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# Protein-Mediated Adhesion of *Lactobacillus acidophilus* BG2FO4 on Human Enterocyte and Mucus-Secreting Cell Lines in Culture

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**The adhesion of *Lactobacillus acidophilus* BG2FO4, a human stool isolate, to two human enterocytelike cell lines (Caco-2 and HT-29) and to the mucus secreted by a subpopulation of mucus-secreting HT29-MTX cells was investigated. Scanning electron microscopy revealed that the bacteria interacted with the well-defined apical microvilli of Caco-2 cells without cell damage and with the mucus secreted by the subpopulation of HT29-MTX cells. The adhesion to Caco-2 cells did not require calcium and involved an adhesion-promoting factor that was present in the spent supernatant of *L. acidophilus* cultures. This factor promoted adhesion of poorly adhering human *Lactobacillus casei* GG but did not promote adhesion of *L. casei* CNRZ 387, a strain of dairy origin. The adherence components on the bacterial cells and in the spent supernatant were partially characterized. Carbohydrates on the bacterial cell wall appeared to be partly responsible for the interaction between the bacteria and the extracellular adhesion-promoting factor. The adhesion-promoting factor was proteinaceous, since trypsin treatment dramatically decreased the adhesion of the *L. acidophilus* strain. The adhesion-promoting factor may be an important component of *Lactobacillus* species that colonize the gastrointestinal tract.**

Microorganisms for yogurt production are generally selected for their growth and flavor characteristics in milk fermentation, whereas other properties must be considered when the bacteria are intended to reside and elicit beneficial effects in vivo (6, 22, 35). One of these properties is the ability to adhere to intestinal cells and mucus, since intestinal attachment is an important prerequisite for colonization of the gastrointestinal tract by many bacterial species. However, thus far only *Lactobacillus gasseri* ADH (7, 23, 28), *Lactobacillus acidophilus* BG2FO4 (19, 23), and *Lactobacillus casei* GG (12) have been studied for this property. Since all lactobacilli do not possess the ability to adhere to human intestinal cells (7, 23), it is necessary to screen and characterize numerous strains to select those that meet this criterion. The use of freshly isolated enterocytes in bacterial adherence assays, however, yields fluctuating results because of the different enterocyte donors (10). The cultured human intestinal cells used previously in adherence studies of lactobacilli were poorly differentiated and possessed an ill-defined brush border. The choice of a cellular model is vitally important to obtain stable and reproducible results for screening strains and investigating the mechanisms by which lactobacilli adhere to human intestinal cells.

Different cell types, e.g., enterocytes, goblet cells, endocrine cells, and Paneth's granular cells, constitute the intestinal mucosa. Recently, several cultured cell lines were used to investigate basic questions related to the organization and function of intestinal cells. The human intestinal epithelial cell lines HT-29 and Caco-2, established from two colonic adenocarcinomas by Fogh et al. (14), have been used extensively in the study of human enterocytic function (40). The advantages of these two cellular models are that they express morphological and functional differentiation in vitro and show characteristics of mature enterocytes, including polarization, a functional brush border, and apical intestinal hydrolases (33, 34). HT-29 and Caco-2 cells form two clearly distinguishable domains, an apical membrane and a basolateral membrane separated by tight junctions (37). With different protein and lipid compositions, these domains are strikingly different in ultrastructure (3). For example, the apical surface (brush border) contains peptidases and disaccharidases (16, 17, 33, 34), the basolateral domain contains several peptide receptors involved in the control of intestinal hydroelectrolytic secretion (25), and the tight junctions contain specific proteins, such as the ZO-1 protein (2). Since the binding of bacteria to mucosal cells involves the interaction of bacterial ligands with specific eucaryotic receptors, human intestinal Caco-2 and HT-29 cells are actually the best available models to study intestinal attachment of bacteria and viruses. With these cell lines, the attachment of *Vibrio cholerae* (32) and enteropathogenic (24) and enterotoxigenic (10, 31) *Escherichia coli* has been described and invasion by *Listeria monocytogenes* (15, 30) and *Salmonella typhimurium* (13) has been investigated. Moreover, the binding and internalization of rotavirus (38) and reoviruses 1 and 3 (1) at the apical surface of Caco-2 cells and apical membrane vesicles have been demonstrated. Recently, an *L. casei* strain (GG) was also reported to adhere to Caco-2 cells (12). However, no comparisons with other adhering strains were made, and the mechanism of adherence was not investigated or discussed.

Currently, little is known about the adhesion of lactobacilli to human enterocytes and goblet cells. Enterocytelike

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Caco-2 and HT-29 cells and a subpopulation of mucus-secreting HT29-MTX cells were used here to investigate the adhesion of human *L. acidophilus* BG2FO4. A mechanism through which *L. acidophilus* strains may interact with eucaryotic receptors is proposed.

## MATERIALS AND METHODS

**Bacterial strains.** The *L. acidophilus* strain used in this study was BG2FO4, a human stool isolate shown previously to adhere in vitro to cultured human fetal intestinal cells (19, 22). Two other *Lactobacillus* strains were also included for comparison: human isolate *L. casei* GG (provided by B. R. Goldin and S. L. Gorbach, Tufts University, Boston, Mass.) and *L. casei* CNRZ 387, a fermenting strain originating from dairy products. Bacterial stock cultures were stored at −80°C in 11% nonfat dry milk containing 10% glycerin. All strains were propagated in MRS broth (11) at 37°C for 18 h before adherence assays were done.

**Cell cultures.** Enterocytelike Caco-2 and HT-29 cell lines were obtained from J. Fogh (Sloan-Kettering Memorial Cancer Center, Rye, N.Y.) (14). The enterocytelike subpopulation of cells that were obtained by selection through glucose deprivation (41) and that maintained their differentiation characteristics (39) when switched back to standard glucose-containing medium (26), and the mucus-secreting HT29-MTX cells (26), were obtained from A. Zweibaum (Unité 178, Institut National de la Santé et de la Recherche Médicale, Villejuif, France).

Cells were routinely grown in Dulbecco's modified Eagle's minimal essential medium (25 mM glucose) (Eurobio, Paris, France) supplemented with 10% (HT-29 and HT29-MTX) or 20% (Caco-2) inactivated (30 min, 56°C) fetal calf serum (Boehringer, Mannheim, Germany) and 1% nonessential amino acids (Caco-2). Monolayers of Caco-2, HT-29, and HT29-MTX cells were prepared on glass coverslips placed in six-well tissue culture plates (Corning Glass Works, Corning, N.Y.). Cells were seeded at concentrations of $2 \times 10^4$ (HT-29 and HT29-MTX) and $1.4 \times 10^4$ (Caco-2) cells per $cm^2$. Cells were maintained and all experiments were carried out at 37°C in a 10% $CO_2$–90% air atmosphere. The culture medium was changed daily. Enterocytelike HT-29, mucus-secreting HT29-MTX, and Caco-2 cells were used at 35 to 60, 20 to 40, and 60 to 90 cell passages, respectively. Cells were used for adherence assays at late postconfluence, i.e., after 15 days (Caco-2) and 20 days (HT-29 and HT29-MTX) in cultures.

**Adherence assay.** The adherence of *L. acidophilus* BG2FO4 to Caco-2 and HT-29 cells was examined as described previously for the *E. coli* adhesion assay (10, 20). In brief, Caco-2 and HT-29 monolayers were washed twice with phosphate-buffered saline (PBS). Lactobacilli (1 ml; $2 \times 10^8$ bacteria per ml in spent culture supernatant, treated supernatant, or fresh MRS broth) were added to 1 ml of the cell line culture medium. This suspension (2 ml) was added to each well of the tissue culture plate, and the plate was incubated at 37°C in 10% $CO_2$–90% air. After 1 h of incubation, the monolayers were washed five times with sterile PBS, fixed with methanol, stained with Gram stain, and examined microscopically. Each adherence assay was conducted in duplicate over three successive passages of intestinal cells. For each monolayer on a glass coverslip, the number of adherent bacteria was counted in 20 random microscopic areas. Adherent bacteria were counted by two technicians to eliminate bias.

In some adherence assays with *L. casei* GG and CNRZ 387, the spent culture supernatant of BG2FO4 was obtained by centrifugation (20,000 × *g*, 1 h at 4°C) and added to the *L. casei* cells.

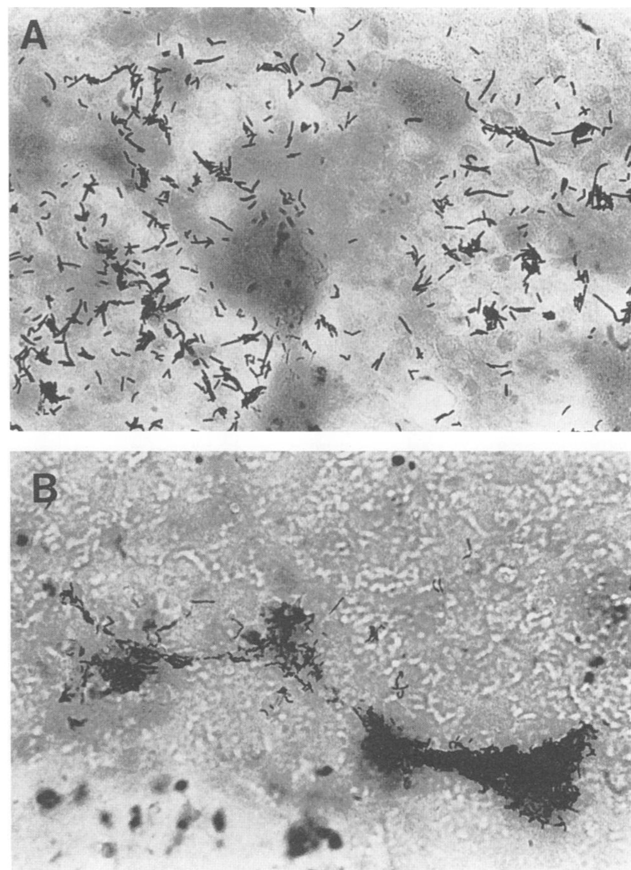


FIG. 1. Adherence of the human stool isolate *L. acidophilus* BG2FO4 to polarized differentiated human intestinal epithelial cells Caco-2 (A) and HT-29 (B). Note the diffuse adhesion to Caco-2 cells versus the dense localized clusters of bacteria on HT-29 cells.

**Physical and chemical treatments of bacteria and spent culture supernatants.** All enzymes and chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). Bacterial cells and spent culture supernatant were separated by centrifugation. The bacterial cells were resuspended in MRS broth and treated with trypsin (2.5 mg/ml for 60 min at 37°C). The spent culture supernatant was also treated with trypsin under identical conditions. Trypsin was inactivated prior to adherence assays by the addition of inactivated (30 min, 56°C) fetal calf serum. In some experiments, the bacteria were preincubated with 1 M metaperiodate for 30 min, centrifuged, washed in PBS, and resuspended in spent culture supernatant at $2 \times 10^8$ bacteria per ml. To determine the influence of divalent cations or calcium on the adherence of strain BG2FO4, we washed the monolayers five times with 20 mM EDTA or the chelating agent of calcium, 20 mM [ethylene-bis-(oxyethylenenitrilo)]tetraacetic acid (EGTA), in PBS after incubation with the bacteria.

**Scanning electron microscopy.** For scanning electron microscopy, the tissue culture cells were grown on glass coverslips. After the bacterial adhesion assay, cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. After two washes with phosphate buffer, cells were postfixed for 30 min with 2%

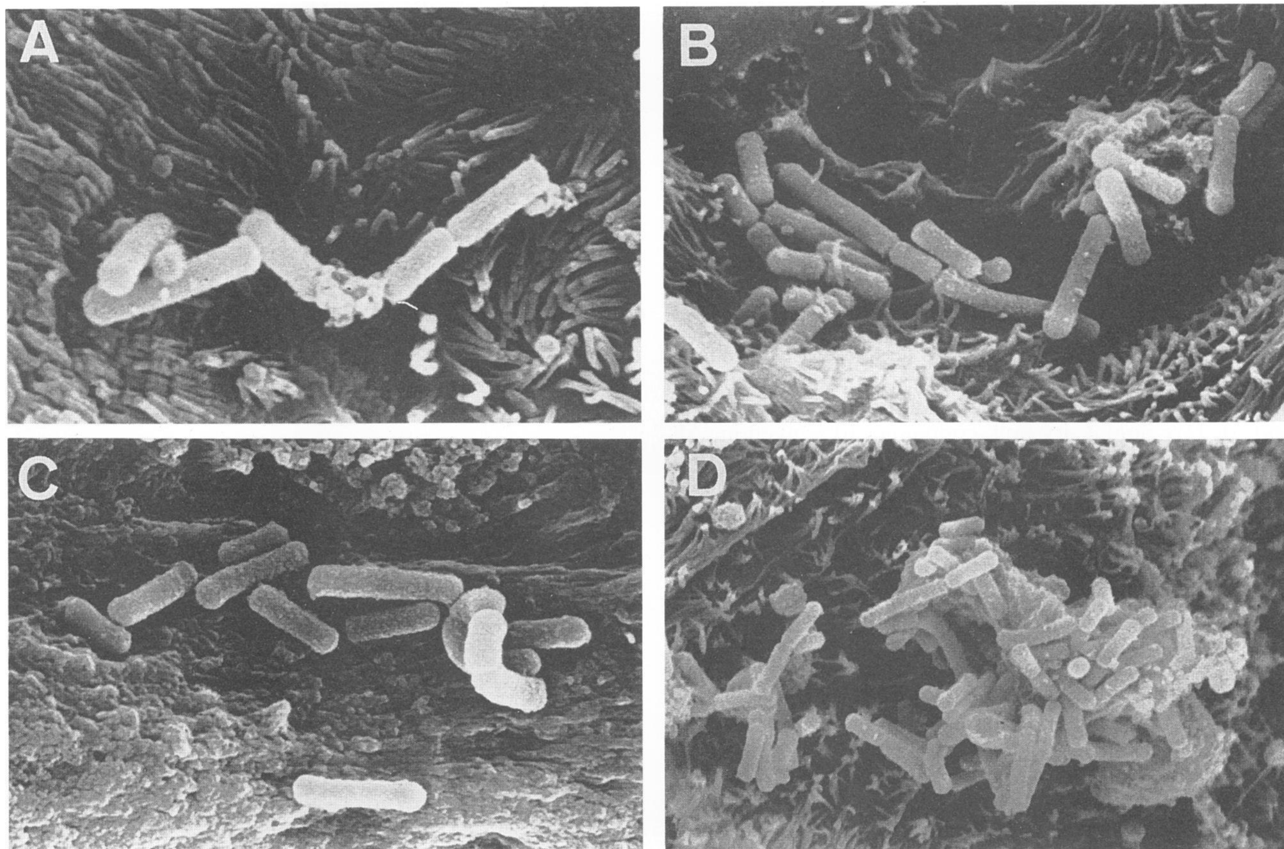


FIG. 2. Low- and high-magnification scanning electron micrographs of differentiated Caco-2 and HT-29 cell monolayers with adhering *L. acidophilus* BG2FO4. (A and C) Interaction with microvilli forming the brush border of Caco-2 cells (magnification, ×12,000). (B and D) Clusters of bacterial cells on HT-29 cells (respective magnifications, ×9,000, ×10,000, and ×6,000).

$OsO_4$ in the same buffer, washed three times with phosphate buffer, dehydrated in a graded series (30, 50, 70, 80, 90, and 100%) of ethanol, and passaged through a graded series (50, 70, 90, and 100%) of amyl acetate. Cells were dried in a critical-point dryer (Balzers CPD030) and coated with gold. The specimens were examined with a JEOL JSM 25S scanning electron microscope.

## RESULTS

*L. acidophilus* BG2FO4 adhered to the enterocytelike HT-29 and Caco-2 cells. Diffuse adhesion was observed with Caco-2 cells (Fig. 1A), whereas dense bacterial clusters that interfered with visual quantitation were observed with HT-29 cells (Fig. 1B). *L. acidophilus* adhesion to differentiated Caco-2 and HT-29 cells was also examined by scanning electron microscopy (Fig. 2). Scanning electron micrographs clearly illustrated that binding between the bacteria and Caco-2 brush border microvilli occurred at the mucosal surface (Fig. 2A and C). Bacteria could be visualized in the microvilli without damage to the microvilli or underlying cell population. After incubation with HT-29 cells, *L. acidophilus* BG2FO4 adhered to the mucosal surface. Bacterial cell clusters were apparent at the mucosal surface, indicating that some cell-to-cell interactions were occurring between the bacterial cells themselves (Fig. 2B and D).

*L. acidophilus* BG2FO4 also adhered to the mucus-secreting HT29-MTX cells. HT29-MTX cells are a homogeneous subpopulation of goblet cells recently selected from the mainly undifferentiated HT-29 cell population after growth adaptation to methotrexate (26). The cell surface was covered entirely by the mucus gel (Fig. 3A). BG2FO4 interacted with the secreted mucus (Fig. 3B) and formed dense clusters (Fig. 3C). Higher numbers of bacterial clusters were observed on monolayers of HT29-MTX goblet cells than on enterocytelike HT-29 cells.

In an attempt to identify the components involved in the adhesion of *L. acidophilus* BG2FO4 to enterocytelike cells in cultures, we subjected the bacteria and spent culture medium to various physical and chemical treatments (Table 1). Washes with EGTA or EDTA after adhesion did not affect the binding of strain BG2FO4. When the spent culture supernatant was discarded and replaced with fresh culture medium, a dramatic reduction in adhesion occurred. Moreover, trypsin treatment of the spent culture supernatant dramatically reduced adhesion over that observed when the bacteria alone were treated with trypsin. These results indicated that an extracellular proteinaceous component is involved in the adhesion of human strain *L. acidophilus* BG2FO4. Following trypsin treatment, BG2FO4 bacteria partially regained their adhesion capacity after two subcultures (data not shown), suggesting that a protein product may accumulate at the bacterial cell surface over time. Metaperiodate treatment of bacteria alone also decreased adhesion, suggesting that carbohydrates may also be partially involved.

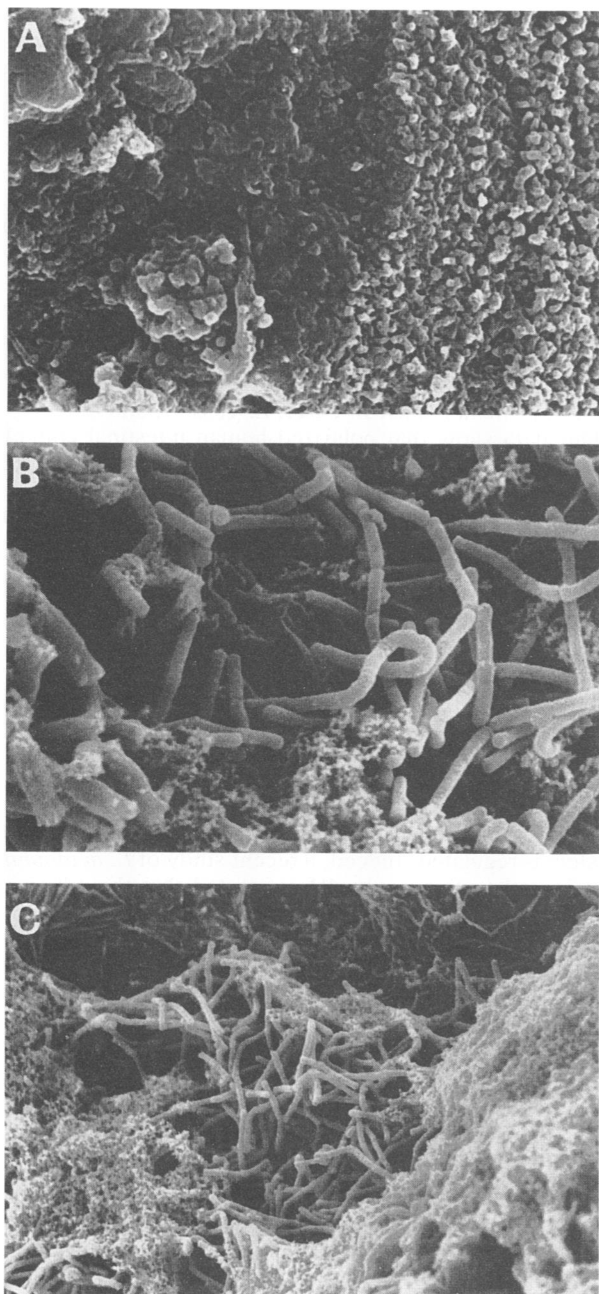


FIG. 3. Scanning electron micrographs of mucus-secreting HT29-MTX monolayers (A) to which *L. acidophilus* BG2FO4 bound (B and C; respective magnifications, ×5,000 and ×2,000). Examination of the cell surface of HT29-MTX cells revealed that the brush border, which is known to be present in these cells (26), was not visible, as it was totally covered by the dense mucus gel produced by these typical goblet cells. Multiple layers of mucus flow covered the cell surface, with abundant budding of mucus.

Not all *Lactobacillus* strains possess the capacity to adhere to human cultured intestinal cell lines (4, 19, 22). We conducted additional experiments with two *L. casei* strains, one of intestinal origin and reported to adhere to Caco-2 cells (12) and one a fermentative strain of dairy origin (Table 2). Human isolate *L. casei* GG adhered to Caco-2 cells in lower numbers than *L. acidophilus* BG2FO4, while *L. casei* CNRZ 387, of dairy origin, failed to adhere to Caco-2 cells. Increased adhesion of *L. casei* GG was observed when the spent culture supernatant of BG2FO4 replaced the spent culture supernatant of *L. casei* GG. In contrast, the adhesion of *L. casei* CNRZ 387 was not improved in the presence of the spent culture supernatant of BG2FO4. These data indicate that the adherence-promoting factor produced by *L. acidophilus* BG2FO4 acts with some specificity, since it did not promote the attachment of the *L. casei* dairy strain.

TABLE 1. Characteristics of adhesion of *L. acidophilus* BG2FO4 to differentiated human intestinal Caco-2 cells in cultures

| Condition | Pretreatment of: | | Mean no. of adhering bacteria/ 100 cells (SD)[a] |
|---|---|---|---|
| | Bacteria | Spent culture supernatant | |
| Control[b] | – | – | 230 (18) |
| Fresh culture medium[c] | – | – | 50 (6)[d] |
| Wash with EGTA[e] | – | – | 190 (25)[f] |
| Trypsin | + | + | 15 (2)[d] |
| Trypsin | – | + | 10 (2)[d] |
| Trypsin | + | – | 50 (15)[d] |
| Metaperiodate | + | – | 110 (18)[d] |

[a] Measured after 2 h of incubation. Twenty randomized microscopic fields per coverslip were counted. Each adherence assay was conducted in duplicate with three successive cell passages. Each treatment was compared with the control. Statistical analysis was performed with a Student *t* test.
[b] Adhesion of *L. acidophilus* in the presence of the spent culture supernatant.
[c] The spent culture supernatant was removed and replaced with fresh culture medium before the adhesion assay was done.
[d] $P < 0.01$.
[e] Similar results were obtained with EDTA washes (data not shown).
[f] No significant difference.

## DISCUSSION

Human polarized intestinal epithelial cell lines in cultures provide a differentiated population of cells that can be used successfully in adherence assays for pathogenic and non-pathogenic bacteria (1, 5, 10, 12, 13, 15, 20, 21, 24, 30–32, 38). These simple assays provide a defined system in which the bacteria, the suspending menstruum, or the epithelial cell monolayer can be altered to investigate both the bacterial

TABLE 2. Effect of a BG2FO4-secreted component on the adhesion of lactobacilli to differentiated human intestinal Caco-2 cells

| *Lactobacillus* strain | Mean no. of adherent bacteria/ 100 cells (SD)[a] |
|---|---|
| Human *L. casei* GG | 62 (15) |
| Human *L. casei* GG with spent culture supernatant of *L. acidophilus* BG2FO4[b] | 200 (18)[c] |
| Dairy *L. casei* CNRZ 387 | 10 (5) |
| Dairy *L. casei* CNRZ 387 with spent culture supernatant of *L. acidophilus* BG2FO4[b] | 11 (7)[d] |

[a] Measured after 2 h of incubation. Twenty randomized microscopic fields per coverslip were counted. Each adherence assay was conducted in duplicate with three successive cell passages.
[b] The spent culture supernatant of *L. casei* was replaced with the spent culture supernatant of *L. acidophilus* BG2FO4. Statistical analysis between the adhesion of *L. casei* in the absence versus the presence of the BG2FO4 supernatant was performed with a Student *t* test.
[c] $P < 0.01$.
[d] No significant difference.

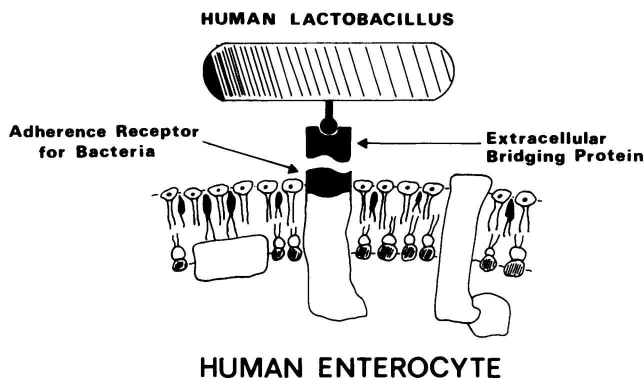


FIG. 4. Model proposed for the adherence of *L. acidophilus* BG2FO4 to human intestinal cells. An extracellular, proteinaceous component produced by adhering lactobacilli provides a divalent bridge that links the bacteria to the intestinal enterocytes. The extracellular bridging protein interacts with a component of the bacterial cell and a receptor on the intestinal epithelium.

adhesins and eucaryotic bacterial receptors. Moreover, since these polarized cell lines mimic the conditions under which in vivo infections with pathogenic bacteria involved in human diarrhea occur, competitive attachment of lactobacilli and intestinal pathogens can be tested directly.

The nature of *Lactobacillus* adhesion determinants differs depending on the bacterial strain studied and the cellular model used in the attachment assays. Studies of intestinal colonization with animal models have identified the following bacterial cell wall components: acidic mucopolysaccharides for epithelial cell adhesion to the chicken crop (3); carbohydrate capsule polymers on lactobacilli that colonize the intestine of pigs (39); and numerous mixed macromolecular complexes of proteins and teichoic acids on *Lactobacillus fermentum* RI, which colonizes the mouse gastric epithelium (36). Only a single anchorage wall protein mediates the adhesion of *L. fermentum* 104-S to porcine gastric squamous epithelium (18), whereas a secreted protein mediates the adhesion of *L. fermentum* 737 to the mouse stomach squamous epithelium (8). For human models, our results (5) and those of others (7, 23) clearly indicate that not all human *Lactobacillus* strains possess the capacity to adhere to human intestinal cells. The characteristics of adhesion observed with *L. acidophilus* BG2FO4 suggest that adhering *Lactobacillus* strains possess specific properties. In contrast to the human uroepithelial cell model, in which macromolecular complexes containing lipoteichoic acid are involved in the adhesion of urogenital *Lactobacillus* strains (4), the human intestinal cell model requires one factor provided by the cell wall and one secreted factor (Fig. 4). The bacterial cell wall factor, which presumably interacts with the secreted factor, involves polysaccharides, since metaperiodate treatment of the cells altered adherence. These data agree well with the results of Hood and Zottola (19), who described the interaction of *L. acidophilus* BG2FO4 with human FHs0074 intestinal tissue cells (ATCC CCL 241). In their experiments, adherence was mediated by a polysaccharide located at the bacterial surface. We have identified a second factor for adherence; this factor was secreted into the culture medium and may provide a bridge between the bacteria and the eucaryotic cell receptors. Trypsin treatment of the spent broth culture supernatant destroyed adhesion, indicating that the extracellular bridging component was proteinaceous. This mechanism of adhesion is similar to that observed in the elegant and comprehensive study by Conway and Kjelleberg (8) for *L. fermentum* 737. This strain adheres to mouse stomach squamous epithelium. The fact that an extracellular component was responsible for 90% of the adhesion capacity of strain BG2FO4 suggests that this component may be an important characteristic of human adherent strains. Indeed, the extracellular component from BG2FO4 significantly improved the adhesion of human strain GG but did not promote adhesion of dairy strain CNRZ 387. Host specificity has been observed for *Lactobacillus* strains isolated from mice (27). However, additional experiments are needed to determine whether this correlation can be extrapolated to more species-specific strains, such as those isolated from pigs and chickens and various human strains, and *Lactobacillus* species, including *Lactobacillus reuteri*, *L. acidophilus*, *L. casei*, *L. gasseri*, and others.

Our data show that polarized human intestinal epithelial cell lines HT-29 and Caco-2 and mucus-secreting HT29-MTX cells provide an excellent system for characterizing how lactobacilli interact with a well-defined brush border and mucus. These stable and reproducible models could promote the selection of *Lactobacillus* strains and allow evaluation of their relative capacities for adhesion in the human intestine, a property thought to be essential for intestinal colonization. Moreover, it would be possible to determine the impact of media, environmental conditions, and temperature on bacterial adhesive properties, since culture media, growth phase, and cellular morphology have long been suggested to influence the adherence of lactobacilli to human epithelial cells (9, 22). Furthermore, it will be of interest to characterize the genetic determinants that encode the bridging protein and determine whether the adherence system is regulated. Indeed, a recent study of *L. acidophilus* 100-33 and *L. fermentum* R1 suggested that the adherence factors were controlled by chromosomal genes (29). Knowledge of the genetic controls and physiological conditions of human adherent *Lactobacillus* cells will be vitally important in the preparation of active cells intended for use as intestinal probiotics or dietary adjuncts.

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