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Characterization of Adhesion of *Bifidobacterium* sp. BGN4 to Human Enterocyte-Like Caco-2 Cells

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Abstract The adhesion of probiotic bacteria to the intestinal mucosa is one of the desirable properties for their colonization in the intestinal tract, where these bacteria constantly compete with other bacteria. The adhesion of different strains of bifidobacteria to Caco-2 cells was compared. Among the strains examined, BGN-4 showed the highest adhesion level and the greatest cell surface hydrophobicity (CSH). No close relationship was found between the adhesion and CSH of the strains. Upon protease and heat treatment, the adhesion of the BGN-4 to the Caco-2 cells decreased significantly. The cells grown at 42°C showed a lower CSH and self-aggregation levels than cells grown at 37°C. The treatment of EGTA did not have any effect on the adhesion. The degree of adhesion did not differ among the experimental groups in which galactose, mannose, or fucose were added in the adhesion assay mixture. The results suggest that the adhesion of the *Bifidobacterium* to the epithelial cells may be affected by the composition and structure of the cell membrane and interacting surfaces.

Key words: Adhesion, *Bifidobacterium*, Caco-2

Bifidobacteria were first discovered in the feces of infants by Tissier [20], who used the name *Bacillus bifidus* communis. Bifidobacteria are generally characterized as Gram positive, nonspore-forming, nonmotile, strictly anaerobes, and V- or Y-shaped bacteria. The G+C content of DNA varies from 55 to 67 mole%. They are saccharoclastic organisms, producing acetic and lactic acids in the molar ratio of 3:2, without CO_2 production except in the degradation of gluconate.

Bifidobacteria are important constituents of the normal intestinal microflora in both humans and animals [2, 21], with various beneficial probiotic effects on the well-being of the host [1, 3, 10]. Also, humans suffering from lactose malabsorption can possibly benefit from reduced lactose contents by the action of *Bifidobacterium* β-galactosidase [15, 19]. Recent studies suggest that *Bifidobacterium* may play a role in the reinforcement of immune functions and improved resistance to cancer [12, 20]. Because of the general belief that bifidobacteria are beneficial to the health of the host in both infants and adults, continuous efforts have been made to improve *Bifidobacterium* strains with enhanced probiotic effect and growth yield during the fermentation process.

For an appropriate application of bifidobacteria in the industry, strains are desired to satisfy several prerequisites. Strains possessing resistance to acid and bile salt are desired. Acid- and bile-resistant bifidobacteria are more likely to survive when they are exposed to gastric low pH and intestinal bile salt. Additionally, the adhesion ability to the intestinal mucosa is one of the desirable properties that have to be selected for their specific use in commercial preparations. Adhesion of *Bifidobacterium* strains to the colon surface may occur by an association of the bacteria with a secreted mucus gel or by adherence to the underlying epithelium [8]. Generally, adhesion can be ascribed to the interplay of attractive and repulsive forces between the approaching surfaces [9, 11]. However, the investigation of adhesion of the *Bifidobacterium* on intestinal mucosa has been scarce, and the exact mechanism of the adhesion has not yet been delineated [6].

The aim of this study was to compare the adhesion to Caco-2 cells by different strains of bifidobacteria isolated from the feces of Korean people, and characterization of the adhesion property. In order to gain insight into the structural properties of the surface of the strains, the relationship between bacterial cell surface hydrophobicity (CSH) and adhesion ability to Caco-2 cells was determined for each strain. By using the highest adhering strain, BGN-4, which also possessed the greatest CSH, the adhesive property was characterized.

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MATERIALS AND METHODS

Bacterial Strains

The bifidobacterial strains used in this study were mostly isolated from the feces of the Korean people by following the method of Mitsuoka [13] and information derived from earlier reports [16]. All of the strains were grown for 24 h at 37°C under anaerobic condition in the MRS broth (Difco, Michigan, U.S.A.) containing 0.05% (w/v) L-cysteine·HCl. Viable cell counts were estimated by a serial dilution process, and 100 µl of each diluted mixture were spread onto BL agar medium (Difco) with 5% horse blood. Then, the plate was incubated anaerobically for 2-3 days at 37°C.

Caco-2 Cell Culture

Caco-2 cells were used throughout this study. The Caco-2 cell line (ATCC, HTB 37) was originally isolated from a human colon adenocarcinoma and obtained from the American Type Culture Collection (ATCC, U.S.A.). The cells were cultured in DMEM (Dulbecco's modified Eagle's Medium) supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were used at passage levels 30-45. For adhesion assays, monolayers of the Caco-2 cells were prepared on coverslips (Nunc™, U.S.A.), which were placed in 24-well Corning tissue culture plates. Cells were seeded at a concentration of $1-2\times10^5$ cells to obtain confluence. The culture medium was changed daily. Cultures at post-confluence after 7 days of culture were used. All experiments and maintenance of cells were carried out at 37°C in a 5% CO_2/95% air atmosphere.

Adhesion Assay of Bifidobacteria

Bifidobacterial strains were grown for 24 h at 37°C under anaerobic condition in the MRS medium containing 0.05% L-cysteine·HCl. The Caco-2 cell monolayers were washed twice with PBS (phosphate buffered saline, pH 7.4) buffer. For each adhesion assay, *Bifidobacterium* suspension (1-3 × 10^7 cfu/ml) was mixed with an appropriate volume of cell-line culture medium and the mixture was added to each well of the cell culture plate which contained the coverslip, and it was then incubated for 1 h at 37°C in a 5% CO_2/95% air atmosphere. After incubation, the medium and nonadherent bacteria were removed by washing five times with PBS. The cell layer and remaining adherent bacteria were then fixed with methanol for 10 min. After fixation, the remaining cells were stained with Gram-stain, and examined microscopically under oil immersion. Each adhesion assay was conducted in duplicate with cells from three successive passages. For each coverslip monolayer, the number of adherent bacteria was counted in 10 random microscopic areas. Adhesion of bifidobacteria was expressed as the number of bacteria adhering to 50 Caco-2 cells.

CSH (Cell Surface Hydrophobicity) Assay

The CSH assay was performed according to Perez *et al.* [17]. Bacterial cells were grown to stationary phase, collected by centrifugation at 2,600 ×g for 15 min, washed twice in PBS, and resuspended in PBS to initial absorbance of OD=0.9±0.05. Three ml of bacterial suspensions were vortexed with 1 ml of xylene for 1 min and the phases were allowed to separate for 20 min. The CSH was calculated by using absorbance values of the aqueous phase before and after mixing with xylene, according to the equation:

$$CSH\ (\%)=100\times(A_i-A_f)/Ai$$

A_i, initial absorbance; A_f, final absorbance.

The higher CSH (%) means that more cells are partitioned from the aqueous phase into the xylene phase.

Enzymatic and Chemical Treatments of the Cultured Cells

To characterize the bacterial determinants involved in *Bifidobacterium* sp. BGN-4 adhesion, the cultured bacterial cells were subjected to various treatments. For EGTA treatment, the bacterial suspension was incubated with the Caco-2 cells in the presence of EGTA (20 mM). Wherever indicated, after monolayers were incubated with *Bifidobacterium* sp. BGN-4, the cells were washed five times with EGTA (20 mM) in PBS. For the heat treatment, bacterial cells were heated at 50, 55, and 60°C for 30 min in a water bath and cooled by immersion in an ice bath. For the protease treatment, bacterial cells were incubated with trypsin (2.5 mg/ml), pronase (2.5 mg/ml), and proteinase K (2.5 mg/ml) for 90 min at 37°C. After treatment, the enzymes were removed by washing 5 times with PBS. After the above various treatments, the treated cells were reacted with cultured Caco-2 cells and the adhesion assay was performed. The effect of sugar was determined by adding D-mannose, L-fucose, or D-galactose into the assay (100 mM, final concentration) medium. All the reagents were from Sigma-Aldrich (St. Louis, U.S.A.).

Scanning Electron Microscopy

Cells for scanning electron microscopy were grown on coverslips. After bacterial colonization assays, the cells were fixed with 2% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 2-3 h at 4°C. After two washes with the same buffer, cells were post-fixed for 2 h with 1% (w/v) OsO_4 in the same buffer. The samples were dehydrated in graded series (30, 50, 70, 80, 95, 100%) of ethanol for 10 min each and passaged through HMDS (hexa methyl disilazame) twice for 15 min each. Cells were dried in hoods and coated with gold. The specimens were then examined with a scanning electron microscope (JEOL, JSM-5410LV, Japan).

Statistical Analysis

Data were analyzed by Duncan's multiple range test. A probability of $P<0.005$ in the two-tailed test was used in the criterion for statistical significance.

RESULTS AND DISCUSSION

Adhesion of *Bifidobacterium* to Enterocyte-Like Caco-2 Cells

The experimental human intestinal cell line Caco-2, a well-characterized cellular model established from a human colonic adenocarcinoma by Fogh *et al.* [7], spontaneously develops characteristics of mature enterocytes with functional brush-border microvilli with an apical membrane and a basolateral membrane that is separated by tight junctions [18]. Adhesion of bifidobacteria onto Caco-2 cells was compared with different strains *in vivo* and *in vitro* [5] and it was shown that the ability of the strain to adhere and colonize the intestinal cells *in vitro* and *in vivo* were similar. In the present study, the adhesion of the various *Bifidobacterium* strains to human enterocyte-like Caco-2 cells was compared. As listed in Table 1, there were considerable variations in their adherence to the differentiated Caco-2 cells among different strains, indicating that adhesive properties are not a universal feature of *Bifidobacterium*. Crociani *et al.* [5] also reported that bifidobacteria adhesion was very heterogeneous between strains of the same genus. Among the *Bifidobacterium* strains tested, *Bifidobacterium* sp. BGN-4 showed the greatest adherence to Caco-2 cells (Table 1). Scanning electron microscopy was used to visualize the physical interaction between *Bifidobacterium* sp. BGN-4 and the surface of cultured human intestinal Caco-2 cells (Fig. 1): The morphology of *Bifidobacterium* sp. BGN-4 showed a typical irregular rod and mostly aggregative forms. Adhesion of BGN-4 in the apical brush-border of the enterocytic Caco-2 cells, and only the bacteria surface facing the microvilli being involved in the adhesion. The BGN-4 interacted with the well-defined apical microvilli of Caco-2 cells without cell damage.

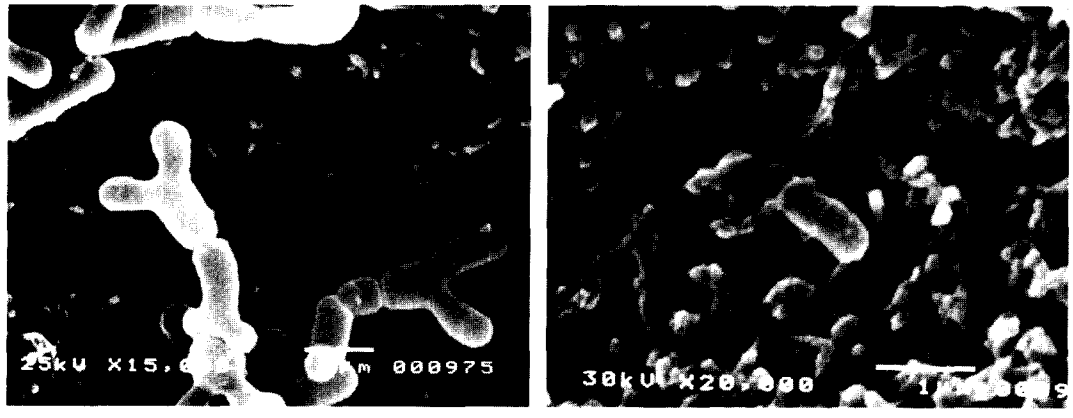
Table 1. Adhesion of the *Bifidobacterium* to Caco-2 cells.

Bifidobacterium strains	Adherent bacteria*
BGN-4	500
E2-18	168
E-15	132
JS-9	84
RD-54	67
SI	61
SH-2	47
RD-60	42
B. bifidum ATCC 2952	35
CN-2	30
SJ-32	26
KJ	24
HJ-30	23
B. animalis ATCC 2552	22
SH-5	17
B. adolescentis ATCC 15703	19
B. infantis ATCC 15697	15
B. longum ATCC 15707	11
M-6	10
MS-1	10

*Mean numbers of adhering bifidobacteria per 50 Caco-2 cells.

CSH of the *Bifidobacterium* Strains

In order to further characterize cell-cell interactions, a correlation between CSH and adhesion was examined, since it was suggested that the CSH and zeta potentials account for the attractive and repulsive forces, respectively, taking place in autoaggregation and adhesion of bacteria to different surfaces [9]. The CSH values of the *Bifidobacterium*



(A) (B)

Fig. 1. Adhesion of *Bifidobacterium* sp. BGN-4 to Caco-2 cells observed by scanning electron microscopy.
Notice that *Bifidobacterium* sp. BGN-4 whole cells interact with apical microvilli of Caco-2 cells [magnification ×15,000 (A) and ×20,000 (B)].

Table 2. Cell surface hydrophobicity of the *Bifidobacterium* strains.

Bifidobacterium strains	CSH (%)
BGN-4	93
KJ	90
HJ-30	90
B. adolescentis ATCC 15703	90
B. animalis ATCC 2552	86
M-6	85
RD-60	69.6
SI	66.3
CN-2	21
B. bifidum ATCC 2952	12
RD-54	7
MS-1	6
SH-5	6
E-15	5
E2-18	-
JS-9	-
SH-2	-
SJ-32	-
B. infantis ATCC 15697	-
B. longum ATCC 15707	-

-: Below the cell surface hydrophobicity value of 5%.

strains examined are presented in Table 2. The CSH of the different *Bifidobacterium* differed considerably among strains. Interestingly, *Bifidobacterium* sp. BGN-4, which showed the highest adherence to Caco-2 cells, also had the greatest CSH value. However, CSH of the overall strains was not closely interrelated with the Caco-2 adhesion. Wadstrom *et al.* [23] reported that some strains in lactobacilli, despite their hydrophilic surface properties, were capable of adhering, suggesting that multiple mechanisms are involved in the adhesion process. Perez *et al.* [17] reported that strains with a nearly identical hydrophobicity value had a wide range of adherence levels. This implies that other factors may also modulate the adhesion that is driven by hydrophobicity.

The Effects of Growth Temperature of the *Bifidobacterium* sp. BGN-4 on CSH and Adherence to Caco-2 Cells

To examine the effect of growth temperature on CSH and adherence to Caco-2 cells, *Bifidobacterium* sp. BGN-4 was used. The CSH and adhesion to Caco-2 cells of *Bifidobacterium* sp. BGN-4 decreased gradually when the growth temperature increased from 30 to 42°C (Fig. 2). When BGN-4's growth temperature was 42°C, *Bifidobacterium* sp. BGN-4's morphology of adhesion to Caco-2 cells changed from aggregative to diffuse (data not shown). The results suggested that CSH of *Bifidobacterium* sp. BGN-4 may be somewhat related to the Caco-2 adhesion. The significance of the CSH of the *Bifidobacterium* to their ability to survive and adapt in the environment needs to be studied further.

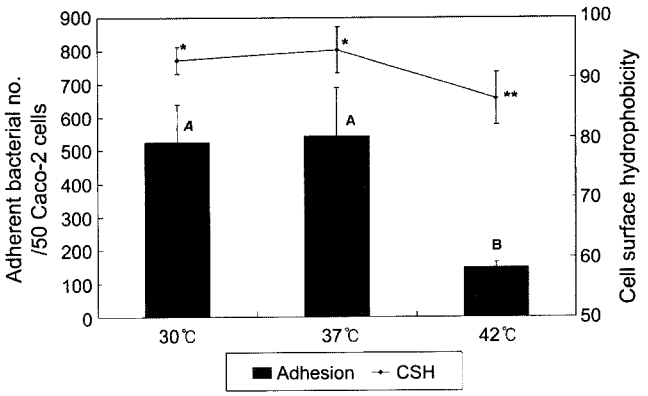


Fig. 2. The effects of growth temperature on cell surface hydrophobicity and adherence to Caco-2 cells of the *Bifidobacterium* sp. BGN-4.
Data are means±SD of triplicate cultures. Values with different superscripts or alphabets were significantly different at $P<0.05$ by Duncan's multiple range test.

The Effects of Heat Treatment of *Bifidobacterium* sp. BGN-4 Cells on the Adhesion to Caco-2 Cells

In order to examine the effect of heat temperature on the adhesion of *Bifidobacterium* sp. BGN-4 to Caco-2 cells, the adhesion of the heat-treated bifidobacterial cells at different temperatures was compared. As shown in Fig. 3, the adhesion of *Bifidobacterium* sp. BGN-4 to Caco-2 cells gradually decreased as heat-treatment temperature increased. Heat treatment at 60°C almost completely abolished the adhesiveness of *Bifidobacterium* sp. BGN-4. This result suggests that the factor(s) involved in the adhesion of *Bifidobacterium* sp. BGN-4 was found to be heat-sensitive.

The Effects of Various Enzymatic and Chemical Treatments on the Adhesion of *Bifidobacterium* sp. BGN-4 to Caco-2 Cells

The presence of simple sugars such as fucose, galactose, or mannose in the adhesion assay mixture decreased the

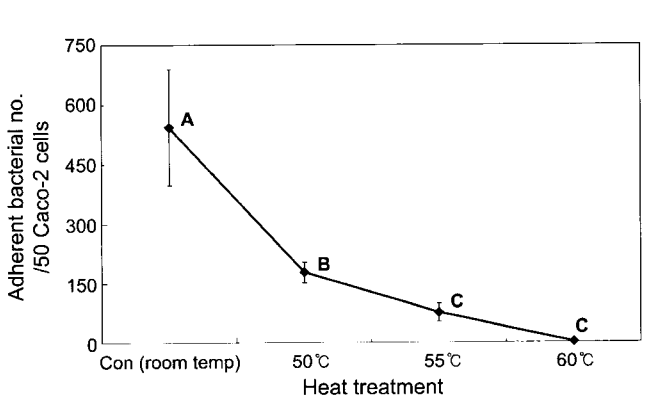


Fig. 3. The effects of heat treatment of *Bifidobacterium* sp. BGN-4 cells on the adhesion to Caco-2 cells.
Data are means±SD of triplicate cultures. Values with different alphabets were significantly different at $P<0.05$ by Duncan's multiple range test.

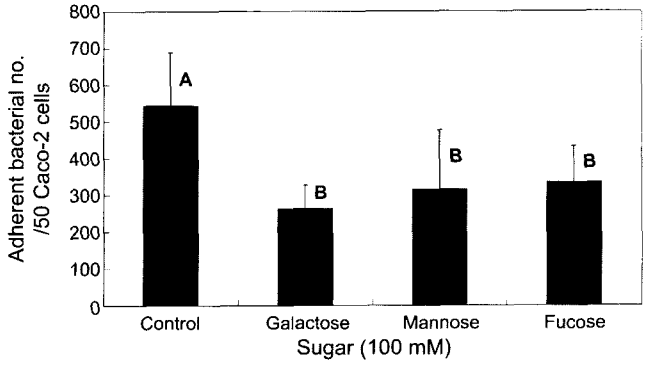


Fig. 4. The effects of simple sugars on the adhesion of *Bifidobacterium* sp. BGN-4 to Caco-2 cells.
Data are means±SD of triplicate cultures. Values with different alphabets were significantly different at $P<0.05$ by Duncan's multiple range test.

adhesion of *Bifidobacterium* sp. BGN-4 by as much as 50%, however, there was no difference between the sugars (Fig. 4). Greene and Klaenhammer [11] showed that enhanced adhesion of lactobacilli to intestinal cells was promoted by the divalent cation calcium, possibly due to an ionic bridge between the surfaces of bacteria and epithelial cells. In this study, treatment of *Bifidobacterium* sp. BGN-4 with EGTA, which is a calcium-chelating agent, did not induce any significant change in the degree of adhesion of *Bifidobacterium* BGN-4 to Caco-2 cells (data not shown). The calcium dependency may vary among different strains, since Chauvier *et al.* [4] reported that, among the adhering lactobacilli of ten strains, five strains had high calcium independent binding capacity to Caco-2 cells. The contribution of protein factors attached to the cell walls was examined by treating the bacterial cells with three proteolytic enzymes; Trypsin, pronase, and proteinase K treatments of bacterial cells with these enzymes had different degree of effects, but always reduced the adhesion of *Bifidobacterium* sp. BGN-4 (Fig. 5). This strongly suggests that the adhesion of *Bifidobacterium* sp. BGN-4

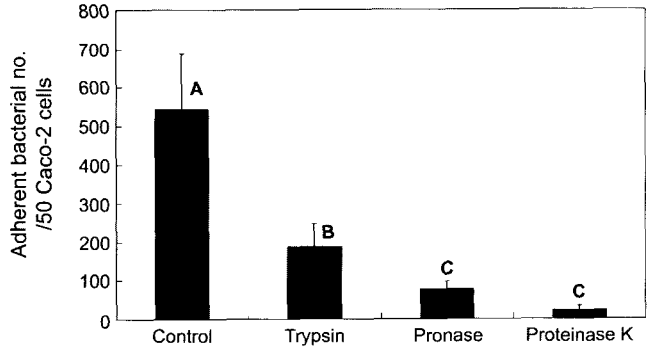


Fig. 5. The effects of proteolytic enzymes on the adhesion of *Bifidobacterium* sp. BGN-4 to Caco-2 cells.
Data are means±SD of triplicate cultures. Values with different alphabets were significantly different at $P<0.05$ by Duncan's multiple range test.

is mediated to some extent by a proteinaceous component of bacterial origin. Also, the involvement of nonproteinaceous cell wall component, lipoteichoic acids (LTA) of Gram-positive bacteria, in the binding to epithelial cells was reported. For example, Op den Camp *et al.* [14] reported that binding of the lipoteichoic acids of *Bifidobacterium bifidum* to human colonic epithelial cells appears to be specific, reversible, and cell concentration and time dependent, and suggested that ester-linked fatty acids are essential for LTA-binding. Thus, the differences in the capacity of adhesion observed in our present study might reflect the strain differences in the physiology and content of the different adhesion factors; i.e, proteinaceous component, polysaccharide, ionic charge, or lipoteichoic acid. BGN-4 showing the strongest adhesion to the epithelial cell among the strains tested, and so may be a good candidate for a probiotic strain in order to improve the human intestinal microbial balance.

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