

Biodistribution of ⁸⁹Zr-DFO-labeled avian pathogenic *Escherichia coli* outer membrane vesicles by PET imaging in chickens

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ABSTRACT Avian pathogenic *Escherichia coli* (APEC) is a serious systemic infectious disease in poultry infections, causing severe economic losses to the poultry industry. Previous studies have shown that secretion of virulence proteins was required for the pathogenicity of APEC through the secretion system. Outer membrane vesicles (OMVs) are a generalized secretion system of Gram-negative bacteria that play a key role in the long-distance delivery of virulence factors, but whether they are associated with the pathogenic mechanism of APEC has not been determined. In this study, OMVs were purified and characterized from AE17 (O2 serotype) by ultracentrifugation and density gradient centrifugation and their protein cargo was identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). In addition, ⁸⁹Zr was labeled after chelating AE17 OMVs by DFO and positron emission tomography PET imaging was used to track ⁸⁹Zr-DFO-OMVs in chickens and to pathologically analyze the distribution sites. This study showed that AE17 OMVs

were membrane vesicles ranging in size from 20 to 200 nm and proteomic analysis revealed the presence of virulence proteins, including adhesion proteins OmpA, OmpC, OmpF, OmpX, FimH, FimC and FigE, and serum resistance proteins OmpT and MliC and immune response regulator proteins (FliC). In addition, in vivo PET imaging to track the biodistribution of AE17 OMVs showed that AE17 OMVs were taken up by the lung region and the gastrointestinal and renal regions but were not detected in other areas. Pathological analysis of the tissue sites where AE17 OMVs were ingested showed inflammatory responses and damage. These findings suggested that AE17 OMVs not only contained a group of virulence proteins associated with AE17 infection but can also deliver these virulence proteins over long distances and caused tissue inflammatory damage. Our study revealed a previously unidentified causative microbial signal in the pathogenesis of APEC that could aid in the development of vaccines and antibiotics effective against APEC.

Key words: avian pathogenic *Escherichia coli*, outer membrane vesicles, proteomics, biodistribution, PET, pathogenicity

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INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) is an extra-intestinal pathogenic *Escherichia coli* (ExPEC) that is transmitted through the respiratory tract and can cause emphysema, multiple plasmacytosis,

septicemia, and other extra-intestinal diseases, as a major cause of morbidity and mortality in poultry (Zhuang et al., 2014; Gao et al., 2015; Biran and Ron, 2018). The virulence proteins of APEC are transported and delivered through the secretory system, causing an inflammatory response in the host and eventually causing systemic infection or death (Guabiraba and Schouler, 2015; Kathayat et al., 2021).

Outer membrane vesicles (OMVs) are a generalized secretion system of Gram-negative bacteria, classified as the type-0 secretion system (TOSS) (Rueter and Bielazewska, 2020). The OMVs range in size from 20 to

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400 nm and are formed in the bacterial outer membrane, wrapping many parental bacterial substances, including outer membrane proteins, periplasmic proteins, phospholipids, lipopolysaccharides (LPSs), peptidoglycans (PGs), and nucleic acids (Bonnington and Kuehn, 2014; Jan, 2017). The OMVs contain virulence factors with numerous functions, such as enterotoxigenic *E. coli* (ETEC) containing the adhesin proteins TibA and CexE and flagellin, which induces IL-8 secretory; enterohemorrhagic *E. coli* (EHEC) containing the apoptosis-inducing proteins Shiga toxin and hemolysin; and *Helicobacter pylori* OMVs containing the mitochondrial apoptosis-inducing protein VacA (Rueter and Bielaszewska, 2020). The OMVs of EHEC and ExPEC were found to cause systemic inflammation and even death in mice in the absence of intact bacterial pathogens through animal models (Park et al., 2010; Jang et al., 2015), but the pathogenicity of OMVs of APEC in chick models have not been reported.

The ability of single photon emission computed tomography (SPECT) based detection of radioisotope-labeled OMVs has been used for in vivo tracing of OMVs of bacterial pathogens (Berzosa et al., 2022). The technique of SPECT has excellent sensitivity for deep tissue imaging and allows quantitative measurements but is not superior in terms of spatial resolution and imaging time (Choi et al., 2022). Positron emission tomography (PET) combines the advantages of SPECT while compensating for the deficiencies in spatial resolution and imaging time and has become a powerful tool for monitoring the biodistribution of vesicles in vivo (Klein et al., 2020). The isotope ^{89}Zr has been used to radiolabel various antibodies, viruses, cells, exosomes, extracellular vesicles (EVs), and nanoparticles (NPs), as the PET isotope of choice (Pandey et al., 2022). This is because ^{89}Zr has a half-life of 78.4 h, which matches well with the biological metabolic cycle of most substances (Yoon et al., 2020). The emitted energy is compatible with PET imaging and is suitable as a carrier for long-term tracking and imaging in vivo (Deri et al., 2013).

There are many successful examples of imaging studies using ^{89}Zr -labeled bioactive molecules. The use of ^{89}Zr for radiolabeling EVs released from stem and cancer cells for in vivo tracking in mice, which assesses the quantitative biodistribution of vesicles in various tissues of animal models, also illustrates the general applicability of this radionuclide for in vivo PET long-term imaging (Choi et al., 2022; Khan et al., 2022). It can easily label the surface of vesicles by using the iron carrier-derived chelator desferrioxamine (DFO) (Zeglis and Lewis, 2015), which contains three chelating metal isohydroxamic acid groups and a primary amine group that couples biomolecules, allowing the formation of very stable complexes (Feiner et al., 2021). The success of these techniques in monitoring and quantifying the in vivo biodistribution of vesicles has set the stage for the study of the in vivo biodistribution of bacterial OMVs.

To investigate the major virulence proteins contained in AE17 OMVs and their biodistribution in host chickens, this study isolated and purified AE17 (O2 serotype)

OMVs and investigated the proteomic profile of OMVs using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The ^{89}Zr was indirectly labeled with AE17 OMVs by DFO and ^{89}Zr -DFO-OMVs were performed within 36 h for in vivo PET imaging and quantitative analysis of the biodistribution of OMVs and pathological analysis of major organs.

MATERIALS AND METHODS

Isolation and Purification of OMVs

The OMVs were prepared as described earlier (Jang et al., 2014). Briefly, Luria-Bertani (LB) medium was aseptically inoculated with APEC strain AE17(O2 serotype) (Fu et al., 2022), stored in this laboratory, which was then cultured in a shaker for 16 h at 37°C and 180 rpm. Bacteria were removed by low speed centrifugation at $5,000 \times g$ for 30 min at 4°C. The culture supernatant was then filtered using a 0.45- μm filter and then concentrated with a 100-kDa ultrafiltration tube (Millipore, Billerica, MA) and then pelleted at $39,000 \times g$ for 3 h at 4°C. To further fractionate OMVs, OptiPrep gradients were layered as: 1.7 mL 45%, 1.7 mL 40%, 1.7 mL 35%, 1.7 mL 30%, 1.7 mL 20%, and 1.7 mL 10%. Isolated OMVs (500 μL) were loaded on top of the OptiPrep gradients. The gradients were ultracentrifuged at $298,700 \times g$ for 6 h at 4°C in a swinging-bucket rotor, and 1 mL of each fraction was obtained. Dilute 8-fold with HEPES buffer and centrifuge at $39,000 \times g$ for 3 h at 4°C to remove residual OptiPrep. The final pellet was suspended in physiological saline and the protein concentration determined with a Micro BCATM Protein Assay Kit (Beyotime, Shanghai, China). The OMV samples were stored at -80°C .

Particle Size Distribution Analysis

The OMVs in Dulbecco's phosphate-buffered saline (dPBS) solutions were diluted in filtered water for nanoparticle tracking analysis (NTA, Malvern NanoSight NS300; Malvern, UK). Diluted OMVs were loaded onto a NanoSight NS300 sizer and counter and the particle size was recorded for 60 s per technical replicate. Five technical replicates and three biological replicates were analyzed per sample.

Transmission Electron Microscopy

For transmission electron microscopy (TEM) analysis, bacterial pellets obtained during OMVs isolation were dissolved in PBS and mixed thoroughly. The diluted samples were placed on a 200 mesh Formvar-coated copper microscopy grid and stained with 2% phosphotungstic acid. After drying, the OMVs morphology was observed by a HT-7700 electron microscope (Hitachi, Tokyo, Japan).

Proteomic Analysis of OMVs by LC-MS/MS

The lyophilized protein powder was dissolved in 10 μL 0.1% formic acid in water as solvent A, and then injected into a home-made 2 cm by 75 μm , 3 μm C18 Nano-Trap column. Peptides were separated in a home-made 15 cm by a 150 μm , 1.9 μm analytical column with a mobile phase of 0.1% formic in 80% acetonitrile as solvent B. Sample elution was performed at a flow rate of 600 nL/min by increasing the solvent B concentration from 6 to 100% over 60 min. The separated peptides were analyzed by a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Scotts Valley, CA), with an ion source of Nanospray FlexTM (ESI), spray voltage of 2.3 kV.

The raw data of MS detection were searched against the UniProt database (<http://www.uniprot.org>). Carbamidomethyl was specified as a fixed modification and oxidation of methionine (M) and acetylation of the N-terminus were specified as a variable modification. The identified protein contained at least one unique peptide with FDR no more than 0.01. The subcellular localization information of all proteins of *E. coli* was downloaded from the uniprot database and then the corresponding subcellular localization information was extracted from a total of 804 proteins according to the three sample proteins in the project. Gene Ontology (GO) analysis was conducted, and the databases of clusters of orthologous groups (COG) were used to annotate the protein family and pathway. The mass spectrometry proteomic data has been deposited in the ProteomeXchange Consortium database (<http://proteomecentral.proteomexchange.org>) via the iProX partner repository, with the dataset identifier PXD029347.

Conjugation of OMVs

The synthesis strategy for ⁸⁹Zr-DFO-APEC OMVs is shown in Figure 1A. Briefly, 100 μL of AE17 OMV solution with 28 mg/mL PBS was added to the reaction flask, 900 μL of 0.1 M Na₂CO₃/NaHCO₃ buffer solution and an appropriate amount of 0.1 M Na₂CO₃ solution were added and the pH of the solution was adjusted to 9. One to 2 mg p-SCN-Bn-DF (Macrocylics, Plano, TX) was dissolved in DMSO to prepare a 10 μM solution, 20 μL of DFO solution was added to the reaction solution and the reaction was conducted at 37°C in a nitrogen blowing apparatus with slow stirring for 90 min. After the reaction, the Df-AE17 OMV solution was purified by a PD10 column to remove unreacted DFO and then concentrated by an ultrafiltration tube.

Radiolabeling of OMVs

A total of 1 to 2 mCi Zirconium oxalate ⁸⁹Zr solution was added to the reaction flask, 9 times the volume of 0.2 M HEPES solution was added to the solution and then 1 M Na₂CO₃ solution at the pH of 10 was used to adjust the pH of the reaction system to 7. A certain volume of Df-AE17 OMV was then added and reacted at 37°C for 30 min. After incubation, the labeled reaction mixture was purified using an activated NAP-5 column with 0.15 M HAc/NaAc as the eluent for purification. The 500 μL of radioactive fractions was collected and analyzed by SEC-HPLC using a 30 cm by 7.8 cm, 5 μm TSK gel 3,000 column, with PBS buffer at a rate of 1 mL per min. The yields of the ⁸⁹Zr labeling reactions were examined by the Radio-iTLC method using activated glass fiber chromatography paper as the carrier

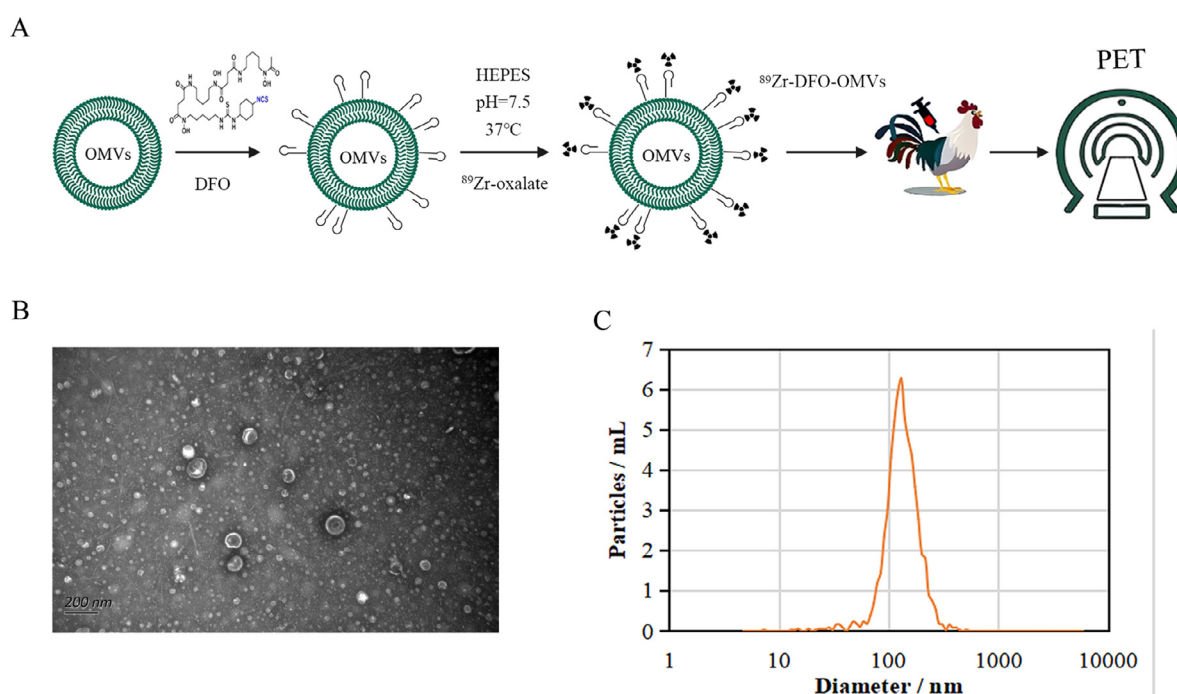


Figure 1. Characterization of Avian pathogenic *Escherichia coli* OMVs. The synthesis strategy for ⁸⁹Zr-DFO-APEC OMVs (A). Transmission electron micrograph of OMVs from AE17 (B). Size distribution of OMVs according to diameter as determined by intensity (C).

and 0.5 M sodium citrate buffer solution at a pH of 5 as the unfolding system. The cut glass fiber chromatography paper was activated in an oven at 60°C for 30 min, then removed and stored in a sealed bag into a desiccator for backup. The sample was taken with a capillary tube to the middle of the initial line of the glass fiber paper, blown dry and placed lightly in an unfolding vial, left to the solvent unfolding the front to the final line, removed, and dried and placed on a Radio-iTLC instrument to analyze the radioactivity ratio.

In Vivo PET Imaging and Biodistribution of ⁸⁹Zr-DFO-OMVs in Chicken Models

⁸⁹Zr-DFO-OMVs were administered into the trachea of three 7-day-old specific-pathogen-free chickens (SPF, Beijing, China). Static imaging was performed for 10 min at 0.25, 1, 2, 3, 12, and 36 h after injection, using a micro-PET scanner (Siemens, Germany) with a 3% volumetric fraction of mixed gas containing isoflurane and oxygen to induce and maintain anesthesia. The imaging time and the residual radioactivity in the syringe were recorded.

Another three 7-day-old SPF chickens were injected with ⁸⁹Zr-oxalate via the trachea as the blank control group for micro-PET imaging. The tracheal, lung, gastrointestinal tract, and kidney regions were delineated by ASIProVM software as regions of interest and the radioactive uptake values %ID/g of each region of interest at separate times were calculated.

Statistical Analysis

Statistical analysis used GraphPad Prism software (GraphPad 8.0 Software). Data are shown as the mean ± SEM.

RESULTS

Characterization of Avian Pathogenic Escherichia coli OMVs

The production of OMVs from AE17 was assessed during in vitro culture. Bacteria were cultured in LB broth and OMVs were purified from culture supernatants by ultracentrifugation. Purified OMVs were recognized by a typical teacup structure with spherical bilayered shape in Figure 1B, which were consistent with previous reports concerning OMVs from other Gram-negative bacteria (Furuyama and Sircili, 2021). Small OMVs, with diameters of 20 to 30 nm, comprised only a tiny proportion, but large-sized vesicles with diameters about 200 nm, were commonly observed as shown in Figure 1C, as the typical size of OMVs produced by Gram-negative bacteria. Taken together, AE17 OMVs were successfully purified.

Identification of AE17 OMVs Proteins by LC-MS/MS and Prediction of Their Subcellular Distribution and Functional Classification of Proteins

The protein cargo of OMVs were next examined by LC-MS/MS analysis and a total of 805 proteins were identified in 3 samples of AE17 OMVs and ranked according to their intensity. The 20 most abundant proteins, along with their gene, subcellular localization, function, and intensity, are listed in Table 1. Several known vesicle marker proteins were identified, including OmpA, OmpF, and OmpW (Lee et al., 2007). Several low abundance outer membrane proteins were also identified, including FHUE, MLTB, and NANC, as well as periplasmic proteins, including AG43, TOLB, YRAP, and SKP (Table 2). Most of the cytoplasmic proteins identified in this study were ribosomal or metabolic proteins, including TNA, LDCL, G3P1, and DLDH (Table 2). The proteins identified by AE17 OMVs were further divided into 5 groups, including cell inner membrane proteins (42.10%), cytoplasm proteins (34.19%), cell outer membrane proteins (9.38%), periplasm proteins (8.46%), and Cell OMV and enriched in cell inner membrane protein, cell outer membrane protein, and cell membrane protein, comprising 57.35% in total in Figure 2A. In contrast, cytoplasm proteins and periplasm proteins typically accounted for 42.65% of the total bacterial proteins. These subcellular localization results showed that OMVs were successfully purified from AE17. To determine the putative functions of the 804 proteins in the AE17 OMVs proteome, they were classified according to their immediate homology group (COG) definitions, shown in Figure 2B. The four largest COG groups identified in the OMVs proteome were found to be energy production and conversion, amino acid transport and metabolism, translation, ribosomal structure and collectively, these findings suggested that the OMV proteome may exhibit specific functions.

Labeling and Stability Detection of Specific Radiotracers

The radiochromatograms obtained from ⁸⁹Zr/⁸⁹Zr-DFO-OMVs solutions always showed one defined peaks, as in Figure 3. The results showed the radiochromatograms of ⁸⁹Zr-DFO-OMVs at different incubation times obtained using 0.5 M sodium citrate buffer solution at pH = 5. It can be seen in Figure 3A that ⁸⁹Zr-DFO-OMV was stable for 96 h. The Rf value for ⁸⁹Zr was about 0.8 as seen in Figure 3B and the Rf value for ⁸⁹Zr-DFO-OMVs was about 0.1, seen in Figure 3A. This indicated that stable ⁸⁹Zr-DFO-OMVs were successfully synthesized in this study.

Biodistribution of ⁸⁹Zr-DFO-OMVs

To analyze the biodistribution of AE17 OMVs (100 μg total protein) in chickens, micro-PET imaging

Table 1. Major proteins identified from native OMVs of AE17.

#UniProt	Gene	Protein	Subcellular localization	Function	LFQ intensity Sample 1	LFQ intensity Sample 2	LFQ intensity Sample 3
P0A910	OMPA	Outer membrane protein A	Cell outer membrane	Cell wall/membrane/envelope biogenesis	1.18E+11	1.26E+11	1.45E+11
P04949	FLIC	Flagellin	Secreted	Cell motility	1.78E+11	1.02E+11	1.32E+11
P0A917	OMPX	Outer membrane protein X	Cell outer membrane	Function unknown	3.11E+10	3.84E+10	3.97E+10
P06996	OMPC	Outer membrane porin C	Cell outer membrane	Function unknown	7.66E+10	1.06E+11	9.99E+10
P69776	LPP	Major outer membrane lipoprotein Lpp	Cell outer membrane	Function unknown	2.04E+10	1.8E+10	1.77E+10
P21420	NMPC	Putative outer membrane porin protein NmpC	Cell outer membrane	Function unknown	3.69E+10	4.98E+10	4.6E+10
P77330	BORD	Prophage lipoprotein Bor homolog	Cell membrane	Function unknown	6.22E+09	7.49E+09	7.44E+09
P0A915	OMPW	Outer membrane protein W	Cell outer membrane	Function unknown	7.97E+09	1.36E+10	1.1E+10
P0A7L3	RL20	50S ribosomal protein L20	Cytoplasm	Translation, ribosomal structure and biogenesis	4.44E+09	5.61E+09	4.98E+09
P0CE48	EFTU2	Elongation factor Tu 2	Cytoplasm	Translation, ribosomal structure and biogenesis	1.33E+10	1.13E+10	1.17E+10
P0CE47	EFTU1	Elongation factor Tu 1	Cytoplasm	Translation, ribosomal structure and biogenesis	1.33E+10	1.13E+10	1.17E+10
P0A7S9	RS13	30S ribosomal protein S13	Cytoplasm	Translation, ribosomal structure and biogenesis	3.6E+09	3.48E+09	3.81E+09
P0A6P9	ENO	Enolase	Cytoplasm	Carbohydrate transport and metabolism	7.63E+09	5.42E+09	3.17E+09
P0A912	PAL	Peptidoglycan-associated lipoprotein	Cell outer membrane	Cell wall/membrane/envelope biogenesis	3.51E+09	4.39E+09	6E+09
P04128	FIMA1	Type-1 fimbrial protein	Fimbrium	Function unknown	2.12E+09	2.2E+09	1.97E+09
P0A6Q6	FABZ	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	Cytoplasm	Lipid transport and metabolism	2.51E+09	9.08E+08	1.62E+09
P0A908	MIPA	MltA-interacting protein	Cell outer membrane	Cell wall/membrane/envelope biogenesis	3.97E+09	4.71E+09	6.12E+09
P0A927	TSX	Nucleoside-specific channel-forming protein Tsx	Cell outer membrane	Function unknown	3.61E+09	4.36E+09	4.06E+09
P62399	RL5	50S ribosomal protein L5	Cytoplasm	Translation, ribosomal structure and biogenesis	3.63E+09	4.03E+09	4.09E+09
P0A7V0	RS2	30S ribosomal protein S2	Cytoplasm	Translation, ribosomal structure and biogenesis	3.38E+09	3.9E+09	3.84E+09

Table 2. Proteins identified from AE17-derived native OMVs.

#UniProt	Gene	Function	Subcellular
		Periplasm	
1	P39180	AG43	Periplasm
2	P0A855	TOLB	Periplasm
3	P64596	YRAP	Periplasm
4	P0AEU7	SKP	Periplasm
5	P0ADV1	LPTA	Periplasm
6	P45955	CPOB	Periplasm
7	P32176	FDOG	Periplasm
8	P0AEQ3	GLNH	Periplasm
9	P09394	GLPQ	Periplasm
10	P0AAX3	YBIJ	Periplasm
11	P0AES9	HDEA	Periplasm
12	P02925	RBSB	Periplasm
13	P0A862	TPX	Periplasm
14	P64534	RCNB	Periplasm
15	P0AFM2	PROX	Periplasm
16	P0ADV7	MLAC	Periplasm
17	P0AFX9	RSEB	Periplasm
18	P24183	FDNG	Periplasm
19	P0AEG6	DSBC	Periplasm
20	P30859	ARTI	Periplasm
21	P75942	FLGJ	Periplasm
22	P76002	PLIG	Periplasm
23	P75777	YBHG	Periplasm
24	P45565	AIS	Periplasm
25	P28249	ASMA	Periplasm
26	P0AEX9	MALE	Periplasm
27	P0AFK9	POTD	Periplasm
28	P0AFL3	PPIA	Periplasm
29	P21170	SPEA	Periplasm

(continued)

Table 2 (Continued)

	#UniProt	Gene	Function	Subcellular
30	P37690	ENVC	Cell cycle control, cell division, chromosome partitioning	Periplasm
31	P33937	NAPA	Energy production and conversion	Periplasm
32	P0AET2	HDEB	Function unknown	Periplasm
33	P39172	ZNUA	Function unknown	Periplasm
34	P0ABZ6	SURA	Posttranslational modification, protein turnover, chaperones	Periplasm
35	P0AAX8	YBIS	Function unknown	Periplasm
36	P45523	FKBA	Posttranslational modification, protein turnover, chaperones	Periplasm
37	P00805	ASPG2	Function unknown	Periplasm
38	P31697	FIMC	Function unknown	Periplasm
39	P23827	ECOT	Function unknown	Periplasm
40	P0AAJ8	HYBA	Energy production and conversion	Periplasm
41	P0AEE5	DGAL	Carbohydrate transport and metabolism	Periplasm
42	P0AD59	IVY	Function unknown	Periplasm
43	P23843	OPPA	Amino acid transport and metabolism	Periplasm
44	P03841	MALM	Function unknown	Periplasm
45	P0AEG4	DSBA	Function unknown	Periplasm
46	P0AEU0	HISJ	Function unknown	Periplasm
			Cytoplasm	
47	P0A853	TNAA	Amino acid transport and metabolism	Cytoplasm
48	P0A9H3	LDCI	Amino acid transport and metabolism	Cytoplasm
49	P0A9B2	G3P1	Carbohydrate transport and metabolism	Cytoplasm
50	P0A9P0	DLDH	Energy production and conversion	Cytoplasm
51	P0A8L1	SYS	Translation, ribosomal structure and biogenesis	Cytoplasm
52	P0ADG4	SUHB	Carbohydrate transport and metabolism	Cytoplasm
53	P33599	NUOCD	Energy production and conversion	Cytoplasm
54	P0A6F5	CH60	Posttranslational modification, protein turnover, chaperones	Cytoplasm
55	P0A7E5	PYRG	Nucleotide transport and metabolism	Cytoplasm
56	P0A817	METK	Coenzyme transport and metabolism	Cytoplasm
57	P05459	PDXB	Function unknown	Cytoplasm
58	P0A6P1	EFTS	Translation, ribosomal structure and biogenesis	Cytoplasm
59	P23893	GSA	Coenzyme transport and metabolism	Cytoplasm
60	P0A8M3	SYT	Translation, ribosomal structure and biogenesis	Cytoplasm
61	P0AG67	RS1	Translation, ribosomal structure and biogenesis	Cytoplasm
62	P0A887	UBIE	Coenzyme transport and metabolism	Cytoplasm
63	P09372	GRPE	Posttranslational modification, protein turnover, chaperones	Cytoplasm
64	P0A749	MURA	Cell wall/membrane/envelope biogenesis	Cytoplasm
65	P0A796	PFKA	Carbohydrate transport and metabolism	Cytoplasm
66	P06987	HIS7	Amino acid transport and metabolism	Cytoplasm
67	P0A850	TIG	Posttranslational modification, protein turnover, chaperones	Cytoplasm
68	P00579	RPOD	Transcription	Cytoplasm
69	P45577	PROQ	Function unknown	Cytoplasm
70	P0A9P6	DEAD	Function unknown	Cytoplasm
71	P0A9×4	MREB	Cell cycle control, cell division, chromosome partitioning	Cytoplasm
72	P75876	RLMI	General function prediction only	Cytoplasm
73	P69441	KAD	Nucleotide transport and metabolism	Cytoplasm
74	P0A9M8	PTA	General function prediction only	Cytoplasm
75	P0A707	IF3	Translation, ribosomal structure and biogenesis	Cytoplasm
76	P0A6B7	ISCS	Amino acid transport and metabolism	Cytoplasm
77	P42632	TDCE	Energy production and conversion	Cytoplasm
78	P0DTT0	BIPA	Signal transduction mechanisms	Cytoplasm
79	Q46868	UBIK	Function unknown	Cytoplasm
80	P07913	TDH	Function unknown	Cytoplasm
81	P0A717	KPRS	Function unknown	Cytoplasm
82	P0A7G2	RBFA	Translation, ribosomal structure and biogenesis	Cytoplasm
83	P26646	ACUI	Function unknown	Cytoplasm
84	P0A9H9	CHEZ	Function unknown	Cytoplasm
85	P05055	PNP	Translation, ribosomal structure and biogenesis	Cytoplasm
86	P21513	RNE	Translation, ribosomal structure and biogenesis	Cytoplasm
87	P0A8×0	YJGA	Function unknown	Cytoplasm
88	P39406	RSMC	Translation, ribosomal structure and biogenesis	Cytoplasm
89	P00962	SYQ	Translation, ribosomal structure and biogenesis	Cytoplasm
90	P0A698	UVRA	Replication, recombination and repair	Cytoplasm
91	P0AC69	GLRX4	Posttranslational modification, protein turnover, chaperones	Cytoplasm
92	P0AE01	TRMJ	Translation, ribosomal structure and biogenesis	Cytoplasm
93	P0A6I6	COAD	Coenzyme transport and metabolism	Cytoplasm
94	P0A9W3	ETTA	General function prediction only	Cytoplasm
95	P25535	UBII	Function unknown	Cytoplasm
96	P60716	LIPA	Coenzyme transport and metabolism	Cytoplasm
97	P0C058	IBPB	Posttranslational modification, protein turnover, chaperones	Cytoplasm
98	P33602	NUOG	Energy production and conversion	Cytoplasm
99	P36979	RLMN	General function prediction only	Cytoplasm
100	P21507	SRMB	Function unknown	Cytoplasm
101	P0A9Q1	ARCA	Function unknown	Cytoplasm
102	P0A6F9	CH10	Posttranslational modification, protein turnover, chaperones	Cytoplasm

(continued)

Table 2 (Continued)

	#UniProt	Gene	Function	Subcellular
103	P0A6R0	FABH	Lipid transport and metabolism	Cytoplasm
104	P63284	CLPB	Posttranslational modification, protein turnover, chaperones	Cytoplasm
105	P0A763	NDK	Nucleotide transport and metabolism	Cytoplasm
106	P0A884	TYSY	Nucleotide transport and metabolism	Cytoplasm
107	P0C0R7	RLME	Translation, ribosomal structure and biogenesis	Cytoplasm
108	P29680	DCUP	Coenzyme transport and metabolism	Cytoplasm
109	P75728	UBIF	Function unknown	Cytoplasm
110	P0AGK4	YHBY	Translation, ribosomal structure and biogenesis	Cytoplasm
111	P0A6Z3	HTPG	Posttranslational modification, protein turnover, chaperones	Cytoplasm
112	P0AA16	OMPR	Function unknown	Cytoplasm
113	P0AGJ9	SYI	Translation, ribosomal structure and biogenesis	Cytoplasm
114	P0AGB6	RPOE	Transcription	Cytoplasm
115	P36938	PGM	Carbohydrate transport and metabolism	Cytoplasm
116	P0A705	IF2	Translation, ribosomal structure and biogenesis	Cytoplasm
117	P0AES6	GYRB	Replication, recombination and repair	Cytoplasm
118	P0A6U3	MNMG	Cell cycle control, cell division, chromosome partitioning	Cytoplasm
119	P0A6T3	GAL1	Carbohydrate transport and metabolism	Cytoplasm
120	P17993	UBIG	Coenzyme transport and metabolism	Cytoplasm
121	P0ABF1	PCNB	Translation, ribosomal structure and biogenesis	Cytoplasm
122	P0A988	DPO3B	Replication, recombination and repair	Cytoplasm
123	P0A993	F16PA	Carbohydrate transport and metabolism	Cytoplasm
124	P0A8J8	RHLB	Function unknown	Cytoplasm
125	P0AES4	GYRA	Replication, recombination and repair	Cytoplasm
126	P0A8A2	YEEN	Function unknown	Cytoplasm
127	P0A7Y0	RNC	Transcription	Cytoplasm
128	P0A9K3	PHOL	Signal transduction mechanisms	Cytoplasm
129	P08839	PTI	Carbohydrate transport and metabolism	Cytoplasm
130	P0A8A0	YEBC	Function unknown	Cytoplasm
131	P0AFM6	PSPA	Function unknown	Cytoplasm
132	P42641	OBG	General function prediction only	Cytoplasm
133	P36929	RSMB	Translation, ribosomal structure and biogenesis	Cytoplasm
134	P00547	KHSE	Amino acid transport and metabolism	Cytoplasm
135	P25534	UBIH	Function unknown	Cytoplasm
136	P0A870	TALB	Carbohydrate transport and metabolism	Cytoplasm
137	P0ACC7	GLMU	Cell wall/membrane/envelope biogenesis	Cytoplasm
138	P0A9A6	FTSZ	Cell cycle control, cell division, chromosome partitioning	Cytoplasm
139	P0A6E4	ASSY	Amino acid transport and metabolism	Cytoplasm
140	P0A6E9	BIOD2	Coenzyme transport and metabolism	Cytoplasm
141	P0A6Y8	DNAK	Posttranslational modification, protein turnover, chaperones	Cytoplasm
142	P0A8V6	FADR	Transcription	Cytoplasm
143	P0A959	ALAA	Amino acid transport and metabolism	Cytoplasm
144	P0A9J0	RNG	Translation, ribosomal structure and biogenesis	Cytoplasm
145	P0A9V1	LPTB	General function prediction only	Cytoplasm
146	P0AE08	AHPC	Posttranslational modification, protein turnover, chaperones	Cytoplasm
147	P0AEI1	MIAB	Translation, ribosomal structure and biogenesis	Cytoplasm
148	P0AEI4	RIMO	Translation, ribosomal structure and biogenesis	Cytoplasm
149	P0AFF6	NUSA	Transcription	Cytoplasm
150	P0AFY8	SEQA	Function unknown	Cytoplasm
151	P0AG24	SPOT	Function unknown	Cytoplasm
152	P0AGL7	RSME	Function unknown	Cytoplasm
153	P21179	CATE	Inorganic ion transport and metabolism	Cytoplasm
154	P23871	HEMH	Coenzyme transport and metabolism	Cytoplasm
155	P69910	DCEB	Amino acid transport and metabolism	Cytoplasm
156	P76536	YFEX	Inorganic ion transport and metabolism	Cytoplasm
157	P0CE48	EFTU2	Translation, ribosomal structure and biogenesis	Cytoplasm
158	P0CE47	EFTU1	Translation, ribosomal structure and biogenesis	Cytoplasm
159	P0A6P9	ENO	Carbohydrate transport and metabolism	Cytoplasm
160	P0A6Q6	FABZ	Lipid transport and metabolism	Cytoplasm
161	P09373	PFLB	Energy production and conversion	Cytoplasm
162	P00509	AAT	Amino acid transport and metabolism	Cytoplasm
163	P0AG86	SECB	Intracellular trafficking, secretion, and vesicular transport	Cytoplasm
164	P12758	UDP	Nucleotide transport and metabolism	Cytoplasm
165	P0A7D4	PURA	Nucleotide transport and metabolism	Cytoplasm
166	P0A6M8	EFG	Translation, ribosomal structure and biogenesis	Cytoplasm
167	P0ABT2	DPS	Inorganic ion transport and metabolism	Cytoplasm
168	P17169	GLMS	Cell wall/membrane/envelope biogenesis	Cytoplasm
169	P0A6R3	FIS	Function unknown	Cytoplasm
170	P0A7G6	RECA	Replication, recombination and repair	Cytoplasm
171	P0A8N5	SYK2	Translation, ribosomal structure and biogenesis	Cytoplasm
172	P0A715	KDSA	Cell wall/membrane/envelope biogenesis	Cytoplasm
173	P21889	SYD	Translation, ribosomal structure and biogenesis	Cytoplasm
174	P13035	GLPD	Energy production and conversion	Cytoplasm
175	P0A8F4	URK	Nucleotide transport and metabolism	Cytoplasm
176	P0A6A8	ACP	Function unknown	Cytoplasm

(continued)

Table 2 (Continued)

	#UniProt	Gene	Function	Subcellular
177	P0A6A3	ACKA	Energy production and conversion	Cytoplasm
178	P0AED0	USPA	Signal transduction mechanisms	Cytoplasm
179	P0A9F3	CYSB	Transcription	Cytoplasm
180	P0AGD7	SRP54	Intracellular trafficking, secretion, and vesicular transport	Cytoplasm
181	P0ACF0	DBHA	Replication, recombination and repair	Cytoplasm
182	P07395	SYFB	Translation, ribosomal structure and biogenesis	Cytoplasm
183	P0A953	FABB	Function unknown	Cytoplasm
184	P23830	PSS	Lipid transport and metabolism	Cytoplasm
185	P0A799	PGK	Carbohydrate transport and metabolism	Cytoplasm
186	P0A9M0	LON	Posttranslational modification, protein turnover, chaperones	Cytoplasm
187	P60906	SYH	Translation, ribosomal structure and biogenesis	Cytoplasm
188	P0A6N4	EFP	Translation, ribosomal structure and biogenesis	Cytoplasm
189	P0A8M0	SYN	Translation, ribosomal structure and biogenesis	Cytoplasm
190	P0A6H5	HSLU	Posttranslational modification, protein turnover, chaperones	Cytoplasm
191	P08312	SYFA	Translation, ribosomal structure and biogenesis	Cytoplasm
192	P0A9M2	HPRT	Nucleotide transport and metabolism	Cytoplasm
193	P0A7I4	RF3	Translation, ribosomal structure and biogenesis	Cytoplasm
194	P60757	HIS1	Amino acid transport and metabolism	Cytoplasm
195	P12295	UNG	Replication, recombination and repair	Cytoplasm
196	P0A825	GLYA	Amino acid transport and metabolism	Cytoplasm
197	P31473	RAVA	General function prediction only	Cytoplasm
198	P00960	SYGA	Translation, ribosomal structure and biogenesis	Cytoplasm
199	P77718	THII	Coenzyme transport and metabolism	Cytoplasm
200	P08622	DNAJ	Posttranslational modification, protein turnover, chaperones	Cytoplasm
201	P30850	RNB	Function unknown	Cytoplasm
202	P0A8N3	SYK1	Translation, ribosomal structure and biogenesis	Cytoplasm
203	P16659	SYP	Translation, ribosomal structure and biogenesis	Cytoplasm
204	P0ABD5	ACCA	Lipid transport and metabolism	Cytoplasm
205	P04036	DAPB	Amino acid transport and metabolism	Cytoplasm
206	P00961	SYGB	Translation, ribosomal structure and biogenesis	Cytoplasm
207	P0A821	SELA	Amino acid transport and metabolism	Cytoplasm
208	P63224	GMHA	Carbohydrate transport and metabolism	Cytoplasm
209	P04693	TYRB	Amino acid transport and metabolism	Cytoplasm
210	P00957	SYA	Translation, ribosomal structure and biogenesis	Cytoplasm
211	P0C093	SLMA	Transcription	Cytoplasm
212	P00963	ASNA	Amino acid transport and metabolism	Cytoplasm
213	P0A9N4	PFLA	Posttranslational modification, protein turnover, chaperones	Cytoplasm
214	P22523	MUKB	Function unknown	Cytoplasm
215	P03004	DNAA	Replication, recombination and repair	Cytoplasm
216	P27247	PLSX	Lipid transport and metabolism	Cytoplasm
217	P00959	SYM	Translation, ribosomal structure and biogenesis	Cytoplasm
218	P0A9L3	FKBB	Posttranslational modification, protein turnover, chaperones	Cytoplasm
219	P0ACF8	HNS	Function unknown	Cytoplasm
220	P07118	SYV	Translation, ribosomal structure and biogenesis	Cytoplasm
221	P07363	CHEA	Function unknown	Cytoplasm
222	P75726	CILA	Energy production and conversion	Cytoplasm
223	P0AFK0	PMBA	General function prediction only	Cytoplasm
224	P07012	RF2	Translation, ribosomal structure and biogenesis	Cytoplasm
225	P0A7B8	HSLV	Posttranslational modification, protein turnover, chaperones	Cytoplasm
226	P0A964	CHEW	Function unknown	Cytoplasm
227	P0A998	FTNA	Inorganic ion transport and metabolism	Cytoplasm
228	P0A9C9	GLPX	Carbohydrate transport and metabolism	Cytoplasm
229	P0A9H7	CFA	Cell wall/membrane/envelope biogenesis	Cytoplasm
230	P0A9K9	SLYD	Posttranslational modification, protein turnover, chaperones	Cytoplasm
231	P22524	MUKE	Cell cycle control, cell division, chromosome partitioning	Cytoplasm
232	P36767	RDGC	Function unknown	Cytoplasm
			Cell outer membrane	
233	P0A910	OMPA	Cell wall/membrane/envelope biogenesis	Cell outer membrane
234	P06996	OMPC	Function unknown	Cell outer membrane
235	P69776	LPP	Function unknown	Cell outer membrane
236	P0A912	PAL	Cell wall/membrane/envelope biogenesis	Cell outer membrane
237	P0A908	MIPA	Cell wall/membrane/envelope biogenesis	Cell outer membrane
238	P0A927	TSX	Function unknown	Cell outer membrane
239	P02931	OMPF	Function unknown	Cell outer membrane
240	P0A905	SLYB	Cell wall/membrane/envelope biogenesis	Cell outer membrane
241	P10384	FADL	Lipid transport and metabolism	Cell outer membrane
242	P02930	TOLC	Function unknown	Cell outer membrane
243	P77774	BAMB	Function unknown	Cell outer membrane
244	P0A940	BAMA	Cell wall/membrane/envelope biogenesis	Cell outer membrane
245	P06971	FHUA	Inorganic ion transport and metabolism	Cell outer membrane
246	P06129	BTUB	Function unknown	Cell outer membrane
247	P0AC02	BAMD	General function prediction only	Cell outer membrane
248	P37194	SLP	Function unknown	Cell outer membrane
249	P0A903	BAMC	Function unknown	Cell outer membrane

(continued)

Table 2 (Continued)

	#UniProt	Gene	Function	Subcellular
250	P46130	YBHC	Carbohydrate transport and metabolism	Cell outer membrane
251	P0A937	BAME	Translation, ribosomal structure and biogenesis	Cell outer membrane
252	P69411	RCSF	Function unknown	Cell outer membrane
253	P76506	MLAA	Cell wall/membrane/envelope biogenesis	Cell outer membrane
254	P45464	LPOA	Function unknown	Cell outer membrane
255	P31554	LPTD	Cell wall/membrane/envelope biogenesis	Cell outer membrane
256	P0A921	PAI	Function unknown	Cell outer membrane
257	P0ADC1	LPTE	Function unknown	Cell outer membrane
258	P0AB10	PQIC	Function unknown	Cell outer membrane
259	P0AB38	LPOB	Function unknown	Cell outer membrane
260	P40710	NLPE	Function unknown	Cell outer membrane
261	P0A6S0	FLGH	Cell motility	Cell outer membrane
262	P28224	MLIC	General function prediction only	Cell outer membrane
263	P02943	LAMB	Function unknown	Cell outer membrane
264	P76115	YNCD	Inorganic ion transport and metabolism	Cell outer membrane
265	P30130	FIMD	Function unknown	Cell outer membrane
266	Q46835	YGHG	Function unknown	Cell outer membrane
267	P0ADE4	TAMA	Cell wall/membrane/envelope biogenesis	Cell outer membrane
268	P0C960	EMTA	Cell wall/membrane/envelope biogenesis	Cell outer membrane
269	P0C066	MLTC	Cell wall/membrane/envelope biogenesis	Cell outer membrane
270	P25894	LOIP	Posttranslational modification, protein turnover, chaperones	Cell outer membrane
271	P75820	AMID	Defense mechanisms	Cell outer membrane
272	P0A917	OMPX	Function unknown	Cell outer membrane
273	P21420	NMPC	Function unknown	Cell outer membrane
274	P0A915	OMPW	Function unknown	Cell outer membrane
275	P13036	FECA	Function unknown	Cell outer membrane
276	P09169	OMPT	Function unknown	Cell outer membrane
277	P61320	LOLB	Function unknown	Cell outer membrane
278	P0A935	MLTA	Cell wall/membrane/envelope biogenesis	Cell outer membrane
279	P75780	FIU	Function unknown	Cell outer membrane
280	P05825	FEPA	Function unknown	Cell outer membrane
281	P16869	FHUE	Function unknown	Cell outer membrane
282	P41052	MLTB	Cell wall/membrane/envelope biogenesis	Cell outer membrane
283	P69856	NANC	Function unknown	Cell outer membrane
Cell membrane				
284	P77330	BORD	Function unknown	Cell membrane
285	P0ADA5	YAJG	Function unknown	Cell membrane
286	P0ADA7	OSMB	Function unknown	Cell membrane
287	P0AEE1	DCRB	Function unknown	Cell membrane
288	P31063	YEDD	Function unknown	Cell membrane
289	P0A8K1	PSD	Lipid transport and metabolism	Cell membrane
290	P28635	METQ	Inorganic ion transport and metabolism	Cell membrane
291	P10100	RLPA	Cell wall/membrane/envelope biogenesis	Cell membrane
292	P0ADN6	YIFL	Function unknown	Cell membrane
293	P0ADT8	YGIM	Signal transduction mechanisms	Cell membrane
294	P0AB26	YCEB	Function unknown	Cell membrane
295	P64451	YDCL	Function unknown	Cell membrane
296	P75818	YBJP	Function unknown	Cell membrane
297	P0AA91	YEAY	Function unknown	Cell membrane
298	P64564	YGGT	Function unknown	Cell membrane
299	P77717	YBAY	Function unknown	Cell membrane
300	P18775	DMSA	Energy production and conversion	Cell membrane
301	P77783	YNFF	Energy production and conversion	Cell membrane
302	P0A7B1	PPK1	Inorganic ion transport and metabolism	Cell membrane
303	P0AFL6	PPX	Function unknown	Cell membrane
304	P17444	BETA	Amino acid transport and metabolism	Cell membrane
305	P64429	YPFJ	General function prediction only	Cell membrane
306	P65292	YGDI	Function unknown	Cell membrane
307	P67244	YQHA	Function unknown	Cell membrane
308	P0ACE0	MBHM	Energy production and conversion	Cell membrane
309	P0AFB1	NLPI	Function unknown	Cell membrane
310	P69741	MBHT	Energy production and conversion	Cell membrane
311	P0ADB7	ECNB	Function unknown	Cell membrane
312	P0AAJ5	FDOH	Energy production and conversion	Cell membrane
313	P0A7E1	PYRD	Nucleotide transport and metabolism	Cell membrane
314	P09152	NARG	Energy production and conversion	Cell membrane
315	P11349	NARH	Energy production and conversion	Cell membrane
Cell inner membrane				
316	P0ABA0	ATPF	Energy production and conversion	Cell inner membrane
317	P0ADZ7	YAJC	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
318	P0ABB0	ATPA	Energy production and conversion	Cell inner membrane
319	P37665	YIAD	Cell wall/membrane/envelope biogenesis	Cell inner membrane
320	P68699	ATPL	Energy production and conversion	Cell inner membrane
321	P0ABJ9	CYDA	Energy production and conversion	Cell inner membrane

(continued)

Table 2 (Continued)

	#UniProt	Gene	Function	Subcellular
322	P64581	YQJD	Function unknown	Cell inner membrane
323	P0AE06	ACRA	Cell wall/membrane/envelope biogenesis	Cell inner membrane
324	P0ADW3	YHCB	Function unknown	Cell inner membrane
325	P0ABK2	CYDB	Energy production and conversion	Cell inner membrane
326	P0ABA4	ATPD	Energy production and conversion	Cell inner membrane
327	P0ADB1	OSME	Function unknown	Cell inner membrane
328	P69428	TATA	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
329	P0ABA6	ATPG	Energy production and conversion	Cell inner membrane
330	P0AC47	FRDB	Energy production and conversion	Cell inner membrane
331	P0ABJ1	CYOA	Energy production and conversion	Cell inner membrane
332	P76576	YFGM	Function unknown	Cell inner membrane
333	P0AFF2	NUPC	Nucleotide transport and metabolism	Cell inner membrane
334	P31224	ACRB	Defense mechanisms	Cell inner membrane
335	P0AAA1	YAGU	Function unknown	Cell inner membrane
336	P07014	SDHB	Energy production and conversion	Cell inner membrane
337	P00363	FRDA	Energy production and conversion	Cell inner membrane
338	P0AG90	SECD	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
339	P0ADY1	PPID	Posttranslational modification, protein turnover, chaperones	Cell inner membrane
340	P69801	PTNC	Carbohydrate transport and metabolism	Cell inner membrane
341	P0AC41	SDHA	Energy production and conversion	Cell inner membrane
342	P0ABC7	HFLK	Posttranslational modification, protein turnover, chaperones	Cell inner membrane
343	P00393	DHNA	Energy production and conversion	Cell inner membrane
344	P0A6E6	ATPE	Energy production and conversion	Cell inner membrane
345	P69805	PTND	Carbohydrate transport and metabolism	Cell inner membrane
346	P09127	HEMX	Function unknown	Cell inner membrane
347	P76372	WZZB	Function unknown	Cell inner membrane
348	P14176	PROW	Amino acid transport and metabolism	Cell inner membrane
349	P0ABC3	HFLC	Posttranslational modification, protein turnover, chaperones	Cell inner membrane
350	P0A8Q0	FRDC	Energy production and conversion	Cell inner membrane
351	P15877	DHG	Function unknown	Cell inner membrane
352	P69786	PTGCB	Carbohydrate transport and metabolism	Cell inner membrane
353	P0AAD6	SDAC	Amino acid transport and metabolism	Cell inner membrane
354	P25714	YIDC	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
355	P0CK95	ACFD	Function unknown	Cell inner membrane
356	P77804	YDGA	Function unknown	Cell inner membrane
357	P0C0S1	MSCS	Cell wall/membrane/envelope biogenesis	Cell inner membrane
358	P0AB98	ATP6	Energy production and conversion	Cell inner membrane
359	P0ADA3	NLPD	Cell wall/membrane/envelope biogenesis	Cell inner membrane
360	P23894	HTPX	Posttranslational modification, protein turnover, chaperones	Cell inner membrane
361	P08395	SPPA	Function unknown	Cell inner membrane
362	P0A742	MSCL	Cell wall/membrane/envelope biogenesis	Cell inner membrane
363	P0AFC3	NUOA	Energy production and conversion	Cell inner membrane
364	P64604	MLAD	Secondary metabolites biosynthesis, transport and catabolism	Cell inner membrane
365	P0CB39	EPTC	Function unknown	Cell inner membrane
366	P77529	TCYP	General function prediction only	Cell inner membrane
367	P0AFA7	NHAB	Function unknown	Cell inner membrane
368	P0ABU9	TOLQ	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
369	P27303	EMRA	Defense mechanisms	Cell inner membrane
370	P0C0L7	PROP	Function unknown	Cell inner membrane
371	P36672	PTTBC	Carbohydrate transport and metabolism	Cell inner membrane
372	P0ABI4	CORA	Inorganic ion transport and metabolism	Cell inner membrane
373	P0A843	TATE	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
374	P0AA47	PLAP	Amino acid transport and metabolism	Cell inner membrane
375	P69423	TATC	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
376	P0AAI3	FTSH	Posttranslational modification, protein turnover, chaperones	Cell inner membrane
377	P08506	DACC	Cell wall/membrane/envelope biogenesis	Cell inner membrane
378	P0ABI8	CYOB	Energy production and conversion	Cell inner membrane
379	P02942	MCP1	Function unknown	Cell inner membrane
380	P29018	CYDD	Function unknown	Cell inner membrane
381	P0AEH5	ELAB	Function unknown	Cell inner membrane
382	P0AC44	DHSD	Energy production and conversion	Cell inner membrane
383	P0A7A7	PLSB	Lipid transport and metabolism	Cell inner membrane
384	P43671	PQIB	Function unknown	Cell inner membrane
385	P60955	LGT	Cell wall/membrane/envelope biogenesis	Cell inner membrane
386	P00803	LEP	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
387	P0C0V0	DEGP	Posttranslational modification, protein turnover, chaperones	Cell inner membrane
388	P07017	MCP2	Function unknown	Cell inner membrane
389	P0AFD6	NUOI	Energy production and conversion	Cell inner membrane
390	P0AA53	QMCA	Posttranslational modification, protein turnover, chaperones	Cell inner membrane
391	P10346	GLNQ	Amino acid transport and metabolism	Cell inner membrane
392	P0AC23	FOCA	Inorganic ion transport and metabolism	Cell inner membrane
393	P27434	RODZ	Function unknown	Cell inner membrane
394	P0ACB4	HEMG	Function unknown	Cell inner membrane
395	P0AC30	FTSX	Cell cycle control, cell division, chromosome partitioning	Cell inner membrane

(continued)

Table 2 (Continued)

	#UniProt	Gene	Function	Subcellular
396	P0ABN1	KDGL	Cell wall/membrane/envelope biogenesis	Cell inner membrane
397	P0AAD8	TDCC	Amino acid transport and metabolism	Cell inner membrane
398	P30845	EPTA	Function unknown	Cell inner membrane
399	P10408	SECA	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
400	Q46798	YGER	Cell wall/membrane/envelope biogenesis	Cell inner membrane
401	P26647	PLSC	Lipid transport and metabolism	Cell inner membrane
402	P0ADV9	LPTC	Function unknown	Cell inner membrane
403	P0ACV0	LPXL	Cell wall/membrane/envelope biogenesis	Cell inner membrane
404	P69874	POTA	Amino acid transport and metabolism	Cell inner membrane
405	P17443	MURG	Cell wall/membrane/envelope biogenesis	Cell inner membrane
406	P11557	DAMX	Function unknown	Cell inner membrane
407	P0AAE8	CADB	Amino acid transport and metabolism	Cell inner membrane
408	P0A830	DCTA	Energy production and conversion	Cell inner membrane
409	P25798	FLIF	Function unknown	Cell inner membrane
410	P02918	PBPA	Cell wall/membrane/envelope biogenesis	Cell inner membrane
411	P60752	MSBA	Defense mechanisms	Cell inner membrane
412	P0ABB8	ATMA	Inorganic ion transport and metabolism	Cell inner membrane
413	P0A6H8	CLSA	Lipid transport and metabolism	Cell inner membrane
414	P0AAF6	ARTP	Function unknown	Cell inner membrane
415	P39396	BTST	Signal transduction mechanisms	Cell inner membrane
416	P10903	NARK	Inorganic ion transport and metabolism	Cell inner membrane
417	P0AG96	SECE	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
418	P24205	LPXM	Cell wall/membrane/envelope biogenesis	Cell inner membrane
419	P0A6A0	UBIB	General function prediction only	Cell inner membrane
420	P0AG00	WZZE	Function unknown	Cell inner membrane
421	P0A8Q3	FRDD	Energy production and conversion	Cell inner membrane
422	P0AE45	YTFL	General function prediction only	Cell inner membrane
423	P60932	UPPP	Defense mechanisms	Cell inner membrane
424	P31801	CHAA	Inorganic ion transport and metabolism	Cell inner membrane
425	P37617	ZNTA	Inorganic ion transport and metabolism	Cell inner membrane
426	P16926	MREC	Cell wall/membrane/envelope biogenesis	Cell inner membrane
427	P0AC78	WECA	Cell wall/membrane/envelope biogenesis	Cell inner membrane
428	P33232	LLDD	Energy production and conversion	Cell inner membrane
429	P0AC75	KDTA	Cell wall/membrane/envelope biogenesis	Cell inner membrane
430	P43341	LPXH	Function unknown	Cell inner membrane
431	P14175	PROV	Amino acid transport and metabolism	Cell inner membrane
432	P11350	NARI	Energy production and conversion	Cell inner membrane
433	P0AAT4	YBDG	Cell wall/membrane/envelope biogenesis	Cell inner membrane
434	P0AE82	CPXA	Signal transduction mechanisms	Cell inner membrane
435	P31680	DJLA	Posttranslational modification, protein turnover, chaperones	Cell inner membrane
436	P56580	PTHB	Carbohydrate transport and metabolism	Cell inner membrane
437	P25744	MDTG	Function unknown	Cell inner membrane
438	P0AEE3	DEGS	Posttranslational modification, protein turnover, chaperones	Cell inner membrane
439	P0A6J5	DADA	Amino acid transport and metabolism	Cell inner membrane
440	P02916	MALF	Carbohydrate transport and metabolism	Cell inner membrane
441	P07117	PUTP	Function unknown	Cell inner membrane
442	P32703	YJCE	Inorganic ion transport and metabolism	Cell inner membrane
443	P60785	LEPA	Cell wall/membrane/envelope biogenesis	Cell inner membrane
444	P09549	DEDD	Function unknown	Cell inner membrane
445	P0A9x1	ZNUC	Inorganic ion transport and metabolism	Cell inner membrane
446	P0AAJ3	FDNH	Energy production and conversion	Cell inner membrane
447	P23930	LNT	Cell wall/membrane/envelope biogenesis	Cell inner membrane
448	P37636	MDTE	Cell wall/membrane/envelope biogenesis	Cell inner membrane
449	P39321	TAMB	Function unknown	Cell inner membrane
450	P29131	FTSN	Function unknown	Cell inner membrane
451	P63183	KUP	Inorganic ion transport and metabolism	Cell inner membrane
452	P09391	GLPG	General function prediction only	Cell inner membrane
453	P0A6J3	CYSZ	Amino acid transport and metabolism	Cell inner membrane
454	P0A9R7	FTSE	Cell cycle control, cell division, chromosome partitioning	Cell inner membrane
455	P0AAE0	CYCA	Amino acid transport and metabolism	Cell inner membrane
456	P0AAH8	SAPF	Function unknown	Cell inner membrane
457	P0ABG7	RODA	Cell cycle control, cell division, chromosome partitioning	Cell inner membrane
458	P0ABP3	DCUC	Function unknown	Cell inner membrane
459	P0ABV6	TOLR	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
460	P0AD65	MRDA	Cell wall/membrane/envelope biogenesis	Cell inner membrane
461	P0AD68	FTSI	Cell wall/membrane/envelope biogenesis	Cell inner membrane
462	P0AEC3	ARCB	Signal transduction mechanisms	Cell inner membrane
463	P0AEJ0	EMRB	Function unknown	Cell inner membrane
464	P0AER0	GLPF	Carbohydrate transport and metabolism	Cell inner membrane
465	P0AFK6	POTC	Amino acid transport and metabolism	Cell inner membrane
466	P0AFY6	SBMA	Function unknown	Cell inner membrane
467	P19934	TOLA	Function unknown	Cell inner membrane
468	P23886	CYDC	Function unknown	Cell inner membrane
469	P24207	PHEP	Amino acid transport and metabolism	Cell inner membrane

(continued)

Table 2 (Continued)

	#UniProt	Gene	Function	Subcellular
470	P25737	LYSP	Amino acid transport and metabolism	Cell inner membrane
471	P28306	MLTG	General function prediction only	Cell inner membrane
472	P30750	METN	Inorganic ion transport and metabolism	Cell inner membrane
473	P37019	CLCA	Inorganic ion transport and metabolism	Cell inner membrane
474	P37637	MDTF	Defense mechanisms	Cell inner membrane
475	P39401	OPGB	Cell wall/membrane/envelope biogenesis	Cell inner membrane
476	P63386	MLAF	Secondary metabolites biosynthesis, transport and catabolism	Cell inner membrane
477	P64606	MLAE	Secondary metabolites biosynthesis, transport and catabolism	Cell inner membrane
478	P68183	MALG	Carbohydrate transport and metabolism	Cell inner membrane
479	P69425	TATB	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
480	P75830	MACA	Cell wall/membrane/envelope biogenesis	Cell inner membrane
481	P76272	YEBT	Function unknown	Cell inner membrane
482	P76298	FLHA	Function unknown	Cell inner membrane
483	P76578	A2MG	General function prediction only	Cell inner membrane
484	P77285	RSXG	Energy production and conversion	Cell inner membrane
485	P77304	DTPA	Amino acid transport and metabolism	Cell inner membrane
486	P77338	MSCK	Cell wall/membrane/envelope biogenesis	Cell inner membrane
487	Q47142	HCAI	Function unknown	Cell inner membrane
488	P0ABB4	ATPB	Energy production and conversion	Cell inner membrane
489	P0ABN5	DCUA	General function prediction only	Cell inner membrane
490	P69054	DHSC	Energy production and conversion	Cell inner membrane
491	P0AEB2	DACA	Cell wall/membrane/envelope biogenesis	Cell inner membrane
492	P0AFE4	NUOK	Energy production and conversion	Cell inner membrane
493	P0ABU7	EXBB	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
494	P0AGA2	SECY	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
495	P0AG99	SECG	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
496	P08194	GLPT	Carbohydrate transport and metabolism	Cell inner membrane
497	P0AB67	PNTB	Energy production and conversion	Cell inner membrane
498	P04846	NLPA	Inorganic ion transport and metabolism	Cell inner membrane
499	P0AFJ7	PITA	Inorganic ion transport and metabolism	Cell inner membrane
500	P0AG93	SECF	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
501	P07001	PNTA	Energy production and conversion	Cell inner membrane
502	P23173	TNAB	Amino acid transport and metabolism	Cell inner membrane
503	P0AFC7	NUOB	Energy production and conversion	Cell inner membrane
504	P0ABN9	DCUB	General function prediction only	Cell inner membrane
505	P0AFD4	NUOH	Energy production and conversion	Cell inner membrane
506	P0AD99	BRNQ	Amino acid transport and metabolism	Cell inner membrane
507	P0ABF8	PGSA	Lipid transport and metabolism	Cell inner membrane
508	P0ABG1	CDSA	Lipid transport and metabolism	Cell inner membrane
509	P21365	YCIC	Function unknown	Cell inner membrane
510	P0AEQ6	GLNP	Amino acid transport and metabolism	Cell inner membrane
511	P0AEL0	FDOI	Energy production and conversion	Cell inner membrane
512	P0AG14	SOHB	Function unknown	Cell inner membrane
513	P02919	PBPB	Cell wall/membrane/envelope biogenesis	Cell inner membrane
514	P0A9C0	GLPA	Energy production and conversion	Cell inner membrane
515	P06282	CDH	Lipid transport and metabolism	Cell inner membrane
516	P09348	MOTA	Cell motility	Cell inner membrane
517	P33607	NUOL	Function unknown	Cell inner membrane
518	P62517	OPGH	Function unknown	Cell inner membrane
519	P0ABV2	EXBD	Intracellular trafficking, secretion, and vesicular transport	Cell inner membrane
520	P13033	GLPB	Amino acid transport and metabolism	Cell inner membrane
521	P0AAH0	PSTB	Inorganic ion transport and metabolism	Cell inner membrane
522	P0AEZ3	MIND	Cell cycle control, cell division, chromosome partitioning	Cell inner membrane
523	P36837	DTPB	Amino acid transport and metabolism	Cell inner membrane
524	P23865	PRC	Cell wall/membrane/envelope biogenesis	Cell inner membrane
525	P0ADC6	LPTG	General function prediction only	Cell inner membrane
526	P37660	YHJV	Amino acid transport and metabolism	Cell inner membrane
527	P0AF98	LPTF	General function prediction only	Cell inner membrane
528	P0A996	GLPC	Energy production and conversion	Cell inner membrane
529	P06974	FLIM	Cell motility	Cell inner membrane
530	P0AA86	DSBE	Function unknown	Cell inner membrane
531	P0AB12	YCCF	Function unknown	Cell inner membrane
532	P0AD27	YEJM	Function unknown	Cell inner membrane
533	P0AE34	ARTQ	Function unknown	Cell inner membrane
534	P0AE74	CITT	Inorganic ion transport and metabolism	Cell inner membrane
535	P0AFF0	NUON	Energy production and conversion	Cell inner membrane
536	P0AFK4	POTB	Amino acid transport and metabolism	Cell inner membrane
537	P23200	MGLC	Carbohydrate transport and metabolism	Cell inner membrane
538	P30844	BASS	Signal transduction mechanisms	Cell inner membrane
539	P31064	YEDE	General function prediction only	Cell inner membrane
540	P31119	AAS	Function unknown	Cell inner membrane
541	P45800	IGAA	Function unknown	Cell inner membrane
542	P63340	YQEG	Amino acid transport and metabolism	Cell inner membrane
543	P64515	YECN	General function prediction only	Cell inner membrane
544	P75958	LOLE	Cell wall/membrane/envelope biogenesis	Cell inner membrane

(continued)

Table 2 (Continued)

#UniProt	Gene	Function	Subcellular
		Bacterial flagellum basal body	
545	P75937	FLGE	Cell motility
546	P0ABX5	FLGG	Cell motility
		Secreted	
547	P33235	FLGK	Cell motility
548	P04949	FLIC	Cell motility
549	P29744	FLGL	Cell motility
550	P24216	FLID	Cell motility
		Fimbrium	
551	P08191	FIMH	Function unknown
552	P04128	FIMA1	Function unknown
553	P08190	FIMG	Function unknown
554	P08189	FIMF	Function unknown

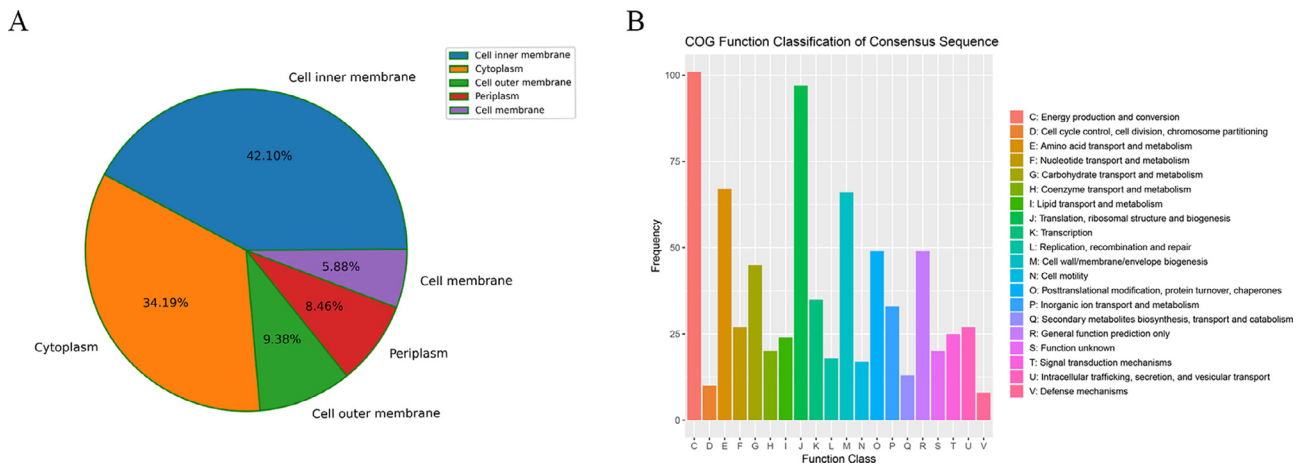


Figure 2. Predicted subcellular locations proportions and functional classification of the AE17 OMVs proteins identified by LC-MS/MS. (A) Predicted subcellular locations of the 544 OMVs proteins that were identified and three independent vesicle preparations that were analyzed (B) The major groups of COG domains that were found in the OMV proteins that were identified and three independent vesicle preparations that were analyzed.

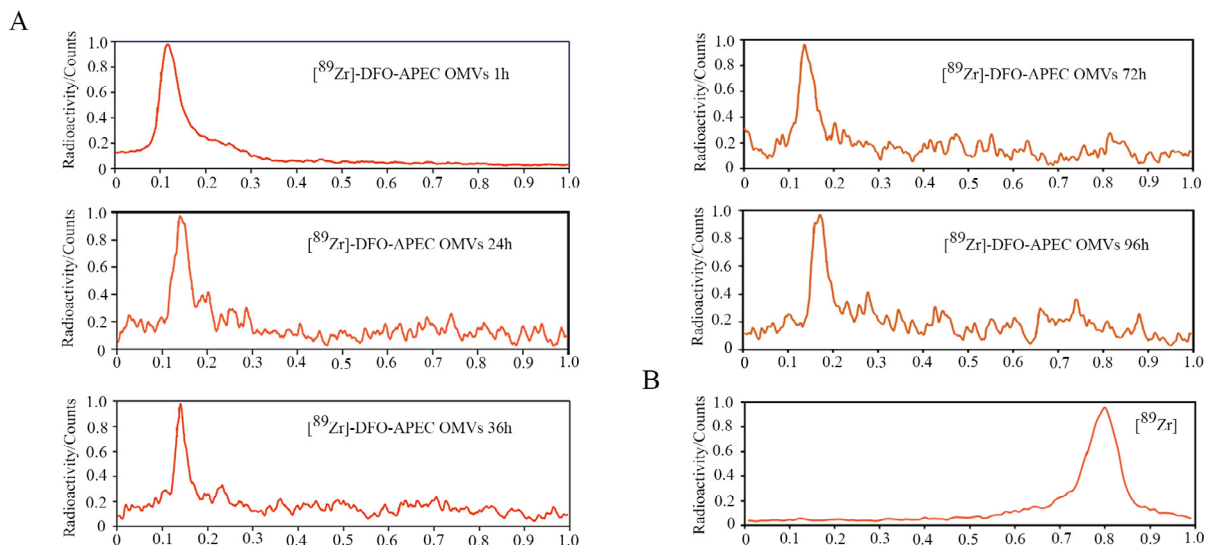


Figure 3. Radiochromatograms (A) ⁸⁹Zr-DFO-OMVs 0.5 M in sodium citrate buffer solution under different incubation times using the Radio-iTLC (B) ⁸⁹Zr in 0.5 M in sodium citrate buffer solution using the Radio-iTLC.

was first performed in Figures 4A and 4B. A strong ⁸⁹Zr signal was detected in the trachea and lung regions within 0.25 h of injection of ⁸⁹Zr-DFO-OMVs. Within 0.25 to 3 h of the injection of ⁸⁹Zr-DFO-OMVs, strong

⁸⁹Zr signals were detected in the gastrointestinal region, while less strong ⁸⁹Zr signals were detected in the renal region and decreased in the tracheal and pulmonary regions. Between 3 and 12 h after injection of ⁸⁹Zr-DFO-

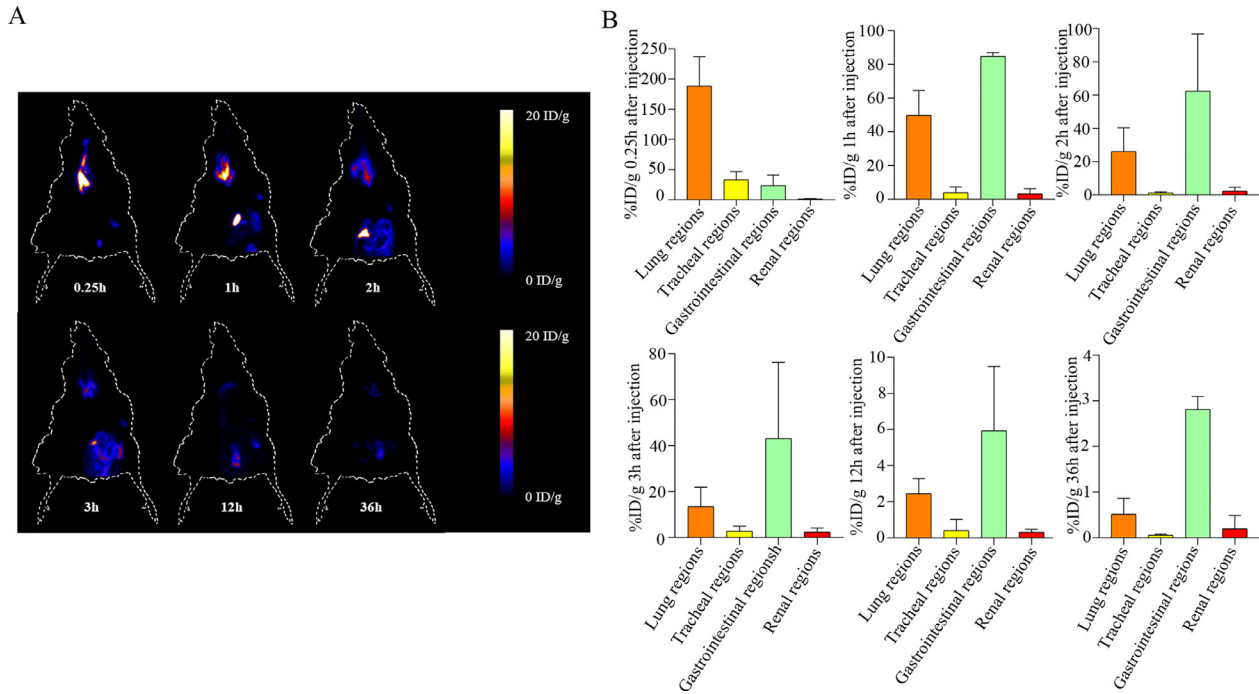


Figure 4. PET images (A) chickens injected with 100 μg of ^{89}Zr -DFO-OMVs obtained at 0.25, 1, 2, 3, 12 and 36 hours (B). PET data quantification was performed for organs uptake at 0.25, 1, 2, 3, 12 and 36 hours after ^{89}Zr -DFO-OMVs injection in three chickens. Data are presented as the mean \pm SD.

OMVs, ^{89}Zr signals were gradually reduced in the tracheal and pulmonary regions as well as in the gastrointestinal and renal regions, but ^{89}Zr signals were still detectable at 36 h after injection. No chickens died after intratracheal injection of ^{89}Zr -DFO-OMVs in SPF chickens (data not shown), indicating that 100 μg of OMVs was a non-lethal dose.

Histological Effects of ^{89}Zr -DFO-AE17-OMV on Major Organs

To determine the histological effects of OMVs in vivo, histological examination of vital organ tissues including intestine, lungs, stomach, and kidneys revealed pathological changes in the chickens treated by AE17 OMVs,

as shown in Figure 5. Histopathological analysis of the intestine showed a large infiltration of inflammatory cells and a loss of intestinal stratification. Histopathological analysis of the lungs showed features of interstitial pneumonia with widening of the interstitium, marked narrowing of the alveolar lumen, massive fibrin exudation, and infiltration of macrophages. Histopathological analysis of the stomach revealed reduced epithelial cells and infiltration of inflammatory cells. Histopathological analysis of the kidney showed degeneration of tubular epithelial granules with partial swelling and rupture, glomerular fibrosis, and inflammatory cell infiltration. These organs all showed a marked inflammatory process, suggesting that AE17 OMVs have inflammatory activity in chickens

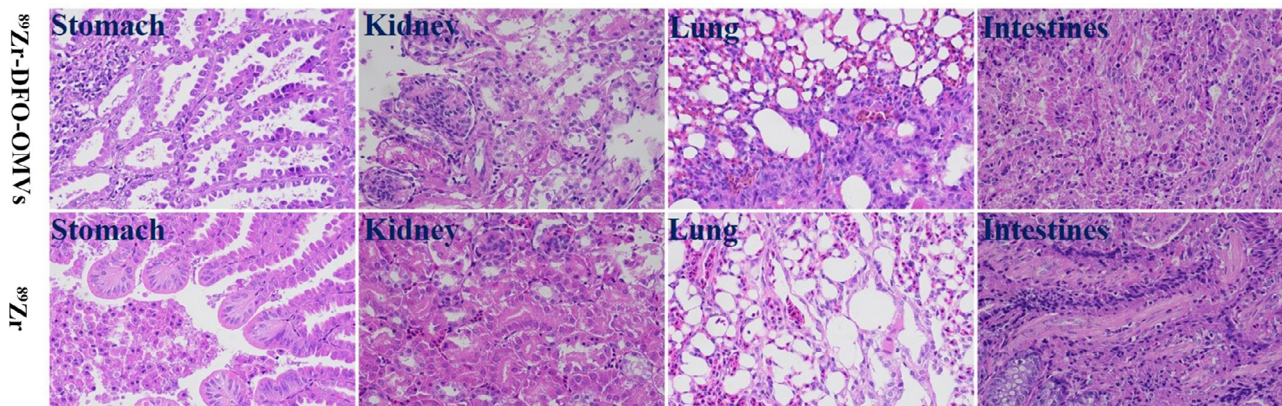


Figure 5. Histology of vital organs. The sections were stained with hematoxylin and eosin (400 \times magnification).

DISCUSSION

The OMVs are proteolipid bodies around 20 to 400 nm in diameter, initially found in nutritionally deficient *Escherichia coli*, but there is growing evidence that a wide range of Gram-negative bacteria can produce such spherical nanostructures (Sartorio et al., 2021). They are derived from the outer membrane, contain most of the contents of the parental bacteria, a mixture of complex composition (Kaparakis-Liaskos and Ferrero, 2015) and exhibit biological activity through associated proteins that promote bacterial survival and the transport and release of virulence (Kulp and Kuehn, 2010).

The present study found that AE17 could secrete OMVs and that the morphology of OMVs was consistent with reported studies. The total protein composition of AE17 outer membrane vesicles was assessed by LC-MS/MS analysis, and among the most abundant proteins, in order of intensity, the majority were outer membrane proteins. Several outer membrane proteins were identified, including OmpA, OmpC, and OmpF, which represent the most abundant proteins and were found in all *E. coli* strains studied (Lee et al., 2008). To date, there has been only one proteomic study on APEC OMVs. The outer membrane lipoprotein (**Lpp**) in that report was the single most abundant outer membrane protein in APEC 078, whereas this study determined that OmpA was the most abundant in AE17, which could be attributed to interstrain variation in APEC bacteria, or OMVs could have been isolated from different growth stages of APEC (Wang et al., 2019). The pathogenic characteristics of OMVs are thought to depend mainly on the role of outer membrane and extracellular proteins (Choi et al., 2011). Several outer membrane proteins OmpA, OmpC, OmpF, OmpX, FimH, FimC, and FlgE have been shown to be important during host infection by APEC and can function as virulence factors for APEC, which adheres to the host cell surface and colonizes through these above virulence proteins (Kathayat et al., 2021). In addition, OmpA and OmpF have immunostimulatory activity and induce leukocyte migration (Ellis and Kuehn, 2010), where flagellin (**FlhC**) can be transported by outer membrane vesicles to the cytosol to trigger the host NLRC4 inflammasome, producing IL-1 β pro-inflammatory cytokines (Yang et al., 2020). Two serum resistance proteins, OmpT and MliC (Vanderkelen et al., 2012; Hejair et al., 2017) were also identified in which OmpT in OMVs may degrade cationic antimicrobial peptides produced by epithelial cells or macrophages (Ramírez-Larrosa and Eckhard, 2022). Notably, the MliC protein is specific to AE17 and has not been previously reported in *E. coli* DH5 α (Lee et al., 2007), suggesting that this protein may be associated with poultry infection. However, further experiments are needed to understand the role of these virulence proteins in the pathogenesis of APEC OMVs.

Several studies have shown that OMVs cause a range of inflammatory diseases in animal models in vivo (Dhital et al., 2021), but few studies have assessed the pathogenic role of OMVs in vivo by looking at their in vivo biodistribution and kinetic behavior. The use of PET imaging allows qualitative and quantitative visualization of the presence of radiometabolites and free radioisotopes through radiotracers while offering the advantage of high sensitivity and noninvasive (Patel et al., 2022). Berzosa et al attempted to develop radioisotope-labeled OMVs to assess biodistribution, but they only showed in-vitro biodistribution over 10 h, which was related to a ^{99m}Tc half-life of only 6 h (Berzosa et al., 2022). Jang et al. used fluorescent Cy7-labeled OMVs to show in vitro biodistribution over 24 h, but optical imaging has limitations in the detection of small organs and deep tissue (Jang et al., 2015). To achieve in vivo radionuclide-labeled OMVs for prolonged imaging under physiological conditions, ^{89}Zr 's was chosen with a half-life of 78.4 h and DFO was used to chelate ^{89}Zr . Due to the protein-rich surface of AE17 OMVs, ^{89}Zr -DFO was able to bind to the surface proteins of OMVs and had good stability for up to 96 h.

The tissue distribution of Extracellular vesicle secreted by eukaryotic cells has been shown to be specific, with a predominant distribution in the liver, spleen, and kidneys (Wiklander et al., 2015; Kang et al., 2021). In contrast, AE17-secreted OMVs are distributed in the tracheopulmonary and gastrointestinal regions, but also in the renal region, which may be related to the fact that EVs and OMVs are excreted through the kidneys. More interestingly tracheal injected AE17 OMVs were ingested at the gastrointestinal tract, which also suggests that AE17 OMVs can deliver virulence factors over long distances. Many studies have shown that OMVs can cause inflammatory responses in mammals. Intranasal administration of *P. aeruginosa* OMVs leads to lung inflammation (Park et al., 2013). *Legionella*-derived OMVs cause inflammation in human lung tissue explants by targeting alveolar macrophages (Jäger et al., 2014). However, studies of OMVs inducing inflammation in poultry are scarce. The considerable number of leukocyte infiltrates in histopathological sections is a strong indicator that OMVs are ingested and trigger an inflammatory response (Kolaczowska and Kubes, 2013). In this study, inflammatory responses in chickens occurred mostly at sites of high uptake of OMVs, but minor inflammatory responses were also detected at other sites. In previous studies, OMVs made the inflammatory system hyperactive, including infiltration of inflammatory cells and increased production of proinflammatory mediators such as tumor necrosis factor (**TNF- α**) and interleukin (**IL-6**) (Park et al., 2010). Based on the evidence provided in this study, AE17 OMVs could cause inflammatory responses in chickens and further research will help understand the regulatory mechanisms of AE17 OMVs-induced inflammatory responses.

CONCLUSIONS

In summary, this study identified 804 proteins located on the OMVs by LC-MS/MS and found several proteins associated with adhesion, serum resistance and immunomodulatory. It also investigated the biodistribution of AE17 OMVs and found that while their distribution in chickens was specific, tracheal injected AE17 OMVs could act on the gastrointestinal tract, suggesting that OMVs could deliver virulence factors over long distances. The AE17 OMVs caused inflammatory damage at the tissue sites where AE17 OMVs were ingested, so the next investigation is to investigate the mechanisms of AE17 OMVs and their molecular cargo-induced inflammation and induction of cell death to provide insights into the pathogenicity of AE17.

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DISCLOSURES

The authors declare no competing interests.

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