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Proteomic analysis of outer membrane vesicles of Aeromonas hydrophila ML09-119

By

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Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Science
in the Department of Comparative Biomedical Science

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2020

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Aeromonas hydrophila ML09-119 is an important fish pathogen that severely affects channel catfish aquaculture. To better understand this strain's virulence factors, outer membrane vesicles (OMVs) were isolated, and their proteome was assessed. Using transmission electron microscopy and dynamic light scattering, OMVs were shown to be monodispersed particles with an average diameter of 120.33 nm. OMV proteins were identified using mass spectrometry, and analysis of the resulting proteome of 74 proteins revealed that many originated from the cytoplasm, but there was an enrichment of outer membrane, periplasmic, and extracellular proteins compared to the total proteome. The majority of the functional classifications were associated with bacterial metabolism. Of the predicted virulence factors, several had a putative function in adherence, and there were type III secretions system proteins as well as three secreted exotoxins. Overall, our data reveal new insights into *A. hydrophila* OMVs and their potential roles in physiology and virulence.

DEDICATION

To my grandmother, Dr. Jane Smink, who continues to inspire me.

"They will dismiss you. Make them listen."

ACKNOWLEDGEMENTS

My loving husband, Christopher Hudson who shares my hopes and dreams.

My parents who have always encouraged and supported my continued education.

Karl F. Smink & Teah Smink

Danny Hudson & Petra Hudson

Robert Lamb and Karl A. Smink who helped redirect me.

My family that have sacrificed weekends and holidays together.

My friends who have excused my prolonged absence and preoccupation.

Countless mentors who have taken a vested interest in my success and kept me from drowning.

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CHAPTER I

GRAM NEGATIVE OUTER MEMBRANE VESICLES

1.1 Introduction

Outer membrane nanovesicles (OMVs) are spherical blebs that are naturally secreted by all Gram-negative bacteria at every stage of growth.[1] OMVs were first observed in electron micrographs in the 1960s.[2] They are heterogeneous in both size and composition, even among the same strain of bacteria.[3] As portions of the cell membrane bleb from the bacterium, they capture portions of the periplasm and cytosol.[4] OMVs are rich in their diversity of virulence factors, including surface proteins, toxins, lipopolysaccharides, and other immunoactivating ligands that enhance the virulence of the pathogen.[5] Vesicles containing these molecules are easily transported to different host tissues.[6] Pathogenic and nonpathogenic Gram-negative bacteria secrete OMVs, indicating that virulence factor transport may not be the sole purpose of these vesicles.[6] OMVs have also demonstrated the ability to initiate host immune response through leukocyte activation and inflammatory cytokine expression independent of parent bacteria.[7-8] Immunomodulatory roles of OMVs have been implicated in sepsis.[7-8] The immune-stimulating properties of OMVs have become an area of interest in preventative medicine as OMVs have been found to activate antigen-presenting dendritic cells as well as prime B and T cell responses.[9] Vaccine potential of OMVs has been investigated, and although vesicles stimulate protective immunity, OMV vaccines have had mixed success.[9-12] Differences in

success are largely due to the structure and molecular composition of these heterogeneous vesicles. OMVs are also difficult and expensive to isolate.

1.2 Structure of OMVs

The Gram-negative cell wall is made up of an outer membrane and an inner plasma membrane. Between these structures is what is known as the periplasmic space filled with a gel-like matrix named the periplasm. These three structures, 1) outer membrane, 2) periplasm, and 3) the plasma membrane, constitute the Gram-negative envelope. OMVs are closed spherical nonreplicating components of the Gram-negative envelope that are excreted at all stages of the bacterial growth cycle.[13-14] OMVs are secreted from bacteria growing in both planktonic or biofilm states, on solid or in liquid media, and in vivo or vitro.[2-3, 7-8]. They are approximately 50-250 nanometers in size and are formed when sections of the outer membrane bleb off between peptidoglycan molecules.[3] Due to their budding mechanisms (Figure 1.1), OMVs are surprisingly stable because the constituents of the outer membrane are situated in the natural manner of the parent bacterium, but on a much smaller scale.[3, 5] OMVs contain surface proteins, toxins, lipopolysaccharide, and autolysins.[3]

1.3 Molecular Composition of OMVs

Gram-negative OMVs are blebs of the outer membrane and periplasm. Molecules found on the outer membrane and within the periplasmic space are included in these tiny bacterial membrane packages. They can include outer membrane proteins, phospholipids, exotoxins, endotoxin, and destructive enzymes such as hemolysins and hydrolases (Table 1.1). Proteomic studies have compared the OMV-associated proteins with the whole bacterial proteome, which revealed differing concentrations or enrichment of some proteins.[14-15]

1.4 Virulence Factors of OMVs

Virulence factors are secreted by bacteria to promote host colonization and bacterial survival. Several highly specialized nanomachines are present in bacteria to secrete proteins, DNA, and other small molecules. Due to the structure of the Gram-negative envelope, molecules in the cytoplasm must traverse the plasma membrane, the periplasm, and the outer membrane to be secreted. Thus, virulence factors of Gram-negative bacteria must be secreted through one of several types of bacterial secretion systems: type I secretion system (T1SS), T2SS, T3SS, T4SS, or T6SS.[16] OMVs are a distinct mechanism that allows bacterial secretion of a complex mixture of proteins and lipids.[14]

1.4.1 Attachment

OMVs are a means of communication and interaction between bacteria and other prokaryotic and eukaryotic cells.[14,16] OMVs mediate adhesion fusion to target eukaryotic cells due to Gram-negative outer membrane expression of adhesins. The strain-specific method of binding and outer membrane fusion is similar to the parent bacteria due to similarity in composition.[14] Some OMVs contain proteins that can act as adhesins.[1, 14] Uptake of OMVs can also be regulated by the interaction between OMV proteins and the target cell.

1.4.2 Exotoxins

Exotoxins are molecules excreted from living bacteria into the surrounding environment. OMVs can transport exotoxin originating from the parent bacterial outer membranes. OMVs are easily disseminated into host tissues that are otherwise difficult for bacteria to gain entry and therefore are an excellent toxin delivery mechanism.[14] Enterotoxigenic *Escherichia coli* (ETEC) produces a heat-labile enterotoxin (LT) responsible for the voluminous watery diarrhea

associated with ETEC infection. LT is located both inside and outside ETEC OMVs, but it is found at enriched volumes in OMVs.[17]

1.4.3 Endotoxin

OMVs can transport endotoxin (lipopolysaccharide) that originates from the outer membrane of parent bacteria. Lipopolysaccharide (LPS) is the outer leaflet of the Gram-negative outer membrane. When released, LPS binds toll-like receptor-4 (TLR4) and initiates production of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNFα) through nuclear factor kappa-B (NF-kB) translocation.[7, 18] Enterotoxigenic *Escherichia coli* (ETEC) OMVs containing LPS stimulate the production of IL-6 and TNFα independent of parent bacteria.[7-8]

1.4.4 Hemolysis

Hemolysins cause the lysis of erythrocytes by membrane destruction. OMVs can transport hemolysins to target cells. Enterohemorrhagic *Escherichia coli* (EHEC) excretes EHEC hemolysin (EHEC-Hly) in both free- and OMV-bound form[19]. EHEC-Hly targets mitochondria and induces cell apoptosis via a dynamin-dependent endocytosis[19]. As EHEC-Hly undergoes endosomal separation, the hemolysin is liberated from the OMVs[19]. It is able to escape the lysosomes, target the mitochondria, and trigger caspase-9, which triggers host cell apoptosis[19].

1.4.5 Hydrolases

Hydrolases are enzymes that catalyze hydrolysis of biomolecules. *Pseudomonas aeruginosa* OMVs were found to contain peptidoglycan hydrolase as well as other periplasmic components such as phospholipase and protease.[20] Peptidoglycan hydrolase (PGase) is an autolysin in OMVs that can lyse both Gram-positive and Gram-negative bacteria.[20]

1.5 OMVs as Immunomodulators

1.5.1 Activation of Endothelium

The endothelium serves as the interface between the blood or lumen and the vascular system. Both pathogenic and nonpathogenic OMVs have demonstrated the ability to activate the endothelium as defined by measuring the NF-kB nuclear translocation.[7] LPS binds TLR4 and causes NF-kB activation by cleaving the inhibitor of kB (IkB) complex from NF-kB-IkB. The released NF-kB is translocated to the nucleus, where it activates transcription of cytokines.

1.5.2 Activation of Leukocytes

Once OMVs activate the endothelium, cytokines TNFα, IL-1, IL-6, IL-8, and IL-12 are expressed and secreted. Following exposure to pathogenic and nonpathogenic bacteria, human umbilical vein endothelial cells (a model for the endothelium) increase expression of IL-6, which activates leukocytes.[7] The ability to activate leukocytes means that OMVs are able to stimulate the innate immune system.

1.5.3 Activation of Antigen Presenting Cells

OMVs can stimulate professional antigen-presenting cells (APCs). OMVs of *Salmonella enterica* Typhimurium stimulate macrophages and dendritic cells to express MHC-II and CD86 as well as increase production of TNFα and IL-12.[9] Stimulation of dendritic cells occurred in TLR-4 independent and dependent models, which demonstrates that OMVs stimulation of APCs is not limited to LPS stimulation.[9] It also indicates there are multiple toll-like receptors responsible for the action of immune stimulation by OMVs.[9] The ability of OMVs to stimulate APCs demonstrate OMVs are immunomodulators of the adaptive immune system.

1.5.4 Stimulation of B and T Cells

Stimulation of B and T cell responses is an essential aspect of the adaptive immune system that is necessary for immune protection. OMVs are antigenically heterogeneous and diverse, with the ability to stimulate both B and T cells. Mice inoculated with OMVs showed immune protection when challenged with *Salmonella enterica* Typhimurium.[9] Additional studies have shown the immunoprotective effects of OMVs in humans and fish.[10, 12]

1.6 Medical Implications of OMVs

1.6.1 Vaccines

Immunomodulatory activities of OMVs make them attractive as potential vaccine candidates. As nonreproducing multiantigenic structures, OMVs are seemingly a better choice than live attenuated vaccines in immune-compromised patients. With a growing population of immunocompromised individuals stemming from long term disease, transplants, and HIV infection, attention is being turned toward nonliving vaccines that cannot replicate in an immunocompromised host. Problems with OMVs arise largely from vast differences in outer membrane composition between strains.[21-22] LPS toxicity is a major concern in OMV vaccines for mammalian species. LPS contained in OMVs can trigger inflammation in mammalian cells and induce sepsis through interaction with mammalian TLR4.[7-8, 18] In this case, strains with low LPS toxicity or chemical treatment that may alter antigenic properties of OMVs are necessary.[1]

1.6.2 Sepsis

Sepsis is a major medical condition, and, in critically ill patients, it is the leading cause of death.[7] Systemic infection activates a cytokine storm resulting in increased capillary permeability and subsequent death by hypovolemic shock. OMVs from ETEC cause alteration of blood flow

resulting in liver and lung damage in mice.[8] Their ability to cause such massive tissue damage is thought to result from their ability to activate the endothelium by increasing production of IL-6.[7]

1.6.3 Research Objectives

Aeromonas hydrophila is a Gram-negative bacteria that produces outer membrane vesicles. Typically considered an opportunistic infection in fish that could be managed using good animal husbandry, a highly virulent isolate emerged in 2009. This new virulent strain of *A. hydrophila* (vAh) affects predominantly market size catfish. Work needs to be done to understand the specific virulence factors that separate opportunistic *A. hydrophila* from highly virulent *A. hydrophila*. OMVs, are potent virulence factors in other bacterial species which leads us to hypothesize that OMVs could be virulence factors of vAh. The objectives of this research are to develop a protocol for the isolation of OMVs from *A. hydrophila*, examine the ultrastructure of OMVs, characterize vesicle size and protein composition, and to identify potential virulence factors that may be associated with the OMVs of vAh.

1.7 Tables and Figures

Table 1.1 Virulence factors in the outer membrane nanovesicles of various bacteria

Bacterial Species	Virulence Factor
Actinobacillus pleuropneumoniae	Proteases, ApxI
Actinobacillus actinomycetemcomitans	Leukotoxin
Bacteriodes fragilis	Hemagglutinin, hydrolytic enzymes
Bordetalla pertussis	AC-Hly toxin, hemagglutinin (FHA), pertussis toxin (Ptx)
Borrelia burgdoferi	OspA, OspB, OspD toxins
Burkholderia cepacia	PLC-N, lipase, PSCP, a 40-kDa protease
E. coli (ETEC)	LT toxin
E. coli (STEC)	Shiga toxin
E. coli (EHEC)	ClyA cytotoxin
Helicobacter pylori	VacA toxin
Legionalla pneumonophila	Mip (Ipg0791), IcmK/IcmX, LaiE/LaiF, hydrolytic enzymes
Moraxella catarrhalis	UspA1/UspA2
Neisseria meningitides	PorA, NlpB, NarE
Pseudomonas aeruginosa	Hemolysin, hydrolytic enzymes, Cif and PQS toxins
Salmonella typhi	ClyA cytotoxin
Shigella flexneri	IpaB, IpaC, IpaD toxins
Shigella dysenteriae	Shiga toxin
Treponema denticola	Proteases, dentilysin
Vibrio angullarum	Metalloproteinase, hemolysin, and phospholipase
Vibrio cholera	RTX toxin
Xanthomonas compestris	Cellulase glucosidase, xylosidase and nonvirulent proteins

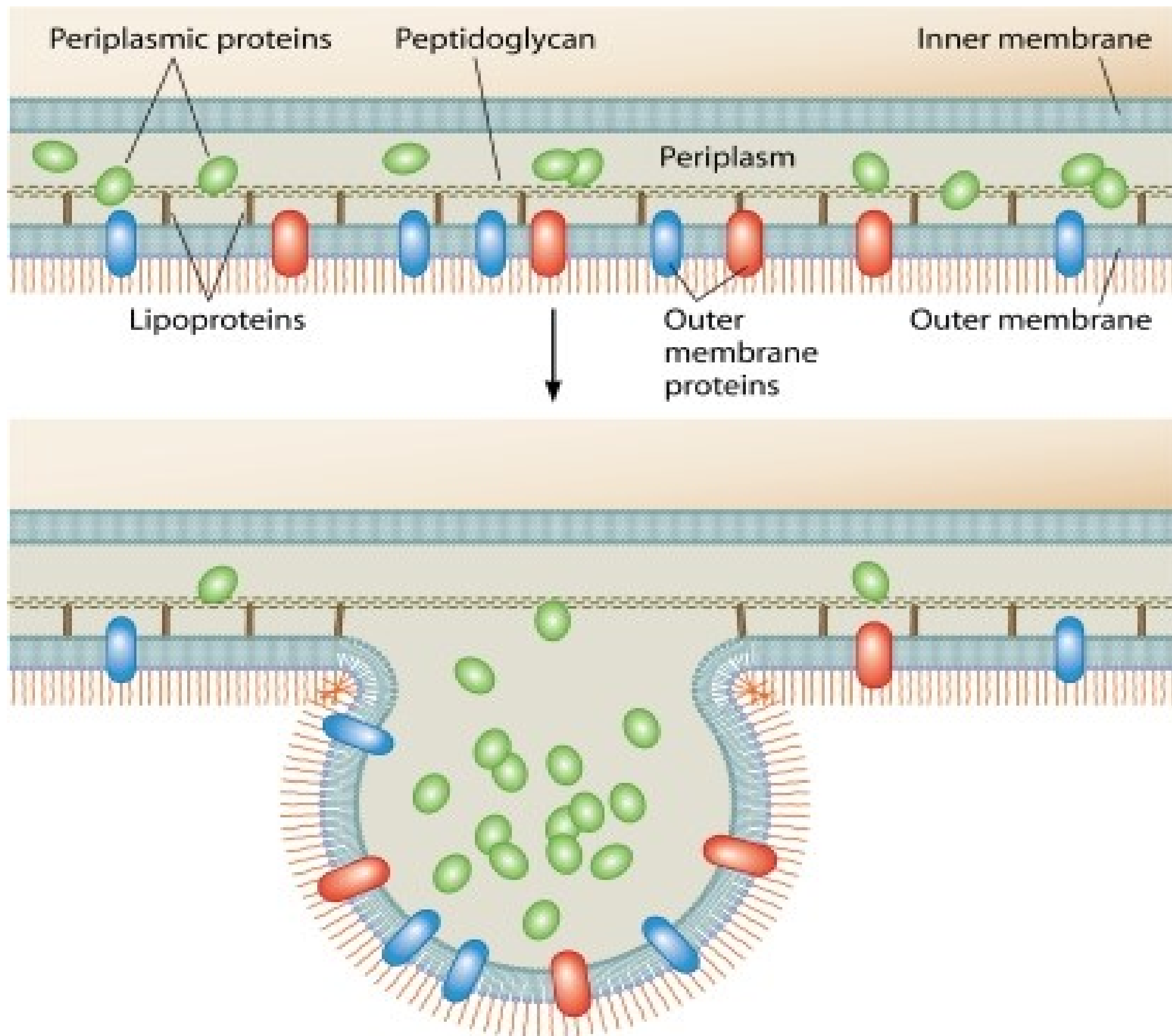


Figure 1.1 **Model of OMV production.** Vesiculation is thought to occur between the spaces of peptidoglycan structures. Budding mechanism results in periplasmic constituents being included inside capsular OMV structures. (*Reproduced with permission from Virulence and Immunomodulatory Roles of Bacterial Outer Membrane Vesicles*).[23]

CHAPTER II

PROTEOMIC ANALYSIS OF OUTER MEMBRANE VESICLES OF *AEROMONAS HYDROPHILA* ML09-119

2.1 Introduction

Aeromonas hydrophila is a causative agent of motile aeromonas septicemia (MAS) that poses a major threat to the channel catfish (*Ictalurus punctatus*) aquaculture industry.[24-27] This Gram-negative, rod-shaped bacteria is ubiquitous in aquatic environments, and it is most typically an opportunistic pathogen resulting from poor health or poor husbandry. However, in 2009 a highly virulent clonal group (virulent *A. hydrophila* or vAh) emerged causing a high incidence of mortality in market size fish. *A. hydrophila* strain ML09-119 is a representative isolate of this clonal group, and it was isolated from an outbreak on a commercial catfish farm.[26] Genome sequencing revealed that vAh strains share > 99.88% average nucleotide identity (ANI), and they are a distinct genetic group compared to other *A. hydrophila*.[26, 28] They have some unique genes and metabolic pathways that enabled development of a real-time PCR method to specifically detect vAh.[25] Work still needs to be done to understand the virulence factors that make vAh different from low-risk opportunistic *A. hydrophila* strains.

Outer membrane vesicles (OMVs) are natural secretions of all Gram-negative bacteria that contain potent multiantigenic immunomodulator molecules unique to each strain of bacteria.[1] OMVs can initiate host immune response through leukocyte activation and inflammatory cytokine expression independent of parent bacteria.[8, 29] OMVs are amorphous and

heterogeneous in size and composition, and they contain portions of the periplasm and the cytosol.[30] The objective of this study was to purify and characterize the outer membrane vesicles of *A. hydrophila* strain ML09-119. Characterizing OMVs of this vAh strain will shed light on its pathogenic mechanisms as well as identify proteins with potential as protective antigens for use in vaccination strategies.

2.2 Materials and Methods

2.2.1 Outer Membrane Vesicle Isolation

A. hydrophila ML09-119 cultures were started from single isolated colonies in brain heart infusion (BHI) broth. Four 5 mL BHI cultures were grown overnight at 37 °C with shaking at 125 rpm. The cultures were used to inoculate four 500 mL cultures in BHI and grown for 18 hours. The resulting cultures were placed on ice to arrest growth and decanted into eight sterile centrifuge bottles. Samples were subjected to centrifugation at 2,652 x g for 10 minutes at 4 °C in a Sorvall RC6+ superspeed centrifuge (ThermoFisher, Waltham MA, USA). The supernatant was removed and filtered using a 0.22 µm SteriCup filter unit (VWR, Randor PA,USA).

The resulting filtrate was then concentrated using a benchtop protein concentrator AKTA Flux (GE Healthcare Lifesciences) using a 4000 kd hollow fiber filter cartridge and a filtration speed of 50 mL/minute. The filtrate was reduced to approximately 50 mL and diafiltrated with 500 mL phosphate-buffered saline (PBS). The concentrated washed solution was then centrifuged at 2,652 x g for 10 minutes at 4 °C (Sorvall RC6+). The supernatant was decanted and filtered using a 0.22 µm sterile syringe filter.

The resulting filtrate was then centrifuged in PBS at 2,315,880 x g for 3 hours at 4 °C in a XL 90 Ultracentrifuge (Beckman Coulter Brea, CA USA). The pellet was resuspended in 1 mL of sterile PBS and streaked onto BHI agar and grown at 37 °C for 48 hours to ensure no viable

bacteria were present. Examination by light microscope and a methylene blue stain was performed to further look for potential bacterial contamination.

Density-dependent ultracentrifugation was used to separate OMVs from other cell debris by layering microvesicles onto a 30-60% (w/v) discontinuous sucrose gradient followed by centrifugation at 4,117,120 x g for 20 hours at 4 °C in an XL 90 Ultracentrifuge. The resulting bands were collected separately and washed with a 20-fold dilution of PBS. Each band was centrifuged at 2,315,880 x g for 3 hours at 4 °C in an XL 90 Ultracentrifuge, and the resulting pellet was resuspended in 1 mL of PBS. Samples from each band were imaged using transmission electron microscopy to determine the presence of OMVs. OMVs were frozen at –80 °C for later use.

2.2.2 Ultrastructural Examination of Outer Membrane Vesicles

OMVs were fixed in 0.5 Karnovsky's fixative in 0.1 M Na cacodylate buffer and then postfixed in 1% osmic tetroxide. The samples were then dehydrated in serial concentrations of alcohol and acetone and embedded in resin. Thick sections (0.5 µm) were cut using an ultramicrotome, stained with toluidine blue, and examined using a light microscope. The ultrathin sections (80 nm) of selected areas were stained with uranyl acetate and lead citrate. The stained sections were examined and photographed using transmission electron microscopy (Jeol TEM-1230).

2.2.3 Outer Membrane Vesicle Size Distribution

The size of OMVs was measured using the Zetasizer Nano (Malvern Instruments Ltd., Grovewood Road, Malvern, United Kingdom) at 25 °C in PBS. The measurements were run in triplicate and averaged.

2.2.4 Protein Quantification

Protein concentration of OMVs was quantified using a Bradford Assay kit (Thermo Scientific: Pierce BCA Protein Assay Kit). Three OMV preparations were measured and averaged together to calculate average protein concentration per preparation. The procedure was conducted following specific manufacturer instructions for the microplate procedure. Absorbance was measured at 562 nm using a Genesys 20 Visible Spectrophotometer Thermo Scientific.

2.2.5 Preparation of Cell Fractions: Whole-Cell Lysates, Periplasmic Proteins, and Outer Membrane Proteins

Cell fractions of whole-cell lysates (WCLs), periplasmic protein (PP), and outer membrane proteins (OMPs) were prepared as described previously.[31] WCLs of *A. hydrophila* ML09-119 were obtained from bacteria grown in BHI broth at 37 °C at 125 rpm to mid-log phase. Bacteria were centrifuged at 5,000 x g for 30 minutes at 4 °C, and the resulting pellet was washed with 1X phosphate-buffered saline and frozen at –20 °C for later use. Bacterial density was adjusted to 0.25 g/mL in 20% (w/v) sterile sucrose dissolved in 20 mM Tris-HCl (pH 8.0) with 0.1 M EDTA and lysozyme (600 µg/g bacteria). Bacteria were incubated on ice for 40 minutes to generate WCLs. Following incubation, 0.5 M $MgCl_2$ was added, and the suspension was centrifuged at 9,500 x g for 20 minutes to remove spheroplasts by pelleting. The resulting supernatant containing the periplasm was stored at –20 °C until used. The pelleted spheroplasts were resuspended in ice-cold 10 mM Tris-HCl (pH 8.0) and then sonicated for purification of OMPs. The spheroplasts extracted using a previous published method.[31]

2.2.6 Cell Fraction Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 15% (w/v) acrylamide separating gel. Each sample (WCL, PP, OMP, and OMV) was resuspended with a sample buffer (3:1 v/w ratio of buffer to sample). The tricine sample buffer contained 2% 2-mercaptoethanol. Samples were boiled at 100 °C for 10 minutes in a mini dry bath and cooled at 22 °C for 10 minutes. Samples were loaded into the gel at 40 µL per well and were run at 100 volts for 1 hour. Following electrophoresis, the gel was stained with Coomassie Blue for 40 minutes and destained with water for two hours. Gel was then imaged on Chemi Doc XRS (Bio-Rad Laboratories Hercules, CA, USA) (Figure 2.5).

2.2.7 Protein Digestion and Identification

OMV pellets were resuspended in 50 µL of 100 mM ammonium bicarbonate/5% acetonitrile and reduced with 1/10 volume of 100 mM dithiothreitol at 65 °C for 15 minutes. Alkylation was done by adding 1/10 volume of 100 mM iodoacetamide and incubating at room temperature for 30 minutes in the dark. OMV pellets were then digested with 1.5 µg of mass spectrometry grade trypsin (Promega, Madison WI) at 37 °C overnight. After 1% formic acid was supplemented to a final concentration of 0.1%, tryptic peptides were freeze-dried (Labconco, Kansas City, MO) and stored at –80 °C. Immediately prior to mass spectrometry, they were resuspended in 20 µL of 2% acetonitrile/0.1% formic acid.

2.2.7.1 Nano-liquid chromatography-tandem mass spectrometry analysis (nLC-MSMS)

Two micrograms of protein tryptic digest were subjected to nLC-MSMS analysis as published previously.[32] Briefly, spectral data were collected using an Orbitrap LTQ Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) linked with an UltiMate 3000

nano flow HPLC system (Thermo Fisher Scientific). Peptides were separated on reversed-phase Acclaim PepMap C18, 75 µm x 150 mm column (Thermo Fisher Scientific) via 60-minute long linear gradient of acetonitrile (in 0.1% formic acid): 2-55% for 35 min, 95% for 10 min, 2% for 15 min. Ions were detected by linear trap mass detector in data-dependent acquisition (DDA) mode with dynamic exclusion being applied. Eight scan events were employed: one MS scan (m/z range: 300–2000) followed by 7 MSMS scans for the 7 most intense ions detected in MS scan.

2.2.7.2 Protein Identification

The raw data files were searched using the SEQUEST algorithm of the Proteome Discoverer software version 1.1 (Thermo Fisher Scientific), as described previously.[33] Variable modifications were considered for: cysteine carbamidomethylation (+57.021), methionine oxidation (+15.995), and methionine dioxidation (+31.990). To allow for calculation of false discovery rates (FDR), experimental MS and MSMS data were matched against target and decoy databases. The target *A. hydrophila* ML09-119 protein database was acquired from the NCBI (www.ncbi.nlm.nih.gov) in May 2018. The reversed copy (created automatically by software) served as a decoy database. To obtain high confidence protein identifications, the search results were filtered by FDR <1%.

We analyzed the *A. hydrophila* ML09-0119 OMV proteome using the NCBI PSORTb database to predict subcellular localization of 74 proteins found across all biological replicates of OMV preparations. The software uses the Hidden Markov Model-based method HMMTOP to identify potential locations of transmembrane alpha-helices and assigns a localization score if three or more helices are identified.

2.2.8 Functional Classification of OMVs According to COG Functional Categories

We analyzed the *A. hydrophila* ML09-119 OMV proteome using the Clusters of Orthologous Groups (COG) database to predict the functional class of 74 proteins found across all biological replicates of OMV preparations utilizing the default parameters for the software.

2.2.9 Identification of Virulence Factors

Identified proteins were analyzed using Virulence Factors Database (https://academic.oup.com/nar/article/47/D1/D687/5160975). Briefly, we downloaded the full dataset from http://www.mgc.ac.cn/VFs/download.htm, and BLAST search was conducted with 74 OMV proteins using CLC Genomic Workbench (version 11.0.1). Only *E-values* $<1\times10^{-20}$ were considered significant matches.

2.3 Results

2.3.1 Outer Membrane Vesicle Isolation

OMVs were isolated from ultracentrifugation resulting in a light pink pellet (Figure 2.1). Culturing of the OMVs showed no evidence of bacterial growth on BHI at 48 hours. Light microscopy examination with methylene blue stain showed no evidence of bacteria in OMV preparations.

2.3.2 Ultrastructural Examination of Outer Membrane Vesicles

Transmission electron microscopy imaging revealed heterogeneous sized OMVs of characteristic amorphic structure. The preparation was visibly free of bacteria and cell fragment contamination (Figure 2.2).

2.3.3 Outer Membrane Vesicle Size Distribution

The Polydispersity Index (PDI) of each OMV replicate was averaged and resulted in a PDI of 0.03. Each sample's Z-averages size was measured and averaged together resulting in a 120.33 nm average particle diameter size (Figure 2.3). An outlying peak occurred on each biological replicate with an average size of 2.68 μm.

2.3.4 Protein Quantification

Absorbance was measured for three different OMV preparations. Absorbance measurements of known standards were plotted in a linear regression with an R^2 value of 0.997. Protein concentrations from OMV preparations were extrapolated using the resulting regression (Figure 2.4). The average of the three OMV concentrations was 4.01 μg/mL.

2.3.5 Preparation of Cell Fractions: Whole-Cell Lysates, Periplasmic Proteins, and Outer Membrane Proteins

Preparations of WCLs, PPs, and OMPs were prepared and demonstrated to be free of bacterial contamination by culturing on BHI plates.

2.3.6 Cell Fraction Electrophoresis

Analysis of bacterial fractions (WCLs, PPs, OMPs, and OMVs) using SDS-PAGE revealed OMVs to have a distinct band pattern differing from other subcellular locations (Figure 2.5).

2.3.7 Protein Identification and Localization

Using nLC-MSMS, 74 proteins were identified with <1% FDR across all biological replicates of OMV preparations (Table 2.1). We analyzed the predicted subcellular localization of these 74 proteins using the PSORTb database and compared the distribution to the predicted

subcellular locations of the entire *A. hydrophila* ML09-119 proteome (Figure 2.6). Relative to the whole proteome, OMV proteins were enriched in extracellular, periplasmic, and outer membrane proteins, and OMV proteins were decreased in the cytoplasmic, cytoplasmic membrane, and unknown categories.

2.3.8 Functional Classification of OMVs According to COG Functional Categories

We analyzed the function of 74 *A. hydrophila* ML09-119 proteins using the COG database (Figure 2.7). Of the 74 proteins assessed, the percent of proteins in each functional category were: 42% catalytic activity, 27% binding, 22% structural molecule activity, and 9% transporter activity.

2.3.9 Virulence Factor Identification

Of the 74 OMV proteins, 27 had significant matches in the Virulence Factors Database (Table 2.2). These 27 proteins were classified into functional categories (Figure 2.8). The largest category was adherence proteins (11), which included four fimbrial proteins and four flagellar proteins. There were also three type III secretion system proteins and three secreted exotoxins.

2.4 Discussion

The protocol described here provides a reproducible method for the isolation of OMVs from *A. hydrophila* ML09-119. Transmission electron microscopy has been used as the preferred method of ultrastructural examination of OMVs.[31] Results of ultrastructural examination revealed the OMVs to be spherical and heterogeneous in size (Figure 2.2), which is consistent with other studies.[1, 14] The preparation was visibly free of bacteria and large cell fragment contamination, which validates our method of OMV isolation.

To quantify the size distribution within the samples, we measured the polydispersity using dynamic light scattering. Polydispersity is the distribution of individual molecular mass values within a sample. The polydispersity index (PDI) of each OMV replicate was averaged and resulted in a PDI of 0.03, making them monodisperse particles. This means that even though the OMVs are heterogeneous, the particles are close to the same size. OMVs of *A. hydrophila* ML09-119 are more uniform in size compared to *Lactobacillus reuteri, Neisseria meningitidis*, and *Escherichia coli*, whose PDIs ranged from 0.4-0.5.[34-36] While the significance of the relative uniformity in size of *A. hydrophila* OMVs compared to other bacteria is unknown, this information can inform future studies of *A. hydrophila* OMVs.

The particle diameter of OMVs was 120.33 nm, which is consistent with OMVs of other bacterial species (20-250 nm).[14, 31, 37]. An outlying peak occurred on each biological replicate with an average size of 2.68 µm, suggesting that a small amount of bacterial flagellar fragments copurified with each OMV purification (Figure 2.3). Copurification of flagellar fragments is common in OMV isolation and is consistent with our OMV proteome analysis, which identified extracellular flagellar proteins (Table 2.1).[38]

The protein profile of *A. hydrophila* ML09-119 OMVs was compared to the protein profiles of other bacterial fractions (WCL, PPs, and OMPs) using SDS-PAGE. The OMV fraction had some similar protein bands compared to the other fractions, but the OMV protein band patterns were visually distinct. This distinct band pattern suggests that the production of OMVs is associated with highly specific protein sorting mechanisms. The differences in OMV protein bands were consistent with other OMV studies in different Gram-negative species.[31]

Using nLC-MSMS, we identified 74 *A. hydrophila* proteins that were consistently present in all OMV preparations. Bacterial OMVs are natural secretions of the Gram-negative cell

envelope and are formed by blebbing of the outer membrane through a budding mechanism between sections of peptidoglycan components.[1] As expected, the OMV proteins were greatly enriched in outer membrane proteins, extracellular proteins, and periplasmic proteins compared to the whole proteome. OMVs are thought to largely depend on the function of outer membrane proteins and extracellular proteins to achieve their pathogenic characteristics. Of the 74 proteins found across three OMV purifications, 22 were found to originate from the outer membrane or extracellular subcellular locations.

However, cytoplasmic proteins still made up the highest percentage of proteins in *A. hydrophila* OMVs (Figure 2.6). The mechanism of OMV formation does not appear to provide an opportunity for large amounts of cytoplasmic protein in comparison to the protein fractions from the outer membrane and the periplasm. Cytoplasmic proteins are likely captured in OMVs because of their high abundance compared to other bacterial compartments (46.6% of the *A. hydrophila* proteome), which is consistent with other studies investigating the proteome of native OMVs.[39-40] For example, many cytoplasmic proteins are present in secreted OMVs of *E. coli* and *Salmonella enterica*.[39-40] In the current study, *A. hydrophila* were grown to stationary phase, which is known to induce a stress response in bacteria. In addition, OMVs that have been grown in a nutrient-rich environment have higher fractions of cytoplasmic proteins in their proteome compared to OMVs isolated in a nutrient-deficient environment.[41] In the current study, *A. hydrophila* was cultivated in BHI broth, which is nutrient-rich.

Through the functional classification analysis of *A. hydrophila* OMVs, 42% of the identified proteins were classified as being involved in catalytic activity, and 39% were involved in metabolism (Figure 2.7). The presence of GADPH (glyceraldehyde 3-phosphate dehydrogenase EC 1.2.1.12), enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11, malate

dehydrogenase (EC 1.1.1.37), and GDP mannose 4,6 dehydratase (EC 4.2.1.47) suggests that the OMVs could be reflective of actively metabolizing bacteria. Glyceraldehyde-3-phosphate dehydrogenase is an enzyme in the glycolytic pathway that dehydrogenates and phosphorylates glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate. Enolase is an enzyme in the glycolytic pathway that converts 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate.[42-43] Both of these enzymes are critical components of the glycolysis and gluconeogenesis pathways. Enolase as a component of OMVs was also found in the OMV proteome of *Borellia burgdorferi*.[44]

One of the roles of OMVs is increasing the survivability of parent bacteria. They can be the delivery mechanism of virulence factors, modulators of the host immune system, and aid in nutrient acquisition.[13, 23, 45-46] Comparison of the 74 *A. hydrophila* ML09-119 OMV proteins to the Virulence Factors Database revealed that 27 OMV proteins have significant sequence identity with known bacterial virulence proteins. The largest functional category of the predicted OMV virulence proteins was adherence (Figure 2.8), which included four fimbrial and four flagellar proteins. Adherence to host cells is a critical step in bacterial pathogenesis that is often mediated by fimbriae with contribution from flagella.[47-53]

OMVs are a potent delivery mechanism of bacterial exotoxins.[8] One of the proteins identified in *A. hydrophila* ML09-119 OMVs was RtxA. RTX toxins are water-soluble cytotoxins that can easily traverse the cell membrane, and many of them have hemolytic activity on red blood cells.[54] Protein Ahh1 is also a known hemolysin produced by *A. hydrophila*.[55] Protein AerA/act is aerolysin, a pore-forming toxin that aggregates to form holes in eukaryotic cells, and it is also considered a hemolysin.[56] *A. hydrophila* is known to be a beta-hemolytic pathogen in which the bacteria can completely lyse red blood cells, producing a clear zone under

colonies on blood agar plates. All three of these exotoxins are considered virulence factors for *A. hydrophila*.[57] The presence of these hemolysins and aerolysin exotoxins in OMVs suggests that OMVs may be used as secretion mechanism and a delivery vehicle to promote lysis of host cells.

Three type III section system (T3SS) proteins were identified in *A. hydrophila* ML09-119 OMVs: T3SS-ATPase, T3SS-YspM, and T3SS-BscD. Type III secretion systems are only present in pathogenic bacteria, and their function is to inject secreted bacterial effector proteins directly into the cytoplasm of host cells. T3SS-ATPase and T3SS-YspM are effector proteins secreted into host cells, and T3SS-BscD is a structural protein that is a component of the T3SS apparatus.[58-60] Another protein in *A. hydrophila* ML09-119 OMVs is Outer membrane protein A (OmpA), which is an abundant OMP in many Gram-negative species. In *Acinetobacter baumannii*, OmpA is essential for adherence to host alveolar epithelium cells.[61] Another of the proteins identified in *A. hydrophila* ML09-119 OMVs, GtrB, is similar to an O-antigen modification enzyme in *Shigella flexneri*, which is an important virulence trait for *S. flexneri*.[62]

2.5 Conclusions

OMVs of *Aeromonas hydrophila* can be successfully isolated using methods of ultrafiltration and protein concentration. The OMVs isolated from *A. hydrophila* are consistent with other bacterial OMVs in size and shape, but not in polydispersity. The polydispersity of the OMVs of *A. hydrophila* ML09-119 reveals them to have a more uniform distribution in size. Due to the low protein concentration found in OMV preparations, it would be difficult to investigate them as a vaccine candidate where doses were dependent on protein concentration. OMVs of *A. hydrophila* ML09-119 have a protein profile that is unique compared to other cell fractions such as outer membrane proteins, periplasmic proteins and whole cell lysate, and they were enriched in outer membrane proteins, periplasmic proteins, and extracellular proteins compared to the

whole *A. hydrophila* ML09-119 proteome. *A. hydrophila* ML09-119 OMVs consistently contained 74 proteins, 27 of which had significant sequence identity with known bacterial virulence factors. Several of the predicted OMV virulence proteins have potential roles in adherence, including fimbrial proteins, flagellar proteins, and outer membrane protein A. *A. hydrophila* OMVs may serve as a secretion mechanism for three potent cytotoxic exotoxins: RtxA, Ahh1, and aerolysin, which contribute to the pathology of MAS in fish.

2.6 Tables and Figures

Table 2.1 *A. hydrophila* OMV proteins identified by nLC-MSMS

Accession	Coverage	# Peptides	# AAs	MW [kDa]	calc. pI	Score	Description	Localization
gi1129422678	2.25	1	888	93.8	5.16	6.86	beta-glucosidase [Aeromonas hydrophila]	Unknown
gi1129422824	34.78	9	115	12.1	7.96	42.96	META domain-containing protein [Aeromonas hydrophila]	Unknown
gi1129422834	55.92	17	152	16.2	4.81	73.89	hypothetical protein [Aeromonas hydrophila]	Unknown
gi1129422859	6.10	1	328	34.6	7.85	9.39	transcriptional regulator [Aeromonas hydrophila]	Periplasmic
gi1129422966	19.67	19	366	40.6	8.13	81.68	phosphatidylcholine-sterol acyltransferase [Aeromonas hydrophila]	Extracellular
gi1129422966	19.67	12	366	40.6	8.13	63.53	phosphatidylcholine-sterol acyltransferase [Aeromonas hydrophila]	Unknown
gi1171827788	9.13	6	635	68.6	5.57	49.91	LppC family lipoprotein [Aeromonas hydrophila]	Cytoplasmic Membrane
gi491445683	7.02	2	242	27.0	7.14	12.60	MULTISPECIES: 30S ribosomal protein S2 [Aeromonas]	Cytoplasmic
gi491447121	22.41	5	174	18.9	5.08	52.03	MULTISPECIES: peptidoglycan-associated lipoprotein Pal [Aeromonas]	Outermembrane
gi491448479	10.90	10	156	17.4	10.30	45.31	MULTISPECIES: 30S ribosomal protein S7 [Aeromonas]	Cytoplasmic
gi491449428	11.04	4	462	50.3	4.98	46.50	MULTISPECIES: ATP synthase subunit beta [Aeromonas]	Cytoplasmic
gi491450210	4.56	2	329	36.2	5.01	16.09	MULTISPECIES: DNA-directed RNA polymerase subunit alpha [Aeromonas]	Cytoplasmic
gi491450678	40.51	7	158	15.8	9.07	43.17	MULTISPECIES: glycine zipper 2TM domain-containing protein [Aeromonas]	Outermembrane
gi491451730	38.14	15	97	10.3	6.10	101.75	MULTISPECIES: molecular chaperone GroES [Aeromonas]	Cytoplasmic
gi491484099	29.17	5	120	12.3	4.56	29.95	MULTISPECIES: 50S ribosomal protein L7/L12 [Aeromonas]	Cytoplasmic
gi498320526	15.00	1	80	8.5	8.15	4.47	MULTISPECIES: hypothetical protein [Aeromonas]	Outermembrane
gi500023597	24.75	9	101	11.5	11.21	63.49	MULTISPECIES: 30S ribosomal protein S14 [Aeromonas]	Cytoplasmic
gi500023598	10.77	1	130	14.0	9.74	8.55	MULTISPECIES: 30S ribosomal protein S8 [Aeromonas]	Cytoplasmic
gi500023601	13.11	6	206	23.4	9.99	27.52	MULTISPECIES: 30S ribosomal protein S4 [Aeromonas]	Cytoplasmic
gi500023774	57.78	45	180	17.9	4.79	208.06	MULTISPECIES: type 1 fimbrial protein [Aeromonas]	Extracellular
gi500024051	11.09	5	433	45.7	5.39	29.50	MULTISPECIES: enolase [Aeromonas]	Cytoplasmic
gi500024086	42.65	64	544	57.1	4.97	352.77	MULTISPECIES: chaperonin GroEL [Aeromonas]	Cytoplasmic
gi500024091	26.67	3	150	16.1	7.08	16.16	MULTISPECIES: pullulanase [Aeromonas]	Periplasmic
gi500024135	13.53	2	266	28.8	5.59	15.78	MULTISPECIES: MetQ/NlpA family lipoprotein [Aeromonas]	Cytoplasmic Membrane
gi500024201	13.07	7	176	18.5	5.26	69.07	MULTISPECIES: DUF4136 domain-containing protein [Aeromonas]	Cytoplasmic Membrane
gi500024220	5.97	1	268	28.7	5.83	24.61	MULTISPECIES: FKBP-type peptidyl-prolyl cis-trans isomerase [Aeromonas]	Periplasmic
gi500024224	2.78	2	612	66.4	5.30	47.39	MULTISPECIES: bifunctional metallophosphatase/5'-nucleotidase [Aeromonas]	Periplasmic
gi500024340	54.21	41	190	20.5	8.57	173.73	MULTISPECIES: hypothetical protein [Aeromonas]	Unknown

Table 2.1 (continued)

Accession	Coverage	# Peptides	# AAs	MW [kDa]	calc. pI	Score	Description	Localization
gi500024343	7.83	2	447	48.6	6.62	28.15	MULTISPECIES: NADH:ubiquinone reductase (Na(+)-transporting) subunit A [Aeromonas]	Cytoplasmic
gi500024696	17.23	11	238	26.9	6.25	58.79	MULTISPECIES: ATP-dependent Zn protease [Aeromonas]	Cytoplasmic
gi500024972	3.42	1	556	61.3	5.00	14.31	MULTISPECIES: 30S ribosomal protein S1 [Aeromonas]	Cytoplasmic
gi500026446	9.74	14	154	17.1	9.69	53.76	MULTISPECIES: hypothetical protein [Aeromonas]	Cytoplasmic Membrane
gi500026640	19.34	9	331	35.2	6.27	69.62	MULTISPECIES: type I glyceraldehyde-3-phosphate dehydrogenase [Aeromonas]	Cytoplasmic
gi500026645	4.08	1	441	48.2	8.72	11.40	MULTISPECIES: protein TolB [Aeromonas]	Periplasmic
gi500026913	13.84	23	354	38.8	4.93	109.84	MULTISPECIES: DUF3103 domain-containing protein [Aeromonas]	Unknown
gi500026976	19.54	14	394	43.4	5.39	81.12	MULTISPECIES: elongation factor Tu [Aeromonas]	Cytoplasmic
gi500027005	7.02	1	171	19.2	8.91	17.84	MULTISPECIES: hypothetical protein [Aeromonas]	Unknown
gi500027025	3.74	1	508	53.0	6.71	34.67	MULTISPECIES: Re/Si-specific NAD(P)(+) transhydrogenase subunit alpha [Aeromonas]	Cytoplasmic Membrane
gi500027194	10.26	2	156	17.0	7.34	25.30	MULTISPECIES: F0F1 ATP synthase subunit B [Aeromonas]	Cytoplasmic Membrane
gi511288694	50.25	137	804	82.6	5.77	774.57	lipase [Aeromonas hydrophila]	Extracellular
gi511288720	26.15	7	218	22.0	9.61	45.62	MULTISPECIES: OmpA family lipoprotein [Aeromonas]	Cytoplasmic Membrane
gi511288896	13.61	1	147	15.2	7.91	6.02	prepilin-type N-terminal cleavage/methylation domain-containing protein [Aeromonas hydrophila]	Extracellular
gi511289029	18.03	3	183	19.3	5.49	16.24	type 1 fimbrial protein [Aeromonas hydrophila]	Extracellular
gi511289145	6.11	1	311	32.1	6.37	13.49	malate dehydrogenase [Aeromonas hydrophila]	Unknown
gi511289224	5.53	3	253	28.5	7.46	44.08	amino acid ABC transporter ATP-binding protein [Aeromonas hydrophila]	Cytoplasmic Membrane
gi511289447	12.62	3	325	36.0	6.35	37.53	undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase [Aeromonas hydrophila]	Cytoplasmic Membrane
gi511289657	32.46	7	268	28.2	6.20	40.96	M48 family peptidase [Aeromonas hydrophila]	Unknown
gi511289676	10.81	4	333	35.0	5.39	48.93	MULTISPECIES: porin OmpA [Aeromonas]	Outermembrane
gi511289677	13.53	34	340	35.9	5.54	170.01	MULTISPECIES: major outer membrane protein OmpAI [Aeromonas]	Outermembrane
gi511289779	4.15	17	4439	469.3	5.55	174.64	MARTX multifunctional-autoprocessing repeats-in-toxin holotoxin RtxA [Aeromonas hydrophila]	Extracellular
gi511289934	21.10	24	621	68.7	6.49	176.69	hemolysin [Aeromonas hydrophila]	Extracellular
gi511290079	17.88	9	302	31.2	5.47	55.57	flagellin-like protein [Aeromonas hydrophila]	Extracellular
gi511290080	18.06	7	299	31.1	5.48	47.61	flagellin [Aeromonas hydrophila]	Extracellular
gi511290085	3.01	1	465	49.1	6.43	58.80	flagellar hook-associated protein 2 [Aeromonas hydrophila]	Extracellular
gi511290250	1.71	1	760	85.0	6.11	29.43	MULTISPECIES: formate C-acetyltransferase [Aeromonas]	Cytoplasmic
gi511290343	45.02	9	251	25.9	6.19	101.33	MULTISPECIES: triose-phosphate isomerase [Aeromonas]	Cytoplasmic
gi511290422	4.59	3	545	58.8	6.65	45.97	GlyGly-CTERM sorting domain-containing protein [Aeromonas hydrophila]	Unknown
gi511290525	8.02	4	1072	113.4	4.82	40.06	nuclease [Aeromonas hydrophila]	Unknown

Table 2.1 (continued)

Accession	Coverage	# Peptides	# AAs	MW [kDa]	calc. pI	Score	Description	Localization
gi511290985	3.40	1	589	64.8	7.97	18.01	lipid A ABC transporter ATP-binding protein/permease MsbA [Aeromonas hydrophila]	Cytoplasmic Membrane
gi511291025	4.06	2	468	48.8	6.92	15.59	alkaline phosphatase [Aeromonas hydrophila]	Periplasmic
gi511291041	6.19	3	339	36.1	5.41	16.76	DUF2066 domain-containing protein [Aeromonas hydrophila]	Unknown
gi511291052	7.51	6	666	70.1	5.03	61.98	flagellar hook protein FlgK [Aeromonas hydrophila]	Extracellular
gi511291244	8.88	24	169	18.1	4.88	95.50	PTS glucose transporter subunit IIA [Aeromonas hydrophila]	Cytoplasmic
gi511291581	10.14	4	355	37.9	5.01	44.03	Zn-dependent exopeptidase M28 [Aeromonas hydrophila]	Extracellular
gi511291751	9.17	6	349	38.0	5.49	56.67	phage major capsid protein, P2 family [Aeromonas hydrophila]	Outermembrane
gi511291874	2.71	1	701	77.5	5.29	43.18	DUF885 domain-containing protein [Aeromonas hydrophila]	Outermembrane
gi511291895	2.37	2	886	98.6	5.73	24.34	pyruvate dehydrogenase (acetyl-transferring), homodimeric type [Aeromonas hydrophila]	Cytoplasmic
gi511291920	8.33	1	192	20.4	9.54	7.77	osmotically-inducible protein OsmY [Aeromonas hydrophila]	Periplasmic
gi511291998	17.47	9	166	17.7	9.42	48.85	MULTISPECIES: 50S ribosomal protein L10 [Aeromonas]	Cytoplasmic
gi511291999	6.87	23	233	24.6	9.61	110.18	MULTISPECIES: 50S ribosomal protein L1 [Aeromonas]	Cytoplasmic
gi511292030	8.82	5	170	18.9	6.00	21.90	hypothetical protein [Aeromonas hydrophila]	Outermembrane
gi759393479	4.46	1	493	54.5	6.10	18.56	aerolysin family beta-barrel pore-forming toxin [Aeromonas hydrophila]	Extracellular
gi759393535	28.65	33	342	37.1	5.36	198.65	fimbrial protein [Aeromonas hydrophila]	Extracellular
gi759394533	6.25	4	368	40.9	5.62	27.09	GDP-mannose 4,6-dehydratase [Aeromonas hydrophila]	Cytoplasmic

Table 2.2 *A. hydrophila* ML09-119 OMV proteins with significant matches in the Virulence Factors Database

Accession	Query	Number of HSPs	Lowest E-value	Accession (E-value)	Description (E-value)
gi1129422966	WP_011704804.1	21	0	VFG010484(gi:54296712)	(htpB) 60 kDa chaperonin (Protein Cpn60)(groEL protein)(Heat shock protein B). [Hsp60 (CVF347)] [Legionella pneumophila str. Paris]
gi1129422966	WP_011707694.1	18	0	VFG016491(gi:26553484)	(tuf) elongation factor Tu [EF-Tu (CVF587)] [Mycoplasma penetrans HF-2]
gi1171827788	WP_016350046.1	133	0	VFG038913(gi:507520991)	(rtxA) structural toxin protein RtxA [RtxA (VF0482)] [Aeromonas hydrophila ML09-119]
gi491445683	WP_016350201.1	19	0	VFG038898(gi:507521195)	(ahh1) hemolysin [Extracellular hemolysin, AHH1 (CVF791)] [Aeromonas hydrophila ML09-119]
gi491447121	WP_016350347.1	246	0	VFG038731(gi:507521385)	(flaA) flagellin [Polar flagella (VF0473)] [Aeromonas hydrophila ML09-119]
gi491448479	WP_016350352.1	81	0	VFG038743(gi:507521390)	(flaH) flagellar hook-associated protein 2 [Polar flagella (VF0473)] [Aeromonas hydrophila ML09-119]
gi491449428	WP_016351252.1	368	0	VFG013250(gi:148825660)	(msbA) fused lipid transporter subunits of ABC superfamily: membrane component/ATP-binding component [LOS (CVF494)] [Haemophilus influenzae PittEE]
gi491450210	WP_016351319.1	163	0	VFG038772(gi:507522553)	(flgK) flagellar hook-associated protein FlgK [Polar flagella (VF0473)] [Aeromonas hydrophila ML09-119]
gi491450678	WP_043118302.1	27	0	VFG038891(gi:117618070)	(aerA/act) hemolysin [aerolysin (TX450)] [Aeromonas hydrophila subsp. hydrophila ATCC 7966]
gi491451730	WP_043119174.1	125	0	VFG002365(gb	YP_001007257) (gmd) GDP-mannose 4,6-dehydratase [O-antigen (VF0392)] [Yersinia enterocolitica subsp. enterocolitica 8081]
gi491484099	WP_043118352.1	93	3.64E-166	VFG038421(gi:507520055)	(fimE) type I pilus assembly protein FimE [Type I pili (VF0486)] [Aeromonas hydrophila ML09-119]
gi498320526	WP_011704769.1	7	2.83E-163	VFG005579(gi:116516768)	(eno) phosphopyruvate hydratase [Streptococcal enolase (CVF153)] [Streptococcus pneumoniae D39]
gi500023597	WP_016350346.1	250	2.76E-142	VFG038731(gi:507521385)	(flaA) flagellin [Polar flagella (VF0473)] [Aeromonas hydrophila ML09-119]
gi500023598	WP_016349296.1	275	9.66E-135	VFG038425(gi:507520056)	(fimF) type I pilus assembly protein FimF [Type I pili (VF0486)] [Aeromonas hydrophila ML09-119]
gi500023601	WP_011704492.1	273	3.07E-109	VFG038408(gi:117621241)	(fimA) fimbrial protein [Type I fimbriae (CVF796)] [Aeromonas hydrophila subsp. hydrophila ATCC 7966]
gi500023774	WP_011704853.1	3	6.90E-106	VFG045346(gb	NP_933683) (IlpA) immunogenic lipoprotein A [IlpA (VF0513)] [Vibrio vulnificus YJ016]
gi500024051	WP_011707358.1	17	1.02E-102	VFG005353(gi:76787761)	(plr/gapA) glyceraldehyde-3-phosphate dehydrogenase [Streptococcal plasmin receptor/GAPDH (CVF123)] [Streptococcus agalactiae A909]

Table 2.2 (continued)

Accession	Query	Number of HSPs	Lowest E-value	Accession (E-value)	Description (E-value)
gi500024086	WP_016349163.1	24	1.92E-76	VFG038578(gi:507519909)	(mshB) MSHA pilin protein MshA [MSHA type IV pili (VF0477)] [Aeromonas hydrophila ML09-119]
gi500024091	WP_016349943.1	108	5.12E-65	VFG001443(gb	AAF37887) (ompA) outer membrane protein A [OmpA (VF0236)] [Escherichia coli O18:K1:H7 str. RS218]
gi500024135	WP_016349944.1	105	2.77E-62	VFG001443(gb	AAF37887) (ompA) outer membrane protein A [OmpA (VF0236)] [Escherichia coli O18:K1:H7 str. RS218]
gi500024201	WP_011704938.1	10	2.73E-50	VFG045705(gi:289165397)	(mip) macrophage infectivity potentiator [Mip (CVF349)] [Legionella longbeachae NSW150]
gi500024220	WP_016349491.1	432	1.61E-44	VFG013192(gi:170717476)	(hitC) ABC transporter related [Haemophilus iron transport locus (CVF501)] [Haemophilus somnus 2336]
gi500024224	WP_016349714.1	158	7.46E-43	VFG000670(gb	NP_706258) (gtrB) bactoprenol glucosyl transferase [LPS (VF0124)] [Shigella flexneri 2a str. 301]
gi500024340	WP_005307215.1	156	1.88E-41	VFG041785(gi:46562141)	(DVUA0119) type III secretion system ATPase [T3SS (SS039)] [Desulfovibrio vulgaris str. Hildenborough]
gi500024343	WP_011704942.1	7	4.65E-31	VFG002420(gb	NP_644838) (adsA) Adenosine synthase A [AdsA (VF0422)] [Staphylococcus aureus subsp. aureus MW2]
gi500024696	WP_075384485.1	23	2.06E-26	VFG041727(gi:123443801)	(yspM) putative lipase/acylhydrolase [Ysa T3SS (SS018)] [Yersinia enterocolitica subsp. enterocolitica 8081]
gi500024972	WP_016348987.1	148	9.24E-21	VFG041154(gi:206558755)	(bscD) putative outer membrane protein [T6SS (SS193)] [Burkholderia cenocepacia J2315]

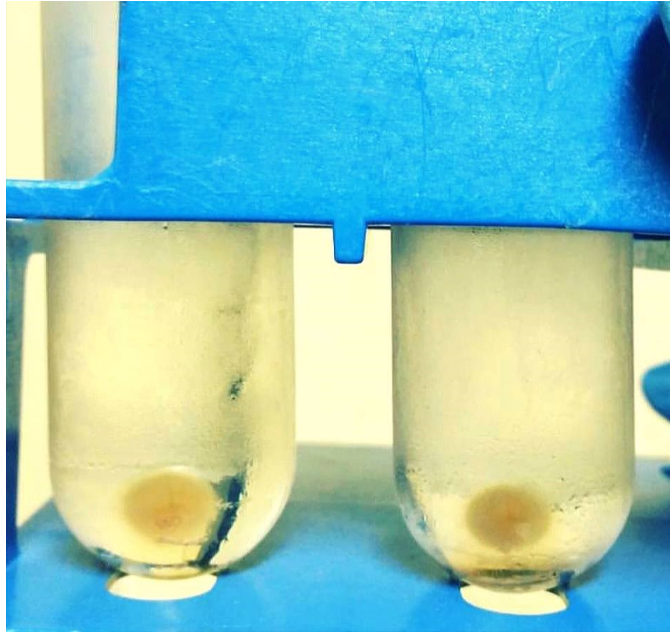


Figure 2.1 Image of OMV pellets from ultracentrifugation.

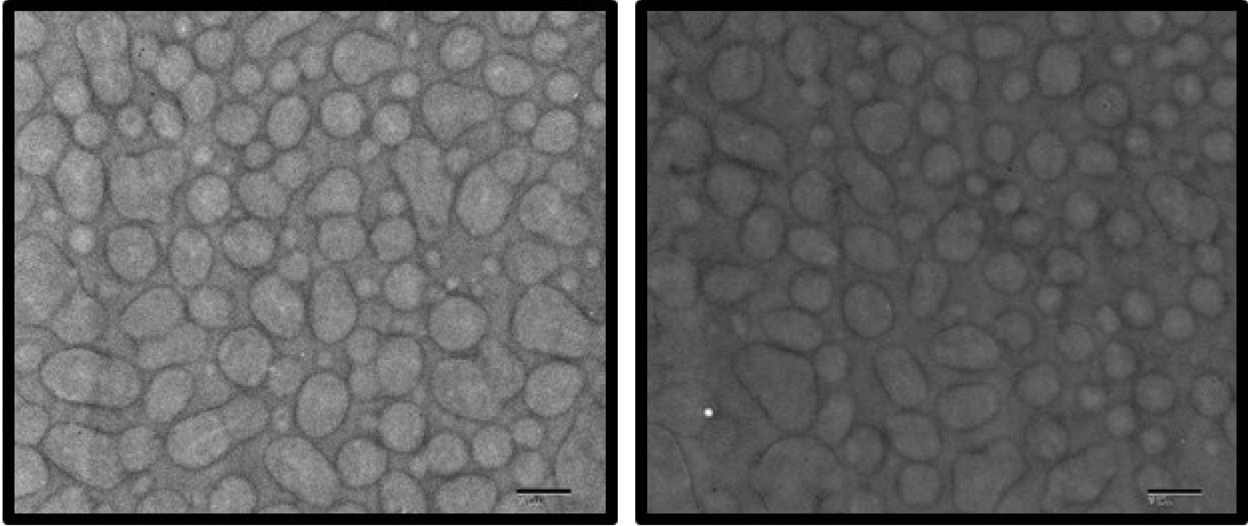


Figure 2.2 Two transmission electron microscopy images of OMVs from *A. hydrophila* ML09-119. Structure of outer membrane vesicles is spherical and is visually free of cell contamination. Image scale (black bar) is 1 µm.

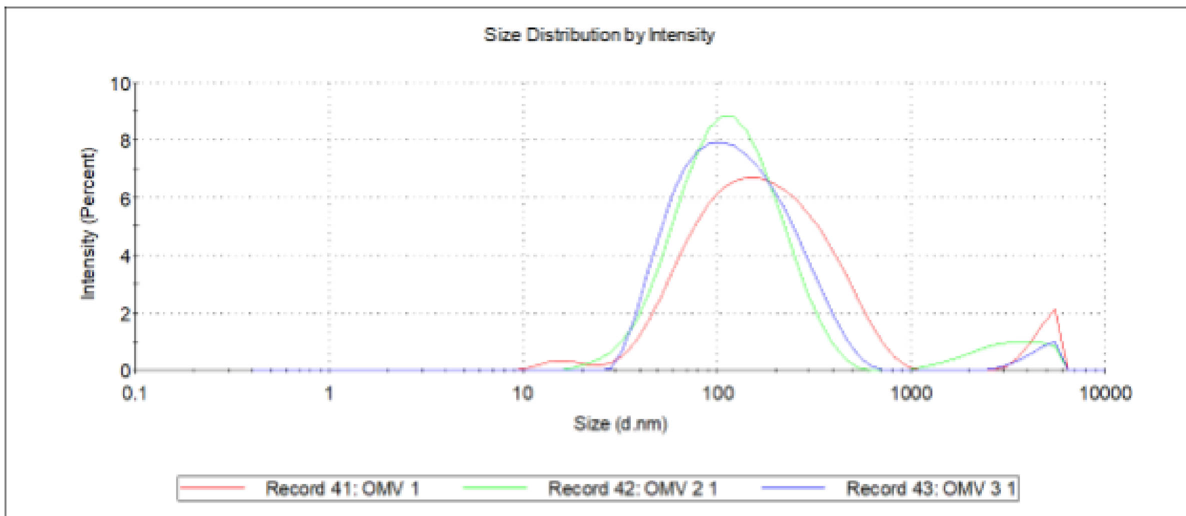
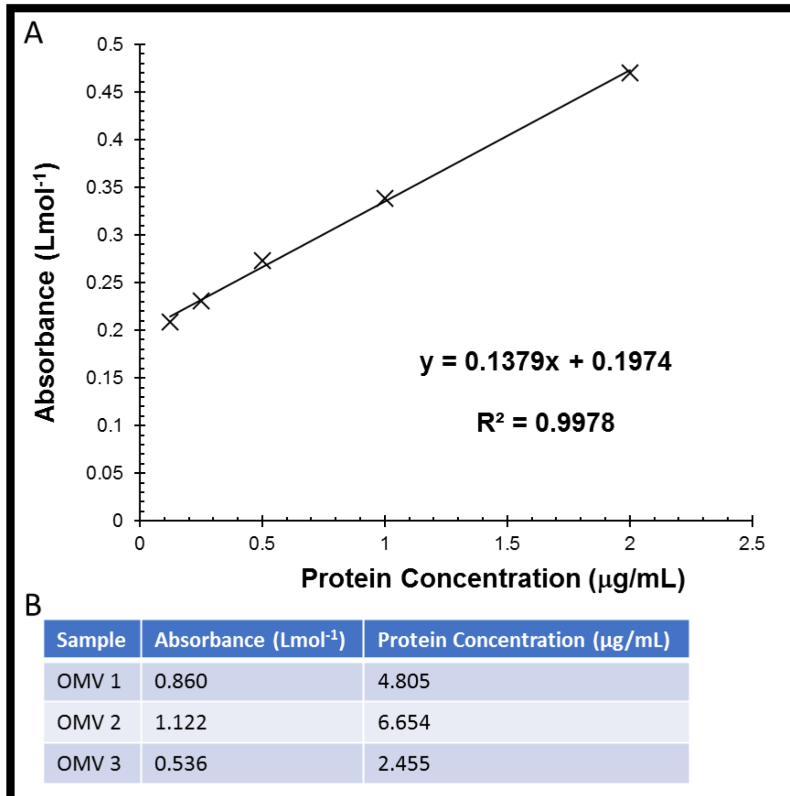


Figure 2.3 Light scattering measurement of OMV size distribution by intensity. Average particle diameter size 120.33 nm. There was a secondary peak with an average particle size of 2.68 µm.



Sample	Absorbance ($Lmol^{-1}$)	Protein Concentration (µg/mL)
OMV 1	0.860	4.805
OMV 2	1.122	6.654
OMV 3	0.536	2.455

Figure 2.4 Measured absorbance from OMV preparations using Bradford Assay. (A). Linear regression from known protein standard (bovine serum albumin). (B). Measured absorbance from OMV preparations.

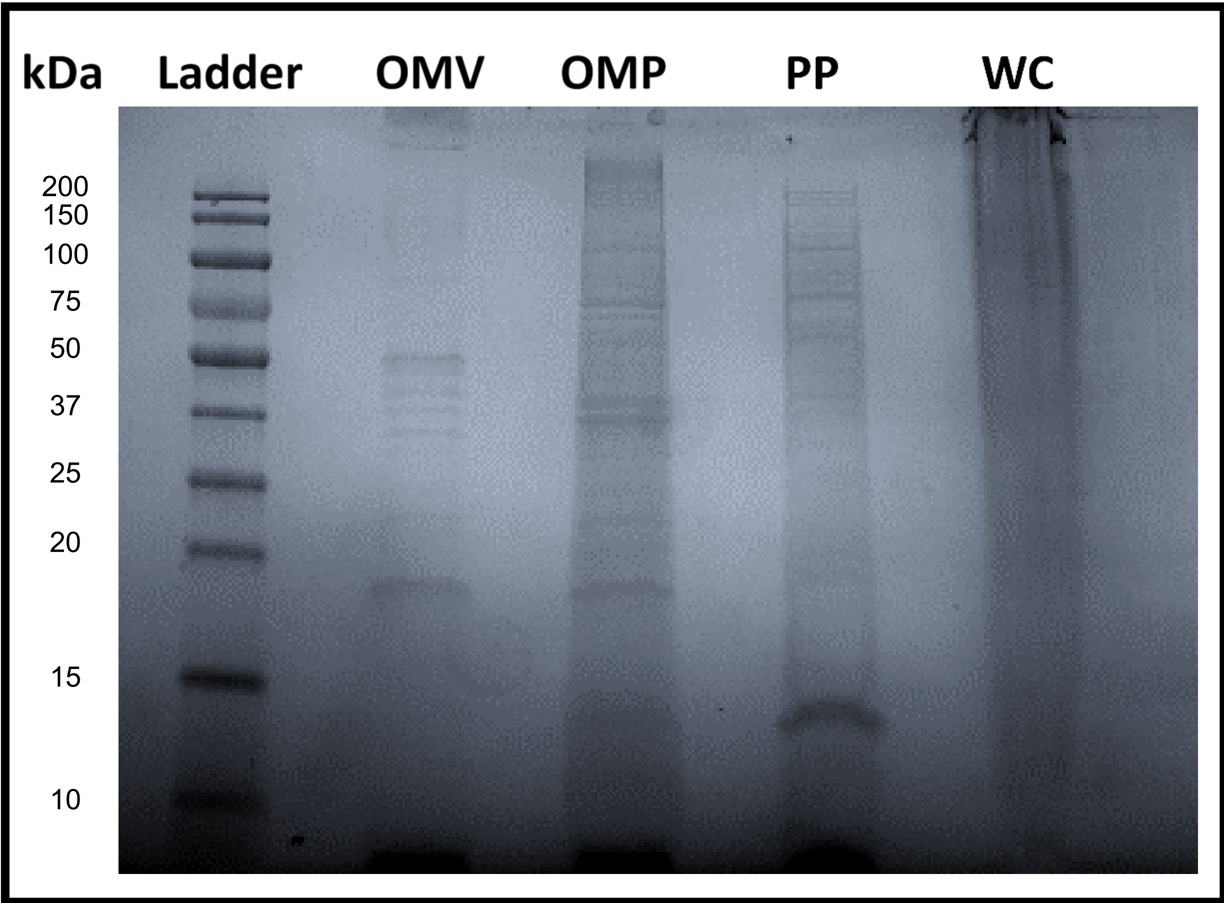


Figure 2.5 Coomassie Brilliant Blue stained SDS-PAGE of *A. hydrophila* ML09-119 fractions: outer membrane vesicles (OMV), outer membrane proteins (OMP), periplasmic proteins (PP), and whole-cell lysate (WC).

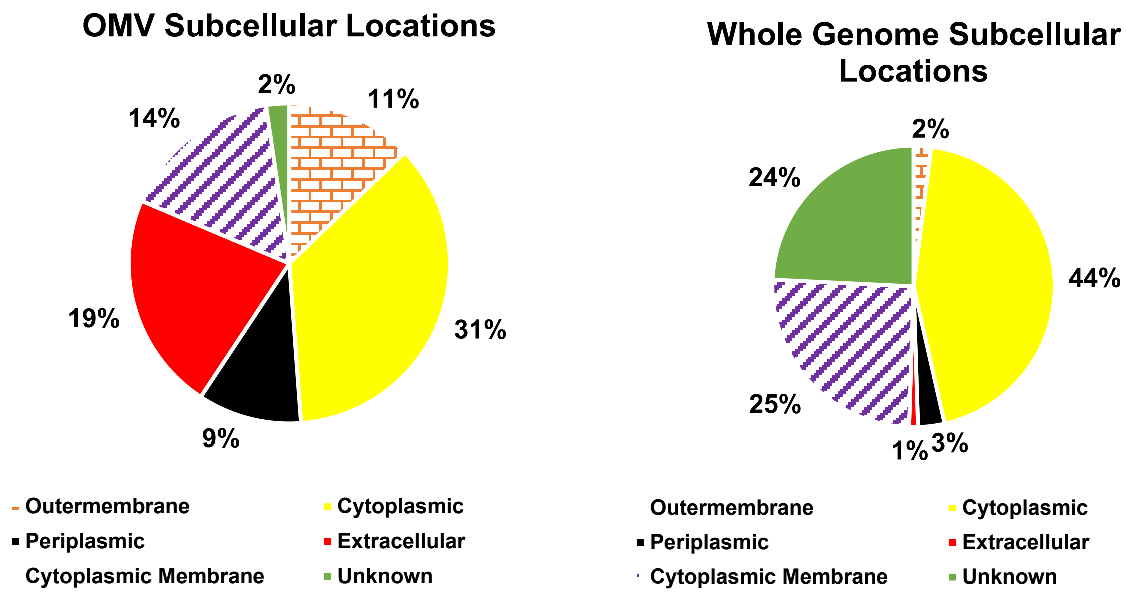


Figure 2.6 Predicted subcellular locations of *A. hydrophila* ML09-119 OMV proteins and the whole predicted *A. hydrophila* ML09-119 proteome using PSORTb.

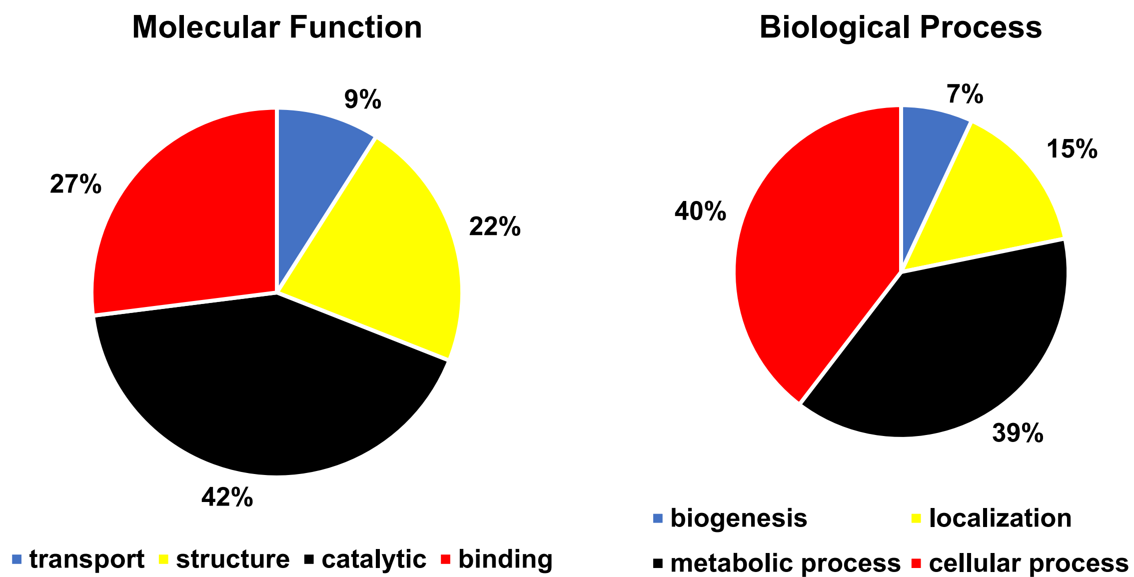


Figure 2.7 Functional prediction of molecular function and biological process of OMV proteins using COG database.

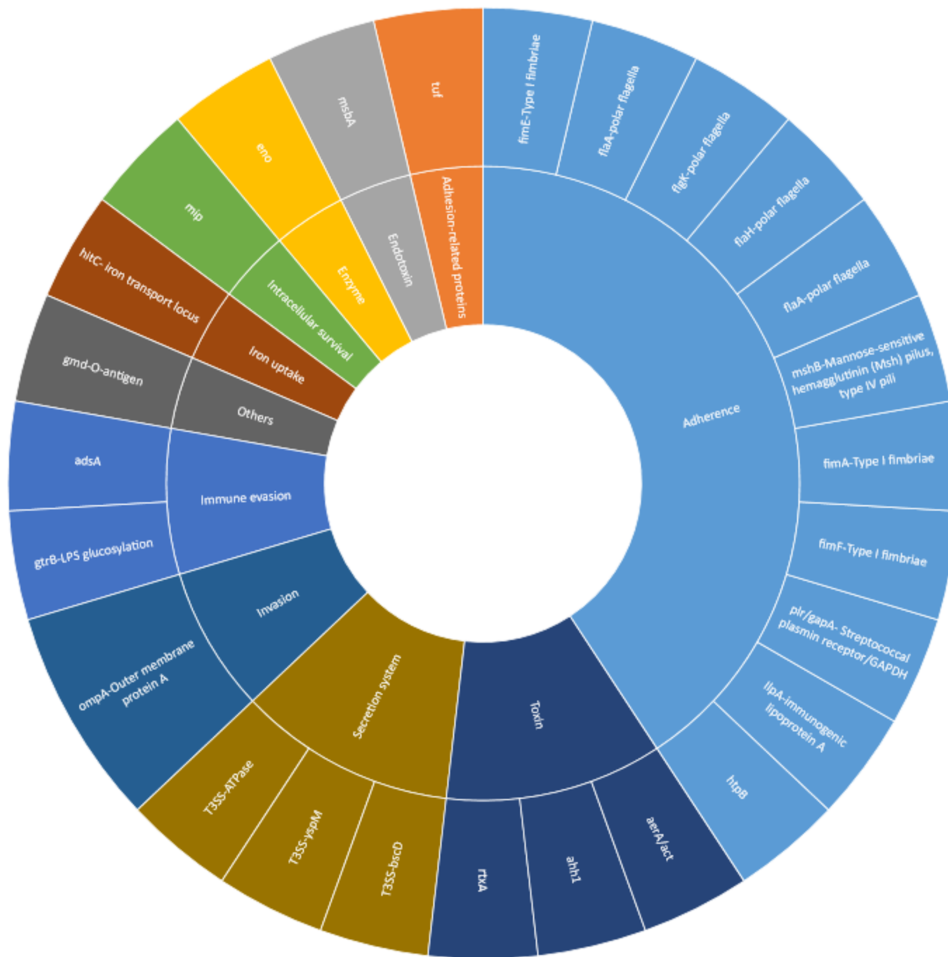


Figure 2.8 Functional categorization of *A. hydrophila* ML09-119 OMV proteins with significant matches in the Virulence Factors Database.

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