APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1997, p. 506–512 Vol. 63, No. 2
0099-2240/97/$04.00+0

Proteinaceous Factor(s) in Culture Supernatant Fluids of Bifidobacteria Which Prevents the Binding of Enterotoxigenic *Escherichia coli* to Gangliotetraosylceramide

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Received 18 March 1996/Accepted 4 November 1996

We have examined the competitive binding of several species of *Bifidobacterium* and *Escherichia coli* Pb176, an enterotoxigenic *E. coli* (ETEC) strain, to gangliotetraosylceramide (asialo GM1 or GA1), a common bacterium-binding structure, and identified a factor(s) in the *Bifidobacterium* culture supernatant fluid that inhibits the binding of *E. coli* Pb176 to GA1. The ETEC strain we used expresses colonization factor antigen (CFA) II, which consists of coli surface-associated antigens CS1 and CS3. Competitive exclusion of ETEC from GA1 molecules by *Bifidobacterium* cells was found by an in vitro thin-layer chromatography overlay binding suppression assay. However, the ETEC cells were less effective in blocking the adherence of *Bifidobacterium* cells to GA1. These findings suggest that the two bacterial species recognize different binding sites on the GA1 molecule and that the mechanism of competitive exclusion is not due to specific blockage of a common binding site on the molecule. The neutralized culture supernatant fluids of *Bifidobacterium* species, including that of *Bifidobacterium longum* SBT 2928 (BL2928), showed remarkable inhibition of the ETEC binding to GA1. Our results suggest that the binding inhibitor produced by BL2928 is a proteinaceous molecule(s) with a molecular weight around or over 100,000 and a neutral isoelectric point. The binding inhibitor produced by BL2928 and other *Bifidobacterium* species is estimated to contribute to their normal anti-infectious activities by preventing the binding of pathogenic strains of *E. coli* to GA1 on the surface of the human intestinal mucosa.

Infections of the gastrointestinal tract are a major health problem for both adults and children worldwide. The major causes of postweaning diarrhea in neonates include the enterotoxigenic *Escherichia coli* strains, which bear colonization factor antigens (CFAs) I and II (14). However, normal intestinal microbes and some probiotic bacteria are believed to enhance the host defense mechanisms against pathogens. Since the first isolation of *Bifidobacterium* species by Tissier (24) from a breast-fed infant, it has been acknowledged widely that bifidobacteria are the predominant species of the intestinal flora of infants (16, 18). A role for Bifidobacteria in host resistance to infection has been repeatedly suggested from various observations (9), but there is limited information on the mechanism by which this might occur. A better understanding of the relationship between the two types of bacteria may help to confirm that there is a beneficial role for bifidobacteria in ensuring the health of the host.

Recently, there has been renewed interest in the age-old concept of using bacterial preparations with inhibitory activity against enteric pathogens for protecting domestic animals and humans. This concept is often referred to as bacterial interference (21, 22). For example, lactose fermenters produce metabolites that are antagonistic to the growth of pathogens. The concept of competitive exclusion of pathogenic microbes by lactic acid bacteria has been reviewed by Conway (3).

Several years ago, Mizutani and Mitsuoka (17) noted that oral administration of several bifidobacterial strains lowered the population of *E. coli* in the bacterial flora of gnotobiotic mice. More recently, our laboratory confirmed that finding by using *Bifidobacterium* strain BL2928 in the same system (5). Those findings suggested the possibility of competitive exclusion between bifidobacteria and *E. coli* in the intestinal flora. Umesaki et al. (26, 27) demonstrated that the intestinal brush border membrane of the mouse is rich in GA1, a neutral sphingoglycolipid, and indicated that *Enterobacteriaceae* have a high affinity for GA1 in vitro. They speculated that fucosylation of the GA1 molecule in the intestinal brush border membrane during the establishment of a germ-free strain of mice enhances host defense mechanisms against infection because it alters the function of the glycolipid (25). Orø et al. (19) showed also that *E. coli* strains harboring CFAs II and IV possess high affinity for GA1.

In the present study, we examined the following two hypotheses: (i) that several strains of both *Bifidobacterium* and *E. coli* have an affinity for GA1 molecules and compete for binding and (ii) that bifidobacteria produce a factor(s) that inactivates or blocks the access of *E. coli* to GA1 receptor sites, with the implication that in vivo the population of *E. coli* would be lowered. To carry out this study, we used thin-layer chromatography (TLC) overlay assays for evaluating the binding of bacteria to the GA1 receptor and biochemical separation techniques to isolate an inhibitory factor from *Bifidobacterium* culture supernatant fluid.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and radiolabeling. The bifidobacterial strains used in this study were isolated from normal human intestinal contents and have been used as the starter strains for making fermented milk. Bifidobacteria were grown in Briggs liver analog (BLA) medium (20 g of tomato juice broth [Difco] per liter, 15 g of protease peptone no. 3 [Difco] per liter, 1 g of yeast extract [Difco] per liter, 15 g of glucose per liter, 5 g of NaCl per liter, 0.2 g of L-cysteine · HCl per liter, 1 g of Tween 80 per liter) under static culture

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TABLE 1. Composition of GPV medium for bifidobacteria

Ingredient	Amt (g/liter)
Ammonium sulfate	3
L-Cysteine HCl · H_2O	0.5
K_2HPO_4	1.1
KH_2PO_4	1.9
$MgSO_4 \cdot 7H_2O$	0.2
Trypticase peptone (BBL)	30
Glucose	25
$MnSO_4 \cdot 7H_2O$	0.25
$FeSO_4 \cdot 7H_2O$	0.015
Sodium acetate	1.5
Sodium citrate	0.05
Tween 80	0.1
Folic acid	0.1
Nicotinamide	0.001
Pantothenic acid	0.001
Pyridoxol	0.001
Riboflavin	0.001

conditions for 2 to 3 days at 37°C. For radiolabeling, the bacteria were cultured in 10 ml of either BLA broth or glucose-peptone-vitamin (GPV) broth (Table 1) in the presence of 18.5 MBq of [^{35}S]methionine (specific radioactivity, 3.7×10^{13} Bq/mmol; ICN Biomedicals, Mississauga, Ontario, Canada) under static culture conditions for 2 to 3 days at 37°C. Enterotoxigenic *E. coli* (ETEC) Pb176 (CFA II; CS1 plus CS3) was used as the indicator strain. The binding specificity of the ETEC strain to GA1 glycolipid has been previously reported by Orø et al. (19). For metabolic labeling, the ETEC strain was cultured in 10 ml of either M9 medium (8) as a minimal medium or tryptic soy broth (Difco, Detroit, Mich.) in a rotary shaker at 150 rpm for 12 h at 37°C with 7.4 MBq of [^{35}S]methionine (specific radioactivity, 3.7×10^{13} Bq/mmol). The binding specificity and affinity of the bacteria for GA1 was the same in both types of culture.

TLC overlay assays. The TLC overlay assays were carried out as described by Karlsson and Stroenberg (12) with slight modifications. TLC chromatography was performed on silica gel-precoated plates (POLY GRAM SILG plastic plate; Brinkmann Instruments Division, Sybron Canada Ltd., Rexdale, Ontario, Canada). The plates were developed in chloroform-methanol-water (60:40:9 or 65:24:4) and then allowed to dry in air. The dried chromatograms were dipped in water in a square petri dish (100 by 100 mm), and the water was removed by aspiration and replaced by Tris-buffered saline (pH 8.0) containing 1% (wt/vol) gelatin and 0.02% sodium azide (gelatin-TBS).

For visualization of glycolipids, an identical plate was sprayed with orcinol reagent and incubated at 100°C for color development. The plate was blocked with gelatin-TBS overnight at 37°C, after which the solution was decanted and replaced with phosphate-buffered saline containing 1% bovine serum albumin (BSA-PBS). The plates were washed three times with BSA-PBS with gentle rotation (50 rpm for 5 min). Subsequently, [^{35}S]methionine-labeled bacteria were washed three times with BSA-PBS by centrifugation (6,000 $\times$ *g* for 10 min) and resuspended in BSA-PBS to give a concentration of 2×10^9 CFU/ml. For bifidobacteria, the concentration was 3×10^8 CFU/ml. The bacterial suspension was poured over the TLC plates, and binding was allowed to proceed during incubation at 37°C with gentle rotation (50 rpm). After 1.5 h, the suspension was removed by gentle aspiration and the plates were washed five times with BSA-PBS by gentle rotation in a petri dish (25 rpm for 5 min). For binding suppression assays, the labeled bacteria were preincubated with an inhibitor at a given concentration for 0.5 h in BSA-PBS at 37°C. In several cases, live bacterial cells were also used as an inhibitor. The bacterial mixture was poured over the TLC plates and further incubated with gentle rotation (50 rpm) at 37°C for 1.5 h.

Once the overlay was completed, the plates were gently washed five times with BSA-PBS by rotation (25 rpm for 3 min for each washing), dried in air, and autoradiographed for 2 to 3 days at −70°C. To quantitate bound bacteria, densitometry of autoradiograms was performed with an LKB Ultroscan XL laser scanner (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden). If the radiolabeling efficiency of the bifidobacteria was poor, the plates were treated with mouse anti-bifidobacterial ascites fluid (1:250) at 37°C for 1 h with gentle rotation (50 rpm). After the plates were washed with BSA-PBS (three times for 3 min each), they were overlaid with horseradish peroxidase-labeled goat-anti mouse immunoglobulin (1:250, vol/vol) (Zymed Laboratories, Inc., San Francisco, Calif.) at 37°C for 1 h. Thereafter, the plates were washed three times (for 3 min each) with BSA-PBS and twice more with PBS. Colour development was performed with the POD immunostain set (Wako Pure Chemical Industries, Ltd., Osaka, Japan), as specified by the manufacturer, until control bacterial binding was clearly detected.

Preparation of culture supernatant fluid. BLA broth was used to cultivate bifidobacteria. A static culture was centrifuged to remove the cells (10,000 $\times$ *g* for 15 min), and the pH of the supernatant was adjusted to ca. 6.0 with 10 N NaOH. The supernatant was filtered through a fiberglass filter to remove large debris and then through a 0.45-mm-pore-size filter unit (Millipore, Bedford, Mass.). The sterilized cultured supernatant was stored at −20°C until used.

Ammonium sulfate fractionation. Solid ammonium sulfate was added to the filtered supernatant by stirring until the solution reached 85% saturation. This solution was kept at 4°C overnight to allow complete precipitation of the protein and then centrifuged at 10,000 $\times$ *g* for 15 min, and the pellet was resuspended in distilled water. The suspension was dialyzed at 4°C against distilled water (molecular weight cutoff, 6,000 to 8,000) with six changes of water. The resulting preparation was stored at −80°C, and a portion of it was lyophilized.

Molecular weight estimation. The lyophilized precipitate was reconstituted to 0.16 to 0.10 of its original volume with PBS containing 0.02% sodium azide and applied to a Bio-Gel P100 column (column volume, 200 ml; 36 by 200 mm; Bio-Rad Laboratories, Richmond, Calif.) which was equilibrated with the same buffer at a flow rate of 0.7 ml/min at 4°C. The void volume fraction (P100V) was collected, dialyzed against distilled water, freeze-dried, and stored at −80°C. The remaining eluate (P100S fraction) was also pooled, dialyzed (molecular weight cutoff, 2,000), and freeze-dried. The P100S fraction was loaded onto a Bio-Gel P10 column (200 ml; 36 by 200 mm; Bio-Rad) equilibrated with PBS containing 0.02% sodium azide. The void volume fraction (P10V) and the remaining fraction (P10S1, which included the column volume) were collected separately. Another 100 ml of eluate (P10S2) was also collected. The three fractions were dialyzed (molecular weight cutoff, 2,000) and freeze-dried.

Deionization of culture supernatant fluid. Three different methods of deionization were used to avoid inactivation of potential inhibitory factors: (i) dialysis with Spectra/Por membrane tubing (Spectrum Medical Industries, Inc., Houston, Tex.) with a molecular weight cutoff of 6,000 to 8,000; (ii) ion-exchange chromatography with a mixed-bed resin, AG501X8 (Bio-Rad Laboratories, pH ca. 6); and (iii) hydrophobic interaction chromatography with Bond Elut C8 (Analytichem International, Inc., Harbor City, Calif.). For the hydrophobic interaction chromatography, the active fraction was eluted with 50% methanol and then dialyzed against cold distilled water.

Proteinase K treatment. Ammonium sulfate precipitates and the P100V fraction were treated with proteinase K at 37°C overnight at enzyme/substrate (E/S) ratios of 1:1, 1:10, and 1:100 in 25 mM HEPES–Hanks balanced salt solution. The reaction was terminated by adding phenylmethylsulfonyl fluoride dissolved in isopropanol (10 mg/ml) to give a final concentration of 100 μg/ml. The resulting reaction mixture was dialyzed against distilled water (molecular weight cutoff, 2,000), lyophilized, and then used in TLC overlay inhibition assays.

Lipid extraction. The lipid fraction was extracted from 100 ml of culture supernatant with chloroform-methanol (1:1, vol/vol), and the resulting chloroform layer was dried with a flash evaporator. The lipid fraction was dispersed in BSA-PBS with a sonic disruptor, and the suspension was used in the binding suppression assay.

Heat treatment. The ammonium sulfate precipitates from the neutralized cultured supernatant of BL2928 were heat treated at various temperatures for 10 min and used in TLC overlay inhibition assays.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Protein profiles of the different fractions obtained were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 7.5% acrylamide gel (13). All the gels were stained either with Coomassie brilliant blue R250 or with silver reagent, using a silver staining kit (Bio-Rad Laboratories).

Determination of protein content. The protein content of all fractions was measured with a protein assay kit (Bio-Rad Laboratories). Bovine serum albumin was used as the standard. The relative protein content was also measured by monitoring the spectrophotometric absorption at 280 and/or 254 nm.

RESULTS

Binding patterns of bifidobacteria to glycolipids. Figure 1 shows the chromatograms of glycolipids developed on TLC plates which were visualized by using orcinol reagent. Figure 2 shows the binding spectrum of several species of *Bifidobacterium* for glycolipids on TLC plates. The binding patterns differed somewhat among various species of *Bifidobacterium.* *Bifidobacterium longum* SBT 2928 (BL2928) bound to all glycolipids tested, including a mixture of globosides, and to phosphatidylethanolamine. The binding patterns of *B. breve* SBT 2803 and *B. infantis* SBT 2852 were relatively similar. *B. bifidum* SBT 0583 showed particularly strong binding to gangliosides. Three of the four species of *Bifidobacterium* tested bound to the GA1 molecule, but the binding was relatively weak except for *B. breve* SBT 2803. In general, binding to all of the negatively charged glycolipids, ganglosides, and sulfatide was observed. All the gangliosides tested exhibited comparable affinity for bifidobacterial cells. Interestingly, however, all of

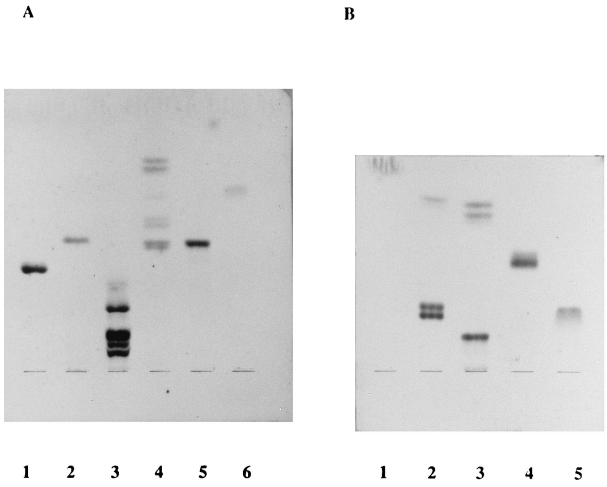

FIG. 1. Thin-layer chromatograms of glycolipids. (A) Lanes: 1, 5 μg of asialo GM1 (Accurate Chemical, Westbury, N.Y.); 2, 5 μg of asialo GM2 (Accurate Chemical); 3, 20 μg of gangloiside mixture from bovine brain (Sigma Chemical Co., St. Louis, Mo.); 4, 20 μg of an equal mixture of globosides (galactosylceramide, lactosylceramide, globotriaosylceramide, and globotetraosylceramide; Matreya, Inc., Pleasant Gap, Pa.); 5, globotetraosylceramide (Accurate Chemical); 6, 5 μg of phosphatidylethanolamine (Sigma). The TLC plate was developed with chloroform-methanol-water (60:40:9, vol/vol/vol). (B) Lanes: 1, 5 μg of ceramide type III and 5 μg of ceramide type IV (Sigma); 2, 5 μg of glucosylceramide (Sigma) and 5 μg of globotriaosylceramide (doublet; Accurate Chemical); 3, 5 μg of galactosylceramide (doublet; Sigma) and 5 μg of globotetraosylceramide (Accurate Chemical); 4, 5 μg of lactosylceramide (Sigma); 5, 5 μg of sulfatide (Sigma). The TLC plate was developed with chloroform-methanol-water (65:24:4, vol/vol/vol). The materials developed on both TLC plates were detected with orcinol reagent.

the *Bifidobacterium* strains also bound to the ceramide moiety itself (Fig. 2B, lanes 1).

Binding patterns of *E. coli* strains to glycolipids. The glycolipid-binding specificity of two strains of *E. coli* (ETEC Pb176 and commensal *E. coli* SBT3242) was examined (Fig. 3). Both strains bound firmly to GA1, whereas binding to other glycolipids and to phosphatidylethanolamine was virtually negligible.

Competition between bifidobacteria and *E. coli* for binding to the GA1 molecule. Figure 4 shows the binding competition between two strains of *E. coli* (ETEC Pb176 and *E. coli* SBT3242) and BL2928. BL2928 clearly inhibits the binding of both *E. coli* strains to GA1 on TLC; however neither of the *E. coli* strains showed much inhibition of BL2928 binding to GA1 (Fig. 4C). Almost identical results were obtained with the other bifidobacteria (*B. infantis* SBT 2852, *B. breve* SBT 2803, and *B. bifidum* SBT 0583 [data not shown]).

Inhibition of the binding of *E. coli* to GA1 by neutralized culture supernatant fluid of bifidobacteria. To check the possibility that bifidobacteria produce factors that reduce the binding affinity of *E. coli* strains to GA1, *E. coli* strains were preincubated with neutralized *Bifidobacterium* culture supernatant fluid before being overlaid onto TLC plates on which GA1 had been developed. Figure 5 shows that culture supernatants of all species of *Bifidobacterium* inhibited ETEC binding to GA1. In subsequent experiments, we focused on the inhibitory activity of BL2928, because *B. longum* is a predominant species in the human intestinal bifidobacterial flora and it also serves as an important starter for producing fermented milk.

Effects of BL2928 culture media and deionization on ETEC-binding inhibition. Table 2 summarizes the effects of culture media and several deionization procedures on the inhibitory activity of neutralized culture supernatant fluid of BL2928. None of the deionization procedures significantly affected the inhibitory activity of the culture supernatant fluid. These results suggest that inhibition of *E. coli* binding was independent of culture medium and ionic strength. The inhibitory material(s) was judged to have almost no charge at about pH 6 (from the results of deionization with the mixed-bed ion-exchange resin) and to have a molecular weight greater than 6,000 to 8,000 (from the results of dialysis).

Recovery of the inhibitory activity by salting out. The ammonium sulfate precipitate (85% saturation) of the neutralized culture supernatant fluid of BL2928 was dialyzed and reconstituted to its original volume with BSA-PBS. The binding-inhibitory activity of this fraction was comparable to that of the original neutralized spent-culture fluid of the strain (data not shown).

Activity in the lipid fraction. The lipid fraction was extracted from the culture supernatant fluid with chloroform-methanol (1:1) and reconstituted in culture supernatant fluid to its orig-

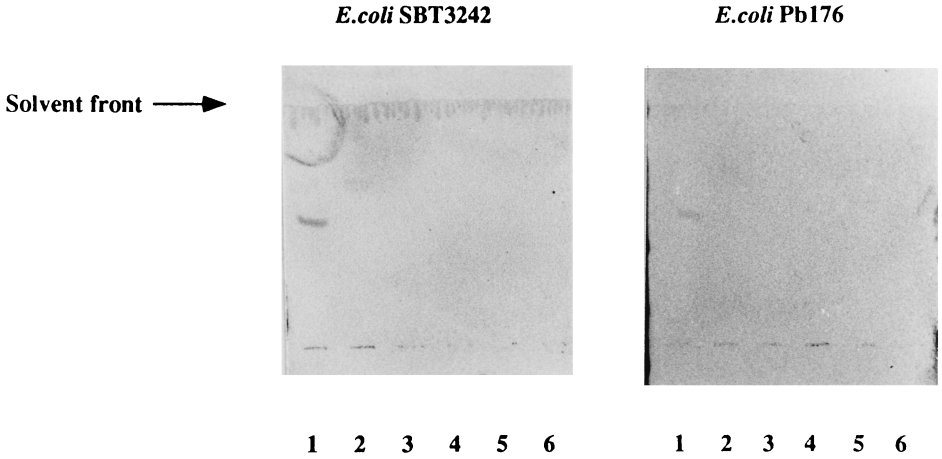

FIG. 3. Autoradiograms of bound ^{35}S-labelled *E. coli* strains. Lanes: 1, 5 μg of asialo GM1 (Accurate Chemical); 2, 5 μg of asialo GM2 (Accurate Chemical); 3, 20 μg of a gangloside mixture from bovine brain (Sigma); 4, 20 μg of an equal mixture of globosides (mixture of galactosylceramide, lactosylceramide, globotriaosylceramide, and globotetraosylceramide; Matreya, Inc.); 5, globotetraosylceramide (Accurate Chemical); 6, 5 μg of phosphatidylethanolamine (Sigma). The TLC plate was developed with chloroform-methanol-water (60:40:9, vol/vol/vol).

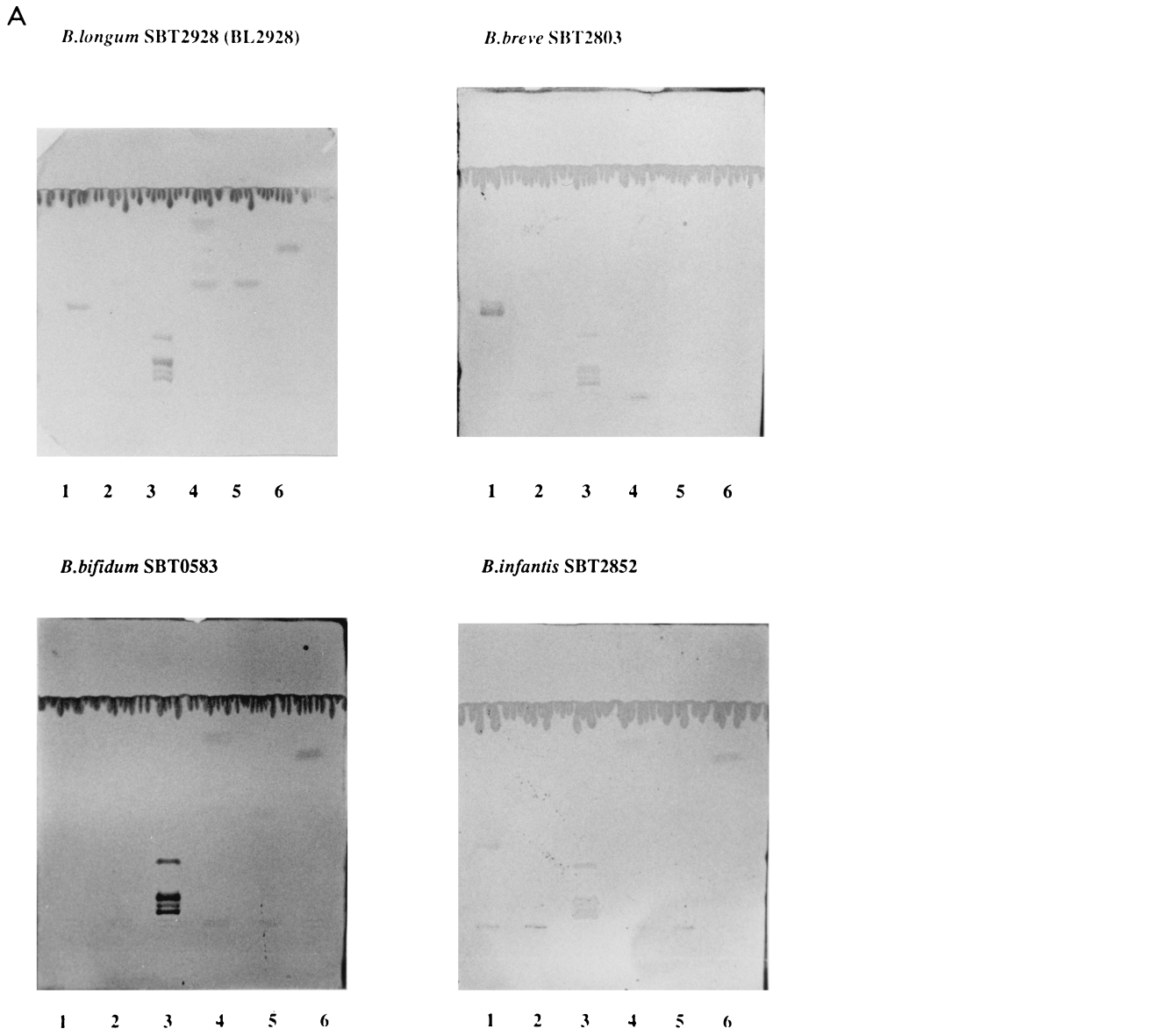

FIG. 2. (A) Autoradiograms of bound ^{35}S-labelled bifidobacteria. Lanes: 1, 5 μg of asialo GM1 (Accurate Chemical); 2, 5 μg of asialo GM2 (Accurate Chemical); 3, 20 μg of gangloiside mixture from bovine brain (Sigma); 4, 20 μg of an equal mixture of globosides (mixture of galactosylceramide, lactosylceramide, globotriaosylceramide, and globotetraosylceramide; Matreya, Inc.); 5, globotetraosylceramide (Accurate Chemical); 6, 5 μg of phosphatidylethanolamine (Sigma). The TLC plate was developed with chloroform-methanol-water (60:40:9, vol/vol/vol). (B) Autoradiograms of bound ^{35}S-labelled bifidobacteria. Lanes: 1, 5 μg of ceramide type III (Sigma); 2, 5 μg of ceramide type IV (Sigma); 3, 5 μg of sulfatide (Sigma); 4, 5 μg of glucosylceramide (Sigma); 5, 5 μg of lactosylceramide (Sigma). The TLC plate was developed with chloroform-methanol-water (65:24:4, vol/vol/vol).

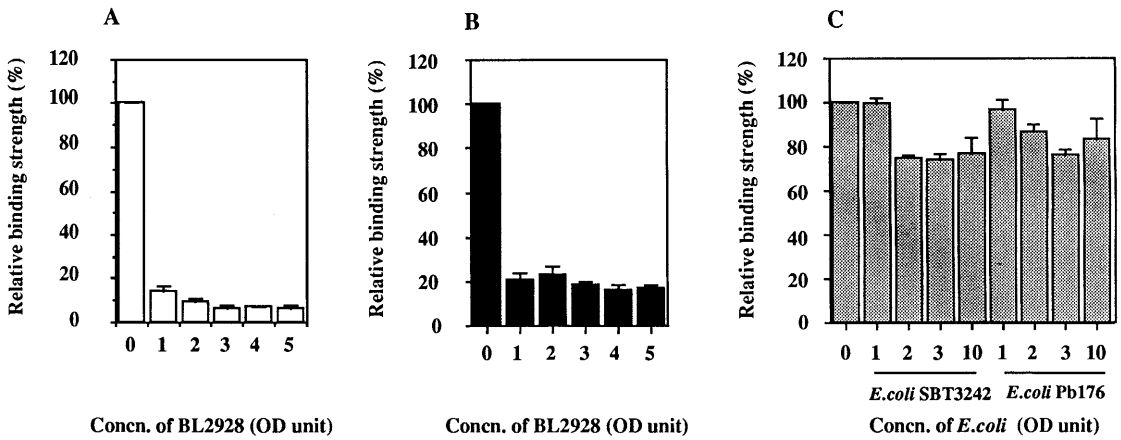

FIG. 4. Competition between *E. coli* strains and BL2928 for binding to GA1. Relative binding strength of a commensal *E. coli* SBT3242 (A) and ETEC Pb176 (B) to GA1 in the presence of various concentrations of BL2928. Values on the *x* axis express the optical density (OD) of BL2928 in the competition assay system. (C) Reverse binding competition between labeled BL2928 and the two strains of *E. coli*. One OD unit corresponds to 2×10^9 CFU/ml for *E. coli* and 3×10^8 CFU/ml for BL2928.

inal concentration. This fraction did not show any inhibitory activity on the binding of ETEC to GA1. Even after reconstitution of the lipid extract in 1/10 of its original volume, there was no inhibitory activity (data not shown).

Effect of heat treatment on the inhibitory activity of the ammonium sulfate precipitates. The inhibitory activity of the precipitates almost disappeared after they were heated at 100°C for 10 min. Heat treatment for 10 min at 60°C caused an approximately 90% loss of activity (data not shown).

Time-dependent production of the binding inhibitory activity. Figure 6 shows that there is a time-dependent increase in the production of inhibitory activity as measured in ammonium sulfate precipitates of BL2928 culture supernatant fluid. Activity could be detected as early as 24 h after culturing BL2928.

Binding-inhibitory activity of BL2928 against various unrelated bacteria which bind to GA1. Figure 7 shows an inhibition spectrum of the ammonium sulfate precipitate prepared from culture supernatant fluid of BL2928 against several bacteria which show affinity for GA1. The ammonium precipitate fraction reduced not only the binding of enterotoxigenic *E. coli* Pb176 to GA1 but also the binding to GA1 of two strains of human commensal *E. coli* (SBT 3146 and SBT 3242), *Pseudomonas aeruginosa* SBT 3092, and *Burkholderia cepacia* 61 (the last bacterium was isolated from sputum of Toronto patients with cystic fibrosis).

Protease treatment of the fraction containing the binding-inhibitory activity. Table 3 shows the effect of proteinase K treatment of ammonium sulfate precipitates of BL2928 culture supernatant fluid on ETEC binding to GA1. Proteinase K treatment reduced the binding-inhibitory activity, and the decrease was more marked as the amount of proteinase K added was increased.

Molecular weight estimation. Molecular weight estimation of the ETEC-binding inhibitory activity of BL2928 was performed by using a combination of two different gel filtration columns (Bio-Gel P100 and Bio-Gel P10). Most of the activity was found in the void volume after gel filtration through the Bio-Gel P100 column (P100V fraction). Proteinase K treatment of this fraction also reduced the ETEC-binding inhibitory activity in a dose-dependent manner (data not shown).

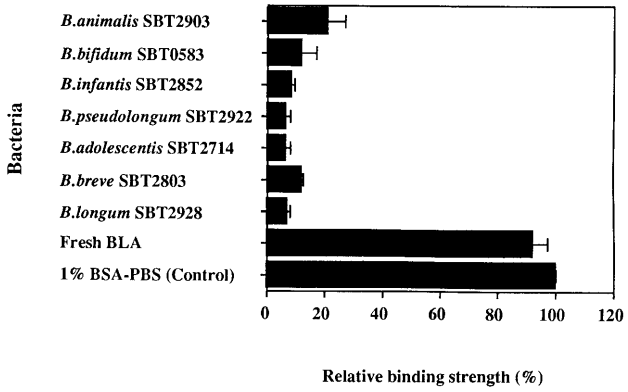

FIG. 5. Inhibitory activity of neutralized cultured supernatants of several species of bifidobacteria on the binding of ETEC (*E. coli* Pb176) to GA1. Radiolabeled ETEC cells were suspended in each neutralized cultured supernatant and overlaid on TLC plates on which GA1 (5 μg) had been developed. The binding strength was measured densitometrically and compared with the binding strength of controls (in BSA-PBS), set to a binding value of 100. Data are expressed as the mean relative binding strength ± standard deviation ($n = 5$).

TABLE 2. Effects of deionization on ETEC-binding inhibition by neutralized spent culture supernatant fluids of BL2928[a]

Medium	% Relative binding in[b]:				
	Uninoculated medium	Neutralized culture supernatant fluid[c]			
		Intact	Deionized with:		
			AG501X8[d]	Octyl-silane	Dialysis
BLA	75.4 ± 8.3	16.5 ± 3.3	23.4 ± 4.7	20.9 ± 2.9	30.3 ± 3.5
MRS	105.0 ± 8.2	37.0 ± 5.0	42.6 ± 3.8	44.5 ± 9.1	52.0 ± 6.6
MRS lacking meat extract	120.0 ± 20.0	56.0 ± 6.4	43.7 ± 7.2	54.8 ± 4.0	52.3 ± 8.4
GPV	85.6 ± 10.7	31.0 ± 6.0	38.0 ± 5.8	40.9 ± 7.7	45.6 ± 4.9

[a] ETEC cells were coincubated with uninoculated medium or intact or deionized neutralized spent culture supernatant fluid (reconstituted to the original volume) and then overlaid onto TLC plates on which GA1 had been developed as described in Materials and Methods. The control binding strength of ETEC to GA1 was measured in BSA-PBS and was set at 100.

[b] Data are expressed as percent relative binding strength ± standard deviation ($n = 5$) compared with control binding.

[c] Neutralization was performed by adding 10 N NaOH until the pH reached 6.

[d] A mixed-bed ion-exchanger supplied by Bio-Rad.

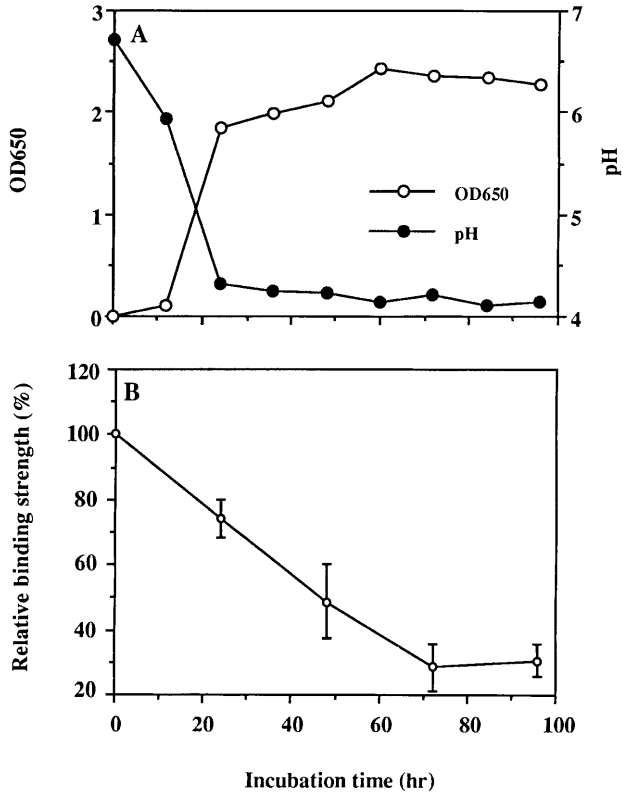

FIG. 6. Growth curve of BL2928 (A) and time-dependent expression of the inhibitory activity for ETEC (*E. coli* Pb176) binding to GA1 (B) in ammonium sulfate precipitates of culture fluids at various culture ages. The binding-inhibitory activity is shown as the relative binding strength ± standard deviation (n = 3) compared with the binding strength (100%) in the presence of the starting (zero time) medium. Ammonium sulfate precipitates were dialyzed and reconstituted to their original volume with BSA-PBS. BLA was used as the culture medium in this experiment. OD, optical density.

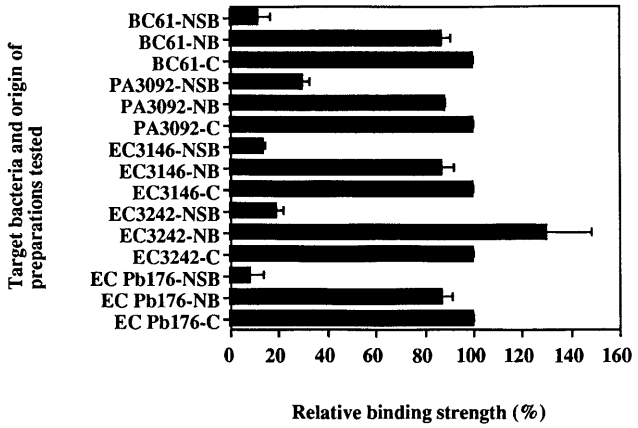

FIG. 7. Spectrum of the inhibitory activity of ammonium sulfate precipitates from cultured BL2928 supernatant on the binding of various bacteria to GA1. Abbreviations: BC61, *B. cepacia* 61; PA3092, *P. aeruginosa* SBT 3092; EC3146, *E. coli* SBT 3146 (commensal strain, human origin); EC3242, *E. coli* SBT 3242 (commensal strain, human origin); ECPb176, *E. coli* Pb176 (enterotoxigenic strain, human origin); C, the assay was carried out in BSA-PBS; NB, the assay was carried out with the ammonium precipitate fraction from fresh BLA (reconstituted to the original volume with BSA-PBS); NB, the assay was carried out with the ammonium sulfate precipitates from neutralized cultured BLA (reconstituted to the original volume with BSA-PBS). Data are expressed as the percent relative binding strength ± standard error of the mean (n = 3) with respect to control (100%) binding.

DISCUSSION

This study provides evidence that several strains of *Bifidobacterium* of human origin, including BL2928, release a proteinaceous molecule(s) which interferes with the adhesion of several strains of bacteria (including strains of ETEC and commensal *E. coli*) to GA1 (asialo-GM1). Binding of *E. coli* strains to GA1 was measured in vitro by TLC overlay binding suppression assays.

ETEC strains are the major cause of bacterial diarrhea, especially in infants (14) and in travelers to developing areas (6). For many years, it has been claimed that ingestion of certain preparations of bacteria, particularly lactose fermenters (including bifidobacteria), can prevent or ameliorate bacterial diarrheas. Recently, interest has been focused on understanding the mode of the defensive action of these bacterial preparations used as probiotics.

The predominance of bifidobacteria in the intestinal flora of newborn babies has been well established (2, 18). Bifidobacteria appear between days 2 and 5 after birth (16) and become dominant within 1 week. At this time, they account for 99% of the fecal microflora. The bifidobacterial biofilm covers the intestinal epithelial wall and provides a defense against pathogenic bacteria and/or opportunistic pathogens (1, 10, 15). Mizutani and Mitsuoka (17) showed that some kinds of lactic acid bacteria, including *B. longum*, lowered the population of *E. coli* in the gut microflora in gnotobiotic mice. Faure et al. (4) reported that *B. longum* conferred complete resistance to colonization by an *E. coli* strain pathogenic to gnotobiotic rats. Yamazaki et al. (28) reported similar protective effects of *B. longum* in gnotobiotic mice. Several possible protection mechanisms have been postulated (20), although the exact mechanism(s) has not yet been elucidated. Bernet et al. (1) have reported competitive binding between bifidobacteria and enteropathogens for cultured Caco-2 cells, but the mechanism of competition was not described.

In the present study, we demonstrated that bifidobacteria, especially *B. longum*, inhibit the binding of *E. coli* strains (including one ETEC strain) to the GA1 binding site. Three of the four *Bifidobacterium* strains that we tested showed affinity to GA1; therefore direct competitive exclusion of *E. coli* strains would be expected. The *E. coli* strains that bound to GA1 blocked the adherence of bifidobacteria to GA1 very slightly, whereas there was a dramatic effect of bifidobacteria on re-

TABLE 3. Effect of proteinase K treatment on the ETEC-binding inhibitory activity of ammonium sulfate precipitates of BL2928 culture supernatant

Treatment	Relative binding strength[a]
Control (BSA-PBS)	100
PPT[b]	17.5 ± 9.2
PK/PPT (1:100)[c]	29.2 ± 10.0
PK/PPT (1:10)	60.0 ± 20.2
PK/PPT (1:1)	95.0 ± 11.8

[a] Data are expressed as relative binding strength ± standard deviation (n = 3) compared with the control binding (100%).

[b] The assay was carried out in the presence of the ammonium sulfate precipitate fraction (reconstituted to one-fifth of the volume with BSA-PBS) from neutralized culture supernatant of BL2928.

[c] Assays were carried out in the presence of proteinase K-treated ammonium sulfate precipitates (reconstituted to one-fifth of the volume with BSA-PBS) from neutralized culture supernatant of BL2928. Numbers in parentheses show the ratio by weight (protein basis) of proteinase K to ammonium sulfate precipitates.

ducing the binding of *E. coli* strains to GA1. In part, this may be explained by the greater affinity of bifidobacteria for the ceramide moiety of glycolipids. It is likely that bifidobacteria form nonspecific hydrophobic interactions with GA1 and that inhibition of *E. coli* binding is due in part to steric hindrance effects, rather than to the attachment to specific carbohydrate epitopes on the GA1 receptor.

As determined in this study, an inhibitory factor(s) in the neutralized culture supernatants of bifidobacteria prevents the binding of *E. coli* strains to GA1. The inhibitory factor(s) is unlikely to be a lipid, as it was not extracted by organic solvents. In addition, the precipitates obtained by salting out from the culture supernatant retained most of the binding-inhibitory activity, indicating that the inhibitor may be a high-molecular-weight electrolyte(s). The activity was found to increase with the time of growth of BL2928, reaching a maximum level about 72 h after the inoculation of BL2928. The involvement of other nonbacterial constituents of the culture media was excluded as contributing to the inhibitory activity. For example, BL2928 produced the active factor(s) even when cultured in GPV medium, a defined medium for bifidobacteria which contains no added proteins or other constituents which would be retained within a dialysis membrane (molecular weight cutoff, 6,000 to 8,000) or eluted in the void volume fraction of Bio-Gel P100. The binding-inhibitory activity produced by BL2928 was destroyed by treatment with proteinase K. Cumulatively, our results argue for a proteinaceous nature of the binding inhibitor.

The factor(s) was partially resistant to organic solvents (chloroform-methanol extraction and methanol elution from a hydrophobic interaction chromatography column) and heat treatment. Fractionation by gel filtration suggested that the active component(s) had a molecular weight of at least 100,000. In addition, the factor(s) seemed to have a neutral isoelectric point, because desalting with a mixed-bed ion-exchanger did not significantly affect its inhibitory activity around pH 6. We do not have enough data as yet to judge whether the inhibitor is a secreted protein(s) from BL2928 or is a loosely associated surface layer protein(s). The exact mechanism(s) by which it interferes with *E. coli* binding remains to be clarified. It has been reported that several other microorganisms, including *P. aeruginosa* and *B. cepacia*, also bind to GA1 (7, 23). Since the binding of both of these strains was reduced by the proteinaceous factor(s) released by BL2928, the factor(s) probably interacts directly with the GA1 molecule rather than with the different bacterial adhesins for GA1.

This is the first study to show that bifidobacteria produce a proteinaceous factor(s) which inhibits the adherence of some *E. coli* (and other) strains to the GA1 molecule in vitro. Ibrahim and Bezkorovainy (11) reported that organic acids of bifidobacteria serve as anti-infectious agents. It is possible, therefore, that organic acids and the proteinaceous factor(s) produced by BL2928 work together in vivo to interfere with the binding of *E. coli* strains to intestinal epithelium. Since the adhesion of pathogenic bacteria to host tissues is regarded as a prerequisite for colonization-based infection, inhibition by bifidobacteria may help to prevent infection in the early stage of colonization.

It is not known yet whether all bifidobacterial strains secrete the same or a similar proteinaceous factor(s). Future research will examine the possible in vivo significance of the GA1-binding phenomenon described and will be directed at the purification and characterization of the inhibitory protein(s).

ACKNOWLEDGMENTS

We thank Umadevi S. Sajjan, Department of Research Biochemistry, The Hospital for Sick Children, Toronto, Canada, for helpful suggestions and technical advice and for supplying the strain of *Burkholderia cepacia* used in this study.

REFERENCES

1. **Bernet, M.-F., D. Brassart, J.-R. Neeser, and A. L. Servin.** 1993. Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interaction. Appl. Environ. Microbiol. **59:**4121–4128.
2. **Bullen, C. L., P. V. Tearle, and M. G. Stewart.** 1977. The effect of "humanised" milks and supplemented breast feeding on faecal flora of infants. J. Med. Microbiol. **10:**403–413.
3. **Conway, P. L.** 1989. Lactobacilli: fact and fiction, p. 263–281. *In* R. Grubbe, T. Midtvedt, and E. Norin (ed.), The regulatory and protective role of the normal microflora. Macmillan Press, New York, N.Y.
4. **Faure, J. C., D. Schellenberg, A. Baxter, and H. P. Wurzner.** 1982. Barrier effect of *Bifidobacterium longum* on *Escherichia coli* in the germ-free rat. Int. J. Vitam. Nutr. Res. **52:**225–230.
5. **Fujiwara, S., T. Hirota, H. Nakazato, T. Mizutani, and T. Mitsuoka.** 1991. Effects of *Bifidobacterium longum* SBT 2928 (BL2928) on spontaneous liver tumorigenesis and intestinal metabolism in gnotobiotic C3H/He male mice. J. Jpn. Soc. Nutr. Food Sci. **44:**261–166. (In Japanese.)
6. **Giannella, R. A.** 1986. Chronic diarrhea in travelers: diagnostic and therapeutic considerations. Rev. Infect. Dis. **8**(Suppl. 2)**:**S223–S233.
7. **Grant, M. M., M. S. Niederman, M. A. Pochlman, and A. M. Fein.** 1991. Characterization of *Pseudomonas aeruginosa* adherence to cultured hamster tracheal epithelial cells. Am. J. Respir. Cell Mol. Biol. **5:**563–570.
8. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual, p. 429–445. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Homma, N.** 1974. Intestinal flora of infants and resistance to infection. Jpn. J. Pediatr. **27:**1267–1275. (In Japanese.)
10. **Homma, N.** 1988. Bifidobacteria as a resistance factor in human beings. Bifidobacteria Microflora **7:**35–43.
11. **Ibrahim, S. A., and A. Bezkorovainy.** 1993. Inhibition of *Escherichia coli* by bifidobacteria. J. Food Prot. **56:**713–715.
12. **Karlsson, K.-A., and N. Stroenberg.** 1987. Overlay and solid-phase analysis of glycolipid receptors for bacteria and viruses. Methods Enzymol. **138:**220–232.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
14. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. **155:**377–389.
15. **Meghrous, J., P. Euloge, A. M. Junelles, J. Ballongue, and H. Petitdemange.** 1990. Screening of *Bifidobacterium* strains for bacteriocin production. Biotechnol. Lett. **12:**575–580.
16. **Mitsuoka, T., K. Hayakawa, and N. Kitamura.** 1974. Die faekal Flora bei Menschen. II. Mitteilung: die Zuzammensetzung der Bifidobakterien-Flora der vershiedenen Altersgruppen. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Grste Abt. Orig. Reihe A **226:**469–478.
17. **Mizutani, T., and T. Mitsuoka.** 1979. Effect of intestinal bacteria on incidence of liver tumors in gnotobiotic C3H/He male mice. JNCI **63:**1365–1370.
18. **Moreau, M. C., M. Thomasson, R. Ducluzeau, and P. Raibaud.** 1986. Cinétique d'établissement de la microflore digestive chez le nouveau-né human en fonction de la nature du lait. Reprod. Nutr. Dev. **26:**745–753.
19. **Orø, H. S., A.-B. Kolstø, C. Wennerås, and A.-M. Svennerholm.** 1990. Identification of asialo GM1 as a binding structure for *Escherichia coli* colonization factor antigens. FEMS Microbiol. Lett. **72:**289–292.
20. **Rasic, J. L.** 1983. The role of dairy foods containing bifido- and acidophilus bacteria in nutritional and health. North. Eur. Dairy J. **49:**80–88.
21. **Reid, G., R. L. Cook, and A. W. Bruce.** 1987. Examination of strains of lactobacilli for properties that may influence bacterial interference in the urinary tract. J. Urol. **138:**330–335.
22. **Reid, G., A. W. Bruce, J. A. McGroaty, K. J. Cheng, and J. W. Costerton.** 1990. Is there a role for lactobacilli in prevention of urogenital and intestinal infection. Clin. Microbiol. Rev. **3:**335–344.
23. **Saiman, L., and A. Prince.** 1993. *Pseudomonas aeruginosa* pili bind to asialo GM1 which is increased on the surface of cystic fibrosis epithelial cells. J. Clin. Invest. **92:**1875–1880.
24. **Tissier, H.** 1923. La putrefaction intestinale. Bull. Inst. Pasteur **21:**361, 409, 577, and 625.
25. **Umesaki, Y.** 1989. Intestinal glycolipids and their possible role in microbial colonization of mice. Bifidobacteria Microflora **8:**13–22.
26. **Umesaki, Y., A. Suzuki, K. Kasama, K. Tohyama, M. Mutai, and T. Yamakawa.** 1981. Presence of asialo GM1 and glucosylceramide in the intestinal mucosa of mice and induction of fucosyl asialo GM1 by conventionalization of germ-free mice. J. Biochem. **90:**1731–1738.
27. **Umesaki, Y., K. Takamizawa, and M. Ohara.** 1989. Structural compositional differences in neutral glycolipids between epithelial and nonepithelial tissue of mouse small intestine. Biochim. Biophys. Acta **1001:**157–162.
28. **Yamazaki, S., H. Kamimura, H. Momose, T. Kawashima, and K. Ueda.** 1982. Protective effect of *Bifidobacterium*-monoassociation against lethal activity of *Escherichia coli*. Bifidobacteria Microflora **1:**55–59.