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A. E. Ballok
Dartmouth College

L. M. Filkins
Dartmouth College

J.. M. Bomberger
Dartmouth College

B. A. Stanton
Dartmouth College

George A. O'Toole
Dartmouth College

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Epoxide-Mediated Differential Packaging of Cif and Other Virulence Factors into Outer Membrane Vesicles

Alicia E. Ballok, Laura M. Filkins, Jennifer M. Bomberger, Bruce A. Stanton, George A. O'Toole

Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire, USA

Pseudomonas aeruginosa **produces outer membrane vesicles (OMVs) that contain a number of secreted bacterial proteins, including phospholipases, alkaline phosphatase, and the CFTR inhibitory factor (Cif). Previously, Cif, an epoxide hydrolase, was shown to be regulated at the transcriptional level by epoxides, which serve as ligands of the repressor, CifR. Here, we tested whether epoxides have an effect on Cif levels in OMVs. We showed that growth of *P. aeruginosa* in the presence of specific epoxides but not a hydrolysis product increased Cif packaging into OMVs in a CifR-independent fashion. The outer membrane protein, OprF, was also increased under these conditions, but alkaline phosphatase activity was not significantly altered. Additionally, we demonstrated that OMV shape and density were affected by epoxide treatment, with two distinct vesicle fractions present when cells were treated with epibromohydrin (EBH), a model epoxide. Vesicles isolated from the two density fractions exhibited different protein profiles in Western blotting and silver staining. We have shown that a variety of clinically or host-relevant treatments, including antibiotics, also alter the proteins packaged in OMVs. Proteomic analysis of purified OMVs followed by an analysis of transposon mutant OMVs yielded mutants with altered vesicle packaging. Finally, epithelial cell cytotoxicity was reduced in the vesicles formed in the presence of EBH, suggesting that this epoxide alters the function of the OMVs. Our data support a model whereby clinically or host-relevant signals mediate differential packaging of virulence factors in OMVs, which results in functional consequences for host-pathogen interactions.**

Pseudomonas aeruginosa is an opportunistic pathogen that employs multiple mechanisms of virulence factor delivery. These mechanisms include secretion into the milieu via numerous secretion systems (1) and direct injection into the host by way of the type III secretion system (2). A third mechanism, virulence factor delivery via outer membrane vesicles (OMVs) (3), was identified late last century, yet the significance of this delivery mechanism has only recently been recognized.

Production of OMVs appears to be a conserved physiological trait among Gram-negative bacteria (4). These versatile vesicles have been shown to contribute to biofilm formation, bacterial defense against toxic compounds, horizontal gene transfer, pathogenic and nonpathogenic interactions, and other bacterial processes (5–10). While bacteria seem to produce vesicles as part of normal growth (11), they also hypervesiculate in response to stressors such as antibiotics, toxic compounds, and membrane perturbations (12–14). Hypervesiculation may serve as an important mechanism of stress tolerance under some conditions (15). It is still not clear whether vesicle formation and packaging of cellular proteins constitute a directed or stochastic process (4).

OMV production is better characterized in *P. aeruginosa* than in many other species. Vesicle formation in this species has been attributed to specific forms of negatively charged lipopolysaccharide (LPS) (16), the membrane-intercalating quorum-sensing molecule *Pseudomonas* quinolone signal (PQS) and its interaction with lipid A (17, 18), and loss of peptidoglycan anchoring by outer membrane proteins (19). Previous work has shown that *P. aeruginosa* OMVs are damaging to epithelia, being both inflammatory and cytotoxic (6, 20). Additionally, bacterial factors important for virulence, such as LPS, phospholipases, proteases, hemolysin, alkaline phosphatases, and the CFTR inhibitory factor, Cif (16, 21), have been identified in OMVs. Furthermore, these vesicles can fuse directly with the eukaryotic membrane to deliver their contents to the host cell cytoplasm (6).

The Cif protein was discovered based on its ability to alter trafficking of CFTR, a mammalian ABC transporter whose loss of function results in the disease cystic fibrosis (CF) (22). Cif, an epoxide hydrolase enzyme, alters the recycling of CFTR to the apical membrane and instead targets CFTR for degradation, although the precise mechanism by which this bacterial protein exerts its effect has not yet been determined (23). In addition to effects on CFTR, Cif induces the degradation of transporter associated with antigen processing 1 (TAP1), which reduces major histocompatibility complex (MHC) class I antigen presentation (24).

Previously, we have shown that production of Cif by *P. aeruginosa* is regulated directly by a TetR-family regulator, CifR (25). The CifR protein binds to two locations in the *cif* promoter to repress transcription (26). In the presence of specific epoxide inducers, CifR presumably binds to these ligands and releases repression of *cif* gene expression. Deletion of this epoxide-responsive regulator dramatically increases *cif* gene expression by >200-fold (26).

Epoxides, while potentially toxic, are produced as a by-product of metabolism in eukaryotes and play key roles in mammalian signaling. For example, epoxide-containing fatty acids are important anti-inflammatory molecules in mammals that inhibit NF-κB activation and also promote tissue regeneration (27, 28). Furthermore, human-derived epoxides are produced by cells such

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Address correspondence to George A. O'Toole, georgeo@dartmouth.edu.

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as leukocytes in the lung (29), a common site of *P. aeruginosa* infections (30).

In this study, we aimed to explore whether epoxides affect packaging of Cif and other proteins into OMVs. The data presented here support our hypothesis that *P. aeruginosa* OMV production is altered by the presence of epoxides and likely other extracellular signals, lending support to the concept of selective packaging of OMV cargo. Furthermore, we propose that that modulation of OMV cargo has functional consequences for host-pathogen interactions and thus may represent an underappreciated target for antimicrobial interventions.

MATERIALS AND METHODS

Strains. Experiments were performed with *P. aeruginosa* UCBPP-PA14 (31), abbreviated here as *P. aeruginosa* PA14, the *cif* and *cifR* gene deletion mutants (21, 25), and transposon mutants from the nonredundant *P. aeruginosa* PA14 transposon mutant library developed by Ausubel and colleagues (32).

OMV purification. Vesicles were purified as described previously with some modifications (20). Briefly, flasks containing lysogeny broth (LB) medium were inoculated at 1:1,000 with overnight cultures of *P. aeruginosa* PA14 and grown with shaking (200 RPM) at 37°C for 12 h. A 1 mM concentration of epibromohydrin (EBH), styrene oxide, epoxyhexane, glycidol, or 3-bromo-1,2-propanediol was added, as appropriate. Cells were pelleted by centrifugation, and supernatants were passed through a 0.45-μm filter. To isolate OMVs, filtered supernatants were concentrated with a 100,000-molecular-weight-cutoff (MWCO) filter (Amicon) and then centrifuged for 1.5 h at 39,000 × *g* to pellet vesicles. Supernatants were removed, and the vesicle pellet was resuspended by pipetting after approximately 30 min of incubation either in phosphate-buffered saline (PBS), followed by use in Western blotting and enzyme assays since additional purification was not required for these assays, or in 50 mM HEPES buffer (pH 6.8), which loosened the OMV pellet, and layered in an Optiprep (iodixanol) gradient (Sigma) (20) for further purification. OMVs layered on the Optiprep gradient were centrifuged for 16 to 20 h at 100,000 × *g*, and fractions containing vesicles based on previous analysis (20) were removed. Vesicles were washed several times in PBS using a 10,000-MWCO filter (Amicon) to remove Optiprep solution.

Cell fractionation. Vesicle-free supernatants were collected after following the OMV concentration protocol (as described above), and these supernatants were concentrated with a 10,000-MCWO filter. Periplasmic and spheroplast sample preparation was based on methods of Jensch and Fricke (33). Periplasmic fractions were obtained by resuspending pelleted *P. aeruginosa* in a solution containing 50 mM Tris HCl (pH 8.0), 200 mM $MgCl_2$, 5 mg/ml lysozyme, and protease inhibitor (Roche) for 30 min at room temperature. Spheroplasts were separated from the periplasmic contents by centrifugation at 11,000 × *g* for 10 min, and then the spheroplasts, consisting of the inner membrane and cytoplasm, were resuspended in 10 mM Tris HCl (pH 7.5) plus 50 mM $MgCl_2$ and lysed by sonication.

Protein quantitation. Protein content of the vesicle and cell fractions were determined using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific), using bovine serum albumin (BSA) as a standard and following the manufacturer's instructions.

MUP assay. 4-Methylumbelliferyl phosphate (MUP) was used to quantify alkaline phosphatase activity, based on methods described previously (34). Briefly, 20 μg of PBS-resuspended vesicles were added to 100 mM Tris-HCl (pH 7.5) and 1 μM MUP and then incubated at 37°C with periodic shaking. Fluorescence was measured on a Spectramax plate reader (Molecular Devices) at 390-nm excitation and 460-nm emission.

NPPC assay. Phospholipase activity was measured using the *p*-nitrophenylphosphorylcholine (NPPC) colorimetric assay (35). Briefly, vesicles in PBS were normalized to 20 μg protein, or alternatively, 12-h culture supernatants were used as a source of OMVs, as needed for each experiment. OMVs were added to a solution containing 2 mM NPPC and 60% (wt/vol) sorbitol in 100 mM Tris-HCl (pH 7.5) in a 96-well plate. The plate was incubated at 37°C with periodic shaking, and absorbance was measured (410 nm) in a Spectramax plate reader. Where appropriate, vesicles were permeabilized with chloroform and SDS.

Protein detection. For detection of proteins from OMVs and cellular fractions, 50 μg total protein was added per well, unless otherwise stated, and electrophoresed on a 4 to 15% or 12% SDS-PAGE gel (Bio-Rad). Cif, OprF, and SadB were detected by Western blotting as described previously (20, 26, 36). Silver staining was performed using the Silver Quest kit (Novex) according to the manufacturer's instructions.

Proteomics. OMVs were isolated for proteomic analysis from three preparations of OMVs from *P. aeruginosa* PA14 and from preparations of OMVs obtained from two clinical isolates using a previously published protocol (6). In brief, LB was inoculated with *P. aeruginosa* PA14 from a glycerol stock and incubated at 37°C using rotation until the bacterial count reached an optical density at 600 nm of 1.5, corresponding to a bacterial count of 5×10^9 CFU/ml (16 to 18 h of culture). Bacteria were harvested by centrifuging cultures at 4,800 × *g* for 30 min at 4°C. Filtered supernatant was then concentrated with a 30-kDa cutoff filter, and OMVs were pelleted and placed in an Optiprep gradient. Gradients were centrifuged at 100,000 × *g* for 16 h at 4°C. After ultracentrifugation, OMVs were removed from the gradient and proteins were precipitated using trichloroacetic acid precipitation. Precipitated proteins were resuspended in Laemmli sample buffer containing 200 mM dithiothreitol and heated for 5 min at 85°C. Samples were then separated using SDS-PAGE and stained with colloidal Coomassie stain (Invitrogen). After overnight destaining in water, gel lanes were divided into 4 sections, excised, and analyzed for protein content using microcapillary liquid chromatography-tandem mass spectrometry (LC/MS/MS) at the Taplin Mass Spectrometry Facility at Harvard University.

For identification of proteins in untreated and EBH-treated fractions, lysed OMV samples were separated by SDS-PAGE in a 4 to 15% gel and silver stained. Bands were excised and destained before trypsin digestion. Samples were analyzed by LC-MS/MS on a linear ion trap (LTQ) mass spectrometer. Five microliters of the material was loaded onto a 100-μm by 120-mm capillary column packed with Magic C_{18} (5-μm particle size, 20-nm pore size, Michrom Bioresources, California) at a flow rate of 500 nl/min. Peptides were separated by a gradient of 5 to 40% CH_3CN–0.1% formic acid over 30 min, 40 to 100% CH_3CN–0.1% formic acid over 6 min, and 100% CH_3CN for 2 min. Product ion spectra were searched using the SEQUEST search engine on Bioworks Browser Rev 3.3.1 SP1 (Thermo Fisher Scientific, Massachusetts) against a *P. aeruginosa* database with sequences in forward and reverse orientations. The database was indexed to allow for full trypsin enzymatic activity, two missed cleavages, and peptides between the molecular masses of 350 and 5,000 kDa. Search parameters set the mass tolerance at 2 Da for precursor ions and 0.8 Da for fragment ions. Cross-correlation (Xcorr) significance filters were applied to limit the false-positive rates to less than 1% in both data sets. The Xcorr values were as follows: (+1), 1.5; (+2), 2.0; (+3), 2.5; (+4), 3.0. Other filters applied were a minimum peptide cutoff of 2, as well as a DeltaCN value (the difference between normalized Xcorr functions) of >0.1. Mass spectroscopic analysis of trypsin-digested proteins was performed at the Vermont Genetics Network Proteomics Facility at the University of Vermont.

TEM of OMVs. Purified vesicles in PBS were spotted onto Formvar grids (Electron Microscopy Sciences) and negatively stained with 1% ammonium molybdate (pH 7.0). Transmission electron microscopy (TEM) images were obtained at the Rippell Electron Microscopy facility at Dartmouth College, at 100 kV, on a FEI Tecnai F20ST field emission gun (FEG) instrument, equipped with a digital camera (XR-41B; Advanced Microscopy Techniques). Eight images were obtained for each condition, randomized, numbered, and given to a third party for vesicle measurement. Vesicle area was quantified as number of pixels per vesicle (ImageJ software).

ELISA and cytotoxicity assays. CFBE41o− cells (ΔF508/ΔF508), derived from a CF patient, were grown to confluence in 24-well plates as described previously (54). Purified OMVs from wild-type (WT) cultures grown with or without 1 mM EBH were added to washed epithelial cells as described previously (6) and incubated for 12 to 24 h at 37°C with 5% CO_2. After incubation, cell culture medium was removed and centrifuged at 13,000 × *g* for 3 min to remove debris. The cytokines interleukin 6 (IL-6), IL-8, Groα, and Groβ were measured in the supernatants by enzyme-linked immunosorbent assay (ELISA) (Promokine), and cytotoxicity was assessed by measuring lactate dehydrogenase (LDH) release using the Cyto Tox 96 nonradioactive cytotoxicity assay (Promega) per the manufacturer's instructions.

Membrane lipid quantification. Vesicle lipids were quantified using FM 4-64 (Invitrogen) as described previously (11). Briefly, vesicles from equivalent volumes of triplicate cultures were incubated with fluorescent dye at a final concentration of 3.3 μg/ml in PBS for 20 min at 37°C, and fluorescence was measured at an excitation wavelength of 506 nm and an emission wavelength of 750 nm.

Growth assay. Overnight cultures of *P. aeruginosa* were subcultured (1:100) into culture tubes containing 1 mM epoxide in 5 ml LB. Cultures were grown at 37°C, and optical density was measured at 600 nm every 30 min for 3.5 h.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays (EMSA) were performed as described previously (25), with the following specifications: purified CifR (160 fmol) was coincubated with 20 fmol of a 5′ biotinylated oligonucleotide (5′CCTCCATTATTTGTAT CGATCACTATAAATTTACTT [26]) for 30 min at room temperature in a volume of 10 μl. EBH, epoxyhexane, or glycidol (1 mM) was added as needed.

cDNA synthesis and qRT-PCR. cDNA was synthesized from 1 μg total RNA isolated from *P. aeruginosa* PA14 using the QuantiTect reverse transcription kit (Qiagen), following prescribed protocols. Quantitative reverse transcription-PCR (qRT-PCR) was performed using cDNA, primers designed for the *plcN*, *katA*, *sodM*, *lexA*, *algD* and *rplU* genes (see Table S1 in the supplemental material), a transcriptional control (37), and Maxima SYBR green qPCR master mix (Fermentas). A Bio-Rad iCycler instrument was used to carry out the reactions, and data analysis was performed using CFX manager software (Bio-Rad). Data are expressed as picograms input cDNA of gene of interest relative to the *rplU* gene transcript control.

RESULTS

Epoxides affect Cif packaging in a CifR-independent fashion. We have previously shown that Cif is packaged into OMVs (21) and that epoxides such as epibromohydrin (EBH) induce expression of Cif through derepression of CifR, a transcriptional regulator (25). We questioned whether epoxides also impacted the amount of the Cif protein packaged into these vesicles. To this end, we grew wild-type *P. aeruginosa* PA14 and the Δ*cifR* mutant in the presence and absence of 1 mM EBH, as described previously (21, 25, 26). We showed previously that this concentration of EBH did not alter the growth of *P. aeruginosa* compared to results with the vehicle control (dimethyl sulfoxide [DMSO]) (26). Cultures were fractionated into supernatant, OMV, periplasmic, and spheroplast fractions (Fig. 1A), concentrated such that 50 μg total protein is loaded per well, and the Cif protein was detected by Western blotting. SadB, a protein previously shown to be cytoplasmically localized (21), served as a control to demonstrate a lack of cytoplasmic contamination from the spheroplast fraction or from the periplasmic fraction (see Fig. S1A in the supplemental material).

Low levels of Cif were noted in all of the fractions from the wild type grown in the absence of EBH, and the level of the Cif protein

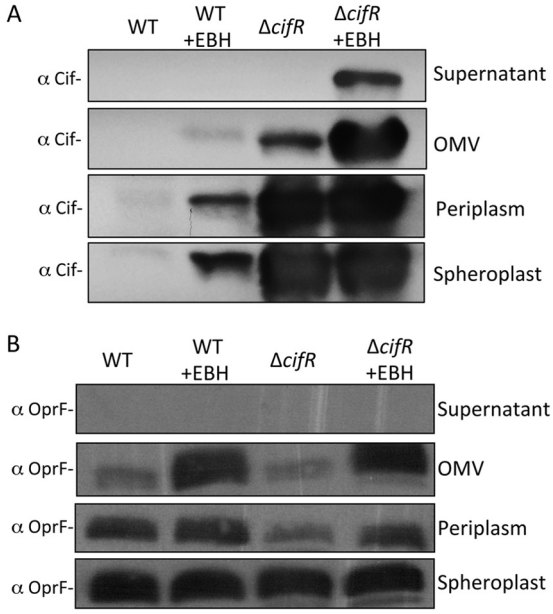

FIG 1 Cif and OprF levels change in response to epoxides. (A) Western blot of fractions of cultures grown overnight in the presence and absence of 1 mM epibromohydrin (EBH), a model epoxide. The *cifR* deletion strain shows increased levels of the Cif protein in the spheroplast and periplasmic fractions due to loss of CifR-mediated repression, as reported elsewhere (26). Note the increase in the Cif protein in the supernatant and OMV fractions for cells grown in the presence of EBH. (B) Western blot of the same samples as in panel A, probed with an OprF antibody. Note that OprF is present in the vesicle fractions and increases in those samples with EBH.

was increased in all fractions isolated from cells grown in the presence of EBH. As expected, the *cifR* deletion mutant had much more Cif in the spheroplast and periplasmic fractions due to loss of CifR-mediated repression, but surprisingly, there was relatively little Cif in the OMV and supernatant fractions given the large amount of Cif produced in the cell-associated fractions. However, in the presence of EBH, a large quantity of the Cif protein was observed in the supernatant and OMV fractions (Fig. 1A). These data suggest that EBH increased Cif packaging into OMVs, and perhaps secretion of the protein, and did so in a CifR-independent manner.

OprF increases in EBH-treated vesicles. OprF is an outer membrane porin previously shown to be important for virulence in an animal model (38), and loss of the corresponding gene was recently shown to increase vesiculation in *P. aeruginosa* (19). Thus, we decided to test whether packaging of this protein into OMVs is affected by the addition of epoxide. In the presence of epoxide, the amount of OprF produced did not change in the periplasmic and spheroplast fractions but did increase to a similar extent for both the WT and the Δ*cifR* mutant in the OMV fraction (Fig. 1B).

The increased OprF and Cif in EBH-treated OMVs suggested that there may be an interrelationship between these two proteins in regard to vesicle packaging. We tested whether mutations of either protein affected packaging of the other. Wild-type and Δ*cif* mutant vesicles purified from cultures grown with and without EBH had similar levels of the OprF protein (see Fig. S1B in the supplemental material). Similarly, OMV levels of the Cif protein remained unchanged in an *oprF* transposon mutant (see Fig.

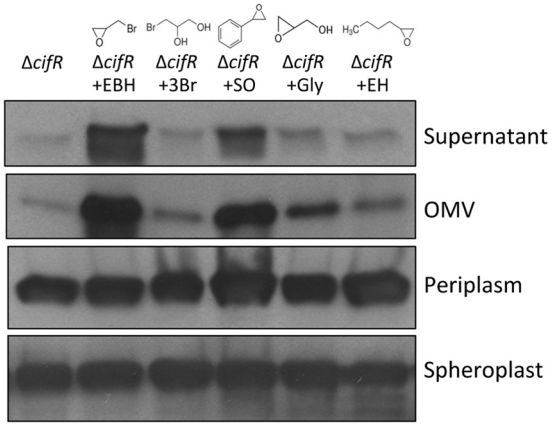

FIG 2 Other epoxides affect Cif packaging. Effects of 3-bromo-1,2-propanediol (3Br) (an EBH hydrolysis product), styrene oxide (SO), glycidol (Gly), and epoxyhexane (EH) on Cif packaging into vesicles was assessed by Western blotting. Chemical structures are depicted at the top. Levels of Cif under the glycidol- and EH-treated conditions are less robust than those observed for the EBH-treated cells; however, the levels of Cif in glycidol- and EH-treated cells are higher than that of OMVs isolated from untreated bacterial cultures.

S1C). These data indicate that while Cif and OprF proteins appear to show similar responses to EBH treatment, they are not dependent on one another for this effect.

Other epoxides alter proteins in vesicles. Previously, we had shown that there was some specificity in epoxide inducers of *cif* gene expression (26). It follows that there may be some specificity with regard to epoxides that induce OMV protein changes. To test this idea, we purified OMVs from the *cifR* deletion strain grown with three epoxides shown to have various effects on Cif protein expression in whole cells (26): styrene oxide (a strong inducer), epoxyhexane and glycidol (both weaker inducers), and the vicinal diol, hydrolysis product of EBH, which does not alter *cif* gene expression (26).

Styrene oxide (SO) had a strong stimulatory effect on Cif protein abundance in vesicles, although slightly less than the effect noted for EBH (Fig. 2). Epoxyhexane (EH) and glycidol (Gly) also increased vesicle packaging in a *cifR* deletion mutant, although to levels far lower than those with EBH (Fig. 2). The vicinal diol of EBH, 3-bromo,1,2-propandiol (3Br), did not dramatically increase packaging of Cif into the OMVs, showing that it is the epoxide rather than its hydrolysis product that induces the response. A growth curve revealed that none of these compounds altered the growth rate in liquid culture (see Fig. S2A in the supplemental material).

As mentioned above, the epoxides EH and Gly tested above could weakly induce expression of the *cif* gene (26). This weak induction could be due to the fact that these compounds may be poor ligands for CifR binding. Thus, we tested whether these weaker inducers could reduce CifR binding to DNA *in vitro*, as shown previously for EBH (25, 26). As determined using EMSA, both glycidol and epoxyhexane do have a minimal effect on CifR-DNA binding, although much less than that with EBH (see Fig. S2B in the supplemental material). These data would support the notion that, as predicted, these small hydrophobic epoxides likely diffuse into the cell but have a small effect on Cif expression due to their limited ability to act as a CifR ligand.

Taken together, these data show that there is specificity in the

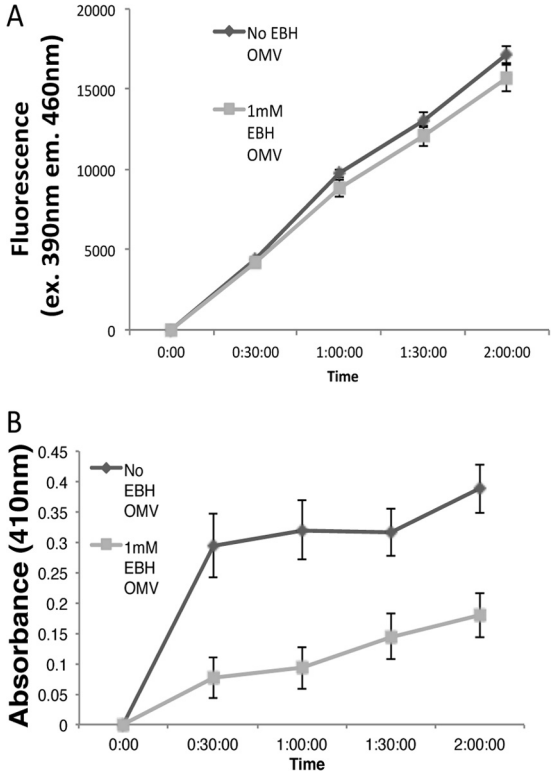

FIG 3 Epoxides alter virulence factor activity of OMVs. (A) Alkaline phosphatase activity associated with OMVs from EBH-treated and untreated bacterial cells. OMVs were normalized by protein concentration, and fluorescence of the 4-methylumbelliferyl phosphate cleavage product was measured over 2 h to evaluate phosphatase activity. No statistical difference between untreated and EBH-treated samples was identified at any time point ($P > 0.05$). Error bars indicate SEM ($n = 3$). (B) Analysis of phospholipase activity of protein-normalized OMVs from untreated and EBH-treated bacterial cells, as measured by colorimetric detection of NPPC cleavage. Error bars indicate SEM ($n = 3$).

epoxides that can mediate changes in OMV protein packaging, with EBH and SO having a strong impact on altering OMV packaging of Cif and the related epoxides EH and Gly having little impact on packaging of Cif.

Epoxide addition reduces phospholipase packaging but does not alter alkaline phosphatase packaging in OMV. Since we had already observed that epoxide treatment alters packaging into OMVs of the OprF and Cif proteins, we decided to test whether other well-documented, OMV-associated proteins are impacted by the addition of EBH (6). We tested the alkaline phosphatase activities of vesicles produced with and without EBH and found no significant difference (Fig. 3).

Next, we assessed the phospholipase activity of these OMVs and found a dramatic reduction in activity upon EBH treatment (Fig. 3B). This effect was not due to EBH inhibition of phospholipase activity, since vesicles incubated with EBH after formation show as much phospholipase activity as untreated vesicles (data not shown). The reduction in activity could not be explained by effects of the epoxide on permeability of the vesicle, since permeabilizing the OMVs did not alter vesicle phospholipase activity (see Fig. S3A in the supplemental material). We also tested culture supernatants to measure phospholipase activity *in toto* and found that there was less activity (see Fig. S3B). qRT-PCR analysis of the

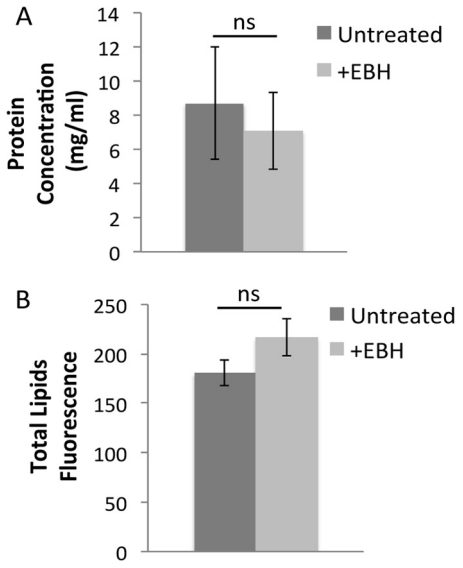

FIG 4 Total protein content and membrane lipids in OMVs are not affected by EBH. (A) BCA measurement of total protein from OMVs isolated from 12 h cultures of *P. aeruginosa* grown in the absence (untreated) or presence of 1 mM EBH. (B) FM 4-64 quantification of the same OMV preparations as in panel A. Error bars indicate SD ($n = 3$).

phospholipases that contribute to this activity indicated no significant decrease in transcript abundance at 12 h (see Fig. S3C).

These data show that unlike the case with Cif and OprF, whose levels in OMVs are increased after epoxide treatment, phospholipase activity decreased. In contrast, not all virulence factors are affected by epoxide treatment, since alkaline phosphatase activity is unchanged in OMVs when cells are treated with EBH. Thus, our findings indicate a degree of specificity in response to epoxide treatment, a point addressed in more detail below.

EBH does not induce hypervesiculation. Treatment of bacterial cells with compounds such as antibiotics, as well as physiochemical stressors, induces hypervesiculation (7). Other groups have tested whether various treatments or mutations alter vesiculation by measuring membrane lipids and protein content of purified vesicles (11, 39). We tested whether epoxides cause hypervesiculation using these reported methods. The protein and membrane lipid levels of the vesicles from EBH-treated cultures were not significantly different from those of untreated cultures (Fig. 4A and B). These data suggest that *P. aeruginosa* does not increase vesicle production in the presence of epoxides.

Vesicle shape and density are altered by EBH. Previous studies with *P. aeruginosa* OMVs have found that a majority of vesicles migrate to a specific fraction of a density gradient during purification (6, 20). After performing the same gradient purification, we observed a difference in the migration pattern of the OMVs from EBH-treated bacteria from that of the untreated control (Fig. 5A). We observe a predominant single band of OMV in the strain grown without EBH (Fig. 5A). For OMV from EBH-grown cells, while some vesicles appeared to fall within the same density region as the untreated vesicles, a second band, corresponding to a higher density, was also observed when cells were treated with EBH. Interestingly, these vesicle density changes occurred only in response to specific epoxides, since glycidol did not alter vesicle density (see Fig. S4 in the supplemental material).

Given the striking changes in density, we suspected that there might also be changes in vesicle morphology. We observed vesicle morphology by TEM following extraction of matched fractions from gradients of treated and untreated vesicles. The vesicles from untreated cultures from fraction 1 were rather small, with little variation in size (Fig. 5B and 6). There were vesicles present in the untreated culture from the second fraction, although fewer and more variable in size, with a smaller average size (Fig. 5C and 6).

Vesicles from the EBH-treated condition had a wider range of sizes in the first fraction and greater irregularity in shape (Fig. 5D and 6). The second fraction from EBH-treated samples contained many more vesicles than the same fraction of the untreated vesicles, although these vesicles were quite variable in size and many were smaller than those observed in the first fraction (Fig. 5E and 6). Taken together, these data show that there are gross changes in the morphology of vesicles produced in the presence of EBH.

EBH-treated vesicle fractions differ in protein profiles. After identifying two vesicle populations for the EBH condition, we speculated that there might be some differences in the protein profiles of the vesicles in each fraction. We analyzed total vesicles from the two fractions described above by Western blotting to assess the amounts of Cif and OprF in each sample using an equivalent amount of loaded protein (Fig. 7A).

Cif was detectable in both EBH fractions but more abundant in the first fraction. Likewise, for both the untreated and treated vesicles, OprF was more abundant in the first fraction than the second, although much more Cif and OprF was present in both EBH fractions than in the untreated control.

Taking this analysis a step further, we analyzed the protein profiles of these fractions by silver staining (Fig. 7B). Initial inspection of the silver-stained gels revealed a number of apparent differences in both the proteins present in each sample and the abundances of particular bands between the EBH-treated and untreated OMVs. We excised several of the bands showing differences between the samples and analyzed the bands by mass spectrometry (Fig. 7; see also Fig. S5 and Table S2 in the supplemental material).

The highly abundant band in untreated fraction 1, near 50 kDa, was identified as the aminopeptidase PepB (PaAP) (40), which is greatly diminished in the same fraction of the vesicles from EBH-treated cells. OprF, the next protein identified, at ~37 kDa, is much more apparent in both of the EBH fractions than in the untreated fractions, verifying the Western blot data described above. A band just below OprF in untreated fraction 1 corresponded to the phospholipase PlcB. A prevalent band near 25 kDa was identified as an *N*-acetyl-anhydromuramyl-L-alanine amidase (N-AA-L-AA), a member of a protein family that is commonly involved in cell envelope biogenesis (41, 42). Although we did not confirm the identification of these proteins by a second method, these data support the conclusion that the proteins present in vesicles grown in the presence of EBH differ from those grown in its absence.

Epoxide response differs from known stress responses impacting OMV production. Recently, multiple groups have shown that stress can dramatically alter vesicle formation in *P. aeruginosa* (15, 43, 44). We suspected that epoxides, which can be quite reactive, might be generating a stress response that could alter vesicle production in a manner similar to that with these previously de-

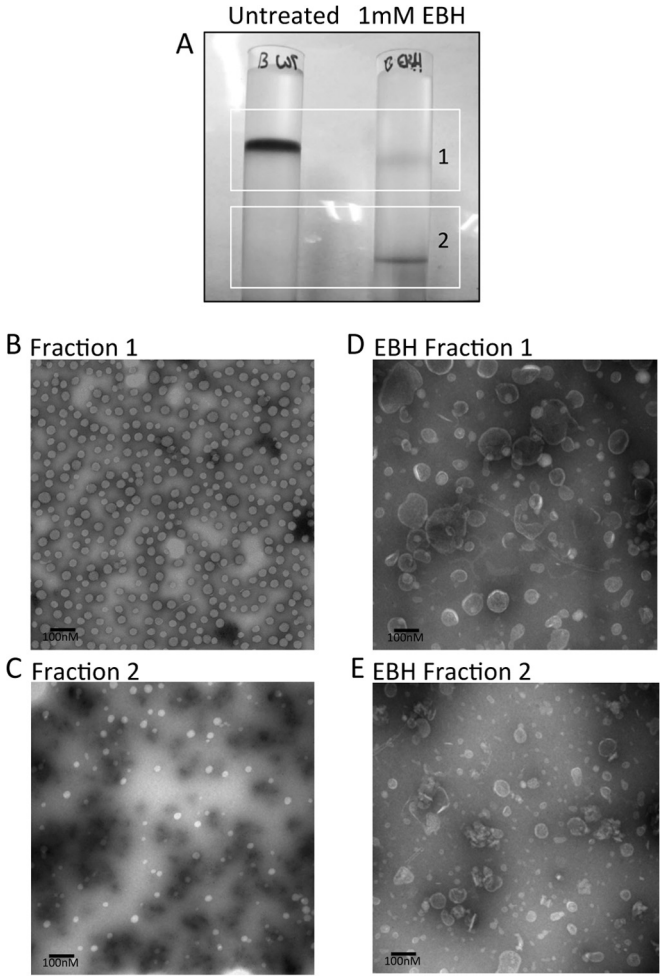

FIG 5 EBH alters OMV shape and density. (A) Image of density gradients after 20 h of centrifugation. The majority of vesicles from untreated cultures fall within a distinct band (left), whereas the OMVs isolated from EBH-treated bacteria migrate in two distinct bands (right). Boxes indicate fractions sampled. (B) TEM of negatively stained vesicles from fraction 1 of untreated bacteria. (C) Vesicles from fraction 2 of untreated bacteria. (D) Vesicles from OMV fraction 1 of EBH-treated bacteria. (E) Vesicles from OMV fraction 2 of EBH-treated bacteria. Note the variability in vesicle size in panels D and E. Scale bar, 100 nm.

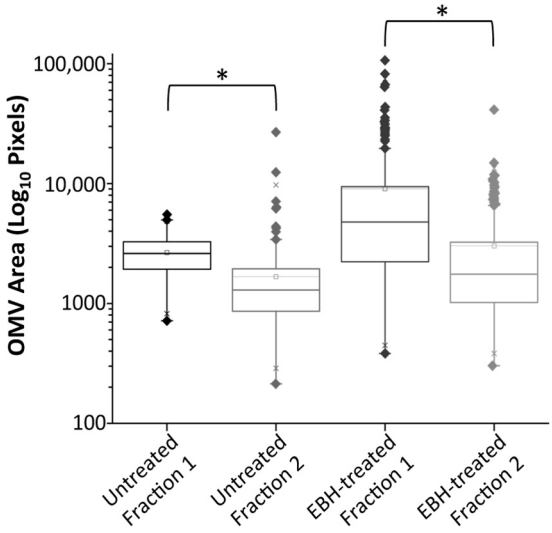

FIG 6 Quantifying OMV size in each gradient fraction. Box-and-whisker plot shows estimated area of vesicles (measured as pixels/OMV). The light-gray line denotes the mean. Note that the vertical axis is on a log scale. The differences in average vesicle size between OMVs from fractions 1 and 2 of untreated cells and between fractions 1 and 2 of EBH-treated cells is statistically significant (*, $P < 0.0001$). Error bars indicate SD.

scribed stressors. We characterized gene expression in a few common stress response pathways to test this possibility.

We examined expression of *lexA* as a marker of SOS response (43) in response to growth in epoxides and found no difference in expression (Fig. 8A). The *sodM* and *katA* transcripts were used as indicators of oxidative stress. These transcripts had levels of expression in the 1 mM EBH-treated cells similar to those in untreated cells. Finally, *algD* expression was measured as an indicator of AlgU-mediated membrane stress response (15, 39). The expression of this transcript was not significantly induced under EBH conditions. Together these data indicate that epoxide-mediated vesicle alteration does not act through the previously identified stress pathways.

Non-epoxide-mediated changes in vesicle cargo. To further test whether any other signals could alter vesicle packaging in a manner similar to that of epoxides, we purified vesicles from cultures grown with subinhibitory concentrations of EBH, gentamicin, D-cycloserine, polymyxin B, cumene hydroperoxide, and plumbagin and assessed changes in vesicle proteins as well as the Cif protein. As can be seen in Fig. 8B, each of the stresses resulted in a unique protein profile as determined by silver staining.

Western blot analysis of Cif abundance showed that treatments targeted at protein synthesis (gentamicin), the cell wall (D-cycloserine), and the cell membrane (polymyxin B) increased Cif packaging relative to results for the untreated control, although not nearly to the levels observed for cells treated with EBH (Fig. 8C). Vesicles formed in the presence of oxidative stress-inducing compounds, including cumene hydroperoxide and plumbagin, showed decreased Cif packaging. These data further support our hypothesis that alterations in Cif packaging are strongly specific to the environmental signal to which the bacteria are exposed.

Proteomic analysis of *P. aeruginosa* OMVs and transposon mutant screen to identify proteins that alter vesicle packaging. We next attempted to identify factors that may contribute to the altered packaging observed with EBH. First, we performed a proteomic analysis of OMVs derived from *P. aeruginosa* PA14, as well as two clinical isolates. A total of 443 proteins were identified in OMVs, with diverse predicted protein localization (see Table S3 and Fig. S6 in the supplemental material). The proteins identified aligned well with OMV proteins found in previous studies in *P. aeruginosa* PA14 and PAO1 (19, 43, 45).

Using the proteomics data as a guide, we selected ~150 proteins either identified in multiple samples or localized to the outer membrane or periplasmic fractions for further analysis. Strains carrying transposon mutations in the genes that coded for these proteins were selected from the previously reported *P. aeruginosa* PA14 mutant library (32), and OMVs from these mutants were isolated. OMVs isolated from the transposon mutants were compared to OMVs from the wild-type strain for both virulence factor (phospholipase, phosphatase, and Cif) and outer membrane protein (OprF and Opr86) abundance (see Table S4 in the supple-

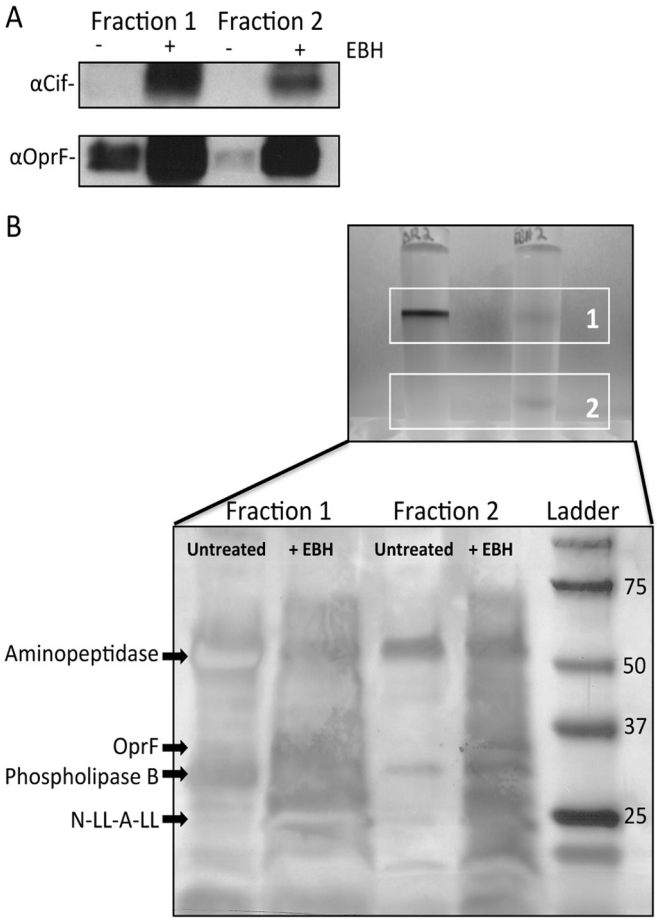

FIG 7 Protein composition is different in OMV fractions. (A) Western blots of vesicle fractions. Cif is abundant in both fractions from EBH-treated cells (top). OprF is present in all fractions with the highest concentration in the OMVs from EBH-treated cells (bottom). (B) Silver-stained polyacrylamide gel of the OMVs fractions indicated at the top of panel B. Note the differences in proteins in each fraction. The predicted identities of proteins based on band excision and analysis via mass spectroscopy are indicated by arrows. Note: in some instances, protein concentrations were high enough to exclude the silver stain, and these bands appear as dark regions with light centers, as can be seen with the aminopeptidase band in fraction 1 from untreated cells and the band marked *N*-acetyl-anhydromuramyl-L-alanine amidase (N-AA-L-AA) in fraction 1 of EBH-treated vesicles. See Fig. S5 and Table S2 in the supplemental material for a complete description of proteins identified.

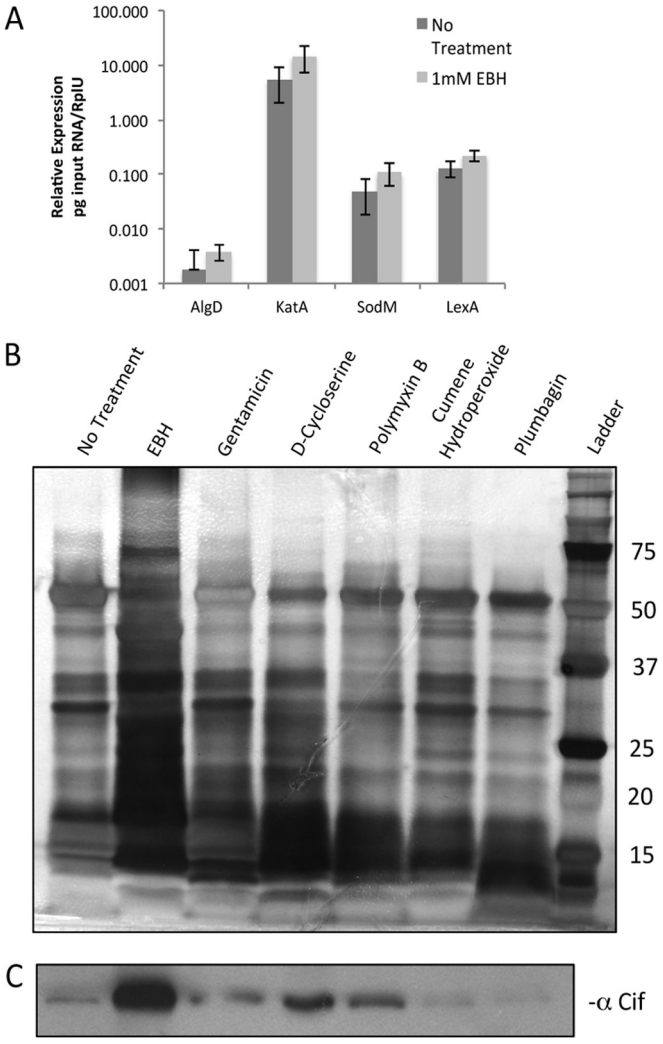

FIG 8 Epoxides do not activate typical stress response pathways. (A) EBH does not increase expression of typical stress response genes. qPCR analysis of four pathways involved in response to DNA damage (*lexA*) and oxidative (*sodM* and *katA*) and membrane stress (*algD*) in *P. aeruginosa* grown in the presence or absence of EBH is shown. Error bars indicate SD ($n = 3$). (B) Silver-stained gel of OMVs harvested from bacteria grown in the presence of subinhibitory concentrations of EBH, gentamicin, D-cycloserine, polymyxin B, cumene hydroperoxide, and plumbagin (20 μg total protein per well). Molecular weight size markers are indicated on the left. (C) Western blot of Cif protein levels of OMVs isolated from Δ*cifR* mutant bacteria grown in the presence of the above stressors. The lane labels correspond to those shown in panel B.

mental material). Most of the mutations had little or no effect on the factors tested. However, several mutations, most notably *mucD*::Tn*M*, *folE2*::Tn*M*, and *ppsA*::Tn*M*, did impact the packaging of several proteins. Unfortunately, none of the mutants fit the profile of the EBH-treated OMVs. Nonetheless, these data do show that the loss of certain proteins identified in *P. aeruginosa* OMVs can impact proper sorting of proteins into OMVs.

OMVs from EBH-grown *P. aeruginosa* are less cytotoxic and elicit an altered cytokine response. We had observed differences in the virulence factors identified in OMVs produced by bacteria grown with EBH, but the functional consequences of such changes in regard to host-pathogen interactions were not clear.

To assess the consequences of changing OMV cargo, we applied purified vesicles, isolated from bacteria grown in the absence and presence of EBH, to a monolayer of immortalized airway epithelial cells and measured lactate dehydrogenase (LDH) release as an indicator of cytotoxicity to host cells (Fig. 9A). After 12 h, OMVs from EBH-treated bacteria had no effect on LDH release by airway epithelial cells compared to results for vehicle (PBS) controls, indicating that OMVs isolated from EBH-treated *P. aeruginosa* PA14 cause little damage to the host cells. However, OMVs from untreated bacterial cultures were highly cytotoxic. Similar results were obtained after 24 h, where the average LDH release from OMVs produced by bacterial cells without EBH was close to 80%. In contrast, LDH released by airway cells treated with OMVs from EBH-grown bacteria was not significantly different from that for the untreated control epithelial cells ($P = 0.089$).

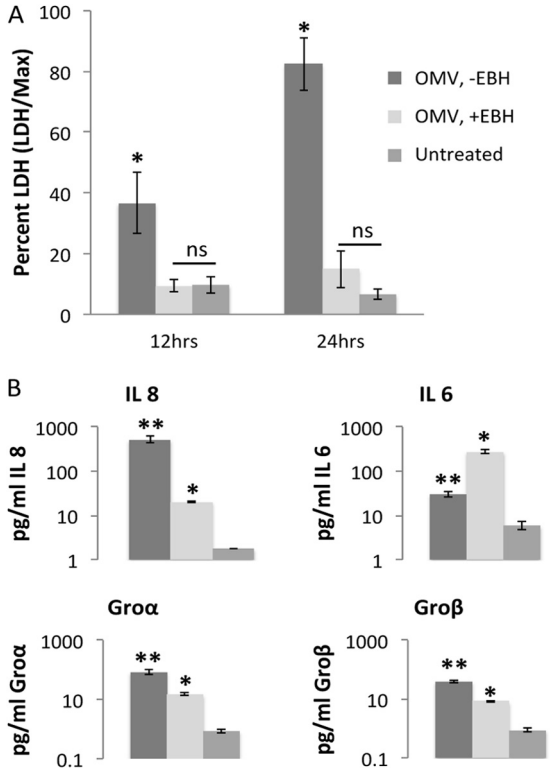

FIG 9 OMVs from EBH-treated cells are less cytotoxic and elicit an altered immune response. (A) Cytotoxicity of OMVs applied to airway epithelial cells. Cytotoxicity is measured as percent LDH released from epithelial cells relative to results for a lysis control (100% LDH release) for epithelial cells treated with OMVs from control cultures (OMV, -EBH), OMVs from bacteria grown with EBH (OMV, +EBH), or the vehicle control (Untreated). "*" indicates statistically significantly different ($P < 0.05$) from the "untreated" condition; ns, not significant ($P > 0.05$). (B) Cytokine production of epithelial cells treated for 24 h with OMVs. ELISAs were performed for IL-8, IL-6, Groα, and Groβ. Note that cytokine measurements are on a log scale. "*" indicates a statistically significant difference ($P < 0.05$) when comparing treatment with OMVs from control cultures (OMV, -EBH) to the vehicle control (Untreated). "**" indicates a statistically significant difference ($P < 0.05$) when comparing treatment with OMVs from EBH-grown cultures (OMV, +EBH) to the vehicle control (Untreated). The legend in panel A also corresponds to the bars in panel B. For both panels, error bars indicate SD ($n = 3$).

The dramatic differences in cytotoxicities of the OMVs isolated from untreated and EBH-treated *P. aeruginosa* PA14 suggested that there may be differences in the immune responses to the two populations of vesicles; thus, we examined expression of four cytokines previously shown to be induced by *P. aeruginosa* (46). After 24 h of incubation with OMVs, large differences were observed in cytokine production in airway epithelial cells exposed to OMVs from EBH-grown bacteria versus vesicles from control bacteria (grown in the absence of EBH). IL-8, Groα (CXCL1), and Groβ (CXCL2) were decreased roughly 5- to 25-fold in the airway epithelial cells exposed to OMVs from EBH-treated bacteria compared to results with OMVs grown in the absence of EBH (Fig. 9B). Interestingly, the opposite response was observed for IL-6 expression, which was higher in airway epithelial cells treated with OMVs from EBH-grown bacteria than in those treated with control OMVs.

The data presented here show that the alterations in OMVs grown in the presence versus the absence of EBH result in strong effects on airway epithelial cell cytotoxicity of, and immune response to, these vesicles and thus result in functional consequences for the host-pathogen interaction.

DISCUSSION

Over the past 20 years, a number of factors that alter vesiculation in some way, including antibiotics, antimicrobial peptides, and quorum-sensing molecules, have been identified (12, 14, 18). Here, we describe the first link of a specific virulence factor inducer, epoxides, to alteration in the virulence factor's packaging into OMVs.

We had previously shown that Cif was packaged into OMVs and OMV-mediated Cif delivery was a highly effective means of transporting this virulence factor into airway epithelial cells (6, 21). We believe the abundance of Cif in OMVs is a physiologically relevant question, since Cif efficacy in impacting host cell biology is 17,000 times greater when delivered by OMVs than with application of the pure protein (6). Here, we tested the ability of EBH, a strong inducer of *cif* gene expression and a model epoxide (25), to affect packaging of this virulence factor into OMVs. Indeed, in the wild-type strain, the Cif protein was increased in the spheroplast and periplasm fractions and in OMVs (Fig. 1A). Surprisingly, deletion of the *cifR* gene, coding for the repressor of *cif* gene expression, had a dramatic effect on Cif expression, boosting Cif levels in the spheroplast and periplasm by >5-fold, but this increase in cell-associated Cif had only a modest effect on Cif packaging. In contrast, growth of the *cifR* mutant in the presence of EBH resulted in a large boost in Cif packaging into OMVs (Fig. 1A). These data support previous suggestions of selective packaging of OMVs (47–49).

In prior studies, we had also characterized Cif production in the presence of a suite of different epoxides and found that there was selectivity of inducers (26). Here, we sought to test whether there was similar selectivity for the packaging response. We selected a strong inducer of *cif* gene expression, styrene oxide, and two poor inducers, glycidol and epoxyhexane, as well as the vicinal diol of EBH (which does not induce *cif* gene expression), as a control for epoxide hydrolysis products contributing to this phenotype. The Cif packaging data trended very closely with the expression data reported previously, and while glycidol is structurally quite similar to EBH (a hydroxyl substituted for a bromine), it had only a limited effect on vesicle packaging (Fig. 2). Note that these studies are performed in a strain lacking the CifR repressor, so the changes in Cif packing are independent of this regulator.

In addition to the changes in Cif, we observed that changes in other vesicle cargo, OprF (Fig. 1B) and a putative envelope biogenesis protein (Fig. 7B), increased in OMVs isolated from bacteria grown with EBH. At this point, we do not understand the mechanism(s) underlying the differential packaging of proteins observed in bacteria grown with EBH. For example, the differences in protein complement in OMVs may reflect changes in membrane composition of the OMVs as a result of EBH treatment or be a secondary consequence of the change in OMV shape and size for EBH-treated cells. It is also possible that the changes in OMV morphology are a consequence of the different proteins they carry.

We considered the possibility that OMVs have specific proteins that recruit cargo into the OMV, analogous to protein trafficking pathways in eukaryotic cells (50). To identify such factors, we performed proteomic analysis of OMVs and screened the OMVs

derived from ~150 strains lacking OMV-associated proteins identified by mass spectroscopy. Unfortunately, none of these mutants showed alterations of protein cargo specific to epoxide-treated OMVs (see Table S4 in the supplemental material). Despite the lack of epoxide-related alterations in packaging, many mutations did alter the screened vesicle cargo. This finding suggests that protein interactions may play a role in vesicle protein sorting, although more study is required to address this point.

Importantly, the fluctuation in virulence factor cargo in the EBH OMVs suggested that there might be functional differences in the OMVs from EBH-grown bacteria. We examined OMV-induced cytotoxicity on airway epithelial cells and found that EBH treatment reduced cytotoxicity of OMVs. EBH treatment contrasts sharply with ciprofloxacin treatment, which has been shown to increase OMV cytotoxicity in *P. aeruginosa* (45). Moreover, IL-8, Groα, and Groβ have been shown to be important for neutrophil recruitment and *P. aeruginosa* clearance (51, 52). The dramatic reduction seen in these cytokines in response to the OMVs from EBH-grown cultures, compared to results with OMVs isolated from vehicle-treated cultures, could indicate changes in the abundance or nature of the pathogen-associated molecular patterns (PAMPs) (such as LPS or flagellin). IL-6 is a chemokine with pleiotropic effects, acting as both a proinflammatory and anti-inflammatory cytokine (53). The increase in IL-6 expression seen with the EBH treatment vesicles relative to that with the untreated vesicles is interesting, since it could indicate that these vesicles may actively limit inflammation or at the very least alter the immune response to *P. aeruginosa*. Thus, the changes in the OMV complement in the presence of epoxides appear to have a functional consequence in regard to host-pathogen interactions. It is tempting to infer from these data that when *P. aeruginosa* encounters epoxides, as it may in the lung, this microbe modulates its OMV cargo to be less cytotoxic and immunogenic as part of its adaptation to a chronic lifestyle.

In summary, it is clear that a variety of environmental factors impact the suite of proteins found in OMVs. And while the mechanism that leads to these differences in OMV content are unclear, modulating the protein content in response to environmental cues could have important implications regarding how OMVs impact host cell function and immune response.

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