶,⁹. Their analysis indicated that different strains of *L. reuteri* colonize certain hosts, suggesting that strains do not adhere to the gut of a host in the same manner. For example, large surface proteins likely involve in the host specificity among *L. reuteri* inhabit rodents, while those bacteria in human have few
of such proteins\(^8\).

In the current study, we are interested in how the adhesins of \textit{L. reuteri} DSM 20016\(^T\) change in the gastrointestinal tract. While changes of gene-expression in responses to substances originated from gastrointestinal tract was reported in few \textit{Lactobacillus} strains\(^16\), those in \textit{L. reuteri} has not been unclear. Since this strain is a human isolate, it is considered to be an appropriate model of probiotics. According to the genome sequence, \textit{L. reuteri} DSM 20016\(^T\) has four known adhesins, MapA, FbpA, EF–Tu, and GroEL at least. Thus, this study attempted to examine how digestive processing could affect those preexisting adhesins and the expression of the corresponding genes of \textit{L. reuteri} DSM 20016\(^T\) under simulated gastrointestinal conditions.

### Materials and Methods

1. **Bacterial cultures and Caco–2 cells**

\textit{Lactobacillus reuteri} DSM 20016\(^T\) was grown in MRS medium (Difco) and cultured anaerobically at 37°C. \textit{Escherichia coli} M15 [pREP4, encoding lacI repressor gene] and \textit{E. coli} SG13009 [pREP4] (Qiagen) were used for production of His-tagged protein. Recombinant \textit{E. coli} was inoculated in LB medium and cultured with aeration and agitation at 37°C. Ampicillin (100 \(\mu\)g/ml), kanamycin (25 \(\mu\)g/ml), and IPTG (Isopropyl \(\beta\)-D-1-thiogalactopyranoside, 1 nM) were added as needed. Dulbecco’s modified Eagle’s medium (DMEM, SIGMA) containing 10% fetal bovine serum (Thermo), 2 mM L-glutamine (Thermo), penicillin (100 U/ml), streptomycin (100 \(\mu\)g/ml), and non-essential amino acids (Thermo) was used to culture Caco–2 cells.

2. **Purification of His-tagged FbpA, EF–Tu, and GroEL proteins and preparation of antisera**

Using chromosomal DNA of \textit{L. reuteri} DSM 20016\(^T\) as a template, the gene fragments \textit{fbpA} (1,683 bp), \textit{tuf} (1,191 bp), and \textit{groEL} (1,629 bp) were amplified using PCR. Amplified gene fragments were digested with \textit{Bam} HI and \textit{Sal} I, ligated into pQE30 and pQE31 (Qiagen), and then introduced into \textit{E. coli} M15 and \textit{E. coli} SG13009. Plasmids were extracted from the transformants, and nucleotide sequences were verified by sequencing. Sequences of Cloning primers specific to \textit{fbpA}, \textit{tuf}, and \textit{groEL} are shown in Table 1. Recombinant \textit{E. coli} producing MapA was constructed in a previous study\(^9\). In accordance with the QIAexpressionist protocol, recombinant His-tagged proteins were produced and purified with affinity chromatography using Ni-NTA columns (Qiagen). Mice or rabbits were immunized with the prepared proteins and protein-specific antisera were prepared (Protein Purify Inc.).

3. **Binding of recombinant protein to Caco–2 cells**

Eighty-percent confluent Caco–2 cells were washed with PBS, and recombinant protein dissolved in PBS (final conc.: 500 \(\mu\)g/ml) was added to Caco–2 cells. The cells were then incubated at room temperature for 1 h. After the cells were washed, PBS containing a 1/1,000 dilution of antiserum was added and the cells were incubated at room temperature for 1 h. PBS containing a 1/500 dilution of Cy3-labeled anti-mouse or anti-rabbit IgG (SIGMA) was added to Caco–2 cells, and cells were incubated at room temperature for 30 min. Confocal laser scanning microscopy (CLSM) was used to identify adhesions molecules binding to the surface of the Caco–2 cells (FV 1200

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
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<tbody>
<tr>
<td>\textit{fbpA} forward</td>
<td>CAG GAT CCG ATG TCT TT TTT GAC GGC TTG TTT</td>
</tr>
<tr>
<td>\textit{fbpA} reverse</td>
<td>TAC GCA GTA AAA CAA TGG CGG CAG ACC</td>
</tr>
<tr>
<td>\textit{tuf} forward</td>
<td>GGG GAT CCA TGG CTG AAA AAG AAC ATG</td>
</tr>
<tr>
<td>\textit{tuf} reverse</td>
<td>TAT CCG ACA TCT TAG ACT AAG TCG ACC CGT</td>
</tr>
<tr>
<td>\textit{groEL} forward</td>
<td>CAG GAT CCG ATG GCA AAA GAA ATT AAG TTG</td>
</tr>
<tr>
<td>\textit{groEL} reverse</td>
<td>TAC CAT TTG ACT AAA ATT TAG TCG ACC CGT</td>
</tr>
</tbody>
</table>

\(\text{For RT-qPCR}\)

| mapA forward | TAA ATG GGA TTC GCT AAT TGC CG |
| mapA reverse | GCC CTT ACG GTC GTC GCT GCA |
| \textit{tuf} forward | CCT GGA CAC GCT GAC TAC |
| \textit{tuf} reverse | AAC CTG AGC AGC AAG AAC |
| \textit{groEL} forward | AAA GCT TTC AGG TGG GGT TGC CG |
| \textit{groEL} reverse | CAG CAC GAG TAG GTC AAG CA |
| 16S rRNA forward | AAG TCG GTG GCC TAA CTT TTA TG |
| 16S rRNA reverse | CTA CGG CTG CCT TGT TAC GAC TT |
| \textit{rpoD} forward | CTG GTG GAT TCG TCA GGC TAT TA |
| \textit{rpoD} reverse | TGG TAA CGG TTC AGG ACC TAA AT |
Recombinant protein dissolved in PBS was added to Caco–2 cells cultured to 80% confluence on 96–well microplates, and the cells were incubated at room temperature for 1 h. The cells were washed with PBS, and then PBS containing a 1/10,000 dilution of antisera was added to Caco–2 cells. The cells were incubated at room temperature for 1 h. The cells were washed, and then PBS containing a 1/50,000 dilution of HRP-labeled anti-mouse or anti-rabbit IgG was added to Caco–2 cells. The cells were then incubated at room temperature for 1 h. After the cells were rinsed with PBS, the cells were incubated at room temperature for 30 min. The cells were washed, and then Sure Blue™ TMB Microwell Peroxidase Substrate (1–Component) (KPL) was added and color developed. Absorbance was measured at 450 nm and 570 nm with a microplate reader.

4. **Inhibition of L. reuteri colonization in Caco–2 cells by recombinant protein or antisera**

An assay of colonization inhibited by recombinant protein, 80% confluent Caco–2 cells were washed with PBS (pH 7.5) and then recombinant protein dissolved in PBS was added to Caco–2 cells. The cells were then incubated at room temperature for 1 h. After the cells were rinsed with PBS, L. reuteri DSM 20016ᵀ that had been cultured for 20 h was prepared at a concentration of 2 × 10⁶ cells/ml and was added to the Caco–2 cells. The cells were then incubated at room temperature for 1 h. In an assay of colonization inhibited by antisera, antisera diluted 100-fold was added to the bacterium and the bacterium was incubated at 4°C for 1 h. The bacterium was washed and then added to Caco–2 cells, and the cells were incubated at 37°C for 1 h. Cells in both assays were washed with PBS and then 300 μl of a 0.1% methylene blue solution was added. The cells were then incubated at room temperature for 5 min. Cells were destained with PBS and then bacteria were counted under a light microscope (× 1000).

5. **Survival of L. reuteri DSM 20016ᵀ in culture medium containing digestive fluid**

L. reuteri DSM 20016ᵀ that was cultured for 20 h was harvested and rinsed with PBS. The bacterial cells were prepared at a concentration of 1 × 10⁹ cells/ml. MRS medium (pH 2.0) containing 0.3% peptin or 1% bile & 1% pancreatin was added to the bacterium. After incubation, the bacterium was cultured on MRS agar and colonies were counted.

6. **Persistence of bacterial surface protein after treatment with simulated digestive fluid**

L. reuteri DSM 20016ᵀ that was cultured for 20 h was harvested and prepared at a concentration of 2 × 10⁶ cells/ml. Simulated gastric juice (0.5% NaCl, 0.3% peptin, pH 2.0) or simulated intestinal juice (0.5% NaCl, 1.0% bile, 1.0% pancreatin, pH 6.5) was added to the bacterial suspension, and the resulting mixture was incubated at 37°C. Bacterial cells were recovered using centrifugation and suspended in methanol. This suspension was then droppedwise into a PVDF membrane. A 1/10,000 dilution of antisera was added to 1.0% BSA/PBS, and this antisera was incubated at room temperature for 1 h. PVDF membranes were washed with PBS and incubated for 30 min in a 1.0% BSA/PBS solution containing a 1/50,000 dilution of HRP-labeled anti-mouse or anti-rabbit IgG. Membranes were washed with PBS and then chemiluminescence was detected with Chemi Doc (BIO–RAD) using a Luminata™ Forte Western HRP Substrate (Millipore).

7. **Analysis of the expression of genes encoding adhesins**

L. reuteri DSM 20016ᵀ was cultured for 1 h in MRS medium (pH 6.5) containing 0.3% peptin, 1% bile & 1% pancreatin, or 0.05% porcine gastric mucin (Sigma-Aldrich). Total RNA was extracted using ISOGEN (Nippon gene), and cDNA was prepared via reverse transcription using the Super Script® VILO™ cDNA Synthesis Kit (Invitrogen). Quantitative real-time PCR (RT–qPCR) was performed for 50 cycles, 5 s at 98°C followed by 4 s at 60°C , using the One Step SYBR® Prime Script® RT–PCR Kit II (TaKaRa Bio). RNA polymerase sigma factor (rpoD) and 16S rRNA served as the internal controls. Sequences of RT–qPCR primers specific to mapA, fbpA, tuf, and groEL are shown in Table 1.
8. Statistical analysis

Experimental data were analyzed using Steel’s test, with $P<0.05$ indicating a significant difference.

Results

1. Adhesion of His–tagged proteins to Caco–2 cells

Purified His–tagged protein (His–MapA, His–FbpA, His–EF–Tu, or His–GroEL) was added to Caco–2 cells and individual proteins were labeled with specific antibodies or fluorescently labeled secondary antibodies. Cells were then observed under a confocal fluorescence microscope. Fluorescence was evident as a result of the addition of each protein, suggesting that the proteins bound to Caco–2 cells (Fig. 1–a). Disparities in binding due to the protein in question and differences in pH were noted. Quantitative measurement with ELISA indicated that each protein bound to Caco–2 cells in a concentration-dependent manner (Fig. 1–b). All of the proteins bound more actively to Caco–2 cells under acidic conditions (pH 5.0) than they did under neutral conditions (pH 7.5).

2. Competitive inhibition of adhesion by the adhesins of L. reuteri

The number of adherent L. reuteri DSMZ 20016T decreased significantly as a result of adding purified His–tagged proteins. An inverse correlation between the number of adherent bacteria and the
Fig. 2 Competitive inhibition of L. reuteri by adhesins or antibodies. Caco-2 monolayers were pre-incubated with purified adhesins (a, b, c and d) or antibodies (e) before adding L. reuteri DSM 20016T to the culture. The cells were also treated with mixture of all antibodies (Ab mix). Values represent Mean ± standard error (SE) (n = 20). *P < 0.05.

3. Survival curves and dot blot analysis of L. reuteri in medium at different concentrations

L. reuteri DSM 20016T was exposed to gastrointestinal fluids, and the viable cell count and the persistence of surface proteins were determined. A decrease in the viable cell count was noted after 30 min after the addition of artificial gastric juice, but substantial changes were not noted after 45 and 60 min (Fig. 3-a). After the addition of simulated intestinal fluid at a concentration of 0.3% or 1.0%, a decrease in the viable cell count was noted after 1 h, but substantial changes were not noted after 2 or 3 h (Fig. 3-c). The sensitivity of surface proteins to digestive fluid was determined using dot blot analysis. MapA, FbpA, and GroEL signals gradually disappeared after the addition of artificial gastric juice (Fig. 3-b). In contrast, an EF-Tu signal was detected 60 min after the addition of artificial gastric juice (Fig. 3-b). All of the surface protein signals disappeared immediately after addition of artificial intestinal fluid (Fig. 3-d).

4. Profile of adhesion gene expression by L. reuteri DSM 20016T in different culture media

L. reuteri DSM 20016T was exposed to gastrointestinal fluids, and changes in levels of expression of MapA, FbpA, EF-Tu, and GroEL were determined using RT-qPCR. Expression of mapA increased in the presence of pepsin and mucin in comparison to that in the control. However, levels of its expression changed little in the presence of bile salts and pancreatin. Expression of fbpA and tuf increased in the presence of pepsin, bile salts, pancreatin, and mucin in comparison to that in the control. Expression of groEL increased in the presence of pepsin, bile salts, and pancreatin in comparison to that in the control. However, levels
Fig. 3 L. reuteri DSM 20016T cells were exposed in simulated gastric juice (HCl/Pepsin) or simulated small intestinal juice (Bile/Pancreatin). Viable cells (cfu) at several time points were plotted (a and c). The adhesins were detected by dot blot assay after the treatment with either simulated gastric juice (b), or simulated small intestinal juice (d). N represents non-treated bacterial cells.

Fig. 4 Transcriptional profile of adhesin genes in L. reuteri DSM 20016T at different culture conditions. Relative mRNA levels were determined by RT-qPCR. Mean ± SE (n = 4). All tests (HCl/Pepsin, Bile/Pancreatin, and Mucin) were statistically significant (P < 0.05) in comparison with each controls (MRS).

of its expression changed little in the presence of mucin (Fig. 4).

Discussion

Over 100 trillion bacteria inhabit the gut, and these bacteria each have their own unique mechanisms of colonization. Lactobacillus strains in the gut are considered beneficial to the host and are also used as probiotics. Thus, determining their mechanism of colonization is crucial. The current study examined how 4 known adhesion factors of L. reuteri DSM 20016T were affected by simulated gastrointestinal conditions. This study cloned 4 protein and purified them as His–tagged proteins. Previous studies reported that these were adhesins of Lactobacillus spp. The current study found that all 4 of these protein adhere to Caco-2 cells in a
concentration- and pH-dependent manner. This finding supported previous studies indicating that _Lactobacillus_ spp. bind to cells in a pH-dependent manner\(^{10,11}\). The current study conducted an assay of competitive inhibition of the adhesion of _L. reuteri_ to Caco-2 cells by adhesins, and results indicated that the number of adherent bacteria decreased depending on the concentration of added protein. Unlike three other proteins, inhibition of the bacterial attachment by GroEL was not clear, thus contribution of the protein on this adhesion might be minor. In addition, an inhibition assay using adhesin-specific antiserum similarly revealed that antisera inhibited the adhesion of _L. reuteri_ to cells. These findings suggest that adhesins could play a role in the adhesion of _L. reuteri_ to intestinal epithelial cells.

Assuming that probiotics will be taken orally, the bacterium will presumably be exposed to substances such as acids, bile, and digestive enzymes. Thus, the current study measured the viable cell count and the persistence of adhesin when the bacterium was exposed to simulated digestive fluid in vitro. _L. reuteri_ DSM 20016\(^{T}\) was resistant to simulated gastric juice but sensitive to simulated intestinal fluid. _L. acidophilus_ NCFM is reported to have genes that render it resistant to bile acids (bile salt hydrolase genes\(^{12}\)), and those genes are found in _L. reuteri_ DSM 20016\(^{T}\), hence _L. reuteri_ DSM 20016\(^{T}\) is presumably somewhat resistant to bile acids. However, the bacterium had a low survival rate in the presence of pancreatin. Adhesins on the surface of the bacterium were relatively persistent in gastric juice but they were immediately digested in simulated intestinal fluid. Taken together, these findings presumably indicate that bacterial surface proteins and/or other cellular components are damaged by the digestive enzymes in pancreatin, leading the bacterium to lose its resistance to bile.

If cells survive despite damage to their cellular mechanisms due to digestive process, then those cells have the ability to regenerate. Thus, RT-qPCR was performed to examine changes in adhesin expression when the bacterium was on culture media containing gastrointestinal fluids. Results indicated that the expression of those adhesins tends to be promoted in comparison to their expression during normal culturing. A previous study reported that _L. plantarum_ binds to mucin, which upregulated the expression of _tuf_ gene\(^{13}\). Other studies have reported that the expression level of some adhesion factors increases when lactobacilli are exposed to bile acids\(^{14-16}\). Taken together, these findings suggest that _L. reuteri_ cells are damaged by the harsh conditions in the upper gastrointestinal tract but part of them could be recovered, allowing _L. reuteri_ to colonize. Further studies are needed to demonstrate that the possible events could be shown in _vivo_.

In conclusion, the _in vitro_ assay of this study suggested that cell-surface adhesins of _L. reuteri_ could be damaged by digestive process but the bacterium may be capable of reproducing those proteins thereafter.

**References**

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Lactobacillus reuteri DSM 20016T が持つ腸管粘膜定着因子の疑似的消化管環境における持続性

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Lactobacillus属細菌の一部は動物消化管に常在し、それらの一部はプロバイオティクスとして利用されている。Lactobacillus reuteri は様々な動物腸管内に分布し、腸内共生微生物のモデルとして定着機構の解明が進められている。これまで、L. reuteri の腸管粘膜定着因子として複数のタンパク質が報告されている。我々は L. reuteri DSM 20016T がもつ腸管粘膜定着因子が消化管環境でどのように変化し得るのかを調べることを目的とし、研究に着手した。疑似的な消化管環境に L. reuteri DSM 20016T を曝した結果、腸管粘膜定着因子タンパク質の発現に対する耐性が比較的高い一方、疑似的腸液に対しては感受性を示した。腸管粘膜定着因子の発現がどのように変化するかを RT-qPCR により調べた結果、それぞれの腸管粘膜定着因子は通常培養時に比べて遺伝子発現が促進される傾向にあることが示された。結論として、本研究の in vitro の試験において、L. reuteri の菌体および菌体表面に存在する腸管粘膜定着因子は消化作用によりダメージを受ける一方、それらの遺伝子発現は増強される得る可能性が示された。