

# Adhesion properties of a putative polymorphic fimbrial subunit protein from *Bifidobacterium longum* subsp. *longum*

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In our previous study, we found that the open reading frame *bl0675* in the genome of *Bifidobacterium longum* subsp. *longum* isolated from human feces encoded a novel putative fimbrial protein, was highly polymorphic, and had five variants (A, B, C, D, and E types). The aim of this study was to evaluate the affinity of these variants to porcine colonic mucins (PCMs). Protein-binding properties were examined using the recombinant BL0675 protein containing a C-terminal 6 × His tag (His-BL0675). Surface plasmon resonance analysis demonstrated that the His-BL0675 A type had strong affinity to PCMs ( $K_D = 9.82 \times 10^{-8}$  M), whereas the B, C, D, and E types exhibited little or no binding. In a competitive enzyme-linked immunosorbent assay, His-BL0675 A type binding was reduced by addition of mucin oligosaccharides, suggesting that the binding occurs via carbohydrate chains of PCMs. The localization of BL0675 to the *B. longum* subsp. *longum* cell surface was confirmed by western blot analysis using A type polyclonal antibodies. Bacterial adhesion of *B. longum* subsp. *longum* to PCMs was also blocked by A type-specific antibodies; however, its adhesion properties were strain specific. Our results suggest that the BL0675 variants significantly contribute to the adhesion of *B. longum* subsp. *longum* strains. The expression and the adhesive properties of this protein are affected by genetic polymorphisms and are specific for *B. longum* subsp. *longum* strains. However, further studies are required on the properties of binding of these putative fimbrial proteins to the human gastrointestinal tract.

**Key words:** adhesion, *Bifidobacterium longum* subsp. *longum*, fimbrial subunit protein, mucin, surface plasmon resonance

## INTRODUCTION

*Bifidobacterium* is one of the major genera of bacteria constituting the gastrointestinal (GI) microbiota in mammals, including humans [1]. The fecal microbiota of infants is characterized by high levels of bifidobacteria [2]. The abundance of bifidobacteria within the human gut decreases with age [3, 4]. Although the bifidobacteria in the adult colon comprise less than 10% of fecal microbes [5], their presence has been associated with beneficial health effects, such as boosting of immune

defenses [6–9] and prevention of infection by pathogens [8, 10].

The mucus layer covering the GI tract is the first line of contact of the intestinal bacteria with the host and provides a habitat for the microbiota [11, 12]. Adhesion is an important prerequisite for the colonization of bacteria in the intestinal mucosa and provides a competitive advantage in this ecosystem [13]. Fimbriae and pili have been established as key structures involved in colonization of the GI tract by intestinal bacteria, not only in pathogens [14] but also in commensal bacteria, including several *Bifidobacterium* strains. For example, sortase-dependent pili from the *Bifidobacterium bifidum* PRL2010 strain have been shown to bind epithelial Caco-2 cells and the extracellular matrix (ECM) proteins fibronectin and laminin [15], while type IVb tight adherence (Tad) pili have been proven to be essential for *Bifidobacterium breve* UCC2003 colonization of the murine gut [16, 17].

*Bifidobacterium longum* subsp. *longum* is one of the

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dominant bacterial species in the intestinal microbiota of adult humans [1, 18]. By sequencing the *B. longum* subsp. *longum* NCC2705 genome, Schell et al. [18] identified a cluster of three fimbria-associated open reading frames (ORFs) *bl0674*, *bl0675*, and *bl0676* possibly involved in fimbria formation. In a previous study performed using *B. longum* subsp. *longum* strains isolated from 89 human feces samples, we found that *bl0675* is highly polymorphic and encodes five variant types: A, B, C, D, and E [19]. Moreover, because the putative amino acid sequence of BL0675 shows 30% homology to that of FimA, the major component of type 2 glycoprotein-binding fimbriae of *Actinomyces naeslundii* [20, 21], we hypothesized that the five BL0675 variant types may have differing affinities to the carbohydrate chains in mucins. The aim of this study was to evaluate the binding affinity of the different types of BL0675 to porcine colonic mucins (PCMs).

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

*B. longum* subsp. *longum* isolates from human feces [19] were cultured in TOS propionate broth or agar (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) supplemented with 0.05% (w/v) L-cysteine hydrochloride monohydrate (Kanto Chemical, Tokyo, Japan) and 0.3% (w/v) L-ascorbic acid sodium salt (Wako, Tokyo, Japan) at 37°C under anaerobic conditions. *Escherichia coli* DH5 $\alpha$  and Rosetta 2 (Stratagene, La Jolla, CA, USA) were grown in Luria-Bertani (LB) broth at 37°C. Ampicillin (100  $\mu$ g/ml), kanamycin (30  $\mu$ g/ml), or chloramphenicol (30  $\mu$ g/ml) were added when necessary.

### Cloning and DNA sequencing

The A and C types of *bl0675* were amplified by PCR with Ex Taq DNA polymerase (Takara Bio, Shiga, Japan) using genomic DNA of *B. longum* subsp. *longum* strains 4-10 and 10-121 as templates and primer sets KS039AF/KS040AR and KS041CF/KS042CR, respectively (Table 1). Purified amplicons were sequenced using primer KS039AF/KS029AC/KS040AR and KS041CF/KS032CC/KS042CR specific for the A and C types, respectively. Nucleotide sequences of the *bl0675* A and C types from *B. longum* subsp. *longum* strains 4-10 and 10-121 were deposited in the DNA Data Bank of Japan under accession numbers LC025937 and LC025938, respectively; those of *bl0675* from strains 1-124 (B type; AB562880), 7-8 (D type; AB562878), and 2-124 (E type; AB562879) have been deposited by Iguchi et al. [19]. The signal peptide and transmembrane domain

Table 1. Primers used for amplification of the *bl0675* variant

Target	Primer	Sequence (5'–3')	Restriction enzyme*
A type	KS013AF	<u>tt</u> aatacccatggaagatgctaccgg	<i>Nco</i> I
	KS014AR	ggatccctgttctcgatctgacgac	<i>Bam</i> HI
	KS039AF	aaccaatgtaaccaactcgaaccg	
	KS040AR	attatattcctccctatcgctcgt	
	KS029AC	ttataaggtgaactccagctcaagt	
B type	KS016BF	tattaataccatggcaggaagccaac	<i>Nco</i> I
	KS020BR	taatggatccgcccttgtgttct	<i>Bam</i> HI
C type	KS018CF	attaatccatggcggatggtg	<i>Nco</i> I
	KS019CR	attatggatccaccggactgttct	<i>Bam</i> HI
	KS041CF	aaccaatgtaaccaactcgaactgg	
	KS042CR	ttctttcgcgaggaaacatgtttt	
	KS032CC	aagatcgggtgatactctgacctt	
D type	KS023DF	aattaccatggataatgccacgctga	<i>Nco</i> I
	KS024DR	tattaattggatccaccggactgttct	<i>Bam</i> HI
E type	KS025EF	taattattaccatggctaccaccgcca	<i>Nco</i> I
	KS026ER	atggatccgcccttctgttttcta	<i>Bam</i> HI

\*Underlining indicates restriction recognition sites

were predicted using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) and TMHMM 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>), respectively.

### Expression and purification of recombinant proteins

The expression vector pET28-b (Novagen, Madison, WI, USA) was engineered to express recombinant BL0675 with a C-terminal 6  $\times$  His tag (His-BL0675). The *bl0675* sequences without the N-terminal secretion signal sequence or the C-terminal sortase recognition site were amplified by PCR using genomic DNA of *B. longum* subsp. *longum* strains 4-10, 1-124, 10-121, 7-8, and 2-124 as templates. The specific primer pairs used were KS013AF/KS014AR for the A type, KS016BF/KS017BR for the B type, KS018CF/KS019CR for the C type, KS023DF/KS024DR for the D type, and KS025EF/KS026ER for the E type (Table 1). The amplified fragments were inserted into the pGEM-T easy vector (Promega, Tokyo, Japan); the recombinant plasmids were then digested with the endonucleases *Nco*I and *Bam*HI and the fragments were ligated into the pET28-b expression vector used to transform *E. coli* Rosetta 2. For recombinant protein expression, transformed bacteria were grown in LB medium at 30°C with shaking until the OD<sub>600</sub> reached 0.5 and then induced with isopropyl- $\beta$ -D-thiogalactopyranoside (0.1 mM) at 30°C for 5 hr. Bacteria were harvested and resuspended in 3 ml lysis buffer (pH 7.0) containing 50 mM Tris-HCl, 1 mM EDTA, 1 mg lysozyme from chicken egg (Sigma-

Aldrich, St. Louis, MO, USA), and protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Crude lysates were fractionated by centrifugation ( $16,000 \times g$ , 10 min,  $4^{\circ}\text{C}$ ), and supernatants were filtered through a  $0.20\text{-}\mu\text{m}$  filter. The samples were purified by  $\text{Ni}^{2+}$ -nitrilotriacetic acid affinity chromatography using HisTrap HP columns (Qiagen, Hilden, Germany) and by ion exchange chromatography using SP-Sepharose Fast Flow (GE Healthcare, Piscataway, NJ, USA). Protein fractions were pooled and dialyzed against 10 mM HEPES buffer (pH 7.4). Protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels, and protein concentration was determined using the BCA method (Thermo Scientific, Wilmington, DE, USA).

#### *Surface plasmon resonance (SPR) analysis*

The binding affinity of the His-BL0675 A type (His-BL0675A) to PCMs was assessed by SPR using a Biacore X instrument (GE Healthcare, Piscataway, NJ, USA) as previously described [22], with the following modifications. Purified PCMs were immobilized on a CM5 dextran sensor chip (GE Healthcare) with 2,706 resonance units (RUs) using amine-coupling reagents (GE Healthcare). The binding of His-BL0675A to the coated surface was determined on Biacore X using HBS-EP (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20; pH 7.4) at flow rate of  $30 \mu\text{l}/\text{min}$ . The analyte His-BL0675A was diluted in HBS-EP buffer and injected over the chip, and dissociation was performed at the same flow rate for 2.5 min. For each binding assay, the specific binding signal was obtained after subtracting the signal of a blank surface. Sensor surface regeneration was achieved by 1-min exposure to regeneration buffer (50 mM Tris-HCl, 2 M NaCl, pH 9.5). The association rate ( $ka$ ), dissociation rate ( $kd$ ), and dissociation constant ( $K_D = kd/ka$ ) were calculated using the BIAevaluation Software version 3.0 (GE Healthcare). Global analysis was performed using a simple 1:1 Langmuir binding model.

#### *Enzyme-linked immunosorbent assay (ELISA)*

ELISA was conducted as previously described [23] with some modifications. His-BL0675A was added to 96-well microtiter plates coated with PCMs and incubated at room temperature for 1 hr. After washing three times with HBS-EP buffer (pH 7.4), an anti-His-tag antibody (1:3,000; Roche) was added and the plates were incubated at room temperature for 40 min. After washing, alkaline phosphatase-conjugated anti-mouse IgG (1:4,500; Dako, Glostrup, Denmark) was added for

40 min at room temperature. Antibody-specific signals were detected with BluePhos MicroWell Substrate Kit (KPL, Gaithersburg, MD, USA) by measuring the absorbance at 620 nm.

#### *Competitive ELISA*

Competitive ELISA was performed to detect the interaction between His-BL0675A and mucin oligosaccharides as previously described [23], with the following modifications. Mucin oligosaccharides isolated from porcine gastric mucin (100, 200, and 400 ng hexose equivalent) were preincubated with His-BL0675A (15  $\mu\text{g}$ ) for 1 hr at room temperature and then added to PCM-coated wells for 1 hr. After washing three times with HBS-EP buffer (pH 7.4), the anti-His-tag antibody was added for 1 hr at room temperature, and the reaction was detected as described above.

#### *Preparation of cell surface proteins*

The *B. longum* subsp. *longum* strains were cultivated anaerobically in 50 ml of TOS broth at  $37^{\circ}\text{C}$  for 12 hr and then centrifuged ( $8,000 \times g$ , 10 min,  $4^{\circ}\text{C}$ ). Pelleted cells were suspended in 300  $\mu\text{l}$  extraction buffer containing 50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 34% (w/v) sucrose, 0.5 mg lysozyme, and 100 U mutanolysin (Sigma-Aldrich) and incubated for 3 hr at  $37^{\circ}\text{C}$ . Bacterial suspensions were centrifuged ( $8,000 \times g$ , 15 min,  $4^{\circ}\text{C}$ ) and supernatants containing cell surface proteins were analyzed by western blotting.

#### *Western blotting*

Polyclonal anti-BL0675 A-type antibodies were raised in rabbits immunized with His-BL0675A. Twenty micrograms of protein samples were separated by SDS-PAGE in 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% (w/v) skim milk in PBS-0.05% Tween 20 (PBS-T) for 24 hr at  $4^{\circ}\text{C}$ , washed with PBS-T, and incubated with anti-BL0675 A-type polyclonal antibodies (1:1,000 in PBS-T) for 1 hr at room temperature. Membranes were then washed and incubated for 1 hr with horseradish peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich) diluted at 1:2,000 in PBS-T. After washing, the TMB Membrane Peroxidase Substrate (KPL) was added to develop the signals.

#### *Bacterial adhesion assay*

Bacterial adhesion to PCMs was assessed using diagnostic glass slides as previously described [24]. Briefly, diagnostic glass slides (Matsunami Glass Ind.

Ltd., Osaka, Japan) were coated with 100 ng of PCMs (40  $\mu$ l/well) for 12 hr at 4°C. After washing three times with PBS containing 0.1% (w/v) bovine serum albumin (BSA), the slides were incubated with 5% (w/v) BSA-PBS for 1 hr at room temperature. Then, 40  $\mu$ l of bacterial suspensions (equivalent to OD<sub>600</sub> = 1.0) was added to the wells and incubated 37°C for 2 hr. The wells were washed three times with 0.1% (w/v) BSA-PBS to remove unbound bacteria and stained with methylene blue. Randomly chosen fields were photographed under an Olympus BX53 microscope (Olympus, Tokyo, Japan), and bacteria were counted in five fields.

To determine whether anti-BL0675 A-type polyclonal antibodies reduced *B. longum* subsp. *longum* binding to PCMs, BL0675 A-type antiserum and normal rabbit serum (control) diluted 1:50 were added to 40  $\mu$ l of bacterial suspensions, which were then incubated for 1 hr at 37°C. The adhesion assay was performed as described above.

#### Statistical analysis

The GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA) was used to perform statistical analysis. Significant differences were determined using one-way analysis of variance (ANOVA) with a post hoc Dunnett's test (for the bacterial adhesion test) or Tukey's test (for the His-BL0675-binding test). The number of individual experiments (n) is indicated in the figure legends. Differences with p values less than 0.05 were considered statistically significant.

## RESULTS

#### Expression and purification of His-BL0675A

His-BL0675A was expressed in *E. coli* Rosetta 2 and purified on HisTrap and ion exchange columns. As evidenced by protein bands detected by SDS-PAGE (Fig. 1), the molecular weights of purified His-BL0675A, B, C, D, and E were consistent with the calculated molecular masses (Table 2).

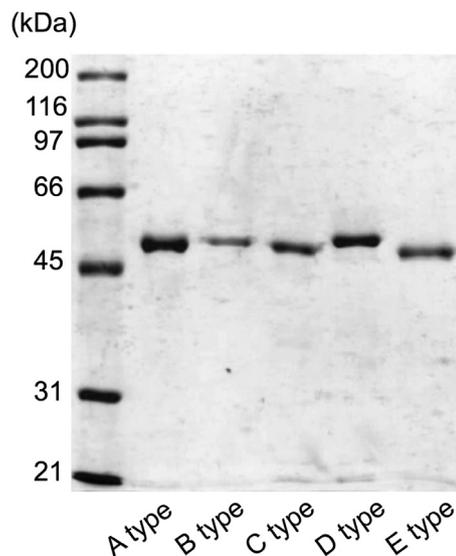


Fig. 1. Expression and purification of His-BL0675. Purified His-BL0675A, B, C, D, and E were separated by SDS-PAGE in a 10% acrylamide gel and stained with Coomassie Brilliant Blue R-250. Molecular mass standards are indicated on the left.

#### Affinity of His-BL0675 for PCMs

The binding of the purified His-BL0675 proteins to PCMs was determined by SPR analysis. Figure 2A shows the RUs at the start of dissociation for different His-BL0675 types at 1,000 nM. His-BL0675A showed significantly greater binding to PCMs than types B, D, and E did; however, binding of His-BL0675C to PCMs was not detected. These results demonstrate that the BL0675 types have differing affinities to PCMs. Moreover, as shown by kinetic analysis, His-BL0675A demonstrated concentration-dependent interaction in the range of 400–1,100 nM, suggesting that the binding was specific (Fig. 2B). The  $K_D$  value for binding of His-BL0675A to PCMs was estimated to be  $9.82 \times 10^{-8}$  M ( $k_a$ ,  $2.84 \times 10^4$  M<sup>-1</sup> s<sup>-1</sup>,  $k_d$ ,  $2.79 \times 10^{-3}$  s<sup>-1</sup>). The chi-square value was 18.5, indicating that the model used

Table 2. Properties of recombinant His-BL0675

Type of BL0675	Deduced number of amino acid residues*	Deduced molecular mass ( $\times 10^3$ )	Gene accession No.
A	460	49.1	LC025937
B	477	51.6	AB562880
C	479	49.4	LC025938
D	470	49.1	AB562878
E	476	50.8	AB562879

\*Not including the N-terminal secretion signal sequence and the C-terminal sortase recognition site

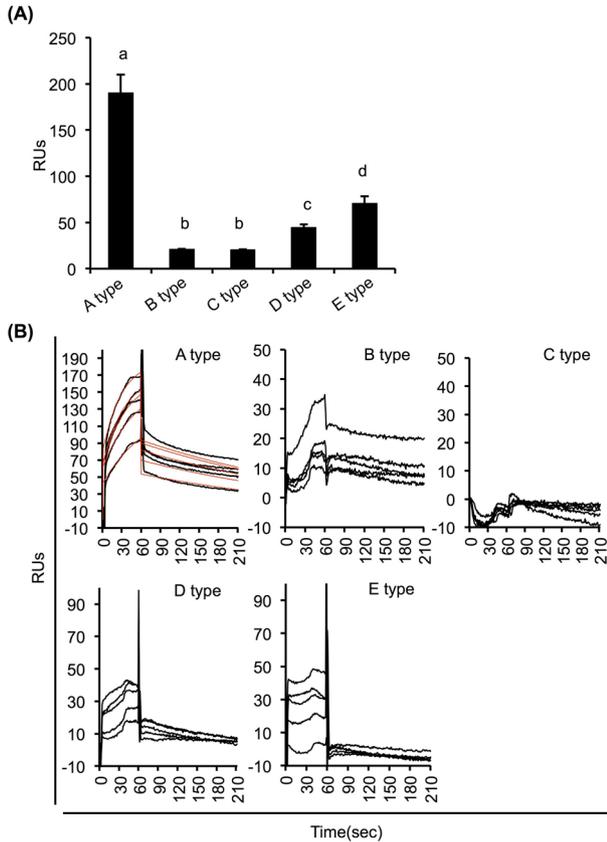


Fig. 2. Interaction of His-BL0675 with porcine colonic mucins (PCMs) analyzed by surface plasmon resonance.

(A) Five types of His-BL0675 (1,000 nM) were injected as analytes over a CM5 sensor chip containing immobilized PCMs. Signals were recorded at the start of dissociation and expressed as resonance units (RUs). The data are presented as the means  $\pm$  SD (n = 3); a–d indicate significant differences (p < 0.05). (B) His-BL0675 were injected at various concentrations (1,100, 1,000, 800, 600 and 400 nM for the A type; 1,200, 1,100, 1,000, 950 and 600 nM for the B type; 1,200, 1,000, 800, 600 and 400 nM for the C, D and E types) over CM5 chip-immobilized PCMs and the signal was recorded over a 60-sec association phase and 150-sec dissociation phase. The measured data (black line) and their global fits are overlaid (red line).

adequately described our data. In contrast, other types of His-BL0675 (the B, C, D, and E types) did not exhibit concentration-dependent and stable sensorgrams (Fig. 2B), suggesting that the interaction was nonspecific and/or that higher binding occurred on the blank surface than the PCM-immobilized surface.

We next examined the binding of His-BL0675A to mucin carbohydrate chains by competitive ELISA. The results demonstrated that His-BL0675 binding to PCMs increased proportionally to its concentration (Fig. 3A) and was inhibited by mucin oligosaccharides in a dose-

dependent manner (Fig. 3B).

#### Evaluation of BL0675-mediated adhesion of *B. longum* subsp. *longum* to PCMs

The cell surface fractions of five *B. longum* subsp. *longum* strains were analyzed for BL0675 expression by western blotting. The BL0675 A type with a molecular mass of approximately 50,000 was detected in all strains except strain 9-5, suggesting that it is expressed on the cell surface in strains 1-1, 1-7, 3-113, and 9-129 (Fig. 4A).

We next examined the effects of polyclonal anti-BL0675 A-type antibodies on the adhesion of *B. longum* subsp. *longum* strains to PCMs. As shown in Fig. 4B, adhesion of the five *B. longum* subsp. *longum* strains demonstrated good correlation with BL0675 expression levels. Treatment with BL0675 antibodies significantly reduced the adhesion of *B. longum* subsp. *longum* strains 1-1, 1-7, 3-113, and 9-129 compared with untreated control cultures. However, pretreatment with normal rabbit serum also reduced the adhesion of *B. longum* subsp. *longum* strains 3-113 and 9-129 to PCMs.

## DISCUSSION

A previous study showed that *bl0675* ORFs encoding a putative fimbria-associated protein are highly polymorphic among *B. longum* subsp. *longum* strains isolated from human feces [19]. However, the affinity of the putative BL0675 proteins to mucosal surfaces remains largely unknown. In this study, we evaluated the binding of five BL0675 types to PCMs and found significant variations in their affinity to PCMs. His-BL0675A exhibited strong binding to PCMs ( $K_D = 9.82 \times 10^{-8}$  M) that higher than the affinity of *Porphyromonas gingivalis* fimbriae to ECM proteins [25], hemoglobin, and fibrinogen [26] as determined by SPR ( $K_D$  values in the micromolar range). These findings suggest that the interaction of the BL0675 A type with PCMs is relatively strong. Moreover, the binding of His-BL0675A to PCMs was dose-dependently inhibited by mucin oligosaccharides. A similar binding pattern was observed in our recent study: the binding of SpaC pilin from *Lactobacillus rhamnosus* GG was markedly reduced by addition of the mucin oligosaccharide, and the carbohydrate moieties of glycoconjugates were found to be important for this binding [27]. Our results strongly suggest that the BL0675 A type has lectin-like properties in that it binds to mucins via oligosaccharides.

Although amino acid sequence identity between the BL0675 A type and other types ranged from 41%

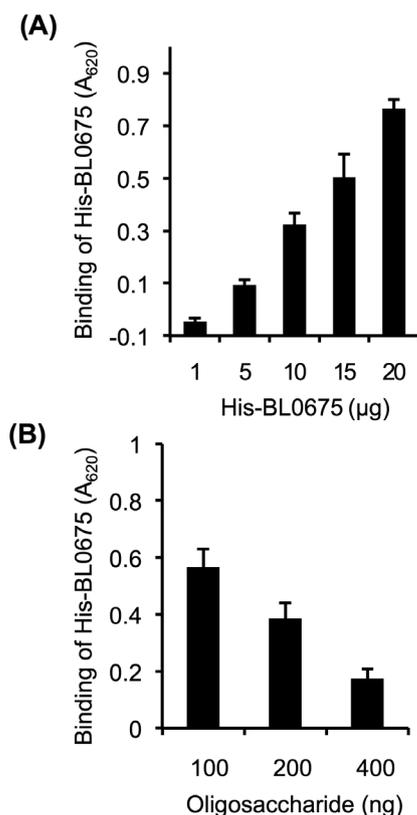


Fig. 3. Competitive inhibition of His-BL0675 binding to immobilized porcine colonic mucins (PCMs) by mucin oligosaccharides.

(A) PCM-coated wells were incubated with 1–20  $\mu\text{g}$  of His-BL0675A. (B) His-BL0675A binding to PCM was analyzed in the presence or absence of mucin oligosaccharide fractions. Oligosaccharides (100, 200 and 400 ng per well by hexose equivalent) were incubated with His-BL0675A as described in the Materials and Methods, and His-BL0675 binding was detected with the anti-His-tag antibody. The data are presented as the means  $\pm$  SD ( $n = 3$ ).

to 50%, the BL0675 B, D, and E types demonstrated weak binding to PCMs, while the BL0675 C type did not show binding. These affinity patterns are similar to those observed in the five BL0675 variant types from 89 human fecal samples in our previous study in which quantitative PCR assays revealed that the A type was the most commonly distributed type (74.2% prevalence), followed by the B, D, E, and C types [19]. Host-specific adhesion is regarded as a desirable property for probiotic bacteria and is thought to be dependent more on the bacterial strain rather than on the host [28, 29]. Although it is not clear whether the distribution of *bl0675* genes affects the BL0675 binding pattern, our results suggest that the BL0675 variant types significantly contribute

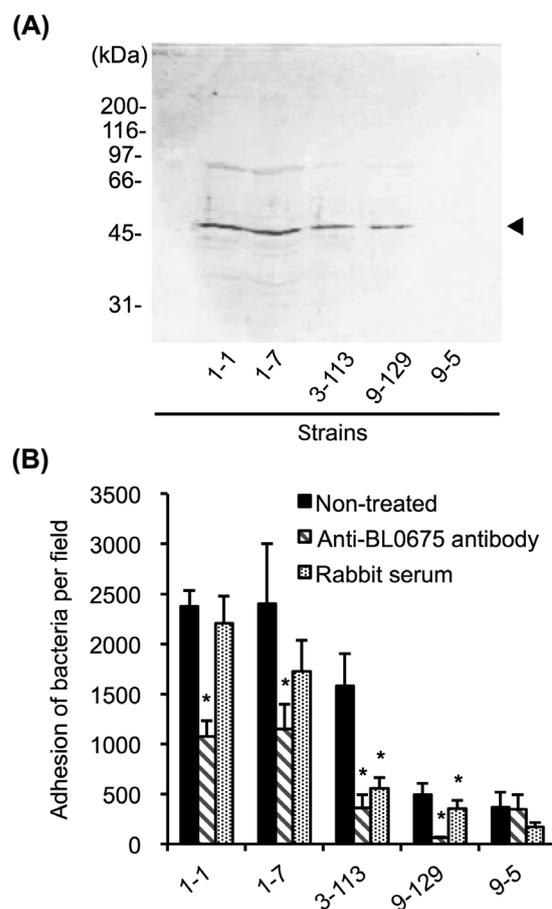


Fig. 4. Expression and adhesion of BL0675 in *B. longum* subsp. *longum* strains.

(A) BL0675 A-type expression in cell wall extracts of *B. longum* subsp. *longum* strains (arrowhead) was detected by western blotting using anti-BL0675 antibodies. Molecular weight standards are indicated on the left. (B) Bacteria were preincubated with polyclonal antibodies against the BL0675 A type or with normal rabbit serum and their adhesion to PCMs was analyzed as described in the Materials and Methods. The data are presented as the means  $\pm$  SD ( $n = 3$ ). \* $p < 0.05$  compared with bacteria preincubated without antibodies.

to the adhesion of *B. longum* subsp. *longum* strains in the human GI tract. Host-specific adhesion patterns have been observed for *Lactobacillus* attachment to the colon. For example, the MucBD-associated domain peptide of *Lactobacillus reuteri* interacts with a specific colonic mucus-secreted component present in humans, rabbits, and guinea pigs, but not in mice [30], and glyceraldehyde-3-phosphate dehydrogenase of *Lactobacillus plantarum* recognizes blood groups A and B expressed on the nonreducing terminal sugar chains of human colonic mucin [31]. Several *Bifidobacterium* species are also known to exhibit differential, strain-specific binding to

carbohydrate moieties of glycolipids or mucins expressed in the GI tract [29, 32, 33]. Since we used porcine, and not human, mucins, it is possible that BL0675 types other than the A type would interact with human mucins via a more stringent carbohydrate recognition process, which we were unable to detect with porcine mucins. Further studies are needed to characterize the specific binding affinity of BL0675 types to mucin carbohydrates.

Reduction in the adhesion of *B. longum* subsp. *longum* strains to PCMs was observed when bacteria were pretreated with the BL0675 antibodies. It is likely that BL0675 is important for the establishment of GI colonization by *B. longum* subsp. *longum* strains; however, this reduction in adhesion was strain specific. Some strains were also inhibited by normal rabbit serum, suggesting that the non-BL0675-based inhibition effect of rabbit serum interferes with adhesion to PCMs through mechanisms such as steric hindrance. Pili and fimbriae are multisubunit protein polymers of high molecular weight that mediate bacterial adhesion via the tip of the polymer structure [21, 34, 35]. Although we detected the BL0675 monomer with a calculated molecular mass of approximately 50,000, we were unable to identify high molecular weight polymers, suggesting that in *B. longum* subsp. *longum*, *bl0675* ORFs encode putative fimbrial proteins, which function as adhesins without polymerization into multiprotein structures such as pili. *Streptococcus* pneumococcal pilus-associated adhesin also binds to host cells even in the absence of polymeric pilus formation [36, 37]. On the other hand, the expression of the fimbrial subunit BL0675 by *B. longum* subsp. *longum* LMG 13197 [38] or sortase-dependent pili by *B. bifidum* PRL2010 [17] is influenced by the presence of complex carbohydrates in the media. Moreover, the GI niche with specific thermal, acidic, and osmotic conditions influences the expression of *B. bifidum* PRL2010 sortase-dependent pili, which is also enhanced by co-culture of PRL2010 with *Lactobacillus paracasei* [39]. Therefore, we hypothesize that the carbohydrate composition of media as well as other environmental factors may affect BL0675 expression and/or pilus polymerization.

Several *Bifidobacterium* strains have the ability to adhere to the GI tract, and some strains of intestinal origin express cell surface adhesion factors, including BopA [40–42], sortase-dependent pili [15], and transaldolase [43] in *B. bifidum* and Tad Pili in *B. breve* [16]. However, to date, the properties of adhesion of *B. longum* subsp. *longum* to the mucosal surface have been largely unknown. We believe that our study of the binding properties of BL0675 related to mucin adhesion

will contribute to the understanding of the mechanism underlying *B. longum* subsp. *longum* attachment to and colonization of the mammalian GI tract.

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