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The mechanism of *Pseudomonas aeruginosa* outer membrane vesicle biogenesis determines their protein composition

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Abstract

Gram-negative bacteria produce outer membrane vesicles (OMVs) and contain bacterial cargo including nucleic acids and proteins. The proteome of OMVs can be altered by various factors including bacterial growth stage, growth conditions, and environmental factors. However, it is currently unknown if the mechanism of OMV biogenesis can determine their proteome. In this study, we examined whether the mechanisms of OMV biogenesis influenced the production and protein composition of *Pseudomonas aeruginosa* OMVs. OMVs were isolated from three *P. aeruginosa* strains that produced OMVs either by budding alone, by explosive cell lysis, or by both budding and explosive cell lysis. We identified that the mechanism of OMV biogenesis dictated OMV quantity. Furthermore, a global proteomic analysis comparing the proteome of OMVs to their parent bacteria showed significant differences in the identification of proteins in bacteria and OMVs. Finally, we determined that the mechanism of OMV biogenesis influenced the protein composition of OMVs, as OMVs released by distinct mechanisms of biogenesis differed significantly from one another in their proteome and functional enrichment analysis. Overall, our findings reveal that the mechanism of OMV biogenesis is a main factor that determines the OMV proteome which may affect their subsequent biological functions.

KEYWORDS
bacterial membrane vesicles, mechanism of biogenesis, outer membrane vesicles, proteome, *Pseudomonas aeruginosa*

Abbreviations: OMVs, outer membrane vesicles; WT-OMVs, wild-type OMVs; B-OMVs, budding OMVs; E-OMVs, Explosive cell lysis OMVs; WT-Bac, wild-type bacteria; B-Bac, budding bacteria; E-Bac, explosive cell lysis bacteria; TEM, transmission electron microscopy; NTA, nanoparticle tracking analysis; LB, Luria Bertani; PES, polyethersulfone; DPBS, Dulbecco's PBS; TEAB, triethylamonium bicarbonate; FA, formic acid; AGC, automatic gain control; maxLFQ, label free qunatifiction; DAVID, database for annotation, visualization, and integrated discovery bioinformatics.

1 | INTRODUCTION

All living organisms release extracellular vesicles as part of their normal growth [1]. In Gram-negative bacteria, extracellular vesicles were initially identified as being produced when a portion of the outer membrane was liberated from the bacterial cell resulting in the production of outer membrane vesicles (OMVs) [2]. It is now well established that all Gram-negative bacteria produce OMVs, and their production appears to occur via a conserved mechanism known as budding [3, 4]. OMV release by budding occurs when the outer membrane of the bacterium bulges out due to changes in the composition or structure of the outer membrane, resulting in a part of the membrane budding off to form an OMV that is then released from the bacterium [3, 5, 6]. There are various known factors that can increase bacterial production of OMVs, which include protein modifications, lipid remodeling, and external stress factors [7]. More recently, a novel mechanism of OMV biogenesis was discovered in *Pseudomonas aeruginosa* and subsequently in *Escherichia coli* and *Shewanella vesiculosa*, known as explosive cell lysis [8–10]. In *P. aeruginosa*, explosive cell lysis can occur during conditions of bacterial stress and is mediated by cryptic prophage tailocins located in the R- and F-type tailocin gene cluster in the *P. aeruginosa* genome [8]. Induction of explosive cell lysis causes the rounding of *P. aeruginosa* which eventually explode, releasing cellular components that are then packaged within membrane fragments as they fuse to create new membrane vesicles (MVs) [8]. Furthermore, it is now understood that Gram-negative bacteria can release MVs consisting of either a single membrane as a result of blebbing from the bacterial outer membrane or containing both an outer and inner membrane bilayer which are referred to as outer inner membrane vesicles (OIMVs) and are produced as a result of bacterial cell lysis [4, 11-13]. Therefore, Gram-negative bacteria produce MVs predominately via two main mechanisms of biogenesis, involving either their release from the outer membrane during normal growth via budding, or their formation as a result of explosive cell lysis, and we collectively refer to all of these types of vesicles as OMVs.

Bacterial OMVs, irrespective of their mechanism of biogenesis, can package a range of biological cargo originating from their parent bacterium. Specifically, OMVs can package DNA [14], RNA [15], and proteins [16] and can therefore act as a delivery vehicle for transporting bacterial material to the surrounding environment [17], neighboring cells [18], and to host cells [19–23]. To better understand the biological functions of OMVs, the proteome of OMVs from a range of pathogenic bacteria has been investigated revealing that OMVs contain a range of virulence proteins [24–27], and can therefore contribute to mediating pathogenesis in the host [28, 29]. More recently, it has been identified that bacteria can alter the protein composition of their OMVs to ultimately modify their functions. For example, bacteria can selectively enrich the packaging of virulence proteins into OMVs to increase their targeted delivery into host cells [30]. In addition, the proteome of OMVs can be altered by a range of factors such as nutrient availability [31], antibiotic stress [32], and planktonic versus biofilm state of growth [33] which highlights the ability of bacteria to tailor the cargo composition of OMVs in response to changes in their environment. Furthermore, the bacterial growth stage from which OMVs are isolated, and the size of OMVs can also determine their protein content and functions [34, 35], highlighting that there are multiple mechanisms whereby bacteria can modify the protein composition of their OMVs to ultimately alter their biological functions. However, despite recent advances in our understanding of how bacterial growth stage, stress, and environmental conditions may affect the proteome of OMVs, we have limited knowledge regarding how the mechanism of OMV biogenesis can alter their production, composition, and their subsequent biological functions.

In this study, we aimed to determine how the mechanisms of OMV biogenesis impacted the quantity, composition, and proteome of *P. aeruginosa* OMVs. To do this, we isolated OMVs from three *P. aeruginosa* strains that each produced OMVs via distinct mechanisms of biogenesis: being either naturally via a combination of budding and explosive cell lysis, by budding alone, or predominately by explosive cell lysis. We found that the production of *P. aeruginosa* OMVs by budding from the cell membrane resulted in significantly fewer OMVs being produced when compared to *P. aeruginosa* strains that produced OMVs naturally via multiple mechanisms. A comparison of the proteome of OMVs produced by all three strains identified that OMVs were enriched for bacterial proteins, irrespective of their mechanism of biogenesis, compared to their parent bacteria. Furthermore, we identified key differences in the proteome of OMVs produced by each of the

Significance of the Study

In this study, we examined if the proteome of *P. aeruginosa* OMVs was determined by their mechanism of biogenesis. We identified significant differences in the production of OMVs when produced by one defined mechanism of biogenesis, as well as significant changes in the proteome of OMVs compared to their parent bacteria. Importantly, we found that OMVs produced via distinct mechanisms of biogenesis have significantly different proteomes when compared to one another. Furthermore, these proteomic differences corresponded to changes in the functional enrichment analysis of proteins, suggesting that OMVs produced by different mechanisms of biogenesis may have altered biological functions. These findings have significant implications regarding our knowledge of the packaging of bacterial cargo into OMVs, as they highlight that OMVs produced by different mechanisms of biogenesis contain proteins derived from different locations within their parent bacterial cell. Therefore, OMVs produced by different biogenesis mechanisms represent unique subpopulations of OMVs that may contribute to independent bacterial processes.

different mechanisms of biogenesis, and observed a selective enrichment of proteins in OMVs based on their mechanism of biogenesis. Our findings revealed that the mechanism of OMV biogenesis determines the number of OMVs produced by bacteria as well as their proteome. This knowledge allows for a greater understanding of the factors that influence OMV production and cargo composition and provides insights into how this may affect their subsequent biological functions.

2 | MATERIALS AND METHODS

2.1 | Bacterial culture conditions

The following *P. aeruginosa* PAO1 strains were used throughout this study: the wild-type PAO1 strain which produced OMVs naturally by both budding and explosive cell lysis, PAO1Δ*lys* a mutant *lys* derivative of PAO1 which cannot undergo explosive cell lysis and therefore produces OMVs by budding only, and PAO1Δ*lys* containing the plasmid pJN105*lys* (PAO1Δ*lys* pJN105*lys*) which produced OMVs predominately by explosive cell lysis due to the overexpression of *lys* [8]. All *P. aeruginosa* strains were routinely cultured on Luria Bertani Agar (LB Agar), consisting of Tryptone (Oxoid, USA), Yeast extract (Oxoid, USA), Sodium Chloride (ChemSupply, Australia) and Agar Base No. 2 (Oxoid, USA), or LB broth, and incubated at 37°C with shaking at 200 rpm for broth cultures as previously described [8]. *P. aeruginosa* PAO1Δ*lys* pJN105*lys* was grown in the presence of 100 μg/mL gentamicin to maintain the pJN105*lys* plasmid.

2.2 | OMV production

For OMV production, all *P. aeruginosa* strains were grown aerobically in 10 mL of LB Broth (Oxoid, USA) overnight and then diluted 1:100 in LB broth without any antibiotic selection and further incubated with shaking at 200 rpm at 37°C for 16 h. L-arabinose (Sigma, USA) was also added to *P. aeruginosa* PAO1Δ*lys* pJN105*lys* broth cultures at a final concentration of 0.2% (w/v) to induce *lys* gene expression under the control of the *araBAD* promotor encoded by pJN105. Viable counts of *P. aeruginosa* cultures at the point of OMV isolation were performed using LB agar, and were incubated overnight at 37°C.

2.3 | OMV isolation and purification

OMVs were isolated from broth cultures using established techniques [19, 20, 34, 36]. Briefly, 2 L of overnight cultures of *P. aeruginosa* PAO1, PAO1Δ*lys*, and PAO1Δ*lys* pJN105*lys* were used to prepare OMVs. Bacteria were pelleted by centrifugation at 4000 × *g* for 60 min at 4°C, the supernatant was then filtered through a 0.22 μm polyethersulfone (PES) filtration unit (Thermo Fisher Scientific, USA) and OMVs were pelleted by ultracentrifugation (Hitachi Ultracentrifuge CP100NX, Japan) at 100,000 × *g* for 2 h at 4°C.

P. aeruginosa OMVs were further purified to remove contaminants using an iodixanol (OptiPrep; Sigma-Aldrich, USA) density gradient [20, 34, 36]. Briefly, OMVs were resuspended in 45% (v/v) OptiPrep and underlaid in a discontinuous OptiPrep density gradient, consisting of 20%, 25%, 30%, 35%, and 40% of OptiPrep solution. The discontinuous gradient was centrifuged at 100,000 × *g* for 16 h at 4°C. A total of twelve 1 mL fractions were collected, with OMVs being contained within fractions 4–10. OMV-containing fractions 4–10 were pooled and washed twice using sterile Dulbecco's phosphate buffered saline (DPBS; Gibco, USA) by centrifugation at 100,000 × *g* for 2 h at 4°C, resuspended in DPBS and stored at −80°C until required.

2.4 | Transmission electron microscopy

TEM was performed as previously described [20, 37]. Carbon-coated 400 mesh copper grids (ProSciTech, Australia) were pre-treated with poly-L-lysine (Sigma, USA). OMV samples were coated onto grids, fixed using 1% (w/v) glutaraldehyde (Sigma, USA) diluted in PBS for 5 min and subsequently stained using 2% (w/v) uranyl acetate (ProSciTech, Australia) pH 7.0 for 5 min and 2% (w/v) methyl-cellulose (Sigma, USA) in 0.4% uranyl acetate pH 4.0 for 10 min. Samples were air dried and imaged using a JEOL JEM-2010 transmission electron microscope (JEOL, Japan) operated at 200 kV fitted with a Valeta 4 MP CCD camera (Emsis, Germany).

2.5 | Nanoparticle tracking analysis

OMV size and concentration was determined using the nanoparticle tracking analyser ZetaView® basic PMX-120 NTA (Particle Metrix, Germany) as previously described [20]. OMVs were diluted in DPBS, and the concentration of OMVs contained within 1 mL of each diluted sample was determined by NTA, which typically contained approximately 1–5 × 10^7 OMVs per mL. The final concentration of OMVs was then calculated with respect to the dilution factor. For quantification of OMVs, instrument calibration was performed using 102 nm polystyrene beads (ThermoFisher, USA) according to manufacturer's guidelines. Measurements were performed using a 405 nm 68 mW laser and CMOS camera. Measurements were taken at 11 cell positions containing approximately 100–200 particles and captured 60 frames per position at 25°C with camera sensitivity 80, shutter speed 100, autofocus and automatic scattering intensity. Data were analyzed using the ZetaView software version 8.05.12 SPI with the following parameters: maximum area: 1000, minimum area: 5, maximum brightness: 255, minimum brightness: 30 and minimum trace length: 15. The average of three biological replicates was plotted as particle size versus number of particles per mL using GraphPad Prism v9.4.1.

2.6 | Qubit™ fluorometric quantification

The DNA, RNA, and protein content of OMVs were quantified using the Qubit™ high sensitivity DNA assay, high sensitivity RNA assay, or protein assay, respectively (ThermoFisher, USA) and were measured using a Qubit™ 3.0 fluorometer. The DNA, RNA, and protein concentrations of OMVs were then normalized to 1×10^8 OMVs.

2.7 | Proteomics sample preparation

P. aeruginosa PAO1, PAO1Δ*lys*, and PAO1Δ*lys* pJN105*lys* bacteria and purified OMVs produced by PAO1, PAO1Δ*lys*, and PAO1Δ*lys* pJN105*lys* ($n = 3$ biological replicates of each) were lysed in 1% (v/v) sodium dodecyl sulphate (SDS), 50 mM HEPES pH 8.0 and quantified by microBCA (Thermo Fisher Scientific, USA). Samples were normalized (10 µg protein) and reduced (10 mM dithiothreitol, DTT) for 45 min at 25°C, alkylated (20 mM iodoacetamide) for 30 min at 25°C in the dark, before the Sera-Mag-based workflow [38]. Magnetic bead slurry was prepared by mixing SpeedBeads™ magnetic carboxylate modified particles (Cytiva, USA; 65152105050250, 45152105050250) at 1:1 (v:v) ratio, washing with MS-grade water and reconstituted to a final concentration of 100 µg/µL. The beads were added to samples at 10:1 beads-to-protein ratio and ethanol (EA043, ChemSupply, Aus) added to a final concentration of 50% (v/v). Protein-bound magnetic beads were washed three times with 200 µL of 80% ethanol and reconstituted in 50 µL of 50 mM triethylamonium bicarbonate (TEAB) pH 8.0. Protein digestion was performed with sequencing grade trypsin (enzyme:substrate 1:50, Promega V5113) overnight at 37°C with agitation (1000 rpm). Peptide digests were acidified to a final concentration of 2% formic acid (FA), centrifuged at 20,000 × *g* for 1 min, the supernatant extracted, frozen at −80°C (30 min) and dried by vacuum centrifugation. Samples were then reconstituted in 0.07% trifluoroacetic acid (TFA), and quantified by Fluorometric Peptide Assay (Thermo Scientific, USA; 23290) with samples normalized for immediate analysis or stored at −80°C.

2.8 | Proteomic liquid chromatography-tandem mass spectrometry

Tryptic peptides for one-shot analyses were analyzed on a Dionex UltiMate NCS-3000RSLC nanoUHPLC coupled to a Q-Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer equipped with nanospray ion source in data-dependent acquisition analysis and positive mode as described [39, 40]. Peptides were loaded (Acclaim PepMap100 C18 3 µm beads with 100 Å pore-size, Thermo Fisher Scientific, USA) and separated (1.9-µm particle size C18, 0.075 × 250 mm, Nikkyo Technos Co. Ltd, Japan) with a gradient of 2%–28% acetonitrile containing 0.1% formic acid over 95 min followed by 28%–80% from 95–98 min at 300 nL min-1 at 55°C (butterfly portfolio heater, Phoenix S&T, USA). An MS1 scan was acquired from 350–1650 m/z (60,000 resolution, 3×10^6 automatic gain control (AGC), 128 ms injection time) followed by MS/MS data-dependent acquisition (top 25) with collision-induced dissociation and detection in the ion trap (30,000 resolution, 1×10^5 AGC, 60 ms injection time, 28% normalized collision energy, 1.3 m/z quadrupole isolation width). Unassigned precursor ions charge states and slightly charged species were rejected and peptide match disabled. Selected sequenced ions were dynamically excluded for 30 s. Data was acquired using Xcalibur software v4.0 (Thermo Fisher Scientific, USA). The MS-based proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository and are available via ProteomeXchange with identifier PXD032996.

2.9 | Database searching and analysis

RAW MS data was processed using MaxQuant [41] (v1.6.14.0) with its built-in search engine Andromeda. Tandem mass spectra were searched as a single batch against the *P. aeruginosa* database (UniProt, UP000002438, Oct 2021; 5564) supplemented with common contaminants. Search parameters were as follows: carbamidomethylated cysteine as fixed modification, oxidation of methionine and N-terminal protein acetylation as variable modifications, trypsin/P as proteolytic enzyme (C-terminal to arginine and lysine) with ≤ 2 missed cleavage sites, Precursor mass tolerance was 20 ppm; product ions were searched at 0.15 Da tolerances; and minimum peptide length was defined at 7, maximum peptide length 144, and max delta CN 0.05, with 1% false discovery rate on peptide spectrum match (PSM) level (5% at protein level) employing a target-decoy approach. Contaminants and reverse identification were excluded from further data analysis. "Match between run algorithm" in MaxQuant (matching time window 0.7, ion mobility window 0.05, alignment time 20 min) and label-free protein quantitation (maxLFQ) was performed. All proteins and peptides matching to the reversed database were filtered out. For clustering analysis, PCA analysis was applied (missing values were imputed from normal distribution; width 0.3, downshift 1.8). For differential analysis, one-way ANOVA and Student's *t*-test were applied. 1D-enrichment analysis (Gene Ontology) based on log2 fold change for sample groups (FDR < 0.01). For enrichment analysis NIH Database for Annotation, Visualization, and Integrated Discovery Bioinformatics Resources 6.7 (DAVID) resources was utilized using recommended analytical parameters [42]. Perseus and R (ggplot2) package, Microsoft Excel and GraphPad Prism were utilized for generating boxplots, volcano plots, heatmaps, and bar charts.

2.10 | Statistical analysis

Data analysis was performed using GraphPad Prism 9.4.1. All data are represented as the mean ± standard error of the mean (SEM) of three biological replicates. Statistical analyses were performed using data from three biological replicates, using the one-way analysis of variance (ANOVA) with Tukey's multiple-comparisons test or a Student's *t*-test as indicated.

3 | RESULTS AND DISCUSSION

3.1 | The mechanism of *P. aeruginosa* OMV biogenesis determines the number of OMVs produced

In this study we aimed to determine whether the mechanism of OMV biogenesis affects the production and protein composition of OMVs. To address this, we isolated OMVs from three *P. aeruginosa* strains that each have a distinct mechanism of OMV biogenesis, being *P. aeruginosa* PAO1 which produced OMVs by both budding and explosive cell lysis (WT-OMVs), *P. aeruginosa* PAO1Δ*lys* which is unable to undergo prophage-mediated explosive cell lysis and therefore produces OMVs via budding only (B-OMVs), and PAO1Δ*lys* pJN105*lys* that harbors a plasmid encoding for the inducible expression of the endolysin responsible for explosive cell lysis and therefore produces OMVs predominately by explosive cell lysis (E-OMVs). The morphology of OMVs produced by all three *P. aeruginosa* strains were examined using transmission electron microscopy (TEM), revealing that OMVs produced by different mechanisms of biogenesis were heterogenous in size (Figure 1A-C).

Next, we examined if the mechanism of OMV biogenesis influenced the number of OMVs produced by *P. aeruginosa*. To do this, the overall quantity of OMVs produced by each *P. aeruginosa* strain with distinct mechanisms of OMV biogenesis was determined using ZetaView Nanoparticle Tracking Analysis (NTA; Figure 1D). Examination of the quantity of OMVs produced by each *P. aeruginosa* strain revealed that significantly fewer OMVs were produced by *P. aeruginosa* PAO1Δ*lys* that produces B-OMVs by budding only, compared to the *P. aeruginosa* strain that produced WT-OMVs ($p < 0.05$, Figure 1D), consistent with previous findings [8]. Quantification of the number of bacteria within the cultures from which OMVs were isolated revealed that there were no differences in the number of bacteria present in the cultures of each of the three *P. aeruginosa* strains examined, suggesting that the significant reduction in the amount of B-OMVs produced was not attributed to reduced bacterial growth (Figure 1E). However, as B-OMVs are produced by the endolysin mutant *P. aeruginosa* PAO1Δ*lys*, a reduction in the amount of B-OMVs produced compared to WT-OMVs suggests that the endolysin, and therefore explosive cell lysis, significantly contributes to the generation of WT-OMVs by *P. aeruginosa* ($p < 0.05$, Figure 1D). In addition, we did not see an increase in the quantity of E-OMVs produced compared to WT-OMVs, suggesting that explosive cell lysis does not increase OMV production by *P. aeruginosa* (Figure 1D). Furthermore, examination of the size distribution of OMVs produced by all three *P. aeruginosa* strains using NTA revealed that OMVs produced by all mechanisms of biogenesis ranged between approximately 50 to 400 nm in size (Figure 1F) with no statistical differences in the size of OMVs produced by all three *P. aeruginosa* strains observed (Figure S1).

Bacterial OMVs contain a range of cargo, including DNA, RNA, and protein. Therefore, we next examined the contribution of the mechanism of OMV biogenesis in regulating the quantity of DNA, RNA, and protein associated with OMVs. Quantification of bacterial cargo associated with *P. aeruginosa* OMVs revealed that WT-OMVs, B-OMVs, and E-OMVs all contained DNA, RNA, and protein (Figure 1G-I). However, E-OMVs were associated with significantly more DNA when compared to both WT-OMVs and B-OMVs ($p < 0.001$; Figure 1G) which could be due to the release of cytoplasmic DNA cargo by bacteria during explosive cell lysis that can be packaged in E-OMVs [8]. In addition, there were no significant differences in the quantity of RNA or proteins associated with OMVs produced by different mechanisms of biogenesis (Figure 1H,I). Collectively, our data shows that the mechanism of *P. aeruginosa* OMV biogenesis determines the quantity of OMVs produced by *P. aeruginosa* and their DNA cargo, but does not affect the overall size distribution of OMVs or their overall quantity of RNA and protein cargo.

3.2 | Global protein comparison analysis revealed that the proteome of OMVs produced via different mechanisms of biogenesis differ significantly to that of their parent bacteria

Bacteria are known to selectively package cargo into OMVs for their delivery to surrounding bacteria or host cells [43, 44]. To determine if there is preferential packaging of proteins into *P. aeruginosa* OMVs produced by different mechanisms of biogenesis, we performed a quantitative global protein comparison and enrichment analysis of all three *P. aeruginosa* bacterial strains and their OMVs. Proteins identified in *P. aeruginosa* bacteria and their OMVs were compared to a *P. aeruginosa* reference genome to reveal selective enrichment of proteins in OMVs [45]. We identified a total of 2983 proteins in samples obtained from all three *P. aeruginosa* strains and a total of 1820 proteins in the OMVs produced by all mechanisms of biogenesis, covering approximately 53% of all *P. aeruginosa* PAO1 predicted proteins (Figure 2A; Table S1). There were 36 proteins only identified at a significant level in OMVs produced by all mechanisms of biogenesis compared to 1199 proteins only identified at a significant level in all three *P. aeruginosa* bacterial strains (Figure 2A; Tables S2 and S3). Of the 36 proteins significantly enriched in OMVs produced by all mechanisms of biogenesis were proteins reported to be involved with the cell surface, or part of integral components of the cell membrane, suggesting that these proteins are localized to the outer membrane of *P. aeruginosa* (Figure 2A; Table S3). Specifically, OMVs produced by all mechanisms of biogenesis were found to be significantly enriched in proteins that comprise part of the ABC transport complex, which is known to facilitate OMV biogenesis [46], and numerous uncharacterized proteins which are thought to contribute to protein secretion and transport, suggesting a functional role for the release of OMVs by *P. aeruginosa* (Table S3). Furthermore, OMVs produced by all mechanisms of biogenesis were found to contain proteins involved in quinone binding, drug transmembrane activity, and metal ion transportation, indicating that *P. aeruginosa* OMVs may play a role in the secretion and transport of metal ions and other molecules to bacteria (Table S3), which has previously been experimentally demonstrated for other bacterial species including *Mycobacterium*

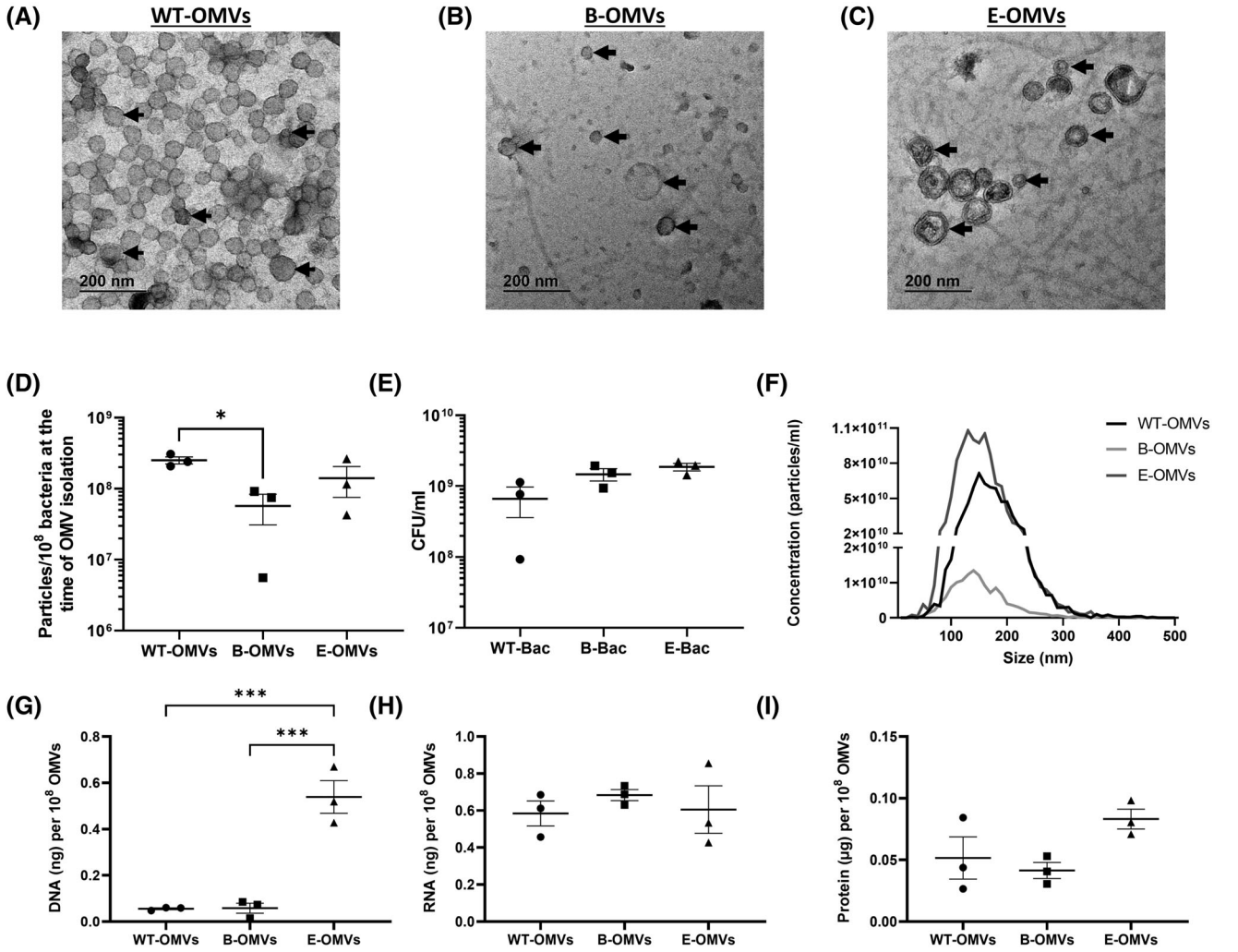


FIGURE 1 Characterisation of *P. aeruginosa* OMVs produced by different mechanisms of biogenesis. *P. aeruginosa* OMVs produced by **(A)** budding and explosive cell lysis (WT-OMVs), **(B)** by budding only (B-OMVs), or **(C)** by explosive cell lysis (E-OMVs) were examined by transmission electron microscopy. Scale bar = 200 nm. Images are representative of three biological replicates. OMVs are indicated by black arrows. **(D)** The number of WT-OMVs, B-OMVs, and E-OMVs produced by *P. aeruginosa* strains was compared using ZetaView Nanoparticle Tracking Analysis (NTA). Data is represented as the mean ± SEM of three biological replicates. $^{*}p < 0.05$, Ordinary one-way ANOVA with Tukey's multiple comparisons. **(E)** The number of viable bacteria present in bacterial cultures at the time of WT-OMV (WT-Bac), B-OMV (B-Bac), and E-OMV (E-Bac) isolation. Data show CFU/mL of individual cultures, and the mean ± SEM of three biological replicates. **(F)** The size and concentration of WT-OMVs, B-OMVs, and E-OMVs were analyzed using Zetaview NTA. Data is represented as the mean of three biological replicates. The **(G)** DNA, **(H)** RNA, and **(I)** protein content associated with 10^8 WT-OMVs, B-OMVs, and E-OMVs was quantified using Qubit fluorometric quantification assays. Data is represented as the mean ± SEM of three biological replicates. Ordinary one-way ANOVA with Tukey's multiple comparison. $^{***}p < 0.001$.

tuberculosis and *Acinetobacter baumannii* and their respective OMVs [47, 48].

Principal component analysis (PCA) of the global proteome profile of OMVs and their parent bacteria revealed that all three strains of *P. aeruginosa* examined were highly similar based on their protein composition (Figure 2B). In contrast, OMVs produced by all mechanisms of biogenesis were distinct in their protein composition from one another and from their parent bacteria (Figure 2B), indicating that there is selective packaging of proteins into OMVs from their parent bacteria that is dependent on their mechanism of biogenesis. Specifically, WT-OMVs and B-OMVs were vastly distinct in their protein composition when compared to their parent bacteria and to one another, suggesting that there may be selective cargo packaging into OMVs when produced by budding from the cell membrane (Figure 2B). However, the broad PCA distribution of the E-OMV population indicates there may be a more indiscriminate incorporation of proteins packaged into E-OMVs due to the explosive nature of their mechanism of biogenesis. The PCA distribution also revealed that E-OMVs were distinct in their protein profile compared to their parent bacteria (Figure 2B).

Comparisons of the proteome of OMVs produced by each mechanism of biogenesis to their parent bacteria revealed that all OMVs,

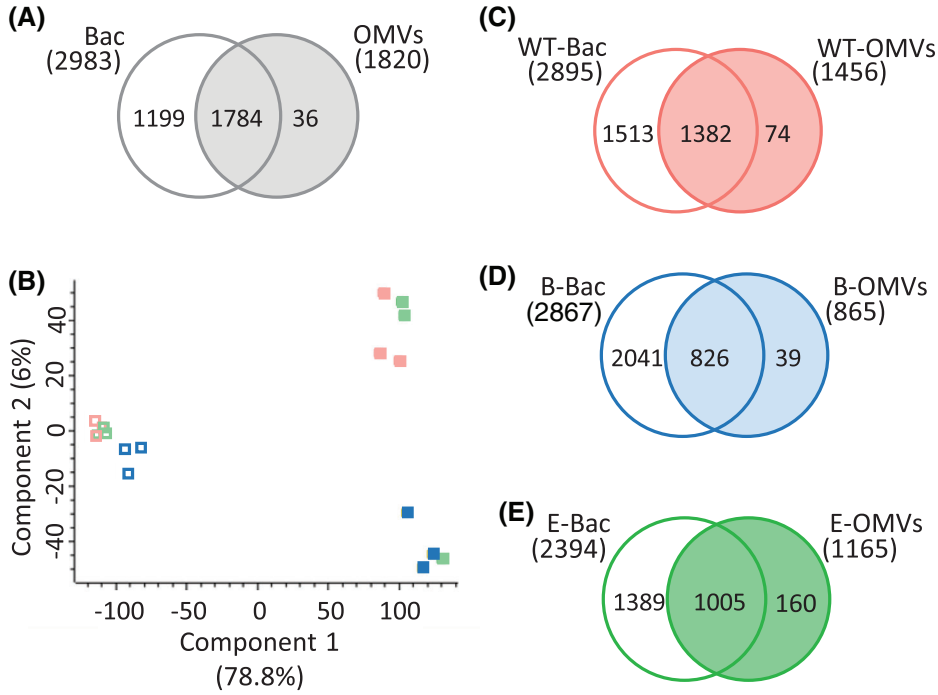
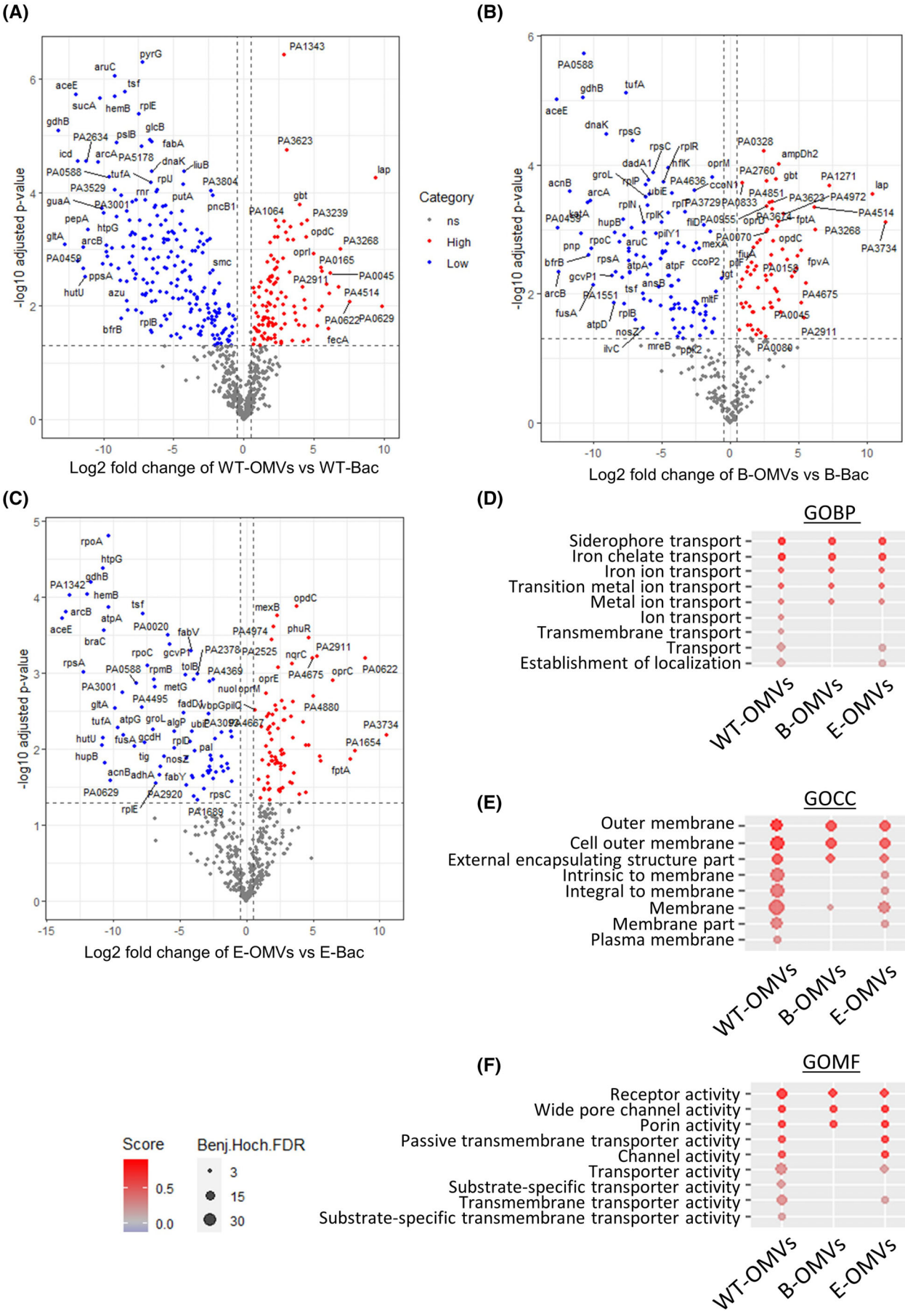


FIGURE 2 *P. aeruginosa* and their OMVs are significantly different in their proteome compared to one another. **(A)** Protein identifications for *P. aeruginosa* bacteria (Bac) and OMVs produced by each mechanism of biogenesis were combined, and comparison between all bacteria and all OMVs was performed. For differential expression LFQ ratio > 2, $p < 0.05$. **(B)** Principal component analysis (PCA) for proteome profiling of *P. aeruginosa* wild-type bacteria (WT-Bac; red open squares), budding bacteria (B-Bac; blue open squares) and explosive cell lysis bacteria (E-Bac; green open squares) and wild-type OMVs (WT-OMVs; red closed squares), budding OMVs (B-OMVs; blue closed squares), and explosive cell lysis OMVs (E-OMVs; green close squares). **(C-E)** Proteome identifications of *P. aeruginosa* and OMVs was performed for each mechanism of biogenesis **(C)** WT-Bac and WT-OMVs, **(D)** B-Bac and B-OMVs and **(E)** E-Bac and E-OMVs. For differential expression LFQ ratio > 2, $p < 0.05$. Shown is the combined data from n = 3 biological replicates.

irrespective of their mechanism of biogenesis, contained a subset of proteins that were not detected at significant levels in their parent bacteria (Figure 2C-E). Specifically, WT-OMVs contained 74 proteins significantly more abundant compared to their parent bacteria, which include proteins reported to be involved with integral components of the membrane and extracellular space (Figure 2C; Figure S2A). B-OMVs contained 39 proteins significantly increased in abundance compared to their parent bacteria, which included proteins reported to be involved in cell division sites and the cell outer membrane (Figure 2D; Figure S2B). Finally, E-OMVs contained 160 proteins that were not detected in their parent bacteria at significant levels, which included integral components of the cell or cytoplasmic membrane (Figure 2E; Figure S2C). The detection of outer membrane associated proteins in WT-OMVs and B-OMVs, and the identification of cytoplasmic membrane associated proteins in E-OMVs suggests that different mechanisms of biogenesis results in the release of OMVs from distinct locations in the bacterial cell. Overall, the identification of proteins significantly abundant in OMVs compared to their parent bacteria reveals a selective incorporation of proteins into OMVs by *P. aeruginosa*, in addition to cargo selection that is dependent on the mechanisms of OMV biogenesis. This data further supports previous studies that have also identified the selective packaging of protein cargo into OMVs [30], indicating that there are ultimately multiple mechanisms harnessed by bacteria to alter the proteome of OMVs.

3.3 | OMVs produced by different mechanisms of biogenesis are enriched in unique subsets of proteins compared to their parent bacteria

We next determined if there were any differences in the enrichment of proteins packaged in OMVs produced by different mechanisms of biogenesis compared to their parent bacteria. Specifically, when compared to their parent bacteria, we found that WT-OMVs were significantly enriched in the tailocin endolysin PA0629, necessary for explosive cell lysis [8], and the tailocin protein PA0622 compared to their parent bacteria (Figure 3A). Furthermore, WT-OMVs were also significantly enriched in the OMV biogenesis proteins TolQ, which is part of the Tol-Pal system that facilitates the production of OMVs by budding [49, 50], and LptD which aids in the assembly of LPS at the outer membrane and can affect OMV biogenesis [51] (Figure 3A; Table S4). In addition, WT-OMVs were significantly enriched in various virulence proteins including the porins OprB, OprD, and OprF, the pyocyanin Pys2 [52], and the multidrug resistance proteins MexA and MexB [53] (Figure 3A; Table S4). Furthermore, AmpDh3 which degrades peptidoglycan has previously been identified in *P. aeruginosa* OMVs and gives rise to lytic activity on murein sacculi zymography [54]. AmpDh3 has also been reported to be enriched in OMVs produced as a result of explosive cell lysis, in addition to being induced by the SOS response [54]. We identified that AmpDh3 was also enriched in WT-OMVs compared to their



parent bacteria, further suggesting that *P. aeruginosa* WT-OMVs may be predominately produced as a result of explosive cell lysis (Table S4). Compared to their parent bacteria, the enrichment of these proteins in WT-OMVs suggests that *P. aeruginosa* WT-OMVs may contribute to enhancing bacterial virulence and pathogenesis.

We then compared the enrichment of proteins in B-OMVs to their parent bacteria to identify any proteins that would indicate the potential role of OMVs produced by budding. We found that B-OMVs were significantly enriched in the peptidoglycan degraders AmpDh2 and AmpDh3, the latter which has previously been identified in *P. aeruginosa* OMVs [54] (Figure 3B; Table S4). In addition, B-OMVs were found to be enriched in the proteolysis protein PA0328 which suggests that B-OMVs may be able to target and degrade bacterial material in the environment (Figure 3B; Table S4). B-OMVs were also significantly enriched in numerous proteins with assigned functions in siderophore transport and metal binding including FpvA, FptA, and the aminopeptidase Iap (Figure 3B; Table S4) suggesting that OMVs produced by budding may contribute to the acquisition of essential metal ions as previously identified for *P. aeruginosa* OMVs [55, 56]. The enrichment of these proteins in B-OMVs indicates that OMVs released from the cell outer membrane via budding may contribute to bacterial survival via targeting competing bacteria and acquiring nutrients.

Furthermore, to understand if OMVs produced by explosive cell lysis were enriched in proteins that would indicate their function, we compared the enrichment of proteins in E-OMVs to their parent bacteria. Our analysis shows that E-OMVs were significantly enriched in the Sec protein translocase complex protein SecF, which aids in the incorporation of proteins into OMVs [57, 58] and the multidrug resistance proteins MexA and MexB (Figure 3C; Table S4). Furthermore, E-OMVs were also significantly enriched in the penicillin-binding protein MrcB and the porins OpdC and PA1271 suggesting that E-OMVs contain a range of cargo that may aid in the survival of bacteria within their environment (Figure 3C; Table S3). Interestingly, as PA0629 is the known causative agent of explosive cell lysis in *P. aeruginosa* [8] and is overexpressed in our E-OMV producing bacteria, we postulated that E-OMVs would be enriched in this protein. However, proteomic analysis revealed that PA0629 levels were significantly reduced in E-OMVs compared to their parent bacteria (Figure 3C), suggesting the protein is not readily packaged into OMVs after inducing explosive cell lysis in *P. aeruginosa*.

Finally, we determined the gene ontology biological process (GOBP), gene ontology cellular component (GOCC), and gene ontology molecular function (GOMF) terms of the proteins enriched in OMVs produced by different mechanisms of biogenesis compared to their parent bacteria (Figure 3D-F). Our analysis revealed similarities in the types of proteins enriched in OMVs produced by different mechanisms of biogenesis, most of which having assigned GOBP terms associated with siderophore and ion transport (Figure 3D), GOCC terms associated with outer membrane and membrane (Figure 3E), and GOMF terms associated with receptor activity or transporter activity (Figure 3F), suggesting that *P. aeruginosa* produces OMVs that can contribute to nutrient acquisition and uptake. Overall, the gene ontology enrichment analysis showed that WT-OMVs and E-OMVs were significantly enriched in proteins with the same GOBP, GOCC, and GOMF terms compared to their parent bacteria, while B-OMVs were not enriched in these types of proteins, suggesting that OMVs produced naturally and by explosive cell lysis were more similar in the types of proteins they packaged compared to B-OMVs produced by budding only (Figure 3D-F). Despite this, our analysis showed that WT-OMVs, B-OMVs, and E-OMVs were all significantly depleted in numerous proteins compared to their parent bacteria, indicating that there is only a small portion of bacterial proteins that are packaged in OMVs (Figure S3). Collectively, our results demonstrate that there is a significant enrichment of *P. aeruginosa* proteins into OMVs compared to their parent bacteria irrespective of their mechanism of biogenesis which may contribute to the roles of OMVs in pathogenesis and survival. In addition, these findings highlight that *P. aeruginosa* WT-OMVs and E-OMVs may be more similar to one another in the types of proteins packaged into OMVs by their parent bacteria compared to the proteins packaged into B-OMVs.

3.4 | The mechanism of OMV biogenesis determines the protein composition of *P. aeruginosa* OMVs

To determine key differences in the proteome of OMVs produced by different mechanisms of biogenesis we examined the protein composition of *P. aeruginosa* OMVs produced naturally, by budding only, or predominately by explosive cell lysis. We revealed that WT-OMVs, B-OMVs, and E-OMVs contained a total of 1456, 865, and 1165 proteins, respectively, with 534 proteins common to OMVs produced by all mechanisms of biogenesis (Figure 4A; Table S5). Of the 1820 total proteins identified, WT-OMVs contained 375 unique proteins, while B-OMVs contained 154, and E-OMVs contained 159 unique proteins, suggesting that the mechanisms of biogenesis resulted in a difference in the proteins packaged within OMVs (Figure 4A). Upon examination of the proteins that were uniquely contained within OMVs produced by different mechanisms of biogenesis, we identified key differences in the types of proteins packaged into *P. aeruginosa* OMVs. Specifically, we identified that only WT-OMVs were highly abundant in the multidrug resistance proteins MexC and PmpM as well as the biofilm

FIGURE 3 *P. aeruginosa* OMVs produced by different mechanisms of biogenesis are specifically enriched in proteins compared to their parent bacteria. Volcano plots of differentially abundant proteins in **(A)** wild-type OMVs (WT-OMVs) versus wild-type bacteria (WT-Bac), **(B)** budding OMVs (B-OMVs) versus budding bacteria (B-Bac), and **(C)** explosive cell lysis OMVs (E-OMVs) versus explosive cell lysis bacteria (E-Bac). $p < 0.05$ & $\log_2$ fold change < -0.5 (blue); $p < 0.05$ & $\log_2$ fold change > 0.5 (red; Student *t*-test) **(D)** Gene ontology biological process (GOBP), **(E)** gene ontology cellular component (GOCC), and **(F)** gene ontology molecular function (GOMF) functional enrichment of proteins based on $\log_2$ fold change of proteins. OMV-enriched terms (score > 0) are demonstrated in red. Shown is the combined data from $n = 3$ biological replicates.

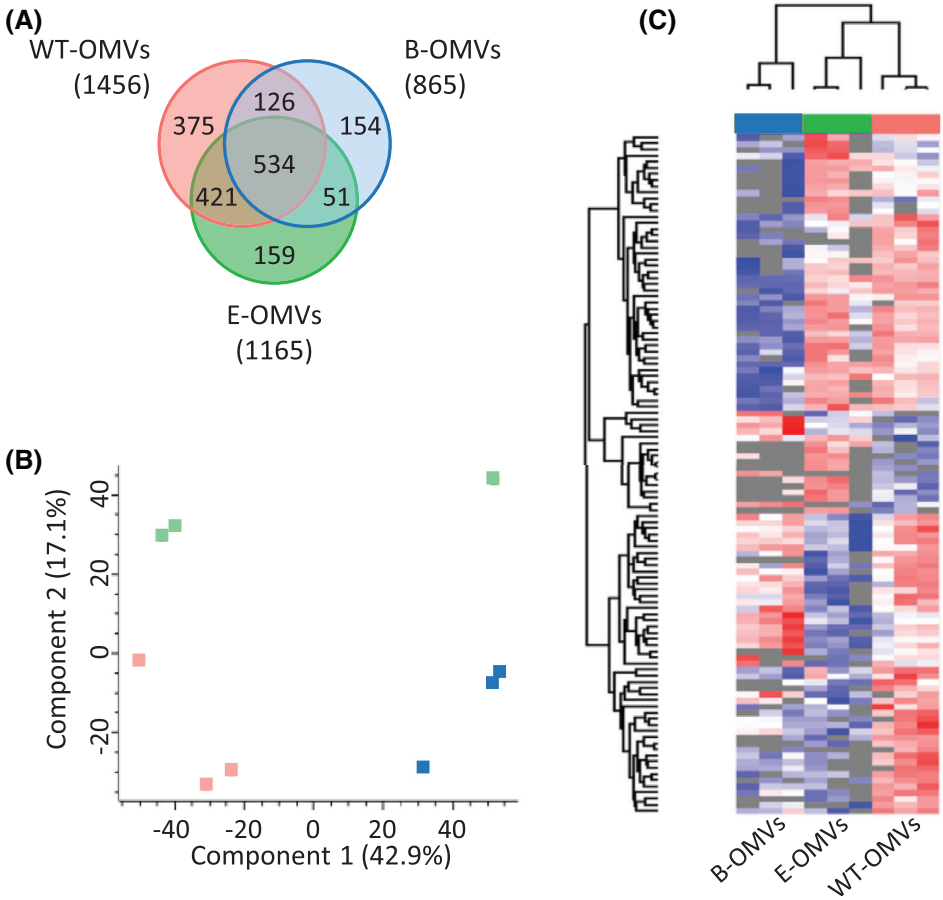


FIGURE 4 *P. aeruginosa* OMVs have distinct proteomes depending on their mechanism of biogenesis. **(A)** Proteomic protein profiling of *P. aeruginosa* wild-type OMVs (WT-OMVs), budding OMVs (B-OMVs), and explosive cell lysis OMVs (E-OMVs) was performed and protein identification for each biogenesis mechanism was compared. For differential expression LFQ ratio > 2, $p < 0.05$. **(B)** Principal component analysis (PCA) for proteome profiling of *P. aeruginosa* WT-OMVs (red closed squares), B-OMVs (blue closed squares), and E-OMVs (green closed squares). **(C)** Protein identification and abundance heatmap of *P. aeruginosa* WT-OMVs, B-OMVs, and E-OMVs ($p < 0.05$, one-way ANOVA). Shown is the combined data from $n = 3$ biological replicates.

forming protein PfpI, and numerous 30S and 50S ribosomal subunit proteins (Table S5). Furthermore, WT-OMVs were highly abundant in cell wall biogenesis and peptidoglycan biosynthesis proteins including UvrA, LpxH, ArnB, MurB, and MurD, which suggests that WT-OMVs may be produced during cell membrane modification events (Table S5).

In comparison, the 154 proteins identified only in B-OMVs consisted of numerous nucleoid-localized proteins, including UvrD, MutL, and FtsK (Figure 4A; Table S5). However, how these proteins can traverse to the outer membrane of the cell for packaging into OMVs via budding from the cell membrane is currently unknown and remains an important question to be elucidated. Additionally, only B-OMVs were found to contain the transcriptional regulatory protein GacA which controls the production of numerous *P. aeruginosa* virulence factors including the bactericidal compound pyocyanin [59, 60], and the antimicrobial toxin protein Tse5 [61] (Table S5), as well as LasI, a component of the LasI-LasR quorum-sensing system which also regulates the expression of virulence genes in *P. aeruginosa* [62, 63]. The inclusion of these proteins in B-OMVs suggests that OMVs produced by budding from the cell membrane are packaged with proteins that can contribute to the regulation of bacterial virulence and are potentially virulent themselves, which forms the basis of future research.

Furthermore, we identified unique proteins in E-OMVs produced by explosive cell lysis that contribute to the toxicity of *P. aeruginosa* within host cells. Specifically, E-OMVs were highly abundant in the cytotoxic protein Exoenzyme T [64] and the virulence protein Hcp1 which is actively secreted by *P. aeruginosa* during chronic cystic fibrosis infections [65], suggesting that this type of OMV may contribute to the virulence and pathogenesis of *P. aeruginosa* in an infectious setting (Figure 4A; Table S5). Additionally, only E-OMVs were significantly abundant in the proteins PA2269 and PA0454, both of which have predicted GOMF transmembrane transporter activity. Furthermore E-OMVs were enriched in the protein TatC which is responsible for both pyoverdine-mediated iron acquisition and bacterial growth inhibition [66], suggesting E-OMVs may have multiple roles in nutrient acquisition and antimicrobial activity. As we had identified a significant increase in the amount of DNA packaged in E-OMVs compared to both WT-OMVs and B-OMVs, we also examined whether E-OMVs contained proteins associated with DNA binding that were not

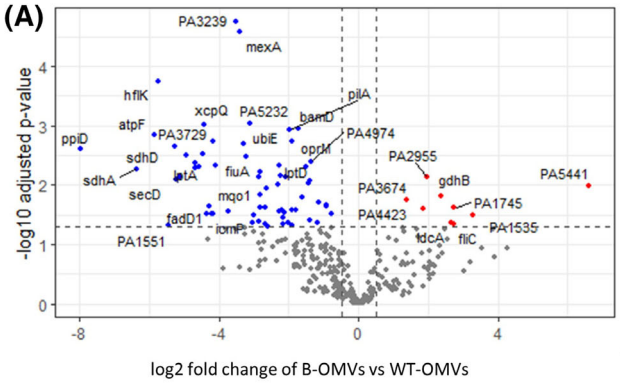
identified in other OMVs types. Upon investigation, we identified numerous DNA binding proteins only in E-OMVs which included AmgR, PA4778, and PA4992, as well as the DNA polymerases PolA and DnaX (Table S5). This suggests that there is an increase not only in bacterial DNA packaged within E-OMVs but also DNA-associated proteins when OMVs are produced by explosive cell lysis.

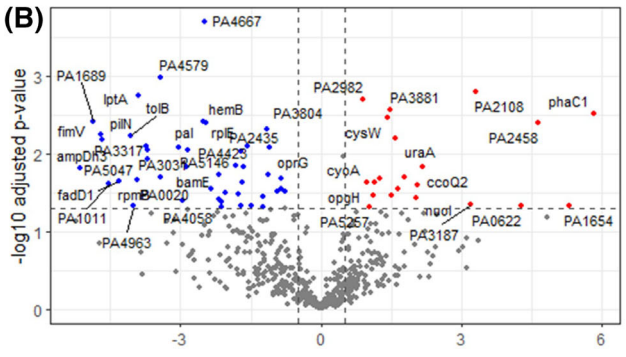
Next, PCA was performed to examine the overall proteome of OMVs produced by different mechanisms of biogenesis (Figure 4B). We identified that WT-OMVs, B-OMVs, and E-OMVs had distinct proteomes, as demonstrated by the three separate populations of OMVs by PCA (Figure 4B). Notably, the spread of E-OMVs observed in the PCA indicates that E-OMVs have greater variation in their proteome than B-OMVs produced by budding from the cell membrane (Figure 4B), which may be attributed to their engulfment of released bacterial cargo during E-OMV formation. Further examination of the proteomes of OMVs produced by different mechanisms of biogenesis supports the hypothesis that OMVs have altered protein profiles depending on their mechanism of biogenesis (Figure 4C). Specifically, the protein abundance heat map revealed significant differences in the abundance of proteins packaged within OMVs from different mechanisms of biogenesis, with both WT-OMVs and E-OMVs enriched in a large subset of proteins that were reduced in B-OMVs, suggesting that the mechanism of OMV biogenesis determines the abundance of their protein cargo (Figure 4C). Furthermore, both B-OMVs and E-OMVs were significantly depleted in a common subset of proteins compared to WT-OMVs, indicating that there are significant differences in the proteome of OMVs when produced predominately via one main mechanism of biogenesis (Figure 4C). Collectively, our data highlights that OMV biogenesis mechanisms determine the proteins that are packaged within OMVs, highlighting that OMV biogenesis is an important regulator of OMV composition and potential downstream biological functions.

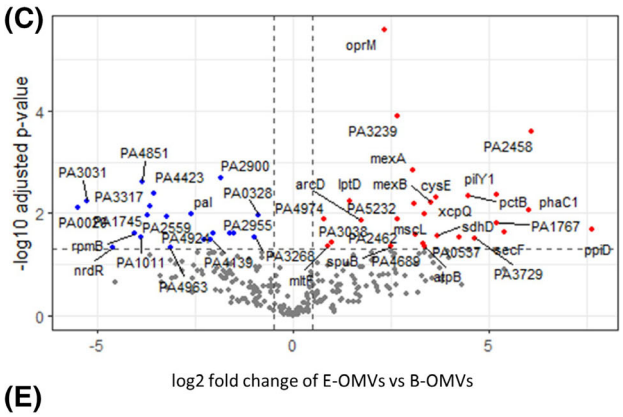
3.5 | OMVs produced by different mechanisms of biogenesis are differentially enriched in proteins compared to one another

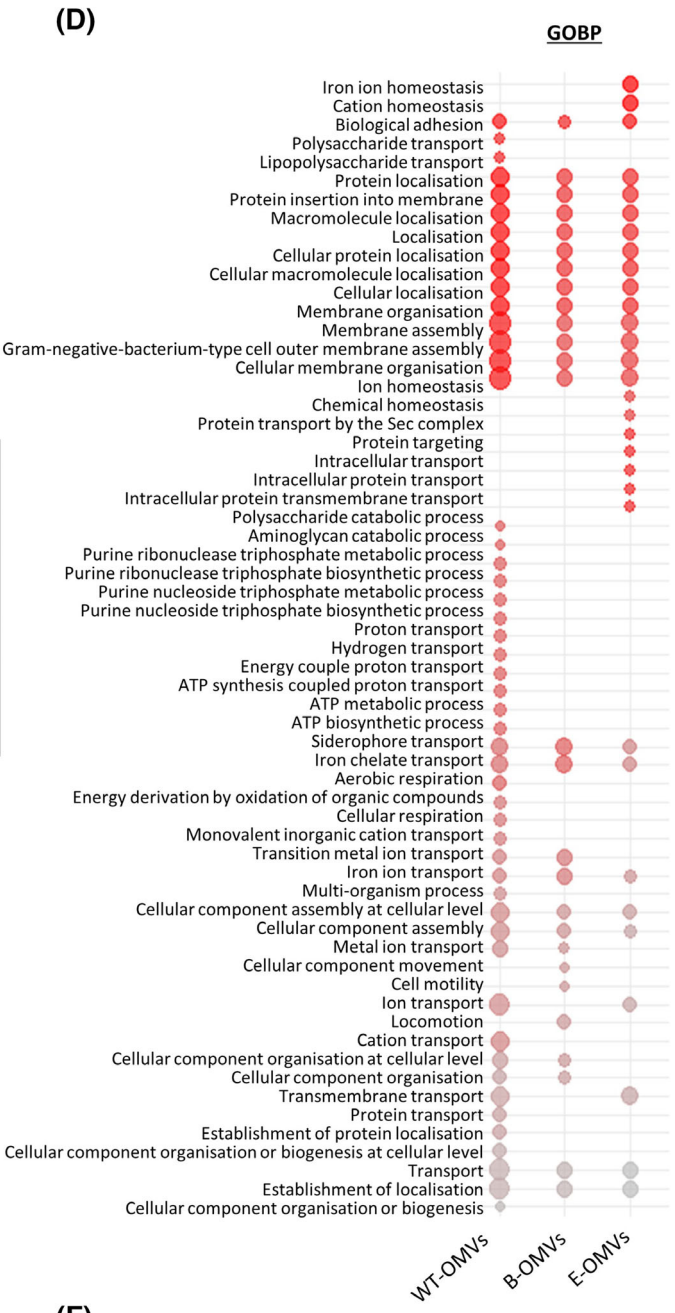
Having identified that the mechanism of OMV biogenesis determines the overall proteome of OMVs, we next sought to determine if the mechanism of biogenesis affected the enrichment of proteins in OMVs. To do so, we compared WT-OMVs, B-OMVs, and E-OMVs to one another and identified a significant enrichment of proteins in each OMV type. Compared to naturally produced WT-OMVs, B-OMVs were significantly depleted in the pathogenic proteins MexA and HflK (Figure 5A). In addition, the majority of proteins significantly enriched in B-OMVs compared to WT-OMVs were uncharacterized proteins with unknown functions, and those with known functions were involved in glutamate synthesis and flagella activity (Figure 5A). Next, the comparison of WT-OMVs to E-OMVs revealed that E-OMVs were significantly depleted in proteins that comprised part of the Tol-Pal complex (Figure 5B). However, E-OMVs were significantly enriched in the tailocin protein PA0622, in addition to the ABC-type transporter protein PA3187 and the protein transporter protein PA2982. We then compared the enrichment of proteins in OMVs produced by a single mechanism of biogenesis. When compared to B-OMVs, we found E-OMVs were significantly enriched in the multidrug resistance proteins MexA, MexB, and OprM, and the porins PA3038 and PA4974, which indicates that explosive cell lysis is responsible for the capture of these proteins by E-OMVs and therefore E-OMVs may have a greater contribution to promoting bacterial survival and antimicrobial resistance, however this remains to be elucidated (Figure 5C).

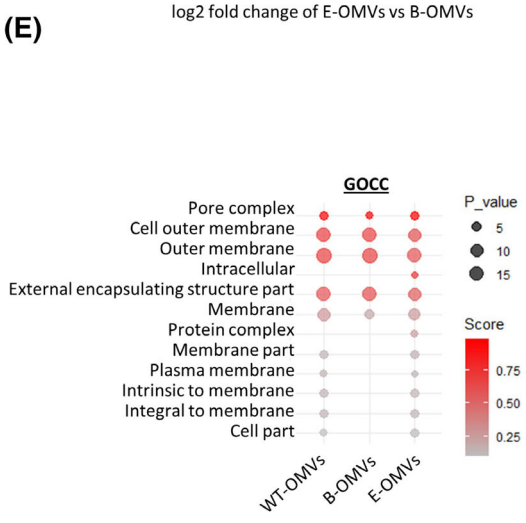
Finally, we performed functional enrichment analyses of the proteins significantly enriched in OMVs produced by different biogenesis mechanisms to better understand their potential functions. All three types of OMVs were significantly enriched in proteins with GOBP terms of protein localisation, cellular localization, siderophore transport, and iron ion transport (Figure 5D). However, only WT-OMVs were significantly enriched in proteins attributed to the GOBP of proton, hydrogen, protein, and cation transport. In comparison, only E-OMVs were significantly enriched in proteins attributed to protein targeting and intracellular protein transport. Interestingly, B-OMVs were not significantly enriched in any unique proteins compared to WT-OMVs and E-OMVs (Figure 5D). Examination of the GOCC of OMVs produced by all three strains of *P. aeruginosa* revealed that all OMV types were significantly enriched in cell outer membrane, outer membrane, and membrane proteins, as expected (Figure 5E). Most importantly, B-OMVs were not enriched in proteins associated with cellular components of the membrane, plasma membrane, or protein complex, indicating that B-OMVs were depleted in proteins that do not localize to the outer membrane as expected, due to being derived from the outer membrane of their parent bacterium (Figure 5E). Moreover, only E-OMVs were significantly enriched in intracellular proteins, supporting the concept that cytoplasmic components of bacteria can only be contained within OMVs produced via explosive cell lysis (Figure 5E), which was also supported by the identification of a significant increase in DNA and the packaging of DNA associated proteins only in E-OMVs (Figure 1G; Table S5). Comparing the GOMF between OMVs produced by different mechanisms of biogenesis revealed that the three types of OMVs may have distinct biological functions as some proteins were found to be only enriched in one type of OMV (Figure 5F). Specifically, WT-OMVs were significantly enriched in a range of cation transmembrane transporter proteins that were not significantly enriched in B-OMVs or E-OMVs (Figure 5F). Furthermore, B-OMVs were significantly enriched in serine hydrolase and peptidase proteins suggesting that B-OMVs may play a role in the degradation of amino acids (Figure 5F), whereas only E-OMVs were significantly enriched in proteins that have a role in oxidoreductase activity on heme indicating they may contribute to bacterial nutrient acquisition during conditions of stress. Notably, all three OMV types were significantly enriched in proteins associated with roles in porin activity and receptor activity suggesting that despite differences in the packaging and enrichment of proteins in OMVs produced by different types of biogenesis, all OMVs may share some conserved biological roles (Figure 5F). Overall, our results show that a shift from a naturally produced population of OMVs to OMVs produced by a single mechanism of biogenesis significantly alters the enrichment of proteins incorporated into OMVs, suggesting

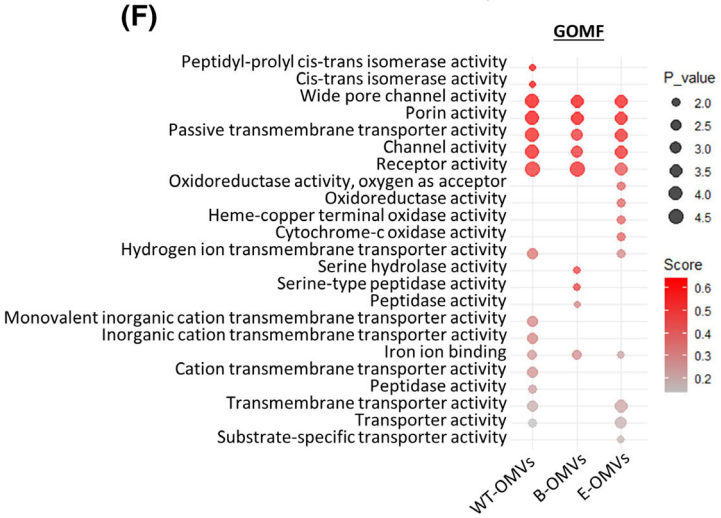












that bacteria may harness different mechanisms of OMV biogenesis to produce OMVs with bespoke functions.

4 | CONCLUDING REMARKS

OMVs are known to package bacterial proteins that can be delivered to host cells or neighboring bacteria [35, 67]. However, the multiple factors which can influence the packaging of select proteins into OMVs is yet to be fully elucidated. In this study, we aimed to understand the role of the mechanism of OMV biogenesis on regulating the production, composition, and proteome of *P. aeruginosa* OMVs. We identified that the mechanism of *P. aeruginosa* OMV biogenesis determined the number of OMVs produced, as significantly fewer OMVs were released via budding compared to the production of wild-type OMVs that are produced via multiple mechanisms of biogenesis. Additionally, E-OMVs were found to contain significantly more DNA than WT-OMVs and B-OMVs, suggesting that the process of explosive cell lysis facilitates DNA packaging into OMVs. Furthermore, global proteomic analysis comparing the proteome of *P. aeruginosa* bacteria and their OMVs revealed that OMVs were significantly enriched in pathogenic proteins and nutrient acquisition proteins compared to their parent bacteria, highlighting that *P. aeruginosa* OMVs may contribute to key processes necessary to facilitate bacterial survival. Interestingly, our analysis showed that OMVs were enriched in bacterial proteins that were not significantly detected in their parent bacteria, suggesting there is a mechanism of enriching cargo in OMVs and further confirming OMVs as a *bone fide* bacterial secretion mechanism that is controlled by bacteria. However, the exact mechanisms by which bacteria can select and traffic specific cargo for their packaging into OMVs remains to be elucidated.

Importantly, we have identified the contribution of the mechanism of OMV biogenesis in regulating the protein composition of *P. aeruginosa* OMVs. We determined that WT-OMVs produced naturally by *P. aeruginosa*, B-OMVs produced by budding from the outer membrane, or E-OMVs produced by explosive cell lysis each contained a unique subset of proteins. Additionally, the unique proteins identified in OMVs produced via distinct mechanisms of biogenesis infer differences in their functions. For example, WT-OMVs were enriched in multidrug resistant proteins that may enable bacterial survival in the presence of antibiotics, while B-OMVs were enriched in bactericidal proteins that could target and kill competing bacteria, and E-OMVs were enriched in pathogenic proteins that could promote inflammation and disease within a host. Identifying key differences in the proteome of OMVs produced via different mechanisms indicates the multi-faceted roles of OMVs, including their function as delivery vehicles for bacterial contents [68, 69], their role in inhibiting bacterial growth [54, 67, 70], and their contribution in mediating inflammation in disease settings [71, 72]. Furthermore, identifying how bacteria may utilize the mechanisms of OMV biogenesis to influence the content of OMVs and potentially alter their functions, may allow for the development of novel techniques to tailor the cargo of OMVs for therapeutic applications. Finally, our findings highlight that the multiple mechanisms of OMV biogenesis can also explain how proteins from distinct bacterial compartments may enter OMVs, as E-OMVs were enriched in cytoplasmic proteins, whereas B-OMVs produced by budding from the outer membrane predominately contained proteins located in the outer membrane. These findings contribute to the growing understanding that not all OMVs package specific bacterial cargo, such as cytoplasmic cargo, due to the variations in the mechanism by which they are produced.

Collectively our findings have identified a novel determinant of *P. aeruginosa* protein packaging into OMVs. This work provides the basis for future research to further elucidate the specific biological roles of OMVs produced by different mechanisms of biogenesis, as changes in their protein content indicates they may have varying abilities to contribute to bacterial survival within their environment or to promote disease within a host. The ability for bacteria to alter the proteome of their OMVs may be an adaption allowing for the survival of bacteria in extreme or stress conditions, and also provides a mechanism by which bacteria can alter their composition through the release of unwanted cargo in response to their changing environment. Additionally, our work supports the hypothesis that OMVs released by bacteria are comprised of a heterogeneous population of OMVs, with distinct proteomes and cargo composition based on their mechanism of biogenesis. These findings advance our current knowledge regarding how bacterial content can be packaged into OMVs and highlight that not all OMVs are the same. Future work enhancing our understanding of the complex regulation of OMV biogenesis and composition will broaden our knowledge concerning how bacteria utilize the release of OMVs for their benefit to package and deliver bacterial cargo, and how this system can be exploited for their use as novel therapeutic or bioremediation tools.

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FIGURE 5 *P. aeruginosa* OMVs produced by different mechanisms of biogenesis are significantly enriched in distinct proteins compared to one another. Volcano plots of differentially abundant proteins in **(A)** budding OMVs (B-OMVs) versus wild-type OMVs (WT-OMVs), **(B)** explosive cell lysis OMVs (E-OMVs) versus WT-OMVs, and **(C)** E-OMVs versus B-OMVs show select differences in the packaging of proteins into OMVs dependent on their mechanism of biogenesis. $p < 0.05$ & $\log_2$ fold change $<$ -0.5 (blue); $p < 0.05$ & $\log_2$ fold change > 0.5 (red; Student *t*-test) **(D)** Gene ontology biological process (GOBP), **(E)** gene ontology cellular component (GOCC), and **(F)** gene ontology molecular function (GOMF) functional enrichment of proteins based on $\log_2$ fold change of proteins identified in WT-OMVs, B-OMVs, and E-OMVs. OMV-enriched terms (score > 0) are demonstrated in red. Shown is the combined data from $n = 3$ biological replicates.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The MS-based proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository and are available via ProteomeXchange with identifier PXD032996.

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