JOURNAL OF BACTERIOLOGY, June 1990, p. 3379–3387
0021-9193/90/063379-09$02.00/0
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Vol. 172, No. 6

Surface Proteins of the Gliding Bacterium *Cytophaga* sp. Strain U67 and Its Mutants Defective in Adhesion and Motility

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Received 28 September 1989/Accepted 15 March 1990

Surface proteins of the gliding bacterium *Cytophaga* sp. strain U67 that make contact with glass substrata were radioiodinated, using a substratum-immobilized catalyst (Iodo-Gen). At least 15 polypeptides were iodinated, fewer than the number labeled by surface biotinylation of whole cells; these polypeptides define the set of possible candidates for the surface protein(s) that mediates gliding-associated substratum adhesion. The labeling of three adhesion-defective mutants exhibited two characteristic patterns of surface iodination which involved addition, loss, or alteration of several polypeptides of high molecular weight. An adhesion-competent revertant of mutant Adh3 and one of Adh2 exhibited the wild-type labeling pattern. Two other Adh2 revertants resembled their adhesion-defective parent. The labeling pattern of surface polypeptides of a nongliding but adhesive cell strain was similar to that of the wild type.

Gliding bacteria demonstrate translocational and other movements when in contact with a substratum. These semirigid rods and trichomes have no obvious external locomotory organelles. Although a variety of mechanistic theories of gliding motility have been proposed, there is no unequivocal evidence for any one model (5, 6, 9, 25).

Cytophaga sp. strain U67, originally described by Henrichsen (15), adheres to glass substrata (7) on which the bacteria glide, flip, and pivot. Like other *Cytophagaceae*, they also actively propel adherent microscopic particles along the cell surface (20, 26). One hypothesis proposed to account for the characteristic movements of this bacterium is that substratum adsorption sites are actively moved within the fluid bacterial outer membrane along tracks fixed to the peptidoglycan layer (20). Although there is currently no direct evidence for this model, gliding motility does, nevertheless, require contact with a substratum. Thus, it is likely that there exists a class of macromolecules that are exposed at the cell surface and maintain cell contact with the substratum during gliding. Some subset of these surface components may also be responsible for transmission of the force generated within the cell envelope that results in translocation of the cell on the substratum.

To identify candidates for such surface components, we have used a glass-immobilized iodination catalyst to label only those cell surface-exposed polypeptides that make contact with the substratum. The array of polypeptides identified by this labeling procedure was compared with those accessible to biotinylation, which should label all surface-exposed polypeptides regardless of their ability to interact with the substratum. For example, if the bacterial surface were characterized by topological uneveness, only the "hills" would be labeled by the immobilized catalyst; both the "hills" and "valleys" would be biotinylated. Alternatively, polypeptides with a large extracellular domain that protrude some distance from the cell surface may be accessible to both iodination and biotinylation, while those polypeptides with a more restricted extracellular domains may not be able to make contact with a glass-immobilized iodination catalyst. The latter species would only be accessible for biotinylation.

To define further the subset of surface polypeptides that may mediate adhesion and, possibly, gliding motility, we have compared the labeling patterns of *Cytophaga* sp. strain U67 with those of several of its mutants defective in adhesion or motility or both and their revertants. Iodinated polypeptides that are missing or altered in the mutant strains become candidates for components that may mediate adhesion or gliding motility or both.

MATERIALS AND METHODS

Bacterial strains. *Cytophaga* sp. strain U67 was originally provided by J. Henrichsen (15). The adhesion-defective mutants Adh1, -2, and -3 were isolated by enrichment for UV light-mutagenized cells that remained in the aqueous phase after mixing the culture with an equal volume of hexadecane (bacterial adherence to hydrocarbon protocol of Rosenberg [31]). Ngl6 is a spontaneous, nongliding but normally adhesive mutant of U67 that was distinguished from the wild type by its colony form: convex with an entire margin. Its colonies were similar in appearance to those of Adh2 and Adh3. Revertants of Adh2, Adh3, and Ngl6 were isolated from flares of gliding cells extending from the periphery of mutant colonies. We have thus far been unable to isolate revertants of Adh1.

Culture conditions. A total of 4×10^5 bacteria in 0.5 ml of C62 medium (0.05% tryptone plus 0.05% yeast extract, pH 7.0 [15]) broth were inoculated onto C62 agar (1.5%) plates and incubated at 25°C for 18 h, at which time each plate yielded approximately 2.5×10^9 bacteria.

Adhesion assay. Bacteria remaining in a Petroff-Hausser counting chamber after flushing out cells that were nonadherent were quantitated. Details of this method are presented in McGrath et al. (24).

Iodo-Gen-catalyzed iodination. The bacteria were harvested in cold 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-[1-ethanesulfonic acid]) buffer, pH 7.0, containing 1 mM $MgSO_4$ (HM buffer) and checked for adhesion and motility in wet mounts by phase-contrast microscopy. The cells were centrifuged at 10,000 × *g* and suspended in HM buffer to a density of 1.2×10^9/ml based on A_{540} of 0.1 being equivalent

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to 10^8 bacteria per ml. A 2-ml portion of cell suspension was placed into each of two scintillation vials that had been derivatized with 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril (Iodo-Gen; Pierce Chemical Co., Rockford, Ill.) by the method of Sullivan and Williams (34). This glass-bound, nonenzymatic catalyst covalently links electrophilic iodine species with aromatic amino acids, tyrosine being the primary site of iodination. The vials were prepared 1 day before the experiment and stored under N_2. Just prior to use, the vials were rinsed seven times with HM buffer.

At time zero, 0.1 mCi of [^{125}I]sodium iodide (specific activity, 13 to 17 mCi/μg; Amersham Corp., Arlington Heights, Ill.) was added to each vial. The vials were incubated for 10 min at room temperature with cycles of agitation (120 rpm, 30 s) interspersed with stationary periods (30 s). Adhesion, motility, and viability were unaffected by this iodination procedure. The reaction was stopped by the addition of 8 ml of 10 mM NaI in HM buffer, after which the bacteria were centrifuged and washed twice with HM buffer at 0°C.

In one control experiment, cells were incubated in the presence of [^{125}I]sodium iodide in an untreated vial. To assure that the iodination catalyst was not removed from the vial surface, bacteria were also incubated for 10 min in an Iodo-Gen-derivatized vial in the absence of the [^{125}I]sodium iodide, after which the suspension was transferred to an untreated vial. [^{125}I]sodium iodide was then added, and the suspension was incubated with cycles of agitation for an additional 10 min in the untreated vial.

Biotinylation. Bacteria were grown and prepared identically to those that were used for iodination. A total of 3×10^9 cells in 4 ml of HM buffer were placed into each untreated scintillation vial to which were added 0.45 ml of a freshly prepared solution of *N*-hydroxysuccinimide ester of biotin (NHS-Biotin; Pierce Chemical Co.) at 10 mg/ml in HM buffer. NHS-Biotin reacts with 1° amines to form amide bonds. All polypeptides exposed at the bacterial surface should be biotinylated. After 10 min of incubation with shaking at room temperature, the bacteria were centrifuged at $10^4 \times g$ and washed once with HM buffer. Biotinylation under these conditions did not affect adhesion, motility, or viability of the cells.

Cell envelopes. The bacteria were washed in HM buffer, suspended in this buffer with the addition of DNase and RNase, both at 100 μg/ml, and disrupted with a French pressure cell at 10,000 lb/in^2. Intact cells were removed by centrifugation at 5,000 × *g* for 10 min. The supernatant fraction was then centrifuged at 10,000 × *g* for 1 h. The cell envelope pellet was suspended in HM buffer and recentrifuged at 100,000 × *g* for 1 h. The entire preparation was carried out at 0 to 4°C.

Preparation of cells for electrophoresis. After iodinated or biotinylated cells were washed twice with HM buffer, they were suspended in a small volume of 0.0625 M Tris hydrochloride, pH 6.8, and mixed with an equal volume of 2× Laemmli sample buffer (19). The samples were boiled for 2 min, cooled, and centrifuged at 15,600 × *g* for 5 min in an Eppendorf Microfuge, model 5412. The supernatant was removed and frozen for subsequent electrophoretic analysis.

SDS-PAGE. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure of Laemmli (19) as modified by Jarvik and Rosenbaum (17) was used. Several types of slab gels were utilized in this study: 4 to 16% and 8 to 16% acrylamide gradients (containing 3 to 6% urea gradient) and uniform 6 and 10% acrylamide gels containing no urea. Gels were loaded for equal numbers of radioactive counts and stained for protein, using Coomassie brilliant blue (for wet acrylamide gels) or amido black (for nitrocellulose blots). Autoradiograms were prepared by exposing dried gels to Kodak XRP X-ray film, usually in conjunction with a Cronex intensifying screen (Dupont).

For detection of biotinylated polypeptides, polyacrylamide gels were electroblotted onto nitrocellulose paper (BA83; Schleicher & Schuell, Keene, N.H.) by the method of Towbin et al. (35). The nitrocellulose paper was gently shaken for at least 1 h at room temperature in phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% Tween 20. They were then incubated in 0.5 μg of horseradish peroxidase-conjugated avidin D (Vector Laboratories) per ml in phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% Tween 20. The nitrocellulose was then washed three times in the same buffer and incubated in 1 mM 3,3′-diaminobenzidine in 100 mM Tris hydrochloride, pH 7.1, containing 0.03% H_2O_2. After sufficient color development, the reaction was terminated by placing the blot into distilled water.

Acrylamide gels, X-ray films, and nitrocellulose blots were photographed on Kodak Pan X 35-mm film, using a red filter for the Coomassie blue-stained gels and amido black-stained nitrocellulose blots and a blue filter for the horseradish peroxidase reaction on nitrocellulose blots of biotinylated proteins.

RESULTS

Iodination and biotinylation of cell surface polypeptides. To identify polypeptides on the cell surface that make direct contact with glass surfaces on which *Cytophaga* sp. strain U67 adheres and glides, we radioiodinated those bacterial polypeptides that interact with a glass-immobilized iodination catalyst (Iodo-Gen). *Cytophaga* sp. strain U67 cells incubated in Iodo-Gen-derivatized glass vials in the presence of [^{125}I]sodium iodide retained their viability and their ability to adhere to and glide along the surface of glass microscope slides. Furthermore, untreated bacteria glided normally on slides coated with Iodo-Gen under conditions identical to those used for preparing the vials. It is, therefore, assumed that the bacterial cells adhered to and exhibited gliding motility along the surface of the derivatized glass vial during the course of labeling.

After 10 min of exposure to the iodination conditions, polypeptides from labeled bacteria were separated by SDS-PAGE and stained with Coomassie blue. The gels were subsequently dried, and autoradiography was performed to identify labeled proteins. At least 15 *Cytophaga* sp. strain U67 polypeptides, ranging in molecular weight from ~16,000 to 120,000, were labeled with immobilized Iodo-Gen (Fig. 1B, lanes c and k). To visualize iodinated proteins to higher molecular weight, a 6% uniform gel was prepared. Long exposure of this gel to X-ray film revealed a diffuse band of ~270 kilodaltons (kDa) (Fig. 2B, lanes c and k) with no obvious Coomassie blue-stained counterpart (Fig. 2A, lanes c and k; Table 1). This ~270-kDa polypeptide was not labeled by biotinylation of whole *Cytophaga* sp. strain U67 cells, a protocol designed to identify all cell surface-exposed polypeptides, including those not in a topological position to contact a solid substratum (Fig. 3, lanes a and b). The absence of staining with Coomassie blue or biotinylation could reflect high carbohydrate content (i.e., a glycoprotein), low abundance, or a polydisperse nature of this ~270-kDa protein.

In some preparations, several of the Iodo-Gen-labeled

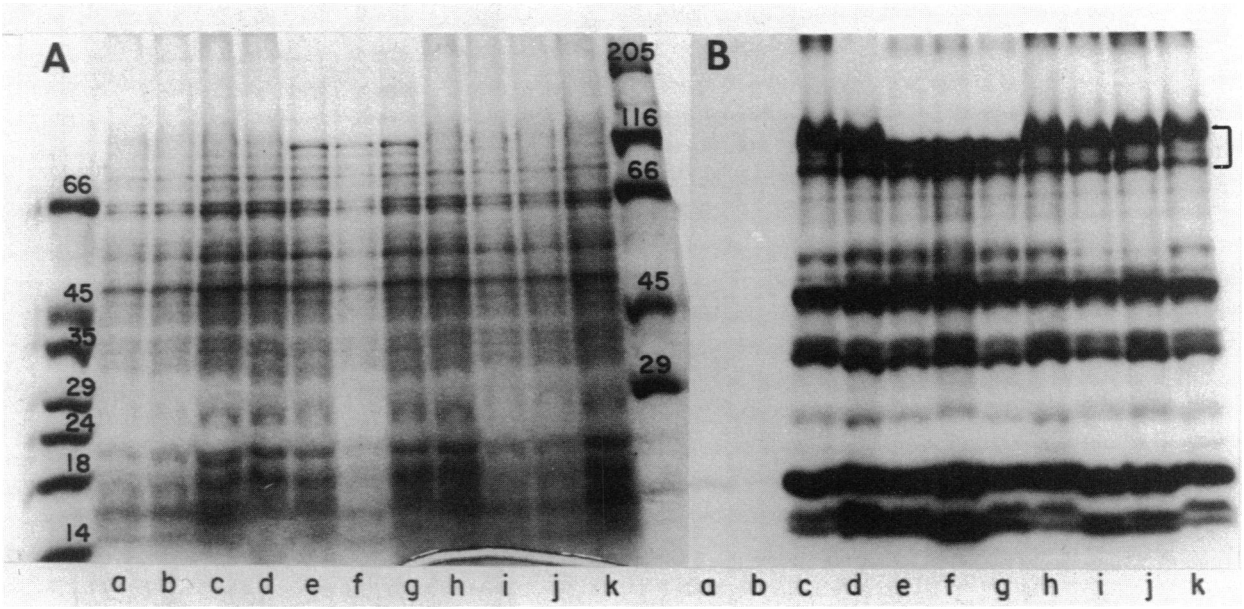

FIG. 1. SDS-polyacrylamide slab gel (8 to 16% gradient) of whole-cell polypeptides of *Cytophaga* sp. strain U67 and its adhesion and motility mutants that were surface labeled with immobilized Iodo-Gen and [^{125}I]sodium iodide. (A) Coomassie blue-stained gel. (B) Autoradiogram. Lanes: a, *Cytophaga* sp. strain U67, control in which cells were sequentially exposed to Iodo-Gen and then [^{125}I]sodium iodide; b, *Cytophaga* sp. strain U67, control in which cells were exposed only to [^{125}I]sodium iodide (no Iodo-Gen); c, *Cytophaga* sp. strain U67; d, Adh1; e, Adh2; f, Adh2Rev1; g, Adh3; h, Adh3Rev; i, Ngl6; j, Ngl6Rev; k, U67. Bracket marks the 95-, 107-, and 113-kDa polypeptides of the signature triplet. Molecular weight markers (10^3) are indicated in panel A.

polypeptides of *Cytophaga* sp. strain U67 were weakly iodinated in a control experiment in which the bacteria were incubated in an Iodo-Gen-treated vial in the absence of ^{125}I before being transferred to an untreated vial to which isotope was added (Fig. 2B, lane a). In other experiments, almost no labeling was detected in this control (Fig. 1B, lane a). Bacteria exposed to [^{125}I]sodium iodide in the absence of Iodo-Gen (Fig. 1B and 2B, lanes b) demonstrated no labeled polypeptides.

In general, the electrophoretic pattern of biotinylated polypeptides (Fig. 3B and 4, lanes n and o) was similar to those of the iodinated polypeptides. The iodinated polypeptides appeared to comprise a subset of those that were biotinylated. This was best seen when polypeptides from iodinated whole bacteria and biotinylated whole bacteria were compared on the same slab gel. In Fig. 4, iodinated *Cytophaga* sp. strain U67 (lane k) and strain Adh3Rev (lane m; see below), whose labeling pattern was always identical to that of the wild type (Figs. 1, 2, and 3), were compared with biotinylated Adh3Rev (lane o). All of the ^{125}I-labeled *Cytophaga* sp. strain U67 polypeptides appeared to have biotinylated counterparts. In addition, there were a number of biotinylated species that did not appear to have ^{125}I-labeled counterparts.

It was expected that all of the iodinated polypeptides would be localized in the bacterial outer membrane. In the absence of a procedure for isolation of a pure outer membrane fraction from *Cytophaga* sp. strain U67, we examined polypeptides from crude cell envelope fractions for their correspondence with iodinated polypeptides from whole bacteria. Cell envelope polypeptides constituted a subset of the total resolvable polypeptides from whole *Cytophaga* sp. strain U67 cells (Fig. 4, lanes a and e). Most of the major iodinated polypeptides (lane k) had corresponding bands in the cell envelope preparation. However, the relative levels of polypeptide iodination did not generally correspond to their relative amounts as judged by the intensity of Coomassie blue staining. This indicates that not all of the iodinatable polypeptides were equally accessible to surface contact and hence surface iodination and/or they differed in their content of tyrosine in the region of the polypeptide making contact with the glass substratum. This effect was particularly noticeable in a triplet of polypeptides of approximate molecular weights 95,000, 107,000, and 113,000 (Fig. 1 and 4). The 95-, and 113-kDa species were heavily iodinated in whole cells (lane k) relative to their representation in the envelope preparation (lane a). In contrast, the ~107-kDa polypeptide was abundant in the cell envelope preparation but was weakly iodinated in whole cells. This signature triplet was reproducibly observed in all of our gels. However, based on comparison with the positions of the molecular weight standard polypeptides, one or more of the signature polypeptides sometimes appeared to migrate at a somewhat different molecular weight than that assigned to it in this paper. For example, in Fig. 1 (lanes c and k), they ran as though their molecular weights were ~89,000, 94,000, and 100,000.

Several polypeptides in the cell envelope fraction had no corresponding iodinated species (e.g., Fig. 4, lanes a and k). This would be expected, as it is unlikely that all envelope polypeptides would contact the substratum. Other polypeptides were iodinated but appeared to lack counterparts in the cell envelope preparation (Fig. 4, lane k). These may be periplasmic proteins that entered the soluble fraction of total cell proteins after disruption of the cells with a French pressure cell. In fact, a number of polypeptides are present among those solubilized during treatment of whole cells with 1 mM EDTA (data not presented), a procedure that releases from *Cytophaga* sp. strain U67 proteins that may be localized in the periplasm (6).

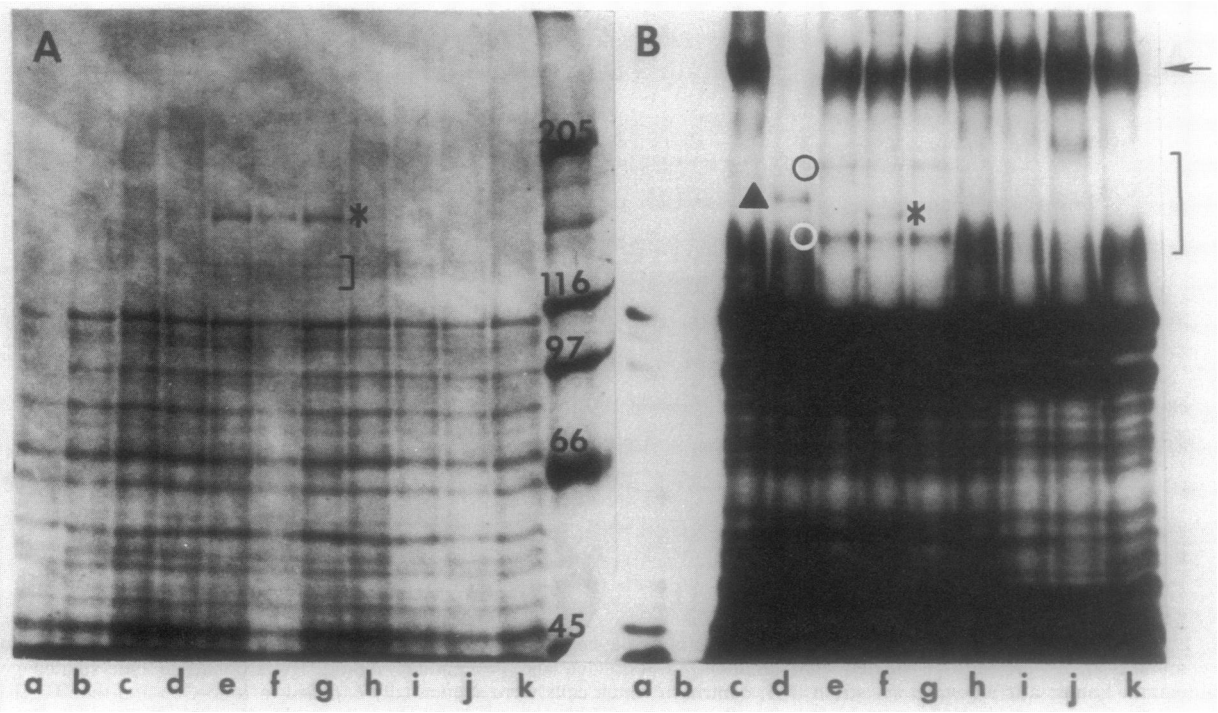

FIG. 2. SDS-polyacrylamide slab gel (uniform 6%) of whole-cell polypeptides of *Cytophaga* sp. strain U67 and its adhesion and motility mutants that were surface labeled with immobilized Iodo-Gen and [^{125}I]sodium iodide. (A) Coomassie blue-stained gel. (B) Autoradiogram. Lanes: a, *Cytophaga* sp. strain U67, control in which cells were sequentially exposed to Iodo-Gen and then [^{125}I]sodium iodide; b, *Cytophaga* sp. strain U67, control in which cells were exposed only to [^{125}I]sodium iodide (no Iodo-Gen); c, *Cytophaga* sp. strain U67; d, Adh1; e, Adh2; f, Adh2Rev1; g, Adh3; h, Adh3Rev; i, Ngl6; j, Ngl6Rev; k, U67. Molecular weight markers (10^3) are indicated in panel A. Arrow in panel B marks the ~270-kDa polypeptide species described in the text. The bracket marks the high-molecular-weight polypeptides that distinguish the mutants from U67 (i.e., 150-, 160-, 170-, and 190-KDa polypeptides are denoted by a white circle, star, triangle, and black circle, respectively).

Adhesion mutants. One approach for identifying those bacterial surface polypeptides that mediate adhesion to substrata is to isolate adhesion-defective mutants and compare their pattern of iodinated surface polypeptides with that of the wild-type parent. Nonadhesive mutants are predicted to lack one or more labeled bands characteristic of the wild type. We isolated such mutants by using the bacterial adherence to hydrocarbon procedure (31), serially enriching for bacteria that remained in the aqueous phase after partitioning between hexadecane and culture medium. Since nonadherent or poorly adherent bacteria would be unlikely to be able to glide, even though the machinery for gliding may be functional, we selected for colonies on C62 agar with a nongliding phenotype by visual screening. On this medium of low nutrient concentration, colonies of nonadherent and nongliding bacteria are convex with entire margins, whereas those produced by wild-type bacteria are flat and spreading.

TABLE 1. Total cell and Iodo-Gen-labeled polypeptides that distinguish *Cytophaga* sp. strain U67 and its adhesion mutants

Polypeptide (kDa)	U67	Adh1	Adh2	Adh2Rev1	Adh3	Adh3Rev
~270	/+[a]		/+	/+	/+	/+
190			/+	/±	/+	
180				/+		
170		/+				
160			+/	±/	+/	
150			/+	/±	/+	
135, 128		±/	+/	±/	+/	±/
95, 107, 113	+/+	+/+				+/+
92, 103, 108			+/+	+/+	+/+	
86	+/±	+/?	+/+	+/+	+/+	+/±

[a] Coomassie blue stained; ^{125}I labeled by Iodo-Gen. +, Present; ±, weakly detectable.

Two of the mutants, Adh2 and Adh3, demonstrated no detectable adhesion to glass (Table 2). They were unstable, reverting to the gliding, adhesion-competent phenotype at high frequency. Revertants were detected as flares of gliding cells that emerged from the periphery of colonies after 2 or more days of incubation. Two of these revertants, Adh2Rev1 and Adh3Rev, demonstrated a degree of adhesion to glass similar to that of *Cytophaga* sp. strain U67, the wild-type strain (Table 2). Mutant Adh1 demonstrated a level of adhesion intermediate between the wild type and the other adhesion-defective mutants. Adh1 and U67 cells adhered to one another when packed by centrifugation at 10,000 × *g*, demonstrable by the cohesiveness of pellets of both strains. Adh2 and Adh3 pellets "ran" under the same conditions. Adh1 colonies were lobate and spread more than those of Adh2 and Adh3. We have not succeeded in isolating revertants of Adh1.

The SDS-PAGE pattern of Coomassie blue-stained, whole-cell polypeptides from the stable mutant Adh1 was indistinguishable from that of the wild type (Fig. 1A and 2A, lanes c & d, respectively). However, autoradiograms distinguished the two strains in that Adh1 demonstrated a unique iodinated polypeptide at ~170 kDa and lacked the diffuse ~270-kDa band (Fig. 2B) that was present in all of the other strains examined (Fig. 2B, lane d). Neither of these polypep-

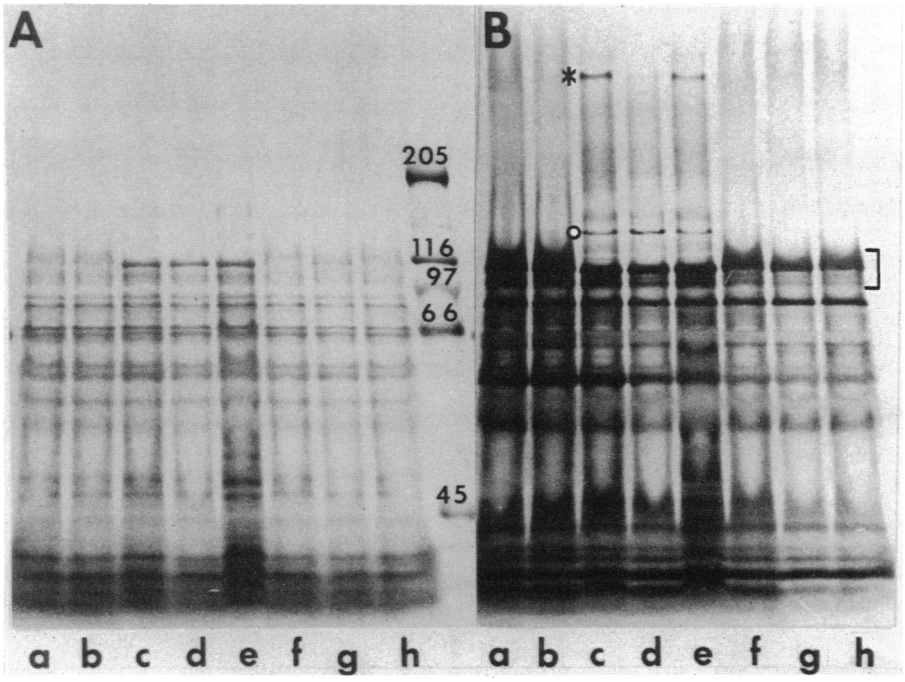

FIG. 3. Whole-cell polypeptides from *Cytophaga* sp. strain U67 and its adhesion and motility mutants. Whole live cells were biotinylated as described in the text, and polypeptides were separated by SDS-PAGE (4 to 16% acrylamide gradient) and electroblotted onto nitrocellulose. Blots were treated with horseradish peroxidase-avidin D and visualized by using 3,3'-diaminobenzidine as substrate. (A) Amido black-stained nitrocellulose. (B) Same gel after reaction to visualize biotinylated proteins. Lanes: a and b, *Cytophaga* sp. strain U67; c, Adh2; d, Adh2Rev1; e, Adh3; f, Adh3Rev; g, Ngl6; h, Ngl6Rev. Bracket marks the signature polypeptide triplet. Polypeptides of 150 and ~450 kDa are denoted by a circle and star, respectively.

tides was detected in Coomassie blue-stained gels. A summary of polypeptide differences among the strains is presented in Table 1.

Polypeptides of the adhesion-defective mutants Adh2 and Adh3 both resembled the wild-type patterns on Coomassie blue-stained gels with a few exceptions. Adh2 and Adh3 had a ~160-kDa polypeptide that was absent in the wild-type cells (Fig. 2A, lanes e and g). In addition, 128- and 135-kDa polypeptides in Adh2 and Adh3 were relatively more abundant compared with the other strains (Fig. 2A, lanes e and g). In most of our gels, the previously mentioned signature triplet of polypeptides of molecular weights ~95,000, 107,000, and 113,000 was shifted or replaced by another signature triplet of approximate molecular weights 92,000, 103,000, and 108,000 (Fig. 4, lanes f and h). However, this shift was not readily detected in all gels (for instance, Fig. 2A, lanes e and g), presumably due to variations in electrophoretic conditions.

Several differences in the pattern of iodinated polypeptides distinguished Adh2 and Adh3 from *Cytophaga* sp. strain U67. The ~92- to 108-kDa signature triplet observed in Coomassie blue-stained gels was iodinated (Fig. 1B, lanes e and g). This can also be seen in Fig. 4 (lane 1) and Fig. 5B (lanes b and d). The shift in mobility of the signature triplet was also evident in biotinylated cells of both mutants (Fig. 3B, lanes c and e). The middle band of the signature triplet in the wild-type strain and at least one of the adhesion-defective mutants (Adh3) was a major cell envelope protein (Fig. 4, lanes a and b). In contrast, the upper and lower bands of the triplet were more heavily iodinated than the middle band (e.g., Fig. 4, lanes k and l) relative to their actual abundance as determined by their level of Coomassie blue staining in the envelope preparation. Adh2 and Adh3 also demonstrated iodinated ~190- and 150-kDa polypeptides, neither of which was observed on Coomassie blue-stained gels (Fig. 2B, lanes e and g); the ~150-kDa polypeptide was also biotinylated (Fig. 3B, lanes c and e). Both of the adhesion-defective mutants carried on their surfaces a ~450-kDa biotinylated polypeptide that was poorly biotinylated in the wild type (Fig. 3B, lanes c and e). Since this polypeptide was not visible in the Coomassie blue-stained gels, it was not possible to determine whether it differed in amount or accessibility to biotinylation between the wild-type and the adhesion-defective mutants. The apparent size of this polypeptide is only an approximation based on extrapolation of the molecular weight standards curve. In some of our gels, an 86-kDa polypeptide was Coomassie blue stained to an equal extent in all strains examined (Fig. 5A, lanes a to d). However, this polypeptide was more heavily iodinated in the Adh2 and Adh3 mutants than in the wild-type parent strain (Fig. 5B, lanes a, b, and d). These differences in band patterns between the wild type and the adhesion-defective mutants are summarized in Table 1.

Adh3Rev, the revertant of Adh3, was indistinguishable from *Cytophaga* sp. strain U67 in its behavior and its SDS-PAGE staining and labeling patterns (e.g., Fig. 1 and 2, lanes h; Fig. 3, lane f; Tables 1 and 2). In contrast, Adh2Rev1, albeit similar to the wild type in its adhesion and motility, demonstrated a polypeptide pattern more like its adhesion-defective parent than the wild-type strain (e.g., Fig. 1 and 2, lane f; Fig. 3, lane d). Minor exceptions to this similarity in pattern between Adh2 and Adh2Rev1 were the somewhat lower relative amounts of the 128-, 135-, and 160-kDa polypeptides in Coomassie blue-stained gels (Fig. 2A, lanes e and f, respectively), the lower level of iodination of the 150- and 190-kDa polypeptides (Fig. 2B, lanes e and f),

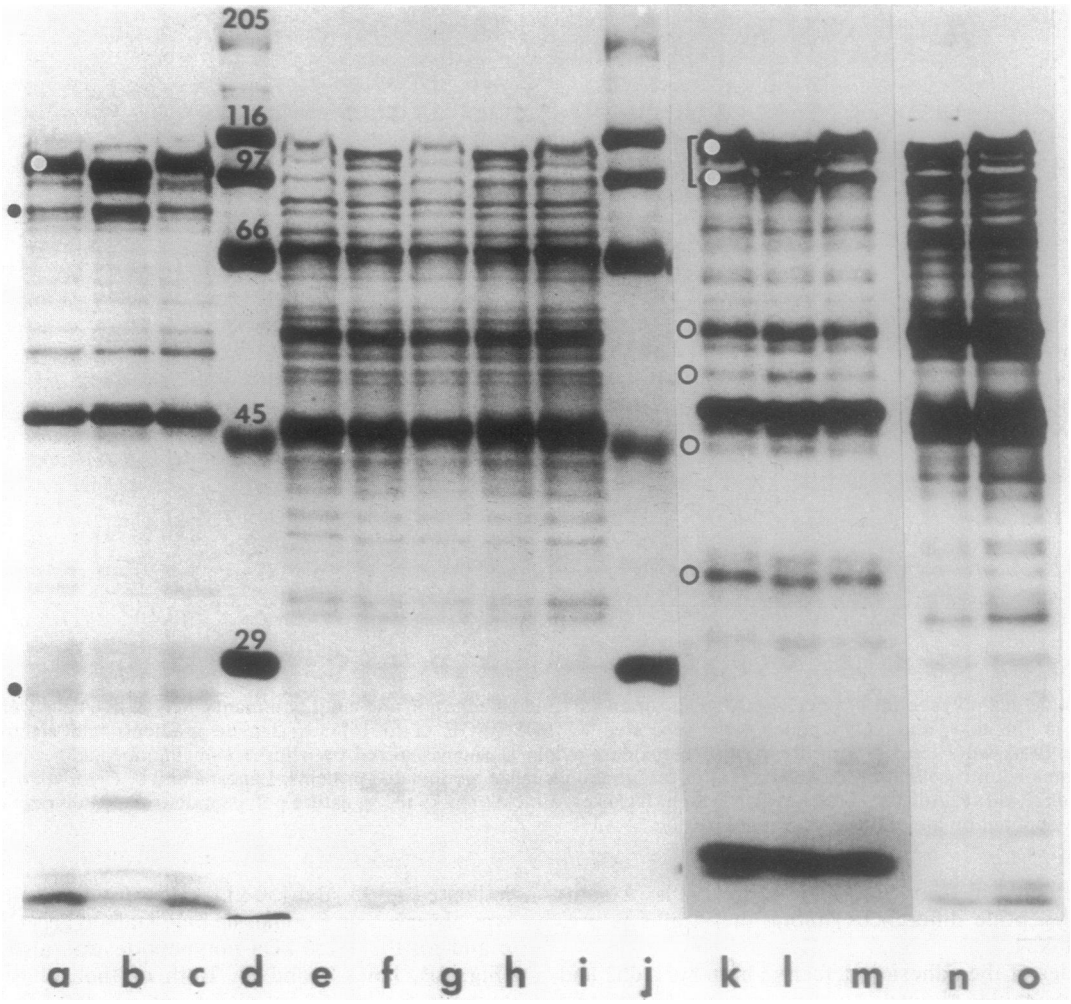

FIG. 4. Comparison of *Cytophaga* sp. strain U67, Adh3, and Adh3Rev polypeptides separated by SDS-PAGE (10% uniform acrylamide gel) and blotted to nitrocellulose. Lanes a to j are stained with Coomassie blue. Lanes a to c contain cell envelope preparations (a, U67; b, Adh3; c, Adh3Rev). Lanes k to m are autoradiograms of lanes e to g which contain whole-cell proteins from iodinated cells (e, k, U67; f, l, Adh3; g, m, Adh3Rev). Lanes n and o are the biotinylated proteins from lanes h and i which contain whole-cell proteins from biotinylated cells (h, n, Adh3; i, o, Adh3Rev). In lane a, the white circle marks the 107-kDa polypeptide. Black circles denote species with no corresponding iodinated bands in lane k. In lane k, the bracket indicates the signature triplet; open circles denote bands lacking counterparts in the cell envelope (lane a); and white circles mark the 95- and 113-kDa species of the signature triplet. Molecular weight standards (10^3) are in lanes d and j.

and the lower level of biotinylation of the ~450-kDa species (Fig. 3B, lanes c and d, respectively) in Adh2Rev1 relative to Adh2. A feature that distinguishes Adh2Rev1 from Adh2 was that the ~160-kDa polypeptide was iodinated only in the former (Fig. 2B, lane f). Recently, we have examined two other independently isolated revertants of strain Adh2, Adh2Rev2, and Adh2Rev4. Adh2Rev4 is characterized by an iodination pattern similar to Adh2Rev1, while that of Adh2Rev2 is identical to that of U67 (Fig. 6).

Nongliding mutants. Ngl6, a nongliding, adhesion-competent mutant of *Cytophaga* sp. strain U67, and a gliding revertant of this strain were also examined by substratum-immobilized iodination and surface biotinylation (Fig. 1, 2, and 3). The iodinated polypeptides of these two strains were similar to each other and to those of the wild-type strain (Fig. 1 and 2, lanes i and j; Fig. 3, lanes g and h), with the possible exception that a ~200-kDa polypeptide appeared to be more heavily iodinated in the Ngl6Rev compared with either the wild-type or the Ngl6 strain (Fig. 2B, lanes i, j, and k). Biotinylation did not reveal any significant differences between *Cytophaga* sp. strain U67 and either Ngl6 or Ngl6Rev (Fig. 3B, lanes a, g, and h).

DISCUSSION

Polypeptides exposed at the cell surface of a variety of gram-negative bacteria have been labeled with 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril (Iodo-Gen) (1, 2, 8, 11, 21–23, 32–34), and the patterns of iodinated polypeptides observed have varied in complexity. The present study provides the first identification of the polypeptides on the surface of a gliding bacterium that make direct contact with a glass substratum. The labeling pattern observed with *Cytophaga*, using Iodo-Gen, is comparable in complexity to that observed in several unrelated gram-negative bacteria (2, 22, 28), in which most or all of the labeled polypeptides are localized in the outer membrane. An outer membrane fractionation protocol has not yet been developed for *Cytophaga*

TABLE 2. Adhesion of *Cytophaga* sp. strain U67 and several adhesion mutants[a]

Strain	No. of bacteria/8 × 10^5 μm³ (mean ± SD)		% Remaining
	Before flush	After flush[b]	
U67	91.3 ± 7.8	87.6 ± 11.7	96
Adh1	119.7 ± 4.5	41.6 ± 5.0	35
Adh2	68.0 ± 7.0	0.3 ± 0.6	0.4
Adh2Rev1	80.0 ± 2.6	86.3 ± 6.7	100
Adh3	66.0 ± 4.6	0	0
Adh3Rev	73.3 ± 6.7	77.6 ± 7.6	100

[a] Bacteria were incubated in a Petroff-Hausser counting chamber for 10 min before three 8 × 10^5-μm volume equivalents were counted. Chamber depth was 20 μm.

[b] Bacteria in the same volume were counted again after three flushes with 5 μl of C62 to displace the fluid in the chamber.

sp. strain U67. Based on the recent finding of at least 10 major polypeptides in the outer membrane of *Cytophaga johnsonae* (12), we predict that most of the iodinated polypeptides of *Cytophaga* sp. strain U67 will be localized in this part of the cell envelope. Previous studies of Iodo-Gen-catalyzed iodination of other gram-negative bacteria have suggested that cytoplasmic membrane and periplasmic proteins may also be labeled (22, 32). Our data (Fig. 4) also indicate that cell envelope proteins other than those localized in the outer membrane of *Cytophaga* sp. strain U67 are accessible to iodination.

We initially planned to compare the Iodo-Gen-labeled polypeptides of *Cytophaga* sp. strain U67 with those iodinated by a soluble lactoperoxidase protocol (16). Such a comparative study permitted the identification of a flagellar surface glycoprotein that mediates cell-substratum interaction in the green alga *Chlamydomonas* (3). For *Cytophaga* sp. strain U67, however, the lactoperoxidase-catalyzed iodination conditions left the bacteria unable to glide and nonviable, although adhesion was unaffected. This may not be surprising in that Klebanoff (18) reported on the bacteriocidal effect of peroxidase-catalyzed iodination on *Escherichia coli*.

As an alternative to lactoperoxidase-catalyzed iodination, we used biotinylation of whole cells, a labeling procedure specific for all surface-exposed polypeptides (14, 27). Comparison of the polypeptide patterns of Iodo-Gen-labeled cells with that of biotinylated cells demonstrated that some surface-exposed polypeptides were not accessible to the glass-immobilized catalyst. This observation suggests that the cell surface topology may not be uniform. Such surface unevenness has been reported by Bayer (Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, J1, p. 144), who described 12-nm-diameter particles or bumps protruding from the *Cytophaga* sp. strain U67 cell surface. Similar structures have also been observed by Poindexter (personal communication) and by us (L. M. Garone, D. W. Pumplin, and R. P. Burchard, unpublished results). It is possible that the cell envelope polypeptides that are localized in these bumps were preferentially labeled by the immobilized iodination catalyst, while biotinylation labeled not only these polypeptides but also those of the planar cell surface domains. Alternatively, all outer membrane polypeptides may be uniformly distributed over the cell surface; polypeptides protruding outward a considerably distance may have screened other surface polypeptides from contacting the glass-immobilized iodination catalyst.

To determine which Iodo-Gen-labeled polypeptide(s) might function in bacterial adhesion to the substratum, we isolated adhesion-defective mutants to compare their surface labeling patterns with that of the wild-type cells. The mutants we have described are similar in their phenotype to the *C. johnsonae* TNM (truly nonmotile) mutants which were also isolated as relatively hydrophilic cells in a hexadecane partitioning protocol (37).

Striking changes in the cell surface labeling patterns accompanied loss of adhesiveness (Table 1). We observed three categories of changes: (i) appearance of new polypeptide species as detected on Coomassie blue-stained gels or the appearance of new iodinated polypeptides or both; (ii) loss of labeled species; and (iii) apparent change in migration (always an increased migration; lower apparent molecular weight) of polypeptide species in SDS-polyacrylamide gels. In the first case (exemplified by the 128-, 135-, 160-, and 190-kDa polypeptides in Adh2 and Adh3), the changes in the pattern of surface labeling could be due to increased protein synthesis, changes in targeting of polypeptides to the cell surface, and/or changes in protein conformation resulting in increased accessibility to labeling. In the second case, typified by the ~270-kDa polypeptide in Adh1, the loss of labeling could be due to a decrease in the actual amount of the polypeptide or to a change in its conformation. Alterna-

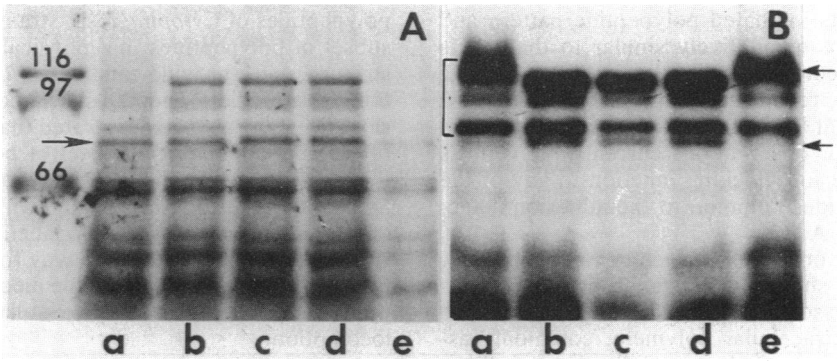

FIG. 5. SDS-polyacrylamide slab gel (4 to 16% gradient) of whole-cell polypeptides of *Cytophaga* sp. strain U67 and its adhesion mutants that were surface labeled with immobilized Iodo-Gen and [^{125}I]sodium iodide. Only a portion of the gel is displayed. (A) Coomassie blue-stained gel. (B) Autoradiogram. Lanes: a, *Cytophaga* sp. strain U67; b, Adh2; c, Adh2Rev1; d, Adh3; e, Adh3Rev. Upper arrow indicates 113-kDa polypeptide of the signature triplet (bracket). Lower arrows mark the 86-kDa polypeptide. Molecular weight markers (10^3) are indicated in panel A.

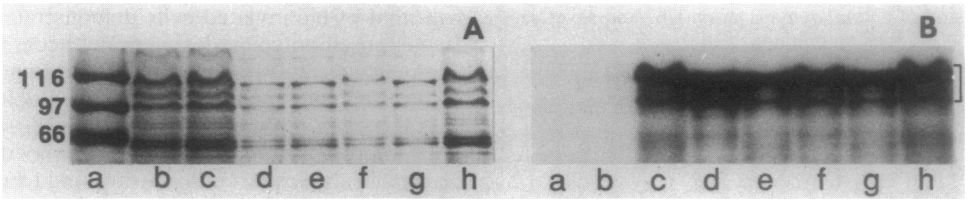

FIG. 6. Section of an SDS-polyacrylamide slab gel (10% uniform) of whole-cell polypeptides of *Cytophaga* sp. strain U67 and its adhesion mutants that were surface labeled with Iodo-Gen and ^{125}I. (A) Coomassie blue-stained gel. (B) Autoradiogram. Lanes: a, molecular weight standards (10^3); b, Iodo-Gen control; c and h, *Cytophaga* sp. strain U67; d, Adh2; e, Adh2Rev1; f, Adh2Rev2; g, Adh2Rev4. Bracket denotes signature triplet.

tively, posttranslational modification may have reduced its accessibility to the iodination catalyst. In the third case, exemplified by a shift in mobility of the signature triplet in Adh2 and Adh3, the apparent increased mobility of the polypeptide species could represent an alteration in a posttranslational modification to the proteins (e.g., proteolytic cleavage or altered glycosylation). A less likely possibility is that the apparent shift in migration of these closely migrating species actually represents the complete loss of several species of protein accompanied by the appearance of new protein species.

The absence of Adh1 of the heavily labeled, diffuse polypeptide band at ~270 kDa suggests that it may function in *Cytophaga* sp. strain U67 adhesion. It may be a minor bacterial protein since it was not visible on Coomassie blue-stained acrylamide gels. Alternatively, it may not be stainable with Coomassie blue, as is the case for some glycoproteins (10, 30). It is likely that other cell surface components also contribute to substratum adhesion since Adh1 demonstrated limited adherence (Table 2).

Interpretation of the polypeptide changes in Adh2 and Adh3 is more difficult because of their complexity. The identity of labeling of Adh3Rev, Adh2Rev2, and the wild type suggests that one or more of the polypeptides that distinguished Adh2 and Adh3 from U67 may account for their loss of adhesion. The high frequency of reversion of Adh3 to the wild-type phenotype and the similarity of the Adh3Rev labeling pattern to that of U67 suggests that a single genetic defect was responsible for the multiple alterations observed, again raising the possibility that an enzyme responsible for the posttranslational modification of several polypeptides destined for the outer membrane might be the primary defect in this mutant. Interpretation of the results is complicated by Adh2Rev1, which demonstrated adhesion and motility that were indistinguishable from those of the wild type. However, its iodinated polypeptide pattern and that of Adh2Rev4 were qualitatively similar to that of its adhesion-defective parent. The distinction between labeling patterns of these two revertants and that of Adh2Rev2 suggests that there are at least two pathways of reversion to wild-type behavior. Our observations currently prevent us from assigning an adhesion blocking function to any of the novel labeled polypeptides common to the adhesion-defective mutants Adh2 and Adh3.

It is conceivable that other cell surface components (e.g., lipopolysaccharide and sulfonolipids) may function in mediating gliding motility-associated adhesion (12). It has also been proposed that extracellular polymers, commonly assumed to be a characteristic product of gliding bacteria, may function in adhesion (4, 25). If these polymers were sandwiched between the gliding bacterium and its substratum, they might block direct contact between the bacterial cell envelope and the substratum. The results of the substratum-immobilized iodination of *Cytophaga* sp. strain U67 polypeptides suggest that, if extracellular polymers do in fact mediate bacterial adhesion, their location is such that direct contact between bacterial envelope proteins and the glass substratum can still occur. It is noteworthy that Lapidus and Berg (20) reported that *Cytophaga* sp. strain U67 did not produce large amounts of extracellular material. The concept that gliding bacteria make close contact with their substratum is further supported by recent interference reflection microscopic studies (13).

The similarity in Coomassie blue, surface biotinylation, and ^{125}I-labeling patterns of *Cytophaga* sp. strain U67 and Ngl6, an adhesion-competent but nonmotile strain, provided no information about polypeptides that might be involved in the machinery of gliding motility. It is quite reasonable to expect that mutant cell lines with normal adhesion but defective gliding motility may have defects in the energy transduction system responsible for the cell being able to apply force onto the substratum during the expression of gliding motility. Since it is known that gliding motility is driven by a proton motive force (4, 29), it is unlikely that all components of such a biological motor would be exposed at the cell surface.

Pate (25) described an electrophoretic analysis of envelope preparations from 63 independently isolated truly nonmotile (TNM) mutants from *C. johnsonae*. He found that 15 major envelope proteins appeared to be related to gliding motility. Although the alterations in the various mutants differed widely, many of the mutants had lost most of their immunoreactivity for monoclonal antibodies directed against a 40-kDa cell envelope protein that is exposed at the surface of *C. johnsonae* (36). A polypeptide of comparable size was labeled by iodination and biotinylation of *Cytophaga* sp. strain U67.

In conclusion, we have characterized the surface-exposed polypeptides of *Cytophaga* sp. strain U67 and identified that subset of polypeptides that physically contacts a solid substratum; these provide candidates for the macromolecule(s) that may mediate gliding-associated cell-substratum adhesion. We have also demonstrated that changes in the pattern of surface labeling of a subset of polypeptides accompany the loss of adhesion in a selected group of mutants. Further, the loss of adhesion is associated with more than one pattern of altered surface polypeptide labeling in this gliding bacterium. These studies pave the way for continued progress in understanding at least one of the mechanisms responsible for procaryotic gliding motility, a still enigmatic form of cell locomotion.

ACKNOWLEDGMENTS

This research was supported by Office of Naval Research contract N00014-88-K-0158 to R.P.B. and by grants from the National

Science Foundation (DCB-8905530) and the Public Health Service National Institutes of Health (R01 GM28766) to R.A.B.

We gratefully acknowledge the expert technical assistance of Cindy-Lou Dull and Nancy Salomonsky and the help of William Fawcett in isolating the adhesion-defective mutants.

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