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Adhesion of an Amylolytic *Arthrobacter* sp. to Starch-Containing Plastic Films

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Cells of the amylolytic bacterium KB-1 (thought to be an *Arthrobacter* sp.) adhered (~70%) to the surface of plastic films composed of starch-poly(methylacrylate) graft copolymer (starch-PMA), but did not adhere (<10%) to films composed of polymethylacrylate (PMA), polyethylene (PE), carboxymethyl cellulose, or a mixture of PE plus poly(ethylene-coacrylic acid) (EAA), starch plus PE, or starch plus PE and EAA. About 30% of the cells adhered to gelatinized insoluble starch. Dithiothreitol (5 mM), EDTA (5 mM), and soluble starch (1%, wt/vol) had little effect on the adhesion of KB-1 cells to starch-PMA films. However, glutaraldehyde-fixed cells, azide-treated cells, and heat-killed cells did not bind to starch-PMA plastic, suggesting that the observed adhesion required cell viability. Culture supernatant from 5-day-old KB-1 cultures contained a proteolytic enzyme that inhibited cell adhesion to starch-PMA plastics. Trypsin-treated KB-1 cells also lost their ability to bind to starch-PMA plastic. When washed free of trypsin and suspended in fresh medium, trypsin-treated bacteria were able to recover adhesion activity in the absence, but not in the presence, of the protein synthesis inhibitor chloramphenicol. These results suggested that adhesion of KB-1 to starch-PMA plastic may be mediated by a cell surface protein. Although KB-1 bacteria bound to starch-PMA plastic, they did not appear to degrade starch in these films. Evidence of starch degradation was observed for starch-PE-EAA plastics, where <10% of the bacteria was bound, suggesting that cell adhesion may not be a prerequisite for degradation of some starch-containing plastics.

Newly developed technologies have made possible the production of plastic materials containing substantial levels of starch in place of more traditional, petroleum-derived polymers. Plastic films have been developed based on starch-based graft copolymers {e.g., starch-poly(methylacrylate) [PMA]} (13), as well as on simple mixtures of conventional hydrocarbon polymers {e.g., polyethylene [PE], poly(ethylene-co-acrylic acid) [EAA]} with either granular (9) or gelatinized (9) starch. In preliminary studies, we observed that cells of a number of known amylolytic bacteria did not bind significantly to starch-containing plastics. Furthermore, commercially available amylases were ineffective in degrading some starch-containing plastics. Recently, we have isolated a number of amylolytic bacteria that are unusually efficient at metabolizing starch in films containing high levels of gelatinized starch (J. M. Gould, S. H. Gordon, L. B. Dexter, and C. L. Swanson, *in* J. E. Glass and G. Swift, ed., *Agricultural and Synthetic Polymers: Utilization and Biodegradability*, Am. Chem. Soc. Sympo. Ser., in press). Bacterial degradation of starch in these films resulted in substantial weight loss and a reduction in tensile strength. Using Fourier transform infrared spectroscopy, we showed that as much as 80% of the starch in some films was metabolized within 25 to 30 days.

Anderson and Salyers (1) recently showed that starch breakdown by *Bacteroides thetaiotaomicron* involves both the starch-binding sites localized on the bacterial outer membrane and the starch-degrading enzymes located in the periplasm of the cell. These investigators suggest that, in *Bacteroides* spp., amylolytic enzymes are not secreted extracellularly and the binding of the starch molecule to the bacterial cell surface appears to be the first step in passing the molecule through the outer membrane into the periplasmic space.

The ability of microorganisms to adhere to the surface of insoluble substrates is critical for their survival in the environment because of intense competition for limited resources. Therefore, the environmental fate of "biodegradable" plastics depends on how successfully amylolytic microorganisms are able to colonize such surfaces. Although soluble amylases may attack starch-plastic films, the ability of amylolytic microorganisms to adhere directly to these substrates should provide an enormous competitive advantage over other, nonadherent amylolytic organisms.

Very little is currently known about the interactions of amylolytic bacteria and starch-containing plastics. For example, it is not known whether intimate contact between the bacterial cell and the film surface is required for starch degradation to occur. In this report, we have examined the ability of a highly amylolytic *Arthrobacter* strain to adhere to and degrade several starch-containing plastic films.

MATERIALS AND METHODS

Chemicals. Unless mentioned otherwise, all chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

Organisms and cultures. The bacterial strain used in this study (KB-1) was originally isolated by Kerr et al., who tentatively identified it as *Arthrobacter* sp. (8). The original isolate was obtained from peanut hulls, and its use to degrade peanut hull lignin has been patented (T. J. Kerr, R. D. Kerr, and R. Benner, U.S. patent 4,643,899, Feb., 1987). Stock cultures were maintained on nutrient agar (Difco Laboratories, Detroit, Mich.). For experimental cultures, cells from the stock culture were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of 5% Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). Cells were allowed to grow for 3 days at 26°C with shaking

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(125 rpm). On day 3, when the culture growth was still in the mid-log phase, 50 to 100 μCi of *trans*-^{35}S label (containing 70% L-[^{35}S]methionine, 15% L-cysteire, 7% L-[^{35}S]methionine sulfide, 3% L-[^{35}S]cysteic acid, and 5% other ^{35}S-labeled compounds; ICN Radiochemicals, Irvine, Calif.) was added to the culture and the cells were allowed to grow overnight. On the following day the labeled cells (mid- to late-log phase) were harvested as described below.

Plastics. The plastic films used in this study were generously provided by C. Swanson and G. Fanta of Plant Polymer Research, Northern Regional Research Center, Peoria, Ill. The starch-PMA graft copolymer films were prepared as described earlier (18). Starch-PE and starch-PE-EAA films were prepared by the semidry method of Otey (9). These plastics were blown into films with a Brabender Plasticorder extruder, as described elsewhere (9).

Adhesion assay. About 100 ml of ^{35}S-labeled cells was harvested by centrifugation (27,000 × *g*, 15 min, 4°C) and washed twice in 50 ml of sterile medium. The final pellet (containing approximately 10^{10} cells and incorporating about 10 to 15% of the total ^{35}S label added to the culture) was suspended in sterile medium, and the volume was adjusted to obtain about 30,000 cpm per 50 μl of cell suspension. Some of this cell suspension was used in experiments the same day. Unused cell suspension was stored at 4°C and washed twice in sterile medium before use within the next 2 days. Cells stored for 2 days at 4°C were viable, and there was little if any leakage (<1%) of labeled material. Disks about 7 mm in diameter were punched from the starch-containing plastic films with a paper punch and then sterilized with a 3% hydrogen peroxide solution followed by extensive washing in sterile distilled water. Each disk was placed flat in the bottom of a 96-well microdilution plate, and a 50-μl portion of the ^{35}S-labeled cells was added. Plates were incubated for 3 h at 37°C in a humid chamber. After incubation, the disks were withdrawn and rinsed by sequential immersion in two test tubes containing 8 ml of sterile media. The disks were then placed into scintillation vials containing 10 ml of Ecolume liquid scintillation fluid (ICN Biomedicals, Inc., Irvine, Calif.) and counted in a liquid scintillation spectrometer. Radioactivity that remained associated with the films was regarded as bound cells. Control experiments indicated <3% cpm quenching by the plastic disks. Experiments were performed in quadruplet and repeated several times. Since preliminary experiments indicated that a pH of 7.4, temperature of 37°C, and incubation time of 3 h were the optimal conditions to obtain maximum adhesion, all subsequent assays were done under these conditions.

Various treatments of KB-1 cells. An appropriate amount of reagent was added to a microcentrifuge tube containing 500 μl of labeled cells. Cells were incubated for 15 min at room temperature (24°C). After incubation, cells were washed twice with sterile media (10,000 x *g*, 5 min; Centra microcentrifuge, IEC) and suspended in fresh media to the original volume. These cells were subsequently used in adhesion assays.

Trypsin treatment of KB-1 cells. A 1-ml amount of KB-1 cells was trypsinized (0.1 mg/ml) for 20 to 30 min and washed three times in a 50-ml volume of sterile medium (27,000 x *g*, 15 min, 4°C; Beckman model J2-21, JA-20 rotor). Cells were suspended in 10 ml of fresh medium and divided into two portions. To one portion 20 μg of chloroamphenicol per ml in 5 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer, pH 7.2, was added. To the other portion only 5 mM PIPES buffer was added to adjust the volume. Cells were incubated for 6 to 7 h, washed two times with sterile medium, and suspended back to the original volume of 0.5 ml. These cells were subsequently used in the adhesion assay described in the text. Cells remained viable after trypsin treatment as well as after trypsin followed by chloramphenicol treatment.

TABLE 1. Adhesion of KB-1 bacteria to plastics

Plastic	% Radioactivity bound (± SD)
Starch-PMA	70 ± 6.4
Starch-PE	6.2 ± 2.8
Starch-PE-EAA	3.0 ± 1.8
Gelatinized cornstarch (see text)	29.0 ± 2.7
PMA	2.8 ± 0.3
PE	8.3 ± 1.4
EAA	4.5 ± 1.3
Carboxymethyl cellulose	5.1 ± 6.2

Scanning electron microscopy. For scanning electron microscopy analysis, portions of the starch-containing plastics were fixed in 1% glutaraldehyde, dehydrated for 10 min each in 50, 75, 90, and 100% ethanol, and mounted on aluminum stubs. Samples were sputter coated with gold-palladium (60:40) and visualized under a scanning electron microscope (International Scientific Instruments model SS-130).

Concentration of culture supernatant. A 100-ml volume of a 5-day-old cell culture (late log phase) was centrifuged (27,000 × *g*, 10 min, 4°C; Beckman model J2-21 centrifuge, JA-20 rotor) to remove the cells. Supernatant was transferred into a dialysis bag (Spectra/por 4, molecular weight cutoff, 12,000 to 14,000; Baxter Scientific Products) and concentrated by placing the dialysis bag on powdered Aquacide II (Calbiochem-Behring, La Jolla, Calif.) overnight at 4°C. Medium was concentrated down to a 10-ml volume and dialyzed against 2 liters of PIPES (Research Organics, Inc., Cleveland, Ohio) buffer, pH 7.4, for about 4 h, and total protein was determined.

The effect of concentrated culture supernatant from KB-1 *Arthrobacter* on its adhesion to a starch-PMA plastic surface was determined as follows. A 400-μl portion of labeled bacteria was first premixed with nonlabeled culture supernatant containing the appropriate concentration of proteins ranging from 0 to 50 μg. The volume was adjusted to 500 μl with cell-free sterile media. Samples were incubated at room temperature (24°C) for 15 min and subsequently used in adhesion experiments as described in the text. Simultaneously, bacteria in control samples were premixed with concentrated culture supernatant that either had been boiled for 5 min or contained a mixture of protease inhibitors (phenylmethylsulfonyl fluoride, 30 μg/ml; pepstatin, 1 mg/ml; apoprotin, 5 mg/ml) before use in adhesion experiments.

RESULTS

When a suspension of KB-1 cells was incubated with starch-PMA plastic film, >70% of the cells bound to the plastic. Only 29% of the cells bound to gelatinized starch, and <10% of the cells bound to starch-PE, starch-PE-EAA, PE, PE-EAA, PMA, or carboxymethyl cellulose films (Table 1). (Gelatinized cornstarch was made as follows. A 0.5-ml portion of 25% soluble starch [CPC International, Englewood Cliffs, N.J.] was dispensed into a 24-well microdilution plate. Gelatinization was accomplished by heating for 10 min in a microwave oven. Plates were cooled overnight at 4°C before use in adhesion assays. At the end of assay, wells

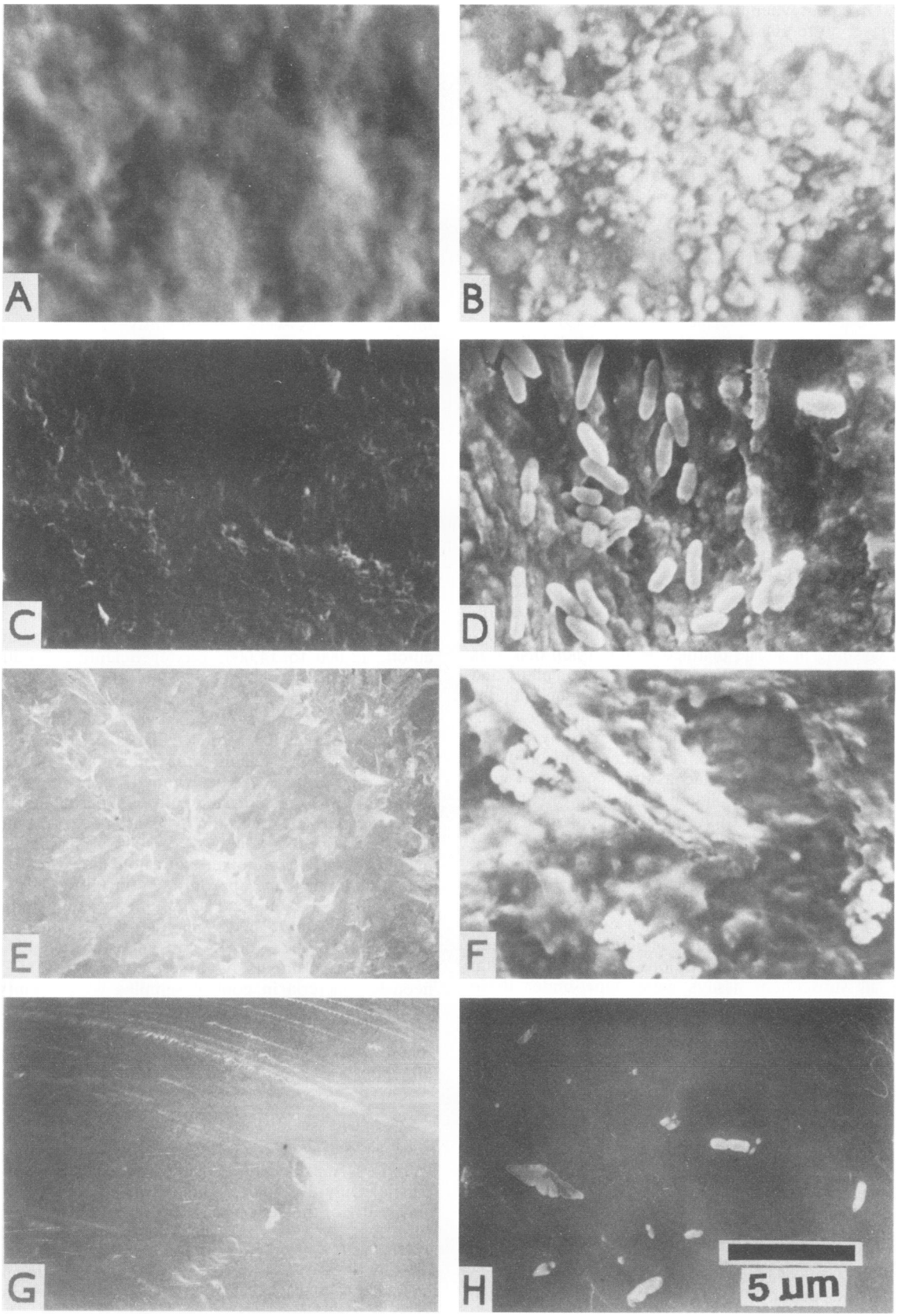

FIG. 1. Scanning electron micrographs showing adhesion of KB-1 *Arthrobacter* to starch-PMA, starch-PE-EAA, and starch-PE plastics and to carboxymethyl cellulose membranes (B, D, F, and H, respectively). Corresponding control surfaces incubated with cell-free sterile media for the same amount of time are shown in panels A, C, E, and G, top to bottom. For experimental details, see Materials and Methods.

TABLE 2. Effect of various additions on adhesion of KB-1 bacteria to starch-PMA plastic

Addition[a]	Concn	% Radioactivity bound (± SD)
Dithiothreitol	5 mM	59.1 ± 4.6
EDTA	5 mM	49.0 ± 6.3
Soluble starch	1% (wt/vol)	54.4 ± 11.1
Control (no addition)		63.0 ± 5.6

[a] An appropriate amount of concentrated stock reagent was added to a microcentrifuge tube containing 500 μl of labeled cells. Cells were incubated for 15 min at room temperature (24°C). These cells were subsequently used in adhesion assays.

were rinsed twice with sterile medium. The gelatinized starch pellet was transferred to a vial containing 10 ml of scintillation fluid and radioactivity was measured.) Examination of the films by scanning electron microscopy after exposure to KB-1 cell suspensions verified that many more cells adhered to the starch-PMA films than to any of the other films tested (Fig. 1). Cells were not observed adhering to films incubated for the same length of time in cell-free (sterile) medium.

The ability of KB-1 cells to attach to starch-PMA plastic film was not significantly altered when 5 mM dithiothreitol, 5 mM EDTA, or 1% soluble starch was present in the attachment assay medium (Table 2).

The attachment of KB-1 cells to starch-PMA films was also unaffected by treatment of the cells with anionic, cationic, or nonionic detergents prior to their introduction into the attachment assay medium (Table 3), indicating that the molecules involved in cell adhesion were not disrupted by these detergents. However, cells treated with heat, glutaraldehyde, or azide prior to their use in the attachment assay showed reduced binding to starch-PMA films, suggesting that adhesion may be an energy-requiring process.

The adhesion of KB-1 cells to starch-PMA films was also inhibited when a concentrated fraction of cell-free culture supernatant from a 5-day-old KB-1 culture was added to the adhesion assay medium (Fig. 2). Compared with control samples, cell adhesion was inhibited 60 to 70% when concentrated supernatant containing 25 μg of protein per ml was added to the incubation mixture. The inhibitory effect of the

TABLE 3. Effect of various treatments of KB-1 bacteria on its adhesion to starch-PMA plastic films

Treatment	Concn (%) (wt/vol)	% Radioactivity bound (± SD)
Anionic		
Sodium dodecyl sulfate	0.01	68.0 ± 2.1
Cationic		
Tetradecyltrimethyl-ammonium bromide	0.01	71.2 ± 2.3
Nonionic		
Triton X-100	0.01	72.4 ± 4.7
Glutaraldehyde	1.0	15.6 ± 1.3
3-min heat inactivated		8.1 ± 2.9
Sodium azide	0.02	3.6 ± 1.0
Control (untreated)		71.0 ± 2.5

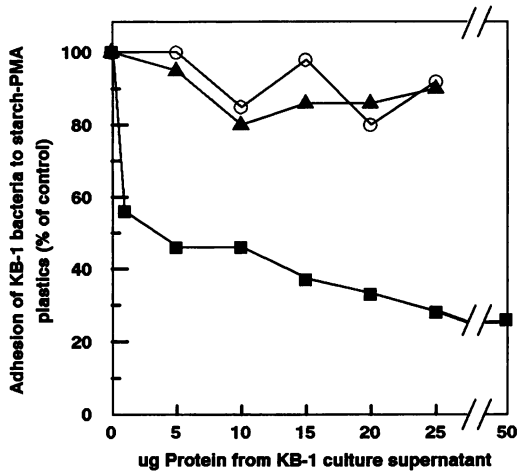

FIG. 2. Effect of concentrated culture supernatant from *Arthrobacter* KB-1 on its adhesion to starch-PMA plastic surface. Symbols: Culture supernatant (■), boiled culture supernatant (▲), and culture supernatant to which protease inhibitors were added (○).

culture supernatant on the attachment of KB-1 cells to starch-PMA plastic was lost when the supernatant was boiled or when protease inhibitors were added, suggesting that the inhibition of cell attachment to the films was the result of proteolytic activity in the growth medium. Some amylolytic bacteria are known to secrete proteolytic enzymes (7).

Treatment of ^{35}S-labeled KB-1 cells with trypsin released only 3% of the total counts into the cell-free supernatant, but significantly ($P \leq 0.01$) inhibited adhesion of the cells to starch-PMA films (Table 4). When trypsin-treated KB-1 cells were washed free of trypsin, suspended in fresh medium, and allowed to grow for 6 to 7 h, the cells recovered their ability to adhere to starch-PMA plastic film (Table 4). When trypsin-treated KB-1 cells were washed and allowed to grow for 6 to 7 h in the presence of the protein synthesis inhibitor chloramphenicol, the cells did not recovery their ability to bind to starch-PMA plastic film (Table 4). Cells remained viable after chloramphenicol treatment. Also, treatments of cells with chloramphenicol alone did not substantially reduce adhesion (Table 4). All of the data presented above suggest that adhesion of KB-1 cells to starch-PMA plastic film is mediated by cell surface proteins.

It has been reported that starch-PMA films showed excellent susceptibility to fungal growth, some samples losing more than 40% of their weight after 22 days of incubation (5). KB-1 cells also secrete amylase activity into their culture medium (unpublished results). Although KB-1 cells bound

TABLE 4. Effect of trypsinized KB-1 bacteria on its adhesion to starch-PMA plastic films and effect of protein synthesis inhibitor on recovery of adhesion activity

Treatment of cells	% Radioactivity bound (± SD)
Control (untreated)	70.0 ± 6.4
Trypsin treated (0.1 mg/ml)	23.3 ± 2.2
Trypsin treated, incubated in fresh medium after trypsin treatment	68 ± 4.9
Trypsin treated, incubated in medium containing chloramphenicol after trypsin treatment	10.0 ± 2.9
Treated with chloramphenicol alone	65.3 ± 39

more readily to starch-PMA plastic films than to other plastic films tested, there was little degradation of the starch-PMA films apparent under the scanning electron microscope, even after 60 days (data not shown). Interestingly, there was obvious degradation of starch-PE-EAA films incubated with KB-1 cells, even though there was little direct adhesion of the cells to the film (data not shown). After 60 to 75 days of incubation with KB-1 cells, starch-PE-EAA plastic films physically disintegrated into smaller pieces. These data suggest that direct adhesion of KB-1 cells to the starch-plastic films was not required for degradation of one or more film components to occur.

DISCUSSION

Adhesion of cells to insoluble surfaces is characteristic of a wide variety of biological systems (3, 4, 6, 10, 12) and plays an important role in influencing or regulating cell growth and development (2, 11) as well as other cellular activities (8, 20). The adhesion of KB-1 cells to the surface of starch-PMA plastic film appears to be an energy-requiring process mediated by cell surface proteins. Furthermore, results indicated that molecules involved in the process of cell adhesion cannot be disrupted by anionic, cationic, and nonionic detergents. Data clearly showed that KB-1 adhesion to starch-plastic required live cells. Also, the presence of EDTA and dithiothreitol did not affect the adhesion process, suggesting that adhesion of KB-1 bacteria to starch-plastic is not a divalent cation-dependent process and that disulfide linkages do not play any apparent role in the adhesion process. That soluble starch did not inhibit the adhesion by blocking the receptor sites on the bacterial cell surface suggests that adhesion of KB-1 bacteria to starch-plastic may not be a simple process. On the other hand, it may be possible that bacteria only recognize the adhesion sites on insoluble starch. This possibility, however, needs to be investigated.

During manufacture of starch-containing plastics, complexation of starch with other components such as PMA, PE, or EAA can affect starch localization within the film and thereby influence its accessibility to an aqueous environment. It is reasonable to conclude that the way in which starch is incorporated into the plastic matrix can influence the ability of amylolytic bacteria to attach to the plastic's surface. This effect is evident in the data presented in Table 1; KB-1 cells preferentially bound to starch-PMA plastic compared with starch-PE, starch-PE-EAA plastics, or gelatinized starch with no plastic components. That KB-1 cells did not adhere to PMA films lacking starch suggests that the starch moiety is the site of cell attachment.

Incorporation of starch into a hydrophobic plastic matrix can also affect the availability of the starch to hydrolytic enzymes. That KB-1 cells adhered to but did not degrade starch-PMA films and degraded but did not adhere to starch-EAA films, suggests that, although more adhesion sites are available in the starch-PMA plastic formulation, the sites sensitive to enzymatic attack are more accessible in the starch-PE-EAA plastic formulation. While we do not know much about the nature of the enzyme-sensitive sites or adhesion sites on the starch granule surface, it appears that adhesion of KB-1 bacteria to starch-PMA film involves a trypsin-sensitive surface protein or proteins that are also sensitive to proteolytic enzymes secreted by KB-1 itself. Teramoto et al. (14) reported that raw starch-digesting α-amylase of *Bacillus subtilis* 65, upon pronase treatment, lost its ability to digest raw starch but retained hydrolyzing ability toward low-molecular-weight substrates. These studies suggest that raw starch-digestive sites on the α-amylase enzyme molecule are localized in the specific region sensitive to pronase.

The fact that adhesion of KB-1 cells to starch-containing plastic films is not prerequisite for enzymatic degradation of the films suggests that starch-hydrolyzing enzymes that are secreted by the bacteria or released upon cell lysis are absorbed onto the film surface. These results also suggest that adhesion to and degradation of starch are probably mediated by different molecules.

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