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In Vivo Labeling of *Escherichia coli* Cell Envelope Proteins with *N*-Hydroxysuccinimide Esters of Biotin

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The primary amine coupling reagents succinimidyl-6-biotinamido-hexanoate (NHS-A-biotin) and sulfosuccinimidyl-6-biotinamido-hexanoate (NHS-LC-biotin) were tested for their ability to selectively label *Escherichia coli* cell envelope proteins in vivo. Probe localization was determined by examining membrane, periplasmic, and cytosolic protein fractions. Both hydrophobic NHS-A-biotin and hydrophilic NHS-LC-biotin were shown to preferentially label outer membrane, periplasmic, and inner membrane proteins. NHS-A- and NHS-LC-biotin were also shown to label a specific inner membrane marker protein (Tet-LacZ). Both probes, however, failed to label a cytosolic marker (the Ω fragment of β-galactosidase). The labeling procedure was also used to label *E. coli* cells grown in low-salt Luria broth medium supplemented with 0, 10, and 20% sucrose. Outer membrane protein A (OmpA) and OmpC were labeled by both NHS-A- and NHS-LC-biotin at all three sucrose concentrations. In contrast, OmpF was labeled by NHS-A-biotin but not by NHS-LC-biotin in media containing 0 and 10% sucrose. OmpF was not labeled by either NHS-A- or NHS-LC-biotin in *E. coli* cells grown in medium containing 20% sucrose. Coomassie-stained gels, however, revealed similar quantities of OmpF in *E. coli* cells grown at all three sucrose concentrations. These data indicate that there was a change in outer membrane structure due to increased osmolarity, which limits accessibility of NHS-A-biotin to OmpF.

N-hydroxysuccinimide (NHS) esters form amide bonds with primary amines (4, 8). The reaction occurs via a nucleophilic attack by an unprotonated amine on the ester, resulting in amide bond formation and release of NHS. The reaction is favored at alkaline pH values, which keep primary amines unprotonated, but also occurs at physiological pH values (4, 8).

NHS-coupled biotinyl compounds have been used to label cell surface proteins in eukaryotic cells. The NHS moiety forms a covalent bond with exposed primary amines of surface proteins (3–6, 8, 9, 11, 12, 16, 18). Biotin-tagged proteins can then be detected or purified with streptavidin-linked reagents. For example, Busch et al. (6) demonstrated that surface proteins in rat hepatocyte cells were preferentially labeled compared with cytosolic proteins when whole cells were exposed to a sulfosuccinimidobiotin (sulfo-NHS-biotin). Sulfo-NHS-biotin has also been used to study the cell surface and junctional plasma membrane domains in the *Dictyostelium discoideum* amebae (12). In addition to these studies with animal and fungal cells, NHS-biotin esters have also been used to study the surface features of carrot somatic embryos (9).

NHS ester-linked biotinyl probes, however, have not been used to label whole gram-negative bacterial cells in vivo. Furthermore, the ability of hydrophobic and hydrophilic NHS coupling reagents to cross different layers of the gram-negative cell envelope has not been examined. In this study, the NHS ester coupling reagents succinimidyl-6-biotinamido-hexanoate (NHS-A-biotin) and sulfosuccinimidyl-6-biotinamido-hexanoate (NHS-LC-biotin) have been used to probe *Escherichia coli* cells in vivo. The sensitivity of both types of probes, the localization of the probes to particular cellular fractions, and the labeling of *E. coli* cells under hyperosmolar conditions were also investigated.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The following *E. coli* strains were used in this study: ATCC 15597, a derivative of *E. coli* K-12 obtained from the American Type Culture Collection; JM101 and HB101, from GIBCO Corp.; DH5α bearing plasmid pUC19, from Promega Corp.; and RV200 (Δ*lacZ*) bearing plasmids pRKH21 (λp_L *tet*-Δ-*lacZ* Amp^r) and pCI857 (λ*c*I857 Kan^r) (10). Strain RV200 bearing pRKH21 and pCI857 was a kind gift from Laura McMurry (Departments of Molecular Biology and Microbiology, Tufts University). *E. coli* DH5α provided a cytosolic protein marker (the Ω fragment of β-galactosidase), and plasmid pRKH21 provided an inner membrane marker protein (involved in tetracycline resistance). All bacterial strains were maintained in Luria broth (LB) medium (14) with appropriate antibiotic selection.

For NHS-biotin labeling, *E. coli* ATCC 15597, HB101, and JM101 were grown in LB medium. For induction of β-galactosidase, DH5α(pUC19) cells were grown in LB medium containing 50 μg of ampicillin per ml and 1 mM isopropyl-β-D-thiogalactoside (IPTG) by following standard protocols (17). To study biotinylation of Tet-LacZ, RV200(pRKH21, pCI857) cells were grown at inducing and noninducing temperatures in LB medium as described previously (10). For hyperosmotic growth, *E. coli* ATCC 15597 cultures were transferred to low-salt LB medium containing 10 g of Bacto Tryptone per liter, 5 g of yeast extract per liter, 5 g of NaCl per liter, and either 0, 10, or 20% sucrose. Ultrapure (ultracentrifuge grade) sucrose was used for these studies. The sucrose used either was added to the medium after being filter sterilized or was added as part of the medium prior to autoclaving (15 min, 15 lb/in^2, 121°C). No differences were

noted between the results obtained with filter-sterilized sucrose and autoclaved sucrose.

In vivo labeling of cell envelope proteins. Cultures (10 ml) of the *E. coli* strain selected for labeling were grown to an A_{640} of 0.6 and pelleted by centrifugation at 5,000 × *g* for 10 min at 4°C. Pelleted cells were washed three times in ice-cold 50 mM sodium phosphate buffer (pH 7.5) (PB) by resuspension and centrifugation (5 min, 12,000 × *g*, 4°C). Washing cells was essential in order to remove residual medium, which interfered with labeling. Pellets were resuspended in 1 ml of ice-cold 40 mM morpholinopropanesulfonic acid (MOPS) (pH 7.5) or PB. Labeling was then performed with either NHS-LC- or NHS-A-biotin as follows. NHS-LC-biotin (50 mM) made fresh in distilled deionized H_2O and 50 mM NHS-A-biotin made fresh in deionized *N,N*-dimethyl-formamide were diluted in ice-cold MOPS and PB, respectively, to a final stock concentration of 10 mM. The stock solutions were then used immediately in individual labeling reactions. MOPS buffer was used for all aspects of NHS-LC-biotin preparation and cell labeling to prevent possible salting out of this reagent in PB. For a given labeling reaction, the 10 mM stock solution was added directly to the cells resuspended in MOPS or PB to give a final concentration of 1 mM. In addition to the NHS-A- and NHS-LC-biotin, unlabeled D-biotin (5 mM) was also added to the labeling mixture to prevent labeling of cytosolic proteins. It was postulated that labeling of *E. coli* cytosolic proteins by NHS-biotin esters in the absence of D-biotin was due to transport of the NHS-biotin by intact *E. coli* cells. During the labeling procedure cell cultures were kept on ice for 20 min with shaking at 50 rpm. Labeling reactions were terminated by addition of 2 volumes of ice-cold 50 mM Tris (pH 7.5) and centrifugation at 12,000 × *g* for 10 min at 4°C. Pellets were then washed three times in ice-cold PB by resuspension and centrifugation. The labeled cells were then used for protein fractionation or for preparation of total proteins as described below.

Cell fractionation and protein preparation. Periplasmic proteins were isolated by modifications of the chloroform shock method of Ames et al. (1). Labeled cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. The supernatant was then decanted off, and the pelleted cells were resuspended in the residual PB which remained. Resuspension was followed by addition of 50 μl of ice-cold chloroform. The cell-chloroform mixture was vortexed for 5 to 10 s and placed on ice for 15 min. During this 15-min period brief vortexing of the cell-chloroform mixture was continued at 5-min intervals. Following chloroform shock, 2 ml of ice-cold 10 mM Tris (pH 8.0) was added, and the chloroform-shocked cells were pelleted by centrifugation at 6,000 × *g* for 10 min at 4°C. Three-quarters of the periplasmic protein-containing supernatant was removed and concentrated by lyophilization. Residual periplasmic proteins were removed by washing the chloroform-shocked pelleted cells two times by resuspension in 2 ml of ice-cold PB, followed by centrifugation as described above. The chloroform-shocked cell pellet contained membrane and cytosolic proteins. Membrane proteins were then prepared by the procedure of Barron et al. (2). Washed chloroform-shocked cell pellets were resuspended in 5 ml of 33 mM Tris–0.5 M sucrose–1 mM EDTA (pH 7.6), and this was followed by digestion of the peptidoglycan by addition of 0.04% lysozyme in 20 mM EDTA (pH 8.0) and incubation of the mixture for 1 h at room temperature. Following lysozyme treatment, phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM. Lysis of cells was completed by brief sonication (10 to 15 s). The membrane fraction (containing both outer and inner membranes) was collected by centrifugation at 130,000 × *g* for 90 min at 4°C. The supernatant contained cytosolic proteins, which were concentrated by lyophilization. The membrane pellet was resuspended in 5 ml of a solution containing 33 mM Tris (pH 8.0), 5 mM $MgCl_2$, 25 μg of DNase per ml, and 25 μg of RNase per ml, incubated for 30 min at room temperature, and centrifuged again at 130,000 × *g* for 90 min at 4°C. All protein samples were resuspended in sample buffer (6% sodium dodecyl sulfate [SDS], 26.4% glycerol, 6% β-mercaptoethanol, 0.19 M Tris [pH 6.8]), heated for 10 min at 90°C, and stored at −80°C prior to SDS-polyacrylamide gel electrophoresis (PAGE).

Total proteins from labeled cells were prepared as follows. Cell pellets were resuspended directly in sample buffer, heated at 90°C for 10 min, and placed on ice for 5 min, and insoluble material was removed by centrifugation at 12,000 × *g* for 10 min at room temperature. The supernatant (containing total proteins) was passed several times through a 20-gauge needle to shear residual nucleic acids. Total protein samples were also stored at −80°C prior to SDS-PAGE.

Western blotting (immunoblotting) (19) by using monoclonal antibodies for the Ω subunit of β-galactosidase was performed to verify the quality of the cell fractionation experiments. Monoclonal antibodies to β-galactosidase (ΔLacZΩ) were purchased from Boehringer Mannheim Biochemicals, and antibody-protein complexes were detected according to protocols provided by the manufacturer.

Analysis of protein biotinylation. Immediately before SDS-PAGE, protein samples were removed from storage at −80°C, thawed on ice, heated to 90°C for 5 min, and centrifuged at 12,000 × *g* for 10 min at room temperature to remove insoluble material. The protein loads used for SDS-PAGE were 25 μg per lane, as determined by the method of Lowry et al. (13). The biotinylated proteins within these gels were transferred from SDS-PAGE gels to nitrocellulose membranes by standard protocols (19). The membranes were then blocked for 2 h at room temperature in a solution containing 2% nonfat powdered milk, 20 mM Tris (pH 8.8), 500 mM NaCl, and 0.1% sodium azide. Following the blocking step, nitrocellulose membranes were incubated for 2 h with shaking (50 rpm) at room temperature in a 1/2,000 dilution of streptavidin alkaline phosphatase conjugate (Fisher Scientific) in a buffer containing 2% nonfat powdered milk, 20 mM Tris (pH 8.8), 500 mM NaCl, 0.1% sodium azide, and 0.01% Tween 20. The nitrocellulose membranes were then washed for 15 min with 20 mM Tris (pH 8.8)–500 mM NaCl–0.01% sodium azide at room temperature. This treatment was followed by three additional 10-min washes at room temperature in 100 mM $NaHCO_3$ (pH 9.8)–1 mM $MgCl_2$. The protein-biotin-streptavidin alkaline phosphatase complexes were visualized by developing the nitrocellulose blots in 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium chloride. Protocols for blot development were provided by Bio-Rad. Identification of outer membrane protein A (OmpA), OmpC, and OmpF was based on comparisons of the protein profiles obtained in our experiments with the profiles of Graeme-Cook (7).

RESULTS AND DISCUSSION

Specificity of protein biotinylation. In vivo protein labeling with NHS-A- and NHS-LC-biotin was compared for total, membrane, periplasmic, and cytosolic protein fractions from three *E. coli* strains. Both probes were found to label the

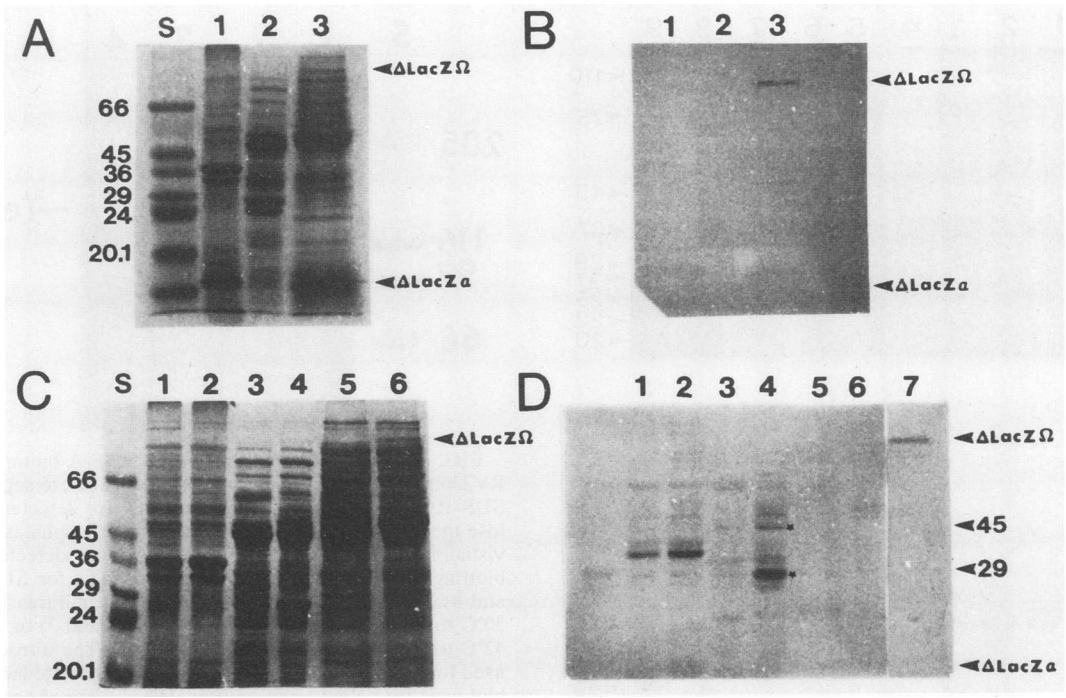

FIG. 1. Membrane, periplasmic, and cytosolic proteins from in vivo NHS-A-biotin-labeled *E. coli* DH5α(pUC19). Proteins (25 μg) were separated on 10% SDS–PAGE gels and analyzed as follows. (A) Coomassie blue detection of membrane, periplasmic, and cytosolic proteins (lanes 1, 2, and 3, respectively) from cells induced with IPTG. (B) Western blot of membrane, periplasmic, and cytosolic proteins (lanes 1, 2, and 3, respectively) from cells induced with IPTG by using monoclonal antibody specific for ΔLacZΩ. (C) Coomassie blue detection of membrane (lanes 1 and 2), periplasmic (lanes 3 and 4), and cytosolic (lanes 5 and 6) proteins from cells that were either uninduced (lanes 1, 3, and 5) or induced with IPTG (lanes 2, 4, and 6). Lane S contained protein standards; molecular weights ($\times 10^3$) are indicated on the left. (D) Detection of biotinylated proteins on nitrocellulose blots by streptavidin alkaline phosphatase colorometric visualization of membrane (lanes 1 and 2), periplasmic (lanes 3 and 4), and cytosolic (lanes 5 and 6) proteins isolated from cells that were either uninduced (lanes 1, 3, and 5) or induced with IPTG (lanes 2, 4, and 6). Lane 7 shows a Western blot of the cytosolic proteins from IPTG-induced cells when a ΔLacZΩ-specific monoclonal antibody was used. No ΔLacZΩ was detected by Western blotting in uninduced cells. The nitrocellulose strip used for the Western blot (lane 7) was cut from the same blot used for detection of biotinylated proteins (lanes 1 through 6). Each gel or blot is representative of four separate labeling experiments.

same proteins in each of the subcellular fractions from three separate *E. coli* strains (ATCC 15597, JM101, and HB101) (data not shown).

Since faint protein bands could be detected by NHS-biotin esters in *E. coli* cytosolic protein fractions (Fig. 1D, lanes 5 and 6, and Fig. 2B, lanes 7 through 9), it was decided that an inducible cytosolic marker should be used to further characterize the labeling specificity of NHS-biotin esters. The inducible cytosolic marker chosen for this analysis was β-galactosidase, since this protein is relatively abundant under induced conditions and not detectable under noninduced conditions. Host *E. coli* DH5α containing plasmid pUC19 served as the source of inducible β-galactosidase. Plasmid pUC19 contains the IPTG-inducible α-fragment of the *lacZ* gene coding for a 15-kDa product (ΔLacZα) and an inducible 5′ deleted *lacZ* gene coding for the Ω fragment of β-galactosidase (ΔLacZΩ), which is a 105-kDa product. This product migrates to a discrete location on a 10% SDS–PAGE gel, providing easy detection with a monoclonal antibody (Fig. 1). Initial analysis of our cell fractionation procedure demonstrated that ΔLacZΩ was localized to the cytosolic fraction (Fig. 1A and B). IPTG-induced and noninduced cells were further analyzed for biotinylation of the inducible β-galactosidase (Fig. 1C and D). The streptavidin-biotin blot of these protein fractions revealed no detectable biotinylation of ΔLacZΩ by NHS-A-biotin (Fig. 1D). A parallel experiment in which NHS-LC-biotin was used as the labeling probe also failed to label the cytosolic ΔLacZΩ marker (data not shown). These results demonstrated that neither NHS-A-biotin nor NHS-LC-biotin labels a cytosolic marker protein.

Periplasmic proteins with molecular masses of 29 and 45 kDa were also intensely biotinylated in DH5α(pUC19) cells cultured under induced conditions (Fig. 1D). The same intensity of biotinylation, however, did not occur under noninduced conditions. Proteins having similar molecular weights are detected in *E. coli* ATCC 15597 grown in LB (Fig. 2B).

In order to further characterize the specificity of labeling by NHS-A-biotin, an *E. coli* inner membrane marker was also studied. This inner membrane marker was a tetracycline-LacZ fusion protein (10) [RV200(pRKH21, pCI857)]. The identification of this protein was aided by its presence behind a temperature-sensitive promoter (induced at 42°C and noninduced at 30°C). When the relative levels of the Tet-LacZ fusion protein were compared, clear induction was observed in Western blots (Fig. 3) and Coomassie-stained gels (data not shown) of total proteins. The detection of the Tet-LacZ fusion protein in uninduced cells was not surprising since these cells have previously been shown to be tetracycline resistant (10). Increased biotinylation of a protein having the same molecular weight as the Tet-LacZ

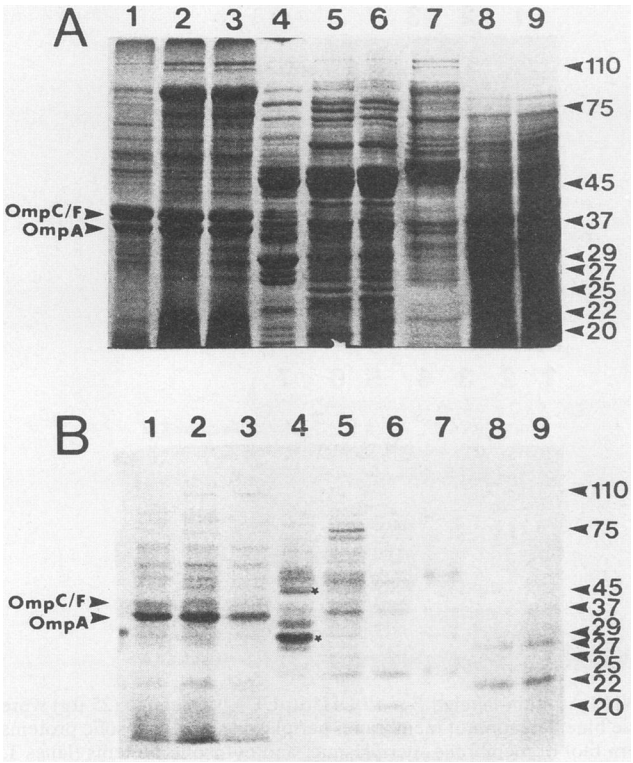

FIG. 2. Membrane, periplasmic, and cytosolic proteins from in vivo NHS-A-biotin-labeled *E. coli* ATCC 15597 grown under hyperosmolar conditions. Proteins (25 μg) were separated by 10% SDS–PAGE and either stained with Coomassie blue (A) or transferred to a nitrocellulose membrane, where biotinylated proteins were detected by streptavidin alkaline phosphatase colorometric visualization (B). Membrane proteins (lanes 1 through 3), periplasmic proteins (lanes 4 through 6), and cytosolic proteins (lanes 7 through 9) were from cells grown in low-salt LB medium containing either no sucrose (lanes 1, 4, and 7), 10% sucrose (lanes 2, 5, and 8), or 20% sucrose (lanes 3, 6, and 9). Molecular masses (in kilodaltons) are indicated on the right. The gel and blot are representative of four separate labeling experiments.

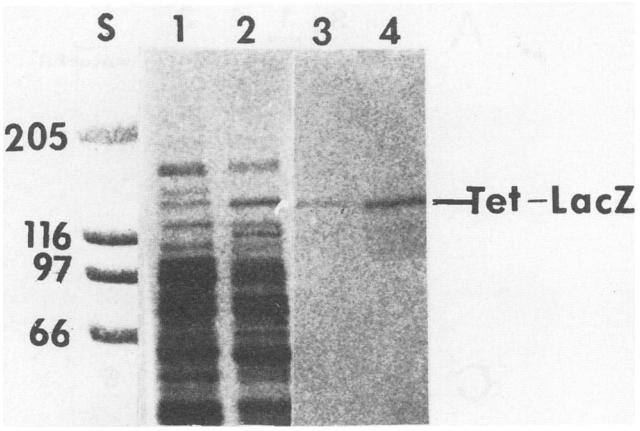

FIG. 3. Total proteins from in vivo NHS-A-biotin-labeled *E. coli* RV200(pRKH21, pCI857). Proteins (25 μg) were separated by 10% SDS–PAGE, and biotinylated proteins were detected on nitrocellulose membranes by streptavidin alkaline phosphatase colorometric visualization (lanes 1 and 2); Tet-LacZ was detected by Western blotting with a monoclonal antibody specific for ΔLacZΩ (lanes 3 and 4). *E. coli* RV200(pRKH21, pCI857) cultures were grown at 30°C to limit Tet-LacZ synthesis (lanes 1 and 3) or were grown at 42°C to induce Tet-LacZ (lanes 2 and 4). The nitrocellulose strips used for the Western blot were cut from duplicate lanes of the same blot used for detection of biotinylated proteins. Lane S contained a protein standard; molecular masses (in kilodaltons) are indicated on the left. Each blot is representative of three separate labeling experiments. Scanning densitometry of Western blots revealed an approximately 10-fold induction of the Tet-LacZ fusion protein.

fusion protein was observed (Fig. 3). It was concluded that this protein was the inner membrane marker since the relative increase in biotinylation was similar to the level of induction detected in Western blots. Several other protein differences were noted between induced and noninduced *E. coli* cells in both biotinylated (Fig. 3) and Coomassie-stained (data not shown) protein profiles. Since biotinylation and protein profiles are dependent upon environmental conditions, it was concluded that these differences more than likely arose because of the different temperatures at which the *E. coli* cells were cultured.

NHS-LC-biotin was also found to label a protein having the same molecular weight as the Tet-LacZ fusion protein (data not shown). However, in these blots the labeling of the putative Tet-LacZ fusion protein was not as intense compared with other biotinylated proteins. Since NHS-LC-biotin is water soluble, it would not be as efficient at crossing into the inner membrane; therefore, the labeling efficiency of NHS-LC-biotin provides additional evidence that the 150-kDa protein is the Tet-LacZ fusion protein.

Protein biotinylation under hyperosmotic conditions. In vivo NHS-A-biotin labeling patterns were compared by using cytosolic, periplasmic, and membrane protein fractions of *E. coli* ATCC 15597 grown in low-salt LB medium supplemented with either 0, 10, or 20% sucrose. Since cells did not grow to the same density for each treatment, adjustments were made to ensure that the same cell concentration was labeled in each experiment (10 mg [wet cell weight] ml^{-1}).

Protein species with molecular masses of 22 and 27 kDa were biotinylated in cytosolic fractions from *E. coli* grown in LB medium supplemented with 10 and 20% sucrose (hyperosmolar conditions) (Fig. 2B). Proteins having similar molecular weights, however, were not detected in protein preparations from low-salt LB medium without sucrose (normal conditions). Although several proteins were biotinylated in the cytosolic fraction from cells grown in LB medium without sucrose, each of these proteins corresponded to a protein with a similar molecular weight in the periplasmic fraction. The 22- and 27-kDa proteins, on the other hand, did not have proteins with corresponding molecular weights in other subcellular fractions. It is possible that these cytosolic proteins were associated with the inner membrane under hyperosmotic conditions and thus were labeled by NHS-A-biotin.

Periplasmic fractions also showed differences in biotinylation under hyperosmolar conditions (Fig. 2B). Proteins with molecular masses of 20, 25, 37, and 75 kDa showed increased biotinylation by NHS-A-biotin in cells grown under hyperosmolar conditions. In cells grown under normal conditions, proteins with molecular masses of 29, 32, and 45 kDa were readily detected in periplasmic fractions. Proteins with similar molecular weights were not detected in fractions from cells grown under hyperosmolar conditions.

NHS-A-biotin biotinylation patterns of membrane pro-

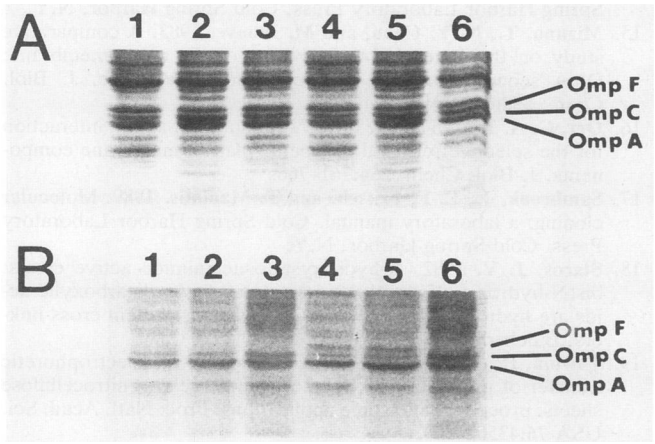

FIG. 4. Membrane proteins from in vivo NHS-LC- or NHS-A-biotin-labeled *E. coli* ATCC 15597 cells grown under hyperosmolar conditions. Proteins (25 μg) were separated by 12% SDS–PAGE and either stained with Coomassie blue (A) or transferred to nitrocellulose membrane, where biotinylated proteins were detected by streptavidin alkaline phosphatase colorometric visualization (B). Proteins were labeled with NHS-LC-biotin (lanes 1 through 3) and NHS-A-biotin (lanes 4 through 6). Labeled cells were grown in low-salt LB medium containing no sucrose (lanes 1 and 4), 10% sucrose (lanes 2 and 5), or 20% sucrose (lanes 3 and 6). The gel and blot are representative of two separate labeling experiments which were performed in addition to the experiments performed to obtain Fig. 2.

teins showed subtle differences in response to osmolarity when proteins were analyzed on 10% polyacrylamide gels (Fig. 2). A 110-kDa protein which appears to be induced by increased osmolarity was found to be biotinylated by NHS-A-biotin (Fig. 2). OmpC and OmpF were not resolved in these 10% polyacrylamide gels, whereas OmpA was resolved (Fig. 2A). OmpA was the primary protein detected by NHS-A-biotin and remained relatively constant with respect to its level of expression under both hyperosmolar and normal conditions. OmpC and OmpF were not as heavily biotinylated as OmpA.

Further analyses were performed to analyze the labeling of OmpC and OmpF under hyperosmolar conditions. In these experiments membranes were prepared directly from cells labeled with NHS-A- and NHS-LC-biotin. These membranes were then separated on 12% SDS–polyacrylamide gels. Coomassie staining of these gels revealed three distinct bands corresponding to OmpA, OmpC, and OmpF (Fig. 4A). Biotinylation of cells with NHS-A-biotin revealed labeling of all three proteins in low-salt LB medium and low-salt LB medium supplemented with 10% sucrose (Fig. 4B). Only OmpC and OmpA, however, were labeled in cells grown in low-salt LB medium supplemented with 20% sucrose. In contrast to these results, NHS-LC-biotin did not label OmpF in cells grown in LB medium supplemented with any concentration of sucrose (Fig. 4B). However, OmpA and OmpC were labeled in cells cultured in low-salt LB medium supplemented with 0, 10, and 20% sucrose. Since the protein biotinylation profiles of the three outer membrane proteins did not correspond to Coomassie-stained protein profiles, it must be concluded that the differences in osmolarity alter the availability of primary amines within each protein. Additionally, since OmpF was labeled by NHS-A-biotin, but not by NHS-LC-biotin, it may be concluded that primary amines on OmpF are not available to NHS-LC-biotin.

NHS-biotin esters can therefore be used to develop inferences about the in vivo organization of proteins within the cell envelopes of gram-negative bacteria. For example, OmpF and OmpC have similarities in amino acid composition and in the number of theoretical labeling sites available to NHS-biotin esters (15). However, OmpF was not detected by NHS-LC-biotin under various osmotic conditions, whereas OmpC was detected (Fig. 4). These data indicate that OmpC must be located in a more hydrophilic region of the cell envelope than OmpF, which is more accessible to NHS-LC-biotin despite the similarities in structure and function shared by the two proteins. These data demonstrate the value of using both hydrophilic and hydrophobic biotin probes to study environmental influences on the gram-negative cell envelope.

Other changes in periplasmic and cytosolic proteins in response to osmolarity further illustrate the usefulness of NHS-biotin esters in the study of environmental responses by bacteria (Fig. 2). In addition to changes in protein biotinylation because of osmolarity in *E. coli* and *Bradyrhizobium japonicum* (unpublished data), we have also observed variation in biotinylation profiles as a result of the temperature or carbon source used to culture these bacteria (unpublished data).

The results in this report present the usefulness of in vivo biotinylation of intact gram-negative cells for (i) rapid localization of certain proteins to the cell envelope, (ii) monitoring changes in response to environmental stimuli for certain cell envelope proteins, and (iii) identification of previously uncharacterized cell envelope proteins. Clearly, NHS-biotin esters can be used to study cell surface proteins from gram-negative bacterial cells in a way similar to the way that they have been used to study eukaryotic cells.

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