Gram-negative bacteria shed outer membrane vesicles composed of outer membrane and periplasmic components. Since vesicles from pathogenic bacteria contain virulence factors and have been shown to interact with eukaryotic cells, it has been proposed that vesicles behave as delivery vehicles. We wanted to determine whether heterologously expressed proteins would be incorporated into the membrane and lumen of vesicles and whether these altered vesicles would associate with host cells. Ail, an outer membrane adhesin/invasin from *Yersinia enterocolitica*, was detected in purified outer membrane and in vesicles from *Escherichia coli* strains DH5α, HB101, and MC4100 transformed with plasmid-encoded Ail. In vesicle-host cell co-incubation assays we found that vesicles containing Ail were internalized by eukaryotic cells, unlike vesicles without Ail. To determine whether luminal vesicle contents could be modified and delivered to host cells, we used periplasmically expressed green fluorescent protein (GFP). GFP fused with the Tat signal sequence was secreted into the periplasm via the twin arginine transporter (Tat) in both the laboratory *E. coli* strain DH5α and the pathogenic enterotoxigenic *E. coli* ATCC strain 43886. Pronase-resistant fluorescence was detectable in vesicles from Tat-GFP-transformed strains, demonstrating that GFP was inside intact vesicles. Inclusion of GFP cargo increased vesicle density but did not result in morphological changes in vesicles. These studies are the first to demonstrate the incorporation of heterologously expressed outer membrane and periplasmic proteins into bacterial vesicles.

Gram-negative bacteria secrete proteins soluble and in association with outer membrane vesicles. All Gram-negative bacteria studied to date, including *Escherichia coli*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Borrelia burgdorferi*, *Shigella flexneri* and *Actinobacillus actinomycetemcomitans*, produce outer membrane vesicles (1–8). Vesicles were first observed by electron microscopy and range in size from 20–250 nm in diameter. Gram-negative bacteria are bounded by an inner and outer membrane that encloses the periplasmic space. During vesiculation, the outer membrane pinches off (1), resulting in a closed proteoliposome composed of outer membrane lipids and proteins and periplasmic components, but not inner membrane or cytosolic components. Virulence factors such as VacA, shiga toxin, heat-labile enterotoxin (LT), leukotoxin, and ClyA are associated with vesicles from pathogenic bacteria (2, 4–6, 8–10).

Bacterial outer membrane vesicles interact with both eukaryotic cells and other bacteria via surface-expressed factors to deliver vesicle components and virulence factors (5, 6, 8, 11–16). For example, LT associated with lipopolysaccharide on the surface of enterotoxigenic *E. coli* (ETEC) vesicles triggers internalization via caveolae and delivers not only catalytically active LT, which intoxicates the eukaryotic cell, but also other bacterial vesicle components. Other studies have suggested that outer membrane invasins IpaB, C, and D may catalyze the internalization of *S. flexneri* vesicles (16).

To date, vesicle components have not been altered by genetic manipulation. Previous studies demonstrated that vesicles could be generated containing periplasmic gentamicin by treatment of cells with gentamicin, but they differed from native vesicles in their composition and size (15–17). Since vesicles are composed of outer membrane and periplasmic components, we hypothesized that expressed heterologous outer membrane and periplasmic proteins should be packaged into vesicles. Furthermore, we wanted to determine whether vesicle properties could be altered by the expression of proteins into the periplasm and outer membrane of bacteria. For instance, green fluorescent protein (GFP) transported to the periplasm and packaged in vesicles was used as a luminal vesicle marker, whereas vesicle incorporation of an outer membrane adhesin/invasin, Ail from *Yersinia enterocolitica*, could alter the adhesion and internalization properties of the vesicles. In this study, we demonstrate that Ail and periplasmic GFP are packaged in vesicles and that these altered vesicles can be used to track vesicle interactions with mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cell Culture—** *E. coli* strains DH5α (Stratagene), MC4100, and ETEC (ATCC strain 43886) were grown in LB or CFA broth (1% casamino acids, 0.15% yeast extract, 0.005% MgSO₄, and 0.005% MnCl₂), respectively. HB101/pVM102 (Ail) was kindly provided by Dr. Virginia Miller (18). Strains were grown in the presence of kanamycin (10 μg/ml) and/or ampicillin (100 μg/ml) as required. Human colorectal HT29 cells (ATCC HTB-38) were grown in McCoy’s 5a media supplemented with 10% bovine calf serum. CHO-K1 cells (ATCC CCL-61) were grown in Ham’s F12K media supplemented with 10% bovine calf serum. All cell lines were grown in the presence of penicillin/streptomycin/ampicillin B antibiotic-antimycotic solution (Sigma).

1 The abbreviations used are: LT, heat-labile enterotoxin; CHO, Chinese hamster ovary; ETEC, enterotoxigenic *E. coli*; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MBP, maltose binding protein; Omp, outer membrane protein; Tat, twin arginine transporter.

2 N. C. Kesty, K. M. Mason, and M. J. Kuehn, submitted for publication.
Heterologous Proteins in Bacterial Vesicles

**Plasmid Construction**—For pNKTAT2 (Tat-GFP), the T7 signal sequence and mutGFP3 sequence were cut out of pJDT1 (19) and inserted into pTrc99A (Amersham Biosciences) using EcoRI and HindIII. The mutGFP3 sequence was replaced with mutGFP2 (20) using MscI and HindIII. pNKTAT (TatABCE) was constructed by partial digestion of pBluescript (21) using EcoRI and HindIII and insertion into the multiple cloning site of pK187 (22). To induce Tat-GFP or TatABCE, overnight cultures were diluted 1:10 and grown at 37 °C for 6 h, unless otherwise mentioned, in the presence of 0.1 mM IPTG. Plasmids were transformed by electroporation into bacteria using a modified CaCl2 protocol (23).

**Vesicle Purification and Labeling**—Vesicles were purified as described previously (2) with the following modifications. Bacteria were pelleted (10 min, 10,000 × g), and vesicles were then pelleted from the supernatant (1 h, 40,000 × g), filter-sterilized (0.45 μm; Millipore), and applied to the bottom of a discontinuous Optiprep (Greiner) gradient (2 ml each of 50, 45, 40, 35, 30, and 25% Optiprep in 10 mM HEPES, pH 6.8, supplemented with 0.85% NaCl). Vesicle-containing fractions were combined, and protein concentration was determined using Coomassie Plus (Fisher). Vesicles were fluorescently labeled by diluting 1:1 with fluorescein isothiocyanate (FITC) (1 mg/ml in 50 mM Na2CO3, and 100 mM NaCl, pH 9.2) and incubated for 1 h at 25 °C. Vesicles were pelleted (52,000 × g, 30 min) and washed three times with sterile 50 mM HEPES, pH 6.8. FITC-labeled vesicles were resuspended in Dulbecco’s phosphate buffered saline supplemented with 0.2 μM NaCl and checked for sterility and protein concentration.

**Results**—To confirm that the 17-kDa band detected in DH5α:Ail vesicles by Coomassie stained SDS-PAGE was Ail, purified DH5α:Ail vesicles (15 μg) were loaded on a 15% SDS-PAGE and transferred to polyvinylidene difluoride, and the N-terminal sequence of the 17-kDa band was determined to be Ala-Ser-Glu-Asn-Ser-Ile-Ser-Ile (Tufts Core Facility).

**Cargo Quantitation**—To determine the percent of GFP or Ail packaged in vesicles, bacteria were pelleted from a culture grown either overnight (Ail) or induced for 6 h (Tat-GFP:pTatABCE). Vesicles were pelleted as described above and filter-sterilized. Bacteria and vesicles were precipitated with trichloroacetic acid (20% final concentration; 4 °C, 30 min), and the proteins were pelleted (10 min, 16,000 × g) and washed with acetone. Samples were then resuspended in 1% SDS in phosphate-buffered saline. Vesicle samples and dilutions of the bacteriapreparation (1:300 for Ail; 1:1000 for GFP) were analyzed by SDS-PAGE and blotted for GFP (rabbit anti-GFP; 1:1000), OmpFC (rabbit anti-OmpFC; 1:1000; MolecImaging), or maltose binding protein (rabbit anti-MBP; 1:6000; New England Biolabs), or E. coli outer membrane antigens (rabbit anti-OM; 1:1000) and detected using horseradish peroxidase-anti-rabbit IgG antibody (1:10,000), ECL reagents (Amersham Biosciences), and autoradiography.

**Negative Staining Electron Microscopy**—To visualize vesicles, samples were applied to carbon-coated 400-mesh copper grids (Electron Microscopy Sciences) and stained with 2% uranyl acetate.

**Quantitative Fluorescence Assay**—To quantitate the amount of vesicles bound by cells, a 96-well fluorescent assay was used. CHO cells were seeded at a concentration of 8 × 10^4 cells/well and incubated overnight (37 °C, 5% CO2) to allow cell adherence. Cells were washed with Hanks’ buffer (2×) and incubated with FITC-labeled vesicles (1 μg in serum- and antibiotic-free media (100 μl)). Following incubation, wells were washed with Hanks’ (2×) to remove unbound vesicles, and 100 μl of Hanks’ containing 1% Triton X-100 was added. Fluorescence was quantitated using a 96-well plate FLUOSTar Galaxy fluorometer (BMG Lab Technologies) with excitation at 485 nm and emission at 520 nm. Relative fluorescence units were converted to micrograms of vesicle protein using separate standard curves that established the relative fluorescence units per microgram values of FITC-DH5α vesicles and FITC-DH5α Ail vesicles (r = 0.99), which were present on each microtiter plate tested.

**Fluorescence and Immunofluorescence Microscopy**—CHO or HT29 (1.6 × 10^5 cells/well) were plated on Permanox microwell chamber slides (Nunc Inc.) and incubated (37 °C) with vesicles (1–5 μg/well) for various times in serum- and antibiotic-free media. Unbound vesicles were then removed, and the cells were washed. Cells were fixed with 4% paraformaldehyde. For immunofluorescence, cells were then permeabilized and blocked for 30 min with 0.1% Triton-X, 5% goat serum, and 0.1% bovine serum albumin. Cells were then incubated for 1 h with rabbit anti-GFP antibody (2.5 μg/ml) and visualized with rhodamine red-X-labeled goat anti-rabbit antibody (2.5 μg/ml; Jackson Immunoresearch Laboratories). Slides were mounted with a coverslip and ProLong Antifade kit (Molecular Probes), and the cells were observed using a Nikon Eclipse TE2000 laser-scanning confocal microscope.

**RESULTS**

**Ail Is Present in E. coli Vesicles**—To determine whether exogenous outer membrane proteins are secreted via vesicles, purified vesicles were prepared from several laboratory strains of E. coli transformed with a plasmid encoding the Y. enterocolitica outer membrane protein Ail under the control of its native promoter (18). Ail was detected in purified outer membrane preparations and in vesicles produced by DH5α:Ail (Fig. 1, A and B), MC4100:Ail, and HB101:Ail (data not shown), and its presence in vesicles was confirmed by N-terminal sequencing. Co-fractionation in Optiprep density gradients of Ail with OmpA and OmpF/C, outer membrane components of native

![Fig. 1. Ail is detected in the outer membrane and in vesicles produced by DH5α:Ail. Coomassie-stained SDSPAGE of 1-μg outer membrane (OM) fraction (A) and purified vesicles (B) from DH5α (1.5 μg) and DH5α:Ail (2 μg). Arrowheads indicate OmpF/C (39 kDa), OmpA (35 kDa), and Ail (17 kDa).](image-url)
vesicles (Fig. 2, A and B), showed that Ail was shed into the culture supernatant in association with other outer membrane vesicle components. Electron microscopy examination of vesicles in low density Optiprep fractions revealed that 90% were closed vesicles (Fig. 2, C and D), not membrane whirls or fragments of lysed vesicles. These data demonstrated that the heterologous outer membrane proteins expressed in E. coli are present in native-like vesicles.

To investigate the effect of a heterologous protein on yield, we compared vesicle production as a function of protein concentration per colony forming unit. After overnight growth, DH5α/Ail produced 2.3-fold more vesicles per colony forming unit than DH5α. A difference in vesicle density was not detected, because vesicles with Ail migrated to the same density fraction as DH5α vesicles (see peak vesicle fractions 4 and 5, Fig. 2, A and B). In addition, no distinguishable difference was detected in the vesicles by negative staining electron microscopy: DH5α vesicles ranged from 22–90 nm (Fig. 2C), and DH5α/Ail vesicles ranged from 22–77 nm (Fig. 2D) in diameter. Therefore, outer membrane vesicle density and size were unaltered due to the incorporation of a heterologous protein into the membrane, but vesicle yield increased.

To determine whether Ail was incorporated differently into vesicles than endogenous outer membrane cargo, we compared the amounts of Ail, OmpF/C, and OmpA packaged into vesicles (Fig. 3). Of the total amount in the bacteria, 0.32% of the Ail, 0.23% of OmpF/C, and 0.14% of the OmpA were packaged into vesicles, which falls in the previously reported range of protein packaged in E. coli vesicles (26, 27). In addition, the Omp to Ail ratio appeared constant in each fraction (0.5 ± 0.03 S.E., fractions 2–7; Fig. 2B). Therefore, heterologous Ail is neither selectively enriched nor selectively excluded from vesicles, and Ail appears to be included in every vesicle.

**Ail Induces Vesicle Internalization by Eukaryotic Cells**—Our previous work has demonstrated that LT, which is externally bound to vesicles because of its association with lipopolysaccharide, induces the association and internalization of vesicles by eukaryotic cells (2, 28). Because Ail is a known adhesin/invasin that can confer an invasive phenotype to HB101 (18), we wanted to determine whether Ail is able to catalyze the internalization of DH5α vesicles. We used fluorescently labeled vesicles to study cell association. Wild type DH5α vesicles displayed minimal eukaryotic cell association (Fig. 4A); however, the presence of Ail in vesicles increased cell association dramatically (Fig. 4B). Quantitation of cell-associated fluorescence revealed that the presence of Ail increased DH5α vesicle-cell association 10-fold (Fig. 4C), and that Ail-dependent vesicle-cell association was vesicle concentration-dependent (data not shown). Interestingly, the unbound DH5α/Ail vesicles removed from the eukaryotic cells contained Ail as shown by Coomassie staining (data not shown). This further supported the finding that Ail was present in every vesicle, because Ail would be expected to be depleted from this fraction if the vesicles contained heterogeneous cargo populations. These data demonstrate that an
exogenous outer membrane protein packaged in vesicles can alter the adherence properties of the vesicles.

**GFP Transport to the Periplasm Is Limited by Endogenous Levels of TatABCE**—Next we wanted to determine whether an exogenous periplasmic protein, which could be utilized in future studies as a lumenal vesicle marker, would also be packaged in vesicles. Due to the reducing environment of the periplasm, GFP transported via the Sec pathway is unable to fold in the periplasm (29); however, GFP folded in the cytoplasm can be transported to the periplasm by the twin arginine transporter system (Tat) using the Tat signal sequence (19, 31).

DH5α and 43886, a pathogenic E. coli strain, were transformed with a plasmid encoding IPTG-inducible GFP (20) fused to the Tat signal sequence (Tat-GFP). As shown previously for Tat-GFP expressed in MC4100 (19, 31), we found that induction of Tat-GFP expression increased the concentration of periplasmic GFP (data not shown). Although GFP-associated fluorescence was detectable, the amount of periplasmic GFP was saturable and could not be increased with longer induction (data not shown). The Tat machinery (TatABCE) has previously been shown to limit the transport of Tat substrate SufI into the periplasm (21, 32). To determine whether this was the limiting factor in Tat-GFP transport, bacteria expressing Tat-

GFP were transformed with a plasmid encoding IPTG-inducible TatABCE. Both periplasmic fluorescence (Fig. 5A) and immunoblot analysis for GFP (data not shown) revealed a significant increase of periplasmic GFP when exogenous TatABCE expression was induced.

Periplasmic GFP was detectable in DH5α and 43886 following induction with 0.1 mM IPTG for indicated times. C, anti-GFP (upper) and anti-MutL (lower) immunoblot analysis of periplasm and spheroplasts for 43886/Tat-GFP/TatABCE and spheroplasts for 43886 following induction as described for panel B. Positions of mature GFP (27 kDa) and MutL (68 kDa) are indicated.

**FIG. 5.** Tat-GFP is transported to the periplasm in DH5α and 43886. A, TatABCE increases Tat-GFP transport to the periplasm of DH5α. Data are represented as fold increase of fluorescence (RFU, relative fluorescence unit) in DH5α/Tat-GFP periplasm ± S.E. with induction of TatABCE using 0.1 mM IPTG for 6 h. Significant difference (*) was determined by Student’s t test (p < 0.001; n = 6). B, anti-GFP (upper) and anti-MutL (lower) immunoblot analysis of periplasm and spheroplasts for DH5α/Tat-GFP/TatABCE and spheroplasts for DH5α following induction with 0.1 mM IPTG for indicated times. C, anti-GFP (upper) and anti-MutL (lower) immunoblot analysis of periplasm and spheroplasts for 43886/Tat-GFP/TatABCE and spheroplasts for 43886 following induction as described for panel B. Positions of mature GFP (27 kDa) and MutL (68 kDa) are indicated.

**FIG. 4.** FITC-labeled DH5α/Ail vesicles associate with CHO cells. Confocal microscopy of CHO cells incubated for 3 h with FITC-DH5α (A) and FITC-DH5α/Ail (B) vesicles. Selected fields from top panels (box) are magnified in bottom panels. Magnification is indicated for pairs of upper and lower panels. C, quantitation of cell-associated vesicles (ng of vesicles per well) following a 3-h incubation of CHO cells with 5 μg of FITC-DH5α/Ail (gray) and FITC-DH5α (black) vesicles. Error bars are ± S.E. Significant difference (*) was determined by Student’s t test (p < 0.001, n = 2). Data are representative of two trials done in duplicate.
5, B and C). These results demonstrated a periplasmic localization of fluorescent GFP in laboratory and pathogenic strains of *E. coli* that is limited by the expression level of the Tat transporter.

**GFP in the Periplasm Is Packaged into Vesicles**—Because Tat-GFP was transported and properly folded in the periplasm, we wanted to determine whether it was packaged into vesicles. Vesicles were purified from cultures of strains expressing Tat-GFP with and without TatABCE after 6 and 12 h of induction. GFP was detectable in both DH5α- and 43886-derived vesicles when TatABCE was expressed (Fig. 6, A and B). Low levels of GFP were detected in the vesicles of 43886 cells expressing only endogenous levels of TatABCE (Fig. 6B). Furthermore, GFP cofractionated with vesicles on an Optiprep gradient during vesicle purification, as observed by the presence of OmpF/C, OmpA, and GFP in the same fractions (Fig. 7A, fractions 4–6). In contrast, soluble GFP and maltose-binding protein (MBP) (Fig. 7B, fractions 8 and 9) or GFP and MBP from lysed vesicles (data not shown) remained in the heavy fractions in an Optiprep gradient. These data showed that heterologously expressed periplasmic GFP copurified during vesicle purification is associated with intact *E. coli* vesicles.

43886 vesicles containing GFP were slightly more dense than those without GFP, because GFP and OmpF/C and OmpA were detected in more dense fractions of the gradient (Fig. 7A; compare Omp peak in fractions 4 and 5, middle panel, with peak in fraction 3, lower panel). Similar results were seen with DH5α vesicles containing GFP (data not shown). Although the difference in density due to the presence of lumenal GFP in vesicles suggested that the lipid to protein ratio was altered (Fig. 7A), we did not detect a significant difference in the size of vesicles purified from strains with and without Tat-GFP/TatABCE (Fig. 7, C and D). 43886 vesicles ranged from 43–165 nm and 43886/Tat-GFP/TatABCE ranged from 20–165 nm in diameter, whereas DH5α vesicles ranged from 22–90 nm and DH5α/Tat-GFP/TatABCE ranged from 25–90 nm in diameter. Note that native DH5α vesicles are approximately one-half the size of 43886 vesicles (Fig. 7, C and D, left panels). Unlike the effect of a heterologous outer membrane protein, inclusion of GFP did not affect bacterial vesicle yield.

We determined that GFP in the culture supernatant was not present as a result of reduced membrane integrity. Unlike bacterial cultures of strains expressing cytoplasmic GFP (data not shown), an RNase detection assay confirmed that bacteria expressing Tat-GFP/TatABCE were not leaking periplasm into the culture supernatant. We had previously determined that vesicles remain intact and do not leak periplasmic components when stored in 0.2 M NaCl or when osmotically buffered in Optiprep (data not shown). To establish whether GFP was a
lumenal component of vesicles rather than attached to their extracellular surface, Pronase was added to intact and solubilized vesicles. GFP was protected from degradation in unsolubilized vesicles, demonstrating that periplasmic GFP is in the lumen of 43886 vesicles (Fig. 8A). Similar results were observed with the endogenous lumenal vesicle component, MBP (Fig. 8A).

To determine whether GFP was incorporated into vesicles to the same extent as endogenous periplasmic cargo, we compared the amounts of GFP and MBP packaged in vesicles (Fig. 8B). Of the total amount in the bacteria, 0.1% of both GFP and MBP were packaged into 43886 vesicles. As with Ail, we were unable to detect any vesicles that did not contain GFP, and subsets of vesicles from 43886/Tat-GFP/TatABCE fractionated by density (lanes 3–6; Fig. 7A) all contained the same ratio of Omps to GFP. Therefore, periplasmic GFP is neither selectively enriched nor selectively excluded from vesicles, and GFP appears to be included in every vesicle.

**GFP-containing 43886 Vesicles Interact with Eukaryotic Cells**—In previous studies, we have examined the internalization of ETEC vesicles by human colorectal (HT29) cells. ETEC vesicles interact with HT29 cells via LT, and FITC-labeled outer membrane vesicle components other than LT were internalized. One goal of packaging GFP inside vesicles was to utilize GFP as a lumenal marker during internalization and trafficking experiments of ETEC vesicles. GFP-containing vesicles were fluorescent; however, the cell-associated fluorescence of GFP-containing vesicles was barely above background, unlike the amount of cell-associated fluorescence of vesicles externally labeled with FITC used in the quantitative assay for Ail-containing vesicles. The fluorescence of the GFP was also too low for confocal microscopy experiments, although punctate green staining was visible (data not shown). Nevertheless, we wanted to determine whether GFP, a lumenal vesicle compo-

![Image 9](image_url)

**Fig. 9.** GFP-containing 43886 vesicles associate with eukaryotic cells. Confocal microscopy of HT29 cells incubated for 8 h with 5 μg of GFP-containing 43886 (A) and DH5α (B) vesicles visualized using anti-GFP and a rhodamine-labeled secondary antibody. Selected cells from upper panels (boxed) are magnified in bottom panels. Magnification is indicated for pairs of upper and lower panels.

**DISCUSSION**

Vesicles are ubiquitously shed by Gram-negative bacteria and have been shown to be vehicles for intercellular transport. They are particularly significant to the study of pathogenic bacteria because they contain and deliver virulence factors to host cells. To understand vesicle formation and vesicle-mediated transport and trafficking inside the host cell, we need to manipulate both vesicle adhesins and vesicle content. Here we show that both membrane and lumenal protein contents of vesicles produced by laboratory and pathogenic strains of *E. coli* can be manipulated such that native vesicles contain active heterologous factors.

Ail is a protein from *Y. enterocolitica* that is abundantly expressed in the outer membrane of *E. coli*. We demonstrate that Ail is also packaged in *E. coli* vesicles without altering the size or shape of the vesicles. Ail expression allows HB101, a normally non-invasive *E. coli* strain, to invade CHO cells (18). Similarly, we found that Ail increased DH5α vesicle association with CHO cells and that the DH5α/Ail vesicles appeared by microscopy to be internalized. Thus, engineered vesicles can be used to investigate host cell association and trafficking mediated by specific outer membrane protein adhesins that are active and presented in their native membrane environment. Although whole bacteria pathogens may activate potentially interesting regulatory effects, vesicles from engineered strains can provide a valuable, novel tool in studying a specific outer membrane protein in its native, membrane context. Vesicles provide a “constant expression” level of an Omp, which can be particularly useful in its initial characterization, whereas changes in the bacterial outer membrane composition induced in response to host cells could confuses results. Thus,
Heterologous Proteins in Bacterial Vesicles

2075

cells by amplifying GFP detection with antibodies to GFP, showing that GFP can be used as a luminal marker protein. More importantly, this result demonstrates that ETEC vesicles carry luminal components into eukaryotic cells.

In eukaryotic cells, signal sequences or protein modifications are necessary signals for protein sorting into different vesicles and organelles. For example, the endoplasmic reticulum retention signal sequence, KDEL, signals for retrograde transport from the Golgi to the ER (34), whereas exosomes are enriched in ubiquitinated proteins (35, 36). Bacterial protein secretion through the inner membrane, such as by the Tat and the sec pathways, also utilizes signal sequences (19, 37, 38). Currently, little is known about protein sorting into outer membrane vesicles produced by bacteria. Previous studies have reported that certain proteins such as LT, leukotoxin, and ClyA are enriched in vesicles, whereas other periplasmic proteins, such as DsbA, are excluded, suggesting that vesiculation is an active and directed process (2, 6, 8). In this study, the percentage of Ail or GFP packaged in vesicles corresponded to the amount of endogenous outer membrane and periplasmic components packaged in vesicles as well as to previous reports of the percentage of protein packaged in E. coli vesicles (26, 27). Presumably, GFP would not contain a vesicle “sorting sequence,” since it is produced by the jellyfish Aequorea victoria and is not a native bacterial protein. These data suggest that a cargo tag is not necessary for protein sorting into bacterial vesicles. Instead, we propose that the previously described toxin enrichment in vesicles may be due to cell envelope “hot spots” for vesicle budding. These enriched areas may occur due to the location of protein secretion machinery, such as the type II secretion machinery necessary for LT secretion through the inner and outer membranes (28, 30, 39).

In conclusion, heterologous outer membrane and periplasmic proteins, such as Ail and periplasmic GFP, are packaged in vesicles and can be used to modify the characteristics of the vesicle. Outer membrane and external proteins such as Ail and LT can be used to direct the interactions of vesicles with eukaryotic cells, and GFP and other luminal vesicle cargo can be used to track soluble transported cargo. Together, these tools provide valuable methods to study the nature of vesicle-mediated transport.

Acknowledgments—We thank S.J. Bauman for the electron microscopv, J. Qi for help with transformations and vesicle purifications, V. Miller for HB101pVM102, C. Robinson for plasmid pJD1T1, B. Cormack for plasmid pEGFP2, T. Yah for plasmid pTatABCE, M. Jobling and R. Holm for plasmid pK86, Y. Takada for anti-OM antibody, P. Moro for anti-MutL antibody, P. Silver for anti-GFP antibody, S. Abrahams for confocal microscopy facilities, and M. Reedy and C. Lucaveche for electron microscopy training and facilities.

REFERENCES

1. Beveridge, T. J. (1999) J. Bacteriol. 181, 4725–4733
2. Horstman, A. L., and Kuehn, M. J. (2000) J. Biol. Chem. 275, 12489–12496
3. Devee, I. W., and Gilchrist, J. E. (1973) J. Exp. Med. 138, 1156–1167
4. Krieger, C., Jey, T., Neal, B., Perez-Perez, G., Allardaye, R., and Bagshaw, P. (2000) FEMS Microbiol. Lett. 182, 259–264
5. Picaux, R., Neechi, V., Sommi, P., Ricci, V., Telford, J., Cover, T. L., and Solcia, E. (1999) J. Pathol. 188, 229–236
6. Kato, S., Kowashi, Y., and Demuth, D. R. (2002) Microb. Pathog. 32, 1–13
7. Wai, S. N., Takade, A., and Amako, K. (1995) Microbiol. Immunol. 39, 451–456
8. Wai, S. N., Lindmark, B., Soderholm, T., Takade, A., Westermark, M., Oscarsson, J., and Jass, J. Richter-Dahlfors, A., Minuzo, Y., and Uhl, B. R. (2003) Cell 115, 25–35
9. Yokoyama, K., Horii, T., Yamashino, T., Hashikawa, S., Barua, S., Hasegawa, T., Watanabe, H., and Ohta, M. (2000) FEMS Microbiol. Lett. 192, 139–144
10. Kolling, G. L., and Matthews, K. R. (1999) Appl. Environ. Microbiol. 65, 1843–1848
11. Demuth, D. R., James, D., Kowashi, Y., and Kato, S. (2003) Cell Microbio 5, 111–121
12. Li, Z., Clarke, A. J., and Beveridge, T. J. (1998) J. Bacteriol. 180, 5478–5483
13. Beerheim, C., Wunderli-Allenspach, H., Groscurth, P., and Filgueira, L. (2000) Cell. Microbiol. 2, 124–131
14. Shoberg, R. J., and Thomas, D. D. (1993) Infect. Immun. 61, 3892–3900
15. Kadaruragamuwa, J. L., and Beveridge, T. J. (1996) J. Bacteriol. 178, 2767–2774
Heterologous Proteins in Bacterial Vesicles

16. Kadurugamuwa, J. L., and Beveridge, T. J. (1998) Antimicrob. Agents Chemother. 42, 1476–1483
17. Kadurugamuwa, J. L., and Beveridge, T. J. (1995) J. Bacteriol. 177, 3996–4008
18. Miller, V. L., and Falkow, S. (1988) Infect. Immun. 56, 1242–1248
19. Thomas, J. D., Daniel, R. A., Errington, J., and Robinson, C. (2001) Mol. Microbiol. 39, 47–53
20. Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996) Gene 173, 33–38
21. Yah, T. L., and Wickner, W. T. (2001) EMBO J. 20, 2472–2479
22. Jobling, M. G., and Holmes, R. K. (1990) Nucleic Acids Res. 18, 5315–5316
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 1.82–1.84, Cold Spring Harbor Press, Cold Spring Harbor, NY
24. Nikaido, H. (1994) Methods Enzymol. 235, 225–234
25. Bernadac, A., Gavioli, M., Lazzaroni, J. C., Raina, S., and Lloubes, R. (1998) J. Bacteriol. 180, 4872–4878
26. Mug-Opstelten, D., and Witholt, B. (1978) Biochim. Biophys. Acta 508, 287–295
27. Hoekstra, D., van der Laan, J. W., de Leij, L., and Witholt, B. (1976) Biochim. Biophys. Acta 455, 889–899
28. Horstman, A. L., and Kuehn, M. J. (2002) J. Biol. Chem. 277, 22538–22545
29. Feilmeier, B. J., Iseminger, G., Schroeder, D., Webber, H., and Phillips, G. J. (2000) J. Bacteriol. 182, 4068–4076
30. Tauschek, M., Gorrell, R. J., Strugnell, R. A., and Robins-Browne, R. M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7066–7071
31. Santini, C. L., Bernadac, A., Zhang, M., Chanal, A., Ize, B., Blanot, C., and Wu, L. F. (2001) J. Biol. Chem. 276, 8159–8164
32. Sargent, F., Gobert, G., De Leeuw, E., Stanley, N. R., Palmer, T., Saibil, H. R., and Berks, B. C. (2001) Eur. J. Biochem. 268, 3361–3367
33. Stanley, N. R., Findlay, K., Berks, B. C., and Palmer, T. (2001) J. Bacteriol. 183, 139–144
34. Pelham, H. R. (1996) Cell Struct. Funct. 21, 413–419
35. Stoorvogel, W., Kleijmeer, M. J., Geuze, H. J., and Raposo, G. (2002) Traffic 3, 321–330
36. Thery, C., Zitvogel, L., and Amigorena, S. (2002) Nat. Rev. Immunol. 2, 569–579
37. Pugsley, A. P. (1990) Microbiol. Rev. 57, 50–108
38. Lory, S. (1998) Curr. Opin. Microbiol. 1, 27–35
39. Scott, M. E., Dossani, Z. Y., and Sandkvist, M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13978–13983