Growth promotion and cell binding ability of bovine lactoferrin to *Bifidobacterium longum*

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Abstract

Lactoferrin, a major whey protein of human milk, is considered as growth promoter for bifidobacteria, the predominant microorganisms of human intestine. In the present study, in vitro growth promotion and cell binding ability of bovine lactoferrin to several strains of *Bifidobacterium longum* have been demonstrated. A dose-dependent as well as strain-dependent growth promotion effect by lactoferrin was observed. Cell binding

ability of lactoferrin was inspected under an inverted confocal laser scanning microscope by incubation bacterial cells with biotinylated bovine lactoferrin and FITC-conjugated avidin. Fluorescence staining showed bovine lactoferrin binding to all tested strains. A lactoferrin-binding protein with a molecular weight of approximately 67 kDa was also detected in the extracted membrane and cytosolic fraction of each *B. longum* strain by far Western blot technique using biotinylated lactoferrin and horseradish peroxidase-conjugated streptavidin. Based on these results, we suggest that existence of lactoferrin-binding protein could be a common characteristic in bifidobacteria. It can also be hypothesized that lactoferrin-binding protein in bifidobacteria is not only involved in growth stimulation mechanism but also could play different role.

Key words: *Bifidobacterium longum*; bovine lactoferrin; lactoferrin-binding protein

Introduction

Since the first isolation by Henry Tissier in 1899-1900, bifidobacteria are thought to be probiotic bacteria because of their potential health benefits to the host [1]. Currently the genus *Bifidobacterium* is represented by over 30 species [2]. In breast-fed infants, this genus represent up to 91% of the total gut flora of which *Bifidobacterium (B.) longum* is one of the most representative species [3]. Consumption of *B. longum* is reported to exert beneficial effects such as antagonistic action towards intestinal pathogens, improve lactose utilization, anticarcinogenic action and control of serum cholesterol levels [4]. Accordingly, the importance of this species has gained wider applications in the formulation of several cultured dairy products, worldwide [5]. Lactoferrin (Lf), a multifunctional iron binding glycoprotein mainly found in milk and exhibits several biological activities normally associated with a host defense system [6].

Although the protein is known for antimicrobial activity as described in recent review [7], it is also considered to stimulate the growth of bifidobacteria. Several studies in vitro [8-13] and in vivo [14] have shown that Lf has the ability to promote the growth of bifidobacteria. Interestingly, the published reports to date (Table 1) reveal that strains of *B. longum* was found to be shown less growth response or inactive against Lf. Lactoferrin-binding protein was previously detected in *Bifidoibacterium* spp. and was thought to be involved in promoting the growth of bifidobacteria by Lf [11,12,15]. It was also reported that one strain of *B. longum* (ATCC 15707) did not express lactoferrin-binding protein, and consequently the growth of this strain was not stimulated by Lf [11]. Conversely, we recently reported lactoferrin-binding protein in *B. longum* ATCC 15708 [16] using the same experimental procedure as described by Kim et al. [15] These results have motivated us to search and compare the existence of lactoferrin-binding protein in *B. longum*. Therefore, an attempt was taken to examine binding ability of bovine Lf (bLf) to several *B. longum* strains including previously reported strains and also to evaluate growth promotional ability by bLf at different concentrations.

Materias and Methods

Protein and other chemicals

Bovine lactoferrin was supplied by Morinaga Milk Co., Ltd. (Zama, Japan). N-hydroxysuccinimide biotin and fluorescein conjugated-avidin (avidin-FITC) were purchased from Sigma-Aldrich Inc. (Saint Louis, Missouri, USA). Polyvinylidene-difluoride (PVDF) membrane (ATTO Chemicals, Tokyo, Japan), bovine serum albumin (BSA; Nacalai Tesque Inc., Kyoto, Japan), streptavidin-labeled horseradish peroxidase (Nichirei Co., Tokyo, Japan), prestained protein markers

(Bio-Rad Laboratories, Hercules, California, USA) and ECL kit (Amersham Biosciences UK Ltd., Buckinghamshire, England) were purchased.

Bacteria

Three strains of *B. longum* (ATCC 15707, ATCC 15708 and kd-5-6) were used in this study. Bacterial strains were the generous gift of Morinaga Milk Co., Ltd. (Zama, Tokyo, Japan). Strains were maintained as frozen stocks at – 80ºC in sterile MRS broth (Merck, Darmstadt, Germany) containing 20% glycerol and 0.05% L-cysteine-HCL. For further use, each bacterium was reactivated by two consecutive subcultures in MRS broth containing 0.05% L-cysteine.HCL under anaerobic condition at 37ºC.

Biotinylation of bovine lactoferrin

Bovine lactoferrin was biotinylated according to a previously reported procedure [15]. The conjugation of biotin to the bLf was confirmed by measuring its electrophoretic mobility.

Sample preparation for confocal laser scanning microscopic (CLSM) observations

Binding assays were performed as described by Rahman et al. [16]. Briefly, after incubation until mid-log phase, bacterial cells were harvested and suspended in phosphate-buffered saline (PBS, pH 7.2). The bacterial suspension was mixed with biotinylated bLf, before being incubated at 37°C for 30 min. The cells were then washed by centrifugation in PBS before being incubated with FITC-conjugated avidin (1:100 dilution in PBS) for 30 min at 37°C. After a final wash in PBS, the cells were examined under an inverted confocal laser scanning microscope LSM 410 (Carl Zeiss Co., Germany). The control specimens were obtained from the cells that were incubated only with FITC-conjugated avidin without prior exposure to biotinylated bLf.

Detection of lactoferrin-binding protein

Extraction of bacterial membrane fractions and detection of lactoferrin-binding protein was carried out according to previously reported protocol [15]. Briefly, bacterial cells (10^8 CFU) were suspended in 50 μl of Dulbecco's phosphate-buffered saline (PBS, pH 7.1) containing protease inhibitor solution (0.1 mM sodium vanadate, 0.5 μg/ml herbimycin A, 50 μg/ml aprotinin, 25 μg/ml leupeptin, 750 μg/ml benzamidine and 1 mM phenylmethane sulfonylfluoride). The cells were disrupted by supersonic waves using a Bioruptor UCD-200 (Cosmo Bio, Co., Ltd., Tokyo). The cytosolic fraction (supernatant) was separated by centrifugation at 11,000 ×g for 10 min. After washing with protease inhibitor solution and cold PBS, the pellet was resuspended in 50 μl of lysis buffer (protease inhibitor containing 1% Triton X-100 and 1% CHAPS), and the membrane-associated fraction (supernatant) was obtained by centrifugation at 13,000 × g for 15 min.

The extracted cytosolic- and membrane-assiciated proteins were separated by polyacrylamide gel electrophoresis (SDS-PAGE) using a discontinuous gel system and Tris-HCl glycine buffer as described by Laemmli [17]. The gels were either stained with Coomassie Brilliant Blue R-250 to visualize resolved protein bands or transferred onto polyvinylidene-difluoride (PVDF) membrane to detect lactoferrin-binding protein by far-Western blot technique. After blocking for 90 min with 2% BSA dissolved in PBS containing 0.01 % Tween 20, the PVDF membrane was immersed in either biotinylated bLf (final concentration 1 μg/ml) or buffer only (as control) overnight at 4°C. The membrane was washed five times with PBS containing 0.5 % Tween 20 (washing buffer), then incubated with streptavidin labeled horseradish peroxidase (final concentration, 0.8 μl/ml) for 30 min at room temperature. Peroxidase bound proteins

were detected using the enhanced chemiluminescence (ECL) system and exposed on X-ray film.

Effects of bovine lactoferrin on growth of Bifidobacterium longum

Bifidobacterium strains were grown under anaerobic condition in MRS broth (Merck, Darmstadt, Germany) containing 0.05% L-cysteiene-HCl at 37°C with or without (control) addition of bLf at different concentration. Lactoferrin solution was prepared by dissolving in sterilized PBS (pH 7.2) followed by filter sterilization (pore size 0.20 μm). The concentration of filter sterilized bLf solution was estimated spectrophotometrically. After sterilization, fresh MRS medium was inoculated with reactivated *B. longum* strain at 1% level and mixed. Aliquots (9 ml) of this mixture were dispensed into 15 ml sterile polystyrene tube. One milliliter of two-fold serially diluted protein solution was then poured into each tube to achieve a final concentration of 4, 2, 1, 0.5 or 0.25 mg/ml. For control cultures, PBS was added instead of bLf solution. Tubes were kept in an airtight, AnaeroPack jar (Mitsubishi Gas Chemical Co., Inc., Tokyo) followed by placement of AnaeroPack-sachet immediately before closing. After 16 h incubation at 37°C, bacterial growth was monitored by measuring absorbance spectrophotometrically at 660 nm with 10 times dilution of the cultured medium. The growth response of bifidobacteria strains by adding bLf was expressed as percent relative growth response level (% RGRL) and calculated using following formula as described by Saito et al. [18]-

% RGRL = A_{660} nm (protein added) / A_{660} nm (control) × 100

Results and discussion

In vitro effect of bLf on the growth of *B. longum* strains is shown in Fig. 1. A heterogeneous growth response of the strains was observed. Among the 3 tested

strains, *B. longum* ATCC 15707 showed less response against bLf. However, at higher concentration (2 and 4 mg/ml) a slight increase in growth response was observed. The two other strains (ATCC 15708 and Kd-5-6) showed less response at lower concentration (0.25, 0.5 and 0.5 mg/ml); On the other hand, at higher concentration (2 and 4 mg/ml) good growth responses by these strains were observed. A comparison of present result with previously reported studies on these strains is shown in Table 1. Our present findings differ with previously published reports. The differences, as shown in Table 1, in media, assay methods, source and concentration of lactoferrin used for measuring the effects of Lf on the growth of bifidobacteria may have produced contradictory results.

Bovine lactoferrin ability to bind each *B. longum* strain was inspected under a confocal laser scanning microscope after exposing bacterial cells to biotinylated bLf and counterstaining with FITC-conjugated avidin (Fig. 2). In the control specimens (unexposed to biotinylated bLf) no such binding was observed. Binding of biotin labeled bLf with bifidobacterial cells generate green fluorescence by binding between FITC conjugated avidin and biotin. Bacterial strains showing green colored fluorescence indicates binding of bLf to bacterial cells. The current results also indicate that lactoferrin-binding proteins in bifidobacteria is associated with lipid bilayer in a way by which the protein entirely or partially located outside of the bilayer and thus, bind with Lf in the environment.

Lactoferrin-binding proteins in both the membrane and cytosolic fractions extracted from each *B. longum* strain are shown in Fig. 3. Lactoferrin-binding proteins were detected by far western blotting using biotinylated bLf as a probe. All strains exhibited a single band and the estimated molecular weight of these bands was

calculated to be approximately 67 kDa (Fig. 3 B). We previously reported that lactoferrin-binding protein was present in the membrane fractions of several *Bifidobacterium* spp. (*B. infantis* ATCC 15697, *B. breve* ATCC 15700, *B. bifidum* ATCC 15696, *B. bifidum* Bb-11, *B. longum 15708*) [11,12,15,16], it was not detected in the membrane fraction of *B. longum* ATCC 15707 [11]. It should be noted that no band was appeared when biotinylated bLf was omitted from the experiment.

Bovine lactoferrin showed the ability to bind all bifidobacteria strains used in this study (Fig. 2). A 67-kDa lactoferrin binding protein was detected in both cytosolic and membrane fractions of all tested strains (Fig.3). Surprisingly, lactoferrin-binding protein was detected in ATCC 1507 (Fig. 3), which was reported to be lack in lactoferrin-binding protein in one of our previous work [11]. The experiment was repeated at least three times to confirm this result. Although the reasons for such contradictory results are unclear; lower extraction rate of lactoferrin-binding protein in membrane fraction could be a reason. For instance, in the present study band correspond to the lactoferrin-binding protein in the membrane fractions of ATCC 15708 was found to be weaker than that of ATCC 15707 and Kd-5-6 (Fig. 3). However, our present data seems to be more reliable due to the supporting evidence of cell binding ability by CLSM (Fig. 2). The binding ability of bLf to bifidobcteria suggests two possible explanations. One is that binding between Lf and bifidobacteria cells could be the result of simple electrostatic interaction between cationic nature of Lf and anionic bacterial cell surface. Another is that presence of lactoferrin-binding protein is a common characteristic in bifidobacteria, and this protein may be either involved in growth promotion mechanism by transferring iron ions or information to the interior of the bacterial cells or may be involved in playing different role.

In the present study, despite presence of lactoferrin-binding protein in all *B. longum* strains, heterogeneous growth promoting pattern was observed (Fig. 1). Bezkorovainy and Topouzian [19] reported that bifidobacteria require iron for growth and they acquire this iron from Lf. Lactoferrin may release iron at lower pH and bifidobacteria utilize this iron for their growth. However, iron requirement may be strain dependent, which produces heterogeneous growth promotion yield by lactoferrin addition. It can also be hypothesized that lactoferrin-binding protein may be activated by bacterial strain according to their requirement.

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Figure legends

Fig. 1. In vitro effects of bovine lactoferrin (bLf) on the growth of *B. longum* strains. Bacteria were grown in MRS medium with or without (control) the addition of bLf at various concentrations. Relative growth promotion level was expressed as the ratio of the absorbance at 660 nm in the presence of bLf to the control absorbance after 16 h of cultivation at 37°C under anaerobic condition. The average absorbance of the control was 0.43, 0.36 and 0.3 for ATCC 15707, ATCC 15708 and Kd-5-6, respectively. The values are the average of triplicate assay.

Fig. 2. The fluorescence detection images of *B. longum* ATCC 15707 (A), ATCC 15708 (B) and Kd-5-6 (C). Bacteria cells were exposed to biotinylated bovine lactoferrin before being counterstained with FITC-conjugated avidin. A transmission detection image of each bacterium is on the left, a fluorescence detection image of the same bacteria is in the middle, and a merged image of the two is on the right.

Fig. 3. (A) Extracted cytosolic- and membrane-associated fractions of *B. longum* strains were separated by SDS-PAGE. Protein bands were stained with CBB-R 250. The concentration of SDS-PAGE separation gel was 10%. (B) Lactoferrin-binding proteins were detected in the both cytosolic and membrane fractions of *B. longum* strains by far-western blot technique with biotinylated lactoferrin as probe. Lane 1, 2, 3 and 4, 5, 6 both in SDS-PAGE (A) and western blot (B) analysis represents *B. longum* ATCC 15707, ATCC 15708 and Kd 5-6, respectively. M indicates prestained protein markers that were used to estimate the molecular weights whereas asterisks indicate lactoferrin-binding protein.

Figure 1

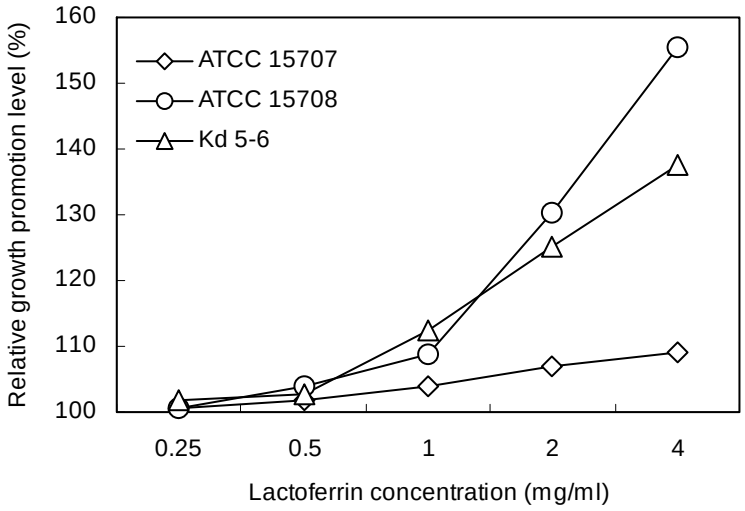
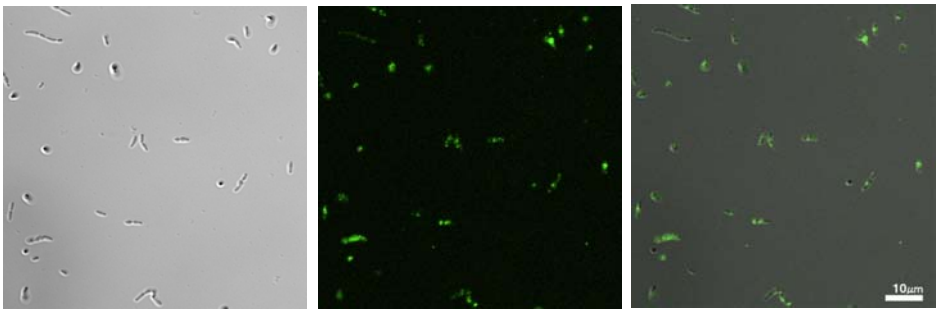
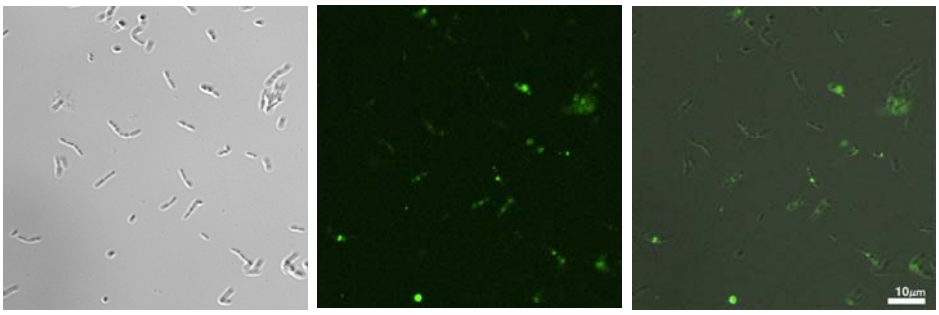

160
150
140
130
120
110
100
Relative growth promotion level (%)
ATCC 15707
ATCC 15708
Kd 5-6
0.25
0.5
1
2
4
Lactoferrin concentration (mg/ml)

Figure 2

(A) *B. longum* ATCC 15707



(B) *B. longum* ATCC 15708



(C) *B. longum* Kd-5-6

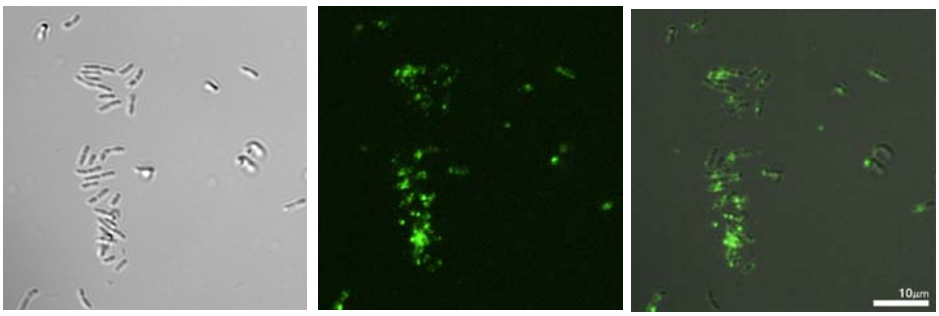
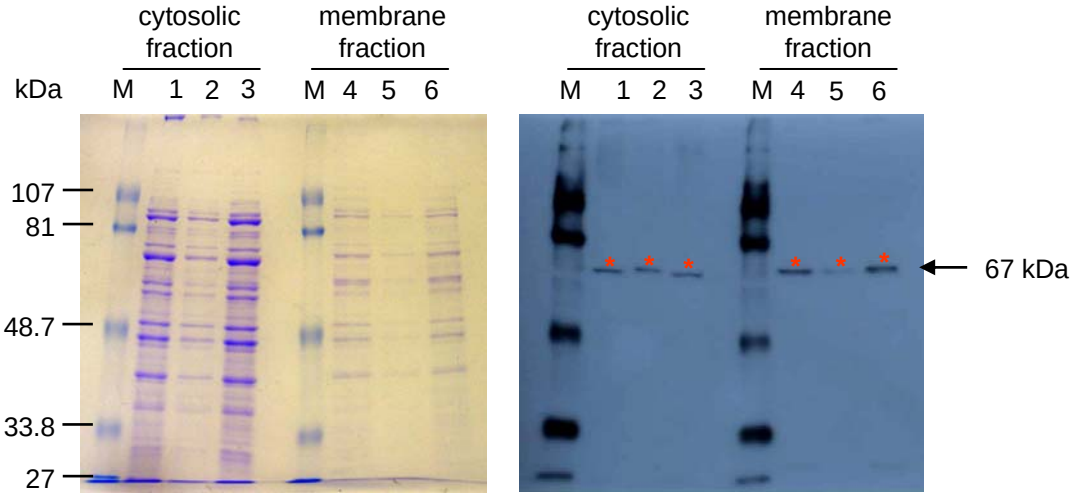


Figure 3


cytosolic fraction
membrane fraction
kDa
M 1 2 3 M 4 5 6
107
81
48.7
33.8
27
cytosolic fraction
membrane fraction
M 1 2 3 M 4 5 6
67 kDa

(A) (B)

Table 1. Comaprison of present result with previous studies in terms of Lf effect on *Bifidobacterium longum*

Strains *of B. longum*	Studies	Lf effect with experimental conditions such as medium, assay system, source of lactoferrin and concentration				
		Medium	assay system	Lf source	Lf concentration	effect
ATCC 15707	Present study	MRS	spectrophotometry	bulk bLf	0.5, 1, 2 & 4 mg/ml	less active even at high concentration (4 mg/ml)
	Kim et al. [11]	MRS	spectrophotometry	apo- & holo-type bulk bLf or hLf	0.01, 0.1 & 1 mg/ml	inactive
	Kim et al. [12]	MRS	viable cell count	bulk bLf	1 mg/ml	active
	Rahman et al. [13]	MRS	spectrophotometry	bulk bLf	1 mg/ml	inactive
	Saito et al. [18]	GAM broth	spectrophotometry	bLf purified from sweet cheese whey	50-2000 PPM	active
ATCC 15708	Present study	MRS	spectrophotometry	bulk bLf	1.0 mg/ml	active at high concentration (2 & 4 mg/ml)
	Petchow et al. [8]	Norris	acid titration	Lf purified from cow milk	0.25, 0.5, 2 & 4 mg/ml	inactive or less active (compared to other strain)
	Rahman et al. [13]	MRS	spectrophotometry	bulk bLf	1 mg/ml	inactive
Kd-5-6	Present study	MRS	spectrophotometry	bulk bLf	0.5, 1, 2 & 4 mg/ml	active at high concentration (2 & 4 mg/ml)
	Rahman et al. [13]	MRS	spectrophotometry	bulk bLf	1.0 mg/ml	inactive
1217	Present study	----------Not done----------				
	Tomita et al. [20]	GAM broth	spectrophotometry	apo- & holo-type blf and hLf	0.3 mg/ml	inactive

Lf, lactoferrin; bLf, bovine lactoferrin; hLf, human lactoferrin; number in the parenthesis indicated selected references