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Growth-promoting effects of lactoferrin on *L. acidophilus* and *Bifidobacterium* spp.

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Abstract

We investigated the effects of lactoferrin on the growth of *L. acidophilus* CH-2, *Bifidobacterium breve* ATCC 15700, *B. longum* ATCC 15707, *B. infantis* ATCC 15697, and *B. bifidum* ATCC 15696. The growth of *L. acidophilus* was stimulated by bovine holo-lactoferrin but not by apo-lactoferrin. With bifidobacteria, bovine lactoferrin stimulated growth of three strains: *B. breve*, *B. infantis* and *B. bifidum* under certain conditions. Both apoprotein and holoprotein had similar effects. However, *B. longum* growth was not affected by lactoferrin. Thus, the mechanism of stimulating growth of bifidobacteria may be different from that of *L. acidophilus*. By far-western blotting using biotinylated lactoferrin and horseradish peroxidase-conjugated streptavidin, lactoferrin-binding proteins were detected in the membrane protein fraction of *L. acidophilus*, *B. bifidum*, *B. infantis* and *B. breve*. The molecular weights of lactoferrin-binding proteins of *L. acidophilus* were estimated from SDS-polyacrylamide gel electrophoresis to be 27, 41 and 67 kDa, and those of the three bifidobacterial strains were estimated to be 67–69 kDa. However, no such lactoferrin-binding components were detected in the membrane fraction of *B. longum*. It is interesting that the appearance of lactoferrin-binding proteins in the membrane fraction of these species corresponds to their growth stimulation by lactoferrin.

Introduction

Lactoferrin is an iron-binding glycoprotein that is present mainly in milk. This protein is known to have many biological functions related to the host defense system (for reviews, see Nuijens *et al.* 1996, Shimazaki 2000, Brock 2002). Antimicrobial activity was the first function of lactoferrin to be discovered (Reiter & Oram 1967, Arnold *et al.* 1977, Reiter 1978). However, lactoferrin not only inhibits the growth of various pathogenic bacteria, but also stimulates the growth of some types of bacteria, including bifidobacteria.

It is well-known that the intestinal flora has significant effects on the health of the host. *Lactobacillus acidophilus*, a Gram-positive, homofermentative, catalase-negative and rod-shaped bacterium, has traditionally been utilized for fermentation of milk products. The probiotic dairy product produced with this bacterium is known as acidophilus milk. Bifidobacteria are Gram-positive, heterofermentative and anaerobic bacteria. As they are naturally predominant among the intestinal tract microflora, their effects on health have been widely recognized (Mitsuoka 1990). Thus, bifidobacteria have been utilized for fermented milk or probiotic diets in the expectation of activating the immune system and enhancing resistance against enteric pathogens. The growth of bifidobacteria can be stimulated by specific compounds, such as oligosaccharides, vitamins, nucleotides and

amino acids (Salminen *et al.* 1998, Crittenden 1999) and by milk proteins such as casein and whey proteins and their hydrolysates (Petschow & Talbott 1990, Gomes *et al.* 1998, Kim *et al.* 1998). Lactoferrin may be one of the proteins that contributes to growth stimulation of lactic acid bacteria and bifidobacteria.

In this report, we investigate the growth-stimulating effects of bovine lactoferrin on *L. acidophilus* and four strains of bifidobacteria. Moreover, we report the presence of lactoferrin-binding proteins in those bacterial strains that are stimulated their growth by lactoferrin. These experiments will help us not only to understand what is happening in the intestine *in vivo* but also to develop a mechanism for a large scale bacterial production.

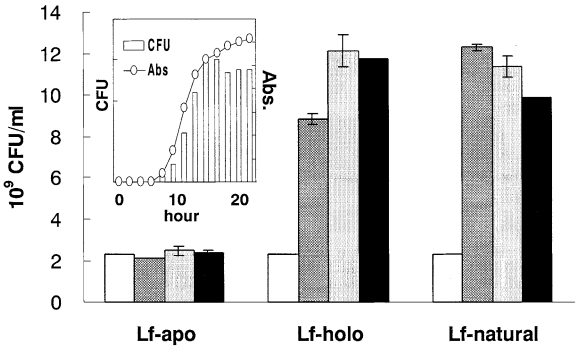

Fig. 1. Effects of bovine lactoferrin on the growth of *L. acidophilus* CH-2 at 16 h incubation. Open bars, control (no lactoferrin); crosshatched bars, 0.5 mg/ml; dotted bars, 1 mg/ml; black bars, 2 mg/ml. The inset graph inserted the relationship between CFU (10^9/ml) counted and the absorbance measured at 660 nm of the *L. acidophilus* culture grown without lactoferrin.

Materials and methods

Bacterial strains, growth conditions and measurements of growth promotion effects

L. acidophilus CH-2 was cultured in MRS broth (Merck, Darmstadt, Germany) or in MRS agar at 37 °C. *B. breve* ATCC 15700, *B. longum* ATCC 15707, *B. infantis* ATCC 15697 and *B. bifidum* ATCC 15696 were cultured under anaerobic conditions at 37 °C in MRS broth containing 0.05% cysteine·HCl. Solutions of lactoferrin were filter-sterilized and added to the autoclaved medium in growth experiments. Bacterial growth was measured by absorbance of the culture media at 660 nm. The growth of *L. acidophilus* was also measured by the number of colony forming units (CFU), which showed good agreement with absorbance data, as shown in Figure 1 (inset). Results are expressed as means ±SD; the significance of differences was determined by Student's *t* test.

Detection of lactoferrin-binding proteins

Extraction of bacterial membrane proteins and the detection of lactoferrin-binding proteins by far-western blot were performed according to our previous report (Kim *et al.* 2002). Briefly, bacterial cells harvested from the culture media were suspended into the protease inhibitor solution (0.1 mM Na-vanadate, 0.5 μg/ml herbimycin A, 50 μg/ml aprotinin, 25 μg/ml leupeptin, 750 μg/ml benzamidine and 1 mM phenylmethylsulfonyl fluoride in Dulbecco's phosphate-buffered saline, pH 7.1) and were processed by supersonic waves and freeze-thaw. Then the precipitate was suspended into the lysis buffer (protease inhibitor solution containing 1% Triton X-100 and 1% CHAPS) and the membrance fraction was obtained by centrifugation at $13,000 \times g$ for 15 min. The membrane-associated proteins were separated by SDS-PAGE performed with a discontinuous gel system using a Tris-HCl glycine buffer (Laemmli 1970) and transferred onto the PVDF membrane. After blocking with 2% serum albumin solution containing 0.01% Tween 20, the PVDF membrane was incubated with biotinylated lactoferrin solution overnight in the refrigerator, and the membrane was washed with saline containing 0.5% Tween 20. Then the membrane was reacted with streptavidin-labeled horseradish peroxidase solution for 30 min at room temperature. After the unbound reagent was removed by washing, the peroxidase activity bound to the proteins on the membrane was detected using the enhanced chemiluminescence (ECL) method.

Proteins and other chemicals

Bovine lactoferrin was supplied by Morinaga Milk Co., Ltd. (Zama). Apo- and holo-lactoferrins were prepared by the method previously reported (Shimazaki & Hosokawa 1991). Biotin-labeled lactoferrin was prepared using N-hydroxysuccinimide biotin. Human lactoferrin, serum albumin and N-hydroxysuccinimide biotin were products of Sigma Chemicals Co. We also purchased the following products from commercial suppliers: streptavidin-labeled horseradish peroxidase (Nichirei Co.), prestained protein markers (New England BioLabs Inc),

Table 1. Effects of lactoferrin on the growth of *Bifidobacterium* spp.

		Ratio of absorbance at 660 nm.[a]			
	concn. (mg/ml)	*B. breve* ATCC 15700	*B. longum* ATCC 15707	*B. infantis* ATCC 15697	*B. bifidum* ATCC 15696
bovine apo-lactoferrin	0.01	$1.11 \pm 0.06^{*}$	0.99 ± 0.01	$1.20 \pm 0.05^{**}$	1.11 ± 0.11
	0.1	$1.14 \pm 0.09^{*}$	0.99 ± 0.01	$1.13 \pm 0.00^{**}$	$1.36 \pm 0.08^{**}$
	1	$1.23 \pm 0.05^{**}$	0.99 ± 0.01	$1.11 \pm 0.02^{**}$	$1.68 \pm 0.16^{**}$
bovine holo-lactoferrin	0.01	1.01 ± 0.05	0.99 ± 0.01	$1.16 \pm 0.03^{*}$	0.96 ± 0.06
	0.1	1.04 ± 0.04	0.99 ± 0.01	1.01 ± 0.02	$1.21 \pm 0.01^{*}$
	1	$1.27 \pm 0.05^{**}$	1.02 ± 0.02	1.09 ± 0.05	$2.64 \pm 0.22^{**}$
natural human lactoferrin	0.01	1.01 ± 0.06	0.98 ± 0.01	$1.16 \pm 0.03^{*}$	1.04 ± 0.06
	0.1	0.97 ± 0.08	0.99 ± 0.03	1.01 ± 0.02	$0.71 \pm 0.10^{**}$
	1	$1.19 \pm 0.10^{*}$	0.87 ± 0.03	1.09 ± 0.05	$1.46 \pm 0.15^{*}$

The values are average and standard deviation calculated from 3 cultures.
* $P < 0.05$, ** $P < 0.01$ in a t test comparing growth with and without lactoferrin.
[a]Ratio of absorbance with lactoferrin to absorbance without lactoferrin at the incubation time of 24 h.

Silver Stain kit Daiichi (Daiichi Pure Chem. Co., Ltd.), ECL kit (Amersham Life Science) and PVDF membranes (Osmonics Lab.).

Results and discussion

We tested the effects of bovine apo-lactoferrin, holo-lactoferrin and natural lactoferrin on growth of *L. acidophilus*. Bacterial cultures were incubated for 16 h with or without lactoferrin; the resulting culture densities at the incubation time of 16 h are shown in Figure 1. Apo-lactoferrin did not affect the growth of *L. acidophilus*, but holo-lactoferrin and natural lactoferrin strongly promoted growth at all concentrations tested. As apo-lactoferrin can capture iron ions, this result suggests two explanations. One is that iron ion in the culture medium is important for the growth of *L. acidophilus*, and apo-lactoferrin removes it and thus inhibits the growth of the bacteria. However, this explanation seems not to be reasonable because apo-lactoferrin should reduce growth of *L. acidophilus* compared to its growth in the absence of lactoferrin according to this explanation. Another is that holo-lactoferrin is beneficial because *L. acidophilus* acquires iron ions through holo-lactoferrin uptake. The other one is that conformational difference between apo- and holo-lactoferrin is concerning with the growth effects.

To determine the effects of lactoferrin on bifidobacteria, growth of four bifidobacterial strains was tested after 24 h incubation in the presence and absence of lactoferrin. Table 1 shows the density of cultures incubated with various forms of lactoferrin relative to cultures with no lactoferrin, as measured by absorbance at 660 nm. Bovine apo-and holo-lactoferrin had a modest growth promotion effect on *B. breve*, *B. infantis* and *B. bifidum*, but not on *B. longum*. Human lactoferrin showed growth promotion effects on *B. infantis* at a lactoferrin concentration of 0.1 mg/ml and *B. bifidum* at 0.01 mg/ml but not on *B. longum* and *B. breve* at a lactoferrin concentration of 0.1 mg/ml. From these results, it seems that lactoferrin stimulates the growth of *L. acidophilus* more than that of *Bifidobacterium* spp. and that the degree of iron saturation of lactoferrin is not important to its effect on bifidobacteria.

Lactoferrin is known to inhibit the growth of many Gram-positive and Gram-negative bacteria, yeasts, molds, and parasites. The bacteriostatic effects of lactoferrin might be due to its ability to sequester environmental iron ions. It is also reported that apo-lactoferrin binds to the outer membrane of Gram-negative bacteria, causing the release of lipopolysaccharide and destroying such cells. Therefore, the environment produced by lactoferrin constitutes an important defense mechanism against invading bacteria. In contrast, some investigators have reported that human or bovine lactoferrin promotes the growth of bifidobacteria *in vitro* (Petschow & Talbott 1991, Tomita *et al.* 1994, Liepke *et al.* 2002) and *in vivo* (Kodama 1983, Hentges *et al.* 1992, Roberts *et al.* 1992, Wharton *et al.* 1994). The ability of lactoferrin to stimulate the growth of bifidobacteria indicates that lactoferrin also contributes to host defense by pro-

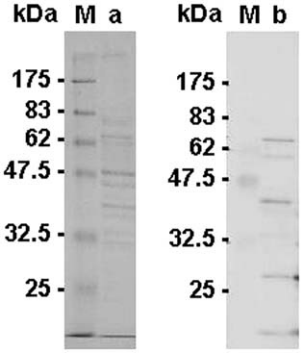

Fig. 2. Membrane proteins (left) and lactoferrin-binding proteins (right) from a membrane fraction of *L. acidophilus*. M, prestained protein markers with the indicated molecular weights; a, protein bands stained with Coomassie brilliant blue R250; b, far-western blot with biotinylated lactoferrin as probe. The concentration of SDS-PAGE separation gel (85×60×1 mm) was 12% and the protein amount applied on each slot was about 100 μg.

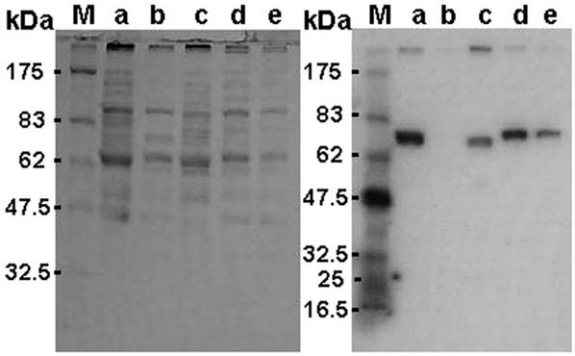

Fig. 3. Lactoferrin-binding proteins from *Bifidobacterium* spp. Membrane proteins (left) were Coomassie-stained, and lactoferrin-binding proteins (right) were detected by far-western blot. M, prestained protein markers with the indicated molecular weights; a, *B. breve* ATCC 15700; b, *B. longum* ATCC 15707; c, *B. infantis* ATCC 15697; d, *B. bifidum* ATCC 15696; e, *B. bifidum* Bb-11. The concentration of SDS-PAGE separation gel used was 10%. Other experimental conditions were the same as in Figure 2.

moting the development of a more favorable intestinal flora. Lactoferrin's growth effects on bifidobacteria are modest compared to its effect on *L. acidophilus* as proved by our experiments, but it is still possible that lactoferrin is one of the factors controlling the intestinal microflora. Moreover, our results confirm the suggestion of a previous report by Gomes *et al.* (1998) that the growth-promoting effects of lactoferrin on bifidobacteria are strain-dependent. Our results differ from those of Miller-Catchpole *et al.* (1997), who reported that *B. breve* ATCC 15700 growth is inhibited by iron-free human lactoferrin and stimulated by iron-saturated human lactoferrin. Our results were obtained using natural human lactoferrin of which iron saturation degree may be 10–30%. In the intestinal tract, most of the proteins in milk proteins should be cleaved to peptides by proteases. It has been reported that such peptides derived from casein and other whey proteins, including lactoferrin, promote growth of bifidobacteria (Petschow & Talbott 1990, Gomes *et al.* 1998, Kim *et al.* 1998, Liepke *et al.* 2002). We have found that lactoferrin hydrolysates prepared by trypsin and pepsin stimulate growth of *L. acidophilus* (manuscript in preparation).

Because lactoferrin-binding proteins or lactoferrin receptors have been found in many microorganisms, and we previously found lactoferrin-binding proteins in *B. bifidum* Bb-11 (Kim *et al.* 2002), we next attempted to detect lactoferrin-binding proteins in membrane fractions of *L. acidophilus* and *Bifidobacterium* spp. Proteins in the membrane fraction of *L. acidophilus* were visualized by dye-staining and by far-western blot using biotinylated lactoferrin as the probe (Figure 2). The blot detected three main bands, located at the molecular weights of 27, 41 and 67 kDa and a minor band at 58 kDa. In bifidobacteria, however, far-western blotting identified fewer bands. Lanes a, d and e in Figure 3 show 69 kDa bands in the membrane fraction of *B. breve* and in two strains of *B. bifidum*. Lane c shows a 67 kDa lactoferrin-binding band in the membrane of *B. infantis*. In contrast, *B. longum* (lane b) showed no or very weak lactoferrin-binding bands.

It is interesting that the bacterial stains whose growth was stimulated by lactoferrin (*L. acidophilus* and three strains of bifidobacteria) also displayed lactoferrin-binding protein or proteins. *B. bifidum* Bb-11 (Figure 3, lane e), in which we found at least one lactoferrin-binding protein (Kim *et al.* 2002), has not been tested for growth stimulation or inhibition by lactoferrin. *B. longum* had no apparent lactoferrin-binding proteins in the membrane fraction, and its growth was unaffected by lactoferrin. These results suggest that the growth-stimulatory activity of lactoferrin may be related to the presence of lactoferrin-binding proteins on the surface of bacterial cells. Some bacteria, such as *E. coli*, secrete chelators to enhance iron uptake. Lactoferrin might provide an alternative means of iron acquisition, if the bacteria have outer membrane protein receptors that recognize lactoferrin complexed with iron, resulting in the internalization of this metal. Indeed, a number of bacterial species have surface receptors capable of specifically binding lacto-

ferrin, with molecular weights ranging from 27 kDa to several hundred kDa. Such cell-surface receptors for lactoferrin are believed to contribute to bacterial growth by facilitating the uptake of iron ions (Otto *et al.* 1992, Gray-Owen & Schryvers 1996, Modun *et al.* 2000). However, the mechanism of iron ion uptake by bacteria in the digestive tract has not yet been resolved, and further studies are needed to test these ideas on *L. acidophilus* and bifidobacteria.

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