APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1994, p. 4487–4494 Vol. 60, No. 12
0099-2240/94/$04.00+0
Copyright © 1994, American Society for Microbiology

Factors Involved in Adherence of Lactobacilli to Human Caco-2 Cells†

JEFFREY D. GREENE AND TODD R. KLAENHAMMER*

Department of Food Science, Southeast Dairy Foods Research Center, North Carolina State University, Raleigh, North Carolina 27695-7624

Received 11 April 1994/Accepted 16 September 1994

A quantitative assay performed with bacterial cells labelled with [^{3}H]thymidine was used to investigate factors involved in the adherence of human isolates *Lactobacillus acidophilus* BG2FO4 and NCFM/N2 and *Lactobacillus gasseri* ADH to human Caco-2 intestinal cells. For all three strains, adherence was concentration dependent, greater at acidic pH values, and significantly greater than adherence of a control dairy isolate, *Lactobacillus delbrueckii* subsp. *bulgaricus* 1489. Adherence of *L. acidophilus* BG2FO4 and NCFM/N2 was decreased by protease treatment of the bacterial cells, whereas adherence of *L. gasseri* ADH either was not affected or was enhanced by protease treatment. Putative surface layer proteins were identified on *L. acidophilus* BG2FO4 and NCFM/N2 cells but were not involved in adherence. Periodate oxidation of bacterial cell surface carbohydrates significantly reduced adherence of *L. gasseri* ADH, moderately reduced adherence of *L. acidophilus* BG2FO4, and had no effect on adherence of *L. acidophilus* NCFM/N2. These results indicate that *Lactobacillus* species adhere to human intestinal cells via mechanisms which involve different combinations of carbohydrate and protein factors on the bacterial cell surface. The involvement of a secreted bridging protein, which has been proposed as the primary mediator of adherence of *L. acidophilus* BG2FO4 in spent culture supernatant (M.-H. Coconnier, T. R. Klaenhammer, S. Kernéis, M.-F. Bernet, and A. L. Servin, Appl. Environ. Microbiol. 58:2034–2039, 1992), was not confirmed in this study. Rather, a pH effect on Caco-2 cells contributed significantly to the adherence of this strain in spent culture supernatant. Our results indicate that adherence assays with Caco-2 cells should be performed under neutral-pH conditions to better mimic the intestinal environment and minimize acid effects that may artificially increase adherence.

Interest in the mechanisms through which pathogenic bacteria colonize the human intestinal tract and cause disease has led to the identification of multiple adherence and invasion genes in a variety of bacterial species (15). The role that these genes play in the progression of bacterial infection has been extensively examined by using molecular genetic methods in combination with in vitro model systems (15). In contrast, there have been few studies of the mechanisms through which the bacterial species that form the normal indigenous microbiota establish and maintain intestinal colonization. The lack of information concerning this process is due primarily to the complexity of the intestinal ecosystem and to the lack of appropriate mutant strains which can be used to test cause and effect relationships.

The normal human gastrointestinal tract is colonized by a variety of gram-positive and gram-negative organisms, including (but not limited to) bacteria belonging the genera *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Peptostreptococcus*, *Clostridium*, *Eubacterium*, *Bacteroides*, *Veillonella*, and *Fusobacter* and *Escherichia coli* (44, 48). *Lactobacillus* species have been isolated from all portions of the human gastrointestinal tract (35), and there is keen interest in the lactobacilli because of their purported health-promoting effects (9, 43). Lactobacilli are major components of the normal microbiota, and determination of the mechanisms through which they colonize the intestinal tract may provide important insights into how the normal microbiota is maintained, as well as provide a basis for rational design of probiotic formulations.

The difficulties involved in performing mechanistic studies on bacterial adherence in vivo, especially in humans, have led to the development of in vitro model systems for studying bacterial adherence to human intestinal cells. Workers have isolated a number of human adenocarcinoma cell lines that have properties of various cell types which occur in the intestinal epithelium (13, 17, 26, 39). One of the cell lines used extensively in studies of bacterial adherence is the Caco-2 cell line (17, 39). Caco-2 cells express several markers that are characteristic of normal small intestinal villus cells and have played a major role in studies on the mechanisms of adherence and invasion of many pathogenic bacteria, including *Salmonella typhimurium* (16, 18), *Listeria monocytogenes* (19, 37), enteropathogenic (28) and enterotoxigenic *Escherichia coli* (12), and *Vibrio cholerae* (38). Caco-2 cells thus provide an excellent system for studying not only the mechanisms through which species in the normal microbiota adhere to the intestine, but also how these bacteria may interact with pathogenic bacteria that compete in the same ecosystem.

Recently, workers have investigated factors involved in the adherence of *Lactobacillus* species to Caco-2 cells and whether these species can compete with various pathogenic bacteria for the same attachment sites on these cells. Reid et al. (42) compared the abilities of urogenital lactobacilli to adhere to urinary and intestinal epithelial cells. Adhesion to urinary epithelial cells was correlated with bacterial hydrophilicity, whereas an extracellular protein and a trypsin-insensitive cell wall factor were involved in adherence to Caco-2 cells. Coconnier et al. (8) concluded that *Lactobacillus acidophilus* BG2FO4 adheres to Caco-2 cells primarily through involvement of a secreted protein found in spent culture supernatant

* Corresponding author. Phone: (919) 515-2974. Fax: (919) 515-7124.

† Paper number FSR94-12 of the Department of Food Science, Southeast Dairy Foods Research Center, North Carolina State University, Raleigh.

and secondarily through involvement of cell surface protein and carbohydrate factors. In a similar study, Chauvière et al. (7) concluded that *L. acidophilus* LB adheres to Caco-2 cells through two mechanisms: a cell surface factor that is protease insensitive and an extracellular factor that is heat stable and protease sensitive. Finally, Chauvière et al. (6) reported that *L. acidophilus* LB, in the presence of spent culture supernatant, could inhibit attachment of *E. coli* to Caco-2 cells. The results of these studies formed the initial basis for the work described in this paper.

In order to identify potential factors through which lactobacilli may adhere to intestinal cells, we used bacterial cells labelled with [^{3}H]thymidine to investigate the biochemical properties of adherence to Caco-2 cells for three human isolates, *L. acidophilus* BG2FO4 and NCFM/N2 and *Lactobacillus gasseri* ADH. The adherence of *L. acidophilus* BG2FO4 in the presence of spent culture supernatant was also reexamined, and the results of additional experiments were compared with the results of previous work (8).

MATERIALS AND METHODS

Bacterial strains. *L. acidophilus* BG2FO4 (27) and NCFM/N2 (1), *L. gasseri* ADH (= MSO2 [27]), and *Lactobacillus delbrueckii* subsp. *bulgaricus* 1489 (51) were obtained from the stock culture collection maintained in the Department of Food Science at North Carolina State University, Raleigh. Bacterial stock cultures were stored at −20°C in MRS broth (Difco Laboratories, Detroit, Mich.) containing 10% glycerol. All strains were propagated in MRS broth at 37°C for 18 h prior to labelling for adherence assays.

Caco-2 cell culture. The Caco-2 human colon adenocarcinoma cell line (17, 39) was obtained from the American Type Culture Collection, Rockville, Md. All chemicals used in preparing the cell culture medium were obtained from the GIBCO/BRL Division of Life Technologies, Inc., Grand Island, N.Y. Cells were routinely grown at 37°C in a 90% air–10% CO_2 atmosphere in Dulbecco's modified Eagle medium (DMEM) containing 25 mM glucose, 1.0 mM sodium pyruvate, 15% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS), 1% nonessential amino acids, 100 U of penicillin G per ml, 100 μg of streptomycin sulfate per ml, and 0.25 μg of amphotericin B per ml. Monolayers of Caco-2 cells, which were used in the adherence assays, were prepared by inoculating six-well tissue culture dishes (Falcon type 3046; Becton Dickinson Labware, Oxnard, Calif.) with 1.3×10^5 cells per well in 4.0 ml of culture medium. The culture medium was replaced every other day, and the monolayers were used in the adherence assay after 15 days of incubation.

Radiolabelling and quantitation of bacterial cells. For most experiments bacterial cells were radiolabelled at 37°C in MRS broth containing 250 μg of 2′-deoxyadenosine (Sigma Chemical Co., St. Louis, Mo.) per ml and 10 μCi of [*methyl*-^{3}H]thymidine (70 to 90 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml; in some experiments (see Tables 2 and 3) Casamino Acids medium (21) was substituted for MRS broth in order to achieve a higher specific activity. Cells were harvested at the stationary phase after 16 h of growth for use in the adherence assays. The numbers of CFU per milliliter in the radiolabelled cultures were estimated by measuring the optical density at 600 nm (OD_{600}) of a 1:6 dilution and using an experimentally derived conversion factor for each strain. The conversion factors were determined by plating stationary-phase cells and determining the number of CFU per milliliter per OD_{600} unit for each strain. The conversion factors for the strains used in our experiments were as follows: *L. acidophilus* BG2FO4, 1.7×10^8 CFU/ml/OD_{600} unit; *L. acidophilus* NCFM/N2, 1.2×10^8 CFU/ml/OD_{600} unit; *L. gasseri* ADH, 4.1×10^8 CFU/ml/OD_{600} unit; and *L. delbrueckii* subsp. *bulgaricus* 1489, 1.8×10^8 CFU/ml/OD_{600} unit. The labelled cells were centifuged at 25°C at 10,000 × *g* for 10 min with a Sorvall model RC-5B centrifuge (Du Pont Co., Wilmington, Del.) or at 16,000 × g for 5 min with an Eppendorf microcentrifuge (Brinkman Instruments, Inc., Westbury, N.Y.), depending on the volume required to yield the necessary number of CFU. The cell pellets were washed twice in 0.8 ml of 6.0 mM Na_2HPO_4–1.5 mM KH_2PO_4–0.138 M NaCl–3.0 mM KCl (pH 7.3) (PBS/7.3) and then resuspended in PBS/7.3 or an appropriate buffer, except when they were treated enzymatically or chemically as described below. The number of CFU in each assay mixture was confirmed by plating appropriate dilutions of washed cells onto MRS agar. The specific activity (in counts per minute per CFU) was determined by counting the activities in aliquots (1×10^8 to 2×10^8 CFU) of labelled cells in 10 ml of Scintiverse BD (Fisher Scientific Co., Pittsburgh, Pa.) with a model LS3801 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Enzymatic and chemical treatments of bacterial cells. Unless indicated otherwise, all enzymes and chemicals were obtained from Sigma Chemical Co. Labelled bacterial cells (2×10^8 CFU) were washed once in PBS/7.3 and then suspended in 0.5 ml of one of the following solutions: PBS/7.3; 5.0 M LiCl in water; 0.1 M citrate-phosphate–0.1 M NaCl (pH 4.5) (buffer A); 0.05 M sodium iodate in buffer A; 0.05 M sodium periodate in buffer A; 0.05 M Tris-HCl–0.1 M NaCl (pH 8.0) (buffer B); 5.0 mg of trypsin per ml in buffer B; 5.0 mg of chymotrypsin (Boehringer-Mannheim, Indianapolis, Ind.) per ml in buffer B; 0.05 M glycine-HCl–0.1 M NaCl (pH 2.2) (buffer C); and 5.0 mg of pepsin (Boehringer-Mannheim) per ml in buffer C. The cells were incubated for 1 h at 37°C, centrifuged at 16,000 × *g*, washed twice in 0.8 ml of PBS/7.3, and resuspended in 1.0 ml of PBS/7.3. In the experiments in which cells were extracted with 5.0 M LiCl and then treated with trypsin, the labelled cells were washed once in 0.8 ml of PBS/7.3, incubated in 0.5 ml of 5.0 M LiCl for 30 min at 37°C, centrifuged at 16,000 × *g*, washed once in 0.8 ml of PBS/7.3, and then incubated for 30 min at 37°C in 0.5 ml of either buffer B containing 5.0 mg of trypsin per ml or buffer B alone. The cells were then washed twice and suspended in PBS/7.3 for use in the adherence assay.

Adherence assays. Fifteen-day-old Caco-2 monolayers were washed twice with 2.0 ml of PBS/7.3 per well before the assay was begun. For the experiments in which we examined the concentration dependence of adhesion, the appropriate numbers of CFU were added to wells in 2.0 ml (total volume) of PBS/7.3. For the experiments in which we examined pH dependence, 2×10^8 CFU was added to each well in 2.0 ml (total volume) of buffer at the appropriate pH. Most of the pH values were obtained by mixing appropriate volumes of 7.5 mM NaH_2PO_4 and 7.5 mM Na_2HPO_4, each containing 0.138 M NaCl and 3 mM KCl; the pH 3 and 4 buffers were obtained by adjusting the monobasic buffer with 1.0 M HCl. For the experiments in which we examined the effects of enzymatic and chemical treatments on adherence, 5×10^7 CFU of treated or untreated cells was added to each well in 1.25 ml (total volume) of PBS/7.3. All preparations were incubated at 37°C for 1 h, and then the bacterial cells were removed and the monolayers were washed four times with 2.0 ml of PBS/7.3 per well. The monolayers with attached bacteria were solubilized with 1.0 ml of 0.1% sodium dodecyl sulfate (SDS)–0.1 M NaOH per well, the wells were washed once with 1.0 ml of PBS/7.3 per well, and the pooled samples were added to 10 ml of Scintiverse BD; this was followed by quantitation with a

liquid scintillation counter. Statistical analyses of adherence data were performed by using the paired *t* test or the Mann-Whitney rank sum test provided by the Sigma Stat computer software (Jandel Scientific, San Rafael, Calif.). The relative levels of bacterial adherence determined by counting radioactivity associated with solubilized Caco-2 cells were confirmed by visually observing adherent bacteria after selected treatments of bacterial cells and spent culture supernatant.

SDS-PAGE of surface proteins from bacterial cells. Untreated bacterial cells or cells which had been extracted with 5.0 M LiCl as described above were boiled for 5 min in 0.1 ml of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (30) and then centrifuged at 16,000 × *g* for 3 min to remove the insoluble debris. Sample buffers containing amounts of protein equivalent to the amounts solubilized from 10^8 CFU of *L. acidophilus* BG2FO4 or NCFM/N2 and 2.5 × 10^8 CFU of *L. gasseri* ADH were subjected to SDS-PAGE by using 12% polyacrylamide gels (30). Protein bands were visualized by staining the gels with Coomasie brilliant blue (33) for *L. acidophilus* BG2FO4 and NCFM/N2 or with silver (36) for *L. gasseri* ADH. Molecular weight markers were obtained from Bio-Rad Laboratories, Richmond, Calif. Gels were photographed by using a model IS-1000 digital imaging system (Innotech Scientific Corp., San Leandro, Calif.).

Spent culture supernatant experiments. *L. acidophilus* BG2FO4 spent culture supernatant was obtained by centrifuging a culture at 16,000 × *g* for 30 min at 4°C. Dialyzed spent culture supernatant was obtained by dialyzing the spent culture supernatant against 4 liters of PBS/7.3 at 4°C for 24 h with four changes of buffer; to do this, we used Spectra/Por (Spectrum Medical Industries, Inc., Houston, Tex.) dialysis tubing with a molecular exclusion limit of 3,500 daltons. In some experiments, spent culture supernatant, dialyzed spent culture supernatant, or fresh MRS medium was either acidified to pH 4.2 with lactic acid (Fisher Scientific Co.) or adjusted to pH 7.3 with 10.0 N NaOH. Spent culture supernatant at either the acid pH or a neutral pH was treated with 2.5 mg of trypsin (Sigma Chemical Co.) per ml for 1 h at 37°C, and the trypsin was inactivated either by adding 2.5 mg of soybean trypsin inhibitor (Sigma Chemical Co.) per ml or by adding FBS to a final concentration of 20% (vol/vol). The controls for these treatments were incubation for 1 h at 37°C without trypsin, followed by addition of soybean trypsin inhibitor or FBS. The spent culture supernatant was boiled by immersing microcentrifuge tubes containing 1.0-ml aliquots in a boiling water bath for 10 min.

Adherence assays involving treated or untreated spent culture supernatants were performed as described above, with the following modifications. Washed, labelled *L. acidophilus* BG2FO4 cells (5 × 10^7 CFU) were suspended in 1.0 ml of either PBS/7.3 or Caco-2 cell culture medium without antibiotics (DMEM) and then mixed with 1.0 ml of test supernatant. The mixtures were then incubated with Caco-2 cells for 1 h. For the experiment in which we investigated the effect of preincubating the Caco-2 cells with either acidified or fresh MRS medium, the Caco-2 cells were preincubated for 1 h with a mixture containing 1.0 ml of acidified or fresh MRS medium and 1.0 ml of Earle's balanced salt solution (pH 7.4) without glucose. After 1 h of preincubation, the Caco-2 cells were washed once with PBS/7.3 and then incubated with 1.0 ml of Earle's balanced salt solution containing 5 × 10^7 CFU of labelled *L. acidophilus* BG2FO4 for an additional 1 h. The mixtures containing PBS/7.3 were incubated in a 37°C incubator under the room atmosphere, whereas the mixtures containing Caco-2 cell culture medium or Earle's balanced salt solution were incubated in a 37°C incubator under a 90% air–10% CO_2 atmosphere. For the final washes and measurements of adherence we used the techniques described above.

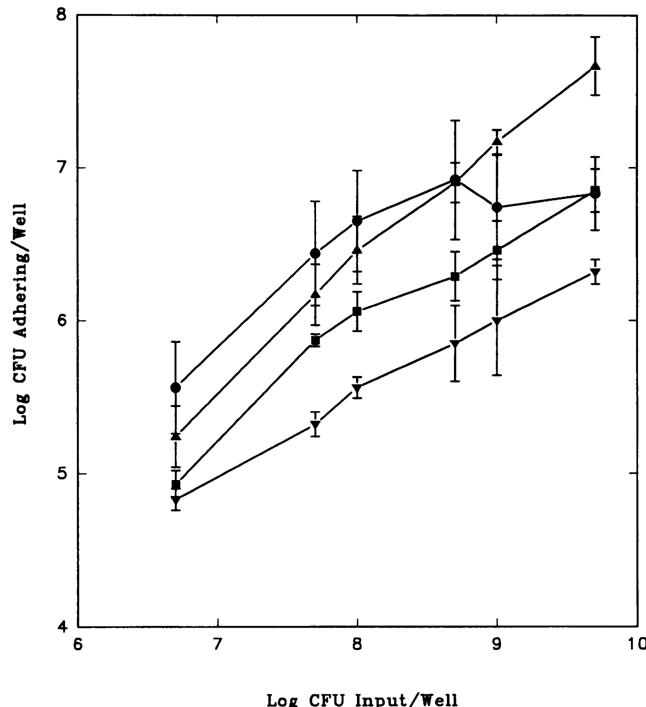

FIG. 1. Concentration dependence of adherence to Caco-2 cells for *L. acidophilus* BG2FO4 (●), *L. acidophilus* NCFM/N2 (■), *L. gasseri* ADH (▲), and *L. delbrueckii* subsp. *bulgaricus* 1489 (▼). The data points are the means and the error bars are the standard deviations for the combined data obtained with duplicate samples in two separate experiments (*n* = 4).

RESULTS

Adherence assays. The strains used in this study all incorporated [*methyl*-^{3}H]thymidine with specific activities ranging from 10^{-3} to 10^{-4} cpm/CFU. The background counts for Caco-2 monolayers solubilized without prior addition of labelled bacteria were generally around 50 cpm per well. The counts obtained with adhering bacteria ranged from approximately 80 to 50,000 cpm per well depending on the number of input bacteria, the strain used, and the assay conditions. Slightly higher specific activities were obtained when the bacteria were labelled in Casamino Acids broth than when they were labelled in MRS broth, but no differences were observed in the absolute levels of adherence (data not shown).

The effects of cell concentration (Fig. 1) and pH (Fig. 2) on adherence were examined by using three human isolates, *L. acidophilus* BG2FO4, and NCFM/N2 and *L. gasseri* ADH, and a single dairy isolate, *L. delbrueckii* subsp. *bulgaricus* 1489. The level of adherence increased steadily with increasing numbers of input CFU per well for all strains except *L. acidophilus* BG2FO4; with this strain preparations became saturated at 5 × 10^8 input CFU per well. The three human isolates all adhered at higher levels than dairy strain 1489 at all of the concentrations tested. The levels of adherence increased substantially for all four strains as the pH decreased from 6 to 3, whereas the levels of adherence were relatively consistent for all of the strains at pH 6 to 8. Although the data in Fig. 2 indicate that more bacteria adhered at acid pH values, the

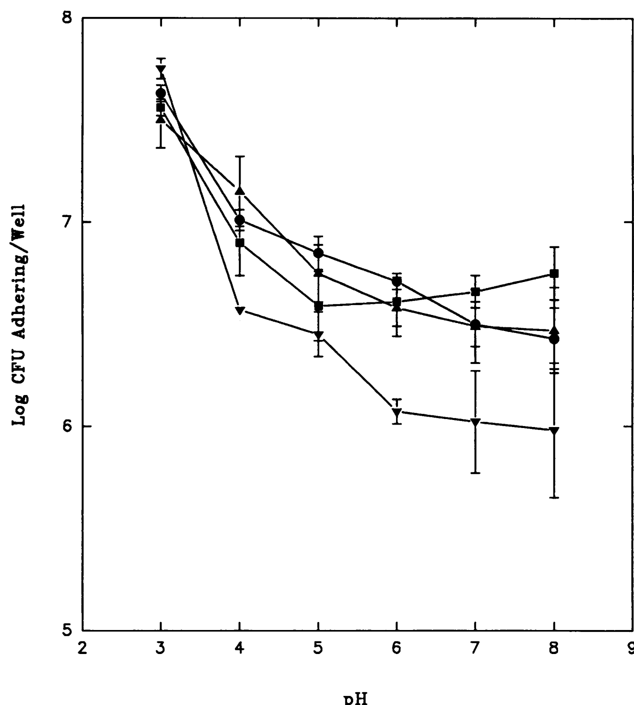

FIG. 2. pH dependence of adherence to Caco-2 cells for *L. acidophilus* BG2FO4 (●), *L. acidophilus* NCFM/N2 (■), *L. gasseri* ADH (▲), and *L. delbrueckii* subsp. *bulgaricus* 1489 (▼). The data points are the means and the error bars are the standard deviations for the combined data obtained with duplicate samples in two separate experiments ($n = 4$).

Caco-2 cell monolayers changed from clear and translucent to white and opaque as the pH of the assay buffer dropped below 5. These changes occurred even in the absence of input bacteria (data not shown). Since the normal pH of the intestinal environment is 6 to 8 (31, 46) and since an acidic pH represents abnormal stress conditions for cells in culture, all subsequent experiments were performed at pH 7.3, unless indicated otherwise (see Table 3), in order to work within the normal pH range and avoid potential problems associated with the effects of low pH on Caco-2 cells.

Biochemical characterization of adherence. The three human isolates were subjected to a variety of enzymatic and chemical treatments in order to characterize and compare the factors involved in adherence of these bacteria to Caco-2 cells. The contribution of protein factors was examined by treating the bacterial cells with three proteases present in the human gastrointestinal tract. The contribution of carbohydrate moieties was examined by oxidizing cell surface carbohydrates with periodate. The results of these experiments are shown in Table 1. For each treatment the absolute level of adherence of the bacteria after incubation in the control buffer was 7 to 25% of the level of adherence found after incubation in PBS/7.3 alone, indicating that the buffers themselves had some effect on adherence. Therefore, to accurately reflect the effects of the treatments on adherence, levels of adherence are reported below as percentages of the control values obtained after incubation in the appropriate control buffers without added chemical or enzyme.

Periodate oxidation of cell surface carbohydrates had no effect on *L. acidophilus* NCFM/N2 adherence, moderately decreased *L. acidophilus* BG2FO4 adherence, and significantly reduced *L. gasseri* ADH adherence. An additional control treatment in which we used an equivalent concentration of iodate, one of the products obtained after periodate oxidation of carbohydrates, had no significant effect on the adherence of any strain. These results indicate that carbohydrate moieties are involved to varying degrees in the adherence of the strains which we examined.

TABLE 1. Effects of chemical and enzymatic treatments on the adherence of lactobacilli to Caco-2 cells

Treatment	% Adherence		
	Strain BG2FO4	Strain NCFM/N2	Strain ADH
Buffer A	100 (19)[a]	100 (4)	100 (6)
Periodate	58 (14)	94 (7)[b]	27 (5)
Iodate	129 (13)[b]	106 (10)[b]	90 (11)[b]
Buffer B	100 (9)	100 (17)	100 (12)
Trypsin	19 (7)	33 (4)	105 (7)[b]
Chymotrypsin	62 (41)[b]	28 (4)	89 (10)[b]
Buffer C	100 (6)	100 (12)	100 (13)
Pepsin	37 (1)	46 (11)	199 (23)

[a] Mean (standard deviation) for duplicate samples in two separate experiments ($n = 4$).
[b] Not significantly different from the control value ($P > 0.05$).

Protease treatment of bacterial cells had different effects on adherence depending on the protease used and the strain examined. Adherence of *L. acidophilus* BG2FO4 was significantly reduced by treatment with trypsin and pepsin but was relatively unaffected by treatment with chymotrypsin. Adherence of *L. acidophilus* NCFM/N2 was significantly reduced by treatment with all three proteases. Interestingly, adherence of *L. gasseri* ADH was not affected by trypsin or chymotrypsin treatment but nearly doubled when cells were treated with pepsin, indicating that this enzyme may exert some type of activating effect on adherence for this strain.

Involvement of S-layer protein in adherence. Schneitz et al. (45) recently described the potential involvement of a crystalline surface layer (S-layer) protein in the adherence of *L. acidophilus* to chicken intestinal epithelial cells. Since protein components appeared to be involved in the adherence of at least two of the three strains used in this study, the surface proteins of all three strains were examined for the presence of S-layer proteins. SDS-PAGE of cell surface proteins revealed the presence of potential S-layer proteins, approximated at 44 kDa, in both *L. acidophilus* BG2FO4 (Fig. 3, lane 2) and *L. acidophilus* NCFM/N2 (Fig. 3, lane 4). No S-layer protein was detected in *L. gasseri* ADH (Fig. 3, lane 6). In order to assess the potential contribution of these proteins to adherence, bacterial cells were extracted with 5.0 M LiCl to remove the putative S-layer protein (32) and then examined for adherence (Table 2) and for alterations in the cell surface protein profiles (Fig. 3, lanes 3, 5, and 7). Extraction with 5.0 M LiCl removed more than 90% of the S-layer protein from both *L. acidophilus* NCFM/N2 and BG2FO4. Removal of the S-layer protein had no effect on the adhesion of *L. acidophilus* BG2FO4 or NCFM/N2. The cell surface protein profile of *L. gasseri* ADH was not altered by the treatment used, and there was no statistically significant effect on adherence.

To confirm that the proteinaceous component involved in adherence was not removed by LiCl extraction, LiCl-extracted cells were incubated in buffer with or without trypsin and tested in the adherence assay (Table 2). Trypsin treatment significantly reduced the adherence of both *L. acidophilus*

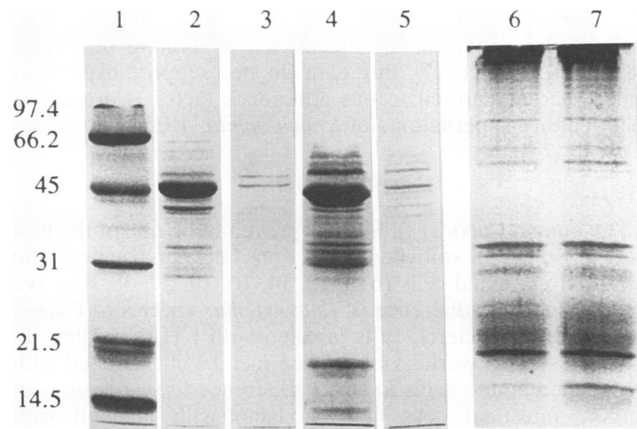

FIG. 3. SDS-PAGE of surface proteins from bacterial cells before and after extraction with LiCl. Lane 1, molecular mass standards (the positions of the standards are indicated on the left, in kilodaltons); lanes 2 and 3, *L. acidophilus* BG2FO4 cell surface proteins from untreated cells (lane 2) and from cells extracted with 5.0 M LiCl (lane 3); lanes 4 and 5, *L. acidophilus* NCFM/N2 cell surface proteins from untreated cells (lane 4) and from cells extracted with 5.0 M LiCl (lane 5); lanes 6 and 7, *L. gasseri* ADH cell surface proteins from untreated cells (lane 6) and from cells extracted with 5.0 M LiCl (lane 7). Lanes 1 through 5 were visualized by Coomasie blue staining, and lanes 6 and 7 were visualized by silver staining.

BG2FO4 and NCFM/N2 but had no effect on the adherence of *L. gasseri* ADH. These data suggest that the S-layer protein is not involved in the adherence of any of the strains which we studied and that some other cell surface protein contributes to adherence.

Secreted protein hypothesis versus pH effects. Several workers have postulated that a secreted protein is involved in the adherence of *Lactobacillus* species to Caco-2 cells (7, 8). Coconnier et al. (8) suggested that *L. acidophilus* BG2FO4 adheres primarily through a secreted protein component present in spent culture supernatant and to a lesser degree through cell surface components. This conclusion was based largely on three observations: (i) adherence in the presence of spent culture supernatant was greater than adherence in fresh MRS medium; (ii) trypsin treatment of spent culture supernatant reduced adherence; and (iii) BG2FO4 spent culture supernatant enhanced the adherence of *Lactobacillus casei* GG. Since our results indicated that adherence of *L. acidophilus* BG2FO4 occurs in the absence of spent culture supernatant, some of the previous experimental results were reexamined and extended by using additional controls.

The levels of adherence of BG2FO4 in spent culture supernatants treated in various ways are shown in Table 3 as percentages of the adherence value obtained with untreated spent culture supernatant (test A), which was arbitrarily assigned a value of 100%. In the adherence assays we used both PBS/7.3 and Caco-2 cell culture media without antibiotics (DMEM). Buffer (PBS/7.3 or DMEM) was mixed with treated or untreated spent culture supernatant at a ratio of 1:1 in order to compare the results obtained with our method with the results obtained with a method used previously (8). The level of adherence in buffer alone (test B) or in fresh MRS broth at pH 6.5 (test C) was significantly less than the level of adherence in spent culture supernatant (test A). The pH of spent culture supernatant from a stationary-phase culture of *L. acidophilus* BG2FO4 was 4.2. When spent culture supernatant was mixed with PBS/7.3 or DMEM at a ratio of 1:1, the level of acid in the spent culture supernatant was sufficient to negate the buffering effects of PBS/7.3 or DMEM. Therefore, we performed additional experiments to investigate the contribution of low pH values to adherence. Spent culture supernatant (pH 4.2) was adjusted to pH 7.3 with sodium hydroxide (Table 3, test E) or dialyzed to a neutral pH against PBS/7.3 (test D). Both treatments significantly reduced the level of adherence compared with the control (tests A, D, and E). Acidification of fresh MRS broth to pH 4.2 resulted in a significant increase in the level of adherence compared with untreated MRS broth at pH 6.5 (tests C and F), but did not fully restore adherence to the level obtained with spent culture supernatant at pH 4.2 (test A). Reacidification of dialyzed spent culture supernatant (test G) also increased adherence, but adherence was not

TABLE 2. Effect of S-layer removal on the adherence of lactobacilli to Caco-2 cells

Treatment[a]	% Adherence [mean (SD)]		
	Strain BG2FO4	Strain NCFM/N2	Strain ADH
PBS/7.3	100 (4)	100 (14)	100 (11)
LiCl	96 (8)[b]	114 (19)[b]	116 (9)[b]
LiCl-buffer B	100 (8)	100 (11)	100 (3)
LiCl-trypsin	28 (8)	36 (16)	88 (12)[b]

[a] Six samples were used for the PBS/7.3 and LiCl treatments, and four samples were used for the LiCl-buffer B and LiCl-trypsin treatments.
[b] Not significantly different from the control value ($P > 0.05$).

TABLE 3. Examination of spent culture supernatant factors on adherence of *L. acidophilus* BG2FO4 to Caco-2 cells

Test	Supernatant[a]	% Adherence [mean (SD)][b]	
		PBS/7.3	DMEM
A	SCS, pH 4.2	100 (4)	100 (8)
B	Buffer alone, pH 7.3 (PBS/7.3 or DMEM)	23 (7)	20 (2)
C	Fresh MRS broth, pH 6.5	8 (1)	11 (1)
D	SCS dialyzed to pH 7.3	8 (2)	25 (12)
E	SCS adjusted to pH 7.3	5 (1)	16 (4)
F	Fresh MRS broth adjusted to pH 4.2	65 (18)	79 (12)
G	SCS dialyzed to pH 7.3, readjusted to pH 4.2	45 (15)	33 (15)
H	SCS adjusted to pH 7.3, readjusted to pH 4.2	99 (22)[c]	53 (6)
I	SCS adjusted to pH 7.3, treated with trypsin inhibitor, readjusted to pH 4.2	97 (28)[c]	60 (7)
J	SCS adjusted to pH 7.3, treated with trypsin, treated with trypsin inhibitor, readjusted to pH 4.2	97 (25)[c]	43 (4)
K	SCS, pH 4.2, treated with trypsin inhibitor	104 (25)[c]	101 (18)[c]
L	SCS, pH 4.2, treated with trypsin, treated with trypsin inhibitor	100 (18)[c]	88 (22)[c]
M	SCS, pH 4.2, treated with FBS	37 (4)	57 (2)
N	SCS, pH 4.2, treated with trypsin, treated with FBS	29 (2)	34 (4)
O	SCS, pH 4.2, boiled	108 (21)[c]	112 (7)

[a] Test supernatants were used at a 1:1 ratio with either PBS/7.3 or DMEM. SCS, spent culture supernatant.
[b] The numbers of samples examined were as follows: test A with PBS/7.3, 24; test A with DMEM, 18; tests B, C, F, G, M, and N, 4; test D, 8; test E, 7; tests H, J, and L, 6; and tests I, K, and O, 5.
[c] Not significantly different from the control value ($P > 0.05$).

increased to the level of adherence obtained with spent culture supernatant alone. Reacidification of spent culture supernatant (test H), which was first neutralized by directly adding base instead of being dialyzed, resulted in a level of adherence of almost 100% when the assay was performed in PBS/7.3 but not when the assay was performed in DMEM (test H). These data indicated that acidic pH values promoted adherence and suggested that the spent culture supernatant contained low-molecular-weight compounds (molecular weight, <3,500) that contributed significantly to the adherence of BG2FO4.

The decrease in BG2FO4 adherence after the spent culture supernatant was neutralized suggested that either a pH-sensitive adherence factor was involved or acidic conditions generally promoted adherence to Caco-2 cells. The effects of low pH values on the Caco-2 cells themselves were examined by pretreating monolayers at different pH values prior to adherence assays. Caco-2 monolayers were preincubated with MRS broth at pH 4.2 (pH adjusted with either lactic acid or 2 N HCl) or MRS broth at pH 6.5, washed with PBS/7.3, and then incubated with labelled *L. acidophilus* BG2FO4 in PBS/7.3. After acid treatment, the levels of adherence on Caco-2 monolayers were 1.99 ± 0.05 (lactic acid-acidified preparation) and 1.88 ± 0.04 (HCl-acidified preparation) times greater than the levels of adherence on monolayers pretreated with MRS broth at pH 6.5 (n = 6). These results suggest that acid pH values result in alterations in the Caco-2 cell monolayers which lead to elevated levels of bacterial adherence. Unfortunately, in this experiment we could not eliminate the possible involvement of an acid-activated factor, since we were not able to directly test for the presence of such a factor in spent culture supernatants because of the impact of acid on the Caco-2 cells themselves. The acid effects appeared to be more generalized, however, because of the positive influence of low pH values on all of the lactobacilli tested (Fig. 2) irrespective of their various types of adherence factors and capabilities.

Coconnier et al. (8) reported that *L. acidophilus* BG2FO4 adherence was reduced when spent culture supernatant was treated with trypsin. However, trypsin has been reported to have little or no activity at pH 4.2 (20). Given the effects of acid pH values on the adherence of BG2FO4 described above, the contribution of a protease-sensitive component in spent culture supernatants was reexamined after treatment with trypsin at neutral or acidic pH values. Following bacterial cell treatment, trypsin was inactivated with soybean trypsin inhibitor or FBS; FBS was used as described previously (8). Table 3 shows that treatment with trypsin plus soybean inhibitor had no significant effect on adherence in PBS/7.3, regardless of the pH at which the trypsin treatment was performed (tests A and I through L). When the assay was performed in DMEM, trypsin treatment at pH 4.2 followed by addition of inhibitor had no significant effect compared with untreated spent culture supernatant (tests A, K, and L). pH adjustments alone did result in a significant decrease (47%) in the level of adherence when the cells were assayed in DMEM (tests A and H). Taking this loss into account, we found that trypsin treatment did not have a negative impact on the adherence of the BG2FO4 cells assayed in DMEM (tests H and J). In all of these experiments, addition of soybean trypsin inhibitor alone did not have an impact on levels of adherence. However, when FBS alone was added to spent culture supernatants at pH 4.2, the level of adherence decreased significantly in both assay buffers (tests A and M); trypsin treatment did not dramatically decrease the level of adherence beyond this point (tests M and N). Therefore, FBS alone can be a major factor which retards adherence if it is added to spent culture supernatants at pH 4.2. Finally, boiling spent culture supernatant had no significant effect on the level of adherence (Table 3, test O). A protein adhesin would probably be denatured or inactivated by boiling or trypsin treatment. Collectively, our data do not support hypothesis that a secreted proteinaceous adherence factor is present in spent culture supernatants of *L. acidophilus* BG2FO4.

DISCUSSION

The human Caco-2 cell line has proven to be one of the best model systems for studying interactions between bacterial and intestinal epithelial cells (6–8, 12, 14, 16, 19, 37, 38, 42). We investigated the adherence of *Lactobacillus* species to Caco-2 cells by using bacterial cells labelled with [^{3}H]thymidine. In previous studies workers have used lactobacilli labelled with ^{3}H-labelled amino acids to study adherence to suspensions of porcine intestinal cells (11) or lactobacilli labelled with [^{3}H]thymidine to study adherence to murine and porcine gastric cells (10, 22, 23, 29). The use of radiolabelled bacterial cells to determine levels of adherence has several advantages over methods in which fixation, staining, and quantification by enumeration of adhering bacterial cells with a light microscope are used. First, this method is faster as it eliminates the time required to count enough microscopic fields to get statistically relevant data. Second, there are no fixation or staining steps, and thus the potential for artifacts due to the fixation process are eliminated, as are the difficulties sometimes encountered in distinguishing individual bacterial cells. Finally, the level of adherence is determined over the entire surface area of a six-well dish, not just a small portion of the surface, which increases the accuracy and reproducibility of the method.

Three human intestinal *Lactobacillus* isolates adhered to Caco-2 cells at levels which were 0.5 to 1 log unit higher than the level of adherence obtained with a control dairy isolate (Fig. 1). Our initial biochemical characterization of the adherence properties of the three human isolates, *L. acidophilus* BG2FO4 and NCFM/N2 and *L. gasseri* ADH, revealed that there are multiple factors on the bacterial cell surface that are involved in the adherence of these strains to Caco-2 cells. *L. acidophilus* BG2FO4 adherence to Caco-2 cells involved protease-sensitive and, to a lesser degree, periodate-sensitive factors on the bacterial cell surface, whereas *L. acidophilus* NCFM/N2 adherence involved only protease-sensitive factors and *L. gasseri* ADH adherence involved only periodate-sensitive factors. These findings are consistent with the findings obtained in previous electron microscopic studies, in which workers described the possible involvement of a layer of ruthenium red-staining, electron-dense material in *L. acidophilus* BG2FO4 (24, 25), which was absent from *L. acidophilus* NCFM/N2. *L. gasseri* ADH (designated Lac 12 in the study of Hood and Zottola [24]) had a variable staining layer. Protein involvement was not investigated in either of the previous studies.

S-layer proteins form a crystalline layer around the cells of many bacterial species, and it has been proposed that these proteins are involved in cell protection, adherence, and surface recognition (47). Both *L. acidophilus* BG2FO4 and NCFM/N2 have proteins that resemble the S-layer proteins identified on the surfaces of other lactobacilli (3, 32, 49). The presence of a crystalline layer was not confirmed by electron microscopy, but the relative abundance of the protein species and their molecular weights indicate that they are probably S-layer proteins. These proteins were not involved in adhesion since removing them with lithium chloride had no statistically significant effect on adherence. The fact that the level of adherence was reduced by protease treatment of cells stripped of their S-layer suggests that another cell-associated protein may be involved in adher-

ence. In bacterial cells S-layer proteins can be glycoproteins (47), but the putative S-layer proteins of *L. acidophilus* BG2FO4 and NCFM/N2 were not stained by periodate-Schiff reagent after SDS-PAGE (data not shown), indicating that neither of these proteins is a glycoprotein. Thus, the carbohydrate involved in *L. acidophilus* BG2FO4 adherence is probably contributed by some other surface structure.

Bacterial adherence in our model system was dramatically influenced by pH. As the pH of the assay buffer decreased, the relative level of adherence generally increased proportionally for all of the strains tested, even though different cell surface factors appeared to be involved in adherence among the strains used. These observations indicated that acidic pH values affected the Caco-2 cells themselves, rather than promoting a pH-dependent effect on adherence factors and ligands present at the surfaces of the bacterial and/or Caco-2 cells. Caco-2 cells have no protective mucus layer, and it is therefore reasonable to assume that exposure to low pH values could result in acid-catalyzed denaturing of proteins or other cell surface components, which could lead to enhanced bacterial adherence.

A cell surface proteinaceous factor was involved in the adherence of *L. acidophilus* BG2FO4. However, on the basis of our data we could not confirm that a protein secreted into spent culture supernatants (8) was primarily responsible for adherence to Caco-2 cells. In a previous report (8), loss of adherence after treatment of spent culture supernatants with trypsin was the major result which supported the hypothesis that a secreted adherence protein was involved. However, FBS was used in that study to inactivate trypsin, and the effects of FBS alone on adherence were not evaluated. In this study, trypsin treatment of spent culture supernatant had no statistically significant effect on adherence, and the presence of FBS alone resulted in a significant reduction in the level of adherence. These data suggest that the reduction in the level of adherence observed in the previous study (8) was primarily due to FBS and not to trypsin-catalyzed proteolysis of a secreted adhesin. Some low-molecular-weight components present in BG2FO4 spent culture supernatants do contribute to adherence at acidic pH values. The nature of these components was not investigated, but it is unlikely that these factors are bacterial adhesions because of their small size, heat stability, and trypsin insensitivity. The results of a variety of experiments indicate that a major factor promoting adherence of *L. acidophilus* BG2FO4 in spent culture supernatants (pH 4.2) is the effect of low pH values on the Caco-2 cells themselves. Conclusions drawn from other studies concerning the adherence of another *Lactobacillus* strain (7), concerning adherence of *Bifidobacterium* species (2), and concerning adherence of urogenital *Lactobacillus* isolates (42) to Caco-2 cells may also be affected by these findings since these studies were based on similar methods in which spent culture supernatants were used.

Bacterial cell-associated protein components were clearly implicated as major factors in the adherence of *L. acidophilus* BG2FO4 and NCFM/N2 to Caco-2 cells. The cell surface factors implicated in the adherence of the *Lactobacillus* strains examined in this study have been implicated previously in studies of adherence of lactobacilli to tissues from other species or to other cell types. Cell wall components have been implicated in the adherence of lactobacilli in the human urogenital tract (5), carbohydrate components have been implicated in the adherence of bacteria to chicken crop epithelium (4) and pig intestine (50), and protein components have been implicated in the adherence of bacteria to porcine gastric squamous epithelium (23), murine gastric squamous epithelium (10), and chicken intestinal epithelial cells (45). Given the variety of adherence factors that have been identified for many intestinal pathogens (15), nonpathogenic bacteria probably also rely on multiple mechanisms involving diverse cell surface structures for intestinal colonization.

To date, none of the components involved in *Lactobacillus* adherence to any species has been purified to homogeneity or characterized at the genetic level. McCarthy et al. (34) described the genetic transformation of the ability to colonize the nonsecreting gastric epithelium of mice into *Lactobacillus* sp. strain 100-33, but no further information concerning the nature of the genetic determinants has followed. Molecular information concerning adhering *Lactobacillus* species is still rare, and many of the techniques used for cloning and expression of DNA have not been developed in the relevant *Lactobacillus* genetic backgrounds. Our own efforts to transform *L. acidophilus* BG2FO4 with functional *Lactobacillus* shuttle vectors have not been successful, whereas genetic transfer systems and vectors are now available for *L. gasseri* ADH (40, 41) and *L. acidophilus* NCFM/N2 (50a). As the requisite techniques and vectors become more widely available, use of the Caco-2 cell model system should facilitate genetic characterization of the various cell surface components involved in adherence of human *Lactobacillus* isolates and provide the necessary mutants to determine the contribution of in vitro adherence to in vivo intestinal colonization in a definitive manner.

ACKNOWLEDGMENTS

This work was supported by the National Dairy Promotion and Research Board and the North Carolina Dairy Foundation.

REFERENCES

1. **Barefoot, S. F., and T. R. Klaenhammer.** 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. **45:**1808–1815.
2. **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 interactions. Appl. Environ. Microbiol. **59:**4121–4128.
3. **Boot, H. J., C. P. A. M. Kolen, J. M. van Noort, and P. H. Pouwels.** 1993. S-layer protein of *Lactobacillus acidophilus* ATCC 4356: purification, expression in *Escherichia coli*, and nucleotide sequence of the corresponding gene. J. Bacteriol. **175:**6089–6096.
4. **Brooker, B. F., and R. Fuller.** 1975. Adhesion of lactobacilli to the chicken crop epithelium. J. Ultrastruct. Res. **52:**21–31.
5. **Chan, R. C. Y., G. Reid, R. T. Irvin, A. W. Bruce, and J. W. Costerton.** 1985. Competitive exclusion of uropathogens from human uroepithelial cells by *Lactobacillus* whole cells and cell wall fragments. Infect. Immun. **47:**84–89.
6. **Chauvière, G., M.-H. Coconnier, S. Kernéis, A. Darfeuille-Michaud, B. Joly, and A. L. Servin.** 1992. Competitive exclusion of diarrheagenic *Escherichia coli* (ETEC) from human enterocyte-like Caco-2 cells by heat-killed *Lactobacillus*. FEMS Microbiol. Lett. **91:**213–218.
7. **Chauvière, G., M.-H. Coconnier, S. Kernéis, J. Fourniat, and A. L. Servin.** 1992. Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. J. Gen. Microbiol. **138:**1689–1696.
8. **Coconnier, M.-H., T. R. Klaenhammer, S. Kernéis, M.-F. Bernet, and A. L. Servin.** 1992. Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell lines in culture. Appl. Environ. Microbiol. **58:**2034–2039.
9. **Conway, P.** 1988. Lactobacilli: fact and fiction, p. 263–281. *In* R. Grun, T. Midvedt, and E. Norin (ed.), The regulatory and protective role of the normal gut flora. Stockton Press, New York.
10. **Conway, P., and S. Kjellberg.** 1989. Protein-mediated adhesion of *Lactobacillus fermentum* strain 737 to mouse stomach squamous epithelium. J. Gen. Microbiol. **135:**1175–1186.
11. **Conway, P. L., S. L. Gorbach, and B. R. Goldin.** 1987. Survival of lactic acid bacteria in the human stomach and adhesion to

intestinal cells. J. Dairy Sci. **70**:1–12.

12. **Darfeuille-Michaud, A., D. Aubel, G. Chauvière, C. Rich, M. Bourges, A. Servin, and B. Joly.** 1990. Adhesion of enterotoxigenic *Escherichia coli* to the human colon carcinoma cell line Caco-2 in culture. Infect. Immun. **58**:893–902.
13. **Dharmsathaphorn, K., J. A. McRoberts, K. G. Mandel, L. D. Tisdale, and H. Masui.** 1984. A human colonic tumor cell line that maintains vectorial electrolyte transport. Am. J. Physiol. **246**:204–208.
14. **Elo, S., M. Saxelin, and S. Salminen.** 1991. Attachment of *Lactobacillus casei* strain GG to human colon carcinoma cell line Caco-2. Lett. Appl. Microbiol. **13**:154–156.
15. **Finlay, B. B., and S. Falkow.** 1989. Common themes in microbial pathogenicity. Microbiol. Rev. **53**:210–230.
16. **Finlay, B. B., and S. Falkow.** 1990. *Salmonella* interactions with polarized intestinal Caco-2 epithelial cells. J. Infect. Dis. **162**:1096–1106.
17. **Fogh, J., J. M. Fogh, and T. Orfeo.** 1977. One hundred and twenty seven cultured human tumor cell lines producing tumors in nude mice. J. Natl. Cancer Inst. **59**:221–226.
18. **Gahring, L. C., F. Heffron, B. B. Finlay, and S. Falkow.** 1990. Invasion and replication of *Salmonella typhimurium* in animal cells. Infect. Immun. **58**:443–448.
19. **Gaillard, J.-L., P. Berche, J. Mounier, S. Richard, and P. Sansonetti.** 1987. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect. Immun. **55**:2822–2829.
20. **Guttfreund, H.** 1972. Enzymes: physical principles, p. 147. John Wiley and Sons, Ltd., London.
21. **Hastings, J. W., M. Sailer, K. Johnson, K. L. Roy, J. C. Vederas, and M. E. Stiles.** 1991. Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. J. Bacteriol. **173**:7491–7500.
22. **Henriksson, A., and P. L. Conway.** 1992. Adhesion to porcine squamous epithelium of saccharide and protein moieties of *Lactobacillus fermentum* strain 104-S. J. Gen. Microbiol. **138**:2657–2661.
23. **Henriksson, A., R. Szewzyk, and P. L. Conway.** 1991. Characteristics of the adhesive determinants of *Lactobacillus fermentum* 104. Appl. Environ. Microbiol. **57**:499–502.
24. **Hood, S. K., and E. A. Zottola.** 1987. Electron microscopic study of the adherence properties of *Lactobacillus acidophilus*. J. Food Sci. **52**:791–792.
25. **Hood, S. K., and E. A. Zottola.** 1989. An electron microscopic study of the adherence of *Lactobacillus acidophilus* to human intestinal cells in vitro. Food Microstruct. **8**:91–97.
26. **Huet, C., M. C. Sahuquillo, E. Coudrier, and D. Louvard.** 1987. Absorptive and mucus-secreting subclones isolated from a multipotent intestinal cell line (HT-29) provide new models for cell polarity and terminal differentiation. J. Cell Biol. **105**:345–357.
27. **Kleeman, E. G., and T. R. Klaenhammer.** 1982. Adherence of *Lactobacillus* species to human fetal intestinal cells. J. Dairy Sci. **65**:2063–2069.
28. **Knutton, S., T. Baldwin, P. H. Williams, and A. S. McNeish.** 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorragic *Escherichia coli*. Infect. Immun. **57**:1290–1298.
29. **Kotarski, S. F., and D. C. Savage.** 1979. Models for study of the specificity by which indigenous lactobacilli adhere to murine gastric epithelia. Infect. Immun. **26**:966–975.
30. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
31. **Lehninger, A. L.** 1982. Principles of biochemistry, p. 687–688. Worth Publishers, Inc., New York.
32. **Lortal, S., J. van Heijenoort, K. Gruber, and U. B. Sleytr.** 1992. S-layer of *Lactobacillus helveticus* ATCC 12046: isolation, chemical characterization and reformation after extraction with lithium chloride. J. Gen. Microbiol. **138**:611–618.
33. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual, vol. 3, p. 18.55. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **McCarthy, D. M., J. H.-C. Lin, L. A. Rinckel, and D. C. Savage.** 1988. Genetic transformation in *Lactobacillus* sp. strain 100-33 of the capacity to colonize the nonsecreting gastric epithelium in mice. Appl. Environ. Microbiol. **54**:416–422.
35. **Molin, G., B. Jeppsson, M.-L. Johansson, S. Ahrné, M. Ståhl, and S. Bengmark.** 1993. Numerical taxonomy of *Lactobacillus* spp. associated with healthy and diseased mucosa of the human intestines. J. Appl. Bacteriol. **74**:314–323.
36. **Morrissey, J. H.** 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. **117**:307–310.
37. **Mounier, J., A. Ryter, M. Coquis-Rondon, and P. J. Sansonetti.** 1990. Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocytelike cell line Caco-2. Infect. Immun. **58**:1048–1058.
38. **Panigrahi, P., B. D. Tall, R. G. Russell, L. J. Detolla, and J. G. Morris, Jr.** 1990. Development of an in vitro model for study of non-O1 *Vibrio cholerae* virulence using Caco-2 cells. Infect. Immun. **58**:3415–3424.
39. **Pinto, M., S. Robine-Leon, M. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum.** 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol. Cell **47**:323–330.
40. **Raya, R. R., C. Fremaux, G. L. De Antoni, and T. R. Klaenhammer.** 1992. Site-specific integration of the temperate bacteriophage ϕADH into the *Lactobacillus gasseri* chromosome and molecular characterization of the phage (*attP*) and bacterial (*attB*) attachment sites. J. Bacteriol. **174**:5584–5592.
41. **Raya, R. R., E. G. Kleeman, J. B. Luchansky, and T. R. Klaenhammer.** 1989. Characterization of the temperate bacteriophage ϕadh and plasmid transduction in *Lactobacillus acidophilus* ADH. Appl. Environ. Microbiol. **55**:2206–2213.
42. **Reid, G., A. L. Servin, A. W. Bruce, and H. Busscher.** 1993. Adhesion of three *Lactobacillus* strains to human urinary and epithelial cells. Microbios **75**:57–65.
43. **Sanders, M. E.** 1993. Effect of consumption of lactic cultures on human health. Adv. Food Nutr. Res. **37**:67–130.
44. **Savage, D. C.** 1977. Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. **31**:107–133.
45. **Schneitz, C., L. Nuotio, and K. Lounatma.** 1993. Adhesion of *Lactobacillus acidophilus* to avian intestinal epithelial cells mediated by the crystalline bacterial cell surface layer (S-layer). J. Appl. Bacteriol. **74**:290–294.
46. **Sernka, T., and E. Jacobsen.** 1979. Gastrointestinal physiology—the essentials, p. 96–101. The Williams and Wilkins Co., Baltimore.
47. **Sleytr, U., and P. Messner.** 1983. Crystalline surface layers on bacteria. Annu. Rev. Microbiol. **37**:311–339.
48. **Tannock, G. W.** 1988. The normal microflora: new concepts in health promotion. Microbiol. Sci. **5**:4–8.
49. **Vidgrén, G., I. Palva, R. Pakkanen, K. Lounatmaa, and A. Palva.** 1992. S-layer protein gene of *Lactobacillus brevis*: cloning by polymerase chain reaction and determination of the nucleotide sequence. J. Bacteriol. **174**:7419–7427.
50. **Wadstrom, T., K. Andersson, M. Sydow, L. Axelsson, S. Lindgren, and B. Gullmar.** 1987. Surface properties of lactobacilli isolated from the small intestine of pigs. J. Appl. Bacteriol. **62**:513–520.

50a. **Walker, C., and T. R. Klaenhammer.** Unpublished data.

51. **Wright, C. T., and T. R. Klaenhammer.** 1984. Phosphated milk adversely affects growth, cellular morphology, and fermentative ability of *Lactobacillus bulgaricus*. J. Dairy Sci. **67**:44–51.