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Effect of Carbon Source on Localized Adherence of Enteropathogenic *Escherichia coli*

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Enteropathogenic *Escherichia coli* (EPEC) strains attach to epithelial cells as discrete clusters of bacteria which are localized at a few sites on the cell surface. Previously, it was shown that this localized-adherence (LA) phenotype is induced by specific growth conditions. We found that wild-type EPEC attached to HEp-2 cells in an LA pattern when the bacteria were grown in Dulbecco's modified Eagle medium (DMEM) containing glucose as the carbon source. In contrast, bacteria incubated in DMEM containing galactose did not adhere to epithelial cells. The latter results were similar to those observed when JPN15, an LA-negative strain, was grown under conditions which promoted bacterial binding. The differences in attachment of wild-type EPEC were independent of the stage of log-phase growth of the cultures and of the number of CFU incubated with the HEp-2 monolayers. Expression of the adherence phenotype by organisms grown in glucose was associated with increased expression of intimin and bundle-forming pilin. In contrast, bacteria grown in medium containing galactose expressed these proteins at levels similar to those observed when JPN15 was grown in medium containing glucose.

Historically, the term enteropathogenic *Escherichia coli* (EPEC) referred to *E. coli* strains of specific serotypes which caused diarrhea in infants (28, 35). The enterovirulent *E. coli* strains are now divided into subgroups according to unique virulence mechanisms (8). Clinical symptoms of EPEC infection result from bacterial colonization of the intestine (39, 47, 51) and the subsequent formation of attaching and effacing (AE) lesions (33). Initially, EPEC strains bind to epithelial cells as discrete clusters of bacteria which are localized at a few sites on the cell surface (4, 41). In vitro, bacteria grown under conditions which promote attachment also secrete several proteins (including the *espA* [25] and *eaeB* [*espB*] [9, 13, 25] gene products) which are associated with virulence (20, 21, 24). Following initial attachment, EPEC activates signal transduction processes involving protein phosphorylation (2, 30, 36) and increased levels of intracellular calcium (3, 11) and inositol phosphates (11, 13, 14) in epithelial cells. The intracellular messengers generated, in turn, cause cytoskeletal rearrangement which results in the effacement of enterocyte microvilli and the accumulation of cytoskeletal components at sites of bacterial attachment (12, 26, 30). Expression of the EPEC *eaeA* gene product (intimin) (19, 22, 23) is required for the organization of cytoskeletal components beneath the bacteria and the subsequent formation of a pedestal-like structure with which the bacteria become intimately associated (26, 33, 36). Recently, intimin was shown to bind to a 90-kDa host tyrosine-phosphorylated protein which accumulates at sites of bacterial attachment (38). McDaniel et al. identified a genetic region, referred to as the locus of enterocyte effacement, in which the genes thus far associated with the AE activity of EPEC are located (32).

Although processes involved in the formation of the AE lesion have been elucidated, less is known about the initial binding of EPEC to epithelial cells. Cravioto et al. demonstrated that EPEC attachment to epithelial cells was mannose resistant, suggesting that type I pili were not responsible for the localized-adherence (LA) phenotype (4). Subsequently, EPEC strains were found to possess a high-molecular-weight EPEC adherence factor (EAF) plasmid which is associated with binding (1). Although several reports described an adhesin(s) which may play a role in attachment (18, 40, 42, 50), the observation that EPEC LA is induced by specific growth conditions helped to identify the factor(s) which mediates binding.

Giron et al. described unique rope-like structures, called bundle-forming pili (BFP), which promoted the formation of microcolonies when the bacteria were grown on blood agar plates or while attached to epithelial cells (17). A role for BFP in LA was supported by results demonstrating that bacteria lacking the EAF plasmid did not produce BFP and that antibodies raised to BFP partially inhibited EPEC binding. Similarly, Vuopio-Varkila and Schoolnik found that EPEC microcolony formation and adherence were induced by transferring bacteria from a relatively undefined bacterial growth medium (Luria broth [LB]) to a defined tissue culture medium (DME with or without fetal bovine serum [FBS]) (49). In those experiments, induction of LA coincided with increased expression of several bacterial outer membrane proteins, including an 18.5-kDa protein which was homologous to the BFP subunit. More recent studies demonstrated that EPEC mutants with Tn*phoA* insertions in the gene encoding the BFP structural subunit (*bfpA*) no longer expressed the LA phenotype (7, 43). Although reintroduction of the *bfpA* gene into these mutants restored BfpA expression, the bacteria were unable to adhere in an LA pattern, suggesting that other genes were required for attachment. Recently, the *bfpA* gene was shown to be part of a gene cluster involved in BFP biosynthesis and LA (44, 45).

Since the LA phenotype is expressed when EPEC is transferred to tissue culture medium (49), we investigated whether specific components of the medium were involved in mediating bacterial attachment. In this report, we describe the effect of the carbon source on EPEC binding. Our results provide a model system to further investigate the role of the carbon source in the regulation of EPEC adherence and virulence.

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MATERIALS AND METHODS

Bacterial strains. Bacterial strains E2348/69, JPN15, *cfm* 27-3-2(1), and *eaeA* 10-5-1(1) were kindly provided by B. B. Finlay (University of British Columbia, Vancouver, British Columbia, Canada). EPEC E2348/69 (O127:H6) is a wild-type strain isolated from an infant with gastroenteritis (29). JPN15 is a derivative of E2348/69 which lacks the EAF plasmid associated with the LA phenotype (23). Strains *cfm* 27-3-2(1) (5, 21, 32, 36) and *eaeA* 10-5-1(1) (5, 22, 23, 32, 36) are Tn*phoA* mutants of E2348/69 which have mutations in the *sepA* and *eaeA* genes, respectively. Strain *bfpA* 31-6-1(1), generously provided by M. S. Donnenberg (University of Maryland School of Medicine, Baltimore, Md.), is also derived from E2348/69 and has a Tn*phoA* insertion in the *bfpA* gene (5, 7). EPEC O111:H2 and O119:H6 are wild-type strains which have been described previously (48). Dulbecco's modified Eagle medium (DMEM) (catalog no. 23800; Gibco, Burlington, Ontario, Canada) was supplemented with 44 mM Na_2CO_3, 40 nM phenol red, and 25 mM (0.45%) glucose or galactose. Throughout all experiments, including coincubations with tissue culture cells, the bacteria were cultured in DMEM without FBS.

All bacterial strains were incubated overnight at 37°C in tryptic soy broth (Difco, Detroit, Mich.) under normal atmospheric conditions. The cultures were then inoculated (1:100) into carbohydrate-supplemented DMEM (preequilibrated to tissue culture conditions by incubation overnight in a humidified atmosphere of 5% CO_2–95% air) and incubated for various periods of time in a CO_2 incubator until their use in experiments.

Tissue culture. HEp-2 cells (CCL-23) were obtained from the American Type Culture Collection (Rockville, Md.). The cells were grown at 37°C in a humidified atmosphere of 5% CO_2–95% air in minimal essential medium supplemented with 10% FBS (Gibco). Subconfluent monolayers were prepared by disrupting HEp-2 cells with a solution consisting of 0.25% (vol/vol) trypsin in FC buffer (0.14 M NaCl, 5.0 mM KCl, 20.0 mM Tris-HCl, 5.0 mM Tris base, 0.5 mM EDTA [pH 7.2]). The cells were resuspended in tissue culture medium, and approximately 10^5 cells were seeded onto 12-mm-diameter glass coverslips in 24-well tissue culture plates. The plates were then incubated at 37°C for use in experiments the following day.

EPEC LA to tissue culture cells. EPEC strains were grown in glucose- or galactose-supplemented DMEM (in the absence of HEp-2 cells) as described above. Subconfluent monolayers of HEp-2 cells were prepared in 24-well tissue culture plates as described above. After the monolayers were washed twice with phosphate-buffered saline (PBS), 900 μl of log-phase bacterial culture (5×10^7 to 5×10^8 CFU grown in DMEM) was added to each well. Depending on the experiment, bacterial cultures either were added directly to the HEp-2 cells or were washed three times and resuspended in fresh preequilibrated DMEM (without FBS and with 25 mM glucose or galactose or no carbon source) immediately before their addition to the tissue culture wells. The latter experiments were performed to determine if wild-type bacteria had to be grown in the presence of a particular carbon source to express the attachment phenotype or if the carbohydrates directly affected bacterial binding. Unless otherwise indicated, mannose (1%, wt/vol) was included during the incubation of bacteria with the HEp-2 cells to inhibit type I mediated bacterial attachment (4). Following incubation of the bacteria with the HEp-2 cells at 37°C for 30 min, the monolayers were washed five times with PBS and then fixed for 10 min with methanol. This relatively short coincubation period was chosen to allow bacterial binding to occur while minimizing a possible influence of HEp-2 cells on the expression of an attachment factor(s) by EPEC. Bacterial adherence was determined by staining the cells with Giemsa stain and viewing the coverslips with the 100× objective lens of a Reichert light microscope. A minimum of 100 randomly chosen HEp-2 cells were observed. HEp-2 cells which had attached microcolonies consisting of more than four bacteria were considered positive for having EPEC with the LA phenotype (49). Experiments were performed three times, each time in duplicate.

Biotinylation of bacterial proteins. Biotinylations were performed by a modified surface-labeling procedure (Pierce, Rockford, Ill.) as described by the manufacturer. Mid-log-phase bacteria were grown in glucose- or galactose-supplemented DMEM (in the absence of HEp-2 cells) as described above. For experiments involving EPEC mutant *cfm* 27-3-2(1), *eaeA* 10-5-1(1), or *bfpA* 31-6-1(1), the bacteria were incubated in tissue culture medium for periods of time (i.e., 3 h) equivalent to those for the wild-type strain E2348/69. The bacteria (approximately 3×10^9 cells) were washed twice with PBS (pH 8.0) and resuspended in 1 ml of PBS (pH 8.0). The samples were transferred to microcentrifuge tubes and 15 μl of biotinamidocaproic acid 3-sulfo-*N*-hydroxysuccinimide ester (20 mg/ml in H_2O) (Sigma, St. Louis, Mo.) was added to each tube. After incubation on an end-over-end rotator for 1 h at room temperature, the cells were washed by centrifugation four times with PBS (pH 6.0) and once with PBS (pH 7.4). Bacterial whole-cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% polyacrylamide) in the presence of 50 mM dithiothreitol and then transferred electrophoretically to Immobilon-P membranes (Millipore, Bedford, Mass.).

Detection of biotinylated proteins. After transfer of the bacterial proteins to membranes, the nonspecific binding sites were blocked by incubating the membranes in a solution consisting of 5% (wt/vol) skim milk in Tris-buffered saline (20 mM Tris-HCl [pH 7.5], 150 mM NaCl) with 0.05% Tween 20 (TBST) for 2 h at room temperature. The membranes were incubated for an additional 2 h at room temperature with a streptavidin-alkaline phosphatase conjugate (Sigma) in 5% skim milk–TBST and then washed three times with TBST. Biotinylated proteins were visualized with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) color development reagents.

Immunoblotting of bacterial proteins. Polyclonal rabbit anti-intimin (22) and anti-BfpA (52) antibodies were generously provided by J. B. Kaper and M. S. Donnenberg (University of Maryland School of Medicine, Baltimore), respectively. Bacterial whole-cell lysates were transferred to Immobilon-P membranes as described above. After being blocked with 5% skim milk–TBST, the membranes were incubated with anti-intimin (1:2,000) or anti-BfpA (1:4,000) antibodies in 5% skim milk–TBST for 2 h at room temperature. The membranes were washed three times with TBST and then incubated with goat anti-rabbit immunoglobulin G antibodies conjugated to alkaline phosphatase in 5% skim milk–TBST. The membranes were washed again with TBST, and proteins were detected with NBT and BCIP color development reagents.

Statistical analysis. The significance of any differences in bacterial binding was determined by Student's *t* test.

RESULTS

Bacterial adherence to HEp-2 cells. Previous results suggested that a component of tissue culture medium was involved in regulating EPEC LA to epithelial cells (49). Preliminary experiments performed in our laboratory revealed that wild-type EPEC grown in DMEM with glucose, fructose, or mannose adhered to HEp-2 cells in an LA pattern, whereas bacteria grown in the presence of galactose did not (data not shown). Based on these results, we selected glucose and galactose as carbon sources to further investigate their role in bacterial attachment. Glucose was chosen (instead of fructose or mannose) to promote the adherence phenotype since most experiments involving EPEC binding to epithelial cells are performed with tissue culture medium supplemented with glucose.

Growth curves were initially prepared for EPEC isolates E2348/69 (O127:H6) (wild type) and JPN15 (a mutant of E2348/69 lacking the EAF plasmid) cultured in glucose- or galactose-supplemented DMEM to confirm that the bacteria were in log-phase growth, since it was shown previously that EPEC attaches to epithelial cells during this stage of growth (49). The ability of the bacteria to utilize glucose or galactose as a carbon source was indicated by a decrease in the pH of the culture medium over time (data not shown). Incubation periods used to grow the bacteria in DMEM (before addition to the tissue culture wells) were selected based on our observation that wild-type E2348/69 grown in DMEM containing glucose attached in significant numbers to HEp-2 cells during early- to mid-log-phase growth. Overall, bacterial binding was examined during the early log to mid-log, mid-log, and late log phases of growth to ensure that any differences in bacterial attachment were not due to differences in the stage of log-phase growth of the cultures. The results of these experiments demonstrate that only E2348/69 grown in glucose-supplemented medium attached to HEp-2 cells (Table 1). Early- to mid-log-phase wild-type bacteria grown in DMEM with glucose attached to 82% of the HEp-2 cells, while bacteria in later stages of log-phase growth attached to approximately 96% of the HEp-2 cells.

In contrast, E2348/69 incubated in DMEM containing galactose did not adhere to HEp-2 cells at any stage of log-phase growth. This result was similar to that seen when JPN15, which also did not bind to HEp-2 cells, was used in these experiments. Although previous reports demonstrated that EPEC lacking the EAF plasmid (i.e., JPN15) is able to adhere to epithelial cells, albeit at reduced levels and requiring prolonged periods of incubation of the bacteria with tissue culture cells (23, 27), no adherence was observed in our assays, presumably because of the relatively short period of coincubation (i.e., 30 min) of the bacteria with the HEp-2 monolayers. The

TABLE 1. EPEC adherence to HEp-2 cells

E. coli strain	Addition to DMEM	Growth phase (log)	Incubation period (h)[a]	% HEp-2 cells with LA EPEC[b]
E2348/69 (wild type)	Glucose	Early to mid	2	82.7 ± 3.7
		Mid	3	96.1 ± 0.2
		Late	4	96.0 ± 0.8
	Galactose	Early to mid	6	0
		Mid	10.5	0
		Late	16	0
JPN15	Glucose	Early to mid	2.5	0
		Mid	3.5	0
		Late	4.5	0

[a] Time of incubation of bacteria in DMEM before their addition to the tissue culture wells.
[b] Means ± standard deviations from three independent experiments.

TABLE 3. Effect of medium washes on adherence of wild-type EPEC E2348/69 to HEp-2 cells

Addition to DMEM for:		% HEp-2 cells with LA EPEC[c]	
Growth[a]	Washing and resuspension[b]	With D-mannose	Without D-mannose
Glucose	Glucose	86.5 ± 4.1	92.4 ± 7.7
	Galactose	81.8 ± 3.7	83.4 ± 9.1
	None	86.3 ± 4.2	90.3 ± 5.6
Galactose	Glucose	0	0
	Galactose	0	0
	None	0	0

[a] Medium used for incubation of bacteria before washing and incubation with HEp-2 cells.
[b] Medium used to wash and resuspend mid-log-phase bacteria immediately before their incubation with HEp-2 cells.
[c] Means ± standard deviations from three independent experiments.

observed differences in bacterial attachment were not due to differences in the number of CFU added to the tissue culture wells or to differences in the pH of the cultures, since these were similar for the various cultures at each stage of log-phase growth (data not shown).

To determine whether the effect of the carbon source on EPEC attachment was unique to isolate E2348/69, we repeated these experiments with wild-type EPEC strains of different serotypes. Using EPEC O111:H2 and O119:H6 isolates, which have been described previously (48), we found that these strains also adhered to HEp-2 cells when the bacteria were grown in DMEM containing glucose but not when the bacteria were grown in DMEM supplemented with galactose (Table 2).

Role of carbon source in bacterial attachment. Additional experiments were performed to determine if it was necessary for wild-type EPEC to be grown in the presence of a specific carbon source in order to express a particular attachment phenotype or if the carbohydrates had a direct function in promoting (glucose) or inhibiting (galactose) bacterial attachment. For this, wild-type EPEC E2348/69 was grown in glucose- or galactose-supplemented DMEM to mid-log phase. The bacteria were then washed and resuspended in fresh DMEM containing either glucose, galactose, or no carbon source immediately before being incubated with the HEp-2 monolayers. We found no significant difference ($P = 0.07$) in the binding ability of E2348/69 initially incubated in DMEM supplemented with glucose, whether the bacteria were washed and resuspended in medium alone or in medium containing glucose or galactose prior to their coincubation with the HEp-2 cells (Table 3). On the other hand, E2348/69 grown in DMEM containing galactose did not adhere to HEp-2 cells regardless of the medium used to wash and resuspend the bacteria.

Similar binding results were obtained when the adherence assays were performed in the absence of mannose (Table 3). This indicated that the pattern of bacterial attachment observed in earlier experiments was not due to the inclusion of mannose during the period of coincubation of bacteria with the HEp-2 cells. Overall, these results suggest that it is the growth of EPEC in the presence of a specific carbon source which influences its attachment to HEp-2 cells, since incubation of the bacteria in the presence of a different sugar (or no sugar) during the 30-min coincubation with HEp-2 cells did not significantly alter their binding to epithelial cells.

Proteins expressed by bacteria grown in media with different carbon sources. Since EPEC LA is associated with the increased expression of several proteins (17, 49), we investigated whether the different attachment phenotypes observed in our experiments also correlated with the expression of specific proteins. Using a surface biotinylation procedure to label bacterial proteins, we observed an increased expression of 94- and 22-kDa proteins in the lysates of adherent E2348/69 grown in DMEM containing glucose compared to nonadherent E2348/69 or JPN15 grown in medium containing galactose or glucose, respectively (Fig. 1A). Although the nonadherent cultures expressed lower levels of a 94-kDa protein, these levels were visible in all experiments. In contrast, a 22-kDa protein was observed in the lysates of nonadherent strains in only some of our experiments and at levels which were difficult to detect (Fig. 1B, lane 4). Wild-type E2348/69 grown in medium with galactose expressed increased levels of 38- to 41-, 46-, and 68-kDa proteins, which were not expressed when the wild-type strain or its derivative JPN15, lacking the EAF plasmid, was grown in medium containing glucose.

Proteins expressed by EPEC mutants grown in medium containing glucose. Mutants of EPEC isolate E2348/69 which are defective at various stages of attachment to epithelial cells were also biotinylated, and the protein profiles were compared to those of the wild-type strain grown under conditions which induced LA. Both the *cfm* 27-3-2(1) and *eaeA* 10-5-1(1) mutants have been characterized previously. The *cfm* 27-3-2(1) mutant (5, 21, 32, 36) has two chromosomal Tn*phoA* insertions. One insertion occurs in the *sepA* gene of the recently described type III secretory pathway of EPEC. Phenotypically, these bacteria do not initiate signal transduction processes in epithelial cells and have greatly reduced fluorescent actin staining activity (which correlates with AE lesion formation

TABLE 2. Adherence of wild-type EPEC of different serotypes to HEp-2 cells

EPEC serotype	Addition to DMEM	Incubation period (h)[a]	% HEp-2 cells with LA EPEC[b]
O111:H2	Glucose	3	86.4 ± 3.4
	Galactose	8.5	0
O119:H6	Glucose	3	57.0 ± 10.7
	Galactose	8.5	0

[a] Incubation of bacteria in DMEM to approximately mid-log phase before addition to the tissue culture wells.
[b] Means ± standard deviations from three independent experiments.

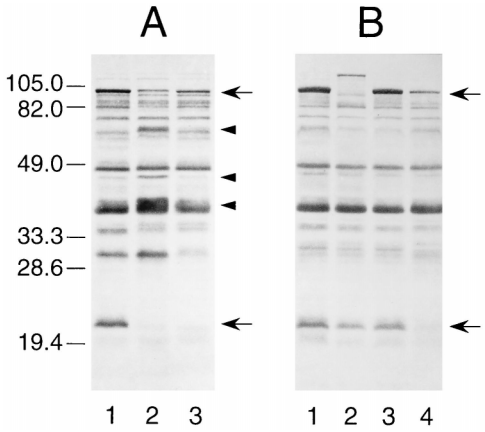

FIG. 1. Analysis of biotinylated EPEC proteins. Mid-log-phase DMEM cultures of wild-type EPEC E2348/69 or mutants of this strain were grown in carbohydrate-supplemented DMEM as described in the text. The bacteria were labeled with biotin, washed to remove unincorporated label, and digested in sample buffer. After separation by SDS-PAGE (12.5% polyacrylamide), the biotinylated proteins were detected by using streptavidin conjugated to alkaline phosphatase. Equivalent amounts of protein were loaded per lane. (A) Lanes: 1, E2348/69 with glucose; 2, E2348/69 with galactose; 3, JPN15 with glucose. (B) Lanes: 1, E2348/69 with glucose; 2, *eaeA* 10-5-1(1) with glucose; 3, *cfm* 27-3-2(1) with glucose; 4, JPN15 with glucose. The mobilities of the prestained molecular size standards (in kilodaltons) are indicated on the left. Proteins expressed at higher levels by E2348/69 with glucose (arrows) and E2348/69 with galactose (triangles) are indicated.

[26]). However, these mutants are still able to attach to epithelial cells in a localized pattern. The *eaeA* 10-5-1(1) mutant (5, 22, 23, 32, 36), on the other hand, has a single Tn*phoA* insertion in the *eaeA* gene, which codes for intimin. Although this mutant is able to attach to epithelial cells in an LA pattern and to generate intracellular messengers in host cells, it is unable to mediate AE lesion formation.

A 94-kDa protein was expressed at higher levels by the LA-positive strains, with the exception of the *eaeA*::Tn*phoA* mutant. This mutant expressed a protein (molecular size of more than 105 kDa) which was not observed in the lysates of the other bacterial strains. Also, there was increased expression of a biotinylated 22-kDa protein in the lysates of all strains which demonstrate the LA phenotype [i.e., E2348/69 and the *cfm* 27-3-2(1) and *eaeA* 10-5-1(1) mutants] (Fig. 1B). A protein with a similar molecular mass was also expressed by the nonadherent (EAF plasmidless) JPN15 strain but at levels much lower than those in adherent bacteria (Fig. 1B).

Effect of carbon source on intimin and BfpA expression. Immunoblotting was performed with wild-type EPEC E2348/69 and JPN15 to determine if the adherence phenotype was associated with the expression of proteins shown previously to be associated with EPEC attachment (15, 17, 38, 49). Overall, the mobilities of proteins detected with anti-intimin or anti-BfpA antibodies were similar regardless of whether the bacteria were biotinylated or not (Fig. 2, lanes 1 to 6). The anti-intimin serum recognized a 94-kDa protein which was expressed at higher levels by adherent bacteria (E2348/69 with glucose) than by nonadherent bacteria (E2348/69 with galactose and JPN15 with glucose). In addition to having the same mobility as intimin, the levels of expression of the biotinylated 94-kDa protein corresponded to the levels of intimin production by the same strains.

Using anti-BfpA antibodies, we observed increased expression of the BFP subunit in the lysates of wild-type E2348/69 grown under glucose-supplemented conditions which promoted attachment. Low levels of the BFP subunit were also detected in the lysates of nonadherent E2348/69 grown in DMEM containing galactose but not in the lysates of JPN15, which lacks the EAF plasmid. Although expression of both BfpA and a biotinylated 22-kDa protein was promoted by glucose, SDS-PAGE revealed a slight difference in the mobilities of these proteins. The molecular size of the protein recognized by BfpA antibodies was calculated to be 20 kDa.

Role for the 22-kDa biotinylated protein in adherence. The slight difference in the molecular sizes of a biotinylated 22-kDa protein and BfpA in some experiments led us to perform additional experiments using the mutant *bfpA* 31-6-1(1) to determine whether expression of the 22-kDa protein was associated with the LA phenotype. The *bfpA* 31-6-1(1) mutant has a single Tn*phoA* insertion in the *bfpA* gene, which codes for the structural subunit of BFP (7). The requirement for additional sequences downstream of the *bfpA* gene to restore the LA phenotype in these mutants, however, suggests that other genes in addition to the *bfpA* gene are affected by this mutation. This mutant was shown previously to be incapable of adhering to epithelial cells in an LA pattern (5). Our results demonstrate that the level of a biotinylated 22-kDa protein expressed by the *bfpA* 31-6-1(1) mutant was lower than that expressed by wild-type E2348/69 and similar to the level observed with the LA-negative strain JPN15 (1, 23) (Fig. 3).

DISCUSSION

The expression of virulence factors by many pathogenic bacteria is environmentally regulated (10, 31, 46). EPEC LA, which is considered to be important for pathogenesis, is an inducible phenotype mediated by specific growth conditions. Previous reports demonstrated that EPEC growth on blood agar plates (17), in tissue culture medium (7, 49), or in the presence of calcium or ammonium (34) regulates the production of BFP or expression of the LA phenotype. Temperature also affects BFP expression (7, 34). In this report, we demonstrate that the carbon source also plays a role in EPEC attachment. Wild-type EPEC grown in DMEM containing glucose

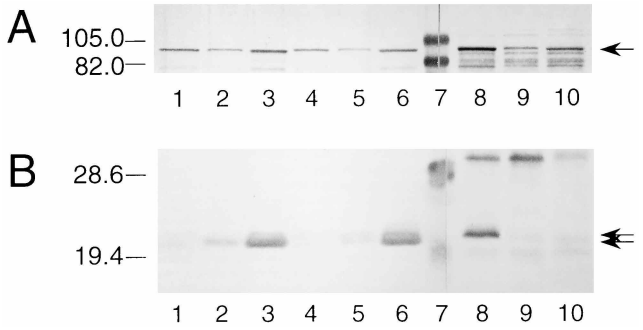

FIG. 2. Alignment of immunoblots with biotinylated proteins of wild-type EPEC E2348/69 and JPN15. Whole-cell lysates of unbiotinylated or biotinylated bacteria were separated by SDS-PAGE (12.5% polyacrylamide) and transferred to Immobilon-P membranes. Proteins were detected with anti-intimin (A) (lanes 1 to 6) or anti-BfpA (B) (lanes 1 to 6) antibodies, and their mobilities were compared to those of biotinylated proteins (lanes 8 to 10). The order of samples loaded in lanes 1 to 3 and lanes 4 to 6 is opposite to the order of samples loaded in lanes 8 to 10 to facilitate comparison of the molecular sizes of proteins. The relative mobilities of the bacterial proteins after realignment of the membranes according to the mobilities of prestained molecular size standards are shown. Lanes: 1, unbiotinylated JPN15 with glucose; 2, unbiotinylated E2348/69 with galactose; 3, unbiotinylated E2348/69 with glucose; 4, biotinylated JPN15 with glucose; 5, biotinylated E2348/69 with galactose; 6, biotinylated E2348/69 with glucose; 7, prestained molecular size standards; 8, biotinylated E2348/69 with glucose; 9, biotinylated E2348/69 with galactose; 10, biotinylated JPN15 with glucose. The mobilities of the prestained molecular size standards (in kilodaltons) are shown on the left. Arrows indicate 94- and 20- to 22-kDa proteins.

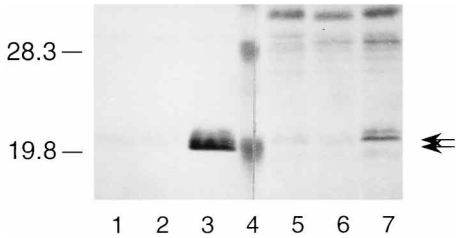

FIG. 3. Association of a 22-kDa biotinylated protein with the LA phenotype. Log-phase bacteria grown in DMEM containing glucose were biotinylated and the cell lysates were separated by SDS-PAGE (12.5% polyacrylamide). After transfer to polyvinylidene difluoride membranes, the biotinylated proteins were detected with either anti-BfpA antibodies (lanes 1 to 3) or streptavidin conjugated to alkaline phosphatase (lanes 5 to 7). Equivalent amounts of protein were loaded per lane. Lanes: 1, JPN15 with glucose; 2, *bfpA* 31-6-1(1) with glucose; 3, E2348/69 with glucose; 4, molecular size standards; 5, JPN15 with glucose; 6, *bfpA* 31-6-1(1) with glucose; 7, E2348/69 with glucose. The mobilities of the prestained molecular size standards (in kilodaltons) are indicated on the left. BfpA and a 22-kDa biotin-labeled protein are indicated by arrows.

adhered to HEp-2 cells, while the attachment of bacteria grown in medium supplemented with galactose was reduced to levels obtained with JPN15, a strain lacking the EAF plasmid which is essentially nonadherent. Expression of the attachment phenotype required the bacteria to be grown in medium containing a specific carbon source, since changing the culture medium prior to incubating the bacteria with the HEp-2 cells did not alter bacterial binding.

An additional factor associated with EPEC virulence is the ability of EPEC to invade epithelial cells (6). Recently, Geyid et al. demonstrated that invasion of wild-type EPEC E2348 (O127:H6) into Caco-2 cells was reduced when the bacteria were grown in LB containing 1% glucose compared to when they were grown in LB alone (16). In their experiments, however, invasion was examined independently from bacterial attachment, since the bacteria were centrifuged onto the epithelial cell monolayers to initiate the invasion process. In view of these results, we repeated our binding assays with DMEM supplemented with 1% glucose to determine if an increased level of glucose in the medium would reduce EPEC attachment to HEp-2 cells. We did not observe a decrease in the LA of EPEC E2348/69 in these experiments compared to that in earlier experiments in which the bacteria were grown in DMEM with 0.45% glucose (data not shown). Because of the differences in the protocols used to investigate attachment and invasion in these experiments, many factors could contribute to the different effects of glucose on EPEC virulence factors, including the presence of other components in the different bacterial growth media (i.e., LB versus DMEM) or the different epithelial cell lines used in the experiments. Alternatively, these results present the interesting possibility that EPEC binding and invasion may be affected differently by the carbon source.

The LA phenotype of EPEC is associated with increased expression of several outer membrane proteins. The best characterized of these proteins are intimin, which is required for intimate attachment (22, 23, 36), and BfpA, which is associated with LA (17, 49). Intimin is a 94-kDa outer membrane protein required for the organization of host cytoskeletal components at sites of bacterial attachment and for the formation of AE lesions. Although intimin is chromosomally encoded, its expression is upregulated by the *per* (plasmid-encoded regulator) region of the EAF plasmid (19). BfpA, on the other hand, is encoded on the EAF plasmid and is associated with the initial binding of EPEC microcolonies to epithelial cells. BfpA is the major structural subunit of BFP and has a predicted molecular size of 18.7 kDa (7, 43).

Using wild-type EPEC strain E2348/69, we found that intimin and BfpA were expressed at higher levels by adherent bacteria than by nonadherent strains (17, 19, 49), which is similar to results reported previously. Furthermore, we observed that the levels of intimin and BfpA expressed by E2348/69 grown in galactose were similar to those observed with JPN15, which lacks the EAF plasmid. Since expression of BFP is required for the LA phenotype (7, 17, 43), these results suggest that the reduced ability of wild-type EPEC grown in DMEM with galactose to attach to HEp-2 cells might also result from lower levels of BfpA expression. Using biotinylation techniques to examine the expression of proteins associated with LA, we identified a 94-kDa protein as intimin based on the similarity of its mobility and relative level of expression by various cultures. Expression of a biotinylated 22-kDa protein was similar to that of BfpA in that it was expressed at higher levels by adherent bacteria than by nonadherent cultures. However, since a slight difference in the mobilities of these proteins was observed in most experiments (Fig. 2B), we are currently performing additional experiments to determine whether these proteins are distinct and, if so, the identity of the 22-kDa protein. Nevertheless, our results indicate that expression of the biotinylated 22-kDa protein is associated with the LA phenotype, since the level of expression of this protein by the *bfpA* 31-6-1(1) mutant was similar to that observed with JPN15.

In addition to the production of BFP (7, 17, 34, 49), protein secretion (20, 24), induction of host tyrosine phosphorylation (24), and AE lesion formation (37) by EPEC are also regulated by growth conditions. The specific mechanisms by which these factors affect the expression of virulence factors, however, has not yet been determined. Overall, these results suggest that the regulation of EPEC virulence factors is likely to be complex and that several environmental parameters may be involved. The observation that EPEC binding is affected by the carbon source provides a model system which can be used investigate the regulation of EPEC attachment. Since colonization of the intestinal mucosa is important for EPEC pathogenesis, a better understanding of the regulation of these processes will assist in the development of potential therapies for EPEC-mediated diarrhea.

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