Towards Understanding the Molecular Basis of Outer Membrane Vesicles Formation

by

Wael Elhenawy

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Abstract

The release of outer membrane vesicles (OMV) is utilized by Gram-negative bacteria to perform a myriad of functions during pathogenesis and symbiosis. Despite their well-recognized roles, the mechanism responsible for OMV formation in bacteria remains elusive. Whether OMV are produced by an active mechanism or by the passive disintegration of the outer membrane (OM) is a still matter of controversy. If OMV are produced as a consequence to cell lysis, then their protein content should not differ from the parent OM. Nonetheless, many studies demonstrated the differences between the proteomes of OMV and the originating membranes, suggesting the presence of a directed mechanism for OMV formation and cargo selection.

In this thesis, we compared the proteomes of OM and OMV purified from *B. fragilis* and *B. thetaiotaomicron.* Using mass spectrometry, we identified the proteins in the OM and OMV of both organisms. Our analysis revealed the presence of more than forty proteins exclusively-packed in *B. fragilis* OMV. In parallel, more than 30 proteins were excluded from OMV and were detected only in *B. fragilis* OM. Sugar hydrolases and proteases constituted a significant fraction of the OMV specific proteins. Using in vitro biochemical assays, we demonstrated the hydrolytic activity of *B. fragilis* OMV. Moreover, we were able to show that *B. fragilis* can induce OMV hydrolases in response to extracellular cues. Similar results were obtained with *B. thetaiotaomicron* OMV analysis.

OMV cargo selection is not restricted to the protein content but was found to include the packed lipids. Previous studies demonstrated the uneven distribution of lipids between the OMV and the parent OM. In the dental pathogen, *Porphyromonas gingivalis*, deacylated lipid A species were preferentially enriched in the OMV. In this thesis, we investigated the role of the

lipid A deacylase, PagL, in OMV production by the prominent enteric pathogen, *Salmonella enterica* serovar Typhimurium. The expression of PagL in vitro resulted in the exclusive accumulation of deacylated lipid A forms in *S.* Typhimurium OMV. Additionally, more OMV production was observed when PagL was recombinantly expressed in *S.* Typhimurium. On contrary, the expression of a catalytically inactive variant of the enzyme did not induce similar vesiculation levels. Moreover, we demonstrated that PagL is involved in OMV formation by intracellular *S.* Typhimurium. Our results suggest a role for PagL-mediated lipid A deacylation in OMV biogenesis. Unraveling the molecular mechanisms mediating OMV formation will greatly improve our understanding of bacterial pathogenesis.

Preface

Portions of chapter 2 of this thesis has been published as "Elhenawy, W., Debelyy, M. O., and Feldman, M. F. (2014). Preferential packing of acidic glycosidases and proteases into *Bacteroides* outer membrane vesicles. MBio 5 (2):e00909-14. doi:10.1128/mBio.00909-14". Debelyy M.O. carried out the proteomic identification of *Bacteroides fragilis* and the in vitro OMV protease assay. Under the supervision of Feldman M.F., I was responsible for the design and performance of the remaining experiments. Data collection and analysis, as well as, writing the manuscript are my original work. Conclusions were drawn under the supervision of Feldman M.F.

Chapter 3 of this thesis is prepared for submission to "*Scientific Reports*" as "Elhenawy, W., Bording-Jorgensen, M.W., Haurat, M.F., Price, N.L., Wine, E., Feldman, M.F. (2015). The role of lipid A deacylation in outer membrane vesicles biogenesis". Cell infection assays and immunofluorescence were carried out by Bording-Jorgensen M.W. and me. The site-directed mutagenesis of PagL was the work of Haurat M.F. The RT-qPCR was carried out by Price N.L. Under the supervision of Feldman M.F., I was responsible for the design and performance of the remaining experiments. Data collection and analysis, as well as, writing the manuscript are my original work. Conclusions were drawn under the supervision of Feldman M.F. Wine E. helped with the discussion and the concluding analysis.

The appendix of this thesis was submitted as "Elhenawy, W., Scott, N.E., Tondo, M.L., Orellano, E.G., Foster, L.J. Feldman, M.F.(2015) Protein *O*-linked glycosylation in the plant pathogen *Ralstonia solanacearum*. Glycobiology (Manuscript number: GLYCO-2015-00120)". Scott N.E. and Foster L.J. carried out the proteomic analysis via mass spectrometry. Tondo M.L. and Orellano E.G. performed the preliminary virulence tests of *Ralstonia solanacearum* against tomato plants. Under the supervision of Feldman M.F., I was responsible for the design and performance of the remaining experiments. Data collection and analysis, as well as, writing the manuscript are my original work. Conclusions were drawn under the supervision of Feldman M.F.

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List of Abbreviations

AMP	Cationic antimicrobial peptides
ANOVA	A one-way analysis of variance
BHI	Brain heart infusion
BSA	Bovine serum albumin
CDT	Cytolethal distending toxin
CFTR	Cystic fibrosis transmembrane conductance regulator
DMEM	Dulbecco's modified eagle's medium
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
2,3-diNAcManA	2,3-diacetamido-D-mannuronic acid
ECA	Enterobacterial common antigen
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
Fuc2NAc	N-acetylfucosamine
GlcNAc	N-acetylglucosamine

Heat-labile enterotoxin	LT
HUS	Haemolytic uraemic syndrome
IM	Inner membrane
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KDO	3-deoxy-D-*manno*-octulosonic acid
LB	Luria-Bertani
LC-MS/MS	Liquid chromatography-Tandem MS
LPS	Lipopolysaccharides
(Man[2NAc3N]) acid	2-acetamido-3-acetamidino-2,3-dideoxy-D-mannuronic
MeUNAG	4-methylumbelliferyl-N-acetyl-β-D-glucosaminide
MOI	Multiplicity of infection
MS	Mass spectrometry
MS/MS	Tandem MS
MurNAc	N-acetylmuramic acid
OM	Outer membrane
OMV	Outer membrane vesicles
PBS	Phosphate buffered saline

PE	Phosphatidylethanolamine
pI	Isoelectric point
PG	Peptidoglycan
PQS	*Pseudomonas* quorum sensing molecule
PSA	Polysaccharide A
PULs	Polysaccharide utilization loci
PVDF	Polyvinylidene fluoride
Q-TOF	Quadrupole orthogonal acceleration time-of-flight
SCV	*Salmonella*-containing vacuole
SDS-PAGE electrophoresis	Sodium dodecyl sulfate polyacrylamide gel
Sif	*Salmonella*-induced filaments
Sus	Starch utilization system
T3SS	Type III secretion system
TBDTs	TonB-dependent receptors
TEM	Transmission Electron Microscopy
THAP	2,4,6-trihydroxyacetophenone monohydrate

Tris-HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol Hydrochloride
TTP	Thrombotic thrombocytopenic purpura

Chapter One

Introduction

Production of membrane vesicles is a biological phenomenon that is widespread among different domains of life. Membrane vesicles play different roles in eukaryotic cells, including the uptake of extracellular particles, biomolecules storage, secretion of proteins, digestion of intracellular components, and signal transduction (Nieuwland and Sturk, 2010). This biological phenomenon was considered for a long time to be restricted to eukaryotic organisms. However, the production of membrane vesicles by other domains is receiving more attention due to the increasing number of studies reporting the production of these structures by bacteria and archaea (Nieuwland and Sturk, 2010; Haurat *et al.*, 2015; Ellen *et al.*, 2009; Kulp and Kuehn, 2010). The production of membrane vesicles by bacteria was observed a long time ago, albeit, they were considered to be an artifact (Bishop and Work, 1965; Knox *et al.*, 1966). Since their discovery, bacterial membrane vesicles production has been a subject of controversy in the scientific community. For many years, it was not clear whether these vesicles are the result of membrane disintegration or a regulated biological process. However, bacterial membrane vesicles recently received more attention due to the different roles they perform (Mashburn-Warren and Whiteley, 2006; Kulp and Kuehn, 2010; Haurat *et al.*, 2015). These roles are determined by the nature of the producing bacterium and the cargo of the secreted vesicles. For example, many pathogens were found to pack toxins into bacterial vesicles for host delivery (Horstman and Kuehn, 2002; Kesty *et al.*, 2004; Bomberger *et al.*, 2009; Bomberger *et al.*, 2011; Wai *et al.*, 2003; Kulp and Kuehn, 2010; Mashburn-Warren and Whiteley, 2006; Haurat *et al.*, 2015). Also, some symbionts utilize vesicles to digest nutrients and secrete immunomodulatory molecules to promote their tolerance by the host immune system (Shen *et al.*, 2012; Rakoff-Nahoum *et al.*, 2014; Stentz *et al.*, 2014; Hickey *et al.*, 2015). Additional roles for bacterial vesicles in horizontal DNA transfer,

quorum sensing, and stress response have been proposed(Mashburn-Warren and Whiteley, 2006; Ellis and Kuehn, 2010; Kulp and Kuehn, 2010; Schwechheimer *et al.*, 2014; Haurat *et al.*, 2015).

In general, membrane vesicles result from the bulging of biological membranes. This process requires free energy of 250-600 KBT, where KBT is the thermal energy, making vesiculation a non-spontaneous process (Bloom *et al.*, 1991). This suggests the presence of a regulated mechanism that controls the process of vesiculation in different organisms. Through such a mechanism, the vesiculating organism can control the amount, as well as, the content of the vesicles produced in response to the surrounding signals. In eukaryotes, vesicles formation is a well-studied process. Through complex mechanisms, the eukaryotic cells have the ability to control the production of vesicles. This includes the remodelling of the membrane at the site of vesicles formation and the selection of the proteins to be packed (Zimmerberg and Kozlov, 2006). Recent data suggested the occurrence of similar processes in bacteria; however, the information available on the biogenesis and regulation of bacterial vesiculation is limited.

In this thesis, we investigated the underlying mechanisms involved in vesiculation of Gram-negative bacteria. We hypothesized that if vesiculation in bacteria is the result of a regulated process, the protein content of the vesicles should vary from that of the parent membrane due to selective packing of the cargo. In our work, we studied the vesicles cargo selection in the prominent members of the human gut microbiota, *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*. Also, we investigated the role of lipid A remodelling in membrane vesicles formation using the enteric pathogen, *Salmonella enterica* serovar Typhimurium, as a model organism. Understanding the biological architecture of the

vesiculation machinery will improve our understanding of bacterial pathogenesis and host-pathogen interactions.

1.1-Vesiculation in Gram-negative bacteria

The Gram-negative cell envelope is a dual membrane barrier surrounding the cytoplasm and providing protection against extracellular threats. The innermost membrane is the cytoplasmic membrane or the inner membrane (IM) which consists of a phospholipid bilayer and proteins, which are either integrated or anchored to the membrane. The outermost membrane is an asymmetric membrane known as the outer membrane (OM). The OM inner leaflet is made of phospholipids while the outer leaflet consists of a glycolipid layer known as the lipopolysaccharides (LPS) (Costerton *et al.*, 1974; Lugtenberg and Van Alphen, 1983; Beveridge, 1999). LPS is made of an essential acylated disaccharide molecule, called lipid A that is covalently linked to an oligosaccharide known as the core. In many Gram-negative bacteria, the core is linked to a glycan polymer known as the O antigen (Whitfield, 1995; Frirdich and Whitfield, 2005; Tran and Whitfield, 2009). The OM provides a hydrophobic environment suitable for the integration of many proteins. Additionally, lipoproteins can be anchored to the OM through an acyl chain (Okuda and Tokuda, 2011). Furthermore, some bacteria possess lipid microdomains in their OM that are enriched in sphingolipids and cholesterol (LaBach and White, 1969; Olsen and Jantzen, 2001; Heung *et al.*, 2006; An *et al.*, 2011; An *et al.*, 2014). The space between the IM and OM is known as the periplasm. The periplasmic space contains proteins, together with, a thin layer of peptidoglycan (PG). PG is an essential biopolymer made of alternating units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) that

are cross-linked by oligopeptides. The IM, OM and the PG can be interconnected by lipoproteins that span the cell envelope(Lugtenberg and Van Alphen, 1983).

The protrusion of the OM leads to the formation of vesicles known as the outer membrane vesicles (OMV). OMV are single bilayer liposomes that share their core components with the OM. OMV outer leaflet is made of LPS while the inner leaflet consists of phospholipids. Similar to the OM, OMV have proteins embedded into their membranes while the vesicular lumen contains periplasmic proteins (Mashburn-Warren and Whiteley, 2006; Ellis and Kuehn, 2010; Haurat *et al.*, 2015).

The production of OMV was first reported in *Escherichia coli,* which was found to produce extracellular LPS under lysine limitation (Bishop and Work, 1965). More work revealed that the extracellular LPS was shed in the form of vesicles from the protruding OM. The extracellular LPS was found to contain higher amounts of phosphatidylethanolamine relative to the cellular LPS (Knox *et al.*, 1966). The production of OMV due to a directed mechanism remained controversial due to the possibility that those structures are a mere result of cell lysis. More work suggested the enrichment of some proteins and lipid forms in OMV compared to the OM (Haurat *et al.*, 2015). Different studies identified the protein content of both, OMV and the parent OM in many bacteria. These studies revealed differences between the two membranous compartments, which indicate the presence of a selective process for packing OMV proteins (Grenier and Mayrand, 1987; Kadurugamuwa and Beveridge, 1995; Kato *et al.*, 2002; Horstman and Kuehn, 2002; Wai *et al.*, 2003; Sidhu *et al.*, 2008; Frias *et al.*, 2010a; Kahnt *et al.*, 2010).

1.1.1-OMV Biogenesis

One of the biggest challenges in the OMV research is the absence of a universal model describing their formation in Gram-negative bacteria. Moreover, a mutant that lacked OMV production was never isolated. Nevertheless, several models have been suggested to describe OMV biogenesis. In the following section, these models will be summarized.

1.1.1.1-Peptidoglycan turnover-derived vesiculation

Peptidoglycan (PG) fragments were detected in OMV of multiple bacteria (Zhou *et al.*, 1998; Kaparakis *et al.*, 2010; Bielig *et al.*, 2011; Wessel *et al.*, 2013; Irving *et al.*, 2014). Therefore, it was suggested that the accumulation of these fragments in the periplasm during PG turnover might contribute to OMV formation. The OM can tolerate a turgor pressure of 3 atm (Koch, 1998). If a higher pressure was exerted against the membrane by the accumulating PG fragments, membrane curvature might occur, leading to OMV formation (figure 1.1.A). Interestingly, *Porphyromonas gingivalis* autolysin mutant strain was found to produce more OMV compared to the wild type strain (Hayashi *et al.*, 2002). Autolysins belong to a family of enzymes that can hydrolyze the bond between N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) units of the PG. They play a role in peptidoglycan turnover, cell wall repair and remodelling (Shockman and Barren, 1983; Doyle *et al.*, 1988; Smith *et al.*, 2000). It was suggested that the *P. gingivalis* autolysin mutant accumulated more PG fragments in the periplasm due to a defect in PG hydrolysis and turnover. The build-up of PG fragments in the periplasm resulted in more vesiculation (Hayashi *et al.*, 2002).

More data supporting the role of low molecular weight PG in OMV formation was provided. An *E. coli* mutant strain that lacks YfgL, a lipoprotein responsible for upregulating lytic transglycosydases (Eggert *et al.*, 2001), was found to produce less OMV compared to the

wild type strain (Rolhion *et al.*, 2005). It was hypothesized that this mutant had lower levels of PG turnover. Therefore, fewer PG fragments would accumulate in the periplasm of the *E. coli* *yfgL*$^{-}$ which will decrease the turgor pressure exerted on the OM and subsequently reduce the levels of OMV produced. The effect of YfgL mutation on OMV production was studied in two *E. coli* strains; K-12 and LF82. However, the authors relied on the levels of secreted outer membrane proteins as a representative of OMV secretion levels, albeit, more accurate methods for OMV quantification are available (Rolhion *et al.*, 2005).

Although the above model suggests a role of PG turnover in OMV formation, it does not explain how the accumulated PG fragments localize in certain regions below the OM. Furthermore, the OMV proteins were found to be selectively packed in many bacteria (Haurat *et al.*, 2015). The PG turnover-derived vesiculation model does not explain how OMV cargo selection occurs. Alternatively, the presence of PG fragments in the OMV of some bacteria can be considered as a virulence mechanism adopted to induce inflammation. The role of OMV-associated PG fragments in inducing inflammation was recently demonstrated in *Helicobacter pylori* and *Pseudomonas aeruginosa* (Zhou *et al.*, 1998; Kaparakis *et al.*, 2010; Bielig *et al.*, 2011; Wessel *et al.*, 2013; Irving *et al.*, 2014). In addition, the release of PG into OMV might be a mechanism through which bacteria relief the stress occurring due to the accumulation of PG fragments in the periplasm. A similar role of OMV in the release of misfolded periplasmic proteins was proposed (McBroom and Kuehn, 2007).

1.1.1.2-Role of membrane-spanning lipoproteins in OMV formation

Analysis of OMV from bacteria like *E. coli* and *P. aeruginosa* revealed reduced lipoproteins content compared to the OM. The lower levels of lipoproteins in OMV suggested a

possible envelope remodelling event preceding OMV biogenesis. Many lipoproteins span the membrane linking the OM to the underlying layers (Okuda and Tokuda, 2011). As shown in figure 1.1.B, it was suggested that in regions where there are less lipoproteins linking the OM to the underlying layers, the OM could outgrow these layers and protrude forming vesicles (Hoekstra *et al.*, 1976; Wensink and Witholt, 1981). This model was supported by the hypervesiculation phenotype displayed by the *E. coli* mutants in Tol-Pal system (Bernadac *et al.*, 1998; Cascales *et al.*, 2002; Iwami *et al.*, 2007). The Tol-Pal system in *E. coli* is a complex system that involves multiple protein components and connects the OM with the PG and IM. The major components of the Tol-Pal system are Pal, which is an OM-anchored lipoprotein associated with the PG, in addition to TolA, TolQ, and TolR, which are located in the IM (Lloubès *et al.*, 2001). The Tol-Pal system is important for OM integrity and mutants lacking some of the Tol-Pal proteins were found to produce more OMV (Bernadac *et al.*, 1998). Similar results were obtained in *S.* Typhimurium strains lacking components of the Tol-Pal system. Furthermore, *S.* Typhimurium mutants lacking the lipoprotein Lpp that links the OM to PG displayed a hypervesiculation phenotype in vitro (Deatherage *et al.*, 2009). Similarly, the PG-interacting OmpA in the nosocomial pathogen, *Acinetobacter baumannii*, was found to be involved in OMV formation. The *A. baumannii* strain lacking OmpA was found to produce higher levels of OMV relative to the wild-type strain. The authors suggested that the OmpA mutant had fewer interactions between the OM and the underlying PG layer which affected OM integrity and increased vesiculation (Moon *et al.*, 2012).

However, the majority of the mutants involved in these studies had a defect in membrane integrity marked by the leakage of periplasmic contents to the supernatant (Bernadac *et al.*, 1998). This makes the hypervesiculation observed confounded by the membrane fragments

released due to cell lysis. It will be useful to couple OMV quantification in these studies with the measurement of OM integrity and cell lysis levels.

1.1.1.3-The role of LPS charge in OMV formation

This model is based on the observations made during *P. aeruginosa* OMV analysis. *P. aeruginosa* expresses in vitro two chemically and immunologically distinct types of LPS; A-band LPS and B-band LPS. The A-band LPS is present in all *P.aeruginosa* strains and is made of short repeats of α-D-rhamnose trimer, and hence neutral in charge. The B-band LPS O-antigen consists of a trisaccharide repeating unit of 2-acetamido-3-acetamidino-2,3-dideoxy-D-mannuronic acid (Man[2NAc3N]), 2,3-diacetamido-D-mannuronic acid (2,3-diNAcManA) and N-acetylfucosamine (Fuc2NAc). At neutral pH, the two mannuronic acid residues acquire a negative charge whereas the N-acetylfucosamine is positive, providing each trimer with an overall single negative charge (King *et al.*, 2009).

The analysis of LPS forms in OMV revealed that only B-band LPS is present in OMV of *P. aeruginosa* (Kadurugamuwa and Beveridge, 1995). Later work by Nguyen et al. suggested that A-band LPS is present in *P. aeruginosa* OMV, albeit, the B-band LPS is more abundant (Nguyen *et al.*, 2003). Further analysis of different *P. aeruginosa* mutant strains that vary in the O-antigen expressed, suggested a role of LPS charge in OMV formation. The strain that expressed only A-band LPS produced the least amount of OMV amid the strains tested (Nguyen *et al.*, 2003). These results suggest the preferential packing of B-band LPS in *P. aeruginosa* OMV. The repulsive forces between the negatively charged B-band LPS might force the OM to bend to relief the energetically unfavorable state. Consequently, OMV might be released as a

result of the OM bending. Other uncharacterized factors might be involved in the process (figure 1.1.C) (Mashburn-Warren and Whiteley, 2006).

Other roles of LPS charge in OMV formation have been suggested. The role of divalent cations like Ca^{2+} and Mg^{2+} in stabilizing the negatively charged phosphate groups of LPS is well-known (Hancock, 1984). Through the formation of salt bridges with the phosphate groups, these divalent cations can stabilize the OM. Compounds, like Ethylenediaminetetraacetic acid (EDTA) that have stronger binding affinities to divalent cations will disturb the salt bridges with LPS leading to OM destabilization (Hancock, 1984; Vaara, 1992). A similar mechanism was suggested to describe the involvement of the *Pseudomonas* quorum sensing molecule (PQS) in OMV formation. Mashburn and Whiteley have observed that *P. aeruginosa* mutants that cannot synthesize the hydrophobic quorum sensing molecule, PQS, produced fewer amounts of OMV compared to the wild type strain. They demonstrated that this phenotype could be rescued by the addition of PQS to the cultures of the *P. aeruginosa* mutants. The authors proposed that PQS can sequester the divalent cations that stabilize the membrane, which will lead to perturbations in the OM and overvesiculation (Mashburn and Whiteley, 2005; Mashburn-Warren and Whiteley, 2006; Mashburn-Warren *et al.*, 2008; Mashburn-Warren *et al.*, 2009; Wessel *et al.*, 2013). Despite the data supporting this model, the occurrence of similar mechanisms in other Gram-negative bacteria remains to be demonstrated.

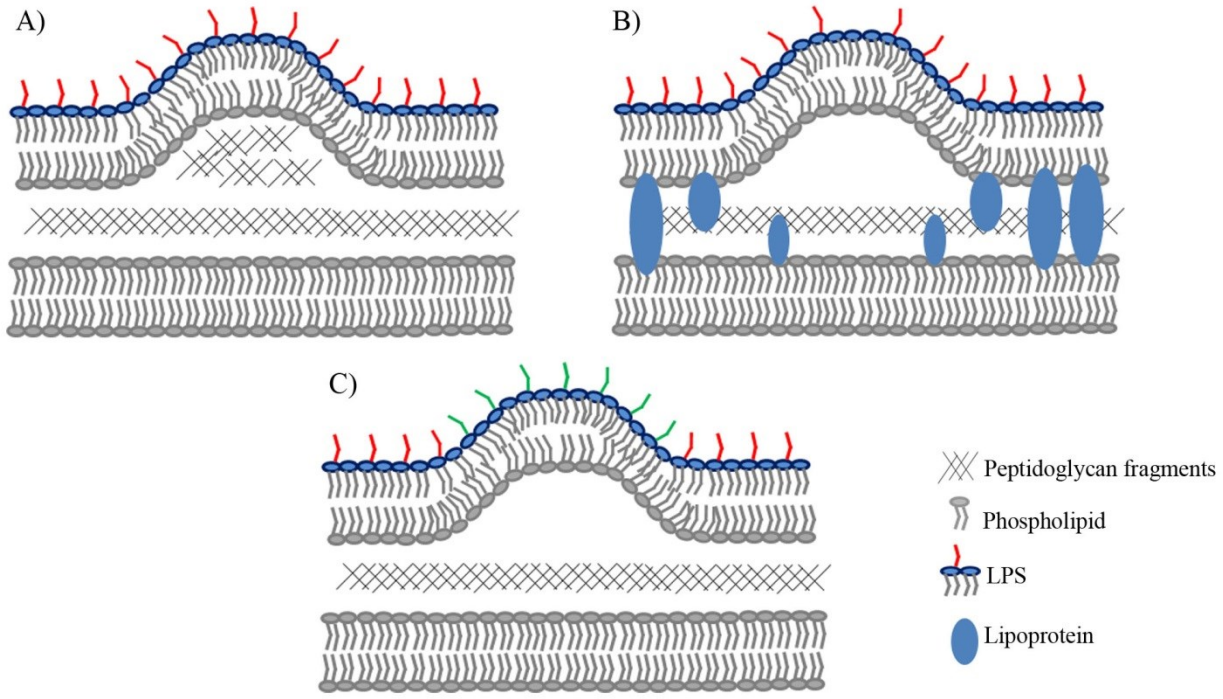

Figure 1.1 Models for OMV formation. (A) OMV formation by peptidoglycan fragments (PG) accumulation. PG fragments accumulate in the periplasm and generate enough turgor pressure to bend the OM. (B) The role of OM-PG interactions remodelling in OMV biogenesis. OMV are formed in regions with reduced OM-PG interactions. Lipoproteins that link OM to PG are excluded from the OMV. (C) O antigen charge repulsion mediates OMV formation. OMV are generated in regions where the negatively charged O-antigen subunits (green) are abundant, and the neutral O-antigen forms (red) are excluded.

1.1.2-Different roles of OMV

1.1.2.1-Role in Pathogenesis

Adhesins, toxins and immunomodulatory molecules have been detected in OMV of different bacteria suggesting a role in pathogenesis (Grenier and Mayrand, 1987; Kadurugamuwa

and Beveridge, 1995; Horstman and Kuehn, 2002; Wai *et al.*, 2003; Kesty *et al.*, 2004; Chitcholtan *et al.*, 2008; Haurat *et al.*, 2015). Packing these biomolecules into vesicles allows them to be delivered to their target host cells in a concentrated fashion (Haurat *et al.*, 2015). Furthermore, the liposomal nature of OMV provides their cargo with stability against extracellular proteases compared to proteins secreted by other mechanisms (Nasseau *et al.*, 2001). Moreover, the conditions in OMV might be optimal for the activation of some toxins like ClyA, which is found in the periplasm of *E. coli* in the form of inactive monomers. The elegant work by Wai et al. revealed that *E. coli* OMV provide the ClyA with the proper redox status essential for its oligomerization, and thus its activation (Wai *et al.*, 2003). *E. coli* DsbA is an oxidoreductase responsible for adding disulfide bonds to multiple proteins in the periplasm including ClyA, keeping it in an oxidized inactive state (Schlapschy and Skerra, 2011). Due to the absence of DsbA in *E. coli* vesicles, ClyA encounters less oxidative environment leading to its oligomerization and activation (Wai *et al.*, 2003).

Additionally, heat labile enterotoxin (LT) from *E. coli* was found to be secreted via OMV, which could be endocytosed by the host cells (Horstman and Kuehn, 2000; Kesty *et al.*, 2004). The LT toxin is associated with OMV by its ability to tightly bind the 3-deoxy-D-manno-octulosonic acid (KDO) core in the LPS (Horstman *et al.*, 2004). Concurrently, LT can bind the ganglioside receptors in the host lipid rafts that will mediate the uptake of the OMV and the subsequent release of the toxin inside the host cell (Horstman and Kuehn, 2002; Saunders, 2004). Interestingly, the LT-bound OMV could induce a protective immune response against enterotoxigenic *E. coli* colonization (Roy *et al.*, 2011). Moreover, *H. pylori*, a well-studied carcinogenic bacterium, was found to utilize OMV in promoting carcinogenesis. The vesicles secreted by *H. pylori* contain VacA, a multifunctional toxin that contribute to the ability of the

bacterium to induce gastric cancer (Cover and Blanke, 2005). The VacA-containing vesicles produced by *H. pylori* were rapidly endocytosed by host cells. Consequently, the host cells developed micronuclei formation and displayed genomic instability in a VacA-dependent manner (Chitcholtan *et al.*, 2008). Shiga toxin is another example of lethal toxins that can be secreted in OMV. The life-threatening Shiga toxin is produced by *Shigella dysenteriae* and *E. coli* O157:H7. The toxin is known for its capacity to induce haemorrhagic colitis, acute haemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) (Griffin and Tauxe, 1991). *S. dysenteriae* utilize OMV to secrete Shiga toxin, whereas the production of toxin-loaded OMV was increased by the addition of antimicrobials (Dutta *et al.*, 2004). Toxin secretion via OMV can sometimes occur in response to the surrounding signals. An interesting example is the secretion of haemolysin via OMV by *S. typhi* in response to norepinephrine (Karavolos *et al.*, 2011). Similarly, cytolethal distending toxin (CDT) of *S. typhi* was detected in OMV when recombinantly expressed in *S.* Typhimurium (Guidi *et al.*, 2013). Interestingly, *Campylobacter jejuni* CDT was found to be secreted and delivered to its target host cells via OMV (Lindmark *et al.*, 2009; Elmi *et al.*, 2012a).

Some bacteria can utilize OMV to interfere with host trafficking pathways. For example, *P. aeruginosa* packs Cif into OMV to promote the degradation of cystic fibrosis transmembrane conductance regulator (CFTR), and thus diminishing mucociliary clearance (Bomberger *et al.*, 2009). *P. aeruginosa* OMV fused with lipid rafts and Cif was released directly into the host cytosol, where it inhibited the deubiquitination of CFTR leading to its lysosomal degradation. *P. aeruginosa* was found to pack other toxins into its OMV including haemolysin and alkaline phosphatase (Bomberger *et al.*, 2009; Bomberger *et al.*, 2011). *Bordetella pertussis* delivers its adenylate cyclase toxin as OMV cargo causing an increase in intracellular cAMP levels of the

host cells (Donato *et al.*, 2012). OMV do not only deliver proteins to the host cells; some species such as *Borrelia burgdorferi* modulate the immune response via the lipids packed in OMV (Crowley *et al.*, 2013). Furthermore, the ability of OMV from different bacteria to activate the immune system in multiple ways is well documented (Kim *et al.*, 2008; Schild *et al.*, 2008; Vidakovics *et al.*, 2010; Ellis and Kuehn, 2010; Nakao *et al.*, 2011; Schaar *et al.*, 2011; Pollak *et al.*, 2012; Roier *et al.*, 2012; Lee *et al.*, 2012; Jun *et al.*, 2013; Kim *et al.*, 2013; Roier *et al.*, 2013; Deknuydt *et al.*, 2014).

1.1.2.2-Role of OMV in interbacterial killing

Many bacteria exist in polymicrobial communities. Due to nutrients limitation, some species evolved to kill other bacteria, and thus decrease food competition. OMV from different bacteria were shown to contribute to interbacterial killing (Mashburn-Warren and Whiteley, 2006; Kulkarni and Jagannadham, 2014; Haurat *et al.*, 2015). Li et al. showed that OMV harvested from 15 different strains that belong to genera; *Escherichia, Pseudomonas, Salmonella, Shigella, Klebsiella,* and *Proteus,* were capable of inducing lysis in many Gram-negative and positive bacteria (Li *et al.*, 1998). Interestingly, *P. aeruginosa* OMV displayed the broadest antimicrobial activity relative to OMV secreted by other species. Further work revealed the presence of a murein hydrolase in *P. aeruginosa* OMV that is responsible for inducing lysis in other bacteria through PG degradation (Li *et al.*, 1996). Additionally, the presence of gentamicin in *P. aeruginosa* OMV was found to contribute to their killing capacity (Kadurugamuwa and Beveridge, 1996). Later, OMV produced by *P. aeruginosa* were shown to contain quinolone molecules, known to have bactericidal activities (Mashburn and Whiteley, 2005). Recently, a bactericidal protein was found to be secreted by multiple *B. fragilis* strains via

OMV. The secreted protein was shown to induce lysis in the prey, possibly through inducing pore formation in the outer membrane. The authors suggested a role for *B. fragilis* OMV in eliminating competitors and shaping bacterial communities in the gut (Chatzidaki-Livanis *et al.*, 2014). Furthermore, the deltaproteobacterium *Myxococcus xanthus* is known to pack hydrolases, like phosphatases and proteases, into its OMV. Packing these hydrolases into OMV would allow *M. xanthus* to predate on other bacteria in soil microbial communities, including *E. coli* (Whitworth, 2011; Evans *et al.*, 2012).

1.1.2.3-Role of OMV in horizontal gene transfer

The main mechanisms for horizontal gene transfer among bacteria are; transformation, conjugation and transduction (Thomas and Nielsen, 2005). OMV delivery to other bacteria was suggested as a possible mechanism for horizontal gene transfer (Mashburn-Warren and Whiteley, 2006). Analysis of OMV from *E. coli* O157:H7 revealed the presence of DNA that was resistant to hydrolysis by extracellular DNAases (Kolling and Matthews, 1999). Further work by Yaron et al. demonstrated that the DNA detected in *E. coli* O157:H7 OMV, originated from different sources. This includes the genomic, phage DNA and a non-conjugative plasmid detected in multiple clinical isolates. Interestingly, the study revealed that OMV-mediated DNA transformation is possible, suggesting that OMV can promote lateral DNA transfer. Similarly, *P. aeruginosa* OMV analysis revealed the presence of intact plasmid among the OMV cargo (Renelli *et al.*, 2004). Recently, the marine cyanobacterium *Prochlorococcus* was found to produce DNA-containing vesicles. Interestingly, the authors were able to harvest these vesicles from ocean water suggesting a role for OMV in marine ecosystems (Biller *et al.*, 2014). The mechanism by which DNA is packed into OMV was not characterized. One possible explanation

is the translocation of DNA from the cytosol to the periplasm by an unknown mechanism followed by packing into OMV. Alternatively, cytoplasmic DNA can be released into the extracellular milieu after cell lysis, and then binds to OMV (Mashburn-Warren and Whiteley, 2006).

Moreover, naturally competent strains of *Haemophilus parainfluenza* displayed higher levels of vesicles on their OM compared to less competent strains. Upon the addition of exogenous linear DNA, the vesicles on the surface of the competent strains disappeared. The authors proposed that *H. parainfluenza* OMV mediated the exogenous DNA uptake by competent strains (Kahn *et al.*, 1982).

1.1.2.4-Role of OMV in biofilm formation

Biofilms are microbial accretions enclosed in a complex matrix of exopolysaccharides, DNA and proteins. Bacteria utilize biofilm formation as a mechanism to resist environmental stressors and to remain attached to biological and non-biological surfaces (Hall-Stoodley *et al.*, 2004). OMV were detected in the biofilms of some Gram-negative bacteria. Microscopical examination of *P. aeruginosa* biofilms revealed the presence of OMV within the biofilm matrix (Beveridge *et al.*, 1997). This suggested a role of OMV in biofilm production. Similarly, OMV were detected in *H. pylori* biofilms. Interestingly, the biofilm-forming capacity of one *H. pylori* strain was increased by the addition of OMV purified from a strain known to produce thick biofilms (Yonezawa *et al.*, 2009; Yonezawa *et al.*, 2011). The exact mechanism by which OMV can mediate biofilm formation remains to be illustrated. However, it was suggested that the secreted vesicles can act as a platform that allows the interaction between the

exopolysaccharides, extracellular DNA, proteins and the adhering surface (Schooling and Beveridge, 2006; Schooling *et al.*, 2009).

1.1.2.5-Role in interspecies communication

Quorum sensing is a biological phenomenon that involves the exchange of chemical molecules between members of the a bacterial population allowing them to coordinate their behaviour in response to cell density (Parsek and Greenberg, 2000). *P. aeruginosa* is employed as a model organism in many quorum sensing studies. 5% of *P. aeruginosa* genes were found to be regulated by quorum sensing molecules (Schuster *et al.*, 2003; Wagner *et al.*, 2003). *P. aeruginosa* has three main quorum signalling molecules; butyryl-homoserine lactone, 3-oxo-dodecanoyl homoserine lactone, and 2-heptyl-3-hydroxy-4-quinolone (known as the *Pseudomonas* Quinolone Signal or PQS). Of the three quorum sensing molecules, only PQS was detected in the OMV of *P. aeruginosa* in vitro. In addition to trafficking in OMV, PQS molecule contributed to the biogenesis of OMV in *P. aeruginosa* in a signalling-independent fashion (Mashburn and Whiteley, 2005; Mashburn-Warren and Whiteley, 2006; Mashburn-Warren *et al.*, 2009). The highly hydrophobic nature of PQS molecules might provide a plausible explanation for its packing in vesicles. Mashburn-Warren et al. demonstrated that PQS molecules integrate into the membrane of *P. aeruginosa* vesicles and interact with its lipid A component (Mashburn-Warren *et al.*, 2008). Furthermore, OMV might provide PQS with a protective environment against degradation by other bacteria in polymicrobial communities that *P. aeruginosa* usually exists in (Mashburn-Warren and Whiteley, 2006). Although PQS is specific to *Pseudomonas*, quorum sensing molecules produced by other bacteria might be packed into OMV. Many Gram-negative bacteria secrete acyl-homoserine lactones as quorum signals. In some bacteria, the

secreted acyl-homoserine lactones are highly hydrophobic. For example, the photosynthetic bacterium, *Rhodobacter capsulatus,* produces N-hexadecanoyl-homoserine lactone (Schaefer *et al.*, 2002). Additionally, the plant symbiont, *Sinorhizobium meliloti,* produces N-octadecanoyl-homoserine lactone (Marketon and González, 2002). The hydrophobic character of the latter molecules implies a possible role of OMV in their secretion, albeit, this remains to be demonstrated.

1.1.2.6-Other roles of OMV

Since the OM will be the first cellular compartment to encounter environmental stressors, like antimicrobials, and harmful chemicals, a possible role of OMV in stress response was hypothesized. In agreement with the latter hypothesis, an increase in vesiculation was observed upon the exposure of different bacteria to various stress factors. The exposure of *P. putida* to toxic concentrations of EDTA, as well as elevated temperature and osmotic shock, resulted in overvesiculation. The authors proposed that OMV were exploited by *P. putida* to get rid of toxic compounds (Baumgarten *et al.*, 2012). Similarly, *P. aeruginosa* produced more OMV in response to antibiotic-mediated stress in vitro (Maredia *et al.*, 2012). Moreover, OMV were proposed to act as a novel stress response used by bacteria to exclude misfolded proteins from the periplasm (McBroom and Kuehn, 2007). Heat shock resulted in an elevated level of misfolded proteins in *E. coli*, and a subsequent increase in OMV production. Interestingly, mutants that displayed an overvesiculation phenotype in vitro showed better survival at elevated temperatures relative to the wild-type strain (McBroom and Kuehn, 2007). However, these studies did not consider the effect of high temperatures on membrane fluidity and how this might impact vesiculation under these conditions (McBroom *et al.*, 2006; McBroom and Kuehn, 2007).

Intrestingly, different observations were made in *Serratia marcescens*. In vitro, *S. marcescens* produced a significant number of OMV at 22°C or 30°C and negligible quantities formed at 37°C. However, the *S. marcescens* mutant defective in the synthesis of the enterobacterial common antigen (ECA) displayed hypervesiculation, supporting the notion that OMV might be produced in response to stress. The hypervesiculating phenotype caused by the mutation in the ECA pathway was suppressed by the inactivation of the Rcs phosphorelay system. This suggested a role for the Rcs phosphorelay in OMV production by this organism (McMahon *et al.*, 2012). Interestingly, OMV were shown to contribute to the bacterial innate immune defenses against bacteriophages under lab conditions (Manning and Kuehn, 2011; Kharina *et al.*, 2015). The authors proposed that OMV might act as decoy structures to which bacteriophages can bind. Intriguingly, Biller et al. demonstrated that bacteria might exploit the same defense mechanism in ecosystems (Biller *et al.*, 2014).

Furthermore, OMV-based vaccines are an important biotechnological application of bacterial membrane vesicles (Bjune *et al.*, 1991; Sierra *et al.*, 1991; Cassio de Moraes *et al.*, 1992). A well-known example is MeNZB, a commercially available vaccine against *Neisseria meningitidis* serogroup B (Davenport *et al.*, 2008). Using OMV as vaccines has multiple benefits. During outbreaks, they can be readily purified from culture supernatants of the pathogen of interest (Oster *et al.*, 2005; Holst *et al.*, 2009). Furthermore, their lipid A content provides them with adjuvant properties that increase the immune response while reducing the amount of antigen needed (Muralinath *et al.*, 2011). Additionally, OMV can be used as a vehicle for recombinantly produced antigens providing a new platform for vaccine development (Chen *et al.*, 2010; Baker *et al.*, 2014).

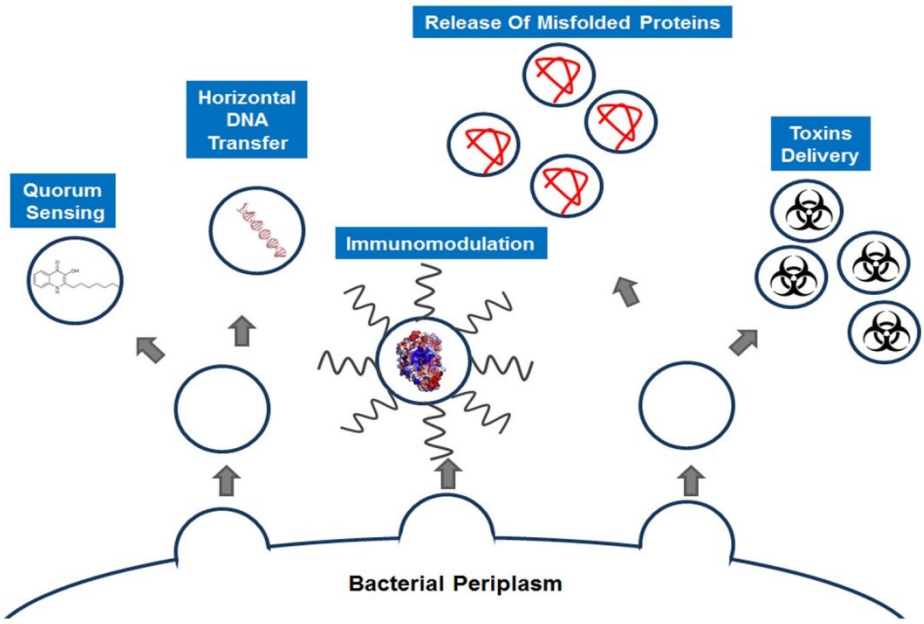

Figure 1.2 Examples of OMV functions in Gram-negative bacteria. Different bacteria were found to pack their OMV with toxins, quorum sensing molecules, misfolded proteins and DNA. Recently OMV were shown to contribute to gut health via immunomodulation of host responses. Figure was adapted from "Prokaryotic Membrane Vesicles: New Insights on Biogenesis and Biological Roles" by Haurat, M.F., Elhenawy, W., and Feldman, M. F., 2014, Biol. Chem. DOI: 10.1515/hsz-2014-0183.

1.1.3-Cargo recruitment in OMV

Understanding the molecular basis of the OMV cargo selection is a pivotal step towards unraveling the underlying mechanism reponsible for bacterial vesiculation. Nonetheless, the molecular details of OMV cargo recruitment remain elusive. Multiple studies detected differences in protein content of both, the OM and OMV, suggesting the presence of an OMV

protein selection mechanism. Grenier and Mayrand reported one of the earliest observations on the differences between OMV and OM in the dental pathogen, *Porphyromonas gingivalis* (Previously known as *Bacteroides gingivalis*) (Grenier and Mayrand, 1987). Similarly, OMV from five different Antarctic bacteria displayed different protein profile compared to their originating membranes (Frias *et al.*, 2010b). Moreover, OMV from different bacteria were enriched in specific toxins. Examples include the LT and CLyA toxins of *E. coli*, leukotoxin of *Actinobacillus actinomycetemcomitans,* and CDT toxin of *S.* Typhi (Horstman and Kuehn, 2000; Kato *et al.*, 2002; Guidi *et al.*, 2013). Moreover, mass spectrometry (MS) was employed to compare the OM and OMV proteomes in various bacteria. In the human pathogen, *Legionella pneumophila*, 33 proteins were found to be OMV specific. The analysis revealed that a significant portion of the OMV proteins is involved in the pathogenesis of *L. pneumophila* (Galka *et al.*, 2008). Similar observations were made in *Francisella novicida* and *Haemophilus influenza* (McCaig *et al.*, 2013; Roier *et al.*, 2015). Comparative proteomic analysis of *Neisseria meningitides* OM and OMV revealed the presence of OMV specific proteins. Interestingly, *N. meningitides* OMV did not contain any proteins linking the OM to PG (Lappann *et al.*, 2013). In addition, MS analysis of *Neisseria gonorrhoeae* and *Campylobacter jejuni* OMV proteins revealed the enrichment of virulence factors in the vesicles of both organisms (Elmi *et al.*, 2012b; Zielke *et al.*, 2014). Recent studies suggested a role for surface glycans in determining OMV protein composition. Sidhu et al. detected protein differences between OM and OMV of the plant pathogen, *Xanthomonas campestris pv. campestris*. Concurrently, the authors detected different forms of LPS in both compartments, which implied a role for LPS in OMV proteins recruitment. However, this claim was not supported by biochemical analysis (Sidhu *et al.*, 2008). Interestingly, *P. gingivalis* mutant strains lacking certain O antigen forms displayed an aberrant

OMV cargo selection. Some of the proteins normally excluded from OMV of the wild-type strain were detected in the OMV of mutant strains (Haurat *et al.*, 2011). These results suggested a role for O antigen in the selection of OMV proteins. One explanation is that the OMV cargo proteins may interact directly with the O antigen via a particular domain. The enrichment of the negatively charged A-LPS in the OMV of *P. gingivalis* suggests a possible interaction between such domain and A-LPS. Proteins lacking this domain could be excluded from the microdomains where the OMV are formed. Alternatively, a sorting factor may recognize the O antigen and the proteins to be packed in OMV, and thus linking them together. This is similar to the role of galectins in sorting exosomal proteins (Delacour *et al.*, 2007). In parallel, the possible presence of an OM retention signal in some proteins might explain their absence from OMV (Haurat *et al.*, 2011). The authors speculated that some proteins might be sorted into the OMV by one of the proposed models, while others might be excluded of the OMV with the help of retention signals. Interestingly, *P. aeruginosa* O antigen mutants displayed variations in their OMV protein content relative to the wild-type strain (Murphy *et al.*, 2014). Moreover, the abundance of certain proteins in *P. aeruginosa* OMV was affected by metabolites from host cells. Ballok et al. demonstrated that the levels of Cif toxin increased in OMV when *P. aeruginosa* was grown in the presence of epoxides produced by eukaryotic cells (Ballok *et al.*, 2014).

The OMV cargo selection is not only obvious in proteomic differences between OM and OMV, but extends to variations in the lipid content of the two compartments. *P. ginigivalis* OMV were enriched in the negatively charged A-LPS. Moreover, the MS analysis of the lipid A component of both, OM and OMV, revealed interesting findings. Deacylated forms of lipid A were enriched in the OMV compared to the OM. Negatively-charged LPS forms were found to be enriched in *P. aeruginosa* OMV (Kadurugamuwa and Beveridge, 1995). In addition, the

phospholipid composition of *P. aeruginosa* OM and OMV was analyzed. Phosphatidylethanolamine, phosphatidylglycerol and phosphatidylcholine displayed variable abundance in OM and OMV. Phosphatidylglycerol was the most abundant phospholipid followed by Phosphatidylethanolamine and phosphatidylcholine in OMV (Tashiro *et al.*, 2011). Furthermore, saturated fatty acids were more abundant in the OMV, unlike the OM that displayed equal distribution of saturated and unsaturated fatty acids (Tashiro *et al.*, 2011). Similarly, *Actinobacillus actinomycetemcomitans* OMV were enriched in cardiolipins (Kato *et al.*, 2002). The variation in lipid content of OMV and their originating membrane might support the notion that a membrane remodelling event precedes OMV formation. Indeed, different studies reported the formation of bacterial lipid domains (Matsumoto *et al.*, 2006; Lopez and Kolter, 2010).

1.2-Vesiculation in Gram-positive bacteria

Gram-positive bacteria have a thick cell wall consisting of cytoplasmic membrane and a dense PG layer. Unlike Gram-negative bacteria, the production of vesicles is not very common among Gram-positive organisms. The Gram-positive bacteria that were reported for vesicles production include *Staphylococcus aureus, Bacillus spp., Streptomyces coelicolor, Clostridium perfringens, Listeria monocytogenes, Thermoanaerobacterium thermosulfurogenes* and *Mycobacterium spp*. (Dorward and Garon, 1990; Mayer and Gottschalk, 2003; Lee *et al.*, 2009; Rivera *et al.*, 2010; Gurung *et al.*, 2011; Schrempf *et al.*, 2011; Prados-Rosales *et al.*, 2011; Lee *et al.*, 2013; Thay *et al.*, 2013; Jiang *et al.*, 2014). The OMV yield in the Gram-positive bacterium, *S. aureus* was found to be about 1000 fold less than the Gram-negative *P. gingivalis* (Haurat and Feldman, unpublished results). Although this difference does not necessarily apply

to the remaining members of the two bacterial groups, it might explain the limited reports on vesicles production by Gram-positive organisms (Lee *et al.*, 2009). Gram-positive vesicles were reported to be 20–250 nm in diameter and contained membrane and cytoplasmic proteins (Lee *et al.*, 2009; Rivera *et al.*, 2010; Schrempf *et al.*, 2011). Among the cargo identified in Gram-positive vesicles are toxins like anthrolysin, anthrax toxin components, coagulases, hemolysins and lipases (Lee *et al.*, 2009; Rivera *et al.*, 2010; Schrempf *et al.*, 2011; Prados-Rosales *et al.*, 2011). Moreover the detection of penicillin- binding proteins and β-lactamases in *S. aureus* membrane vesicles could explain the increment of antibiotic resistance associated with infections by this organism (Lee *et al.*, 2009). Furthermore, *M. tuberculosis* was found to release vesicles inside macrophages. The amounts of produced vesicles increased under conditions mimicking the environment inside granulomas (Prados-Rosales *et al.*, 2011; Prados-Rosales *et al.*, 2014). Recent data suggested a possible regulatory mechanism of vesiculation in *M. tuberculosis* (Rath *et al.*, 2013). There are increasing evidence that membrane remodeling might contribute to vesiculation in Gram-positive bacteria. *S. coelicolor* was found to release vesicles containing proteins with a possible common signal that might contribute to their sorting into vesicles (Schrempf *et al.*, 2011). Furthermore, lipid analysis of *B. anthracis* vesicles suggested the enrichment of unsaturated fatty acids into the secreted vesicles (Lee *et al.*, 2009; Rivera *et al.*, 2010). Similarly, *M. tuberculosis* vesicles were enriched in diacyl and triacyl glycerides and PE, while lacking many lipid species that were detected in the cytoplasmic membrane (Prados-Rosales *et al.*, 2014). It is tempting to speculate that vesicles in Gram-positive bacteria are formed at membrane microdomains, albeit, more lipid analysis of membranes and vesicles in different Gram-positive bacteria remains to be demonstrated. Nevertheless, what occurs to the thick PG layer during vesiculation remains unclear. One possibility is the degradation of PG in

the regions of the membrane where vesicles pinch off. In agreement with the previous hypothesis, the peptidoglycan layer was degraded and vesicles were formed when *T. thermosulfurogenes* was grown under starch limitation (Mayer and Gottschalk, 2003). Similar to OMV, the Gram-positive vesicles have a potential biotechnological application as vaccines. A group of mice immunized with *Bacillus anthracis* vesicles displayed better survival rates when challenged with the pathogen compared to non-immunized groups (Rivera *et al.*, 2010).

1.3-Main organisms studied in this thesis

1.3.1-*Bacteroides*

The human colon harbors the majority of microorganisms in the body with the anaerobic bacteria constituting the highest percentage among the gut microbiota (Wexler, 2007). 25% of these anaerobes belong to genus *Bacteroides*. Members of this genus are obligate anaerobic, Gram-negative, rod-shaped bacteria that belong to family *Bacteroidaceae,* order *Bacteriodales* in the phylum *Bacteroidetes* (previously known as *Cytophaga-Flavobacteria-Bacteriodetes*). Both, *B. fragilis* and *B. thetaiotaomicron,* are the most prominent members of the genus (Wexler, 2007). Members of genus *Bacteroides* appear in the gastrointestinal tracts of humans, ten days after birth. Gut colonization with *Bacteroides* species is beneficial to the host due to their extensive ability to digest a broad spectrum of glycans that the host can not degrade (Pumbwe *et al.*, 2006b; Comstock, 2009). In addition to its role as a commensal, *B. fragilis* is a frequent isolate of intraabdominal abscesses and bacteremia (Willis, 1991).

Under normal conditions, the intestinal barrier separates the gut microbiota from the blood stream. However, the disruption of this barrier, either due to a disease or surgery, will give the intestinal bacteria access to the blood stream. Initial tissue destruction occurs by aerobic members of the gut microbiome, like *E. coli*, leading to a decrease in oxygen levels in those tissues. Following the reduction in oxidation-reduction potential, anaerobic bacteria, like *B. fragilis* can thrive and cause disease (Willis, 1991; Pumbwe *et al.*, 2006b).

Bacteroides members have complex surface structures that contribute to the ability of the bacteria to either, establish symbiosis in the human host or cause opportunistic infections. Discussed below are examples of various surface structures that are involved in gut colonization and pathogenesis.

1.3.1.1-Pili and adhesins

Out of 19 *B. fragilis* strains examined, 16 were found to possess pili. Microscopical examination of the piliated strains revealed a peritrichous distribution of pili around the body of the bacteria. When piliated *B. fragilis* strains were treated with trypsin, the ability to cause haemagglutination and adhesion to intestinal cell lines was compromised. This suggested an important role for pili in cell adhesion (Ferreira *et al.*, 1999). On the contrary, other studies showed that treating *B. fragilis* with proteases did not affect adhesion to intestinal cells. Nevertheless, when these strains lost their ability to form capsules, adhesion to host cells was inhibited. It worth mentioning that pili formation was not affected in the capsule mutants (Oyston and Handley, 1990). One possible explanation for the discrepancy between these studies, is the differences between the cell lines used. Different cell lines might have different levels of mucus production and variable surface receptors, which will affect the adhesion of the

bacteria to the cell surface. Moreover, other surface structures with adhesion properties might be present in *B. fragilis*. Genomic analysis of multiple *Bacteroides* members suggested the presence of large number of lipoproteins with possible OM localization and a putative function in adhesion. Additionally, surface lectins were identified in *B. fragilis* and were found to bind host sugars (Pumbwe *et al.*, 2006b). Furthermore, a putative fimbrial assembly protein from *B. thetaiotaomicron* was crystallized, which suggested the presence of a functional fimbrial system in the bacterium (Xu *et al.*, 2010).

1.3.1.2-Capsule

All *B. fragilis* strains analyzed to date were found to be encapsulated. *B. fragilis* has 8 loci coding for 8 different capsular polysaccharides (designated PSA to PSH). The expression of the different polysaccharides is phase variable due to the presence of inverted promoters upstream of all loci except PSC. Within an in vitro *B. fragilis* population, bacterial cells expressed variable capsule glycans (Coyne *et al.*, 2008). Interestingly, each polysaccharide locus was found to encode a transcriptional terminator for other heterologous loci (Chatzidaki-Livanis *et al.*, 2010). The expression of at least one capsular form was found to be important for gut colonization. A mutant lacking capsule formation was outcompeted by the wild-type strain in a gnotobiotic mouse colonization model (Coyne *et al.*, 2008).

The role of capsular polysaccharides in immunomodulating the host responses was demonstrated. The injection of purified PSA or PSB was sufficient to induce abscesses in mice (Pumbwe *et al.*, 2006b). Moreover, PSA was found to play a significant role in the gut by actively suppressing the host immune system via toll-like receptors (TLR) signalling. Such immunomodulation is pivotal to promote immunologic tolerance towards *B. fragilis*, which is

necessary for long-term colonization of the host (Round *et al.*, 2011). Interestingly, PSA was found to be delivered to the immune cells via OMV (Shen *et al.*, 2012). On contrary, little is known about the capsule structures in *B. thetaiotaomicron*. Nevertheless, eight loci were predicted to code for capsular polysaccharides (Xu *et al.*, 2007).

1.3.1.3-Lipopolysaccharides

B. fragilis was found to produce LPS that is 10-1000 times less toxic than LPS purified from *E. coli* (Delahooke *et al.*, 1995). The lipid A portion of LPS isolated from *B. fragilis* was found to be penta-acylated and mono-phosphorylated. This is different from *E. coli* LPS that has the hexaacylated dephosphorylated lipid A as the predominant form. The fatty acids present in *B. fragilis* LPS were 15, 16, or 17 carbon units long, with some of them branched. *B. fragilis* attached a higher percentage of 3-OH-17 fatty acid compared to *B. thetaiotaomicron* (Wollenweber *et al.*, 1980; Berezow *et al.*, 2009). Although both species produced less toxic LPS than *E. coli*, *B. thetaiotaomicron* LPS displayed slightly more immunostimulatory properties than that of *B. fragilis* (Berezow *et al.*, 2009). Structural analysis of LPS in *B. fragilis* NCTC 9343 revealed the presence of a core and a short O-antigenic chain. Similar structure was suggested for other strains of *B. fragilis*, while the structure of *B. thetaiotaomicron* LPS remains to be elucidated (Lindberg *et al.*, 1990).

1.3.1.4-Polysaccharide utilization loci (PULs)

The amount of dietary polysaccharides that reaches the human gut is enormous and mainly has a plant origin. These polysaccharides are usually originating from plant cell wall and storage polysaccharides, like inulin (Flint *et al.*, 2008). Many of the glycosyl hydrolases required

for the digestion of these complex polysaccharides are absent in the human genome. As a result of the symbiotic life style of *Bacteroides* members in the human gut, they evolved to produce a large number of glycoside hydrolases capable of digesting complex polysaccharides (Comstock, 2009). Genes coding for 236 glycoside hydrolases and 15 polysaccharide lyases were detected in *B. thetaiotaomicron* VPI-5482 genome (Xu *et al.*, 2003; Flint *et al.*, 2008).

The pioneering work by Salyers and colleagues provided the first mechanistic insight into polysaccharide utilization by *B. thetaiotaomicron.* They demonstrated that *B. thetaiotaomicron* relies on an eight-gene locus to utilize starch as a nutrient (D'Elia and Salyers, 1996; Cho and Salyers, 2001; Koropatkin *et al.*, 2012). This locus encodes for outer membrane and periplasmic proteins that are required for the binding and hydrolysis of starch. Gene disruption studies revealed that 3 genes (*susC,susD,susG*) in the starch utilization locus (Sus) are essential for growth on starch as a sole carbon source. SusD is an outer membrane protein involved in starch binding, while SusC is a TonB-dependent transporter required for starch binding and the import of starch oligosaccharides into the periplasm. The OM neopullulanase, SusG, is responsible for the hydrolysis of OM-bound starch into oligosaccharides that can be imported into the periplasm (Reeves *et al.*, 1997; Flint *et al.*, 2008).

The starch utilization locus in *B. thetaiotaomicron* represents a universal paradigm for glycan acquisition systems, known as polysaccharide utilization loci (PULs). PULs constitute about 18% of *B. thetaiotaomicron* str. VPI-5482 genome (Koropatkin *et al.*, 2012). Moreover, paralogues of these systems are ubiquitous among *Bacteriodetes* members. These PULs were found to be involved in the utilization of a variety of glycans, and some of them are important for human host colonization (Koropatkin *et al.*, 2012). In *B. thetaiotaomicron*, two PULs are

important for the ability of the bacterium to forage human glycans (Martens *et al.*, 2008). Moreover, *B. fragilis* has 47 PUL orthologues, and one of them was shown to be involved in the acquisition of host N-glycans (Cao *et al.*, 2014).

1.3.1.5-Role of *Bacteroides* OMV in gut health

Recently, the role of OMV secreted by microbiota members have received more attention. OMV produced by members of genus *Bacteroides* were found to play a significant role in immunomodulation (Haurat *et al.*, 2015). OMV from *B. fragilis* were found to deliver polysaccharide A (PSA) to dendritic cells (Shen *et al.*, 2012). PSA is zwitterionic polysaccharide and is one of eight capsular forms expressed by *B. fragilis* (Pumbwe *et al.*, 2006a; Chatzidaki-Livanis *et al.*, 2008). Upon stimulation by PSA, regulatory T cells would be activated to release interleukin-10 that has been shown to protect against inflammation (Ruiz-Perez *et al.*, 2005; Mazmanian and Kasper, 2006). PSA carried by OMV resulted in a different cascade of immune response compared to pure PSA. This shed light on the role of OMV in the delivery of immunomodulators in the colon. The OMV-mediated delivery of PSA to dendritic cells was shown to be important for immunotolerance and long-term survival of *B. fragilis* in the gut (Shen *et al.*, 2012).

B. thetaiotaomicron was shown to utilize OMV in a multifaceted fashion to promote gut colonization (Haurat *et al.*, 2015). A homologue of an eukaryotic inositol phosphatase was detected in the OMV of *B. thetaiotaomicron.* The detected inositol phosphatase was found to interefere with Ca^{2+}-dependent signaling inside host cells. It was proposed that packing the inositol phosphatase into OMV provides protection against proteases in the gut (Stentz *et al.*, 2014). However, the exact role of *B. thetaiotaomicron* inositol phosphatase in gut colonization

remains to be demonstrated. Moreover, Stentz et al. showed that *B. thetaiotaomicron* OMV harbor β-lactamases that conferred protection to other commensals against β-lactams that might be encountered by the gut microbiota during host colonization (Stentz *et al.*, 2015). Recently, an elegant study by Hickey et al. demonstrated the role of OMV in colitis induction by *B. thetaiotaomicron* in susceptible mice. The authors showed that *B. thetaiotaomicron* utilize OMV sulfatases to degrade mucin sulfates. Following mucin desulfation, OMV can gain access to immune cells and trigger an inflammation response preceding colitis (Hickey *et al.*, 2015).

1.3.2-*Salmonella enterica*

Members of genus *Salmonella* belong to family Enterobacteriaceae. They are motile Gram-negative bacteria that can cause enteric diseases in many animals. Over 2500 serovars belong to the species *Salmonella enterica*. These include typhoidal and non-typhoidal strains. The typhoidal strains are *S. enterica* subsp. *enterica* serovars Typhi and Paratyphi (Fàbrega and Vila, 2013). Only humans can be infected with the typhoidal serovars that cause abdominal pain and fever (typhoid fever). Non-typhoidal strains, like *S. enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium), can infect a broad range of hosts causing acute gastroenteritis that can develop to a systemic disease in immunocompromised individuals. Oral ingestion is the major route of infection by *S.* Typhimurium. The bacterium has the ability to survive gastric acidity and access the intestinal epithelia, where it elicits a strong inflammatory response (Fàbrega and Vila, 2013; LaRock *et al.*, 2015). *S.* Typhimurium can resist killing by macrophages, albeit, can be easily eradicated by neutrophils. To avoid neutrophils-mediated killing, *S.* Typhimurium adopted an intracellular lifestyle (Cheminay *et al.*, 2004). Initially, the bacterium traverses the intestinal epithelial barrier by invading the microfold cells. Subsequently,

S. Typhimurium can invade different mammalian cell types, including macrophages. Intracellular *S.* Typhimurium can resist lysosomal killing and reside in a specialized compartment, called *Salmonella*-containing vacuole (SCV). Intravacuolar survival is an essential step for *S.* Typhimurium pathogenesis and necessary for the dissemination of the infection to neighboring cells (Garcia-del Portillo and Finlay, 1994; Rathman *et al.*, 1997; Fàbrega and Vila, 2013; LaRock *et al.*, 2015).

Discussed below are examples of the main virulence factors of *S.* Typhimurium:

1.3.2.1-Adhesins

S. Typhimurium LT2 genome encodes 13 distinct fimbrial loci that include two loci expressed in vitro (McClelland *et al.*, 2001). Under standard laboratory conditions, only type I fimbriae and curli fimbriae are expressed, while the remaining fimbrial systems displayed poor expression. Interestingly, the expression of all fimbrial systems was increased in vivo, indicating an important role in pathogenesis (Humphries *et al.*, 2003). Different studies demonstrated the role of *S.* Typhimurium fimbriae in adhesion to epithelial cells and intestinal persistence in mice (Bäumler *et al.*, 1996; Sukupolvi *et al.*, 1997). However, the deletion of individual fimbrial loci resulted in a moderate decrease in virulence towards mice, whereas combining genetic inactivation of multiple fimbrial systems caused a significant virulence reduction (van der Velden *et al.*, 1998; Fàbrega and Vila, 2013). These results suggested the presence of functional redundancy among the different fimbrial systems of *S.* Typhimurium. Furthermore, the presence of non-fimbrial adhesins was reported in *S.* Typhimurium. The autotransporter protein, MisL, was found to bind the host extracellular matrix protein, fibronectin. Additionally, SiiE is an

adhesion exported by type I secretion system and mediated adhesion of *S.* Typhimurium to epithelial cells (Fàbrega and Vila, 2013).

1.3.2.2-Type three secretion systems (T3SSs)

T3SS is a needle-like machinery that is assembled by many bacteria to translocate effectors directly into host cells (Mattei *et al.*, 2011). The pathogenesis of *S.* Typhimurium is highly dependent on two distinct T3SSs that are encoded in two different pathogenicity islands; *Salmonella* pathogenicity island-1 (SPI-1) and *Salmonella* pathogenicity island-2 (SPI-2) (LaRock *et al.*, 2015). *S.* Typhimurium relies on SPI-1 T3SS for invading the non-phagocytic cells, like intestinal epithelia. This invasion requires the occurrence of cellular events marked by actin cytoskeleton rearrangement. This occurs through the modulation of the activity of actin-organizing GTP-binding proteins that belong to the Rho subfamily of GTPases (Keestra-Gounder *et al.*, 2015). Rho GTPases function as molecular switches to control actin dynamics in the cell. Rho proteins can exist in two forms; the GTP-bound active form and the GDP-bound inactive form, which occur when the intrinsic GTPase activity of the Rho proteins is stimulated. Different proteins regulate the conversion of Rho proteins from one form to the other (Mao and Finnemann, 2015). Various SPI-1 T3SS effectors are injected into the host cells and mediate Rho GTPase-dependent actin rearrangements at the cell membrane leading to its ruffling and bacterial uptake. SopB, SipA, SopE and SopE2 are SPI-1 T3SS effectors that trigger actin rearrangement via distinct mechanisms (Fàbrega and Vila, 2013; Keestra-Gounder *et al.*, 2015; LaRock *et al.*, 2015). SopB acts as a phosphatidylinositol phosphatase to generate secondary messengers that indirectly activates Cdc42 and RhoG, which are members of the Rho subfamily (Mao and Finnemann, 2015). Concurrent with SopB-mediated cytoskeletal rearrangements, SopE and

SopE2 activate Rho GTPases through their guanine nucleotide exchange factor (GEF) activity (Friebel *et al.*, 2001). Furthermore, SipA inhibits actin depolymerisation and stimulates actin bundling, and thus promotes the internalization of the bacterium (Zhou *et al.*, 1999). Interestingly, SipC plays a dual role in *S.* Typhimurium uptake. In addition to its role in pore formation as T3SS translocator, it can nucleate actin at the site of insertion (Myeni and Zhou, 2010). Intriguingly, *S.* Typhimurium can reverse the cytoskeletal rearrangements after cell invasion, and thus restores the normal architecture of the host cell membrane. This is mediated by SptP, an SPI-1 T3SS effector that can activate the GTPase activity of Rho proteins leading to their inactivation (Fu and Galán, 1999).

Many of the SPI-1 T3SS effectors (including SopB, SopE, SopE2, SipA, SipC and SopA) mediate the inflammatory response that occur during early stages of *S.* Typhimurium infection (Keestra-Gounder *et al.*, 2015; LaRock *et al.*, 2015). Gut inflammation is beneficial to *S.* Typhimurium due to the release of growth factors, like tetrathionate and lipocalin-2, which give *S.* Typhimurium an advantage over gut microbiota. Unlike microbiota, tetrathionate can be used by *S.* Typhimurium for respiration. Lipocalin-2 can sequester the microbiota siderophores, while *S.* Typhimurium siderophores will escape sequestration. Therefore, the release of lipocalin-2 will deplete the microbiota members from their iron supply, favoring *S.* Typhimurium outgrowth (LaRock *et al.*, 2015).

SPI-2 T3SS plays an essential role in the intracellular survival of *S.* Typhimurium . *S.* Typhimurium mutants lacking SPI-2 T3SS were severely attenuated in virulence towards mice (Fàbrega and Vila, 2013). Inside the phagocytic vacuole, the presence of antimicrobial peptides, the low pH and Mg^{2+} limitation will activate the PhoPQ regulatory system, leading to a

subsequent activation of SPI-2 T3SS (Bijlsma and Groisman, 2005). SPI-2 T3SS effectors mediate the vacuolar modifications that occur to SCV immediately after *S.* Typhimurium invasion. Consequently, *S.* Typhimurium can survive and replicate in the SCV despite its fusion with lysosomes. Moreover, the SCV can traffic along the microtubular network as a result of SPI-2 T3SS effectors activities (LaRock *et al.*, 2015). SseJ and SifA are the best characterized effectors of SPI-2 T3SS. SseJ indirectly modifies the cholesterol and phospholipids composition of the SCV. The changes in the lipid properties of SCV dramatically alter the protein content of its membrane (LaRock *et al.*, 2012). SifA is responsible for linking the SCV to the microtubular network via its interaction with host proteins, like kinesin-interacting protein (Boucrot *et al.*, 2005). Together with other SPI-2 T3SS effectors, SifA induces the formation of *Salmonella*-induced filaments (Sifs). Sifs are actin-independent microtubule-dependent endosomal tubule extensions formed by intracellular *S.* Typhimurium. Although the exact physiological role of Sifs remains to be determined, mutants defective in Sifs formation displayed attenuated virulence in mouse models. One possible explanation is that Sifs allow intracellular *S.* Typhimurium to harvest more nutrients, together with the concurrent dilution of harmful lysosomal enzymes (Ramsden *et al.*, 2007; LaRock *et al.*, 2015).

1.3.2.3-LPS modifications

LPS is a major component of the Gram-negative OM and a prominent target for detection by the immune system. The mammalian immune system can detect LPS via Toll-like 4 (TLR-4) receptors, triggering a potent inflammatory response (Tran and Whitfield, 2009). *S.* Typhimurium deacylates lipid A, the hydrophobic core of LPS, to avoid detection by TLR-4. Two OM enzymes mediate the deacylation of lipid A by *S.* Typhimurium; PagL and LpxR. PagL

removes the hydroxymyristate group at 3-positon of lipid A, while LpxR removes the 3'-acyloxyacyl moiety (Trent *et al.*, 2001; Kawasaki *et al.*, 2012). Under standard lab conditions, both OM deacylases of *S.* Typhimurium are downregulated. The activation pathway of LpxR remains to be elucidated, while PagL is activated via the PhoPQ regulatory system. Intravacuolar cues, like low pH and high AMP levels, stimulate the PhoPQ system, leading to PagL-mediated lipid A deacylation (Trent *et al.*, 2001; Kawasaki *et al.*, 2004; Kawasaki *et al.*, 2012).

Moreover, the innate immune system targets the negatively-charged LPS with cationic antimicrobial peptides (AMP), like defensins. The positive charges on the AMP allow them to bind to the negatively charged bacterial surfaces and thereby disrupt the bacterial membrane integrity. *S.* Typhimurium has evolved to sense the presence of AMP and remodel the OM to resist their lethal effects (Matamouros and Miller, 2015). During intravacuolar life, the PhoPQ regulatory system is stimulated and subsequently activates the PmrAB system, which upregulates many genes involved in LPS modifications (Gunn and Miller, 1996). *S.* Typhimurium lipid A has negatively charged phosphate groups at 1 and 4' positions of the disaccharide (Tran and Whitfield, 2009). The phosphate groups of lipid A are modified by the PmrA-activated, ArnT and EptA, through the addition of a cationic aminoarabinose and zwitterionic phosphoethanolamine groups respectively. These modifications aim at decreasing the overall negative charge on LPS, and thus diminishing the electrostatic interaction between LPS and AMP (Zhou *et al.*, 2001; Lee *et al.*, 2004; Matamouros and Miller, 2015). Additionally, the activation of PhoPQ system results in the acylation of lipid A and OM phosphatidylglycerol by the palmitoyl transferase, PagP. Consequently, the OM fluidity will decrease due to the increase in the overall hydrophobicity, and thus delaying the entry of AMP to the cell (Dalebroux *et al.*, 2014; Matamouros and Miller, 2015).

1.4-Thesis objectives

Understanding the molecular basis for OMV formation and cargo selection in Gram-negative bacteria are the main objectives of this thesis.

1.4.1-Investigation of OMV proteins sorting in *Bacteroides*

Previous reports suggested the presence of OMV cargo selection machinery in many Gram-negative bacteria, including *P. gingivalis* (Haurat *et al.*, 2011; Haurat *et al.*, 2015). The first objective of this thesis is to determine if the selective packaging of OMV proteins occurs in members of genus *Bacteroides*, which are genetically close to *P. gingivalis* (Nelson *et al.*, 2003). Moreover, members of genus *Bacteroides* are easier to be genetically manipulated relative to *P. gingivalis*. OM and OMV proteomes were identified and compared using MS. We employed immunoblotting to validate our MS results. Furthermore, genetic screening was performed to understand the molecular mechanisms underlying OMV biogenesis and cargo recruitment in *Bacteroides*.

1.4.2-Studying the role of lipid A remodelling in OMV formation by *S.* Typhimurium

The differences in lipid composition between OM and OMV were previously reported. In *P. gingivalis*, OMV were found to be enriched in deacylated lipid A species (Haurat *et al.*, 2011). In this thesis, we proposed a role for lipid A deacylation in OMV biogenesis. We employed the enteric pathogen, *S.* Typhimurium, as a model organism to study the effect of lipid A deacylation on vesiculation. *S.* Typhimurium has a well-characterized lipid A deacylase in the OM, known as PagL (Trent *et al.*, 2001). We hypothesized that PagL-mediated deacylation might be involved in OMV production by *S.* Typhimurium. To test this hypothesis, we employed immunofluorescence

to study the role of PagL in vesiculation during intracellular life of *S.* Typhimurium. Furthermore, we used a biochemical approach to quantify OMV production in vitro when PagL was expressed. In addition, we studied the distribution of different lipid A species between the OM and OMV of *S*. Typhimurium during PagL expression.

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Chapter Two

Preferential packing of acidic glycosidases and proteases into *Bacteroides* outer membrane vesicles

Portions of this chapter have been published.

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2.1-Introduction

The human intestine is colonized by a dense population of bacteria commonly known as the microbiota. The microbiota is beneficial to the host in many ways. These benefits include resistance to invasive pathogens, stimulating cell turnover, and aiding in the digestion of complex nutrients to provide the host with extra energy and essential vitamins (Boleij & Tjalsma, 2012). The majority of simple sugars and oligosaccharides ingested by the human host are absorbed through the small intestine (Bond *et al.*, 1980). Other sugars reach the colon intact where they serve as substrates for the microbiota (Cummings *et al.*, 1989, Gibson & Roberfroid, 1995). Besides, the host itself produces fermentable products including glycoproteins and polysaccharides that are consumed by the gut microbiota (Gibson & Roberfroid, 1995, Macfarlane & Gibson, 1991, Roberton & Stanley, 1982, Salyers *et al.*, 1982, Salyers *et al.*, 1977). Members of genus *Bacteroides* represent the most abundant polysaccharide utilisers in the colon (Macy & Probst, 1979, Salyers *et al.*, 1977). 10%-20% of the commensal population in the colon consists of *Bacteroides* genus representatives (Fletcher *et al.*, 2009). Among these is *B. fragilis*, an opportunistic pathogen and the most commonly isolated anaerobe from human infections such as intra-abdominal and brain abscesses (Brook, 1989, Duerden, 1980, Sherwood *et al.*, 2011, Wexler, 2007). Under normal symbiotic conditions, *B. fragilis* is beneficial to the host and contributes to the modulation of the host immune response through the polysaccharide A (PSA) (Mazmanian *et al.*, 2008), delivered to the host cells via outer membrane vesicles (OMV) (Shen *et al.*, 2012). Recently, OMV from *B.fragilis* and other *Bacteroides* members were found to participate in the establishment of a cooperative ecosystem in the gut. In the model proposed by Rakoff-Nahoum *et al*, some species secrete OMV, which are able to breakdown

polysaccharides for the benefit of the other species present in the community (Rakoff-Nahoum *et al.*, 2014).

OMV are small blebs originating from the outer membrane (OM) of Gram-negative bacteria. OMV contain lipopolysaccharide (LPS), outer membrane proteins, phospholipids, and periplasmic components (Ellis & Kuehn, 2010). The presence of toxins in OMV from different bacteria has led to suggestions that vesicles are long distance delivery tools (Bomberger *et al.*, 2009, Chitcholtan *et al.*, 2008, Ellis & Kuehn, 2010, Jin *et al.*, 2011, Kadurugamuwa & Beveridge, 1997, Kato *et al.*, 2002, Kolling & Matthews, 1999). Interestingly, ClyA toxin of enterohemorrhagic *E. coli* was found to be active only in vesicles, where the proper redox potential supports its oligomerization (Wai *et al.*, 2003). Additional roles in stress response and quorum sensing have been attributed to OMV (Mashburn & Whiteley, 2005, McBroom & Kuehn, 2007). Despite the different roles suggested for vesicles, the absence of a clear model for OMV biogenesis has led to disagreement on whether vesiculation is a directed process or just the result of passive membrane disintegration. If vesicles are formed by the latter mechanism, OM and OMV should have the same protein composition. However, previous reports have shown the enrichment of toxins in vesicles from different pathogens supporting the notion of cargo selection in vesicles (Ellis & Kuehn, 2010, Haurat *et al.*, 2011, McMahon *et al.*, 2012, Wai *et al.*, 2003). A proteomic analysis of OM and OMV in the oral pathogen *Porphyromonas gingivalis* showed that some OM proteins were enriched in OMV whereas other OM proteins were excluded from vesicles, and that mutations in the LPS resulted in aberrant cargo selection, providing evidence of the presence of selection machinery that is capable of packing specific cargo into OMV (Haurat *et al.*, 2011).

In this work, we carried out a proteomic analysis of both the OM and OMV of *B. fragilis*. We found multiple OM proteins that were absent in OMV and an exceptionally high number of proteins exclusively present in vesicles. Many of the OMV proteins were acidic and annotated as hydrolases, mainly proteases and glycosidases, some of which were shown to be active *in vitro*. Similar results were obtained when the content of the OMV from *B. thetaiotaomicron* was analyzed. These results strongly advocate for the existence of yet uncharacterized machinery dedicated to OMV production in *Bacteroides*.

2.2-Experimental Procedures

2.2.1-Bacterial Strains and Growth Conditions

Bacteroides fragilis NCTC 9343 and *Bacteroides thetaiotaomicron* VPI-5482 were grown either on blood agar plates containing 5% defibrinated horse blood or brain-heart infusion broth supplemented with hemin (5 µg/ml) and meniadone (1µg/ml) in an anaerobic atmosphere of 90% N_2, 5% H_2, and 5% CO_2. When needed, *B.fragilis* was grown on basal medium supplemented with 0.5% of either glucose or fucose (Sigma Aldrich). For screening assays, *B. thetaiotaomicron* was grown on basal medium supplemented with 0.5% of either fructose or N-acetyl glucosamine (Sigma Aldrich). For conjugation experiments, *E. coli* S17-1 λ pir was grown on LB, while *B. thetaiotaomicron* was grown on TYG medium (Goodman *et al.*, 2009).

2.2.2-OMV Purification

250 ml of 24-h cultures of *B. fragilis* were centrifuged at 10,000 rpm at 4 °C. In order to remove residual cells, the supernatant was filtered using a 0.45 µm followed by 0.2 µm pore-size polyvinylidene difluoride (PVDF) membrane (Millex GV; Millipore). The filtrate was subjected

to ultracentrifugation at 100,000 x g for 2 h (Optima L-90K ultracentrifuge; Beckman Coulter). The supernatant was discarded, the pellet was washed with sterile PBS, and the ultracentrifugation step was repeated. The vesicle pellet was resuspended in distilled water or 50 mM Tris-HCl buffer (pH 6.5) and quantified using 2D-quant kit (GE Healthcare Life Sciences).

2.2.3-Membranes Purification

Cells of overnight cultures were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. The pellets were gently resuspended in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 50 mM $MgCl_2$ containing complete EDTA-free protease inhibitor mixture (Roche Applied Science) followed by sonication. Total membranes were collected by ultracentrifugation at 100,000 x g for 1hr at 4°C. The OM was isolated by differential extraction with the same buffer and 1.5% (v/v) Triton X-100 and incubated at 4 °C overnight. The OM fractions were recovered by centrifugation at 100,000 x g for 1 h at 4°C.

2.2.4-Transmission Electron Microscopy

3 µl of the OMV preparations were adsorbed onto the formvar support film of carbon-coated copper grids (Ted Pella Inc.) for 3 min. Liquid excess was discarded, and the samples were negatively stained with 2% (w/v) uranyl acetate for 3 min and evaluated in a Morgagni (FEI) transmission electron microscope. OMV sizes were determined using the images obtained.

2.2.5-Mass spectrometry analysis of the OM and OMV

Purified OM and OMV were run on 10% SDS-PAGE and stained with Coomassie Brilliant Blue (Haurat *et al.*, 2011). Protein bands were excised from gels. The excised protein bands were in-gel digested using sequencing grade modified trypsin (Promega).Peptide

fragments were eluted from the gel piece, desalted using ZipTipC18 columns (Millipore) according to the supplier protocol, and dissolved in 0.1% formic acid. For in-solution digestion of membranes, lipids were removed from lyophilized pellets using trifluoroethanol. In-solution trypsin digestion was then carried out as before. A hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer, Q-TOF Premier (Waters), equipped with a nanoACQUITY Ultra performance liquid chromatography system (Waters) was used for MS/MS analyses of the peptides, and the resulting mass spectra were used for the identification of the proteins by the Mascot search engine using the NCBI nr data base. In all search attempts, the significance threshold was set to a P value of <0.05 resulting in an identity ion score threshold between 60 and 62. Only results with ion scores greater than or equal to the identity threshold were considered significant.

2.2.6-OMV Zymography

OMV were solubilized in urea buffer and the total protein content was quantified using 2D-quant kit (GE Healthcare Life Sciences). Zymogram of OMV (10 μg) were performed using Novex® Zymogram Gel (10% Tris-Glycine gel with 0.1% gelatine as the substrate) according to the manufacturer protocol (Life Technologies™). Following separation at denaturing conditions, proteins are renatured to allow substrate cleavage. Colloidal Coomassie Brilliant Blue G-250 staining of gel was used to visualize areas where the substrate (gelatin) was digested by proteases.

2.2.7-Detection of OMV peptidase activity

The peptidase enzyme activity was determined by incubating 10 µg of the quantified OMV proteins in a 50 mM Tris-HCl buffer, pH 6.5, with 200 µl of 50 mM L-lysine-p-nitroanilide dihydrobromide, L-alanine-p-nitroanilide hydrochloride, L-leucine-p-nitroanilide or pyroglutamic acid-p-nitroanilide (Sigma Aldrich), for 1 hour at 37°C. Reactions were stopped by the addition of 100 µl 1M Na_2CO_3. The release of the p-nitroanilide group was then detected spectrophotometrically by measuring the absorbance at 405 nm. All experiments were done in triplicates.

2.2.8-Detection of OMV and OM glycoside hydrolase activity

Bacteroides vesicles were tested for the presence of β-galactosidases using 50 µl 50mM 4-nitrophenyl β-D-galactopyranoside as a substrate. Purified OMV and OM were normalized according to protein content, and 10 µg were incubated with the substrate separately for 1 hour at 37°C. 50 µl 1M Na_2CO_3 were added to stop the reaction. α-L-Fucosidase activity was measured by incubating 50 µl of 50mM 2-Chloro-4-nitrophenyl-α-L-Fucopyranoside with 10 µg of different membrane fractions for 2.5 hrs at 37°C. In both assays, absorbances of the hydrolyzed products were measured spectrophotometrically at 405nm. Standard curves were generated using the same substrates and commercial enzymes (*Escherichia coli* β-galactosidase from Sigma Aldrich and *Thermotoga maritima* α-L-Fucosidase from Megazyme). Sugar hydrolyzing activities of OMV and OM were calculated using the corresponding A_{405} readings and standard curves. All experiments were done in triplicates. To measure chitinolytic activity of OM and OMV fractions, 20 µg of purified membrane proteins were incubated for 1 hr at 37°C with 1 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (MeUNAG) dissolved in 0.1 M phosphate buffer (pH 7.8). At the end of incubation, 50 µl of 10% sodium bicarbonate were

added to enhance fluorescence. Chitinolytic activity was determined fluorometrically by measuring the emission of the released fluorophore at 460 nm following its excitation at 360 nm.

2.2.9-Cloning of BF1581 and BF0018

BF1581 and BF0018 of *B.fragilis* NCTC 9343 were PCR amplified using the primers listed below (table 2.1.). BF1581 PCR product was digested with BamHI and SacI then inserted into pFD340 using the same cut sites to yield pBF1581. BF0018 PCR product was digested with BamHI and HindIII and cloned into pEXT20 then subcloned into BamHI and SmaI sites of pFD340 to yield pBF0018. pFD340 was a generous gift from Comstock L.E.

Table 2.1. Primers used in cloning BF1581 and BF0018. Restriction sites are in bold. Histidine tags and stop codons are underlined

Primer	**Orientation**	**Sequence**
BF1581BamHI	Forward	CCCC**GGATCC**ATGAAAAAAATAAATGCTTTAATTACT
BF1581SacI	Reverse	CCCC**GAGCTC**TTAGTGGTGGTGGTGGTGGTGGTGG TGGTGGTGTTTAAAAACTATGCTTGAAGG
Bf0018BamHI	Forward	CCCC**GGATCC**ATGAAGCAAATTTATAGTACCC
Bf0018HindIII	Reverse	CTTC**AAGCTT**TTAGTGGTGGTGGTGGTGGTGGTGG TGGTGGTGAAAATGGTAAGAGATTACTGCTG

2.2.9-Protein localization assays in OM and OMV

pLEC280 expressing His-tagged BACOVA_04502 from *B.ovatus* was a generous gift from Comstock LE (Rakoff-Nahoum *et al.*, 2014). The recombinant vectors, pBF1581 and pBF0018, were transferred to *B.fragilis* via *E.coli* harboring pRK231. In brief, the donor and the recipient strains were grown overnight under proper antibiotics selection. In the following day, the donor *E. coli* was subcultured in 10 ml LB without antibiotics, while the recipient *B.fragilis* was subcultured in 100 ml of antibiotic-free basal medium. Both strains were allowed to grow to mid-log phase, and then cell pellets were harvested and resuspended in 100 µl of antibiotic-free basal medium. The mixed pellets suspension was spotted on BHI agar without antibiotics and the plate was incubated overnight aerobically at 37ºC. To select for conjugants, the lawns formed were streaked on BHI agar plates supplemented with gentamicin (200 µg/ml) and erythromycin (10 µg/ml) and were incubated anaerobically for 2 days at 37ºC.

As described above, OM and OMV were harvested from the recombinant strains then normalized according to their total protein content using 2D-quant kit (GE Healthcare Life Sciences). Both compartments in each strain were run on SDS-PAGE followed by western blotting into nitrocellulose membranes. Localization of proteins was detected using anti-His rabbit antibody (primary antibody) followed by AlexFluor680-labelled anti-rabbit goat antibody (secondary antibody). Images were taken using LI-COR Odyssey Imaging system.

2.2.10-MALDI-MS analysis of Lipid A

Lipid A from vesicles and cells was prepared in duplicates using 10 mg of sample for each preparation according to the procedure of Yi and Hackett (Yi & Hackett, 2000). The

purified lipid A was resuspended in 6 µl of methanol:dichloromethane (1:1). 1µl of the mixture was loaded on the MALDI plate followed by addition of 0.5ul of 2,4,6-trihydroxyacetophenone monohydrate (THAP) as the matrix. MALDI MS was then performed on a Bruker Daltonics (Bremen, Germany) UltrafleXtreme MALDI TOF/TOF mass spectrometer in linear negative mode.

2.2.11-Generation of transposon-mediated library in *B. thetaiotaomicron* VPI-5482

A mariner transposition system, which provides erythromycin resistance to *B. thetaiotaomicron*, was used to generate a library of 10,000 mutants. The transposon is carried on the suicide vector, pSAM, which is propagated in *E. coli* S17-1 λ pir. Conjugation was used to transfer pSAM from the donor *E. coli* strain to *B. thetaiotaomicron* VPI-5482 as described before (Goodman et al., 2009). In brief, donor and recipient strains were grown for 16 hrs under normal growth conditions. 100 µl of the donor strain were mixed with 1 ml of the recipient strain, followed by washing in TYG medium with no antibiotics. The final pellet was resuspended in 400 µl of TYG without antibiotics, and then spotted on an antibiotic-free BHI agar. The agar plate was incubated aerobically at 37ºC for 12 hrs with the agar side down. The wet surface of the agar promoted the growth of a thick *E. coli* lawn. As the *E. coli* grew, it made the underlying regions of the lawn more anaerobic, allowing the *B. thetaiotaomicron* to grow and serve as a conjugation recipient. After the incubation period, the lawn was resuspended in 4 ml TYG medium without antibiotics, followed by plating 100 µl of the suspension on BHI plates containing gentamicin (200 µg/ml) and erythromycin (25µg/ml). The plates were incubated anaerobically for 2 days till the appearance of colonies. *B. thetaiotaomicron* mutant colonies were picked manually and sorted individually, under sterile conditions, into 96 well plates

containing basal medium with the proper antibiotics. In each plate, one well was inoculated with the reference strain (mutant Tn397, which has an transposon insertion in a non-coding region), while another well was left without inoculation to serve as a blank for the fluorometric assay. The library was preserved by the addition of 20% sterile glycerol and stored at -80°C till the time of screening.

2.2.12-Screening the *B. thetaiotaomicron* transposon library for altered chitinolytic levels in the cell-free supernatants

The *B. thetaiotaomicron* transposon library was grown overnight anaerobically in round-bottom 96-well plates with fructose-containing basal medium. Next day, the library was subcultured into basal medium containing 0.5% N-acetyl glucosamine and incubated for 16 hrs under standard growth conditions. At the time of the assay, the OD_{600} values of the cultures were determined spectrophotometrically at 600 nm. The plates were centrifuged at 4000 rpm for 10 mins to pellet down the cells, followed by the transfer of the supernatants to 0.22 µm hydrophilic low protein-binding membrane MultiScreen HTS filter plates (Millipore). 150 µl of the filtered supernatants were transferred to new sterile 96-well plates. The fluorogenic substrate, 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (MeUNAG), was dissolved in dimethylformamide (DMF) at a final concentration of 25mM, and then diluted 1:25 in 0.1 M phosphate buffer (pH 7.8). 50 µl of the latter solution were added to the filtered *B. thetaiotaomicron* supernatants and the plates were incubated for 1 hr at 37°C. After 1 hr, 50 µl of 10% sodium bicarbonate aqueous solution were added to stabilize the released fluorophore. Chitinolytic activity was determined fluorometrically by measuring the emission of the released fluorophore at 460 nm following its excitation at 360 nm. The blank reading in each plate was subtracted from the remaining readings. Relative fluorescence values were normalized by the

OD_{600} values of the corresponding cultures. Finally, the obtained values were divided by that of the reference strain per plate to calculate fold production. Mutants displaying 3-fold change in activity compared to the reference strain were considered for further analysis.

2.2.13-Secretome analysis of the *B. thetaiotaomicron* mutants isolated from the chitinase-based screening assay

Reference strain and the mutants of interest were grown anaerobically overnight under standard growth conditions. Following incubation, cultures were centrifuged at 5000 rpm for 10 min. 500 µl of the cell-free supernatants were concentrated to 20 µl by centrifugation at 10,000 X g for 20 min using Amicon Ultra-0.5 mL centrifugal Filters for DNA and protein purification and concentration (Millipore). The concentrates were washed twice with sterile water, and then eluted at 1000 X g for 1 min. The eluted concentrates were boiled in 1X Laemmli buffer for 5 min then loaded on 10% SDS PAGE, followed by Coomassie staining to visualize the protein bands.

2.2.14-Semi-random PCR for the amplification of transposon-genome junctions

To sequence the *B. thetaiotaomicron* genomic regions surrounding the transposon insertion, semi-random PCR was employed to amplify the transposon-genomic junctions according to the protocol of Goodman et al (Goodman *et al.*, 2009, Goodman *et al.*, 2011). In brief, mutants of interest were grown anaerobically in TYG medium overnight in the presence of the proper antibiotics. Genomes were extracted from the cultures using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. First round of PCR was performed using the mutant genome as a template, and the transposon-specific primer SAMseq1 "ACGTACTCATGGTTCATCCCGATA", together with the random primer AR1A

"GGCCACGCGTCGACTAGTACNNNNNNNNNNGTAAT", where N represent any nucleotide. The product of the first PCR round was purified using the PCR-purification kit (Qiagen) and eluted with 20 µl TE buffer. The purified PCR product was used as a template for a nested PCR using primers; SAMseq2 "GCGTATCGGTCTGTATATCAGCAA" and AR2 "GGCCACGCGTCGACTAGTAC". PCR product was purified with the PCR-purification kit and sent to be sequenced (Molecular Biology Service Unit, department of biological sciences, University of Alberta). Sequencing data was analyzed to identify the transposon insertion genomic site.

2.3-Results

2.3.1-*B. fragilis* produces spherical, uniformly sized OMV

Previous reports showed that, similarly to other Gram negative bacteria, *B. fragilis* produces OMV (Patrick *et al.*, 1996). Accordingly, we wanted to ensure that our method of vesicles purification resulted in a cell-free OMV preparation. *B. fragilis* vesicles were harvested from cell-free supernatant of overnight culture. The OMV were resuspended in sterile water then examined using transmission electron microscopy (TEM). As shown in figure 2.1, *B. fragilis* vesicles were spherical and uniform in size with their diameter ranging from 30 to 80 nm. This range is narrower than what was reported previously for some other gram negative bacteria (Nguyen *et al.*, 2003). No *B. fragilis* cells were observed in our OMV preparations.

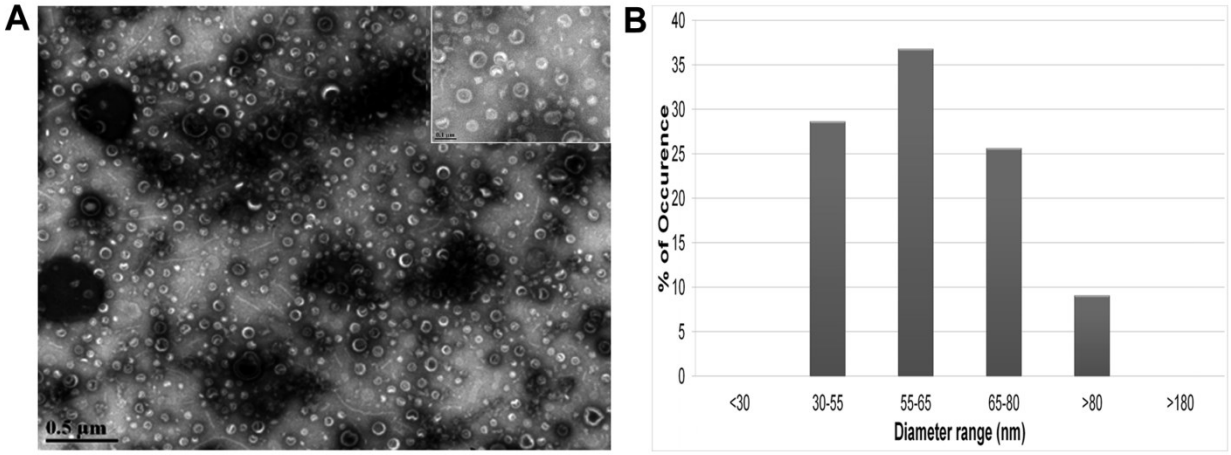

Figure 2.1. *B. fragilis* produces OMV.(A)Transmission electron microscopy of *B. fragilis* OMV at two different magnifications; X 28,000 and X 140,000 (inset). Scale bars represent 500 nm and 100 nm, respectively. (B) Quantification of the size range of *B.fragilis* OMV as observed under the electron microscope.

2.3.2-OMV show different protein profile from OM in *B. fragilis*

Recent work has shown that OMV from *P. gingivalis*, *Serratia marcescens* and *Neisseria meningitidis* carry, to a variable extent, different proteins compared to the OM from which they originated (Haurat *et al.*, 2011, Lappann *et al.*, 2013, McMahon *et al.*, 2012). We tested whether OMV cargo selection occurs in *B. fragilis*, which is an important member of the gut microbiota and genetically close to *P. gingivalis* (Fletcher *et al.*, 2009, Nelson *et al.*, 2003). To study protein sorting in *B. fragilis* OMV, both OM and OMV proteins were separated by SDS-PAGE followed by Coomassie staining. *B. fragilis* OMV exhibited a very different protein profile compared to the OM of the same cells (figure 2.2). Bands were excised from gel, tryptically digested, and analyzed by mass spectrometry for protein identification. A total of 115 proteins were detected in

OMV while 102 proteins were found in the OM of *B. fragilis*. Only predicted outer membrane and periplasmic proteins were found in OMV with no inner membrane or cytosolic proteins detected.

69 proteins were common between OMV and OM (Table 2.4, figure 2.3.A). Among the common proteins were several proteins annotated as "SusD-like". SusD proteins belong to the "starch utilization system" (Sus) family, composed by a very large number of glycan binding proteins. Sus proteins can bind to starch and its derivatives like maltooligosaccharides, amylose, amylopectin, and pullulan (Cho & Salyers, 2001, Koropatkin & Smith, 2010, Shipman *et al.*, 2000). In *Bacteroides thetaiotaomicron*, an important human gut symbiont that is genetically related to *B.fragilis*, the Sus proteins account for about 18 % of the genome (Koropatkin & Smith, 2010). Some of the Sus components were predicted to be surface-exposed lipoproteins (Martens *et al.*, 2008).

46 proteins were found exclusively in OMV (Tables 2.2 and 2.3, figure 2.3.A). Although enrichment of a few proteins in OMV has been previously described, the dramatic difference in the compositions of OM and OMV observed in *B. fragilis* has not been previously reported. For those proteins which are not annotated in GenBank, we relied on BLASTP (Altschul *et al.*, 1997), PFAM (Bateman *et al.*, 2004) and HHPRED algorithms (Soding *et al.*, 2005) for homology-based annotation of the identified proteins. Interestingly, we found about one quarter of OMV proteins to be homologous to hydrolases (figure 2.3.C). These included 11 glycosidases, 11 peptidases and one lipase. Most of these glycosidases (8/11) and peptidases (7/11) were not detected in OM (Table 2.2). Such abundance of hydrolytic enzymes was only previously reported in OMV of *Myxococcus xanthus* (Kahnt *et al.*, 2010, Whitworth, 2011). On the other hand, MS analysis of OM proteins revealed 33 unique proteins that were not detected in vesicles

(Table 2.4). Most proteins in the OM fraction are involved in ligand binding and transport, albeit some putative hydrolases were also detected but at lower abundance compared to OMV. The majority of the OM proteins were TonB-dependent receptors (TBDTs). TBDTs are bacterial outer membrane proteins that bind and transport different substrates like siderophores, carbohydrates and vitamin B12 using energy provided by the inner membrane proton motive force (Noinaj *et al.*, 2010). Other proteins involved in heme binding and transport like HmuY were also found in the OM (Table 2.4, figure 2.3.C).

To purify the OM and separate it from the inner membrane, we used a Triton X-100-based method (Haurat *et al.*, 2011). To exclude the possibility that the differences in the protein contents were due to the treatment with this detergent, we repeated our analysis using total membrane preparations, which includes inner and outer membrane proteins. After removing the lipids, the total membrane proteins were digested with trypsin. The resulting peptides were analyzed by LC-MS/MS to identify the proteins. None of the 46 proteins unique to OMV were found in the total membrane, indicating that the presence of proteins only in OMV is not an artifact of the preparation (Data not shown). Taken together, these results indicate that mechanisms for sorting and exclusion of proteins into OMV must exist in *B. fragilis*.

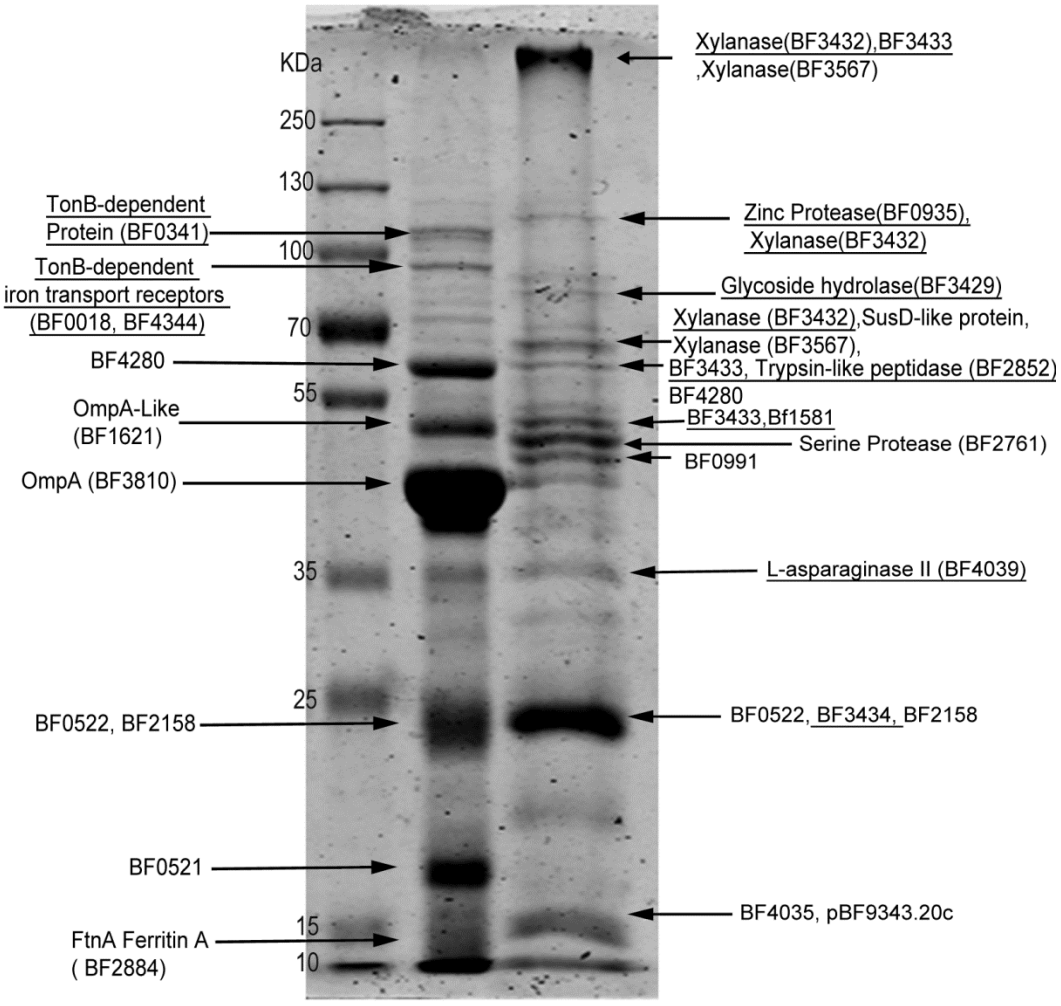

Figure 2.2. OMV show different protein profile from that of OM. 10 µg of purified OM (left lane) and OMV (right lane) proteins were run on 10% SDS-PAGE followed by Coomassie staining. Protein bands were excised from the gel and digested with trypsin. The resulting peptides were enriched using ZipTip C18 columns then analyzed via liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) followed by protein identification with Mascot search engine using the NCBInr data base. The arrows point to the

most abundant proteins in each band. Underlined proteins were detected only in the corresponding compartment.

Table 2.2. Putative Hydrolases identified by MS/MS exclusively in OMV of *B. fragilis*

Number	Function	Transmembrane domain or Lipo signal	pI
BF3432	Xylanase	Yes	Acidic
BF3429	Glycosyl Hydrolase	No	Acidic
BF3562	Xylanase	Yes	Acidic
BF0339	Beta-Glucosidase	Yes	Acidic
BF3793	Beta-Galactosidase	Yes	Acidic
BF0810	Alpha-L-Fucosidase	Yes	Alkaline
BF4060	Beta-Glucanase	Yes	Acidic
BF3083	Alpha-L-Fucosidase	No	Alkaline
BF0935	Zinc Protease	Yes	Acidic
BF2852	Trypsin-Like Peptidase	No	Acidic
BF2757	Clostripain-Related Peptidase	No	Acidic
BF2157	Peptidase	Yes	Acidic
BF3010	Trypsin-Like Peptidase	Yes	Acidic
BF1408	Dipeptidase	Yes	Acidic
BF4039	L-asparaginase II	Yes	Alkaline

Table 2.3. Proteins identified by MS/MS exclusively in OMV of *B. fragilis*

Number	Function	Transmembrane domain or Lipo signal	pI
BF3804	Fimbrillin-A Associated Anchor Lipoprotein	Yes	Acidic
BF1578	Fimbrillin-A Associated Anchor Protein	No	Acidic
BF4084	Fimbrillin-A Associated Anchor Protein	No	Acidic
BF4165	Phospholipase	Yes	Alkaline

BF2707	Heme-Binding Lipoprotein HmuY	Yes	Acidic
BF3139	Putative Exonuclease/Phosphatase	No	Acidic
BF3433	Hypothetical Protein	Yes	Acidic
BF3434	Hypothetical Protein	Yes	Acidic
BF2257	Hypothetical Protein	No	Acidic
BF1581	Hypothetical Protein	Yes	Acidic
BF1215	Hypothetical Protein	Yes	Acidic
BF2163	Hypothetical Protein	Yes	Acidic
BF3067	Hypothetical Protein	Yes	Acidic
BF1363	Hypothetical Protein	Yes	Acidic
BF1629	Hypothetical Protein	Yes	Acidic
BF3428	Hypothetical Protein	Yes	Acidic
BF3256	Hypothetical Protein	No	Acidic
BF3942	Hypothetical Protein	No	Acidic
BF2301	Hypothetical Protein	Yes	Acidic
BF0540	Hypothetical Protein	Yes	Acidic
BF1881	Hypothetical Protein	Yes	Acidic
BF4343	Hypothetical Protein	Yes	Acidic
BF3669	Hypothetical Protein	Yes	Acidic
BF1641	Hypothetical Protein	No	Acidic
BF2571	Hypothetical Protein	Yes	Acidic
BF3250	Hypothetical Protein	Yes	Acidic
BF1383	Hypothetical Protein	Yes	Alkaline
BF2100	Hypothetical Protein	Yes	Alkaline
BF2968	Hypothetical Protein	Yes	Alkaline
BF1214	Hypothetical Protein	Yes	Alkaline
BF4352	Hypothetical Protein	Yes	Alkaline

Table 2.4. Proteins identified by MS/MS in OM of *B. fragilis*

Number	Function	Transmembrane domain or Lipo signal	pI	Found in OMV
BF1618	RagA-Like TonB-linked Outer Membrane Protein	Yes	Alkaline	No
BF0341	RagA-Like TonB-linked Outer Membrane Protein	Yes	Alkaline	No
BF2195	RagA-Like TonB-linked Outer Membrane Protein	Yes	Alkaline	No
BF3444	RagA-Like TonB-linked Outer Membrane Protein	No	Alkaline	No

BF3146	RagA-Like TonB-linked Outer Membrane Protein	Yes	Acidic	No
BF0018	TonB-Dependent Outer Membrane Receptor	Yes	Alkaline	No
BF4344	TonB-Dependent Outer Membrane Receptor	Yes	Alkaline	No
BF1029	TonB-Dependent Outer Membrane Receptor	No	Alkaline	No
BF2541	TonB-Dependent Outer Membrane Receptor	No	Alkaline	No
BF2697	TonB-Dependent Outer Membrane Receptor	No	Acidic	No
BF1583	TonB-Dependent Outer Membrane Receptor	No	Alkaline	No
BF0551	TonB-Dependent Outer Membrane Receptor	No	Alkaline	No
BF3178	TonB-Dependent Outer Membrane Receptor	Yes	Alkaline	No
BF3068	TonB-Dependent Outer Membrane Receptor	Yes	Alkaline	No
BF0154	Outer Membrane Efflux Lipoprotein	Yes	Alkaline	No
BF1959	OmpA-Family peptidoglycan-associated lipoprotein	Yes	Alkaline	No
BF0586	Lipid-Associated Lipoprotein	Yes	Alkaline	No
BF0156	Biotin-Lipoyl Outer Membrane Efflux Transport Protein	Yes	Alkaline	No
BF2529	HlyD family secretion lipoprotein	Yes	Alkaline	No
BF2476	Biotin-Lipoyl Outer Membrane Efflux Transport Protein	No	Alkaline	No
BF3077	OmpH-Like Outer Membrane Lipoprotein	Yes	Alkaline	No
BF0899	Dipeptidyl Peptidase	Yes	Alkaline	No
BF1378	Disulfide Reductase PdiA	Yes	Neutral	No
BF3366	Outer Membrane Efflux Protein	Yes	Alkaline	No
pBF9343.38c	Plasmid Transfer Protein	No	Alkaline	No
BF3759	Outer Membrane Efflux Protein	No	Alkaline	No
pBF9343.35c	Outer Membrane Peptidoglycan-Associated Lipoprotein	Yes	Neutral	No
BF2694	Thioredoxin	Yes	Alkaline	No
BF1285	Hypothetical Protein	Yes	Alkaline	No
BF1390	Hypothetical Protein	Yes	Alkaline	No
BF1622	Hypothetical Protein	Yes	Alkaline	No
BF2622	Hypothetical Protein	Yes	Alkaline	No
BF3319	Hypothetical Protein	Yes	Alkaline	No

BF3810	OmpA	No	Alkaline	Yes
BF1028	Putative Heme-Binding Transport Lipoprotein HmuY	Yes	Acidic	Yes
BF2761	Heat Shock-Related Serine Protease	Yes	Acidic	Yes
BF2494	Putative plasmid transporting protein	No	Acidic	Yes
BF3042	NigD-like lipoprotein	Yes	Acidic	Yes
BF2776	Lipocalin-like lipoprotein	Yes	Alkaline	Yes
BF3567	Sugar Hydrolase (Xylanase)	Yes	Acidic	Yes
BF3888	Peptidyl-Prolyl Cis-Trans Isomerase	Yes	Acidic	Yes
BF2475	Outer Membrane Efflux Protein	Yes	Alkaline	Yes
BF2706	Lipocalin-Like Protein	Yes	Acidic	Yes
BF1979	Serine peptidase	Yes	Acidic	Yes
BF1285	OmpA-like lipoprotein	No	Alkaline	Yes
BF2739	Thioderoxin	Yes	Acidic	Yes
BF4040	Outer Membrane Phosphate-Selective Porin	No	Alkaline	Yes
BF0448	OmpH-Like Cationic Protein	Yes	Acidic	Yes
BF3152	Outer Membrane Efflux Protein	No	Acidic	Yes
BF1670	Sporulation related lipoprotein	Yes	Alkaline	Yes
BF2639	DNA-Uptake Lipoprotein ComL	No	Alkaline	Yes
BF2528	Outer Membrane Efflux Protein	No	Acidic	Yes
BF4216	TonB-Dependent Outer Membrane Receptor Protein	Yes	Acidic	Yes
BF0811	Peptide-N-glycosidase	Yes	Acidic	Yes
BF3236	Sugar Hydrolase (Mannosidase)	Yes	Acidic	Yes
BF2884	Ferritin A	No	Acidic	Yes
BF4308	Peptidyl-Prolyl Cis-Trans Isomerase	No	Acidic	Yes
BF3964	Outer Membrane Efflux	No	Acidic	Yes
BF4309	Peptidyl-Prolyl Cis-Trans Isomerase	Yes	Alkaline	Yes
BF3342	Beta-Lactamase	Yes	Acidic	Yes
BF2064	Aminopeptidase	Yes	Neutral	Yes
BF3528	ExbD Biopolymer Transport Protein	Yes	Acidic	Yes
BF0657	Aminopeptidase	Yes	Neutral	Yes
BF1184	Putative META-domain containing lipoprotein	Yes	Alkaline	Yes
BF1621	OmpA-Like Outer Membrane Protein	Yes	Alkaline	Yes
BF1987	Collagen-like protein	Yes	Acidic	Yes
BF1957	SusD-like protein	Yes	Acidic	Yes
BF0595	SusD-like protein	Yes	Acidic	Yes
BF0340	SusD-like protein	Yes	Acidic	Yes
BF2196	SusD-like protein	Yes	Acidic	Yes
BF0589	SusD-like protein	Yes	Acidic	Yes
BF3145	SusD-like protein	Yes	Acidic	Yes
BF1619	SusD-like protein	Yes	Acidic	Yes

BF1802	SusD-like protein	Yes	Acidic	Yes
BF1687	Hypothetical protein	Yes	Acidic	Yes
BF0991	Hypothetical protein	No	Acidic	Yes
BF4280	Hypothetical protein	Yes	Alkaline	Yes
BF4035	Hypothetical protein	Yes	Alkaline	Yes
BF2158	Hypothetical protein	Yes	Alkaline	Yes
BF1004	Hypothetical protein	No	Acidic	Yes
pBF9343.20c	Hypothetical protein	No	Acidic	Yes
BF0922	Hypothetical protein	Yes	Acidic	Yes
BF0521	Hypothetical protein	Yes	Alkaline	Yes
BF2161	Hypothetical protein	Yes	Acidic	Yes
BF2566	Hypothetical protein	Yes	Acidic	Yes
BF0883	Hypothetical protein	Yes	Acidic	Yes
BF4230	Hypothetical protein	Yes	Acidic	Yes
BF2334	Hypothetical protein	No	Alkaline	Yes
BF1345	Hypothetical protein	Yes	Alkaline	Yes
BF3563	Hypothetical protein	No	Alkaline	Yes
BF2026	Hypothetical protein	No	Acidic	Yes
BF0522	Hypothetical protein	Yes	Acidic	Yes
BF0876	Hypothetical protein	Yes	Acidic	Yes
BF3547	Hypothetical protein	No	Alkaline	Yes
BF2105	Hypothetical protein	Yes	Acidic	Yes
BF1542	Hypothetical protein	No	Alkaline	Yes
BF3144	Hypothetical protein	Yes	Acidic	Yes
BF0451	Hypothetical protein	Yes	Acidic	Yes
BF0941	Hypothetical protein	Yes	Acidic	Yes
BF1151	Hypothetical protein	Yes	Alkaline	Yes
BF1885	Hypothetical protein	No	Alkaline	Yes
BF3423	Hypothetical protein	Yes	Acidic	Yes

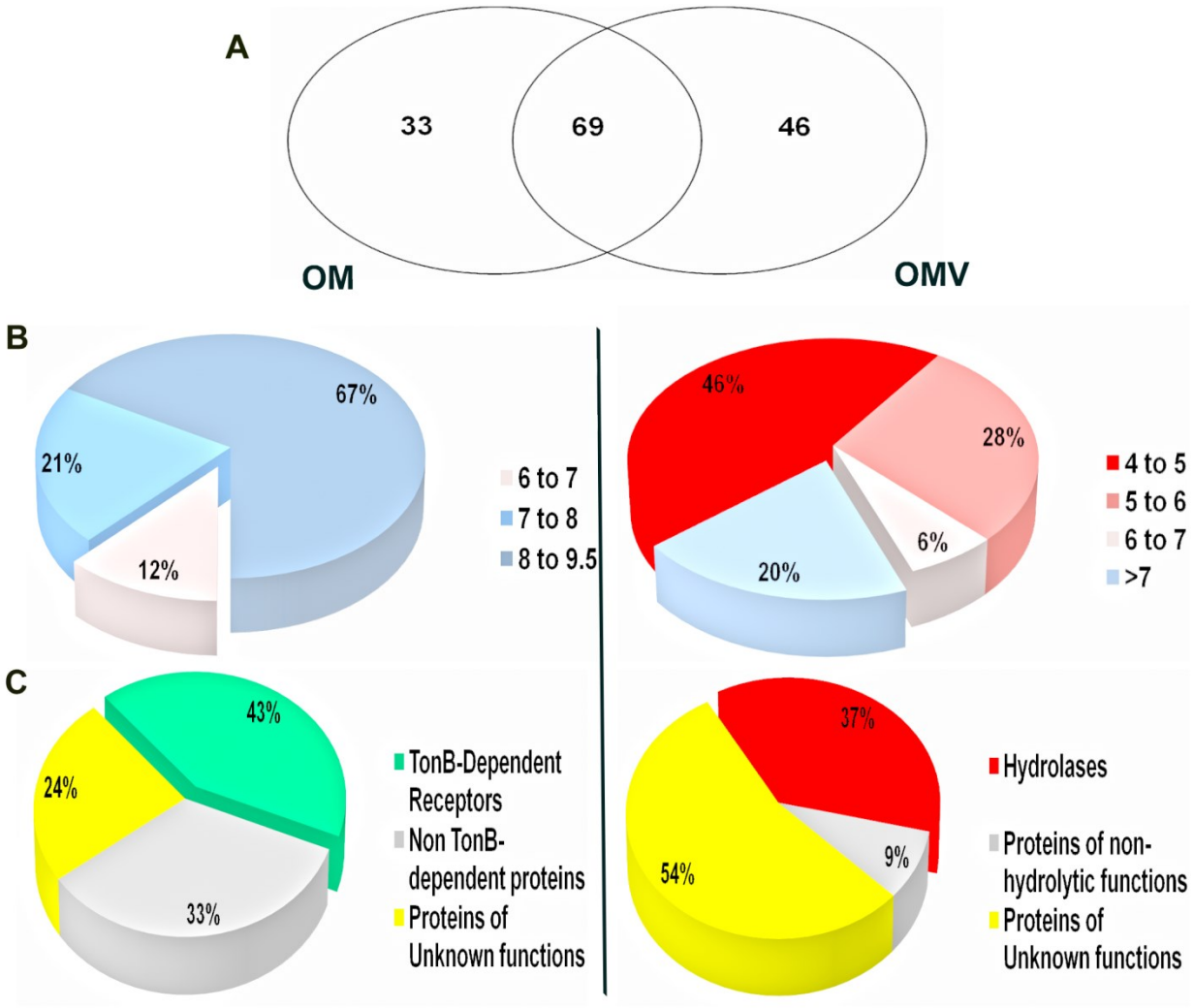

Figure 2.3. *B. fragilis* OMV and OM display different distribution of proteins based on pIs and functions (A) Schematic representation of protein compartmentalization in both OMV and OM. Proteomic analysis identified 69 proteins common to OM and OMV, and 46 proteins only detected in OMV. (B) Pie charts showing the distribution of OM and OMV exclusive proteins according to their pI. OMV displayed preferential sorting towards acidic proteins while alkaline proteins resided in the membrane. (C) Pie charts showing the functional distribution of OM and OMV exclusive proteins. OMV were enriched in hydrolases compared to OM which displayed a large percentage of TonB-dependent receptors.

2.3.3-OMV exclusive proteins are mostly acidic, OM unique proteins are mostly basic

Another interesting trend discovered in this analysis is related to isolectric point (pI) of OM and OMV proteins. According to their calculated pIs, about 80% of OMV-specific proteins are acidic (Tables 2.2 and 2.3, figure 2.3.B). In contrast with vesicular proteins, only 2 of the 33 OM unique proteins were found to have acidic isoelectric points, while the majority was found to be alkaline. As shown in Tables 2.2 and 2.3, the majority of the OMV proteins were predicted to have either a lipoprotein signal or transmembrane domains. This suggests that these proteins are genuine membrane proteins, which must be targeted to the OM before their inclusion in OMV, and not secreted proteins interacting with OMV after OMV are formed.

2.3.4-OMV show proteolytic activity

As previously mentioned, we identified eleven proteins in OMV that were annotated as proteases. Therefore, we tested if they display activity via a zymogram analysis. OMV were harvested from *B. fragilis* and run on a SDS-PAGE gel containing gelatin as a protease substrate. Following renaturation and incubation at 37ºC, the gel was stained with Coomassie to detect proteolytic activity. As shown in figure 2.4.A, several clear bands were visualized after staining the gel, indicating that gelatin was digested at these sites. This allowed us to conclude that at least some of the proteases detected in the vesicles are active. In addition, this experiment was repeated with heat-inactivated OMV and no hydrolase activity was observed (data not shown). To confirm the protease activity of vesicles, we performed *in vitro* protease assays using amino acids (Lysine, Alanine, Leucine, Glutamate) linked to *p*-nitroanilide. *p*-nitroanilide is a

chromogenic compound that will show maximum absorbance at ʎ 405 nm when the substrate is cleaved. All 4 substrates were cleaved by the OMV, showing higher activity when using *p*-nitro anilide alanine as substrate (figure 2.4.B).

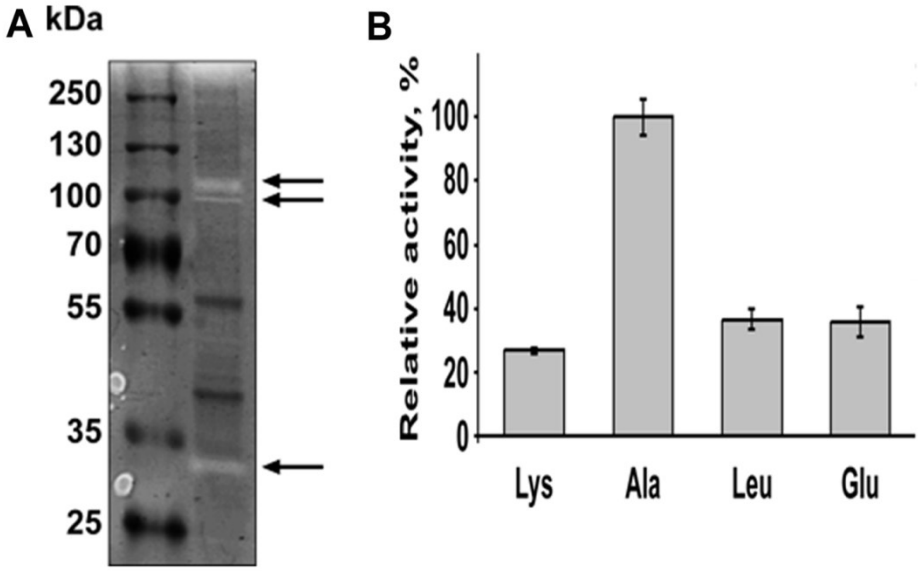

Figure 2.4. OMV display protease activity. (A) 10 µg of OMV proteins were loaded on 10% Tris-Glycine gel with 0.1% gelatin as the substrate. Following separation at denaturing conditions, proteins are renatured then incubated at 37º C for 2 days to allow substrate cleavage. Colloidal Coomassie Brilliant Blue G-250 was used to stain the gel. Clear bands marked by the arrows indicate the digestion of gelatin by proteases. (B) Peptidase activity of *B. fragilis* vesicles was tested using different p-nitroanilide-linked amino acids. 10 µg of purified OMV proteins were incubated with different substrates for 1 hour at 37°C. Activities were determined by measuring the absorbance of the released p-nitroanilide at 405 nm. All activities are represented relative to the alanine-peptidase activity. All experiments were done in triplicates.

2.3.5-Sugar hydrolase activity of OMV

The proteomic analysis of OMV revealed the presence of several proteins annotated as sugar hydrolases (Table 2.4). These included α-1,2-mannosidase (BF3236), xylanase (BF3567) and a peptide-*N*-glycosidase (BF0811). The previous proteins were detected in both the OM and OMV fractions. However, OMV possessed several unique putative sugar hydrolases that were not detected in the OM (Table 2.2). These hydrolases were two xylanases (BF3432, BF3562), two α -L-fucosidases (BF0810, BF3083), putative glycoside hydrolase (BF3429), β-glucosidase (BF0339), β -galactosidase (BF3793), and β-glucanase (BF4060). Since these proteins are annotated based on *in-silico* analysis, we decided to test the activity of some of them. We compared the β–galactosidase and the α-L-fucosidase activities in both OMV and OM. For these experiments, we employed specific sugars linked to a chromogenic group that is released upon enzymatic cleavage, allowing absorbance to be measured at ʎ 405nm. Enzymatic activities of different membrane compartments were determined by comparison against standard curves produced using commercial enzymes incubated with the same chromogenic substrates (for details see Material and Methods). Both OMV and OM displayed comparable β–galactosidase activity, which was severely reduced by heat treatment (figure 2.5.C). Interestingly, α-L-fucosidase activity was detected exclusively in OMV, with both the OM and heat inactivated OMV showing no fucosidase activity (figure 2.5.D). It has been previously established that α-L-fucosidase activity in *Bacteroides* can be induced in presence of fucosylated human milk oligosaccharides (64). We hypothesized that *B. fragilis* can sense fucose monomers, inducing secretion of fucosidases in OMV. To test this hypothesis, *B. fragilis* was grown on defined media using either glucose or fucose as carbon source. OM and OMV were collected in both conditions and α-L-fucosidase activity was determined. In presence of fucose, OMV fucosidase activity was

induced about 7-fold compared to OMV collected from *B. fragilis* grown on glucose (figure 2.5.E). This suggests the hydrolase content of the OMV is regulated to optimize the breakdown of the nutrients.

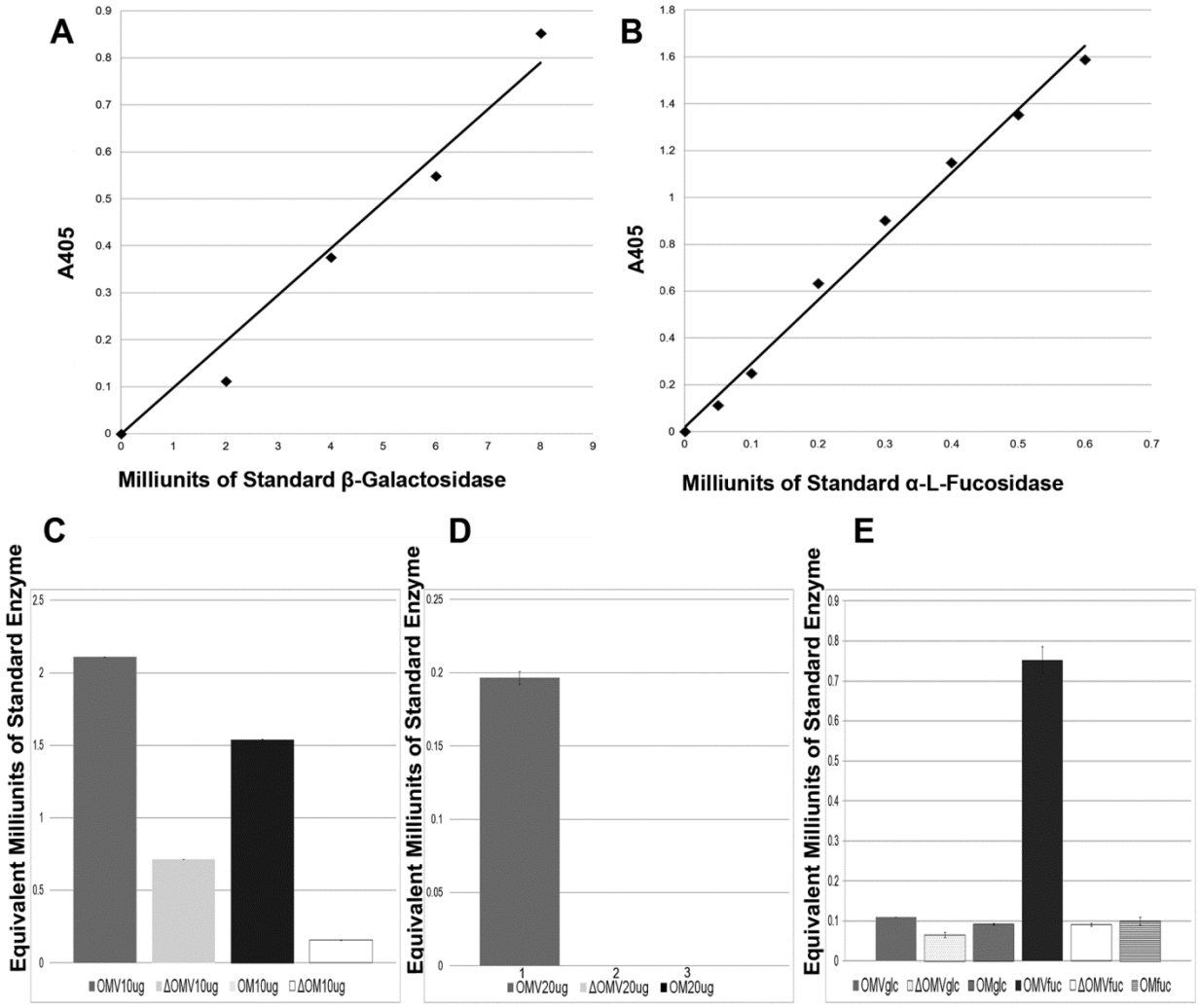

Figure 2.5. *B. fragilis* vesicles possess sugar hydrolyzing activity. OMV were tested for the presence of β-galactosidase and α-L-fucosidase activities. (A) A standard curve for β-galactosidase activity was developed using commercial β-galactosidase from *E. coli* and 4-nitrophenyl β-D-galactopyranoside as a substrate. (B) A standard curve for α-L-fucosidase

activity was developed using commercial α-L-Fucosidase from *T. maritima* and 2-Chloro-4-nitrophenyl- α-L-fucopyranoside as a substrate (Upper panel). (C) 10 µg of purified OMV and OM proteins were incubated with 4-nitrophenyl β-D-galactopyranoside for 1 hour at 37° C. Reactions halted by the addition of 50 µl 1M Na_2CO_3 followed by reading absorbance at ʎ=405. β-galactosidase activities of OMV and OM were calculated from the standard curve using A_{405} readings then plotted (Lower panel). (D) α-L-fucosidase activities of OM and OMV were measured by incubation with 2-Chloro-4-nitrophenyl- α-L-fucopyranoside for 2.5 hrs at 37°C. A_{405} readings of OM and OMV were used to calculate α-L-fucosidase activities from the standard curve. (E) Fucosidase activity of cells grown in defined media with glucose or fucose as carbon source. Heat-killed controls are marked by "Δ". All experiments were done in triplicates.

2.3.6-Acidic hydrolases are also packed in *Bacteroides thetaiotaomicron* OMV

Since *B. fragilis* OMV were enriched in acidic proteins including hydrolases, we investigated whether this phenomenon is specific to *B. fragilis* or a common feature among the genus *Bacteroides*. We extended our proteomic analysis to OMV and OM of *B. thetaiotaomicron* which counts for 12% of all Bacteriodetes in the adult colon (65). As shown in figure 2.6, *B. thetaiotaomicron* OMV protein profile was different from that of OM. The MS-based protein identification revealed the packing of 84 proteins in *B. thetaiotaomicron* OMV that were not detectable in OM. 82% of these 84 OMV proteins are acidic. Additionally, 25 of the OMV unique proteins in *B. thetaiotaomicron* were predicted to be hydrolases. With the exception of one protein, all the OMV unique hydrolases have acidic isoelectric points (Table 2.5).

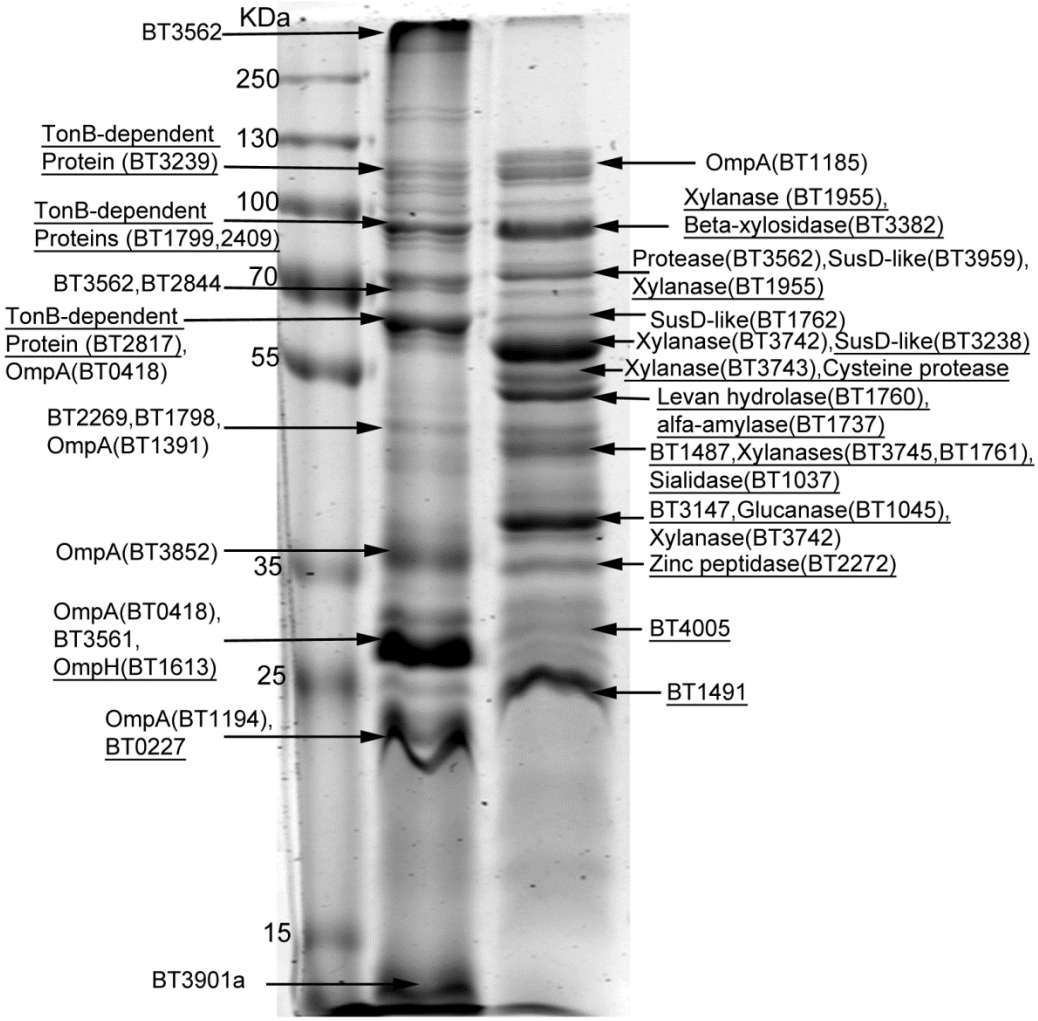

Figure 2.6. *B. thetaiotaomicron* OMV display different protein content from OM. 10 µg of purified OM (left lane) and OMV (right lane) proteins were run on 10% SDS-PAGE. Coomassie-stained bands were excised from the gel and tryptically digested. The resulting peptides were analyzed via liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) followed by protein identification with Mascot search engine using the NCBInr data base. The arrows point to the most abundant proteins in each band. Underlined proteins were detected only in the corresponding compartment.

Table 2.5. Putative hydrolases identified by MS/MS exclusively in OMV of *B. thetaiotaomicron*

Number	Function	Transmembrane domain or Liposignal	pI
BT_1955	Xylanase	Yes	Acidic
BT_3382	Beta-xylosidase	Yes	Acidic
BT_3727	Glycosyl hydrolase	Yes	Acidic
BT_0278	Glucanase	Yes	Acidic
BT_3859	Hydrolase	Yes	Acidic
BT_3742	Xylanase	Yes	Acidic
BT_3743	Xylanase	Yes	Acidic
BT_3960	Cysteine proteinases	Yes	Acidic
BT_1760	Levan hydrolase	Yes	Acidic
BT_1737	Alfa-amylase	Yes	Alkaline
BT_3987	Endo-β-N-acetylglucosaminidase	Yes	Acidic
BT_3523	Glucanases	Yes	Acidic
BT_3381	Beta-xylosidase	Yes	Acidic
BT_1037	Alpha-sialidase	Yes	Acidic
BT_3745	Xylanase	Yes	Acidic
BT_1761	Xylanase	Yes	Acidic
BT_3985	Chitinase	Yes	Acidic
BT_1038	Chitinase	Yes	Acidic
BT_1045	Glucanases	Yes	Acidic
BT_3861	Glucanases	Yes	Acidic
BT_2239	Protease	Yes	Acidic
BT_3577	Serine protease	No	Acidic
BT_2272	Zinc peptidase	No	Acidic
BT_3522	Glucanases	Yes	Acidic
BT_3237	Zinc peptidase	Yes	Acidic

Table 2.6. Proteins identified by MS/MS exclusively in OMV of *B. thetaiotaomicron*

Number	Function	Transmembrane domain or Liposignal	pI
BT_0656	OspA	Yes	Acidic
BT_1956	Cell surface protein	No	Acidic
BT_3313	Carbohydrate binding protein	No	Acidic
BT_3740	Putative cell adhesion protein	No	Acidic
BT_3148	Putative polysaccharide binding protein	No	Acidic
BT_3147	Fimbrillin-A associated anchor proteins	No	Acidic
BT_1896	cell surface antigen	No	Acidic
BT_1954	Protein-binding surface layer protein	Yes	Acidic
BT_0316	hemin receptor	Yes	Acidic
BT_2095	surface layer protein	Yes	Acidic
BT_2317	Fimbrial protein	Yes	Acidic
BT_3949	peptidyl-prolyl cis-trans isomerase	Yes	Acidic
BT_3911	malate dehydrogenase	Yes	Acidic
BT_1512	surface protein	Yes	Acidic
BT_1538	Hemagglutinin	Yes	Alkaline
BT_4005	NigD-like protein	Yes	Acidic
BT_0177	NigD-like protein	Yes	Acidic
BT_4719	NigD-like protein	Yes	Acidic
BT_1040	TonB-dependent outer membrane receptor	No	Acidic
BT_3983	TonB-dependent outer membrane receptor	No	Alkaline
BT_3240	TonB-dependent outer membrane receptor	No	Acidic
BT_3569	TonB-dependent outer membrane receptor	No	Acidic
BT_1552	TonB-dependent outer membrane receptor	No	Alkaline
BT_2364	TonB-dependent outer membrane receptor	No	Alkaline
BT_4246	SusD-like protein	Yes	Acidic
BT_3520	SusD-like protein	Yes	Acidic
BT_3238	SusD-like protein	Yes	Acidic
BT_3568	SusD-like protein	Yes	Acidic
BT_3241	SusD-like protein	Yes	Acidic
BT_1039	SusD-like protein	Yes	Acidic
BT_2365	SusD-like protein	Yes	Acidic
BT_1439	SusD-like protein	Yes	Acidic
BT_2263	SusD-like protein	Yes	Acidic
BT_2259	SusD-like protein	Yes	Acidic
BT_1553	SusD-like protein	Yes	Acidic
BT_4306	Hypothetical protein	Yes	Acidic
BT_3413	Hypothetical protein	Yes	Acidic
BT_3236	Hypothetical protein	Yes	Acidic

BT_1036	Hypothetical protein	Yes	Acidic
BT_1574	Hypothetical protein	Yes	Acidic
BT_4368	Hypothetical protein	No	Acidic
BT_4367	Hypothetical protein	Yes	Alkaline
BT_1486	Hypothetical protein	Yes	Acidic
BT_1487	Hypothetical protein	Yes	Acidic
BT_1579	Hypothetical protein	Yes	Alkaline
BT_1488	Hypothetical protein	Yes	Acidic
BT_0323	Hypothetical protein	No	Alkaline
BT_2438	Hypothetical protein	Yes	Acidic
BT_1395	Hypothetical protein	Yes	Acidic
BT_0766	Hypothetical protein	Yes	Alkaline
BT_0174	Hypothetical protein	No	Acidic
BT_2437	Hypothetical protein	No	Acidic
BT_0225	Hypothetical protein	Yes	Alkaline
BT_0410	Hypothetical protein	Yes	Alkaline
BT_1895	Hypothetical protein	Yes	Alkaline
BT_2753	Hypothetical protein	Yes	Alkaline
BT_1287	Hypothetical protein	Yes	Acidic
BT_3222	Hypothetical protein	Yes	Acidic
BT_2170	Hypothetical protein	Yes	Alkaline

Table 2.7. Proteins identified by MS/MS in OM of *B. thetaiotaomicron*

Number	Function	Transmembra-ne domain or Lipo signal	pI	Found in OMV
BT_3239	TonB-dependent outer membrane receptor	No	Alkaline	No
BT_1799	TonB-dependent outer membrane receptor	Yes	Alkaline	No
BT_2409	TonB-dependent outer membrane receptor	No	Alkaline	No
BT_2260	TonB-dependent outer membrane receptor	No	Alkaline	Yes
BT_2758	Phosphate-selective porin	No	Alkaline	No
BT_1440	TonB-dependent outer membrane receptor	Yes	Alkaline	Yes
BT_2817	TonB-dependent outer membrane receptor	Yes	Acidic	No
BT_2268	TonB-dependent outer membrane receptor	Yes	Acidic	Yes

BT_2264	TonB-dependent outer membrane receptor	No	Acidic	Yes
BT_3958	TonB-dependent outer membrane receptor	Yes	Acidic	Yes
BT_1763	TonB-dependent outer membrane receptor	Yes	Acidic	Yes
BT_0658	conjugal transfer protein	Yes	Alkaline	No
BT_3560	Porin	Yes	Alkaline	Yes
BT_0418	OmpA	No	Alkaline	Yes
BT_1194	OmpA	Yes	Alkaline	Yes
BT_1391	OmpA	No	Alkaline	Yes
BT_1613	OmpH	Yes	Acidic	Yes
BT_1185	OmpA	Yes	Acidic	Yes
BT_3852	OmpA	No	Acidic	Yes
BT_3562	Lys-gingipain	Yes	Acidic	Yes
BT_3742	Xylanase	Yes	Acidic	Yes
BT_3959	SusD-like protein	Yes	Acidic	Yes
BT_1762	SusD-like protein	No	Acidic	Yes
BT_2269	SusD-like protein	Yes	Acidic	Yes
BT_3901a	Hypothetical protein	Yes	Alkaline	Yes
BT_1414	Hypothetical protein	Yes	Alkaline	No
BT_0227	Hypothetical protein	Yes	Alkaline	No
BT_2844	Hypothetical protein	No	Alkaline	Yes
BT_1328	Hypothetical protein	Yes	Alkaline	Yes
BT_0645	Hypothetical protein	Yes	Alkaline	Yes
BT_4614	Hypothetical protein	Yes	Alkaline	Yes
BT_0646	Hypothetical protein	Yes	Alkaline	Yes
BT_1502	Hypothetical protein	Yes	Acidic	No
BT_1798	Hypothetical protein	No	Acidic	Yes
BT_3561	Hypothetical protein	Yes	Acidic	Yes
BT_1491	Hypothetical protein	Yes	Acidic	Yes
BT_2262	Hypothetical protein	No	Acidic	Yes

2.3.7-*B. fragilis* selectively packs non-native acidic hydrolases into OMV

BACOVA_04502 is an inulinase that was shown to be secreted in OMV of *B.ovatus* (Rakoff-Nahoum *et al.*, 2014). We tested if this enzyme is also recognized and selectively packed in OMV produced by *B. fragilis.* We expressed a His-tagged version of this inulinase in *B. fragilis* and determined the presence of the protein in whole cells, OM and OMV. As controls

we employed His-tagged versions of two proteins identified in our proteomic analysis, BF1581 and BF0018. BF1581 is an acidic protein detected only in OMV while BF0018 is an alkaline TonB-dependant protein that was found only in OM of *B. fragilis*. OM and OMV were collected from the *B.fragilis* strains, each expressing one of the three proteins. Samples were normalized according to total protein content and separated on SDS-PAGE followed by Western blot. As shown in figure 2.7, BF1581 only appeared in OMV while BF0018 was detected only in OM confirming the MS results. The *B. ovatus* acidic inulinase was greatly enriched in the OMV of *B. fragilis* (figure 2.7.C). This suggests that *B. fragilis* machinery is capable of recognizing and sorting non-native acidic hydrolases into the OMV.

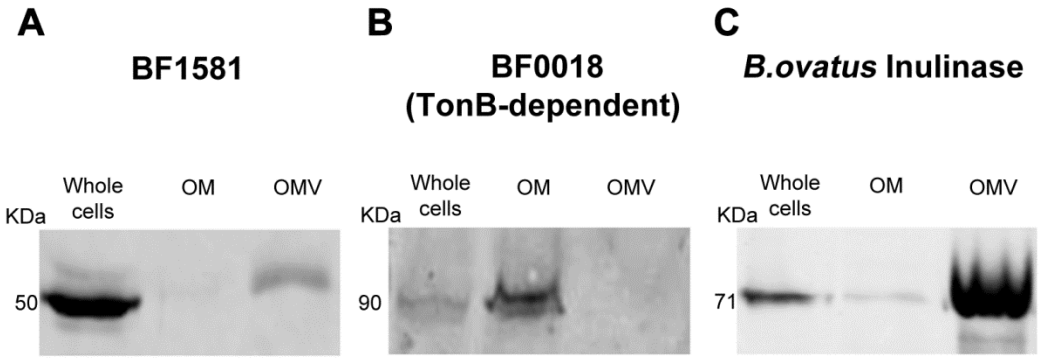

Figure 2.7. *B. fragilis* packs *B. ovatus* inulinase into its OMV. Western blots showing the distribution of BF1581 (A), BF0018 (B) and B. ovatus acidic inulinase BACOVA_04502 (C) in OM and OMV of *B. fragilis*. 10 µg of OM and OMV proteins of each strain were run on 10% SDS-PAGE followed by Western blot.

2.3.8-Lipid A is similar in OMV and OM

In previous work, we showed that deacylated lipid A accumulated in OMV of the dental pathogen *P. gingivalis* (Haurat *et al.*, 2011). Since *B. fragilis* and *P. gingivalis* are

evolutionarily-related, we investigated if the same phenomenon occurs in *B. fragilis* by comparing the lipid A composition in OM and OMV using MS (Nelson *et al.*, 2003). We used the Trizol method to purify LPS from *Bacteroides* cells and OMV. Lipid A was then obtained through mild acid hydrolysis of LPS (Berezow *et al.*, 2009, Yi & Hackett, 2000). As shown in figure 2.8, MALDI-TOF MS analysis of the purified lipid A showed no difference between OM and OMV. Both spectra displayed a cluster of peaks (m/z 1632, 1646, 1660, 1674, 1688, 1702) representing the penta-acylated lipid A of *B. fragilis*. The 14 amu differences are caused by fatty acids length variations known to occur in *B. fragilis* (Berezow *et al.*, 2009).

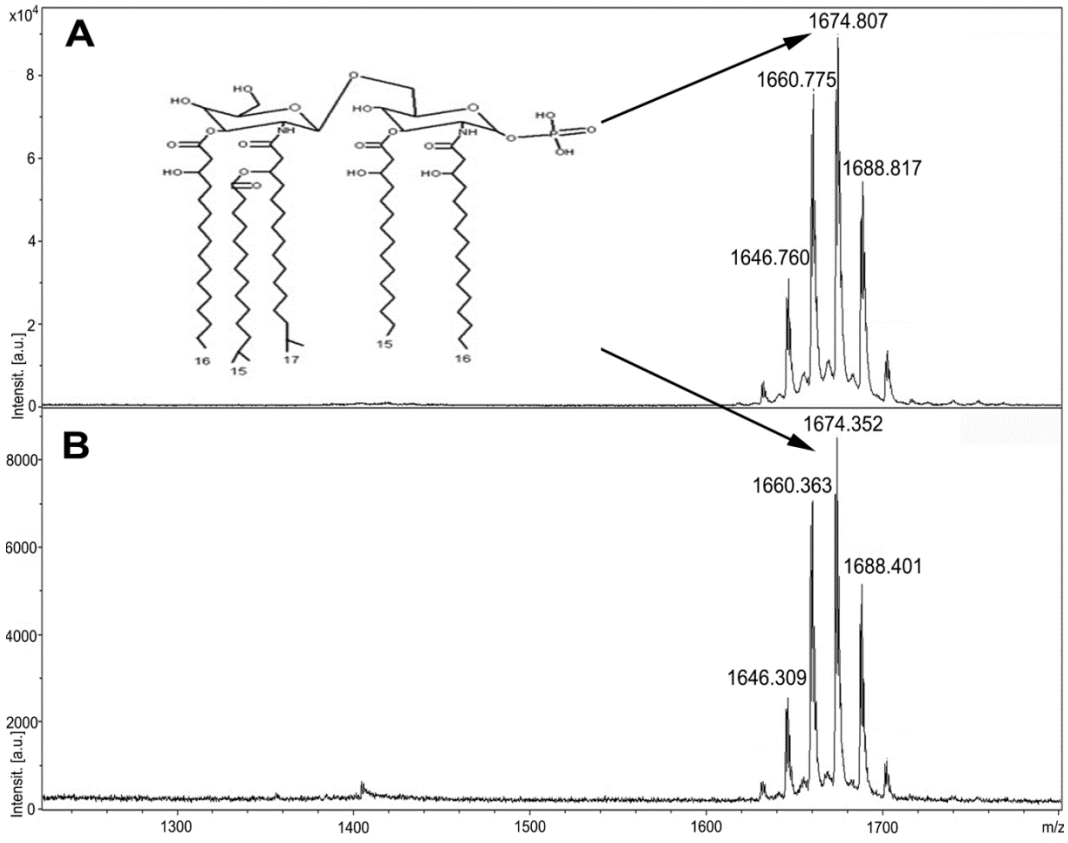

Figure 2.8. OMV and OM share the same lipid A species. Lipid A was extracted from both whole cells and vesicles of *B. fragilis* by mild acid hydrolysis of LPS in 1% SDS/10mM sodium acetate pH 4.5. Lipid A was dissolved in water and mixed with 2',4',6'-

trihydroxyacetophenone monohydrate matrix (THAP) in 1:1 ratio followed by MALDI-TOF MS analysis in negative linear mode. MS analysis of lipid A from cells (A) and vesicles (B) showed the same cluster of peaks representing the penta-acylated monophosphorylated lipid A. The 14 amu differences are caused by fatty acids length variation.

2.3.9-Identification of factors involved in *Bacteroides* OMV biogenesis and proteins sorting

In this chapter, it was shown that members of genus *Bacteroides* can selectively pack hydrolases into their OMV. Moreover, our experiments demonstrated that *B. fragilis* can induce OMV fucosidase activity in response to growth on fucose. Unlike OMV, growth on fucose did not induce the fucosidase activity of the OM. These results suggest that vesiculation in this genus is mediated by a regulated mechanism. However, the mechanisms responsible for OMV formation and cargo selection remain to be elucidated. To further investigate this process, a transposon mutant library of either *B. fragilis* or *B. thetaiotaomicron* could be generated. The transposon library could be grown in 96-well plates under conditions that induce the packing of OMV with sugar hydrolases. Therefore, the incubation of the cell-free supernatants with a fluorogenic substrate would allow the fluorometric determination of the enzymatic activity. In this regard, the library would be screened for mutants displaying a significant change in the enzymatic activity of their OMV-containing supernatants relative to the wild-type strain. This screening might allow the isolation of mutants with altered vesiculation levels or aberrant OMV cargo selection.

2.3.9.1-Induction of chitinolytic activity in the supernatants of *B. thetaiotaomicron*

As shown in table 2.5, our MS analysis of *B. thetaiotaomicron* OMV proteins suggested the presence of three OMV unique proteins with predicted chitinolytic activity (BT_3987, BT_3985, and BT_1038). Chitinolytic enzymes are hydrolytic enzymes that can degrade chitin (Adrangi *et al.*, 2010). Chitin is the second most abundant naturally-produced carbohydrate polymer. It is present in fungal cell walls and exoskeleton of different invertebrates. Chitin is formed by the polymerization of N-acetylglucosamine monomers through β-(1 → 4) linkages (Adrangi & Faramarzi, 2013). Chitinolytic enzymes are classified mainly into two categories; chitinases that randomly cleave chitin at internal sites of the polymer, and β-N-acetylhexosaminidases which catalyze the hydrolysis of N-acetylglucosamine units from the non-reducing end of the polymer (Adrangi & Faramarzi, 2013, Adrangi *et al.*, 2010). We hypothesized that growing *B. thetaiotaomicron* in the presence of N-acetylglucosamine would induce the OMV chitinolytic activity, similar to the induction of OMV fucosidase activity obsereved when *B. fragilis* was grown on fucose. Chitinolytic activity can be measured in vitro using the fluorogenic substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (MeUNAG) (Linko-Lopponen & Makinen, 1985). Therefore, *B. thetaiotaomicron* wild type strain was grown overnight on fructose and N-acetylglucosamine separately in a 96 well-plate. The cell-free supernatants were incubated with the fluorogenic MeUNAG, followed by fluorometric determination of chitinolytic activities. As shown in figure 2.9, the chitinolytic activity in the supernatant of *B. thetaiotaomicron* grown on N-acetylglucosamine was induced by 8-fold compared to that grown on fructose.

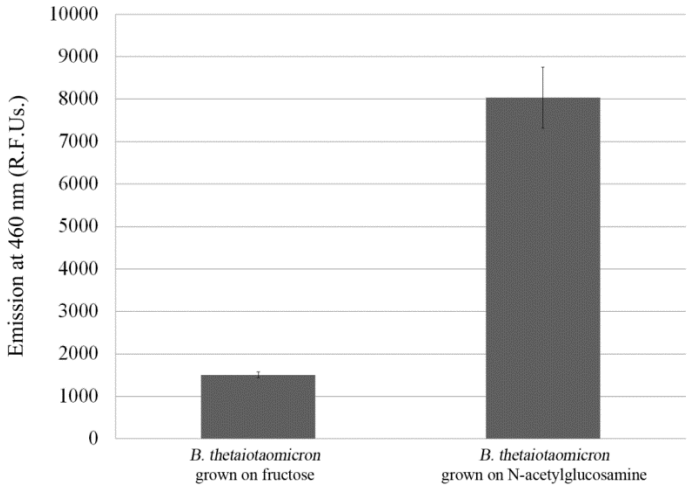

Figure 2.9. Growth of *B. thetaiotaomicron* on N-accetylglucosamine induces the chitinolytic activity in the supernatant. MeUNAG was added to 150 µl of cell-free supernatants obtained from the cultures of *B. thetaiotaomicron* grown on different sugars. After 1 hr incubation at 37ºC, chitinolytic activity was determined fluorometrically, with excitation at 360 nm and emission at 460 nm. Presented are the averages of the relative fluorescence units (R.F.Us.) obtained from 3 biological replicates of each growth condition.

2.3.9.2-Determination of chitinolytic activity in the OM and OMV of *B. thetaiotaomicron* grown on different carbon sources

As shown above, growing *B. thetaiotaomicron* on N-acetylglucosamine induced the chitinolytic activity in the supernatants. Therefore, more chitinolytic enzymes might be packed into OMV under inducing conditions. To test this hypothesis, *B. thetaiotaomicron* was grown on defined media containing either fructose or N-acetylglucosamine as a carbon source. OMV were harvested from both cultures, and compared for chitinolytic activity in vitro using MeUNAG as a substrate. Moreover, we harvested the OM in both conditions to test whether the chitinolytic activity will be induced in the parent membrane. As shown in figure 2.10, the OMV chitinolytic

activity was induced by 7-fold when *B. thetaiotaomicron* was grown on N-acetylglucosamine compared to fructose. Under the same inducing conditions, *B. thetaiotaomicron* OM displayed about 3-fold increase in chitinolytic activity.

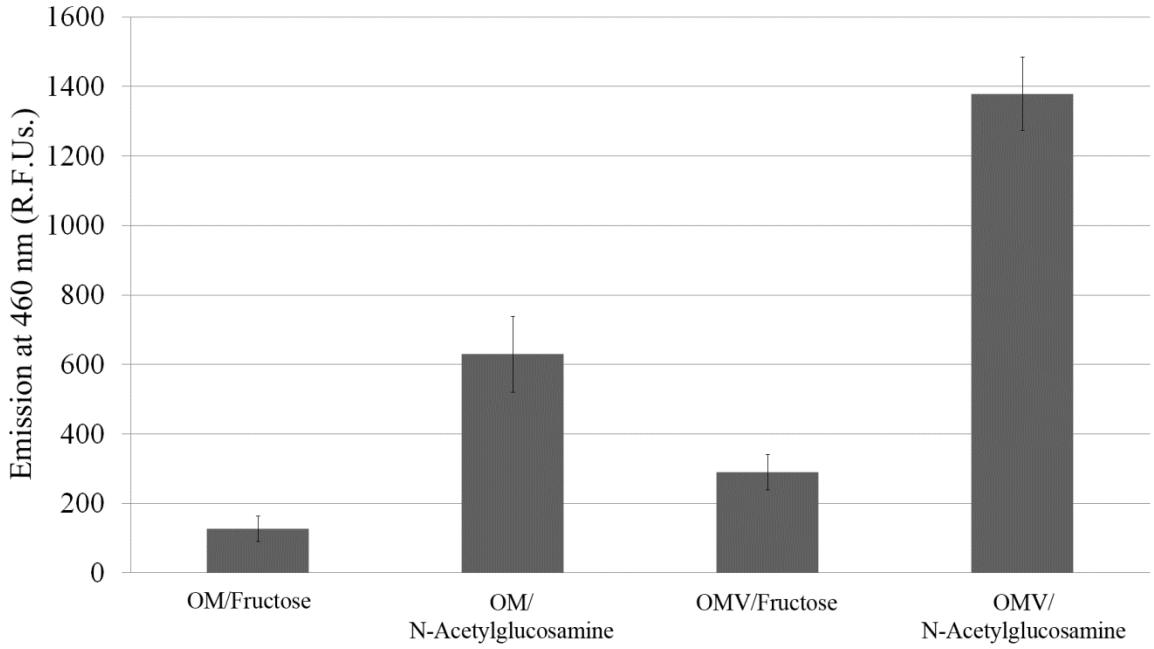

Figure 2.10. The utilization of N-acetylglucosamine by *B. thetaiotaomicron* as a carbon source results in inducing the chitinolytic activities of the OM and OMV. OM and OMV proteins were purified from *B. thetaiotaomicron* grown on different carbon sources. MeUNAG was added to 20 µg of each membrane compartment obtained from the different cultures of *B. thetaiotaomicron*. After 1 hr incubation at 37ºC, chitinolytic activity was determined fluorometrically, with excitation at 360 nm and emission at 460 nm. Presented are the averages of the relative fluorescence units (R.F.Us.) obtained from 2 biological replicates of each growth condition.

2.3.9.3-Establishment of high-throughput screening assay for the isolation of *B. thetaiotaomicron* mutants with altered vesiculation levels

The above results suggested that measuring the chitinolytic activity in *B. thetaiotaomicron* cell-free supernatants can be exploited to screen for mutants that over- or under-produce OMV compared to the wild type strain. We hypothesize that the overvesiculating mutants might display higher chitinolytic activity in their supernatants relative to wild type. On contrary, undervesiculating mutants will show diminished enzymatic activity in their supernatants compared to the parent strain. Nevertheless, alterations in enzymatic activity could also arise from aberrant sorting of proteins in the mutants OMV. A library of 10,000 *B. thetaiotaomicron* VPI-5482 mutants was generated using pSAM, a mariner transposon delivery vector. This transposon system was shown to disrupt 3435 of the 4779 predicted open reading frames in the genome of *B. thetaiotaomicron* VPI-5482 (Goodman *et al.*, 2009). Mutants were sorted into 96-well plates and grown on defined media containing N-acetylglucosamine as carbon source. A mutant with a transposon inserted in a non-coding region (mutant Tn397) was used as the reference strain in each plate, to account for any pleiotropic effects caused by the transposon. The library was grown overnight in chitinase-inducing conditions, followed by the measurement of OD_{600} values spectrophotometrically. Enzymatic activity of the cell-free supernatants was determined fluorometrically using MeUNAG as a substrate. To account for any defects in growth, the obtained fluorescence units were normalized by the OD_{600} values of the corresponding mutants. Subsequently, all values were divided by that of the reference strain to obtain the fold enzymatic activity. Mutants with at least 3-fold difference in their enzymatic activity relative to the reference strain were considered for further analysis. A total of 5000 mutants were screened using this method, and about 100 candidates were isolated. Due to the large number of candidates, we employed a secondary screening to compare the secretomes of the isolated mutants to the reference strain. Mutants were grown in 500 µl overnight cultures,

followed by the harvesting of cell-free supernatants. Centrifugal filters were used to concentrate the cell-free supernatants to 20 µl. Accordingly, the concentrated proteins were separated on 10 % SDS-PAGE, followed by Coomassie staining. The reference strain secretome was included in each gel for comparison. Using this analysis, we searched for mutants that secreted more or less proteins compared to the reference strain. Additionally, we searched for mutants that might display a different secretome compared to the reference strain. Surprisingly, the vast majority of the mutants analyzed displayed the same secretome as that of the reference strain. Our analysis revealed one mutant (referred to as *B. thetaiotaomicron* mutant strain 35A7) with higher protein content in its supernatant compared to the reference strain (figure 2.11). Semi-random PCR analysis was used to amplify transposon-genome junction in *B. thetaiotaomicron* mutant strain 35A7. Sequencing the amplicon suggested that the transposon was inserted in the BT1526-BT1525 region. BT1526 encodes a putative myo-inositol-1-phosphate synthase, while BT1525 is annotated as phosphatidyl-glycero-phosphatase A.

A)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.494824	1.642882	1.25414	1.468667		1.944683	3.718794	1.915745		3.131828	3.459818	3.143138
B	1.391514	2.92979	3.038522	1.538852	1.203611	1.21631	3.161657	0.816228	3.313648	1.447303	2.654152	1.472332
C	0.895374	0.623609	1.904247	2.255967	1.132909	5.549713	0.998712	0.122491	1.797107	1.792697	3.070555	0.841034
D	1.140129	1.425364	1.989451	1.390916	1.294469	1.407943	0.51809	1.473584	1.790339	1.272939		1.859517
E	1.290798	1.179423	2.04137		2.140262	1.539955	2.639489	1.752289	0.631017	1.544171	2.787299	2.04607
F	2.278112	3.407462	1.664077	2.027593	1.431816	2.028718	1.835399	1.751768	2.547933	3.494103	1.880621	2.163044
G		1.875424	3.107095		1.887845	1.848001	0.123573	0.327754	1.113772	1.103858	0.195057	2.032981
H	1.733315	1.814618	1.666792	3.108111	1.308871	1.300368	2.060254	2.010516	1.149103	2.237258	1	

B)

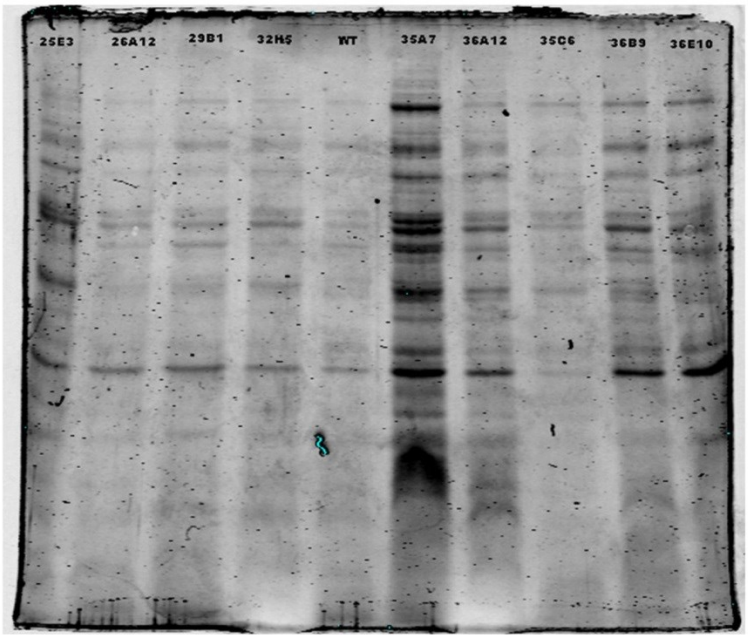

Figure 2.11. Screening a transposon library of *B. thetaiotaomicron* for the alteration in the chitinolytic activity of cell-free supernatants. A) Mutants library was grown overnight in 96-well plates under chitinase-inducing conditions, followed by fluorometric determination of chitinolytic activity in the supernatants. Relative fluorescence units were corrected by subtracting the blank value, and the resulting values were normalized by the OD_{600} readings of the corresponding cultures. Presented are the fold change values in chitinolytic activity relative to the reference strain (well H11). Heat map was generated based on the distribution of the fold change values relative to the reference strain (i.e. blue for values lower than 1 and red for higher values). Mutants that failed to grow were excluded from the analysis and are shown as white squares. B) Concentrated cell-free supernatants of different mutants were separated by SDS-

PAGE, followed by Coomassie staining to analyze the differences in the secretomes of these strains. Each strain was given a number based on its position in the screening plate. Numbers are presented on the top of each lane, with the reference strain denoted as "WT".

2.3.9.4-Protein analysis of OMV produced by *B. thetaiotaomicron* mutant strain 35A7

The previous results suggested that *B. thetaiotaomicron* mutant strain 35A7 might secrete more proteins, and possibly more OMV, into its supernatant. To investigate this possibility, we sought to compare OMV proteins purified from *B. thetaiotaomicron* mutant 35A7 and the reference strain. Three colonies were tested for each strain. OMV were harvested from OD_{600}-normalized cultures of different strains, followed by separation of the OMV proteins by SDS-PAGE and Coomassie staining. Unlike our results from the secretome analysis, there were only slight differences between the OMV proteomes of *B. thetaiotaomicron* mutant 35A7 and the reference strain (figure 2.12).

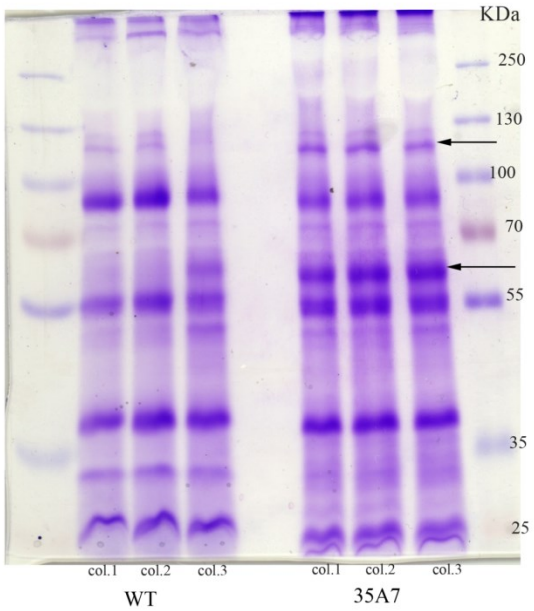

Figure 2.12. *B. thetaiotaomicron* mutant strain 35A7 OMV display slight differences compared to the OMV of the reference strain. OMV were harvested from different *B. thetaiotaomicron* overnight cultures. 3 colonies from each strain were analyzed. OMV proteins were separated on SDS-PAGE, followed by Coomassie staining. Only slight differences (marked by arrows) were observed between the OMV proteomes of both strains. *B. thetaiotaomicron* mutant strain 35A7 is denoted as "35A7", while the reference strain is marked as "WT".

2.4-Discussion

Membrane vesicles in eukaryotic cells are well known for their role in storage, trafficking and digestion of cellular components according to their location and function (Nieuwland & Sturk, 2010). Vesiculation appears to be ubiquitous among Gram negative bacteria, which suggests OMV play an important physiological role (Kulp & Kuehn, 2010, Mashburn-Warren & Whiteley, 2006, Schwechheimer *et al.*, 2013, Whitworth, 2011). Different functions have been assigned to vesicles including toxin delivery, interbacterial communication, biofilm formation, horizontal gene transfer and disposal of misfolded proteins (Bomberger *et al.*, 2009, Dutta *et al.*, 2004, Horstman & Kuehn, 2000, Horstman & Kuehn, 2002, Kadurugamuwa & Beveridge, 1995, Keenan *et al.*, 2008). In this work we characterized the protein content of OMV from *B. fragilis* and *B. thetaiotaomicron.* One of the most striking characteristics of these vesicles is the abundance of hydrolytic enzymes, mainly glycosidases and proteases. Previous reports showed the presence of hydrolase activity in the vesicles of *Bacteroides*, albeit none of these studies included a total proteome investigation of OMV (Forsberg *et al.*, 1981, Patrick *et al.*, 1996). It is well-recognized that glycan metabolism plays an important role in the establishment, the composition and the balance of the gut microbiota (Koropatkin *et al.*, 2012). Species from the genus *Bacteroides* carry multiple systems to bind and degrade polysaccharides known as "Sus-

like proteins". SusCDEFG form a surface localized complex where SusG is an α-amylase, SusC is a TonB-dependent porin and SusDEF are glycan-binding proteins (Martens *et al.*, 2009). Glycan metabolism is very important for these bacteria, to the point that Sus-like proteins can constitute up to 20% of their genome. The presence of active glycosidases in OMV, some of them selectively packed, suggests that *B. fragilis* secretes hydrolytic OMV to maximize the chances to degrade polysaccharides and other glycoconjugates. While the Sus-like proteins would be carrying a "selfish" activity, because the polysaccharides are degraded at the bacterial surface and the sugars are immediately uptaken, the hydrolases in OMV may carry a "social" function, as the resulting oligo- and mono-saccharides would be available for other bacteria to utilize. Recently it has been described that members of the genus *Bacteroides* participate in a complex polysaccharide utilization network based on the release and use of OMV, which act as "public goods" (Rakoff-Nahoum *et al.*, 2014). The authors showed that OMV produced from a microorganism can support the growth of another bacterium which is unable to degrade a given polysaccharide. Thus, OMV produce nutrients that can be utilized by other members of the microbiota. However the sugars liberated by OMV may also be exploited by pathogenic bacteria. For instance, *C. jejuni* does not produce fucosidases, but can utilize fucose liberated by other bacteria in the gut (Yu *et al.*, 2013). Finally, it has been recently demonstrated that *Salmonella typhimurium* and *Clostridium difficile* are also capable of utilizing sugars such as fucose and sialic acid liberated by *B. thetaiotaomicron* to successfully colonize the gut (Rakoff-Nahoum *et al.*, 2014). Our results suggest that OMV produced by *Bacteroides* play an important role in sugar metabolism in the gut. Several sugar hydrolases are only induced in the presence of the specific polysaccharide (Macfarlane & Gibson, 1991). We showed the levels of fucosidase activity packed in OMV are increased in presence of fucose, suggesting that the content of OMV

is also dependant on the nutrients. Interestingly, similar observations were made when *B. thetaiotaomicron* was grown in N-acetyl glucosamine, the end product of chitin digestion.

Fucose is of utmost importance for *Bacteroides* because it is incorporated in both its capsular polysaccharides and glycoproteins. The *Bacteroides* mutant lacking fucose in its proteins/capsule was found less fit in competitive colonization experiments with wild type *Bacteroides* (Coyne *et al.*, 2005). We detected two fucosidases (BF0810, BF3083) exclusively in *Bacteroides* vesicles. BF0810 is in the same operon with BF0811 which is annotated as a peptide-N-glycosidase and was found in OMV as well. This suggests that the two hydrolases might be functionally-related. BF0811 might cleave the human glycans from glycoproteins allowing BF0810 to cleave the fucose. Similar to *B. fragilis*, *B. thetaiotaomicron* OMV displayed an enrichment in acidic hydrolases. Among these is the levan hydrolase BT_1760, which was shown to be involved in supporting the growth of other *Bacteroides* unable to degrade levan (Rakoff-Nahoum *et al.*, 2014). In an interesting analogy, OMV produced by the bacterial predator *Myxococcus xanthus* were shown to contain a high number of digestive hydrolases. These OMV played a role in predation and possessed lytic activity against *E. coli*. (Kahnt *et al.*, 2010, Whitworth, 2011). Another example for hydrolase-containing OMV is the cellulolytic rumen bacteria *Bacteroides succinogenes,* which was shown to have 50% of its cellulose digesting enzymes associating with vesicles (Groleau & Forsberg, 1981). Additionally, these hydrolases might also target human molecules, such as mucus glycans, allowing *Bacteroides* to modulate host pathways. *P. gingivalis* OMV are rich in proteases that can degrade some of the host molecules leading to impairment of normal cellular function (Furuta *et al.*, 2009).

The lack of an accepted mechanism for OMV biogenesis has installed the controversy: are OMV the result of a directed and still poorly understood active cellular process, or are OMV

formed by passive membrane disintegration or membrane lysis? Several of our results favour the first hypothesis for OMV biogenesis in *Bacteroidetes*. First, the simple comparison of the OMV and OM proteins (figures 2.2 and 2.6) show a dramatically different profile. Second, the MS analysis identified more than 40 proteins in the OMV that were not present at detectable amounts at the OM. Third, a significant percentage of the proteins that are preferentially packed into OMV are mostly hydrolytic enzymes. Random clustering of such functionally related enzymes is unlikely. Fourth, proteins directed to the OMV are predominantly acidic.

Previous reports showed that OMV and OM vary in terms of lipid and protein composition. As evidence for lipid variation, different lipopolysaccharides (LPS) were detected in membrane and vesicles of the same bacteria. For example, vesicles from *Pseudomonas aeruginosa* were found enriched in the negatively charged LPS species, which was suggested to play a role in OMV biogensis through charge repulsion (Nguyen *et al.*, 2003). In *Serratia marcescens*, unique proteins were detected in vesicles but not in the outer membrane. Among the proteins detected in *Serratia* vesicles are lipases, phospholipases and chitinases which play a role in virulence (McMahon *et al.*, 2012). *N. meningitidis* provides another example of specific proteins enrichment into OMV. Lappann M *et al.* compared the protein content of both OM and OMV of *N. meningitidis* quantitatively. Some proteins were found to be enriched in OMV compared to OM and vice versa. Both preparations contained cytoplasmic proteins (Lappann *et al.*, 2013). In addition, we previously showed that vesicles of *P. gingivalis* varied from the OM in their protein composition. Multiple proteins in *P. gingivalis* OM were excluded from the vesicles. Concurrently, a few other proteins were enriched in vesicles compared to the membrane suggesting the presence of a protein sorting mechanism. In an interesting homology to the role of galectins in the eukaryotic system, LPS was suggested to participate in the cargo selection

process occurring during vesiculation of *P. gingivalis* (Haurat *et al.*, 2011). More proteomic studies on OMV of various organisms showed that they were enriched in different virulence factors (Schwechheimer *et al.*, 2013). However, the dramatic differences between OM and OMV protein composition determined in *B. fragilis and B. thetaiotaomicron* are unprecedented. Furthermore, for the first time, a common signature, acidity, was found for the proteins preferentially sorted into the OMV. The observation that an acidic inulinase from *B. ovatus* can be selectively packed in OMV by *B. fragilis* indicate that whatever mechanism is employed, is likely common within the genus *Bacteroides*. Another component that is variable between OMV and OM are the capsular polysaccharides. In *B. fragilis*, an important member of the human gut microbiota, Polysaccharide A and B (PSA and PSB) were detected in vesicles. PSA is known to have an immunomodulatory role in the human gut where it indirectly activates regulatory T cells (T_{reg}) to secrete interleukin-10. Interestingly, it was shown that *B. fragilis* uses its OMV to deliver PSA to dendritic cells which subsequently lead to T_{reg} cells activation (Shen *et al.*, 2012). Unlike the protein differences that we found between OMV and OM in *B. fragilis*, no variation in lipid A between the two compartments was found. Previously, we showed that the lipid A component of LPS was different between vesicles and membrane of *P. gingivalis.* The latter observation implied the presence of a lipid selection mechanism in vesicles additional to the protein selection. Although *P. gingivalis* is genetically close *to B. fragilis*, they seem to behave differently when packing lipids into their vesicles (Nelson *et al.*, 2003). This indicates that the vesiculation process might be variable among gram negative bacteria even in closely related species.

Rakoff-Nahoum *et al.* demonstrated that OMV produced by members of the microbiome can breakdown complex polysaccharides supporting the growth of other bacterial species unable

to degrade those polysaccharides. Diverse species appear to have co-evolved to be responsible for the degradation of specific polysaccharides and now may rely on each other's OMV for efficient utilization of the polysaccharides present in the gut. Our results supports the model that OMV produced by all these bacteria contribute to the syntrophy required for the establishment and maintenance of the human microbiota. Moreover, several reports suggested an immunomodulatory role for *Bacteroides* OMV in the gut (Hickey *et al.*, 2015, Shen *et al.*, 2012, Stentz *et al.*, 2014).

Our work shows that sugar hydrolases and proteases are preferentially packed into OMV produced by members of genus *Bacteroides*, in which appears to be a pI-dependent mechanism. To unravel the OMV biogenesis mechanism, we developed a genetic screening of a transposon-mediated library of *B. thetaiotaomicron* VPI-5482. In the presence of N-acetyl glucosamine as a carbon source, *B. thetaiotaomicron* induces the chitinolytic activity of its OMV. Therefore, a mutant with higher or lower chitinolytic activity in the supernatant might be defective in one of the factors required for the regulation or biogenesis of OMV. Our primary analysis of the mutants library resulted in the isolation of a large number of candidates with altered levels of enzymatic activity relative to the reference strain. Unfortunately, screening the secretomes of the isolated mutants did not show a significant difference when compared to the reference strain. Moreover, *B. thetaiotaomicron* mutant strain 35A7 displayed a different secretome from that of the reference strain. However, further analysis revealed that the OMV proteomes of strain 35A7 and the reference strain did not vary significantly. There are different explanations for the latter observations. It is possible that the vesiculation machinery is phase variable, consequently, some of the analyzed strains might display altered vesiculation levels that are due to phase variability rather than the lack of an underlying genetic factor. It is known that phase variation significantly

affects capsule synthesis in *Bacteroides* (Chatzidaki-Livanis *et al.*, 2008). Similarly, the vesiculation machinery might be affected, albeit, a possible crosslink between vesiculation and phase variation remains to be explored. Furthermore, growth defects might provide plausible explanation for the variability in the enzymatic levels displayed by the mutants when compared to the reference strain. We normalized the fluorescence readings according to the OD_{600} values of the cultures. Nonetheless, this approach did not account for mutants with different growth rates compared to the reference strain. Therefore, a mutant with delayed growth will reach the same culture density as that of the reference strain in longer time, and thus accumulating less OMV during its growth. This would result in less chitinolytic activity in the supernatant, however, the phenotype resulted from delayed growth rather than an impairment of the vesiculation machinery. Additionally, there might be technical issues with the screening method that requires further optimization to make it more stringent.

In summary, our results suggest the presence of a regulated mechanism involved in *Bacteroides* OMV biogenesis. We demonstrated that fluorogenic substrates could be employed to perform an enzymatic activity-based screening for the underlying genetic factors involved in OMV formation by members of genus *Bacteroides*. However, more work will be required to optimize the screening protocols towards a more stringent assay. Unraveling the mechanism responsible for OMV formation in *Bacteroides* will significantly improve our understanding of their role in gut health.

2.5-References

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Chapter Three

The Role Of Lipid A Deacylation In Outer Membrane Vesicles Biogenesis

3.1-Introduction

Salmonella enterica serovar Typhimurium (referred to as *S*. Typhimurium) is a leading cause of gastroenteritis, with approximately 3 million deaths reported annually worldwide (Chimalizeni *et al.*, 2010). *S*. Typhimurium possesses a plethora of virulence factors that are required for disease development. Many of these virulence factors are encoded within horizontally-acquired genomic islands called, *Salmonella* pathogenicity islands (SPI) (Galán, 2001). Amid the different SPIs in *S*. Typhimurium, SPI-1 and SPI-2 are the main virulence determinants. Each of them encodes a different type III secretion system (T3SS), which is a nanomachinery capable of injecting bacterial effectors across the eukaryotic plasma membrane into the host cell cytosol (Cornelis, 2006; Galán and Wolf-Watz, 2006; Haraga *et al.*, 2008; McGhie *et al.*, 2009; Abrusci *et al.*, 2014). To minimize fitness trade-offs in vivo, *S*. Typhimurium relies on two-component regulatory systems to spatially and temporally regulate the expression of genes within different SPIs (Fass and Groisman, 2009). Following phagocytosis by macrophages, *S*. Typhimurium resides in a specialized compartment, named *Salmonella* containing vacuole (SCV). The intravacuolar milieu activates the two-component system, PhoPQ, in *S*. Typhimurium (LaRock *et al.*, 2015). Once activated, PhoPQ mediates lipid A modifications, and outer membrane (OM) remodelling (Gunn, 2008; Dalebroux *et al.*, 2014; Dalebroux *et al.*, 2015). Moreover, PhoPQ activates SPI-2 T3SS to mediate translocation of effectors across the SCV to the host cell cytosol (Ochman *et al.*, 1996; LaRock *et al.*, 2015). In addition to T3SS, intracellular *S*. Typhimurium can secrete its proteins via outer membrane vesicles (OMV) (Guidi *et al.*, 2013).

OMV are liposomal structures protruding from the OM of Gram-negative bacteria. They were found to mediate numerous functions in environmental bacteria, pathogens and symbionts (Kulp and Kuehn, 2010; Haurat *et al.*, 2015). These roles involve; quorum sensing, horizontal gene transfer, interbacterial killing, toxins delivery, and secretion of misfolded proteins to relieve cell envelope stress (Yaron *et al.*, 2000; Renelli *et al.*, 2004; Mashburn and Whiteley, 2005; McBroom and Kuehn, 2007; Kulp and Kuehn, 2010; Chatzidaki-Livanis *et al.*, 2014; Haurat *et al.*, 2015). Recently, OMV produced by members of genus *Bacteroides* were shown to immunomodulate the host and contribute to gut health (Shen *et al.*, 2012; Elhenawy *et al.*, 2014; Rakoff-Nahoum *et al.*, 2014; Stentz *et al.*, 2014; Hickey *et al.*, 2015). Several models for OMV biogenesis were proposed, albeit, a universal model explaining their formation remains to be elucidated (Mashburn-Warren and Whiteley, 2006; Kulp and Kuehn, 2010; Haurat *et al.*, 2015). OMV share the basic components with the originating OM, including OM proteins, phospholipids and the lipopolysaccharides (LPS). Despite the similarity in general composition between the two membrane compartments, the OMV protein cargo in many bacteria was found to be distinct from the parent OM (Sidhu *et al.*, 2008; Lappann *et al.*, 2013; Galka *et al.*, 2008; Kato *et al.*, 2002; Haurat *et al.*, 2011; Elhenawy *et al.*, 2014; Haurat *et al.*, 2015). Moreover, lipid analysis of the two compartments suggested the enrichment of specific lipid species in OMV compared to the OM in several bacteria (Kadurugamuwa and Beveridge, 1995; Kato *et al.*, 2002; Tashiro *et al.*, 2011). For example, OMV produced by the dental pathogen, *Porphyromonas gingivalis,* were found to be enriched in deacylated lipid A forms compared to the lipid A purified from the cells (Haurat *et al.*, 2011). Lipid A is an acylated disaccharide and represents the hydrophobic anchor of LPS in the OM (Frirdich and Whitfield, 2005). The previous result suggested a role for lipid A deacylation in OMV formation.

Herein, we study the role of the lipid A deacylase, PagL, in *S*. Typhimurium OMV biogenesis. PagL is localized in the OM and is tightly regulated by the PhoPQ system (Trent *et al.*, 2001). Using immunofluorescence, we monitored the intracellular OMV production in *S*. Typhimurium wild type strain and a mutant lacking PagL. Interestingly, we were able to detect OMV inside the macrophages infected with the wild-type strain, but not in those infected with the *pagL*$^{-}$ strain. Moreover, we monitored the effect of PagL expression on OMV formation by *S*. Typhimurium in vitro. Overexpression of PagL resulted in more OMV production by *S*. Typhimurium, compared to a catalytically inactive variant of the enzyme. Furthermore, we exploited mass spectrometry (MS) to compare the lipid A content of *S*. Typhimurium OMV and cells. Our MS analysis revealed an exclusive accumulation of deacylated lipid A forms in *S*. Typhimurium OMV when PagL is expressed. Our results suggest a possible role for PagL-mediated lipid A deacylation in OMV formation by *S*. Typhimurium.

3.2-Experimental Procedures

3.2.1-Cell culture and media

J774A.1 mouse macrophages were maintained in maintained in Iscove's Modified Dulbecco's Medium (IMDM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humid atmosphere of 5% CO2. *S. enterica* subsp. *enterica* serovar Typhimurium 14028s was routinely cultured in Luria–Bertani (LB) medium. When needed, antibiotics were supplemented at the following concentrations; ampicillin (100 μg.ml^{-1}) and spectinomycin (100 ug.ml^{-1}).

3.2.2-Construction of Δ*pagL* strain

An isogenic *S.* Typhimurium mutant lacking *pagL* was created as described before (Datsenko and Wanner, 2000). In brief, the FRT-flanked kanamycin-resistance cassette was PCR amplified from pKD4 using primers; PagLKOFw (5'-CCATAGGGTCGATAACGATCGGCTATTCACAACACGTTTTGTAGACAACGTACGGTGATTAATTACTCCTTCAGCCAGCAACTCGCTAATTGTTATTCAACTTCAGAACATATGAATATCCTCCTTAGTTCCTATTCCG) and PagLKORv (5'-GTAGTGTGGATGCTATATCAGCCGTTTCTGTGAGCGTAAGCGTGGCGTAGAAAATTTTAAATATGTTAGCCGGTTAAAAATAACTATTGACATTGAAATGGTGGTGGAAGCGATTGTGTAGGCTGGAGCTGCTTCG). The latter primers included around 100 bp (underlined) homologous to the sequences flanking *pagL* in the genome. The amplified product was used to electroporate *S.* Typhimurium 14028s wild type strain expressing Red recombinase from pKD46. The recombinase-mediated allelic exchange resulted in the replacement of *pagL* by the kanamycin-resistance cassette. Km-resistant transformants were selected and cured from pKD46 by growth at 37 °c. The kanamycin-resistance cassette was excised by the expression of FLP recombinase from pFLP2. Δ*pagL* strain was cured from pFLP2 by growth on 10% sucrose, which will induce the expression of the toxic SacB. The clean deletion of *pagL* was confirmed by PCR amplification of its flanking regions in the genome followed by sequencing of the amplified product.

3.2.3-In vitro cell culture infection model

Cell line J774A.1 mouse macrophages (ATCC TIB-67, Rockville, MD, USA) were seeded in a 24 well tissue culture plate in dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at a density of 5 x 10^5 cells per well overnight

on 13mm coverslips. The media was changed to serum-free DMEM and infection was carried out as shown before with slight modifications (Bjur *et al.*, 2006). Different *S.* Typhimurium strains were cultured overnight in Luria-Bertani (LB) broth at 37^{o}C. Next day, the strains were subcultured until early log phase. 1 mL of the culture was washed and resuspended in DMEM. For opsonisation 4 µL of polyclonal anti-*Salmonella* rabbit serum were added for 30 min. The macrophages were infected at a MOI (multiplicity of infection) of 10: 1 for 1 hr in fresh DMEM with high concentration of gentamicin (100 µg/mL) to kill extracellular bacteria. Next, the media was changed to lower the gentamicin concentration (50 µg/mL) and incubation was continued for 2 hr. After incubation, the cells were fixed with 4% para-formaldehyde for 20 min, blocked in 2% Goat Serum and 1% Bovine Serum Albumin (15 min). The intracellular *Salmonella* were stained with primary monoclonal antibody directed against abequose (mouse monoclonal, 1:300 dilution in 0.1% Triton, 0.2% Goat Serum and 0.1% BSA) for 1 hr, followed by an incubation with the secondary antibody (anti-mouse Alexa 488 1:500 dilution 0.1% Triton, 0.2% Goat Serum and 0.1% BSA). Actin was stained with phalloidin 546 (1:40 dilution, 0.1% Triton, 0.2% Goat Serum and 0.1% BSA) for 30 min and DAPI (1:1000 dilution, 0.1% Triton, 0.2% Goat Serum and 0.1% BSA) was used for staining the nucleus. Slides were examined using an Olympus IX-81microscope with a Yokagawa spinning disk confocal head and a Hamamatsu EMCCD camera. Images were taken and analyzed with Volocity imaging software. Illustrations were formatted, for noise reduction and increased sharpness, using Image J. To count the intracellular vesicles, LPS-positive particles at the size of 250 nm and below were counted and normalized by the number of intracellular bacteria. The intracellular localization of all the counted subjects was confirmed by confocal microscopy examination .A total of 30 images were used to assess intracellular vesicles production by each strain. LPS-positive particles between

250 nm and 0.5 μm were considered lysed fragments and the macrophages that contain those fragments were excluded from counting.

3.2.4-OMV Purification

Cultures from different *S.* Typhimurium strains were normalized by their OD_{600} readings. Next, cells were pelleted down at 5000 rpm 4°C. In order to remove residual cells, the supernatant was filtered using a 0.45μm followed by 0.2μm pore-size polyvinylidene difluoride (PVDF) membrane (Millex GV; Millipore). The filtrate was subjected to ultracentrifugation at 100,000 x g for 3 hrs (Optima L-90K ultracentrifuge; Beckman Coulter). The supernatant was discarded, the pellet was washed with sterile PBS, and the ultracentrifugation step was repeated. The final vesicle pellet was resuspended in PBS and quantified using 2D-quant kit (GE Healthcare Life Sciences).

3.2.5-MALDI-MS analysis of Lipid A

Lipid A from vesicles and cells was prepared in duplicates using 10 mg of sample for each preparation according to the procedure of Yi and Hackett (Yi and Hackett, 2000). The purified lipid A was resuspended in 6 μl of methanol:dichloromethane (1:1). 1μl of the mixture was loaded on the MALDI plate followed by addition of 0.5ul of 2,4,6-trihydroxyacetophenone monohydrate (THAP) as the matrix. MALDI MS was then performed on a Bruker Daltonics (Bremen, Germany) UltrafleXtreme MALDI TOF/TOF mass spectrometer in linear negative mode.

3.2.6-Cloning of PagL constructs

PagL was PCR amplified from the genome of *S.* Typhimurium 14028s using primers; PagLEcoRIFw (ccccgaattcATGTATATGAAGAGAATATTTATATATC) and PagLBamHIRv (ccccggatccttagtggtggtggtggtggtgGAAATTATAACTAATTGAAGCACC). The reverse primer included extra sequence coding for 6 histidine residues at the c-terminal of the protein. To include the native promoter of PagL in the construct, primer PagLEcoRIFw was replaced by PagLpromEcoRIFw (aattgaattcACAATGTGACATAACAGAAGTG). The amplified products were restricted with EcoRI and BamHI, followed by cloning in pExt20 (Dykxhoorn *et al.*, 1996) to yield pWel1(PagL expressed from tac promoter) and pPagL (PagL expressed from native promoter). To generate a catalytically-inactive variant of PagL, site-directed mutagenesis was used. pWel1 was used as template for primers PagLH163AS165AFw"ACAGAAGCTTATATCCGGGCCTTCGCGAATGGATCACTTACGG" and PagLH163AS165ARv "CCGTAAGTGATCCATTCGCGAAGGCCCGGATATAAGCTTCTGT" to change the histidine and serine residues, at positions 163 and 165 respectively, to alanine. Similarly, pPagL was used as a template to generate $pPagL_{inactive}$ that codes for an inactive variant of PagL downstream of its native promoter. The mutation was confirmed by sequencing and the new construct was named pMFH19. For expression in *S.* Typhimurium, *pagL* and $pagL_{inactive}$ were subcloned from pWel1 and pMFH19 using the same restriction sites into the low copy number vector, pExt21 (Dykxhoorn *et al.*, 1996), to yield pWel2 and pWel6 respectively.

3.2.7-RT-mediated qPCR

RT-mediated qPCR was carried out as described before with some modifications(Rehl *et al.*, 2013). Total RNA was harvested from different strains at mid-log phase. cDNA was

synthesized using a SuperScript II cDNA Synthesis kit according to manufacturer's instructions (Invitrogen). Quantitative realtime PCR (RT-qPCR) was used to determine differences in the expression of *rpoE, cpxR* and *rcsC* using the housekeeping gene, *rpoD*, for normalization. Relative quantification qRT-PCR was done as described previously and analyzed using the ΔΔCT method (2001). For each experiment, two biological replicates were included and the average was presented. Primers used are listed in the table below (table 3.1).

Table 3.1 Primers used in the RT-qPCR analysis of different genes involved in the bacterial envelope stress response

rpoEfw	GAAAGAAATTTCGAACCCTGAGAA
rpoErv	TCCCGTAAGGTGATTGCCATA
cpxRfw	GGGCGCGGACGACTATT
cpxRrv	AGTGGGAACGGCGCAAA
rcsCfw	TCGCGAACTGGTACTCAAAAAA
rcsCrv	CCAGGACTAAATCTACAGGCACAGA
rpoDfw	GCGGCTACAAATTCTCCACCTA
rpoDrv	CAATCATATGCACCGGAATACG

3.2.8-OMV protein identification by mass spectrometry

Purified OMV were run on 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Protein bands were excised from the gel and digested using sequencing grade modified trypsin (Promega). The released peptides were eluted from the gel pieces, and desalted using ZipTipC18 columns (Millipore), followed by resuspension in 0.1% formic acid (Haurat *et al.*, 2011; Elhenawy *et al.*, 2014). A hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer, Q-TOF Premier (Waters), equipped with a nanoACQUITY Ultra performance liquid chromatography system (Waters) was used for MS/MS analyses of the peptides. The

generated mass spectra were used for the identification of the proteins by the Mascot search engine using the NCBI database.

3.2.9-In vitro lipid A deacylation assay

The deacylase activity of different PagL constructs was tested as shown before with some modifications (Trent *et al.*, 2001). In brief, total membranes were purified from different *E. coli* DH5α strains carrying pWel1, pMFH19 and pEXT20 grown to early stationary phase. Protein content of different membrane preparations was determined using 2d quant kit (GE health). *S.* Typhimurium 14028s LPS was extracted and used as a substrate for 5 µg of the membrane preparations obtained from different *E. coli* strains. The reaction was completed to 10 µl with 50 mM Hepes, pH 8.0, 0.1% Triton X-100, 0.5 M NaCl, 0.5mM phenylmethylsulfonyl fluoride. All reaction were incubated at 30ºC overnight, followed by lipid A extraction and MS analysis as stated above.

3.2.10-Immunoblotting

To test expression of PagL constructs in *S.* Typhimurium, whole cells pellets were obtained from different strains after normalization based on OD_{600} values. The harvested cell pellets were solubilized by boiling in 1X Laemlli buffer, then loaded on 12% SDS-PAGE gel. Following separation, proteins were transferred to a nitrocellulose membrane and the PagL constructs were visualized using anti-His rabbit antibody (primary antibody) followed by AlexFluor680-labelled anti-rabbit goat antibody (secondary antibody). To monitor cell lysis in different *S.* Typhimurium strains, 1 ml of cell-free supernatants were collected from OD_{600}-normalized cultures. Next, proteins were precipitated by trichloroacetic acid followed by

washing with acetone. Protein pellets were boiled in 1X laemlli buffer, then loaded on 12% SDS-PAGE gel. Immunoblotting was performed as mentioned above using mouse monoclonal anti-RNA polymerase (1:2500, RNAP α-subunit; Neoclone). Membrane was then probed with IRDye conjugated anti-mouse. Images were taken using LI-COR Odyssey Imaging system (LI-COR Biosciences, Lincoln, NE).

3.2.11-KDO-based quantification of OMV

To compare the amount of OMV produced by different *S.* Typhimurium strains, OMV were purified essentially as described above with some modifications. Cultures were grown to early exponential phase, followed by 4 hrs of induced expression of PagL constructs from pWel2 and pWel6 using 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). After 4 hrs, OD_{600} readings were determined. OMV were harvested from different strains after normalizing them according to the OD_{600} readings of the cultures. Final OMV pellets were resuspended in 100 µl of PBS and OMV production was determined by quantifying the 3-deoxy-D-manno-octulosonic acid (KDO) content in different preparations using the method of Lee and Tsai (Lee and Tsai, 1999). In brief, 50 µl of OMV preparations were boiled for 8 min with 0.5 M H_2SO_4 to release the KDO content, followed by oxidation with 50 µl of 0.1 M periodic acid. The latter reaction will convert KDO to formylpyruvic acid. The addition of 0.2 M sodium arsenite and freshly prepared thiobarbituric acid (0.6%), followed by boiling, will yield a chromagen. The reaction product can be extracted using n-butanol and measured spectrophotometrically at 552 nm and 509 nm. Commercially-available KDO (Sigma-Aldrich) was used as a standard. To measure KDO concentration in OMV preparations, the 552 nm readings were subtracted by absorbance at 509 nm and the standard curve was used to calculate the KDO content. The results were obtained from two

independent experiments under the same conditions. A one-way analysis of variance (ANOVA) followed by Tukey test to assess the significance of differences between groups were performed. The differences were considered significant when $p < 0.05$.

3.3-Results

3.3.1-Proposed model for OMV biogenesis in intracellular *S.* Typhimurium

Previous mass spectrometry (MS) analysis of the lipid A, purified from *P. gingivalis* cells and OMV, revealed interesting findings. *P. gingivalis* OMV were enriched in deacylated lipid A species compared to the parent membrane, suggesting a role for lipid A deacylation in OMV biogenesis (Haurat *et al.*, 2011). The effects of lipid A deacylation on its topological structure are well-studied (Schromm *et al.*, 2000; Seydel *et al.*, 2000). Hexacylated lipid A is conical in shape while the deacylated forms tend to acquire cylindrical to inverted cone-shaped structures (Schromm *et al.*, 2000). The influence of geometrical lipid properties on the eukaryotic membranes curvature is well-recognized (McMahon and Boucrot, 2015). Lipids with inverted-cone shaped structure, like lysophosphatidylcholine, favor the formation of positive membrane curvatures (Zimmerberg and Kozlov, 2006). Based on the previous studies, we proposed that the shape modifications imposed by lipid A deacylation might result in the curvature of the OM, and thus mediate OMV formation.

We chose *S.* Typhimurium as a model organism to study the effect of lipid A deacylation on OMV production. *S.* Typhimurium has a characterized OM deacylase, PagL, which removes the β-hydroxymyristoyl chain in the 3-position of lipid A (Trent *et al.*, 2001). This modification makes lipid A, and hence *S.* Typhimurium, less detectable by toll-like receptor 4 (TLR-4) of the

innate immune system (Kawasaki *et al.*, 2004). As shown in figure 3.1, we hypothesized that the intracellular environment will result in PagL activation. This activation might be mediated by the PhoPQ system or an uncharacterized pathway. When PagL is active in the OM, lipid A will be deacylated. Consequently, the hydrophobic cross-section area of the molecule will decrease, deforming its topology towards a cylindrical or inverted-cone shaped form. The accumulation of the conically-shaped lipid A in the OM will drive the formation of membrane curvature, a step preceding OMV formation.

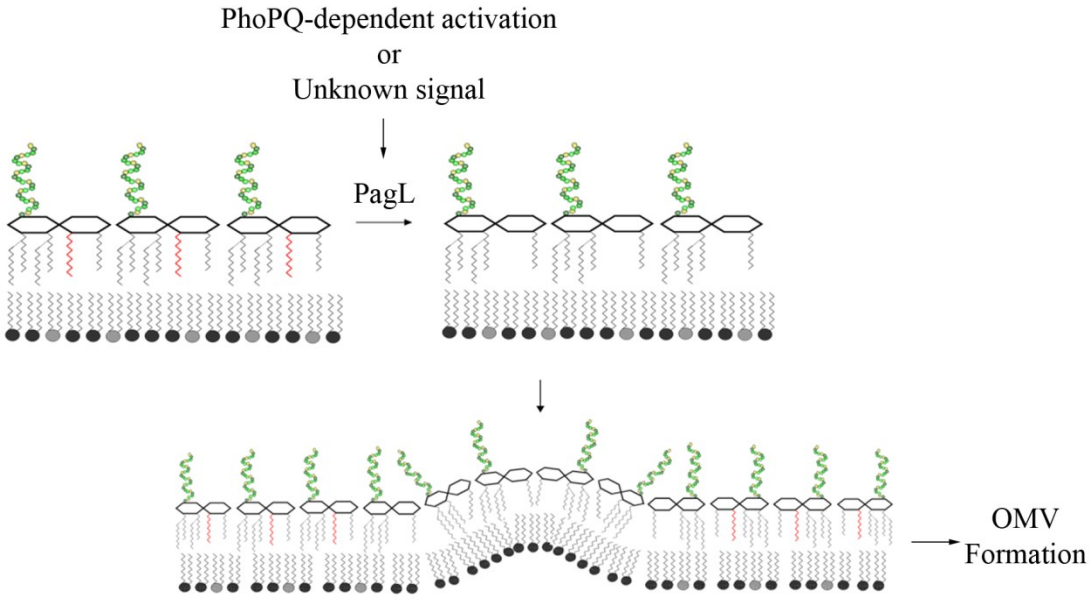


Figure 3.1. Hypothetical model for PagL-mediated vesiculation in *S.* Typhimurium. During its intracellular life, *S.* Typhimurium will activate PagL in PhoPQ-dependent or independent pathway. PagL will deacylate lipid A, leading to a change in its topology. As a consequence to the decrease in hydrophobic cross section area, lipid A will adopt an inverted cone shape, leading to membrane curvature and OMV formation.

3.3.2- *S.* Typhimurium OM and OMV share the same lipid A profile under PagL noninducing conditions

In *P. gingivalis*, OMV displayed a different lipid A profile compared to the OM. Most of the detected lipid A forms in *P. gingivalis* OMV were deacylated, whereas OM lipid A displayed higher degree of acylation (Haurat *et al.*, 2011). Therefore, we sought to compare the lipid A content of the OM and OMV in *S.* Typhimurium. OMV and cells were harvested from *S.* Typhimurium grown under standard lab conditions. Next, lipid A was extracted from both fractions and analyzed by MS (Yi and Hackett, 2000). Our MS analysis of lipid A revealed identical profiles in both, cells and OMV. As shown in figure 3.2, the bis-phosphorylated hexaacylated lipid A was the predominant peak (m/z 1796) in both spectra. In agreement with previous reports, we did not detect any PagL-mediated deacylated forms of lipid A (Manabe *et al.*, 2010). It is known that PagL is under the tight control of the PhoPQ system, which is activated by host cues in the SCV (Trent *et al.*, 2001; Kawasaki *et al.*, 2004). Therefore, our results indicate that *S.* Typhimurium does not modify OMV lipid A under normal growth conditions in vitro.

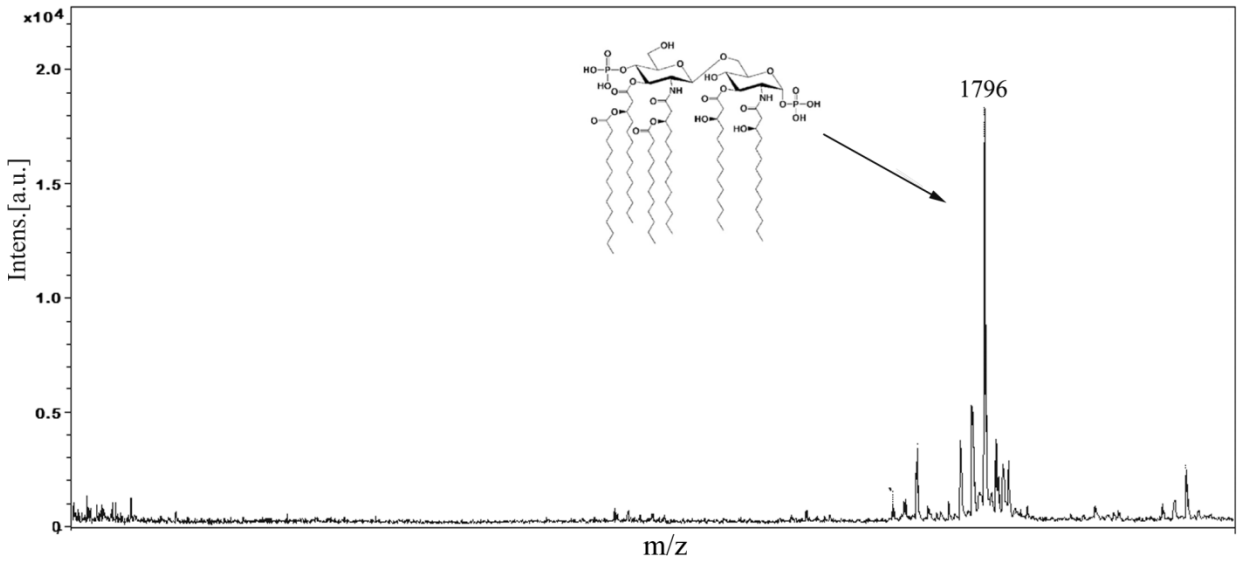


Figure 3.2. *S.* Typhimurium OMV lacks deacylated lipid A forms under standard laboratory conditions. Lipid A was extracted from cells and OMV of *S.* Typhimurium wild type strain, followed by MALDI-MS analysis. Lipid A from both fractions displayed the bisphosphorylated hexaacylated lipid A form (m/z 1796) as the most abundant form. None of the known deacylated lipid A species were detected in both fractions.

3.3.3- PagL is secreted in S. Typhimurium OMV without affecting their protein content

To study the effect of PagL on vesiculation in vitro, we adopted a biochemical approach. Instead of growing the bacteria under PhoPQ-activating conditions, we expressed PagL in trans in *S.* Typhimurium. This approach will allow us to study the effect of PagL on OMV formation in the absence of other PhoPQ-mediated lipid A modifications (Prost *et al.*, 2007; Prost and Miller, 2008). Next, we wanted to examine whether the expression of PagL affected the OMV proteome. To explore this possibility, we harvested OMV from the *S.* Typhimurium strain

expressing PagL in trans and the strain carrying the empty vector. Both OMV preparations were separated by SDS-PAGE followed by Coomassie staining to visualize the protein bands. With the exception of one band corresponding to a 15KDa protein, PagL expression did not alter the OMV proteome (figure 3.3). All protein bands were excised and tryptically digested, then the produced peptides were analyzed by MS for protein identification. Previous studies have identified the OMV protein content in *S.* Typhimurium (Deatherage *et al.*, 2009; Bai *et al.*, 2014). We detected the same set of proteins in the OMV preparations obtained from both *S.* Typhimurium strains (Table 3.2). Most of the proteins we detected were previously reported to be part of the *S.* Typhimurium OMV proteome (Deatherage *et al.*, 2009; Bai *et al.*, 2014). Our MS analysis revealed that the 15 KDa protein detected above is PagL. These results indicate that PagL migrates to OMV without affecting their protein composition.

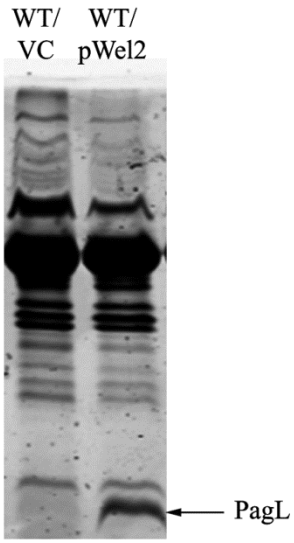


Figure 3.3. Expression of PagL does not alter the protein content of OMV. OMV proteins were purified from the *S.* Typhimurium strain expressing PagL (WT/pWel2), and the vector control strain (WT/VC). The purified proteins were run on 12% SDS-PAGE followed by

Coomassie staining. Gel bands were excised from the gel, followed by trypsin digestion. The digested peptides were separated by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Proteins were identified from the generated spectra using Mascot search engine and the NCBI database.

Table 3.2. List of proteins identified by MS in the OMV purified from different *S.* Typhimurium strains

Accession number	Gene locus	Protein name	Description
325530278	STM14_3483	SipC	Cell invasion protein
267995013	STM14_3484	SipB	Translocation machinery component
267993946	STM14_2378	FliC	Flagellin
267995010	STM14_3481	SipA	Type III secretion effector protein
267994353	STM14_2797	OmpC	Outer membrane porin protein
267992822	STM14_1214	OmpA	Outer membrane protein
267992583	STM14_971	OmpX	Outer membrane protein
267992739	STM14_1130	OmpF	Outer membrane protein
267996660	STM14_5207	GroEL	Chaperonin
267992123	STM14_0489	Tsx	Nucleoside channel
267992844	STM14_1237	SopB	Type III secretion effector protein
267995006	STM14_3477	SptP	Type III secretion effector protein
267993264	STM14_1671	Lpp	Murein lipoprotein
267996436	STM14_4965	BtuB	Vitamin B12/cobalamin outer membrane transporter
267994598	STM14_3051	NlpB	OM lipoprotein
267992951	STM14_1348	FlgE	Flagellar hook protein
267996238	STM14_4760	PldA	Phospholipase A
267993108	STM14_1513	Hypothetical	Putative outer membrane lipoprotein
267995055	STM14_3527	NlpD	OM lipoprotein
267992525	STM14_0912	YbhC	OM pectinesterase
267993484	STM14_1898	NmpC	Putative outer membrane porin
267992999	STM14_1397	PotD	Spermidine/putrescine ABC transporter periplasmic substrate-binding protein
267995271	STM14_3750	AnsB	L-asparaginase II
267995563	STM14_4052	Mdh	Malate dehydrogenase
267993373	STM14_1785	YdgH	Putative periplasmic protein
267995625	STM14_4119	RpsD	30S ribosomal protein S4
267993623	STM14_2040	PspA	Phage shock protein
267990133	STM14_5621	TraT	Conjugative transfer protein

267994804	STM14_3264	YfiO	Outer membrane protein assembly complex subunit
267996252	STM14_4774	Udp	Uridine phosphorylase
267992473	STM14_0854	SdhB	Succinate dehydrogenase
267991915	STM14_0266	YaeT	Outer membrane protein assembly factor
267991764	STM14_0112	LptD	LPS assembly outer membrane complex protein
267995460	STM14_3946	YraM	Putative transglycosylase
267991878	STM14_0228	FhuA	Ferrichrome outer membrane transporter
267996451	STM14_4990	RpoB	RNA polymerase β-subunit
267992204	STM14_0572	Hsp90	Heat shock protein 90
267992489	STM14_0870	TolB	Translocation protein
267994496	STM14_2942	FadL	Long-chain fatty acid outer membrane transporter

3.3.4- PagL expression did not induce envelope stress in *S.* Typhimurium

To examine whether PagL mediates vesiculation in *S.* Typhimurium, we sought to quantify OMV production in the *S.* Typhimurium transformant expressing PagL. Previous studies have suggested a role for specific proteins in the vesiculation of Gram-negative bacteria (Kitagawa *et al.*, 2010; Moon *et al.*, 2012). However, it remains to be demonstrated in many cases whether the changes in vesiculation levels observed are correlated with the activities of these proteins, and not due to cell stress that leads to lysis. It was proposed before that OMV production can occur as a response to envelope stress in bacteria. To correlate between PagL-mediated lipid A deacylation and the vesiculation levels in *S.* Typhimurium, we have to consider any possible stress that the overexpression of PagL might cause in the OM. To address this possibility, we monitored three of the main systems involved in the bacterial envelope stress response; σ^E, Cpx and Rcs pathways (Raivio, 2005; Rowley *et al.*, 2006). We used RT-qPCR to measure the effect of PagL induction on the expression levels of a representative for each pathway; *rpoE*, *cpxR* and *rcsC* (Raivio, 2005; Huang *et al.*, 2006; Rowley *et al.*, 2006). *rpoE* codes for the main transcription factor required for the upregulation of the σ^E-regulon during

envelope stress. CpxR is the response regulator in the Cpx pathway, while RcsC is a key histidine kinase in the Rcs phosphorelay system. It is known that all three genes are upregulated in response to the activation of their corresponding pathways (Raivio, 2005; Huang *et al.*, 2006; Rowley *et al.*, 2006). None of the previous genes was upregulated when PagL was overexpressed, suggesting that the elevated levels of PagL might not cause cell envelope stress in *S.* Typhimurium (figure 3.4).

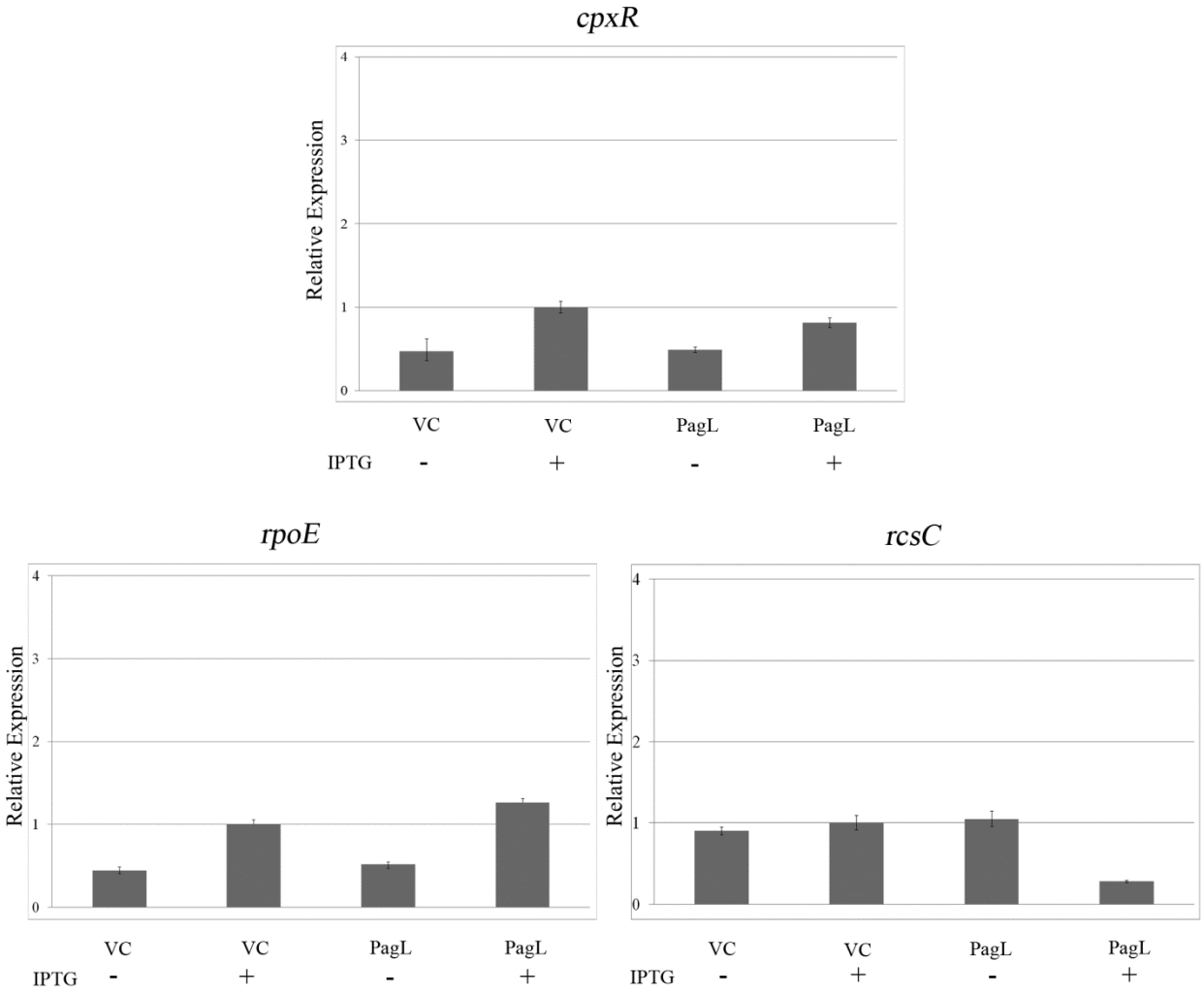


Figure 3.4. PagL expression does not activate σ^E, Cpx or Rcs pathways in *S.* Typhimurium. Total RNA was purified from different *S.* Typhimurium strains at mid-log phase using phenol extraction method. Following cDNA synthesis, relative expression of different genes was determined by qPCR using SYBR green. *rpoD* was employed as an endogenous control. PagL was expressed from pWel2. VC denotes the vector control, pExt21. Two biological replicates were employed for each strain and the average was presented. The experiment was done in the absence and presence of IPTG induction.

3.3.5- Construction of catalytically inactive variant of PagL

To investigate whether PagL enzymatic activity is required for OMV formation, we created a catalytically inactive variant of PagL as a control. Geurtsen et al. identified the conserved residues among PagL homologues in different bacteria (Geurtsen *et al.*, 2005). Another study revealed the catalytic mechanism for PagL and demonstrated the key residues that are essential for activity (Rutten *et al.*, 2006). Therefore, we exploited site-directed mutagenesis to change the histidine (position 163) and serine (position 165) residues to alanine, which should abolish the deacylation activity. To confirm that the PagL variant was enzymatically inactive, we tested its activity using a modified in vitro assay for lipid A deacylation. We expressed PagL and its modified variant in *E. coli,* which lacks a homologue for *pagL* (Trent *et al.*, 2001; Geurtsen *et al.*, 2005). Both PagL and the modified variant were expressed at similar levels in *E. coli* and were localized in the OM (Data not shown). Membrane preparations of different *E. coli* strains were incubated with purified *S.* Typhimurium LPS overnight. Following incubation, lipid A was extracted from the different reactions and analyzed by MS. Only the expression of wild type PagL resulted in the detection of a peak corresponding to the 3-*O*-deacylated bis-phosphorylated

lipid A (m/z 1570). Conversely, the mutated variant of PagL (now referred to as $PagL_{inactive}$) lost its ability to deacylate lipid A as indicated by the absence of the m/z 1570 peak (figure 3.5).

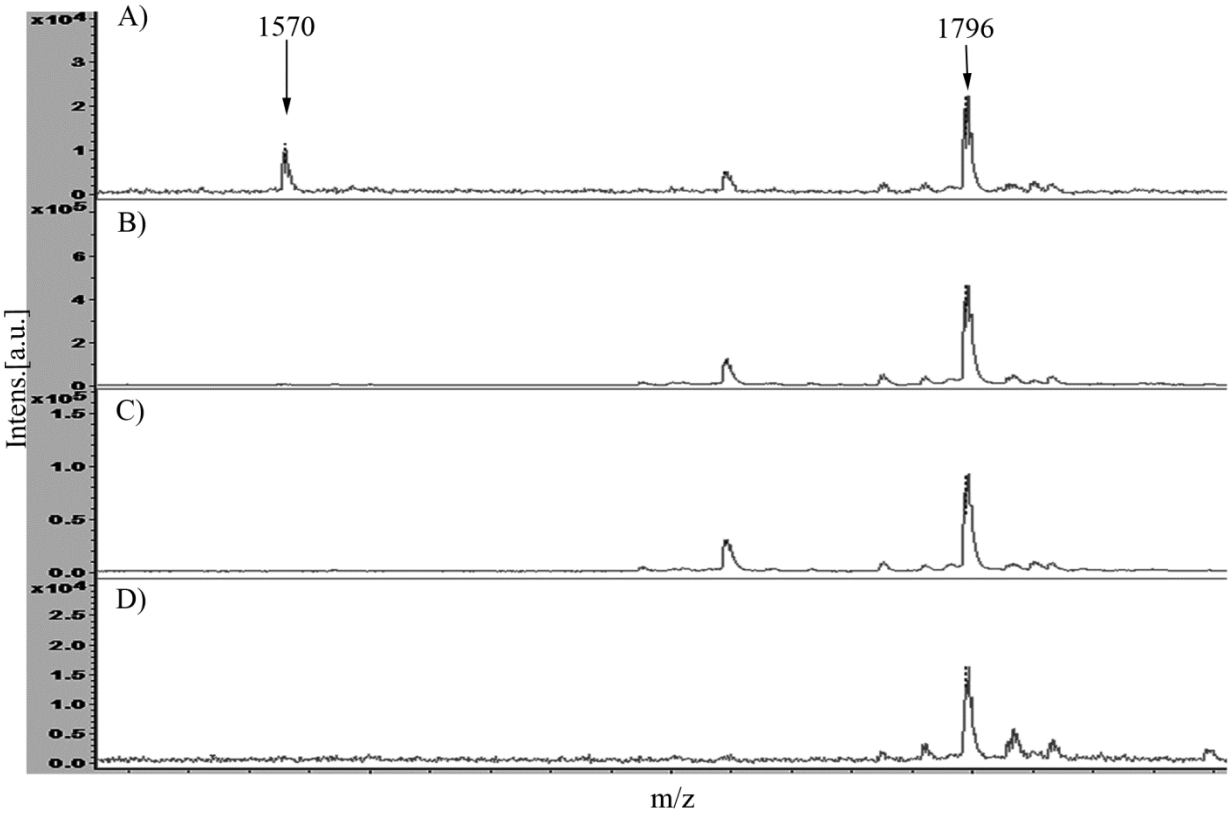


Figure 3.5. The loss of the deacylase activity in $PagL_{inactive}$. Total membranes were purified from different *E. coli* DH5α strains carrying pWel1 (A), pMFH19 (B) and pEXT20 (C). *S.* Typhimurium LPS was used as a substrate to test the deacylase activity of different membrane preparations. One reaction contained only *S.* Typhimurium LPS without the addition of *E. coli* membranes as a control (D). Following overnight incubation, lipid A was purified from different reactions and analyzed by MS.

3.3.6- PagL-mediated lipid A deacylation might contribute to OMV formation in *S.* Typhimurium

To assess the role of lipid A deacylation in *S.* Typhimurium vesiculation, we sought to measure OMV production in *S.* Typhimurium transformants expressing PagL and $PagL_{inactive}$. Using immunoblotting, we verified that both proteins are expressed at similar levels in *S.* Typhimurium (figure 3.6A). Next, different *S.* Typhimurium strains were grown under standard lab conditions and the expression of PagL and $PagL_{inactive}$ was induced for 4 hrs from a low copy number vector. Subsequently, OMV were harvested from cell-free supernatants of OD_{600}-normalized cultures, followed by quantification. Other studies relied on measuring the protein concentration of OMV preparations as a representative of vesiculation levels (McBroom and Kuehn, 2007; Kitagawa *et al.*, 2010; Moon *et al.*, 2012; Schwechheimer *et al.*, 2015). Nevertheless, this approach does not discriminate between overvesiculation and the enrichment of proteins in OMV without a change in their production level. Moreover, some proteins might be non-specifically associated with OMV and will result in higher readings that do not represent a true overvesiculation phenotype. To avoid any confounding effects that proteins might have on measuring the vesiculation levels, we quantified the 3-deoxy-D-manno-octulosonic acid (KDO) content of *S.* Typhimurium OMV. KDO glycoasylates lipid A at position 6' in different bacteria (Frirdich and Whitfield, 2005). In *S.* Typhimurium, two molecules of KDO covalently modify each molecule of lipid A (Munson *et al.*, 1978). Since lipid A is a principle component of OMV, KDO quantification will be representative of the OMV amounts produced. Using a colorimetric assay, we quantified the KDO content of OMV produced by *S.* Typhimurium transformants expressing PagL and $PagL_{inactive}$, relative to the control strain carrying the empty vector (Lee and Tsai, 1999). Interestingly, PagL expression resulted in about 4-fold increase in the KDO content

of OMV compared to that of the vector control. In accord with losing activity, $PagL_{inactive}$ expression led to a slight increase in OMV KDO content that did not vary significantly from the vector control (figure 3.6B). As mentioned above, the overexpression of PagL did not activate any of the cell envelope stress pathways examined. However, we predicted that the induction of PagL and its inactive variant might create stress in the OM that is not sufficient to activate the cell envelope stress response. To address the previous concern, we monitored the levels of the cytoplasmic RNA polymerase released into the cell-free supernatants of different *S.* Typhimurium transformants using immunoblotting. The expression of PagL, and to a less extent $PagL_{inactive}$, resulted in higher levels of cell lysis compared to the vector control (figure 3.6C). Together, the previous results suggest a possible role for PagL activity in OMV production. Unfortunately, our experiments do not discriminate between the OMV produced due to PagL activity and the OM fragments released due to cell lysis. However, our results indicate that the overvesiculation phenotype observed correlates with PagL enzymatic activity.

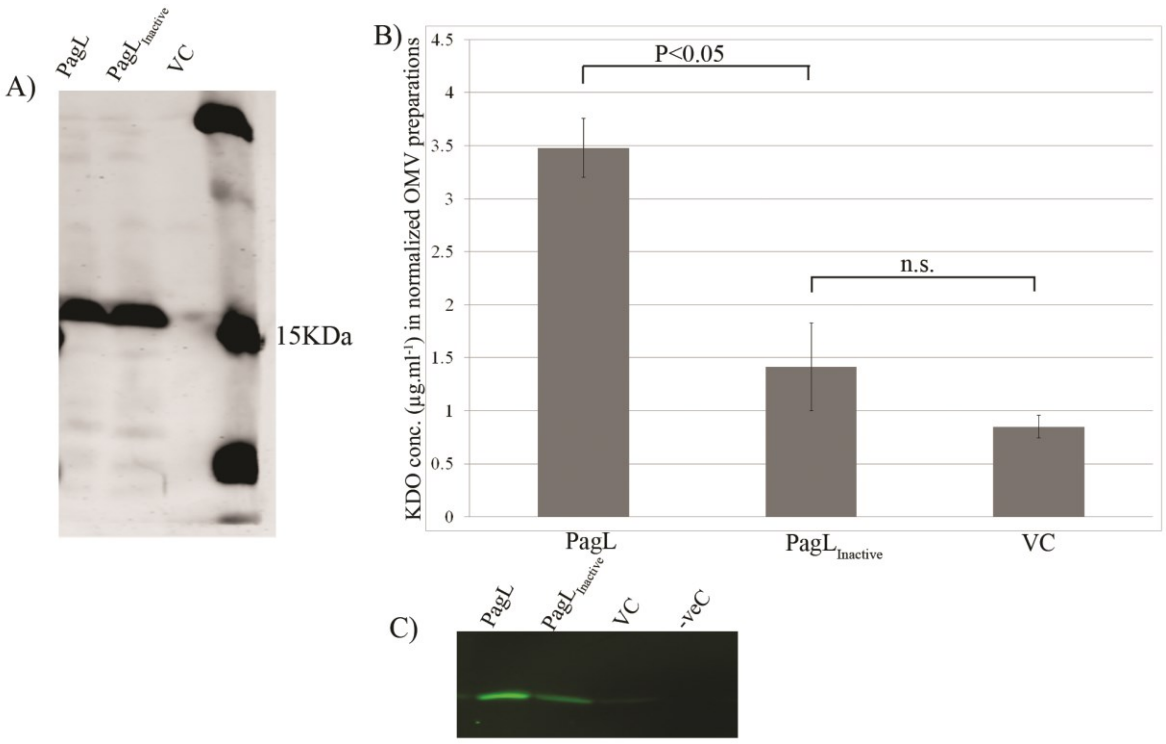


Figure 3.6. In vitro expression of catalytically active PagL induces OMV production in *S.* Typhimurium. (A) Cell lysates were obtained from *S.* Typhimurium strains carrying pWel2 (expressing PagL), pWel6 (expressing $PagL_{inactive}$) and empty vector (VC), followed by SDS-PAGE separation. The expression levels of PagL and $PagL_{inactive}$ in *S.* Typhimurium were monitored by immunoblotting using anti-His rabbit antibody (primary antibody) followed by AlexFluor680-labelled anti-rabbit goat antibody (secondary antibody). As shown, both proteins were expressed at similar levels. (B) Shown are the KDO concentrations ($\mu g.ml^{-1}$) in OMV preparations harvested from OD_{600}-normalized cultures of different *S.* Typhimurium strains. The results were obtained from two independent experiments under the same conditions. Presented are means ± SEM. (C) Immunoblotting was used to monitor RNA polymerase levels in the cell-free supernatants of different strains using mouse monoclonal anti-RNA polymerase (1:2500,

RNAP α-subunit) followed by AlexFluor800-labelled anti-mouse goat antibody (secondary antibody). As a negative control, one lane of the gel was loaded with PBS buffer (-veC).

3.3.7- PagL expression accumulates deacyated lipid A in S. Typhimurium OMV

Our results suggested a possible role of PagL deacylation activity in OMV formation. Therefore, we sought to compare the lipid A content of OM and OMV of *S.* Typhimurium. The *S.* Typhimurium strain expressing PagL was grown in vitro, together with the strain carrying an empty vector as a control. Lipid A was purified from the cells and OMV of both *S.* Typhimurium strains, followed by MS analysis. In the control strain carrying the empty vector, both fractions displayed the same lipid A species (data not shown). Intriguingly, the expression of PagL resulted in striking differences between the cellular lipid A and that purified from OMV. Despite the expression of PagL in the cells, the deacylated lipid A species were exclusively accumulated in the OMV compared to the cell fraction (figure 3.7). The predominant lipid A form in the PagL-expressing *S.* Typhimurium cells was the bis-phosphorylated hexaacylated lipid A (m/z 1796). Other less predominant forms were detected in the cells, including the monophosphorylated hexaacylated lipid A (m/z 1716) and the bis-phosphorylated heptacylated lipid A (m/z 2035). The formation of the heptaacylated lipid A is mediated by another OM enzyme, called PagP, which transfers a palmitoyl group from a phosphatidylglycerol donor to the 2-position in lipid A (Guo *et al.*, 1998). On contrary, the predominant lipid A forms in the cell were all deacylated in the OMV by the loss of the β-hydroxymyristoyl group at the 3-position. The bis-phosphoorylated pentaacylated lipid A (m/z 1570) was the most predominant form in the spectrum. Furthermore, the mono-phosphoorylated pentaacylated lipid A (m/z 1490) and the bis-phosphoorylated hexaacylated lipid A (m/z 1808) were also detected in OMV. These lipid A

modifications were all PagL-dependent since we did not detect these forms in the *S.* Typhimurium strain carrying the empty vector. Interestingly, these results are consistent with our previous findings in *P. gingivalis* (Haurat *et al.*, 2011) and suggest that lipid A deacylation might be involved in bacterial OMV formation.

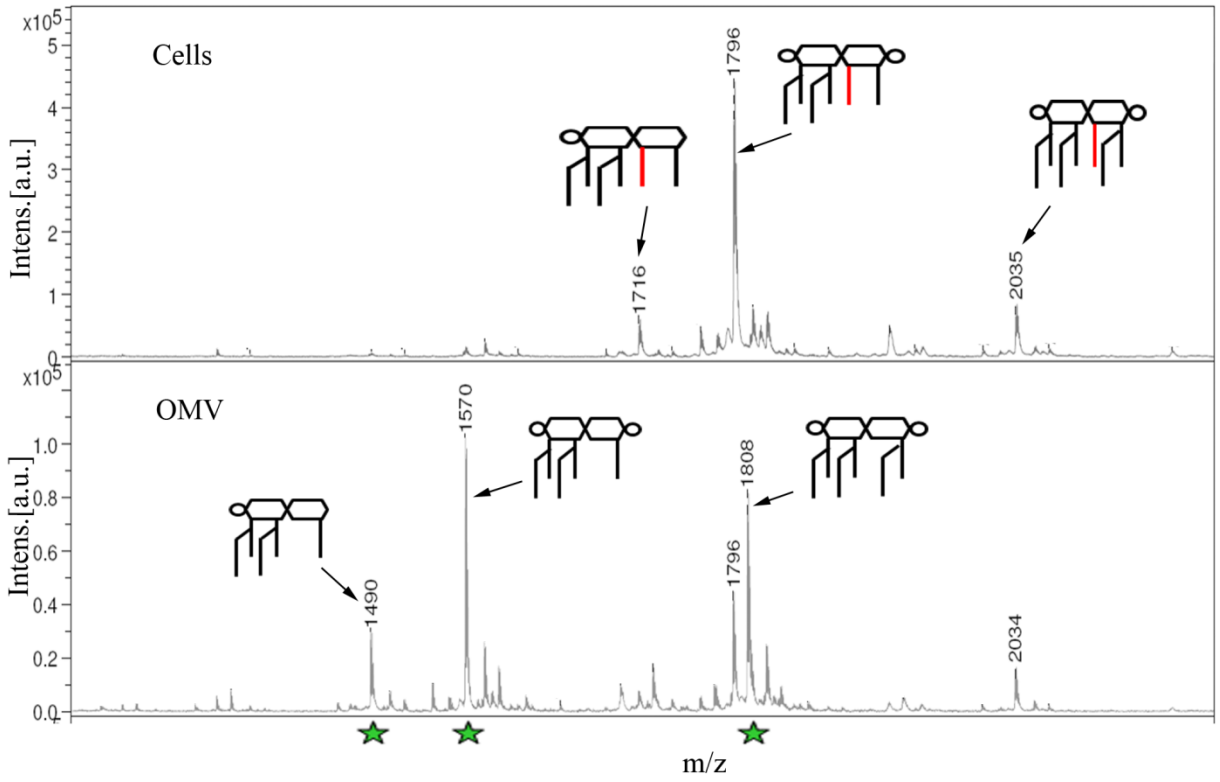


Figure 3.7. Deacylated lipid A is preferentially packed into *S.* Typhimurium OMV when PagL is expressed. Lipid A was purified from cells and OMV of *S.* Typhimurium strain expressing PagL from pWel2. MS analysis of the purified lipid A revealed the accumulation of deacylated lipid A species (marked by green stars) in OMV compared to cells. The major deacylated lipid A species detected in OMV are; bisphosphorylated hexaacylated lipid A (m/z 1808), bisphosphorylated pentaacylated lipid A (m/z 1570) and monophosphorylated pentaacylated lipid A (m/z 1490). On contrary, the major lipid A forms that were detected in

cells had the β-hydroxymyristoyl group at the 3-position of lipid A. The main cellular lipid A species detected were bisphosphorylated heptaacylated lipid A (m/z 2035), bisphosphorylated hexaacylated lipid A (m/z 1796) and monophosphorylated hexaacylated lipid A (m/z 1716). These results were obtained from two independent experiments.

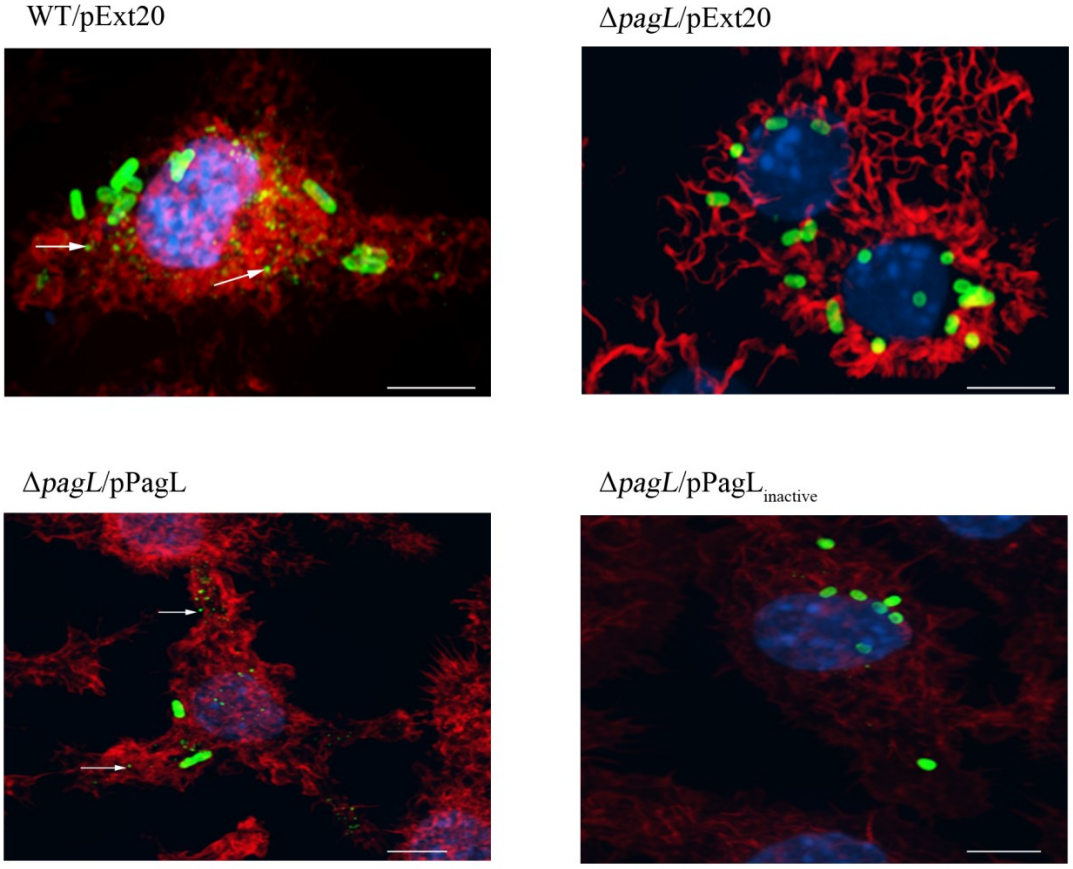
3.3.8- Detection of OMV in S. Typhimurium-infected macrophages

Next, we sought to monitor OMV secretion by intracellular *S.* Typhimurium wild type and *pagL*$^{-}$ strains. The secretion of LPS-positive vesicles by intracellular *S.* Typhimurium has been reported before (Garcia-del Portillo *et al.*, 1997; Guidi *et al.*, 2013). A previous study demonstrated the release of LPS by *S.* Typhimurium into the infected epithelial cells. The secreted LPS were detected in multiple cellular compartments including, the SCV and the host cell cytosol (Garcia-del Portillo *et al.*, 1997). Recently, Guidi et al showed that *S.* Typhimurium can secrete OMV packed with cytolethal distending toxin (CDT) inside infected epithelial cells. The secreted vesicles tested positive for *S.* Typhimurium LPS (Guidi *et al.*, 2013). Together, these results suggested that *S.* Typhimurium produces OMV during its intracellular life.

To examine the role of PagL in intracellular vesiculation, we compared a wild type *S.* Typhimurium strain, and a mutant lacking *pagL*, for intracellular OMV secretion. Both strains were used to infect J774A.1 mouse macrophages, followed by immunofluorescence to visualize the intracellular LPS using a specific monoclonal antibody. We used opsonization to normalize the number of intracellular bacteria. Moreover, we employed confocal microscopy to examine OMV production by intracellular bacteria. Interestingly, our microscopy analysis of the

intracellular wild type strain revealed the presence of nanosized vesicles that tested positive for LPS, suggesting that they are OMV. These vesicles were detected at a close vicinity and apart from the intracellular bacteria. On contrary, cells infected with the Δ*pagL* strain displayed significantly lower levels of intracellular OMV secretion relative to wild type (figure 3.8A). To quantify the levels of intracellular OMV production by different *S.* Typhimurium strains, we used Image J software to count LPS-positive vesicles of size 250 nm and below, followed by normalizing the detected OMV by the number of intracellular bacteria. Furthermore, any macrophages with LPS-positive particles between 250 nm and 0.5 μm in size were considered to contain lysed cells, and thus, were excluded from our analysis. Intracellular OMV production was restored by the in trans expression of PagL from its native promoter. On contrary, the expression of inactive PagL variant in the Δ*pagL* strain did not increase intracellular vesiculation to wild type levels. Our results suggest a role for PagL-mediated lipid A deacylation in the intracellular OMV production by *S.* Typhimurium.

A)


WT/pExt20
ΔpagL/pExt20
ΔpagL/pPagL
ΔpagL/pPagL inactive

B)

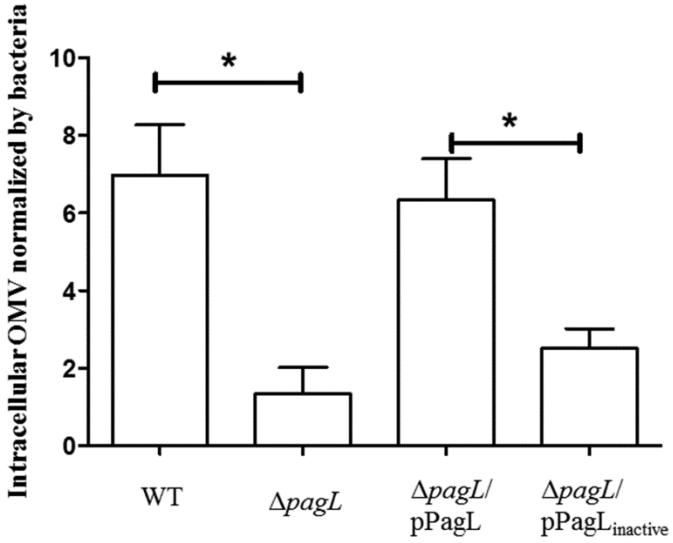

Intracellular OMV normalized by bacteria
10
8
6
4
2
0
*
*
WT
ΔpagL
ΔpagL/ pPagL
ΔpagL/ pPagL inactive

Figure 3.8. PagL might play a role in intracellular vesiculation of *S.* Typhimurium. Fluorescence imaging of OMV (marked by white arrows) production in J774A.1 mouse macrophages infected with different *S.* Typhimurium strains (A). After incubation, cells were fixed with 4% PFA, stained with monoclonal anti-LPS (**green**), actin (**red**) and DAPI (**blue**). Images were taken with Volocity imaging software using an Olympus IX-81microscope (numerical aperture 1.42) with a Yokagawa spinning disk confocal head and a Hamamatsu EMCCD camera and formatted using Image J. Intracellular OMV of different strains were counted using Image J and normalized by the number of producing bacteria (B). Each image shown represents a set of 30 macrophages. The experiment was repeated twice. Scale bars represent 7 µm. Asterisks denotes significant differences ($P<0.01$).

3.4-Discussion

OMV production is a common feature among Gram-negative bacteria, suggesting that these liposomes are key players in bacterial pathogenesis. Extensive work has been reported on the different functions of OMV (Mashburn-Warren and Whiteley, 2006; Kulp and Kuehn, 2010; Haurat *et al.*, 2015). Despite the well-recognized functions of OMV, the mechanism underlying their formation remains to be elucidated. Various studies have provided compelling data that suggest the presence of selective machinery responsible for vesiculation in bacteria. Many bacteria were found to preferentially pack proteins into their OMV(Haurat *et al.*, 2011; Lappann *et al.*, 2013; Elhenawy *et al.*, 2014; Sidhu *et al.*, 2008; McCaig *et al.*, 2013; Roier *et al.*, 2015). Other lines of evidence suggested that the OMV cargo selection is not restricted to proteins, but extends to lipids as well. Multiple studies demonstrated the enrichment of specific lipid species in OMV of different bacteria, suggesting a membrane remodeling event preceding vesiculation

(Kadurugamuwa and Beveridge, 1995; Kato *et al.*, 2002; Tashiro *et al.*, 2011; Haurat *et al.*, 2011). For example, *P.* gingivalis preferentially packs deacylated lipid A forms into its OMV(Haurat *et al.*, 2011). This suggested a possible role for lipid A deacylation in vesiculation. In this work, we investigated the role of the OM lipid A deacylase, PagL, in OMV formation by *S.* Typhimurium. PagL is regulated by the PhoPQ two component system, which is activated by *S.* Typhimurium inside the host cells (Trent *et al.*, 2001). Previous reports suggested that *S.* Typhimurium produces OMV during its intracellular life. Garcia-del Portillo et al. showed that *S.* Typhimurium can shed extracellular LPS into the intracellular milieu. It was proposed that *S.* Typhimurium exploits the secreted LPS as signaling molecules to interfere with the host cellular pathways (Garcia-del Portillo *et al.*, 1997). Guidi et al. observed the intracellular secretion of CDT toxin in *S.* Typhimurium OMV using immunofluorescence, whereas LPS colocalized with CDT in vesicular structures (Guidi *et al.*, 2013). In this study, we employed immunofluorescence and confocal microscopy to compare the wild type *S.* Typhimurium and a mutant lacking PagL for the intracellular production of OMV. In agreement with previous reports, we detected the production of OMV by the S. Typhimurium wild type strain. On contrary, the strain lacking PagL displayed a dramatic decrease in the production of intracellular vesicles. In trans expression of PagL from its native promoter restored intracellular vesiculation in the mutant, whereas expressing the inactive variant of PagL did not. In this regard, a possible role for PagL in the intracellular vesiculation of *S.* Typhimurium can be surmised.

To study the role of lipid A deacylation in OMV formation in vitro, we recombinantly expressed PagL in *S.* Typhimurium. In addition to PagL activation, growing *S.* Typhimurium under PhoPQ-inducing conditions will activate other lipid A-modifying enzymes, which might affect our analysis (Prost *et al.*, 2007; Prost and Miller, 2008). Moreover, some of these

modifications were shown to inhibit PagL activity (Manabe and Kawasaki, 2008). Furthermore, PagC, another PhoPQ-activated protein, was proposed to provoke OMV formation in *S.* Typhimurium (Kitagawa *et al.*, 2010). Under standard lab conditions, *S.* Typhimurium OMV shared the same lipid A species with the originating OM (figure 3.2). Intriguingly, the expression of PagL resulted in the accumulation of deacylated lipid A species exclusively in the OMV (figure 3.7). The latter observation is similar to what was shown in the OMV of the dental pathogen, *P. gingivalis* (Haurat *et al.*, 2011). Previous studies detected PagL in *S.* Typhimurium OMV under PhoPQ-activating conditions (Kitagawa *et al.*, 2010; Bai *et al.*, 2014). Similarly, the recombinantly expressed PagL migrated to *S.* Typhimurium OMV, as suggested by our MS analysis. Nevertheless, the OMV proteome did not vary with PagL expression (figure 3.3, table 3.2).

Our MS analysis of lipid A supported a model in which PagL activity might be correlated with OMV biogenesis in *S.* Typhimurium. We hypothesized that the PagL-mediated deacylation would change the topology of lipid A facilitating membrane curvature, and thus OMV formation (figure 3.1). The effect of the acylation degree on the shape of lipid A is well-studied, whereas the number of acyl chains affects the angle by which lipid A is tilted to the membrane. Moreover, reducing the number of lipid A acyl chains will decrease the hydrophobic cross-section area of the molecule, shifting its shape from a cone to a cylinder or an inverted cone (Schromm *et al.*, 2000; Seydel *et al.*, 2000). Therefore, we propose that the accumulation of the structurally-modified deacylated lipid A in the OM will disturb its stability, forcing it to bend. The effect of lipids topology on membrane curvature is well-studied in eukaryotes (McMahon and Boucrot, 2015). Lipids like lysophosphatidylcholine and phosphatidylinositol phosphates mediated the formation of positive membrane curvatures due to their inverted conical shape.

Conversely, lipids with small polar head groups relative to their hydrophobic moiety, mediate negative membrane curvature (McMahon and Boucrot, 2015). In this regard, phospholipase A2 enzymatic activity can generate membrane curvatures in the Golgi through the accumulation of the inverted-cone shaped lysophospholipids (de Figueiredo *et al.*, 1998; Brown *et al.*, 2003). Phospholipase A2 is an enzyme that catalyzes the hydrolysis of *sn*-2 ester bond of glycerophospholipids, releasing lysophospholipids. The topological changes in the membrane induced by phospholipase A2 enzymatic activity were shown to mediate membrane budding (Staneva *et al.*, 2004).

To investigate whether PagL might play a similar role in *S.* Typhimurium, we sought to quantify the changes in OMV production associated with PagL expression in vitro. We quantified the LPS content of different OMV preparations to determine the OMV production levels in *S.* Typhimurium strains. In trans expression of PagL for 4 hours from a low copy vector, resulted in approximately 4 fold increase in vesiculation by *S.* Typhimurium. Interestingly, the inactive variant of PagL was unable to induce similar vesiculation levels in *S.* Typhimurium (figure 3.6B). OMV secretion was proposed as a response adopted by bacteria to relieve cell envelope stress (McBroom and Kuehn, 2007). Therefore, we investigated whether PagL expression resulted in an envelope stress that led to vesiculation. We monitored the expression levels of key players in the σ^E, Cpx and Rcs pathways, involved in bacterial envelope stress response (Raivio, 2005; Rowley *et al.*, 2006). Our RT-qPCR analysis suggested that none of the previous pathways was activated when PagL was expressed (figure 3.4). However, some cell lysis was observed with the expression of the PagL constructs (figure 3.6C). It is possible that the overexpression of PagL and $PagL_{inactive}$ resulted in membrane stress that is not sufficient to initiate a general stress response. Nonetheless, this does not explain the higher lysis levels

detected with PagL expression. One possible explanation is that PagL-mediated deacylation is one of many factors required for OMV formation in *S.* Typhimurium. Therefore, the activation of only one component of the machinery might cause some stress in the OM. According to the Bilayer Couple hypothesis, the changes in one leaflet of a bilayer membrane will force the other leaflet to accommodate such changes (Sheetz and Singer, 1974). In this regard, PagL-mediated deacylation might result in the bending of the outer leaflet of the membrane that will force the inner leaflet to bend as well. It is tempting to speculate that cone-shaped lipid species, like cardiolipins, might be needed to accumulate in the OM inner leaflet to alleviate the PagL-mediated stress. Interestingly, PhoPQ activation results in the accumulation of cardiolipins in the OM of *S.* Typhimurium (Dalebroux *et al.*, 2015). However, the role of OM cardiolipins in vesiculation will be the scope of our future studies.

Together, the above results suggest a role for lipid A deacylation in OMV biogenesis. In analogy to the role of phospholipase A2 in eukaryotes, we propose that PagL is involved in remodeling the OM through lipid A deacylation, an event leading to membrane curvature. Nevertheless, our results do not eliminate the possibility that other factors might be involved in OMV biogenesis in *S.* Typhimurium. A previous study suggested that the envelope remodelling events which occur during *S.* Typhimurium growth and division might be responsible for OMV release (Deatherage *et al.*, 2009). However, our study suggests a new mechanism for OMV formation in *S.* Typhimurium. The dissemination of PagL homologues in different bacteria highlights its possible involvement in the vesiculation of many bacteria in addition to *S.* Typhimurium (Geurtsen *et al.*, 2005). Unraveling the underlying mechanisms responsible for OMV formation will improve our understanding of bacterial pathogenesis. Furthermore, it will provide us with new targets for antimicrobial therapy in the postantibiotic era.

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Chapter 4

Discussion

Outer membrane vesicles (OMV) secretion is ubiquitous among Gram-negative bacteria. These proteoliposomes were found to play a myriad of functions in pathogenic and symbiotic bacteria (Mashburn-Warren and Whiteley, 2006; Kulp and Kuehn, 2010; Haurat *et al.*, 2015). However, the mechanism underlying OMV biogenesis remains uncharacterized. Consequently, there is controversy regarding the nature of OMV and whether they are released due to cell lysis. In this thesis, we investigated the mechanisms involved in OMV biogenesis and protein cargo selection. We chose members of genus *Bacteroides* to study how bacteria pack proteins into OMV. Moreover, we examined the role of lipid A remodeling in OMV formation using *Salmonella enterica* serovar Typhimurium (referred to as *S*. Typhimurium) as a model.

4.1-Evidence for OMV cargo selection in *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*

Previous studies showed that bacterial OMV varied in their protein content from the parent outer membrane (OM) (Haurat *et al.*, 2015; Grenier and Mayrand, 1987; Haurat *et al.*, 2011; Lappann *et al.*, 2013; Kahnt *et al.*, 2010). These observations support a model in which bacteria can selectively pack proteins into OMV. However, the molecular basis of OMV cargo selection remains to be elucidated. In the dental pathogen, *Porphyromonas gingivalis*, some OM proteins were always excluded from OMV (Haurat *et al.*, 2011). The excluded proteins were mainly OM receptors. In chapter two of this thesis, we studied OMV protein sorting in members of genus *Bacteroides*, which are genetically related to *P. gingivalis* (Nelson *et al.*, 2003; Fletcher *et al.*, 2009). We analyzed the proteomes of OMV and the parent OM in two prominent members of the gut microbiome; *B. fragilis* and *B. thetaiotaomicron*. Both organisms displayed drastic differences between their OM and OMV proteins (figures 2.2 and 2.6). We employed mass

spectrometry (MS) to identify the protein content of both compartments in each organism. We detected more than hundred proteins in the OMV of both *B. fragilis* and *B. thetaiotaomicron* separately. Interestingly, a significant number of the OMV proteins were not detected in the parent membrane of each organism. These results strongly supported the presence of OMV protein sorting machinery in *Bacteroides*. About 40% of the unique OMV proteins were predicted to be hydrolases while the majority of the OM proteins belong to the TonB-dependent family of receptors (TBDTs). TBDTs are OM receptors responsible for the translocation of different nutrients into the bacterial cell (Noinaj *et al.*, 2010). We have observed the same pattern of proteins distribution between the OM and OMV in both, *B. fragilis* and *B. thetaiotaomicron*. Although this is similar to what was found in *P. gingivalis* (Haurat *et al.*, 2011) and other bacteria (Haurat *et al.*, 2015), the differences between the OM and OMV proteomes in *Bacteroides* were unprecedented. The symbiotic lifestyle of *Bacteroides* in the gut might provide an explanation for the high abundance of hydrolases in the OMV. Members of genus *Bacteroides* are known for their ability to degrade a wide variety of glycans, including host-derived oligosaccharides (Martens *et al.*, 2008; Koropatkin *et al.*, 2012). As a consequence for their nutritional needs, they evolved to rely on complex systems for glycans digestion and acquisition, named the polysaccharide utilization loci (PULs) (Martens *et al.*, 2009). The prototype is the starch utilization system (Sus) in *B. fragilis*, which consists of surface localized receptors, hydrolases and a TBDT (Koropatkin and Smith, 2010). Collectively, these proteins function to sequester starch polymers and hydrolyze them into oligomers that can be translocated by the TBDT to the periplasm. The periplasmic hydrolases can now access these oligomers to degrade them to monomers that can be utilized by the bacteria (Martens *et al.*, 2009). Genomes of different members belonging to the *Bacteroides* genus encode multiple PULs that are specific for

different polysaccharides. For example, Sus-like proteins represent around 18% of *B. thetaiotaomicron* genome (Koropatkin and Smith, 2010). The tremendous ability of these bacteria to digest a broad range of nutrients allowed them to survive in a dynamic niche like the human gut. However, the high efficiency of the PULs is associated with a fitness burden on the bacteria due to the complexity of these systems. Therefore, it is challenging for one species to have enough PULs to cover the broad spectrum of glycans in the colon. Rakoff-Nahoum et al. demonstrated how different members of genus *Bacteroides* rely on each other for nutrients acquisition (Rakoff-Nahoum *et al.*, 2014). Some species were lacking certain glycan-hydrolyzing enzymes that were supplied by other members of genus *Bacteroides*. OMV were found to play a fundamental role in this ecological network. The vesicles secreted by one species were packed with enzymes that can digest glycans supporting the growth of another (Rakoff-Nahoum *et al.*, 2014). Therefore, this might explain the large number of hydrolases detected in *Bacteroides* OMV. On contrary, it was not surprising that nutrients receptors constituted the majority of OM proteins. The role of TBDTs in nutrients translocation obligates their localization in the OM. Therefore, packing hydrolases into OMV serves an altruistic behavior that provides other bacteria in the gut with nutrients. Furthermore, OMV secretion gives *Bacteroides* access to the out-of-reach nutrients. Additionally, the liposomal nature of OMV confers enzymes more stability compared to the soluble form.

Approximately 80 % of the unique proteins in the OMV of *B. fragilis* and *B. thetaiotaomicron* were acidic (i.e. had low pI). Conversely, the majority of proteins in the OM of both organisms were alkaline. Despite OMV cargo selection was reported in many studies, this is the first time to detect a common signature among the proteins that were selectively packaged into bacterial vesicles. Based on our results, we propose the hypothetical model

presented in figure 4.1. In this model, acidic hydrolases are recruited to certain regions of the OM by a still uncharacterized, positively charged recruiting factor. The recruiting factor could be a protein or group of proteins, a glycan or a lipid. The sorting factor would recruit specific proteins based on their charge, and thus mediate protein compartmentalization in the OM. OMV would be formed in these regions by an unknown machinery that would generate OM curvature. The secreted OMV containing the hydrolytic enzymes may accomplish a "social" function by degrading polysaccharides, glycoconjugates, and proteins, allowing the same bacteria and others to utilize the resulting peptides, amino acids, mono- and oligo- saccharides. Furthermore, we were able to demonstrate the ability of *Bacteroides* members to sense the nutrients available and modify the OMV hydrolases in response. Growing *B. fragilis* on fucose as a carbon source upregulated the OMV fucosidases. Similarly, *B. thetaiotaomicron* secreted more chitinolytic enzymes into OMV in the presence of N-acetyl glucosamine, the building monomer of chitin (Adrangi and Faramarzi, 2013). Interestingly, these results suggested the presence of a regulatory mechanism involved in OMV protein recruitment.

To identify the molecular factors responsible for OMV biogenesis and/or cargo selection, we developed a genetic screening assay. By growing *B. thetaiotaomicron* mutant libraries on N-acetyl glucosamine, we stimulated the secretion of chitinolytic enzymes into OMV. Using fluorogenic substrates, we were able to determine the chitinolytic activity of OMV produced by different mutants relative to the reference strain. We hypothesized that mutants with lower OMV chitinolytic activities than that of the reference strain might be defective in vesiculation or OMV proteins sorting. Conversely, a hypervesiculating mutant might display elevated enzymatic levels in OMV compared to the reference strain. Unfortunately, our screening method was not stringent enough to isolate mutants with defects in OMV biogenesis. As previously discussed in chapter

two, there are different factors that might have affected the stringency of our method. This included phase variation, growth defects, and technical factors. Therefore, the optimization of the screening method will be the scope of our future studies.

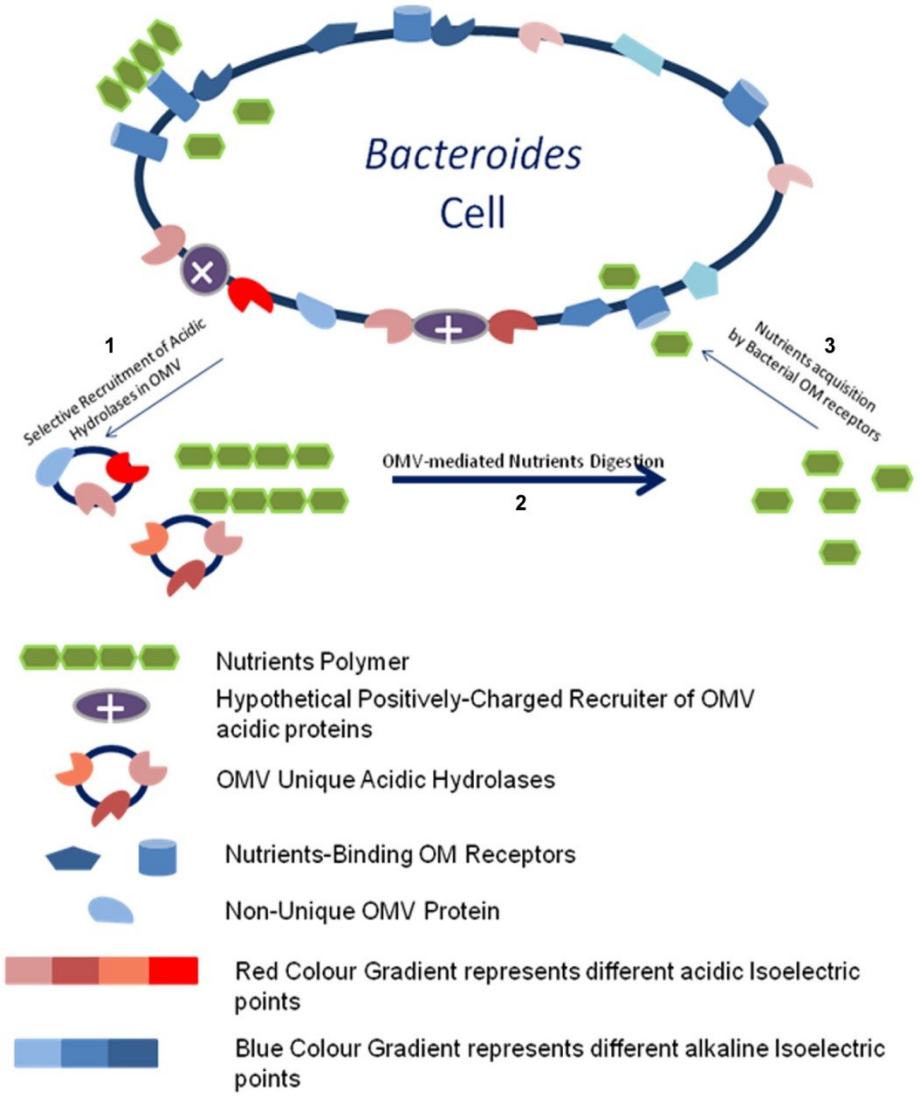

Figure 4.1. Hypothetical model for selective protein packaging in *Bacteroides* OMV. A positively charged component in the OM might recruit acidic hydrolases and glycan-binding

proteins to OMV based on their charge. Once secreted, OMV will encounter the nutrients that the bacteria cannot reach and digest them into readily absorbable components that can be captured by the TonB-dependent receptors found exclusively in the OM.

4.2- Possible role for lipid A remodeling in OMV biogenesis

MS analysis of lipid A purified from OMV and cells of *P. gingivalis* revealed interesting results. There was an uneven distribution of lipids between the OMV and the parent OM in *P. gingivalis*. Pentaacylated and tetraacylated lipid A were the predominant forms in the cells of *P. gingivalis*, while most of the lipid A forms detected in the OMV were triacylated (Haurat *et al.*, 2011). These results suggested a possible role for lipid A deacylation in OMV formation. In chapter 3, we investigated the latter hypothesis using the well-characterized enteric pathogen *S.* Typhimurium as a model organism. *S.* Typhimurium is known for its ability to modify its lipid A in different ways. Most of these modifications are mediated by the two-component regulatory system PhoPQ, which is activated by host cues during the intracellular phase of infection (Guo *et al.*, 1998; LaRock *et al.*, 2015). PagL is a PhoPQ-dependent lipid A deacylase that is localized in the OM. When activated by PhoPQ system, PagL mediates the removal of the β-hydroxymyristoyl chain from 3-position of lipid A, a modification that makes *S.* Typhimurium less detectable by the immune system (Trent *et al.*, 2001; Kawasaki *et al.*, 2004). We hypothesized that the role of PagL is not restricted to the evasion of immune detection but includes OMV formation. The PagL-mediated deacylation will decrease the hydrophobic cross section area of lipid A. Changing the acylation status of lipid A forces the molecule to undergo topological changes towards an energetically favorable shape. Therefore, the deacylation of hexaacylated lipid A will convert its structure from a cone shaped to a cylindrical or an inverted

cone shaped molecule (Schromm *et al.*, 2000; Seydel *et al.*, 2000). We proposed that PagL-mediated deacylation would trigger topological changes in lipid A, hence forcing the OM to bulge. Previous studies reported the intracellular OMV secretion by *S*. Typhimurium (Garcia-del Portillo *et al.*, 1997; Guidi *et al.*, 2013). To examine the possible role of PagL in vesiculation inside the host, we monitored OMV formation by *S*. Typhimurium inside infected macrophages. We employed immunofluorescence to compare the wild type strain with a mutant lacking PagL for intracellular OMV production. We detected OMV secretion inside macrophages infected with the wild-type strain. On contrary, macrophages infected with the mutant displayed significantly less intracellular OMV. Only the expression of enzymatically-active PagL restored the OMV production by the Δ*pagL* strain intracellularly. To examine the role of PagL in OMV production in vitro, we adopted a biochemical approach. The expression of PagL in *S*. Typhimurium in vitro resulted in an exclusive accumulation of deacylated lipid A forms in the OMV. We could not detect any deacylated forms in the cells. Intriguingly, these findings are similar to what was observed in *P. gingivalis* OMV (Haurat *et al.*, 2011). To quantify OMV production during PagL expression, we measured the 3-deoxy-D-manno-octulosonic acid (KDO) content of OMV purified from different *S*. Typhimurium strains. There are two KDO groups decorating each LPS molecule in *S*. Typhimurium (Munson *et al.*, 1978). Since LPS is a basic component of OMV, KDO quantities should reflect the amount of vesicles produced by each strain. Using our KDO quantification assay, we observed higher levels of OMV production by the *S*. Typhimurium transformant expressing PagL relative to the control strain carrying the empty vector. We questioned whether the elevated vesiculation levels observed with PagL expression are due to its lipid A deacylase activity and did not result from envelope stress. A previous study suggested that OMV are produced by bacteria to exclude misfolded proteins and alleviate envelope stress

(McBroom and Kuehn, 2007). In this regard, PagL expression might be causing stress in the OM that led to vesiculation. Therefore, we adopted several strategies to address the latter possibility. First, we monitored the activation levels of σ^E, Cpx and Rcs pathways that represent the main envelope stress response pathways in Gram-negative bacteria (Raivio, 2005; Huang *et al.*, 2006). Our RT-qPCR analysis revealed that none of the previous pathways were activated when PagL was expressed. Second, we created a catalytically inactive variant of PagL ($PagL_{inactive}$) that was expressed at equal levels in *S.* Typhimurium. Using an in vitro lipid A deacylation assay, we confirmed that $PagL_{inactive}$ had lost its deacylase activity. The levels of OMV production, when $PagL_{inactive}$ was expressed in *S.* Typhimurium, did not vary significantly from that of the control strain. The above results suggested that PagL-mediated deacylation might mediate OMV formation in *S.* Typhimurium. However, we observed elevated levels of cell lysis when PagL constructs were expressed in *S.* Typhimurium compared to the control strain. This suggested that the expression of the PagL constructs triggered some stress in the OM, albeit, not sufficient to activate the main envelope stress response pathways. However, the expression of PagL resulted in more cell lysis compared to that resulting from $PagL_{inactive}$ expression. One possible explanation is that PagL is one of many factors required for vesiculation in *S.* Typhimurium. Therefore, the overexpression of only one component of the machinery might create stress in the OM. The Bilayer Couple hypothesis states that the changes in one leaflet of a bilayer membrane would trigger changes in the opposite leaflet to accommodate any deformation in the membrane shape (Sheetz and Singer, 1974). The expression of PagL might mediate curvature formation in the LPS outer leaflet of the OM at the site of OMV formation. Therefore, the inner leaflet of the OM might be forced to bend in response, which might create stress in the OM. It is tempting to speculate that the accumulation of deacylated lipid A forms in the OM outer leaflet requires the

enrichment of cone-shaped lipids, like cardiolipins, in the opposite leaflet of the membrane. In addition to PagL induction, PhoPQ activation was shown to mediate the accumulation of cardiolipins in the OM (Dalebroux *et al.*, 2015). Therefore, it will be important in the future to study the involvement of OM cardiolipins in OMV formation.

4.3-Concluding remarks

In agreement with previous studies, the results obtained from our work suggest that OMV production in bacteria is the outcome of a regulated mechanism. If OMV are produced due to cell lysis, it would be expected to share the same protein content of the parent OM. Nevertheless, our proteomic analysis of OM and OMV fractions in *Bacteroides* suggest the presence of protein sorting machinery responsible for packing specific proteins into OMV and/or excluding others by retaining them in the OM. Hydrolases represented a significant fraction of the OMV specific cargo, with the possible presence of a charge-based mechanism for their recruitment. Moreover, we demonstrated that members of genus *Bacteroides* can modify their OMV cargo in response to the surrounding environment by packing the necessary hydrolases for the degradation of the presented glycan. Furthermore, our work in *S.* Typhimurium supports a model which correlates OM lipid remodeling with OMV formation. Previous studies have reported the presence of different lipid species in the OMV compared to the OM of various bacteria (Kadurugamuwa and Beveridge, 1995; Kato *et al.*, 2002; Tashiro *et al.*, 2011; Haurat *et al.*, 2011). The role of membrane lipids remodeling in vesiculation is well-studied in eukaryotes. For example, exosomes had different lipid composition compared to the parental cell membrane. Sphingolipids were enriched in exosomes, albeit, phosphatidylcholine was less abundant in the exosomal fraction relative to the originating membrane (Laulagnier *et al.*, 2004). Moreover, phospholipase

A2-mediated deacylation of membrane phospholipids was found to contribute to the Golgi tubule formation. As a consequence of phospholipase A2 activity, the inverted cone shaped lysophospholipids will accumulate in the membrane, mediating its budding (de Figueiredo *et al.*, 1998; Brown *et al.*, 2003; Staneva *et al.*, 2004). Our work in *S.* Typhimurium suggested a similar role of lipid remodeling in vesiculation. Our results demonstrated the involvment of PagL in intracellular OMV formation. Moreover, we were able to demonstrate that OMV and OM did not share the same lipid A species when PagL was expressed. Together, the previous results indicate that OMV cargo selection includes both, protein and lipid, components of the secreted vesicles.

4.4-Future directions

With the advances in MS-based proteomics, the OMV protein content in many bacteria was analyzed. This improved our understanding of the different roles carried out by OMV during bacterial life, albeit, there are many aspects of OMV formation that remain undetermined. Different groups investigated the mechanisms underlying OMV formation; nevertheless, a non-vesiculating mutant was never isolated. It is possible that vesiculation is essential to bacteria, and without vesiculation OM proteins might accumulate causing high levels of envelope stress that are beyond the capacity of canonical stress response pathways. However, it will be important to understand how vesiculation is regulated. Moreover, it is not known how bacteria secure the energy needed for vesiculation with the absence of nucleoside triphosphates in the periplasm. Lipid remodeling can mediate membrane curvature formation in eukaryotes. Therefore, it might be possible that the same process occurs in bacteria. Indeed, more lipidomic analysis of OMV in different bacteria is required to address the latter possibility. Deciphering the mechanisms of OMV formation in bacteria will not only improve our understanding of bacterial pathogenesis

but will also provide us with new targets for antimicrobial therapy in a time when antibiotic resistance became widespread.

4.5-References

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Appendix

Protein *O*-linked Glycosylation in the plant pathogen *Ralstonia solanacearum*

This appendix was submitted to Glycobiology on July 10th, 2015 as "Wael Elhenawy, Nichollas E. Scott, M. Laura Tondo, Elena G. Orellano, Leonard J. Foster, Mario F. Feldman.(2015) Protein O-linked Glycosylation in the plant pathogen *Ralstonia solanacearum*. Glycobiology (Manuscript number: GLYCO-2015-00120)".

X.1-Abstract

Ralstonia solanacearum **is one of the most lethal phytopathogens in the world. Due to its broad host range, it can cause wilting disease in many plant species of economic interest. In this work, we identified the *O*-Oligosaccharyltransferase (*O*-OTase) responsible for protein *O*-glycosylation in *R. solanacearum*. An analysis of the glycoproteome revealed 20 glycosylated proteins, including type IV pilins. Although multiple glycan forms were identified, the majority of the glycopeptides were modified with a pentasaccharide composed of HexNAc-(Pen)-dHex$_3$, similar to the O antigen subunit present in the LPS of multiple *R. solanacearum* strains. A mutant strain unable to glycosylate proteins displayed lower biofilm levels compared to wild-type. Preliminary virulence tests suggested a possible role for protein *O*-glycosylation in the pathogenesis of *R. solanacearum*. A comparative proteomic analysis was carried out to mine for global changes in protein expression when protein *O*-glycosylation is lost. Our proteomic analysis suggested that in absence of glycosylation, the levels of one of the glycosylation targets, the type IV pilins, are diminished. At the same time, disruption of glycosylation triggers an increase in the levels of a surface lectin homologous to *Pseudomonas* PA-IIL. These results reveal the important role of glycosylation in the pathogenesis of *R. solanacearum*.**

X.2-Introduction

Ralstonia solanacearum is a soil-borne β-proteobacterium known to cause lethal wilts in more than 200 plant species in all continents leading to enormous economic losses (Genin & Denny, 2012, Peeters *et al.*, 2013). In soil, the pathogen relies on flagellar-driven swimming to access the plant vascular system via the roots (Tans-Kersten *et al.*, 2001, Yao & Allen, 2006). Once inside the host, *R. solanacearum* rapidly colonize the xylem tissues and produce large amounts of extracellular polysaccharides (EPS) (Orgambide *et al.*, 1991, Saile *et al.*, 1997). Accumulation of the released polysaccharides is required for the establishment of colonization as well as vascular occlusion of the infected plant (Araud-Razou *et al.*, 1998, Denny & Baek, 1991, Husain & Kelman, 1958, Kao *et al.*, 1992). Additionally, *R. solanacearum* uses its type III secretion system (T3SS) to hijack host cellular pathways in order to avoid detection by the plant immune system (Coll & Valls, 2013, Peeters *et al.*, 2013, Erhardt *et al.*, 2010). *R. solanacearum* is known to secrete a large number of T3SS effectors, many of which were acquired by horizontal gene transfer (Poueymiro & Genin, 2009). Other virulence factors in *R. solanacearum* include a type II secretion system, extracellular cellulases and pectinases, and type IV pili (Peeters *et al.*, 2013). Type IV pili are involved in many biological processes including adhesion, twitching motility, biofilm formation and horizontal gene transfer (Fussenegger *et al.*, 1997, Merz *et al.*, 2000, Strom & Lory, 1993). These filamentous appendages are formed by the polymerization of pilin monomers that can reach a few micrometers in length (Fernandez & Berenguer, 2000, Strom & Lory, 1993). Type IV pili were found to be dispensable for the virulence of some phytopathogens like *Xanthomonas campestris* (Ojanen-Reuhs *et al.*, 1997). However, the loss of type IV pili in *R. solanacearum* resulted in attenuated virulence in a tomato

plant model. Both twitching motility and biofilm formation were impaired in *R. solanacearum* mutants lacking type IV pili (Liu *et al.*, 2001, Kang *et al.*, 2002).

Pilin proteins are *O*-glycosylated in many bacteria, including *Neisseria meningitidis, N. gonorrhoeae*, *Pseudomonas aeruginosa, Francisella tularensis*, *Acinetobacter nosocomialis*, *A. baylyi* and *Burkholderia cenocepacia* (Marceau *et al.*, 1998a, Jennings *et al.*, 2011, Castric, 1995, Lithgow *et al.*, 2014, Egge-Jacobsen *et al.*, 2011, Harding *et al.*, 2015). Protein *O*-glycosylation is a post-translational modification that is widespread among bacteria. Bacteria can utilize cytoplasmic glycosyltransferases to *O*-glycosylate their proteins by sequential addition of sugars. Alternatively, the glycan can be assembled on a lipid carrier in the inner membrane and then transferred to the acceptor protein in the periplasm via *O*-Oligosaccharyltransferases (*O*-OTase) (Iwashkiw *et al.*, 2013). OTase-dependent glycosylation systems have been described in many bacteria including *Neisseria spp*., *Bacteroides fragilis*, *F. tularensis*, *Acinetobacter baumannii* and *B. cenocepacia* (Marceau *et al.*, 1998a, Fletcher *et al.*, 2009, Balonova *et al.*, 2012, Iwashkiw *et al.*, 2012, Lithgow *et al.*, 2014). Moreover, functional OTases were found in *Vibrio cholerae* and *B. thailandensis* (Gebhart *et al.*, 2012). Although protein *O*-glycosylation is required for the virulence of many bacteria, its exact role remains unclear (Iwashkiw *et al.*, 2013).

O-OTases share the Wzy_C domain with the closely related WaaL enzymes involved in lipopolysaccharide synthesis. Indeed, both bioinformatics and biochemical approaches can be employed to differentiate between the two enzymes (Schulz *et al.*, 2013, Gebhart *et al.*, 2012). The WaaL enzyme in *R. solanacearum* GMI1000 was recently identified (Li *et al.*, 2014). *In silico* analysis of *R. solanacearum* GMI1000 genome revealed the presence of a protein carrying

the Wzy_C domain (Power *et al.*, 2006). The putative *O*-OTase (Rsc0559) is located downstream of PilA, the Type IV pilin subunit protein. This suggested the presence of a functional *O*-glycosylation system in *R. solanacearum,* with pilin as one of the glycoproteins. In this work, we demonstrate that *O*-glycosylation in *R. solanacearum* extends to 20 proteins. Our assays show that *O*-glycosylation is important for biofilm formation and might be involved in the virulence of *R. solanacearum* towards tomato plants. Furthermore, we analyzed the changes in the proteome of *R. solanacearum* in response to disruption of glycosylation.

X.3-Experimental procedures

X.3.1-Bacterial strains and growth conditions

R. solanacearum GMI1000 strains were grown on BG medium at 30ºC as previously described (Kang *et al.*, 2002). When needed, tetracycline (Tc) was added at concentration 3 µg mL^{-1}. *E. coli* CLM24 was grown on LB medium at 37 ºC.

X.3.2-In vivo protein glycosylation assay

Rsc0559 was amplified from the genome of *R. solanacearum* GMI1000 via PCR using primers; GMOtaSmaIFw (5'-AATTCCCGGGATGTTGTGGCCGGTCTGG**)** and GMOtaHindIIIHisRv (5'-TGGT AAGCTTTTAGTGGTGGTGGTGGTGGTGATCTACACCGACAACCAAGT), then cloned into SmaI/HindIII sites of pEXT20. The *in vivo* glycosylation experiment was carried out as described by Gebhart C *et al*, 2012 (Gebhart *et al.*, 2012). Briefly, *E. coli* CLM24 strains (lacking WaaL ligase), expressing bacillosamine biosynthesis genes from *Neisseria*, were grown at 37ºC. At mid-log phase, 0.1 mM IPTG and 0.2% arabinose were added to induce the

expression of different *O*-OTases and DsbA respectively. Cells were incubated until stationary phase and glycosylated proteins were detected via immunoblotting using the monoclonal anti-Histidine and the polyclonal anti-bacillosamine antibodies (Gebhart *et al.*, 2012).

X.3.3-Construction of *O*-OTase mutant in R. solanacearum GMI1000 and complementation

The suicide vector pTOK3 was used to make clean deletions in *R. solanacearum*. pTOK3 was obtained after cloning *sacB* from pFLP2 into pTOK2 (Kang *et al.*, 2002, Iwashkiw *et al.*, 2012). Briefly, pTOK2 was cut using BamHI, then both the vector and *sacB* were blunt-ended using klenow fragment (ThermoScientific) followed by ligation. In order to make a clean deletion of Rsc0559 in *R. solanacearum* GMI1000, about 500 bp of the flanking regions around Rsc0559 were PCR amplified using primers; GMOtaUpFwSmaI (5'-AATTCCCGGGTATTCTGGCTGCGATTGCC) and GMOtaUpRv (5'-AATTTTCTTTTTGAGACGCAATCCCAGGGAACGATGAACTGGAA) for the upstream region, while primers GMOtaDwnFw (5'-AATTTTCCAGTTCATCGTTCCCTGGGATTGCGTCTCAAAAAGAA) and GMOtaDwnRvHindIII (5'-TATTAAGCTTAGCGGACGTCGGATTTGATC) were used to amplify the downstream region. Both regions were cloned into pTOK3 using SmaI and HindIII sites, and the resulting plasmid was used to transform *R. solanacearum* using electroporation. Tc resistant colonies were grown on BG broth overnight then subcultured on sucrose-supplemented minimal medium (Liu *et al.*, 2005). Sucrose resistant colonies were screened by PCR to confirm the second recombination event followed by sequencing to confirm the deletion of Rsc0559. For *in cis* complementation, Rsc0559 plus the upstream and downstream regions were amplified

using primers; GMOtaUpFwSmaI and GMOtaDwnRvHindIII, and cloned into pTOK3. A silent mutation was introduced into Rsc0559 by changing the codon for the serine residue at position 380 from "TCG" to "TCA" as a scar to discriminate the *in cis* complemented and the wild type strains.To complement the mutation *in trans*, Rsc0559 was amplified via PCR using primers; GMOtaSacIFw (5'-AATTGAGCTCATGTTGTGGCCGGTCTGG**)** and GMOtaBamHIHisRv (5'-TGGTGGATCCTTAGTGGTGGTGGTGGTGGTGATCTACACCGACAACCAAGT), then cloned into pHC60, a vector that is stable in planta, using SacI and BamHI sites (Cheng & Walker, 1998)..

X.3.4-LPS analysis by silver-stained SDS-PAGE gel

LPS was prepared from *R. solanacearum* strains using 10 mg of dried cells using the method of Yi and Hackett (Yi & Hackett, 2000). LPS was run on a 15% SDS-PAGE and visualized by the silver staining method described by Tsai and Frasch (Tsai & Frasch, 1982).

X.3.5-Digestion of membrane enriched samples of *R. solanacearum*

Peptide lysates for glycopeptide enrichment and quantitative analysis were prepared according to Lithgow *et al* with minor modifications (Lithgow *et al.*, 2014). Briefly, 2 mg of dried membrane enriched protein samples were solubilized in 6 M urea, 2 M thiourea, 40 mM NH_4HCO_3 and reduced with 10 mM Dithiothreitol (DTT) followed by alkylated with 25 mM iodoacetamide (IAA) for one hour in the absence of light. The resulting alkylated protein mixture was then digested with Lys-C (1/100 w/w) for 4 hrs, diluted 1:5 in 40 mM NH_4HCO_3 and digested with trypsin (1/50 w/w) overnight at 25°C. Digestion was terminated with the addition of 1% trifluoroacetic acid (TFA) and peptide digests were purified using the C_{18} empore (Sigma-

Aldrich, St. Louis MO) STop And Go Extraction (STAGE) tips to remove primary amide and salts which can interfere with dimethyl labeling and ZIC-HILIC glycopeptide enrichment protocols described below.

X.3.6-Enrichment of *R. solanacearum* glycopeptides using ZIC-HILIC purification

ZIC-HILIC enrichment was performed according to Scott NE *et al*, 2011 with minor modifications (Scott *et al.*, 2011). Micro-columns composed of 10 μm ZIC-HILIC resin (Sequant, Umeå, Sweden) packed into p10 tips containing a 1 mm^2 excised C_8 Empore™ disc (Sigma) were packed to a bed length of 0.5 cm. Prior to use, the columns were washed with ultra-pure water, followed by 95% acetonitrile (ACN) and then equilibrated with 80% ACN and 5% formic acid (FA). Samples were resuspended in 80% ACN and 5% FA, and insoluble material was removed by centrifugation at 16,100 × *g* for 5 min at 4ºC. Samples adjusted to a concentration of 3 μg/μL and 150 μg of peptide material were loaded onto a column and washed with 10 load volumes of 80% ACN, 5% FA. Unbound fractions were collected, pooled and dried by vacuum centrifugation. ZIC-HILIC bound peptides were eluted with 3 load volumes of ultra-pure water and concentrated using vacuum centrifugation. Biological replicates of *R. solanacearum* strains were subjected to ZIC-HILIC independently using freshly prepared reagents.

X.3.7-Identification of glycopeptides using reversed-phase LC-MS, CID MS-MS and HCD MS-MS

Purified glycopeptides/peptides were resuspended in Buffer A (0.5% acetic acid) and separated using reversed-phase chromatography on either an Agilent 1290 Series HPLC (Agilent

Technologies, Mississauga, ON) coupled to LTQ-Orbitrap Velos (Thermo Scientific, San Jose CA) for qualitative analysis of glycopeptides or a EASY-nLC1000 system coupled to a Q-exactive for quantitative studies. For qualitative analysis of *R. solanacearum* glycopeptides, a packed in-house 20 cm, 75 µm inner diameter, 360 µm outer diameter, ReproSil – Pur C_{18} AQ 1.9µm (Dr. Maisch, Ammerbuch-Entringen, Germany) column was used, while for quantitative studies a house packaged 45 cm, 50 µm inner diameter, 360 µm outer diameter, ReproSil – Pur C_{18} AQ 1.9µm column was used. In both systems, samples were loaded onto a trap column, an in-house packed 2cm, 100 µm inner diameter, 360 µm outer diameter column containing Aqua 5µm C18 (Phenomenex, Torrance, CA), at 5 µL/min prior to gradient separation and infused for mass spectrometry. A 180 min gradient was run from 0% buffer B (80% ACN, 0.5% acetic acid) to 32% B over 140 min, then from 32% B to 40% B in the next 5 min, then increased to 100% B over 2.5 min period, held at 100% B for 2.5 min, and then dropped to 0% B for another 20 min. Unbound fractions from ZIC-HILIC glycopeptide enrichment were subjected to analysis using the same instrumental set up as qualitative analysis of glycopeptides. Both instruments were operated using Xcalibur v2.2 (Thermo Scientific) with a capillary temperature of 275°C in a data-dependent mode automatically switching between MS, CID MS-MS and HCD MS-MS for qualitative analysis as previously described and using a top 10 data-dependent approach switching between MS (resolution 70k, AGC target of $1x10^6$), and HCD MS-MS events (resolution 17.5k, AGC target of $1x10^6$ with a maximum injection time of 60ms, NCE 28 with 20% stepping) for quantitative studies. High resolution CID analysis was performed on an LTQ-Orbitrap Velos with CID fragmentation (NCE 35, 10 msec activation) analyzed within the orbital trap (resolution 7.5k, AGC $5.0x10^4$), to enable the detection of high m/z ion, the high mass range setting was used (Scott *et al.*, 2011). Raw files were processed as previously described by Scott

NE *et al*, 2011 (Scott *et al.*, 2011). Briefly, Proteome Discoverer v. 1.2 (Thermo Scientific) was used to search the resulting glycopeptide data using MASCOT v2.4 against the *R. solanacearum* GMI1000 database (obtained from UNIPROT, http://www.uniprot.org/, 2013-04-4, Taxon identifier: 267608 containing 5,014 protein sequences). Mascot searches were performed using the following parameters: peptide mass accuracy 20 ppm; fragment mass accuracy 0.02 Da; no enzyme specificity, fixed modifications - carbamidomethyl, variable modifications - methionine oxidation and deamidated N, Q. The instrument setting of MALDI-QUAD-TOF was chosen as previous studies show quadrupole–like fragmentation within HCD spectra OLSEN JV 2007. Scan events that did not result in peptide identifications from MASCOT searches were exported to Microsoft Excel (Microsoft, Redmond WA). To identify possible glycopeptides within exported non-match scans, the MS-MS module of GPMAW 8.2 called 'mgf graph' was used to identify HCD scan events that contained the 204.08 *m/z* oxonium of HexNAc. All scan events containing the oxonium 204.08 *m/z* ion were manually inspected to identify possible glycopeptides. To facilitate glycopeptide assignments HCD scan events containing the 204.08 oxonium were manual inspected to identify potential deglycosylated peptides ions. Within these HCD scans the MS features (*m/z*, charge and intensity), which corresponded to masses below that of the deglycosylated peptide were extracted using the Spectrum list function of Xcalibur v2.2. The resulting numerical values of the detected MS features were scripted into mgf files and the peptide mass set to that of the deglycosylated peptide mass. The resulting mgf files were then searched using the MASCOT setting described above. All spectra were searched with the decoy option enabled and no matches to this database were detected; the false discovery rate (FDR) was 0%. To further validate all glycopeptide matches, all HCD spectra were annotated using the Expert Annotation tool (http://www.biochem.mpg.de/mann/tools/) .

X.3.8-Quantitative dimethylation of *R. solanacearum* membrane extracts

Quantitative dimethylation of WT, *O*-OTase$^-$ and the complemented strains were undertaken using dimethylation as outlined by Boersema *et al,* 2009 (Boersema *et al.*, 2009). Two biological replicates of each strain were used in the analysis. Briefly, 1 mg of peptide lysate from each strain was resuspended in 30 ul of 100 mM Tetraethylammonium bromide and mixed with the following combinations of 200 mM formaldehyde (30 uL) and 1 M sodium cyanoborohyride (3 uL) isotopologues. For diplex experiments, wild type samples were labeled with light formaldehyde (CH_2O) and light sodium cyanoborohyride ($NaBH_3CN$) and *O*-OTase$^-$ samples with medium formaldehyde (CD_2O) and light sodium cyanoborohyride. For triplex experiments wild type samples were labeled with light formaldehyde (CH_2O) and light sodium cyanoborohyride ($NaBH_3CN$), *O*-OTase$^-$ samples with medium formaldehyde (CD_2O) and light sodium cyanoborohyride and the complemented strain samples with heavy formaldehyde ($^{13}CD_2O$) and heavy sodium cyanoborodeuteride ($NaBD_3CN$). Reagents were mixed and samples incubated at room temperature for 1 hour. Dimethylation reactions were repeated twice to ensure complete labeling of all amine groups. Dimethylation reactions were terminated by the addition of 30 uL of 1 M NH_4Cl for 20 minutes at room temperature. Samples were acidified by addition of 5% (v/v) acetic acid and allowed to equilibrate in the dark for 1 hour before pooling of the three samples in at 1:1:1 ratio. Pooled samples were then STAGE tip cleaned up, lyophilized and stored at -20ºC.

X.3.9-Quantitative proteomic comparison of *R. solanacearum* strains

MaxQuant (v1.4.1.2) was used for identification and quantification of the resulting experiments COX j 2008. Database searching was carried out against the UniProt *R.*

solanacearum GMI1000 database (Taxon identifier: 267608 containing 5,014 protein sequences) with the following search parameters: carbamidomethylation of cysteine as a fixed modification; oxidation of methionine, acetylation of protein N-terminal trypsin/P cleavage with a maximum of 2 missed cleavages. A multiplicity of two and three was used for diplex and triplex experiment respectively, with each multiplicity denoting one of the dimethylation channels (light, medium and heavy respectively). The precursor mass tolerance was set to 6 ppm and MS/MS tolerance 20 ppm in accordance with previously reports with a maximum false discovery rate of 1.0% set for protein identifications. The resulting protein group output was processed within the Perseus (v1.4.0.6) analysis environment to remove reverse matches and common proteins contaminants prior to analysis with Matlab R2012a (http://www.mathworks.com).

X.3.10-Preparation of cell lysates for SDS-PAGE and immunoblotting

Whole cells pellets were obtained from overnight cultures of different *R. soalnacearum* strains after normalization based on OD_{600} values. The harvested cell pellets were then solubilized in urea buffer at 37°c for 30 minutes then loaded on 15% SDS-PAGE gel. Following separation, proteins were transferred to a nitrocellulose membrane and pilin proteins were probed using rabbit polyclonal antibody. As a loading control, cytoplasmic RNA polymerase levels were monitored using mouse monoclonal anti-RNA polymerase (1:2500, RNAP α-subunit; Neoclone). The membrane was incubated with IRDye conjugated anti-mouse and anti-rabbit antibodies to visualize the bands using Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

X.3.11-Biofilm assays

Biofilms were measured as described before (Siri *et al.*, 2014). Briefly, different *R. solanacearum* strains were grown overnight then diluted to OD600 0.1 mL^{-1} (10^8 CFU mL^{-1}) and 20 µL were used to inoculate 200 µL of BG broth in 96 wells plate. Cells were grown for about 48 hrs at 30ºC. The formed biofilms were washed gently with sterile water and stained with 0.1% crystal violet for 30 mins. Excess crystal violet was removed and the plate was washed with water. Stained biofilms were solubilized in 100 uL 95% ethanol, then quantified spectrophotometrically by measuring absorbance at λ=590nm. Readings were normalized by the OD600 values of the corresponding strains after 48 hrs. The comparative statistical analysis of results was performed using SigmaPlot® for Windows (Systat Software, CA, USA). A one-way analysis of variance (ANOVA) followed by Holm-Sidak post hoc test to assess the significance of differences between groups were performed. The differences were considered significant when $p < 0.05$.

X.3.12-Virulence assays in tomato plants

To test the virulence of *R. solanacearum* strains on tomato plants, seeds (*Solanum lycopersicum* Mill., cultivar Platense) were germinated in commercial soil mix and 2-week old seedlings were transplanted into 10 cm-plastic pots and incubated in a growth chamber at 25ºC for 16 hrs photoperiod for 4-5 weeks before inoculation. To prepare the inocula, strains were grown overnight at 28ºC in liquid BG supplemented with appropriate antibiotics. Cells were pelleted by centrifugation, suspended in sterile distilled water and adjusted to 10^6 CFU/mL. Inocula concentrations were checked by plating on BG-agar supplemented with glucose (5 g/L) and triphenyltetrazolium chloride (50 mg/L) to observe typical smooth colonies after 2-day incubation at 28ºC. Leaf petioles were inoculated by making a cut in the first leaves above the

cotyledon (0.5 to 1 cm from their base), then immediately applying a 3 µL droplet of the bacterial suspensions to the wounded surfaces. A set of 6 tomato plants were inoculated with each strain and placed in separated trays in order to prevent cross contamination. After inoculation, plants were incubated in a growth chamber at 28ºC and 16 hrs photoperiod. The experiment was repeated twice under the same conditions to a total of 3 independent experiments.

X.4-Results

X.4.1-*Ralstonia O*-OTase is functional in *Escherichia coli*

Power *et al* (2006) and Schulz *et al* (2013) suggested the presence of an *O*-OTase (Rsc0559) in the genome of *R. solanacearum* GMI1000 (Schulz *et al.*, 2013, Power *et al.*, 2006). Although some *O*-OTases are specific to pilin, others are classified as general *O*-OTases with a relaxed specificity towards their protein targets (Iwashkiw *et al.*, 2013). Our phylogenetic analysis suggested that Rsc0559 is a general *O*-OTase similar to *Neisseria* PglL (figure X.1). To test the activity of the putative *O*-OTase in *R. solanacearum*, we first employed an *in vivo* enzymatic assay (Gebhart *et al.*, 2012). Rsc0559 was recombinantly expressed in *E. coli* CLM24, a W3110 strain that lacks the WaaL O-antigen ligase (Feldman *et al.*, 2005). Deletion of the *waaL* gene eliminates the competition between the O-antigen ligase and the *O*-OTase for the lipid-linked glycans. Concurrently with Rsc0559 expression, both an acceptor protein and the genes coding for the biosynthesis of undecaprenyl-linked 2,4-di-*N*-acetylbacillosamine (diNAcBac) were co-expressed *in trans*. This sugar was shown to be a good substrate for *O*-OTases and a specific antibody for diNAcBac is available. We employed a C-terminal hexahistidine-tagged DsbA (Ng_1706) from *N. meningitidis* as the acceptor protein in this assay

(Vik *et al.*, 2009). Cell lysates of *E. coli* CLM24 strains expressing Rsc0559, *Neisseria* PglL, or an empty vector were separated by SDS-PAGE and analyzed by Western blot to detect glycosylated DsbA. We used a monoclonal anti-hexaHistidine antibody (green channel) to detect DsbA expression while diNAcBac was visualized using a polyclonal antibody (red channel). Overlapping the two signals will yield a yellow colour that is indicative of DsbA glycosylation with bacillosamine. DsbA was glycosylated only when Rsc0559 or PglL from *N. meningitidis* ($PglL_{Nm}$) were expressed (figure X.2). These results suggest that Rsc0559 is a functional *O*-OTase. We therefore named Rsc0559 as $PglL_{Rs}$.

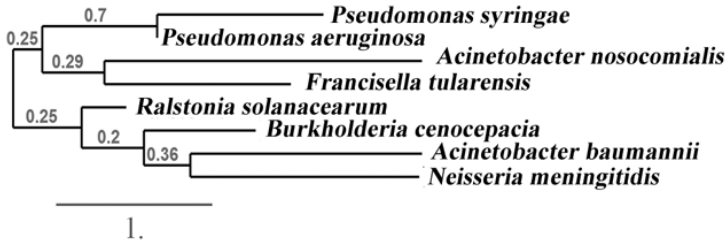

Figure X.1. Phylogenetic tree including known *O*-OTase genes from different bacteria. Bootstrap values (ratios) shown in grey.

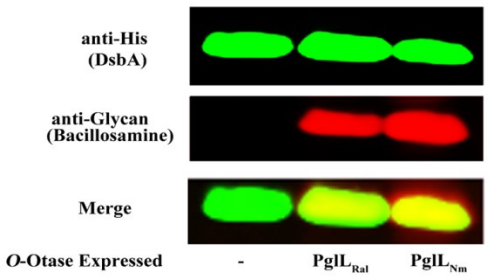

Figure X.2.Glycosylation of *Neisseria* protein (DsbA) by *Ralstonia O*-OTase ($PglL_{Rs}$) in *E. coli*. Whole-cell lysates of *E. coli* CLM24 cells expressing DsbA as an acceptor protein, together with bacillosamine and different *O*-OTases, were separated by SDS–PAGE and analyzed by

western blotting. His-tagged DsbA was detected using the monoclonal anti-His antibody (green). The bacillosamine sugar was detected using specific antibody (red). The overlapping signals are shown in yellow (merge). Both *O*-OTases from *Ralstonia* ($PglL_{Rs}$) and *Neisseria* ($PglL_{Nm}$) were able to glycosylate DsbA (Lanes 2 and 3 respectively).

X.4.2-$PglL_{Rs}$ is essential for *O*-glycosylation in *R. solanacearum*

To further demonstrate the *O*-OTase activity of $PglL_{Rs}$ we constructed a clean deletion mutant of the gene. In addition, we complemented the mutation by expressing $PglL_{Rs}$ *in cis* in the mutant strain. To exclude any possible effect that the mutation might have on O antigen assembly, we purified and analyzed the LPS from the wild type and mutant strains using SDS-PAGE followed by specific staining. LPS appeared identical in the two strains, excluding a role of $PglL_{Rs}$ in O antigen biosynthesis (figure X.3).

To identify the glycoproteins in *R. solanacearum* and their decorating glycan structures, glycopeptide enrichment coupled to MS/MS identification was utilized. Using ZIC-HILIC enrichment, which exploits the hydrophilic properties of glycans to enable their enrichment from complex lysates(Lithgow *et al.*, 2014, Iwashkiw *et al.*, 2012, Nothaft *et al.*, 2012), and multiple MS/MS fragmentation approaches, a total of 37 unique glycopeptides were identified within wild type *R. solanacearum* GMI1000 representing 20 glycoproteins (Table X.1) (Nothaft *et al.*, 2012, Scott *et al.*, 2011). The detected glycoproteome includes type IV pilin protein (PilA), pilin assembly protein (PilN), and the putative peptidase (Q8Y2I4_RALSO) which posseses a PDZ-domain and is homologous to the periplasmic chaperone, Prc of *Xanthomonas oryzae* (Deng *et al.*, 2014).

Within the identified glycopeptides eight glycans were observed (figures X.4, X.5) ranging in size from a single HexNAc (N-acetylhexoseamine) (figure X.5.A) to an octasaccharide composed of HexNAc-(Pen)-$dHex_3$-Pen_3 (pen; pentose) (figure X.5.F). The majority of the glycopeptides were found modified with a pentasaccharide composed of HexNAc-(Pen)-$dHex_3$ which is similar to the O antigen subunit characterized in the LPS structures of multiple *R. solanacearum* strains (figure X.4) (Varbanets *et al.*, 2003). Multiple glycoforms displayed an atypical carbohydrate of 188 Da potentially corresponding to the mass of an acetylated deoxyhexose sugar (figure X.5.C,E). Interestingly, some glycoproteins were decorated with different glycans at the same serine residue. As expected, no glycopeptides were detected in the Δ$pglL_{Rs}$ strain proteome while the non-glycosylated forms of the same peptides were observed confirming that protein *O*-glycosylation in *Ralstonia* is *O*-OTase dependent. Conversely, the in-genome expression of $PglL_{Rs}$ in the Δ$pglL_{Rs}$ strain led to the restoration of all glycoproteins.

Table X.1. Summary of the glycoproteins detected in *R. solanacearum* GMI1000 showing the detected glycans masses and the glycosylation sites

Uniprot Number	Protein Annotation	Gene Locus or Name	Mass of Attached Glycan/s	Glycosylation Sites
HBOH_RALSO	D-(-)-3-hydroxybutyrate oligomer hydrolase	RSc1334	773.32	undetermined
Q8XQM5_RALSO	Putative membrane fusion protein	RSp1197	773.31	S^{418}
			815.32	S^{418}

			815.31	S^{442}
Q8XR30_RALSO	Probable lipoprotein transmembrane	RSp1038	773.31	S^{120}
Q8XRR4_RALSO	Probable transmembrane protein	RSp0767	773.30	S^{160}
Q8XRR8_RALSO	Probable transmembrane protein	RSp0763	773.34	undetermined
Q8XS78_RALSO	Probable serine protease protein	RSp0603	773.34	undetermined
Q8XSI7_RALSO	Probable m20-related peptidase	RSp0487	773.34 905.36	undetermined
Q8XV57_RALSO	Probable fimbrial type-4 assembly membrane transmembrane protein PilN	RSc2974	773.30	undetermined
Q8XVC9_RALSO	Probable lipoprotein	RSc2902	773.31 815.32 905.34 947.36	undetermined
Q8XWI3_RALSO	Hypothetical signal peptide protein	RSc2491	641.26 815.31 773.30	S^{61}
Q8XX43_RALSO	Probable polysaccharide	*ragB*	773.31	S^{346}

	transport system component			
Q8XXY5_RALSO	Probable transmembrane protein	RSc1978	815.33	undetermined
Q8XZ41_RALSO	Peptidyl-prolyl cis-trans isomerase	RSc1565	773.31	undetermined
Q8Y030_RALSO	Probable transmembrane protein	RSc1214	773.31	undetermined
Q8Y078_RALSO	Probable tpr domain signal peptide protein	RSc1166	203.08	S^{210}
Q8Y1X9_RALSO	Type 4 fimbrial pilin signal peptide protein PilA	RSc0558	1037.40 203.09 1169.43 905.35	undetermined
Q8Y2P9_RALSO	Probable cell division FtsN transmembrane protein	*ftsN*	947.37	undetermined
			773.32 905.35 815.33	S^{78}
Q8Y3G9_RALSO	Probable acriflavin resistance lipoprotein	*acrA*	773.31 815.32	S^{393}
Q8Y2I4_RALSO	Probable peptidase transmembrane protein	RSc0352	773.31 815.32	undetermined
Q8XVI0_RALSO	Cell division protein FtsL	*ftsL*	773.32	undetermined

			905.35	

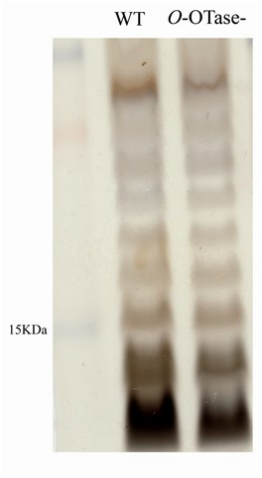

Figure X.3. LPS synthesis is not affected by the loss of protein *O*-glycosylation in *R. solanacearum*. LPS was harvested from wild type and *O*-OTase$^-$ *R. solanacearum* strains followed by separation on 15% SDS-PAGE and silver staining.

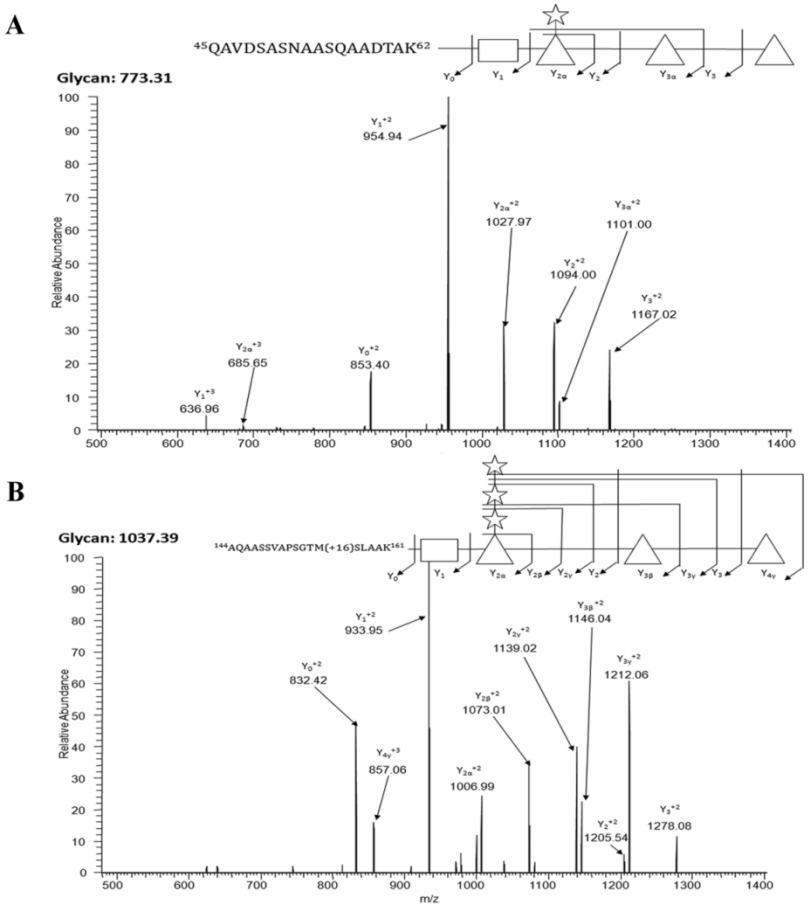

Figure X.4. Major *O*-glycan structures identified in *R. solanacearum* glycoproteins. *O*-Glycan structures were identified using ITMS-CID fragmentation of *Ralstonia* glycopeptides. A) a pentamer glycan of HexNAc-(Pen)-dHex$_3$ attached to ^{45}QAVDSASNAASQAADTAK^{62} of Q8XVC9_RALSO. B) a heptamer glycan of HexNAc-(Pen$_3$)-dHex$_3$ attached to ^{144}AQAASSVAPSGTM(+16)SLAAK^{161} of Q8Y1X9_RALSO.

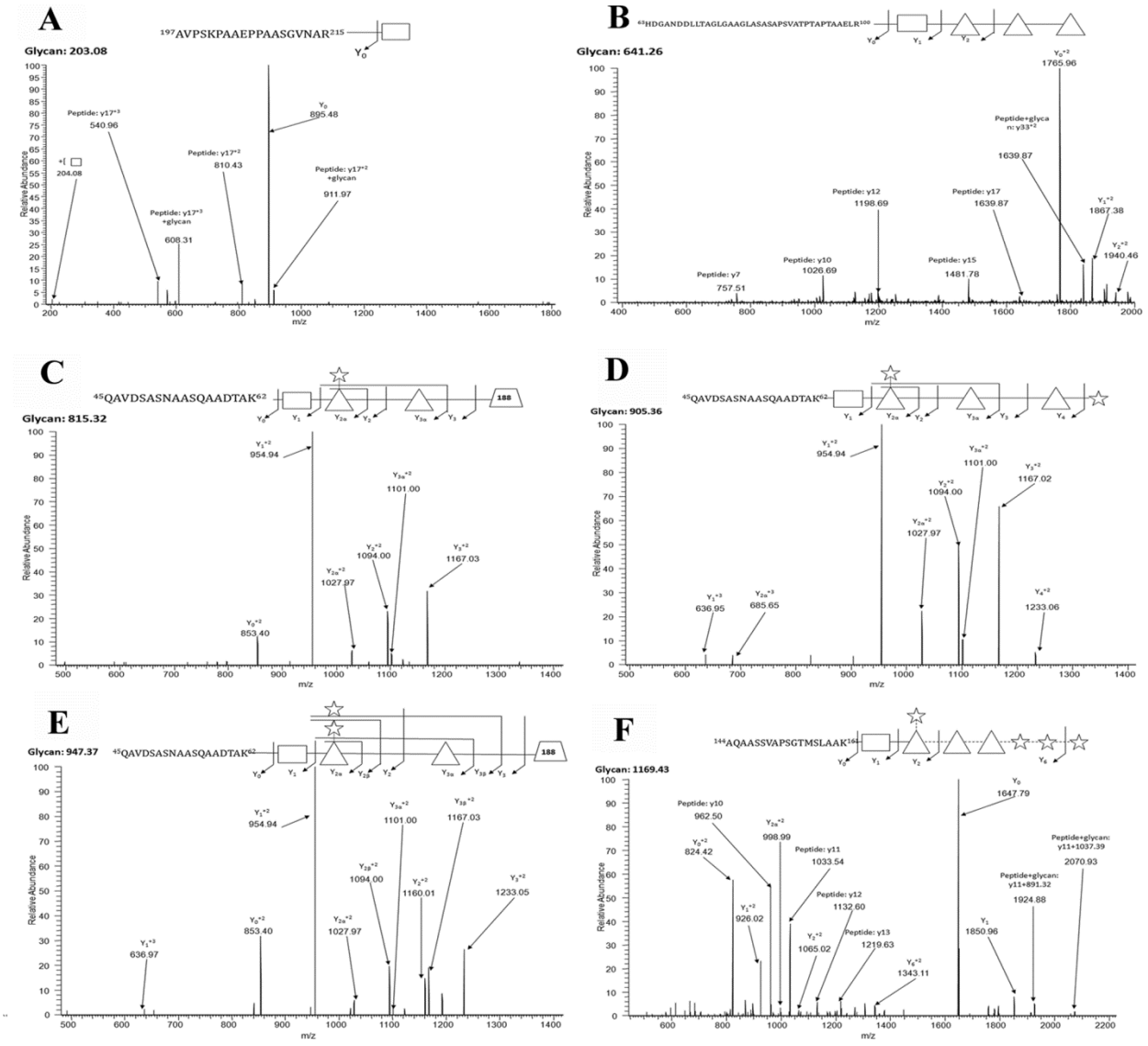

Figure X.5. Different *O*-glycan structures identified in *R. solanacearum* glycoproteins. *O*-Glycan structures were identified using ITMS-CID fragmentation of *Ralstonia* glycopeptides. A) A single HexNAc residue attached to ^{197}AVPSKPAAEPPAASGVNAR^{215} of Q8Y078_RALSO. B) a tetramer glycan of HexNAc-dHex$_3$ attached to ^{63}HDGANDDLLTAGLGAAGLASASAPSVATPTAPTAAELR^{100} of HBOH_RALSO. C) a pentamer glycan of HexNAc-(Pen)-dHex$_2$-188. D) a hexamer glycan of HexNAc-(Pen)-dHex$_3$-

Pen. E) a hexamer glycan of HexNAc-(Pen_2)-$dHex_2$-188. All attached to ^{45}QAVDSASNAASQAADTAK^{62} of Q8XVC9_RALSO. F) an Octamer glycan of HexNAc-(Pen)-$dHex_3$-Pen_3 attached to ^{144}AQAASSVAPSGTMSLAAK^{161} of Q8Y1X9_RALSO.

X.4.3-Lack of *O*-Glycosylation affects biofilm formation in *R. solanacearum*

The glycoproteome of *R. solanacearum* included type IV pilin which was shown to be important for biofilm formation in many bacteria, including *R. solanacearum* (Kang *et al.*, 2002, van Schaik *et al.*, 2005, Flemming & Wingender, 2010). Biofilm formation aids *R. solanacearum* in evading the host defenses and the entrapment of nutrients from the xylem flow (Yao & Allen, 2007, Álvarez *et al.*, 2010). To test the role of *O*-glycosylation in biofilm formation, we compared both Δ$pglL_{Rs}$ and complemented strains to the wild-type strain in a standard microtiter plate-based biofilm assay. The three strains were grown in non-shaking conditions to allow attachment and matrix formation. The resulting biofilms were stained with crystal violet and quantified spectrophotometrically. Less biofilm was produced in the Δ$pglL_{Rs}$ strain compared to the wild-type (Figure X.6). Expressing $PglL_{Rs}$ from its native promoter in-genome restored biofilm formation in the mutant. Since lacking *O*-glycosylation affected the biofilm forming ability of *R. solanacearum*, we hypothesized that the Δ$pglL_{Rs}$ strain would be defective in virulence towards the plant host. To test this hypothesis, we employed a tomato plant infection model to compare the virulence of the *R. solanacearum* wild type and Δ$pglL_{Rs}$ strains. The two strains were inoculated into wounded petioles and the infected plants were monitored for wilting symptoms (figure X.7) The WT caused the typical disease symptoms: initial chlorosis of the leaves and then loss of firmness and wilting. On the other hand, the

Δ*pglL*$_{Rs}$ mutant did not display wilting symptoms under the conditions tested. Expressing PglL$_{Rs}$ *in trans* restored virulence in tomato plants suggesting a role of *O*-glycosylation in *R. solanacearum* pathogenesis.

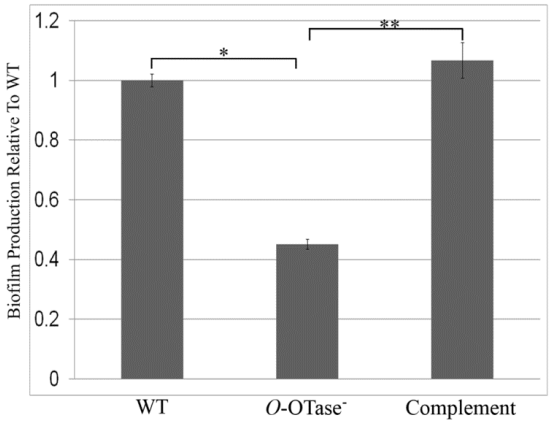

Figure X.6. *Ralstonia O*-OTase mutant is defective in biofilm formation. Different *R. solanacearum* strains were grown for 48 hrs in 96 well plates at 30ºC. The formed biofilms were washed and stained with 0.1% crystal violet, then dissolved in 95% ethanol. Biofilm formation was measured spectrophotometrically by measuring absorbance at ʎ=590nm. Readings were normalized by the OD_{600} values of the corresponding strains after 48 hrs. Biofilm production in *O*-OTase$^-$ and complemented strains was presented relative to WT. The data shown was obtained from 3 independent experiments (n=3). * $p<0.01$, ** $p<0.001$.

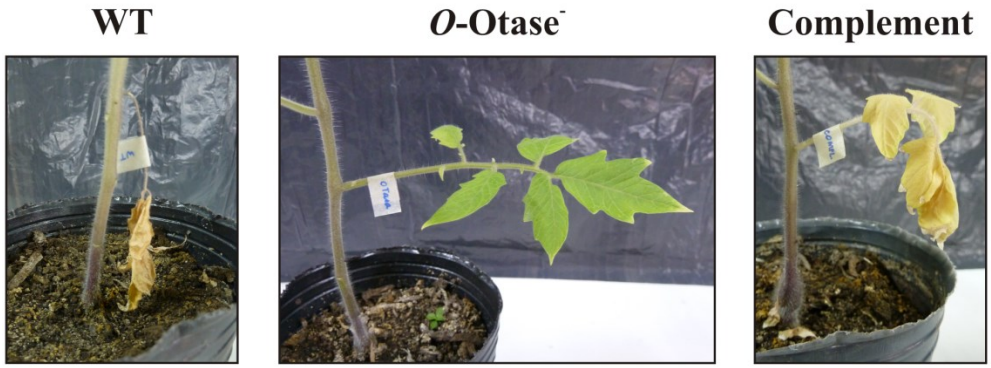

Figure X.7. *O*-Glycosylation might be involved in the virulence of *Ralstonia* towards tomato plants. Different *Ralstonia* strains were grown overnight at 30ºC, then diluted to 10^6 CFU/mL using sterile distilled water. Inocula concentrations were checked using serial dilution plating on BG agar. Leaf petioles were inoculated with 3 µl of the normalized inocula from the 3 *Ralstonia* strains. A set of 6 tomato plants were inoculated with each strain and placed in separated trays in order to prevent cross contamination. After 16 hrs photoperiod incubation at 28 ºC, only plants infected with WT and the complemented strains caused wilting symptoms. The experiment was repeated twice under the same conditions to a total of 3 experiments.

X.4.4-Quantitative proteomics reveals that lack of glycosylation produces changes in the levels of some proteins in *R. solanacearum*

The detection of multiple glycoproteins in *R. solanacearum* suggested an important role of *O*-glycosylation in this bacterium. Therefore, we hypothesized that the identification of proteins over or under expressed in the absence of glycosylation might provide hints about the role of glycosylation in the physiology or the pathogenesis of this bacterium. The total proteomes of wild-type, Δ$pglL_{Rs}$ and the complemented strains were analyzed by the peptide stable isotope dimethyl labelling technique (Boersema *et al.*, 2009).

The proteomic comparison of the *R.solanacearum* strains revealed changes in the levels of several proteins in Δ$pglL_{Rs}$ relative to the wild-type and the complemented strains. However, when proteins that displayed at least a two-fold change respect to wild type levels were considered, only three proteins exhibited significant differences in their expression. PilA, the major pilin subunit, displayed about 4 fold reduction in Δ$pglL_{Rs}$ compared to wild type strain. Similarly, the putative type VI secretion protein (Q8XRT8_RALSO) showed a 2 fold reduction in the Δ$pglL_{Rs}$ strain. Conversely, the RS-II lectin levels in Δ$pglL_{Rs}$ were about 3.5 fold more than in the wild type strain. RS-II was shown to bind mannose and fucose sugars, which are widely distributed among the plant polysaccharides (Sudakevitz *et al.*, 2004, Kostlanova *et al.*, 2005). Expressing $pglL_{Rs}$ *in cis* from its native promoter restored the wild type levels of the three proteins in Δ$pglL_{Rs}$ strain as shown by our proteomic analysis. Additionally, we monitored the levels of PilA using immunoblotting in the 3 *R. solanacearum* strains. As shown in figure X.8, the pilin subunit displayed a weak signal in the Δ$pglL_{Rs}$ strain compared to the wild type and complemented strains, confirming the MS data Moreover, PilA band appeared at a lower size in the strain lacking *pglL* confirming that PilA is glycosylated in *R. solanacearum* as shown by our MS analysis. An antibody against RNA polymerase was included as loading control.

Table X.2. List of proteins that displayed altered levels in OTase⁻ compared to wild type strain

Uniprot number	Protein Annotation	Gene Locus	Relative Protein Abundance In *O*-Otase⁻ vs WT Strain [a]	Relative Protein Abundance In *O*-Otase⁻ vs WT Strain [a]
Q8Y1X9_RALSO	PilA	RSc0558	0.23	1
Q8XRT8_RALSO	Putative type VI secretion protein	RSp0743	0.46	0.96
Q8XUA5_RALSO	RS-II lectin	RSc3288	3.45	0.9

a: Presented is the average of 2 readings

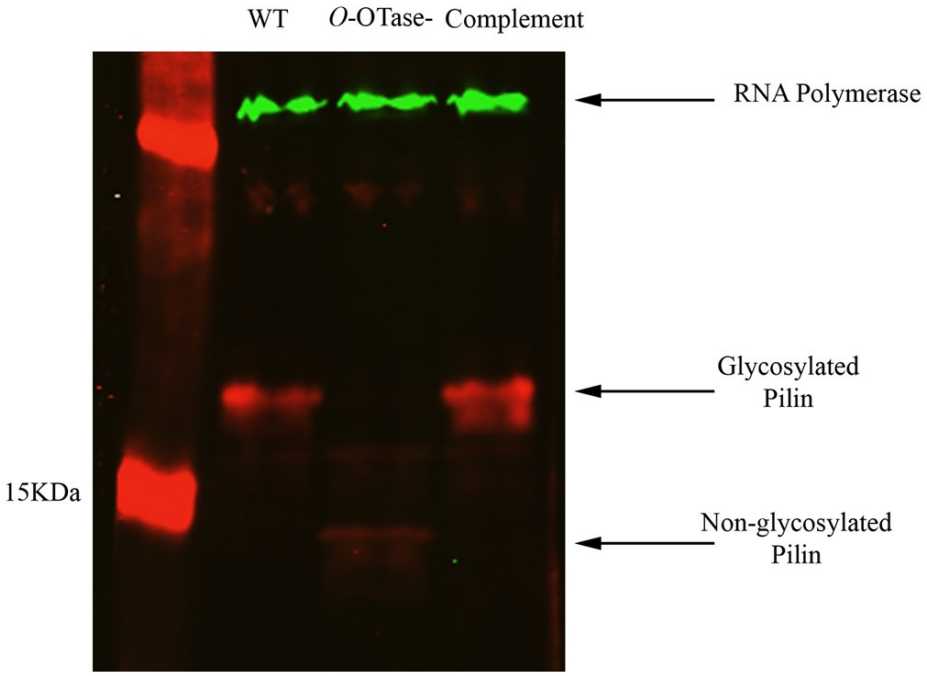

Figure X.8. Loss of *O*-Glycosylation altered the levels of pilin in *R. solanacearum*. Whole cell lysates of different *R. solanacearum* strains were run on SDS-PAGE gel followed by immunoblotting using rabbit polyclonal anti-pilin and mouse monoclonal anti-RNA polymerase (1:2500, RNAP α-subunit; Neoclone). Membranes were then probed with IRDye conjugated anti-mouse and anti-rabbit antibodies and visualized on an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

X.5-Discussion

Protein glycosylation is a well-known posttranslational modification that is widespread in bacteria. Bacteria can glycosylate their proteins by sequentially adding sugars to their target proteins via cytoplasmic glycosyltransferases. Alternatively, the lipid-linked glycans can be transferred *en bloc* to the target proteins by an OTase. Although initially thought to be limited to *N*-glycosylation, it was shown that many bacteria exploit OTases to *O*-glycosylate their proteins (Nothaft & Szymanski, 2010, Iwashkiw *et al.*, 2013). Some *O*-OTases are pilin-specific, like PilO/TfpO in *Pseudomonas*, while other *O*-OTases evolved to be more promiscuous towards their protein substrates (Castric, 1995, Kus *et al.*, 2008). Examples of OTase-dependant general *O*-glycosylation systems include *A. baumannii*, *Neisseria spp.*, *B. cepacia* complex, and *F. tularensis* (Marceau *et al.*, 1998a, Power *et al.*, 2003, Borud *et al.*, 2011, Jennings *et al.*, 2011, Iwashkiw *et al.*, 2012, Balonova *et al.*, 2012, Lithgow *et al.*, 2014). Some *Acinetobacter* strains evolved to keep both a pilin-specific and a general *O*-OTase (Harding *et al.*, 2015). Protein *O*-glycosylation was shown to be important for the virulence of many pathogens, albeit, the exact role of *O*-glycosylation in pathogenesis remains unknown (Iwashkiw *et al.*, 2013). In this work,

we identified a general *O*-glycosylation system in the important phytopathogen *R. solanacearum*, one of the leading causes of plant diseases worldwide (Genin & Denny, 2012). Our MS-based proteomic analysis of the strain GMII1000 revealed twenty glycoproteins in *R. solanacearum* including type IV pilin and the type IV fimbrial membrane-assembly proteins (Table X.1). *R. solanacearum* genome analysis revealed the presence of a protein homologous to the *Neisseria* *O*-OTase. We demonstrated that $PglL_{Rs}$ was able to transfer a foreign glycan (diNAcBac) to a *Neisseria* protein when all the components of the reaction were co-expressed in *E. coli* (figure X.2). This indicates that, like previous characterized *O*-OTases, $PglL_{Rs}$ has relaxed glycan specificity and reinforces the concept that *O*-OTases from different bacteria recognize similar sequences in their target proteins. Within the detected glycoproteins, we observed heterogenous glycans decorating the proteins. The most abundant oligosaccharides detected were similar to the O-antigen characterized in a number of *R. solanacearum* strains (Varbanets *et al.*, 2003). Varbanets *et al*. (2003) detected N-acetyl glucosamine, rhamnose (deoxyhexose) and xylose (pentose) in the O antigens characterized. Our *in silico* analysis of *R. solanacearum* GMI1000 revealed a homologue of a rhamnosyltransferase (RSc0687) within a typical glycan biosynthetic cluster that also contain enzymes usually involved in the synthesis of the O-antigen. The same gene cluster included a homologue of undecaprenyl phosphate N-acetylglucosaminyltransferase transmembrane protein (RSc0689). This protein belongs to the initiating glycosyl transferases family, which is responsible for linking the first sugar in the O-antigen glycan to the lipid carrier in the inner membrane, a step essential for O-antigen synthesis. Interestingly, Li et al demonstrated that the deletion of RSc0689 resulted in the loss of O antigen formation in in *R. solanacearum* GMI1000 (Li *et al.*, 2014). Additionally, a UDP-4-keto-pentose/UDP-xylose synthase (Uxs) was previously identified in *R. solanacearum* GMI1000 (Gu *et al.*, 2010),

suggesting that xylose is the pentose contained in the O-glycans attached to proteins. However, this gene is not encoded in the cluster containing RSc0687. A differential regulation of these genes could explain the detection of heterogeneous glycan structures in *R. solanacearum* glycoproteins. Our results suggest that *R. solanacearum* shares its O antigen between two different pathways; LPS synthesis and protein *O*-glycosylation. This is in agreement with what was observed in a number of bacteria and was proposed to be a strategy by which bacteria can save energy and resources (Cuccui & Wren, 2013, Lees-Miller *et al.*, 2013). The role of the putative initiating glycosyl transferase (RSc0689) in protein glycosylation remains to be demonstrated.

Protein *O*-glycosylation was completely abolished in the Δ*pglL*$_{Rs}$ strain, which produced less biofilm. Our preliminary virulence test on tomato plants suggested a possible role for *O*-glycosylation in the pathogenesis of *R. solanacearum.* Nevertheless, a more comprehensive infection model will be required to support this finding. MS data showed that the expression of PglL$_{Rs}$ *in cis* restored *O*-glycosylation. Type IV pilin was among the restored glycoproteins in the complemented strain. The work by Kang *et al* showed that type IV pili are important for autoaggregation, biofilm development and pathogenesis of *R. solanacearum* (Kang *et al.*, 2002). Therefore, it is possible that the Δ*pglL*$_{Rs}$ mutant might be defective in biofilm formation and virulence due to the impaired function of the non-glycosylated pilin.

The glycoproteome of *R. solanacearum* proved to be relatively large compared to that of other bacteria (Iwashkiw *et al.*, 2013). Therefore, we hypothesized that the lack of *O*-glycosylation might impact other physiological functions in *Ralstonia*. To investigate additional effects of glycosylation loss, we performed a comparative proteomic analysis between wild-type

and Δ*pglL*$_{Rs}$ strains, using peptide stable isotope dimethyl labelling (Boersema *et al.*, 2009). Surprisingly, our analysis revealed a change in the expression levels of only three proteins as response to the lack of glycosylation. These proteins are PilA, RS-II lectin and a putative type VI secretion protein.. Using immunoblotting, we were able to validate our proteomic analysis by monitoring the levels of pilin proteins in different *R. solanacearum* strains. In addition to being unglycosylated, *R.solanacearum* pilin was detected at lower levels in the Δ*pglL*$_{Rs}$ strain relative to wild type as suggested by our MS analysis. Native pilin protein levels were restored upon *cis* complementation (figure X.8). One possible explanation is that in *R. solanacearum*, when *O*-glycosylation is absent; pilin is more susceptible to degradation by proteases. The role of protein glycosylation in protection against degradation is well-established (Iwashkiw *et al.*, 2013). Nonetheless, levels of pilin protein do not appear to be reduced in glycosylation deficient strains of other bacterial species such as *Neisseria* and *Acinetobacter* (Marceau *et al.*, 1998b, Harding *et al.*, 2015). Due to its role in biofilm formation, the reduced pilin levels might contribute to the lower levels of biofilm displayed by Δ*pglL*$_{Rs}$ strain in vitro (figure X.6). However, our experiments do not show which of the two, loss of pilin glycosylation and lower pilin levels, has more impact on biofilm formation in Δ*pglL*$_{Rs}$ strain. We observed higher levels of RS-II lectin in the OTase mutant via quantitative proteomics. RS-II is fucose/mannose binding lectin in *R. solanacearum* that is homologous to PA-IIL in the phylogenetically related *P. aeruginosa.* PA-IIL was shown to contribute to host cell adhesion and biofilm formation (Sudakevitz *et al.*, 2004). A similar role was suggested for RS-II in *Ralstonia* pathogenesis towards plants (Valls *et al.*, 2006). It is tempting to speculate that the lectin upregulation might act as a compensatory attempt of the cells to restore interbacterial or host-bacterial interactions mediated by the *O*-glycans and the pilin in wild-type bacteria. We also detected a 2-fold decrease in the levels of a

putative type VI secretion protein (Q8XRT8_RALSO) in Δ*pglL*$_{Rs}$ strain (Table X.2). Type VI secretion system is a newly discovered system in Gram negative bacteria that is capable of delivering effectors to prokaryotic and eukaryotic cells (Jani & Cotter, 2010). Recently, a type VI secretion system was identified in *R. solanacearum* and was shown to be involved in biofilm formation and virulence (Zhang *et al.*, 2014). It remains to be demonstrated if the type VI machinery in the Δ*pglL*$_{Rs}$ strain is affected by the reduction in Q8XRT8_RALSO levels. To our knowledge, this is the first report studying global protein changes related to a deficiency in protein glycosylation in a prokaryote. Given the extensive glycoproteome present in *R. solanacearum*, we expected significant changes in the levels of multiple proteins in the absence of glycosylation. However, only a few proteins were altered in our experimental conditions. It is possible that in other condition such as stress, biofilms, or during plant infection, protein glycosylation plays a more important role and therefore additional changes it the proteome of *R. solanacearum* occur in these conditions. Further work will be necessary to understand the role of protein glycosylation in the biology of this important phytopathogen.

X.6-References

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